Sachin Kumar Mandotra
Atul Kumar Upadhyay
Amrik Singh Ahluwalia *Editors*

Algae

Multifarious Applications for a Sustainable World

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Preface

The meteoric growth of the human population and their ever-increasing thirst for natural resources are posing several appalling threats to the environment as well as to mankind. The post-industrialization era witnessed various anthropogenic activities. Eutrophication, heavy metal contamination, and acidification resulted in polluting large freshwater bodies. Therefore, it is imperative to search for alternative resources to satisfy human needs within environmental limits. From this perspective, algae could provide a solution to meet our energy demands and other value-added products in a sustainable manner.

The edited volume "Algae: Multifarious Applications for a Sustainable World" is a compilation of useful characteristics of algae with reference to its innovative natural products. It covers the present and future prospects of algal biofuel, algae-derived pharmaceuticals and nutraceuticals, bioremediation of heavy metals, and algae-mediated nanoparticle synthesis. Furthermore, this book also describes comprehensively about the cyanobacterial blooms and cyanotoxins and the application of diatoms (class Bacillariophyceae) in forensic analysis.

Chapter 1 presents a brief outlook about the nutrient management of municipal and agricultural wastewater using microalgal biomass followed by potential applications of algal biomass in bioenergy, biofertilizers, human food supplements, and animal feed. Chapter 2 discusses the synergistic application of algae and constructed wetlands in the maintenance of diversity and their role in wastewater treatment via bioremediation and energy production in a sustainable manner. Chapters 3 and 4 mainly focused on heavy metals such as mercury, lead, arsenic, and cadmium, their sources and their entry into the ecosystem. The authors have provided a brief overview about the impact of these heavy metals on microorganisms, plants, and animals, besides this, the chapter also reviews various conditions affecting heavy metal biosorption, morphological and physiological responses of microalgae to survive heavy metal toxicity, and a brief insight into the utilization of algal biomass in biofuel production.

Chapter 5 provides detailed insight into the organic and inorganic components of wastewater together with its microbial composition. The later part of the chapter deals with wastewater treatment, heavy metal removal by algae, and potential application of algal biomass in value-added products. Chapter 6 deals with the phycoremediation of Cr(VI) using microalgal isolate. The chapter comprehensively

describes the isolation, identification, and cultivation of isolated microalgal strain. The isolated strain was subjected to a different set of conditions such as time of contact between contaminated wastewater and microalgae, initial concentration of Cr(VI) in water, inoculum size, and pH of the media to study the phycoremediation of Cr(VI) using artificial neural network and simulated annealing. Chapters 7 and 8 provide detailed information about pharmaceutical, nutraceutical, and cosmeceutical properties of microalgal compounds. These chapters provide state-of-the-art applications of algae in therapeutics and cosmetic industry. The information about strain selection, strain improvement, growth condition optimization, extraction methods of value-added compounds from algal biomass, and operational expenditures at commercial scale have been discussed thoroughly.

In the last few decades, natural food supplements have gained significant interest due to increased public concern towards health issues and disease prevention. The search for potential food supplements unravels various natural sources. Chapters 9 and 10 provide a detailed review of the food supplements and fucoxanthin production from *Spirulina* and diatoms, respectively. In Chap. 9, the authors reviewed literature pertaining to the nutritional and antioxidant value of *Spirulina*, its domestic and commercial cultivation and favorable growth conditions. The later part of the chapter gives an overview of economic importance and global *Spirulina* market. Chapter 10 mainly focuses on fucoxanthin biosynthesis in diatoms, various abiotic factors affecting its production, and genetic engineering strategies to improve fucoxanthin yield.

Chapters 11 and 12 mainly focused on algal biofuel. Chapter 11 provides state-of-the-art information about various technologies to convert algal biomass into fungible biofuel. Potential solutions to algal biofuel challenges and operational costs associated with commercialization have also been thoroughly discussed. Chapter 12 reviews the defense responses under UV-B radiation along with UV-B radiation-based lipid alteration in microalgae. Chapters 13 and 14 provide an overview of the algae-mediated nanomaterial synthesis. The authors briefly describe various classes of nanoparticles, mechanism of their synthesis, and factors affecting the synthesis of nanoparticles such as algal extract, contact time, pH, and temperature. Various applications of microalgal-synthesized nanoparticles have also been discussed.

Chapter 15 deals with the cyanobacterial harmful algal blooms, ecological factors that help in their occurrence, and various methods of their detection. The chapter also deals with the treatment of intracellular and extracellular cyanotoxins along with harmful algal bloom management. Chapter 16 presents a comprehensive outlook on the importance of golden-brown algae (diatoms) in forensic analysis. The chapter starts right from the basic structure of diatoms followed by the significance of diatoms in forensic limnology. The penetration of diatoms in the victim's body and various extraction methods to recover diatoms are thoroughly discussed. The later part of the chapter describes the current status and controversies associated with solving forensic cases using diatoms.

The purpose of this book is to give an alternate perspective to students and researchers and to encourage them to understand more about the application of

algal technologies to various emerging fields. We hope that this book will fill up the various gaps in the abovementioned fields.

We thank all the contributors for their valuable input and their timely contribution. We also express our gratitude to Dr. Madhurima Kahali and Raagai Priya Chandrasekaran (Springer Nature) for their time to time help and kind cooperation during the preparation of this book.

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Valorization of Wastewater via Nutrient Recovery Using Algae-Based Processes

1

Pfano Musetsho, Nirmal Renuka, Sachitra Kumar Ratha, Ismail Rawat, and Faizal Bux

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Abstract

Increasing anthropogenic activities have amplified the unregulated wastewater discharge into water bodies, leading to the accumulation of nutrients and subsequent destruction of freshwater resources and aquatic habitats. Exploration of energy competent, cost-effective systems, which are able to sequester nutrients in wastewaters, has become a requisite. Recently, microalgae–bacterial consortia are gaining attention as an environmentally friendly and economically viable option for wastewater treatment and biomass production. Co-cultivation of these microorganisms promotes mutual growth and enhances nutrients removal from wastewater. Furthermore, several studies have projected the potential applications of wastewater-grown microalgal biomass for the production of renewable energy. Recently, wastewater-grown microalgae biomass has also been explored for its

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applications in aquaculture nutraceutical and agricultural nutrient management sectors. Thus, the use of wastewater for microalgal biomass production could form a sustainable biorefinery via wastewater treatment vis-a-vis multiple products development through valorization of biomass. The focus of this chapter is to provide the status and prospects of nutrients sequestration from wastewater using microalgae and potential applications and challenges of wastewater-grown biomass.

Keywords

Microalgae · Wastewater · Phycoremediation · Algae–microbe interaction · Biomass · Value added products

1.1 Introduction

In the present century, technological advancement with upliftment of world's economy particular to middle and low income countries tends to enhance the comfortability of human life, which has led to production of more wastewater as compared to past. Moreover, world is confronted with wastewater management issues due to extensive industrialization, urbanization, commercialization, and an increase in population (Pizzera et al. 2019). Wastewater produced from domestic, industrial, and farming activities on daily basis constitutes a major cause of contamination in receiving aquatic environment, which is a great concern for water quality management services (Singh et al. 2019). Excess amounts of phosphorus (P) and nitrogen (N) in wastewater result in the eutrophication of receiving aquatic bodies, and lead to the reduced lifespan and destruction of the ecosystem, and increased water toxicity, which is unsuitable for human consumption (Renuka et al. 2014b; Shahid et al. 2019). Furthermore, pathogenic microorganisms, heavy metals, endocrine disruptors, and organic matter are some of the additional contaminants that are released into freshwater bodies (Rajasulochana and Preethy 2016).

Eutrophication is a serious environmental concern, and since the twenty-first century, it has become more prevalent. The extraction of excess nutrients and other contaminants within acceptable limits before disposal and reuse of wastewater is a major requirement for wastewater treatment (Cai et al. 2013). The new era water reuse could be a beneficial approach. The existing wastewater treatment processes across the globe are facing issues related to cost economics and energy consumption to minimize the nutrients load. The conventional wastewater treatment processes are mostly based on physical and chemical treatments that are neither environmentally friendly nor satisfactorily cost-effective (Li et al. 2019a). Conventional wastewater treatment and chemical methods such as aluminum and iron salts precipitation produce large amounts of sludge that generate additional challenges for their disposal (Gonçalves et al. 2016). In this regard, microalgae-mediated wastewater treatment designated as "phycoremediation" has recently gained more attention, which is one of the most promising technologies for the nutrient recovery and insinuated as advanced treatment of wastewater. This is an environmentally friendly

technology for nutrient recovery with additional benefits of CO_2 sequestration and production of microalgal biomass feedstock for value-added products, thus has gained an enhanced commercial interest (Renuka et al. 2018; Li et al. 2019b; Mandotra et al. 2020). Significant efforts have been made in recent decades to study microalgae production in wastewater. Numerous researchers have shown the feasibility of using microalgae for tertiary wastewater treatment in advanced treatment of agricultural, industrial, and municipal wastewater for its high efficiencies for nutrient removal. Controlled cultivation of microalgae has shown more promise for efficient biological wastewater treatment (Shahid et al. 2019).

Microalgae are a broad category of photosynthetic microorganisms, which are ubiquitous in nature, and are potential feedstock for new generation biofuels, biofertilizers, food, and feed production (Kumar et al. 2016; Mandotra et al. 2019). However, the microalgae cultivation requires excessive levels of nutrients, nitrogen, and phosphorus to develop desirable quantity of feedstock within economical time-period. In addition, the use of chemical fertilizers for production of microalgal biomass is projected to result in an unsustainable cycle, even increasing competition for nutrient availability between algae-based commodities and food crops. In order to improve the economics and sustainability of microalgae-based commodities, a strategy should be created to use waste/wastewater nutrients as low-cost nutrients and reduce the use of chemical fertilizers (Renuka et al. 2013; De Bhowmick et al. 2019). Consumption of nutrients (carbon, nitrogen, phosphorus, etc.) from wastewater for microalgal biomass production could significantly reduce the overall cost of production (Goswami et al. 2019; Chinnasamy et al. 2014). Therefore, cultivation of microalgae combined with the removal of N and P from wastewater to manufacture biofuels, aquaculture nutraceuticals, or other bio-based green chemicals could form a sustainable biorefinery (Bag et al. 2019; Kumar et al. 2020; Laurens 2017; Renuka et al. 2018).

In wastewater system, microalgae could promote the growth of heterotrophic bacteria by releasing organic compounds and oxygen through photosynthesis and protect them from harmful chemicals and adverse environmental conditions. Alternatively, bacteria provide CO_2 required for photosynthesis, and organic growth promoters, which enhance the growth of microalgae (Gonçalves et al. 2016). However, the synergetic connection between bacteria and microalgae in wastewater treatment was revealed long back by Oswald et al. (1957). Recently microalgae–bacteria consortia have been explored for their efficiency for nutrient recovery and biomass production. A symbiotic relationship between the cultivation of autotrophic and heterotrophic/mixotrophic microalgae–bacteria consortium has been reported to achieve greater wastewater treatment efficiencies (Qu et al. 2019). The microalgae–bacteria consortium method is energy-efficient, cost-effective and has great potential in reducing the carbon footprints of wastewater treatment process (Gonçalves et al. 2016; Laurens 2017; Boelee et al. 2014). This could also be a beneficial strategy for enhancing microalgal growth, since some of the bacteria have the ability to improve microalgal growth and biomass production through synergistic interactions (Huo et al. 2020). This chapter discusses the current status of utilizing microalgae to remove nutrients from different types of wastewater, the importance and interaction

between algae and microbes in wastewater, and applications of wastewater-grown biomass for bioenergy, feed, and biofertilizer production.

1.2 Current-Status of Nutrient Recovery Using Microalgae-Based Processes

Microalgae have shown to be dynamic in the extraction of nitrogen, phosphorus, and other contaminants from a broad range of wastewater. Immense studies on microalgae cultivation in agricultural, domestic, and industrial wastewater have been reported (Renuka et al. 2014b). The significance of microalgae cultivation using wastewater in high rate ponds (HRP) was revealed since 1950s (Oswald et al. 1957). Numerous studies have proved the ability of different microalgal species to remove nitrogen, phosphorus, and other wastewater elements (Markou and Monlau 2019). Such studies have reported substantial amount of nutrient removal and algal biomass production in wide range of wastewater. Microalgal biomass seeks application in various industrial sectors such as feed, food, biofertilizers, and renewable fuels. Presently microalgae-based biofuels production is not commercially viable because of high biomass production cost. This is due to the requirement of expensive chemical fertilizers and excess freshwater resources, and therefore more novelties for green production are needed. In this context, combining microalgal cultivation with wastewater treatment is a sustainable alternative to decrease the use of expensive chemical fertilizers and limited freshwater resources.

1.2.1 Municipal Wastewater

Generally, municipal wastewaters are a combination of domestic (80–95%) and industrial (5–20%) influents. It mainly contains nutrients, microorganisms, and other organic waste, household, and industrial chemicals; however, most significantly, the percentage composition of municipal wastewater differs based on local community activities. The distinctive components of such types of wastewater involve nutrients, COD (chemical oxygen demand), metals, pathogenic microorganisms, and organic and inorganic materials. Ammonia (NH_4-N), phosphate (PO_4-P), and other nutrients essential for microalgal growth are the most dominant nutrients in municipal wastewater. Conversely, the activities of some localized small-scale factories, a significant level of heavy metals such as cadmium (Cd), copper (Cu), chromium (Cr), lead (Pb), zinc (Zn), mercury (Hg), arsenic (As), etc. are also present in raw municipal sewage. The common nutrients content in untreated domestic wastewater mixed with moderate level of industrial wastewater is given in Table 1.1. It is of great significance to examine wastewater characteristics for the determination of its suitability for microalgal propagation (Renuka et al. 2014a). Komolafe et al. (2014) indicated that characterization of wastewater serves to examine wastewater treatment process during the microalgal cultivation period, and also gives an understanding on the requirement of dilution or nutrient

Table 1.1 Common nutrients content in untreated domestic wastewater

Parameters	Wastewater characteristics (low–high nutrient level)
pH	7–8
Total suspended solids (mg L^{-1})	250–600
Volatile suspended solids (mg L^{-1})	200–400
Biological oxygen demand (mg L^{-1})	140–350
Chemical oxygen demand (mg L^{-1})	300–900
Total nitrogen (mg L^{-1})	30–100
NO_3-N (mg L^{-1}) + NO_2-N (mg L^{-1})	0.1–0.5
NH_4-N (mg L^{-1})	20–75
Organic nitrogen (mg L^{-1})	10–25
Total Kjeldahl N (mg L^{-1})	30–100
PO_4-P (mg L^{-1})	4–15
Total phosphorus (mg L^{-1})	6–25
Organic phosphorus (mg L^{-1})	2–10
Aluminum (μg L^{-1})	350–1000
Copper (μg L^{-1})	30–70
Chromium (μg L^{-1})	10–25
Lead (μg L^{-1})	25–80
Mercury (μg L^{-1})	1–3
Cadmium (μg L^{-1})	1–4

Modified from Guldhe et al. (2017) and Henze and Comeau (2008)

supplementation in wastewater for the growth of microalgae. Research studies based on the usage of municipal wastewater for microalgal cultivation have been done extensively. Different microalgal species have shown capability to recover wastewater nutrients and production of substantial biomass, which can be exploited for wider applications (Table 1.2).

The research studies on growing microalgae in municipal wastewater for nutrient removal process and biomass production are conducted extensively. Several strains of microalgae have been utilized, and have proven to possess the potential of removing nutrients from wastewater. However, nutrients removal efficiency and biomass production potential are prominently dependent on nature of strains, cultivation conditions, and type of wastewater. The retrieval of nitrate (NO_3-N) and PO_4-P from wastewater is of great significance in the view of tightened discharge regulations. In a study conducted by Ramsundar et al. (2017) evaluated the suitability of different wastewater streams within a domestic wastewater treatment plant for the cultivation *Chlorella sorokiniana*. Promising biomass production of 72.5 mg L^{-1} day^{-1} and 77.14 mg L^{-1} day^{-1} was achieved in *C. sorokiniana* with 89.13% and 94.29% of NH_4-N removal in influent and anaerobic centrate wastewater, respectively, under mixotrophic cultivation conditions. However, *C. sorokiniana* showed a lower NO_3-N removal rate of 19.23% and 28.67% in influent and anaerobic centrate, respectively. Higher COD (Chemical oxygen demand) removal was obtained in heterotrophic cultivation as compared to mixotrophic growth. In

Table 1.2 Nutrient removal and biomass production by different microalgal species in municipal wastewater

Algal strain	Type of wastewater	Removal efficiency (%)	Biomass yield (Y) and productivity (P)	References
Halochlorella rubescens	Primary and secondary municipal wastewater	TP-70 TN-99	6.3 g m^{-2} day^{-1} (P)	Shi et al. (2014)
Nannochloropsis gaditana	Municipal wastewater (MW)	NA	0% MW −0.92 g L^{-1} (P) 30% MW −1.87 g L^{-1} (P) 60% MW −1.44 g L^{-1} (P) 100% MW −0.94 g L^{-1} (P)	Onay (2018)
Chlorella zofingiensis	Municipal wastewater supplemented with pig biogas slurry	TN-93 TP-90	2.5 g L^{-1} (Y)	Zhou et al. (2018)
Dunaliella salina	Tertiary treated municipal wastewater	COD-52 NH_4-N-70.7 NO_3-N-88 PO_4-45.7	0.209 g L^{-1} day^{-1} (P)	Liu and Yildiz (2018)
Chlorella sorokiniana	Diluted municipal and industrial wastewater	COD-52.1 TN-57.5 TP-68.8	1.524 g L^{-1} day^{-1} (P)	De Francisci et al. (2018)
Chlorella sorokiniana	Diluted effluent mixed with piggery wastewater	COD-NA NH_4-N-100 NO_3-N-NA PO_4-P-40–60 DIC-46–56	1.0 g L^{-1}(Y)	Leite et al. (2019)
Wild-algae and *Scenedesmus*	Simulated municipal wastewater	COD-NA NH_4-N-NA NO_3-N-87 PO_4-P-19	0.278 g L^{-1} (Y)	Qu et al. (2019)
Scenedesmus sp. 336 and activated sludge symbiotic system	Synthetic municipal wastewater	COD-100 NH_4-N-87.13 NO_3-N-100	0.81 g L^{-1} (Y); 0.115 g L^{-1} day^{-1}	Chen et al. (2019)

(continued)

Table 1.2 (continued)

Algal strain	Type of wastewater	Removal efficiency (%)	Biomass yield (Y) and productivity (P)	References
		PO_4-P-99.82		
Microalgae (*Scenedesmus*) and bacteria (Sphingobacteria, Flavobacteria, and Proteobacteria) consortium	Municipal wastewater	COD-92 NH_4-N-NA TN-95.8 TP-98.1	1.8 g L^{-1} (Y)	Lee et al. (2016)
Chlorella sorokiniana, Nitrosomonas, and *Dechloromonas*	Synthetic municipal wastewater	COD-88 NH_4-N-NA NO_3-N-98 PO_4-P-96	2.5 g L^{-1} (Y)	Fan et al. (2020)
Chlorella vulgaris and activated sludge	Municipal wastewater	COD 55–64 TN-94–95% PO_4-P-NA	1.42 g L^{-1} (Y)	Leong et al. (2019)
Scenedesmus obliquus and wild yeast	Municipal	COD-95 NH_4-N-100 NO_3-N-100 PO_4-P-92.6	2.74 g L^{-1} (Y)	Walls et al. (2019)
Microalgae–bacteria consortia	Influent (pre-settled municipal wastewater)	COD-87 NH_4-N-99 NO_3-N-NA PO_4-P-16	NA	Foladori et al. (2018)
Microalgae–bacteria consortia	Municipal wastewater	COD-50 TN-36 PO_4-P-92 (high irradiance) COD-89 TN-60 PO_4-P-28 (low irradiance)	NA	Arcila and Buitrón (2017)

NA not available, *COD* chemical oxygen demand, *NH_4-N* nitrogen in the form of ammonium, *NO_3-N* nitrogen in the form of nitrate, *TN* total nitrogen, *PO_4-P* phosphorus in the form of phosphate, *TP* total phosphorus, *DIC* dissolved inorganic carbon

contrast, Li et al. (2011) conducted a study based on the evaluation of an adapted strain of *Chlorella* sp. to highly concentrated municipal wastewater for its efficiency for nutrient removal and biodiesel production. The results indicated the removal efficiency of 93.9%, 89.1%, 80.9%, and 90.8%, of NH_4-N, TN, TP, and COD correspondingly on 14 day of batch culture. Shi et al. (2014) fabricated a prototype TL-PBR (Twin-Layer photobioreactor) system with immobilized *Halochlorella rubescens*, microalga for the removal of nitrogen and phosphorus from primary and secondary municipal wastewater. The average growth of *H. rubescens* was reported to be 6.3 g m^{-2} day^{-1}. Phosphorus and nitrate concentrations in the effluents could efficiently be reduced by 70–99% after treatment. Nevertheless, under precise functioning conditions a pilot scale test should be implemented at wastewater treatment plants to assess the economical, ecological, and technical insinuations of this innovative technology for efficient nutrient removal from wastewater.

The strategies for diluting highly concentrated wastewater with weak wastewater have been proved promising. Reyimu and Özçimen (2017) supplemented wastewater with seawater for the cultivation of *Nannochloropsis oculata and Tetraselmis suecica* under similar culture conditions. *N. oculata* had the highest specific growth rate of 0.5430 day^{-1} (75% of wastewater) followed by *T. suecica* with growth rate of 0.4778 day^{-1} (25% of wastewater). Onay (2018) cultivated *N. gaditana* in various ratios of municipal wastewater for the production of bioethanol. Findings of their study showed that *N. gaditana* showed the highest biomass productivity in 30% wastewater. Furthermore, Zhou et al. (2018) studied the potential application of municipal wastewater mixed with pig biogas slurry as a supplement for the cultivation of *Chlorella zofingiensis.* Results revealed that pig biogas slurry (8%) in municipal wastewater had a substantial outcome on microalgal growth. *C. zofingiensis* obtained 2.5 g L^{-1} biomass yield and removed 93% and 90% of total nitrogen (TN) and total phosphorus (TP), respectively. Leite et al. (2019) blended municipal wastewater with piggery wastewater for the cultivation of *C. sorokiniana* in a UASB (Upflow anaerobic sludge blanket reactor) reactor. The UASB reactor showed >90% of organic matter removal with substantial biomass production of 1 g L^{-1}, with average removal of 40–60%, 100%, and 46–56%, of PO_4-P, NH_4-N, and dissolved inorganic carbon (DIC), respectively. Municipal and piggery wastewater combination utilized for *C. sorokiniana* cultivation presented tremendous potential to resolve the wastewater dilution problem from centralized wastewater (Leite et al. 2019). Their findings illustrated a pertinent NH_4-N removal via air stripping, reducing the N:P ratio during microalgal cultivation and, hence, improving the nutrient recycling efficiency and biomass production. Further studies on reducing the ammonia stripping and improving nutrient assimilation in microalgal biomass could be useful for improving the nutrient recovery process.

Municipal wastewater contains adequate amount of different nutrients required for algal growth; however, the limiting factors and undesirable components of wastewater should be taken into consideration. Low concentrations of nutrients content and excessive, and/or varying organic load in municipal wastewater are limiting factors that could affect microalgal growth. Raw wastewater with higher

concentrations of TSS (Total suspended solids) would hinder light penetration and scattering, which could have a negative influence on microalgal growth under phototrophic conditions. High concentration of heavy metals in municipal/urban wastewater and other emerging organic contaminants are the research areas that still need to be explored in-depth. Some metals can act as micronutrients for microalgal growth, while others are dispensable and can exert toxic impact at high concentrations (Renuka et al. 2014b). Nevertheless, the effect of heavy metals on microalgae can vary among different microalgae species and is extremely dependent on the type of compounds (Suresh Kumar et al. 2015). Microalgal species that possess high affinity for polyvalent metal ions can be exploited for the remediation of such metals from wastewater.

Furthermore, abundant indigenous pathogenic microorganisms and bacteria found in municipal wastewater may also affect the growth of microalgae primarily via cell meditated contact and/or secondarily through extracellular compounds (Kouzuma and Watanabe 2015). These indigenous microorganisms can compete with inoculated microalgae for essential macro- and micronutrients. Conversely, microalgae can pose detrimental effect on the microbial growth and activity due to an increase in pH, dissolved oxygen (DO), and/or excretion of growth inhibiting metabolites (Kouzuma and Watanabe 2015). Additionally, beneficial association between different microalgal and bacterial species in wastewater treatment processes has also been revealed by various studies (Bohutskyi et al. 2019; de Bashan et al. 2004; Ji et al. 2018; Leong et al. 2019). Although extensive investigations need to be done for identification of bacteria that possess symbiotic relationship with potential microalgal strains, and exploration of their potential role in such cultivation systems.

1.2.2 Agricultural and Industrial Wastewater

Agriculture, one of the main industries around the globe, generates a variety of wastewater rich in N and P. Agricultural wastewater is deliberated as a suitable nutrients source for microalgae cultivation because of similarity between microalgal nutrition requirements, and nutrients concentration and easy availability. Several microalgal strains have revealed the ability to utilize nutrients and grow in a range of agricultural wastewater (Abou-Shanab et al. 2013; Lowrey et al. 2015). Agricultural wastewaters also contain solid wastes such as animal compost, plant materials, etc. (Chiu et al. 2015). The nutrient composition of these wastewaters however purely depends upon the source of origin (Table 1.3). Wastewaters from animal husbandry industry are one of the most polluting sources of wastewater because of the high concentration of organics (Olguín 2012). However, location, age, productivity, animal diet, management, and usage have a great influence in the characteristics of animal's wastewater. The N:P ratio in swine, beef feedlot, and dairy wastewater is approximately 2–8 (Cai et al. 2013). Regardless of high nutrients content; proficient growth of microalgae on agricultural wastewater has been demonstrated in various studies (Table 1.3). Additionally, different studies have also revealed the capability of microalgae in eliminating nitrogen and phosphorus from agricultural wastewater.

Table 1.3 Microalgal biomass production and nutrient removal in different types of agro-industrial wastewater

Algal strain	Type of wastewater type	Removal efficiency (%)	Biomass yield (Y) or productivity (P)	References
Chlorella pyrenoidosa and *Rhodotorula glutinis*	Piggery wastewater	CO-85 NH_4-N-NA NO_3-N-83 PO_4-53	1.0 g L^{-1} (Y)	Li et al. (2019a)
Chlorella, Klebsiella and *Acinetobacter*	Dairy farm wastewater	COD-90 NH_4-N-NA NO_3-N-84.7 PO_4-P-NA	2.87 g L^{-1} (Y)	Makut et al. (2019)
Chlorella and *Scenedesmus*	Anaerobically digested piggery wastewater	COD-44 NH_4-N-98 NO_3-N-NA PO_4-P-NA	2.96 g L^{-1} (Y)	Raeisossadati et al. (2019)
Scenedesmus obliquus	Brewery effluent	COD-71 NH_4-N-NA NO_3-N-88 PO_4-P-30	NA	Ferreira et al. (2019)
Tribonema minus	Tofu whey industry wastewater	COD-86.7 NH_4-N-NA NO_3-N-92.8 PO_4-P-72	0.432 g L^{-1} day^{-1} (P)	Wang et al. (2018)
Ascochloris sp. (ADW007)	Raw-dairy wastewater	COD-95 NH_4-N-NA NO_3-N-78 PO_4-P-98	0.102–0.107 g L^{-1} day^{-1} (P)	Kumar et al. (2019)
Parachlorella kessleri	Agro-waste	COD 39 NH_4-N- NA NO_3-N->98 PO_4-P-59	0.062 g L^{-1} day^{-1} (P)	Koutra et al. (2018)

Chlorella	Sea-food processing wastewater	COD-NA NH_4-N-NA NO_3-N-94.5 PO_4-P-68.4	0.077 g L^{-1} day^{-1} (P)	Gao et al. (2018)
Scenedesmus obliquus	Secondary brewery effluent	COD-74 NH_4-N-NA NO_3-N-94 PO_4-P-NA	0.200 g L^{-1} day^{-1} (P)	Marchão et al. (2018)
Coelastrum sp.	Cattle farm wastewater	COD 42 NH_4-N-NA NO_3-N-80 PO_4-P-100	0.281 g L^{-1} day^{-1} (P)	Mousavi et al. (2018)
Chlorella vulgaris	Aquaculture and pulp wastewater	COD-75.5 NH_4-N-NA NO_3-N-76.5 PO_4-P-92.7	0.187 g L^{-1} day^{-1} (P)	Daneshvar et al. (2019)
Scenedesmus and *Trichoderma reesei*	Seafood processing wastewater	COD-74 TN-44 TP-93	2.17–6.64 g L^{-1} (Y)	Srinuanpan et al. (2018)
C. vulgaris and *Yarrowia lipolytica*	Yeast industry	COD-80 NO3-N-80	1.23–1.56 g L^{-1} (Y)	Qin et al. (2019)
Ankistrodesmus falcatus	Aquaculture wastewater	COD-NA NO_3-N-80.85 NH_4-N-86.45 PO_4-P-98.52	0.161 g L^{-1} day^{-1} (P)	Ansari et al. (2017)
Leptolyngbya and *Ochromonas*	Winery and raisin industry wastewater	COD-93 TN-78 TP-99	1.3 g L^{-1} (Y)	Tsolcha et al. (2018)

NA not available, *COD* chemical oxygen demand, *NH_4-N* nitrogen in the form of ammonium, *NO_3-N* nitrogen in the form of nitrate, *TN* total nitrogen, *PO_4-P* phosphorus in the form of phosphate, *TP* total phosphorus, *DIC* dissolved inorganic carbon

For instance, Hena et al. (2015) showed that a consortium of native microalgae was capable of eliminating >98% nutrients from dairy wastewater with 153.5 t ha^{-1} $year^{-1}$ and 16.9 % of biomass and lipid production, respectively. Microalgal biomass generation using wastewater from animal husbandry industry could form a sustainable biorefinery, since the generated biomass could be further utilized as a protein supplement in animal feed production. A study by Ansari et al. (2017) investigated the potential of aquaculture wastewater as a nutrient source supplemented with sodium nitrate for cultivation of *Scenedesmus obliquus*, *Chlorella sorokiniana,* and *Ankistrodesmus falcatus* and their nutrient removal efficiencies. The biomass acquired in their study demonstrated adequate protein, lipid, and carbohydrates productivities to be utilized as feed supplement. *A. falcatus* showed the highest biomass and lipid yields among the selected strains. Cultivation of microalgal strains in aquaculture wastewater showed high nutrients removal efficiencies for NO_3-N (>75%), PO_4-P (>98%), and NH_4-N (>86%). The composition of generated biomass was found to be suitable for its use as feed supplement. Whereas Zhu et al. (2017) examined the microalgal biomass and lipid production in livestock waste. The livestock waste compost medium provided optimal nutrients concentration for the cultivation of *Chlorella* sp., and obtained 288.84 mg L^{-1} day^{-1} and 104.89 mg L^{-1} day^{-1} of biomass and lipid productivities, respectively. Thus, these biorefinery concepts have great potential to benefit the agriculture sector via value-addition of wastewater-grown microalgal biomass.

Although agricultural wastewaters contain considerable amount of nutrients and indicate immense potential as a source of nutrients. The excessive nutrients content such as high ammonia concentration, turbidity, and suspended solids challenge their direct utilization for microalgae cultivation and nutrient recovery. The high concentration of nutrients in these wastewaters could negatively affect microalgal growth, therefore, the use of diluted wastewater was found to be a suitable strategy to achieve high nutrients recovery using microalgae strains (Pittman et al. 2011). Diluted piggery wastewater (COD of 1900 mg L^{-1}) was found optimal for the recovery of nutrients, and microalgal lipid and biodiesel production (Zhu et al. 2013). Although agricultural wastewater could serve as a suitable source of microalgal growth, there are some major challenges with regard to cultivation of microalgae in these wastewater. These include increased turbidity due to the occurrence of suspended solid that could affect light penetration during autotrophic microalgal cultivation, and imbalanced nutrients ratio, which could hinder microalgal growth (Chen et al. 2015; Zhao et al. 2014). For example, total phosphate (TP) and total nitrogen (TN) concentrations in piggery wastewater can differ from 185 to 3213 mg L^{-1} and 30–987 mg L^{-1}, respectively (Guldhe et al. 2017). The large fraction of the nutrients/carbon sources in agro-industrial wastewater is embedded into the insoluble organic compounds and therefore is not available to microalgae. Therefore, huge amount of freshwater resource is required to dilute the concentrated agro-industrial wastewater unless the use of wastewater with low nutrients, water recycling, and reuse is facilitated. The identification of high performing microalgal strains adapted to such adverse conditions in agro-industrial wastewaters still need more investigations and screening at larger scale.

1.3 Algae–Microbe Interaction in Wastewater and Its Significance

The use of algae–bacteria consortia is an emerging trend in algae-based wastewater treatment processes and biomass production (Rada-Ariza et al. 2017). Microalgae–bacteria consortia can be applied effectively for polishing wastewater, as co-cultivation of these microbes can support mutual growth and improve the nutrients recovery efficiency (Kang et al. 2018). A hybrid culture system of bacteria and microalgae is found to be more effective in nutrient removal and biomass production as compared to microalgae monocultures (Fig. 1.1). In an algae–bacteria consortia system, the activities of aerobic bacteria and photosynthetic microalgae promote the liberation of oxygen, and thus increase the capacity of microalgae to assimilate carbon compounds and promote the excretion of growth promoting substances such as vitamins and hormones by bacterial partners (Huo et al. 2020). Additionally, microalgae may serve as a habitat for bacteria, protecting them against adverse environmental conditions, and stimulating bacterial growth by releasing extracellular metabolites (i.e. extracellular polymeric material). The extracellular metabolites play a key role in the microalgae–bacteria relationship, allowing the bacterial cell to bind directly to microalgal cell surface. However, the consortia method should preserve its long-term cultivation structure and stability (Kang et al. 2018). Despite their low operational costs and environmental impacts, wastewater treatment using algae–bacteria consortium systems has rarely been deployed at full scale (Pizzera et al. 2019). Hard-to-control variables such as temperature variation, light availability, and fluctuating properties of wastewater remain obstacles to the scaling up of algae–bacteria consortium systems in practical aspects. In large-scale operations, photo-synthetically produced O_2 could be one of the main factors for algal–bacterial consortia influencing organic carbon removal and nitrification.

Significant research interest has been attracted by algal–bacterial consortia symbiotic systems to extract nutrients from wastewater. Recently, many studies have been carried out using consortial approach (Ji et al. 2018; Maza-Márquez et al. 2014). Study conducted by de Bashan et al. (2010) revealed that bacterium *Azospirillum brasilense* improved the growth of *Chlorella sorokiniana*. The removal efficiencies of NH_4-N and P were found 100% and 75%, respectively. Furthermore, a study conducted by Makut et al. (2019) reported that *Chlorella sorokiniana* DBWC2 and *Chlorella* sp. DBWC7 and two strains of bacteria, viz. *Acinetobacter calcoaceticus* ORWB3 and *Klebsiella pneumonia* ORWB1 could be a potential microalgae–bacteria consortium. Moreover, a significant improvement in microalgal biomass production and nutrient removal was observed in consortium grown in artificial and raw dairy wastewater as compared to microalgae monoculture. Average biomass titer, nitrate reduction, COD removal efficiency were found to be 2.84 g L^{-1}, 93.59%, 82.27% and 28.7 g L^{-1}, 84.69%, 90.49%, respectively. They concluded that the selected consortium of microalgae bacteria might be a potential platform for sustainable biomass production in wastewater (Makut et al. 2019). Liang et al. (2013) reported that co-culture of *Chlorella vulgaris* and *Bacillus licheniformis* was proved to be promising in efficient NH_4-N (86%) and TP (93%)

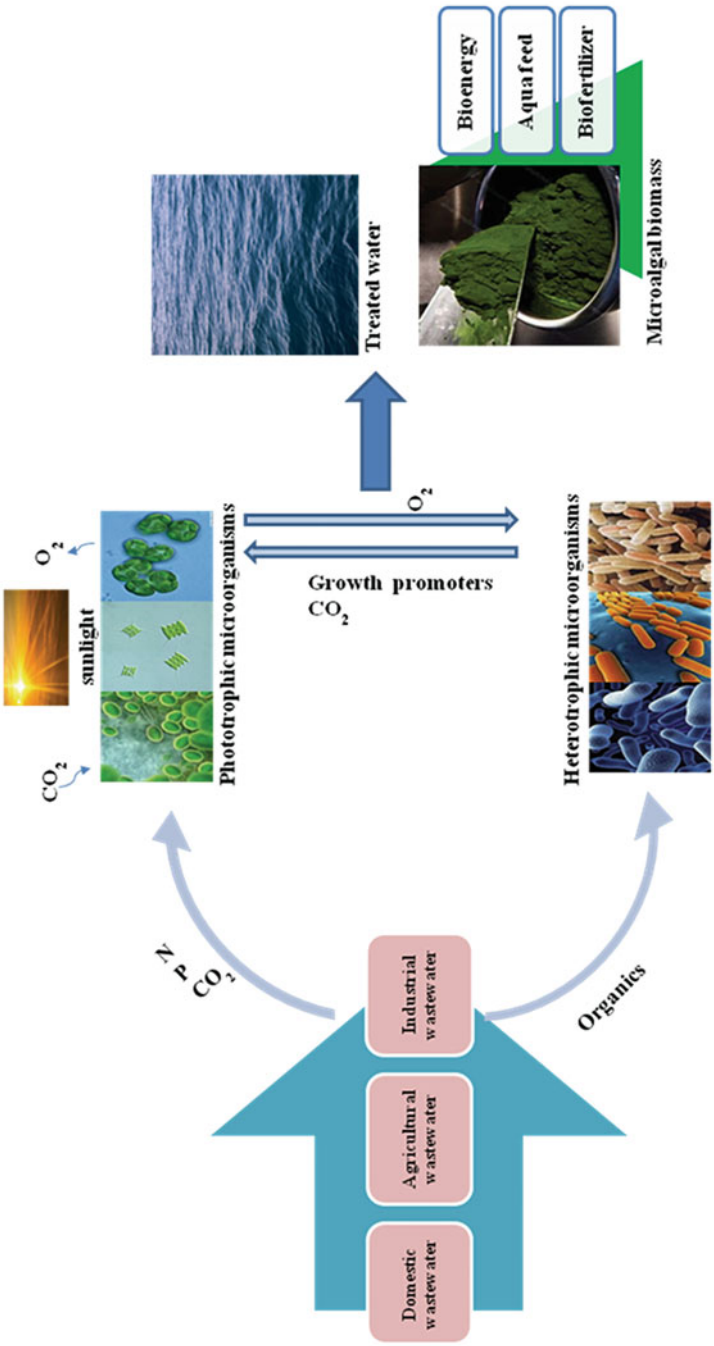

Fig. 1.1 Schematic representation of hybrid culture system for efficient nutrient removal using microalgae-based wastewater treatment process

removal from synthetic wastewater. In another study conducted by Lananan et al. (2014) a percent nutrient removal of 99.15% (PO_4-P) and biomass productivity of 0.534 mg L^{-1} day^{-1} were achieved by *Chlorella* sp. and a symbiotic microorganism EM-1 from aquaculture wastewater, in contrast to 49.73% PO_4-P removal and biomass productivity of 0.130 mg L^{-1} day^{-1} while using traditional *Chlorella* sp. Furthermore, when co-cultivated with selected bacterial strains, the concentration of *Scenedesmus obliquus* biomass was also increased by 3.5–24.8%. The co-cultivation method also increased the microalgal lipid content and contributed to improvements in both fatty acid and protein compositions (Ma et al. 2014). A consortium of *S. obliquus* and wild yeast cultivated in municipal wastewater resulted in 95%, 100%, 100%, and 92.6% reduction in COD, NH_4-N, NO_3-N, and PO_4-P, respectively (Walls et al. 2019). Nevertheless, low nutrient removal rates (60% NO_3-N, 53% NH_4-N, and 46% PO_4-P) and biomass yields (0.98 g L^{-1}) were attained under the contaminated conditions. Microalgae and yeast co-cultures also achieved high biomass concentrations of 3.7 and 4.2 g L^{-1} (Walls et al. 2019).

Light intensity has been found to be one of the vital factors for the production of extracellular polymeric substances, formation of microalgae-bacteria aggregates and their efficiency for nutrient removal. Thus, Arcila and Buitrón (2017) conducted a study on the effect of solar irradiance level on microalgae-bacteria aggregates growth in municipal wastewater. The findings of their study presented a deprived wastewater treatment performance associated with low removal rate of total COD (50%) and TN (36%) under high radiance. Conversely, the higher removal rate of PO_4-P (92%) was observed under these conditions. High removal efficiencies for total COD (89%), TN (60%), and PO_4-P (28%) were obtained under low level of irradiance.

At present, the area of use of microalgae–bacteria consortia in the wastewater treatment processes and biomass production is still at an early stage. The practical application of such consortia in wastewater treatment could be very promising due to the possibilities of wide variety of possible combinations of microalgae with indigenous microorganisms. However, compatible strains have to be identified at wider scale, which has synergistic effect on microalgal growth and nutrient recovery efficiency. Extensive studies are required to evaluate the long-term stability of such consortia.

1.4 Perspectives of Wastewater-Grown Microalgal Biomass

Microalgal biomass quests potential applications in the production of bioenergy, feed, biofertilizer, bioactive compounds, pigments, and synthesis of various compounds for the nutraceutical, cosmetics, and pharmaceutical industries (Elrayies 2018; Renuka et al. 2018). Production of microalgal biomass for various commercial applications using different wastewater has both environmental and economic benefits. However, more emphasis has mainly been given on the production of bioenergy from wastewater-grown algal biomass. Owing to concerns related to possible heavy metal and microbial contamination in wastewater, wastewater-

grown microalgal biomass remains untapped for other applications such as food, feed, and biofertilizer production, which needs comprehensive studies.

1.4.1 Bioenergy

Bioenergy production in the form of biodiesel, biocrude, biomethane, biochar, etc. from microalgal biomass feedstock has attracted strong interest among researchers over the past decade (Roles et al. 2020). Successful usage of microalgae biofuels in transportation sector and power generation has the potential to improve energy security and may help to minimize serious environmental concerns related to the use of fossil fuels. Extensive studies on the production of biofuels from wastewater-grown biomass are available. Among different biofuels, biodiesel production combined with wastewater treatment is proved as a promising approach to reduce the cost of production. The combination of wastewater remediation with processing of algal biomass is potentially one of the most economically and environmentally safe ways of producing bioenergy and bio-products. Research studies on the use of wastewater to produce microalgal biomass for biodiesel production have revealed that wastewater provides cheap source of nutrients and could increase the accumulation of lipids under natural stress conditions of wastewater; however, it can vary depending upon the cultivation conditions (Elshobary et al. 2019; Marella et al. 2019). Marella et al. (2019) reported that lipid productivities in wastewater-grown microalgal biomass varied seasonally. Highest lipid productivity of 9.2 g m^{-2} day^{-1} was observed in summer season along with superior biodiesel quality with high cetane number. Lipids C18:3 in high amounts are not favorable since they have an adverse effect on the biodiesel oxidative stability, therefore, the maximum limit of 12% for C18:3 has been set by the European standards for biodiesel. Soydemir et al. (2016) utilized secondarily treated domestic wastewater effluent for the cultivation of mixed cultures for biodiesel production, which gave 26.2% lipid yield. The fatty acid profile analysis showed that FAME (fatty acid methyl ester) composition mainly consisted of stearic, oleic, palmitic, linoleic, palmitoleic, and linoleic acid methyl esters that had FAME composition and biodiesel conversion efficiency comparable to pure cultures and monoculture grown in a synthetic culture media. Moreover, Mata et al. (2013) analyzed the possibility of cultivating *Scenedesmus obliquus* in brewery wastewater as a prospective candidate for biodiesel production. In their study, the results revealed an average lipid content of 27% of dry-weight biomass and an average biomass and lipid yield of 0.90 and 0.24 g L^{-1}, respectively. They concluded that oil extracted from *S. obliquus*, containing 56.4% of saturated esters (C16:0) and less than 12 % of the linolenate unsaturated ester (C18:3), could be used to produce biodiesel. In another study Guldhe et al. (2019) reported that the fatty acid profile and biodiesel properties of wastewater-grown microalgal biomass were comparable to the microalgal biomass grown in standard culture medium.

Microalgal carbohydrates are also an important feedstock for variety of commercial products. The processing and recovery of carbohydrates are dependent upon the carbohydrates solubility. Martin-Juarez et al. (2019) illustrated the feasibility of

microalgae biomass grown in domestic, piggery, and synthetic medium for the production of fermentable monosaccharides. They revealed that bacterial content in the wastewater-grown microalgal biomass did not exert adverse effect on carbohydrates solubilization, however improved the degradation of carbohydrates in piggery and domestic wastewater-grown biomass as compared to the synthetic medium. This study revealed efficacy of wastewater-grown biomass for the production of biofuels through fermentation process.

Thermochemical conversion is one of the important methods for biomass up-gradation for bioenergy production, which include different processes such as gasification, pyrolysis, and direct liquefaction (Chiaramonti et al. 2015; Jena et al. 2011). Biomethane is an important form of bioenergy, which could be produced through the anaerobic digestion of wastewater-grown microalgal biomass. An algae–bacteria consortium was reported to contain 34–38% of protein and 18–28% of lipids in biomass grown in wastewater in an open raceway pond (Bohutskyi et al. 2018). The methane production through the anaerobic digestion of the biomass varied significantly (by 30%) with the highest value of 0.34 L g VS^{-1} depending upon the lipid content. Net Energy Ratios and Net Energy Efficiency ranged from 1.6–2.2 and 60–70%, respectively, for large-scale anaerobic digestion process, and an optimal hydraulic retention time of 20–30 days (Bohutskyi et al. 2018). However, Bohutskyi et al. (2019) demonstrated that the biomethane production from lipid extracted wastewater-grown microalgal biomass though anaerobic digestion can be improved through the hydrothermal-pretreatment and co-digestion with sewage sludge, which can lead to 30-50% increase in methane yield and approximately four folds increase in the energy output. A study by Shahid et al. (2019) demonstrated the potential of *Chlorella* sp. and *Bracteacoccus* sp. for the treatment of urban wastewater and biomass production. The wastewater-grown biomass showed significant potential for energy production through pyrolysis process with 159–190 kJ mol^{-1} of Gibbs free energy and 43–81 J mol^{-1} of entropy values.

Among different technologies of algal biomass conversion, hydrothermal liquefaction (HTL) process has been found to be energy efficient method, since it minimizes the dewatering requirements and have higher conversion rates as compared to other technologies. HTL process has been reported to contain biocrude yield of approximately 60% with HHV (high heating value) of 36–40 MJ kg^{-1} (Cheng et al. 2018). Recent studies on the biocrude formation from wastewater-grown microalgal biomass through the HTL process have revealed the significant biocrude conversion efficiency (Arun et al. 2018; Cheng et al. 2019). Cheng et al. (2019) demonstrated the potential of two microalgal strains (*Galdieria sulphuraria* algae, 5587.1 and SOOS) grown in municipal wastewater. However, the biocrude oil yields were lower in the municipal wastewater as compared to biomass grown in synthetic medium, which showed the need of further studies on the optimization of HTL conditions for enhancing the production of biocrude. Recently, Naaz et al. (2019) compared the bioenergy production from wastewater-grown microalgal biomass (*Chlorella pyrenoidosa* and *Phormidium* grown in municipal wastewater) through anaerobic digestion and HTL process. HTL process showed 43% conversion into biocrude with net energy value of 0.08, which was significantly higher than

anaerobic digestion process (0.007). HTL process has been found an energy efficient process for bioenergy production; however, research in this direction is in early stage, which needs further optimization and life cycle assessment studies.

1.4.2 Biofertilizer

Microalgal bioinoculants and/or fertilizers have been reported to improve the quantity and quality of agricultural crops (Renuka et al. 2018). The usage of biofertilizer reduces the requirements of synthetic N fertilizers to agricultural lands, therefore assist in the mitigation of harmful effects of chemical fertilizers, and increasing the yield of agronomic crops. N, P, and potassium (K) are the most important nutrients needed for crop nutrition. Microalgae cultivation coupled with the removal of excess nutrients in wastewater could improve the economic viability of algae-based bioinoculants. Microalgae have the potential to assimilate wastewater nutrients into their biomass, such generated biomass can be utilized as biofertilizer/organic fertilizer for agronomic crops. Wuang et al. (2016) examined the technical viability of growing *Spirulina platensis* using aquaculture wastewater for algae fertilizer production. The use of algae-based fertilizer showed higher amounts of iron (Fe), zinc (Zn), calcium (Ca), and magnesium (Mg), however, low amount of NPK in comparison with chemical fertilizer. They reported that wastewater-grown *S. platensis* biomass exhibited the ability to improve the growth of leafy plants (*Amaranthus gangeticus*, *Brassica rapa* ssp. *Chinensis*, *Eruca sativa*) and enhance the seed germination of Kai Lan (*Brassica oleracea alboglabra*) and the Chinese Cabbage (*B. rapa* ssp. *chinensis*). In another study Renuka et al. (2016) demonstrated a significant improvement in the productivity and yield of wheat crop with the inoculation of two wastewater-grown microalgal consortia of filamentous and unicellular strain as compared to recommended synthetic fertilizer. A significant increase in the N, P, and K contents of shoots, grains, and roots was also obtained with wastewater-grown microalgal bioinoculant. Another approach is the use of residual nutrient rich microalgal biomass (after the extraction of compounds and digested biomass) as organic fertilizer. The use of residual biomass has been reported to act as soil conditioner (Doğan-Subaşı and Demirer 2016). These studies revealed the efficiency of such wastewater-grown microalgae as bioinoculant and/or fertilizer for enhanced plant growth, yield, and soil fertility. This can also assist in integrated nutrient management practices of agronomic practices. The presence of toxic metals and pathogenic microorganisms in different types of wastewater could have detrimental effect on the quality of microalgal biofertilizers. Moreover, there are limited studies on assessing the effect of wastewater-grown algal biomass as biofertilizer in agronomic crops and its ecological implications. Most of studies are conducted either at lab-scale or under controlled conditions. Therefore, field level investigations on wastewater-grown biomass as bioinoculant are required to prove their practical and economical feasibility.

1.4.3 Food and Feed

Microalgae find potential applications in human food supplements and animal feed, since they are a rich bioresource of pigments, proteins, and essential fatty acids (Yarnold et al. 2019; Apandi et al. 2019). Polyunsaturated fatty acids (PUFA), such as docosahexaenoic acid (DHA) and eicosapentanoic acid (EPA) are naturally produced by microalgae, could boost immune system, and also used in treating inflammatory and heart diseases (Sims et al. 2019). While in aquaculture, these organisms can be used as live feed and/or feed supplement. Microalgae also assist in boosting the immune system and improving overall health. The protein and fatty acid content in many microalgal species have been found to be substantial and comparable to the conventional sources of fish feed (Sims et al. 2019). Microalgae-based aquaculture feed has been found to increase organism health, productivity, and yield. Therefore, there is an increased interest for the production of microalgae-based aquaculture feed in the recent times. However, studies on the evaluation of wastewater-grown microalgal biomass as human food supplement and aquaculture (fish) feed are very limited (Ahmad Ansari et al. 2020; Apandi et al. 2019). There are few studies on the production of microalgal biomass in aquaculture wastewater, which could act as a basis for setting up microalgae-aquaculture wastewater based biorefinery for aqua feed production (Apandi et al. 2019; Tossavainen et al. 2019). Tossavainen et al. (2019) studied the growth, fatty acid (FA) profile, tocopherol content, and nutrient recycling in a consortium of *Euglena gracilis* and *Selenastrum* cultivated in aquaculture wastewater from catfish (*Clarias anguillaris*) and pikeperch (*Sander lucioperca*). They revealed that the highest biomass yield of (1.5 g L^{-1}) with substantial EPA and DHA content, and efficient nutrient removal were achieved in sludge amended aquaculture wastewater. However, the tocopherol and arachidonic acid content were higher than the required standard for the fish feed. Tocopherol at high amount may result in poor growth, toxic liver reaction, and death. Therefore, in their study microalgae proved to be a promising substitute for eradication of nutrients from wastewater while offering an alternative for fish oil. While Ahmad Ansari et al. (2020) recently investigated the use of *Scenedesmus obliquus* whole and lipid extracted biomass for Nile tilapia growth. In their study, the economic feasibility of algal assisted aquaculture wastewater involving the direct utilization of algal biomass for fish production and use of algae to produce biodiesel trailed by the usage of LEA (Lipid extracted algal biomass) in fish feed. Nile tilapia was able to ingest and use both LEA and whole algae biomass, the substitution of 7.5% LEA in fish feed showed the highest fish growth. However, there are limited studies on the evaluation of wastewater-grown microalgal biomass as aqua feed. Therefore, extensive studies are required to assess the potential of wastewater-grown biomass as feed on model organisms. The challenges related to the microbial loading and heavy metal contamination in wastewater-grown biomass need to be addressed, which needs field scale life cycle assessment studies.

1.5 Future Outlook and Conclusions

Extensive literature on the nutrient recovery by different microalgal species has demonstrated beyond doubt that these organisms can be effectively applied for the recycling of nutrients from wastewater along with the production of valuable biomass. However, more studies are required on-site at larger scale to study the practical and economical feasibility of process at the wastewater treatment plants. The effect of increasing concentrations of heavy metals and emerging organic and inorganic contaminants needs to be explored broadly. Recently, microalgae–bacteria consortia have shown tremendous potential for improved nutrient removal efficiency and biomass production in wastewater. However, comprehensive studies are required to assess their stability, biochemical and biological properties, to establish their potential for feed, biofertilizer, and bioenergy production. Wastewater-grown microalgal biomass has been most commonly assessed for its applications in bioenergy production. However, the areas such as aquaculture feed and biofertilizer production need in-depth studies and field scale trials. Challenges related to heavy metal contamination and microbial loading still need to be addressed, and require extensive studies on model organisms for their applications in agriculture sector.

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Constructed Wetland and Microalgae: A Revolutionary Approach of Bioremediation and Sustainable Energy Production

2

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Abstract

The advent of industrial revolution brings the problem of land, wastewater, and biodiversity. Constructed wetland, the mega reservoir of biodiversity are shrinking due to encroachment in search of food and shelter which indirectly enhances the requirement of energy, creating a panic situation of biodiversity as well as civilization lost in coming decade. Therefore, researcher, policymaker, thinker, and scientist keenly focused towards managing the demand of food, energy, shelter, fresh water in cost effective and sustainable manner to protect the world. Applying the duel functionality of microalgae in waste reduction and energy production in CW not only maintain the biodiversity but also provides all the basic services of human. In this view, present chapter discusses the suitability of CW in bioremediation and energy production in sustainable and cost effective manner.

Keywords

Microalgae · Constructed wetland · Energy · Sustainability · Bioremediation

2.1 Introduction

Energy is the primary requirement for the survival of human being on the earth. This energy demand is achieved by fast dwindling finite reserve of fossil fuels (Gasparatos et al. 2017). Finite reserves are exhausted at a faster rate due to increasing human population. Besides, increasing population load brings water pollution. Water present on the earth is mainly contaminated by anthropogenic induced activity like generation of sewage, contributed >70% of water pollution. Thus, rising world's energy demand and its sustainable fulfillment are two key issues for the increasing population. At the end of 2050, the population projected to grow to approx 10 billion, resulting into high demand of resource, which need more fuel, food, and water by ~50%, 70%, and 50%, respectively (Godfray et al. 2010). The algae driven solar biotechnology could be a potential alternative to tackle to situation from to be more catastrophe.

Over the years, microalgae act as a bridge in the production of bioenergy and phytoremediation (Singh et al. 2019; Stockenreiter et al. 2016). Algae are the dominant photosynthetic organism on the earth planet. Various group of microalgae have been applied for the treatment of variety of wastes including human waste, sewage, livestock wastes, industrial and agro-industrial waste (Shelef et al. 1980; Ibraheem 1998; Mandotra et al. 2020).

Constructed wetlands (CWs) are green clean technology employing different aquatic plants and microbes for successful remediation of waste. In CW, macrophytes play a central role degrades, detoxify and accumulate the pollutants thorough all the physical, chemical, and biological processes (Upadhyay et al. 2016). Macrophytes richness decides the functioning of the wetland. Wetland provides a variety of ecosystem services including, feed, shelter, pollution treatment and

recreational opportunity, etc. (Upadhyay et al. 2017). The ecosystem services of wetland largely depend on the how plant diversity influences the biomass and nutrient retention (Engelhardt and Ritchie 2001).

2.2 Constructed Wetland

Constructed wetland (CW) is a green, engineered ecofriendly, and artificial ecosystem technology of waste reduction and resource rehabilitation. The idea of CW initially was focused on the removal of waste which has now been extended to different services such as habitat reorientation, waste reuses, food production, and recreational opportunities. The benefited aspect of CW to human societies depends on the macrophytes richness, biomass production, and nutrient recycling as species richness might enhance the wetland functioning. In CW different emergent, submerged, rooted, and floating plants were employed to produce better output (Upadhyay et al. 2016). The fundamental criteria for selection of plants in wetlands are plant used in the CW should have the following uniqueness (Kadlec et al. 2000):

- Plant should be indigenous as they are easy to handle and culture
- Plant should be perennial
- Plant should have profuse root system
- The most important is high biomass and fast growth rate and able to stand in adverse condition
- Plants have high translocation factor

The vegetation present in wetland should not be intense. Dense vegetation can inhibit water circulation. Intense vegetation allows water to retain for longer period of time and thus performance of CW. Densely grown wetland plants slow down the water velocity, to allow water to flow longer course, frictional resistance to water flow to enhance sedimentation. However, high vegetation in wetland reduces sediments resuspension in CW through wind.

2.2.1 Role of Plants in CW

Plant present in CW provides a suitable habitat for the different types of microorganism present in wetland. The extensive root systems of plants absorb large amounts of nutrients, accumulate, and sequester toxic element in their roots and aboveground part. In the root system, rhizospheric area of plants secretes various substance and chelating molecules which degrade the contaminants and chelate metals and absorbed through the root via different transporter present in the roots (Salt et al. 1995). Following are some important functions of plants given:

1. Provide oxygen to sediments.

2. Submerged portion of the plants supports for biofilms formation, facilitates the organic flocculation, nutrient transformations, pollutants filtration, and sedimentation.
3. The emergent parts protect the plants from the wind and shading and assist in decreasing water temperature and algal growth in constructed wetland.
4. Offer varied habitats for different aquatic plants and animal and thus assist in biodiversity.

2.2.2 Designing of Constructed Wetland

Prior to design CW, it should be acknowledged the type of wastewater that will be treated in wetland. The standard for wetland design includes selection of site, water hydrology analysis, source and quality of water and wastewater, plant material, soil, and geology. Some of the aspects that are important during designing or construction of wetland include retention time, hydraulic loading rate, organic loading rate, wetland shape, water depth, and aspect ratio. A characteristic range of different criteria is presented below (Crites 1994; Hammer 1989; Kadlec et al. 2000; Watson and Hobson 1989) (Table 2.1).

2.2.3 Treatment and Operation

In CW wastewater treatment occurs through the movement of wastewater in wetland medium and the plant rhizosphere. Plants grown in wetlands have profuse root branching with hollow stem which facilitate the high trapping of oxygen from the atmosphere and subsequently its transfer in the rhizospheric area through hollow stem system. In this way a thin biofilm of aerobic and anaerobic condition develops. Development of redox environment inside the rhizospheric zone assists in the production of aerobic and anaerobic bacteria around the rhizospheric area. Thus, thin aerobic films are formed covering the root hair, creating a zone of aerobic. This

Table 2.1 Designing characteristics in the development of constructed wetland

Range of design criteria	
Factor	Range
Retention time (for dissolved pollutants), day	5–14
Retention time (for suspended pollutants), day	0.5–3.0
Maximum BOD_5 loading rate (kg ha^{-1} day^{-1})	80–112
HLR (hydraulic loading rate) (m day^{-1})	0.01–0.05
Area requirement (ha m^{-3} day^{-1})	0.002–0.014
Aspect ratio	2:1–2:10
Water depth—average condition (m)	0.1–0.5
Bottom slope (%)	0.5

The basic designing criteria for both surface flow and subsurface flow CW are alike but differ by the macrophytes types, hydraulic rate, and direction of flow

aerobic zone is formed due to the leakage of oxygen from the roots, rhizomes, and rootlets. Organic matter present in wetlands is decomposed via the aerobic and anaerobic micro-organisms present around the root surface. The reduction of nitrogen from waste is important because of nitrogen derived production of ammonia and other substances which harm the fishes and aquatic microbes if discharged into water bodies. The organically bind nitrogen is released through the process of mineralization and releases as inorganic nitrogen as nitrates, nitrites, ammonia, and ammonium, which further accumulated, absorbed, and assimilated by plants, fungi, and bacteria (Boyd 2020; Vymazal 2007). Wastewater also contains phosphorus both organic and inorganic forms. Phosphorus present in wetland is coprecipitated by iron, aluminum, and calcium compounds present in the root-bed medium (Vohla et al. 2011). The removal and storage of phosphorus can only occur within the CW by the chelation of phosphorus with organic matter, incorporation into living biomass, and precipitation of iP via Fe, Ca, and Al present in wetland (Vymazal et al. 1998; Upadhyay et al. 2016). Macrophytes in wetland systems also act as temporary nutrient storage space uptake nutrients during the growth period and releasing at senescence (Rai et al. 2013; Upadhyay et al. 2019). Suspended solids are filtered by self-settling in the surface flow wetlands or mechanical filtered in subsurface flow wetland cells.

2.3 Algae and Bioremediation

Microalgae are fast growing, photosynthetic microphytes, and potential resource of bioremediation and lipid mediated biofuel production. Algae intensive remediation, i.e., phytoremediation employs for the reduction and biotransformation of different pollutants present in wastewater (Olguín 2003; Bag et al. 2019; Upadhyay et al. 2020). High degree of adaptation to varied environment, fast growth rate, high surface area, and tolerance to extreme stressor makes algae an ideal candidate for the removal of pollutants. In 1957, first successful implementation of algae was done in waste stabilization pond by Palmer (1974) using different group of microalgae including *Chlamydomonas, Oscillatoria, Micractinium, Chlorella, Ankistrodesmus, Scenedesmus, Euglena,* etc. Pham et al. (2014) use thirty algal species belonging to phyla (Chlorophyta, Cyanobacteria, Chrysophyta, Euglenophyta, and Cryptophyta) for bioremediation and treatment processes. Following are the characteristics which need to address prior choosing species for bioremediation.

1. High growth rates (corresponds to high bioremediation capability) (Upadhyay et al. 2019).
2. Ability to grow across a wide range of conditions (de Paula Silva et al. 2012).
3. Species should be indigenous (Matthews and Endress 2008).

In wetland, large populations of cyanobacteria and algae are present and reduce the pollution level by absorption, adsorption, degradation, and uptake. Algae are

important in wetland as they provide the foundation of widespread food web established in wetland community.

Although nitrogen and phosphorous are fundamental macronutrients for algal growth, but yet maybe a serious pollutants if present in high concentration. Algae can flourish in nitrogen and phosphorus rich environments common to many wastewaters (Pittman et al. 2011) and not only remove, but also detain nutrients as agricultural fertilizer (Patel et al. 2012; Neori et al. 2004). In addition, algae have capacity to reduce metal load from the waste as reported by various authors (Megharaj et al. 2003; Shamsuddoha and Ali 2006; Bursali et al. 2009; Al-Homaidan et al. 2011). Microalgae remove heavy metals from polluted water through the process of metabolism dependent accumulation and biosorption which is a non-active adsorption process (Matagi et al. 1998).

The most common systems for treatment of wastewater are High Rate Algal Ponds (HRAP) (Oswald 1988) and the patented Algal Turf Scrubber (ATS) (Craggs et al. 1996). Recently several species of microalgae have been identified as frequently used in reduction of heavy metals like algae *Phormidium* (Ni, Cu, Cd, Zn, and Pb), *Caulerpa racemosa* (Boran), *Nannochloropsis* sp., *Chlorella vulgaris,* etc. (Wang et al. 1998; Bursali et al. 2009; Upadhyay et al. 2016; Rai et al. 2013). The high metal accumulation ability in microalgae is due to presence of different negatively charged functional groups like phosphoryl (−PO), hydroxyl (−OH), amino (−NH), sulfhydryl (−SH), and carboxyl (−COOH) on its cell wall. Since, metals present in water are cation, adsorbed onto the cell surface and thus enhanced the accumulation (Skowronski and Ska 2000).

2.4 Strategies of Bioremediation

2.4.1 Phytoextraction: (Phytoaccumulation, Phytoabsorption, or Phytosequestration)

In phytoextraction process, plants and algae accumulate contaminants in the roots and shoot system (Ali et al. 2013; Yoon et al. 2006; Rafati et al. 2011). After a certain time period plants are extracted from the CW and thus reduces the pollution load. However, in this technology, utilization of phytoremediated biomass is major issue which can either disposed or dumped into the ground.

2.4.2 Phytodegradation

It is the process of degradation of organic pollutants present in waste with the help of plants. This process does not require rhizospheric microbes (Vishnoi and Srivastava 2007). Plants and algae can accumulate the organic compounds from contaminated environments and detoxify through metabolic activities.

2.4.3 Phytostabilization

Phytostabilization is a technique in which plants trim down the mobility and relocation of contaminant and stabilize them by binding with algae (Singh 2012). Leachable constituents are adsorbed and bound into root surface, forming a stable mass and thus make the contaminant immovable.

2.4.4 Rhizofiltration

In the process of rhizofiltration, as the term suggests the contaminant gets filtered from the waste with the help of the root. Plant roots uptake the contaminant by the absorption and sequestered it into vacuole thereby reducing the pollution level. Rhizofiltration is used to decrease pollution in estuary areas and natural wetlands.

2.4.5 Phytovolatilization

Phytovolatilization refers to uptake of volatile pollutants like metals from soil and water by plants and algae. The plants and algae uptake the volatile contaminants and successively release them into the atmosphere. In this approach only a limited amount of pollutant can be removed from the contaminated sites. A general mechanism concerned in remediation of pollutants in CW is summarized in Table 2.2.

Table 2.2 General mechanism involved in the remediation of different pollutants in CW

S. no.	Parameters	Mechanism of removal
1	Suspended solids	Filtration and sedimentation Flocculation
2	Biological oxygen demand	Microbial degradation Organic matter deposition at the bottom
3	Nitrogen	Nitrification and denitrification Ammonia volatilization Anammox process Cannon process Plant uptake
4	Phosphorus	Sorption Plant uptake Biological mineralization
5	Pathogen	Sedimentation/filtration UV radiation Natural die off
6.	Metals	Uptake and sequestration Adsorption Transformation and volatilization

2.5 Algae as Source of Renewable Energy

Transition towards renewable energy has become the global priority due to dwindling fossil fuel reserves, fast industrial insurgency, and climate change. The aquatic ecosystems are recipient of all kinds of pollutants (inorganic and organic) and experience degradation due to the unabated anthropogenic processes. Besides, the chemicals used in different industries contain organic pollutants such as azo dyes, phenolic compounds, pesticides, surface-active compounds, sulfonated oils, etc. have genotoxic and cytotoxic nature which are not fully degraded through the secondary CETP process and released without any treatment. Thus, continuous releases of these pollutants will develop a disastrous problem in the environment.

In the changing climatic condition, algae have been identified as a sustainable feedstock for renewable energy and waste treatment. Currently, research is keenly focused on the development of genetically modified algal strain, one of the best alternatives of non-renewable energy and waste remediator with capacity to generate high yield of combustible lipid under different environmental conditions and wastewater remediation. In the changing climatic condition, algae have been identified as a sustainable feedstock for renewable energy and waste treatment (Abomohra et al. 2016). High growth rate potential to produce energy rich compounds and ability to rapidly strain improving capacity in harsh environment make algae an exciting organism for sustainable green energy alternatives without competing the arable land (Jambo et al. 2016; Mandotra et al. 2019). In addition, degradation of waste with the help of specific/modified algae offers remediation of particular pollutants present in wastewater. The dual use of naturally occurring algae in waste remediation and green energy generation is still in infancy and restricted to some group of the algae and genetic engineering.

2.6 Extraction and Production of Lipid by Algae

The extraction of lipid from microalgae is basically achieved by mechanical and chemical method. The chemical method includes accelerated solvent extraction, supercritical fluid extraction, Soxhlet extraction. The mechanical methods include microwave assisted extraction, oil expeller, ultrasonic assisted extraction. Some of them are mentioned below.

2.6.1 Folch Extraction Method

In the Folch extraction methods, lyophilized algae biomass is homogenized in the chloroform/methanol mixture (2:1 v/v) (Folch et al. 1957) to obtain endogenous algal lipid. In the extraction process homogenized samples are further agitate at room temperature for 20 min followed by centrifugation. The supernatant is used for the next step by washing with 0.9% NaCl solution. After the washing, the samples were again vertexed for few minutes and thus two layers are formed. The lower layer

contains the desired content of lipid in chloroform which is further evaporated by rotary evaporator to get the desired results.

2.6.2 Bligh and Dyer Method

Bligh and Dyer method of lipid extraction is one of the widely used strategies (Bligh and Dyer 1959). This method is very much similar to Folch extraction methods, but the ratio of solvent/biomass and solvent/ solvent is different.

Recently the modification of the above two methods was done by Matyash et al. (2008), which are the most reliable approach of lipid extraction. In this method of lipid extraction almost all the classes of lipid are extracted from the algae. In this method the solvent Methyl-*tert*-butyl ether (MTBE) is used for extraction and homogenization purposes. Levine et al. (2010) also demonstrated the in situ transesterification and in situ lipid hydrolysis approach for the extraction of lipid.

2.6.3 Microwave Assisted Extraction

Microwave assisted extraction was first established in mid-1980 for the extraction of lipid and pesticides from different sources like seed, food, feed, etc. (Ganzler et al. 1986). However, this technology is not safe for the extraction of lipid in algae due to high cost, unsafe, and time taking.

2.6.4 Ultrasound Assisted Extraction

Ultrasound assisted extraction (UAE) is advantageous as compared to conventional mechanical approaches due to low costs, fast, and high percentage purity of the end product. In UAE ultrasonic waves are used. In UAE cells are ruptured by means of cavitation producing microbubbles around the cell. The eventual crumple of these bubbles releases a shockwave and breaks cell wall, hence discharge the intracellular contents (Mubarak et al. 2015).

In microalgae lipid is present in the range from 20 to 50% of cell dry weight which may altered on account of different environmental conditions. Some algal species have high potential to accumulate significant amount of lipid, such algae are known as oleaginous species. Algae of the genera *Dunaliella, Nannochloropsis, Chlorella, Schizochytrium, Scenedesmus,* and *Phaeodactylum* are known to contain 20–50% DCW of lipid (Bellou et al. 2014). The lipid production in microalgae may be enhanced through the supplementation of modified culture medium and other abiotic stresses (Mandotra et al. 2014, 2016; Singh et al. 2018). Generally, lipid contains glycerol and unsaturated fatty acids with carbon chain length $>C_{12}$. Fatty acids with chain length of C_{14}–C_{20} are used in the production of biodiesel (Kumar et al. 2016). Chain length >20 is polyunsaturated fatty acids such as EPA and DHA, etc.

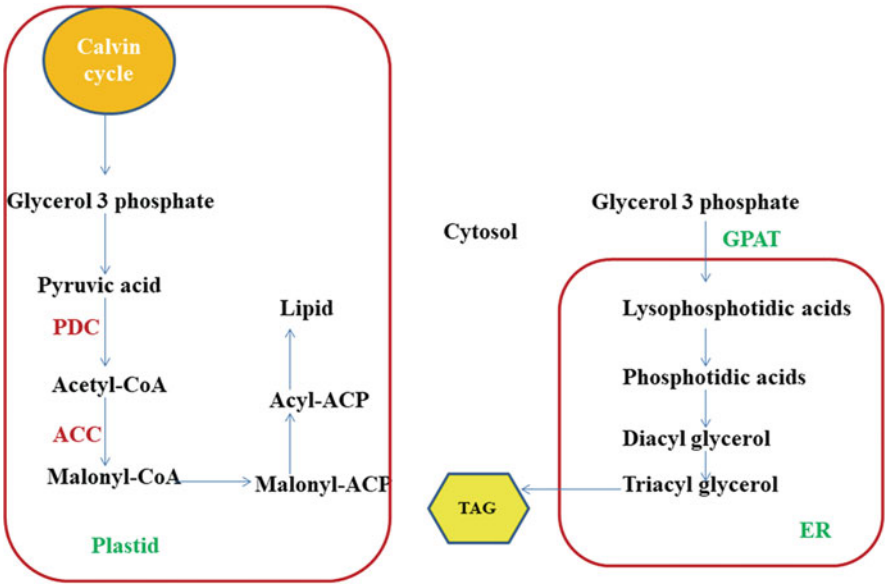

Fig. 2.1 A generalized view of lipid biosynthesis in the cells. Abbreviations: *ACC* acetyl-CoA carboxylase, *ACP* acyl-carrier protein, *PDC* pyruvate dehydrogenase complex, *TAG* triacylglycerol

Glycerol 3-phosphate is the precursor of lipid in the algae. The synthesis of glycerol 3-phosphate in microalgae takes place by reductive assimilation of CO_2 through the process of photosynthesis. Glycerol 3-phosphate converted to acetyl-CoA through sequential conversion catalyzed by pyruvate dehydrogenase complex. The acetyl-CoA formed further initiates the lipid biosynthetic pathway (Bellou and Aggelis 2013; Lei et al. 2012). Acetyl-CoA with the help of enzyme ACC (acetyl-CoA carboxylase) converted into malonyl-CoA and acyl–acyl carrier protein (ACP) which ultimately converted in structure lipid (Baba and Shiraiwa 2013) (Fig. 2.1).

2.7 Potential Aspects and Future Prospects of Algae and Constructed Wetland

Overcrowding of earth inhabitants causes rapid shrinkage of wetlands and increased pollution load. Wetlands as a natural purifier and house of millions of animals and plants and microbes, scientist keenly focused on the rehabilitation of degenerated wetland to save the life of earth. As the wetland provides the basic ecosystem services to human being, its conservation and expansion are urgent issue. In addition, widening the area of wetland could reduce the water pollution in cost effective and sustainable manner. The combined idea of wetland and algae might be very useful for the scientist with duel benefits of pollution remediation as well as green energy production. Growing algae in wetland not only reduces the pollution load but

also generates renewable biofuel for the sustainable world. Various studies have been reported that enhanced biomass and lipid contents in algae can be induced by the application of physical as well as physiological stress (salinity, light, metals, temperature, nutrients, etc.). The stressors used for enhancing yield might change the genotype and adaptive responses of the algae during the course of time, which leads to alteration in ecology, genetics, and biodiversity as well as waste treatability potential of the algae.

Nevertheless, despite of studied for over 50 years, now, there are still large untapped potential of microalgae are present which still need to be explored. The production of biofuel through algae is still not so economical at large scale and therefore research should be more focused on cutting down the price involved in extraction and biofuel production. It is necessary to conduct techno-economic assessment prior to large scale cultivation and biomass production.

2.8 Conclusion

In conclusion this chapter highlights the importance of designed CW for treatment of waste in a cost effective manner. Designing of such low cost CW could be used in individual, community as well as large scale level before its direct discharge into large reservoirs. The developed CW will definitely help developing or underdeveloped countries. Further, the research should be paying attention on the utilization of high biomass producing algae for phytoremediation of waste as well as biofuel production. The post-harvested algal biomass could be further utilized in the production of important value added products, production of different fatty acids and biofuel. The duel approach of conserving wetland and high biomass production of algae will definitely tackle the global energy crisis and water problems.

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Mitigation of Heavy Metals Utilizing Algae and Its Subsequent Utilization for Sustainable Fuels

3

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Abstract

Urbanization and industrialization have accelerated the heavy metal release into the environment, thereby creating a major threat to the life forms on the earth. This pollution has been a challenge for a long time, although various methods for removal of heavy metals have been implemented but the results are still not

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satisfactory. Recently, there has been an upsurge in using green technology for this remediation through biosorption by photoautotrophic microalgal organisms. This chapter deals with the advantages of employing algal system for sorption over the conventional methods and also precisely reviews the severe impact of different heavy metals on microorganisms, plants and humans. Mechanisms for defence to survive under metal stress by algae are discussed. This chapter also highlights the conditions affecting the biosorption capacity of the algae and a brief insight for utilization of algae in production of biofuel.

Keywords

Algae · Bioremediation · Heavy metals · Biofuel · Pollution

3.1 Introduction

Earth exists as the third planet from the Sun in the solar system and holds a unique feature of anchoring diverse forms of life. It provides habitat to several living organisms in form of islands, shrubs, trees, mangroves, rivers, oceans, etc. A single drop of water from the ocean contains an invaluable amount of organisms. Thus Earth holds a special status as it provides varied forms of conditions to support the needs of different life forms. This irreplaceable planet has been tormented by the humans and has led to ruination. Increasing human population is demolishing the environment and its vital resources. In recent years many synthetic products have been devised by the humans to welcome ease in their lifestyle, but it has taken a toll on the environment. These inventions include plastics, fertilizers, pesticides, etc. The expeditious growth and vastness of industrialization and globalization have proved to be immensely prejudicial for the environment. The processing of the synthetic substances lead to release of noxious by-products which affect the delicate life forms severely. It releases large amounts of unwanted substances in the environment which are known as pollutants. Pollutants bring an unacceptable change in the environment and leads to deterioration of the natural environment and thus leading to nasty effects to the surroundings and as well as to the living forms. Such methods often aggravate the concentration of heavy metals in the environment. These metals are lethal in low concentrations and have high relative density. These are spontaneously arising components of the Earth. Anthropogenic activities and industries like tanneries, mining, agricultural practices, urban and industrial waste have accentuated heavy metal concentration in the environment and are thus causing bioaccumulation and also they are non-degradable (Masindi and Muedi 2018). The bioaccumulation leads to persistence of such harmful elements in the environment which lead to several nasty effects on the existing life forms. This leaves the environment in a degrading state which will be baleful for the organisms residing in it. It leads to neurotoxicity due to overexposure but some are required by human body in very low amounts. Extensive pollutants in treated wastewaters, industrial, agricultural wastewater are the heavy metals. Trace amounts of such metals is required by plants and animals but unbridled amounts accumulate in different organs, such as the brain. The calamitous

intracellular events induced by upraised metal levels include endoplasmic reticulum (ER) stress, DNA fragmentation, oxidative stress, protein misfolding and various others (Chen et al. 2016). The conventional method of discarding the waste to the dumping sites or in landfill includes the hazards of spreading the contamination during transportation apart from increasing contamination at one site (Tangahu et al. 2011). This has heightened the imbalance in natural environment which is already in a man-made catastrophic situation. Primarily, the industries release their effluents into nearby water sources damaging the submerged flora and fauna in that stretch, this leads to increase in higher concentration of disastrous metals into plants which ultimately ingresses the food chain. This has led to some serious concerns regarding the heavy metal concentration in the biosphere which need to be addressed. For these concerns, technologies like physical, chemical and biological methods are used. Physical methods include mechanical screening, electrostatic separation, flotation, magnetic separation; chemical methods include chemical precipitation, ion exchange, coagulation and flocculation, electrochemical treatments, membrane filtration and electrodialysis are used while biological methods include activated sludge, trickling filters, stabilization pond and bioremediation (Gunatilake 2015). Bioremediation is the recruitment of living organisms mainly microorganisms (bacteria, algae, fungi, etc.) for degradation of hazardous waste into non-toxic substances. Phytoremediation refers to using plants for heavy metal sequestration at a location and plants are also used to acquire heavy metals from them known as phytomining (Rascio and Navari-Izzo 2011). Present remediation technologies are less reasonable, tedious, time exhausting and deliver accessory waste to the environment (Masindi and Muedi 2018). Bioremediation currently is the most appropriate method for removal of toxic pollutants as it is an eco-friendly approach to detoxify the environmental pollutants and the pollution is being devoured by the nature itself. Fungal biomass particularly of *Aspergillus*, *Penicillium*, *Rhizopus*, *Saccharomyces*, *Fusarium* and *Mucor* has been used for uptake of heavy metals like Cr, Ni, Zn, Pb, As and U (Bishnoi 2005). Coconut shell charcoal has also been used as a potent adsorbent of Cr (VI) from wastewater of electroplating industries (Babel and Kurniawan 2004). It is presently the cheapest and most fitting method for destruction of heavy metals with no harmful residue. It proves to be of high success rate without much expensive investment. The sustainability of this method is at par with any other method for remediation as it is almost natural and biomass is easily available in abundance. The organism recruited for the process is not harmed in but is enriched in return with nutrients which are used by it as an energy source. The conventional physical and chemical methods are futile in low metal concentration, whereas bioremediation can work in low concentrations (Hlihor et al. 2017). Being microscopic, microorganisms are extremely efficient in contacting with the pollutant molecules and result in productive outcomes, moreover it is not location specific. The need of the hour is to employ strategies which are sustainable, cost effective, more productive, easy to assemble and employ less time consuming and non-polluting.

3.2 What Are Heavy Metals?

Heavy metals are ill-defined elements which naturally prevail in the environment and are highly toxic elements even at low concentrations. They have high density. Duffus (2002) cited that in the past, the definition categorizing an element as a heavy metal has been remould several times. The threshold density specified for a metal to be considered as a heavy metal has been restored to different limits, i.e. ranging from 3.5 to 7 g/cm^3. In his review, he also mentioned that different groups have defined heavy metals with reference to density, atomic mass or weight, atomic number, chemical properties and other non-chemical terms. But biologically, heavy metal is a metal which has high density and is potentially noxious or deadly. There are potential pollutants of freshwater and terrestrial ecosystems. They are found in earth's crust naturally released by volcanic eruptions, fossil fuel burning and leaching at waste disposal site, etc. (Pachana et al. 2010). They are recuperated from the different chemical forms of their ores in the rocks such as oxides of aluminium, antimony, manganese, gold and selenium and sulphides of nickel, iron, arsenic, lead-zinc, cobalt, gold-silver, while others exist as ores of sulphide and oxide like iron, cobalt and copper (Duruibe et al. 2007). The weathering of rocks gradually releases the metals to the surface which either through the action of wind gets mixed with the air and finally land in water or are taken up by plants and this is how it enters human body or animals. According to the description of Sharma and Agrawal (2005) there are about 40 heavy metals some of which are Pb (lead), Hg (mercury), Al (aluminium), Cr (chromium), Cd (cadmium), Ni (nickel), Co (cobalt), Zn (zinc), Ur (uranium), Se (selenium) and As (arsenic) which is a metalloid, etc. (Tangahu et al. 2011; Ojuederie and Babalola 2017). Heavy metals are classified into essential and non-essential heavy metals. Micronutrients like Cu, Ni, Zn, Fe, Mn, Mo and Co are essential for plants and animals, for example: iron is a component of haemoglobin but excess amount is poisonous. As the name micronutrients say that they are required in less concentrations, but in elevated concentrations they are considered heavy metals as they now behave as toxic components. They are required for numerous physiological and structural operations in the cell (Rengel 2004). Non-essential heavy metals do not have any role in cellular mechanisms of living mechanisms and are comprised of As, Pb, Cd, Hg (Ali and Khan 2018).

3.2.1 Entry of Heavy Metals into Ecosystem

Heavy metals reside in earth's crust but with some processes their entry in ecosystem can be hastened increasing their concentration exceeding the permissible limits. The factors can be natural as well as anthropogenic. Natural factors include weathering of rocks due to their contact with acidic water. The major contributor to the anthropogenic factor is mining activity. Mining is a destructive activity as it alters the ecosystem of the particular area causing loss of biodiversity. It exposes these poisonous metals from the Earth's crust and brings them to the exterior which

through rain and snow enters the water bodies and does harm to the aquatic life, this leads to bioaccumulation.

3.2.1.1 Mercury

Mercury exists in the environment in three forms mainly (Mahurpawar 2015). (a) Elemental mercury, it is the liquid mercury that we commonly see which is liquid at room temperature; (b) Inorganic mercury, it comprises mercurous and mercuric forms which are more toxic than elemental form; and (c) Organic mercury, it comprises alkylmercury compounds which include methylmercury, ethylmethyl salts and dimethylmercury, out of which methylmercury is majorly found in ecosystem (Rice et al. 2014). The organic form is the most toxic amongst ionic, metallic and organic forms.

The common natural source of elemental mercury is via dental amalgam, thermometers, sphygmomanometer, batteries, etc. (Rice et al. 2014). Organic mercury mainly methylmercury exposure occurs via fish and seafood (Rice et al. 2014; Mahurpawar 2015). Release rate of amalgam fillings is increased with chewing in the form of mercury vapours (Matta and Gjyli 2016). In some cases lightning creams, soaps and other traditional practices are the sources of inorganic and elemental mercury (Mahurpawar 2015).

3.2.1.2 Lead

Lead has proved to be a toxic metal and is common in human vicinities thus often contactable by humans. Lead enters the body through food, water and inhalation in inorganic form (Duruibe et al. 2007). Leaded gasoline, paints, toys, batteries are some of the sources for lead exposure. Solders are sometimes used to make tins and cans for packaging food materials by several companies (Mason et al. 2014). Often lead is also a constituent for pipes which carry water in households, etc. which also greatly increases the lead exposure. Anthropogenic activities such as industrial processes (such as mining, tanneries) and fossil fuel burning contribute to the lead load of the environment. Plants can also accumulate lead from either air or soil which ultimately ends up in animal and human systems producing critical results. Frolicking children and infants in parks or gardens are at extended risk of lead exposure via the soil, the effects of lead vary on the basis of type of exposure as it can be either acute or chronic. Headache, arthritis, sleeplessness, vertigo and loss of appetite are some of the symptoms of acute exposure while dyslexia, autism, birth defects, mental retardation, paralysis, muscular weakness are some symptoms of chronic lead exposure (Jaishankar et al. 2014; Matta and Gjyli 2016).

3.2.1.3 Arsenic

Arsenic is one of the most toxic and carcinogenic heavy metal (Jaishankar et al. 2014). It naturally occurs inside earth's crust (Chung et al. 2014). Exposure of arsenic can be natural like volcanic eruption and soil erosion as well as anthropogenic (Tchounwou et al. 2012). Fossil fuel combustion and use of synthetic pesticides and fertilizers, industrial processes, mining are some of the anthropogenic factors (Chung et al. 2014; Matta and Gjyli 2016). The two deadly forms of arsenic

are the inorganic forms such as arsenite and arsenate (Matta and Gjyli 2016; Jaishankar et al. 2014) which are readily taken up by the gastrointestinal tract, although the organic forms of arsenic in food and seafood are less harmful. Taiwan, India, Mexico, Bangladesh, Chile, Uruguay are some of the countries where arsenic concentration in groundwater has been really high (Abdul et al. 2015; Tchounwou et al. 2012; Chung et al. 2014). Drinking water and food are the main sources for arsenic exposure. Arsenic content has been found in numerous fish species like salmon and tuna which are commonly eaten, also in sardines, chub mackerel, horse mackerel, mullet, carps (Matta and Gjyli 2016; Jaishankar et al. 2014; Chung et al. 2014). Numerous health supplements use fish liver and other parts to fortify health leading to arsenic exposure. Molecular size of arsenic is an important criterion of arsenic exposure through inhalation. Pentavalent compounds are less water soluble and hence less toxic as compared to the trivalent compounds of arsenic. Chronic exposure to arsenic through different means leads to arsenic poisoning called arsenicosis. Artificial ripening agent like calcium carbide contains traces of arsenic and phosphorus hydride and is extensively used to ripe fruits like mangoes and bananas. Some countries have also banned the use of calcium carbide for such purposes (Siddiqui and Dhua 2010).

3.3 Impact of Heavy Metals on Microorganisms

Ecosystem consists of a diversity of organisms from microscopic to macroscopic ranging from few millimetres to several feet in size. Heavy metals are generally present in the environment and so does the microscopic life hence there must be some synergistic connection between them. Heavy metals present in the ecosystem affect organisms and are required even by microorganisms in certain amounts but beyond threshold level often harm the microbes. Microbes are an essential part of our environment and perform crucial activities for maintenance of life on Earth. Heavy metal pollution in their environment often adversely affects their population. The concentration and form of heavy metal present in the environment greatly affect the kind of microbe present in the soil. Microbes are equipped to remediate heavy metals into harmless substances through various processes like bioprecipitation, biosorption, etc. But stress caused by heavy metals produces unfavourable environment for microorganisms causing inhibition in their normal functioning. Due to the hostile environment the energy requirement of the microbes increases and out of the large amounts of consumed carbon only a bit is used for production of organic material and most of the part is released in the form of CO_2 (Abdu et al. 2017). Soil contains a diverse variety of microorganisms each group reacting differently to the variety of heavy metals. Soil, fresh and marine water ecosystems consist of variety and abundance of microscopic life having different tolerance abilities to heavy metals. Sensitivity of a microbial group to a particular metal can affect its population and thus can lead to changes in demograph of that ecosystem, such that microbial biomass (C) dropped on treatment with Cd and Ti, while treatment with Pb produces no significant changes in the microbial biomass. Hence microbial diversity in the soil

can change under heavy metal stress. Heavy metals are detrimental to growth, biochemical activities, morphology and diversity of microorganisms in soil. The bioremediation capability of microorganisms is harmed by Cd and Cr. Cadmium also causes decline in population of oligotrophic bacteria, oligotrophic sporulating bacteria, copiotrophic and copitrophic sporulating bacteria in soil. Shape of the bacterial cells is also disturbed by the existence of heavy metals. The bacterial cell envelope has Penicillin Binding Proteins (PBPs) which determine the shape of the cells, are hampered due to heavy metal toxicity. Replacement of Ca^{2+} by other divalent metals on the bacterial cell also affects the microbial cell as Ca^{2+} helps in binding of the cell to other proteins (Chakravarty et al. 2007). Ahmad et al. (2005) reported that aerobic heterotrophic bacterial populations showed sensitivity to several heavy metals in an orderly manner, where maximum to Ni & Cd chased by Cu, Cd, Hg, Mn, Cr and minimal to Zn. He also reported sensitivity of asymbiotic nitrogen fixers to metals like Cd, Hg, Pb followed by Cu, Cr, Mn, Ni and least sensitivity to Zn. Actinomycetes showed less tolerance to heavy metal toxicity unlike aerobic heterotrophic bacteria and asymbiotic nitrogen fixers (Ahmad et al. 2005). Some essential cellular metabolites of bacteria and protozoa are also obstructed by heavy metal stress (Madoni and Romeo 2006).

3.4 Impact of Heavy Metals on Plants

Plants are the life givers of our planet as they carry out several functions beside photosynthesis. The effect of heavy metal pollution on plants depends upon several factors like age of the plant, species of the plant, sensitivity to the metal ion form and environmental conditions, etc. The nutrient source for plants in the soil is becoming highly polluted due to extensive use of fertilizers, pesticides and also due to dumping of sewage waste and landfills. It causes mainly heavy metal stress to the soil which ultimately enters the plants causing adverse changes. Heavy metals which are not required by plants always prove to be harmful to plants unlike other heavy metals which are needed in minute amounts. Ghani (2010) studied effect of six heavy metals on growth of maize plant and concluded that each metal has a potential for toxicity in the order Cd □ Co □ Hg □ Mn □ Pb □ Cr, this toxicity is based on the extent of reduction of biomass of shoot, root and seed yield. This study also showed the synergistic effect of the heavy metals on the protein content of the maize seed. Mercury is a known toxic to plants and is also a constituent of sludge, manures, fertilizers which are extensively used in agricultural fields. Mercury exists in different ionic forms in soil particles but Hg^{2+} is a chief occurring ionic form. Disruption of water channel proteins occurs as Hg^{2+} binds with them thus preventing smooth water flow, also causing stomata to close due to low turgor pressure. Excessive Hg^{2+} leads to formation of ROS which causes oxidative stress and impaired mitochondrial activity. Excess mercury can affect rice (*Oryza sativa*) in several ways such as reduced plant height, tiller and panicle formation and lower crop yield. Additionally, in tomato (*Lycopersicon esculentum*) seed germination percentage drastically reduced, reduction in plant height, slashed flowering and fruit weight and chlorosis

of whole plant were some effects seen. Lead affects physiological processes by adversely affecting enzymes. Seedling growth, root and shoot elongation and leaf expansion were all inhibited by lead. It is also known to cause abnormal morphology in different plant species. Lignification in parenchymal cortex, irregular radial thickenings in endodermal cell wall are some of the injurious effects reported in pea roots. Like mercury it also causes oxidative damage by formation of ROS species. Plant biomass, germination percentage and protein content were reportedly reduced in maize (*Zea mays*). In *Avena sativa,* enzyme activity deteriorates affecting CO_2 fixation. Arsenic toxicity in *Lycopersicon esculentum* causes fruit reduction, stunted growth and wilting was reported in *Brassica napus*. In *Oryza Sativa*, reduced leaf size, stunted seedling height and germination were some of the effects of arsenic (Asati et al. 2016). Root and shoot length were reduced with increasing concentration of arsenic in *Phaseolus vulgaris.* Inorganic arsenic showed more toxicity to growth than organic arsenic (Chandra et al. 2018). Thus several biochemical and physiological mechanisms of plants are affected due to heavy metals like disruption of enzymatic activities, minimized biomass, stunted growth, disrupted fruit production and premature leaf fall, etc. (Ayangbenro and Babalola 2017).

3.5 Impact of Heavy Metal on Humans

Heavy metal pollution has been a crucial concern for environment as well as human health. It is currently proving to be a dominant contributor to many 'modern diseases'. Several heavy metals unknowingly sweep into our food chain through surface soil and groundwater. The sources of such noxious metals are varied. There are numerous heavy metals which affect humans in different ways and target different body organs, thus producing variety of symptoms and diseases. Cu, Co, Cr, Cd, Fe, Zn, Pb, Mn, Ni, Mo, V and W come under the category of heavy metals (Table 3.1).

3.5.1 Mercury

Methylmercury is a potent carcinogen and can seep through the placental barrier and blood–brain barrier, thus mercury can pass from mother to foetus and also to infant through mother's milk. Mercury has been proved to be neurotoxic and damaging to developing brain, kidneys, thyroid, lungs, liver, endocrine and reproductive system (Masindi and Muedi 2018; Mahurpawar 2015; Matta and Gjyli 2016). After oxidation, mercury vapour can become lipid soluble, bioaccumulates in renal cortex, liver and especially in brain (Rice et al. 2014). Methylmercury being highly lipophilic makes it easier to penetrate through the phospholipid bilayer of the plasma membrane. Gastrointestinal tract absorbs most of the organic mercury entering the body. Enzyme containing sulphydryl (thiol) groups are the target site for mercury, as it readily binds with these groups and hence affect the body functioning. The enzymes containing sulphydryl groups are present in inner mitochondrial membrane and thus

Table 3.1 Toxic effect of different heavy metals on humans (Bilal et al. 2018; Abbas et al. 2014; Ayangbenro and Babalola 2017; Masindi and Muedi 2018; Salama et al. 2019; Gunatilake 2015; Chen et al. 2016; Dixit et al. 2015)

Heavy metal	Major source	Toxic effect
Arsenic (As)	Pesticides, fungicides, herbicides, smelting, mining, atmospheric deposition	Cardiovascular/ peripheral diseases, immunological, neurological disorders, developmental abnormalities, carcinogen, diabetes, portal fibrosis, bronchitis, dermatitis, bone marrow depression, haemolysis, haepatomegaly, brain damage, conjunctivitis, skin cancer, visceral cancer, affects ATP synthesis, induces mitochondrial oxidative stress, hampers intracellular Ca^{2+} , neuronal cell death, affects cytoskeletal morphology
Cadmium (Cd)	Metal industry, paint pigment, fertilizer, cigarette smoking, food, plastic, welding, pesticides, mining, refining	Pulmonary and gastrointestinal irritation, carcinogenesis, kidney and liver damage, bronchitis, bone marrow damage, hypertension, lung insufficiency, weight loss, itai-itai disease, coughing, emphysema, headache, prostate cancer, lymphocytosis, nausea, reproductive toxicity, mutagenic, endocrine disruptor, impaired Ca^{2+} regulation, induces oxidative stress, suppressed gene expression, inhibits DNA repair and apoptosis
Chromium (Cr)	Anticorrosives, industrial welding, chrome electroplating, tanneries, wood preservatives, textile, dyeing, paint pigments, steel fabricating, electroplating, and textile	Carcinogen, gastrointestinal ulcers, damage to sperm, male reproductive system malfunction, anaemia, mutagenic, teratogenicity, upper abdominal pain, nausea and vomiting, severe diarrhoea, lung tumours, bronchopneumonia, emphysema, headache, skin irritant, itching of respiratory tract, liver disease, lung cancer, renal failure, hair loss, insomnia, Wilson disease
Copper (Cu)	Fertilizers, leather industry, photovoltaic cells, plating, copper polishing, paint, printing operations	Carcinogenic, neurodegenerative disorder, complications in diabetes, atherosclerosis, dizziness, diarrhoea, abdominal pain, headache, liver and kidney damage, metabolic disorders, nausea and vomiting, gastrointestinal irritation, severe poisoning with possible fatalities, dermatitis, chronic asthma, coughing, brain damage, further induce liver cirrhosis

(continued)

Table 3.1 (continued)

Heavy metal	Major source	Toxic effect
Lead (Pb)	Lead batteries, lead paint, devices to shield X-rays, mining, paint, pigments, electroplating, burning of coal	Nervous system disruptor, male reproductive system, microvascular endothelium, impairs mammalian spermatogenesis, inhibits sperm function, anaemia, brain damage, anorexia, malaise, loss of appetite, liver and kidney damage, gastrointestinal damage, mental retardation in children, anorexia, chronic nephropathy, damage to neurons, high blood pressure, insomnia, learning disability, reduced fertility, renal system damage, risk of Alzheimer's disease, difficulties in concentrating, damage to foetal brain, circulatory and nervous system damage, dwindled intelligence, amnesia, coordination problem, impaired development in children, decreased intelligence, processing speed, visual and motor skills, impaired verbal memory and visual memory, lower decision making speed
Mercury (Hg)	Oceanic emission, biomass burning, power plants, metal industry, gold mining, batteries, paper industry, mining, coal combustion, geothermal activity, volcanic eruption, weathering of rocks	Alzheimer's disease, Parkinson's disease, respiratory illness, damage to nervous system, protoplasm poisoning, corrosive to skin, eyes and muscles, dermatitis, kidney damage, ataxia, attention deficit, deafness, blindness, dizziness, dysphasia, loss of memory, reduced immunity, sclerosis, pulmonary edema, decrease in fertility, gastrointestinal irritation, rheumatoid arthritis, damage to circulatory and nervous system, autoimmune diseases, depression, drowsiness, fatigue, hair loss, insomnia, restlessness, temper outbursts, tremors, brain damage, mitochondrial dysfunction, DNA damage, generation of ROS, hindered dopamine synthesis
Zinc (Zn)	Oil refinery, mining, brass manufacturing, plumbing	Lack of muscle coordination, depression, gastrointestinal irritation, haematuria, icterus, impotence, kidney and liver failure, lethargy, macular degeneration, metal fume fever, prostate cancer, seizures, vomiting, electrolyte disturbances, dizziness, vascular type dementia, ROS production in mitochondria, brain trauma

after forming covalent bond with methylmercury it perturbs the functioning of mitochondrial membrane. The microtubule in the human body is made up of tubulin protein which consists of sulphydryl groups which readily combines with the mercury on exposure hence mercury exposure has dismantling effect on the microtubules. Mercury also proves to be a protoplasm poison (Abbas et al. 2014). Also known to cause gingivitis, it has degenerating effects on the brain as it is neurotoxic thus causing dizziness, dementia and makes it difficult to concentrate and pay attention (Ayangbenro and Babalola 2017).

3.5.2 Lead

Through many studies it has been found that lead is a carcinogen. The effects of this metal rely on age, duration of contact and extent of exposure. Firstly after entering the vascular system, i.e. the blood it is excreted via urine and then the additional amount remaining via red blood cells enter the soft tissues and finally get stored in the bones as insoluble phosphates (Jaishankar et al. 2014; Mason et al. 2014). Approximately 95% of the total lead is concentrated in bones and teeth. Lead exposure at workplace is linked to possibilities of lung cancer and stomach cancer (Mahurpawar 2015). It moves systemically in blood and can thus affect several systems in the body and can also easily pass through the placenta causing neurotoxicity to the foetus (Mason et al. 2014; Matta and Gjyli 2016). Lead negatively affects the joints, kidneys, reproductive system, cardiovascular system, central nervous system and peripheral nervous system and this is further promoted by calcium, iron and zinc deficiencies. Bloody urine is an outcome of lead induced damage to gastrointestinal tract and urinary tract (Duruibe et al. 2007; Mason et al. 2014). In calcium deficiency the concentration of lead is increased through its retention and thus the gravity of ill effects is enhanced. Iron is a major component of haemoglobin in red blood cells, its deficiency combined with lead exposure negatively affects haematopoiesis in pregnant women and children. Lead absorption is upgraded in zinc deficiency. Lead exposure adversely affects the memory and causes difficulty to recall in adults above 55 years of age and severe exposure can cause memory problems also in adults below 55 years of age. Duruibe et al. (2007) found that lead causes reduced development of grey matter in children thus contributes to lower intelligence, poor grammatical reasoning and command following skills. Individuals with blood lead concentration of 40 μg/dL and above showed slow decision making. Lead is also a major contributor to anxiety, depression and phobias which is in agreement with studies of bone lead level. Antisocial, violent and aggressive behaviour are also an outcome of lead exposure in adults which positively correlates with the blood lead level (Mason et al. 2014). Anaemia, brain damage and renal diseases are some of the adverse effects of lead exposure (Sati et al. 2016).

3.5.3 Arsenic

Arsenic is a known carcinogen and has multiple adverse effects on health through different means of exposure. Arsenic exposure in humans is interpreted by urinary arsenic concentrations (Ng et al. 2003). It takes approximately one hour for the effects to start show after ingesting heavy quantities of arsenic. Numerous disorders are related to high dose or chronic arsenic exposure which can be occupational also. It is a potent neurotoxic to central nervous system and peripheral nervous system (Adeniji 2004). The keratin fortified tissues of the body are the dumping sites for long term arsenic exposure and hence its levels can be easily detected from hair, nails and skin (Matta and Gjyli 2016). Long term exposures to arsenic can cause skin lesions, hyper pigmentation, respiratory defects, cardiovascular defects and renal defects. Cancers of lung, bladder and skin are some of the effects of arsenic exposure. The outbreak of Black Foot Disease in Taiwan was due to the chronic exposure of arsenic via drinking water (Ng et al. 2003). In this disease blood vessels of the extremities are injured causing a gangrenous effect and eventually death. The endocrine system is adversely affected by arsenic exposure mainly thyroid, pancreas and gonads. It also causes a decline in the lifespan of red blood cells (Abdul et al. 2015). Psychiatric disorders are commonly encountered in arsenicosis patients, mainly anxiety and depression. In a study by Tyler and Allan (2014) it was found that language and vocabulary, intelligence, reasoning, memory and maths skills deteriorated in children of age group 6–8 years. Difficulty in swallowing food through oesophagus, blood vomiting, metallic taste, dry mouth and burning sensation on lips are some of the symptoms of acute gastrointestinal syndrome (Abernathy et al. 2003).

3.6 Algae as a Potential Candidate for Bioremediation

Algae represent a diverse group of oxygen producing organisms inhabiting both land and water. Aquatic life forms can be found in both marine (seas, salt lakes and oceans) and freshwater (lakes, ponds, rivers and streams) surviving in extreme temperatures like on snow and in thermal springs (Sati et al. 2016). So, they can be manipulated to a certain extent in an experiment and can survive in laboratory conditions without much of hassle. Also, algal biomass production does not require too much of space as compared to the number of cells produced. Several functional groups are present on the algae such as carboxyl, carbonyl, sulphate, amino groups; they can easily dissociate the protons providing negative charge on the cell wall, for potential biosorption. Algae accumulate the complex toxic compounds and dissociate it into less toxic simple forms or inculcate it to boost their growth (Singh et al. 2017). Amongst all brown algae are considered premium biosorbent because of its excellent biosorption capacity due to copious alginate content. (Abbas et al. 2014; Ayangbenro and Babalola 2017).

The uptake of heavy metals by algae occurs via two steps, viz. primary, passive and rapid uptake and secondary, active slow uptake. During the primary process,

within a few seconds or minutes, the metal ions are adsorbed on the algal cell wall and this process is energy independent. The secondary process is slow as the metal ions are transported across the plasma membrane into the cytoplasm and this process is energy dependent as energy is required to cross the bilayer membrane. Adsorption on cell wall of algae takes place through some binding components on the algal cell wall. These functional groups on the cell wall require a specific pH to dissociate into corresponding proton and anion which are negatively charged and thus easily bind positively charged metal ions onto them. The cell wall components of algae vary with the group to which it belongs. Cyanobacterial cell wall is made up of peptidoglycan which has carboxyl functional group ($-CO_2$) for metal binding (Mehta and Gaur 2005). The chlorophycean members include various compositions of the cell wall mainly cellulose and pectin.

3.6.1 Defence Mechanism of Algae Against Heavy Metals

Algae have several mechanisms and systems to protect it from the degenerative effects of heavy metals. One of them includes glutathione which acts as antioxidant to the manufacturing of reactive oxygen species (ROS) and lipid peroxidation during the heavy metal stress. Glutathione reductase activity spiked during mild heavy metal stress. Oxidative stress is controlled by ascorbate and glutathione up to a certain level but at higher concentrations of heavy metals the antioxidant army itself becomes non-functional due to exuberant hydrogen peroxide and its ROS. Multiplication of carotenoid in *A. obliquus* cultures was also attributed to a kind of tolerance mechanism by green alga as it acts as scavenger of chlorophyll (Piotrowska-Niczyporuk et al. 2015). Chelators are also produced by the algae which form complexes with the metal and these are then dumped inside the metal complexes and help in tackling obnoxious side effects. Metallothioneins are cysteine rich metal binding proteins which maintain the intracellular amount of metals. Presence of sulphydryl groups explains its affinity towards metal ions. They are reported from simplest prokaryotic to most complex eukaryotes (Mantzorou et al. 2018).

Phytochelatins are the other kind of chelators present as polypeptides present in higher plants and algae but could not be found in animals. The polypeptide is made up of three amino acids: glutamic acid, cysteine and glycine with the latter joined to cysteine by γ- carboxamide linkage. Here also the presence of sulphydryl group in cysteine contributes to the metal binding property. The production of phytochelatins is induced by the presence of several heavy metals. These organometallic complexes are then stored in vacuole, pyrenoids and thylakoids (Mantzorou et al. 2018).

3.6.2 Effect of pH on Biosorption

pH is a strong determinant as it is responsible for providing of ionic sites during biosorption process. pH balances the process of protonation and deprotonation to maintain the required sites for biosorption. Biosorption increases with increasing pH

but only up to an optimum level. Low pH of the media solution indicates highly acidic environment, thus spare H^+ and H_3O^+ ions, which because of repulsive forces does not bind to the positive metal ions for functional groups like carboxylic acid. For amine, carboxyl and hydroxyl functional groups, deprotonation resurfaces negative charge due to formation of hydroxide and anions with increasing pH up to a certain level, after that it leads to precipitation and therefore low biosorption (Bilal et al. 2018). Metal toxicity increases as the pH is increased due to the reduction in anionic sites on the cell surface (Arunakumara and Zhang 2008). Ionic strength is also associated with the pH as it is inversely proportional to the pH of the solution (Halder 2014). Teimouri et al. (2016) examined the biosorbent capacity of macroalga *Gracilaria corticata* on copper, lead, zinc and cadmium and found that biosorption was optimum at pH of 6.0–8.0. The alga poorly adsorb at extremely acidic and extremely alkaline conditions.

3.6.3 Effect of Temperature on Biosorption

Temperature is an important criterion for sorption process. The nature of reaction can be exothermic or endothermic. Biosorption of the metal ion increases with increase in temperature during the endothermic reaction as high temperature provides active sites attributed to bond breakage. In the exothermic process, biosorption decreases with increase in temperature, also very high temperature dismantle the cell wall and biomass structure. Prasher et al. (2004) conducted temperature and sorption study with Cu and Pb and observed that Cu uptake increased with increasing temperature till 60 °C but the uptake of Pb decreased with further increase in temperature. Although relationship between temperature and biosorption has been correlated but in different studies variable results have been obtained. Some reports have also suggested that temperature does not affect sorption process (Mehta and Gaur 2005).

3.6.4 Impact of Contact Time

Teimouri et al. (2016) conducted a study using *G. corticata* as sorbent to study the effect of contact time on biosorption capacity of four different heavy metals. He observed that during the first 40 min of contact time the sorption was rapid and slow sorption took place in next 20 min. For all the four metals, namely copper, lead, zinc and cadmium, sorption was achieved more than 90% during the 60 min of contact time. As a result of this study it can be concluded that the affinity of sorbent for every metal differs which could be due to difference in the contact sites of sorbate and sorbent, distinct ionic radius of every metal and available active sites on the sorbent. Demey et al. (2018) also reported slower uptake of sorbate with increasing time. Population of algal cells in the sorption medium also affect the uptake of heavy metal ion, as the initial metal ion concentration is also responsible for the time taken to saturate the active sites of the sorbent (Imani et al. 2011; Teimouri et al. 2016).

Biosorption using algae is a promising method for removal of heavy metals from their environment. Utilization of algae for biosorption process has several advantages over other conventional methods such as: (a) this process has minimal investment; (b) reduced requirement for expensive reagents and chemicals; (c) rapid multiplication ability; (d) abundant in nature; (e) can purify large quantities of samples; (f) treats trace amounts of toxic metals which is difficult to remove by conventional methods; (g) has high affinity for heavy metals if provided optimum pH; (h) recovery and reuse of toxic metals are uncomplicated and (i) different species of algae can work under variety of temperature, pH, thus biosorption can operate over a range of conditions (Abbas et al. 2014).

Instead of having so many advantages as a potential biosorbent, algae still have certain shortcomings such as its inability to work in high metal concentration, as oxidative stress exceeds beyond repair and requirement of specific conditions throughout the year. These shortcomings, however, can be overcome by a little modification in the culture conditions (Bwapwa et al. 2017).

3.7 Algal Biofuel Production

Apart from having feasible, eco-friendly and cost effective bioremediation capabilities, algae have several other benefits of producing variety of value added products, out of which one is biofuel. The process of bioremediation of heavy metals could be made more economical by using algal biomass as a feedstock for biofuel production. With the little modification in the growth conditions and minimal cost input the algal biomass could serve as a potential biofuel feedstock (Mandotra et al. 2019). In the latter section we will discuss potential of algae as a biofuel feedstock separately.

The commercial energy producing oils like diesel and petrol are nature's reserves, which are produced over a million of years. The speed at which these oil reserves are being consumed we are not far away from the day we deplete these reserves leading to a standstill as we single handedly depend on them. Every transportation means, certain machines run on diesel mainly. As a pattern we could see a subsequent rise in prices of this fuel as the reserves are depleting the prices are soaring because the demand is increasing. There are tensions amongst countries and politics revolving around the authority of fuel trade. To reduce our dependence on such limited fuels we need to find an alternate source of such fuels. Biodiesel from natural sources like plants and algae is becoming popular globally and steps to make them fully functional are under trial (Mandotra et al. 2016).

3.7.1 Cultivation of Algae

For commercial production of biodiesel alga must have certain properties to produce the fuel feasibly. The alga must multiply at a rapid rate to produce enough biomass and should produce abundant lipid, which is the actual biodiesel producing

component of the alga. So, the first step is to select an alga that is easy to grow and give maximum output. After isolating the specific alga it must be grown in huge numbers to produce sufficient biomass. The cultivation is the next important step where the alga under production requires certain conditions to give desirable results. There are several methods for the cultivation of algae.

3.7.1.1 Open Raceway Ponds

The shape and build-up of this pond are like a race course with rounded edges, they are not very deep (~0.25 m in depth), so as to provide each and every algal cell a uniform and sufficient light intensity. One of the most significant features of the raceway pond (Fig. 3.1) is the present of paddle wheels, they continuously agitate the algae and keep them flowing to adequately distribute nutrients for favourable biomass production. The nutrients are provided near the paddle wheel for uniform distribution and one outlet is present for harvesting. They are lined by cement, plastic or clay (Golinski 2015).

3.7.1.2 Photobioreactor

It is a type of close system used to cultivate algae in transparent containers (glass or fibre) of different shapes (Fig. 3.2). They are most suitable when it comes for the commercial production as they have controlled conditions and thus it is easy to maintain the required temperature, pH, salinity and nutrient concentration and keep the contamination at bay to some extent (Mandotra et al. 2014). However cleaning is quite tricky in some photobioreactors (PBRs) due to their shape. Agitators present in PBRs could increase the biomass productivity. One disadvantage associated with PBRs is that they are quit costly.

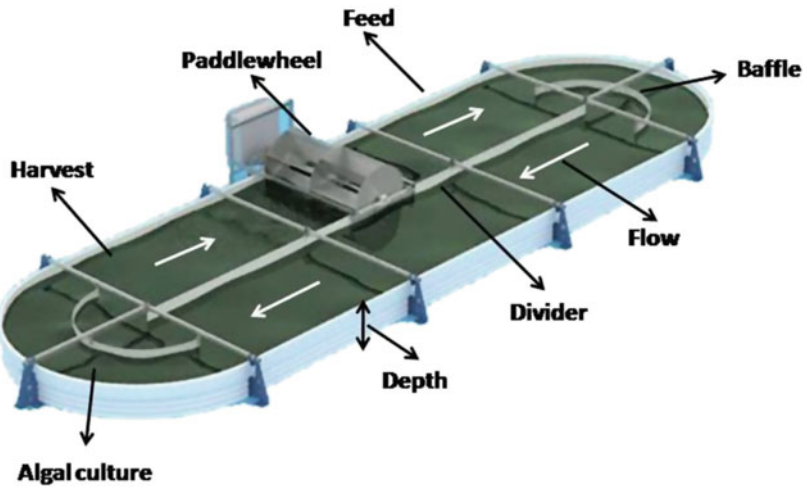

Fig. 3.1 Schematic diagram of algal raceway pond

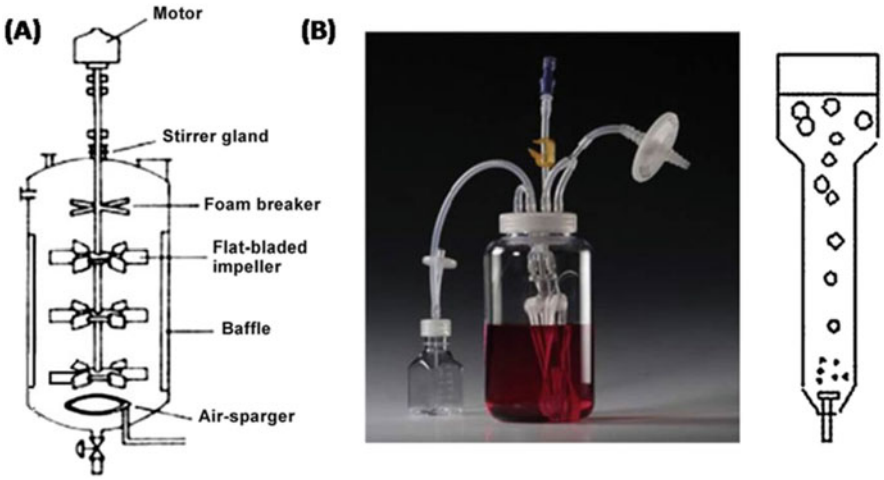

Fig. 3.2 Schematic diagram showing (**a**) Stirred tank photobioreactor, (**b**) Bubble column reactor (Singh and Sharma 2012)

3.7.2 Harvesting Methods

After desired growth, algae are harvested to produce algal oil. Harvesting is an important step during the production of biofuels as plentiful biomass will result into successful extraction of algal oil. Traditional harvesting method is through centrifugation; however, in large scale cultivation it is not feasible as it add up in huge cost investment. Algal biomass can also be harvested by the process of auto-sedimentation, during this process of harvesting, the algal cultures/suspension are kept un-agitated for some time, as a result, algal biomass tends to settle down by the influence of gravity. Flocculants are also the major vehicles to drive the extraction of biomass and fruitfully clusters the alga together to increase its size for easy separation. Due to the opposite charges on the flocculant and algal cell wall, the algal cells are aggregated together. Flocculation is of different types based on the nature of the flocculants.

3.7.2.1 Chemical Flocculation

Chemical flocculation is achieved with the assistance of two different types of flocculants, viz. organic and inorganic flocculants. Organic flocculants include okra mucilage, Greenfloc-120, combination of starch and chitosan, chitosan and modified cationic chitosan-polyacrylamide, whereas inorganic flocculants are aluminium sulphate ($Al_2(SO_4)_3$), ferric sulphate ($Fe_2(SO_4)_3$), ferric chloride ($FeCl_3$) and lime ($Ca(OH)_2$) (Chen et al. 2011; Suali and Sarbatly 2012).

3.7.2.2 Microbial Flocculation

As the name suggest, microbial flocculation is achieved by the use of microorganisms. Bacteria are used for clustering algae, whereas consortium of

fungi and bacteria can also be used. This type of flocculation is commonly seen in variety of wastewater, where bacteria and fungi are present naturally (Tredici et al. 1991).

3.7.2.3 Electroflocculation

During this process, generally iron or aluminium electrodes are used; the flocculation is achieved by the metal ions which are electrolytically released from the anode. This process is more or less similar to the conventional metal salts; however, electroflocculation offers numerous advantages such as requirement of low doses, wide working range of pH, efficient harvesting and absence of contaminants (coupled anions). The amount of metal contaminants in biomass is much lesser in this process compared to conventional metal salts (Kumar et al. 2013; Branyikova et al. 2018).

3.7.3 Oil Extraction

After biomass harvesting, oil is extracted from the algal cells by the help of various methods. One of the conventional methods of oil extraction is through mechanical press. It is a feasible method of extraction where the oil extracted is without any additive impurity as no solvent is required. As the name suggests a high pressure is applied on the cells disrupting the cell wall and releasing the oil. The main disadvantages, however, associated with this method are the lower oil yield.

Solvent extraction is one of the widely used oil extraction methods from algal cells. In this method, cell wall degenerating solvents are used, which dissolve the cell wall thus leaving the cell without any boundary and releasing the oil. Solvents used are cyclohexane, acetone, benzene, hexane, chloroform and methanol. These solvents are either used individually or in combination with each other under different ratios. Traces of solvents, however, can be found in this kind of algal oil as additives are used and the purity is little less than the former method.

Supercritical fluid extraction is yet another oil extraction method that is being widely used. In this method a special type of carbon dioxide is used as a solvent. Firstly, pressure is applied to CO_2 to liquefy it followed by heating to such an extent that it behaves partially as gas and partially as liquid. This method is one of the best to maintain the quality of oil. The biggest disadvantage associated with this method is the requirement of sophisticated equipment and high cost input (Kumar et al. 2013).

3.7.4 Transesterification of Algal Oil

After the extraction, transesterification of the oil is done to adjust the viscosity of oil as viscosity is an important factor to label oil as fuel (Topare et al. 2011; Fathi et al. 2013; Kumar et al. 2016) (Fig. 3.3).

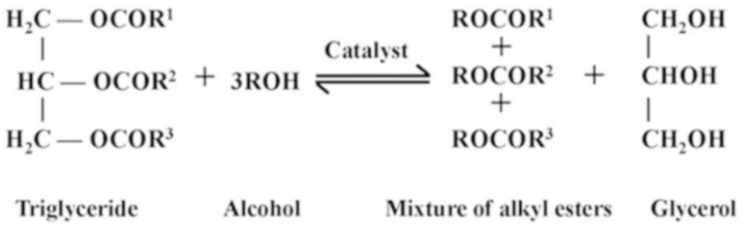

Fig. 3.3 General reaction of transesterification (Mandotra et al. 2020)

For transesterification, algal oil and methanol undergo a reaction in the presence of a catalyst which can be KOH or NaOH to give a fatty acid ester (biodiesel) and glycerine as a biproduct. The glycerine in the reaction is taken out at intervals through gravity separation or centrifugation. After successfully removing glycerol, methanol and soap remain in the solution. Methanol is removed through vapourization. If soap concentrations are high in the solution, then after removal of methanol it will precipitate in the form of sludge as methanol is a solvent for soap. After some series of washing to remove the traces of methanol, soap and catalyst, the biodiesel is reacted with methanol in the presence of an acid mainly sulphuric acid to produce a methyl ester and water. Water produced in this step tends to be problematic, thus it is removed from the process at intervals. The methyl ester obtained in the final step is then dried to achieve the final product (Topare et al. 2011).

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Adaptive and Tolerance Mechanism of Microalgae in Removal of Cadmium from Wastewater

4

Shweta Tripathi and Krishna Mohan Poluri

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Abstract

Cadmium contamination in aquatic bodies is one of the major environmental issues that impact the deterioration of water and food quality, hindering its availability to human society. Unchecked release of cadmium in effluents from electroplating, textile, battery, and fertilizer industries is leading to serious threats to human lives as well as plants and animals. There is a mounting interest in exploring microalgae for removal of such heavy metals due to their efficiency and ability to cope with metal toxicity and stress. Phycoremediation is an eco-friendly and cost-effective approach having an edge over conventional technologies for

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treating metal contaminated wastewater. Microalgae respond to toxic metals like cadmium by modulating their biochemical components and synthesizing stress proteins, compounds like phytochelatins and metallothioneins, etc. The present chapter will be focused on microalgae based cadmium remediation from polluted sites and industrial effluents. Molecular responses of microalgae in order to survive cadmium toxicity as well as utilization of generated biomass for production of bioenergy and other commercial compounds will also be discussed. In summary, the current chapter provides detailed mechanistic insights into the thriving behavior of microalgae in cadmium exposure, and its efficiency in mitigating heavy metal contaminated water.

Keywords

Algae · Wastewater · Cadmium · Remediation

4.1 Introduction

Over the past few decades, globalization and rapid industrialization have led to a sudden escalation of environmental pollutants in nature. Heavy metal pollution is one of the most pervasive issues throughout the globe. Heavy metals are generally categorized as a group of metals or metalloids having density higher than 4 gm/cm^3, atomic number >20, and cause severe toxicity even at small doses (Yan-de et al. 2007; Suresh Kumar et al. 2015). According to this definition, there are around 53 metals under this category, but As (arsenic), Cd (cadmium), Hg (mercury), and Pb (lead) are considered as the most toxic metals. Among all, teratogenic, carcinogenic, and mutagenic nature of cadmium increases risk to all life forms (Cai et al. 2019). Cadmium is a hazardous pollutant which has an acclaimed history of toxicity since its discovery by the German chemist F. Strohmeier in 1817 (Nordberg 2009). It is a post-transition metal (atomic number = 48), situated between Zn (zinc) and Hg (mercury) in the periodic table, having chemical properties similar to zinc and mercury (Bernhoft 2013). The very first case of its pernicious behavior was confirmed while diagnosing acute gastrointestinal and respiratory problems in workers exposed to cadmium carbonate used for polishing (Sovet 1958). High bioavailability and mobility of cadmium leads to its incorporation into higher trophic levels in the food web (Ghoneim et al. 2014). Cadmium poisoning is becoming a major concern worldwide due to its wide applications in various industries like electroplating, smelting, mining, pigment and paint synthesis, Ni–Cd batteries. In fact, International Agency for Research on Cancer (IARC) has categorized cadmium as Group 1 carcinogen on the basis of increased risk of lung cancer in people working at such industries (McElroy and Hunter 2019).

Cadmium is primarily released into the water bodies and soil through wastewaters. Generally, relevant concentration of cadmium in natural bodies ranges from 0.11 to 25 μg/L but may reach up to 5–200 mg/L in acid mine drainages and industrial wastewaters (Cui et al. 2012; Yang et al. 2015; Yu et al. 2018). According to a recent study, a total of 6.4 tons of cadmium is released into the aquatic water

bodies from iron and steel plants, disposal of rechargeable cadmium–nickel batteries, mining, and phosphate fertilizers, which badly deteriorate the aquatic life and eventually get incorporated into the plants and human body (Abinandan et al. 2019). To avoid this risk, WHO (World Health Organization) has decided 5 ppb cadmium as the minimal permissible limit in drinking water (Suresh Kumar et al. 2015). Cadmium, being a toxic and persistent metal, readily binds to the organic molecules by forming bonds with sulfur and nitrogen,thereby inactivating proteins capable of causing a broad range of adverse effects in microbes, plants, animals, and humans (Vig et al. 2003; Ghoneim et al. 2014). This allows exposure of Cd to human body leading to the development of lethal diseases like cancer, cardiovascular, urinary, and reproductive disorders. Additionally, displacement of Ca^{2+} by Cd^{2+} in skeletal system may lead to osteomalacia, a well-known disease in Japan (Itai-itai) (Rahimzadeh et al. 2017).

Numerous physico-chemical techniques such as precipitation, ion exchange, membrane filtration, and electroplating are widely employed for the removal of heavy metals from wastewater (Arora et al. 2017). However, the above methods accompany limitations like lower efficiency, generation of more concentrated metal laden discards along with high operating and maintenance costs (Cheng et al. 2019). In order to overcome such demerits, phycoremediation emerged as an eco-friendly and cost-effective approach having an edge over the conventional technologies for treating metal contaminated wastewater. Microalgae binds to heavy metals by the virtue of functional groups on their cell surface resulting in biosorption and further internalization in the cell, that leads to intracellular detoxification via vacuolar compartmentalization or chloroplast mitochondrial sequestration (Perales-Vela et al. 2006; Yang et al. 2015). They respond to such toxicity by modulating their biochemical components along with synthesis of stress proteins like phytochelatins, metallothioneins, etc. (Perales-Vela et al. 2006).

In light of the present scenario, the current chapter provides a comprehensive overview of utilizing microalgae in cadmium mitigation. Response of microalgae cells in cadmium toxicity, mechanism of detoxification as well as adaptive behavior of cells to thrive in such situation is also highlighted. Preventive role of enzymatic and non-enzymatic antioxidant molecules inside the cells under cadmium toxicity has also been reviewed. Further, the utilization of Cd laden biomass for biodiesel production is also discussed in detail.

4.2 Sources of Cadmium Pollution

Both natural and anthropogenic sources contribute towards cadmium release into the environment. Indeed, the predominant utilization of cadmium in every sector of human life has created a situation of prime concern. Cadmium is primarily released into the environment through volcanic eruptions, smelting, weathering, mining and refining (Hayat et al. 2019). Despite the acclaimed history of toxicity, cadmium exhibits several interesting physical and chemical properties that attracts industries towards its utilization. Properties such as anti-corrosion and tolerance to chemicals,

make cadmium the requisite element for paints, pigments, and alloy manufacturing industries. The excellent electrical conductivity and resistance to high temperature make cadmium suitable element for its application in semiconductors, manufacturing of glass and ceramics (Morrow 2000). In order to contribute to cadmium reduction policies, it is essential to make a holistic approach by listing sources of cadmium emission into the atmosphere.

4.2.1 Natural Sources of Cadmium Emission

Abundance of cadmium in the Earth's crust is merely 0.1–0.5 ppm, but natural activities like weathering of rocks and volcanic eruptions may lead to its accumulation in sedimentary rocks (Hayat et al. 2019; Morrow 2000). It might reach to a concentration of 500 ppm, as reported for phosphorites and marine phosphates (Jackson and Macgillivray 1995). Indeed, volcanic eruptions along with weathering of rocks are known to contribute around 1500 metric tons of cadmium into the water bodies on an annual basis (Richards and Mullins 2013; Kesler et al. 2015; Rahman and Singh 2019), whereas other fluxes come from forest fires, aerosols, and windborne particles (Nriagu 1990).

4.2.2 Anthropogenic Sources of Cadmium Emission

The release of cadmium into air, water, and soil is mainly through the industrial waste run-offs containing cadmium. Coal mining, combustion of fossil fuels, smelting of ferrous and non-ferrous elements, incineration of municipal waste, run offs from fertilizer, cement, electroplating industries, and disposal of Ni–Cd batteries are the major anthropogenic sources of cadmium (Hayat et al. 2019; Suresh Kumar et al. 2015; Abbas et al. 2018). In fact, an annual consumption of cadmium in pigment synthesis crossed over 2500 tons (Faulkner and Schwartz 2009). On an average, cadmium consumption could be categorized as a percentage based on their applications in different industrial sectors. Annually, Ni–Cd batteries consume around 86% of total cadmium consumed globally, followed by pigments and coatings with plating industries consuming 8% and 7%, respectively. The remaining 2–3% is utilized in alloys, stabilizers, solar cells, etc., (Faroon et al. 2012; Yuan et al. 2019). Cadmium is commonly seen as an impurity present in zinc ores; mostly isolated as a byproduct during mining, refining, or smelting of zinc (Zn) (Bi et al. 2006).

According to a report by the European commission in 2014, more than 50% of the total cadmium emission (58.3 tons) was contributed by the domestic (28 tons) and commercial sectors (6 tons). Cadmium emission by the domestic sectors basically involves municipal waste incineration. This waste constitutes of natural substances like food, vegetables, plants, colored glasses, ceramics, iron, cement, and alloys such as steel (Hasselriis and Licata 1996). Other processes contributing to cadmium emission through air involve combustion of fossil fuels, plastics, and Ni–Cd batteries

(Sahmoun et al. 2005). Furthermore, the small dust particles released through chimneys of alloy industries get collected in the air, eventually reaching the Earth through rain, thus resulting in air, soil, and water pollution. Also the cadmium content adsorbed in soil is accumulated in plants like rice, paddy, and leafy vegetables which eventually reaches the human body causing cadmium poisoning (Council 2003). Oceans have an average concentration of 5 ng/L cadmium released through ship oils and paints (Bro-Rasmussen 1996). Furthermore, leaching from galvanized steel pipes used in metro cities for transporting water from a source to different colonies as well as a poor drainage system also adds cadmium content into the drinking water. Another important source of cadmium contamination in soil and food is the use of phosphate fertilizers, herbicides, and pesticides in agricultural sector. High cadmium content ranging from 30 to 150 μg/L has been reported in some green leafy vegetables like spinach, lettuce along with contamination in cereals and potatoes (Hayat et al. 2019). A large population of the world is fond of sea food, but it itself is a major threat of Cd contamination. Approximately 5–40 μg/L of cadmium is found in fishes and other edible aquatic creatures (Satarug et al. 2003), through which Cd finds an easy route to travel into the human body.

4.3 Microalgae: A Potential Candidate for Cadmium Mitigation

Numerous physical and chemical techniques are used for removing heavy metals from water bodies. The physical methods include adsorption, ion exchange, membrane filtration, and reverse osmosis, whereas chemical methods include chemical precipitation, flocculation, coagulation, solvent extraction, and electrochemical treatments (Monteiro et al. 2012b; Cheng et al. 2019). These conventional methods are widely employed by many industries, but certain shortcomings such as the generation of heavy metal laden discards and their disposal, inefficient removal of metals, initial capital costs and maintenance costs limit the utilization of these techniques (Monteiro et al. 2012b). All these associated limitations of existing technologies urge the need for more affordable and efficient alternative technologies like utilizing the biomass originated from plants, algae, bacteria, and fungi. Recently, microalgae emerged as the most suitable candidate for the removal of heavy metals. It has attracted the attention of researchers worldwide due to its easy cultivation and high growth rate in minimal nutrients without any seasonal limitations (Marcano et al. 2009; Yang et al. 2015; Cheng et al. 2019; Mandotra et al. 2014). Their large surface area to volume ratio facilitates around 10% of biomass area to bind metals. Additionally, the presence of negatively charged functional groups improves selectivity and efficiency for metal removal in an aqueous media (de Bashan and Bashan 2010; Monteiro et al. 2012a; Kumar et al. 2015; Mandotra et al. 2019).

There are several studies assessing the potential of microalgae biomass as a biosorbent for removal of Cd ions from contaminated water (Peña-Castro et al. 2004; Perales-Vela et al. 2006; Brinza et al. 2007; de Bashan and Bashan 2010). The removal efficiency of any microalgae species largely depends on abiotic factors such as pH, amount of biomass, along with bioavailability and speciation of the

concerned metal ions in the media. Live cells mitigate metal ions from the media by the cumulative effect of biosorption and bioaccumulation. In such cases, high concentration of metals like Cd adversely hampers the essential regulatory networks in microalgae cells (Priyadarshini et al. 2019). The concentration of heavy metal that inhibits the growth of microalgae by half is regarded as IC_{50} (inhibitory concentration) or LC_{50} (Lethal concentration). Determination of this concentration is quintessential for exploiting the complete potential of live microalgal cells in tolerating high concentrations of metal ions. For example, live marine microalgae *Tetraselmis suecica* used for Cd removal showed EC_{50} of 7.9 mg/L and removed more than 60% of Cd from 45 mg/L Cd culture through bioadsorption (Pérez-Rama et al. 2002). The toxicity assessment of Cd in *Chlorococcum* sp. depicted IC_{50} value of 11.2 mg/L, whereas *Tetraselmis tetrathela* showed IC_{50} value of 9.8 mg/L (Satoh et al. 2005). Similarly, both the dead and live biomass of *Chlorella minutissima* was used for performing biosorption and bioaccumulation of Cd ions in the media (Yang et al. 2015). There are several studies listed in Table 4.1 focusing on the removal and adsorption efficiency of both live and dead microalgae biomass.

Over the past few years, modified algal biomass emerged as an alluring technique to combat increased heavy metal pollution in industrial wastewaters (Bag et al. 2019). One of the most fascinating areas is the production of nanoparticles from algal extracts. Microalgae derived nanoparticles are preferred over plant-based nanoparticles due to the higher growth rate and photosynthetic ability of microalgae (Anirudh et al. 2018; Agarwal et al. 2019). A recent review by Cheng et al highlighted the prospects of chemical modifications, engineering the metal binding proteins, and immobilizing algae to enhance heavy metal removal (Cheng et al. 2019). To highlight the enhanced Cd removal capacity of microalgae cells, microalgal-biochar immobilized complex (MBIC) was made by using *Chlorella* cells and water hyacinth biochar (WHB). MBIC improved the adsorption capacity of the complex to 217.41 mg/g in comparison to *Chlorella* cells (169.92 mg/g) and WHB (95.82 mg/g), when used separately (Shen et al. 2017). Another study showed the utilization of *Chlorella vulgaris* immobilized in Ca-alginate beads leading to enhanced adsorption of Cd from aqueous media (El et al. 2019). Zhang et al used *Selenastrum capricornutum* and *Microcystis aeruginosa* as bioreactors for immobilizing Cd ions and co-incubating them in selenium (Se), thus leading to the formation of fluorescent CdSe NPs. This study also demonstrated the utilization of these CdSe NPs as a probe for rapid detection of Hg^{2+} in medium encouraging the applicability of microalgae as a biosensor (Zhang et al. 2019). All these above representative studies demonstrated that microalgae with modifications could create a paradigm shift in biosorption of Cd.

4.4 Mechanism of Cadmium Remediation by Microalgae

Interaction of metals with the cell surface of microalgae is quite complex and largely depends on several factors such as type of metals, pH of the media, and presence of other competing metals/contaminants, etc. Low pH makes the cell surface more

Table 4.1 Summary of cadmium removal (mg/g dry cell weight), IC_{50} value, adsorption efficiency, and time of incubation by various microalgae

Microalgae	IC $_{50}$ value	pH	Type of biomass	Initial metal concentration (ppm)	Time of contact	Metal uptake (mg/g)	% Removal	References
Pseudochlorococcum typicum	–	7	Live	0.0032	24 h	5.48	76	Shanab et al. (2012)
Desmodesmus pleiomorphus	–	4	Live	0.5	24 h	61.2	98.9	Monteiro et al. (2010)
D. pleiomorphus (ACOI)	–	4	Live	0.5	24 h	85.3	98.1	
Scenedesmus obliquus	–	–	Non-loving	150	2 h	60.8	32.4	Monteiro et al. (2011b)
D. pleiomorphus	–	–	Non-living	150	2 h	58.6	31.9	
Isochrysis galbana	121.6 µM	–	Live	–	96 h	–	–	Liu et al. (2011)
Tetraselmis chuii	37.8 µM	–	Live	–	96 h	–	–	
S. obliquus	–	6	Non-living	50	–	68.6	100	Chen et al. (2012)
Tetraselmis chuii	–	8	Non-living	0.5	15 min	13.46	–	Sjahrul and Arifin (2012)
S. quadricauda		5	Non-living	50	10 mg/L	135.5	66	Mirghaffari and Moeini (2015)
Chaetoceros gracilis	2370 µg/L	7	Live	–	96 h	–	–	Suratno et al. (2015)
Isochrysis sp.	490 µg/L	7	Live	–	96 h	–	–	
Chlorella vulgaris	–	–	Non-living	100	105 min	16.65	95	Cheng et al. (2017)
Scenedesmus sp.	–	–	Live	0.0073	18 days	–	93.06	Apandi et al. (2018)
Chlorella sp.	8 ppm	–	Live	1	7 days	–	8.07	Duque et al. (2019)
	–	–	Live	7	7 days	–	8.60	
Scenedesmus sp.	20.89	–	Live	1	7 days	–	5.13	
	–	–	Live	7	7 days	–	32.7	

positive with higher number of H^+ ions and repels the attachment of positively charged metals like cadmium in the medium (Samadani et al. 2018). Under such conditions negatively charged anions are preferred for interactions. Bioavailability of the metal ions largely depends on the fraction of free ions since they are known to be the most toxic and available forms. Additionally, reduced uptake of Cd^{2+} in the presence of Zn^{2+} and Ni^{2+} ions by *C. vulgaris* cells suggests the hindrances caused due to competitive metals during biosorption (Monteiro et al. 2011a). In general, the overall mechanism followed by microalgae to detoxify heavy metals can be elucidated in four sequential steps. The very first step is (1) the attachment of metal cations like cadmium to different functional sites on the cell surface, followed by (2) intracellular bioaccumulation and sequestration, (3) detoxification by organic chelation to metals, and (4) efflux of excess metals from cells to the surrounding (Priyadarshini et al. 2019) (Fig. 4.1).

Biosorption is the prime step towards the attachment of metal ions onto the cell wall of microalgae. The cell wall of microalgae is composed of proteins, polysaccharides, and lipids which provide attachment sites through functional groups like hydroxyl, carboxyl, sulfyhydryl, phosphate, amino, etc. These groups are responsible for overall negative charge and high binding affinity (Monteiro et al. 2012b). These interactions are mainly dominated by ionic interactions (ion exchange). However, microprecipitation, complexation, covalent, electrostatic, Vander-Waal bonding and/or combination of these may play a major role in biosorption (Mehta and Gaur 2005; Kumar et al. 2015; Priyadarshini et al. 2019). Biosorption by microalgae biomass is a cost-effective, eco-friendly approach and is reported to remove around 100 mg/L of heavy metals from industrial effluents (Rangsayatorn et al. 2002). Microalgae biomass has an advantage over other microbial biomass due to its easy cultivation, higher biomass productivity without any secondary toxin production (Das et al. 2008). Several studies reporting the removal of cadmium by using microalgae biomass as biosorbent are listed in Table 4.1. For instance, lyophilized biomass of *C. minutissima* was used for removing cadmium which showed 38.8% removal in initial Cd concentration of 0.6 mM with algae adsorption capacity of 303.03 mg/g. Further, bioaccumulation in live cells under same concentration showed efficiency of 38.8 mg/g (Yang et al. 2015). Similarly, *Dunaliella salina* showed 45% removal of cadmium from media containing 25 ppm Cd, where 99% of the total Cd was removed by bioadsorption, whereas remaining 1.17% was accumulated inside the cells (Belghith et al. 2016). *Scenedesmus quadricauda* was able to remove 66% of Cd ions with biosorption capacity of 135.5 mg/g dry cell weight (Mirghaffari and Moeini 2015). Current studies are focusing on modified algae biomass by immobilizing it in appropriate systems. Dry and immobilized biomass of *C. vulgaris* was utilized to remove cadmium ions in range of 20–100 ppm from the media (El et al. 2019). Authors also demonstrated the effect of cadmium concentration, pH, time, agitation speed, and biomass dosage on removal efficiencies. These results depicted that dry biomass showed maximum removal efficiency of 65% in 75 mg/L Cd at a pH of 6 with an adsorption capacity of 8.53 mg/g dry cell weight, whereas immobilized biomass showed improved removal

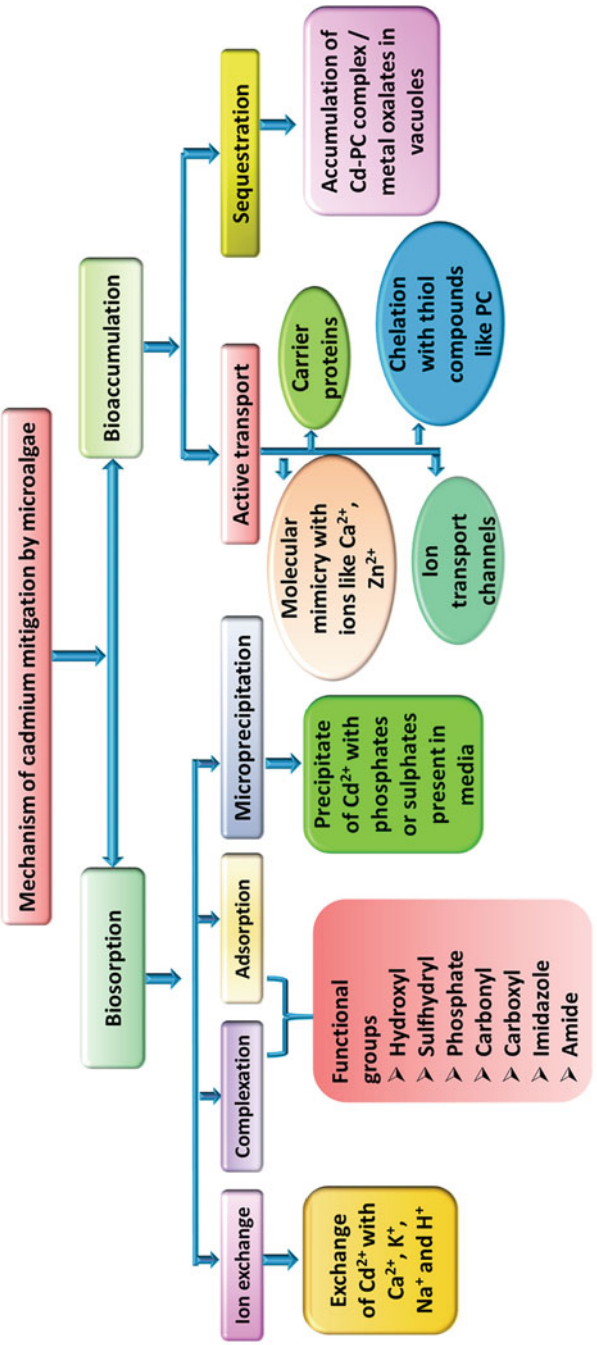

Fig. 4.1 Flowchart showing the mechanism of cadmium detoxification by microalgae

of 76.4% of Cd with biosorption efficiency of 1.168 mg/g dry cell weight with 0.025 g of initial algal biomass (El et al. 2019).

Bioaccumulation is an energy dependent process aided by metabolically active cells for intracellular uptake of heavy metals (Ahalya et al. 2005). Several mechanisms have been proposed to explain this phenomenon. One is dedicated to saturation of binding groups on the cell surface, eventually aiding the transportation of metals inside the cells. Molecular similarity of toxic heavy metals with essential metals allows their entry through the constitutive ions channels in cell wall. Once inside the cells, the most common method of intracellular detoxification is activated via synthesis of phytochelatins or metallothioneins (III) and their complexation with metal ions. These organometallic complexes help in sequestration of toxic metal ions into compartments like vacuoles to neutralize cytosolic toxicity. Other important strategy for detoxification of heavy metals is the secretion of oxalic acids which help to stabilize the metal in its soluble metal oxalate, aiding its accumulation in cell organelles (Priyadarshini et al. 2019). Along with organic acids and other metabolites, polyphosphate bodies present in algae cells interact with the divalent ions transporting them to the vacuolar system (Monteiro et al. 2012a).

Phytochelatins (PC) are low molecular weight peptides with general structure of (γ-Glu-Cys) *n*-Gly, where n varies from 2 to 11 depending on the metal and species (Monteiro et al. 2012b). They are cysteine rich in nature and bind to heavy metal ions by their thiol groups (Priyadarshini et al. 2019). PCs were first discovered in higher plants; Stokes discovered phytochelatin as a metal chelating agent in *Scenedesmus acutiformis* in 1977 (Stokes et al. 1977). Cadmium is a well-known activator of phytochelatins in microalgae followed by lead, zinc, and other heavy metals. Several studies reported induction of phytochelatin synthase (PCS) at a threshold of 20 μM cadmium in *Chlamydomonas* cells (Gekeler et al. 1988; Howe and Merchant 1992). Presence of cysteine in PC is the key part of chelating core. It plays an important role in activating phytochelatin synthase thereby promoting synthesis of phytochelatins as the prime mechanism behind detoxification of heavy metals in plants and algae (Oven et al. 2002; Gekeler et al. 1988). Based on the presence of sulfide ions in PC-metal complexes they are generally categorized into two groups, namely low molecular weight complex (LMWC) and high molecular weight complex (HMWC) (Hu et al. 2001; Perales-Vela et al. 2006). Sulfide ions are incorporated in the form of nanosized particles in HMWC enhancing its stability with metal ions (Scarano and Morelli 2003). LMWC chelates ions through their thiol groups and get converted to HMWC to sequester Cd–PC complex in a more stable manner (Hu et al. 2001). Another study in freshwater microalgae *Scenedesmus vacuolatus* reported the concentration dependent (from 10^{-14} to 10^{-7} M) intracellular accumulation of Cd compensated by induction in thiol compounds such as glutathione, PCs, and γ-glutamylcysteine (Le Faucheur et al. 2005). Comparative sensitivity of two different freshwater microalgae *C. reinhardtii* and *Pseudokirchneriella subcapitata* exposed to free cadmium concentration ranging from 0.73 to 253 nM was demonstrated along with subcellular partitioning within organelles, granules, cellular debris, heat stable proteins (HSP), and heat denaturable proteins (HDP). The authors reported *C. reinhardtii* to be more tolerant than *P. subcapitata* by having

maximum fraction of Cd accumulated in organelles (40%) followed by HSP (30%), HDP, and cell debris when exposed to 0.7 nM of free cadmium. On the contrary, opposite trend at high Cd concentration (221 nM) was observed with 70% fraction in HSP followed by 20% in organelles (Lavoie et al. 2009). A recent study done by Belgith et al. showed an increase in total metallothionein concentration in a dose dependent manner ranging from 25 to 150 mg/L cadmium in *D. salina* (Belghith et al. 2016). Some studies also established the accumulation of cadmium inside cells as black deposits in TEM micrographs and EDX analysis in *Ankistrodesmus falcatus, Chlorella pyrenoidosa*, *S. quadricauda*, and *C. reinhardtii* (Silverberg 1976; Samadani et al. 2018). All these studies suggested the importance of phytochelatins as chelating agent in microalgae in response to cadmium ions. In order to maintain the regulatory networks of algal cells, the metallic complexes are hydrolyzed and excess metal ions are pumped out through efflux active transporters in their cell wall. This phenomenon also facilitates the cell to adapt and survive under continuous exposure of toxic metals (Levy et al. 2008; Priyadarshini et al. 2019).

4.4.1 Physiological and Morphological Response of Microalgae to Cadmium Toxicity

Microalgae fix atmospheric CO_2 via photosynthesis and produce a variety of energy molecules such as carbohydrates, lipids, and proteins which are the basis for their utilization as feedstock for energy production (Ran et al. 2019). They are capable of removing nutrients and heavy metals from polluted waste streams (Rodgher et al. 2012; Marchello et al. 2018). Microalgae are the primary link which transfers the metal cations to higher trophic levels in the food chain. Heavy metals such as Cd interfere with the normal cycle of CO_2 fixation and the production of energy reservoirs. Cd ions primarily react with carboxyl, sulfhydryl, and imidazole groups of important enzymes to inhibit processes such as growth, photosynthesis, photorespiration, starch biosynthesis, protein degradation, and lipid degeneration (Wang and Wang 2009; Belghith et al. 2016). For example, Cd toxicity in *D. salina* showed inhibitory action on chlorophyll synthesis and growth. The study also showed remarkable reduction in both soluble and insoluble carbohydrates, whereas protein content increased. This increase in protein content was corroborated with enhanced metallothioneins against Cd accumulation inside the cells (Belghith et al. 2016). Similarly, *Synechocystis* sp. showed 68% inhibition in growth when exposed to 8 mg/L of Cd ions in media leaving pronounced effect on chlorophyll a, carotene, and phycocyanin content (Arunakumara and Xuecheng 2009). Recent studies in *Scenedesmus rotundus* and *Monoraphidium* sp. also followed the similar trends of increased protein content under Cd stress along with reduction in growth and photosynthetic pigments (Shivaji and Dro 2019; Zhao et al. 2019). *Monoraphidium* sp. showed reduction in carbohydrate content compensated by an increase in lipid accumulation. This dynamic shift in energy reservoirs clearly represents the adaptive capability of microalgae cells to cope in Cd toxicity (Zhao et al. 2019). In contrary, *D. salina* showed a decrease in protein content when assessed at both physiological

and molecular end. This study depicted effect of 0.5 and 2.5 mg/L Cd on microalgae cells after 6 and 96 h showing prevalence of time-dependent cadmium toxicity. Increased synthesis of photosynthetic pigments along with an active antioxidant system showed instant protective mechanism adapted by the cells. In total 30 ribosome genes in *D. salina* were up-regulated after 6 h of Cd exposure, whereas down-regulated after 96 h, thus reducing the protein content. This study suggested ribosomes as the direct target of Cd ions providing a better understanding towards temporal dynamics and an adaptive strategy of cells against metal toxicity (Zhu et al. 2019).

In order to understand the detailed molecular response of cadmium toxicity, few studies were conducted in heterotrophic and mixotrophic mode to mimic the real conditions of wastewater. Wastewaters comprise a significant quantity of organic contaminants influencing the cultivation of microalgae in the open environment (Kováčik et al. 2017; Marchello et al. 2018). *S. acutiformis* was cultivated with 1 and 10 μM Cd under a heterotrophic condition by adding glucose and casein in the media. This led to 13–16% increase in biomass of *S. acutiformis* suggesting the positive influence of 10 μM Cd. In contrary to this, a little depletion in chlorophyll autofluorescence was observed due to the absence of light, whereas protein content remained constant (Kováčik et al. 2017). This study indirectly suggests an adverse effect of Cd ions on carbon assimilation in phototrophic conditions resulting in loss of pigments (Arunakumara and Xuecheng 2009). Ecotoxicological effects of Cd were assessed by comparing the phototrophic and mixotrophic cultivation of microalgae. This study manifests the role of mixotrophy in lowering the inhibitory effects of Cd on cells. Glucose as an organic carbon source showed positive effects in mixotrophic systems representing an unaffected photosynthetic efficiency (Fv/Fm) until encountering high Cd concentration (10^{-5} mol/L). Although toxic effects of Cd on carbohydrate content were similar in both cultivation modes, mixotrophic mode was more efficient in maintaining cell viability, chlorophyll content, and protein: carbohydrate ratio (Marchello et al. 2018). Pronounced degradation of carbohydrates in Cd toxicity inside the microalgae cells could be explained by considering the interactions of these heavy metal ions with reactive sites in enzymes such as ribulose biphosphate carboxylase involved in glycolysis (Stiborová et al. 1987). Protein degradation might be the result of the displacement of essential cofactors in enzymes like Cu, Fe, and Mn involved in photosynthetic electron transport (Miazek et al. 2015).

Increase in cell size of *C. acidophila* along with deposition of polyphosphate bodies was observed on cadmium accumulation inside the cells (Nishikawa et al. 2003). *Chlamydomonas* strains were extensively studied for deleterious effects of Cd. The wild strains of *Chlamydomonas* showed an increase in the cell size (Jamers et al. 2009; Samadani et al. 2018). On the contrary, acid-tolerant strain of *Chlamydomonas* CPCC 121 does not show any significant changes in cell size under Cd toxicity (Samadani et al. 2018). Another study on Cd resistant freshwater microalgae *Dictyosphaerium chlorelloides* showed a significant decrease in the cell surface exposed to cadmium varying from 1.82 μm^2 in wild type cells to 0.71 μm^2 in the mutant strain under 100 μM Cd (Bartolomé et al. 2016). All these studies showed

the physiological and morphological variations of microalgae cells in response to Cd toxicity.

4.5 Indicators of Cadmium Stress in Microalgae: Enzymatic and Non-enzymatic Markers

Microalgae possess both extracellular immobilization and intracellular detoxification mechanisms to cope in toxic concentrations of cadmium (Branco et al. 2010). Once cadmium ions enter the cells, they competitively bind to functional groups and disrupt the essential metabolic activity of enzymes causing disintegration of key molecules like lipids and proteins, thus affecting the molecular machinery (Priyadarshini et al. 2019). Generally, there are a series of aerobic reactions executed inside the cells resulting in the generation of ROS (reactive oxygen species) as well as its removal at the same rate (Zhu et al. 2019). Accumulation of cadmium inside the cells leads to production of excess ROS resulting in membrane damage, lipid peroxidation, inactivation of enzymes, and eventually cell death (Piotrowska-Niczyporuk et al. 2012). To ensure survival in such toxic conditions, microalgae have developed unique and robust antioxidant machinery which comprises both enzymes and metabolites (Sytar et al. 2013). These two components work hand in hand to scavenge ROS species to prevent expected damage to the cells. All these events are composed of different and complex reactions, therefore, attracting our interest to unveil the ROS scavenging mechanism along with stress biomarkers.

ROS mainly constitutes $^1O_2$ (singlet oxygen), H_2O_2 (hydrogen peroxide), $O^{\bullet-}{}_2$ (superoxide radical), and $OH^{\bullet}$ (hydroxyl radical) (Das and Roychoudhury 2014). Among these, the major threat to photosynthetic organisms is due to superoxide radicals generated during electronic reduction of molecular oxygen in the electron transport chain. Many essential trace metals are involved as a cofactor of different enzymes and metalloenzymes such as Mn-superoxide dismutase (Mn-SOD), Fe-superoxide dismutase (Fe-SOD) in thylakoid to scavenge the produced ROS (Pinto et al. 2003). Cadmium actively displaces the metals in Mn-SOD and Fe-SOD preventing the detoxification process, thereby elevating oxidative stress. Resultant superoxide radical generated during oxidation–reduction in PS I eventually diffuses to stroma and gets dismuted into O_2 and H_2O_2. Further the interaction of reduced metal ions with H_2O_2 forms $OH^{\bullet}$ (Takeda et al. 1995; Pinto et al. 2003). In addition to this, polyunsaturated fatty acids present in chloroplast membrane being prone to peroxidation undergo severe damage (Halliwell and Gutteridge 1999). Different heavy metals follow different sequence of reactions to elevate ROS generation. Heavy metals like Fe^{3+} and Cu^{2+} produce $OH^{\bullet}$ by Haber–Weiss cycle (Winterbourn 1982), whereas Cr^{+6} utilizes Fenton-type mechanism (Shi and Dalal 1990). Cd^{2+} being a non-redox element exclusively targets the reduction of glutathione (GSH) pool, and interferes with the calcium and iron mediated processes inside the cells leading to $^1O_2$ and $O^{\bullet-}{}_2$ production (Asada 1987; Nowicka et al. 2016).

The antioxidant system comprises low molecular weight compounds like carotenoids, tocopherols, glutathione (GSH), phenols, ascorbate, flavonoids, etc.,

along with high molecular weight antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), glutathione peroxidase (GPX), and glutathione *S*-transferase (GST) (Pinto et al. 2003; Liu et al. 2017). Carotenoids that are present in thylakoid membranes are dedicated for protective functions (Mallick and Mohn 2000). They play major roles in light harvesting to scavenge excess ROS generated in the chloroplast protecting the cell apparatus from photochemical damage (Frank and Cogdell 1996; Pinto et al. 2003). For example, a recent study showed an increase in carotenoid content under 0.5 and 2.5 mg/L cadmium stressed *D. salina* cells suggesting a compensatory mechanism (Zhu et al. 2019). Few other studies reported a decrease or no change in carotenoid content in response to cadmium. For instance, 3–7 mg/L of cadmium resulted in 72% reduction in the total carotenoid content of *Chlorella vulgaris* (Cheng et al. 2016). Another study in *C. reinhardtii* showed no change in carotenoid contents, whereas a total increase in plastoquinone was reported (Nowicka et al. 2016). Other important low molecular weight metabolites are glutathione and ascorbate. GSH is non-proteinaceous and basically represents the pool of reduced sulfur (Mallick and Mohn 2000). It is a tripeptide thiol (γ-Glu-Cys-Gly) having broad redox potential and ROS scavenging capacity (Branco et al. 2010). Sulfhydryl (thiol) group of GSH also helps to reduce peroxides formed during the partial reduction of O_2 (Pinto et al. 2003). Ascorbate present in both cytosol and chloroplast plays a vital role in removing excess H_2O_2 via ascorbate–glutathione cycle. It acts as an antioxidant by maintaining the cycle of reduction and oxidation by consuming the reducing equivalents like (nicotinamide adenine dinucleotide phosphate hydrogen) NADPH along with GSH to maintain ascorbate pool (Mallick and Mohn 2000). Flavonoids are another group of antioxidant molecules widely distributed in plants categorized as secondary metabolites. They have an important role in scavenging ROS molecules by inhibiting the lipid peroxidation of chloroplast membrane (Das and Roychoudhury 2014). Flavonoids bound to metal ions through their hydroxyl group of phenols reducing the toxicity of heavy metals in the cell (Pinto et al. 2003). Another lipophilic antioxidant molecule is tocopherol, vital for preventing the lipid peroxidation by scavenging singlet oxygen and superoxide radicals (Stahl et al. 2019). *C. reinhardtii* in presence of 20–100 μM cadmium showed an increase in tocopherol along with plastoquinone as an antioxidant response to prevent oxidative damage (Nowicka et al. 2016).

With regard to enzymatic antioxidant systems, SOD acts as the primary line of defense catalyzing the disproportion of superoxide radicals to oxygen and hydrogen peroxide. It is a metalloenzyme with two different isoforms in *C. reinhardtii*, one as Mn-SOD in mitochondria and the other as Fe-SOD in chloroplast (Wu et al. 2009). Few recent studies reported the enhanced expression of both forms of SOD in response to cadmium in *C. reinhardtii* (Nowicka et al. 2016). In contrary, *D. salina* exhibited reduced SOD activity in cadmium exposed cells (Zhu et al. 2019). CAT is a heme containing enzyme which catalyzes dismutation of H_2O_2 to H_2O and O_2. It is a ubiquitous enzyme but its absence in chloroplasts needs active APX (Mallick and Mohn 2000). GR alleviates the stress by maintaining GSH/GSSG pool which regulates the cell metabolism especially in case of metal toxicity

(Schaedle and Bassham 1977). GPX is a cytosolic enzyme catalyzing conversion of H_2O_2 to H_2O and its respective alcohol, whereas GST is involved in reduction of hydroperoxides into less harmful alcohols and maintains a redox homeostasis inside the cells by regenerating ascorbates (Hossain et al. 2012). All these molecules act in a regulatory manner compensating to prevent oxidative damage under heavy metal toxicity.

For example, exposure to 0.5 and 1 mg/L of Cd led to an increase in SOD activity by 34.2%, CAT activity by 38.79%, and GR activity by 92.4%. However, further increase in Cd concentration led to decreased activity of SOD and GR suggesting severe damage to the algae system (Cheng et al. 2016). Similarly, time and concentration dependent study of cadmium exposure to *D. salina* showed an increased activity of SOD and GST in all concentrations (0.1–2.5 mg/L) just after 6 h, whereas increase in GR, GPX, and APX activity was observed at higher doses. On the contrary, prolonged incubation for 96 h led to reduced SOD and GR activity under 2.5 mg/L of Cd (Zhu et al. 2019). Another study on cadmium stressed *Nitzschia palea* highlighted an increase in GSH and proline content along with SOD and CAT activity as a survival mechanism to minimize damage (Branco et al. 2010). On a similar note, *Monoraphidium* sp. QLY-1 under 40 μM cadmium showed a significant increase in ROS species and high level of lipid peroxidation (Zhao et al. 2019). The possible toxic effects of Cd and the adaptive response of microalgal cells have been shown in Fig. 4.2. These studies show that oxidative stress and antioxidant response of microalgae cells are specific to every other microalgae species and need to be explored more to unravel the underlying ROS scavenging mechanism.

4.6 Integration of Cadmium Mitigation with Bioenergy Production

Recent advancements in industrialization and technologies accompany several considerable issues that are gaining attention among scientific community throughout the globe. Especially, the anthropogenic activities with excessive generation of CO_2 interfere a lot with nature leading to remarkable climate change (Raheem et al. 2018). Further, the ungenerous utilization of fossil fuels incentivizes development of better alternatives outcompeting petroleum based fuels. In fact, 87% of the total CO_2 emitted globally is due to utilization of fossil fuel (coal, oil, and natural gas) (Raheem et al. 2018). Over the past decades, biofuels have gained momentum towards establishment of sustainable and eco-friendly approach. Various biomass sources including crops and lignocellulosic plants were regarded as first and second generation biofuels, respectively (Sayed et al. 2019). Utilizing food crops such as wheat, maize, rapeseed, soybean for bioethanol and biodiesel production led to food versus fuel dilemma (Doshi et al. 2016), whereas pretreatment steps required in converting lignocellulosic biomass to biofuel (bagasse for bioethanol, Jatropha, palm oil for biodiesel) were expensive (Sayed et al. 2019). Both first and second generation biofuels suffer from certain limitations like requirement of arable land

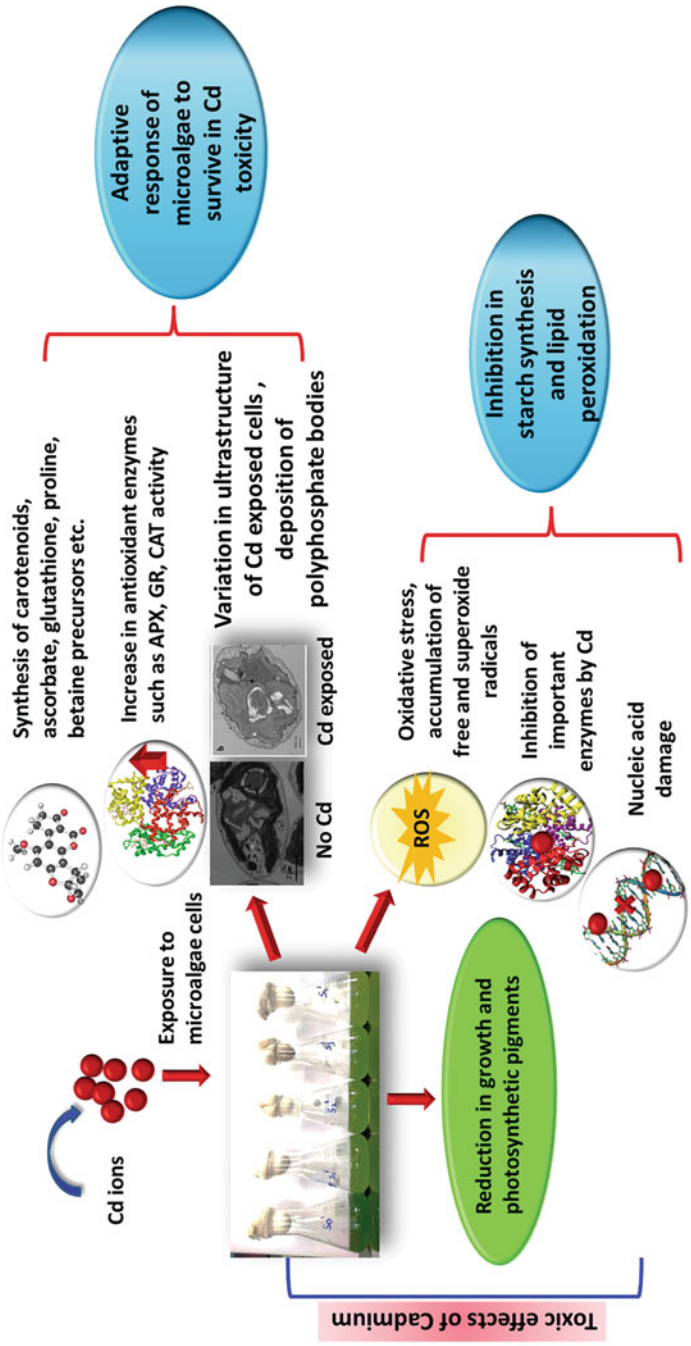

Fig. 4.2 Toxic effect and adaptive response of microalgae in presence of cadmium

and high generation time leading to competitive situation between food crops and cost associated with non-edible sources of biomass (Maity et al. 2014).

Recently, microalgae have emerged as the most promising feedstock for sustainable production of biofuel and are categorized as third generation biofuel. Microalgae encompass large reservoirs of energy in the form of carbohydrates, lipids, and proteins along with other commercially valuable metabolites like antioxidants and carotenoids (Sayed et al. 2019). Microalgae biomass confers advantages such as short generation time, ability to grow in wastewater throughout the year without any pretreatment make them preferable over terrestrial plants (Stephens et al. 2010). In spite of all these advantages, the cost associated with microalgae derived biofuel is quite high in comparison to present petroleum based fuel. Currently, global consumption of 82.5 million barrel oil is reported per day which is expected to rise at least by 60% in near future (Godfray et al. 2010; Arora et al. 2018). In such scenario, microalgae biofuel holds the capability to fulfill the expected rise of 16% energy in transportation sector by 2040, provided with reduced cost (IEAS 2017). A recent report estimated that implication of new technologies has reduced the cost associated with algae derived biofuel to US$2.80/L in comparison to the past years. It could be further reduced by adopting integrated systems for producing high value products (animal feed, food supplement) and treating wastewater along with fuel production (Chia et al. 2018; Correa et al. 2019; Sayed et al. 2019).

Among these approaches, integrating wastewater treatment with biofuel production prospects a cost-effective method (Mandotra et al. 2020). There are many studies for removal of nutrients (nitrate, phosphate, TOC (total organic carbon), COD (chemical oxygen demand), and BOD (biochemical oxygen demand)) by microalgae as a cost-effective approach of biodiesel (Kothari et al. 2012; Arora et al. 2019), but a little attention is paid towards heavy metal containing wastewaters due to their associated toxicity (Cao et al. 2014; Yang et al. 2015; Tripathi et al. 2019). Microalgae tend to accumulate lipid bodies in response to abiotic stress such as light, salinity, nutrient deprivation, having plentiful studies online (Pal et al. 2011; Gao et al. 2013; Takeshita et al. 2014; Mohan et al. 2014). Recently, few researchers have tried to explore microalgae for remediating heavy metals such as arsenic, chromium, cadmium, copper, lead, and zinc along with biodiesel production (Yang et al. 2015; Arora et al. 2017; Zhao et al. 2019). Yang et al suggested that wastewater-algae biofuel-heavy-metal integrated utilization (WABHMIU) technology could reduce the cost of algae biofuel production, improving the wastewater treatment efficiency (Yang et al. 2015). Earlier few studies demonstrated the synergistic effect of cadmium, nitrogen, and phosphorus in *Chlorella vulgaris* and suggested the modulatory effect of cadmium on lipid contents (Chia et al. 2013a, b). This study demonstrated effect of different combination of varying cadmium (2×10^{-8}; 10^{-7}M) and nitrogen (2.9×10^{-6} to 1.1×10^{-3} M) concentration led to dominance of TAGs along with phospholipid (PL) and acetone mobile polar lipids (AMPL) (Chia et al. 2013b). Schematic showing pathway of lipid induction in microalgae cells has been depicted in Fig. 4.3.

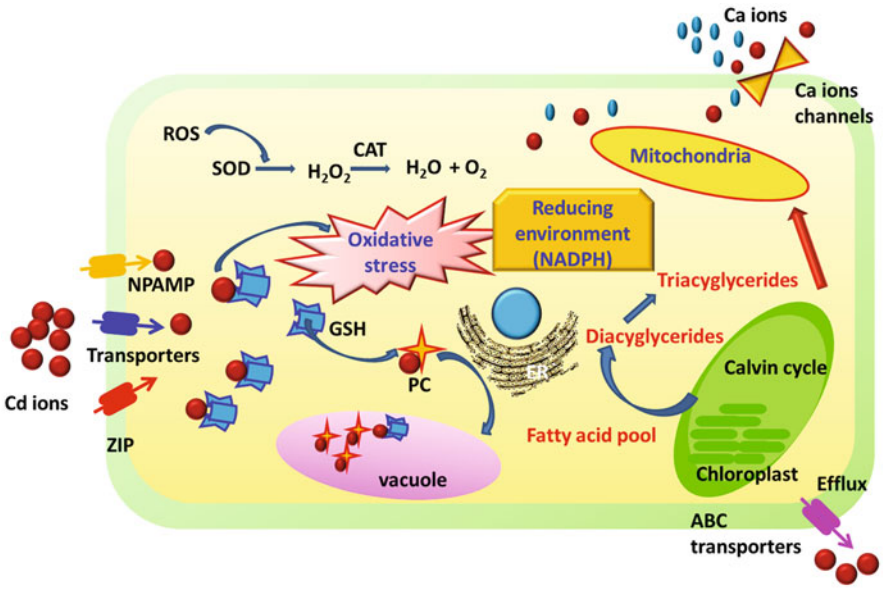

Fig. 4.3 Schematic showing the integrated approach of cadmium chelation and lipid production using microalgae cells

Cadmium being a toxic and non-essential element is known to inhibit growth of microalgae by disrupting essential metabolic activities, leading to lipid and protein degradation. But few recent studies reported positive influence of cadmium stress lipid accumulation in microalgae. For example, *C. minutissima* UTEX2341 accumulated 21% lipid content under 0.4 mM cadmium along with a removal efficiency of 74%. In contrast, higher concentration caused degradation of lipid content to 15% (Yang et al. 2015). Similarly, *Monoraphidium* sp. QLY-1 showed significant induction in lipid portion under 40 μM of cadmium in the media. The fatty acid profile of the QLY-1 under cadmium stress showed development of biodiesel having higher cetane number and eventually good fluidity and combustion property (Zhao et al. 2019). Another study demonstrated ability of two microalgae species, *Desmodesmus* sp.MAS1 and *Heterochlorella* sp. MAS3 grown in acidophilic condition to mimic the real scenario of leaching mine areas at pH 3. These microalgae species show rapid growth in presence of cadmium in the same condition and produced lipid rich biomass (Abinandan et al. 2019). This was also confirmed by the TEM images of *D. salina* having dark depositions under cadmium stress in comparison to control cells (Zhu et al. 2019). Furthermore, transcriptome data of *Auxenochlorella protothecoides* UTEX234 illustrated the role of different transporters involved in the uptake and efflux of Cd, thus preventing damage of lipid biosynthesis pathway.

Transporters like resistance-associated macrophage proteins (Nramp), Multi-antimicrobial extrusion protein (MATE), Zrt-Irt like proteins (ZIP), and Ca ion

channels allow Cd ions across the cell membrane leaving the cell in shock. It induces the activation of antioxidant system and relieves the cytotoxicity by emanating the excess Cd via efflux transporters such as ATP-binding cassette (ABC) present on the cell membrane (Lu et al. 2019). Figure 4.3 represents the schematic showing the possible mechanism of lipid induction in presence of Cd inside microalgae cell. Thus, this integrated approach is quite useful for production of microalgae biomass rich in neutral TAGs and aliphatic fatty acid hydrocarbons that could be converted as sustainable biofuels (Kumar et al. 2016). In order to execute such system, selection of potent microalgae strains from extreme environment having ability to thrive under toxic conditions is also of prime importance.

4.7 Conclusions and Future Perspectives

Ungenerous release of toxic pollutants such as cadmium is leading to deadly diseases like cancer, renal disturbances, liver, and kidney damages in human. Emergence of microalgae for removal of heavy metals as the most sustainable, eco-friendly, and cost-effective alternative make them jewels of nature. Other than utilization of sole microalgae biomass as biosorbent, modification by immobilization with alginate beads and biochar also enhanced the removal efficiency, making the overall process more impactful. Recent studies evidenced the key pathway of cadmium detoxification with primary genes targeted by Cd inside the cells. However, the unique behavior of different microalgae strains in the presence of Cd does not allow a single hypothesis to be universal. Further, understanding the expression of stress biomarkers also enhances our understanding towards the molecular mechanism of Cd detoxification. Integrating the Cd mitigation with production of TAG molecules as a source of bioenergy will be a cost-effective and sustainable energy conservation methodology.

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Algae as Miniature Wastewater Scavengers

5

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Abstract

Anthropogenic activities are accountable for the release of various inorganic and organic substances into the environment leading to severe environmental pollution. Effluents released from various industrial, domestic, and agricultural sources ultimately find their way into water bodies rendering the water unfit for use. There is a pressing need to treat this water in order to meet the prerequisites of the growing human population. The primary and secondary treatment of wastewater removes the settled particles and organic matter providing apparently clear water.

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The secondary effluvium is although laden with inorganic forms of nitrogen (NO_3^- – N; NO_2^- – N; NH_4^+ – N) and phosphorus (PO_4^{3-} – P) which causes eutrophication and other indelible issues due to the occurrence of heavy metals and refractory organic substances. Microalgae act as a very significant tool in treatment (tertiary) of wastewater and provide large quantities of invaluable multi-purpose algal biomass. They are very efficient in sequestering inorganic nutrients from wastewaters due to their high productivity rate and lesser space requirements compared to other terrestrial plants. Their property of sequestering toxic substances and heavy metals does not lead to the secondary pollution. Thus microalgae act as scavengers of the environment removing the pollutants from the wastewater and making it reusable at lower investments.

Keywords

Anthropogenic activities · Eutrophication · Secondary pollution · Heavy metals · Sequestration

5.1 Introduction

The sewage produced during wastewater treatment, causing severe environmental impacts has gained tremendous recognition. Sewage is basically, the waterborne domestic and industrial waste. It is composed of organic and inorganic material either in soluble or suspended form. The substances which are either organic or inorganic in nature, when released into the surroundings due to industrial, agricultural or domestic activities are responsible for organic and inorganic pollution (Mouchet 1986; Lim et al. 2010). The aquatic pollution has become a burning issue for its direct impact on the health, environment, and economy of the organisms. The sewage needs to be treated aerobically before its disposal because of the foul smell produced during its anaerobic digestion. Moreover, it is a rich source of many inorganic nutrients like nitrogen, sulfur, potassium, and phosphorus. If the sewage is treated properly, it can also serve as fertilizer. Environmental laws and their applicability have become increasingly strict (Abdel-Raouf et al. 2012). Pollution caused due to the presence of pathogens, organic pollutants, and heavy metals in the sewage when compared to the cost for disposal of residual sludge which is about 25–60% of the overall expense of handling a treatment plant for wastewater (Zhang et al. 2009).

Algae, however, play a very significant part in the oxidative sewage breakdown. This process is carried out in small tanks and the oxygen required by bacteria is provided by algae during their photosynthesis. Currently, the wastewater purification using microalgae-based processes is under limelight for their tremendous capabilities of metal uptake and nutrient sequestration from wastewater. This process also involves the huge microalgal biomass production and that too inexpensively, that later on can be utilized for the production of fertilizers or biofuels (Van Wagenen et al. 2015; Mandotra et al. 2020; Bag et al. 2019). During this whole process, the algae supply O_2 required for bacterial respiration, in return the bacteria provide the CO_2 needed for their photosynthetic process (Munoz and Guieysse

2006). Also, the algal-bacterial biomass assimilates the nitrogen and phosphorus loaded in the wastewater efficiently (Decostere et al. 2016).

5.2 Wastewater Composition

Water bodies receive pollutants from numerous inputs varying both in their power and extent. Wastewater is the offshoot generated from any activity or process. It could be from factories, industries, households, textile industries, landfills, aquaculture, agriculture, petrochemical industries or any other source (Abdel-Raouf et al. 2012). Organic and inorganic pollution in these wastewaters is a regular trend. The presence of huge quantities of organic compounds is known as organic pollution and in case of inorganic pollution the inorganic substances are present in bulk (Kamyab et al. 2014; Priac et al. 2017). The sources of these organic compounds are from domestic sewage, agriculture, treatment plants, urban run-off, aquaculture, and industrial effluents, such as food processing, paper, and pulp making wastes. Pesticides, hydrocarbons, fertilizers, plasticizers, phenolic compounds, biphenyls, greases, oils, detergents, and pharmaceuticals are some of the usual organic pollutants (Ali et al. 2012). Heavy metals, fluorides, and arsenides are some of the common inorganic toxic pollutants (Ali 2010; Cao and Li 2014) received from industries, such as paint manufacturing, agriculture, etc. (Rule et al. 2006). They also include calcium, bicarbonate, sodium, potassium, chlorine, magnesium, ammonium salts, sulfur, phosphate, and heavy metals (Lim et al. 2010). Sewage water is a composite blend of natural organic, inorganic, and synthetic substances. The dumping sites of these pollutants are responsible for several health related problems and hence a solution needs to be sought (Horan 1990). Water scarcity, energy, and food needs are the forces behind the exploration of various wastewater recycling techniques to meet the growing demands of the human population (De la Nouë and De Pauw 1988).

5.2.1 Sewage Microbial Composition

The microorganisms in wastewater form well structured and functional communities in which they are bound to the organic and inorganic particles by extracellular polymeric compounds. The organic contaminants from wastewater are removed by various groups of heterotrophic microorganisms. The removal of biogenic elements (phosphorus and nitrogen) is carried out by phosphate accumulating organisms and nitrite and ammonium oxidizing bacteria (Kallistova et al. 2014). Since sewage water provides a favorable environment for the growth of wide variety of microbes including bacteria, viruses, and protozoa. A large group of these micro-organisms are non-toxic and act as an important tool for biological sewage water treatment, but few of them are pathogenic also, and are excreted by a symptomatic carrier or sick individuals. Different diseases caused by microbes including bacterial diseases (typhoid, cholera, and tuberculosis); viral diseases (hepatitis); protozoan diseases

(dysentery) are all found in wastewater (Glynn Henery 1989). Moreover, eggs of parasitic worms are also present in such waters (Shaaban et al. 2004). The extent with which the total coliform organisms are eliminated estimates the efficiency of disinfecting sewage (Sebastian and Nair 1984).

5.3 Methods of Treatment

5.3.1 Conventional Method

The main focus in conventional wastewater treatment method was on removal of nutrients particularly dissolved nitrogen and phosphorus (in the form of nitrate, nitrite, ammonium, and phosphate) as they occur in higher quantities in wastewater. Moreover, the elimination of coliform bacteria, suspended solids, biochemical oxygen demand (BOD), and toxic substances is the main motive for getting decontaminated sewage water (Abdel-Raouf et al. 2012). Their higher concentrations is due to various anthropogenic sources causing eutrophication in water bodies (Camargo and Alonso 2006), which leads to an escalated frequency of algal blooms (Liu et al. 2013) and ultimately causing hypoxia (Zhu et al. 2011). In most of marine and fresh water ecosystems, eutrophication causes a diminution in coral reef health and communities, increases fish mortality rate, and decreases water transparency (Smith and Schindler 2009). There are various other consequences of excess nitrogen concentrations ($>45\ gm^{-3}$) in drinking water including methemoglobinemia, virulence caused due to non-ionized ammonia to aquatic animals particularly fishes and interference with disinfection where chlorination is done (Lincolin and Earle 1990).

5.3.2 Preliminary Sewage Treatment

The initial step of wastewater treatment includes the removal of coarse biodegradable material and debris from the waste stream in order to stabilize the wastewater by equalizing or adding chemical to it. Primary treatment mainly refers to a sedimentation process prior to secondary treatment. It separates larger sized materials that could clog the flow through the treatment plant. These include solid objects such as wood, rags, fecal matter, and gravel particles. The sewage is passed among bars separated by a distance of 20–60 mm to remove larger sized objects; the left over material is gathered from the bars at uniform periods of time (Tebbutt 1983). Grit which is the heavier inert matter in wastewater and does not decompose in treatment processes is detached by decreasing the velocity of the flow to a point which makes the grit and silt to settle. This velocity of the flow ranges between 0.2 and 0.4 m/s leaving the organic matter in suspension (Gray 1989).

5.3.3 Primary Sewage Treatment

The elimination of coarse material is followed by the passing of sewage into the sedimentation tanks. It removes the settleable solids by gravity (about 70%). About 40% of BOD as settleable solids can be removed by a well designed sedimentation tank (Horan 1990). Pathogen removal varies highly as different organisms are reported to have different removal rates (Pescod 1986; Gray 1989). Preliminary and primary processes remove approximately 25% of the organic load and virtually all of the non-organic solids in case of domestic wastewater, whereas in industrial waste treatment, these treatments include adjustment of pH, equalization of flow or addition of chemical that is paramount for the entire treatment process.

5.3.4 Secondary Sewage Treatment

The secondary treatment reduces the biological oxygen demand (BOD) employed by the diminution of substances which are organic in nature. This is carried out by adding a mixture of organic matter utilizing heterotrophic bacteria which employ the wastewater for their growth, development, and energy requirements. The aerophilic oxidation of BOD is carried out at many available biological unit operations. On the basis of the microbial populations, these operations can be categorized into fixed film or dispersed growth processes. In case of fixed film reactors, the organic compounds are separated by their adsorption to the biofilms which are attached to a firm surface and get degraded oxygenically by microorganisms. The examples of such growth processes are trickling filters and rotating contractors. In case of suspended growth reactors, the suspension of wastewater and microorganisms is done by using air diffusers or by mechanical agitation (Horan 1990). Examples include activated sludge systems, ponds, and lagoons. Studies have revealed that about 90% of pathogenic bacteria are removed by biological oxidation systems from sewage waters; however, this figure varies markedly in case of viruses. The later are effectively removed by adsorption method. The suspended growth reactors give 90% of removal due to mixing of solids flocs and sewage, whereas film reactors show varied reduction rates due to smaller biological adsorption sites (Gray 1989; IAWPRC Study Group 1991).

5.3.5 Tertiary Sewage Treatment

Tertiary process of sewage treatment points at removing all ions which are organic in nature. It can be carried out chemically or biologically. The chemical tertiary treatment is too expensive compared to the biological one which performs comparatively well and can be implemented in most places and there are usually chances of secondary pollution in case of chemical treatment. Moreover, each supplementary step of treatment significantly enhances the total expenditure in a wastewater system (Oswald 1988). An entire tertiary process is about four times more costly than first

level of treatment for removing ammonium, nitrate, and phosphate in wastewater (De la Nouë et al. 1992). Likewise next treatment step will be greater than eight times more costly than previous one. These different levels of treatment are done for the removal of soluble minerals, organic substances, and heavy metals (Oswald 1988). Further treatment levels are based on complicated methods such as ozonation, chemical precipitation, carbon adsorption or reverse osmosis. These techniques are meant for removal of a selected nutrient from the wastewater.

5.4 Microalgal Wastewater Treatment

The micro- as well as macroalgae has been cultured since ages due to its application in wastewater treatment plants involving the mass scale production of algal species like *Dunaliella* and *Chlorella*. Macroalgae known as seaweeds also play a significant role of acting as an adsorbent to replace the functional activated carbon in wastewater treatment process. Biological treatment using microscopic-algae is highly striking (De la Nouë and De Pauw 1988) because of the unique algal photosynthetic abilities responsible for conversion of sun energy into valuable biomasses and absorbing eutrophication-causing nutrients into their biomasses. This initiative of using microalgae in treatment process was launched by Oswald and Gotaas (1957) has since then been thoroughly experienced throughout the world (Goldman 1979; Shelef and Soeder 1980; De Pauw and Van Vaerenbergh 1983).

The vast distribution of microalgal genera in wastewater ponds was studied by Palmer (1974). The algae found in their study, in the order of the frequency of abundance were *Chlorella*, *Ankistrodesmus*, *Scenedesmus*, *Euglena*, *Chlamydomonas*, *Oscillatoria*, *Micractinium,* and *Golenkinia*. The attempts are being made to develop hyper-concentrated algal cultures in the wastewater treatment systems as the space requirements of such systems are sizeable (De Pauw and Van Vaerenbergh 1983). This will help in efficient removal of nutrients from the wastewater and that too in very short time intervals of less than 1 h of time (Lavoie and De la Nouë 1985). Algae can treat almost all types of anthropogenic wastes including human sewage, agro-industrial wastes, livestock wastes, and wastes from industrial sources (Lincoln and Hill 1980; Phang 1990, 1991; Ibraheem 1998; Kaplan et al. 1988). It may also include piggery effluent, food processing effluents from factories, and other agricultural wastes which have been considered by many workers (Martin et al. 1985a; Pouliot et al. 1986; Rodrigues and Oliveira 1987; Phang and Ong 1988). Furthermore, algal based technique for the exclusion of noxious substances like arsenic, bromine, cadmium, lead, mercury, scandium, and tin have also being devised (Hammouda et al. 1995; Cai-XiaoHua et al. 1995). Algal based treatment has also been used as a tertiary process, traditionally (Lavoie and De la Nouë 1985; Martin et al. 1985b). They have been anticipated as a possible secondary wastewater treatment system (Tam and Wong 1989). The eight algal genera which were found to be most tolerant to the organic pollutants include *Chlorella*, *Chlamydomonas*, *Euglena*, *Oscillatoria*, *Nitzschia*, *Navicula*, *Scenedesmus* and *Stigeoclonium* (Palmer 1969).

The unique potential of microscopic algae of using inorganic nitrogen and phosphorus for their growth and development provide a unique resolution to tertiary and quaternary wastewater treatments (Tam and Wong 1995). They are also known to have tremendous capacity of removing heavy metals as well as toxic compounds of organic nature (Rai et al. 1981; Redalje et al. 1989), hence preventing secondary pollution. Among important characteristics of microalgae is their production of oxygen during photosynthesis, raising the medium pH and thus having a disinfecting impact (Mara and Pearson 1986; De la Nouë and De Pauw 1988). Algal wastewater treatment can also be utilized for a fulfilling variety of other targets; some of them are the elimination of coliform bacteria, eutrophication-causing nutrients, diminution of both biochemical and chemical oxygen demand, and sequestration of heavy metals.

5.4.1 Heavy Metal Removal by Algae

Microalgae play a pivotal role in the heavy metal sequestration process (Rai et al. 1981). The release of harmful substances to wastewater systems has escalated reportedly with the onset of industrialization. The heavy metal sequestration from water bodies has drawn significant attention due to their toxic nature, exceeding the regulatory standards, affecting both the living organisms as well as the environment (Adamu et al. 2015). However, some heavy metals are toxic and carcinogenic, even at lower concentrations. They are non-biodegradable and get accumulated in the tissues of living organisms (Badruddoza et al. 2013). Heavy metal build-up in groundwater and soil is a major concern (Al Raisi et al. 2014) and their sources are either lithogenic (soil parent material) or anthropogenic which include paint industry, leather tanning, metal smelter, fertilizers, agricultural processes, electroplating, and other disposed industrial waste materials (Ozay et al. 2009; Alloway 2013).

Algae have been used as an effective tool in the separation of heavy metals from the sewage waters. Several workers concluded that the method involving the separation of algae (saturated with metals) from the medium is a productive method for metal sequestration, resulting in high class reusable wastewater (Bhat et al. 2008; Pandi et al. 2009). Various algal species (live and dead) have the capacity of removing huge quantities of intoxicating metal ions from aqueous media. Algal heavy metal sequestration occurs by various methods depending on metal ions, state of affairs of the solution, algal strain and the condition if they are living or nonliving. It has been reported that trace elements (such as Ca, Co, Mg, Mo, Zn, Cr, Pb, Cu, and Se) were stored intracellularly in living algal cells through active transport (Yuce et al. 2010; Tuzen and Sari 2010; Kiran and Thanasekaran 2011; Singh et al. 2012). The live microalgae have been reported to have a considerable role in detoxifying heavy metals in wastewater of minery (Gale 1986). Algal species like *Coelastrum proboscideum* removes 100% and 90% of Pb from 1 ppm aqueous solution maintained under varied lab conditions (20 h at 23 °C and only after 1.5 h at 30 °C). The pyrethroid insecticide Deltamethrin produced as an extracellular esterase is degraded by green alga *Dunaliella bioculata* (Baeza-Squiban et al. 1990; Schimdt

1991). Several workers like Cerniglia et al. (1980); Carpenter et al. (1989) reported that algal cultures degraded the hydrocarbons found in oily wastes.

5.5 Algal Production and Utilization of Algal Biomass

Waste-grown microalgae provide huge quantities of algal biomass for the production of biofuel (Fig. 5.1). However, most of the pond systems meant for treating wastewater does not use harvested algal biomass. The ones which use it return the biomass to the pond floor resulting in its decomposition, methane emission and hence degrading the grade of water (Chaiprasert 2011). Contrarily, the algal biomass can be utilized for lipid extraction, which can be used as fuel for vehicles, or it can be processed for making biogas anaerobically (Brune et al. 2009; Kumar et al. 2016).

Microalgae have two major plus points with respect to production of biofuels over higher plants. Firstly, the microalgae have significantly higher biomass productivities (Mandotra et al. 2016, 2019), with productivities of about 70 MT/ha year in high rate ponds (Sheehan et al. 1998). Their productivity is higher when compared with land crops (e.g., 9 MT/ha year for corn, 3 MT/ha year for soybeans) (Perlack et al. 2005). Secondly, the microalgal cultivation does not require fresh water or arable land; it can be executed in ponds with shallow depths using brackish or saline water on hardpan soils. Relatively lesser work has been carried out on the microalgal digestion done anaerobically (Sialve et al. 2009). Earlier work on

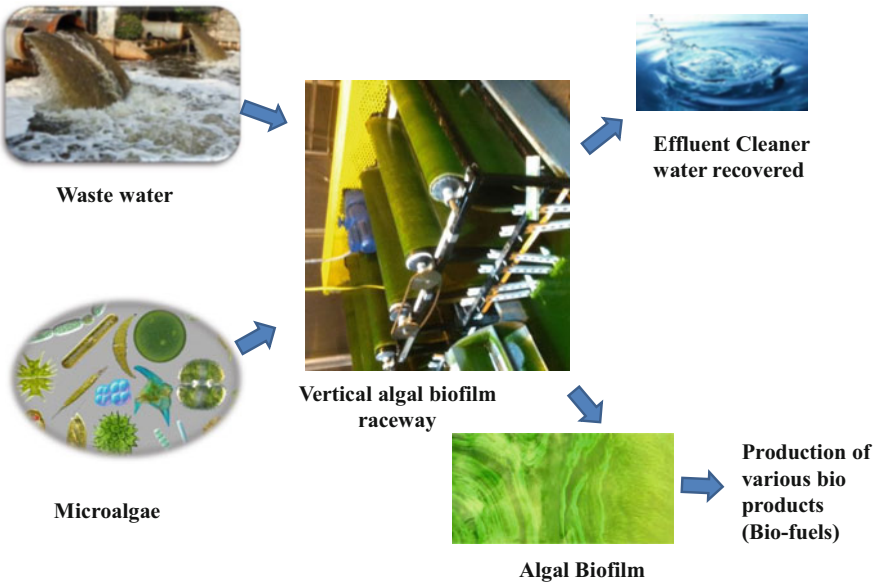

Fig. 5.1 Wastewater treatment, algal production, and biomass utilization (Modified and adapted from Zhang et al. 2018)

domestic sludge digestion was done using green microalgae, *Chlorella* and *Scenedesmus* biomass retrieved from ponds (Golueke et al. 1957). They recorded that these algae can yield within a range of 0.25–0.50 L CH_4/g in comparison to the input when incubated at 35–50 °C, at an 11-day retention time. They suggested that the cell walls resist bacterial degradation which is responsible for relatively low yield of microalgal biomass. In the case, where equal masses of *Spirulina* biomass and sludge were co-digested, methane emission and productivity were doubled (Samson and LeDuy 1983). Likewise, waste paper (50% w/w) was added to algal sludge making the Carbon Nitrogen ratio to about 20–25:1 which increased the rate of methane yield (from 0.6 L/L day to 1.2 L/L day maintained at 35 °C and 10 days of hydraulic retention time) (Yen 2004; Yen and Brune 2007).

5.6 Conclusion

Algae can be utilized in the management of wastewater for removing various toxic substances and acting as scavengers of wastewater pollutants including the reduction of Biological Oxygen Demand, absorption of Nitrogen and/or Phosphorus, elimination of coliforms and sequestration of heavy metals. Since the concentration of Nitrogen and Phosphorus is higher mainly in wastewaters which signifies that they can be used as inexpensive nutrient sources for mass scale production of algal biomass. The biomass can be utilized for the production of methane, compost formation, manufacture of variety of fuels which are in liquid state, apart from this the algal biomass can also be used in culture of aquatic organisms or as animal fodder as well as for fine chemical production.

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6 Parametric Modeling and Optimization of Phycoremediation of Cr(VI) Using Artificial Neural Network and Simulated Annealing

Sushovan Sen, Abhilasha Rai, Jitamanyu Chakrabarty, Sandip Kumar Lahiri, and Susmita Dutta

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Abstract

The present study focuses on the abatement of Cr(VI) from synthetic wastewater using *Chlorococcum* sp. The effects of different operating variables like pH (7–11), inoculum size (2–10%), and initial concentration of Cr(VI) (5–40 mg/L) have been examined following one-factor-at-a-time approach. The most effective condition at which maximum removal of Cr(VI) has been obtained as observed during OFAT analysis is as follows: initial concentration of Cr(VI): 20 mg/L, initial pH: 9, inoculum size: 10% and under such conditions the test strain removes up to 67.3 ± 0.03% of Cr(VI) after 12 days. Further, variation of removal of Cr(VI) with time has been investigated at different operating conditions. Simultaneous studies on production of biomass and biomolecules like protein, carbohydrate, and lipid have also been done. To assess the mechanism of metal removal, the distribution of Cr(VI) through biosorption and bioaccumulation has been determined. The biomass has been characterized using Scanning Electron Microscopy (SEM), Energy Dispersive Spectroscopy (EDS), and Fourier Transform Infrared (FTIR) spectrometer. Finally, Artificial Neural Network (ANN) and Particle Swarm Optimization techniques have been used to model and optimize the phycoremediation of Cr(VI).

Keywords

Green algae · Chromium · Biosorption · Bioaccumulation · Phycoremediation · Biomolecules

6.1 Introduction

The main anthropogenic sources of water pollution include the disposal of sewage and industrial wastewater into the water bodies. The increase in human population along with excessive industrialization contributes a major part of water pollution. The effluents from different industries contain significant organic and inorganic pollutants in combination with heavy metals and other chemicals which contaminate the water bodies. Specific gravity of heavy metals is five times greater than H_2O (Shanab et al. 2012). Some of the important heavy metals discharged as a result of improper treatment of industrial effluent include chromium, copper, nickel, lead, iron, mercury, etc. Among the several heavy metals, which pollute the water reservoir globally, the contamination of Cr(VI) has been acknowledged as one of the main problems because of its consistent use in the industries (Liu et al. 2012). In a recent study, Yen et al. (2017) reported Cr(VI) as the second most common heavy metal pollutant in the environment with the concentration ranging from 0.008 to 173 mM in groundwater. The toxic character of chromium largely depends on the oxidation states, in which it is present in the water. Generally, chromium is present with two different oxidation state in water like chromium(III) and chromium(VI). However, Cr(VI) is highly soluble and can easily penetrate through the cell membrane. Acute exposure to chromate leads to several health problems in human

beings, like gastrointestinal disorders, hemorrhagic diathesis, and convulsions. In extreme cases, it may also lead to cardiovascular shocks, followed by death. Therefore, proper treatment of industrial effluent to remove Cr(VI) is essential from pollution control point of view. Several conventional techniques such as membrane separation, solvent extraction, chemical precipitation, etc., are in practice, however, all these technologies have their own limitations. Researches are now trending towards the biological treatment process using bacteria, algae, fungi, etc. Microalgae have been proven as one of the most efficient bioremediants in comparison to other microorganisms due to their higher mucilage volume, greater binding affinity, etc. Moreover, it can grow in wastewater and can remove the pollutants up to a significant level without producing any toxic sludge during the process. Biosorption and bioaccumulation are the major steps for bioremoval of metals. While biosorption is a metabolically independent rapid process in which metals are bound on the surface of living or non-living biomass, bioaccumulation is a metabolically dependent slow process where uptake of metals occurs inside the living cell (Sen et al. 2018). Therefore, to assess the proper mechanism of metal removal, the estimation of biosorbed and bioaccumulated percentages is essential to know.

In the present study, a green microalgal strain has been used to phycoremediate Cr(VI) from its simulated solution. The effect of Cr(VI) on the proliferation of microalgal strain in terms of its biomass and biomolecules production has been investigated. Further, Artificial Neural Network (ANN) modeling technique is applied to model the non-linear complex relationship between chromium removal percentage with various input parameters like pH of the medium, inoculum size, time of contact, and initial concentration. After developing a reliable and accurate model, the input parameters are optimized using simulated annealing technique to achieve maximum removal of Cr(VI). As far as known, the numerical modeling and optimization of bioremoval of Cr(VI) is terse and here lies the novelty of the present study.

6.2 Materials and Methods

6.2.1 Isolation, Identification, and Cultivation of Isolated Strain

The algal sample was collected from the effluent of a coke-oven plant in West Bengal, India. The wastewater generated during coking and by-product recovery processes contains an array of pollutants starting from organic pollutants to heavy metals. The reason for selection of such contamination site lies in the fact that the collected strain will be having the metal resistance property. The collection, isolation, and identification were done by other group in the same laboratory and were described by Mistry et al. (2019).

6.2.2 Growth Study of Microalgal Isolate

The strain was inoculated in BG-11 medium (10% inoculum size (IS)) and placed in algal-incubator under the germ-free condition at temperature 25 ± 2 °C and light intensity 2400 lux for 16/8 h (light/dark). The culture was withdrawn after every 2 days and the wet biomass was harvested by centrifugation (Model No. TC8100F, Eltek, India) at 5500 revolution per minutes for 20 min. Washed pellet was dried at 60 °C overnight to get cell dry mass. The growth was observed in terms of cell dry mass. Biomolecules such as protein, carbohydrate, and lipid were extracted and estimated using standard protocols (Sen et al. 2017; Upendar et al. 2018).

6.2.3 Characterization of Collected Microalgal Strain

Characterizations of both untreated and treated (with Cr(VI)) samples were done using Scanning Electron Microscopy (SEM) (SEM; Hitachi–S-3000N, Japan), Energy Dispersive Spectroscopy (EDS) (EDS; Hitachi–S-3000N, Japan), and Fourier Transform Infrared (FTIR) spectrometer (NicoletiS10, Thermo Fischer Scientific, USA). For getting treated microalgal biomass, the test strain was exposed with Cr(VI) (initial concentration: 20 mg/L) in BG 11 medium (10% inoculum size) taken in 500 mL Erlenmeyer flasks. These were then placed in a microalgal incubator under specified condition. After a time period of 14 days, samples were collected and centrifuged. The pellets were collected and dried (Sen et al. 2017; Upendar et al. 2018). The dry biomass was taken for further analysis.

6.2.4 Effect of Input Variables on Bioremoval of Cr(VI) Using OFAT Approach in Batch Study

The influence of different factors on phycoremediation of Cr(VI) using the present strain was assessed through OFAT (one factor at a time) analysis. Initial pH of the medium (7–11), initial concentration (IC) of Cr(VI) (5–40 mg/L), and IS (2–10%) were altered in a pragmatic manner. At first, IC was varied keeping both IS (10%) and pH (7) constant. The pH was altered next, keeping the IC of Cr(VI) at 20 mg/L as obtained from previous experimental set and IS at 10%. At last, IS was varied and other two factors were maintained at pH 9 and 20 mg/L IC of Cr(VI). All the above experiments were carried out for 12 days. The culture was withdrawn after 12 days and centrifuged. The cell mass was used for cell dry biomass and biomolecules estimation while liquid part was examined for remaining Cr(VI) concentration using Atomic Absorption Spectrophotometer (iCE 3000, Thermo Fisher Scientific). Biomolecules like carbohydrate, lipid, and protein concentrations were measured from microalgal biomass using the standard protocol. The values of input variable at which maximum removal of Cr(VI) was attained were judged as most effective one.

6.2.5 Variation of Bioremoval of Cr(VI) and Production of Biomass and Biomolecules with Time at Different Operating Conditions

Variation of bioremoval of Cr(VI) with time was studied at different IC of Cr (VI) (10–25 mg/L) keeping other parameters (pH and IS) constant at their most effective values. Since in previous (OFAT) studies, less growth was observed at 30 mg/L Cr(VI) solution, the range of IC of Cr(VI) was fixed 10–25 mg/L. After inoculation, the solutions were cultured for 14 days. Samples were collected after certain time interval and analyzed for residual Cr(VI) concentration, biomass concentration, and different biomolecules contents. In the next stage, the growth of test strain was investigated at different initial pH of the solution keeping IC of Cr(VI) and IS constant at their most effective values. Finally, the same study was performed at various IS keeping other parameters constant at their most effective values. To assess the mechanism of Cr(VI) removal using test strain, the estimation of biosorbed and bioaccumulated percentages is required to know. The biosorbed Cr(VI) onto the cell surface and the bioaccumulated Cr(VI) in intracellular space were measured following the method as described by Sen et al. (2018).

6.3 Theoretical Analysis

6.3.1 Artificial Intelligence Based ANN Modeling

As stated above, detail experimentation was carried out to know the effect of different operating variables like IS (2–10%), pH (7–11), and IC of Cr(VI) (10–25 mg/L) on the abatement of Cr(VI) from simulated solution using *Chlorococcum* sp. The purpose of modeling is to find the quantitative relation between chromium removal with different input variables stated above. Since, there is no reliable first principle-based model for this kind of microalgae, ANN based model is selected to find out the relations.

In the last decade ANN algorithm has gained popularity as "effortless computation algorithm" and applied in different domains successfully and lot of application of ANN has been reported in diverse fields. ANN is proven to be very effective to find a non-linear relationship between input and output parameters in similar complex systems where exact process phenomenology is less understood. From literature survey and experimentation following four parameters were shortlisted as input parameters which have large effect on efficiency of Cr removal by microalgae:

1. Time of contact between contaminated wastewater and microalgae.
2. Initial concentration of Cr(VI) in water.
3. Inoculum size
4. pH of media.

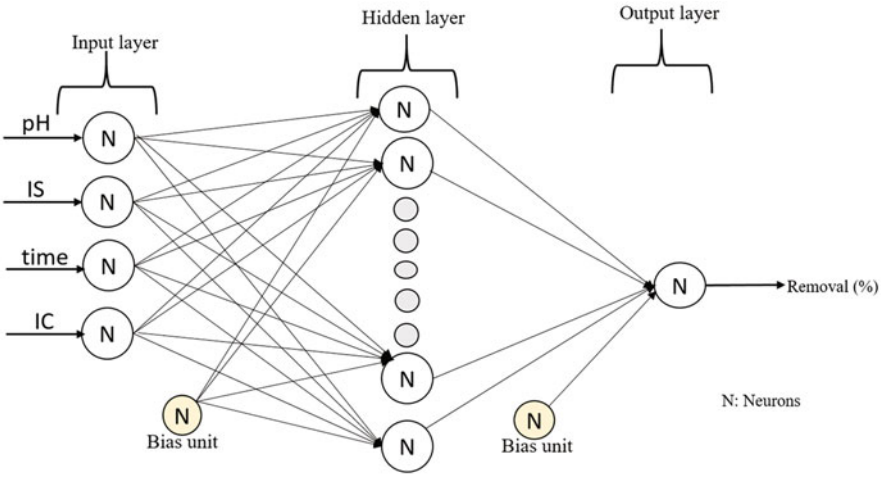

Fig. 6.1 A schematic diagram of Artificial Neural Network (ANN) for Cr(VI) removal

Percentage of chromium removal in effluent water after treatment is considered as model output.

ANN mimics human nervous system. Typically an ANN consists of three layers, namely input layer, hidden layer, and output layer as shown in Fig. 6.1.

In this model, input layer consists of four nodes corresponding to four input parameters, while output layer consists of one node corresponding to one output parameter as shown in Fig. 6.1. Nodes in hidden layer are not known a priori and it varies systematically during model building phase to reduce MSE on test data. Each node in hidden layer and output layer consists of a non-linear activation function (e.g., sigmoidal function) which is also supplied by user. Each node in input layer is connected with each hidden layer node by weights. Also each connection of hidden layer nodes to output layer nodes is done by weights, which represents the dominance/influence of each input on final output. 80% of experimental input–output data set (chosen randomly) used for training and 10% used for validation and rest 10% for testing purposes. During model building phase, neural network algorithm updates various weights in each iteration based on training error and tests the model performance with unseen validation data set and test data set. This is commonly known as back-propagation algorithm. Neural regression continues to do so until validation error starts increasing. At that time training stops and different weights in input and output layer freeze. The final performance of neural network model is judged by the mean squared error (MSE) and coefficient of determinations (R^2) on unseen test set data with the last frozen weights. MSE and R^2 are defined as follows:

Coefficient of determination:

Table 6.1 Various ANN Training algorithm and activation function available in literature

Training algorithm	Activation function
Levenberg–Marquardt algorithm	Log-sigmoid
BFGS quasi-Newton back propagation	Hyperbolic tangent sigmoid
Conjugate gradient back propagation with Powell–Beale restarts	Linear function
Resilient back propagation	Radial basis
Conjugate gradient back propagation with Fletcher–Reeves updates	Triangular basis
Conjugate gradient back propagation with Polak–Ribiére updates	
One-step secant back propagation	
Gradient descent with momentum and adaptive learning rate back propagation	

$$R^2 = \frac{\sum_{i=1}^{N} \left(Z_{i,\exp} - \overline{Z_{\exp}}\right)\left(Y_{i,\text{pred}} - \overline{Z}_{\text{pred}}\right)}{\left(\sqrt{\sum_{i=1}^{N} \left(Z_{i,\exp} - \overline{Z_{\exp}}\right)}\right)\left(\sqrt{\sum_{i=1}^{N} \left(Z_{i,\text{pred}} - \overline{Z}_{\text{pred}}\right)}\right)} \tag{6.1}$$

Mean Square Error

$$\text{MSE} = \frac{1}{N}\sum_{i=1}^{N} \left(Z_{i,\text{pred}} - Z_{i,\exp}\right)^2 \tag{6.2}$$

where N = Number of experimental data point, $Z_{i,\text{pred}}$ = output for ith experimental data point, $Z_{i,\exp}$ = experimental output for ith experimental data, $\overline{Z}_{\text{pred}}$=mean value of model predicted output, $\overline{Z_{\exp}}$=mean value of experimental output.

Over the last 20 years ANN basic algorithm undergoes lot of modifications and in literature lot of new improved ANN algorithms are available which are applied in diverse fields as shown in Table 6.1.

These training algorithms are characterized by some exceptional improved features over normal back propagation algorithm and developed by various researchers over the last decade. However, there is no single universal algorithm which can be applied in any data set and perform best and there are no guidelines in literature for choosing an algorithm for a particular data set. Hence, choosing a particular ANN algorithm for model building needs detail knowledge of both data set and ANN algorithm and cannot guarantee the best performance of the developed model. Also, there are five different activation functions (Table 6.1) which are commonly used in ANN hidden and output layer nodes. Here also, no definite guideline is available for selection of best one for these experimental data.

Number of hidden layer nodes also has to be supplied by users during model building phase, and currently in literature trial and practice approach is taken to find out the optimum number of hidden layer nodes for a specific data set. Hence choosing the best algorithm and best activation function in hidden layer and output

layer along with optimum number of hidden layer nodes is not an easy task for novice users. To overcome this issue, exhaustive searches of all combinations of algorithms and activations are made in this study to find out the best ANN model from universe.

Following exhaustive search algorithm is applied to find out the most effective ANN model:

1. At the most outer layer all the eight ANN algorithms are calculated by one by one.
2. Second nested layer consists of five activation functions as present in hidden layer nodes.
3. Same five activation numbers are used in output layer nodes in the third nested layer.
4. Number of hidden layer nodes change from one to hundred in the fourth nested layer.
5. Each calculation is done 10 times to avoid ANN solution to get stuck in local minima in the innermost nested layer.

All these five steps are carried out in Artificial Neural Network (ANN) model in Matlab environment and lowest mean square error (MSE) in test data and highest R^2 model is chosen as the final best model.

With this algorithm, all available ANN models in literature is searched, all known activations functions are evaluated, and a final model is generated which has best prediction capability among universe. Although a large number of ANN models are calculated in this method but with the advent of high speed computer, the execution time in Intel i-5 processor is less than one hour. So, in brief, ANN topology considered four input parameters and one output parameter as shown in Fig. 6.1. Optimum nodes in hidden layer, best ANN algorithm, optimum activation function in hidden layer and output layer are calculated during ANN model building time by an exhaustive search. The generated best model for this data set is further used for model prediction.

6.3.2 Application of Simulated Annealing (SA) Optimization

Once a reliable and accurate ANN model is developed the next job is to optimize the input parameters, namely time, initial concentration, inoculum size, and pH so that the percentage removal of Cr(VI) is maximized. Since the ANN equations are complex and consist of various weights and exponential terms, traditional optimization techniques are difficult to apply on ANN model. Simulated Annealing Optimization technique is applied in the present study due to its simplicity and ease of implementation. SA is considered as very efficient, generic, Meta heuristic algorithm for solving global optimization problem.

6.3.2.1 Simulated Annealing at a Glance

This optimization technique mimics the slow cooling of metal commonly known as annealing in Metallurgical Engineering. As per kinetic theory, there are chaotic movements of atoms of molten metal at very high temperature. But these random chaotic movements of the atoms get restricted as the temperature is gradually reduced. If the temperature is further reduced, there is a significant reduction of chaotic movements and finally crystals are formed having least energy state. Slow rate of cooling is the main determining factor to reach crystalline state having absolute minimum energy.

6.3.2.2 Algorithm of SA

The simulated annealing optimization procedure tries to mimic the slow cooling rate to obtain the global minimum of a function. A temperature-like parameter is introduced in SA optimization algorithm to mimic the slow cooling of metals, where Boltzmann probability distribution is followed. This expression enforces low probability to remain in a high energy state at low temperature which is normally observed in actual molten metal annealing. The algorithm of SA is given below as per reference (Dutta 2016).

Step 1: Algorithm starts with user-defined values of starting temperature (T_0), number of iterations in each temperature step (n), number of cycles (k), and final temperature (T_F).

In the present work, starting temperature is calculated by taking mean value of the objective function in the first generation (Dutta 2016).

Step 2: Program starts with random initial value of decision variables (X_0). Objective function value f(X_0) is calculated next. In this problem, objective function is $f = 1/(1+\%$ removal of Cr) is calculated using a trained ANN model. Since we want to maximize the Cr removal, we take inverse for minimization problem.

Step 3: In next step, a new neighbor solution is calculated in the vicinity of current solution by

$$X_{i+1} = X_i + N(0, \sigma_i)$$

where $N(0, \sigma_i)$ is a random Gaussian number with zero mean and σ_i standard deviation. Objective function value $f(X_{i+1})$ is calculated in this new point. Iteration counter $i = 1$ and cycle counter $k = 1$ are set to value 1.

Step 4: Difference in objective function value is calculated as $\Delta f = f(X_{i+1} - X_i)$

To mimic the metal cooling process, following probabilistic approach is used (Metropolis criteria) to decide whether the new design point is accepted or rejected.

$$P(f) = \begin{cases} 1 & \Delta f \leq 0 \\ e^{\frac{-k\Delta f}{T}} & \Delta f > 0 \end{cases}$$

New design point is accepted if the probability $P(f)$ is within acceptable range. If it is not within acceptable range, new design point is rejected and reverted back to previous design point.

As seen from above equation, probability value is 1 for the case $\Delta f \leq 0$, which essentially means that the new point X_{i+1} is always accepted. In the function minimization context, this is obvious because the new point X_{i+1} generates superior results than older one X_i and the new point must be accepted. Complexity in decision-making arises when $\Delta f > 0$, which essentially means that the function value at X_{i+1} is worse than that at X_i. As per most traditional function minimization algorithms, the new point X_{i+1} must be discarded in this situation. Metropolis algorithm does not reject the new point out rightly even it is worse but attaches a finite probability to accept it. However, this probability of acceptance of the worse solutions varies as per situation. This probability, as per Metropolis criteria is $e^{\frac{-k\Delta f}{T}}$ and greatly influenced by the relative magnitude of $k\Delta f$ and T values. When the parameter T is very large, the probability term $e^{\frac{-k\Delta f}{T}}$ is high for points with largely disparate function values. This ensures that high probability of acceptance of inferior solutions for a large value of T. On the other hand, probability of accepting an arbitrary point is drastically reduced when the parameter T is small. This ensures, for small values of T, the new points with only a small deviation in the function value are accepted.

Step 5: Return back to Step 3, increase the counter $i = i + 1$; Repeat the following sub steps for n times.

Step 6: Terminate the calculations if temperature reaches to final temperature ($T_k = T_F$). Otherwise, slowly reduce the temperature in each step as per $T_{k+1} = s(T_k)$, mimicking the metal annealing procedure where value of s is in the range [0.8,0.99].

Increase the counter $k = k + 1$; and go to Step 3.

It is observed that the quality of final solution of SA is greatly influenced by the initial temperature and the subsequent rate of cooling. From literature it can be concluded that these choices require some trial-and-error efforts and still remains an art (Dutta 2016).

6.4 Results and Discussions

6.4.1 Growth Study of Isolated Algal Strain

The isolated sample has been identified as single green microalgal culture of *Chlorococcum* sp. (Mistry et al. 2019). The variation of concentrations of biomass and biomolecules like protein, carbohydrate, and lipid has been studied in BG-11 media and has been shown in Fig. 6.2. It is seen that adaptive phase has extended up to 4 days and the strain has attained the stationary phase after 12 days. Between these, the active growth takes place. Dry biomass, carbohydrate content, protein content, and lipid content during exponential or active growth period have increased 3.03, 1.52, 4.1, and 2.94 fold, respectively. Initially, the growth is low due to the

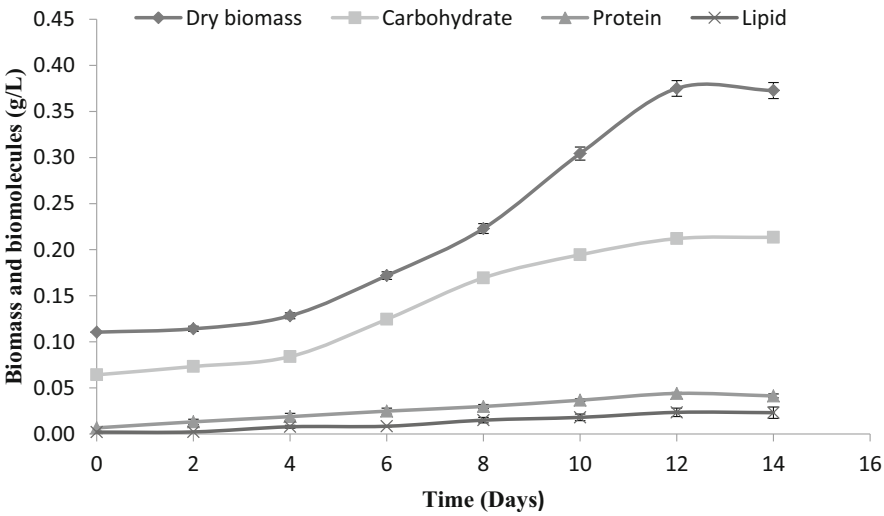

Fig. 6.2 Growth study and concentrations of biomolecules of microalgal strain

sudden introduction into the new environment and the increase in the biomass afterwards may be due to the attainment of capability to consume the nutrient from the culture medium. The utilization of these nutrients enhances the biomass of microalgal sample (Kushwaha et al. 2014).

6.4.2 Characterization and Identification of *Chlorococcum* Sp.

From the micrographs of SEM of the test strain before (Fig. 6.3a) and after Cr (VI) treatment (Fig. 6.3b), it can be stated that, the structure of the native microalgal cell is of granular type with lots of pores present, whereas after treatment, the surface of biomass has been appeared to be very rough. Figure 6.3c, d provides the EDS spectra of the microalgal biomass before and after Cr(VI) treatment, respectively. In the EDS spectra of native strain, there is no trace of Cr(VI), whereas, in the case of treated biomass, the presence of Cr(VI) has proved the binding of chromium onto the microalgal surface.

FTIR spectra of both native (Fig. 6.3e) and treated microalgal biomass (Fig. 6.3f) reveal different functional groups responsible for interaction between Cr(VI) and microalgal strain. Microalgal cell wall carries different characteristic functional groups which are mainly active for the binding of pollutants (Onyancha et al. 2008). The functional groups present in the naive microalgal cell are C=C bending (670 cm^{-1}), C-O stretching (1076 cm^{-1}), C=C stretching (1653 cm^{-1}), C≡N (2365 cm^{-1}), and O-H stretching (3432 cm^{-1}). In case of treated microalgal biomass C=C bending functional group disappears and C=C stretching and O-H stretching bonds have shifted to the 1654 cm^{-1} and 3425 cm^{-1} wavenumber, respectively.

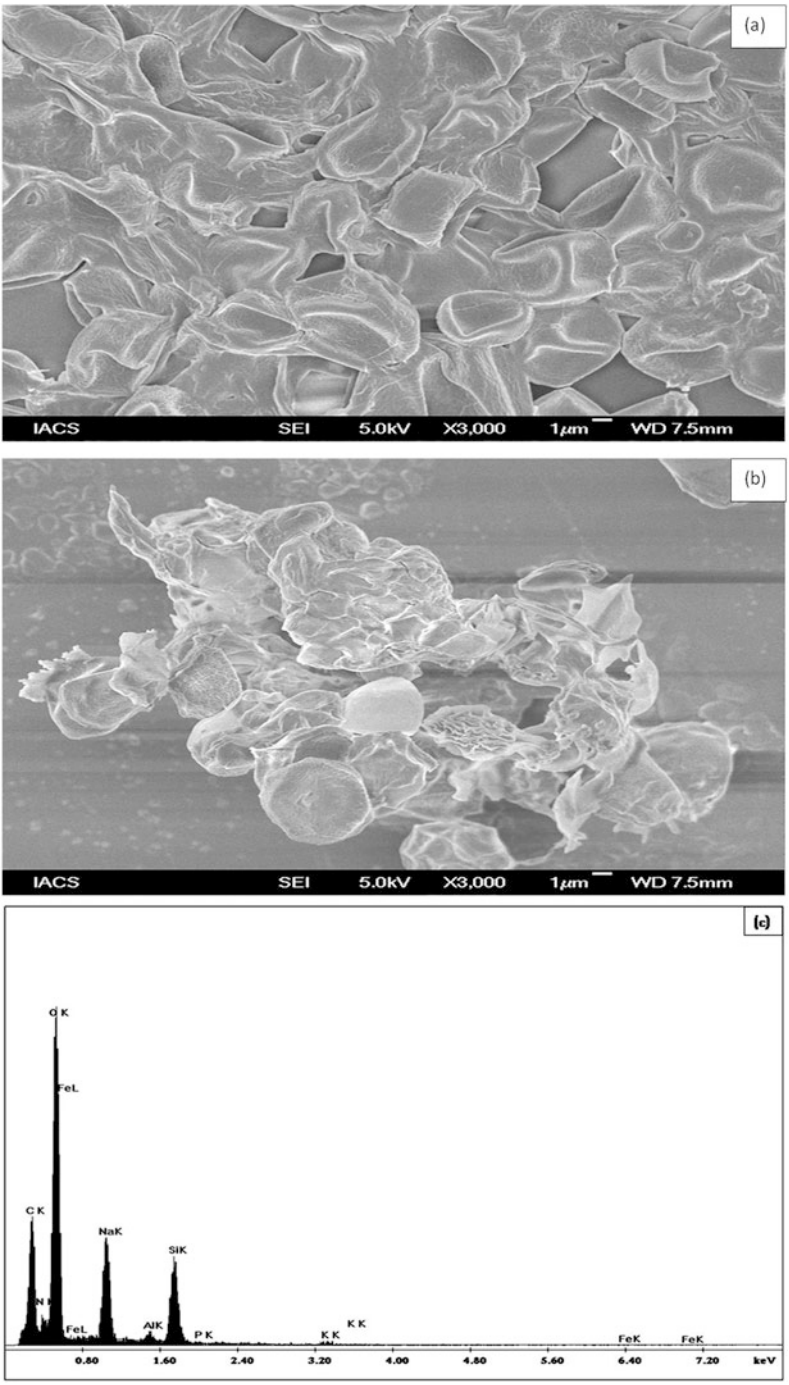

Fig. 6.3 (**a**) SEM image of microalgal biomass. (**b**) SEM image of microalgal biomass after Cr(VI) treatment. (**c**) EDS spectra of native microalgal biomass. (**d**) EDS spectra of Cr(VI) loaded microalgal biomass. (**e**) FTIR spectra of native microalgal biomass. (**f**) FTIR spectra of Cr(VI) loaded microalgal biomass

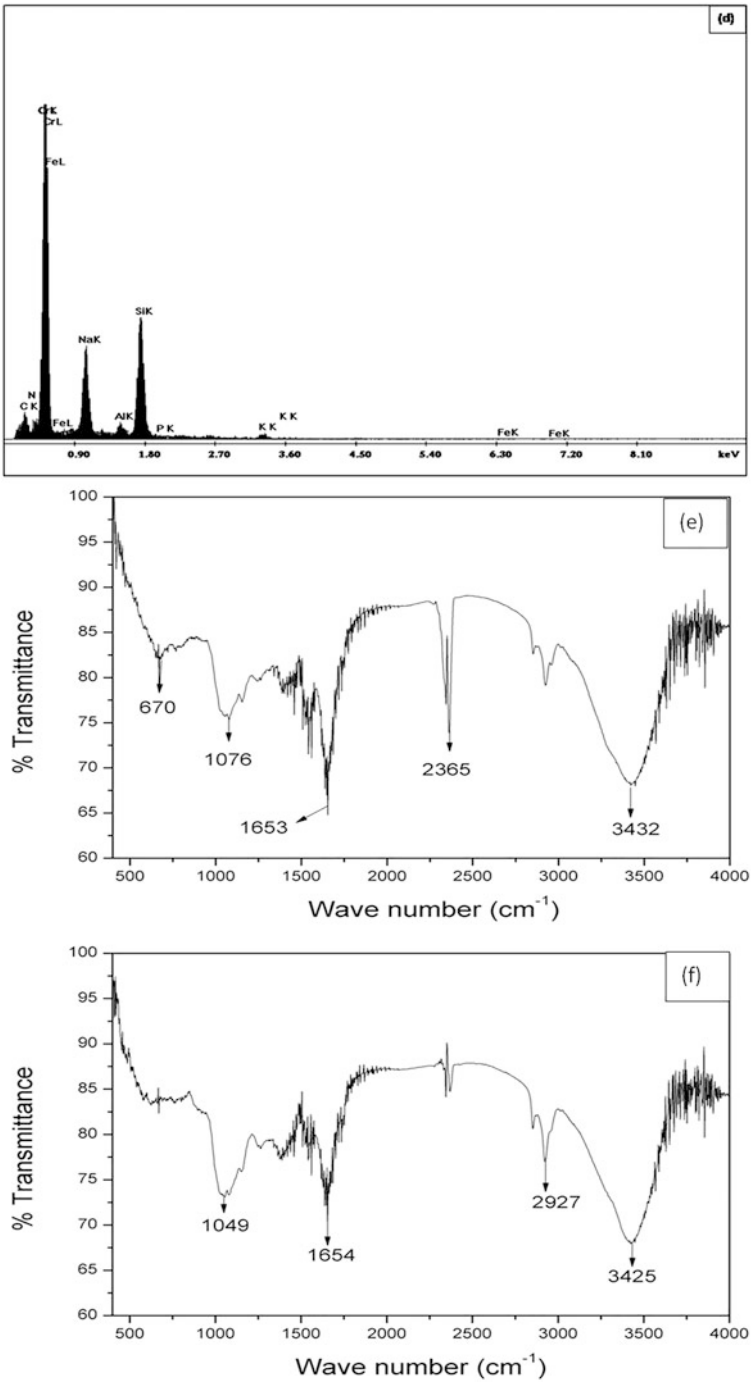

Fig. 6.3 (continued)

Furthermore two new functional groups C-N and C-H stretching have appeared at wave number of 1049 and 2927 cm^{-1}, respectively. The shifting of these groups signifies the microalgae–metal interaction.

6.4.3 Effect of Input Variables on Bioremoval of Cr(VI) Using OFAT Approach in Batch Study

6.4.3.1 Effect of IC

The fractional bioremoval of Cr(VI) at different IC of Cr(VI) (5–40 mg/L) after 12 days of inoculation at constant pH (7.0) and inoculum size (10%) is shown in Fig. 6.4a. No growth has been observed at 40 mg/L. Therefore, data have been shown up to 30 mg/L The fractional bioremoval of Cr(VI) has been increased from 0.298 ± 0.019 to 0.624 ± 0.040 with the increase in IC of Cr(VI) up to 20 mg/L and thereafter, it gradually decreases to 0.199 ± 0.003 at 30 mg/L Cr(VI) concentration. The concentrations of biomass and different biomolecules have been shown in Fig. 6.4b. BG-11 media without Cr(VI) has been considered as control. The production of biomass is less in control than in Cr(VI) supplemented medium up to 20 mg/L initial concentration. The carbohydrate and protein contents follow the same pattern as that of biomass, however, the lipid content is always higher in Cr (VI) contaminated solution than that in control. This result suggests that under metal stressed condition microalgae can produce higher lipid (Markou and Nerantzis 2013). The maximum concentrations of biomass (0.892 ± 0.02 g/L), carbohydrate (0.442 ± 0.014 g/L), protein (0.087 ± 0.006 g/L), and lipid (0.107 ± 0.014 g/L) have been obtained at 20 mg/L initial concentration. When the test strain has been grown in normal BG-11 media, the concentrations of biomass (0.375 ± 0.009 g/L), carbohydrate (0.212 ± 0.011 g/L), protein (0.044 ± 0.004 g/L), and lipid (0.023 ± 0.003 g/L) are less after 12 days of incubation than that in Cr(VI) loaded condition. Since the maximum bioremoval of Cr(VI) and maximum biomass concentration have been obtained at 20 mg/L initial concentration, it can be said that microalgal strain can uptake Cr(VI) as nutrient up to a certain dose and thereafter, the higher concentration of Cr(VI) may restrict the growth. It is worthy to note that, fractional bioremoval of Cr(VI) has been found to be more for *Chlorococcum* sp. than that obtained by cyanobacterial consortium of *Limnococcus* sp. and *Leptolyngbya* sp. (44 ± 0.070% at 15 mg/L) and by cyanobacterial strain *Limnococcus* sp. (60.27 ± 0.002% at 20 mg/L) (Sen et al. 2018). Enhanced biomass production at much less concentration of Cr(VI) was observed by Hörcsik et al. (2006).

6.4.3.2 Effect of pH

The fractional bioremoval of Cr(VI) has increased from 0.624 ± 0.040 to 0.673 ± 0.068 with increase in pH from 7 to 9. The test strain has showed a decrease in fractional bioremoval (0.593 ± 0.037) with further increase in media pH to 11 (Fig. 6.4c). Similar type of observation was made by Miranda et al. (2012) during removal of lead at different pH of solution ranging from 3 to 7. According to their

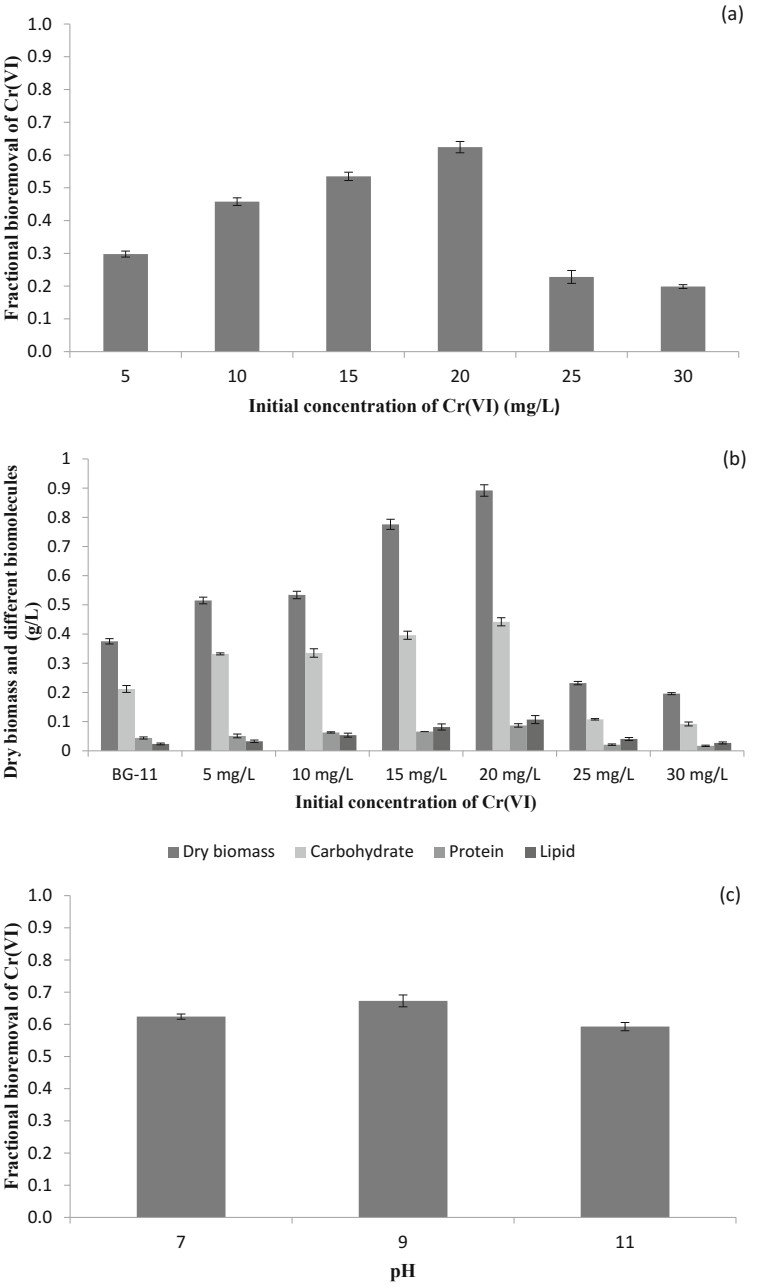

Fig. 6.4 (**a**) Fractional bioremoval of Cr(VI) using microalgal strain during OFAT analysis at different concentrations of Cr(VI). (**b**) Concentrations of biomass and biomolecules using microalgal strain during OFAT analysis at different concentrations of Cr(VI). (**c**) Fractional bioremoval of Cr(VI) using microalgal strain during OFAT analysis at different pH. (**d**) Concentrations of biomass and biomolecules using microalgal strain during OFAT analysis at different pH. (**e**) Fractional bioremoval of Cr(VI) using microalgal strain during OFAT analysis at different inoculum size. (**f**) Concentrations of biomass and biomolecules using microalgal strain during OFAT analysis at different inoculum size

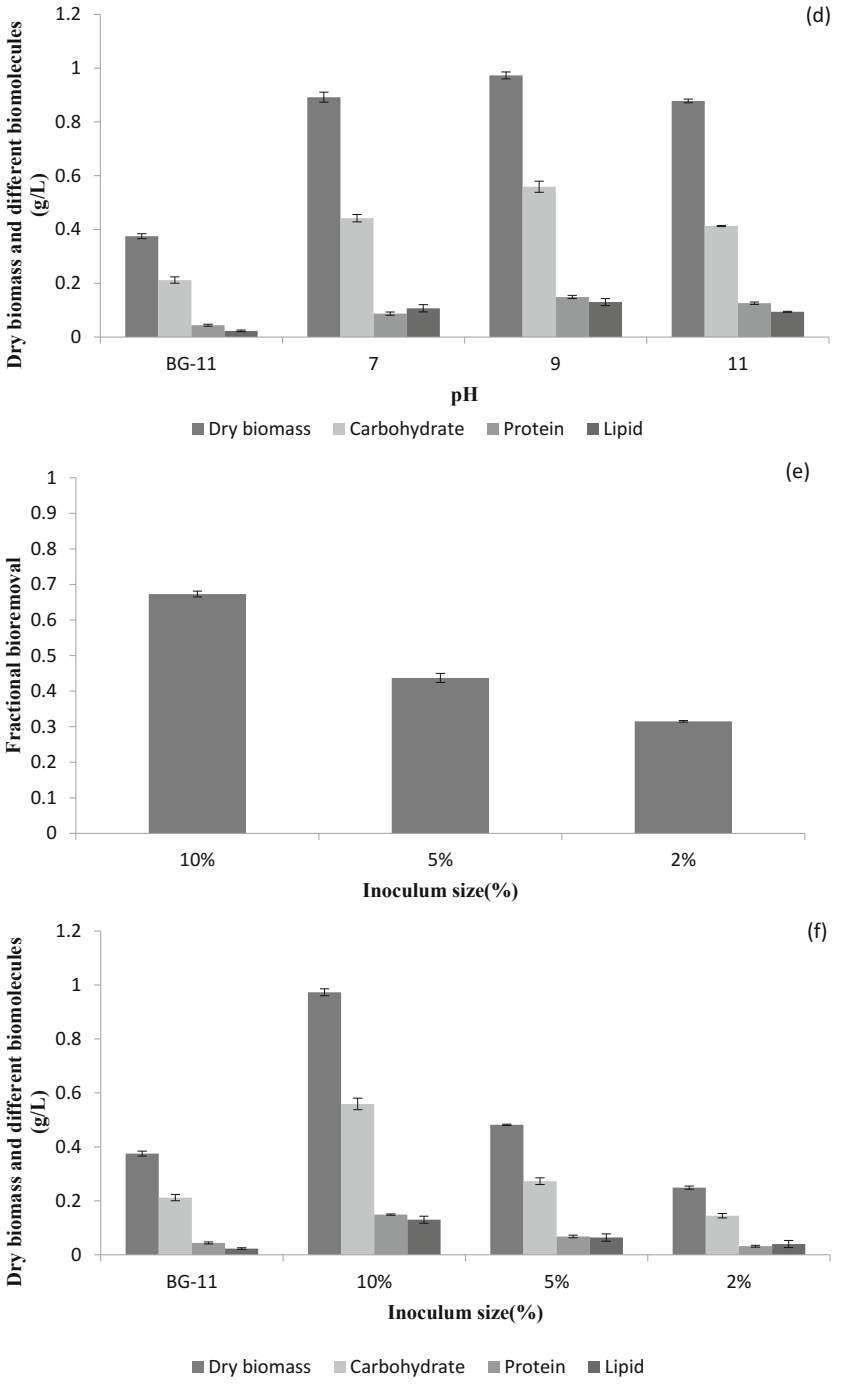

Fig. 6.4 (continued)

study, the removal increased from pH 3 to 5 and thereafter, it decreased. The less removal at higher pH (pH 11) may be for the low bioavailability of Cr(VI) owing to the complexation of the different ions in the medium (Miranda et al. 2012). The growth of test strain in BG-11 media has been considered as control. The maximum production of biomass (0.973 ± 0.012 g/L) and biomolecules (carbohydrate (0.559 ± 0.020 g/L), protein (0.149 ± 0.006 g/L) and lipid (0.13 ± 0.012 g/L)) have been found at pH 9 (Fig. 6.4d). Enhanced production of biomass and biomolecules has been obtained in Cr(VI) supplemented solution. Thus, it can be stated that, when the test strain is grown in 20 mg/L Cr(VI) solution, the production of biomass and correspondingly concentrations of different biomolecules are higher than that in control which reconfirms the utilization of Cr(VI) as nutrient for growth.

6.4.3.3 Effect of IS

The bioremoval of Cr(VI) and the production of biomass and different biomolecules for different IS have been depicted in Fig. 6.4e, f. It is evident from the figure that the higher inoculum size enhances the Cr(VI) removal. The bioremoval of Cr (VI) increases from 0.315 ± 0.002 to 0.673 ± 0.068 with increase in inoculum size from 2 to 10%. Initial pH and initial concentration of Cr(VI) have been maintained at 9 and 20 mg/L, respectively, as found most effective in the previous studies. The production of biomass and biomolecules is also higher when inoculum size increases from 2 to 10% due to the higher initial cell density. At higher inoculum size, the growth of cell increases due to the availability of more cells vis-à-vis more active sites which in turn bind more Cr(VI) from solution leading to enhanced removal. When inoculum size increases from 2 to 10%, the biomass, carbohydrate, protein, and lipid increase 3.91, 3.86, 4.66 and 3.25 fold, respectively. Higher removal efficiency at higher IS was reported by several researchers (Shukla et al. 2002; Rahman et al. 2007; Silva et al. 2012).

6.4.4 Variation of Bioremoval of Cr(VI) and Production of Biomass and Biomolecules with Time at Different Operating Conditions

Cr(VI) removal has been studied at different IC of Cr(VI) (10–25 mg/L), pH (7–11), and IS (2–10%). At first IC of Cr(VI) has been changed from 10 to 25 mg/L keeping pH and IS constant at 9.0 and 10%, respectively (Fig. 6.5a). With the increase in IC of Cr(VI) from 10 to 20 mg/L, the removal has increased from 55.93 ± 4.78% to 73.39 ± 6.28% after 14 days. Beyond this concentration, the removal becomes less (22.25 ± 1.90 at 25 mg/L). The variation of dry biomass, carbohydrate, protein, and lipid contents with time is shown in Fig. 6.5b–e, respectively. From Fig. 6.5b it can be stated that the production of biomass is higher in Cr(VI) supplemented solution than that in pure BG-11 media up to 20 mg/L IC of Cr(VI), whereas production of biomass is less at 25 mg/L IC of Cr(VI) than that in the control. Therefore, it can be inferred that the microalgal strain may accumulate Cr(VI) as a nutrient up to 20 mg/L Cr(VI) concentration, which leads to higher production of biomass and thus the

removal efficiency increases due to accessibility of more active sites. From the figure it can be stated that contents of biomolecules have followed the similar pattern as that of biomass. For all IC of Cr(VI), the carbohydrate content is higher than that of protein and lipid content. At different IC of Cr(VI) 10, 15, 20, 25 mg/L, the increase in biomass, carbohydrate, protein, and lipid contents has been found to be 4.43, 6.58, 8.03, 2.16 fold, 4.2, 5.06, 6.95, 1.44 fold, 16.76, 18.08, 21.62, 6.26 fold, and 28, 51, 64.5, 23 fold, respectively, after 14 days of growth.

In the next stage, the time variation of percentage removal (Fig. 6.6a), dry biomass (Fig. 6.6b), carbohydrate (Fig. 6.6c), protein (Fig. 6.6d), and lipid (Fig. 6.6e) have been studied at different pH (7–11) keeping IC of Cr(VI) and IS constant at 20 mg/L and 10%, respectively. From the figures, it is evident that the production of biomass and different biomolecules is higher at pH 9. Furthermore, the test strain has also showed higher removal efficiency at pH 9 due to the generation of higher biomass. With the increase in pH from 7 to 9 the removal has increased from 46.62 $\pm$ 0.88% to 73.39 $\pm$ 6.28% after 14 days. Beyond this pH, the removal becomes less (55.72 $\pm$ 3.30 at pH 11). At different initial pH of 7, 9, 11 the increase in biomass, carbohydrate, protein, and lipid contents has been found to be 4.73, 8.03, 5.1 fold, 3.40, 7.26, 5.13 fold, 13.48, 22.27, 19.24 fold, and 14, 64.5, 22.5 fold, respectively, after 14 days of growth.

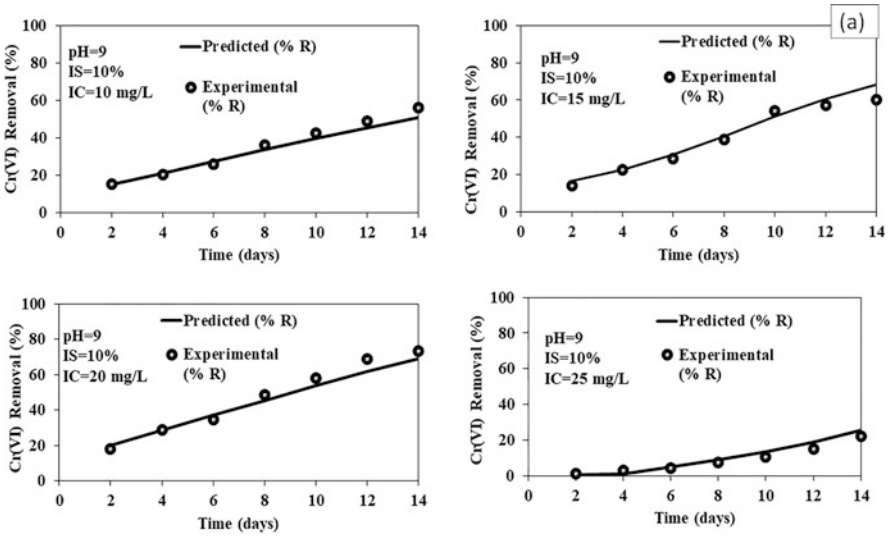

Fig. 6.5 (**a**) Experimental vs. ANN model prediction of percentage removal of Cr(VI) for different initial metal concentration and time (pH: 9 and inoculum size: 10%). (**b**) Dry biomass for different initial metal concentration with time (pH: 9 and inoculum size: 10%). (**c**) Carbohydrate content for different initial metal concentration with time (pH: 9 and inoculum size: 10%). (**d**) Protein content for different initial metal concentration with time (pH: 9 and inoculum size: 10%). (**e**) Lipid content for different initial metal concentration with time (pH: 9 and inoculum size: 10%)

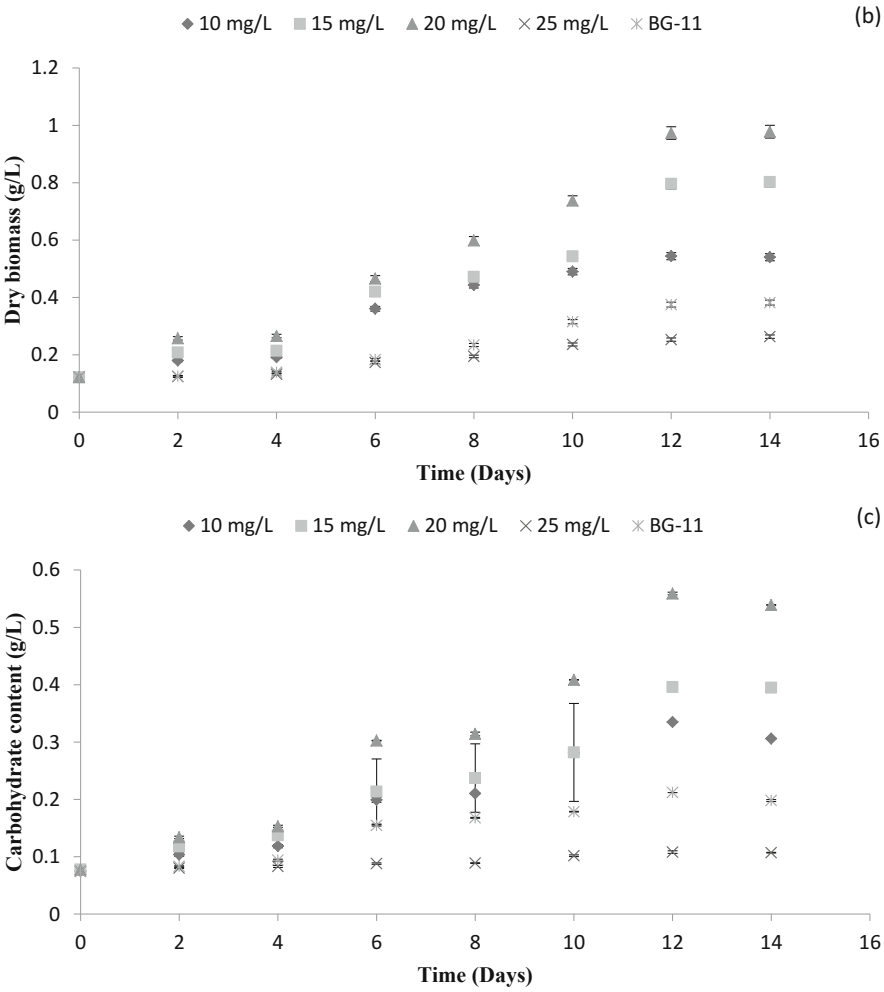

Fig. 6.5 (continued)

Finally, inoculum size has been varied in the range of 2–10% keeping initial concentration of Cr(VI) and pH constant at 20 mg/L and 9, respectively. Figure 6.7a–e represents the variations of percentage removal of Cr(VI), concentrations of dry biomass, carbohydrate, protein, and lipid with time, respectively. From the diagram, it can be stated that as the percentage of inoculum increases, both dry biomass and corresponding biomolecules content increase. The higher cell density due to higher inoculum size favors the interaction of Cr(VI) with the microalgal cells and thus the percentage removal increases. With the increase in inoculum size from 2 to 10%, the removal has increased from 30.88 ± 1.43% to 73.39 ± 6.28% after 14 days. At

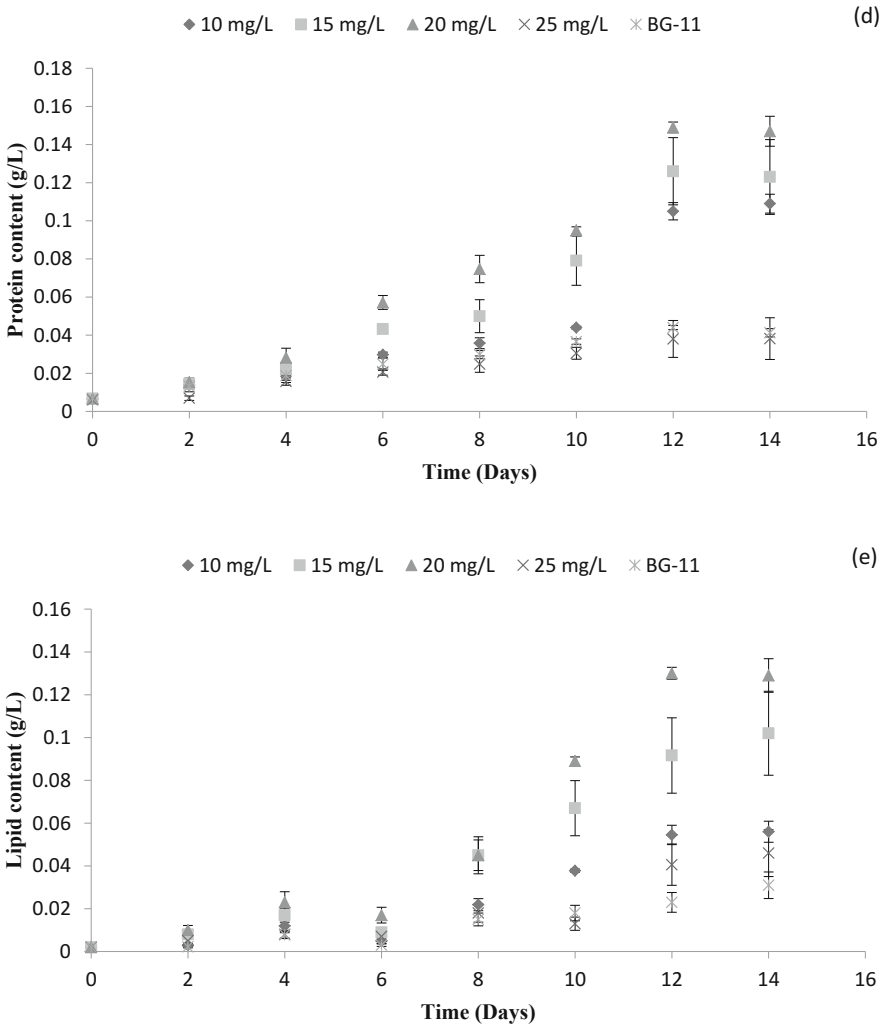

Fig. 6.5 (continued)

different inoculum size 2, 5, 10%, the increase in biomass, carbohydrate, protein, and lipid contents has been found to be 16.05, 13.69, 8.03 fold, 5.31, 5.69, 7.26 fold, 24.99, 22.05, 22.27 fold, and 28, 22.5, 64.5 fold, respectively, after 14days of growth.

The percentage of biosorbed and bioaccumulated Cr(VI) with time for different IC of Cr(VI) is represented in Fig. 6.8a–d. The bioremoval of Cr(VI) occurs through biosorption onto the cell wall followed by slow uptake at the cytoplasmic space through bioaccumulation. For 10 and 15 mg/L Cr(VI) solution, the biosorption is

dominant initially and later on, Cr(VI) is accumulated inside the cell. Initially Cr (VI) binds onto the cell surface and thus biosorption process is rapid compared to bioaccumulation. However, at higher concentration of Cr(VI), due to higher driving force, both bioaccumulation and biosorption occur simultaneously from the very beginning of the experimentation (Shuler and Kargi 2008).

6.4.5 ANN Model

Table 6.2 shows some typical input–output data used in ANN training of phycoremediation of Cr(VI). Table 6.3 summarizes the performance of the developed model. Very high R^2 (0.99926) and very low MSE (1.18563) for Cr (VI) removal of this developed model show that ANN successfully captures the relation between percentage removal of the contaminants with various input parameters like time, initial concentration, inoculum size, and pH.

Accuracy of developed ANN model can be judged by Figs. 6.5a, 6.6a, 6.7a, and 6.9. Figure 6.5a shows experimental vs. ANN model prediction of percentage removal of Cr(VI) for different initial metal concentration and time. Similarly Fig. 6.6a summarizes the experimental vs. ANN model prediction of percentage removal of Cr(VI) for different initial pH of the solution and time.

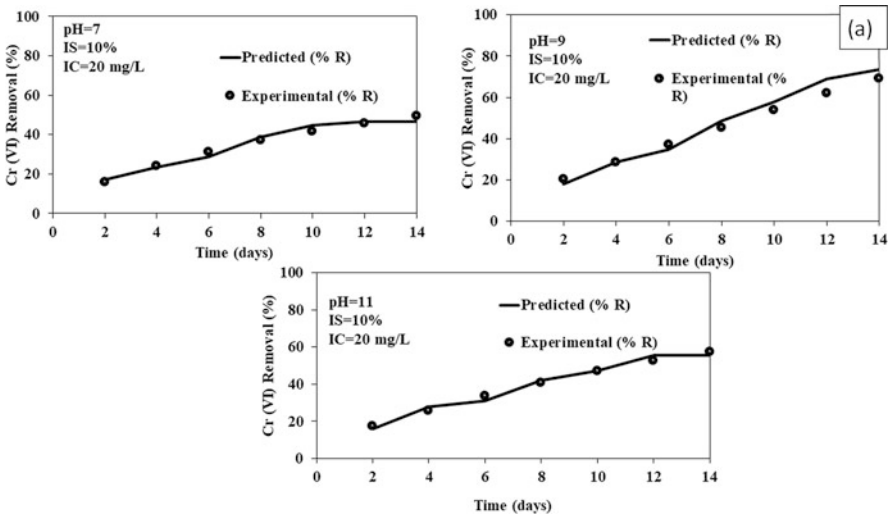

Fig. 6.6 (**a**) Experimental vs. ANN model prediction of percentage removal of Cr(VI) for different initial pH of the solution and time (Initial concentration: 20 mg/L and inoculum size: 10%). (**b**) Dry biomass for different initial pH of the solution with time (Initial concentration: 20 mg/L and inoculum size: 10%). (**c**) Carbohydrate content for different initial pH of the solution with time (Initial concentration: 20 mg/L and inoculum size: 10%). (**d**) Protein content for different initial pH of the solution with time (Initial concentration: 20 mg/L and inoculum size: 10%). (**e**) Lipid content for different initial pH of the solution with time (Initial concentration: 20 mg/L and inoculum size: 10%)

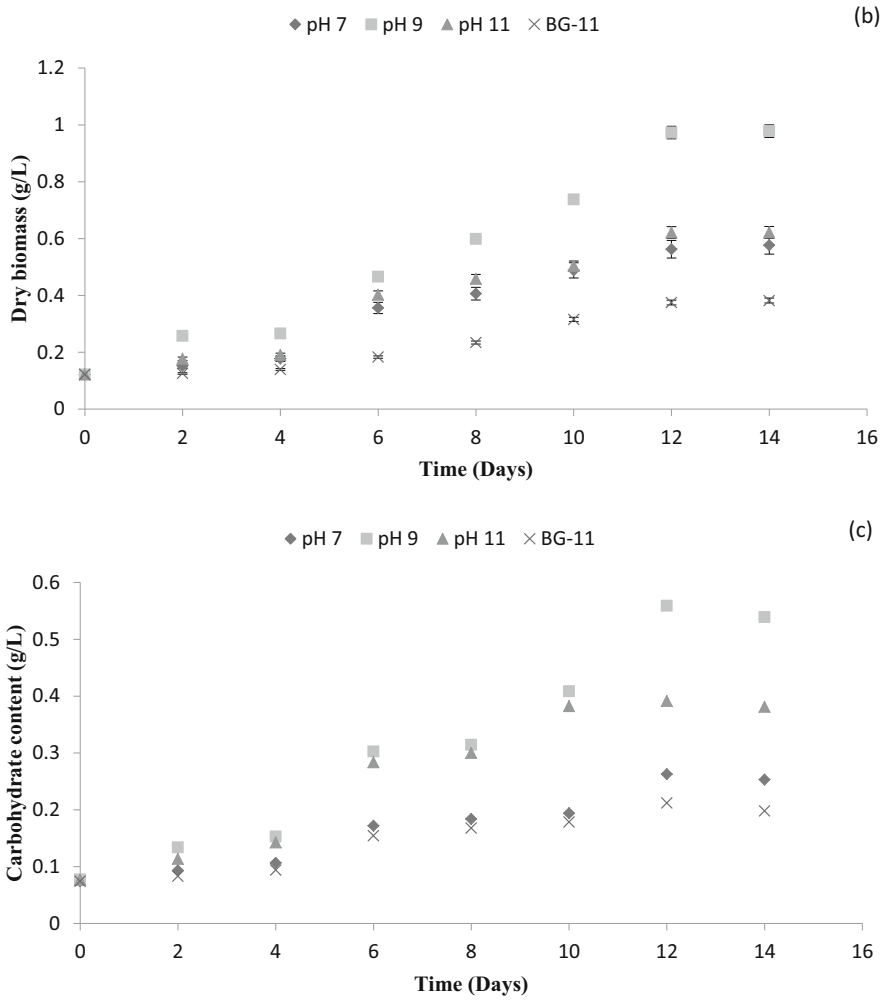

Fig. 6.6 (continued)

Experimental vs. ANN model prediction of percentage removal of Cr(VI) for different inoculum size and time is depicted in Fig. 6.7a. Figure 6.9 shows the close fit of experimental vs. Model predicted plot for phycoremediation of Cr(VI) for all the experimental data.

From these four figures, it is evident that there is very close fit between model prediction and experimental data and thus it can be concluded that ANN model truly capture the relationship.

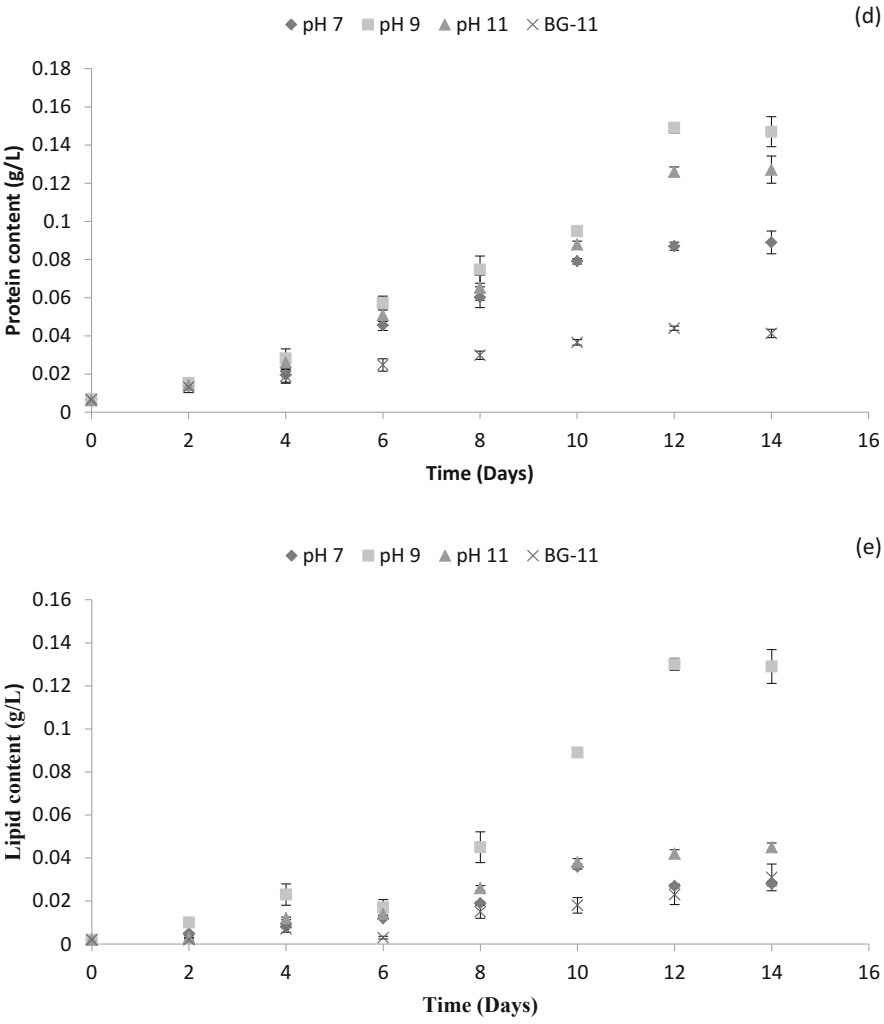

Fig. 6.6 (continued)

6.4.6 Simulated Annealing Optimization

Simulated annealing technique is applied in the present study to find out optimum value of four input variables (namely, pH, initial concentration of Cr, IS, and time of contact), so that Cr removal is maximized. Three different case studies were run to optimize the input parameters at different limiting conditions as shown in Table 6.4.

Case Study 1 In this case study minimum and maximum value limits of input parameters are fixed as per original experimental results as shown in Table 6.4. SA algorithm assumes any values of four input parameters within their high–low limit and passes it on to trained ANN model to get the percentage Cr removal. Each iteration SA algorithm modifies the input parameter as per its algorithm until the minimization of objective function is achieved.

SA algorithm keeps on searching the whole space of input parameters that until percentage Cr removal is maximized. It is concluded that 84.5% removal of Cr is possible under present study if the operating conditions are made as per given in Table 6.4. It is to be noted that maximum Cr removal obtained during experimentation was 73.4%. The benefit of such optimization study is that it will suggest what operating parameters need to be kept so that this Cr removal percentage increased up to 84.5%.

Case Study 2 In case study 1, it is observed that inlet concentration of Cr is 10 mg/L. Now we want to investigate how much percentage of Cr can be removed if inlet Cr concentration is kept at its highest, i.e., 25 mg/L. For that, low and high values of inlet concentration of Cr are kept 25 mg/L and optimization algorithm was run with this limit. Results showed that percentage removal drastically reduced to 32.11%.

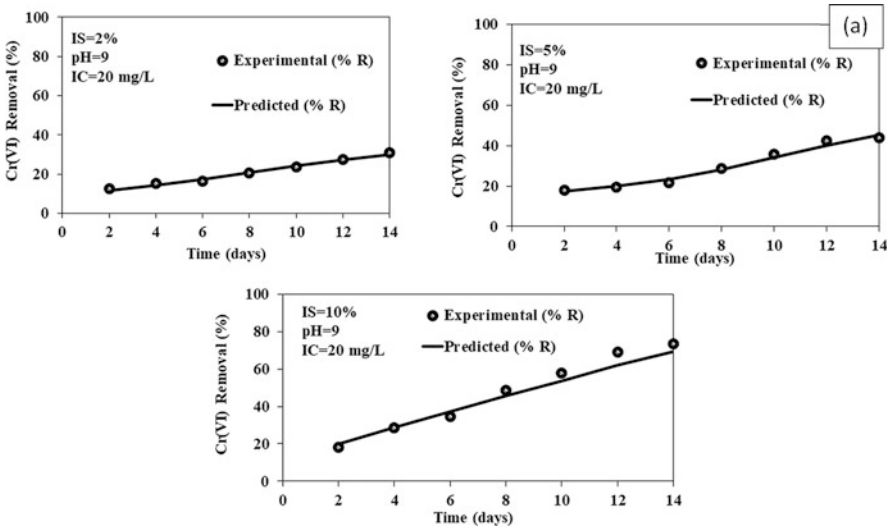

Fig. 6.7 (**a**) Experimental vs. ANN model prediction of percentage removal of Cr(VI) for different inoculum size and time (Initial concentration: 20 mg/L and pH: 9). (**b**) Dry biomass for different inoculum size with time (Initial concentration: 20 mg/L and pH: 9). (**c**) Carbohydrate content for different inoculum size with time (Initial concentration: 20 mg/L and pH: 9). (**d**) Protein content for different inoculum size with time (Initial concentration: 20 mg/L and pH: 9). (**e**) Lipid content for different inoculum size with time (Initial concentration: 20 mg/L and pH: 9)

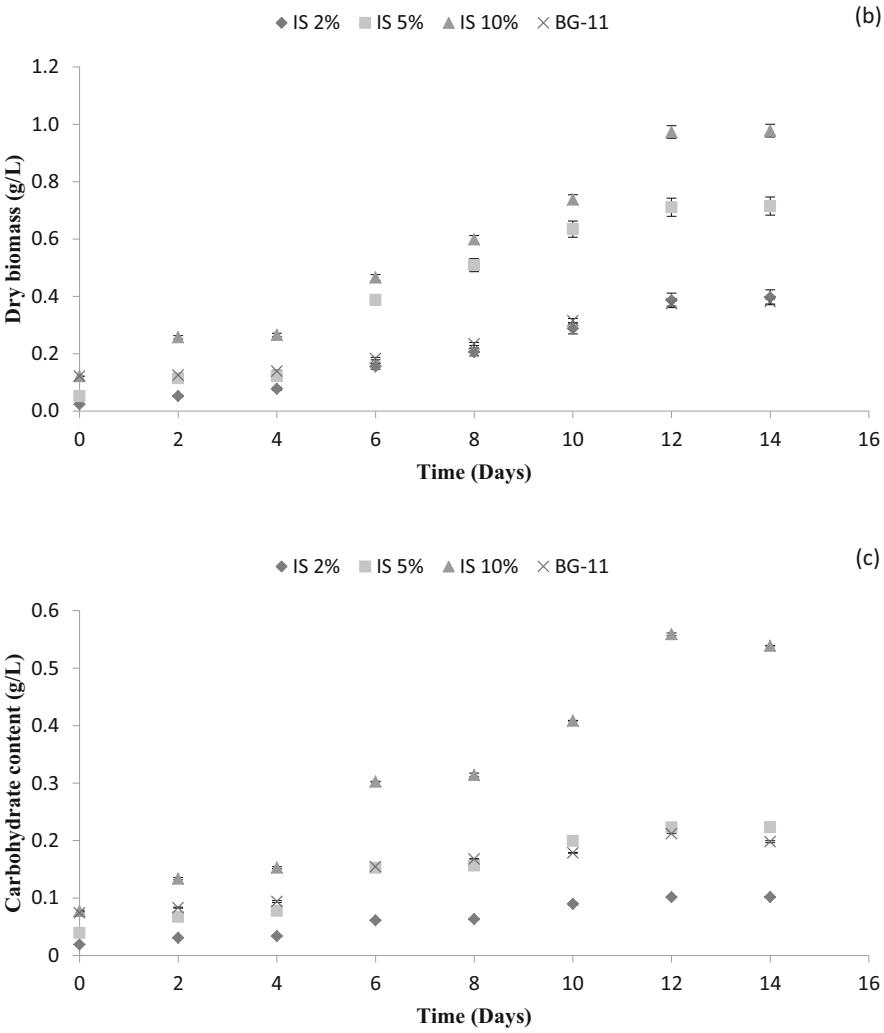

Fig. 6.7 (continued)

Optimum conditions of other parameters is calculated by SA and shown in Table 6.4. Note that maximum percentage removal at 25 mg/L inlet concentration during experimentations is 25.3%. It means by adjusting other input parameters 7% removal can be increased.

Case Study 3 For industrial applications, time is a crucial parameter. In this case study, time limit is maintained between 2 and 5 days. It will ensure maximum removal within 5 days instead of 14 days. Under this time limit condition, it is

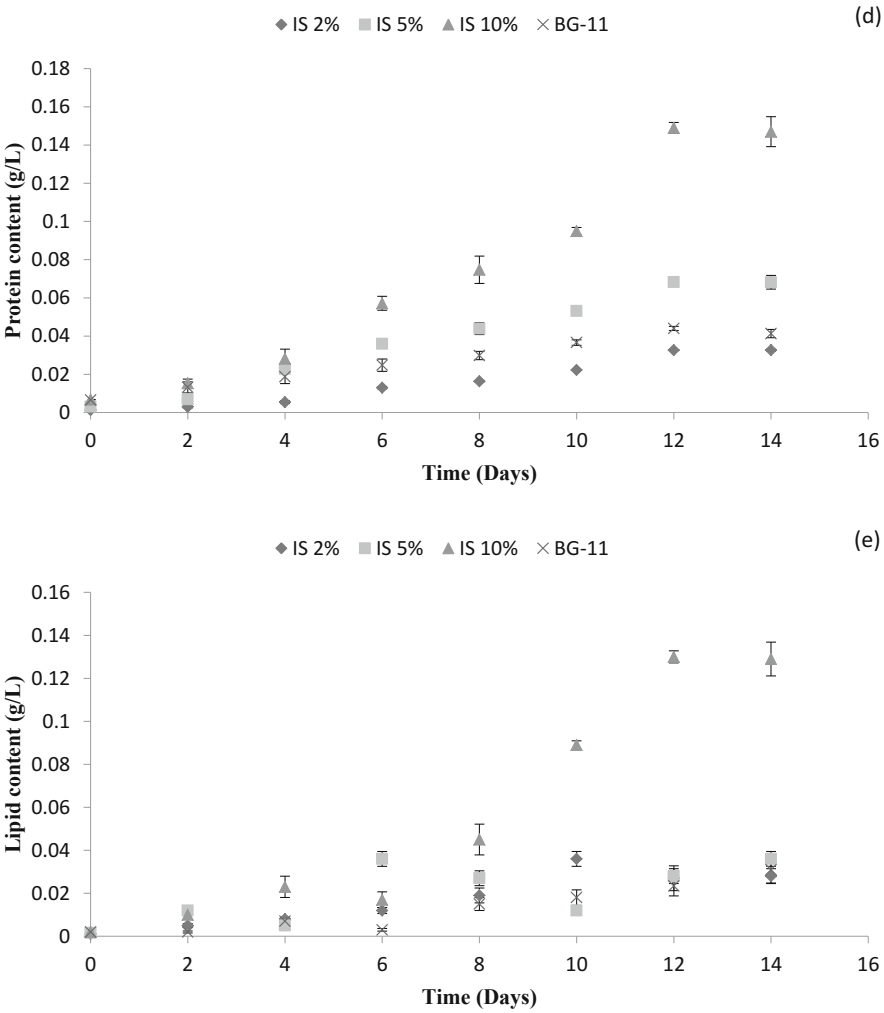

Fig. 6.7 (continued)

found that around 80% removal is possible within 5 days. For that other parameters to be kept at their optimum value as given in Table 6.4.

6.5 Conclusion

The microalgal strain of *Chlorococcum* sp. has been found efficient for abatement of Cr(VI) from synthetic wastewater. A detailed experimentation is carried out to know the effect of different process parameters on Cr(VI) removal efficiency. An accurate,

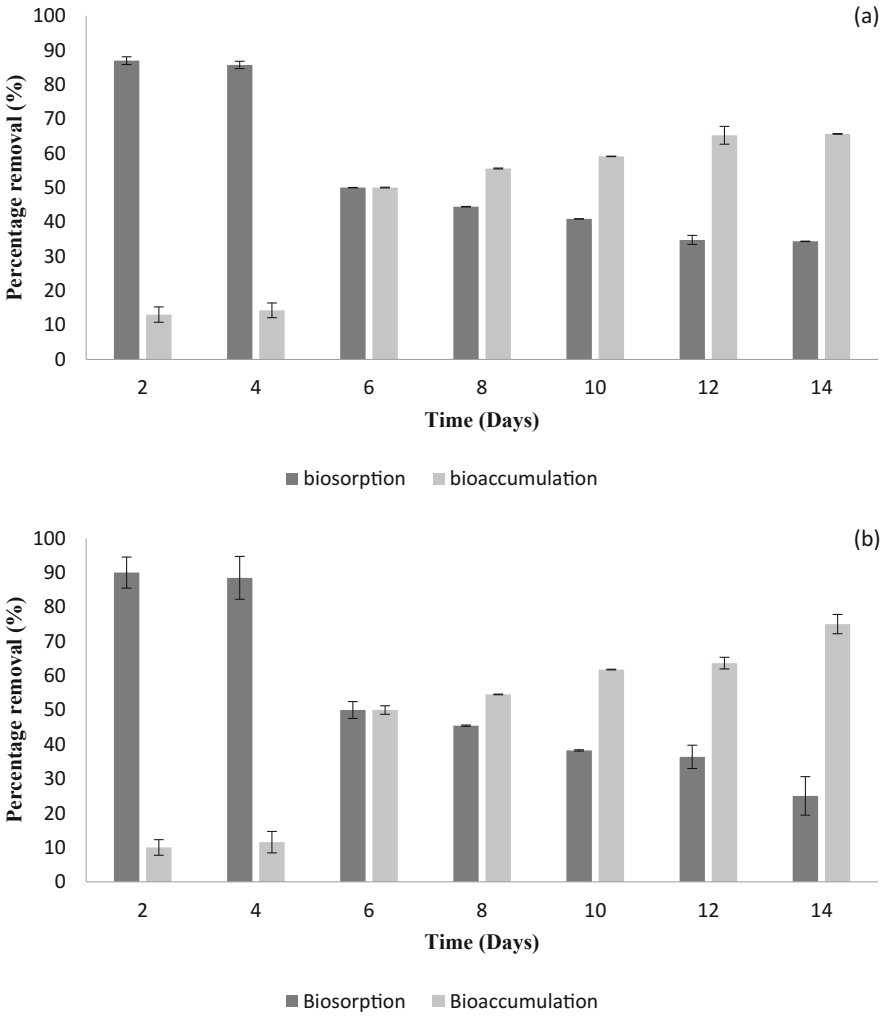

Fig. 6.8 (**a**) Biosorption and bioaccumulation study for Cr(VI) removal at (**a**) 10 mg/L initial concentration. (**b**) Biosorption and bioaccumulation study for Cr(VI) removal at 15 mg/L initial concentration. (**c**) Biosorption and bioaccumulation study for Cr(VI) removal at 20 mg/L initial concentration. (**d**) Biosorption and bioaccumulation study for Cr(VI) removal at 25 mg/L initial concentration

reliable ANN model is developed in the present work by an exhaustive search of all available ANN algorithms and activation functions available in literature. This will relieve the novice users to know the detail intricacy of all ANN algorithms and choose the best algorithm automatically on its own. The developed ANN model is then used to optimize the input space so that Cr(VI) removal can be maximized by simulated annealing technique. Experimentally maximum removal of

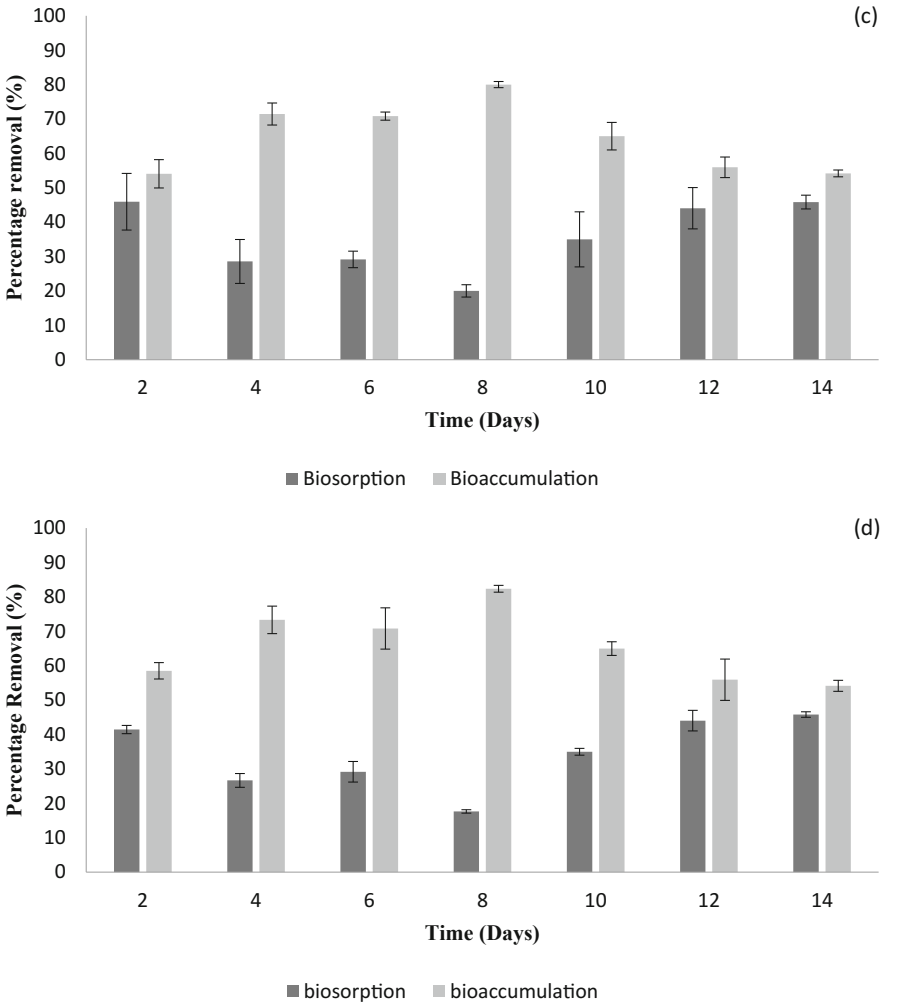

Fig. 6.8 (continued)

73.39 ± 6.28% has been obtained with initial chromium concentration of 20 mg/L, pH 9, and inoculum size 10% after 14 days of treatment. However, with the help of ANN modeling and simulated annealing optimization technique removal percentage can be increased up to 84.5%. The modeling and optimization framework used in the present study is generic and the methodology can be extended to any such experimental case study where the relationship is complex and process phenomenology is not fully known.

Table 6.2 Typical input–output data for ANN training for removal of Cr (VI) from synthetic wastewater using *Chlorococcum* sp.

SI. no	Time (days)	Initial concentration (mg/L)	Inoculum size (%)	pH	% Removal of Cr (VI)
1	2	10	10	9	15.08
2	14	10	10	9	55.93
3	4	15	10	9	22.69
4	12	15	10	9	56.97
5	8	20	10	9	48.77
6	12	20	10	9	68.97
7	12	25	10	9	15.03
8	14	25	10	9	22.25
9	6	20	10	7	28.78
10	8	20	10	7	38.79
11	10	20	10	7	44.79
12	8	20	10	11	42.08
13	14	20	10	11	55.73
14	12	20	2	9	27.69
15	14	20	2	9	30.88

Table 6.3 ANN model performance

Parameter	ANN model to predict removal of Cr(VI) from synthetic wastewater using *Chlorococcum* sp.
Total no of experimental data point	56
Optimum no of node	15
Activated function	Hyperbolic tangent sigmoid (hidden layer) and linear(output)
Training algorithm	BFGS quasi-Newton back propagation
Prediction accuracy	$R^2 = 0.99$ MSE = 1.27

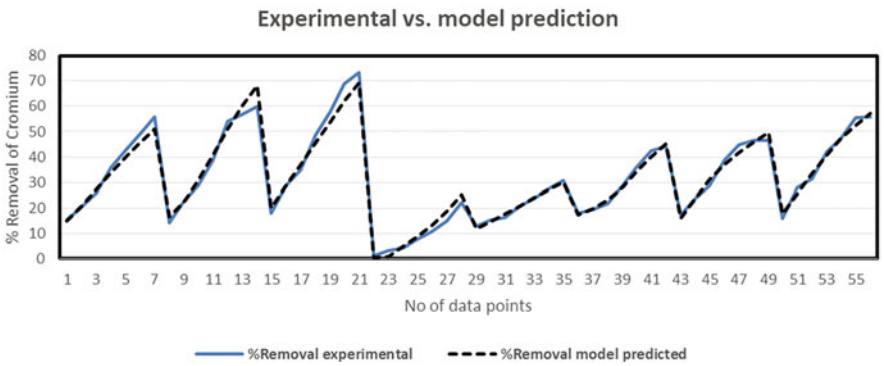

Fig. 6.9 Experimental vs. ANN model prediction of Cr(VI) removal percentage

Table 6.4 Optimum values of input parameters to maximize Cr removal

SI. no.	Time (days)	Initial concentration (mg/L)	Inoculum size (%)	pH	% Removal of Cr (VI)
Case study 1					
Low limit	2	10	2	7	
High limit	14	25	10	11	
Optimum value	11.08	10	2.05	9.3	84.50
Case study 2					
Low limit	2	25	2	7	
High limit	14	25	10	11	
Optimum value	4	15	10	9	32.11
Case study 3					
Low limit	2	10	2	7	
High limit	5	25	10	11	
Optimum value	5	10	2.09	9	80.10

Conflicts of Interest The authors declare that they do not have any conflict of interest.

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7 An Insight into the Potential Application of Microalgae in Pharmaceutical and Nutraceutical Production

K. Dhandayuthapani, S. Malathy, Sikandar I. Mulla, and Sanjay Kumar Gupta

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Abstract

Microalgal biomasses (MAB) is the most abundant source of various natural value-added biomolecules and bioactive compounds (BACs), therefore, considered as the best promising feedstock for the food and pharmaceutical industries. Microalgae-based BACs such as carotenoids, peptide molecules, phycocyanins, polyphenols, and polyunsaturated fatty acids (PUFAs) have significant application as functional ingredients in the pharmaceutical and nutraceutical industries. Due to the awareness of the consumers about the nutraceuticals in combating the occurrence of lifestyle and chronic diseases, the demands for the algal-based nutraceuticals have recently increased by several folds. Microalgae pigments, such as phycobiliproteins, chlorophylls, and carotenoids, have enormous possibilities for commercialization due to its therapeutic activities, which include antimicrobial, antioxidant, anti-inflammatory, antiproliferative, and anti-atherogenic activities. As per the estimates, the nutraceuticals market would cross about $278.96 billion by the end of 2021. Similarly, microalgae-based polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid, EPA, docosahexaenoic acid, arachidonic acid, etc. are also an important commercial product, and its global market is around USD 9.0 billion/Year. This chapter provides a comprehensive overview of bioactive compounds of microalgae and its pharmaceutical and nutraceutical properties. Various strategies used for the profitable production of microalgae biomass and extraction of bioactive compounds from algal biomass are discussed in detail. The commercial potential of algae BACs, associated safety, and regulatory issues are also discussed.

Keywords

Microalgae · Bioactive compounds · Pharmaceutical · Nutraceutical · Carotenoids

7.1 Introduction

Microalgae are photosynthetic microscopic unicellular or filamentous/colonial forms. This simple structure makes them utilize their full energy for growth and reproduction rather than maintaining body structures. Microalgae can live in complex habitats like high nutrients, salinity, and temperature. Thus, they can quickly

acclimatize to new environmental circumstances to live and produce various secondary metabolites that do not occur in organisms (Rodríguez-Meizoso et al. 2010). In addition to this, by the use of light energy and carbon dioxide (CO_2), the microalgae can produce high biomass compared to terrestrial plants. Besides, they can grow in land and climates that are not suitable for agricultural purposes. Therefore, microalgae never compete with food crops cultivation land. The control of BACs, the production by manipulation of microalgae culture conditions, is an additional benefit (Brennan and Owende 2013; Singh and Dhar 2011; Singh et al. 2011). Among the plant kingdoms, the microalgae are considered to be unadulterated natural bioreactors for making various non-synthetic bioactive compounds (BACs) and also the best choice to synthetic BACs of commercial concern (Plaza et al. 2009).

Microalgae are photosynthetic microorganisms, and their metabolic activity is similar to higher plants. Microalgae can produce a wide range of BACs. Therefore, these microalgae are considered one of the best promising sustainable feedstocks for pharmaceutical and nutraceutical applications. The growth rate of microalgae is comparably superior to higher plants. Microalgae can easily grow on limited nutrients, water, and light as these are good sequester of natural light compared to land plants (Gupta et al. 2018, 2019). Algae can efficiently utilize atmospheric CO2, and various micro- and macronutrients present in the water and wastewater. Therefore, they do not battle with food crops (Brennan and Owened 2010). Microalgal biomasses (MAB) have significant applications in the food and pharmaceutical industries due to their high value-added biomolecules (Saha and Murray 2018). The MABs are considered as a repository of BACs which have marketable nutraceutical and pharmaceutical properties (Ansari et al. 2017, 2017a, Saha et al. 2015).

The MABs used by human beings are century-old. Mass cultivation of microalgae was begun soon after World War II in countries like the USA, Germany, and Japan as an alternative food resource. Since the 1960s, the MABs are being used as a natural resource for health and medical purposes (Walker et al. 2005). In the past few decades, the MABs have also been used for wastewater treatment, biofuels production, mitigation of greenhouse gases (Benemann and Oswald 1996). Microalgae, which cover approximately 75% of algae species, contribute about 40% of the O_2 in the atmosphere. Nearly 40,000 microalgae species have been identified (Singh and Dhar 2010). The composition of MABs is very complex that includes vitamins, minerals, and several primary and secondary metabolites. Therefore, MABs have broad applications and uses for humans, i.e., nutritional to anti-aging properties (Nicoletti 2016). Microalgae-based BACs such as carotenoids, peptide molecules, phycocyanins, polyphenols, and polyunsaturated fatty acids have significant application as functional ingredients in the pharmaceutical and nutraceutical industries. These compounds are synthesized during primary or secondary metabolism and get accumulated in the algal cells (de Morais et al. 2015). Due to the awareness of the consumers about the nutraceuticals in combating the occurrence of lifestyle and chronic diseases, the demands for the algal-based nutraceuticals have recently increased by several folds. As per the estimates, the growth rate of the nutraceuticals market is increasing by 7.3% annually, and it would

cross about $278.96 billion by the end of 2021. The world's nutraceuticals market is majorly governed by some of the major companies like E. I. du Pont de Nemours, Groupe Danone S.A, BASF SE., Royal DSM N.V., Nestle, etc. Three microalgae species, such as *Spirulina*, *Chlorella*, and *Aphanizomenon*, have majorly revolved in the global nutraceuticals market for BACs production (Nicoletti 2016). About 11–12% increase is expected in the world market size of microalgae-based polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid (C20 ω-3:5) (EPA), docosahexaenoic acid (ω-3 C22:6) (DHA) and arachidonic acid (ω-6 C20:4) (ARA). The sales would surpass up to US$4 billion by 2022. Microalgae such as *C. vulgaris* and *Nannochloropsis* and many other species as well are believed to be the key constituents in the nutrition of sportsman thus are highly priced ranging from 18000 to 36000 US$ t^{-1} (Jha et al. 2017).

Microalgae-based BACs have enormous possibilities for commercialization due to its therapeutic activities, which include antimicrobial, antioxidant, anti-inflammatory, and antitumor activities. Traditionally terrestrial plants are exploited for BACs than the microalgae. In the past few decades, the focus has been shifted toward the microalgae as the diversity of BACs in microalgal biomasses is comparably several times higher than the terrestrial plants. Still, various aspects of microalgae are underexploited for BACs production. Only very few studies studied among the tens of 1000 species survive global (Salehi-Ashtiani et al. 2015). Due to these advantages, it is important to focus on pharmaceutical discovery and the production of microalgae-based medicinally critical natural products (Richmond and Hu 2013).

7.2 Microalgal and Their Bioactive Compounds

There is a growing interest in using microalgal biomasses (MAB) for the production of nutritional supplements, nutraceuticals, and pharmaceuticals (Olasehinde et al. 2017a, b). The biotechnological significance of MAB is similar to bacterial and fungal biomasses. Microalgae can rapidly flourish; thus, the production of algal metabolites is comparably higher than the terrestrial plants. Due to numerous potential health benefits, the algal metabolites are used in the cosmetic, food, and pharmaceutical industries (Pangestuti and Kim 2011; Skjanes et al. 2013). However, each fraction of cellular content differs in the algal species, and thus the concentration of the nutrient. The cellular composition of microalgae also depends on the various factors such as photoperiod and intensity of light, temperature, pH, etc. (Brown et al. 1993; Carvalho and Malcata 2000). These biotic and abiotic stresses can induce the secretion of various secondary metabolites in the microalgae species. Hence, MAB is considered a reservoir of multiple BACs (Table 7.1).

MAB is a promising source of the sustainable production of various natural pigments that have positive health effects. Microalgae pigments, for example, phycobiliproteins, chlorophylls, and carotenoids, are used in pharmaceutical products, cosmetics, and as food additives (De Jesus Raposo et al. 2013). Among various other pigments, carotenoids, which is a lipid-soluble pigment, have several

Table 7.1 Bioactive compounds of microalgae

Bioactive components	Microalgal species	References
β-Carotene	*Dunaliella salina*, *Chlorella Sorokiniana*	Matsukawa et al. (2000), Enzing et al. (2014)
Astaxanthin	*Haematococcus pluvialis*, *chlorella zofingiensis*, *Botryococcus braunii*, *Scotiellopsis oocystiformis*	Ghosh et al. (2015), Shah et al. (2016)
Lutein	*Scenedesmus* spp., *Muriellopsis* sp., *Chlorella sorokiniana*	Matsukawa et al. (2000)
Fucoxanthin	Diatoms	Yi et al. (2015)
Canthaxanthin	*Chlorella vulgaris*	Cha et al. (2008)
Echinenone	*Botryococcus braunii*	Tonegawa et al. (1998)
Violaxanthin	*Chlorella ellipsodea*	Cha et al. (2008)
GLA	*Spirulina*	Ramaraj et al. (2019)
α-Tocopherol	*Chlorella Sorokiniana*	Matsukawa et al. (2000)
ARA	*Porphyridium purpureum*, *P. cruentum*, *Parietochloris incisa*	Su et al. (2016), Solovchenko et al. (2008), Giménez et al. (1998)
EPA	*Nannochloropsis* sp., Phaeodactylum tricornutum, Porphyridium cruentum	Asgharpour et al. (2015), Molina et al. (1999), Sukenik (1999)
DHA	*Crypthecodinium cohnii*, *Schizochytrium limacinum*, *Monoraphidium* sp.,	Horrocks and Yeo (1999)
Phycobiliproteins	*Spirulina platensis*, *Phormidium autumnale*	Rodrigues et al. (2015)
Bioactive peptides	*Chlorella vulgaris*, *Spirulina platensis*	Xie et al. (2018)
Polyketide	*Scytonema pseudohofmanni*, *Nostoc* ATCC 53789	Ishibashi et al. (1986), Schwartz et al. (1990)
Superoxide dismutases	*Porphyridium*, *anabaena*	De Jesus Raposo et al. (2013)
Sterols	*Chaetoceros*, *Pavlova lutheri*, *Tetraselmis* sp., *Thalassiosira* sp.	Santhosh et al. (2016), Ahmed et al. (2015), Luo et al. (2015)

biological activities. Hence, it can be used as anticarcinogenic and antimutagenic agents. It also has antioxidant, anti-inflammatory antiproliferative, and anti-atherogenic properties. This pigment also functions as an accessory light-harvesting pigment in photosynthetic organisms (Lamers et al. 2008). This pigment is classified into carotenes and xanthophylls (IARC 1998). As of now, carotenoid is one of the most popular microalgal products in the market. More than 1200 tons of β-carotene are produced every year from a unicellular halophytic green microalga *Dunaliella salina* (Coesel et al. 2008; Enzing et al. 2014; Minhas et al. 2016). Therefore, countries like Australia, Israel, the USA, and China are pioneers in β-carotene production from algal biomass (Del Campo et al. 2007). The natural β-carotene can exist either in the *trans* or *cis* configuration. The all-*trans*, 9-*cis*, 13-*cis*, and

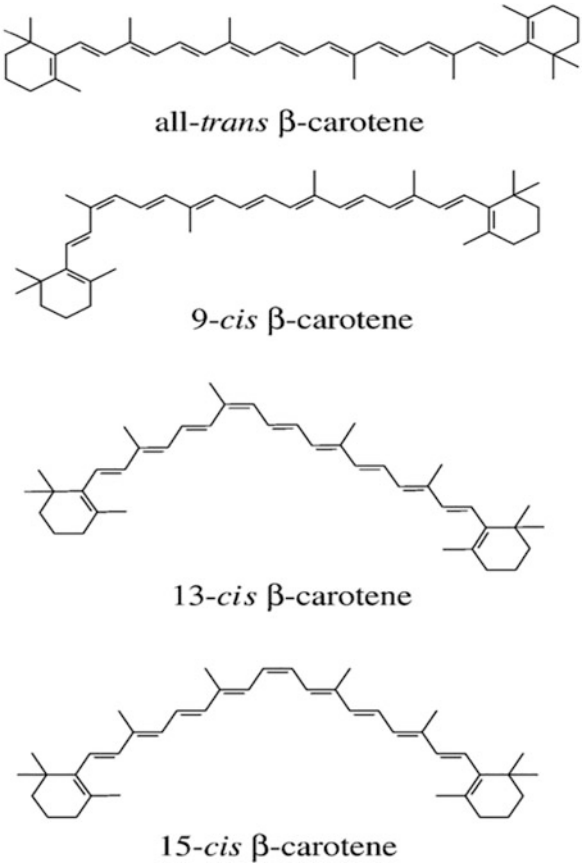

Fig. 7.1 Different chemical structures of β-carotene of *Dunaliella* sp. (Reproduced from Ben-Amotz et al. 1988)

15-*cis* stereoisomers have been identified in the natural β-carotene (Fig. 7.1). The β-carotene stereoisomers have different biokinetics and biological activities. Green microalga *Dunaliella* can biosynthesize up to 40% of 9-*cis* and 50% of all-*trans* stereoisomers β-carotene (Ben-Amotz et al. 1988).

Astaxanthin is another commercially vital carotenoid, which is mainly produced by microalga *Haematococcus pluvialis* (Shah et al. 2016). Other than this species, many other microalgae are also considered as an excellent natural resource for commercial production of astaxanthin (Ranga Rao et al. 2013). Astaxanthin is a powerful antioxidant agent like other nutraceuticals and used for boosting the immune system (Lee et al. 2015). Economically, astaxanthin is considered as the third most important carotenoid after β-carotene and lutein. Chemically this pigment exists in three different configurational isomers such as 3R, 3′R and 3S, 3′S (enantiomers), and a 3R, 3′S (meso form) (Fig. 7.2). However, the 3S, 3′S is the most abundant isomers in nature, whereas, in the synthetic astaxanthin, the configurational isomers occur as a racemic mixture (Turujman et al. 1997). Some microalgae species, such as *Chlorococcum* sp., *Chlorella zofingiensis*, and

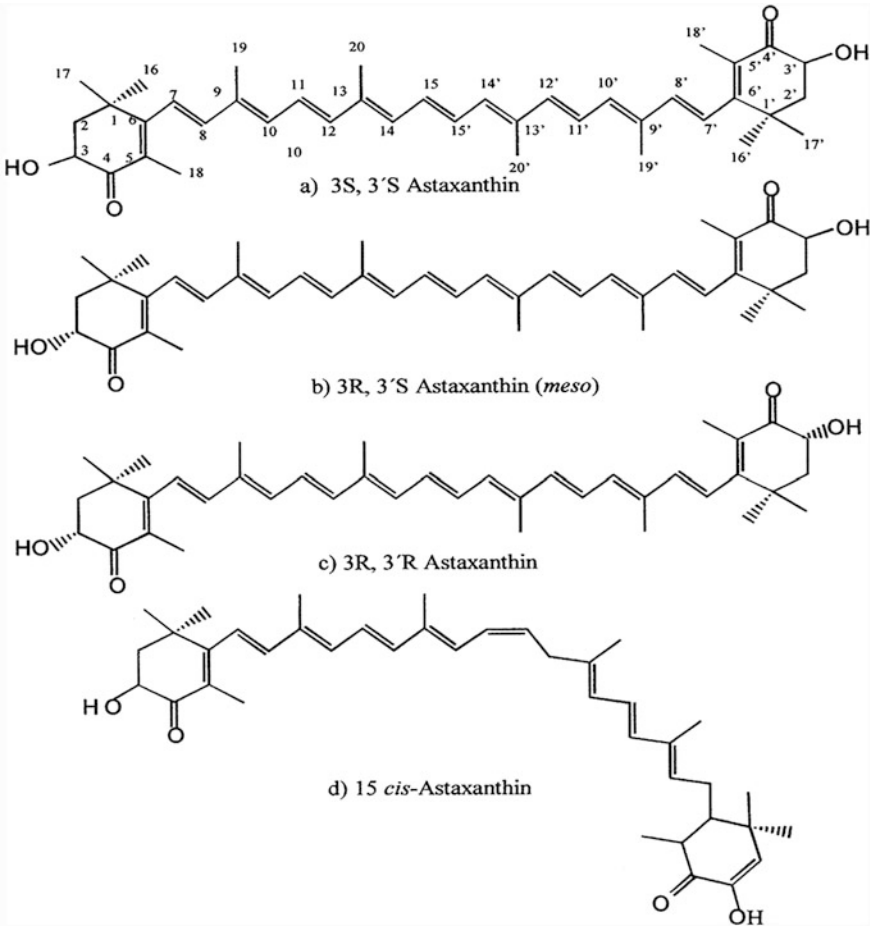

Fig. 7.2 Astaxanthin configurational isomers (**a**–**c**) and a geometric *cis* isomer (**d**) (Reproduced from Turujman et al. 1997)

Haematococcus pluvialis, are considered as potential producers of astaxanthin (Ambati et al. 2014).

Among the marine carotenoids, the fucoxanthin is a major one, which contributes about 10% of total carotenoids production (Peng et al. 2011). Diatoms and brown seaweed are rich sources of fucoxanthin. Chemically this pigment differs from other common carotenoids as structurally, this molecule includes a conjugated carbonyl, 5, 6-monoepoxide, and acetyl groups. Because of this unique property, it has high-energy transfer efficiency, i.e., ~80% (Kajikawa et al. 2012) and hence enhanced bioactivities. Therefore, presently, fucoxanthin is also an essential BAC due to its crucial roles in human health (Sathasivam and Ki 2018). The culture conditions and seasonal changes directly influence the fucoxanthin synthesis in microalgae (Fariman et al. 2015; Fernandes et al. 2016). Generally, 0.2–2.08 mg g^{-1} of

fucoxanthin can be obtained from wet, and 2.24–59.2 mg g^{-1} from dried microalgae biomasses. Due to high salinity tolerance, the MAB is considered as comparably better feedstock for fucoxanthin production than seaweeds (Gómez-Loredo et al. 2015; Ishika et al. 2017).

Microalgae-based polyunsaturated fatty acids are also considered as an important commercial product, and its market value is estimated at 140 USD/kg (Borowitzka 2013). The chances of contamination in microalgae PUFAs are comparably lesser than fish oils (Gerber et al. 2012; Ryckebosch et al. 2014). Some of the microalgal species, such as *C. cohnii* and *S. limacinum*, are heterotrophically cultivated in commercial-scale bioreactors for the production of DHA, which is commonly used as a dietary supplement in child food. The estimated global market of PUFAs is approximately USD 9.0 billion/Year (Qu et al. 2013; Wynn and Ratledge 2005; Barkia et al. 2019). The EPA is another essential fatty acid in the integrity of human tissues. The therapeutic application of γ-Linolenic acid (GLA) in cosmetics is to rejuvenate the skin and slow down the aging process. Linoleic and linolenic acids are essential for enhancing the human immune system and tissue regeneration. Besides, linoleic acid is also used for hyperplasia treatment (De Jesus Raposo et al. 2013). Some of the studies have been revealed that DHA and EPA can control heart disease, strokes, arthritis, and hypertension (Mimouni et al. 2012). DHA also plays a vital role in nervous system development. Furthermore, ARA and EPA are good platelet aggregators, vasodilators, and vasoconstrictors. They have antiaggregatory action on endothelium and chemostatic response in neutrophils (De Jesus Raposo et al. 2013).

Nutraceuticals and pharmaceutical biomolecules such as amino acids, proteins, vitamins, and polysaccharides can be obtained from MAB. All these biomolecules have a better effect on antibodies and cytokines production. Polysaccharide obtained from *S. platensis* has enhanced the proliferation of macrophages as well as T- and B-cell, and also can improve the confrontation of the organism. Besides, the same biomolecule of *S. platensis* also has the properties to improve erythrocytes, granulocytes, monocytes, and fibroelastosis proliferation in the bone marrow and thus the immune systems (Cheng-Wu et al. 1994).

Proteins are one of the vital macrometabolites of microalgae. The algal proteins have significant therapeutic potential as bioactive peptides and used as a food supplement for human beings (Ibanez and Cifuentes 2013). The amino acids and peptides present in the algal proteins not only provide nutritional benefits but also act as immunomodulators and play a significant role in the repair of injured and impaired tissues. De Jesus Raposo et al. (2013) reported that due to specific amino acid profile *Chlorella* and *Spirulina* (*Arthrospira*) can be used as functional foods and nutraceuticals to prevent the various types of human diseases.

7.3 Pharmaceutical and Nutraceutical Properties of Microalgal Compounds

7.3.1 Anticancer Properties

Cancer is the second leading reason for death globally (International Agency for Research on Cancer, IARC). In the year 2018, 18.1 million new cases and 9.6 million deaths were recorded worldwide. Approximately 70% of deaths by cancer occur in low- and middle-income countries. Globally, one in 8 men and one in 11 women die of cancer (IARC-WHO 2018). Chemotherapy is used as a primary treatment for cancers, but this treatment has many side effects in humans, such as baldness, appetite loss, etc. Therefore, tremendous efforts are being made for the hunt for new anticancer compounds from natural resources. Previous studies have demonstrated that plants contain various metabolites that can be used as an essential resource for the treatment of several forms of tumors (Cragg and Newman 2005). Microalgae-based phytochemicals are having comparably better potential anticancer activities than the terrestrial plant phytochemicals. Microalgae also contain a variety of phenolic groups, chlorophylls, and carotenoids, which are quite different from medicinal plant species (Prabakaran et al. 2018; Villarruel-López et al. 2017).

The bioactive compounds, such as phycobiliproteins, β-carotene isomers, and astaxanthin of microalgae *Spirulina*, *Dunaliella*, *and Haematococcus*, respectively, can serve as an anticancer drug. Microalga *Spirulina* contains a high amount of GLA, which serves as a potential nutraceutical product (Ramaraj et al. 2019). Previous studies have shown that GLA can kill tumor cells without damaging any healthy cells (Ben-Amotz 2004). Similarly, the β-carotene of the halotolerant *Dunaliella*, also well-known anticancer compound (Raja et al. 2018). The *Dunaliella* powder is widely used as a source of β-carotene (1–3%) in the form of tablets, capsules, and fortified nutritional blends for human health dietary supplements and animal feed. The oil extract β-carotene is used as a coloring agent (Hemaiswarya and Raja 2010), whereas water-extract β-carotene is used for beverages coloring.

Astaxanthin can prevent the cellular proliferation of two human gastric cancer cell lines, such as KATO-III and SNU-1 cell lines, by seizing the G0/G1 phase of the cell cycle. In addition, it can increase the p27 expression in stomach cell lines due to tilt of the extracellular signal-regulated kinase and cell cycle proteins (Joo et al. 2009). Astaxanthin is used as an anticancer agent (Walker et al. 2005), and it is being sold under various brand names; for example, in Hawaii, astaxanthin is sold in the name of BioAstin by Cyanotech Corporation (Raja et al. 2018). The various microalgae *Botryococcus braunii*, *Chlorella vulgaris*, *Chlorella zofingiensis*, *Chlorococcum* sp., *Diacronema vlkianum*, *Euglena rubida*, *Haematococcus lacustris*, *Haematococcus pluvialis*, *Neochloris wimmeri*, and *Scotiellopsis oocystiformis* are rich in natural astaxanthin, which has beneficial effects in human metabolism due to high antioxidant properties. Therefore, these algal species are being used as a nutraceutical product and food supplement for humans (Ambati et al. 2014; Ghosh et al. 2015).

Marine blue-green algae (BGA) are potential producers of BACs and effective destroyer of cancer cells through inducing apoptotic death/affecting cell signaling via activation of protein kinase C enzyme (Boopathy and Kathiresan 2010). Potential anticancer compounds such as polyketide-derived macrolides scytophycins, cryptophycin, and scytonemin have been isolated from blue-green algae *Scytonema pseudohofmanni* (Ishibashi et al. 1986), *Nostoc* ATCC 53789 (Schwartz et al. 1990), and *Stigonema* (Stevenson et al. 2002), respectively. The calothrixins A and B are phenanthridine alkaloids, which are extracted from the *Calothrix* sp., belong to BGA. It has been established that this compound kills the human HeLa cells at the molecular level (Pang et al. 2010). DHA of microalgae also induced many changes in mitochondria to enhance the cellular apoptosis of human colonic tumor cell line HT29 (Siddiq and Dembitsky 2008). Similarly, fucoxanthin produced from *Cylindrotheca closterium* and *Phaeodactylum tricornutum* also serves as a potential anticancer agent (Maeda et al. 2007).

7.3.2 Antioxidant Properties

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) cause oxidative stress and are accountable for the degradation of proteins, DNA, and lipids (Pérez-Rodríguez 2009). Though the human body has robust mechanisms to control oxidative stress through antioxidants but a higher concentration of both ROS and RNS can induce various chronic and degenerative ailments in humans. In the human body, this process takes place via different in-situ and ex-situ processes. In-situ, the human body synthesizes an endogenous antioxidant with the help of enzymatic and non-enzymatic pathways, while exogenous antioxidants cannot be synthesized in the human body. These can only be supplemented through foods in the form of vitamin E and C, carotenoids, flavonoids, DHA, selenium, manganese, zinc, etc. (Pham-Huy et al. 2008). Both types of antioxidants function as free radical scavengers and repairing cell damages caused by ROS and RNS. Thus improve the immune system and reduce the risk of cancer (Valko et al. 2006; Chatterjee et al. 2007).

Plants are a good source of a wide range of natural antioxidants. Similar to higher plants, lower non-vascular plant group, such as algae, also represents a significant resource for antioxidant compounds (Rodriguez-Garcia and Guil-Guerrero 2008; Kumar et al. 2019). Algae are a precious resource of a mixture of pigment and antioxidants, thus have an excellent marketable outlook (Vigani et al. 2015). Furthermore, ease of algal cultivation, manipulation of growth environment made it one of the most significant resources for antioxidants (Giorgis et al. 2017). Microalgae are also able to produce numerous components by single species. For example, 0.69% of dry matter of *C. Sorokiniana* contains 112, 600, and 4300 μg g^{-1} of α-tocopherol, β-carotene, and lutein, respectively. These compounds have a high antioxidant property (Matsukawa et al. 2000). Hence, MAB is considered a natural resource for antioxidants.

The α-carotene, β-carotene, astaxanthin, lutein, phycobiliproteins, and Vitamin E extracted from microalgae contain higher antioxidant activity. However, the

efficiency of these compounds is comparably low than synthetic antioxidants butylated hydroxyanisole and Butylated hydroxytoluene (Natrah et al. 2007). Various studies have demonstrated that the phycobiliproteins of *S. platensis* also function as free radical scavengers and can save the DNA from ROS (Bhat and Madhyastha 2001; Bermejo et al. 2008). Rodrigues et al. (2015) showed the antioxidant ability of phycobiliproteins obtained from *Phormidium autumnale* at 274 μmol Trolox g^{-1} of dry biomass weight.

Carotenoids are found in both plants and algae, while some are limited only to algae (Takaichi 2011). Algae carotenoids exhibit different properties including singlet oxygen quencher and antioxidant activity. Carotenoids of microalgae are considered as potent antioxidants since it can scavenge singlet molecular oxygen and peroxyl radicals (Sies and Stahl 2004). Due to awareness among the consumers, there is a massive demand for natural antioxidants. The bioavailability of natural antioxidants is higher than synthetic antioxidants (Gouveia et al. 2008; Spolaore et al. 2006). Therefore, MAB has significant use in pharmaceuticals, functional foods, and beverages for human consumption (Christaki et al. 2011). Various microalgae, such as *D. salina*, *C. vulgaris*, *C. protothecoides*, *H. pluvialis*, and *Sarcina maxima*, are rich in carotenoids and thus are a suitable candidate for commercialization of carotenoids (Han et al. 2012; Lordan et al. 2011).

Some of the microalgae produced phenolic compounds as secondary metabolites with antioxidants and other biological activities. Though these compounds are not directly involved in primary algal processes, such as photosynthesis, cell division, and reproduction, but most of these compounds play an essential role in algal cell defense against abiotic and biotic stress. Purified phenolic compounds exhibit many activities such as anti-radical, UV-protection, metal chelation, e.g., copper and antifouling. However, the main bioactivity associated with phenolic compounds such as hesperidin, morin, caffeic acid is the antioxidant activity for human beings, if consumed as a food (Suganya et al. 2016). Microalgae *Porphyridium* and *Anabaena* produce superoxide dismutases (SOD), which are metalloenzymes having iron, manganese, or copper/zinc. These enzymes function against cytotoxic superoxide free radicals as antioxidants and protect cells from the effect of ROS (De Jesus Raposo et al. 2013). The fucoxanthin of *Cylindrotheca closterium* and *Phaeodactylum tricornutum* is also a potent antioxidant agent (Maeda et al. 2007a, b).

7.3.3 Antihypertensive Properties

The WHO reported that >17.5 million people died every year by CVD like hemorrhagic stroke, myocardial infarction, chronic kidney disease, peripheral vascular disease, and premature death. Hypertension (high blood pressure) is a significant reason for these diseases; therefore, it is also known as the silent killer. In human beings, blood pressure is regulated by the renin-angiotensin system (RAS) and kallikrein bradykinin system (KKS) (Deng et al. 2018; Faria et al. 2008). Currently, calcium channel blockers, diuretics, and RAS inhibitors are used for the treatment of

hypertension. In the RAS, the renin enzyme is used to convert the inactive angiotensinogen into active angiotensin-I. Then this vasodilator is converted into angiotensin-II by the angiotensin-I converting enzyme (ACE-I). Hence, these two inhibitions are now considered as primary targets for the treatment of hypertension. Some of the previous studies have shown that natural compounds act as an inhibitor for the renin-angiotensin system (Hong et al. 2008; Jarari et al. 2016; Lee and Hur 2017). However, presently, captopril, enalapril, lisinopril, and fosinopril are used as common ACE-I inhibitors. Most of these drugs cause serious side effects and add the additional cost of health care (García-Mora et al. 2017). Due to this rationale, the researcher's interest increased to develop natural ACE-I inhibitor peptides, which does not pose any toxicity, so that the synthetic antihypertensive drugs can be replaced (Aluko 2015). Some of the studies showed that dietary intake of the *Chlorella* biomass could decrease blood pressure in human beings, but the mechanism is still unexplained. ACE-inhibitory peptides present in *C. vulgaris* and *S. platensis* can also regulate the RAS and lower the blood pressure (Murakami et al. 1987; Suetsuna and Chen 2001; Xie et al. 2018).

7.3.4 Anti-Obesity Properties

Obesity is a multifactorial metabolic disorder, which can increase several health complications due to adipogenesis and may cause diseases like cancer, diabetes mellitus, and cardiovascular disease (CVD) (Kopelman 2000; Kong et al. 2009; Wang et al. 2008). In addition, ROS and NOS play a role in adipogenesis, therefore, also involved in the development of obesity. Due to the presence of BACs, including antioxidants, MAB is considered as an anti-hyperlipidemic and fat reducing agent. Hayato et al. (2006) reported that the fucoxanthin and fucoxanthinol can arrest the proliferation of 3T3-L1 cells into adipocytes by downregulating the peroxisome proliferator-activated receptor-C. Okada et al. (2008) also reported that the microalgal pigment, neoxanthin, and fucoxanthin could inhibit the fat accumulation in mouse fed on MAB. Fucoxanthin notably reduced fat in obese persons in a clinical trial (Abidov et al. 2010; Kim et al. 2012). Maeda et al. (2007a) also demonstrated anti-obesity properties of the fucoxanthin of *C. closterium* and *P. tricornutum*.

Obesity is directly linked to CVD and diabetes. A 4-week study evaluating the effects of *S. platensis extract* on obesity revealed that the *S. platensis* supplement (2.8 g) reduced the body weight by lowering the fats level in obese humans (Becker et al. 1986; Mani et al. 2000). In another similar study, Park et al. (2008) reported that the intake of *S. platensis* biomass might decrease hyperlipidemia nephrotic syndrome by lowering the triacylglycerol levels and increasing high-density lipoprotein levels. The C-phycocyanin component of blue-green algae also has a hypocholesterolemic effect, and it inhibits the bile acid reabsorption in the ileum and cholesterol reabsorption in the jejunum (Yeganeh et al. 2014; Nagaoka et al. 2005). Such properties directly and/or indirectly save the cardiovascular system by reducing diastolic and systolic blood pressure (Torres-Duran et al. 2007; Samuels et al. 2008). The PUFAs of *Monoraphidium* sp., *Scenedesmus* sp., and *Nannochloropsis* sp. are

used in the human diet to control obesity. The results revealed that the PUFAs, the EPA, and DHA play a significant role in lowering the blood cholesterol as well as the rejuvenation of the fetal brain (Jha et al. 2017).

7.3.5 Anti-Inflammatory Properties

Inflammation is a multifaceted physiological process in the human body, which occurs due to invasion of pathogenic microbes or physical injury (Calder 2006). The synthetic pharmaceuticals that are used for inflammation may cause metabolic diseases like CVD, type 2 diabetes, as well as gastrointestinal side effects (Esser et al. 2015; Sostres et al. 2010). The use of natural anti-inflammatory agents is desirable as a long-term approach to curb chronic inflammation and alleviate disease-associated symptoms. Microalgal BACs include peptides and polyphenols, which have anti-inflammatory effects on mammalian cells, with several other potential health benefits (Lordan et al. 2011; Robertson et al. 2013). Therefore, in the recent past, microalgae biomass has attracted attention as an anti-inflammatory agent (Robertson et al. 2015; Soontornchaiboon et al. 2012). In numerous studies, microalgae pigments such as chlorophyll-*a*, β-carotene, fucoxanthin, and zeaxanthin have been proved as an anti-inflammatory agent (Nidhi et al. 2015; Robertson et al. 2015).

Some microalgae contain high LC-PUFAs (EPA and DHA) up to 60% of their total fatty acids, which have been used as an anti-inflammatory to reduce inflammation in both in vitro and in vivo studies (Calder 1851). *These compounds act* as a precursor to potent anti-inflammatory mediators, which stop the inflammatory cytokine production and also the expression of the inflammatory gene (Wall et al. 2010). Therefore, LC-PUFA has been considered as a dietary therapeutic ingredient for the treatment of various chronic inflammatory diseases (Calder 2013). The anti-inflammatory effect of *S. platensis* is explained as an inhibitive effect of gamma-linolenic acid, which controls inflammation (Nichols and Wood 1986; Remirez et al. 2002). Chen et al. (2012) observed that the C-phycocyanin extract from *S. platensis* could regulate the cytotoxicity and inflammation-associated factors like COX-2, tumor necrosis factor-α, etc. Similarly, the fucoxanthin of *C. closterium* and *P. tricornutum* can also reduce the inflammation (Maeda et al. 2007a).

7.3.6 Anti-Cardiovascular Disease Properties

High blood pressure, hyperlipidemia, and hyperglycemia; each one is a risk factor for CVD (Foroughi et al., 2013). Low-density lipoproteins (LDL) and very-low-density lipoproteins (VLDL) levels are the most crucial independent danger factor for cardiovascular events. At the same time, low levels of high-density lipoproteins (HDL) and elevated triglycerides (T.G.) are also associated with residual risk factors for CVD (Sharma et al. 2009). The use of microalgae BACs may reduce the risk of CVD by decreasing LDL/VLDL, T.G. and increasing HDL cholesterol. Therefore,

algal BACs can be used for preventing and mitigating the damage caused by such types of disorders (de Lorgeril et al. 1999; Ku et al. 2013).

The carotenoids from microalgae have the ability to prevent cardiovascular disease due to their antioxidant properties. The carotenoids can stop oxidative stress (O.S.) by shielding against lipid peroxidation and following damage to proteins and DNA by triggering genes accountable for regulating enzymes such as superoxide dismutase (SOD), catalase, and lipid peroxidase. Green microalgae *D. salina* and *B. bardawil* contain a mixture of *cis*- and *trans*-β-carotene antioxidant compounds that can protect against O.S. by blocking lipidic free radicals or chelating metal ions, scavenging or quenching ROS or by absorbing UV radiation (Cuvelier 2001; de Raposo and de Morais 2015). The *Spirulina* extracts have hypolipidemic activity, which reduced total serum cholesterol, LDL, and VLDL fractions. Besides, it can also increase the HDL cholesterol levels and decrease atherogenic indices and triacylglycerol levels. Nagaoka et al. (2005) suggested that the phycocyanin of *Spirulina* is a dynamic element and responsible for hypolipidemic activity. Derivatives of microalgae PUFAs, such as γ-ALA, EPA, DPA, and DHA, are also being used for the treatment of CVD (Jha et al. 2017).

7.3.7 Antimicrobial Properties

Pathogenic bacteria and fungi cause several types of diseases in both plants and animals, including humans. The synthetic antibiotics that are used to control these organisms have many side effects. Therefore, tremendous efforts are being made to find out new natural antibiotics having a broad action spectrum. Generally, natural antibiotics have reasonably no side effects. The hunt for new antimicrobial compounds is motivated by an increase of antibiotic resistance in humans due to regular clinical uses of several antibiotics. Microalgae have a wide variety of BACs that are efficient as antimicrobial compounds. Therefore, MAB is considered as an important resource due to the presence of various compounds such as phenols, sulfur-containing heterocyclic compounds, halogenated aliphatic compounds, sterols, acrylic acids, fatty acids, terpenoids, acetogenins, and carbohydrates. Almost all of these compounds have excellent antimicrobial properties (Prakash et al. 2011).

Washida et al. (2006) reported first microalgae-based antibacterial products obtained from *Chlorella*, which considerably inhibited the growth of gram-positive and gram-negative bacteria. Some BGA also produce antifungal compounds (Volk and Furkert 2006). The exo-metabolites of cyanobacteria *Nodularia harveyana* and *Nostoc insulare* such as Norharmane (9H-pyrido(3,4-*b*)indole) and 4,4-′-dihydroxybiphenyl have also had antimicrobial activities. The okadaic acid and ciguatoxin of *Prorocentrum lima* and *G. toxicus* are active antifungal agents as well.

The antimicrobial action of MAB is noteworthy because of their potential to produce α- and β-ionone, β-cyclocitral, neophytadiene, phytol, γ-linolenic acid, EPA, hexadecatrienoic acid, DHA, palmitoleic acid, lauric acid, oleic acid, lactic acid, and arachidonic acid. These compounds act as an antimicrobial agent against various human pathogens like *Escherichia coli*, *Pseudomonas aeruginosa*,

Staphylococcus aureus, and *S. epidermidis* (Amaro et al. 2011; Smith et al. 2010). Lipid metabolites of *Chaetoceros lauderi* have been found to inhibit different bacterial pathogens. The crude extract *of M. aeruginosa* contains several toxic metabolites that display high antimicrobial activity as well (Khalid et al. 2010). The crude extract of *D. salina* drastically inhibits the growth of *S. aureus*, *Klebsiella pneumoniae*, *E. coli*, *Candida albicans*, *P. aeruginosa*, *and Aspergillus niger* (Mendiola et al. 2008). Extracts of *D. primolecta* also act as an excellent antibacterial against human pathogen *S. aureus* (Pane et al. 2015).

Studies have shown that bioactive compounds of MAB exhibit antiviral activity against herpesviruses, togaviruses, rhabdoviruses, and human immunodeficiency viruses (HIV). Polysaccharides play an essential role in the biomedical and pharmaceutical industries because they act as antiviral compounds that can stop the penetration and replication of viral particles in human cells. These readily available polysaccharides are non-toxic, safe, biodegradable, and biocompatible (Ahmadi et al. 2015; Falaise et al. 2016). Extracellular sulfated polysaccharide (ESP) of microalgae is a potential therapeutic agent as well. Highly sulfated antiviral polysaccharides from microalgae consisted of xylose, glucose, and galactose (Raposo et al. 2014). Sulfated polysaccharides inhibited HIV replication at 10 ng mL^{-1} concentration without any side effect on host cells (Schaeffer and Krylov 2000). A sulfated polysaccharide of *S. platensis* showed the excellent antiviral activities against herpes simplex virus type 1 (HSV-1) and immunodeficiency virus type 1 (HIV-1). A similar molecule of *A. platensis* is also used for the treatment of viral disease without any cytotoxic effects (Hayashi et al. 1996; Rechter et al. 2006). Naviculan of diatom *Navicula directa and Cochlodinium polykrikoides* extract such as A_1 and A_2 have potent antiviral activity against enveloped viruses like HIV-1, HSV-1, and influenza virus type A (Hasui et al. 1995; Yim et al. 2004).

Steroids and glycolipids obtained from MAB have potent antiviral activity against HIV. Boyd et al. (1997) showed that cyanovirin-N, a polypeptide, extracted from *N. ellipsosporum*, was interacted with the envelope of virus and inhibited drug-resistant HIV-1. Polysaccharide calcium of *Spirulina* sp. also had broad activity against herpes simplex virus, influenza, and HIV. Nostoflan extracted from *N. flagelliforme* inhibited herpes simplex virus replication by inhibition of virus-host interaction (Kanekiyo et al. 2005).

7.3.8 Antidiabetic Properties

Diabetes is a shocking endemic of the twenty-first century. It is becoming the third killer of human health subsequent to cancer and CVD. The world diabetic population would be expected up to 366 million by 2030. Diabetes Mellitus (DM) is a metabolic disorder characterized via chronic hyperglycemia due to carbohydrate metabolism characterized by low insulin secretion and/or insulin resistance (ADA 1999). The primary complications of DM are retinopathy and neuropathy. There are two major DM types, Type 1 or insulin-dependent DM and Type 2 or non-insulin dependent

DM. Both have different clinical and pathophysiologic features (Bhattacharjee et al., 2014).

Apart from antioxidant activity, some of the marine algal species have antidiabetic properties and can inhibit some of the enzymes like α-glucosidase, aldose reductase, and protein tyrosine phosphatase. Fucoxanthin of marine diatoms can induce the DHA synthesis in the liver and reduces weight by stopping the accumulation of white adipose tissue fat. About 0.02% of fucoxanthin is enough to reduce the body weight. It can also lower plasma concentrations of hepatic triglyceride, adipocyte fatty acid synthesis, hepatic fatty acid synthesis, and triglyceride synthesis. Experiments on animals have shown that this compound can slow down blood glucose levels and regulate plasma-insulin in obese mice. Fucoxanthin of *C. closterium* and *P. tricornutum* is a potential antidiabetic agent (Maeda et al. 2007b). Besides, fucoxanthin controls the tumor necrosis factor-α, monocyte chemoattractant protein-1, interleukin-6, and also regulates the insulin/glucagon ratio (Bhattacharjee et al. 2014). Some of the studies showed that the food supplemented with *S. platensis* had a positive effect on diabetic patients as the gamma-linolenic acid of this alga has the antidiabetic properties, which is attributed to the reduction in hyperglycemia (Becker et al. 1986; Ben-Amotz 2004). Derivatives of microalgae PUFAs, such as ALA, DHA, and EPA, were used for the treatment of type 2 diabetes.

7.3.9 Alzheimer's Disease

Neurological disorder such as Alzheimer's disease (AD) is a complex pathophysiological disorder resulting in memory loss and cell death (Adefegha et al. 2016; Oboh et al. 2016). The death rate of patients suffering from AD is increasing all over the world. As per the recent report, >46 million people are living with AD, and this figure is expected to be >130 million by 2050 (World Alzheimer's Report 2015). Currently, rasagiline, rivastigmine, and donepezil are used to treat AD by inhibiting the cholinesterase and β-secretase (BACE-1) inhibitors (Vassar 2014).However, these drugs have side effects. Therefore, the use of natural functional foods and nutraceuticals may be an alternative therapeutic agent to manage AD (Olasehinde et al. 2017a, b).

Various neuroprotective products obtained from algal biomass have shown beneficial effects on nerve cells. Such nutraceuticals may save the nerve cells from multiple neurodegenerative diseases. Various studies have shown that extracts of *B. braunii*, *N. oculata*, *C. minutissima*, *Tetraselmis chuii*, and *R. salina* have potential inhibitory effects on AChE activity and improve the cholinergic transmission in AD patients (Pangestuti and Kim 2011; Custódio et al. 2012). Similarly, Pereira et al. (2015) reported that methanol extracts of *Nannochloropsis* sp., *Picochlorum* sp., and *Desmochloris* sp. could inhibit the BChE activity. The DHA and EPA of some microalgae also have inhibitory effects on AChE and BChE activity. Several epidemiological studies proved that the omega-3 PUFA reduces the AD risk (Calon and Cole 2007; Lim et al. 2005).

Nannochloropsis oceanica extract protected neuronal cells against β-amyloid (Aβ). In a similar study, Lai (2015) reported that extract *of N. ocaenia* could also protect the neuronal cells from β-amyloid (Aβ) -induced oxidative stress (). A compound "Biochanin A (isoflavone)'" obtained from C. *vulgaris* has been identified as neuroprotective, which can protect PC-12 cells from Aβ-induced neurotoxicity (Tan and Kim 2016). Furthermore, carotenoids like astaxanthin, canthaxanthin, and β-carotene can also protect the neurons from Aβ-induced neurotoxicity (Chang et al. 2013). Among the different BGA, *S. platensis* and *S. maxima* are considered as a good source for neuroprotective compounds that have neuroprotective abilities and being used for the prevention of neurodegenerative diseases and neurotoxicity of MPTP (1-methyl4-phenyl-1, 2, 3, 6-tetrahydropyridine) (Jha et al. 2017). Microalgal astaxanthin can cross the blood–brain barrier in humans and has been found to improve its antioxidant benefits. Therefore, astaxanthin can help to stop Alzheimer's disease.

7.3.10 Functional Materials in Cosmetics

Developing novel products from natural resources is the recent trend of the cosmetics industry to meet out the demand of consumers, which have multiple benefits and without side effects. Pharmaceuticals used in cosmetics are generally known as cosmeceuticals. Cosmeceuticals are those bioactive compounds which are used in the cosmetic products that can therapeutically improve the biological function of the skin (Arora et al. 2012). Currently, MAB plays a significant role in this field due to the presence of wide verities of BACs that can be successfully used for the treatment of skin disorders like aging, tanning, and pigmentation (Wang et al. 2015; Das et al. 2011). The color of the skin, hair, and eyes depends on a compound known as melanin. Various algal BACs can inhibit the function of the tyrosinase enzyme, which is responsible for the formation of melanin, therefore, widely being used in the fairness creams. For example, *Spirulina* sp., *Chlorella* sp., and *Arthrospira* sp. are some of the very common microalgae used in the cosmetic industries (Fabrowska et al. 2015). The fucoxanthin and phloroglucinol are other important derivatives of marine microalgae, which are used as a skin whitening agent because these compounds can inhibit the tyrosinase enzyme. Therefore, extracts of marine MAB are considered as promising tyrosinase inhibitor agents. Skin wrinkling is caused by development of matrix metalloproteinase that degenerates skin collagen due to the oxidative stress of ROS. The phenolic compounds of marine microalgae have the ability to inhibit the matrix metalloproteinase formation, which can slowdown the skin aging. Similarly, polysaccharides of microalgae can also be used for various cosmetic functions (Thomas and Kim 2013). Phycobiliproteins obtained from Spirulina and *Aphanizomenon flos-aquae* are commercially used in cosmetic industries for making perfumes and eye-makeup powders (Odjadjare et al. 2017).

The extract of *Arthrospira* and *Chlorella* is used for the manufacturing of various cosmetics products like sun protection cream, skincare, cream, and hair care cream

(Spolaore et al. 2006). *Ascophyllum nodosum*, *C. vulgaris*, *Alaria esculenta*, *Chondrus crispus*, *Mastocarpus stellatus*, *S. platensis*, *D. salina*, *N. oculata*, etc. are the common algal species used in the cosmetics (Rizwan et al. 2018). Exsymol company is preparing various anti-aging consumer products by the use of a protein-rich extract from *Arthrospira*. Similarly, *C. vulgaris* extracts stimulate the collagen formation in the skin, which results in the revival of the tissues and decreased skin wrinkle formation (Dermochlorella, Codify, St.Malo, France). Recently, Pentapharm, Basel, Switzerland, has launched two new products, named as "Pepha-Tight" and "Pepha-Ctive." The first one is obtained from *N. oculata* and used as a skin tightening agent. The next one is prepared using extracts of *D. salina* and used as a cell growth enhancing agent and also used to improve the skin energy metabolism (Rizwan et al. 2018; Stolz and Obermayer 2005).

7.4 Strategies of Profitable Production of Microalgae Biomass for Pharmaceutical and Nutraceutical products Production

The commercial-scale production of algae-based value-added products require pilot-scale production of microalgal biomass, For scale-up biomass production, isolation of specific strains, optimization of the growth of strains of interest and achieving economic viability of the biomass production are some of the important aspects which need more focused research (Koussa et al. 2014).

7.4.1 Strain Selection and Improvement

Selecting new or better strains with optimized growth conditions is one of the most important aspects to fulfill the accelerated demands of algal BACs. The selection of particular microalgae among the vast diversity in microalgae species is challenging as the growth characteristics and metabolic pathways of biomolecules synthesis are different among the different microalgal species. Hence, the screening is the first step of the selection of microalgae for specific attributes, e.g., value-added product production. Several vital factors direct the selection criteria of microalgae for pilot-scale cultivation. Temperature, light, salinity, flue-gas components; the probable growth rates and the strain of product; and effortlessness of harvesting as well as downstream processing, etc. are the crucial factors. These *contemplations* are normaly based on prior experience however, pilot-scale microalgae cultivation strategies majorly depending on local circumstances (Adesanya et al. 2014).

Compared to the fungi, yeast, and bacteria, the discipline of algal biotechnology is quite new. By the 1970s, apprehensions associated with the climate change and ecological crisis shifted the algal research toward sustainable development of algal biofuels and green energy. Identification, enumeration, and genetic manipulation of the identified algal strains were possible only with the help of algal biotechnology. Recombinant DNA (rDNA) technology, enzymes, metabolites engineering, etc. played a crucial role in the desired genetic manipulations and development of

Table 7.2 Value-added products of genetically modified microalgae

Products	Microalgae	References
Hepatitis B antigen protein (HBsAg)	*Dunaliella salina*	Sun et al. (2003)
Human growth hormone (HGH)	*Chlorella vulgaris*, *Chlorella sorokiniana*	Hawkins and Nakamura (1999)
Erythropoietin; human fibronectin 10FN3 and 14FN3; interferon β; Proinsulin; human vascular endothelial growth factor (VEGF); high mobility group protein B1 (HMGB1)	*C. reinhardtii*	Rasala et al. (2010)
Bovine lactoferricin (LFB)	*C. reinhardtii*	Li and Tsai (2009)
Avian and human metallothionein type II; antigenic peptide P57; antigenic proteins VP19,24,26,28; foot and mouth disease virus VP1 protein; Antiglycoprotein D of herpes simplex virus; anti-rabbit IgG; human tumor necrosis factor; bovine mammary-associated serum amyloid; classical swine fever virus E2 viral protein; human glutamic acid decarboxylase 65; human erythropoietin; anti-anthrax protective antigen 83 antibody; D2 fibronectin—Binding domain	*C. reinhardtii*	Griesbeck et al. (2012)
Flounder growth hormone (FGH)	*Synechocystis*	Liu et al. (2008)

algal strains for specific purposes (Scott et al. 2010; Radakovits et al. 2010). Enhanced microalgal BACs and energy production are only possible just because of recent biotechnological developments. Microalgal biomass is an excellent natural resource for food commodities, BACs, and biofuels production (Guiheneuf et al. 2011; Draaisma et al. 2013; Mandotra et al. 2019). Since 1989, genetic transformation of around 30 algal species has been established (Hallmann 2007; Song and Jiang 2012). Genetic manipulation is a valuable tool to improve identified microalgal species to accomplish new and economical practical production systems (Table 7.2) (Wijffels and Barbosa 2010). In general, there are three types of goals for genetic alteration in algae: (1) improvement in the photosynthetic efficiency; (2) improvement in the production of selected products; (3) development of new G.M. algae products. Numerous methods for DNA deliverance have been successfully applied to microalgae. These processes consist of micro-particle bombardment (or biolistic), cell disturbance with microparticles or macro-particles (e.g., glass beads), protoplast alteration with polyethylene glycol, protoplast, or complete cell alteration using electroporation and finally *Agrobacterium*-mediated transformation (Coll 2006). Cell forms of the late logarithmic growth phase are commonly used for transformation (Song and Jiang 2012).

7.4.2 Suitability of Medium

Microalgae have been considered as renewable, pollution-free, and robust candidates for the production of nutraceutical and pharmaceutical products. Microalgae are diverse organisms that are characterized by different physiological attributes. Therefore different microalgal species have very different growth requirements. The environmental conditions can significantly influence on the dynamic growth of microalgae and their BACs production. Microalgae are the fastest-growing organisms; they can complete an entire growing cycle every few days if adequate amounts of sunlight, water, CO_2, and nutrients are available. However, the selection of growth mediums for the pilot-scale production of microalgal biomass must be made in a way so that the overall production should be economically viable. Nowadays, instead of the conventional growth medium, various types of industrial wastewater (Gupta and Dhandayuthapani 2019; Letry et al. 2019; Osundeko et al. 2019), domestic wastewater (Gupta et al. 2016a; Gupta et al. 2016b; Balasubramani et al. 2016; Gupta et al. 2017), aquaculture wastewater (Ansari et al. 2020, 2019a, b) are being used as an alternative growth medium for the commercial-scale production of a range of microalgal biomass. The wastewater streams contain a high amount of organic and inorganic nutrients that can be able to give better resolution to save water and nutrients for the cultivation of microalgae.

The intensive production of microalgae is a feasible technology for the appliance of commercial-scale nutrients removal from wastewater and as a basis of sustainable biomass (Letry et al. 2019; Ashok et al. 2019a; 2019b; Osundeko et al. 2019). These are extending the ecological sustainability of treating the wastewater industry, simultaneously supporting to meet the rising insistence for food and energy (Cole et al. 2016). From the past few decades, a variety of wastewater streams, together with municipal, industrial, and agricultural wastewaters as well as effluents, centrates, and also anaerobic digestion effluents, are being used as an appropriate nutrient media for the cultivation of microalgae. However, nutrient variability and the presence of some potential inhibitors in the specific type of wastewater or effluent influence the microalgal growth (Ji et al. 2014; Yang et al. 2015). There are still some challenges being faced for the management of low-level nutrients, high-level turbidity, bacterial contagion, and the presence of toxic materials to a variety of wastewaters. Therefore, based on the type of wastewater and the modes of cultivation (photoautotrophy, mixotrophy, and heterotrophy) specific treatment strategies are applied for the optimum production of algal biomass.

7.4.3 Conditions Optimization

Almost 40,000 microalgae varieties have been identified and reported. For economically viable algal biomass production, in a culture, the biomass growth rate should be >30 g/m^2-day. Microalgae culture depends on several aspects like nutrient accessibility (nitrogen, phosphorus, etc.), temperature, pH, salinity, inorganic carbon, oxygen, light intensity, and CO_2 (Mandotra et al. 2016). Other essential factors

that determine the culture conditions are rousing and mixing, girth and deepness of the bioreactor, crop regularity, and intensity rate (Mata et al. 2010). Throughout the cultivation, the algae cells overtake through different stages such as lag stage, exponential stage, stationary stage, and death stage. The essential requirement is different for different microalgae species. However, the primary needs such as carbon basis, nitrogen, phosphorus, iron, etc. are similar for approximately every type of algae (Grobbelaar 2004; Khan et al. 2018).

Different microalgae required different light duration and intensity for optimum biomass production. Light is the primary requisite for ATP and NADPH synthesis in microalgae. Therefore, there is a straight connection between microalgae growth and light intensity and up to the diffusion point. Hence, optimum light is required by every cell to exploit optimum CO_2 absorption and photorespiration (Ye et al. 2012). Extremely low or high light intensities affect the growth of microalgae due to photo-inhibition and, thus, the biomass yield (Mata et al. 2010).

In the same way, the temperature is also an imperative factor for the development of microalgae. Optimal growth temperature is unique for each of the microalgal species. Rising temperatures to the optimal level exponentially raise microalgal growth to the saturation point, but further increase or decrease in the temperature directly affects the microalgae growth (Bechet et al. 2017). The optimal temperature ranges between 20 and 30 °C for most of the species (Singh and Singh 2015). However, thermophile microalgae like *Anacystis nidulans* and *Chaetoceros* can sustain up to 40 °C, whereas some of the microalgae of warm spring can sustain close to 80 °C (Covarrubias et al. 2016). Non-optimal temperature, especially for external culture systems like open ponds or raceways, affects the biomass yields (Lee et al. 2015).

The pH of the algal culture medium is another critical factor that directly affects the growth of microalgae. Similar to the light and temperature, the pH requirements are also different for different species. Most of microalgae species are pH-sensitive. Nevertheless, pH range between 6 and 8.7 is quite suitable for most of the algal species. However, a few of the microalgae species, such as Chlorella and Scenedesmus, can tolerate a broad pH range (Lam and Lee 2012). Though some of the species have high pH tolerance; however, the optimum growth and biomass yield are unique for most of the species. For example, the optimum growth rate for chlorella is achieved at pH 9–10 (Daliry et al. 2017). The salinity of the culture medium increases when pH increase, which harmfully affects the culture (Juneja et al. 2013).

7.5 Extraction of Pharmaceutical and Nutraceutical Products from Algal Biomass

The microalgal based BACs are used in various commercial sectors like biomedical, cosmeceutical, pharmaceutical, nutraceutical, and chemical industries. Therefore, a standardized extraction of algal metabolites from biomass is imperative for economic sustainability. Therefore, the selection of appropriate techniques for

extracting the BACs from microalgal biomass is a vital step. The algal cell wall is quite rigid, which makes it difficult to extract specific algal metabolites. Cellular disruption is compulsory to get the cellular components and facilitate the extraction method. The extraction step is very crucial as the quality and quantity of the extracted biomolecules depend on the cell-disruption method and the equipment used. Therefore, using an appropriate method and equipment is a key factor in increasing the biomolecule extraction efficiency. Thus, choosing an efficient method for extraction and purification is the most important goal of present research and development (Sosa-Hernandez et al. 2018). Various studies have demonstrated the importance of optimized solvent extraction on the yield of primary metabolites of microalgae (Ansari et al. 2017a, 2017b).

Conventional methods such as solid-liquid extraction and liquid-liquid extraction require a considerable quantity of organic solvents, and more importantly, the process yields depend on the skill of operators. Therefore, these methods are not accurately reproducible. Nowadays, advanced extraction methods have been developed by automation that requires less quantity of organic solvents, more eco-friendly, highly selective, and competent. Currently, supercritical fluid extraction, pressurized fluid extraction, ultrasound-assisted extraction, microwave-assisted extraction, enzymatic treatment, ionic liquids extraction, and various combined techniques have been developed and are used for the extraction of BACs (Hernandez-Ledesma and Herrero 2014).

7.5.1 Supercritical Fluid Extraction

Supercritical Fluid Extraction (SFE) is an excellent green chemistry technique for extracting the BACs from the microalgal biomass. The SFE is mostly used for the extraction of heat-sensitive BACs to reduce their thermal degradation. This technique is not using any toxic solvent. The CO_2 is mainly used in SFE to separate compounds since it is a green solvent and comparably inexpensive (Sanchez-Camargo et al. 2011). It may be considered as an alternative to conventional extraction techniques, but it is required to promise thermal constancy and high-quality products.

Furthermore, during the separation, CO_2 need not to be separated for the purification of compounds from the organic solvent as at room temperature CO_2 released as a gas. Hence, SFE is an eco-friendly technique as well to extract high value-added compounds from various samples. Carotenoids and fatty acids from several microalgae species such as *D. salina*, *H. pluvialis C. vulgaris*, *Nannochloropsis gaditana*, *Synechococcus* sp., *and S. almeriensis* have been successfully extracted by SFE (Di Sanzo et al. 2018; Macias-Sanchez et al. 2010; Molino et al. 2019).

7.5.2 Ultrasound

Ultrasound-assisted extraction is another efficient extraction method due to shorter extraction time stimulated by its acoustic cavitation power. The ultrasound irradiation generates a mechanical effect, which amplifies the solvent penetration into the biomass to speed up extraction (Rostagno et al. 2003). Ultrasound techniques have been used effectively for various compounds extraction and are easy to implement for large-scale industrial applications (Hadiyanto and Suttrisnorhadi 2016; Rocha et al. 2014). The cavitation is the main phenomenon that is characterized by the violent collapse of bubbles in an alternating pressure field. Cavitation bubbles in the aqueous suspension of microalgae cells produce severe and localized short-term pressure increase as well as micro-streaming effects (movement of liquid around gas bubbles formed by cavitation) and shock waves that promote the rupture of microalgae cells (Greenly and Tester 2015).

Currently, ultrasound is widely applied for extracting the pigments phycocyanin (Setyoningrum and Nur 2015), astaxanthin (Zhu et al. 2013), β-carotene and phycobiliproteins from biomass of different microalgal species (Abalde et al. 1998; Furuki et al. 2003; Johnson et al. 2014). Moreover, it is also recently used as an efficient technique to extract low molecular weight compounds from plant tissues (Hromadkova et al. 2002). However, important variables such as temperature, time, and frequency of the ultrasound-assisted extraction process need to be optimized. Temperature is one of the very important parameter for the extraction of phycocyanin as the pigment and protein content of phycocyanin are highly temperature-sensitive (Setyoningrum and Nur 2015). The temperature above 80 °C can damage the phycocyanin structure and reduce its antioxidant activity due to the coagulation of phycobiliproteins (Antelo et al. 2008; Sarada et al. 1999). Besides, the extraction time is also considered as another important parameter for BACs extraction (Kadam et al. 2015). In addition, the selection of power is the next important parameter for ultrasound-assisted extraction of BACs, which directly can affect the extraction efficiency. Vuong et al. (2012) observed that the power range has no positive effect on the extraction efficiency, while contrary to it, Kadam et al. (2015) reported dissimilar results, in which BACs extraction was positively affected by ultrasound power. However, the optimum quantity of phycobiliproteins was extracted from *S. platensis* by ultrasound-assisted extraction at 25 kHz for 30 min (Rodrigues et al. 2018).

7.5.3 Microwave

The microwave-assisted extraction (MAE) technology is also considered as an advanced extraction technique to reduce issues that are associated with conventional extraction techniques and minimize energy costs with the least environmental impact. This emerging technology is mainly used to extract the polysaccharides, phenolic compounds, oils, pigments, and proteins from various higher plants, seaweeds, microalgae, agricultural wastes, and lignocellulosic biomass. In MAE,

the penetration of the solvent into biomass is accelerated due to the absorption of microwave energy by polar molecules. The MAE has been used for the extraction of various organic compounds from different sources. This process is energy efficient and needs a very short period for the extraction as well as the requirement of the solvent is comparably less. Besides, this method improves the energy transfer and promotes the disturbance of weak hydrogen bonds. The effects of microwave energy depend on numerous factors like the nature of solvents, solid matrix, the nature of the target compound, and solvent dielectric constants (Duarte et al. 2014).

Compared to other extraction methods, the significant advantage is the reduced extraction time. Usually, a few minutes is enough to extract the BACs with only a few ml of solvent. Other significant advantages are enhanced extraction yield, improved accuracy, and precision, appropriateness to the extraction of thermolabile compounds, potential for extraction of minor/trace components from a few milligrams of the sample. Two chief MAE systems are commercially available, in which the agitation is provided during extraction to enhance the mass transfer. In the *closed extraction vessels*, extraction is carried out under controlled temperature and pressure, whereas, in the *focused microwave-assisted solvent extraction* (FMASE), only a part of the extraction vessel has the sample, which is irradiated by microwaves (Donato et al. 2015).

In 2017, Esquivel-Hernandez et al. advocated that the MAE is an alternative green extraction method for extracting BACs from microalgae. This is an efficient technique for disruption of the cell wall of microalgae at relatively low energy input with fast treatment time, and more importantly, without using any hazardous extraction solvents (Al Hattab and Ghaly 2015). Besides this technique, chlorophylls and carotenoids can be easily extracted by breaking of the rigid cell wall of microalgae. MAE is also considered an effective method for extracting microalgae pigments, which requires high mechanical resistance. MAE can accomplish similar yields as hot soaking in 30–60 minutes (Pasquet et al. 2011a). Vernès et al. (2016) reported efficient extraction of phycocyanin from *S. platensis* using MAE compared to the freeze–thaw process or hot soaking. MAE is the best method for fucoxanthin extraction from *Cylindrotheca closterium* than maceration and UAE due to the microwave's potential to break frustule of diatoms. It was also reported that no fucoxanthin degradation even at 56 °C of extraction temperature (Pasquet et al. 2011b).

7.5.4 Ionic Liquids (I.L.S)

Ionic liquids (I.L.s) are salts composed of loosely seized cations and anions and, unlike inorganic salts, are liquid over a wide range of temperatures. I.L.s are very solvating non-coordinating mediums, in which different organic and inorganic solute can quickly dissolve. Additionally, I.L.s have widely tunable properties, particularly their high solvency power, which makes them as designer solvents flexible to a plethora of techniques and technologies. They are considered as environment cooperative substitutes for typical organic solvents due to their pretty

physiochemical properties for extraction, which is mainly exaggerated by the dimension and brand things to see their cation and anion segment (Liu et al. 2005). Such notable properties include ease of miscibility with various solvents, non-flammability, low substance reactivity, thermal stability, and insignificant vapor pressure. It is applied widely in division science and applicable to extraction procedures such as liquid-stage miniaturized scale extraction, layer partition, and solid-state extraction (Zhang et al. 2014). I.L.s pre-treatment has numerous advantages, such as its non-usage of autoclave reactor due to short reaction time, low vapor pressure, high yield, and reuse of ionic liquid. Besides, I.L.s can capably replace organic solvents that can disrupt the cellular structure and promote efficient extraction/purification process because of their capacity to dissolve cellulose.

Currently, I.L.s are being used as extracting agents and as a replacement of various organic solvents for extracting different components from microalgae. The I.L.s easily break the hydrogen bonds of the cell wall and enhance the lipid extraction (Choi et al. 2014). For example, >70% of natural food colorant astaxanthin was extracted from *H. pluvialis* after permeabilizing their cells using hydrophilic I.L.s such as 1-ethyl-3-methylimidazolum di-butylphosphate (EMIM DBP) under 45 °C and then subsequently extracted using ethyl acetate, while the cell wall is kept intact (Desai et al. 2016). The I.L.s can disrupt the microalgae cell wall, thus promoting the release of some specific components. The I.L.s are easy to recycle for inferring the economic feasibility of the process. Moreover, other than cell disruptors, I.L.s can also be used to fractionate the microalgae biomass for the separation of lipids, proteins, and carbohydrates (Orr et al. 2016).

7.5.5 Combined Technique

Biomass of *C. vulgaris* is pretreated with microwaves before supercritical CO_2 extraction, which resulted in a high yield of unsaturated fats. The oil throughput from microwave-assisted supercritical CO_2 extraction contained large amounts of α-linolenic, linoleic, oleic, and palmitic acids than the extraction by only supercritical liquid extraction without microwave pre-treatment. Alternatively, I.L.s can also be used as an extraction medium in MAE systems. Here, the I.L.s can simply absorb microwaves and scatter energy quickly throughout the I.L.s medium (Liu et al. 2012). Then, the expansion of microwave illumination gives quick heat exchange, expands yield, and decreases side residuals.

Furthermore, the rapid increase of heat and pressure inside the sample empowers target compounds to be liberated successfully from the samples. Pan et al. (2016) extracted the lipids from biomasses of *C. sorokiniana*, *N. salina*, and *G. sulphuraria* by MAE using an imidazolium-based I.L. such as 1-butyl-3-methylimidazolium hydrogen sulfate ([BMIM][HSO_4]). In this process, the BMIM HSO_4 was interacted with microwaves and increased extraction rate by ten times. Comparing with the solvent extraction system, the BMIM HSO_4 delicately broken the cell wall of the microalgal biomass. The combination of sonication and microwaves is also another combined duo technique for extracting lipids from vegetables and microalgae

biomass. Ultrasonication alone, microwave irradiation alone, or combination of both methods gave outstanding extraction efficiencies in terms of yields and time, with a 10-fold decrease in the time required with conventional methods, and yields amplified from 50 to 500% (Cravotto et al. 2008).

7.6 Capital and Operational Expenditures of Pharmaceutical and Nutraceutical Products from Microalgae

Microalgae have a wide range of active natural products, which are pharmacologically important. Phycobilins, carotenoids, polysaccharides, fatty acids, vitamins, and sterol are major pharmaceutical and nutraceuticals natural products that are derived from various microalgae. These products have high human and animal health significance. However, the production of pharmaceutical and nutraceutical products from microalgae is very costly. There is no optimized operational technique for pilot-scale production of microalgae-based pharmaceuticals.

The demand of natural pharmacologically active compounds (PACs) and antibiotics has increased in the recent past. Due to the presence of comparably higher concentrations of PACs and antibiotics in algae and cyanobacteria, it has high demand in the market. A wide range of pharmaceutical and nutraceuticals obtained from algal biomass are used in several drugs as antimicrobial, antivirals, and antifungals, and also as food supplements as therapeutic proteins, and neuroprotective products. Increasing the pharma application of algae biomass is an attractive revenue source for commercial points of view. The demand for physiologically active nutraceuticals obtained from algae is incredibly high. These can be directly consumed as food supplements and also have broad application and use in various consumer products. Some of the unique and economically important algal products are PUFA, carotenoids, β-Carotene, pigments, etc. (Rahman 2020).

7.7 The Commercial Potential of Algae-Based Pharmaceutical and Nutraceutical

In the past years, consumer's interest in using "healthy foods" or "superfoods" has increased by several folds. The superfoods are very nutritional foods, which have additional health benefits and can cure many chronic diseases. This has motivated researchers for the assessment of various natural resources for the production of healthy functional foods (Seyidoğlu and Galip 2014). The usage of microalgal biomass or its BACs has become a novel move toward the development of the production of superfoods (Gouveia et al. 2008). Among the microalgal-derived food, the production of *Spirulina* is the maximum which is around 12,000 tons/year, followed by other microalgal species such as *Chlorella* sp, *D. salina*, *A. flos-aquae*, *H. pluvialis*, *C. cohnii*, *and Shizochytrium* with 5000, 3000, 1500, 700, 500, and 20 tons per year, respectively (García et al. 2017). However, the production of algal biomass is comparably low than the terrestrial crops, which is

around 40 million tons/ year (Wijffels and Barbosa 2010). China, Indonesia, and South Korea are the three topmost countries in the world in exporting algae-based products. In the year 2010–2012, these countries exported algae-based products costing more than 125 million dollars/year. China was a leading exporter to E.U., with 13 million dollars/year of algal-based products, followed by Indonesia and the USA. Similarly, Ireland was the second-largest exporters to E.U. in the year 2010–2012, France and the Netherlands, but with values of one order of magnitude lower than China. The USA, Australia, and South Africa are also importing the algal-based products from the E.U. All over the world, nearly fifty private companies are producing the algae-based food/feed products and nutrients (Vigani et al. 2015). High-value molecules production from algal biomass is geographically dispersed than dried algae (Table 7.3). The total market size of microalgae-based food and feed products and their production volumes are very smaller than the conventional commodity crops. However, since the beginning of 2000 microalgae, the demand for algae-based food and feed showed a 5-fold increase (Ismail 2010).

A report by credence research market states that the compound annual growth rate (CAGR) is expected to cross 5.8% with the stand market value at USD 53.43 billion by 2026 for microalgal products (Rahman 2020). Due to the increasing demand of microalgae products, mainly *Spirulina*, for uses in cosmetics and natural colorants,

Table 7.3 Global private companies producing microalgae-based commercial food and feed products (Vigani et al. 2015)

Country	Number of companies	Products
Australia	04	Food-dried algae
Canada	02	Feed-dried algae, algal paste
China	05	Food-dried algae, EPA/DHA, beta-carotene
France	01	Food-EPA/DHA, other dietary supplements
Germany	03	Food-dried algae, EPA/DHA, beta-carotene, astaxanthin, phycocyanin Feed-dried algae, astaxanthin, aquaculture feed
India	02	Food-dried algae, astaxanthin, beta-carotene
Israel	03	Food-dried algae, astaxanthin, beta-carotene
Japan	06	Food-dried algae, beta-carotene, astaxanthin, phycocyanin
Mexico	01	Feed-dried algae
Mongolia	01	Food-dried algae
Myanmar	01	Food-dried algae
Netherland	02	Food-dried algae, EPA/DHA, beta-carotene
New Zealand	01	Beta-carotene, EPA/DHA
Portugal	01	Feed-dried algae, aquaculture feed
Switzerland	01	Food-EPA/DHA
Sweden	02	Food & feed-Astaxanthin
Thailand	01	Food-dried algae
Taiwan	02	Food-dried algae
USA	11	Food-food-dried algae, astaxanthin, EPA/DHA

its CAGR is expected to be approximately 10% with an anticipated value of USD 2000 million by 2026. On the other hand, the CAGR of *Chlorella* ingredients market is expected to be 25.4%, reaching USD 700 million by 2022. The world market of astaxanthin, which is mainly used as nutraceuticals, is also expected to increase by many folds (Garcia et al. 2017).

In 2017, the global market of microalgae products was dominated by Europe **as** the market was led by the nutraceutical application sector. Expanding demand for nutraceuticals in Europe has a high demand for microalgae products. Therefore, increasing demand of microalgae products for the nutraceutical and pharmaceutical industry is one amongst factors that are endorsing the growth of microalgae products (Rahman 2020). The Asia Pacific is also likely to exhibit remarkable growth in algae products in the future. India and China maintain enormous growth possibilities due to growing health awareness among the people. China is one of the leading markets in the Asia Pacific that decide the overall market trend. Further, increasing awareness about wellbeing among the general public is also another factor for the high demand of microalgae products in India and other countries (Rahman 2020).

The fatty acids such as DHA and EPA have a world market value of 700 Million USDD/year, followed by pigment β-carotene with 261 Million USD/year. The astaxanthin has a world market value of 240 Million USD/year, closely followed by lutein with 233 Million USD/year, and finally phycobiliproteins with 60 million USD/year (Markou and Nerantzis 2013). The worldwide demand for carotenoids is anticipated to increase more than 1.8 billion USD by the end of the year 2019 (Bhalamurugan et al. 2018).

7.8 Safety and Regulatory Issues of Algal Pharmaceutical and Nutraceutical Products

MAB has been used as a human nutritional supplement for 100 years. BGA, such as *A. platensis* and *A. maxima*, are used by Aztecs from Mexico Lake of Texcoco circa AD 1300. In the year 1974, in the United Nations world food conference and world health organization (Geneva, Switzerland June 8, 1993) declared that the *Spirulina* is the best food for the future due to the protein affluent and can be given to the children. In the 16th general assembly, session of U.N. (agenda item 52) and the international institution for the use of microalgae *Spirulina* against malnutrition (IIMSAM) initiated a modified draft resolution for the use of *Spirulina* to fight food shortage and undernourishment and to assist accomplish sustainable development. As a pursue-up on this decision, the United Nations Food and Agriculture Organization (FAO) released a draft on *Spirulina*, in the year 2008 (FAO 2011).

Three European regulations, such as regulation on safety food, regulation on novel food and novel food ingredients, and regulation on nutrition and health claims for a food, regulate the marketing of microalgae or its components. Among the three, European Community Regulation on Food Safety (EC 178/2002) and Regulation on Novel Foods and Novel Food Ingredients (EC 258/97) mainly control the production and commercialization of microalgae-based food and feed products (Vigani et al.

2015). Regulation on GMOs for food and feed production was disqualified from the list since, at that time, there were no recombinant microalgae at the commercial phase. In 2002, a general food law regulation (E.C. No. 178/2002) was adopted by the European Parliament, and it is Council for designing the general principles and necessities of food law. Besides an independent agency European Food Safety Authority (EFSA) was set up to advise and support the scientific groups. Novel Food means food, which was not consumed by humans at a significant level in the E.U. before 1997. When the first law on novel food came into force, between 1997 and 2014, nearly 170 applications received for approval across the E.U. However, in the November 2015 a new novel food regulation was adopted by E.U. [Regulation (E.U.) 2015/2283, repealing regulation (E.C.) No. 258/97], but it came to effect from January 2018. The main aim of this regulation was to enhance the efficiency of the approval procedure, to facilitate faster delivery of safe novel food to market, to eliminate the unnecessary trade barriers, and to ensure food safety. In E.U., many microalgae products have been permitted according to the regulations in force. For example, in 2002, *O. aurita* and in 2004, *T. chuii* were allowed as novel foods. Similarly, oils of *Schizochytrium* and astaxanthin of *H. pluvialis* were also considered as a novel food ingredient.

The United States Food and Drug Administration (FDA) regulates food safety, including algal products. The Center for Food Safety and Applied Nutrition (CFAN) classified the algal biomass as an additional nutritional supplement. The biomass of *Chlorella*, *C. cohnii*, *Dunaliella*, *Haematococcus*, *Schizochytrium*, *Spirulina*, and *P. cruentum* are generally recognized as safe category (GRAS) food sources. Further products obtained from microalgae are also classified by the FDA as GRAS. For instance, oils of *Schyzochitrium*, whole microalgal biomass protein powder, *Chlorella* lipid, etc. are considered as GRAS food sources (García et al. 2017).

Some other developed countries also have specific regulations on microalgal food. For example, Food Standards Australia New Zealand (FSANZ) regulates the use of novel microalgae-based food ingredients. Similarly, Food Standard Code authority grants consent for using the dried powder of *Schizochytrium*, which is a rich source of DHA and a novel food ingredient. Health Canada advises consumers to take some care while taking non-*Spirulina* cyanobacterial products. In addition, it does not allow any therapeutic claims for substances, which are sold as food (García et al. 2017). Recently, a study on the effect of microalgal nutritional on humans reported that utilization of microalgae up to 20 g/day had no adverse effects even if the ingestion is extended for a longer period. Microalgal based diets have a momentous contribution to human health improvement. However, merely little data are emphasizing the risks and safety of consuming microalgae-based products (Bhalamurugan et al. 2018).

7.9 Future Prospects

Increasing demand for microalgae products from the nutraceuticals and pharmaceutical industries is one of the main factors that augment the market growth of microalgae products. Microalgae are emerging as one of the most gifted sustainable resources with potential health benefits, together with pigments, omega-three fatty acids, carbohydrates, proteins, peptides, and antioxidants. The nutraceuticals and pharmaceutical potential of various microalgae species have gained ever-increasing interest. Nutraceutical and pharmaceutically bioactive components have been obtained from various microalgae and used as human supplements for different purposes like an anticancer drug, antioxidant agent, antidiabetic agent, etc. In the future, microalgae based astaxanthin, β-carotene, Phycocyanin, EPA, and DHA will become available as ingredients in food and feed market worldwide. Because very recently, microalgae have become much more extensively cultivated and harvested at huge industrial scale for BACs production. However, still, many key challenges remain to be addressed in bioactive compounds production from microalgae (Fig. 7.3).

Nowadays, compared to the synthetic pigments that cause different chronic diseases in humans, natural pigments have gained much attention because of its wide applications in medicine, cosmetics, feedstock, and food industry. The up-and-coming field of microalgal biotechnology could lead to the most important industrial production of low-cost natural pigments with less time consumption, using microalgae. For example, currently, the pigment astaxanthin production is very less than β-carotene, even though it has a high market value (Panis and Carreon

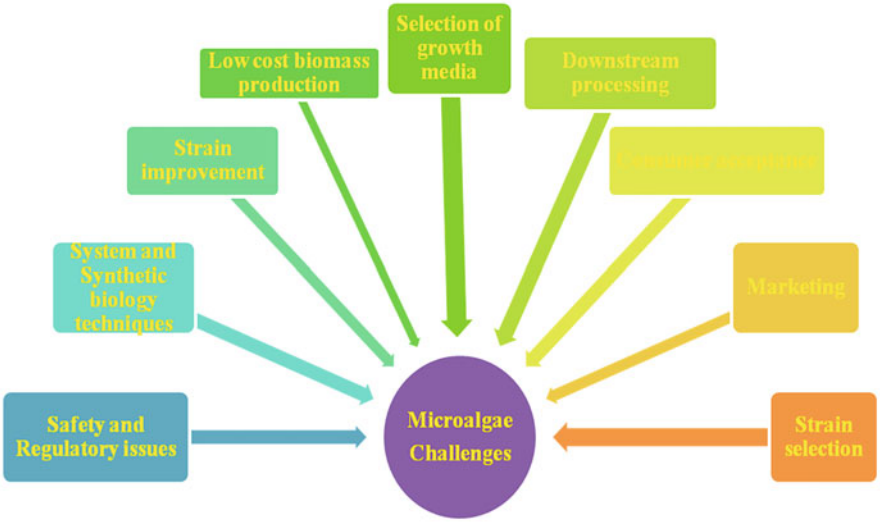

Fig. 7.3 Key challenges for improving the production of pharmaceutical and nutraceutical products in microalgae

2016). The higher production cost is a major drawback for the slow commercialization of astaxanthin and the lack of a pre-established market as a nutraceutical for human consumption (Borowitzka 2013).

7.10 Conclusion

However, still, a lot of positive health benefits are possible to be discovered with their increased use as feed and food additives. Their potential for treating numerous types of diseases be supposed to improve the interest and encourage research activities into their value, mostly for human wellbeing. Thus, additional intensive investigations on finding novel functional compounds from microalgae biomasses would offer added health benefits to humans. Microalgae continue to be one of the most unexplored microorganisms in the planet; additional research in bioprospecting is required. For instance, microalgae can be utilized as a substitute and sustainable source of PUFAs; however, extremely a small number of species are used for the production of PUFAs, and more comprehensive investigation study and continuous isolation of novel strains are necessary.

Omics technologies, systems biology, and synthetic biology are the key areas, which require further more attention on microalgae research (Gimpel et al. 2015). Though over 30 microalgae species DNA have been sequenced so far (Guarnieri and Pienkos 2015). However, more *omics* data are needed to recognize and illustrate the metabolic pathways, which are involved in various nutraceuticals products production. Elaborated research is still needed on the gene manipulation and transformation in microalgal species to improve the high-level output of target BACs.

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The Budding Potential of Algae in Cosmetics

8

Barasa Malakar and Kaustubha Mohanty

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Abstract

Algal extracts contain biologically active components that are used in cosmetic industry. These compounds are often employed in cosmetic industry as antioxidants, thickeners, water binders, emollients, etc. These extracts are found in skin and face care formulations as anti-aging cream, masks, lotions, etc., ultraviolet (UV) protection formulations as sunscreens, and hair care products. Several studies have been reported where bioactive algal compounds have exhibited their potential to be skin shielding agent, which includes safeguard from harmful UV rays and wrinkles.

Keywords

Algae · Cosmetics · Antioxidant · UV protection · Anti-aging

8.1 Introduction

8.1.1 Structure, Function, and Composition of the Skin

Skin is the prime organ in the human body. It is a complex array of cells and tissues that regulates heat generation and water loss from the body and is very complex. Skin has many significant roles in physical functions such as prevention of invasion of toxic compounds and microbes. Epidermis, dermis, and hypodermis are the prime structural divisions of skin (Pimentel et al. 2018). Epidermis is differentiated into five distinct layers: basal, granular, lucid, spinous, corneum and lucid. The predominant keratinocyte cells in the epidermis repair damage of skin. Melanocyte cells possess melanin in that determines the skin color and provides UV protection (Brenner and Hearing 2007). A degree of immunity is provided by Langerhans cells, which are a type of dendritic cell, originated from the bone marrow (Pereira 2018; Wollenberg et al. 1996). Receptors available detect different environmental stimuli and accordingly respond, while thermo receptors detect heat and mechanoreceptors detect sensations. For maintaining temperature homeostasis and to get rid of waste, the receptors present in the skin causes sweat glands to sweat (Denda et al. 2007). The subcutaneous layer of the skin provides insulation to the body as it stores fat.

The stratum corneum present on peak of the skin is responsible for preventing dehydration as it is a significant obstacle to passive water diffusion from the skin and hence allowing individuals to dwell in air, and it also acts as a hindrance to certain skin irritants as well. Under the epidermis, the second layer dermis is present, which mainly comprises connective tissues that include blood vessels, collagen, nerves, fibroblasts, sweat glands, and elastin. Hyaluronic acid is a crucial element of the dermis, which is involved in tissue repair as well. Elastin and collagen are cross linked and provide support to the skin. However, collagen and hyaluronic acid break down with skin aging, causing skin to lose its firmness and making wrinkles to appear (Wang et al. 2015). The final layer, the hypodermis, is a layer of loose

connective tissue and is comprised of fat majorly. It mainly supplies insulation to the human body (Benbow 2009).

8.1.2 The Cosmetic Industry

The Federal Food, Drug & Cosmetic Act (FDA) of the USA and article L5131-1 of the French Public Health Code defines that, a cosmetic item is any product or formulation that is to be applied, sprayed, sprinkled, or rubbed, to the exterior parts and surface of the body, particularly the skin, hair, lips, nails, and teeth as the substance cleanse, safeguards, alters appearance, perfumes, and helps to diminish body odor. Hence it can be concluded that it is an array of cosmetic treatments and procedures to ensure beautification and personal hygiene. Prerequisites for cosmetic items are to ensure that such preparations are natural, safe, protective, and effective with good sensory value.

According to the recent financial analysis about the world beauty market, documented by Eurostaff, a French-based company, the cosmetic market has an estimated turnover of US$170 (Arora et al. 2012). It is indicated in the study that the cosmetic market will continue to flourish with the development of the world. However, despite this flourishing and hopeful future, further analysis and investigations have to be done to boost the cosmetic items, by utilizing natural ingredients to meet the demand from consumers from different parts of the world.

8.1.3 The Need for Natural Substitutes

The usage of cosmeceuticals is driven by the pursuit of beauty. However, customers today have become doubtful regarding chemical ingredients used in cosmeceuticals; hence, the need for alternatives has arisen. An enhanced urge for natural and environmentally sustainable compounds, such as derivatives of algae, has emerged, that have a notable market demand. Reports suggest that marine algal extracts and compounds can be employed as cosmeceuticals (Wang et al. 2015).

8.2 Algal Species Used in Cosmetic Industry

A variety of cosmetic products in the present day involve commercial use of algae, viz., *Spirulina*, *Chlorella*, *Dunaliella*, *Haematococcus*, *Phaeodactylum*, *Botryococcus*, and *Porphyridium* (Ariedea et al. 2017). Red microalgae are used as colorants for cosmetic preparations such as lipstick, makeup, and eye shadow. Due to the presence of various biological elements, viz., vitamins, minerals, sugars, amino acids, lipids, red and brown macroalgal species are used in cosmeceuticals (Sanghvi and Lo 2010; Skjånes et al. 2012).

8.2.1 Microalgae

Microalgae are prokaryotic, microscopic, and unicellular organisms with size ranging from 1 to 50 μm. Microalgae are rich in iron, phosphorous, calcium, vitamin A, B, C, E, B12, folic acid, biotin, beta-carotene, and pantothenic acid. They are mainly phototrophic, while some can grow heterotrophically as well (Fabregas and Herrero 1990). Microalgal chemical composition makes them a highly valuable bio-sustainable element for an extensive array of applications. (Pimentel et al. 2018). Microalgae are present in all habitats, terrestrial and aquatic. They have higher productivity, minimal seasonal variation, and are relatively easy to extract. They can also multiply quickly and survive under stress and harsh environmental conditions such as cold, heat, salinity, etc. They are rich source of valuable components like carbohydrates, protein, enzymes, fiber, polyunsaturated fatty acids, tocopherols, sterols, antioxidants, and pigments such as chlorophyll and carotenoids. Already commercialized algal species belong to the green algae family Chlorophyceae such as *Hematococcus pluvialis*, *Dunaliella salina*, *Chlorella vulgaris*, and also the intermediate species between plants and bacteria such as *Spirulina* and *Aphanizomenon flos-aquae* (Christaki et al. 2013).

8.2.2 Macroalgae

Macroalgae are eukaryotic, macroscopic multicellular algae. They grow under optimal availability of light in marine environment and are widely known as seaweeds. Macroalgae are one of the most economically and ecologically significant living assests of the marine environment. Macroalgae are an important natural source of some of the significant nutrients, viz., fiber (15–76% dry weight, dw), protein (1–50% dry weight, dw), important amino acids and minerals, and trace elements (ash: 11–55% dry weight, dw). The macroalgae are easy to grow in a shorter period of time and their growth conditions can be controlled and thus it is feasible to exploit their chemical composition comprising polyphenol, protein, and pigments (Pereira 2018; Pimentel et al. 2018). Macroalgae grows abundantly in coastal areas and have simple structure which makes them different from terrestrial plants as they do not possess specific parts like stems, roots, or leaves. Few popular macroalgae are the red seaweed *Porphyra umbilicalis* commonly known as Nori, the long brown seaweed *Macrocystis pyrifera* commonly known as kelp, and the green seaweed *Ulva lactuca*, generally known as sea lettuce (Christaki et al. 2013).

Macroalgae are classified into the following main groups based on their pigment content (Joshi et al. 2018):

- Chlorophyceae, Green algae
- Rhodophyceae, Red algae
- Phaeophyceae, Brown algae

8.2.2.1 Green Algae

The green color of the algae is because of a photosynthetic pigment known as chlorophyll, which is able to capture light energy and produce food. The chlorophyll is present in the exposed surface of the algal species that provides a moisturizing environment by supplying oxygen and thus prevents it from drying up. It has anti-inflammatory properties as well. Few Green algal species utilized in cosmeceuticals are *U. lactuca*, *C. vulgaris*, etc. Beta-carotene extracted from *D. salina* can be utilized as colorants in various cosmetic industries (Joshi et al. 2018).

8.2.2.2 Red Algae

Along with photosynthetic pigment chlorophyll, the red algae possess a red protein pigment known as phycoerythrin. Due to its light-harvesting property, it imparts color to red algae, where the red light is reflected and the blue light is absorbed (Rossano et al. 2003). The protein establishes a covalent bond with phycobilins containing chromatophores, enabling these algal species to carry out photosynthesis. Few red algal species utilized in cosmetics are Irish moss, *Porphyra*, *Gracilaria*, etc.

8.2.2.3 Brown Algae

The chloroplast of brown algae contains a supplementary pigment called fucoxanthin, which has tyrosinase inhibitory effects. It possesses anti-inflammatory effect, also helps to reduce skin pigmentation, and aids in preventing natural skin aging by assisting in the generation of the structural protein collagen that tends to disperse with aging. Fucoxanthin moisturizes the skin as well (Shimoda et al. 2010). Few brown algal species utilized in cosmetics are *Laminaria digitata*, *Postelsia palmaeformis*, *Isochrysis* spp., etc.

8.3 Use of Algae in Cosmetic Industry

The intention behind using cosmetics is to beautify appearance, promote attractiveness, or cleanse. Carrageenan, agar, and alginates are extracted from seaweed and are very significant to the cosmetic industry. Algae have many benefits. It provides moisture to the skin, aids in circulation of blood, triggers the renewal of cell, balances the function of sebaceous gland, promotes anti-inflammatory effect, and enhances the resistance of the skin. Seaweed produces secondary metabolites such as pigments, sterols, vitamins, phenolic metabolites, and other bioactive agents (Pereira 2018).

Fucus vesiculosus (a type of brown seaweed) extract is employed to minimize the emergence of dark circles under the eye. The anti-inflammatory and antioxidant activities of the extract in topical preparations could enhance the appearance of the skin under the eyes, and trigger production of collagen that could aid in minimizing the appearance of wrinkles and fine lines in the skin (Ariedea et al. 2017). Microalgae produce secondary metabolites which can reduce blemishes, repair skin damage, treat seborrhea, inhibit inflammation of the skin, promote skin healing, and maintain moisture of the skin (Kim et al. 2008). Red micro algal extract are

found in skin care formulations, UV protection, hair care products, and anti-aging creams. Algae are majorly included in cosmetic preparations as thickener, water-binder, and antioxidant agent (Ariedea et al. 2017).

Algal derivatives are incorporated in the cosmetic market as antioxidants, sunscreens, sensitizers, thickening and moisturizing medium to improve the proficiency of skin against abrasions, skin tanning, etc., (Table 8.1). The secondary metabolites generated under stress or harsh conditions have antimicrobial effect against pathogenic fungi and viruses. Algal biomolecules and pigments are isolated and incorporated in various cosmetic products. Algal polysaccharides such as agar, alginates, and carrageenan, extracted from *Phaeophyceae* and *Rhodophyceae* act as gels in certain lotions, shampoos, and creams. The extracts of macroalgae contain stabilizing, preserving, and organoleptic properties. *Spirulina*, *Chlorella* are unicellular microalgae which have a vital role in the cosmetic market, as the pigments and metabolites created by them add value to the cosmetics. Algal proteins, amino acids, and vitamins contain natural moisturizing properties which are employed for keeping the hydration of the skin intact and prevent skin drying. Algal compounds such as lipids like carotenoids and sterols, phycobili proteins like phycocyanin, terpenoids, and pigments, possess anti-oxidant and anti-inflammatory properties. These metabolites also act as stabilizing medium in emollients (Bedoux et al. 2014).

Spirulina, *Dunaliella*, *Chlamydomonas* species produce value added metabolites such as astaxanthin, various proteins, MAA (mycosporine-like amino acids) that provide nourishment to the hair and skin and. Red algae contain cleansing properties that clarify the skin thereby aiding in the enhancement of the overall skin fitness (Joshi et al. 2018).

8.3.1 Industrial Applications of Algae

Cosmeceuticals that contain algae or their derivatives are very popular and have a huge demand in the market. *C. vulgaris* extract triggers collagen production in the skin thereby assisting in cell regeneration and wrinkle reduction (Stolz and Obermayer 2005).

Preservatives must be incorporated in cosmetic items to eliminate microbial contamination. Hence, it is immensely important to generate safe and secure antimicrobial preservatives. Extracts of macroalgae and microalgae are promising antimicrobial compounds. It has been reported that macroalgal extracts of *Himanthalia elongata* and *Synechocystis* showed inhibiting properties against *Staphylococcus aureus* and *Escherichia coli* bacterium (Plaza et al. 2010). Studies demonstrate that microalgal extracts of *Isochrysis galbana*, *D. salina*, *Pavlova lutheri*, *Nannochloropsis oculata*, and *Chlorella marina* showed antibacterial properties against *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* (Srinivasakumar and Rajashekhar 2009). *Arthrospira* and *Chlorella* are some of the prominent microalgae in the market of skin care. *D. salina*, *Mastocarpus stellatus*, *Alaria esculenta*, *C. vulgaris*, *Spirulina platensis*, *N. oculata*, *Ascophyllum nodosum*, and *Chondrus*

Table 8.1 Products made from algae available in the cosmetic market

Serial no.	Product	Brand	Algae	Cosmetic application	Cosmetic effect	References
1	Detoxifying Sea algae mask	Prolixr	Seaweed algae	Detoxifying mask	Improves skin tone, rejuvenates, hydrates, and moisturizes skin	https://www.nykaa.com
2	Nutritious microalgae pore minimizing lotion	Estee Lauder	*Chlorella vulgaris*, *Spirulina platensis*	Sheer lightweight moisturizing lotion	Hydrates skin, minimizes pores, controls oil	https://www.nykaa.com
3	Pure clay mask	Loreal Paris	Red algae	Exfoliating clay mask	Exfoliate and refines pores	https://www.lorealparis.co.in
4	Nutritious microalgae purifying cleanser	Estee Lauder	*Chlorella vulgaris*, *Spirulina platensis*	Cleansing gel	Removes makeup and impurities from skin	https://www.nykaa.com
5	Spa of the World Brittany coast algae body wash	The Body Shop	Sea algae	Shower gel	Body wash	https://www.nykaa.com
6	UV shield sunscreen face wash gel	Oshea Herbals	*Prophyra umbilicalis* (red algae)	Sunscreen face wash gel	Skin purifying and whitening properties	https://www.nykaa.com
7	Whitening algae peel off mask	Blisskin	Sea algae	Peel off mask	Minimizes dark spots, whitens, brightens, and lightens skin tone, promotes epidermis healing, has antioxidant properties, slows down melanin synthesis	https://www.nykaa.com

(continued)

Table 8.1 (continued)

Serial no.	Product	Brand	Algae	Cosmetic application	Cosmetic effect	References
8	Seborregulating algae peel off mask	Blisskin	Sea algae	Peel off mask	Unclogs pores, suitable for oily and acne prone skin	https://www.nykaa.com
9	Hydrating algae peel off mask	Blisskin	Sea algae	Peel off mask	Suitable for dry dehydrated skin, restores skin's elasticity and softness	https://www.nykaa.com
10	Marine algae sea weed pack	Roots to Roses	Sea weed algae	Face pack	Hydrates and conditions skin	https://www.nykaa.com
11	Calming algae face mask	Anatomicals	Sea weed algae	Face mask	Clarifies skin, detoxifies, conditions, softens, and improves skin texture	https://www.nykaa.com
12	Algae serum body lotion	Iraya	Spirulina	Body lotion	Moisturizes skin	https://www.nykaa.com
13	Chocolate algae peel off mask	Blisskin	Sea algae	Peel off mask	Soothes sensitive skin, evens skin tone, fights skin aging, improves skin's elasticity	https://www.nykaa.com
14	Daily repair serum	Shankara	Algae extract	Serum	Wrinkle reduction, supports skin tissue repair and renewal	https://www.nykaa.com
15	Organic algae soap	Rustic Art	Grade I Algae/ Cyanelles powdered 600 µm	Bathing soap	Moisturizes skin	https://www.nykaa.com

16	Sea algae gel	Rahul Phate's	Sea algae	Oil free moisturizer	Anti- aging properties, hydrates hair	https://www.purplle.com
17	Italian mood mud peel off mask kit with black and white algae mask	O3+	White and black algae	Detoxifying and glow boosting peel off mask	Tan removal	https://www.purplle.com
18	Regenerative repair shampoo	Dove	Red algae	Shampoo	Repairs damaged hair, restores hair strength	https://www.purplle.com

crispus are majorly used algal species in the cosmetic industry (Priyadarshani and Rath 2012).

External factors such as UV radiation, harsh climatic conditions, and air, water, and environmental pollutants can adversely affect the skin and advances its aging prematurely (Lecas et al. 2016). Aging of skin brings several alterations: skin gets thinner, fragility is increased, and gradually loses its elasticity and capability to hold hydration (Wang et al. 2015). The primary functions of natural ingredient are to have collagen boosting property, or even antioxidant and anti-inflammatory activity in cosmetic preparations. These bioactive compounds are included in formulations meant for anti-aging care, including shielding against free radicals, wrinkle reduction and prevention, anti-photoaging, moisturizing, skin whitening and photoprotection against UV rays (Ariedea et al. 2017; Bedoux et al. 2014).

8.3.2 Extracts of Algae as Ingredient in Cosmetics

Algae are an important resource in the field of cosmeceuticals because of the existence of number of phytochemicals such as polysaccharides like agar, carrageenan, alginates, fucoidans, laminarins, ulvans; proteins and their derivatives like amino acids, cyclic peptides, lectins; lipids like fatty acids, phytosterols, galactoglycerolipids; pigments like chlorophylls, phycobiliproteins, carotenoids, and phenolic compounds like phlorotannin, terpenoids, bromophenols. Polysaccharides extracted from algae are mostly used as chelating agents, protective colloids, gels, moisturizers, thickening and emulsion stabilizers. They have antioxidative, anticellulite, antimicrobial, anti-inflammatory, anti-aging properties. Algal proteins and their derivatives possess antioxidative, chelating, antimicrobial, and anti-inflammatory activities. They function as moisturizing and natural sun screening agent. Phenolic compounds, lipids, and pigments extracted from algae have antioxidative, antiallergic, anti-aging, antiwrinkle, anti-inflammatory, and antimicrobial activities. Phenolic compounds extracted from algae also act as natural UV screening agent. Algae are utilized in micronized form or as extracts in cosmetics. Algal cosmetics can be anti-aging cream, moisturizing cream, serum, antiacne products, peelings, face and body scrubs, anticellulite body lotions, hair mask, and slimming creams (Michalak et al. 2017).

8.4 Algal Pigments Used in Cosmetic Industry

The green, brown, red algae contain different and specific kinds of pigments in them. Carotenoids are natural isoprenoid lipid-soluble pigments which give photoprotection against UV light induced photooxidation in the skin cells. Marine microalgae contain up to 0.2% carotenoids, which act as potent antioxidants. Microalgae belonging to the Chlorophyceae family are rich in carotenoids. *Dunaliella* accumulates maximum amount of beta-carotene, and *H. pluvialis* contains the maximum content of xanthophylls such as astaxanthin. Microalgae

produce xanthophylls such as violaxanthin, antheraxanthin, zeaxanthin, neoxanthin, lutein, loroxanthin, astaxanthin, and caraxanthin. Fucoxanthin, diatoxanthin, and diadinoxanthin are synthesized in brown algae. A carotenoid pigment, astaxanthin, demonstrates stronger antioxidant activities than beta-carotene and vitamin E. Reports suggests that topical application of astaxanthin formulation exhibited protective effects against UV induced skin cell injury (Berthon et al. 2017).

Astaxanthin, extracted from the green algae *H. pluvialis* has an effective defense against UV photooxidative injury thereby refining the skin texture by preventing wrinkles. It is reported that in vitro protective effect of astaxanthin seems to be powerful compared to lutein and beta-carotene. Violaxanthin, a natural xanthophyll pigment derived from *Chlorella ellipsoidea*, is found to possess anti-inflammatory activities (Berthon et al. 2017).

Fucoidan, laminarin, and alginate extracted from the brown algae *Turbinaria conoides* have antioxidative activities, which can be employed to prohibit aging of the skin. Alginate also acts as a water-binding and thickening agent in cosmeceuticals. The red algae *Pyropia yezoensis* contains certain low molecular weight polysaccharides which have several biological properties, including antifatigue, and antioxidant, anti-inflammatory properties, and is also reported to be effective against UVA-induced photoaging (Lee et al. 2016).

Some microalgae accumulate lipid almost up to 90% of their dry weight and this lipid content is of specific interest in the cosmetics domain (Li et al. 2008a, b). Microalgae are a rich origin of pigments, mostly carotenoids such as beta-carotene (yellow-orange colored), canthaxanthin, astaxanthin (red-pink colored), lutein, lycopene (red colored), and cryptoxanthin, of vitamins such as A, B1, B2, B6, B12 and C (Carballo-Cárdenas et al. 2003), and of phycobiliproteins such as phycocyanin, phycoerythrin (Guillerme et al. 2017).

Astaxanthin and carotenoids are derived from marine algal species are investigated for cosmetic formulations. Mycosporine glycine, asterina-330, shinorine, palythinol, palythine, porphyra-334, palythene are few photoprotective components synthesized from various species of macroalgae. A cream carrying Mycosporine-like amino acids (MAA) derived from *P. umbilicalis*, a red alga, effectively protected the skin against UVA in in vitro studies. Algal extracts such as carotenoids, phycobilin pigments of *Chlorella*, *Spirulina*, *Haematococcus*, and *Dunaliella*, can be found in an array of cosmetics. Glutathione (GSH), an antioxidant found in all macroalgal species, is used as a skin whitening agent orally. Brown seaweed extract possessing fucoidan fractions can be used in cosmetology treatments aimed at esthetics, such as, in antiwrinkle treatments or in the inhibition of aging of the skin. The methanol extracted from *Corallina pilulifera* accomplished a commendable antioxidant activity and exerted protective impact on UVA-induced oxidative stress of the human dermal fibroblast cell. These results demonstrated that macroalgae extract may be a significant raw material for natural anti-photoaging components. The lipids derived from the green algae *Nannochloropsis* are utilized in cosmeceutical and skin care formulations (Michalak and Chojnacka 2015).

8.4.1 Algae as Moisturizing Agent

Moisturization and hydration are vital for skin to maintain its elasticity and health, and also to act as a barrier to stressful environmental conditions (Bedoux et al. 2014). Polysaccharides have a very significant part in cosmetic preparations as moisturizers and humectants. These macromolecules have an elevated water storage capability and can be linked through hydrogen bonds to keratin, which in turn improves skin moisturization (Leelapornpisid et al. 2014; Bedoux et al. 2014). Polysaccharides derived from *Saccharina japonica* suggest that they could be an interesting constituent for cosmetics (Wang et al. 2013). A cosmetic preparation containing 5–10% derivative of *Laminaria japonica* is reported to improve skin moisture in a study conducted on a batch of volunteers (Choi et al. 2013).

Moisturizers aid in maintaining the moisture of the skin thereby blocking formation of wrinkles and dryness (Bonté 2011). Alginate, agar, carrageenan, and fucoidans are some polysaccharides found in certain algae that help to maintain the water diffusion in the skin. These polysaccharides are mostly toxin free, cheap, and immensely found in the algae and can be used as a substitute to silicone based or oil-based agents used in cosmetics. It is reported that polysaccharides derived from algae such as *S. japonica*, *Codium tomentosum*, and *C. crispus* aid in water or moisture absorption and provide soothing effect (Wang et al. 2013). Proteins and hydrolyzates extracted from algae *Spirulina*, *Porphyra*, and *Chlorella* have the ability of moisture retention in skin and hair making them glossier and shiny. Algal peptides are used in cosmetic items for skin and hair care, such as lotion, cream, shampoo, hair revitalizer, and bath agents. Squalene, extracted from algae *Thraustochytrium*, *Aurantiochytrium*, and *Schizochytrium* is found to be non-toxic, nonsensitizing and non-irritating, and can be used as an emollient in moisturizing creams (Ariedea et al. 2017).

8.4.2 Algae as Thickening Agent

Thickeners are being used in cosmetic formulations to prevent inconsistency in the product. Polyethylene glycol and vegetable gum are few notable thickeners utilized in the cosmetic industry since a long time (Kadajji and Betageri 2011). Agar isolated from the cell wall of red algae such as *Gracilaria* and *Gelidium* acts as binder. Another kind of thickening and stabilizing agent is Carrageenan, obtained from the organism *C. crispus*. Algae are rich in pigments such as phycocyanin, carrageenan, vitamin A, proteins, and sugars that are effective and beneficial for skin and hence they are inculcated in cosmetics as skin sensitizers. Biomass from *Macrocystis pyrifera* is used as a thickening agent in cosmeceuticals (Skjånes et al. 2012).

8.4.3 Algae in Hair Care

Algal extracts of *Thalassiosira*, *Monodus*, *Chaeloceros*, and *Chlorococcum* could regulate melanogenesis (the process through which the pigment melanin is produced) in hair and skin, enhancing the proliferation of hair and hair follicles thereby preventing hair loss. Species of microalgae *Chlorella* has been reported to have the capability to soften both hair and skin. Alguronic acid, created from a mixture of polysaccharides isolated from *Chlorella protothecoides* and *Parachlorella*, has emerged as an innovative cosmetic item able to improve skin appearance, health, and fitness (Ariedea et al. 2017). Vitamins extracted from algae *Spirulina maxima* and *C. vulgaris* help in toning of skin, dark circles healing, skin purification, and encouraging hair growth by dandruff treatment (Joshi et al. 2018).

8.5 Other Benefits of Algae

8.5.1 Antimicrobial Properties

Antimicrobial activities of algae have been investigated since ancient times and hence they are of crucial interest for significant usage in cosmetic preparations. Seaweeds or macroalgae have the capability to eliminate bad bacteria and fungi, balancing the skin flora. Algal extract can act as preservatives to stop the development of harmful microbes that may damage the cosmetic formulation or even harm the customers. *Padina pavonica* (brown algae) and *Rhodomela confervoides* (red algae) were reported to have antifungal activity against *Mucor ramaniannus* and *Candida albicans*, respectively, that are two crucial classes of microbes, found on the skin. *S. aureus* is naturally harmless but when the skin is punctured, the secan invade the wound causing infections. When the staphylococcal toxins are contacted on the injured skin, they can give rise to blisters and pimples on the skin. The red algal extract Laurinterol, extracted from *Laurencia pacifica* can be used to tend to the infections of *S. aureus*. Algae *H. elongata* and *Synechocystis* are reported to have antimicrobial activities on four microbes, *S. aureus*, *C. albicans*, *E. coli*, and *Aspergillus niger* (Wang et al. 2015).

8.5.2 Skin Anti-Aging

A complex biological activity, skin aging, is distinguishably marked by the loss of elasticity, creases, ridges, wrinkles, enlarged pore formation, dryness, fine line formation, and discoloration on the skin (Ganceviciene et al. 2012). Peroxides, superoxide, hydroxyl radical, and singlet oxygen are reactive oxygen species (ROS), and they are the most common reason of aging of the skin (Thomas and Kim 2013). Vitamin E from algae, a fat-soluble antioxidant and beta-carotene, a pigment found in red and green algae can rejuvenate the skin and enhances immunity towards skin aging (Keen and Hassan 2016; Schagen et al. 2012). UVA, UVB, and

UVC are the three segregation of Ultraviolet (UV) light. UVA is responsible for penetrating through the dermis thereby triggering wrinkles and other skin symptoms. UVB burns the skin at a rate that is thousand times rapid than UVA and is more carcinogenic. UVC, on the other hand, presents no threat to the skin as it is obstructed by the ozone layer (Wang et al. 2015). Algal derivatives of *Turbinaria ornata*, *Gracilaria*, *Ahnfeltiopsis*, *Padina*, *Halymenia*, *Hydroclathrus*, *Polysiphonia*, *Laurencia*, *Colpomenia* are reported to exhibit anti-aging actions (Kelman et al. 2012). Mycosporine-like amino acid (MAAs) found in *P. umbilicalis* safeguards against UVA, as UVA results in premature skin aging and severe skin damage.

Skin aging is influenced by extrinsic factors such as harsh environmental conditions, smoking, pollution, and exposure to UV rays and toxins, and intrinsic factors such as natural or genetic changes. The brown macroalgae *Macrocystis pyrifera* possess antioxidant compounds such as phlorotannins, phloroeckol, and tetrameric phloroglucinol, which aid in preventing skin aging. *Monodus* and *Chlorococcum* extracts are used in anti-aging formulations to boost synthesis of collagen. Extract of *C. vulgaris* stimulates synthesis of collagen thereby elevating reduction of wrinkles and tissue regeneration. *Chlamydocapsa* extract, also known as snow algae, are used in photoaging care formulations and even for hair protection (Ariedea et al. 2017).

Protein hydrolysates and residual biomass of the three green microalgae *Tetraselmis suecica*, *Nannochloropsis*, and *Dunaliella tertiolecta* have been reported to display antioxidant and anti-aging properties (German-Báez et al. 2017; Norzagaray-Valenzuela et al. 2017). Macroalgae such as *Ecklonia cava*, *Scytosiphon lomentaria*, *Codium fragile*, *Petalonia binghamiae*, *Botryocladia wrightii*, *Piropya dentata* (red algae), *Umbraulva japonica* (green algae), and *Undaria pinnatifida* (brown algae) have attracted huge popularity in the field of cosmeceuticals as the bioactive compounds of algae are used in anti-aging, wrinkle reduction, skin whitening, and pigmentation reduction formulations (Wang et al. 2015). *Laminaria*, *Fucus* (brown algae), and *Chondrus* (red algae) are few algae routinely used in cosmeceuticals due to their capability to nourish and refine the skin. Fucoidan triggers the synthesis of Heparin growth factor (HGF) that enhances the growth and activity of a broad diversity of tissues and cells, thereby enhancing hydration and elasticity of cells and hence, commercially exploited by the Japanese company, Takara-Bio (Fitton et al. 2015; Fujimura et al. 2002).

Beta-carotene, synthesized by the halotolerant microalgae *D. salina* is utilized in anti-aging products, as provitamin A. Mixture of algal extract of *Meristotheca dakarensis* and *J. rubens* is found to stimulate glycosaminoglycans (GAGs), keratin, and collagens I and III synthesis. The brown algae *Macrocystis pyrifera* can synthesize hyaluronic acid, which is used in anti-aging products (Guillerme et al. 2017).

8.5.3 Skin Whitening

Melanin is a complex polymer pigment that bestows color to the skin and acts as a protective obstruction for skin cells as well. When constant exposure between skin and UV radiation is established for an extended period of time, the radiation is absorbed by melanin resulting in tanning. Algal pigments like fucoxanthin derived from brown algae *Laminaria japonica*, *Alaria*, *chorda*, and *Macrocystis* aid to diminish the tyrosinase activity and melanogenesis (Joshi et al. 2018). The major determinant of skin color, melanin, absorbs UV transmission and hinders the formation of free radicals, thus providing skin protection from sun injury and aging. The abnormal generation of melanin, however, can be a severe cosmetic issue and dermatological condition (Pimentel et al. 2018).

A whitening agent that regulates the process of melanogenesis to diminish brown spots and develop an even complexion has been launched. Whitonyl® is mainly composed of oligosaccharides and extracted from the red algae *Palmaria palmata*. It is reported that direct sun contact leads to photoaging, which is distinguished by the formation of wrinkles, loss of skin elasticity, and pigment formation such as freckles or brown spots. Whitonyl® is reported to provide these freckles a clear, homogeneous, and even complexion (Pereira 2018).

A phlorotannin named 7-phloroeckol, extracted from the brown seaweed *E. cava*, has been reported as a whitening agent because of its anti-tyrosinase activity. Extract of *Chlorella* proposed by the company Codif is reported to minimize skin pigmentation by more than 10% (Yoon et al. 2009). Pure extract zeaxanthin, an anti-tyrosinase ingredient, derived from the algae *N. oculata*, was patented to be used in creams (Ariedea et al. 2017).

8.5.4 UV Protection

Sunlight damages our skin on each day of the year, eventually leading to premature aging of the skin, known as photoaging as well. Solar radiation that lands up at the earth's surface ranges between 290 and 4000 nm and it is segregated into UVB (290–320 nm), UVA (320–400 nm), visible light (400–700 nm), and infrared radiation (700–4000 nm). A large number of biological systems get affected by UV emission. The high level of energetic photons in these wavelengths causes harm to the macromolecules such as DNA, membrane lipids, and proteins. However, Nature has developed a few defense mechanisms to cope with these harmful radiations which include extraction of UV screening components. Specified epidermal cells, melanocytes, produce melanin in response to UV light in humans. But protection by melanin is mostly inadequate. Higher energy UVB causes acute sunburn after exposure to direct sunlight. UVA can penetrate the dermis where it is accountable for the premature effects of skin aging caused by the sun. The fact that the skin on exposed parts such as the face and hands show aging signs much before clearly indicates UV as the primary etiologic agent in premature aging of skin. Since it is established that UVB is totally absorbed in the epidermis, it is UVA that rises

aging symptoms such as wrinkles, dryness, and pigments. UVB is mostly dependent on the season, daytime and cloudiness, whereas UVA is relatively constant throughout the year as it is independent of any season or time of the day, and it probes rather well through clouds and glass. Therefore, skin requires shielding against UVA exposure. However, cosmetic ingredients meant for daily application must be absolutely safe to use. Mycosporine-like amino acids (MAA) are UVA sunscreens generated by the red alga *P. umbilicalis*, a seaweed commonly known as Nori (Daniel et al. 2004). Sunscreen formulations made from the extracts of *P. umbilicalis* (red algae) which offers distinct effects in terms of protection from DNA damage based on its UVA protective property which is quite alike to synthetic UV filters. Hence it can be considered as a substitute for diminishing the amount of synthetic filters in photoprotective preparations. Vitamins A, E, C, and *G. biloba* together with the red algae *P. umbilicalis* can enhance the action of the sunscreen in preventing DNA damage and inflammation thereby acting on the cell renewal and anti-aging properties (Mercurio et al. 2015).

The human skin possesses natural antioxidant agents that are able to limit cell destabilization by blocking reactive oxygen species (ROS). However, when the amount of ROS is enhanced by UV exposure, these skin defense mechanisms can be overrun. ROS accumulation due to UV radiation could be responsible for photoaging complications, such as skin inflammation like skin drying, melanoma, and skin cancer. Polysaccharides such as laminaran, fucoidan, and alginate extracted from brown algae *F. vesiculosus* and *T. conoides* have antioxidative activities, and can be applied to hinder cutaneous diseases. Antioxidants also aid in maintaining the organoleptic properties of cosmetic formulations by avoiding lipid oxidation, hence limiting changes in its odor, appearance, and flavor (Wang et al. 2015).

Few algal species synthesize components with particular chemical structures which absorb UV rays and reduce actions on melanin synthesis as well. Extract of *Chlorogloeopsis* provides benefits to the keratin by preventing damages resulting from chronic UVA and UVB transmission. It aids in avoiding photoaging, wrinkles formation, and skin sagging. Algae *Isochrisis* could inhibit UV radiation with the same effect as a preparation containing only organic and inorganic filters with SPF 15 (Sun protection factor). Algae *Nannochloropsis* were also found beneficial against UVA and UVB radiation (Ariedea et al. 2017).

Sporopollenin and Scytonemin are produced by certain microalgae which can be used as UV protectants (Priyadarshani and Rath 2012).

8.6 Thalassotherapy: An Algal Treatment

Unfolded at the onset of the eighteenth century, this Greek term means “thalasso” (sea) and “therapia” (cure). This treatment includes therapy with sand, seawater, and other marine organisms like bacteria and macroalgae (Charlier and Chaineux 2009). Originated at Ireland, thalassotherapy includes a bath of seaweed, mostly *Fucus serratus* (Phaeophyceae) prepared in warm fresh water. It has been found that

F. serratus and *F. vesiculosus* have an extensive range of effective compounds, which includes fatty acids, antioxidants, and iodine.

Few benefits of thalassotherapy are given below (Pereira 2018):

- Skin permeability is increased.
- Enables the diffusion of the cosmetic elements into the skin during or immediately after the therapy.
- Gives a rested appearance to the skin thereby relaxing tight muscles.
- Seborrheic secretion is normalized.
- Hyperhidrosis, which means excessive sweating, is reduced.

8.7 Conclusion

Aging is a natural process. The skin being the prime organ where aging appears more apparent, it gradually loses few of its natural purpose. As millions of customers across the world are becoming more concerned about their appearance regarding beauty and youthful look, cosmetic products have become an indispensable part of their lives. Hence, cosmetics are an extensively competitive industry in a continuous state of evolution. Cosmetic industry is devoted in developing and finding natural, sustainable, and economical organic ingredient, to provide novel and innovative items that meet costumers' needs and demands. Algae are thus being actively employed in the diverse cosmetic items as a trust-worthy natural and organic constituent to rise the value of these products. The leading algal compounds are the pigments fabricated by these photosynthetic organisms. The compounds produced by algae such as polysaccharides, proteins, pigments have immense purpose and utilization. These components act as antioxidant, anti-inflammatory, anti-aging, anti-wrinkling, moisturizing, photoprotective, slimming, whitening and melanin-inhibiting, and collagen boosting agent thereby enhancing the health and appearance of the skin**.** However, commercialization of algal based products is a complex matter and needs crucial evaluation of the items assessing their safety and efficacy for human use.

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Food Supplements Formulated with *Spirulina*

9

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Abstract

Microalgae *Spirulina* is widely known after NASA successfully used it as a space travel nutritional supplement for astronauts. Humanity has not utilized the natural populations of *Spirulina* until now. However, its potential as an anticancer, antiviral, antioxidant, and antiallergic agent is being realized lately. From an industrial or commercial perspective, *Spirulina* has the potential in pharmaceutics, food industry, agriculture, perfumery, medicine, and environmental applications. Proper identification of strains, culture conditions, and growth technique are needed for successful commercialization. In the present chapter *Spirulina* productions from domestic to commercial scale along with its potential application and nutritional benefits are reviewed. The challenges in large-scale cultivation, market potential, and business opportunities are also discussed.

Keywords

Spirulina · Nutritional · Antioxidants · Domestic · Commercial

9.1 Introduction

Photosynthetic microorganisms are proficient in generating organic compounds through natural photosynthesis process. These organisms include plants, algae, and cyanobacteria. *Spirulina* are the richest source of protein and other nutrients. *Spirulina* sp., *Chlorella* sp., *Dunaliella* sp., and *Haematococcus* sp. are the fundamental microalgae species formulated for nutritional products as of now. Large-scale cultivation of microalgae has more than 40 years of history with the major species grown being *Dunaliella salina* and *Haematococcus pluvialis* for carotenoid processing, *Spirulina* for health supplements, and many other species for aquaculture. Half of the production of microalgae occurs in mainland China, and much of the rest occurs in Japan, Taiwan, and the USA. While, India and Australia, are minor producers of different microalgal products (Shi et al. 2016).

Spirulina is a multicellular and filamentous blue-green microalgae belonging to the Oscillatoriaceae family. Its most widely available species belongs to two separate genera, *Spirulina* and *Arthrospira*, which grow in alkaline water in latitudes between 35°S and 35°N in Asia, North America, South America and Africa, areas of incident solar irradiation between 600 and 850 KJ/cm^2. *Spirulina* having high economical value is the only microalgae that is viable for industry scale or commercial cultivation. It is highly photosynthetically efficient and having doubling time of 24 h which can easily be adapted to the wide variety of environments (Lanlan et al. 2015).

Spirulina sp. standout among the most cultivated microalgae that has been utilized as complete nourishment and additive since 1970s because of its rich nutrient supplements such as proteins, carbohydrates, minerals, and vitamins (De Bhowmick et al. 2014; Chen et al. 2011). It has gained significant acceptance in the food industry in the various countries of the world. *Spirulina* has become a major source of protein, human health food, and vitamin supplement to aquafeeds.

Despite the fact that its commercialized production, as complete diet and value-added products is beneficial, more capable and advanced methods are required for its economical production. *Spirulina* cultivation is exclusively done in open ponds and photobioreactors. Among these open pond cultivation system is easy to assemble and do not require particular control of ecological parameters such as light and temperature. The disadvantage of this systems is the lower biomass productivity, under 12 g m^2 day^{-1} as a result of contamination and excess water evaporation due to open conditions in surrounding environment (Chen et al. 2011; Xu et al. 2009; Shimamatsu 2004). Another cultivation method for *Spirulina* sp. is the use of an enclosed photobioreactors (PBRs), different PBRs include bubbling, tubular, horizontal, vertical, airlift reactors, poly bags, etc. This system has controlled set of environmental parameters as nutrients, light, temperature, and it also has lesser contamination and water evaporation. This all results in better biomass productivity and superior quality of products. On the other hand, PBRs are expensive and also difficult in maintaining (Xu et al. 2009; Shimamatsu 2004; Seyidoglu et al. 2017; Sudhakar and Premalatha 2015).

9.2 Morphology of *Spirulina*

Kingdom: Bacteria

Phylum: Cyanobacteria

Order: Oscillariales

Class: Cyanophyceae

Genus: *Spirulina*

Species: flavovirens *S.fusiformis, S. gracilis, S .gomontii, S.platensis*

Habitat: Soil, marshes, freshwater, seawater (salinity >30 g/L) with high pH (8.5–11.0), and thermal springs. High solar irradiance levels, particularly in the tropics (Liu et al. 2013).

Shape: Rod-disk shaped.

Main feature: Filamentous noticeable by the organization of the multicellular cylindrical trichomes in helical shaped filaments. The blue-green non-heterocyst filaments are solitary and free-floating under a light microscopy, and exhibit gliding motility.

Environmental factors: Temperature, physical, and chemical conditions might affect the geometry of the helix.

Arthrospira platensis is a helical colony or trichome (Fig. 9.1). The prevalent occurrence of straight trichomes indicates that plasmids carry the helicity trait.

The following characteristics (Mohan et al. 2014; Milano et al. 2016) describe trichomes:

Pitch: pitch is quite analogous to wavelength. It is the distance in the trichome between two peaks. The pitch may range from 7 to 50.

The Eq. (9.1) determines the pitch of the helix (h) (Shen et al. 2014).

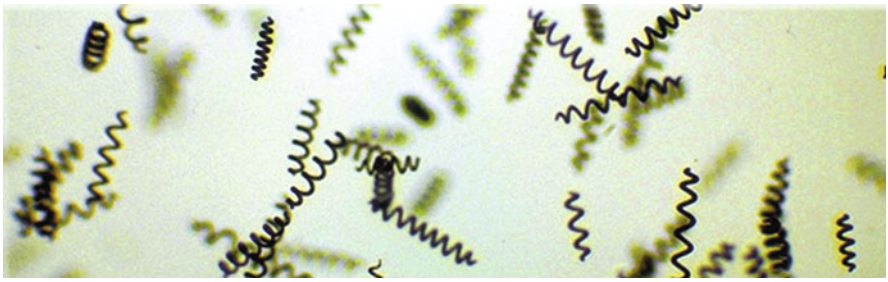

Fig. 9.1 Light micrograph of *Arthrospira maxima* (Soni et al. 2017)

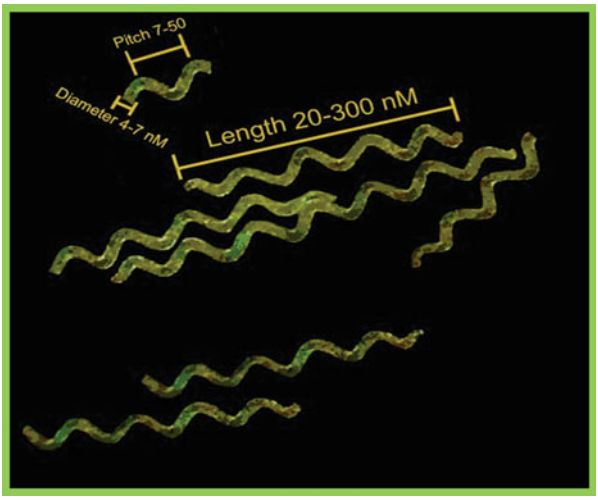

Fig. 9.2 Helical colony or trichome of *Spirulina* (Sudhakar et al. 2013a)

$$h = 2\pi r \cot \alpha \tag{9.1}$$

where, r is the radius of cylindrical surface to which the helix belongs; α is the angle formed by the helix and the cylinder generatrices which represents the slope of the helix curve.

Colony Length: 20–300 nm. Harvestable 60–200-nm long.

Diameter: The width of the trichome's individual cells is 4–7 nm.

Width: The width of the trichomes ranges in a multitude of types from about 6–12 μm (16 μm) (Fig. 9.2).

9.3 Functions of *Spirulina*

Spirulina is a conventional food consumed by some African people and Mexicans. It is a blue-green planktonic algae found in the alkaline water of volcanic lakes.

9.3.1 Nutritional Function

Arthrospira (*Spirulina*) is indeed one of the highest protein sources. Its protein content is around 65–71% (Liu et al. 2013; Shen et al. 2014; Shi et al. 2014) which is extremely beneficial compared to other protein sources (Table 9.1).

Spirulina is the highest, most potent, and most digestible protein source known, and this is what makes it one of the most valuable sources of proteins. It contains all of the eight essential, as well as conditionally essential and non-essential amino acids (Table 9.2).

Spirulina has no toxicity and it has been studied during animal experimentations. Authors (Sharma et al. 2012) have found that *Spirulina* did not demonstrate any harmfulness even with raised doses of human consumption (Zhu et al. 2014). *Spirulina* is also a good supplement for iron deficiencies during pregnancy and lactation. Studies done by Kapoor and Mehta (Soni et al. 2017), Nutritional

Table 9.1 Comparison of different types of protein sources and the amount of protein (Sudhakar and Soni 2017)

Source	Nutritional ingredient	Protein percentage (%)
	Egg (whole)	13.3
	Soyabean (whole)	43.2
	Wheat germ	29.2
	Peanuts	25.3
	*Spirulina*	65

Table 9.2 Amino acids in *Spirulina* (Sudhakar and Soni 2017)

Essential amino acids	Non-essential amino acids	Conditionally essential amino acids
Isoleucine	Alanine	Cysteine
Leucine	Arginine	Histidine
Lysine	Aspartic acid	Tyrosine
Phenylalanine	Glutamic acid	
Methionine	Glycine	
Threonine	Proline	
Tryptophan	Serine	
Valine		

supplement by *S. platensis* during the first stage of pregnancy and lactation, was found to increase the iron storage of rats, superior to anything accomplished from the mixture of casein and wheat gluten diets (Chen et al. 2016; Lupatini et al. 2017). Very limited studies have been performed on the health benefits of *Spirulina* on humans and animals (Piero Estrada et al. 2001). The *Spirulina* product is safe and does not have side effects in animals or humans. In human feeding experiments, *Spirulina* protein is adequately absorbed when fed to adults and results in a low level of uric acid in the serum and a moderate increase in fecal nitrogen. With third-degree malnutrition-stricken children, *Spirulina* has been shown to be better than soy and as good as whole cow's milk and human milk. Table 9.3 shows the nutritional facts of *Spirulina*/100 g. Nutritional tests have shown that *Spirulina* algae have a protein efficiency ratio (PER) of 2.2–2.6 (74–87%) and a net protein use (NPU) of 53–61% (85–92%) and a digestibility of 83–84%. This has a strong pigmenting effect on the skin and egg yolks in hens. Study results are interesting and one day might deserve a look from Pharmaceutical Companies. *Spirulina* is 10–15% of polysaccharide carbohydrates, since our cells can absorb them easily, these (polysaccharides) make *Spirulina* a great source of fast energy.

The mineral content of *Spirulina* biomass and culture conditions of the alga are given in Table 9.4.

Spirulina has approximately 63% amino acid content and is the world's richest natural source of vitamin B12 and also includes phytopigments of carotene and xanthophyll. It has a flexible cell wall which contains complex protein and sugars. *Spirulina* has achieved much popularity because of its nutraceuticals and pharmaceuticals properties (Sharma et al. 2012).

Spirulina can be used as a food supplement for weight loss as it is complete and natural food. It is used as a protein supplement in kids and adults to eradicate malnutrition. It can also be used as an energy booster. Good quality *Spirulina* has green color indicating excellent chlorophyll and phycocyanin. Carbohydrates are produced by phycocyanin through the absorption of sunlight to increase energy levels. *Spirulina* is the sustainable superfood that can be a full diet. Dietary benefits of *Spirulina* are given in Table 9.5.

In addition to the macro and micronutrients, *Spirulina* contains a few important phytonutrients like chlorophyll, phycocyanin, and carotenoids. These phytonutrients

Table 9.3 Nutritional Facts of *Spirulina* 100 g (Sudhakar et al. 2013a)

Nutrients	Percentage
Moisture content	4.0–5.0%
Energy	2.9 Kcal/g
Protein	60–71 g
Fatty acids	4–5 g
Carbohydrate	15–18 g
Chlorophyll	1–2 g
Mixed carotenoids	350–450 mg
Beta carotene	180–190 mg
Phycocyanin	8–12 g
GLA	1–2 g
Calcium	400–600 mg
Iron	50–100 mg
Potassium	200–2000 mg
Magnesium	200–300 mg
Zinc	1–2.0 mg
Folate (folic acid)	0.1 mg
Vitamin A (beta-carotene)	100–200 mg
Vitamin B1 (thiamine)	1.5–4.0 mg
Vitamin B2 (riboflavin)	3.0–5.0 mg
Vitamin B6 (pyridoxine)	0.5–0.7 mg
Vitamin B12 (cyanocobalamin)	0.05–0.2 mg
Vitamin E (A-tocopherol)	5.0–20 mg

Table 9.4 Mineral content of *Spirulina* and culture conditions (Sudhakar et al. 2013a)

Minerals (to be supplied in growth medium)	Dry weight (g/kg)	Growth conditions	
Carbon (from CO_2)	550	pH	9–11
Nitrogen (from NO_3)	100	Salinity	3.5–8.2%
Sulfur	30	Average temperature in ponds	17–35 °C
Potassium	30	Average sunlight	1–10 klx
Magnesium	2		
Calcium	1		
Micronutrients	–		

are basic for the synthesis of different compounds that are required for various metabolic processes. For instance, chlorophyll plays a key role in maintaining hemoglobin in the body. Hemoglobin is present in the red blood cells, contains iron (heme), and is required for the transportation of oxygen. The structure of chlorophyll is similar to that of hemoglobin. The only difference is that at its center,

Table 9.5 Functions of *Spirulina* and its benefits (Sudhakar et al. 2013b; Soni et al. 2017; Ishimi et al. 2006)

Benefits	Nutrients	Function	Suitable for
Resists fatigue	Vitamins, minerals, proteins	Rebuild the body's depleted nutrients	Work fatigue, low immunity, hormonal imbalance, nervous disorder, lack of energy, tire easily, emotional instability, insomnia
Food for brain	Nucleic acid (DNA/RNA), SOD	Help produce high-quality brain tissues, increases the ability of RBC to carry oxygen to the brain	Everyone especially unborn babies and children
	AFA	Effectively balance functions of right and left brain, enhances memory	Students, strenuous mental activity
Antiaging	Nucleic acid (DNA/RNA),	Helps repair genetic elements in injured cells, slows down the aging process	Everyone especially middle-aged to elderly individuals
	SOD	Antioxidant, delay aging, revive energy levels, prevent wrinkle formation/gray hair/ pigmentations, helps to restore energy levels, memory, and youthfulness	
	C/E, Beta carotene	Fights oxidative stress delays premature aging	
	Enzyme	Directs cellular growth, renewal, and repair	
Improves Anemia	Iron, protein, fat, chlorophyll, B12	Vitamin B12 250% higher than in any food, helps to build blood components, alleviates anemia	Children, elderly, weight loss, women, pregnancy, vegetarians
	MVTs, minerals, proteins, pigment	Six main nutrients provide balanced elements for blood formation, prevents anemia due to nutrient deficiency	
	MVT, protein, iron	Maintains balanced nutritional intake	Restricted diets, athletes

chlorophyll contains a magnesium ion while hemoglobin contains an iron molecule. If chlorophyll is ingested with iron, it is proposed that the magnesium can be displaced and formation of hemoglobin occurs. This is how *Spirulina* intake can work out to be beneficial in anemia (a condition in which hemoglobin concentration in the body is reduced). Chlorophyll also has beneficial effects on the digestive system. It normalizes the secretion of acids in the stomach. It stimulates the movements of intestines and can facilitate the passage of stools. It also helps in the regeneration of injured cells. Chlorophyll is also known for its effects on the proper functioning of heart since it can increase the efficiency of the pumping function.

Phycocyaninis, pigment that gives *Spirulina* its blue color. Phycocyanin contains both magnesium and iron. It is involved in various body functions like digestion of amino acids, stimulation of defense system, and the building of blood cells. *Spirulina* also contains numerous carotenoids like alpha-carotene, beta-carotene, xanthophylls, cryptoxanthin, echinenone, astaxanthin, and lutein. Carotenoids are the precursors of vitamin A, i.e., they are capable of producing vitamin A (hence *Spirulina* can offer all the benefits of Vitamin A). The added advantage of carotenoids from *Spirulina* is that these are converted to Vitamin A only when the body has a demand for it. This minimizes the chances of a vitamin A overdose.

9.3.2 Antioxidant Function

Oxygen is very important to live. Although oxygen is essential for metabolism, it releases products called free radicals when combined with complex metabolic molecules in the body. The stress levels of modern living, pollution, and food produced by masses, as well as poor lifestyle and diet tend to increase the number of free radicals in the body to dangerous levels (Bahadar and Bilal Khan 2013). The effect is cellular damage that causes premature aging, wrinkles, poor skin, dark rings under the eyes, and often severe diseases. Antioxidants are a generic term for the body's nutrients that cope up and neutralize free radicals. *Spirulina* contains many antioxidants, including vitamins E and C, and carotenoids such as astaxanthin (Chu et al. 2010; Aruoma 1994).

Besides the importance of *Spirulina* as a supplementary dietary protein food additive, there are also many potential for medical and therapeutic applications (Sudhakar et al. 2012a; Vadiveloo et al. 2016). For instance, it also exhibits hepatoprotective role (Borowitzka and Vonshak 2017). This role, which has to do with *Spirulina*'s antioxidant activity, has been asserted by various researchers before hand. Its antioxidant activity is correlated to the presence of two phycobiliproteins: phycocyanin and allophycocyanin, as determined by its action against ascorbate/iron/H_2O_2-generated OH radical. The activity was found to be proportional to the phycobiliprotein concentration and was mainly caused by the phycocyanin content (Slocombe and Benemann 2016). As an antioxidant effect, phycocyanin and phycocyanobilin from *Spirulina* inhibited oxygen stress which resulted in protection against diabetic nephropathy (Papadaki et al. 2017; Zheng et al. 2013).

It is now well known that increased production of oxygen radicals and other oxygen derivatives frequently causes damage to tissues. There has recently been an increasing rise in the use of dietary supplements with antioxidants. Epidemiological evidence indicates that ingestion of some vitamins , minerals, and other constituents of foods may help protect against heart disease, cancer, and the aging process, and that antioxidants may have a protective impact either in preventing these diseases or in reducing the severity of the diseases upon their occurrence. Much of their activity is mediated by reactive oxygen species (ROS) produced during the oxidative burst (Sudhakar and Premalatha 2012a; Sudhakar et al. 2012b; Griffiths et al. 2016; Olasehinde et al. 2017).

Several in vitro studies show that *Spirulina* polysaccharides improve synthesis of DNA repair and enzyme activity in the cell nucleus. Also, *Spirulina* is a great immune tonic. Food studies show that even small quantities of *Spirulina* build up both the immune system's humoral and cellular mechanisms (Kedik et al. 2011; Nuhu 2013; Guldhe et al. 2017; Geada et al. 2017; Mobin and FirozAlam 2017; Deamici et al. 2016). *Spirulina platensis* also produces phycobilisomes as light processing protein-pigment complexes. Phycobilisomes are composed predominantly (80–85%) of beautifully colored polypeptides called phycobiliproteins (Marco et al. 2014; Soni et al. 2019; Santos et al. 2016; Gutiérrez-Salmeán et al. 2015; Martelli et al. 2014). Phycocyanin and allophycocyanin are the two more important biliproteins in this microalgae, both having the same chromophoric group (Vo et al. 2015). An increase in the amount of phycocyanin is associated with an increase in antioxidant activity in the different fractions, and thus phycobiliprotein and phycocyanin are the components primarily responsible for the antioxidant activity of the *S. platensis* protein extract (Sudhakar and Premalatha 2012b). This is the reason for NASA to use *Spirulina* and the Food and Agriculture Organisation (FAO) to call it as the "food for future" (Sudhakar and Premalatha 2012c).

9.4 Domestic and Commercial Cultivation of *Spirulina*

Spirulina grows normally in alkaline ponds around the globe. Historical records archive conventional people groups harvesting and consuming *Spirulina* from lakes in Mexico, Africa, and Asia (Fig. 9.3).

A portion of the largest natural *Spirulina* lakes are around Lake Chad in Central Africa, and along the Great Rift Valley in East Africa. For a tremendous amount of time, algae pioneers have envisaged harvesting algae from these lakes to feed a large number of hungry people and promote a sustainable economy. In the past decade, many companies have attempted to manufacture *Spirulina* commercially. Many commercial production attempts have failed, although some successful producers are still restricted to the domestic markets. *Spirulina* was also first commercially produced from natural lakes in Myanmar in 1988.

Fig. 9.3 World map showing lakes with natural *Spirulina* blooms (Soni et al. 2019)

9.4.1 Domestic Cultivation

Major components required to grow *Spirulina* at the domestic level are a tank, water, culture medium, harvesting equipment, *Spirulina* mother culture, and Drying Racks. *Spirulina* flourishes in the salty, alkaline atmosphere. Any watertight, open tank that resists corroion can be used to grow *Spirulina*. Its shape is irrelevant though it should be kept away from sharp edges to encourage cleaning and agitation. Its depth usually is 40 cm. It may very well be as small as 1 m^2 but more conventional are 5, 20, or 100 m^2. Size is limited only by the need for agitation and cleaning access. To encourage emptying the base should have a slight slant and a break. For practical reasons two lakes are superior to only one. The most affordable ponds are made of 0.5 mm or greater U.V.-resistant plastic film (PVC or polyethylene), with blocked sides or a wooden frame or metal tubing. To cover it, a layer of dry ash needs to be placed under the film in addition to a layer of sand. Ponds made of concrete are obviously a decent solution where experienced manpower is accessible. Prior to grow the culture, the concrete should be very much restored and whitewashed. A green house over the ponds offers numerous favorable advantages, if it tend to be aerated and shaded. Infact, covering the ponds is practically important.

9.4.2 Important Parameters

Light, temperature, agitation, culture medium, correct medium, and water source are very important factors for growing *Spirulina*. *Spirulina* can be one of several algal species, under normal water conditions. The more alkaline water becomes, the more inhospitable it is to other life forms, thereby allowing *Spirulina* to thrive as a single species. *Spirulina* thrives in alkaline lakes where other microorganisms are difficult or impossible to survive. Because the level of bacteria in alkaline water is quite low, the count of *Spirulina* bacteria, harvested, and dried without further processing, is low. Algae microfarms in temperate climates are small-scale commercial farms within the greenhouses. Modern smart algae microfarms are also facilitated with the supply of artificial lighting that can provide high growth rates even in cloudy days (Fig. 9.4). They may be the future of greenhouses, hydroponics, and urban agriculture. *Spirulina*, in less area can produce higher incomes for farmers than conventional foods, vegetables, and herbs.

9.4.3 Climatic Factors

It is widely acknowledged that light and temperature are the two fundamental factors for the development of open pond systems. Light has been thought to be the primary constraining factor for development in generally warm climates, where the greater part of the algal biomass production sites are found. Numerous investigations have been completed attempting to improve the accessibility of light to algal cells developed outdoors. Temperature is the most important climate factor affecting

Fig. 9.4 Smart *Spirulina* microfarm, Olympia, Washington, USA (Courtesy: Robert Henrikson) (Chojnacka and AndrzejNoworyta 2004)

Spirulina growth rate. The optimal growth temperature is between 20 °C and 35 °C. Growth occurs only in light (photosynthesis), but lighting is not recommended for 24 h a day. Chemical reactions occur in *Spirulina* during the dark time, such as protein synthesis and respiration. *Spirulina* cannot withstand a strong light below 15 °C. Light is a significant factor, but the best lighting rate may not be full sunlight: 30% of full sunlight is really better, except that more may be needed to heat up the morning culture quickly.

Individual *Spirulina* filaments are disrupted by prolonged intense illumination ("photolysis"). Rain is useful to mitigate evaporation, but it must not be permitted to bring runoff to the culture pool. Wind may bring dirt, but it is good for agitation and aeration. Artificial light using fluorescent tubes and halogen lamps can illuminate and heat the culture simultaneously, although they are not economical.

9.4.4 Commercial Cultivation

Since 30 years, commercial *Spirulina* production has begun with shallow open raceway lakes intended to be agitated by paddlewheels. The size of the ponds ranges around 5000 m^2 or even more, while the depth ranged from 20 to 25 cm. Earthrise Nutritionals, California , USA was established in 1981 and expanded to cover approximately 108 acres over the next decade. Commercial manufacturing has also been reported in Australia, Chile, Cuba, Israel, Vietnam, Bangladesh, the Philippines, Peru, Martinique, Brazil, Portugal, Spain, Chad, and other nations. *Spirulina* farms are multiplying around the globe with their company potential. *Spirulina* (*Arthrospira*) is a commercially cultivated filamentous cyanobacterium for meat and feed and as a dye and additive for meat. There are currently many firms manufacturing *Spirulina* at a rate of 3000 tonnes per year in distinct nations (Schreiber et al. 2017).

- *Earthrise Farms, USA*
  Earthrise Farms is the world's first and biggest food grade *Spirulina* manufacturing farm founded in the USA. The farm is currently a DIC subsidiary

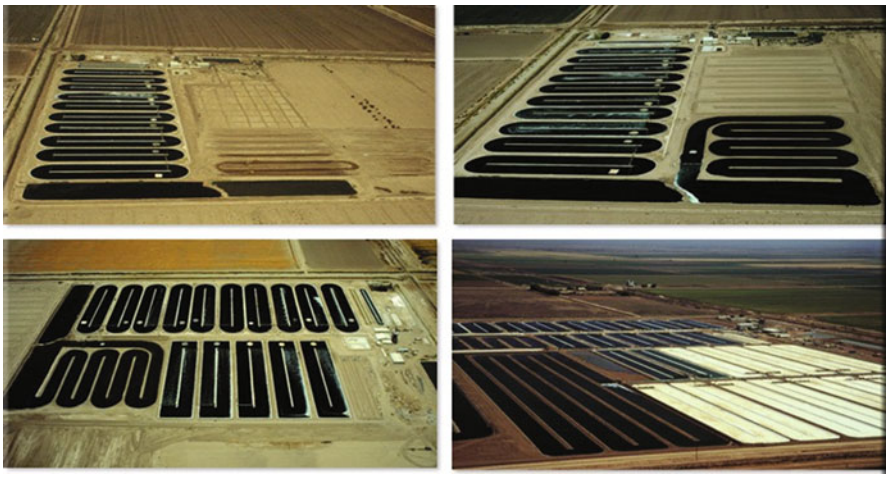

Fig. 9.5 Earthrise farm, USA (Courtesy: Robert Henrikson) (da Silva Vaz et al. 2016)

and supplies the Pacific Basin as well as North and South America, Europe, and Japan (Fig. 9.5). There are a total of 15 microalgae cultivation ponds each with a region of around 5000 m^2. The complete area of the pond is approximately 75,000 m^2. There is also a distinct *Spirulina* manufacturing facility for feed grade with a pond region of approximately 40,000 m^2. Earthrise Farms has the world's biggest output of food grade with an annual capability of over 200,000 kg. They also have experimental lakes that range from 2 to 1000 m^2 in area (Sudhakar et al. 2011). Earthrise Farms represents approximately 60% of the total region, excluding Sosa Texcoco, a semi-natural production plant, and producers account for approximately 45% of the total annual production sold to the USA and the global market. Together, the two farms belonging to Dainippon Ink & Chemicals (Earthrise Farms and Siam Algae Company) make up about 75% of the total area and about 70% of total production (da Silva Vaz et al. 2016).

- *Parry's Nutraceuticals, India*
  Parry Nutraceuticals is a subsidiary of EID Parry (I) Ltd. and a member of the US$4.4 billion Murugappa Company, one of the largest conglomerates of Indian companies (Fig. 9.6). Headquartered in Chennai, India, E.I.D. Parry has been associated with dynamism since 1788. Market leaders in a range of business fields, including Electronics, Abrasives, Finance, General Insurance, Cycles, Cotton, Farm Inputs, Fertilizers, Seeds, Bio-Products and Nutraceuticals, the Company has 28 manufacturing facilities across 13 Indian states (Tobon-Velasco et al. 2013). As a world leader in microalgae technology, Parry Nutraceuticals focuses on excellent health by harnessing the wealth of nature supported by technology in agriculture, extraction, and formulation. Its products have become an essential component of several significant brands in more than 40 nations. North America , Europe, South East Asia, and the Far East are the main markets for many years now. Parry Nutraceuticals has been a pioneer in the market for

Fig. 9.6 Parry Nutraceuticals *Spirulina* farm (Tobon-Velasco et al. 2013)

Fig. 9.7 Cyanotech, Hawaii *Spirulina* farm (Makhlouf and Makhlouf 2012)

nutritional supplements for microalgae cultivation and has been instrumental in the marketing of algae-based goods such as *Spirulina*, Astaxanthin, and Natural Mixed Carotenoids. Algae cultivation remains a proprietary knowledge known only to a few organizations, and mastering this expertise was the corner stone of Nutraceuticals' dominant presence in this sector.

- *Cyanotech, Hawaii, USA*
  Over many years of ongoing cultivation, a chosen strain of *Spirulina platensis*, *Spirulina pacifica*, was created in Hawaii (Fig. 9.7). The growth of strain with beneficial outcomes began in 1984. Their cultures in Hawaii embody the world's oldest continually grown and harvested *Spirulina* (Makhlouf and Makhlouf 2012). Some University of Hawaii scientists in molecular biology have recognized a distinctive enzyme that is present only in *Spirulina pacifica*.

Fig. 9.8 Hydrolina Biotech *Spirulina* farm (Soni et al. 2019)

Spirulina pacifica is a source of highly absorbable protein, mixed carotenoids, and other beta vitamins, phytonutrients, essential amino acids and GLA.

- *Hydrolina Biotech, India*
  Hydrolina Biotech is a worldwide company with the main goal of producing goods related to nutraceutically important natural resources extracted specifically from algae, fungi, and bacteria (Fig. 9.8). The company is currently engaged in "Vitalinaa" *Spirulina* Dried Powder, tablets, and capsules processing and export. The firm has produced steady strides by gaining a reputable name through its proactive board with the capable guidance and precious knowledge. Healthy product "Vitalinaa" forms in the balanced ratio the construction blocks required for balanced nutrition, which is also available in a total biologically active form.
- *Australian Spirulina, Australia*
  Spirulina Farm in Australia are situated in the Northern Territory's pollution-free surroundings and ice-clear mineral water. Australian *Spirulina* is the Australia's only *Spirulina* company (Fig. 9.9).

  Using state-of-the-art technology with distinctive and special spray drying technique (Protected by a 20-year Australian patent), they dry *Spirulina* in less than 10 s. Australian *Spirulina* is 100% pure without any binder or artificial additives.

Based on current findings in China and elsewhere, the realization of *Spirulina*'s prospective therapeutic impacts has in recent years attracted the attention of several Chinese producers. During the past couple of years alone, several facilities were freshly built in the People's Republic of China, while some were being developed. The enhanced knowledge of Spirulina's advantages for human and animal use will undoubtedly lead to a continuous rise in the establishment of new installations worldwide (Chojnacka and AndrzejNoworyta 2004).

Fig. 9.9 Australian *Spirulina* farm (Chojnacka and AndrzejNoworyta 2004)

9.4.5 *Spirulina* Harvesting

The best time to harvest *Spirulina* is early in the morning as the lower temperature facilitates and comforts the working conditions. There will be more sunlight availability for drying the product. The protein percentage is highest during the early morning time.

Harvesting involves following two steps:

Filtration—To obtain biomass containing 10% dry matter and 50% residual culture medium.

Removal of the residual culture medium by washing to obtain freshly prepared wet biomass of *Spirulina* for consumption. After further drying it contains 20% of dry matter and is completely washed off the residual culture medium.

Filtration is done by using a fine woven cloth, where gravity is used as the major driving force. Synthetic fiber cloth having mesh size of 100–400 μm size is preferable. Fine netwill is used to support the filtration cloth. The netwill can be put over the pond with filter cloth to recycle the filtrate directly. The harvested culture is passed through the sieve or mesh cloth to remove the foreign matters.

Dewatering—Filtration is done on a 100–400 μm mesh sheet. After filtration the biomass gets agglomerates and the cloth remains clean. Now the biomass contains 10% dry mass with soft consistency. At the later stage dewatering is done by enclosing the biomass in the mesh cloth and then further pressing it by heavy weight so that all the liquid is expelled out of the biomass. Usually pressing is done about 15–20 min. After pressing the pH of the biomass is reduced to eight due to the deterioration of some *Spirulina* cells. Germs can also be introduced by washing; unpressed biomass contains half the dry mass to that of the pressed one. Also unpressed biomass takes more time for drying. Pressed biomass has the firm

consistency as that of a cheese and can be cut easily through a knife and also it can be consumed in the same form.

9.4.6 Drying

For drying process, biomass should be thin enough to dry before it gets fermented. The dewatered and pressed biomass is brought to rods or spaghetti shape. It must be sturdy enough to maintain its shape.This can be done in sun drying or green house drying for about 7–8 h. Some precautions should be taken so that the biomass may not get contaminated by dust or insects. The final water percentage should be less than 9%. After completely drying these rods are grinded to powder form . These powders are generally stored under vacuum in heat sealed, aluminized plastic bags. For large scale or in industries spray dryer is mostly used. Spray drying is done at very high temperature and with low apparent density, it yields extremely fine powder. Sun drying is done in small scale, but it should not be done for larger time period as the chlorophyll in *Spirulina* might get reduced and also the final product might appear blue.

9.5 Economic Importance and Commercial Value of *Spirulina*

Recent studies show that *Spirulina* processing for algal biofuels is not economically feasible, so there is more stress on microalgae as a source of higher value-added items (Piero Estrada et al. 2001). Over the last few years, the global spirulina market is projected to record of an annual growth of an investment (CAGR) of 10%. The market is expected to grow from US$700 million in 2016 to US$2000 million by 2026 due to the growing applications of *Spirulina* in cosmetics. Today, *Spirulina* can be used as a natural coloring agent since the EU government has prohibited the use of synthetic colors in the food industry. So, it is expected that it will increase the demands and hereby boost the sales of *Spirulina* in different industries for different applications in coming years. As the new applications and various value-added products of *Spirulina* are brought up, many new initiatives taken by the government for the growth of the global *Spirulina* market is projected to take place during the coming years. Governments of different countries have attempted to popularize the *Spirulina* products and their benefits in order to boost the needs and developing a source of income for rural communities (https://www.persistencemarketresearch.com/market-research/spirulina-market.asp). Local *Spirulina* market is also supported by government of Angola, India, and Ghana, which are actively participating for bringing *Spirulina* and its major benefits for the development of society. EFSA, the EU food safety regulatory agency, has prohibited the Usage of synthetic colors in items consumed in EU countries to boost natural color market growth. The Blue-green *Spirulina* pigment has become the most attractive color in the world due to its characteristics and texture, and is primarily used in the food and beverage industry. *Spirulina* has hypolipidemic, antioxidants, and antiinflammatory characteristics as a

result of which the food and beverage and cosmetics sectors are experiencing a large increase in demand (Quader et al. 2013). Antioxidants defend the body against harm created by damaging molecules (free radicals) that enhance a person's health. *Spirulina* comprises a big amount of vitamin A, C, and E and beta-carotene with a minimum amount of Lutein Zeaxanthin; all these are antioxidants that assist to cure multiple illnesses and medical situations. Changing consumer preferences to nutritious and safe products is the latest trend for which companies are launching innovative products which complement the needs of contemporary society. Naked Juice, for instance, introduced their 100% juice variety consisting of *Spirulina* as a main ingredient (Soni et al. 2017). Major developments noticed on the worldwide *Spirulina* market are increased production of ready to drink smoothies prepared from *Spirulina* and increased demand for *Spirulina* in the biofuel sector expected to stimulate *Spirulina* demand during the following years.

9.6 Business Opportunities

Various value-added products and solutions which are based on *Spirulina* bring exciting opportunities in our country as well as globally. *Spirulina* provides opportunities in various sectors as food, feed, biopolymers, coloring agents, nutraceuticals, pharmaceuticals, biofuels, and many more (http://www.consult.eai.in/algae-products).

9.6.1 Global *Spirulina* Market

Spirulina is primarily consumed in forms of powder, tablet, and capsule. Among the different product types of *Spirulina* used, the powder form was projected to report the highest peak value of CAGR of 10.4% during the following years. This is the consequence of the high and increasing use of *Spirulina* powder for food purposes. The segment of powder has been projected to reflect the highest value share of 73.5% in 2016 and the segment is expected to gain substantial market share by the end of 2026. The capsule and tablet types account for the second highest CAGR of 8.8% in terms of value over the expected period after powder (https://www.persistencemarketresearch.com/market-research/spirulina-market.asp).

The natural color that *Spirulina* generates has excellent potential in the worldwide market. *Spirulina* has developed numerous natural colors, and there are still many more to come. Spirulina Blue, a natural color created from blue-green *Spirulina*, was approved by the FDA in 2013, opening a whole new segment for *Spirulina* products (Table 9.6). The natural blue and green color of *Spirulina* is in high demand, and has seen good consumer growth over the past two years. Lots of businesses such as GNT Holdings B.V., DIC Company, DDW Inc., Chr. Hansen Holding A/S, and Sensient Technologies Corp. manufacture Spirulina Blue which is still finding more producers due to increasing demand. DDW Inc. launched a revolutionary *Spirulina* blue color that improved light stability by up to 40%.

Table 9.6 *Spirulina* market segmentation (https://www.persistencemarketresearch.com/market-research/spirulina-market.asp)

By-product form				By application type		
Powder form		Tablets		Nutraceuticals	Food and beverage	Animal feed
Capsules	Liquid	Gels and granules		Biofuel	Face packs	Cosmetics
Key regions/countries covered						
North America	Latin America	Western Europe	Eastern Europe	Asia Pacific excluding Japan		Japan

9.6.2 Commercial *Spirulina* Innovative Products

Spirulina microalgae exemplifies the cyanobacteria biomass that humans and many animals can consume. There are two main types of *Spirulina*, which are further processed for different applications. This includes *Arthrospira plantesis* and *Anthrospira maxima*. *Arthrospira* is produced worldwide and served as nutritional supplements and as a complete food as it provides all the nutrients. It also meets its implementation in kids, pregnant women, and lactating mothers who need more protein and iron in their diet to fight against anemia. Other applications include personal care products for skin and hair, such as scrubs, masks, creams, shampoos, and cleansers. *Spirulina* are an outstanding complement containing 60–65% protein, are simple to digest, and are known to help easily recover from malnutrition. *Spirulina* products can significantly expand the variety, sustainability, and reliability of aquaculture products to satisfy increasing food safety requirements while reducing CO_2 emissions. In the aquarium, aquaculture, and poultry sectors, *Spirulina* is also commonly used as a feed substitute (https://www.persistencemarketresearch.com/market-research/spirulina-market.asp; Paniagua-Castro et al. 2011; Rodríguez-Sánchez et al. 2012; El-Desoky et al. 2013). The protein content, final product color, and *Spirulina*'s consistency are a deciding factor in evaluating its market price, the higher the micro-nutrient/protein content, the higher the economical value . Some of the known commercial *Spirulina* products are *Spirulina* Energy Drink (100% Natural organic Drink), *Spirulina* Soap, *Spirulina* Cereal, *Spirulina* Toothpaste, etc. Other than this, wide range of products are offered by gerophyta nutraceuticals company such as *Spirulina* Powder, *Spirulina* flakes, *Spirulina* tablets, *Spirulina* Capsules, Spiruvita-C, Dr. *Spirulina* Diavita-C, *Spirulina* herbal face pack, *Spirulina* drinks, *Spirulina* chocolates (Rabelo et al. 2013). Some of the products of *Spirulina* are illustrated in Fig. 9.10.

9.7 Challenges in *Spirulina* Production

Optimizing a *Spirulina* production system involves issues such as strain choice, choice of nutrient media, cultivation system layout, and harvesting techniques into a practical and economically feasible design. Though it seems simple to cultivate microalgae, there are many obstacles, including:

Fig. 9.10 Different products of *Spirulina* from Gerophyta *Spirulina* farm, Illupur (i) Powder form (**a**) Spiruvita-C, *Spirulina* with amla (**b**) Dr. *Spirulina* (ii) Capsule form (**c**) Diavita-C, *Spirulina* with gymnema (**d**) Spiruvita-C, *Spirulina* with amla (iii) (**e**) *Spirulina* herbal face pack (**f**) *Spirulina* sweets (Soni et al. 2017)

1. *Minimizing contamination and production costs*:
   Spirulina has lengthy filaments organized in an elongated helix shape, and this shape helps to harvest and allows easy screen filtration. It also enables for the use of different depths of the pond and reduced cell rates consistent with economic harvest. Use a baffle system and recirculation and mixing equipment for ponds

that are typically more than 20 cm in depth. Flow mixing is usually more realistic and cost-effective than paddle agitator as it relies entirely on rpm and can also split filaments. The former often causes the water mass to circulate through the raceways at a speed of 0.03–0.06 m/s thereby preventing thermal stratification.

2. *Efficient provision of light*:
   Prolonged strong illumination ("photolysis") destroys the Individual *Spirulina* filaments. Through agitation, photolysis is reduced. Elevated light intensity may result in photoinhibition, production of toxic photographic products like H_2O_2, OH, and triple chlorophyll, and even damaging the Photosystem II reaction core. Low light intensities, on the other hand, may limit photosynthetic activity that lowers the rate of development and hence the biomass productivity. Although many algae may become somewhat accustomed to distinct intensities of light, the level of light sensitivity varies with species.
3. *Minimizing space requirements/controlling cultivation conditions*:
   Open cultivation systems are located outdoors and they totally rely on sunlight or natural light. As the open systems are less expensive, they have many disadvantages. Due to the open cultivation system, culture can get easily contaminated and maybe out-compete with the undesired species of algae (Oncel and Vardar Sukan 2008). During past times, algae was grown or cultivated in natural waters or artificial ponds, more recently closed photobioreactors have been introduced and used.

   On the other hand, closed photobioreactors are being used to cultivate photosynthetic microorganisms such as cyanobacteria, microalgae, plant cells, and photosynthetic bacteria for different research purposes as well as for biotechnology and food applications. Illumination is achieved artificially or naturally for the production of microalgae. The algal cultivation systems with naturally illuminated large areas include open ponds, flat-plate, horizontal/serpentine tubular airlift, and inclined tubular photobioreactor. Laboratory-scale photobioreactors are typically artificially lit with fluorescent, ultraviolet, or other forms of lamps. Closed reactors may be installed indoors or outdoors, and provide better and more regulated culture conditions. Major disadvantage associated with photobioreactors is that, they are expensive, but the quality and quantity of the product are better than open pond cultivation system. Intensive research is going on to make closed reactors cost-effective (Oncel and Vardar Sukan 2008).
4. *Efficient provision of carbon dioxide*:
   The adequate absorption of CO_2 for algal cultivation is another significant challenge. Algae are photo-autotrophs which use carbon dioxide as a source of carbon during photosynthesis, which may help mitigate the environment's CO_2 emissions and reduce global warming. Enhancing the cost-effective mass transmission of CO_2 to cells in a aqueous environment is not a trivial problem for large-scale aquatic production systems as required for outdoor algal growing. Moreover, *Spirulina* is grown using photosynthesis process, presence of reduced carbon can lead to the faster growth of microbes which affects the growth of *Spirulina*. Subsequently, the distribution of carbon to *Spirulina* mass cultivation

systems poses a significant problem and limits the cost-effectiveness of *Spirulina* biomass production (Oncel and Vardar Sukan 2008)

5. *Problems in scaling up*:
   Upgrading from small to large is a dynamic activity that involves knowledge and expertise. Maintaining stability, higher biomass productivity, uncontrolled outdoor or natural conditions of variable illumination, different climatic contidions with varying temperatures are major challenges to overcome, as these are not much expertise for small scale or laboratory cultivation conditions.

9.8 Environmental Benefits of *Spirulina*

Spirulina, the superfood not only does wonder to the human body but it also tremendously helps the environment. It is known to be the best and effortlessly grown nutritious food and the richest proteinous food. The environmental benefits of *Spirulina* to plants are:

- *Conservation of soil*: The organic cultivation of *Spirulina* can be done on land areas where other crops hardly grow. It also helps in soil erosion. *Spirulina* is most proteinous food. Because of its faster growth rate it doubles itself in 24 h. In comparison to soybeans, it provides about 20 times more protein per acre and 40 times more protein than corn, and sometimes even 200 times more than beef. *Spirulina* can be cultivated easily in saline conditions and does not require fertile land area (https://www.persistencemarketresearch.com/market-research/spirulina-market.asp).
- Efficient water use: *Spirulina* requires less quantity of water when compared with crops. The water used for harvesting of *Spirulina* can be recycled. Major water loss is in summer due to evaporation only. *Spirulina* needs only 25% of water as that of soya, 17% of corn and 2% that of beef.
- *Good oxygen generator*: *Spirulina* recycles around 6.4 tons of CO_2 per hectare annually, and the trees recycle around 1–4 tons of CO_2 per hectare per year in comparison (Schreiber et al. 2017). *Spirulina* also produces about 16.8 tons of oxygen. The carbon dioxide fixation in blue-green algae follows higher plant C3-metabolism. The carbon dioxide fixation Calvin cycle is operational in *Spirulina* sp. A significant amount of RUBISCO (ribulose bisphosphate carboxylase/oxygenase) is also present in most species of *Spirulina*, which reflects the low affinity to CO_2.
- *High yield*: Like many other microorganisms, in comparison with higher plants, *Spirulina* has a greater specific growth rate (Sharma et al. 2012). With a protein content of about 60–65%, the greater development of *Spirulina* implies providing approximately 20 times additional protein per unit area than soybeans, 40 times more than maize, and even 200 times more than beef. Versatile food products—*Spirulina* is becoming a major food product with high protein content worldwide. More than 50 products have been commercialized. Mostly powder, capsules, and tablet forms are prefered. *Spirulina* is also being used in salads, drinks, cereals,

chocolates, cookuies, cakes, etc., where it is basically used to increase the protein content.

9.9 Conclusion

This chapter indicates that, despite its extensive publicity on health advantages, microalgae *Spirulina* has not yet received severe consideration. The following are the main findings:

- In coastal and alkaline fields where conventional agriculture is struggling, *Spirulina* can be a prospective main product, particularly as salinity and water scarcity have increased impact. The role of domestic governments and inter-governmental organizations is to reconsider the capacity of *Spirulina* to meet both its own food security requirements, as well as a resource for its overseas growth and emergency response initiatives.
- International organizations continuing to work with *Spirulina* should start implementing a functional manual for the manufacture of small-scale *Spirulina*, which could be used as a reason for increasing and advancing techniques. Small-scale production should be aimed at providing nutritional and dietary supplements for extensive use in rural and urban communities or regions with poor or inadequate staple diets.
- New lighting technologies and their control and scale-up are anticipated to make closed culture systems cheaper, more controllable, and more effective. *Spirulina*'s future looks bright, and creative technologies and products are expected to emerge in the coming years.

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Fucoxanthin Production from Diatoms: Current Advances and Challenges

10

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Abstract

Increasing public attention to health issues and disease prevention has created a favorable global market for naturally derived nutraceuticals. This has led to a dramatic increase in demand for carotenoids in general and fucoxanthin (FX) in particular. This orange-colored compound has an array of health stimulating properties, including antioxidant, anticancer, anti-obesity, and anti-diabetic. It is currently isolated from seaweeds, but fast-growing diatoms, a class of microalgae, synthesize FX at higher levels, making them a promising candidate for sustainable FX production. Still, to produce diatom FX cost-effectively at large scale, significant improvements in productivity are quintessential. In the present chapter we provide an overview of FX biosynthesis by diatoms and the effect of various abiotic growth factors on FX production. Eventual commercial deployment of diatoms will depend on genetically constructing superior FX producing strains and optimizing the diatom cultivation conditions.

Keywords

Fucoxanthin · Diatoms · Abiotic factors · Genetic engineering

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10.1 Introduction

The new health paradigm towards well-being and disease prevention has led to an expansion of naturally derived compounds for the nutraceuticals and functional foods industry. Nutraceuticals have dual benefits of nutritional and pharmaceutical value and are intended to provide long-term health benefits, thereby improving a person's quality of life. Among nutraceuticals, carotenoids are a rapidly emerging class that is naturally synthesized by algae, plants, yeasts, and bacteria (Mikami and Hosokawa 2013). Carotenoids (tetraterpenes) are pigment compounds with a characteristic eight isoprene (5-carbon) unit creating a linear 40 carbon backbone with up to 11 conjugated double bonds (Novoveská et al. 2019). Based on the presence of oxygen in their molecular structure, carotenoids are divided into two main categories: (1) Carotenes made of carbon and hydrogen, including α/β carotene and lycopene; and (2) Xanthophylls, which are oxygenated carotenes, such as fucoxanthin, astaxanthin, zeaxanthin, and lutein (Mohamadnia et al. 2019). In algae, xanthophylls are further classified as primary xanthophylls, which are located in the chloroplasts, and secondary xanthophylls, which are located in lipid vesicles inside either the plastid or cytosol (Bauer et al. 2019). The primary xanthophylls serve as structural and functional components of the algal cell photosynthetic apparatus with light harvesting antennas, whereas the secondary xanthophylls, including fucoxanthin, are metabolites synthesized by the algal cells in response to environmental stressors, such as nutrient depletion, light intensity, temperature, and salinity (Mohamadnia et al. 2019).

Among xanthophylls, fucoxanthin is a pro-vitamin A (can be converted to vitamin A by the human body) carotenoid with reportedly numerous biological activities and health stimulating properties, including antioxidant, anti-inflammatory, cardiovascular, anti-obesity, anti-diabetic, anti-angiogenic, and anti-malarial activity (Mohamadnia et al. 2019; Aslanbay Guler et al. 2020). It has also demonstrated protective effects against dermal, ophthalmic, bone, cerebrovascular, and cardiovascular disorders and is even consumed as a dietary supplement for weight loss (McClure et al. 2018). Moreover, it has been reported to exhibit broad anticancer and anti-proliferative activity in leukemic (H-60), epithelial colorectal adenocarcinoma, prostate cancer, urinary bladder cancer, breast cancer, lung cancer, and gastric cancer cell lines (Karpiński and Adamczak 2019). These biological activities of FX are due to its unique molecular structure that includes an allenic bond, a conjugated carbonyl with a 5,6-momoepoxide, and an acetyl group (Mohamadnia et al. 2019). Thanks to the broad spectrum of health benefits of fucoxanthin (FX), the global FX market is expected to increase from $88 million in 2019 to over $100 million over the next 5 years (Report, Global Fucoxanthin Market 2020).

In nature, FX is the orange-colored pigment found in Chromophyta (Heterokonphyta or Ochrophyta), including brown seaweeds (Phaeophyceae) and diatoms (Bacillarophyta) (Peng et al. 2011). Presently, FX is commercially produced from the brown macroalgae (seaweeds) *Saccharina japonica*, *Undaria pinnatifda*, *Sargassum fusiforme,* and *Eisenia bicyclis,* which contain 0.1–1.0 mg of FX per

gram of macroalgal mass (Bauer et al. 2019; Sahin et al. 2019). However, brown seaweeds cannot meet the global demand for FX due to their slow growth, low yield, need for cell growth regulators, and quality concerns associated with heavy metal contamination of the oceans (Gómez-Loredo et al. 2016; Guo et al. 2016). As a result, diatoms are seen as far more promising organisms for the production of FX, since they yield 2.0–26.6 mg/g of FX, which is more than 10× higher than brown seaweeds, have shorter doubling time, can be cultivated in closed and controlled systems free of heavy metals, and are not affected by seasonal variations unlike seaweeds (Bauer et al. 2019).

The present chapter analyzes the studies reported to date on the production of FX from diatoms with emphasis on the effects that key abiotic factors, like light intensity, nutrient availability, and carbon sources, have on production of the pigment. Moreover, we review efforts to improve FX production in diatoms via genetic engineering of these algae.

10.2 Fucoxanthin Biosynthesis in Diatoms

Diatoms encompass a taxonomic group of about 200,000 different species responsible for an astounding 40% of natural carbon fixation via photosynthesis (Ikeda et al. 2008). They are the major ecological players in the biogeochemical cycling of carbon, nitrogen, and silicon (Depauw et al. 2012). Since their first appearance approximately 180 million years ago as a result of endosymbiosis of red algae and heterotrophic flagellates, they inhabit both marine and freshwater ecosystems (Veith et al. 2009; Zulu et al. 2018). Diatoms belong to the Stramenopile and Heterokont phyla under the Bacillariophyceae class (Zulu et al. 2018). Their most extraordinary feature is their ability to precipitate soluble silicic acid and incorporate it into a highly patterned cell wall (frustule) composed of silica, $(SiO_2)_n(H_2O)$, organized as nanostructured valves (Siaut et al. 2007; Veith and Büchel 2007; Baldisserotto et al. 2019). Compared to plants, they possess additional genes and a complex plastid with four membranes instead of two (Zulu et al. 2018).

Based on their frustule morphology, diatoms can be broadly classified into pennate (fresh water), radial centric (marine with circular valves), and bipolar centric (marine with bipolar valves) (Baldisserotto et al. 2019). Furthermore, although the photosynthetic apparatus of diatoms resembles that of higher plants and cyanobacteria, it has distinct thylakoids, which are not divided into discrete grana and stroma lamellae, but are instead organized into bands containing three thylakoids each with no distinction between photosystem I (PSI) and photosystem II (PS II) (Veith and Büchel 2007). Whereas in plants the core of PSII consists of 25 subunits surrounded by membrane proteins referred to as light harvesting centers (LHC), in diatoms PSII consists of fucoxanthin-chlorophyll (a and c)proteins (FCPs) responsible for harvesting sunlight and conducting energy transfer during photosynthesis (Büchel 2003; Bauer et al. 2019). FX absorbs visible light at 450–570 nm, which is blue green to yellow green with maximum absorbance between 510 and 525 nm, giving the diatoms their characteristic golden-brown color (Peng et al. 2011;

Telussa et al. 2019). Three different classes of FCP genes have been characterized to date in diatoms: (1) fcp1-5 in *Cyclotella cryptica* and fcpA-F in *Phaeodactylum tricornutum*; (2) fcp4 in *C. cryptica* and a homologue gene in *Thalassiosira pseudonana*; and (3) i818 in *Chlamydomonas reinhardtii* and fcp6, 7, and 12 in *C. cryptic* (Veith and Büchel 2007). Among the diatoms, only a few genera have been studied to date, including *Thalassiosira*, *Chaetoccros*, *Coscinodiscus*, *Skeletonema*, *Phaeodactylum, Nitzcia,* and *Cyclotella* (Baldisserotto et al. 2019). However, only two of them, *P. tricornutum* (pennate) and *T. pseudonana* (centric), have been completely sequenced.

The biosynthesis of FX in the diatom *P. tricornutum* reportedly occurs via three different putative pathways: (1) the violaxanthin (Vlx) cycle; (2) the diadinoxanthin (Ddx) cycle; and (3) the β-cryptoxanthin cycle (Fig. 10.1). *P. tricornutum* utilizes the methylerythritol-4-phosphate (MEP) plastidic pathway, also known as the non-mevalonate pathway, for isoprenoid biosynthesis (Bauer et al. 2019). The pathway starts with the formation of 1-deoxy-D-xylulose 5-phosphate from glucose-derived glyceraldehyde-3-phosphate, catalyzed by 1-deoxy-D-xylulose 5-phosphate synthase (DXS). This is followed by the formation of isopentenyl diphosphate (IPP) and dimethyl allyl diphosphate (DMAPP). Then, the formation of geranylgeranyl phosphate (GGP) initiates biosynthesis of β-carotene, which is the starting molecule for all the three hypothesized pathways for FX biosynthesis. Phytoene synthase (psy) catalyzes the conversion of GGP to phytoene, followed by desaturation of phytoene to ξ-carotene using phytoene desaturase (pds). Then, ξ-carotene desaturase (zds) catalyzes the formation of prolycopene followed by conversion to lycopene using lycopene β-cyclase and then to β-carotene (β-Car), which is the actual precursor molecule for FX biosynthesis, as depicted in Fig. 10.1 (Depauw et al. 2012).

The Violaxanthin (Vlx) cycle starts with the sequential conversion of β-Carotene to Zeaxanthin (Zea), followed by reversible conversion to Antheraxanthin (Atx) and Violaxanthin (Vlx) with both reactions being catalyzed by Zeaxanthin epoxide (ZEP). Vlx is subsequently converted to Neoxanthin (Nex) and then either to FX or to Diadinoxanthin (Ddx) (Cui et al. 2019). The Ddx cycle follows the same pathway as the Vlx cycle, i.e. β-Car→Zea→Atx→Vlx. Subsequently, Vlx gets converted to Ddx, which can then be converted to FX or diatoxanthin (Dtx). The third pathway results in the conversion of β-Car to β-cryptoxanthin (β-Cpx) and then to β-cryptoxanthin 5, 6-epoxide followed by conversion to Vlx, thereby resulting in the formation of Ddx, which in turn is converted to FX or Dtx (Cui et al. 2019). However, these pathways have not be fully elucidated or experimentally validated yet due to missing information and lack of characterization of several enzymes involved in the conversion of intermediate products, as depicted in Fig. 10.1.

10.3 Abiotic Factors Affecting Fucoxanthin Production

The cultivation conditions significantly impact the growth characteristics and biochemical composition of diatoms. Important abiotic factors are light intensity and wavelength, nutrient depletion/deprivation, and carbon utilization mode

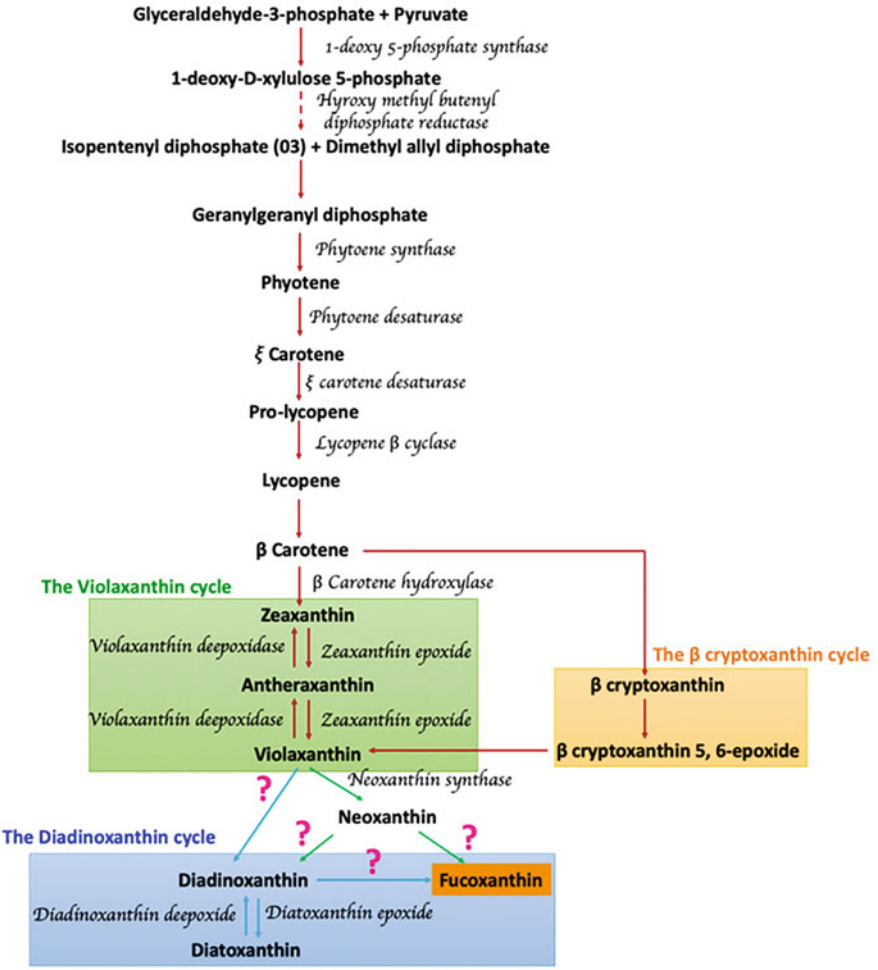

Fig. 10.1 Schematic of fucoxanthin biosynthesis paths in diatoms. The question marks signify unidentified enzymes

(autotrophic, heterotrophic or mixotrophic). Other factors include temperature, agitation speed, salinity, and mode of cultivation (batch, continuous or fed batch). Carotenoids in diatoms are potent antioxidant molecules that are synthesized in large quantities during any environmental stress in order to enable diatom cells to quench the formation of reactive oxygen species (ROS) and re-establish cellular homeostasis (De Jesus Raposo et al. 2015). To this end, various abiotic stress strategies have been utilized to augment FX production in a range of diatoms (Table 10.1).

Since FX is involved in light harvesting and photoprotection of diatoms, light intensity and spectra significantly impact its biosynthesis. It is under low light

Table 10.1 Effect of abiotic growth factors on biomass and fucoxanthin production by diatom strains

Diatom	Media composition	Abiotic factor	Culture conditions	PBR scale (L)	Dry cell weight (g/L)	Fucoxanthin (mg/g)	References
Isochrysis zhangjiangensis	F/2	Light (40 μmol photons m^{-2} s^{-1})	Bubble column PBR, 14 h/10 h (light/dark cycle), 25 °C	0.7	2.4	23.29	Li et al. (2019)
Isochrysis sp.		Nitrogen (100 mg/L)	Flask, 20 μmol photons m^{-2} s^{-1}, continuous light, 22 °C	–	3	17.5	Sun et al. (2019a)
		Phosphorous (4.50 mg/L)			2.75	15	
		Light (30 μmol photons m^{-2} s^{-1})	Flask, continuous light, 22 °C		2.15	13.5	
Isochrysis galbana		Light (13.5 μmol photons m^{-2} s^{-1}) + Aerated culture	Flask, continuous light, 25 °C		–	0.17	Gómez-Loredo et al. (2016))
	Conway media				–	0.26	
Nitzschia laevis	F/2 + glucose (5 g/L)	Mixotrophic	Flask, 15 μmol photons m^{-2} s^{-1}, continuous light, 22 °C	–	2.34	15.6	Lu et al. (2019)
		Heterotrophic			2.31	10	
	LDM + glucose (5 g/L)	Mixotrophic	Flask, 10 μmol photons m^{-2} s^{-1}, continuous light, 23 °C	–	1.91	11.1	Lu et al. (2018)
Nitzschia closterium	F/2	UVA365 (3 days)	Flask, 54.80 μmol photons m^{-2} s^{-1}, 18 h/6 h (light/dark cycle), 22 °C	–	–	2.96	Huang et al. (2018)
Nitzschia sp.		Nitrogen rich (2 g/L)	Flask, 140 μmol photons m^{-2} s^{-1}, continuous light, 20 °C	–	2.66	7.83	Sahin et al. (2019)
		Iron rich (0.0158 g/L)			1.868	6.16	
Nanofrustulum shiloi	BG-11	Nitrogen rich (2 g/L)			3.03	5.71	
		Nitrogen deficient (0 g/L)				3.12	
		Iron rich (0.0158 g/L)			2.42	7.83	
		Iron deficient (0 g/L)				5.23	

Odontella aurita	L1	Light (150 µmol photons m^{-2} s^{-1}) + low nitrogen (6 mM)	Glass PBR, continuous light, 22 °C, 1% CO_2	75	3.95	5	Xia et al. (2018)
		Light (300 µmol photons m^{-2} s^{-1}) + high nitrogen (18 mM)			5.84	12.5	
		Light (150 µmol photons m^{-2} s^{-1}) + high nitrogen + supplementary nitrogen (36 mM)			4.13	25	
		Nitrogen replete (18 mM) + high light (300 µmol photons m^{-2} s^{-1})	Flask, continuous light, 22 °C	–	6.36	20.83	Xia et al. (2013)
		Nitrogen replete (18 mM) + low light (100 µmol photons m^{-2} s^{-1})			4.26	16.71	
Phaeodactylum tricornutum	F/2	Light (100 µmol photons m^{-2} s^{-1})	Flask, continuous light, 20 °C, 1% CO_2	–	0.23	42.8	McClure et al. (2018)
		Light (150 µmol photons m^{-2} s^{-1})			0.29	23.2	
		10× F/2			0.59	26.7	
		10× nitrogen			0.37	59.2	
		2% CO_2			0.17	21.1	
		Airlift PBR	65 µmol photons m^{-2} s^{-1}, continuous light, 22 °C	7	1.93	1.4	Aslanbay Guler et al. (2019)
		Flat panel PBT			3.03	2.42	
		Stirred tank PBR			2.09	0.82	
		Iron rich (10 µM)	Flask, continuous light, 22 °C	–	–	40.39 fgcell^{-1}	Kosakowska et al. (2004)
	Spent yeast	–			1	5.97 mg/L	Yuan et al. (2019)

(continued)

Table 10.1 (continued)

Diatom	Media composition	Abiotic factor	Culture conditions	PBR scale (L)	Dry cell weight (g/L)	Fucoxanthin (mg/g)	References
	Palm oil effluent (POME)-30%	Medium light (100–200 µmol photons m^{-2} s^{-1}) + temperature (20 °C)	Flask, 16 h/8 h (light/dark cycle)	–	–	700 µg/L/D	Nur et al. (2019)
	F/2	Light intensity (30 µmol photons m^{-2} s^{-1})	PBR, continuous light, 23 °C	20	1.56	7.5	Wang et al. (2018)
		Nitrogen (300 mg/L)	PBR, 70 µmol photons m^{-2} s^{-1}, continuous light, 23 °C		1.61	5.4	
		Salinity (20%)			1.49	7.4	
Cylindrotheca fusiformis		Light intensity (30 µmol photons m^{-2} s^{-1})	PBR, continuous light, 23 °C		1.38	6.5	
		Nitrogen (300 mg/L)	PBR, 70 µmol photons m^{-2} s^{-1}, continuous light, 23 °C		1.52	6.1	
		Salinity (30%)			1.64	6.3	
Navicula sp.	Sea water	Nitrogen source (nitrate)	Outdoor, maximum light 2700 µmol photons m^{-2} s^{-1}, 21–35 °C	–	–	2.61	Telussa et al. (2019)
Cyclotella cryptica	SK media + glucose	Heterotrophic	Flask, continuous light, 22 °C	–	0.3	7.7	Guo et al. (2016)
		Nitrogen (1 g/L) + light (30 µmol photons m^{-2} s^{-1})			1.72	11.62	
		Nitrogen depleted + light (30 µmol photons m^{-2} s^{-1})			0.9	0.6	
		Nitrogen (1 g/L) + dark			1.0	0.8	
		Nitrogen depleted + dark			0.7	0.5	

intensity that biosynthesis of FX is activated in order to help the cellular metabolism capture and transport photon energy as efficiently as possible (Nymark et al. 2009). FX binds to specific proteins present in the photosynthetic apparatus (Li et al. 2019). In contrast, under high light intensity, the FCPs become supersaturated, resulting in photoinhibition and oxidative stress (Nymark et al. 2009). This stimulates synthesis of secondary carotenoids, such as astaxanthin and β-carotene, at the expense of FX through modulation of the Ddx cycle (Fig. 10.1). Hence, low light intensity is favorable to FX production, but the exact intensity for maximum FX production varies among diatom species. In *P. tricornutum,* maximum FX yield (productivity) of 42.8 mg/g was reported when the culture was illuminated with 100 μmol photons m^{-2} s^{-1}, whereas at 150 μmol photons m^{-2} s^{-1} FX productivity dropped to half (23.2 mg/g) (Table 10.1).

Although cultivation at low light intensity increases FX yield, it slows down the overall metabolism and growth of diatoms reducing biomass productivity and hence resulting in a trade-off between biomass production (cell growth) and FX biosynthesis. For diatoms to serve at large scale as a viable source for commercial FX production, the use of outdoor cultivation systems will be imperative from a process and economic standpoint, necessitating the use of diatom strains that can synthesize high levels of FX under natural high light intensities. One such promising strain is *Odontella aurita,* which produced 12.5 mg/g of FX when cultivated under 300 μmol photons m^{-2} s^{-1}at a peak biomass concentration of 5.84 g/L (Li et al. 2019).

In addition to light intensity, nitrogen also has a major effect on FX yield of diatoms, as nitrogen is an essential nutrient involved in the biosynthesis and regulation of cellular metabolites (Guo et al. 2016). In algae, including diatoms, nitrogen is assimilated in the form of ammonium (NH_4^+) by glutamate synthase/glutamine synthetase leading to formation of glutamate, which is the precursor for amino acids and chlorophyll synthesis (Alipanah et al. 2018). The genomes of both *P. tricornutum* and *T. pseudonana* contain transporter proteins for the uptake of inorganic and organic nitrogen compounds. The most common nitrogen source used for the cultivation of diatoms is nitrate, which is first reduced to nitrite by nitrate reductase and is then converted to ammonium by nitrite reductase (Arora et al. 2019).

A directly proportional relationship has been established between nitrogen availability and FX production in diatoms, as increasing the nitrogen concentration in the growth media resulted in enhanced FX productivity (Table 10.1). This could be due to the upregulation of chlorophyll biosynthesis in high nitrogen presence, thereby also promoting FX production (Guo et al. 2016). When the effect of nitrogen depleted (0 g/L) and nitrogen-rich media (2 g/L) was studied with two diatom strains, *Nitzschia* sp. and *Nanofrustulum shiloi, Nitzschia* sp. was not able to grow under nitrogen depleted conditions, while *N. shiloi* grew, but both its biomass production and FX yield were significantly lower than in nitrogen-rich media (Sahin et al. 2019). Moreover, the combined effect of nitrogen and light intensity has been studied in various diatom strains as listed in Table 10.1. In *Odontella aurita*, five different combinations of light and nitrogen were studied: low light with low nitrogen, high light with high nitrogen, and high light with high nitrogen and

supplementary nitrogen (Table 10.1). Among these combinations, the latter with supplementary nitrogen resulted in maximum FX productivity of 25 mg/g (Xia et al. 2018). These results confirm that continuous supply of nitrogen boosts FX biosynthesis in diatoms and should be used at large scale to achieve high FX yields.

Besides nitrogen, phosphorous and iron may also have an effect on FX production as they are essential components of the photosynthetic and cellular mechanisms of diatoms (Table 10.1). Phosphorous is an important nutrient aiding in nitrate absorption, photosynthetic respiration, energy transfer, signal transduction, and biosynthesis of nucleic acids, lipids, and other metabolites (Arora et al. 2015; Alipanah et al. 2018). To date, only one study has studied the effect of phosphorous on FX production in *Isochrysis* sp. (Sun et al. 2019b). The authors reported no significant changes in biomass or FX production irrespective of phosphate concentration in the growth media (1.13, 2.25, and 4.50 mg/L) (Table 10.1). However, this could be a species-specific response and more detailed studies are imperative to establish the possible effect of phosphorous on FX biosynthesis in other diatom species.

Iron (Fe) is responsible for the biosynthesis of a protoporphyrin precursor, δ-aminolevuline acid, which is involved in chlorophyll synthesis (Kosakowska et al. 2004). Furthermore, iron is a component of both cytochromes b and c, the electron transport chain (ferrodoxine), photorespiration, enzymes involved in nitrogen assimilation, and activators of peroxidase and catalase (Kosakowska et al. 2004).Similar to nitrogen, deficiency in Fe led to a decrease in both biomass and FX production in *Nitzschia* sp. and *N. shiloi* (Sahin et al. 2019). Iron deficiency appears to result in photooxidation and activation of a photoprotective cycle triggering the biosynthesis of secondary carotenoids and Dtx, as opposed to FX and light harvesting pigments.

Another important factor in FX production is the mode of carbon utilization by diatoms. Diatom growth can be autotrophic (assimilation of inorganic carbon (CO_2) + light), heterotrophic (utilization of organic carbon + dark), and mixotrophic (uptake of both CO_2 and organic carbon + light). The preferable mode for maximum cell growth and FX production is species-specific, but overall it appears that algae grown mixotrophically achieve higher biomass productivity, since photorespiration does not impact growth. Cultivation of *Nitzschia laevis* under mixotrophic conditions resulted in higher biomass and FX yield as compared to heterotrophic conditions (Table 10.1). Interestingly, *C. cryptica* could grow heterotrophically with similar FX production, 7.7 mg/g, as autotrophically (Guo et al. 2016). However, when the authors also explored the synergistic effect of nitrogen and heterotrophy, higher FX productivity was achieved in the nitrogen-rich media under light conditions indicating that for this particular strain concentration of nitrogen is more crucial than carbon utilization mode for FX production (Table 10.1).

In order to reduce growth media cost, some new low-cost media were recently tested with diatoms in an attempt to improve the economics of future scale up and commercial production of FX (Table 10.1). The list of media included palm oil mill effluent, sea water, and spent yeast cell hydrolysate (Ishika et al. 2019; Nur et al. 2019; Yuan et al. 2019). Unfortunately, none of these media was able to match the

FX productivity obtained when diatoms were cultivated in the costlier standard media F/2.

10.4 Genetic Engineering Strategies to Improve Fucoxanthin Productivity

Among the numerous diatom strains producing FX, only a few have been successfully genetically engineered, including *P. tricornutum*, *Cylindrotheca fusiformis*, *C. cryptica*, *Navicula saprophila*, *Fistulifera solaris*, *T. pseudonana,* and *Halamphora coffeaeformis* (Poulsen and Kröger 2005; Velmurugan and Deka 2018). Furthermore, although there is extensive literature on the overproduction of lipids from *P. tricornutum*, very few studies have attempted to specifically enhance FX production (Lavaud et al. 2012; Kadono et al. 2015; Eilers et al. 2016). In order to successfully introduce heterologous (foreign DNA) or overexpress the endogenous genes in the genome of diatoms, the development of an appropriate genetic toolbox is quintessential (Huang and Daboussi 2017). Such a toolbox should include: (1) an expression vector system with all the essential elements, namely promoters, ribosome binding sites, terminators, and 5′ UTR (untranslated region) and 3′ UTR sites; (2) selectable markers for isolation and identification of transformed cells; and (3) efficient transformation techniques for DNA delivery, homologous recombination, and strain stability.

Both constitutive and inducible promoters have been identified and tested for the overexpression of desired genes in diatoms. To date, various endogenous constitutive promoters have been identified in diatoms, such as the promoters associated with the expression of numerous genes, including the light harvesting complex protein (lhcf 1-15), the histone gene (h4), the elongation factor 2 (ef2) gene, the ammonium transporter (amt) gene, and the purine permease (pup) and diacylglycerol acyltransferase (dgat1) genes (Huang and Daboussi 2017; Adler-Agnon (Shemesh) et al. 2018; Watanabe et al. 2018). The Lhcf promoters, in particular, have been widely used for overexpression of various genes in several diatom strains, but their dependence on light availability makes them unsuitable for FX gene expression under dark conditions (Huang and Daboussi 2017). On the other hand, although the h4 promoter is light independent, it yields low levels of expression compared to the Lchf promoters. The ef2 promoter does not require light and has proven to be effective in terms of expression (1.2 fold higher than Lchf2) (Huang and Daboussi 2017).

Endogenous inducible promoters include those for nitrate reductase (nr), iron starvation induced protein 1 (Isi 1), ferrichrome binding protein 1(fbp1), flavodixin (fld genes), and a CO_2 responsive promoter derived from the carbonic anhydrase gene (ca1) (Huang and Daboussi 2017). Although inducible promoters offer the advantage of controlled gene expression, their use at industrial scale is problematic because of operational issues (extensive cleanliness needed to remove any trace metals that could untimely induce the promoters) and the need to cultivate the diatoms in inducer-free media, which may generate stress on the cells. Unlike the

plethora of promoters, only a few endogenous terminators have been identified in diatoms, namely Lhcf1, Lhcf9, Lhcr14, nr, and rubisco small subunit (rbcL) (Huang and Daboussi 2017).

The genetic transformation methods available for diatoms include biolistic, electroporation, and conjugation (Bozarth et al. 2009). Among them, the biolistic method is the most successful one achieving ~90% transformation efficiency in the chromosome of diatoms, when using Zeocin as selection marker (Velmurugan and Deka 2018). Apart from the cell nucleus, chloroplast transformation has also been successfully reported in *P. tricornutum,* particularly for overexpressing genes of prokaryotic origin (Bozarth et al. 2009).

In another genetic attempt to enhance FX production, the violaxanthin De-epoxide gene (VDE) was silenced in *P. tricornutum* (Lavaud et al. 2012). The VDE gene catalyzes the de-epoxidation of violaxanthin (Vlx) to Zeaxanthin (Zea) and then its subsequent conversion to Dtx, thereby channelling the flux of Zea eventually to FX (Fig. 10.1). However, this knockdown did not result in overproduction of FX, possibly due to the presence of multiple pathways for FX production, as outlined earlier (Lavaud et al. 2012). In another study, the psy gene obtained from *P. tricornutum* and dxs1 gene obtained from corn were overexpressed in *P. tricornutum* (Eilers et al. 2016). The authors reported an increase in FX yield to 24.2 mg/g in the psy transformed cells and 18.4 mg/g in dxs1 transformed lines, as compared to the wild type (10 mg/g). In contrast, when the psy gene was overexpressed in *P. tricornutum*, no FX augmentation was observed (Kadono et al. 2015).

10.5 Conclusion and Future Perspectives

The health-boosting benefits of FX supplementation in several preclinical and a few clinical studies have significantly increased its demand worldwide. Among the potential sources for FX production, diatoms represent one of the most promising groups of microorganisms that could be commercially exploited thanks to their fast growth rate, high FX yield, and ability to grow in enclosed controlled systems compared to currently used seaweeds. Indicative of the diatom potential is the recent expansion of commercial FX production from *P. tricornutum* by an Israeli biotechnology company that cultivates the diatom in closed photobioreactors under natural light conditions to meet global demand (algatech.com). Over the last few years, researchers have filled crucial gaps in diatom physiology and FX biosynthesis. Nevertheless, there is still (1) from a genetic standpoint, a need for full elucidation of the carotenoid and FX biosynthesis mechanisms in diatoms, and (2) from a process engineering standpoint, a need for better understanding of the effect of additional abiotic factors, such as temperature, inoculum size, bioreactor operation, and salinity, on FX production. All this knowledge is imperative to propel diatom-based FX production from lab scale to commercial scale.

Although there are about 200,000 diatom species, identification of efficient FX producing strains and optimization of nutrient and abiotic parameters remain a big

challenge. Progress on those issues can be achieved by screening and down-selecting more diatom strains possessing high growth rate, high FX yield, resistance to algal predators, ability to grow in saline water or wastewater, and ability to easily flocculate for cost-effective downstream processing and extraction and purification of FX. It is rather unlikely that a single diatom strain will naturally possess all these characteristics, hence genetic engineering tools will be required to further enhance promising natural strains. Recent advances in genetics, such as suppression/knock-down of target genes using RNAi silencing, CRISPR-Cas9, and transcription activator-like effect or nuclease, present a great opportunity to design improved diatoms. However, such tools can be best used once there is in-depth understanding of carotenoid and FX biosynthesis, which can benefit from the integration of OMICS technologies, such as genomics, transcriptomics, proteomics, metabolomics, and fluxomics. In parallel with genetic engineering approaches, we recommend the use of genome modification techniques, such as chemical mutations and adaptive laboratory evolution (ALE), to select for high FX yield diatoms among both natural and genetically modified strains.

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Liquid Biofuels from Algae

11

Devinder Singh and Giovanna Gonzales-Calienes

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Abstract

Extensive uses of fossil fuels are posing several threats to the environment as well as human health. They have been used in such a way that in coming few decades, the finite sources of fossil fuels will be completely exhausted. Algae are one of the most primitive microorganisms on the Earth. They are small photosynthetic organisms that have an ability to completely replace the need of conventional fossil fuel for energy demand. They are robust microorganisms and can be grown

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in photo-bioreactors, open ponds, sewage or industrial waste without the need of arable land. Microalgal biomass can be converted to variety of biofuels via biochemical and thermochemical methods, they can also be used for the production of high value nutraceuticals at industrial scale. The present chapter deals with the various conversion technologies of algal biomass to biofuel, resource requirements, research gaps and operational costs associated with it.

Keywords

Algae · Biofuel · Transesterification · Lipids

11.1 Introduction

Some of the earliest commercial applications of algae were in the areas of high value nutraceuticals and health food additives like β-carotine, astaxanthin, polyunsaturated fatty acids, etc. For these applications microalgae like *Chlorella vulgaris*, *Arthrospira*, *Haematococcus pluvialis* have been produced commercially since the 1960s–1980s by companies like Cyanotech Corp. (USA), Japan Algae Co., Ltd, Parry Nutraceuticals (India), etc. (Spolaore et al. 2006). Algae as a source of biofuels started gaining considerable attention in the late 1970s due to its promising attributes compared to terrestrial sources of biomass:

- Faster growth rates than terrestrial plants. Microalgae double their biomass in 24 h, and in usually as low as 3.5 h during exponential growth (Chisti 2007). This when combined with high lipid content of microalgae gives the productivity of biodiesel at around 52,000–86,000 kg of biodiesel produced per hectare of land mass per year (Fig. 11.1), higher by a factor of 100 compared to oleaginous crops like soybean, camelina, etc.
- The use of arable land for biofuels in some cases has been associated with food insecurities and increased greenhouse gases caused by indirect land use change

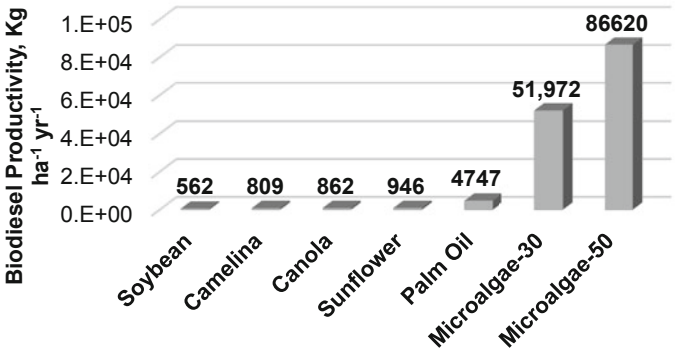

Fig. 11.1 Comparison of biodiesel productivities of oleaginous crops with microalgae (30% and 50% lipid content) (Chisti 2007; Mata et al. 2010)

Table 11.1 World production and price of high value products from algae compared to the production and value of transportation fuels (IEA 2019; Spolaore et al. 2006; Kalghatgi 2016).

	World production (tons year^{-1})	Price (USD kg^{-1})
High value products from algae		
B-carotene	1200	300–3000
Astaxanthin	300	2500
Algae derived poly unsaturated fatty acids		80–60
Dried microalgae chlorella	2000	
	World production (1000 L year^{-1})	**Price (USD L^{-1})**
Demand for transportation fuels (2015)		
Gasoline	1.04E+09	~0.3–1.9 (gasoline)
Diesel/gas oil	1.37E+09	
Jet fuel/kerosene	3.34E+08	

effects (Searchinger et al. 2008; Robertson et al. 2017). Microalgae can grow on land not suitable for agriculture and would alleviate these concerns.
- Algae can grow on brackish or wastewater not suitable for agriculture. It has also been shown to remediate wastewaters, as demonstrated in various studies (Venkata Mohan et al. 2015; Li et al. 2019).

These attributes prompted United States Department of Energy to fund Aquatic Species Program (from 1978 to 1996) for the production of biodiesel from algae using carbon dioxide form point source emissions like coal fired power plants. Similar projects were funded in Japan during the 1990s. However, the shift from algae as a source of high value, low volume health product market with total volume of around few thousand tonnes per year to a transportation fuel market with a total demand six orders of magnitude higher and average selling price in the range 0.3–1.9 USD L^{-1} (see Table 11.1) has posed numerous challenges and constraints for the production of biofuels from algae:

- The technologies chosen should be sustainable with a net favourable energy balance. Although commercial technologies are available for the transformation of bio-feedstocks and bio-oils to biofuel, these technologies may not be sustainable when applied to algae.
- The low price of fuels compared to traditional high value algae products makes these processes and biofuel products economically unattractive.
- As the scale of production for transportation fuels is six orders of magnitude higher than the traditionally produced high value products from microalgae, any significant replacement of the fuel market with algal biofuels would require optimum use of the resources.

Recent research for production of liquid biofuels from algae has focussed on overcoming the challenges discussed above. This chapter gives an overview of some of these challenges, potential solutions, and research directions.

11.2 Conversion Technologies and Products

A major distinction between terrestrial bio-feedstocks and microalgae that influences the choice of conversion technologies and pathways is that at cultivation stage, microalgae consists of 0.5–200 μm (Roy and Mohanty 2019) sized cells suspended in water at concentrations ranging from around 0.3 g (dry weight) per litre in open raceway ponds to around 1.7–2.1 g (dry weight) per litre in photo-bioreactors (Norsker et al. 2011). Due to these dilute concentrations in water some of the traditional thermochemical technologies like pyrolysis and gasification that require drying of the algae feedstock pose significant energy penalties on the process. Some studies (Brentner et al. 2011; de Boer et al. 2012; Du et al. 2015) suggest that algae to biofuel conversion scenarios that require drying of the algae feedstock renders the process energetically unfeasible, requiring more energy than the process produces. Conversion technologies that obviate the need for drying the algae have a clear advantage for sustainable algae to biofuel process. Some of these technologies for the production of liquid biofuels include: wet lipid extraction to produce biodiesel or green diesel and hydrothermal liquefaction to obtain algal-crude which could then be further upgraded to multiple fuels and chemicals as with petroleum crude. These conversion technologies are the focus of this chapter and are discussed below.

11.2.1 Lipid Extraction

The extraction of lipids from oil seeds using processes like mechanical pressing, hexane extraction, etc. are well established technologies. These commercial oil extraction processes are described elsewhere (Wang et al. 2012). Some authors (Lardon et al. 2009) have compared the energy associated with the extraction of lipids using dry and wet extraction processes. The total net energy balance associated with the production of 1 kg of biodiesel was calculated to be in the range of −2.6–12 MJ for dry processing and 66–105 MJ for wet processing methods, illustrating the enormous energy advantage realized using technologies that can handle wet extractions.

A typical process for obtaining lipids from microalgae involves the following steps: cultivation in open ponds or photo-bioreactors followed by harvesting of algae using technologies like sedimentation, flocculation, filtration, centrifugation, etc. Depending on the process and conditions, primary harvesting concentrations in the range of 2–8 wt % solids are obtained (Milledge and Heaven 2013). This is generally followed by secondary harvesting using technologies like filtration or centrifugation. Algae concentrations after secondary harvesting are in the range of 20–27 wt %. Depending on specific technology chosen, feedstocks resulting from primary or

secondary harvesting process steps are subject to lipid extraction. Most common methods for the extraction of lipids from microalgae include: solvent extraction, direct transesterification, and algae that secrete products directly into the growth medium (milking).

Solvent extraction of lipid from algae involves the following steps: disruption of the algae cell wall, solvent penetration of the cell wall, contact with lipids and solvation of the lipid material, and recovery of the lipids from the solvent (Mandotra et al. 2019). During solvent extraction from wet algal biomass, water is a barrier for effective contact between the lipids and non-polar organic solvents like hexane. So, a combination of polar and non-polar solvents has been used. Solvents and their combinations studied include: hexane, ethanol, 1-butanol, dimethyl ether, mixtures of hexane with 2-propanol, methanol, ethanol, mixtures of dichloroethane with ethanol/methanol (Shen et al. 2009; Yoo et al. 2012; Dejoye Tanzi et al. 2013; Zbinden et al. 2013). Most studies have focused on specific solvents or their combinations with cell disruption methods. In a study carried out by Shen et al. (2009), wet milling followed by hexane extraction yielded best recoveries for *S. dimorphus* (recovered lipid content of 25.3%) while bead-beating followed by hexane extraction was most effective for *C. protothecoides* (recovered lipid content of 18.8%). The differences in cell shape, size, and structure between algae species were attributed for different lipid recoveries. Dejoye Tanzi et al. (2013) used terpenes as natural solvents for the extraction of lipids from wet algae obtaining yields comparable with Bligh and Dyer method (Bligh and Dyer 1959), a benchmark for the comparison of extraction methods. Zbinden et al. (2013) demonstrated the use of pulsed electric filed as a method for aiding cell disruption and enhancing lipid extraction using ethyl acetate as a solvent. Kwak et al. (2020) demonstrated a high-shear assisted solvent extraction of wet *Nannochloropsis* sp. using a combination of hexane, ethanol, and acid. High lipid yields (>90%) were obtained with lower energy consumption compared to industrial methods. Some of these cell disruption methods used to assist extractions like shear-force disruption, pulsed electric field disruption, microwave, chemical disruption, etc. are reviewed in detail elsewhere (Jeevan Kumar et al. 2017; Lee et al. 2017).

Supercritical carbon dioxide (SC CO_2) has also been extensively studied for the extraction of algal lipids and hydrocarbons (Mendes et al. 1995; Cheng et al. 2011; Soh and Zimmerman 2011; Santana et al. 2012) as it is an environmentally benign solvent and its selectivity can be altered by varying the conditions. In a comparative study of different solvents (ethyl acetate/methanol, toluene/methanol, hexane/methanol) and supercritical CO_2 for dried algae, FAME extraction yields for supercritical extraction with bead-beating, ethyl acetate/methanol mixed solvent, and Soxhlet method were 99%, 98%, and 62%, respectively (Cheng et al. 2011). SC CO2 extraction (at 250 bar and 50 °C) and Bligh Dyer method yielded comparable extraction yields: 17.6 wt % with SC CO_2 compared to around 18.2 wt % using Bligh Dyer method (Santana et al. 2012).

Direct transesterification for the production of biodiesel from microalgae has been studied by several authors (Levine et al. 2010; Patil et al. 2011; Cao et al. 2013; Velasquez-Orta et al. 2013; Abedini Najafabadi et al. 2015). These methods combine lipid extraction and conversion to fatty acid methyl ester in a single step where

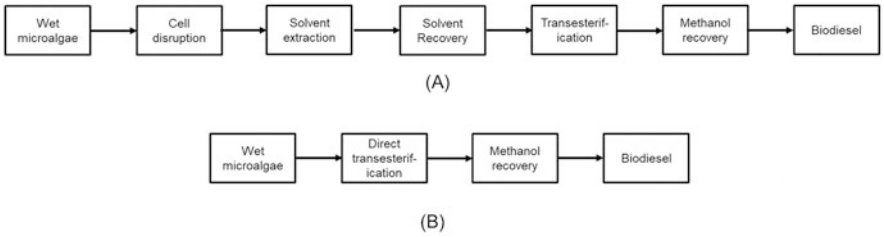

Fig. 11.2 Schematic comparing steps for the conventional transesterification (**a**) with direct transesterification (**b**) for the production of biodiesel from wet microalgae

methanol is used both as a solvent for lipid extraction and a reactant for the transesterification step. Illustrated in Fig. 11.2, direct transesterification process has a potential to minimize processing steps thus being more cost and energy effective.

Using this method biodiesel yields in excess of 90% have been obtained using algae with 80–90% moisture content (typical concentrations after harvesting steps) and have been demonstrated for several species of algae. Most of these studies were performed at around 90–120°C using acid catalysts or at supercritical ethanol/methanol conditions with the use of catalyst. Some studies at lower temperatures (Velasquez-Orta et al. 2013) show a strong dependence of biodiesel yields on water content, with yields decreasing from 92% to 61% with the increase in microalgae biomass moisture from 0% to 10%. An energy comparison of several lipid extraction and transesterification technologies revealed best case scenario as supercritical methanol direct transesterification of wet biomass, representing 85% savings in energy, relative to the base case involving drying of microalgae, followed by mechanical pressing, hexane extraction, and transesterification (Brentner et al. 2011). The net energy balance for the best case scenario, however, was still slightly negative, indicating need for additional improvements in other areas of the overall process.

Milking of oils from specific algae species like *Botryococcus braunii* involves secretion of oils, which are stored extracellularly, into the growth medium without disruption of the cell wall. These oils can then be solvent extracted and recovered. The lipids are being reproduced with the algae and the process can be repeated. It has a lot of potential for improving the overall energy and economic aspects of algae to biofuel process (Chaudry et al. 2018) but is still a nascent technology requiring significant research and development in areas of strain, species selection/development and study of process parameters for milking. These are the subject of some recent studies (Chaudry et al. 2019; Mehta et al. 2019) and reviews (Ramachandra et al. 2009; Jackson et al. 2017).

Given the complex differences in algae species (shape, size of cell walls, complex mixtures of algal oils with significant extractable non lipid components) suitable combination of cell disruption techniques, lipid extraction solvents for specific strains are some areas which need further investigation. Most of the extraction

studies are performed on laboratory scale. Data on extraction processes at larger scales is also needed for rapid commercial adaptation of these technologies.

11.2.2 Hydrothermal Liquefaction

Hydrothermal Liquefaction (HTL) involves the conversion of wet algae (at around 10–30 weight %) in hot liquid water at around 250–374 °C and 4–22 MPa (Elliott et al. 2013). It is a promising technology for the conversion of algae and has received considerable attention due its following attributes:

- It obviates the need for drying thereby improving the sustainability and economics of algae to biofuel process. Several studies have identified the requirement of drying the algae as the major energy consuming step.
- HTL could use very low lipid algae as a feedstock. It is not necessary to promote lipid accumulation rather focus is on improvements in biomass productivity. The conversion of the whole algae via HTL leads to bio-crude yields that are higher than the lipid content of algae.
- Depending on the overall process being considered, HTL could also be used as a technology that takes in leftover algae after extraction of lipids or other high value compounds as the input feedstock.
- HTL is also one of the promising pathways for the recovery and recycle of essential nutrients (nitrogen and phosphorous) (Biller et al. 2012). As discussed in the following sections of the chapter, this is an essential component for sustainable development of algae to biofuel.

The properties of water under subcritical conditions are very different from that of normal water and play a key role in hydrothermal reactions. Compared to properties at 25 °C and 1 atm, the density of water is decreased by 40%, dielectric constant decreases by around 80% thereby increasing the solubility of hydrophobic organic compounds, and the increase in ionic product accelerates many acid and base catalysed reactions. These properties relative to normal water are compared in Table 11.2. Thus hot compressed water acts as a reaction media, a benign solvent

Table 11.2 Properties of water at normal and subcritical conditions

	Normal water	Subcritical water	
Temperature (°C)	25	250	350
Pressure (MPa)	0.1	5	25
Density (g cm^{-3})	1	0.8	0.6
Dielectric constant (F m^{-1})	78.5	27.1	14.1
Ionic product (pKw)	14.0	12.0	11.2
Heat capacity (kJ kg^{-1} K^{-1})	4.22	4.86	10.1
Dynamic viscosity (mPa s)	0.89	0.11	0.064

Adapted from Kruse and Dinjus (2007), Toor et al. (2011)

for organic biomolecules, and also an acid or base catalyst as its ion concentrations are order of magnitude higher than normal water.

The major reactions during hydrothermal conversion include depolymerisation, hydrolysis, decarboxylation, dehydration, condensation, repolymerization, etc. Some of the possible reaction pathways for various components of algae (carbohydrates, proteins, and lipids) are presented in Fig. 11.3 (Gai et al. 2015; Bhujade et al. 2016; Déniel et al. 2017; Hu et al. 2019). Lipid molecules are hydrolysed to fatty acids (FAs) and glycerol, FAs undergo decarboxylation to produce straight chain hydrocarbons. Some of these FAs also end up as amides and nitriles following amidation and dehydration reactions. Proteins undergo hydrolysis to form amino acids which end up as amines or organic acids following decarboxylation or deamination reactions. Depolymerization of carbohydrates lead to the formation of saccharides which end up as furanics, cyclopentanones, monoaromatics, etc. Some of these aromatics and polycyclics repolymerize to form higher molecular weight compounds or char. The product composition of the obtained bio-crude oil (referred to as algal-crude here) is a mixture of these organic molecules that end up in the oil phase and reflected in the gas chromatography mass spectrometry (GC/MS) analysis of the algal-crude samples as shown in Fig. 11.4, showing a combination of these molecules and their relative abundance. Yields of algal-crude are dependent on factors such as type of algae, reaction, and process conditions. In continuous reactors, yields of algal-crude in the range of around 38–53 wt % were obtained (see Table 11.3) with a calculated high heating value of around 40 MJ $kg^{-1}$for most cases (Elliott et al. 2013; Singh 2016). For the studies tabulated in Table 11.3, algal-crude was separated from the aqueous phase without using any solvents, which would be the case for commercial processing. Although hydrothermal liquefaction experiments in batch reactors using solvents for the recovery of algal-crude are more common in the literature (Biller and Ross 2011; Duan and Savage 2011; Yu et al. 2011; Jena et al. 2012; Valdez et al. 2012; Toor et al. 2013; Neveux et al. 2014; Xu and Savage 2014; He et al. 2020; Ma et al. 2020), these processes are less likely to be used in commercial settings, and serve to understand the influence of various parameters, catalysts, process conditions on yields and properties of algal-crude.

Compared to traditional petroleum crude, algal-crude is higher in heteroatoms like oxygen (5–9 wt % in algal-crude, 0.4–1.7 wt % in petroleum crudes) and nitrogen (around 5 wt % in algal-crude, 0.5–2 wt % in petroleum crudes (Yui 2008, Arcelus-Arrillaga et al. 2017)), and an order of magnitude lower in sulphur. The removal of heteroatoms is the focus of most upgrading studies and is discussed in the following section. As far as various distillate fractions are concerned, algal-crude is generally heavier than traditional petroleum crude but lighter than a heavy unconventional petroleum oil like bitumen. These differences are presented in Fig. 11.5. The majority of the components in algal-crude lie in the jet fuel (15 wt %), diesel (45 wt %), and heavy gas oil (21 wt %) range while traditional petroleum crude is rich in naphtha (27 wt %), jet fuel (16 wt %), and diesel (26 wt %) range components. The majority of the components in bitumen lie in the heavy gas oil range.

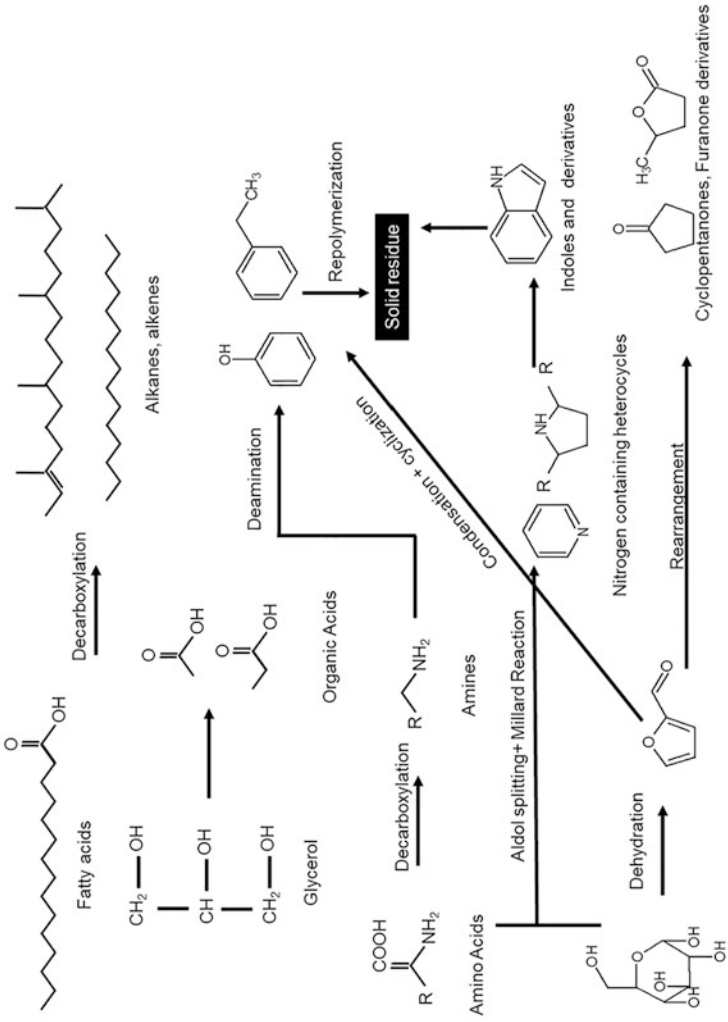

Fig. 11.3 Possible reaction pathways for various components of algae (Gai et al. 2015; Bhujade et al. 2016; Déniel et al. 2017; Hu et al. 2019)

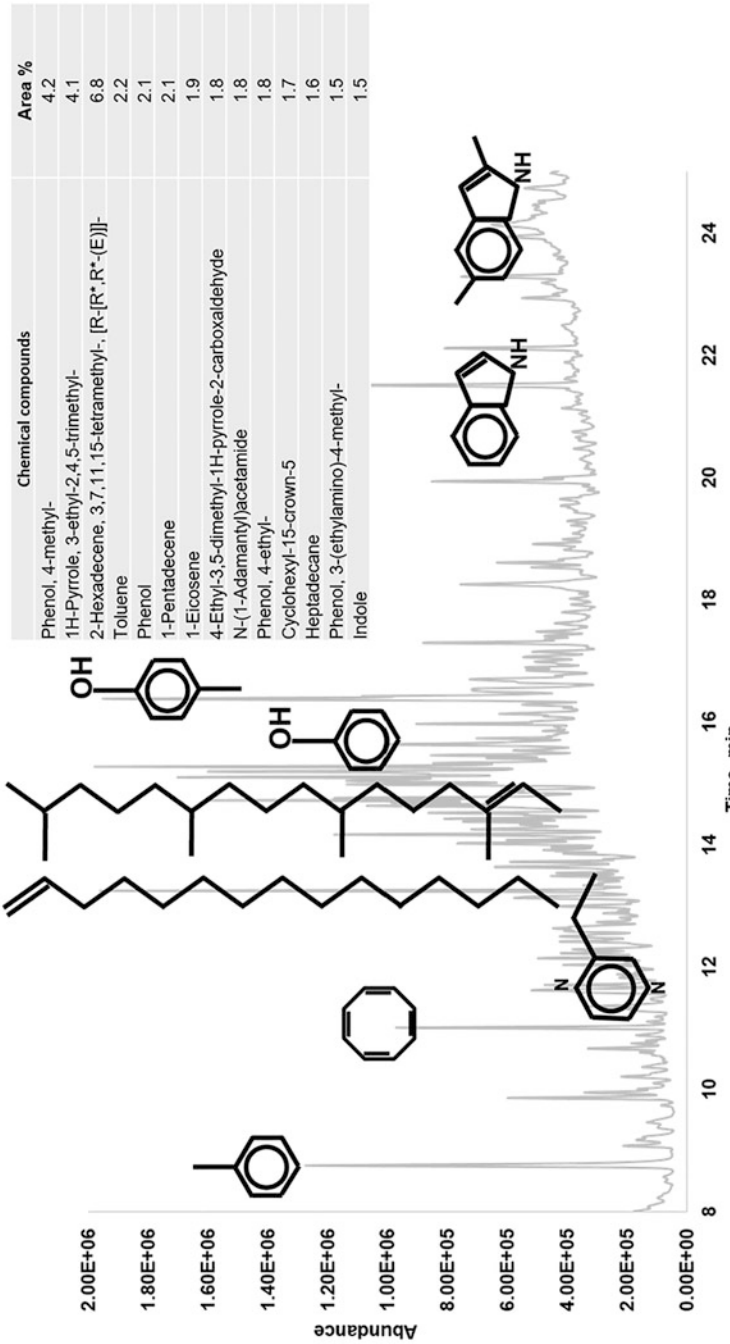

Fig. 11.4 Chemical compounds identified using GC-MS in algal-crude produced by hydrothermal liquefaction of chlorella vulgaris (Singh 2016)

Table 11.3 Algal-crude yields and properties obtained using different species of algae in continuous systems at around 35 °C and 3000 psig (Elliott et al. 2013, Singh 2016)

	NB238	Lipid extracted algae	*Chlorella vulgaris*
LHSV	1.5–2.2	1.5–2.2	0.5
Algal-crude yields (wt %)	38	53.2	43
Carbon (wt %)	79.2	78.6	74.4
Hydrogen (wt %)	10.0	10.4	11.4
Oxygen (wt %)	5.7	5.3	8.8
Nitrogen (wt %)	4.7	4.2	4.6
Sulphur (wt %)	0.5	0.5	0.6
HHV (MJ kg^{-1})[a]	40.1	40.3	40.0
Density (kg m^{-3})	0.946	0.96	–
Kinematic viscosity (cSt@40 °C)	205	114	–
Ash content (wt %)	–	–	0.2

[a]HHV was calculated using Boie's formula (Fassinou et al. 2011)

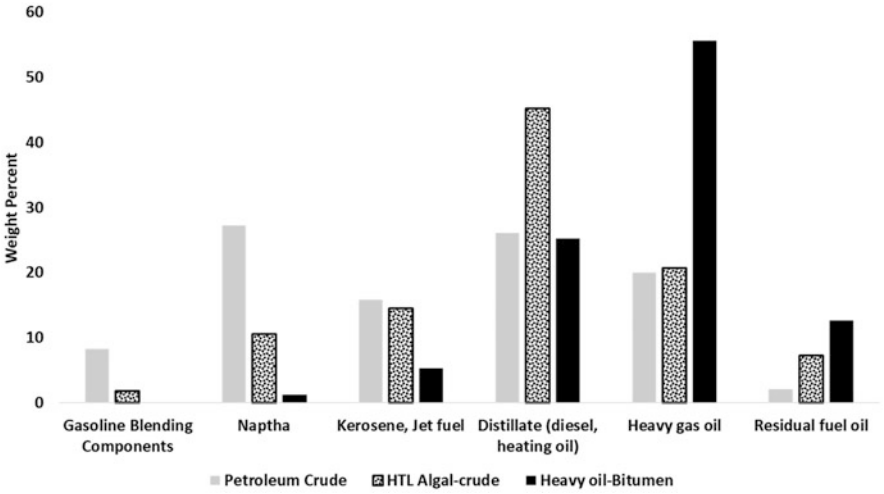

Fig. 11.5 Comparison of fractions from petroleum crude (Gulf of Mexico crude oil standard), bitumen (Alberta), and HTL algal-crude (Singh 2016) based on thermo gravimetric analysis (TGA)

Advanced hydrothermal liquefaction concepts using catalysts for improving the yields and properties of algal-crude have also been the subject of several lab scale batch studies (Duan and Savage 2011; Zhang et al. 2013; Yang et al. 2014; Xu et al. 2019). Although improvement in algal-crude yields and quality was observed, heteroatom content was still high (around 9% oxygen and 4% nitrogen), requiring additional upgrading (Duan and Savage 2011). Catalyst deactivation during hydrothermal processing due high concentrations of heteroatoms and solid impurities are critical issues that need to be addressed.

11.3 Upgrading Technologies for Fungible Biofuels

The upgrading aspects of algae-based bio-oils or their fractions involve processes to transform the components of oils such that the properties of biofuels obtained are within standard specification of conventional fuels and biofuels. Standard specifications of biodiesel and diesel are listed in Table 11.5. Most common methods for upgrading algae derived lipids/ crude oils include: transesterification of lipids to biodiesel, catalytic upgrading of lipids/algal-crude, and thermal upgrading of algal-crude. These upgrading methods are discussed below.

11.3.1 Transesterification of Lipids

The transesterification for the production of biodiesel involves reaction of triglycerides with methanol in the presence of a catalyst to produce fatty acid methyl esters (Fig. 11.6).

The process has been commercially used for the production of biodiesel from oleaginous crops (Kumar et al. 2016). Industrial process is carried over an acid or alkali based catalysts using excess of methanol to shift the equilibrium towards formation of fatty acid methyl esters. Yields greater than 98% are generally obtained (Fukuda et al. 2001). The transesterification process for the production of biodiesel is elaborated in various reviews (Ma and Hanna 1999; Fukuda et al. 2001), Van Gerpen 2005, Meher et al. 2006). The properties of biodiesel (cetane number, viscosity,

Table 11.5 Apparent nitrogen and phosphorous removal, specific growth rates, and final biomass yields of *Scenedesmus* sp.

Growth media	Apparent N removal (%)	Apparent P removal (%)	Specific growth rate (day^{-1})	Final biomass yield (g dw L^{-1})
HTL-recovered growth media	>99	68.3 ± 5.7	1.2 ± 0.1	1.0 ± 0.1
Modified Bold's basal	63.2 ± 31.1	6.4 ± 5.0	1.2 ± 0.1	1.0 ± 0.3

AMD Dusing recovered nutrients (N and P) from HTL wastewater. Nitrogen was recovered using air stripping and phosphorous using struvite precipitation (McGinn et al. 2019). Algae growth with using recycled media was comparable to positive control (Bold's basal medium)

Fig. 11.6 Transesterification of triglycerides to produce biodiesel

Table 11.4 Standard specifications for biodiesel fuel (ASTM-D6751 in the USA and EN-14214 in Europe)

Property	ASTM-D6571 (biodiesel)	EN-14214 (biodiesel)	EN-590 (diesel)
Density at 15 °C (g cm^{-3})	–	0.86–0.9	0.82–0.845
Viscosity at 40 °C (mm^2 s^{-1})	1.9–6.0	3.5–5.0	2.0–4.5
Flash point (°C, min)	130	120	55
Cetane number (min)	47	51	51
Oxidative stability (h/g m^{-3})	3 (h)	6 (h)	25 (g m^{-3})
Sulphur (mg kg^{-1})	15.0 max	10.0 max	10.0 max
Acid number (mg KOH g^{-1})	0.8 max	0.5 max	–
Iodine value	–	120 max	–
Copper corrosion (3 h at 50 °C)	No. 3	No. 1	No. 1
Distillation 90% recovered (°C)	360 max	–	<65% (V/V) recovered at 250 °C, min 85% (V/V) recovered at 350 °C, 95% point 360 °C max

oxidative stability, cloud point, etc.) are highly dependent on the profile of fatty acids (R_1, R_2, R_3 in Fig. 11.4) attached to the glycerol backbone (Mandotra et al. 2016). As an example, methyl stearate (C18:0, carbon length of 18 with no double bonds) has a cetane no. 101, melting point 37.7 °C, and oxidative stability >24 h, while methyl-linoleate (C18:3, carbon length of 18 with three double bonds) has a cetane no. 22.7, melting point of −52 °C, and oxidative stability 0 h and (Knothe and Dunn 2009) (see Table 11.4 for specifications). Compared to plant based sources of triglycerides, algal derived triglycerides often have a higher fraction of high melting point saturated fatty acids or polyunsaturated fatty acids like C16:3ω4, C22:6ω4 or both (Yee 2016; Ferreira et al. 2019). The FA composition in algae derived lipids unfavourably influences the cold flow properties and oxidative stability of biodiesel. The fuel properties of various FAME's are listed elsewhere (Knothe 2011). Apart from lipid content of algae species, other important considerations that need much attention include the fatty acid profile of lipid and optimum growth conditions for a favourable profile (Knothe 2011). A few recent studies (Hariram and Mohan Kumar 2013; Tüccar et al. 2014; Islam et al. 2015; Rahman et al. 2015) have also focused on emissions from diesel engines when using biodiesel derived from microalgae. A reduction of power sand increase in NO*x* emissions are generally reported. The use

of emulsions instead of neat biodiesel was suggested for reduction of CO_2 and NO*x* emissions. Some of the recent papers produced contradictory results (Piloto-Rodríguez et al. 2017), more studies in this area are warranted to ascertain the influence of deviation from plant derived biodiesel on engine performance and emissions.

11.3.2 Catalytic Upgrading

Catalytic upgrading of algal-crudes or lipids involves removal of heteroatoms like oxygen, nitrogen, and sulphur using an appropriate catalyst and hydrogen. These contaminants if not removed have detrimental effects on the equipment, catalysts, and quality of the end products (Speight 1999). Some of the reactions involved during catalytic upgrading of lipids/algal-crude include hydrodeoxygenation, hydrodecarboxylation, hydrodesulphurization, and hydrodenitrogenation. These reactions are exemplified in Fig. 11.7 using common molecules found in algae/bio-derived lipids/bio-crudes. Reactions (1)–(3) show the hydrodeoxygenation and hydrodecarboxylation reactions of a palmitic triglyceride, a common molecule in algal lipid, to C16 and C15 hydrocarbons with the evolution of CO_2 and H_2O. Reactions (4)–(6) show the hydrodeoxygenation, hydrodenitrogenation, and hydrodesulphurization of phenol, pyridine, and thiophene, respectively, to hydrocarbons with the evolution of H_2O, NH_3, and H_2S (Graça et al. 2013; Ma et al. 2015). The specific pathways involved in each case are much more complex involving several reactions, intermediates and are the subject of various studies and

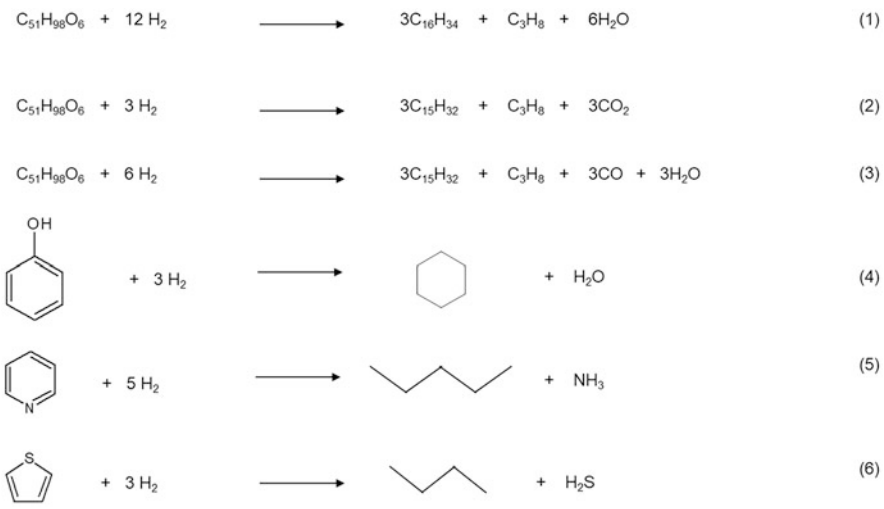

Fig. 11.7 Examples of hydrotreating reactions for the hydrodeoxygenation, hydrodenitrogenation, and hydrodesulpherization of bio-derived molecules (Saidi et al. 2014; Ancheyta et al. 2016; Vásquez et al. 2017; Bandyopadhyay and Upadhyayula 2018)

reviews (Saidi et al. 2014; Elliott 2015; Ramirez et al. 2015; Patel and Kumar 2016; Vásquez et al. 2017).

Catalytic upgrading of lipids for the production of green diesel and jet biofuel has been extensively studied, especially for the plant seed derived lipids and waste lipids has received considerable attention (Krár et al. 2010; Kubička and Horáček 2011; Sotelo-Boyás et al. 2011) and the technology is considered mature(Vásquez et al. 2017). Some of the recent work has focused on the upgrading of algal-crude, a more complex mixture of molecules (see Figs. 11.3 and 11.4) with high levels of nitrogen. Li et al. (Li and Savage 2013) studied the upgrading of algal-crude obtained from hydrothermal liquefaction of *Nannochloropsis* sp. using HSZM-5 catalyst at 400–500 °C, 4 h residence time in batch reactors. Although a better quality bio-oil was obtained, only around 50% reduction in nitrogen levels was obtained. Other studies using platinum, palladium, and Ru based catalysts have reported similar results (Xu et al. 2018). Biller et al. (2015) studied the hydrotreating of algal-crude using nickel-molybdenum and cobalt-molybdenum catalysts with a 60% reduction in nitrogen and 85% reduction in oxygen at 405 °C. The gasoline and diesel fraction were reported to be around 24% and 50–54%, respectively. Further reduction in heteroatoms was suggested using solvent extraction. Elliott et al. (2013) demonstrated the catalytic hydrotreating of algal-crude at around 410 °C and 1950 psig hydrogen pressure, liquid hourly space velocity of 0.14–0.2 L/L/h with molybdenum sulphide catalyst supported on fluorinated-alumina. Depending on the species of algae used to produce algal-crude, the liquid product yields from hydrotreating were in the range of 80–85 wt %, with oxygen reduced to around 0.8–1.8 wt %, nitrogen in the range of 0.05–0.25 wt %, and sulphur <50 ppm. The viscosity of the oil reduced from around 114–355 cSt@ 40 °C for the algal-crude to around 2.5–4.5 cSt@ 40 °C for the hydrotreated oil. Most of the focus of recent studies has been around demonstrating upgrading of algal-crude using specific catalysts, analysing products, and yields. Some areas that need further research and understanding include: studies on long-term activity and stability of specific catalysts, optimization of operating parameters with these catalysts, and studies regarding deactivation and regeneration procedures.

11.3.3 Thermal Upgrading

Thermal upgrading of crudes involves its decomposition to lighter molecules or its rearrangement by the application of heat. It is a common primary upgrading step for heavy oils like bitumen or heavier fractions of conventional crudes. Examples of thermal upgrading processes include: delayed coking, fluid coking or visbreaking. Details of these processes as applied to petroleum crudes can be found elsewhere (Speight 1999). There are limited studies on thermal upgrading of algal-crudes. Roussis et al. (2012) studied its thermal upgrading at temperatures in the range of 350 °C–450 °C. An improvement in the quality of the oil was observed with yields in the range of around 87 wt % at 350 °C to around 41 wt % at 450 °C. Increase in temperature of thermal treatment leads to an increase in carbon, decrease in

heteroatoms (oxygen, and sulphur), and increase in lighter fractions. Thermal treatment at 450 °C decreased the oxygen from 5.7 wt % to 1.6 wt %, sulphur from 0.6 wt % to 0.1 wt %, and increased the carbon content from 77.7 wt % in the algal-crude to around 84.0 wt % in the upgraded liquid product. Extraction methods, variation in processes and feedstocks, lead to differences in the chemical composition of the algal-crude which could potentially influence the thermal cracking behaviour and liquid product yields.

11.4 Co-processing of Algae Derived Bio-Oils

Given the enormous scales at which traditional petroleum refineries operate to maintain profits, resource limitations of algae biofuels with current technologies constraining its scale, and interest from major oil companies in algae derived biofuels, co-processing of algae derived oils with petroleum crudes for producing transportation fuels and products with biogenic carbon would provide economies of scale for algae derived biofuels, help accelerate its adoption, and is thus an attractive option in the near term. As discussed previously, compared to petroleum crudes, bio-crudes from algae are generally high in heteroatoms like nitrogen, oxygen, and low in sulphur compared to petroleum feedstocks.

A very important aspect of co-processing is at what point in a petroleum refinery algae derived bio-oils/bio-crudes should be blended to maximize yields while minimizing operational issues. A typical upgrading scheme in a petroleum refinery is shown in Fig. 11.8 with potential intersection/blending points for algal-crude: at the atmospheric distillation unit (algal-crude$_{AD}$), thermal processing/delayed coker process unit (algal-crude$_{DC}$), at hydrotreating of middle distillates process step (algal-crude$_{HYD}$), and at the fluid catalytic cracking feed (algal-crude$_{FCC}$). Each of the potential intersection points also presents their own advantages and risk. Very limited studies are available in the area of co-processing of algae derived bio-oils or bio-crudes. Some of these studies are either conceptual while others have used non-algae feedstocks. These co-processing scenarios and studies are discussed next.

Co-processing at the atmospheric distillation unit (algal-crude$_{AD}$): At the atmospheric distillation unit, components of the algal-crude would distribute itself among various boiling fractions (naphtha/heavy gas oil/residuum, etc.) to be upgraded in the respective downstream upgrading steps. One of the potential risks of the approach is the distribution of heteroatoms in all the processing units posing challenges or requiring process modifications for each unit. Intersection at the atmospheric distillation unit was studied by Lavanya et al. (2016). The properties of different atmospheric distillation cuts obtained after blending algal-crude from marine and fresh algae with petroleum crude (10% bio-crude and 90% petroleum crude) were evaluated. Depending on a specific fraction and the type of algae biomass used (freshwater/marine). In the naphtha fraction, the amount of nitrogen increased from around <0.01 wt % in petroleum crude to around 0.04–0.21 wt %. For the diesel fraction, the amount of nitrogen increased from around 0.05 wt % in petroleum crude to around 0.2–0.4 wt % in the blend. Specific gravity also increased from around

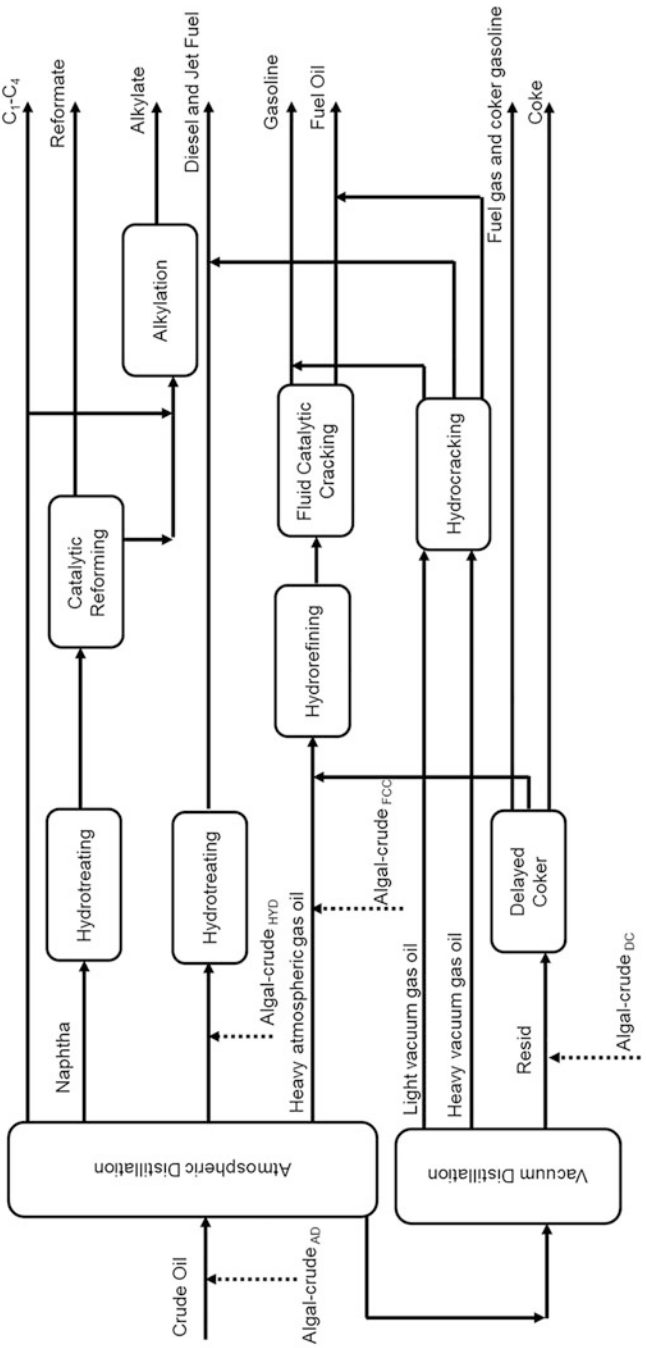

Fig. 11.8 Schematic overview of a typical petroleum refinery (Speight 1999). Dashed arrows show some potential integration points of algae derived bio-crude

0.81 to around 0.85 (kerosene fraction) for the 10% blend. To minimize process upsets several pre-treatment steps were proposed before the distillation unit if this option was to be implemented. These pre-treatment steps included: removal of impurities, desalting, and removal of heteroatoms (Lavanya et al. 2016).

Co-processing at the fluid catalytic cracking feed (algal-crude$_{FCC}$): Over 50% of the refinery's heavy crude goes through FCC unit for processing, using zeolite catalysts and no external hydrogen, high molecular weight hydrocarbons are converted into more valuable lighter products like gasoline. FCC catalysts are zeolite catalysts which are continuously regenerated by burning off coke deposits on the catalysts. These catalysts are generally more tolerant to heteroatoms. Fogassy et al. (2012) determined the fraction of renewable hydrocarbons in the end product of FCC utilizing a blend of a bio-derived oil (hydrodeoxygenated pyrolysis oil) and vacuum gas oil using caron-14 tracking (as fossil is essentially ^{14}C free). The distribution of bio-carbon was somewhat unfavourable being richer in the coke and gaseous produced than the liquid products. Increase in the coke yields while using bio-oils were observed by other studies as well. Some key considerations for excess coke formations potential damage caused by higher than usual temperatures. The variability of bio-crudes and bio-oils with source and type of process would limit application of some of these results to algae derived bio-crudes and should be studied further. US DOE labs (Los Alamos National Laboratory, Pacific Northwest National Laboratory, National Renewable Energy Laboratory) are following a similar approach studying various feedstocks including algae, catalyst performance and lifetime, tracking biogenic carbon, and understanding the fate of oxygenates during co-processing (Bladwin et al. 2019). Some initial results with fast pyrolysis oils were reported but most of bio-intermediates (including algae derived crudes/oils) remain unexplored.

Co-processing at the thermal processing/delayed coker process unit (algal-crude$_{DC}$) (algal-crude$_{DC}$): Co-processing at this intersection point would involve upgrading of the mixture of heavier fraction of the petroleum crude (or heavy unconventional petroleum crude like bitumen) and algal-crude in a unit similar to a delayed coker where feed is heated to cracking temperatures of 450–500 °C and the volatile products are removed. It is generally used in petroleum refineries to upgrade residuum into liquid and gaseous products and is the most preferred process for residue processing. Depending on the feedstock and process coke yields are generally in the range of 20–30 wt %, and liquid yields are around 60–70 wt %, while gas yields are $<$10 wt % (Tian et al. 2012). Potential advantages of this approach include: reduction in heteroatoms without the use of catalysts or hydrogen, reduction in impurities and metals as most of the impurities would end up in the coke fraction, and reduction in viscosities of the liquid product. This approach presents the least process risk but offers lower liquid yields and a relatively low refining margin. Although thermal upgrading has been demonstrated for algal-crude as discussed in the previous section, thermal upgrading of algal-crude in blends with heavy petroleum crudes/fractions remains unexplored or unreported.

Co-processing at the hydrotreater (algal-crude$_{HYD}$): Hydrotreating involves removal of heteroatoms at relatively mild temperatures in the range of

260–345 °C, 500–1000 psig hydrogen pressures and catalysts like tungsten-nickel sulphide, cobalt-molybdenum-alumina, nickel oxide-silica-alumina, platinum-alumina(Speight 1999). Reaction pathways during hydrotreating are discussed in the previous section (Fig. 11.7). Co-processing of vegetable oils and animal fats with petroleum feedstocks like atmospheric gas oil, light gas oil, vacuum distillate, and diesel has been studied extensively(Huber et al. 2007; Donnis et al. 2009; Sebos et al. 2009; Šimáček et al. 2011; De Paz Carmona et al. 2020). In some studies, hydrotreating lipids diluted with heavy vacuum oil (5 wt % lipids and 95 wt % heavy vacuum oil) increased the yields of straight chain alkanes without having any detrimental effect on the rate of desulfurization (Huber et al. 2007). Hydrotreating studies involving blends of algae derived crudes/oils with petroleum intermediates are less common in literature.

Current petroleum refineries are an integration of several complex manufacturing plants involving several conversion, separation, and finishing processes. Due to the nature of this complexity, influence of different intersection points on various downstream operations, long term influence on various process catalysts, and on final product quality are important areas that have received less attention. Most of the research focus in this area has been on the upgrading aspects of technologies, however, stability and compatibility of crudes, bio-crudes and their fractions are important considerations for co-processing of crudes. Blending of incompatible crudes could cause operational issues or negatively influence the downstream operations and product quality. Incompatible and unstable blends would lead to undesirable phase separations, negatively influencing the operations of a refinery (Stark and Asomaning 2003; Rodríguez et al. 2016). The compatibility of different petroleum crudes has been the subject of various academic and industrial studies (Wiehe and Kennedy 2000; Mendoza De La Cruz et al. 2015; Guzmán et al. 2017; Kumar et al. 2018), however, the compatibility of more complex algal-crudes or algal oils with different petroleum crudes or their fractions although important for co-processing scenarios is another area that has not yet been explored. Ultimately the decision regarding co-processing at specific intersections points in a refinery should take into account all the potential risks and benefits associated with co-processing a bio-derived feedstock with the petroleum crude. More testing and analysis at appropriate scales is needed to ascertain product quality over long term, yields at specific insertion points and provide refiners the confidence to co-process algal-crudes/oils in existing petroleum refineries.

11.5 Resource Requirements

Although several technologies have been tested for the production of algae derived biofuels, the extent to which algae can replace fossil fuels is dependent largely on the availability of resources for the production of substantial amount of algae feedstock. Some of these limiting resources as identified in several studies (Pate et al. 2011; Chisti 2013) include: carbon dioxide, phosphorous, and nitrogen nutrients. One kilogram of algae requires 1.83 kg carbon dioxide, around 0.088 kg nitrogen, and

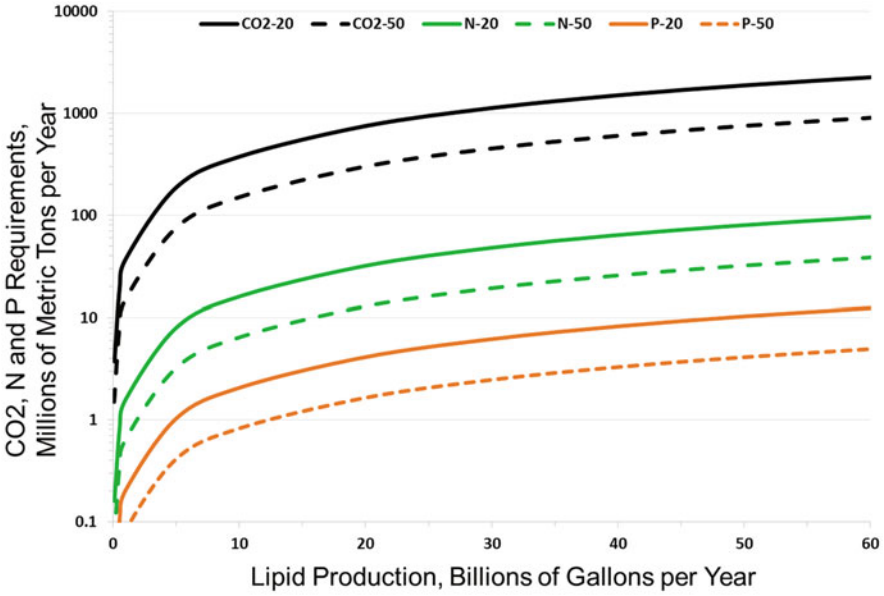

Fig. 11.9 CO_2, nitrogen, and phosphorous resource requirements as a function of target lipid production. CO_2-20, CO_2-50, N-20, N-50, P-20, P-50 are the resource requirements at algal biomass lipid content of 20 and 50% weight (Pate et al. 2011)

0.012 kg of phosphorous (Pate et al. 2011). Resource requirements as a function of lipid production are illustrated in Fig. 11.9. As an example, lipid production levels of around 10 billion gallon per year would replace around 15% of total US diesel fuel demand. Meeting this demand level would require 375 million metric tons per year of carbon dioxide, 16 million metric tons per year of nitrogen (which is the same as demand for nitrogen fertilizer in North America: 14.5 million tonne in 2019), and around 2 million metric tons per year of phosphorous which is more than twice the phosphorous fertilizer demand in North America: around 5.2 million metric tons per year (FAO 2017). Although supply of these nutrients through wastewaters has been demonstrated, it would only cover a fraction of the total nutrient requirement (Chisti 2013). Clearly any technological solution for substantial replacement of fossil fuels by algae derived biofuels would require almost complete recycle of nitrogen and phosphorous.

Recycling of algal nutrients has been the subject of several recent studies. Most of the studies (Biller et al. 2012; Garcia Alba et al. 2013; Bagnoud-Velásquez et al. 2015; Leng et al. 2018) have focused on recycling of the HTL aqueous phase. Almost half of the nitrogen, most of the phosphorous (85–100%), and some fraction of organics produced during hydrothermal conversion of algae end up in the aqueous phase (Leng et al. 2018). So aqueous phase is not only a source of nutrients but it is also potential inhibitory compounds like phenolics, pyridines, piperidones and their derivatives. Most common strategies employed to minimize their inhibitory effect

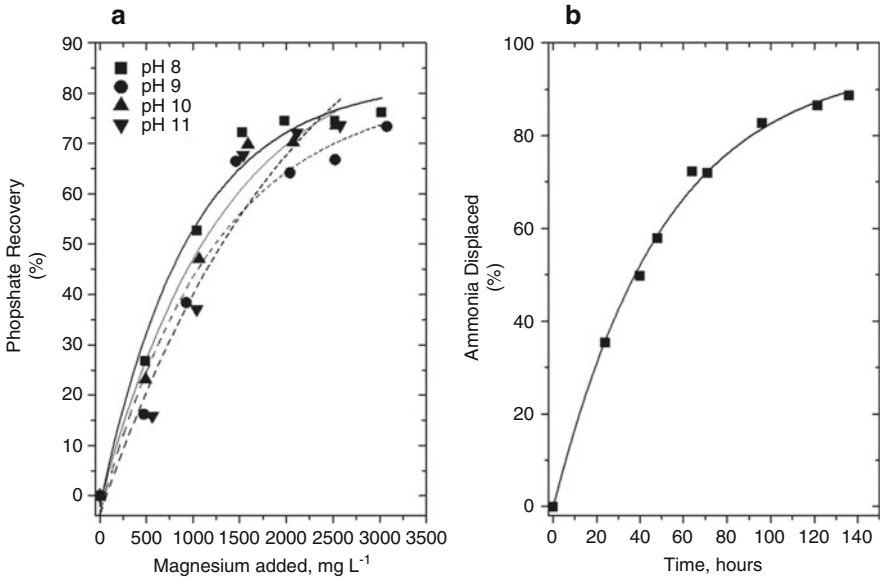

Fig. 11.10 Recoveries of phosphorous and ammonia from HTL wastewater using struvite precipitation and air stripping, respectively (McGinn et al. 2019)

involve dilution of the aqueous phase. Dilutions by a factor of up to 500× have been used (Chernova et al. 2019). Some of the higher dilutions studied have been able to minimize the inhibitory effects while in other studies algae growth was species dependent and only observed for species that could adapt to these inhibitors (Bagnoud-Velásquez et al. 2015). An elaborate review of these approaches has been presented elsewhere (Leng et al. 2018). Due to factors such as: complex mixtures of inhibitory compounds in the aqueous phase, its strain dependent response, and buildup of contaminants with multiple recycling steps, these approaches are less likely to be adaptable at a commercial scale. McGinn et al. (McGinn et al. 2019) employ an approach which essentially decouples the nutrients from HTL aqueous phase and its inhibitory compounds by recovering most of phosphorous as struvite and nitrogen as ammonia. Phosphorous was recovered by precipitation as struvite by adding a source of magnesium and ammonia was recovered by air stripping (see Fig. 11.10). Most of ammonia and around 75% of phosphate was recovered using this approach. The recovered nutrients were used for the growth of algae with comparable growth rates and final biomass yields as synthetic medium (see Table 11.5). This approach offers a more reliable and scalable process for the recovery and recycling of HTL water nutrients. The overall process scheme is depicted Fig. 11.11. Depending on factors such as fraction of organics in the nutrient depleted aqueous phase, energy, and economic aspects of the process, the nutrient free aqueous phase could be further treated as outlined in these studies (Elliott 2008; Si et al. 2019).

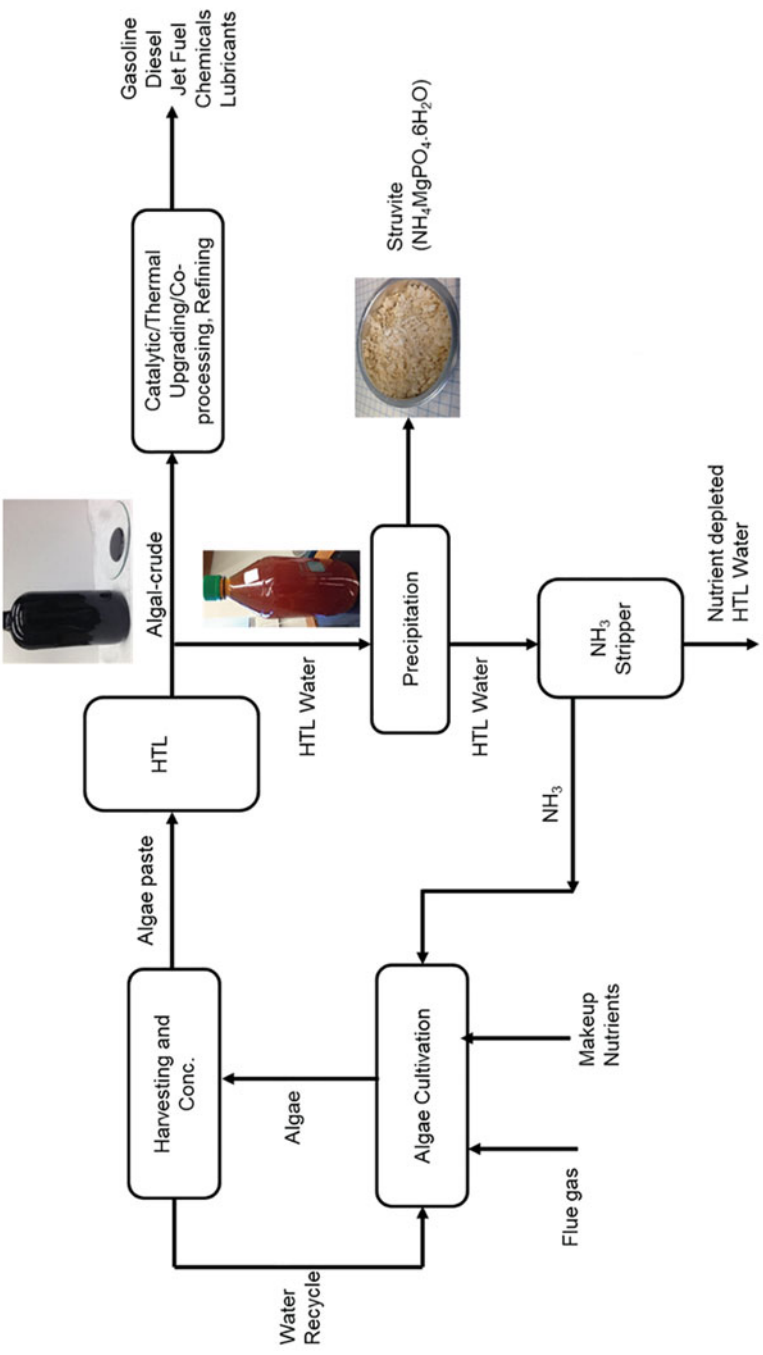

Fig. 11.11 Algae to liquid biofuel process schematic via hydrothermal liquefaction with nutrient recycling

Sustainable production of biofuels from algae requires almost complete recovery and reuse of nitrogen and phosphorous (Chisti 2013). Most of the approaches discussed above involve the recycle of nutrients that end up in the water phase. These approaches have a potential to recover most of the phosphorous but not nitrogen since a significant portion of it forms part of the bio-crude phase. A further improvement to this process would entail the recovery of nitrogen lost in the oil phase (algal bio-crude), which has a nitrogen content in the range of around 4–5 wt % (Elliott et al. 2013). This process would involve recycling ammonia produced from the upgrading of algal-biocrude (as discussed in Fig. 11.5) and using a similar approach as followed by McGinn et al. (2019) to recycle nutrients. Such processes although feasible have not been demonstrated due to complex and multidisciplinary nature of the work involved. The development and demonstration of these processes are essential for close to complete recycle of nutrients and for substantial replacement of fossil fuels by algae derived biofuels.

11.6 Financial Feasibility

The economic and financial feasibility of biofuel pathways based on microalgae as feedstock are extensively evaluated using techno-economic analysis (TEA), a well-known assessment tool in understanding cost benchmarks for algae technologies.

The economic aspects of algae to liquid biofuel production system deployments comprises a cost analysis of algae production and conversion into biofuel, taking into account the capital and operational costs over plant lifetime. These costs are then evaluated under a discounted cash flow analysis to determine the minimum fuel selling price (MFSP) of the liquid biofuel produced needed to meet an overall 10% rate of return when the net present value is equal to zero (Jones et al. 2013).

11.6.1 Capital and Operating Costs

The total capital and operating (CAPEX and OPEX) costs for liquid biofuel production comprises the CAPEX and OPEX for both the algae biomass production and the conversion processes.

According to a recent study (Pankratz et al. 2019), the capital costs related to algae biomass cultivation and harvesting include land, infrastructure/equipment, engineering and contingency costs. Land requirements include cultivation system space for open ponds systems, buildings, roadways, administration, processing, and laboratory requirements. For open raceway ponds (ORP) systems, this would also include civil work, installation of piping, pumps, paddle wheels, settling area, inoculum ponds, while for PBRs, construction and installation with pumps, piping, light emitting diode (LED) lighting systems, chillers, and the buildings that would enclose them, would be included. Dewatering assets would include membrane filtration units, centrifuges, and chillers. Before dewatering, algae-flocculent (primary harvesting) is generally used to accumulate the algae and increase the

efficiency of secondary harvesting units like settling, clarification, filtrations, and centrifugation operations. However, the cost of flocculation is negligible (Silva et al. 2013). Operating costs would include electricity costs, water costs, staff salaries, system maintenance, and transport of algae biomass for downstream processing (Pankratz et al. 2019).

The conversion processing from algae biomass to biofuel would follow either a bio chemical pathway via fermentation integrated with a thermochemical process like HTL or a direct thermochemical pathway via either HTL or transesterification (Prabandono and Amin 2015; Sun et al. 2019). Several studies consider that hydrothermal liquefaction process as one of the most promising algae upgrading processes for production of liquid biofuels and is more competitive for algae conversion to algal-crude in terms of economic return of fuel production and product quality than other thermochemical pathways (Dimitriadis and Bezergianni 2017; Tzanetis et al. 2017; Skaggs et al. 2018; Kumar et al. 2019). The HTL conversion process cost includes capital and operating costs. The capital cost includes major equipment such as furnace, reactor, and pre-heater (Tzanetis et al. 2017), and is usually calculated using equations of purchased equipment cost as function of equipment size factors (Magdeldin et al. 2017; Tzanetis et al. 2017). Operating costs related to the conversion process comprises variable costs, which include feedstocks, utilities, and catalysts, and fixed costs such as labour, administrative, maintenance, local taxes, insurance, and plant overhead (Tzanetis et al. 2017; Kumar et al. 2019).

Transesterification with prior lipid extraction is another cost effective thermochemical pathway to convert algae feedstock to liquid biofuel (Gallagher 2011; Silva et al. 2013). The extracted algae oil must be converted into biofuel via a separate chemical process because algae oil consists of triglycerides that have high viscosity are incompatible for using in most diesel engines. Transesterification is the most common process to convert algae extracted oil to commercial biofuel for transportation (refer to previous sections for details). The major capital costs for this process include chemical reactors and heat exchangers that contribute 82% and 14%, respectively, to the total cost; operating costs comprises raw materials such as feedstock and catalyst, labour, and utilities (Silva et al. 2013).

11.6.2 Cost Breakdown

The cost of feedstock production has the biggest impact on overall economics to obtain algae derived biofuels. Overall, it can range from US$445 to US$3711 per ash free dry weight (AFDW) tons of algae biomass from different types of cultivation systems (DeRose et al. 2019). A recent study (Clippinger and Davis 2019) estimated the minimum biomass selling price (MBSP) for different PBR cases in a range of $639–2014/AFDW tons of algae biomass. To understand this broad variation in PBR economics, key cost drivers were identified such as material of construction for helical tubes (glass versus plastic), plastic lifetime for the flexible plastic PBR designs, and the necessity for external cooling of the system.

Table 11.6 Key cost factors for algae biomass production of 172,365 AFDW metric tons per year

Parameter	Cultivation system	
	ORP[a]	PBR[b]
Average productivity (g/m^2/day)	25	25–52
Land required (m^2/t/year)	179	74–245
CO_2 demand (t/day)[3]	1148	1148[d]
Anhydrous ammonia demand (t/day)[c]	10	10[d]
Diammonium phosphate (DAP) demand (mg/g algae)[c]	5	5[d]
Process water demand (m^3/t)	51.3	0.231[e]
Power required (kWh/ha/day)	116[2]	192–1644
Total installed capital cost (US$$_{2014}$/annual t)[f]	1355	1561–7426
MBSP (US$$_{2014}$/t)[f]	532	704–1976
Facility size (acres, cultivation ponds only)	5000	2428–5148
Total land area (acres)	7615	3169–10,444
Annual cultivation days	330	230

Adapted from Pankratz et al. (2019)
[a]Davis et al. (2016)
[b]Clippinger and Davis (2019)
[c]CO_2 and nutrients requirements levels for these models surpass 10% and 20% of stoichiometric demands (Davis et al. 2016; Zhu et al. 2018; Clippinger and Davis 2019)
[d]PBR cases assume the same targeted algae compositions as set for the open pond design Clippinger and Davis (2019)
[e]Pankratz et al. (2019)
[f]2011 and 2014 US$ Chemical Engineering Plant Cost Index 585.7 and 576.1; Ratio: 0.983609

The breakdown of the algae (feedstock) production costs considers cultivation, harvesting, and dewatering costs. ORP cultivation systems present high resources costs, including water, nitrogen, and carbon dioxide (DeRose et al. 2019), while photo bioreactors (PBR) systems costs decrease the use of water but it is a capitally intensive cultivation scheme. An alternative growth system is the attached growth systems, such as algal turf scrubber (ATS) where algae is used to clean nutrient-rich wastewater surfaces, reducing the use of nitrogen, phosphorous, and carbon dioxide (DeRose et al. 2019). The capital cost of any cultivation system (ORP and PBRs) is the largest contributor to the MBSP in a range of 37–74% (Clippinger and Davis 2019). Regarding feedstock production operating costs, PBR systems present higher variable operating costs than OPRs, in part due to PBRs use more electricity power for mixing, aeration, and cooling than the OPRs. Likewise, PBR systems have higher fixed operating costs due to higher labour costs as well as maintenance costs (Clippinger and Davis 2019). Table 11.6 shows the main cost factors for algae production from case studies developed by the Renewable Energy Laboratory (NREL), where 172,365 AFDW metric tons of dewatered algae biomass production per year were modeled using both ORP and PBRs cultivations systems (Davis et al. 2016; Clippinger and Davis 2019). All associated costs were scaled to 2014 dollars using the Chemical Engineering Plant Cost Index.

Mixing ponds electricity demand represents 48% of total power requirement in an ORP system and an average of 70% for PBRs cases. In all PBRs cases, mixing power

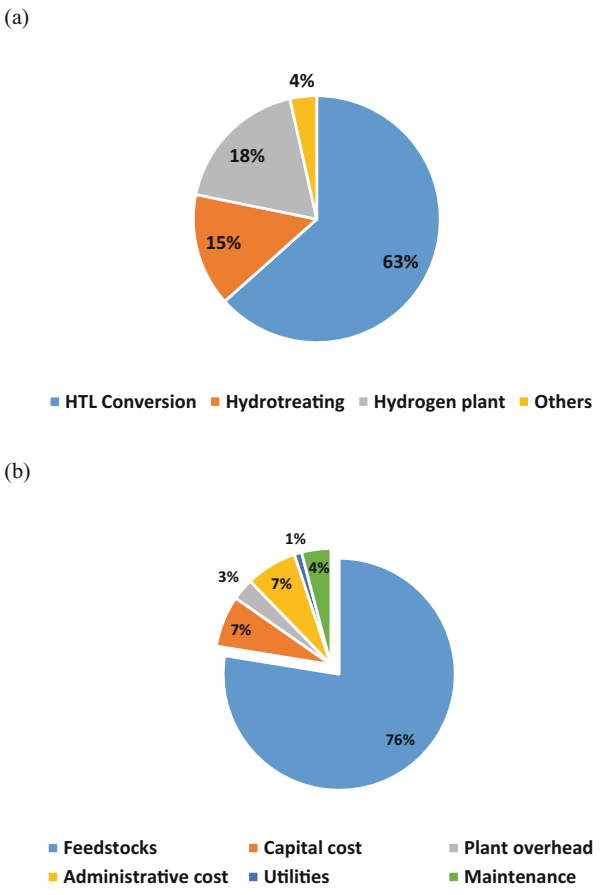

Fig. 11.12 Breakdown of (**a**) total purchased equipment cost (TPCEC) and (**b**) operating costs for hydrothermal plant facility for an algae biomass flow rate of 2200 AFDW t per day. Adapted from Kumar et al. (2019)

is higher than for open pond circulation that is a relevant metric for evaluation of global warming environmental impact given the life cycle assessment dependencies on circulation power demand even for ORP cultivation systems (Sun et al. 2019).

Related to the cost breakdown of HTL conversion costs, the highest contribution to total capital costs comes from total purchased major equipment costs for the thermochemical conversion process as it is shown in Fig. 11.12a. The algae biomass (feedstock) production cost represents 76% of total operating costs for a HTL plant facility with an algae biomass flow rate of 2200 AFDW t per day as it is shown in Fig. 11.12b.

In the case of transesterification process, (Silva et al. 2013)'study evaluated the overall economics of the whole biofuel supply chain from producing 4091 tons of algae per day by dry weight using ORP system cultivation, CO_2 sequestration, and harvesting with chitosan as a flocculent and dissolved air flotation until to obtain biodiesel at a scale of 175 millions of gallons per year via lipid extraction and transesterification. Their results concluded that cultivation and harvesting represent

98% of total capital costs of the entire biofuel production meanwhile lipid extraction and transesterification biofuel conversion processes only contribute to 2%. Regarding operating costs, feedstock production and catalyst purchase represent 77% and 17%, respectively. Lipid extraction using microwave extraction represents 4% and labour and utilities 2%.

11.6.3 Cost Drivers

Several economic studies identified that the main cost drivers are the following:

- *Feedstock costs*
  - *Average algae biomass production yields.* According to Pankratz et al. (2019) an increase (decrease) of 60% in feedstock yields would decrease (increase) the biomass price by 17% (33%) and 21% (45%) for ORP and PBR systems, respectively. Same as open ponds, MBSPs for PBRs follow an exponential cost curve as a function of productivity, with MBSP reducing more gradually as productivity increases (Clippinger and Davis 2019).
  - *Land costs.* In ORP systems, the land price has a crucial impact in the biomass production cost (Pankratz et al. 2019).
  - *High ash content* is a challenge that affects the overall algae biomass production costs (DeRose et al. 2019).
- *Type of cultivation technology.* High MFSPs are associated to PBRs systems (Davis et al. 2011; Richardson et al. 2012)
- *Total capital investment.* Since several parameters from algae biomass cultivation to biofuel production affect the amount of capital to be invested, there is a potential of fluctuations on capital cost would significantly impact the biomass price and MFSP (Pankratz et al. 2019).

In addition to these cost drivers, climatic conditions where the algae is cultivated can also generate different techno-economic impacts on the algae biomass production phase. Pankratz et al. (2019) compared the performance and techno-economic results of two ORP systems based on very different climatic conditions (hot and cold climate) and then provided a comparison in performance between the open pond versus a PBR technology cultivation system that does not have reliance on geography, both sited in central Alberta, Canada. Cold climates like Canada are suitable for algae biomass cultivation using PBR cultivation technologies since the minimum biomass selling price (MBSP) of $549/ton is more economical than the MBSP of $1288/ton using an ORP system as it is estimated in (Pankratz et al. 2019) based on a 30-year facility lifetime and an internal rate of return (IRR) of 10%.

11.6.4 Minimum Fuel Selling Price

A number of TEAs studies combine engineering-based process modeling with economic estimates and financial assessment to quantify minimum fuel selling prices, typically on a dollar-per-gallon basis (Quinn and Davis 2015). Table 11.7 shows a comparison of minimum algae-based biofuel selling prices from different studies. It is important to note that the MFSP calculated in these studies show a high variability mainly due to technical and economic considerations assumed to model growth and biofuel production pathways. For comparison of these studies, results have been scaled to 2018 US dollars using the correspondent Chemical Engineering Plant Cost Index.

Differences on MFSP of algae-based biofuels depend on the type of the conversion pathway, the type of algae biomass cultivation, different productivities projections, lipid concentrations, total purchased equipment cost, and subsidies and other economic conditions (DeRose et al. 2019; Gallagher 2011; Quinn and Davis 2015; Zhu et al. 2013). Considering changes in these factors, Zhu et al. (2013) estimated a variation of the minimum fuel selling price of HTL bio fuel of algae

Table 11.7 Minimum fuel selling price review

Sources	MFSP (US_{2018}/GGE)[a]	Cultivation system	Conversion process with upgrading
Gallagher (2011)	3.26[b]	ORP	Transesterification with lipid extraction
Davis et al. (2011)	10.63	ORP	Solvent extraction and Hydrotreating
Davis et al. (2011)	22.18	PBR	Solvent extraction and Hydrotreating
Richardson et al. (2012)	14.48	ORP	Solvent extraction and Hydrotreating
Richardson et al. (2012)	36.59	PBR	Solvent extraction and Hydrotreating
Silva et al. (2013)	4.21[c]	ORP	Transesterification with lipid extraction
Zhu et al. (2013)	2.13–7.32	ORP/PBR	HTL
Jones et al. (2014)	4.62	ORP	HTL
Davis et al. (2016)	4.48	ORP	Algae lipid extraction and upgrading
Davis et al. (2016)	4.63	ORP	HTL
Juneja and Murthy (2017)	6.76[d]	ORP	HTL
DeRose et al. (2019)	10.72	ATS	Direct HTL

GGE gallon of gasoline equivalent, *ASP* algal turf scrubber, *ORP* open raceway ponds
[a]The product value was estimated through discount cash flow of rate of return for a 30-year plant life and an assumed internal rate of return 10%
[b]10% discount cash flow of rate of return for a 20-year plant life
[c]10% discount cash flow of rate of return for a 15-year plant life
[d]10% discount cash flow of rate of return for a 20-year plant life

biomass between US$2.13 and 7.32/GGE. Likewise, Gallagher (2011) and Silva et al. (2013) estimated the MFSP of algae-based biofuel via transesterification with lipid extraction in a range of US$3.26–4.21/GGE. Regarding a comparison between type of biofuel conversion processes, HTL and transesterification with lipid extraction provide more competitive MFSPs than solvent extraction with hydrotreating process.

Direct biofuel cost comparisons associated with growth cultivation systems, ORP and PBR, are studied by Davis et al. (2011) and Richardson et al. (2012) concluded that ORP systems are economically advantageous by more than a factor of 2 assuming PBR systems have similar productivities than ORP, however, it is expected that large-scale PBR systems could improve productivities and culture stability than ORP (Pankratz et al. 2019; Quinn and Davis 2015). Biodiesel prices calculated by Richardson et al. (2012) are higher than Davis et al. (2011) due to Richardson et al fully accounting for financial costs and risk which such as debt servicing costs on CAPEX, dividend payments to investors, risk on production and prices, annual interest for cash flow deficit re-financing, cash flow deficit loan repayment, annual replacement and financing of machinery and equipment, and federal income taxes (Richardson et al. 2012).

Jones et al. (2014) and Davis et al. (2016) process design and economic analysis reports from Pacific Northwest National Laboratory (PNNL) and National Renewable Energy Laboratory (NREL), respectively, combine an engineering approach, i.e. process modeling using commercial process simulation software tools, with economic and financial assumptions to estimate MFSPs. These reports also take into account aspects such as carbon dioxide injection/recycling and water and nutrients recycling that improve economics and life cycle performance. Davis et al. (2016) primarily conducted a TEA of algae biomass production in ORP systems and estimated a MFSP (best case result). It was suggested that CO_2 delivery from power plant bulk flue gas and recycling back of CO_2 and nutrients such as P and N to the algae production process reduce MBSP (base case) by 8% and 10%, respectively, that accounts for a best case MSFP. Jones et al. (2014) focused on algae HTL conversion and considered recycling of water, CO_2, nitrogen, and phosphorous from the HTL conversion plant back to the algae pond. Water comes from the hydrotreating and catalytic hydro-gasification (CHG) processes, CO_2 is recovered from the hydrogen plant flue gas and the CHG water stream, nitrogen as ammonia is recycled from HTL water streams meanwhile phosphorous is recovered from HTL solids phase.

DeRose et al. (2019) calculated a high MFSP primarily due to variables such as ash content, biomass costs, and reaction yields. In the case of ATS systems ash reduction has potentially significant effects on reducing capital and operational costs by reducing associated oversizing of downstream equipment.

The techno-economic studies reviewed in this chapter indicate that several factors influence to obtain high biofuel production costs. Feedstock costs play a significant role to reduce/increase MFSP this is also associated with biomass productivities. Other variables that contribute to the cost effectiveness of algae-based biofuels

production include high capital costs of algae growth and harvesting, type of growth cultivation system, and lipid concentrations in bio-crude (triglycerides).

Techno-economic assessment is a useful tool that provides a better understanding of the current commercial viability of algae-based biofuel production systems. A large variability exists in results primarily attributed to differences in growth cultivation systems, conversion pathways, financial inputs, and productivity assumptions (Quinn and Davis 2015). More experimental data at appropriate demonstration scales are needed to ascertain various assumptions and decrease the variability around economics of algae to biofuel processes.

11.7 Commercialization Efforts

As interest in algae-based biofuels has grown, several start-ups and demonstration projects have launched worldwide during the past decade. Some examples of these demonstration projects (Zhang 2015) utilizing CO_2 from flue gas for algae growth are discussed below:

- Australia: Algae Tec and Bays water Power Station (New South Wales, Australia), with plans to remove 1.3 Mt CO_2 per year once fully operational; MBD Energy and Tarong Power Station (Queensland, Australia) investigating to use power station waste stream (flue gas and ash dam water from a 1400 MW power plant) to grow local algae strains.
- Canada: Pond Biofuels in partnership with National Research Council of Canada's demonstration of a 25,000 L photo-bioreactor at St Mary's Cement plant (Ontario, Canada) using CO_2 from plant operations.
- China: Seambiotic and Penglai Power Station's Hearol project (China) for commercial cultivation of algae using flue gas from the coal fired power plant.
- Germany: E.ON Hanse AG and Hamburg-Reitbrook Power Station (Hamburg, Germany), microalgae were grown in outdoor systems using CO_2 from an on-site boiler.
- India: National Aluminium Company Limited (NALCO) and Angul Captive Power Plant (Angul, India) where treated flue gas from the furnace was used to grow algae in 728 m^2 open pond system. The current carbon capture rate of this demonstration facility is 56 tonne per hectare which algal biomass generation of about 37 ton per hectare per year.
- Israel: Seambiotic and Rutenberg Power Station (Israel) utilizing flue gas to grow algae in a 1000 m^2 open pond system, producing food grade dry algae. This is the only facility that is commercially producing algae using flue gas from a coal fired power plant.
- United States: University of Kentucky Center for Applied Energy Research (CAER) and East Bend Power Station (USA) using a vertical tube photo-bioreactor demonstrating the successful technical feasibility of the project. Other algae-based companies funded through the US Bioenergy Technology

Office (BETO) include: Solazyme, Inc., Sapphire Energy, Inc, Algenol Biotech, and BioProcess Algae.

The interest in algae has also led to several petroleum oil majors investing in algae in partnership with algae technology companies. Some of these major partnerships include: ExxonMobil and Synthetic Genomics with Synthetic Genomics focusing on engineering algae strains(Ajjawi et al. 2017) to create scalable biofuels while ExxonMobil focusing on processing aspects and large scale deployment of the developed technology ; Chevron with Solazyme Industrials with Solazyme focusing on heterotrophic algae strain selection that can convert sugars into oils; Shell with Cellana, its core technology ALDOU™, being a photosynthetic production system that combines closed-culture photo-bioreactors with open ponds thereby avoiding contamination by undesirable algae strains (Knoshaug et al. 2018). Cellana's Kona demonstration facility on Big Island, Hawaii is a 6 acre production and research facility, which has produced over 20 metric tons of whole algae (dry weight) (Su et al. 2017). Reliance Industries Limited with Genifuel and Pacific Northwest National Laboratory focusing on the demonstration and scale up of hydrothermal liquefaction of algae to produce algal-crude for upgrading to transportation fuels (Bhujade et al. 2016). Although technical feasibility of algae to biofuel processes has been demonstrated, its commercialization still has lot of challenges for the production of sustainable and cost-competitive biofuels. Meanwhile algae companies (Pond Biofuels, Synthetic Genomics, Solazyme, etc.) are reducing costs by focusing on offering diversified products including animal feed, nutraceuticals, fish feed with biofuel as one of the product offerings. This helps these companies sustain as several aspects of algae technology develop to an extent where it is sustainable and cost effective for the biofuel market.

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UV-B Coupled Lipid Induction: A Strategy Towards Economical Biofuel Production Through Algae

12

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Abstract

Sustainable production of the renewable energy is being keenly debated globally since the first generation biofuels, mainly produced from food crops, and mostly oil seeds are limited in their aptitude to fulfill the demand of biofuel, climate change, and economic growth. These anxieties have developed the interest towards second generation biofuels produced from non-food feedstocks which potentially offer acute opportunities in the longer term. This chapter covers the status and capacity of microalgae used for lipid production under the UV-B environment. The microalgae oriented biodiesel production and its advantages are presented and described comprehensively in comparison with other available biodiesel feedstocks.

Keywords

UV-B · Microalgae · Renewable energy · Biofuel

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12.1 Introduction

Non-renewable fuels such as petroleum, oil, and coal have been worldwide recognized as major contributor to increasing demand of global energy supply (Oh et al. 2018). Such non-renewable energy sources are largely considered to be the prominent cause of environmental pollution, global warming, diversity loss, and climate change (Rahman et al. 2017; Mandotra et al. 2016). Currently, about 90% of total energy demand is fulfilled by fossil fuels and remaining ~10% from renewable resource such as biofuel, biodiesel, solar energy, etc. (Milano et al. 2016). Based on the current status of the per capita energy consumption, there will be no more oil reserves after 2050 (Miller and Sorrell 2013). Application of fossil fuel enhances the pollution load in water, air, and soil (Tripathi et al. 2016).

The aquatic ecosystem is one of the mega reservoirs of all type of pollutants emitted from various resources into the atmosphere. Therefore, efforts should be made for the development of sustainable energy solution from renewable resources such as plants and microalgal biomass (Hari et al. 2015; Mandotra et al. 2019). Application of microalgal biomass as a sustainable resource of biofuel has captured considerable interest as it has the huge potential to be a next generation feed stock (Pandey et al. 2018). Biofuels have greater potential to meet the global energy demand and considered to be most viable alternative sources of energy owing to its renewable and environment-friendly nature (Mandotra et al. 2014; Chew et al. 2017). The capacity and strength of algal biomass is becoming a great victory towards industrial applications due to its fast growth rate, ability to adopt to diverse habitat, no competition with food crops and land, and easy to culture and multiply in waste waters as well as bare lands (Chen et al. 2011; Mandotra et al. 2020). The high photosynthetic capacity of algae is not only used for lipid production, but also plays a pivotal role in oxygen production, carbon sequestration and nutrient recycling, etc. (Shen 2014). Microalgae are known as metaphor of "green gold mines" due to predominance of triglycerides (TAGs) in their lipid bodies (Shen et al. 2016). Lipid and biofuel production have several advantages such as reduction of country's reliance on crude oil imports, employment and source of farmer's income, etc. (Gheewala et al. 2013). The biofuel derived from microalgae lipid is also non-toxic, biodegradable, and does not contribute to net CO_2 or sulfur emissions (Atabani et al. 2012; Shuba and Kifle 2018).

Lipids are energy reserves in microalgae cells and the raw materials for biofuel production (Chew et al. 2017). Although the biofuel production from microalgae are much improved than the other raw materials, but it is not successfully employed as energy resource for commercial purpose due to low lipid productivity (Chen et al. 2015; Rai et al. 2015). Various studies have been reported that the biomass and lipids production in microalgae can be enhanced by different environmental stresses and through genetic engineering technology (Minhas et al. 2016; Zhu et al. 2016). Various stress factors such as pH, temperature, intensity of light, nitrogen, salt and UV, etc. are known to influence the lipid production, biomass productivity, and tolerance responses in microalgae (Lin and Wu 2015; Rai et al. 2015; Schnurr

and Allen 2015; Benavente et al. 2016). However, mechanism behind stress induced stimulation in lipid production is still unexplored.

Under environmental stress, reactive oxygen species (ROS) are produced as a consequence of oxidative damage in algae. ROS produced free radicals that are derivative product of oxygen (Das and Roy Choudhury 2014). ROS are known to produced and accumulated in algae due to various environmental stressors like temperature, salt, heavy metal, light intensity and others nutritional stresses (Chokshi et al. 2015). The ROS includes both radical (superoxide radicals ($O_2^{\bullet -}$); hydroxyl radical ($OH^{\bullet}$), perhydroxy radical and alkoxy radicals ($RO^{\bullet}$) as well as non-radicals (H_2O_2 and $^1O_2$) (Gill and Tuteja 2010). ROS like $O_2^{\bullet}$, $OH^{\bullet}$ and H_2O_2 are produced via partial reduction of O_2 that is an essential aspect of aerobic life (Sharma et al. 2012a). Free radicals are believed to be as slow toxin due to their harmful effects on biologically important macromolecules (Droge 2002). It has been anticipated that about 1% of oxygen consumed by the plants are transformed into ROS in various subcellular parts such as mitochondria, chloroplasts, and peroxisomes (Sharma et al. 2012a). ROS are well recognized in current research era for dual role as beneficial as well as deleterious species, depending upon their concentration in the cells (Sharma et al. 2012a). At higher concentration, ROS damages the algal cell biomolecules, while at low or moderate concentration, acts as a secondary messenger in signaling cascades (Sharma et al. 2012a). The threshold point of ROS causes peroxidation of membranes lipid, which in turn adversely affects balanced cellular functioning (Redza-Dutordoir and Averill-Bates 2016). ROS is injurious to algal cells affecting algal growth, development, and physiochemical reactions (Sharma 2016) due to oxidation of endogenous target molecules which are also considered as reactive oxygen species reporter molecules. For example, increase in MDA, thymine dimers of DNA (Ayala et al. 2014), products of ROS coupled oxidation of polyunsaturated lipids and DNA is respectively indicating the presence of ROS (Gaschler and Stockwell 2017). Primary evidence for the generation of superoxide radicals ($O_2^{\bullet -}$) was observed in algae visible to UV-B radiation (Xue et al. 2005). Increasing secondary evidences corroborate the involvement of ROS mediated damage developed by UV-B radiation (De Jager et al. 2017) to lipid in algae (He and Häder 2002). High production of ROS in algae cells exposed at maximum intensity UV-B exhibits harmful effects on enzyme activities and gene expression, which finally hints to cellular injury and automatic cell death (Xie et al. 2019).

Lipid peroxidation worsens the effect of oxidative stress by production of several lipid-derived radicals that affects the proteins and DNA (Sharma et al. 2012b). Increased lipid peroxidation has been reported in various plants growing under different environmental stresses (Sharma and Dubey 2005; Han et al. 2008). Malondialdehyde (MDA) is the end product of peroxidation of the unsaturated fatty acids of cell membrane (Gaschler and Stockwell 2017). Oxidative stress may also act as inducer for high lipid production and accumulation in algae like *Dunaliella salina* (Yilancioglu et al. 2014), *Chlorella pyrenoidosa* (Duan et al. 2012), and *Chlorococcum humicola* (Singh et al. 2018).

12.2 Impact of UV-B Radiation on Algae

Industrial revolution and population growth is the major contributor of environmental deterioration (Zhang et al. 2007). Anthropogenic activities directly or indirectly add to the harmful substance into the atmosphere destroying the stratospheric and ozone layer. All aquatic organisms, including microalgae, are susceptible to UV-B, but to dissimilar extents (Sinha and Häder 2002). Studies have been showed that green microalgae treated with UV-R exhibited tolerance to a high intensity of UVR, which might be due to the occurrence of extremely effective escape mechanisms (Rastogi et al. 2014). Photosynthetic pigments of algae are more prone to UVR and the photosynthetic pigment acts as photosensitizer and produces reactive oxygen species under excess of UV/visible light radiation. The destruction of chlorophyll content as well as decreasing photosynthesis process results in the overall drop in biomass productivity of the algae (Xue et al. 2005). In *Dunaliella bardawil*, UV-B radiation causes main injury to cells with respect to photo-bleaching by damaging the CO_2 fixation process, i.e., RUBISCO (Xue et al. 2005). Lesser et al. (2001) studied that *Scenedesmus* sp. growing in Antarctica regions have developed potential to ensure their survival against UVR by replacing the injured D1 protein or RuBisCO, and repair of UV-B encouraged DNA damage. In another study Leya et al. (2003) reported the production of red colored hypnoblasts as protectants from UV-B irradiation in microalga *Chlamydomonas nivalis*. In the unicellular algae *Haematococcus pluvialis*, production of excess astaxanthin protects the chloroplasts against UVR stress (Shah et al. 2016). There is a noticeable effect of UV-B on algal physiology as due to UV-B effect on various morphological, physiological, biochemical, and molecular alteration under UV exposure. Holzinger et al. (2006) studied that algae *Prasiola crispa* exposed to 24 h UV-B (2.0 W m^{-2}) showed high degree of dilations in the thylakoids of chloroplast, deceptive reduction in the number of plastoglobuli and quantum yield (Fv/Fm).

It has been observed that low doses of UV-B induce synthesis of proteins, significant for protection and maintenance of photosynthetic apparatus via UVR gene signaling (Kataria et al. 2014). The UVR8/HY5 (HYH) based UVR signaling pathway indicates to the expression of SIG5 that encodes sigma factor of RNA polymerase, involved in D2 protein synthesis and Early Light Inducible Protein (ELIP1) related with D1 protein of photosystem II (Mellenthin et al. 2014). In continuation, ELIP1 is encouraged in chloroplasts during maturation, protecting photosynthetic machinery from photooxidative damage (Rossini et al. 2006). Musil and Wand (1994) observed that stimulation of net CO_2 acclimatization rates and growth in *Dimorphotheca pluvialis* L. was observed under very low, ambient doses of UV-B stress.

12.3 UV-B and Photosynthetically Active Radiation (PAR)

Numerous observations have expressed the role of photosynthetically active radiation (PAR) (400–700 nm) in modulation of algal sensitivity and photomorphogenic response to UV-B radiation and vice versa. Direct mechanism in nature viz.,

increased photorepair, photoreactivation, and levels of photoprotective compounds, as well as indirect mechanisms in nature have been postulated to clarify the UV-B protective effects of high photosynthetically active radiation light conditions (Bolink et al. 2001; Krizek 2004; Hoffmann et al. 2015). An interactive effect such as production of various metabolites and chemical substance shown by the plants under PAR and UVB was reported by several authors (Götz et al. 2010; Barnes et al. 2013; Vidovic et al. 2015), suggested a common acclimation response of algae and plants to light signals (Wargent et al. 2015). Vidovic et al. (2015) has reported on the basis of previous observation that under high intensity, UV-B increases the rate of photosynthesis in *Plectranthus coleoides* via enhancing stomatal conductance, rate of CO_2 assimilation, and internal CO_2 concentration whereas under natural sunlight, photo-inhibition in *Cucurbita pepo* has been also suggested to be caused by the UV-A, but not the UV-B of solar radiation (Hakala-Yatkin et al. 2010). When both UV-B and PAR light are applied, maximum flavonoids is detected in old leaves. For example, high PAR induced the accumulation of flavonoids in young leaves of *Hordeum vulgare* and coupled treatment with UV-B increased the production of flavonoids in older leaves (Klem et al. 2012). Similarly, Morales et al. (2013) reported high flavonoid production in young and older leaves of *Betula pendula* plants grown in ambient PAR and UV-B.

12.4 Microalgae and Lipid Production

Lipids are stored and produced in all the living entities and play the key structural and functional role, including cell membranes generation, carbon and energy storage (Escriba 2017) in algae and plants. However, few other microorganisms like cyanobacteria, yeasts, and molds can accumulate lipids ~20% of their DCW (Donot et al. 2014). Microalgae are emerging alternative sustainable source for the lipid yields and various other bioactive compounds (Laoteng et al. 2011). High lipid accumulation and biomass productivity are the two main desired phenotypes in microalgae preferred for sustainable biodiesel production (Yilancioglu et al. 2014; Kumar et al. 2016). Increase of reactive oxygen species under varied environmental conditions is helpful in increasing the accumulation of lipid yields in the microalgae (Li et al. 2010). The production of lipid yield can be enhanced in economical way by using various substrates as a source of nutrients for the microalgae (Sun et al. 2018). Microalgae are much capable to accumulate cellular lipids in the range of 25–70% of dry cell weight (DCW) under different environments including high light stress, UV-B and nutrients deprivation (Donot et al. 2014; Fan et al. 2014). Microalgae mainly accumulate neutral lipids (about 90% of their lipid storage) (Donot et al. 2014). Lipid yield, formation, and accumulation in the microalgae depend on the availability of nutrient elements required for growth. In the presence of essential elements, the cells of microalgae rapidly divide, synthesize membrane lipids and chloroplast lipids (Solovchenko 2012). When one of the growth factor becomes limiting but photosynthetic CO_2 fixation continues, that process called lipogenic phase characterized by slowdown or cessation of cell division that leads to the

reduction of the photosynthetic machinery and accumulation of lipids (Solovchenko 2012). In addition to the well-known function of lipid in carbon and energy storage, lipids may act as antioxidants or defense molecules under varied stress condition (Hu et al. 2008). Satpati et al. (2016) reported on the basis of previous research article that microalgae treated with high UVR increased the microalgae cellular content via improving the TAG formation. Microalgae adopted different mechanism including uptake, membrane fluidity, efflux, and Na^+ retention inside and outside of the cell under UV-B exposure to produce fatty acids of different composition and functions (Srivastava et al. 2017). The increases in concentration of cellular lipids can also be accounted as a defense mechanism towards the cell injury due to osmotic stress (Sharma et al. 2012b). The generation of low ROS initiates adaptive responses, such as lipid accumulation and biosynthesis (Singh et al. 2018). In contrast, high ROS levels may cause cell level toxicity, and initiates the consumption of stored lipids as energy source to maintain the cellular homeostasis (Singh et al. 2018). Microalgae are capable of de novo PUFA (polyunsaturated fatty acid) synthesis and represent the untapped resource of these fatty acids which could be used in the treatment of various ailments in human and animal. In addition, PUFA may act as a green resource of health supplements for the betterment of human civilization (Robertson 2013). PUFA plays a significant role in inflammatory disorders, rheumatoid arthritis, obesity, diabetes mellitus, and cardiovascular disease (Robertson 2013). Thus, growing microalgae in devastating UVR rich bare lands are crucial and one of the best practices for economic success of the nation via biomass utilization in production of lipid yields and other value added products for sustainable development.

12.4.1 Strategies of UV-B Based Lipid Alteration in Microalgae

UV-B light can bring significant alteration in the chemical composition of algae. It is vital for photosynthesis at low intensity and together with photoperiod is a dangerous factor for the algal growth (Wahidin et al. 2013). Light intensity of 100–200 $\mu E/m^2/s$ is commonly used for lipid production via generation of high rate biomass in algae (Zhao and Su 2014). Increasing the light intensity from 200 to 400 $\mu E/m^2/s$ increased the growth in microalgae (Radakovits et al. 2010). In *Mychonastes homosphaera*, *Chlorella vulgaris, Raphidocelis subcapitata*, and *Scenedesmus* sp., lipid productivity has been shown to be increased with increase in light intensity from low to high (Tang et al. 2011; Liu and Hu 2013). Khotimchenko and Yakovleva (2005) explain the high PUFA content under low light intensity whereas high light intensity resulted in maximum storage of lipid.

In case of algal lipid production, they have different requirement of light intensity and several other important parameters for the overall energy balance considerations. It may be feasible to use high light intensities for enhanced production of lipids yields, biomass as well as suitable fatty acid profile for improving biofuel potential. Under high light intensity, the cells experience photo-inhibition which needs to be considered before applying to get the desired lipid content in algae. Thus, microalgal

cultivation is adopted under two phase system. In the first phase cells are cultivated and grown under low light and transferred to high light in second phase. This might overcome the limitation caused by high light. This strategy for lipid yields may improve the overall lipid productivity and biomass and addresses properly the concern of photo-inhibition.

12.4.2 Defense Responses in Algae Under UV-B Radiation

Formation of ROS is one of the major characteristics of algae under UV-B stress (Das and Roy Choudhury 2014). A controlled balance between ROS production and destruction is essential if function and metabolic capacity are to be maintained in both optimal and stress environment (Poljsak et al. 2013). There is a wide range of ROS generation in algae shown in the (Fig. 12.1). The electron transport system of mitochondria and chloroplast are two major sources of ROS production (Sharma et al. 2012b). In the chloroplast, environmental stress such as UV-B and salt may limit CO_2 fixation and reduce the $NADP^+$ regeneration by the Calvin cycle (Tausz 2004). Chloroplast is highly sensitive to UV-B radiation and extreme radiation of UV may lead to over saturation of the light reactions of photosynthesis, which eventually may cause photo-inhibitory injury to the photosynthetic apparatus (Kataria et al. 2014).

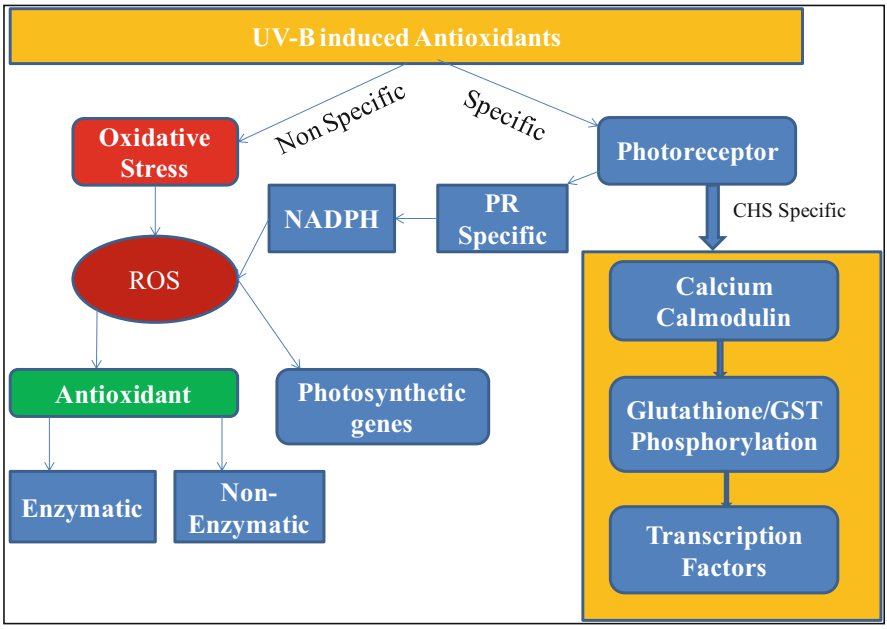

Fig. 12.1 Generalized view of UV induced toxicity and responses mechanism in the algal cells

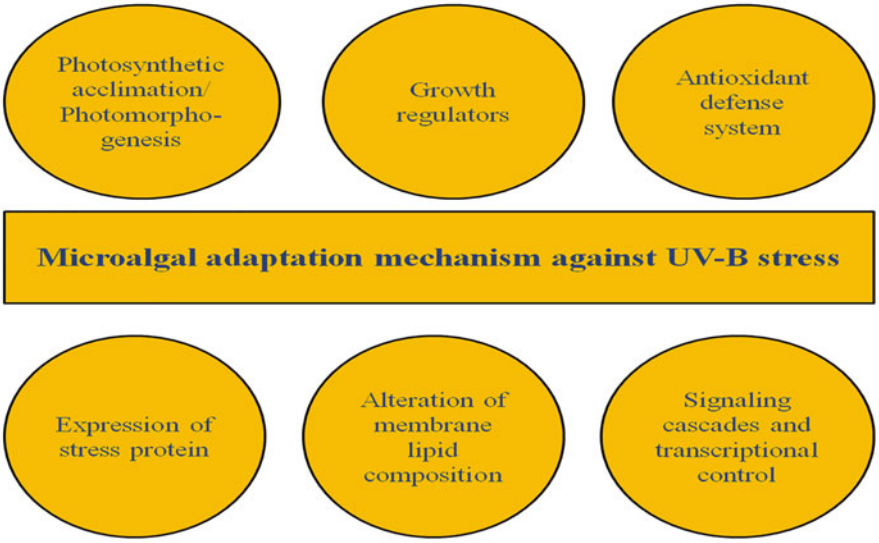

Fig. 12.2 Different adaptation mechanism in microalga under UV-B stress

Algae exposed to UV-B radiation accumulate H_2O_2 in cells, due to stimulated production of oxidative products and it is countered by stronger adaptive responses present inside the cells (Shiu and Lee 2005). This suggests that the UV-B radiation induced oxidative bursts of H_2O_2 are responsible for the injury and degradation of photosystem II (PSII). It is extensively accepted that UV-B radiation damages the donor side of photosystem II by deactivating the Manganese (Mn) cluster of water oxidation (Schmidt et al. 2016). Peroxide radical is the main reactive oxygen induced by UV-B radiation in the thylakoids of chloroplast (Das and Roy Choudhury 2014). Effective antioxidant defense systems, including enzymes and antioxidants have been developed by algae to counteract the toxicity of ROS (Mallick and Mohn 2000) (Fig. 12.2). Bowler et al. (1992) have concluded that UV-B radiation produces $O_2^{\bullet -}$, •OH, and H_2O_2 radicals.

12.5 Conclusion and Future Prospects

Increasing the temperature of the earth and subsequent high incidence of UV radiation and global warming causes adverse impact on living organism which leads to climate change. This climate change alters the vegetation and biodiversity of plants. The plants used so far to supply the energy and food demand could not meet the desired level and failed in the initial level. Therefore, there is an urgent need for the development of sustainable alternative for food and fuel. Algae being grown in high light condition (with high UV-B incidence) could be a protective measure to meet the energy demands and production of different value added products such as

medicine, food supplements, fodder, fertilizer, food, etc. However, ecotoxicological study, selection of suitable algal species and their consortia and genetic engineering might be done prior to its large scale cultivation and biomass production. Genetic engineering in microalgae could not only enhance the biomass production and lipid yield but also develop adaptation to withstand harsh environments without decreasing the biomass.

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Microalgae Mediated Nanomaterials Synthesis

13

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Abstract

Algae are well familiar for their CO_2 sequestration nature and as a potential alternative source of energy. At the same time algae have an equal potential to act as a green source to develop a new method of the nanoparticles synthesis. Although there exists number of different physical and chemical synthesis techniques but over their limitations green synthesis has been emphasized for last two decades. This chapter will briefly summarize the synthesis of the nanomaterials by different green process. Algae mediated synthesis processes are significantly explored for the different nanomaterials synthesis. Finally different characterization techniques and application of the algae mediated nanomaterials have been summarized.

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Keywords

Green synthesis · Algae · Nanomaterials · Intracellular mechanism · Extracellular mechanism

13.1 Introduction

Nanotechnology is one of the important interdisciplinary branch of science and technology that deals with manufacturing and applications of sub-100 nm materials and devices. The idea of nanotechnology was initially proposed by Noble laureate Richard Feynman in his famous talk "There is plenty of room at the bottom" at the California Institute of Technology (CalTech), 29th December, 1959. Among several aspects of nanotechnology, synthesis, characterizations, and applications of nanoparticles are important areas that have been studied extensively during the last few decades with gradual increasing activities as well as exciting discoveries. Materials having size sub-100 nm in any of its defined dimensions are termed as nanomaterials either it will be nanorod/nanotube (1D), nanosheet (2D), or nanoparticles (3D). Most importantly because of the small size these materials show different properties compared to the bulk material. Especially it shows quantum confinement effect and very high specific surface area and both are highly essential considering any specific application especially in catalysis and imaging. Among the different nanomaterials metallic nanoparticles especially noble metals are the division of metals which resists corrosion, oxidation, or attack by acids in humid ambience and have a wide range of application because of their excellent optical (González et al. 2008; Kadkhodazadeh et al. 2019), electronic (Fratoddi et al. 2017), electro-catalyst (Liu et al. 2015), photothermal (Jain et al. 2008), photocatalytic (Baba et al. 2017), catalytic, and electrical properties. The metals in Group 8 to 11 (Ru, Rh, Pd, Ag, Os, Ir, Pt, and Au) are considered as noble metals with exciting properties and wide application in distinct areas like medicinal, ornamental, imaging, electronics, garnishing items, coining, economical trade exchange, etc. Other than the metallic nanoparticles, oxides and metals salts especially chalcogenide semiconductors are equally important because of their catalytic and semiconductor properties. So in that situation it is always required an appropriate green technology to synthesize these materials with precise controlled size and shape for suitable applications.

The nanomaterials are synthesized by either "Top Down" or "Bottom Up" approaches. The "Top Down" approach is started from the bulk material whereas the "Bottom Up" approach is just the reverse, i.e., atoms/molecules are bind together to attain nanosize. In both approaches there are some advantages with some essential limitations. Generally in the Top Down approach by employing external power sources, the bulk materials are grounded to reduce the size in the nanodimension. Whereas, the Bottom up approach starts from atom to enhance the size in nanodimension. In current situation there are plenty of studies that are related to the synthesis of these nanomaterials by conventional (physical or chemical) and advanced processes. But all these processes have some limitations which are related

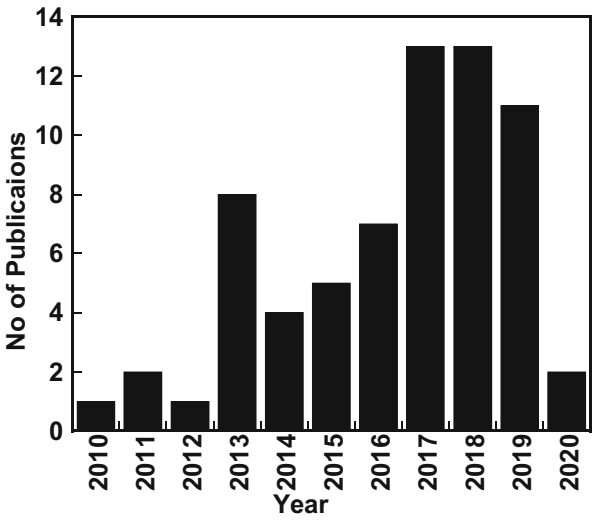

Fig. 13.1 Publications per year for "Nanomaterials Synthesis" and "Algae" keywords during the period 2010–2019 (data collected from the Scopus Database)

to either use of high energy or highly adverse condition or presence of any nondegradadable highly toxic chemicals. But due to increasing need of nanoparticles in myriad of application fields, it is essential to develop an alternative eco-friendly and economic process for the nanomaterials synthesis. So researches are now trending towards the usage of biomaterials starting from bio-waste to various microorganisms including, bacteria, fungi, algae, etc. for biogenic synthesis of nanoparticles. Algae, being autotrophic in nature, play a vital role in ecosystem. It has advantages of low nutritional requirement and less production of toxic metabolites over other microbes (Priyadarshini et al. 2019). Therefore, algae mediated nanoparticle synthesis vis-à-vis "phyconanotechnology" can be considered as the potential route of the biogenic synthesis of nanoparticles. A large number of research works was carried out to synthesize metallic nanoparticles like silver, gold, zinc, iron, copper, palladium, platinum, cadmium, etc., whereas, studies on algae based synthesis of metal oxide nanoparticles such as copper oxide, iron oxide, zinc oxide, etc. are also reported in literature. Figure 13.1 clearly shows a statistical data analysis on the published research papers and it indicates this area is not completely cultivated but in last five years a clear increasing trend is observed. These data were collected from Scopus Database using the keywords "Nanomaterials Synthesis" and "Algae."

Therefore considering the importance this chapter aims to highlight the progress of research advancement on the algae mediated nanomaterials synthesis. This is new area for the synthesis of nanomaterials which is yet to be explored fully. So here initially the conventional process and other green process are briefly described. Then the overall synthesis routes of different nanomaterials by algal biomass are considered with their detailed mechanism and finally a few important applications out of several others are also discussed to show the importance of these nanoparticles.

13.2 Different Methods of Nanomaterials Synthesis

13.2.1 Conventional Physical and Chemical Synthesis Methods

An array of Top Down and Bottom Up approaches are present which are still being practiced because of their efficacy. These approaches are associated with certain merits and demerits. For example in Top Down approach large scale production without any additional purification of certain particles size (10–1000 nm) is possible but at the same time obtained nanoparticles are in different shape with wide size distribution and possible impurities. Another limitation in Top Down approach is its highly expensive nature. Whereas with Bottom Up approach ultrafine nanoparticles with narrow size distribution (1–20 nm) is possible but again chemical purification is required with the limitation of large scale production. As these approaches have been categorized as physical, chemical, and biological synthesis methods, some of those methods have been taken up here. Physical methods mostly consist of gas/vapor phase mechanical fabrication techniques like ball milling (De Castro and Mitchell 2002), cutting, grinding, etching, lithographic techniques (Krämer et al. 2003; Haynes et al. 2002, 2001), and electrical discharge method (Tseng et al. 2013). Gas phase mostly contains Physical Vapor Deposition (PVD) using sputtering, laser ablation, thermal evaporation, electron beam evaporation, and electric arching but beside these methods, laser ablation and evaporation-condensation are the important physical tactics (Colfen 2003).

Basically in laser ablation method one metal rod (solid) is ablated with high power laser in a closed chamber consisting of Ar gas. The resulting plasma of respective metal atoms is then evaporated and condensed on specific substrate resulting in corresponding metal nanoparticles. With this method metal decomposition rate is high but quality is low. Efficacy of this method depends on various factors like laser light wavelength, laser pulse duration, etc. This method requires high temperature and low pressure which again is very costly (Jiménez et al. 2010; Barcikowski and Mafuné 2011). Arc discharge often called as evaporation-condensation method utilizes electric arc produced by the application of high voltage around 80–100 V which causes vaporization of material between 2 electrodes. Again the generated plasma is further evaporated and condensed on specific substrate to get the desired metal nanoparticles (El-Shall et al. 1996; Gouriet et al. 2009).

The chemical methods consist of liquid phase fabrication techniques like CVD using microwave (Francis et al. 2018) and wet chemical synthesis using sol–gel, micro-emulsion, and spray pyrolysis. Most importantly the wet chemical processes can be useful for the large scale production of nanomaterials (Tan and Zhang 2015). In Chemical Vapor Deposition (CVD) technique high performance and high purity materials can be synthesized, but this technique is little bit expensive. Here the nanoparticles are deposited on the inert substrate through the decomposition or reaction between one or more gas phase precusor. This is a low temperature process with which high purity is achieved (Palgrave and Parkin 2006). Whereas considering colloidal synthesis method, here different solutions (containing distinct ions) are mixed in controlled pressure and temperature to get desired insoluble precipitates

(Yang et al. 2016) in nanodimension. Generally the metallic nanoparticles are synthesized by the reduction reaction using either any conventional reducing agents like $NaBH_4$ (Dong et al. 2010; Male et al. 2008), hydrazine, tri-sodium citrate (Pillai and Kamat 2004), ascorbic acid (Zielińska et al. 2009), ethylene glycol, dimethylformamide (DMF), glucose, hydrazine hydrate, etc., whereas metal oxides are synthesized by sol–gel, hydrothermal or solvothermal process, and metallic salt materials are synthesized by precipitation reaction.

Micro-emulsion method results in either Reverse-Micelle structures or Langmuir–Blodgett films (Zhang and Chan 2003). Generally micro-emulsion is categorized either as water-in-oil (W/O) or oil-in-water (O/W) or bicontinuous system and can be used to synthesize nanoparticles with narrow size distribution. These W/O microemulsions are composed of nanosized water droplets stabilized by surfactants, co-surfactant, and dispersed in the continuous oil media. These droplets serve as a nanoreactor to form nanoparticles. However by this method small size particles with specific controlled size and narrow size distribution can be achieved. The size of the particles in reverse micelles preferably depends on dispersion medium, water to surfactant ratio, and the presence of co-surfactant. In this process the major limitations are difficulty in large scale production, higher synthetic surfactant consumption, difficulty in separation and purification of the materials (Eastoe et al. 2006).

13.2.2 Biological/Green Methods

In last two decades the synthesis of the nanomaterials by appropriate green/biological method is an alternative technique to overcome or minimize the limitations in the conventional techniques. Under this method generally natural green biogenic routes are being utilized to develop an alternative ecological and economical process for achieving the growing demand of nanomaterials. Eco-friendly nature, utilization of green resources in wet process, and simplicity in the process can make it scalable for bulk production. But the only limitation in this process is its slow reactivity and due to that polydispersed nanomaterials are formed. At the beginning different plant extracts are mostly used for the green synthesis of metal nanoparticles, but later on other greener source like algae, bacteria, fungi, actinomycetes and yeasts are considered to use as a source of require chemicals to produce nanomaterials (Narayanan and Sakthivel 2010). In terms of mechanism of the particle formation, both plants (Makarov et al. 2014) and microbes (Zhang et al. 2011) mediated synthesis follow two mechanisms i.e. extracellular and intracellular path mechanism. Moreover extracellular mechanism is bit easier than intracellular because the cells need not require any rupturing processes followed by purification steps such as centrifugation, filtration, etc. Hence downstream processes are reduced down by extracellular mechanism. Although large number of studies were performed on the green synthesis of the nanoparticle but most often mechanistic hypothesis is being discussed to explain the biochemical process and the complete

mechanism is yet to explored. Some different metallic nanoparticles synthesized by either plant extract or by microorganism assisted process are listed in Table 13.1.

13.2.2.1 Plants Assists Synthesis Method

In phytosynthesis either whole plant or parts of it (fruit, leaf, flower, stem, root, fruit peel, shoot) has been taken as direct biomass or extract or as phytochemicals for synthesizing nanoparticles. The plants assists method is mainly used for the synthesis of the noble nanoparticles because most of the plants are rich in biomolecules such as flavonoids, citric acid, amino acid, dehydrogenase, hydrogenase, NADP reductase, proteins, polyols, saponin, and many more which often acts as reducing and capping/stabilizing agents to yield stable noble metal nanoparticles when reacted with metal salt precursors. However Akhtar et al. have reviewed all 4 Ag, Au, Pt, and Pd metal nanoparticles synthesis using plants/plant parts (Akhtar et al. 2013). They have also discussed about the effect of pH, temperature, and incubation time to yield small sized nanoparticles with high absorbance peaks. Dauthal and Mukhopadhyay (2016) have also reviewed noble metals nanoparticles synthesis mediated by plants elucidating the live plant biomass, plant extract, and phytochemicals based mechanisms. They have further tabulated different antifungal, antibacterial, anticancer, antiparasitic activities revealed by plant mediated noble metal nanoparticles. Generally three different mechanisms i.e., intracellular, extracellular or phytochemical (as shown in Fig. 13.2) are exist depends on the source of the reducing agents.

13.2.2.2 Bacteria Assists Synthesis Method

These are prokaryotic organisms and biosynthesis of noble metal nanoparticles by utilizing bacteria may be observed either intracellularly or extracellularly depending upon the location where the reductive component is positioned. If the reductive components or enzymes are present within the cells and its interaction with metal ions occurs within those cells, then it is termed as intracellular mechanism. Here metal ions need to be transported within the cell. Basically these bacteria yield nanoparticles due to the energy which effluxes out during the decomposition of ATP to ADP by using ATPase. This efflux energy carries out the further chemical reaction and resists the toxicity of heavy metals. So as the result of this resistance towards toxicity, metal ions get transformed to metal nanoparticles. Moreover an intracellularly obtained nanoparticle requires additional downstream processing steps over extracellular mechanisms such as ultra-sonication, centrifugation, washing, and other purification steps.

But if these reductive enzymes are positioned at cell wall, then the nanoparticles would be obtained extracellularly and here the metal ions need to be trapped on the cell surface. Overall the bacterial reduction depends on either nitrate reductase or NADH reductase enzymes. This mechanism is mostly preferred commercially over intracellular mechanism because of its wider applications in bio-imaging, optoelectronics, sensor technology and in electronics, etc.

Table 13.1 Some microbial and plant species employed to synthesize noble metal nanoparticles

S. No	Plant/ microbes	Species name	Metal nanoparticles	Mechanism of particles formation	Morphology	References
1	Plant	*Diospyros kaki*	Pt	Extracellular	2–12 nm	Song et al. (2010)
2	Plant	Alfalfa Plant	Au	–	6–10 nm	Peralta-Videa et al. (2002)
3	Weedy plant	*Chenopodium album*	Ag and Au	Extracellular	Quasi-spherical/ 10–30 nm	Dwivedi and Gopal (2010)
4	Plant	Water hyacinth	Pt	–	Spherical/3.7 nm	Leo and Oluwafemi (2017)
5	Seed	Coffee and tea	Pd and Ag	–	20–60 nm	Nadagouda and Varma (2008)
6	Plant	*Cinnamomum zeylanicum*	Pd	–	15–20 nm	Sathishkumar et al. (2009)
7	Fruit	Banana peel	Pd	–	50 nm	Bankar et al. (2010)
8	Bacteria	*G. stearothermophilus*	Ag and Au	Extracellular	Au: 5–8 nm and Ag: 5–35 nm	Mohammed Fayaz et al. (2011)
9	Bacteria	Protein extract	Pt	–	Square, rectangle/ 50–300 nm	Riddin et al. (2010)
10	Bacteria	*Escherichia coli* and *Desulfovibrio desulfuricans*	Pd	Intracellular and Periplasmically	1–5 nm and 20–30 nm	Gomez-bolivar et al. (2019)
11	Yeast	*P. jadinii*	Au	Intracellular	Various	Gericke and Pinches (2006)
12	Fungi	*Neurospora crassa*	Ag, Au, and, bimetallic	Intracellular	Spherical/11 nm-Ag and 32 nm-Au	Castro-Longoria et al. (2011)
13	Fungi	*V. luteoalbum*	Au	Intracellular	10 nm/Spherical	Gericke and Pinches (2006)
14	Yeast	Yeast strain MKY3	Ag	Extracellular	2–5 nm	Kowshik et al. (2003)

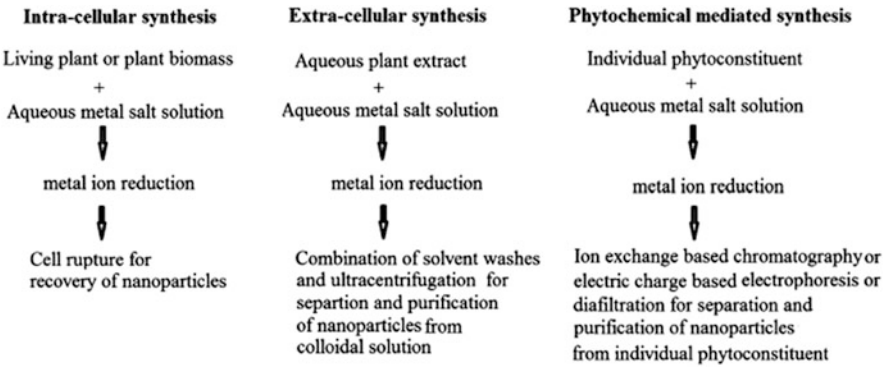

Fig. 13.2 Plant based nanoparticles synthesis approach. Reprinted with permission from Dauthal and Mukhopadhyay (2016). Copyright (2016) American Chemical Society

13.2.2.3 Fungi Assists Synthesis Method

Fungies are eukaryotic organisms which forms decomposing creature and compare to the other microbes or plant materials, the fungus growth rate is rapid. Fungus mycelia can endure oscillation in the pressure and flow rate inside bioreactors which enhance the rate of particles formation. Like bacteria, fungi also reflect intracellular and extracellular mechanisms. Enormous number of reducing chemical often secrets from the cell so extracellular mechanism is very common in fungi assist synthesis process. One important thing to notice is that certain fungal species capable of producing different metal nanoparticles does not mean that all varieties of same species can produce all noble metal when incubated with respective salt solution. Narayanan and Sakthivel (2010) have discussed about *Fusarium* species i.e. *F. moniliforme* is capable of producing silver nanoparticles. Beside this they have also tabulated lots of fungi, biomolecules, bacteria, yeast, virus, and actinomycetes which are utilized for nanoparticles synthesis by intracellular or extracellular mechanism. Moghaddam et al. (2015) have done an outstanding work by reviewing the biosynthesis of nanoparticles (Ag/Au) intensively using fungi and yeast. They have tabulated potential yeast and fungi species employed for the biosynthesis of AgNPs and AuNPs. Along with this several distinct merits of fungi have been displayed such as preferably high wall linking ability, economic livability, etc.

13.2.2.4 Yeast Assists Synthesis Method

Yeast (eukaryote) is another microorganism which possesses a special feature of growing with simple nutrients and producing various enzymes in any laboratory conditions. Similar to the other microbes, yeast has a potential to synthesize the nanomaterials. Moghaddam et al. (2015) have reviewed different major fungi and some yeasts which can synthesize the bio-fabricate metallic nanoparticles.

They mentioned the basic biomolecules for nanoparticles synthesis are quinones and oxidoreductase enzymes. Herein they have tabulated lots of fungi and yeast of biological importance. However Apte et al. (2013) have synthesized AgNP using

marine strain named *Yarrowia lipolytica* (NCYC 789) from which they have extracted melanin pigment and using this they synthesized AgNPs which showed excellent antimicrobial activity (as shown in Fig. 13.3). But Mourato et al. (2011) used extremophilic yeast from mine drainage for biosynthesizing crystalline AuNP and AgNP. They got AgNPs of size <20 nm and AuNPs between 30 and 100 nm using fungal biomass. Also they elucidated that yeasts release protein which acts as reducing and stabilizing agents.

13.2.2.5 Actinomycetes Assists Synthesis Method

These microbes share significant characteristics of bacteria and fungi. However it is classified as prokaryote which is capable of producing secondary metabolites which often serve as antibiotics. Generally actinomycetes and fungi are rich in enzymes and actinomycetes contain large amount of cytosine with guanine inside their DNA due to which they are often used for catalyzing particular reactions. Their growth is branched filamentous. Sastry et al. (2003) have synthesized 7–10 nm gold nanoparticles by using *Thermomonospora* sp. which is an extremophilic actinomycete. Here the reaction temperature was kept at 50 °C and pH equals to 9 which is extreme condition for any microbe to sustain. Abdeen et al. (2014) synthesized AgNP using Actinomycete isolated from 2 distinct soils. As per their study the biological method can produce smaller size particles (10–20 nm) compared to the chemical method (60–80 nm) and these small sized particles are more effective to act as an antibacterial agent.

These plant extract and microbes consist of varieties of biomolecules like enzymes, proteins, amines, phenolic compounds, alkaloids, and vast range of pigments which supports biosynthesis of nanoparticles by either acting as a reducing or/and stabilizing agents. Although plant based synthesis method have been quite explored but still the requirement of desired nanoparticles with specific morphologies is yet to be achieved. Meanwhile terrestrial microbes are capable enough of growing in harsh, light, or dark conditions when exposed to few amount of nutrients. But it becomes very difficult to deal with such microbes as their growth is difficult to control under normal conditions and they require specific parameters like temperature, pH, media composition to sustain in laboratory conditions.

13.3 Algae Mediated Nanoparticle Synthesis

Similar to the different microbes such as bacteria, fungi or actinomycetes, algae is another option to utilize as a source of appropriate chemicals for the nanomaterials synthesis. Over decades fewer significant work has been done by researchers considering algae which came out as useful support with desired results because of their richness in biomolecules which efficiently reduces metal ions to metal nanoparticles. Microbes (excluding algae) are terrestrial in nature and have a tendency to contaminate or make environment pollution whereas algae can be considered as safest one to deal with for the green synthesis of nanoparticles. Algae are eukaryotic photosynthetic organisms which may be unicellular (Diatoms or

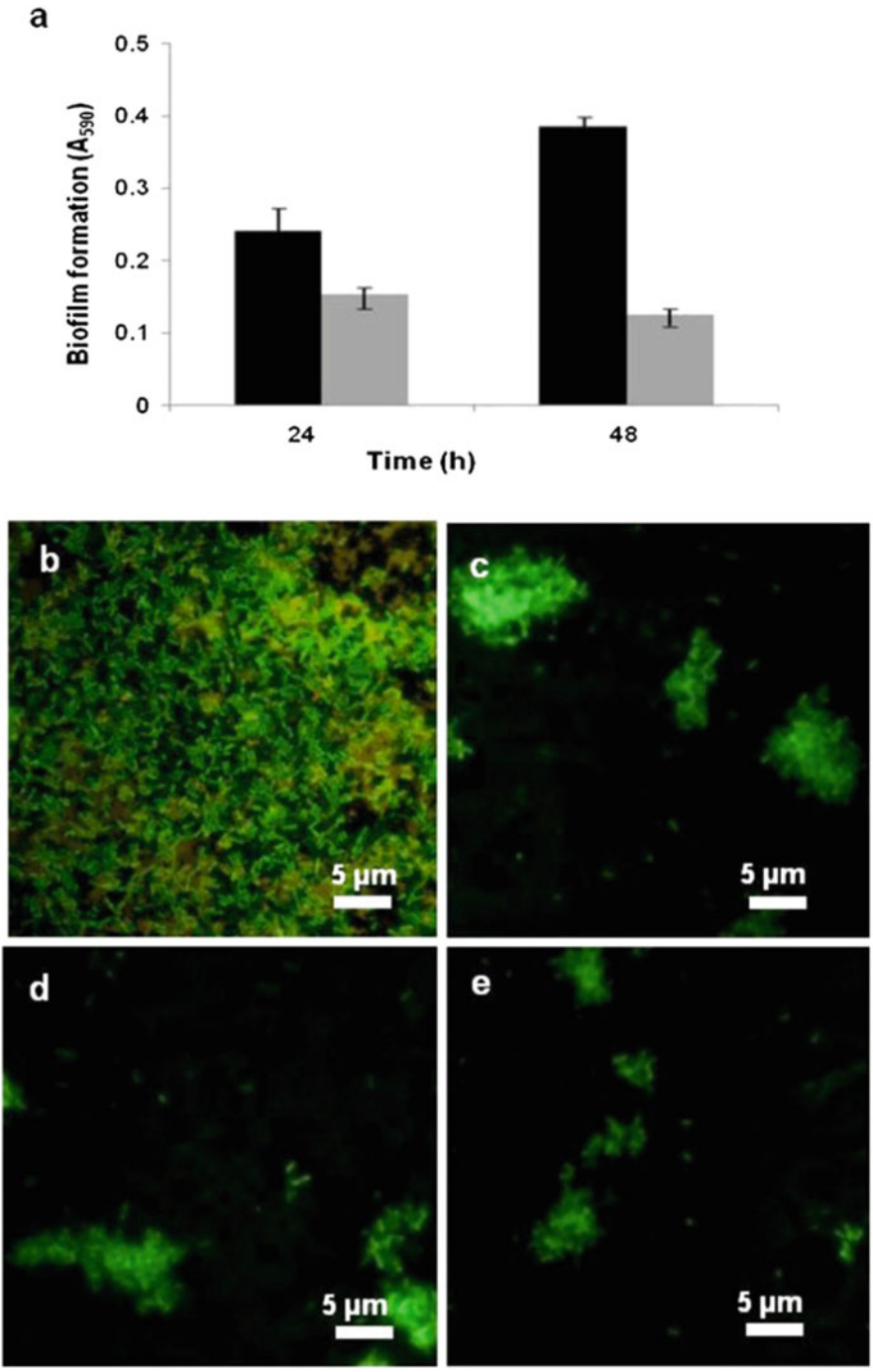

Fig. 13.3 Representative results of antibiofilm activities of the melanin mediated silver nanoparticles. (**a**) Inhibition of *Salmonella paratyphi* biofilms by AgNPs in microtiter plates (control: black bars; test: gray bars). Fluorescent microscopy images of *S. paratyphi* biofilms (**b**)

Chlorella) or multicellular (Brown alga or Kelp) and aquatic autotrophs which can survive easily in natural aquatic systems. They possess chlorophyll as photosynthetic pigment and the chloroplast which reveals similar structure as that of cyanobacteria (blue green algae). According to Barsanti and Gualtieri, "the term algae has no formal taxonomic standing" (Barsanti and Gualtieri 2014) or Guiry "generally algae are considered to be aquatic, oxygen-evolving photosynthetic autotrophs that are unicellular, colonial or are constructed of filaments or composed of simple tissues." (Guiry 2012); the algae can be good alternative and safest option to get nanomaterials. Algae have chlorophyll pigment and because of that it is autotrophic photosynthetic eukaryotes which are being aquatic and have been exposed to varieties of organic and inorganic elements for over millions of years. Due to this, algae are adopting the reducing nature and are successfully investigated to biologically synthesize noble metal nanoparticles but the entire biochemical and molecular mechanism is yet to be explored precisely (Thakkar et al. 2010). Several works carried out so far and as per report the experimental process and morphologies of the nanomaterials are well described but none has elucidated about biomolecules which is mainly involved in the reaction to get the nanomaterials.

Also over terrestrial organisms, aquatic organisms are found to possess biomolecules which do not limit the reaction with metals or metal oxides or metal sulfide. But comparative to microbes other than algae, the literature support is quiet less for algal based synthesis. As per algae based synthesis method is concerned, this can be categorized in different ways: firstly, based on types of algae whether macro- or micro- or cyanobacteria, further, whether freshwater or marine water algae; secondly, based on place of occurrence whether intracellular or extracellular space; thirdly, types of chemicals responsible for biosynthesis—whether algal extract or intracellular enzyme, vitamin, saccharides, etc. In this section, the role of chemicals in biosynthesis of nanoparticles will be discussed. Several algae were reported to be used for synthesis of gold nanoparticles. Some of them are *Sargassum wightii, Laminaria japonica, Gracilaria corticata*, etc. (Khan et al. 2019). At the same time, cyanobacteria such as *Spirulina platensis, Calothrix* spp., etc., were also used for the same purpose. The cell extract constituting of several chemicals such as glutamic acid, andrographolide, oleic acid, hexadecanoic acid, gallic acid, stearic acid, epigallocatechin catechin, 11-eicosenoic acid, etc., were used to synthesize metallic nanoparticles. The said chemicals may act as reducing as well as stabilizing or capping agent (Khan et al. 2019). Porphyran, a sulfated carbohydrate and derived from red seaweed was used for synthesis of gold nanoparticle. The intracellular production of gold nanoparticles is also being reported. The enzyme present in cell wall of the algae *Tetraselmis kochinesis* acted as reducing agent for intracellular production of gold nanoparticles. *Fucus vesiculosus*, a brown alga, was used for bioreduction of Au (III) to Au (0). The bioreduction occurred with sharp decrease in pH and redox potential. The hydroxyl group present in the algal polysaccharides

Fig. 13.3 (continued) control, (**c**–**e**) with 2.5, 5.0, 10.0 mM AgNPs, respectively. Reprinted with permission from Apte et al. (2013). Copyright (2013) Springer

played the key role in the bioreduction process. The reducing capabilities of hydroxyl group present in the sugar molecule of algal cell reduce the metal precusor to metallic nanoparticles and at the same time these bulky groups act as a capping agent to reduce the growth of the particle and maintain particles size in nanodimension. The metabolites (terpenoids and flavonoids) present in algal broth (*Chaetomorpha linum*) acted as effective capping and stabilization agent for synthesis of AgNP. Similar to metal nanoparticles metal oxides are also synthesized by the algal extract. In case of ZnO nanoparticles synthesis, the water soluble pigment present in the algal extract acts as reducing and stabilizing agent. Thylakoids as present in algae play key role in biosynthesis of materials like sugar molecules; the role of other biomolecules like protein is also established in biosynthesis of metal nanoparticles. Amino acids, the building block of protein molecules, help in fabrication of nanostructures. Arginine, *Tryptophan,* Glutamine, Methionine, Tyrosine, etc., are few examples of amino acids involved in the process of nanoparticle synthesis. The protein present in *Lemanea fluviatilis* played vital role in synthesis of AuNP by acting as templating and stabilizing agent. The microsphere of calcium carbonate was formed by biomineralization of CO_2 using *Chlorella* sp. KR-1. Such microsphere was used as economical matrix for synthesis of AgNP (Sharma et al. 2019).

In general it can be said that the mechanism for phycosynthesis of NPs is yet to be fully understood. It is rational to consider the role of biomolecules such as enzymes, proteins, and polysaccharides present in algal membrane as reducing agent. Such molecules reduce the metal salts to zero valent metal. Further, owing to their large structure and amphiphilic nature, these compounds act as capping agents to enhance the surface charge density (zeta potential) on the nanomaterial for enhancing the dispersibility. Based on the properties of the materials, the algae mediated synthesis can be classified in three different categories: (1) metallic, (2) metallic salts, and (3) metal oxide nanoparticle synthesis.

13.3.1 Metallic Nanoparticles Synthesis Using Algae

Among the different nanomaterials, metallic nanoparticles synthesized by algae mediated process is the most common practice and among different metals noble metals especially Au and Ag are extensively studied by the researchers because of their potential applications in different areas due to their excellent optical and catalytic properties. The synthesis of metallic nanoparticle by any green process is required a suitable metal salt precursor, reducing agent (from green source), capping/stabilizing agent to control the particles size and shape. Whereas in case of microbes mediated process the microbes secrete reductive enzymes or metabolites which directly reduce the metal precursor. So in case of algae mediated synthesis process

either algal extract or biomass is exposed to the corresponding metal salt precursor to obtain respective metal nanoparticles. The most important features of microalgae for employing them for the biosynthesis process is their surface charge, generally negatively charged algae act as appropriate capping agent to control growth which ultimately give smaller sized nanoparticles in less expensive way so that there is a possibility of large scale production. Some examples of different metallic nanoparticles synthesized by the algae are listed in Table 13.2.

Algae are rich sources of polysaccharides and proteins, so the algal extract can be used as alternative source of reducing chemicals for synthesis of nanoparticles. Figure 13.4 schematically shows the metallic nanoparticles synthesis by algae extract. Algae are mostly found to flourish under water that may be contain nutrients and heavy metals which are toxic to algae. These heavy metals are capable of altering the bimolecular activities inside the algae by either blocking the exposure of functional groups with the outside metal ions or by replacing the binding of important metal ions with those heavy metals only and due to which they have the ability to sustain in different habitats. Hence they affect the green synthesis of noble metal nanoparticles. However algae are potentially competent to withstand against toxicity of heavy metals due to the resistive mechanisms against heavy metals attained by algae (Priyadarshini et al. 2019). Figure 13.5 shows the schematic description of the resistive mechanism of the algae. Here the algal resistance against toxicity caused by heavy metals is explained in four steps. Firstly Biosorption of heavy metals by algae occurs at the cell wall which limits their diffusion into the cell. It is basically an extracellular tactic against the heavy metals. It primarily occurs because of electrostatic interaction (because of negative charge on cell surface due to polysaccharides such as cellulose and metal ions are positively charged) caused by algal surface and heavy metal ions out of electrostatic force (either chemical bonding or *van der Waal* force or sometimes both). Secondly, if cells remain alive out of the toxicity and stress caused by heavy metals so due to metabolic activities and physiochemical changes, they act upon heavy metals and result in their bioaccumulation as these metals keep on precipitating different organelles on cell surface. Thirdly for surviving under stress condition these alga secretes detoxifying or chelating agents/biomolecules such as peptides (Phytochelatin or metallothioneins), proteins, enzymes, etc. and that makes a comple with these metal in different cellular organelles which reduce the toxicity of these metals. Fourthly due to excessive accumulation of heavy metal ions which becomes toxic for algae, a non-specific efflux/transport system or defense mechanism is created by algae itself to maintain metal concentration inside the cell. So few metal ions get bio-transformed to complex metal and escape out of cell while other metal ions get transported out due to the efflux energy liberated by bimolecular conversion of NATP to NADP.

Beside these *Cystoseira baccata,* a brown macroalgae, (González-Ballesteros et al. 2017) was studied and found capable of producing spherical polycrystalline AuNPs within size scale of 6.2–12.6 nm. It was also tested for treatment against cancer cells activity in in vitro condition which proved to be nontoxic for the respective application. On the other hand *Chlorella pyrenoidosa* was successfully

Table 13.2 Algal species utilized for green synthesis of noble metal nanoparticles

S no.	Algal species	Nanoparticle	Mechanism of particles formation	Morphology	References
1	Brown alga/ *Stoechospermum marginatum*	Au	–	Spherical, hexagonal, triangle/ 18.7–93.7 nm	Arockiya Aarthi Rajathi et al. (2012)
2	*Sargassum bovinum* alga	Pd	–	Octahedral/ 5–10 nm	Momeni and Nabipour (2015)
3	*Fucus vesiculosus*/ Brown alga	Au	Biosorption/ bioreduction	Au(III)-Au (0)	Mata et al. (2009)
4	*Sargassum wightii*/ Marine alga	Au	Extracellular	8–12 nm	Singaravelu et al. (2007)
5	*C. crispus*/Red alga and *S. insignis*/ Green alga	Au and Ag	Extracellular	30–50 nm/ spherical	Castro et al. (2013)
6	*Ecklonia cava*/ marine brown alga	Au	Extracellular	Spherical, triangle/ 29.75–30.25 nm	Venkatesan et al. (2014)
7	*Galaxaura elongate*/ powder or extract	Au	–	Spherical, triangle, rod/3.85–77.13 nm	Abdel-Raouf et al. (2017)
8	*Ulva fasciata*/Green seaweed extract	Au	Extracellular	7–13 nm/ spherical	Kumari et al. (2014)
9	*Sargassum swartzii*/ Brown algae	Au	Extracellular	Spherical/ 35 nm	Dhas et al. (2014)
10	*Kappaphycus alvarezii*/Marine alga	Au	Extracellular	Spherical/ 10–40 nm	Rajasulochana et al. (2010)
11	*Turbinaria ornata*/ Brown Sea weed	Au	Extracellular	Spherical/ 7–11 nm	Ashokkumar and Vijayaraghavan (2016)
12	*Padina gymnospora*/ Brown marine macroalga	Au	Extracellular	53–67 nm	Singh et al. (2013)
13	*Spirulina platensis*/ Blue green algae	Au	Extracellular	Spherical/ 20–30 nm	Cheng et al. (2014)
14	*Prasiola crispa*/ Green algae	Au	Extracellular	Spherical/ 5–25 nm	Sharma et al. (2014)
15	*Sargassum muticum*/ marine microalgae	Au	–	Spherical/ 5.67–6.7 nm	Namvar et al. (2015)
16	*Tetraselmis cochinensis*	Au	Intracellular	5–35 nm	Senapati et al. (2012)

(continued)

Table 13.2 (continued)

S no.	Algal species	Nanoparticle	Mechanism of particles formation	Morphology	References
17	*Caulerpa racemosa*/ marine algae	Ag	Extracellular	5–25 nm	Kathiraven et al. (2015)
18	*Cystophora moniliformis*/brown marine algae	Ag	–	75 nm	Prasad et al. (2013)
19	*Chlamydomonas reinhardtii*/algal extract	Ag	–	4–6 nm	Indu et al. (2011)
20	*Chlorella vulgaris*/ algal extract	Au	–	Spherical/ 2–10 nm	Annamalai and Nallamuthu (2015)
21	*Plectonema boryanum* UTEX 485/cyanobacteria	Pd	Extracellular	Spherical/ <30 nm	Lengke et al. (2007)
22	*Chlorella pyrenoidosa*	Ag	–	5–20 nm	Aziz et al. (2015)
23	*Plectonema boryanum UTEX 485*/cyanobacteria	Au	Extracellular	Octahedral/ <10 nm	Lengke et al. (2006)
24	*Calothrix algae*/blue green algae	Au	–	Spherical/ <100 nm	Kumar et al. (2016)
25	*Phormidium valderianum, Microcoleus chthonoplastes, P. tenue*/ cyanobacteria	Au	Intracellular	Triangular, hexagonal/ <100 nm	Parial et al. (2012)
26	*Gracilaria corticata*/ marine algae	Au	Extracellular	Spherical/ 45–57 nm	Murti et al. (2015)
27	*Pithophora oedogonia*	Au	–	Spherical/ 32.06 nm	Li and Zhang (2016)
28	*Rhizoclonium hieroglyphicum*	Au	Intracellular	Spherical/ <20 nm	Chakraborty et al. (2009)
29	*Chaetomorpha linum*	Ag	–	Clusters/3–44 nm	Kannan et al. (2013)
30	*Spirogyra varians*	Ag	–	Quasi sphere/ 35 nm	Salari et al. (2016)
31	*Scenedesmus* sp.	Ag	Both intracellular and extracellular	Spherical crystalline/ 15–20 nm	Jena et al. (2014)
32	*Ulva lactuca*	Ag	–	Cubical/20–35 nm	Murugan et al. (2015)
33	*Laminaria japonica*	Ag	Extracellular	Spherical, oval/32 nm	Kim et al. (2016)

(continued)

Table 13.2 (continued)

S no.	Algal species	Nanoparticle	Mechanism of particles formation	Morphology	References
34	*Chaetomorpha linum*	Ag	–	Clusters/3–44 nm	Kannan et al. (2013)
35	Diatoms (*P. antarctica/A. kerguelensis*)	Ag	Extracellular	Spherical/6–13 nm	Shivaji et al. (2011)

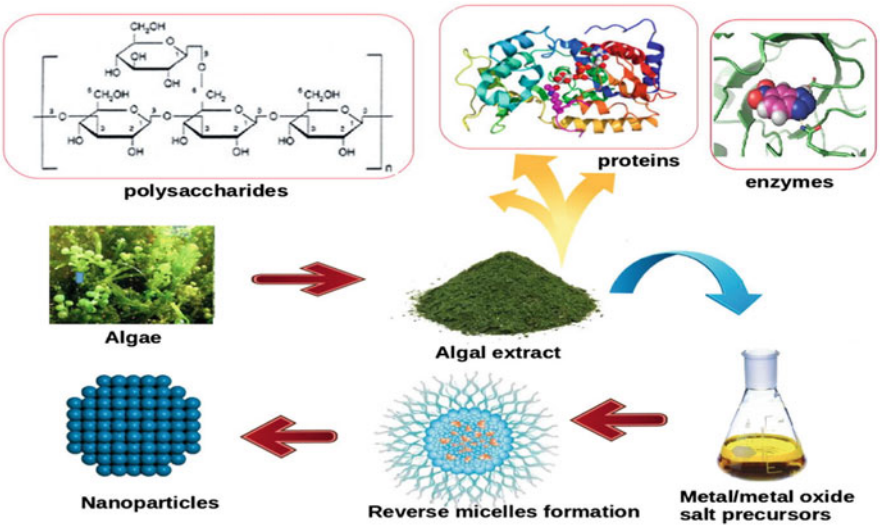

Fig. 13.4 General nanoparticles synthesis mechanism by employing algae. Reprinted with permission from Sharma et al. (2019). Copyright (2019) Elsevier Limited

tested for synthesis of AgNPs and revealed significant antibacterial properties with enhancement in photocatalysis (Aziz et al. 2015). However Khanna et al. (2019) have reviewed different types of green, red, and brown algae species employed for the synthesis of AgNPs, AuNPs, PtNPs, and PdNPs. Asmathunisha and Kathiresan (2013) have reviewed nanoparticle's biosynthesis by considering marine microorganisms where they have discussed about *Fucus vesiculosus*, *Navicula atomus*, *S. wightii*, and *Diadesmis gallica* algal species to get inorganic metal nanoparticles. As per their study a few marine, seaweed, and a brown alga are capable of synthesizing AgNPs and AuNPs. As marine microbes are in deep down the ocean for a significant time period with verities of organic and inorganic minerals and elements, so a distinct reducing potential have been developed within these algae and that can reduce metal ion to metal nanoparticles. However they have described that *S. wightii* produces stable AuNPs in size scale of 8–12 nm and *Fucus*

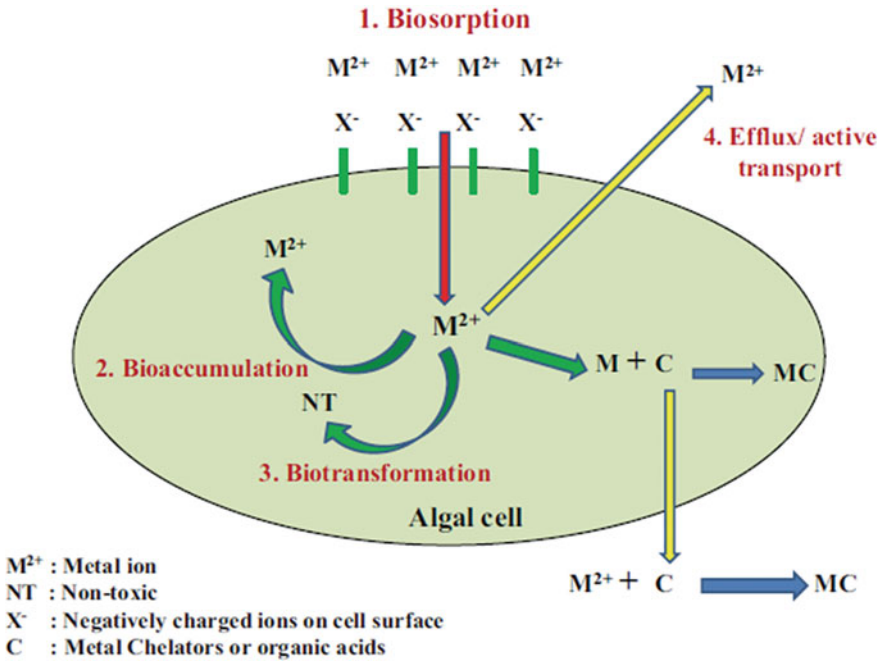

Fig. 13.5 Resistive mechanism of algae against heavy metals toxicity. Reprinted with permission from Priyadarshini et al. (2019). Copyright (2019) Springer

vesiculosus reduces Au (III) via biosorption followed by bioreduction. Whereas *Navicula atomus* was found capable of producing AuNPs along with Silica-Au nanocomposites. Moreover it has been found that the nanoparticles synthesized by using same algal biomass and extract reveal dissimilar characteristics. Talking about filamentous *Cyanobacteria*, *P. boryanum* was employed for AuNPs synthesis with gold chloride solution. Here this cyanobacteria successfully reduced Au (III) to Au (I) specie having morphology as octahedral platelets in size range of 10 nm–6 μm along with an intermediate gold-sulfide (Hulkoti and Taranath 2017).

Among the other metallic nanoparticle PdNPs is also synthesized (as shown in Fig. 13.6) by a green microalgae and these PdNPs shows the excellent catalytic activity (Gahlawat and Choudhury 2019). Here we can observe that the photosynthetic algae produce reducing agents such as NADPH, ATP, ADP, RuBiSCO, Fd, etc. which carries out the biochemical reaction between $Na_2[PdCl_4]$ with the biomolecules (acted as reducing and stabilizing agents) to produce PdNPs, PdO, and immobilized Pd particles on nanofiber mat (chitosan) which further used as catalyst. Below schematic diagram depicts the whole process occurring within the algal cell.

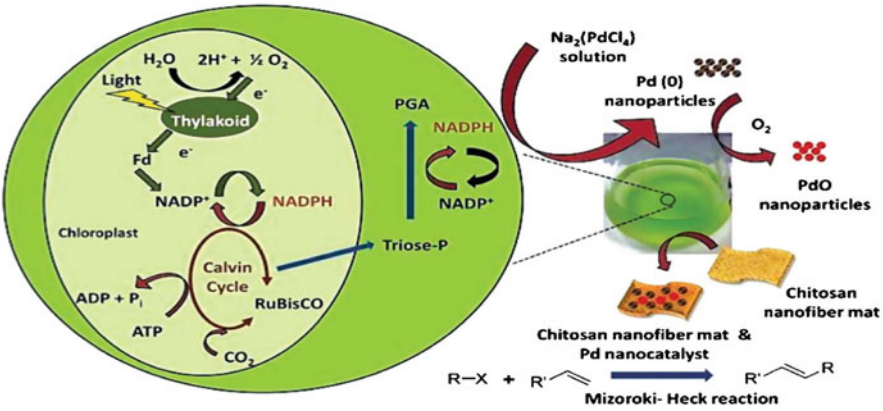

Fig. 13.6 Green microalgae based PdNPs synthesis mechanism. Reprinted with permission from Sharma et al. (2019). Copyright (2019) Elsevier Limited

13.3.2 Metal Salt Nanoparticles Synthesis Using Algae

Beside metallic nanoparticles, algae are found to be capable of synthesizing different metallic salts when exposed to the respective metal precursors. Because of the vast range of biomolecules present in the algal species that could easily react upon the respective metal precursors to precipitate the corresponding metal salts without formation of any toxic by-product. However peptide like phytochelatins found in *Phaeodactylum tricornutum* (phytoplanktonic alga) was useful in detoxifying the cellular mechanism carried out to form CdS nanocrystals. Earlier alga was exposed to Cd which formed Cd-phytochelatins complex then S ion was introduced which acted as stabilizing agent leading to the formation of CdS nanocrystallites (Scarano and Morelli 2003). Beside those algae which are utilized for green synthesis of metallic nanoparticles, there also exist some harmful algae which have not been utilized for biosynthesis purposes. Cui et al. (2019) have studied about the harmfulness of algae/algal blooms which can synthesized Fe/C composites by using ferric nitrate as metal salt precursors. These obtained Fe/C composites were found capable of efficiently removing Cr (VI) as a result of adsorption and reduction. 5 nm spherical CdS nanoparticles were synthesized by using *Chlamydomonas reinhardtii* (Rao and Pennathur 2017). These metallic nanoparticles shows excellent optical properties and photocatalytic activity to degrade 90% of synthetic dye without producing any additional toxicity which makes algae mediated this process will be an appropriate less expensive and eco-friendly alternative to treat the dye containing wastewater.

13.3.3 Metal Oxide Nanoparticles Synthesis Using Algae

Beside metals and metal salts biosynthesis, few algae can synthesize metal oxide nanoparticles with their desired properties. So far metal oxides are considered

especially zinc, titanium, and copper oxides nanoparticles were widely used in surface coating for its catalysis, sensing, and electronics properties to develop sensor devices or catalytic applications. Recently Sadek et al. (2019) have worked out with *Sargassum muticum*, a brown algae to synthesize spherical CuO and ZnO nanocomposites of 15–20 nm. These nanocomposites were tested for anticorrosion and antifouling property which they revealed successfully. Whereas Abboud et al. (2014) have reported the use of a new brown algae which is *Bifurcaria bifurcate* for biosynthesizing crystalline CuO nanoparticles (5–45 nm) and this crystalline CuO possesses antibacterial property against 2 specific bacterial strains viz. *Staphylococcus aureus* and *Enterobacter aerogenes*. Another case of photocatalytic activity by *Chlamydomonas reinhardtii* against methyl orange was studied by synthesizing nanoflowers shaped ZnO which could be assembled to form nanorods or nanosheets (Rao and Gautam 2015). Biomedical applications of FeO encapsulated diatoms (Todd et al. 2014) have also been demonstrated.

13.4 Characterization of the Nanomaterials

Characterization of the as synthesized materials is the most important task in nanotechnology before performing any kind of application because the properties of the nanomaterials are completely dependent on the size and shape of the materials. The characterization of the nanomaterials will give a clear idea about their morphologies, compositions, crystal structures, surface area, surface energy, surface charge, porosity, and associated functional groups to apply those materials for their respective applications. The most common characterization are listed in Table 13.3 to get minimum information about synthesized materials.

However Modena et al. have nicely elucidated the whole nanoparticles characterization techniques by considering dry powder as well as suspended dispersion and as per their proposal this improved the performance of individual techniques (Modena et al. 2019). The properties like shape, size, porosity, and surface charge of the nanoparticles directly affect their functionality with the respective purposes. Most common characterization techniques are UV absorption spectroscopy, microscopic analysis, scattering analysis, electro kinetic properties, and thermal gravimetric

Table 13.3 Different characterization techniques for nanomaterials (Quadros and Marr 2010)

S. no.	Characteristics	Technique
1	Morphology	• Transmission electron microscopy (TEM)
2	Crystal structure	• Scanning electron microscope (SEM) • X-ray diffraction (XRD)
3	Composition	• Energy dispersive X-ray spectroscopy (EDS)
4	Functionality and surface composition	• Fourier transform infrared spectroscopy (FTIR) • X-ray photoelectron spectrometry (XPS)
5	Size distribution (aerosol)	• Transmission electron microscopy (TEM)
6	Surface charge	• Aerosol electrometer

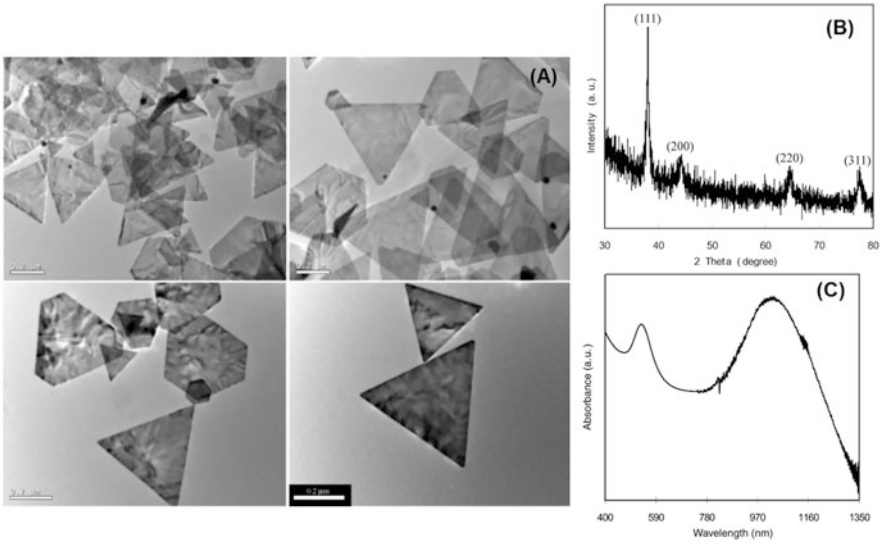

Fig. 13.7 (**a**) TEM pictures of AuNPs produced under the following conditions: 5 mL of 1-day aged *Sargassum* sp. seaweed extract, 5 mL H_2O and 1 mL $HAuCl_4$ (10 mM), at pH 7 and RT for 3 h of reaction time. (**b**) XRD pattern of AuNPs obtained under the same conditions as (**a**) except for the age (5 days) and reaction time (5 h). (C) UV–Vis spectrum of AuNPs obtained under the same conditions as (**a**) except for the age (7 days). Reprinted with permission from Liu et al. (2005). Copyright (2005) American Chemical Society

analysis which will give a clear idea about size, shape, and stability of the nanomaterials (Ghosh Chaudhuri and Paria 2012). UV–Vis absorption spectroscopy is the common initial characterization technique especially for the noble metals, because individual materials have specific absorption spectrum which is completely identical. Figure 13.7b shows UV–Vis spectrum of AuNPs obtained by *Sargassum* sp. seaweed extract. Similarly XRD analysis will provide the detailed crystalline property, Fig. 13.7c the XRD pattern of same AuNPs for the age 5 days, the sharp peaks indicate the crystalline nature. Microscopy is the most important direct characterization related to the particle size and shape (as shown in Fig. 13.9). Figure 13.7a shows the TEM image of the AuNPs produced in 5 mL of 1-day aged *Sargassum* sp. seaweed extract, 5 mL H_2O, and 1 mL $HAuCl_4$ (10 mM), at pH 7 and RT for 3 h of reaction time, which clearly indicate the size and triangular shape of the particles. Figure 13.8a shows the SEM image of AgNPs, whereas Fig. 13.8b shows EDX analysis of highlighted section in SEM image to determine the composition (elemental) of the nanomaterial. Almost 80% of AgNPs are encountered. Figure 13.9 shows AuNPs of different shapes like (a) nanoplates, (b) triangular, spherical, hexagonal, (c) spherical, (d) triangular, spherical, (e) oval shaped, (f) multiple shapes in presence of filtrate (live cell), (g) triangular, hexagonal, (h) membrane bound clusters or isolated specific size AuNPs synthesized by Blue Green Algae. However Aziz et al. (2015) have worked with *Chlorella pyrenoidosa* algae to synthesize AgNPs which are characterized by different characterization

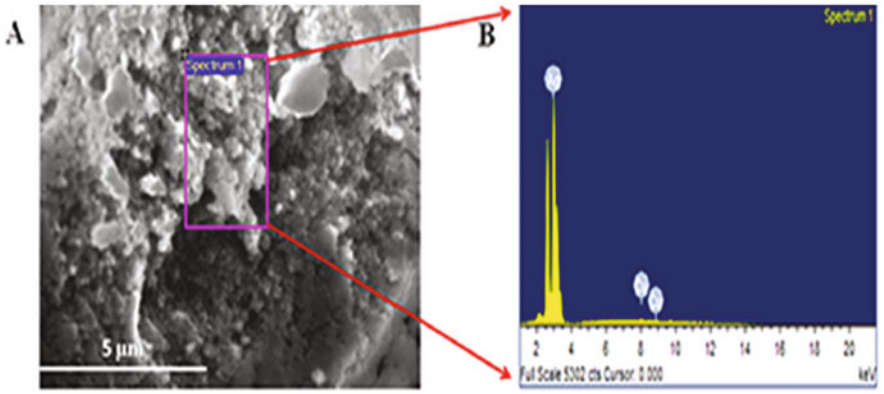

Fig. 13.8 SEM image and EDX spectra of AgNPs. Reprinted with permission from Aziz et al. (2015). Copyright (2015) American Chemical Society

techniques (UV absorption, SEM, TEM, XRD, and FTIR) and studied its photocatalytic and antibacterial properties. Beside these common characterization techniques the researchers also prefer to analyze the materials by Raman scattering and X-ray photoelectron spectroscopy (XPS) to perform the elemental analysis.

13.5 Application of Nanoparticles Synthesized by Microalgae

Nanomaterials are in so much demand because of their intensive physical, chemical, and biological properties. Nanoparticles synthesized by plants/microbes have got wide areas of applications such as biomedical (Zhao et al. 2018), catalysis (Shen et al. 2017), optics, electronics (Khan et al. 2017), water purification (Pradeep 2009), sensor (Priyadarshini and Pradhan 2017), etc. Figure 13.10 shows distinct biomedical application of green synthesized noble metal nanoparticles. Algal based synthesized nanomaterials are utilized significantly in different major areas related to biomedical (Baskar et al. 2015), electronics, catalysis (Zhang et al. 2016), sensors (Momeni and Nabipour 2015), photocatalysis, optics, antibacterial (Venkatpurwar and Pokharkar 2011), dye degradation (Sriramulu and Sumathi 2018), and lot more to be explored yet.

13.6 Conclusions and Future Prospect

Green synthesis of the nanomaterials is an important part of nanotechnology to overcome the adverse effect of conventional physical and chemical methods. Under the green synthesis process different plant extract and microbes are used for synthesis of the nanomaterials, but the plants and microbes like bacteria, fungi, and actinomycetes are not exposed to varieties of metal salts for longer duration and being terrestrial organism they lack this exposure. Whereas, the algae mediated

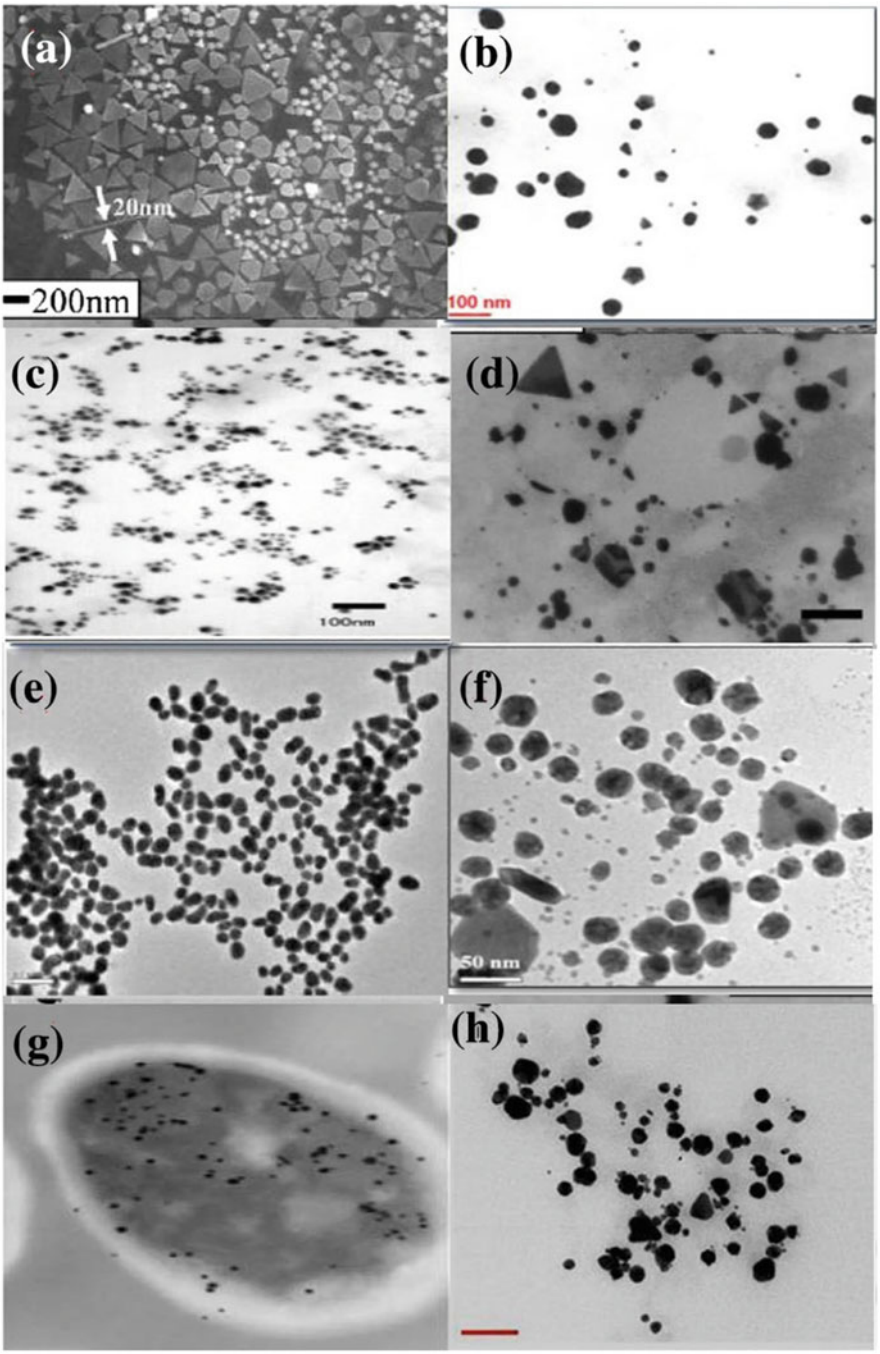

Fig. 13.9 Different types and morphologies of AuNPs synthesized by different algal species. Reprinted with permission from Khan et al. (2019). Copyright (2019) Springer

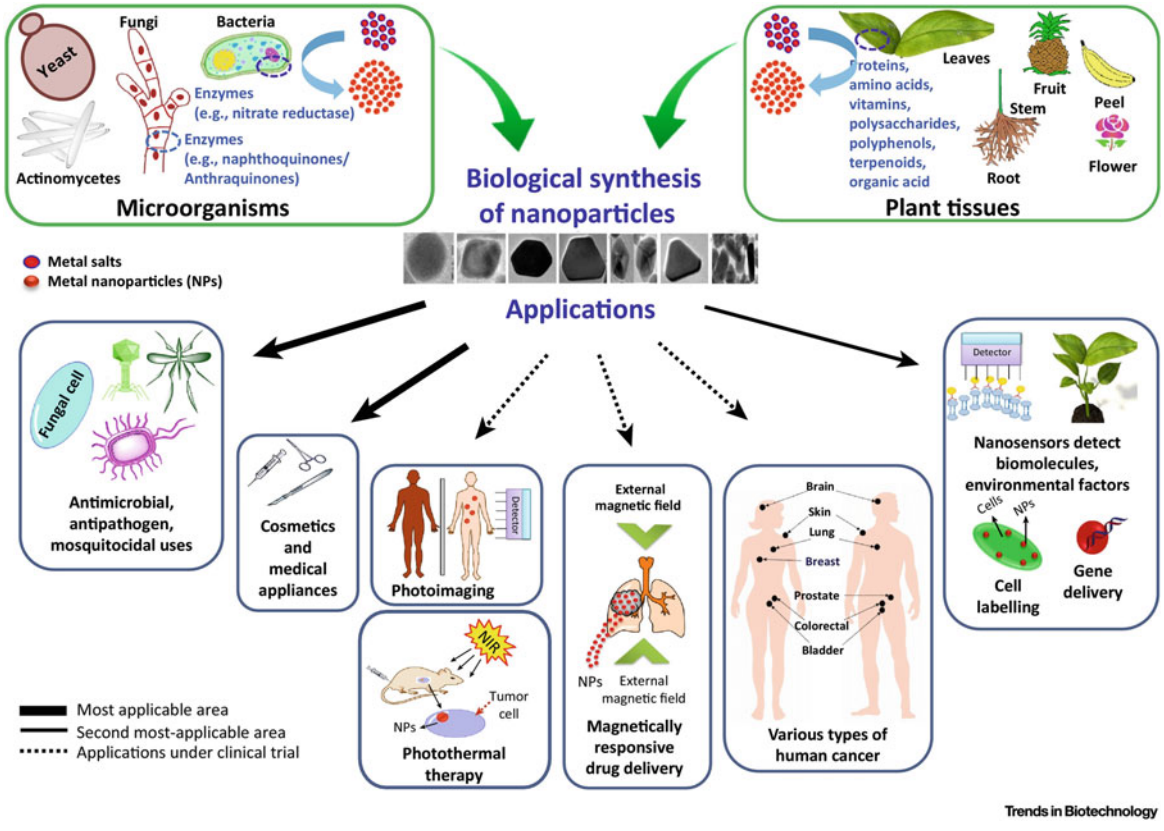

Fig. 13.10 Different biomedical applications of noble metal nanoparticles. Reprinted with permission from Singh et al. (2016). Copyright (2016) Elsevier Limited

nanomaterials synthesis process is one of the environment friendly and economical process. As the algae are being aquatic and terrestrial so it have been in contact with the numerous useful and heavy metals and because of that algae have induced distinct characteristics within themselves to sustain out of the stress caused by the toxicity of heavy metals. Due to that algae will be highly useful even in the presence of the other water impurities for the synthesis of nanomaterials. So an array of green, brown, red, micro-, and macroalgae have been found potent of synthesizing noble metal nanoparticles. Also algae based synthesized nanoparticles/nanomaterials have got major applications in significant fields like biomedical (Baskar et al. 2015), electronics, catalysis (Zhang et al. 2016), sensors (Momeni and Nabipour 2015) photocatalysis, optics, antibacterial (Venkatpurwar and Pokharkar 2011), dye degradation (Sriramulu and Sumathi 2018), and lot more to be explored yet.

As algae are both aquatic and terrestrial microbes so they have got wide range of exposures with organic and inorganic elements due to which they have divergent synthesis mechanism which is yet to be precisely investigated and elucidated compared to other microbes/plants based synthesis. Among the different types of the materials, metallic nanoparticles are widely studied, but metal salts or metal oxides are not properly focused. Few literatures are available on these materials but those are not consider the detailing of the mechanism of the particle formation. Especially algae consist of different varieties of biomolecules like enzymes, proteins, amines, phenolic compounds, alkaloids, and vast range of pigments, but which is participating in the nanomaterials formation reaction is not clear most of the time. The kinetic of the particles formation is too slow in the algae mediated synthesis process. So for the bulk production with proper control size and shape it is always required to understand the reaction mechanism. So more studies are required to develop the reaction mechanism so that the process will be simplified and scaled up easily. Finally, it is clear that there is still an enormous scope for future to establish the algae mediated nanomaterials synthesis process.

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Algae-Mediated Biological Synthesis of Nanoparticles: Applications and Prospects

14

Akhilesh Kumar Shukla, Atul Kumar Upadhyay, and Lav Singh

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Abstract

Synthesis of nanoparticles (NPs) through algae has been assumed a few significant roles in nano-based medications for human life improvement. Recently, there are few reports on the synthesis of NPs using algae, which are being utilized as a bio-processing plant for the blend against the microbes in various manners. Besides, NPs can be produced by physical and chemical techniques, for example, attrition, pyrolysis, etc. The physical and chemical strategies have different disadvantages, for example, significant expense of production, requiring high energy information, and generation of harmful side effects. To beat this, few biological techniques are utilized in the synthesis of nanoparticles. The biological techniques are generally cost-effective, non-toxic, and eco-friendly. This chapter deals with the algae-mediated metallic NPs synthesis and its applications.

Keywords

Metallic nanoparticles · Biosynthesis · Characterization · Algae · Biomedical applications

14.1 Introduction

Nanoscience has become a fast growing, charming, and attractive area in various fields of science. Nanoparticles (NPs) may be ordered into three types: natural, accidental, and engineered NPs (Buzea et al. 2007). The huge surface-to-volume proportion of nanoparticles, their capacity of simple interface with other particles, and a few different highlights make them a striking utensil in different fields of science (Gaurav et al. 2014). Interestingly, owing to the remarkable physicochemical characteristics of MNPs and its shape, researchers are keenly interested for NPs application in different scientific area of research such as natural bioremediation, biomedical science, bioimaging, engineering, and drug delivery (Guo and Wang 2011; Mandotra et al. 2018). The creation of NP with controlled crystallographic and physiochemical properties and surprising highlights has become an outstanding area to centre. The synthesis of NPs is accomplished by both physical as well as chemical methods. NPs are commonly synthesized by a conservative technique of wet method. Till date, metallic NPs, for example, nano-silver and nano-gold, are the popular nanomaterials made up by exploiting the capability of microalgae to reduce metals. Synthesis of nanoparticles is grown up in a fluid medium containing a blend of reactants prevalently reducing agents, including potassium bitartrate, $NaBH_4$, etc. (Kim et al. 2007; Tan et al. 2003). Some stabilizing agents, for example, sodium dodecyl benzyl sulphate and polyvinyl pyrrolidone (Li et al. 1999; Tan et al. 2003), are mixed with the reaction mixture to prevent nanoparticles agglomeration.

Chemical methods commonly used include chemical reduction (Balantrapu and Goia 2009), electrochemical and photochemical reactions (Rodriguez-Sanchez et al. 2000; Taleb et al. 1997). In addition, the physical methods include attrition and pyrolysis. The process of attrition involves particles grinding via size-reducing

mechanism. However, in the both appraoches various toxic substances are generated during the synthesis. Thus, there is pressing need to omit toxic ingredients in synthesis of NP to make it more safe, sound, cost-effective and sustainable. To tackle this, scientists are focusing towards NP synthesis by biological method. Biologically derived NPs are cost-effective, non-toxic, and eco-friendly (Thakkar et al. 2010). Till now, a number of plant extracts, algae, bacteria, and fungi have been used for the synthesis of NPs (Ali et al. 2011; Balaji et al. 2009; Gilaki 2010; Saifuddin et al. 2009; Schneidewind et al. 2012).

Algae are eukaryotic aquatic oxygenic photoautotrophs (Castro et al. 2013). They are unicellular or multicellular forms staying in various conditions, such as freshwater, wastewater, marine water, agriculture waste, surface of moist rocks, etc. (Momeni and Nabipour 2015; Oscar et al. 2014) and play a central role in pharmaceutical, clinical, farming, cosmetics applications, and aquaculture. It is also an important resource for different commercial products, viz. natural dyes and biofuels (Johansen 2012; Sing et al. 2013). Microalgae of different groups including Phaeophyceae, chlorophyceae, Rhodophyceae, and Cyanophyceae etc. (diatoms and euglenoids) have been used (Sharma et al. 2016) for the synthesis of nanomaterials. The high metal accumulation potentia with less toxicity impacts makes algae as super contender for NPs biosynthesis. Algae, such as *Chlorella vulgaris*, *Lyngbya majuscule*, and *Spirulina platensis*, were used as a low-cost method for the synthesis of silver NPs (Chakraborty et al. 2009). Comparing with other microorganism, viz. fungi, yeast, and bacteria, algae being largest photosynthetic organisms are considered as a potent source for a number of metabolites including proteins, flavin, terpene, pigments, etc. These secondary metabolites can be served as biofactories of metal NPs synthesis (Ali et al. 2011; González-Ballesteros et al. 2017; Namvar et al. 2015). Generally, unicellular green microalgae were found to have high binding capability towards tetrachloroaurate ions/$AgNO_3$, to form microalgal bound silver/gold which was later reduced to Ag (0)/Au (0). The dried *C. vulgaris* reduced ~88% of gold in metal state and accumulated gold crystals in the both outer and inner parts of cell surfaces with decahedral, icosahedral, and tetrahedral morphology (Luangpipat et al. 2011). Govindaraju et al. (2009) reported that the dried blue tetrahedral green alga *S. platensis* was used in the synthesis of monometallic (Ag, Au, and bimetallic nanoparticles).

14.2 Classification of Nanoparticles

Nanoparticles have wider application and can be used in sunlight-based and power devices for effective vitality creation, for clinical treatment and in air and water channels to decrease contamination. They can also be utilized as a catalyst in current assembling procedures to abolish the use of hazardous materials. The most common conventional approach used to synthesize NPs is wet method. This method uses physical and chemical methods and is considered under top-down and bottom-up methodologies. The physical methods comprise attrition and pyrolysis. The live cells of microalgae are used to synthesize metallic NPs via one-step technique. This

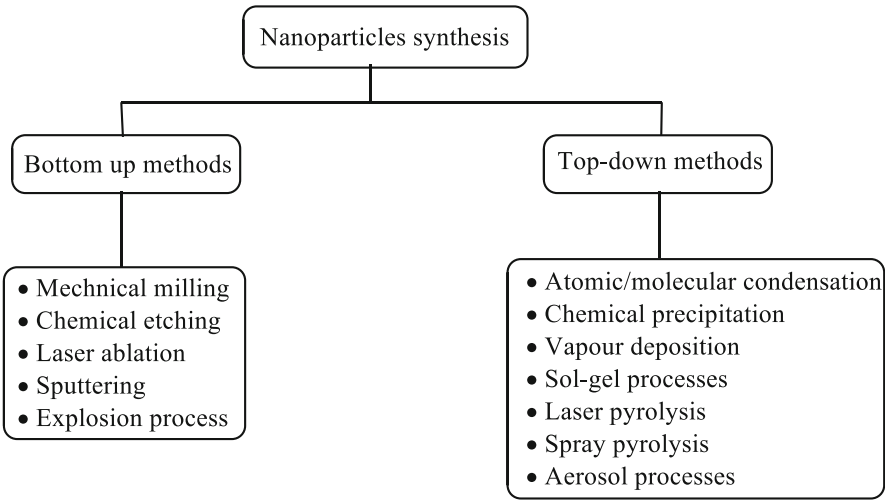

Fig. 14.1 Nanoparticles (NPs) synthesis (via bottom-up and top-down methods)

technique contains an aqueous solution of metallic salts added directly on the cells grown under culture condition. After the synthesis of NPs, it is discharged into the culture wrapped inside the framework which is further liable for formation of colloids (Fig. 14.1).

14.3 Types of Metallic Nanomaterials (NPs)

Fundamentally, two approaches of NPs synthesis are involved, i.e., top-down and bottom-up approaches. In top-down approaches, pieces of voluminous size materials were reduced into nanoscale size. It regularly uses microfabrication process, where distantly controlled tools are being used to cut the plants, algae, and other objects and shape them into ideal structure (Wong et al. 2009; Ghosh Chaudhuri and Paria 2012). A number of NPs were manufactured by top-down processes such as etching, laser ablation, electro-explosion, and sputtering (Arole and Munde 2014; Cheng et al. 2016; Liu et al. 2015.

The bottom-up approach also known as molecular nanotechnology includes gathering of definite structure and there coherent joining in the order of atom, molecule, bunch or self-organization (Thakkar et al. 2010). In bottom-up approach, self-assembled property of single molecule is exploited to construct composite conformations at nano level (Medintz et al. 2003). Nanoscale structures which have been accounted to be synthesized through bottom up method includes synthesis of supercritical fluid (Byrappa et al. 2008), use of templates, frame spraying synthesis, pyrolysis, sol–gel process, vapour deposition; molecular condensation, chemical reduction and green synthesis etc (Bhaviripudi et al. 2010; D'Amato et al. 2013;

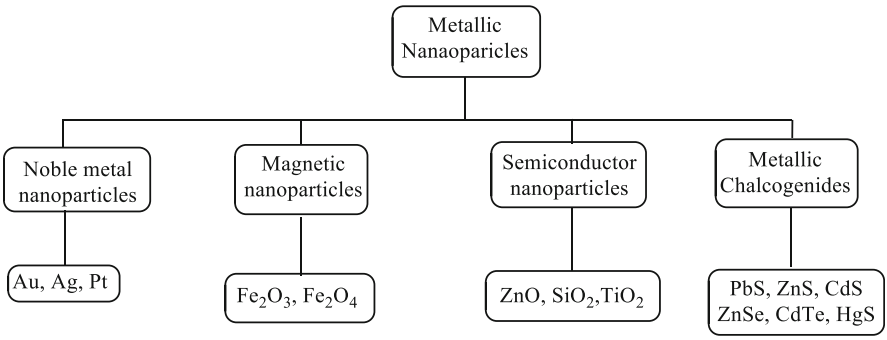

Fig. 14.2 Types of metallic nanomaterials (NPs)

El-Ghwas et al. 2020; González-Ballesteros et al. 2017; Guzmán et al. 2009; Gurentsov et al. 2007; Johnston and Shah 2004; Sangeetha and Saravanan 2014; Sekine et al. 2009) (Fig. 14.2).

In top-down methods, physicochemical procedures are included that may prompt to surface imperfection which influence the properties of NPs. Likewise, in bottom-up, NPs are bunch together from smaller unit. Thus, in both processes, development of NPs is regulated by kinetics that decides size and shape of the NPs. The growth rate and vitality of the crystals are observed by introducing companionable stencils or surfactant which may limit the interfacial energy (Schneemann et al. 2018; Sharma et al. 2011). There are varieties of metal NPs relying on their magnetic and metallic properties (Fig. 14.3). Until now, different commercial surfactants (sodium dodecyl sulfate, cetyltrimethylammonium bromide (CTAB), sodium hexametaphosphate (SHMP), mercaptoethanol (ME), thioglycerol) have been utilized as capping agent, which legitimately change the surface structure of NPs during the synthesis (Rahdar 2013).

14.4 Synthesis of Nanoparticles

14.4.1 Intracellular Mode of Nanoparticles Synthesis

Intracellular NPs synthesis refers the process of synthesis which takes place inside the cells. There is no prerequisite of any primary or pre-treatment of microalgae on the grounds. The pathway of green NPs synthesis depends on the metabolic pathways of algae for example nitrogen fixation, photosynthesis and respiration (Sharma et al. 2016), in which the reducing agents might be nictoinamide adenosine dihydrogen phosphate or NADPH dependent reductase throughout the process of photosynthesis or may be electron transport system (ETS) (Senapati et al. 2012).

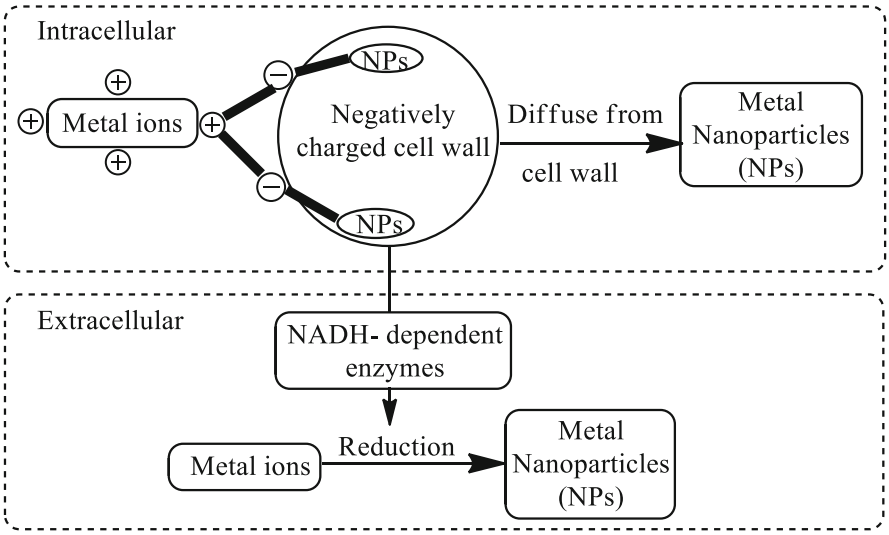

Fig. 14.3 Intracellular and extracellular mode of synthesis of NPs

14.4.2 Extracellular Mode of Nanoparticles Synthesis

Extracellular NPs synthesis occurs outside of the cell that supported via the different exudates including pigments, metabolites, ions, enzymes, and non-protein entities (nucleic acid hormones, lipids, antioxidants, etc.) (Vijayan et al. 2014). The biomass of algal is subjected to exposed simple pre-medications, for example washing and the blending. Parial and Pal (2014) also reported the extracellular gold NPs synthesis by *Lyngbya* sp. and *Spirulina subsalsa*, where massive biotransformation of Au^{3+} to Au^{0}, leads to production of Au-NPs. The FT-IR spectrum clearly signifies the extracellular synthesis of organic moieties that may be apt to check agglomeration and facilitate the synthesis. The researchers believed that an extracellular path was also responsible as shown in purple colour with a peak at wavelength 535 nm (Sharma et al. 2014a, b).

Besides, the extracellular and intracellular modes of NPs synthesis, researchers reported that both process of NPs synthesis occurs simultaneously (Jena et al. 2014; Parial et al. 2012a, b).

14.5 Green Microalgae and NPs Synthesis

Over the last decades, researchers have been demonstrated the urgency of algae in synthesis of silver nanoparticles (Ag-NPs) (Xie et al. 2007a). Annamalai and Nallamuthu (2016) utilized the extract of *C. vulgaris* for the silver nanoplates synthesis. This process is dynamically controlled where hydroxyl group of tyrosine

residues is most active and accountable for reduction of Ag+ and anisotropic development. The shape of Ag-NPs is controlled by COOH groups of glutamic acid and aspartic acid of the protein fraction (Xie et al. 2007b).

14.5.1 Silver Nanoparticles Synthesis from pheophyceaen Algae

A fast and basic technique for the reduction of Pd^{+2} to the synthesis of Pd NPs was done by liquid extraction method in algae *C. vulgaris* (Arsiya et al. 2017). Incessant change in the colour of solution from yellow to deep brown indicates the Pd-NPs formation. The reaction of Pd-NPs synthesis was done in 10 min. This was determined by the disappearance of characteristic peak of Pd^{+2} at wavelength of 410 and 420 nm. Moreover, the arrangement of Pd-NPs was confirmed through SPR peak which lies in range of 370–440 nm. The amide and polyol groups of the concentrate were thought to be accountable for reduction and stabilization reaction as evident by the presence of strong and intense peaks at wavelength of 1051 cm^{-1} (C-O-C of polysaccharides, Nucleic acid and other PO_4^{-3} containing compounds, 2922 (polyols C−H stretching), 3417 cm^{-1} (polyols O−H group) and 1641 cm^{-1} (stretching vibrations of aromatic rings; amide or C=C) in FT-IR spectrum (Arsiya et al. 2017).

14.5.2 Green Algae and Gold Nanoparticles Synthesis

The intracellular microalgal synthesis of gold nanoparticles was reported in algae *C. vulgaris* (Ting et al. 1995). Later on, Xie et al. in 2007 also developed a triangular, single-crystalline, gold nanoplate from *C. vulgaris* and found that gold forming protein (GSP: 28 kDa) present in algae was responsible for the bioreduction and shape guideline (Xie et al. 2007b).

14.6 Spectroscopic and Diffractographic Techniques

The spectroscopic and diffractographic techniques are monitored by X-ray photoelectron spectroscopy (XPS), UV–visible spectroscopy, XRD, energy dispersive spectroscopy (EDS), dynamic lights scattering (DLS), Fourier transform infrared spectroscopy (FT-IR), and Raman spectroscopy (Menon et al. 2017). These techniques are the indirect approaches used to analyse the composition, organization, and crystal phase. NPs and its properties are significantly altered by morphological structure using microscopic techniques like TEM, SEM HR-TEM, and AFM. SEM presented the data about particles at the nanoscale and helps in deducing surface morphology and the mass of NPs dispersion. TEM is used for the size and shape. In addition, it also gives the information about the number of material layers. However, when TEM are conjunct with EDS, it facilitate to find out the localization and tissue specific orientation of the metals (Oza et al. 2012). With intracellular NPs

synthesis, metal localization is examined via SEM and TEM. TEM mainly provides a 2D image of 3D nanoparticles. Further, to establish the precise size, shape, and the crystalline structure, HR-TEM is utterly required. On the other hand, AFM assists in finding the surface topography of NPs. AFM technique of microscopy can be used to get three-dimensional data on NPs synthesis (Quester et al. 2013).

14.7 Mechanism of Nanoparticles Synthesis

Algae are well-known hyperaccumulator of heavy metal with an outstanding capacity to transform them into different forms (Fawcett et al. 2017). On account of these attractive characteristics, microalgae have been expected as model entities for manufacturing biological nanomaterials. Algae contain carbohydrates, minerals, proteins, oil, polyunsaturated fatty acids, fats, and bioactive compounds including pigments, antioxidants (polyphenols, tocopherols), chlorophylls, phycocyanin, and phycoerythrin (Michalak and Chojnacka 2015) which acts as reducing as well as stabilizing agents (Fig. 14.4). The microalgae-based synthesis of NMs includes preparation of (1) microalgal extract, (2) metal solution, (3) incubation of extract of microalgae with metal solution (Sharma et al. 2016). The response is started by mixing the microalgal extract and solution of metal precursor. Generally, change in the mixture colour demarcated the (1) visible sign of initiation and nucleation, (2) growth of NPs in which the adjacent nucleonic particles associated together, and (3) formation of thermodynamically stable NPs of various shape and size (Fawcett et al. 2017; Prasad et al. 2016).

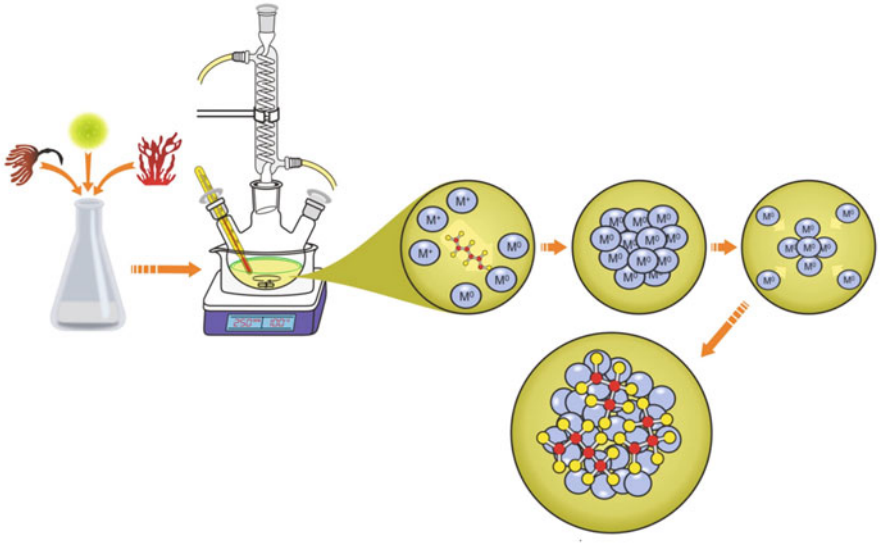

Fig. 14.4 Mechanism of nanoparticles (NPs) synthesis from algae

14.8 Factors Controlling Synthesis of Nanoparticles

The factors, viz. pH, concentration of precursor, exposure time, response time, and temperature, control all the process of NPs synthesis, i.e., formation, nucleation, and adjustment. These components can be modified to alter the size, structure, and morphology as well as agglomeration properties of NPs (Dahoumane et al. 2014a, b; Dahoumane et al. 2012; Parial and Pal 2015). The impact of pH, time, extract, and temperature were analysed through UV spectroscopy (Fig. 14.5) for optimization and the production of Ag-NPs by *Caulerpa serrulata* (Aboelfetoh et al. 2017).

14.8.1 Effect of Microalgal Extracts

The 5–25% extract of microalgae *C. serrulata* was mixed with 10^{-3} M silver nitrate at 25 °C (Aboelfetoh et al. 2017). After a certain period of 24 h, effect was measured

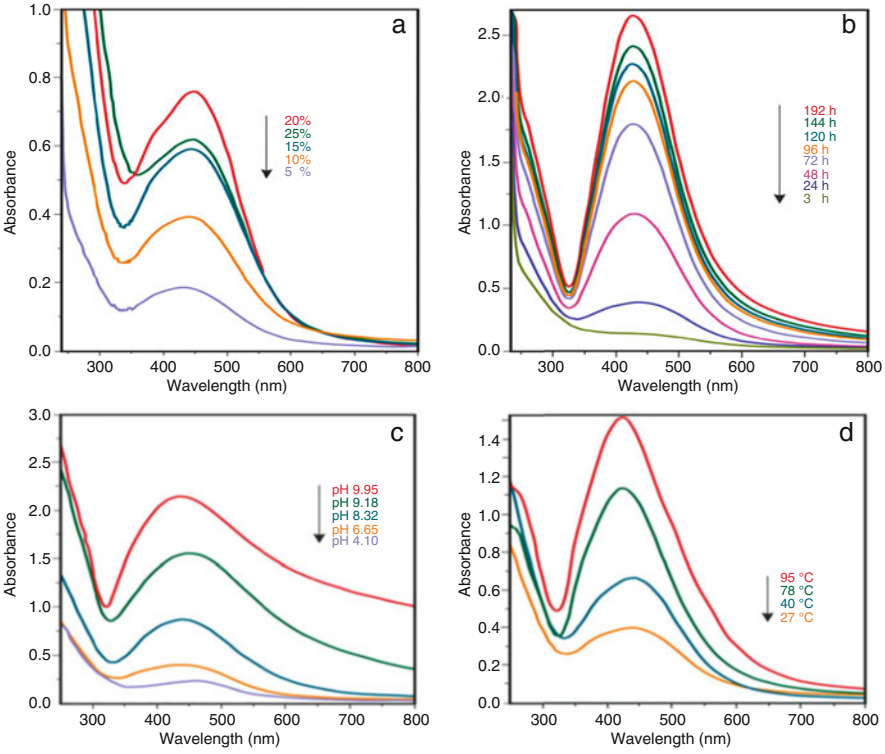

Fig. 14.5 UV–Visible spectral image of Ag-NPs formed with various extract concentrations (**a**), 10% extract as a function of contact time (**b**), 10% extract and pH values (**c**) and 10% extract at varied temperatures (**d**) (Abdel-Raouf et al. 2017)

by Ag-NPs synthesis. The increase in extract concentration from 5 to 20% led to an increase in the intensity of SPR band that causes a blue shift towards wavelength of 435 nm. This shift indicates the overall decrease in average Ag-NPs size (Fig. 14.5a). However, with the increased extract concentration (25%), reduced SPR band intensity was observed which may be due to particle agglomeration (Velammal et al. 2016).

14.8.2 Effect of Contact Time

The extract (10%) of *C. serrulata* and silver ion was allowed to mix together for 8 days period at 25 °C (Aboelfetoh et al. 2017). As the contact time increased, there was a gradual increase in SPR peak intensity with no disturbance leading to fast Ag-NPs synthesis. This confirmed the Ag-NPs constancy without agglomeration (Fig. 14.5b).

14.8.3 Effect of pH

pH plays a significant role in NPs synthesis and decides the colour of the reaction mixture and SPR peak intensity in the solution. The stabilizing and reducing power of *C. serrulata* extract enhances at basic pH. With the increase in pH (slight acidic to basic; pH 9.995), a narrow band of SPR at 427 nm wavelength was observed along with the increased absorbance as reported by Aboelfetoh et al. (2017) (Fig. 14.5c). On the other hand, in acidic pH (4.10), a broad SPR band intensity at 470 nm, reflects the agglomeration potential of Ag-NPs. In addtion, increase in small size, specify the synthesis of high quantity of Ag-NPs at high pH (Siddiqui et al. 2018).

14.8.4 Effect of Temperature

Temperature acts as a key player in Ag-NPs synthesis. With the increase in temperature, the rate of reaction increases leading to the formation of small size NPs (Ibrahim 2015). Aboelfetoh et al. (2017) reported that by increasing the temperature up to 95 °C, a less intense SPR peak was observed at 440 nm (Fig. 14.5d).

14.9 Applications of Microalgal Nanoparticles

According to some ongoing research, algae, such as *Scenedesmus quadricauda* and *C. vulgaris*, are very much suitable for the accumulation and degradation of polycyclic aromatic hydrocarbons. Algal frameworks have customarily been utilized as a tertiary wastewater treatment process. Algae can be utilized in management of a variety of functions, including the removal of coliform bacteria, reduction in biological oxygen demand (BOD), COD removal of nitrogen, phosphorus, total

solids, and the evacuation of substantial metals (Rawat et al. 2016). The biomedical applications of algal NPs comprise wound healing, antibacterial, antifungal, anticancer activities, etc.

14.10 Conclusion

Synthesis of metal NPs through algae employed a glimpse to some simpler nanoparticles which are being currently modified for their potential application. Low-cost, least time consuming, and environment-friendly production decreases the application of dangerous chemicals which make algae a best alternative stand for the NPs synthesis. However, the mechanistic facet of the biological synthesis of NPs is doubtful. Thus, future research must focus on the various aspects which directly or indirectly associated with the synthesis of NPs. In addition, the concern with reference to environmental vulnerability due to heavy metals necessitates to diminish the ill effect of NPs. Emerging techniques would make easy and might support the wise collection of algae-based NPs. Based on the findings and report mentioned in this chapter, in prospect, a remarkable advancement may witness in microalgae-based NPs synthesis with enormous potential in agriculture, pharmaceutics, medicine, cosmetics, etc.

Conflicts of Interest The authors report that there are no conflicts of interest.

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Cyanobacterial blooms and Cyanotoxins: Occurrence and Detection

15

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Abstract

Enormous increase in anthropogenic activities results in nutrient loading into the environment causing eutrophication of aquatic bodies. The increased eutrophication of freshwater and marine water bodies has intensified the algal growth which is commonly known as algal blooms. Prokaryotic blue green algae/cyanobacteria are one of the most common bloom causing algae in aquatic ecosystem, commonly known as cyanobacterial harmful algal blooms (CHABs). Some cyanobacteria can produce toxins called as cyanotoxins, which not only hinder recreational use of water bodies but also adversely affect microalgae,

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invertebrates, fish, birds, plants, and mammals. The current chapters offer an overview of occurrence of CHABs and their toxins. Further, emphasis has been given on types of cyanotoxins, their measurement and removal from aquatic ecosystem.

Keywords

Algal bloom · Aquatic ecosystem · Bloom management · Cyanobacterial harmful algal blooms (CHABs) · Cyanotoxins

15.1 Introduction

Harmful algal blooms (HABs) comprise of phytoplankton which naturally produce bio-toxins that have harmful effects on resident population, as well as human beings (Carpenter et al. 1998; Harrness 2005). HABs can alter food chains, release potent toxins, and disrupt ecosystems (Paerl 1988). They broadly cause death of aquatic organisms either due to their toxins or by depleting the nutrients for other neighboring organisms. Despite knowing these familiar characteristics, HABs vary in terms of organisms causing bloom formation, type of impact, and bloom dynamics.

Cyanobacteria, one of the major contributors of HABs are ubiquitous and therefore found commonly in lakes, rivers, and other surface waters. Few examples of bloom forming cyanobacteria are *Lyngbya wollei*, *Cylindrospermopsis raciborskii*, *Anabaena bergii*, *Raphidiopsis curvata*, *Aphanizomenon flos-aquae*, *Anabaena lapponica*, and *Microcystis aeruginosa*. Like other algal species, cyanobacteria multiply rapidly in aquatic ecosystem and result in bloom formation when conditions are favorable. In freshwater systems, cyanobacteria are the major cause of HABs (Anderson et al. 2002). Their fast growing property leads them to occupy water surfaces and thereby impeding the sunlight from reaching other co-occurring photosynthesizing organisms (Horne 1972; Huisman et al. 1999) and resulting into enhanced pH levels far from the tolerable limit to other phytoplankton (Mogelhaj et al. 2006). The dominating cyanobacterial harmful algal blooms (CHABs) replace the more diverse and nutritious phytoplankton (Bernardi and Giussani 1990; Müller-Navarra et al. 2004). Depending on nutrient levels and light conditions, some cyanobacteria can float to different levels of water surface due to their feature of having gas filled cavities. This also helps cyanobacteria to establish themselves in the water body, causing "scum formation."

Various abiotic and biotic factors that influence CHAB formation and its persistence include intensity of light, availability, and type of nutrient (mainly phosphorus), pH, water temperature, and precipitation. Though the seasonal and yearly fluctuations in the cyanobacteria levels are the result of interrelationship of these factors but the major cause of the extensive proliferation of CHABs among all the factors is considered to be the eutrophication of aquatic bodies (Steffen et al. 2014; Gobler et al. 2016; Ndlela et al. 2016; Miller et al. 2017).

In present chapter we are providing an overview of cyanoblooms and cyanotoxins in terms of their occurrence and detection.

15.2 Toxic CHABs and Cyanotoxins

Historically, evidence of toxic blooms of cyanobacteria persists in Europe for over five hundred years (Codd and Beatie 1991). The first report of CHAB in killing livestock by toxic algae in lakes of South Australia was published in Nature (Francis 1878). Recurrent and acute CHABs have been reported world-wide over past one fifty years (Paerl and Huisman 2009). Different cyanobacterial genera are found in different continents of the world (Table 15.1), making algal bloom as a worldwide phenomenon. The dominance of CHABs in the different part of the world is mainly contributed by the presence/release of their toxins.

Cyanobacteria/BGA produce various types of cyanotoxins, which has been structurally divided into three major groups such as alkaloids (anatoxin-a, cylindrospermopsin, saxitoxins, and lyngbiatoxin-a), cyclic peptides (microcystin and nodularin), and lipopolysaccharides. However, on basis of biological response, the cyanotoxins can be majorly grouped into four categories: cytotoxins, hepatotoxins, neurotoxins, and dermatotoxins, (Kaebernick and Neilan 2001; Codd et al. 2005). Among them hepatotoxins and neurotoxins are very common. Hepatotoxins include microcystin, cylindrospermopsin, and nodularin. However, cylindrospermopsin has both neurotoxic and cytotoxic potentials (Corbel et al. 2014; Kaplan et al. 2012; Kaebernick and Neilan 2001), whereas anatoxins and saxitoxins are neurotoxins by different cyanobacterial species (Neilan et al. 2013).

15.3 Ecological Factors

The growth of CHABs is affected by many ecological factors like temperature, pH, intensity of light, and nutrient conditions. Among them quality of light and its intensity are among the vital factors in the growth of phytoplankton. The cyanobacteria being prokaryotic contain phycobiliproteins as light harvesting mechanism, which allows light absorption from wider spectrum of light (Oliver et al. 2012). Apart from light intensity and quality, temperature is one of the major factors that promotes cyanobacterial bloom formation and plays an essential role in toxins production and assimilation of nutrients (Davis et al. 2009; Mowe et al. 2015; Wang 1974). For example, after examining one hundred forty three lakes along latitude from subarctic part of Europe to southern area of South America Kosten et al. (2012) found that for the formation of cyanobacterial biomass, temperature and total nitrogen (TN) concentrations were the most important variables. Likewise Beaulieu et al. (2013) examined the proliferation of cyanobacterial species in 1147 freshwater bodies in the USA and showed that the linear regression model with multiple variables was best indicated by temperature and TN of the water bodies. Therefore, with increasing global warming, temperature of water will be increased, and cyanobacteria will gain an advantage over other phytoplankton and flourish in the water bodies.

The higher diversity of non-nitrogen fixers (*Microcystis* sp.) can be found with higher pH, while nitrogen fixers dominate at low pH (Whitton and Potts 2012). The

Table 15.1 List of dominant CHABs found across the world

Continent	Dominant cyanobacterial species	References
Africa	*Microcystis flos-aquae*, *M. wesenbergii*, *Dolichospermum* sp., *Oscillatoria* sp., *Anabaenopsis* sp., *Lyngbya* sp.	Ndlela et al. (2016), Mowe et al. (2015), Codd et al. (2005), Blaha et al. (2009)
America	*M. aeruginosa*, *Cylindrospermopsis* sp., *Dolichospermum* sp., *Lyngbya* sp., *M. viridis*, *M. wesenbergii*, *Aphanizomenon schindleri*, *D. flos-aquae*, *D. planctonicum*, *D. lemmermannii*, *D. smithii*, *D. viguieri*, *D. circinale*, *C. raciborskii*, *Nodularia* sp., *P. rubescens*, *Lyngbya majuscula*, *L. wollei*, *Phormidium* sp., *Woronichinia naegeliana*	Paerl and Otten (2013), Mowe et al. (2015), Codd et al. (2005), Blaha et al. (2009), Schanz et al. (1979), Pick (2016), Nguyen-Quang et al. (2018)
Antarctica	*Oscillatoria* sp., *Phormidium*, *Nostoc commune*, *Aphanocapsa hyalina*, *Aphanocapsa holastica*, *Arthronema* sp., *Geitlerinema deflexum*, *Nodularia harveyana*, *Oscillatoria subproboscidea*, *Phormidium murrayi*, *Phormidium pseudopriesteyi*, *Calothrix* sp.	Pandey et al. (2004), Taton et al. (2006)
Asia	*Aphanizomenon ovalisporum*, *Planktothrix rubescens*, *P. agardhii*, *M. aeruginosa*, *Dolichospermum* sp., *Nodularia spumigena*, *Synechocystis* sp., *Aphanizomenon* sp., *Cylindrospermopsis* sp., *Planktothrix* sp., *Microcystis* sp., *Merismopedia* sp., *Nostoc* sp.	Codd et al. (2005), Blaha et al. (2009), Paerl and Otten (2013), Meriluo et al. (2017)
Australia	*Chrysosporum ovalisporum*, *Anabaena circinalis*, and *Lyngbya majuscula*	John et al. (2019), Mitrovic et al. (2011), Paul (2008)
Oceania	*M. aeruginosa*, *Aphanizomenon tenuicaulis*, *C. raciborskii*, *A. ovalisporum*, *A. issatschenkoi*, *P. rubescens*, *Kamptonema formosum*, *M. panniformis*, *D. planctonicum*, *D. circinale*	Mowe et al. (2015), Codd et al. (2005), Blaha et al. (2009), Meriluo et al. (2017)
Europe	*Gloeotrichia* sp., *Cylindrospermopsis* sp., *Microcystis* sp., *Dolichospermum* sp., *Aphanizomenon* sp., *Planktothrix* sp., *Nodularia* sp., *Phormidium* sp., *Anabaenopsis* sp.	Paerl and Otten 2013, Meriluo et al. 2017, Codd et al. (2005), Svirčev and Simeunović (2007)

formation of bloom also depends upon the complex structure of the lake. Therefore, a thorough study of individual water body has to be done to study the toxic bloom formation as all these factors need extra attention to make water body exclusive.

The toxin content in some marine harmful cyanobacteria (Stolte et al. 2002; Glibert and Burford 2017) under nutrient limiting conditions was higher. On the

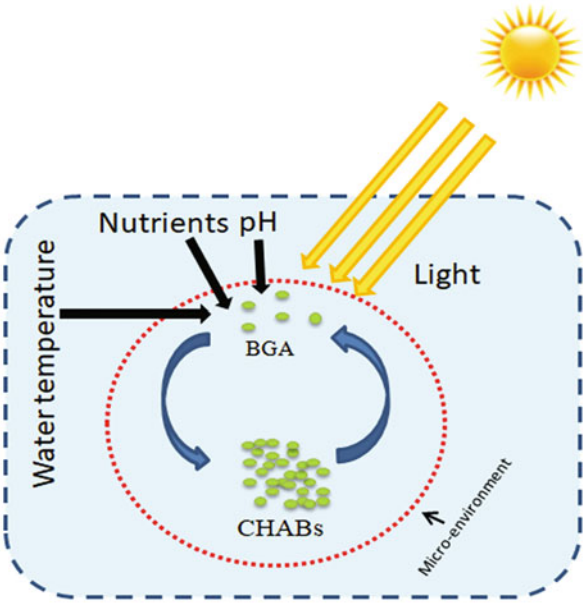

Fig. 15.1 Different ecological factors responsible for CHABs formation. Abbreviation: *BGA* blue green algae, *CHABs* cyanobacterial harmful algal blooms

basis of rigorous experimental evidence, it has been shown that phosphorus limitation controlled the release of allelochemicals externally in the cyanobacterium *Trichormus doliolum* (Von Elert and Jüttner 1997). There was an increase in production of microcystin and nostophycin by toxic *Nostoc* sp. under stress conditions of temperature, light, phosphate, and nitrate (Kurmayer 2011) (Fig. 15.1).

Since last few decades anthropogenic activities are increasing tremendously that causes the loading of nutrient in water bodies. Therefore, it is highly important to understand the effects of one or more environmental factors such as nutrients which drive the formation of CHABs.

15.3.1 Nutritional drivers of CHABs

The most studied environmental factor associated with CHABs is the natural and anthropogenic influx of nutrients into the freshwater ecosystems. The formation of CHABs was supposed to be mainly because of concentration of the nutrients (Piehler 2008). Although it is well known that nutrient input plays a symbolic part in proliferation of CHABs, still the type of nutrients is under debate (Piehler 2008).

In one of the primarily done extensive CHAB studies, (Smith 1983) while on his study on 17 lakes worldwide, interpret that CHAB formation and the TN: TP ratio are firmly associated and found that blooms do not favor higher ratios of TN: TP to multiply in water bodies. On same hand, Trimbee and Prepas (1987) analysis on 16 lakes of Canada supported the fact of low TN: TP concept. They found that TP

alone is the most potent predictor of cyanobacterial biomass than the total nitrogen to total phosphorus ratio.

Several studies depicted significant association between cyanobacterial harmful algal bloom proliferation and the availability of nutrients. Jacoby et al. (2000) reported that bloom formation and high TP concentration are strongly associated, while conducting his study on formation of cyanobacterial harmful algal blooms in Steilacoom Lake, Washington. Similarly, Anderson et al. (2002) in his investigation supported the association between influx of phosphorus and freshwater harmful blooms of cyanobacteria as well as between influx of nitrogen and marine CHABs. Correspondingly a positive relationship was analyzed between cyanobacterial biomass formation and TN:TP concentration (Giani et al. 2005). While investigating San Francisco Estuary, Lehman et al. (2005) also found the association of TN and TP with cyanobacterial biomass. While observing the 28 year old data set of Floridian lake, Havens et al. (2003) concluded that over the period of study total nitrogen to total phosphorus ratio declined from 30:1 to 15:1 corresponding an increase in cyanobacterial biomass.

Fisher et al. (1992) found that the nutrients for phytoplankton biomass in estuaries deviate from silicon and phosphorus limitation during spring season to nitrogen during summers. Maximum freshwater runoff during winter and summer precipitation drives the estuary towards cyanobacterial biomass controlled by phosphorus as in freshwater system, whereas the estuary has nitrogen-correlated algal biomass during low summer precipitation typical of a marine ecosystem. A study conducted on Western Lake Erie shows that increasing concentration of non-nitrate N over the last few decades (Spearman's rank correlation coefficient (ρ) = 0.68, p = 0.001) is significantly responsible for cyanobacterial bloom biomass (Newell et al. 2019).

15.3.2 Nutrient Drivers for Release of Extracellular Metabolites/ Toxins

The nutrient availability is recognized as one of the important components to control algal biomass and growth. Therefore proliferation of a specific species, while forming harmful algal bloom, is its ability to compete for limiting nutrients. The ability to compete for the nutrients available in the water body leads to dominance of particular species and thereby release of toxins in that environment.

Increase in release of extracellular compounds may occur under nutrient limiting conditions (Von Elert and Jüttner 1997). Grosse et al. (2018) observed that nitrogen limited communities exhibited substantially slower production of essential amino-acids, while with addition of nutrients in short-term experiments, this trend was contrary to previous result immediately after N addition to the levels found under not limiting conditions.

However, reports about relations between concentrations of nitrate and microcystin production have provided conflicting results. Einhellig (1995) concluded that limiting amount of distinct resource results in increasing allelochemical production. Ginn et al. (2010) explained that the N-limitation leads

to expression of *ntcA and mcy*B in *M. aeruginosa* (microcystin producer). Tonk et al. (2008) in the study conducted bring about the effect of N presence on MC production as addition of leucine and arginine increased the synthesis of microcystin LR and microcystin-RR, respectively, in *Planktothrix agardhii*. Van de Waal et al. (2009) also observed *Microcystis* blooms capable of producing microcystin-RR variants at higher levels mainly due to CO_2 and nitrogen enrichment. Cyanobacterial community structure consisting of *M. wesenbergii*, *Aphanizomenon flos-aquae*, and *M. aeruginosa* producing MC-LA, MC-YR, and MC-RR, respectively, was influenced by different forms of nitrogen source determining the toxicity of the bloom (Monchamp et al. 2014).

Nodularia spumigena blooms were reported to dominate the water body under conditions with higher phosphorus but low N:P and moderate salt concentrations (Mazur-Marzec et al. 2006). Similarly, Lehtimaki et al. (1997) examined that the synthesis of NOD is influenced by concentrations of nitrogen and phosphorus. While higher concentration of phosphorus (200–5500 μg L^{-1}) enhances the synthesis of nodularin but NOD production got inhibited by higher salinity and inorganic nitrogen concentrations.

Inconsistency in studies was also reported regarding cylindrospermopsin (CYN) production and different N-sources (NO_3^-, N_2,NH_4^+). Saker and Neilan (2001) were the first ones to start an investigation in *C. raciborskii* to study the correlation among CYN and type of N source for which *C. raciborskii* cultures were grown in absence of a fixed source of nitrogen resulting into slower growth rate but increased CYN while with changed nitrogen source in the form of NH_4^+, CYN was less and growth rate was higher. CYN production also depends upon phosphate limitation but contradictory reports regarding the same are found in literature. The decreased and enhanced expression of CYN secretion in *Aphanizomenon ovalisporum* have been observed under phosphate limitation in separate studies conducted by Bacsi et al. (2006) and Bar-Yosef et al. (2010), respectively. These studies clearly indicate that various environmental factors affect the production of CYN in several cyanobacteria.

Anatoxins (ATXs) synthesis was reported to vary with different factors like nutrient limitation, light, temperature, and different phases of growth (Kaebernick et al. 2000; Peary and Gorham 1996; Gupta et al. 2002; Rapala et al. 1993; Bumke-Vogt et al. 1996). Like MC, the growth of cell and ATX production were not related to each other in the study conducted by Long et al. (2001). N limitation could raise ATX production just like other cyanotoxins as already mentioned above (Neilan et al. 2013).

Saxitoxin (STX) production in cultures of *C. raciborskii* got influenced with N:P ratio as treatment with higher ratio resulted into increased STX production when compared to treatment with low N:P (Chislock et al. 2014). However, concentrations of nitrogen had reverse effect as with higher N concentrations, a lower amount of STX secretion has been found in *Raphidiopsis brookii* (Yunes et al. 2009).

It is evident that toxicity increases under nutrient stress and the activity of the toxins under such conditions provides us an advantage to suspect that allelopathic compounds play an important role in the growth and biology of toxic algae.

15.4 Methods of Detection

In order to measure the presence of cyanotoxins, various methods are available in the literature such as bioassays, molecular analysis, biochemical and chemical assays (Fig. 15.2). Enzyme-linked immunosorbent assay (ELISA) test kits are distinctive and accepted test methods to detect cyanotoxins (Gaget et al. 2017), as extensive training or expensive equipment are not required. ELISA kits can detect microcystin-LR, saxitoxin, and cylindrospermopsin precisely, while for anatoxin-a detection, a more rapid receptor-binding assay kit is available. Although they provide rapid results, there are certain limitations associated with ELISA kits as they are not congener specific.

LC/MS or liquid chromatography with mass spectrometry can precisely analyze specific congeners of microcystin with the use of available standards; this method in particular has been designed in a way to reduce matrix interference. In HPLC-PDA methods the quantification of toxin is trickier mainly because of less specificity and interference by matrix and are therefore less efficient than LC/MS methods. However, standards available for analytical toxins confirmation could assess the resolution of the congeners present.

15.4.1 Sample Handling

To ensure reliable results, samples must be handled properly. Guidelines for design and sampling for cyanobacterial toxin and taste-and-odor studies in lakes and reservoirs (2008) provided by The United State Geological Survey (USGS)

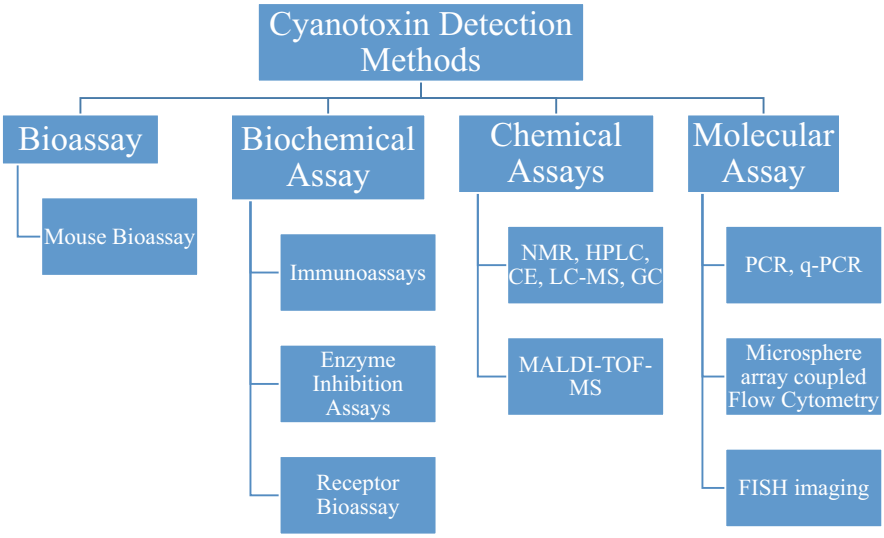

Fig. 15.2 Overview of Methods available for Cyanotoxin detection

sampling protocol can be consulted for establishing such procedures. The three most important sample handling consideration steps are (1) collection of samples, (2) quenching of samples using appropriate quenching agents, and (3) chilling of samples till further analysis at the laboratory.

15.4.2 Sample Analysis

While estimating both intracellular and extracellular toxins, cell lysis is generally done to rupture the cyanobacterial cells and to damage the cell wall and secrete the toxins into the solution. Most commonly used lysing technique is freeze/thaw cycling, though various other approaches such as sonication, bead beating, lyophilization are also used depending on analytical methods (Kim et al. 2009). The effectiveness of raw-water lysing using microscopic examination for intact algal cells is yet to be confirmed by analysts.

Variety of different extraction techniques and cell disruption methods are available for cyanotoxin detection from various cyanobacteria. Generally following steps of protocol are followed on algal samples: Extraction method, Selection of Solvent, Cell Disruption method, Centrifugation and Cyanotoxin detection.

15.5 Cyanotoxin Treatment and Bloom Management

Cyanobacteria and cyanotoxins identified in the surface water can be removed or inactivated in many different ways. After identification of cyanobacterial species that dominates the bloom and understanding its growth pattern as well as the properties of its intracellular and extracellular cyanotoxins, suitable treatment processes will help in employing adequate management schemes. As observed by Kim et al. (2018), oxidation of microcystin depends on temperature, pH of the water. Applying the wrong treatment process could damage the cells causing cell rupturing and release of cyanotoxins rather than their removal.

Table 15.2 compiles different types of methods for treatment of water to remove intracellular and extracellular toxins along with intact cells of most important cyanobacteria.

15.5.1 Developing an Exigency Strategy

Concerned authorities should develop effective strategies to get rid of cyanobacterial harmful bloom occurrence. The planning methods should approach various ways to rule out the possible risk involved with each action to eradicate algal bloom as all blooms are not toxic. Successful application of such planning involves many factors such as monitoring programs to determine sampling sites, frequency of sampling, sample volume, sampling methods for both cyanobacterial cells and specific

Table 15.2 Cyanotoxin treatment methods

Treatment method	Efficacy
Intracellular cyanotoxins	
Pre-treatment oxidation method	Causes cell lysis of cyanobacterial cells releasing the toxin into the surrounding water. Prefer lower doses of an oxidant (potassium permanganate). Otherwise, higher doses may destroy total toxins present
Coagulation	When cells which are assembled in sludge are confined from the plant
Membrane method	Study data limited. Ultra and microfiltration are useful when cells do not assemble on membranes
Flotation method	Dissolved Air Flotation (DAF) is most effective as most of the toxin producing cyanobacteria are buoyant
Extracellular cyanotoxins	
Membrane method	Based upon quality of water and pore size of membrane. Nanofiltration and reverse osmosis filtration are most efficient for removal of extracellular microcystin and cylindrospermopsin, respectively
Addition of Potassium Permanganate	Efficient method for oxidation of extracellular anatoxins and microcystins
Ozone method	Mainly to oxidize extracellular microcystin, anatoxin-a, and cylindrospermopsin
Chlorination method	Adequate method below pH 8 and not effective for anatoxin-a
Ultraviolet Radiation method	Degrade microcystin and cylindrospermopsin at higher application
Addition of activated carbon	Powdered activated carbon (PAC) and Granular activated carbon (GAC). Effectiveness of the method depends on carbon type and pore size. Microcystin are best adsorbed by wood-based activated carbons but carbon cannot adsorb saxitoxin

cyanotoxins, analytical screening method to be used, and appropriate conditions to send samples to laboratory for affirmation.

15.6 Future Prospects and Conclusion

With the growth of industrialization and uncontrolled use of natural resources, the healths of water bodies are severely affected in terms of biotic and abiotic factor. These imbalances are creating the bloom formation in water bodies, which eventually affect the survival/health of aquatic organisms. Therefore, it is very important to get a holistic overview of events/factors which control the bloom formation. Attempts have been made to do it but they are not quite enough to tackle this problem. In nut shell the time has come to make interdisciplinary efforts by ecologist/chemist/algologist for comprehensive understanding as well as to find out novel approaches to deal with this social issue worldwide.

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Potential of Golden Brown Algae in Forensic Analysis: A Review

16

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Abstract

Algae are eukaryotic photosynthetic organisms found in freshwater, marine, or brackish water. They are capable of surviving in harsh conditions such as in snow, deserts, hot water spring, and in mutual relationships with other organisms like lichens, making them as cosmopolitan species in environment. Algae play vital role in every aspect may it be as primary producers in food chain or fixing maximum amount of carbon dioxide in nature. But talking about applications of

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algae in this paper, we have focussed on Diatoms (class Bacillariophyceae) which are golden brown algae. Their ubiquitous presence, small size, and silicious frustule with peculiar ornamentation add uniqueness to their identity. Nowadays diatoms are considered as prime evidence in solving cases of drownings in the fields of forensics. Diatoms from the drowning site can tell about the physico-chemical nature of environment and help in knowing the mode of death of a drowned victim. As during drowning, these small entities enter inside the body through body openings and once penetrated inside the body they get circulated and deposited in various organs till the victim dies. These penetrated diatoms are then recovered as evidence by various techniques and then their microscopic studies further help in their identifications. This helps in linking the crime scene and the victim during the investigation and thus helping the crime mystery of drowning to be solved. Because of their vast role in the drowning case investigation they are regarded as golden standards in the fields of forensics.

Keywords

Algae · Diatoms · Bacillariophyceae · Forensics · Drowning

16.1 Introduction

Diatoms (Bacillariophyceae; division Heterokontophyta) are the unicellular golden brown algae present worldwide, generally as unicells or sometimes found as pseudocolonies or pseudofilaments. The single cell ranges from 2 to 200 μm in size (Tomas 1997a, b). A diatom cell is composed of chloroplasts with two additional chloroplast endoplasmic reticular membranes, tubular mitochondria, and storage materials like chrysolaminarin and oil (Lee 2018a, b). The chloroplast contains chlorophyll "a," "c_1," and "c_2" along with some pigments likes beta-carotene, fucoxanthin, diatoxanthin, and diadinoxanthin. Their peculiar color (golden brown) is due to the presence of fucoxanthin and silica. They are photoautotrophic cosmopolitan species which are distributed abundantly in a variety of habitats i.e. oceans, lakes, estuaries, soil, and other moist places. They are capable of growing on various substrata including muddy sediments (epipelic), sand (episammic), rocks (epilithic), plants (epiphytic), animals (epizoic), water (planktonic), and even in ice (cryophilic) (Tomas 1997a; Karthick et al. 2013). Few species of centric diatoms were found and identified from the Jurassic era of earth (Tomas 1997a). According to their suitable habitat, they have been grouped further as terrestrial, freshwater, marine, and air borne diatoms. The growth of diatoms in a particular area can be specific, as they are dependent for their growth on certain physicochemical features of the surrounding environment like pH, salinity, temperature, duration of light, and other organic matter (Round 1981; Stevenson et al. 1996; Reynolds 2006). These organisms have a distinctive feature in their outer cell wall called "Frustule," which is composed of silica. This has various structural and micro-structural structures that provide them a particular shape and pattern of ornamentation adding uniqueness to their identity. Because of their remarkable

beautiful appearance, they are also recognized as "Jewels of the sea." According to Mann and Vanormelingen (2013) around 120,000 species of diatoms have been identified on the basis of their structural variations but many species still exist which are to be discovered yet. These reproduce generally by asexual reproduction (cell division) during favorable conditions but undergo sexual reproduction when the cell size becomes smaller beyond a critical limit due to continued division.

In terms of organic CO_2, diatoms contribute 20% of the total primary productivity on earth and their total primary productivity in oceans has been around 40% (Tréguer et al. 2018). Their fossils are major contributors of crude oil worldwide to the tune of about 30% (Ramachandra et al. 2009).

16.2 Structure of a Diatom Cell

As their name suggests, dia-tom i.e. "cut in half," the morphology of diatom varies on the basis of structural variations of siliceous frustule. The silicified cell wall of diatoms is hard and porous which is arranged into two unequal halves namely "epitheca"—the larger outer half and "hypotheca"—the smaller inner half which are attached to each other by a "cingulum" or also known as "set of girdle bands."

The valves have several patterns of ornamentations like ridges, pores, spines, and elevations which help in their identification. On the basis of their shape and symmetry, they are further classified into two main orders i.e. *centrales* (having radial symmetry) and *pennales* (having bilateral symmetry) (Fig. 16.1). An important feature of their structure is the presence of *raphe*, a shallow groove like area on the valve surface connecting both the polar nodules and the central nodule in pennate diatoms. It is also responsible for locomotion in diatoms. Table 16.1 shows the diatoms classified with reference to cell morphology and frustules ornamentation.

16.3 Diatoms in Forensic Limnology

Diatoms are proving as effective supportive evidence in solving even the complex drowning cases. Drowning occurs when a person is forcefully or accidently gets submerged in any fluid medium. Whenever a victim gets drowned in a water body, the water along with some of its debris gets inside the body and ultimately reaches some of the organs and body tissues (Guy 1861). Drowning is very common worldwide and according to World Health Organization (WHO 2014) report, approximately 42 drowning deaths occur per hour every day globally. In solving a forensic casework of a drowning related death, the diagnosis of the drowned victim soon after the death is easier. However, if the corpse is recovered after a prolonged time interval or it is in a state of dismembered condition, the diagnosis becomes relatively critical. Longer duration of the corpse leads to putrefaction and onset of postmortem changes that further lead to hindrance in the investigation of crime. Moreover, the body recovered after long interval of time may attain some injuries or loss due to the predation of some zooplanktons present on or inside the water body

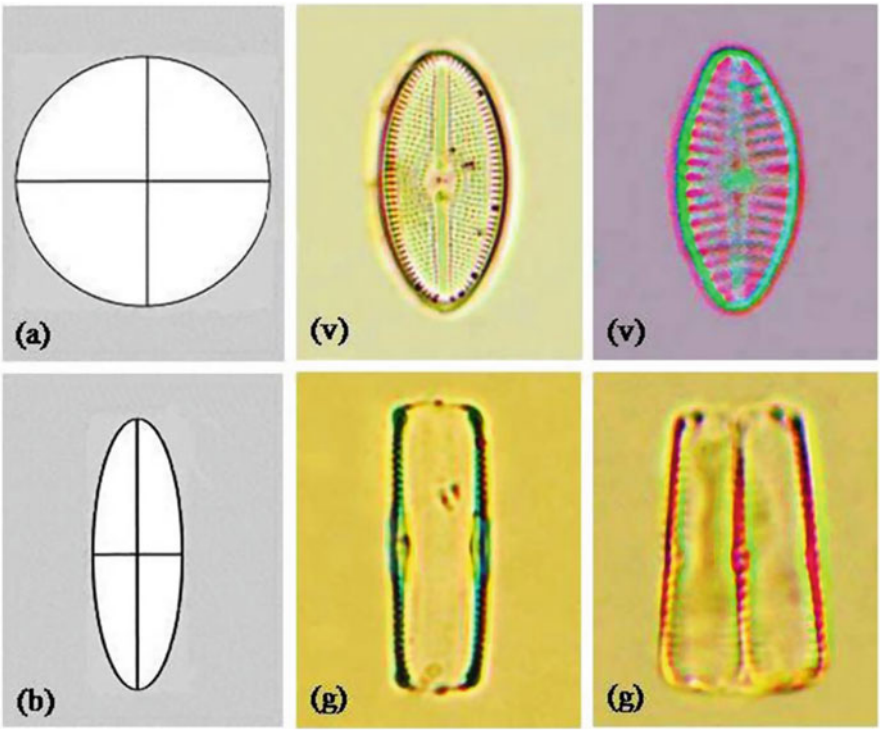

Fig. 16.1 Diagrammatic representation of valve view of Diatom symmetry: (**a**) Radial symmetry of a centric diatom, (**b**) Bilateral symmetry of Pennate diatom; pictorial representations of different shapes of diatoms in their valve (**v**) and girdle view (**g**)

and may mislead the diagnosis resulting in falsified judgments. However, in such circumstances, diatoms comprise major evidence in solving such crime mysteries.

The preference for selecting diatoms among many other unicellular algal forms is due to their small size, wide abundance, and ubiquitous distribution which make them suitable to easily penetrate inside the body both through aspiration and openings like nostrils, ears, and mouth. Their silicified frustules make the cells resistant to undergo any sort of decomposing changes occurring in the body after death, in addition, the frustules are resistant to enzymatic and acidic tests performed during postmortem examination of the organs or body tissues. It is not possible for many other microorganisms present in the body of the victim. The microstructure and peculiar ornamentation of cell wall (frustule) make the identification of diatoms easier and tenable. The types of diatoms present inside the body of a victim correlate the physiochemical nature of the environment in which it is residing. Apart from this, in skeletonized or dismembered body samples, diatoms constitute the major evidence to solve the possible crime. It can be figured out from the forensic point of view that diatoms play a significant role due to which they are termed as "Golden Standards" in forensic language for solving drowning cases.

Table 16.1 With reference to cell morphology and frustules ornamentation classification of diatoms (Round 1981)

Characteristics	Centrale diatoms	Pennate diatoms
Habitat	Mostly marine and planktonic	Mostly in freshwater streams, planktonic, epiphytic, and benthic forms
Shape	Discoid/cylindrical	Elongated
Symmetry (valve view)	Radial	Bilateral
Orders	Biduphiales	Bacillariales
Suborders	I. Coscinodiscineae—valves with marginal ring of processes, primarily no polarities II. Rhizosoleniineae—absence of marginal ring processes, unipolar arrangement of valves III. Biddulphiineae—Absence of marginal ring processes, bipolar arrangement of valves	I. Fragilariineae—Araphid (without raphe) diatom II. Bacillariineae—Raphid (presence of raphe) diatom
Motility	Generally non-motile	Motile(some raphid diatoms)
Examples	*Cyclotella*, *Melosira*	*Pinnularia*, *Navicula*

16.4 Course of Penetration of Diatoms Inside the Body of a Drowned Victim

Perusing about the penetration of diatoms inside the body of a victim, it has been found that when a body gets submerged whether intentionally or unintentionally, the person must be respiring just before one's death. During this time period, with the pressure of water stream, diatoms residing in the drowning medium sneak inside the body through aspiration and get inserted inside the lung cavity through respiratory tract and few settle in stomach through mouth opening. From there, they are further immobilized to many distant organs through systemic circulation till death. Hofmann (1878) was the first to detect presence of diatoms in lung fluid. In 1904, Revenstrof solved a drowning case with the help of diatoms. Incze (1942) demonstrated the process of circulation of diatoms through lungs and successfully detected their presence in blood and parenchymatous cells of various organs. Tamaska (1949) stated that when the diatoms are found in an enclosed tissue like bone marrow, this made the confirmation of death from drowning. Thomas et al. (1961) supported the idea of presence of diatoms in lungs as very strong corroborative evidence to suggest that the victim has taken few breaths during submersion. The analysis of diatoms in the bone marrow and other organs provide the glimpse of *antemortem* drowning (Porawski 1966).

Geissler and Gerloff (1966) and Hendey (1973) threw light on the application of diatoms in the area of Forensics. Timperman (1972) described the process in a manner that as a person drowns, the water enters the lungs through respiration

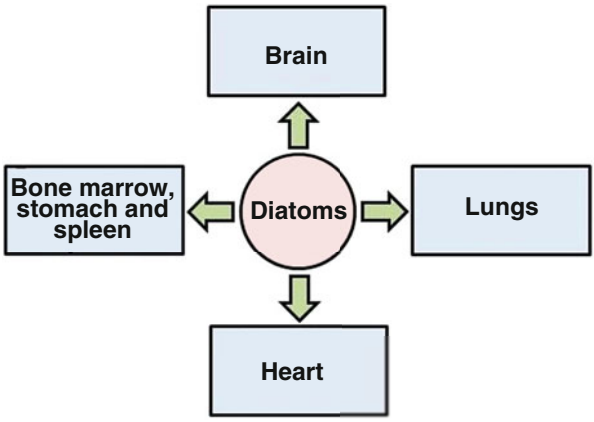

Fig. 16.2 Distribution of diatoms in different body organs in a drowning death

until the lung cavity gets filled with water which further exerts some amount of pressure on the alveolar walls. This pressure on the lung walls facilitates the rupturing of peripheral alveoli that will allow the water and its contents to enter inside the blood stream. Through the blood stream, they get distributed in the distant parts of the body through systemic circulation till the person dies and the circulatory system stops. Aquatic bodies have diatoms (mainly of small size) which get circulated to kidney, brain, spleen, and bone marrow through this process (Fig. 16.2). The diatoms which are able to penetrate inside the body of a drowned victim are called "drowning-associated diatoms" (DAD). Several types of experiments have been done by various scientists that included investigation of different parts of human body to confirm the detection of diatoms in internal organs like liver, kidney (Matsumoto and Fukui 1993; Taylor 1994; Ludes et al. 1999; Hürlimann et al. 2000), and stomach (Peabody 1977; Hürlimann et al. 2000). Aghayev et al. (2005) found that left ventricular blood can be taken into account for performing the diatom test whereas Pachar and Cameron (1993) described the detection of diatoms in brain, liver, and kidney. Apart from the *antemortem* drowning cases, the presence of diatoms in distant organs in postmortem drowning cases, where the body is thrown in water after the victim has died, is found to be very low or negligible. But in such cases of postmortem immersion of the body, journey of the diatoms gets restricted within the lungs only where they reach through the process of passive respiration or by the hydrostatic pressure of water.

Scientists like Mueller (1952), Peabody (1977), Ludes et al. (1994), Pollanen et al. (1997), Hürlimann et al. (2000), and Kakizaki et al. (2018) have contributed a lot in the field of forensic diatomology by performing different experiments and then applied their utility in solving the complex cases. In case of detecting the putative drowning site, the water sample along with the organ sample of the victim recovered from that site is collected and diatoms identified and compared by performing various tests. Thus, quantitative and qualitative analysis of the diatoms is an important criterion for the analysis of both the manner and site of death, respectively.

16.5 Extraction Methods

The extraction of diatoms from the tissue of a victim and water sample is most usually done by acid digestion method though other methods like enzymatic, Microwave Digestion Vacuum Filtration Scanning Electron Microscopy, Soluene-350 digestion, ash digestion, molecular approaches like PCR and whole slide imaging are also used for their examination. An account of evolution in methods for improvement in extraction of diatoms and analytical data has been discussed.

16.5.1 Chemical Digestion Method

This method includes digestion of organic/inorganic material of diatoms with the help of strong oxidizing chemicals. It is the oldest and the most widely adopted method used for cleaning of diatoms and making them suitable for identification. In this method the commonly used reagents are Nitric acid (HNO_3) or Hydrochloric acid (HCl) or Sulfuric acid (H_2SO_4) or Hydrogen Peroxide (H_2O_2); they are easily available in laboratories and from the records of many past experiments they proved suitable for the removal of organic and inorganic matter present inside diatom cells. This is helpful for making the representation of structural ornamentation of the frustule view clear for the purpose of qualitative identification. Many scientists have used this technique and supported this method for the analysis under proper surveillance. Few workers have used this method with little modifications. Peabody (1977) performed the rigorous acid test by adding concentrated HCl for the removal of $CaCO_3$ and other carbonates. Concentrated H_2SO_4 was added gently until charring occurred. The sample solution was then allowed to cool for some time and solid sodium nitrate ($NaNO_3$) was added. The color of the solution then changed from yellow to transparent which indicated that the production of HNO_3 has oxidized carbon to carbon dioxide. Washing the resultant sample solution and resuspension in acetone were done. The refractive index of clear diatom silica wall is 1.44, so permanent slides were made by using Naphrax (refractive index 1.74) as a mounting medium for the exact morphological examination.

Auer and Möttönen (1988) conducted acid digestion during the case study of 107 drowned victims by dissolving the water sample from the putative drowning medium and thin strips of tissue samples i.e. lungs, liver, brain, and kidney separately in Kjeldahl flask and boiled the solutions for few minutes in distilled water maintaining the contamination free conditions. After this, HNO_3 (10 mL) and H_2O_2 (30%) were added to the samples and allowed to boil. This addition was continued gradually until the tissues samples dissolved properly. The samples were then cooled and washed using distilled water through centrifugation at 3000 rpm. Fresh slides of the sediment leftover were made with the coating of Canada balsam and bromonaphthalene. The diatoms were identified and compared quantitatively and qualitatively for forensic analysis. Ludes et al. (1994) did quantitative and qualitative analysis of diatoms by comparing acid digestion and enzymatic methods. Their study was conducted on 12 putrefied bodies recovered after drowning deaths in

Strasbourg, France. The diatoms from water samples of suspected immersion sites were digested by using H_2O_2 whereas those present in the organs (lungs, liver, kidneys, and brain) were treated with HNO_3. The final sediment (100 μL/mL) retained after several washings and centrifugation at 2500–3000 rpm was air dried and mounted with Naphrax for the microscopic study and recording observations.

Pollanen (1998a, b) carried out the investigation of six homicidal drowning cases. In a flask the bone marrow from femur was collected as a sample (~50 g) and nitric acid (50 mL) was added in it. The marrow with the acid solution was boiled gently for 48 h. After the boiling process, the acid-marrow suspension was allowed to cool. Centrifugation and washing was done with distilled water by discarding the supernatant and cleaning the residue pellet again with the same procedure till the acid got washed away properly. The residue was then air dried and used in making fresh slides of diatoms. The diagnosis of these slides was done through phase contrast microscopy. Comparative study of the samples and fluid from maxillary sinus were also examined by using the same procedure for getting more information. Yange et al. (1999) made an instrument called "Can" to overcome the drawbacks of the digestion method. It contained three parts: an outer cover, an inner cover, and a body. A thread is interconnected to them for the maintenance of pressure. Teflon of good quality was used in manufacturing of "can" where the degradation of organic matter has to be done. It works on the principle that after the addition of strong acid and keeping high temperature, the solid organic material gets converted into liquid in the sealed "can." The liquid thus formed contains diatoms which do not get destroyed due to their hard silica wall. Therefore, they put 3 g of tissue in the "can" body to which 2 mL of pure concentrated HNO_3 was added and both the covers (inner and outer parts) were tightly closed. In this specific condition, it was placed in dry box at 120 °C for 100 min. It was cooled thereafter and opened. The desirable liquefied material was then transferred to centrifuge tubes for centrifugation. The last process applied to the sediment left after centrifugation was to use the residue for making microscopic slides for examination. This proved to be successful in overcoming the shortcomings of traditional digestion method due to its qualities like good capacity, resistance to heat and corrosion with no leakage.

Gruspier and Pollanen (2000) investigated the case of five amputated lower limbs recovered from Lake Ontario, Lake Erie, and Niagara River in Ontario. Diatom analysis helped to analyze the cause of death. For this, 50 g of femoral bone marrow was taken in a flask to which HNO_3 was added for the digestion of organic and inorganic material from the diatoms. The sample acid suspension was allowed to cool and centrifuged repeatedly. The supernatant was discarded off and the residual pellet was used for making microscopic slides for the diatom analysis. The same process was used for the water sample taken from the putative drowning medium for a comparative study. Bhatt et al. (2001) studied the diatom community in Kistobazar Nala in Purulia, West Bengal. They scrapped epilithic diatoms from the rocks and boulder surface. These were cleaned with HNO_3 and $K_2Cr_2O_7$. After cleaning the diatom samples, these were subjected to centrifugation for 10–30 min at 1000 rpm. The supernatant was carefully discarded and the pellet was washed twice using isopropanol and once with xylene. Permanent slides were made using Canada balsam.

Krstic et al. (2002) performed acid digestion on laboratory rats, drowned corpse of victims, and control samples. In their digestion method, H_2O_2 was poured first in tissue samples and kept for 24 h. H_2SO_4 was added to the samples thereafter for the proper removal of organic material. Then saturated permanganate solution was poured which made the color of the sample solution violet. Discoloration was performed with saturated solution of oxalic acid and sedimentation was initiated for 48 h. Finally centrifugation was done at 3000 rpm for 20 min followed by the microscopic observations for further analysis. Yen and Jayaprakash (2007) carried out extraction of diatoms from five types of non-vegetarian food items namely fish, prawns, chicken, anchovy, and clams. Sample (50 mL) was made by blending the food items after being washed with distilled water and then dried. The blending was done till the residue appeared pulpy in nature. Distilled water was added to the residue to make the final volume up to 100 mL. Then 100 mL of concentrated HNO_3 was added and the solution was allowed to boil. This method was repeated till the acid residue suspension becomes yellow in color. This yellow residue was extracted out in 100 mL flask and heated until reduced to 20 mL. Then 1.5 mL of the extract was centrifuged at 1000 rpm for the time limit of 15 min. The supernatant was then discarded and the sediment was used for microscopic examination.

Thakar and Singh (2010) made diatomological map of 10 different water bodies of Punjab, India. The water samples were collected for two consecutive years covering different seasons of both years (2005–2007). The test for the detection of diatoms was performed by taking 200 mL of water sample and adding 40–45 mL of concentrated HNO_3 and a pinch of potassium dichromate ($K_2Cr_2O_7$) acting as an oxidizing agent in this acid digestion treatment. The sample acid suspension was kept undisturbed for 2 h. Then consecutive washings were done with centrifugation at 3000 rpm using distilled water. Diatom examination was performed using the pellet left after centrifugation.

Three methods for extraction of diatoms from clothing were used by Uitdehaag et al. (2010). They took two T-shirts, one used (washed) and the other was unused (both of 100% cotton). These T-shirts were then dipped separately in a canal and freshwater ditch, respectively. For the reference sample, water was collected from the middle and edge of both the water bodies. They compared three methods for the diatom extraction from the clothing materials. The methods included: rinsing clothing in water (RW), in 70% ethanol (RE), or dissolving the clothing (DI). The DI method was successful in yielding the highest number of diatoms. This method comprised of acid digestion of clothing material in Cem-Star system-6 for temperature dependence. An amount of 97% H_2SO_4 (2.5 mL) and 65% HNO_3 (12 mL) was added to the sample material and heated to 130 °C. Again at the same temperature, of 65% HNO_3 (12 mL) and of 30% H_2O_2 (20 mL) was added to the solution for complete digestion of organic / inorganic residues of the diatoms. Washing was done repeatedly and then microscopic observations were recorded.

Ago et al. (2011) examined nine postmortem cases of bathwater drowning and one case of the victim who died because of ischemic heart disease in bathwater. Lungs, liver, spleen, and kidney were taken as samples for the acid digestion process. The samples were treated with 10% formalin, diluted with distilled water,

and kept undisturbed for several days. They were subjected to thin sectioning and washed frequently with distilled water. 10 mL of H_2SO_4 was then added which gradually blackened the sample solution. To make this solution clear, 10–15 mL of HNO_3 was poured gradually. Centrifugation and washing were done and the slides of the extract were made for the diatoms.

A comparative study of both the acid digestion and enzymatic extraction methods has been made by DiGiancamillo et al. (2011). The tests were performed on organs/tissues of 15 slaughtered pigs along with mud full of freshwater diatoms. Various combinations of acids of variable concentrations were used and their results were compared. In first treatment, 10 mL of 10%, 20%, and 37% HCl was poured on the minced samples of pig tissues along with 1 mL of mud sample. Then digestion of the sample was initiated at 95 °C for 15 min. In the second test, 1 g of tissue sample was mixed with 2 mL of H_2O_2 at the room temperature and then 10 mL of 10% or 20% of HCl was poured along with 1 mL of mud sample rich in diatoms. The sample was boiled in the same manner. In the third test, to 1 g of pig specimen, 10% or 20% of 5 mL (HNO_3 and H_2SO_4) or the mud sample was mixed in 10 mL of 90% HNO_3 and digestion was initiated. The fourth and fifth tests for the diatom extraction were of enzymatic digestion method. For better results, all the tests performed were repeated ten times and a control sample (having no diatom) was also kept to check the chance of any cross contamination with each test samples. Acid digestion with 20% HCl proved highly significant in yielding good amount of intact diatoms in a short time as compared to other studies.

Xu et al. (2011) examined the validity of acid digestion technique on 56 rats. These were divided in seven groups with eight in each group and then submerged in river to mimic drowning death. Autopsy was carried out in aseptic conditions. Lungs, liver, kidneys, and bone marrow were extracted at different time intervals i.e. 0.5, 1, 6, 12, 24, and 48 h after drowning. Thin sections of the tissues were cut and employed further for digestion with concentrated HNO_3. The digestive fluid after the acid digestion was subjected to centrifugation for 5 min at 5000 rpm. Microscopic analysis of the diatoms was done thereafter for a comparative study. Nadia Fucci (2012) studied the traditional acid digestion method with newly produced digestion method using diluted minimal amount of H_2SO_4 for the extraction of diatoms. Dead bodies (10) recovered from the drowning site under criminal investigation were used for the experiment. The samples included kidney, lungs, and liver of the victims along with water and available sediments from drowning sites for a comparison. Samples were dipped in a strong mixture of HNO_3 and H_2SO_4 and left undisturbed overnight on one hand whereas on the other hand 10 mL of 30% diluted H_2SO_4 was added to the samples. Negative control with both the extraction methods was also kept. Centrifugation of both the test samples was done at 4000 rpm and further washing was done using distilled water. Slides were made from the final residue and examined under microscope. This study led to the conclusion that the acid digestion using diluted acid is one of the best methods, as there was no formation of any precipitate.

Singh and Deepa (2013) authenticated acid digestion technique by HNO_3 for the re-examination of four water bodies for diatomological mapping (D-mapping). The

seasonal collection of water samples was done during 2011–2012. The samples collected (200 mL of water) from the collection site were digested by pouring 40 mL of HNO_3. This acidic sample solution was centrifuged for 10 min at 3000 rpm. The supernatant was discarded off and the residue was used for microscopic study using DPX as mounting material. Magrey and Raj (2014) described acid digestion technique as one of the best methods for the extraction of diatoms in case studies related to drowning deaths. They performed acid digestion process for the diagnosis of 31 drowning cases in Jammu and Kashmir, India. The parts taken were sternum, clavicle, femur, and lungs with the water sample from the suspected site of drowning. The exhibits were digested with 50 mL of HNO_3 for the proper removal of inorganic/organic materials present inside the diatoms. The solution was kept undisturbed overnight after the addition of acid to the samples. The acid sample solution was then boiled till yellow color appears. Centrifugation was performed thrice at 4000 rpm for 10 min. Permanent slides of the diatoms were made for further analysis.

Lin et al. (2014) carried out experiments on 100 autopsy cases (September 2009–2012) of victims who were considered to have died due to drowning. The sample taken was fluid extracted from sphenoid sinus and lower lobe of lung tissue. The acid digestion was performed by using mixture of glacial acetic acid: Concentrated H_2SO_4 (9:1) for sphenoid sinus fluid whereas HCl: HNO_3 (1:3) was used for the extraction of diatoms from lung tissues. The diatom examination was performed for their microscopic analysis. Scott et al. (2014) highlighted the efficiency of H_2O_2 to study the extent of transfer of diatoms and their extraction from clothing. The section of 100% unused cotton T-shirt was immersed in the soil and water samples taken from sampling sites (aquatic and terrestrial), respectively. The immersion was performed for variable durations beginning with 3 min to 24 h to allow proper transfer of diatoms. Then digestion was initiated using H_2O_2 and thereafter final clear slides were made using Naphrax as the mounting medium.

Wang et al. (2015) performed a test for the comparison of traditional acid digestion technique and a newly developed Lefort's aqua regia technique. The sample taken was 20 minced kidneys mixed with water rich in diatoms. The Lefort's aqua regia HNO_3: HCl (15 mL: 5 mL) was added to samples with the addition of H_2O_2 (1 drop per 3 s). This addition of H_2O_2 was stopped when the occurrence of bubbles in the sample solution were found to be the least; the acid sample solution was left undisturbed in thermostatic water tank for 50 min at 85 °C. Then heating of this solution was done with the addition of H_2O_2 (1 drop per 3 s) whereas in the traditional acid digestion method, 20 mL of concentrated HNO_3 with the addition of 5 mL of 30% H_2O_2 was used. The samples were analyzed with Scanning Electron Microscope (SEM). Lefort's aqua regia gave better results with the clearer background and more diatom recovery with intact features. A seasonal diatom database of Douro river estuary was preferred and 37 cases of drowning death in Oporto River were studied from November 2012 to March 2014 by Coelho et al. (2016). They treated the water samples with 96% (W/W) of 20 mL H_2SO_4 and the tissue samples (assembled from the drowning case victims) were treated from 37% (W/W) of 20 mL HCl. On the completion of digestion process, microscopic study of the

final residue left was done using Naphrax as the mounting medium. The diatoms examined from the tissue samples were compared with those found in water samples.

Fucci et al. (2017) demonstrated the importance of acid digestion test in the veterinary context. They performed successful test of diatom extraction on ten species of wildlife animals. Lungs, liver, kidney, brain, heart, bone marrow, spleen, and putative drowning medium were taken as samples for the examination of cause and manner of death. The samples were digested carefully by using 40% H_2O_2 and 20% HCl with subsequent centrifugations at 1000 rpm. Finally qualitative and quantitative examination of the diatoms was done under microscope with the aliquot left after centrifugation. Pal et al. (2017) examined 66 cases of drowning deaths from three districts of north range of Himachal Pradesh, India. The samples collected comprised of bones like sternum, femur, and tibia along with tissues (lungs, spleen, and heart) from the bodies of drowned victims along with the water from the suspected drowning sites. Acid digestion of all these samples was done using H_2SO_4 as an oxidizing agent for the extraction of matter present inside the diatoms. Once the digestion process was complete, washing was done with distilled water for avoiding any cross contamination and centrifugation at 4000 rpm was performed for the complete removal of the acid. Then analysis of the residue was done microscopically under proper surveillance of a forensic investigator.

Scott et al. (2019) studied the transfer of diatoms to various types of clothing under various environmental conditions and clothing types. This test was executed by using nine types of natural and synthetic clothing fabrics immersed in the river water, under consideration. The sample collection was done three times in a year to study diversity of diatoms with seasonal variations i.e. in early spring, late spring, and in winter. Reference control sample was also taken in a wide range to ensure the possibility of all types of diatoms present at the site of collection. Acid digestion of the water samples recovered was done successfully by using 10% HCl and 30% H_2O_2. Finally repeated centrifugation was done for the proper cleaning of the sample solution and slides of the residue were made for the microscopic examination.

16.5.1.1 Limitations of Acid Digestion Method

These digestion processes for the extraction of diatoms have few shortcomings. Working with concentrated acids (H_2SO_4 or HNO_3) may lead to some burns or injuries during an experiment; the reaction with these acids occurs violently which can destroy some fragile diatoms thus may lead to error in examination and investigation. Moreover, during the subsequent washings and centrifugations at high rpm, there can be a chance of cross contamination or mislaying of diatoms present. But apart from these few shortcomings, it can be believed that acid digestion method was the most commonly used in forensic drowning from the past many years to date. This method proved beneficial as it can be feasible due to easier availability of all the reagents and digestion of the organic material and can be achieved in a short duration of time. It can also be applied to all types of samples recovered during a drowning investigation. Therefore, digestion of diatoms is a process of careful handling and it should be carried out under the guidance of an experienced investigator taking all the precautions.

16.5.2 Enzymatic Method

During the last few years, some enzymatic methods have been reported for the digestion and analysis of diatoms for forensic purposes (Ludes et al. 1994; Kakizaki and Yukawa 2015; Kakizaki et al. 2018). Ludes et al. (1994) were the first to try enzymatic method for diatom analysis on the 12 corpses retrieved from different water bodies of Strasbourg area, France. This method was carried out by using 10 g of well cleaned lungs, liver, kidney, and brain of the victims. These tissues were treated by taking 500 µL of Proteinase K (10 mg/mL), 100 mL of 0.01 M of Tris-HCl buffer solution (PH-7.5), and 2% of SDS. This solution was then incubated at 50 °C and then kept overnight. Again 500 µL of Proteinase K was mixed and the solution was diluted by using 100 mL of distilled water. Once the enzymatic digestion is completed, the sample solution was centrifuged at 3000 rpm for 15 min. Supernatant was finally discarded and the residue was observed microscopically. Overall this method proved useful and has shown better results for quantitative and qualitative analysis of diatoms.

Kakizaki and Yukawa (2015) examined fresh and marine diatoms in 20 lung samples isolated from 10 drowned victims using digestion method with Qiagen Proteinase K, Qiagen Buffer ATL, and 5 N HCl. The lung tissue was minced in a petri dish and transferred to polymethylpentene centrifuge tubes with 6 mL of Buffer ATL for tissue lysis and 1 mL of 20 mg/mL of Qiagen Proteinase K. Negative control of 1 mL ultrapure water was also taken to check any cross contamination from air or reagents. Digestion of the sample solution was carried out at 56 °C for 15–60 min using a block incubator followed by centrifugation. The supernatant was discarded and the residue was centrifuged repeatedly with ultrapure water. The residue was then mixed with 13 mL of 5 N HCl and heating of the sample solution was initiated for 15 min at 75 °C. Centrifugation and washing with ultrapure method was repeated. Depending upon the amount of residue left, 13–14 mL of 99.5% of ethanol was added and centrifuged. After washing, the sample was analyzed for diatom examination.

Recently Kakizaki et al. (2019) extracted diatoms from 80 cadavers found at various aquatic sites including rivers, paddies, near estuaries, and sea using new enzyme called papain. This enzyme has been extracted from the plant *Carica papaya*. This protocol was used for the digestion of samples including 1 g lung, 10 g of kidney and liver, respectively. The first test was employed on the liver tissue of Porcine using different concentrations of papain (0.08, 0.25, 0.75, and 100 mg/mL) at various temperatures (30, 40, 50, 60, and 70 °C). This enzyme works actively with a sulfhydryl group which was allocated by L-Cysteine (0.2, 0.5, 10, and 2.5%) with pH of 6.0–7.0. Porcine liver (1 g) was taken as a sample. Activity of Papain and Proteinase K was studied at various temperatures and concentrations with the presence of standard detergent sodium dodecyl sulfate 2% (SDS). The digestion of the tissue was found to be the best at 50 °C performed for 1 h for papain at a concentration of 0.5 mg/mL. The papain digestion was performed to detect diatoms in the tissues of 80 cadavers died due to drowning. This method was superior to Proteinase K digestion but could not match chemical digestion method.

16.5.3 Combined Approach of Microwave Digestion, Vacuum Filtration and Scanning Electron Microscopy

Hu et al. (2013) performed diatom analysis by using a combined approach of microwave digestion, vacuum filtration followed by scanning electron microscopy (SEM). They performed an experiment using 20 mL of water and 2 g of thin organs (lungs, liver, and kidney) and transferred them to microwave digestion system (MW3000) with 6 mL of HNO_3 and 2 mL of H_2O_2. To liquefy the samples, microwave power was increased for 5–10 min. The fluid obtained was transferred for vacuum filtration, made using a nylon membrane of fine 0.45 μm pore size which was further connected to automatic scanning mode using SEM. This method proved to be efficient than acid digestion as it was safer, faster, and time saving and was easy to perform with very little, if any, chances of contamination.

Diatoms were analyzed in the tissues obtained from 128 drowned victims. The samples under examination were lungs, liver, and kidneys of the victims and water samples from the suspected drowning sites were recovered for comparison (Zhao et al. 2017). The samples were cleaned properly with distilled water, cut into thin sections, and then placed in a microwave digestion system with 8 mL of concentrated HNO_3 and 20 mL of 30% H_2O_2 for the oxidization of organic and inorganic materials present inside the diatoms. This sample solution was filtered using Millipore membrane. The membrane was examined by scanning electron microscope. This study suggested that in drowning cases, chances of having diatoms in lungs are 100% and 97% in other distant organs.

16.5.4 Soluene-350 Digestion

Matsumoto and Fukui (1993) developed a method of digesting diatoms using a solubilizer Soluene-350; samples from lungs, liver, and kidneys of rats (5 g each) were mixed with 45 mL of water enriched with diatoms. Centrifugation of the sample was done at 3000 rpm for 20 min. The residue left after centrifugation was transferred to 30 mL glass tube to which 20 mL of Soluene-350 was added. The mixture was placed in an ultrasonic cleaner where it was exposed to ultrasonic waves followed by centrifugation for 5 min at 3000 rpm. The residue left was analyzed microscopically for detection of diatoms. The diatom enriched water sample was also analyzed by using same procedure. The results obtained from this technique were better and the time consumed was lesser as compared to conventional acid digestion.

Yoshimura et al. (1995) used Soluene-350 test for the detection of diatoms in two drowned bodies of victims found in Yodo River in Japan. The lungs, liver, and kidneys were extracted and cut into thin sections. These sections were subjected to centrifugation at 3000 rpm for 5 min. Washing of the centrifuged sample was done using Mili Q water free of plankton to avoid any contamination and centrifugation was repeated. The supernatant was discarded off and the pellet was mixed with Soluene-350 and incubated at 50 °C for 2 h and kept overnight at room temperature.

After the incubation, centrifugation was done at same speed for 60 min; the residue left was analyzed microscopically. Sidari et al. (1999) used Soluene-350 on freshwater samples of Rosandra stream and seawater from Adriatic Sea. They took 30 mL of both the water samples for washing process using distilled water. Then centrifugation was done at 3000 rpm for 5 min. The pellet formed was treated with 8 volumes of Soluene-350. This sample solution was incubated at 50 °C and centrifuged. It was concluded from the microscopic study of the samples that this technique has been more efficient for the freshwater as compared to seawater samples. This was due to the fact that the seawater diatoms are not so resistant because of lesser silica present in their frustules. Overall this technique has shown promising outcome for freshwater diatoms.

16.5.5 Ash Digestion Method

Ludes et al. (1994) performed ash digestion method by taking 10 g of the tissue material. The sample was dried in a muffle furnace, first at 80 °C for 8 h followed by at 200 °C for another 8 h. The sample was converted to ash finally at 550 °C. This ashed sample was then subjected to acid digestion and the final residue was microscopically examined.

This technique has not been accepted widely because it usually destroys the sample and has limited usage for the organs present in large quantity which is generally not found. Moreover, ashing method cannot be used for the water samples.

16.5.6 Polymerase Chain Reaction (PCR) Method

Rutty et al. (2015) performed PCR on the study of diatom analysis in twenty cases of victims found to have died due to drowning but later it was learnt that only twelve of them died due to drowning. They took different body tissues and kept them at 4 °C without any preservative and maintained the sterile conditions for the extraction of DNA and tissue lysis. The isolated DNA was stored again at 4 °C. PCR proved successful in the analysis of diatoms on genetic level but the rate of contamination in this technique is quite high. Moreover, it is also a time-consuming and laborious process.

16.5.7 Whole Slide Imaging

It is one of the latest and finest methods for diatom analysis. Zhou et al. (2019) performed first diatom analysis by whole slide imaging using convolutional neural networks. The rate of identification through this mechanism was quite high as it was based on artificial intelligence. The diatoms were examined successfully using this whole slide scanning mechanism. Thus, this technique paved a way to use this in future for the examination of diatoms in a short time without any error and labor.

16.6 Status of Diatom Test in Solving Forensic Cases

In the field of forensics, validity of diatom test has been making progress nationally and internationally. There are variety of drowning death crime scenes whose mystery was solved using diatoms as supportive corroborative evidence. Some of these are discussed below.

A case of a 15 year old school boy of Panipat, Haryana (India) was solved due to application of diatoms in forensics (Vinayak et al. 2011). The victim's body was found beheaded in a canal in Rohtak, Haryana. At first sight the case was thought to be of honor killing and the samples (clavicle, sternum, and skull) collected from the crime scene were sent to Forensic state lab (FSL) Madhuban, Haryana for examination. The diatom test was performed and the results showed that the reason of death was suicidal drowning.

Bąk et al. (2018) performed a case study of a victim (40-year-old women) who was found missing between her home and workplace. During the initial police investigation, the rescue dog took them to the concrete tank near the workplace but the body was not found. Then after two months, victim's body was found in a riverside 1 km away from her residence. According to autopsy report, it was an accidental drowning but the site of crime was not confirmed. So to assure the crime site, diatom test was performed by taking lungs, liver, kidney, and rib marrow samples along with water samples from both sites (concrete tank and the river body), respectively. Diatom test was performed and it was concluded that the victim died in the concrete tank but with the flow of water her body passively moved to riverside. Therefore, it can be observed from the above case reports that diatoms are beneficial supportive evidence in solving cases related to forensic drowning. But the examination should be carried out with extensive care under the guidance of a well experienced forensic examiner. In putrefied bodies, the sample is limited and the diatoms are so minute entities thus making their examination more difficult. Thus to have a correct analysis, avoid such, a knowledgeable forensic expert should be approached for the examination.

16.7 Controversies in the Validation of Diatom Test in Solving Cases

With the various benefits and significance of diatoms in the drowning cases, several controversies also appeared pointing questions on the authenticity of the test. Presence of diatoms in the *antemortem* drowning cases denotes that the person was alive during his entry to the water body and then died due to drowning. However, there are several cases where diatoms occurred in the body organs of victims who died due to a reason other than drowning. Moreover, the absence of diatoms in several *antemortem* drowning case findings had raised controversies on their application in the fields of forensics. Hürlimann et al. (2000) showed that the diatom density goes on decreasing from the factor of 10–100 from the drowning medium to the lungs and further keeps on decreasing (100–1000) during their

transportation to other organs. Under normal conditions, this density gradient depicts that the diatoms in drowning medium decide the fate of their presence in the organs of drowned victims.

It was concluded that the study based on the density gradient will be relevant only if the drowning medium has plenty of diatoms in it. Langer et al. (1971) found that some small diatoms or diatom fragments were present in the low quality cigars. The composition of the cheap cigars consisted of tobacco leaf sheets which have traces of diatoms spicules or their fragments which were also observed from their smoke after burning. It was deduced therefore that the presence of diatoms in the respiratory organ of a person might occur due to smoking of such cigars. After air, then comes the source of diatoms in the digestive tract through food and drinks.

Yen and Jayaprakash (2007) stated that food preferences of a person can also be responsible for the presence of diatoms inside the body. They concluded that anyone habitual of eating prawns and clams also consumes about two million diatoms in a single year. Lunetta et al. (2013) proposed that diatom penetration inside the non-drowned victims may take place due to the strong hydrostatic difference of water, through wounds or injuries and due to the forced submersion of the body in the drowning medium. According to the findings of Ludes et al. (1994), positive diagnosis of diatoms from the samples of non-drowned victims can occur through contamination from the glasswares used during experimental examination or sometimes from the reagents. Ludes (2013) also stated that in some of the cases they can occur due to cross contamination during autopsy. Therefore, the test has to be conducted with great care and precision.

Despite all these controversies, the validity of diatom test is still recognized successful in solving most of the drowning cases. Contamination or cross contamination can be avoided by using disposable instruments and gloves during the autopsy or in laboratories. In case of washing the sample, double distilled water should be used. With each diatom analysis, a blank control and a negative control sample should be used to check the cross contamination from the lab apparatus or from the reagents used. However, if the diatom count in the sample is low, even though the cause of death due to drowning cannot be ruled as sometimes the diatom content may be less at a particular site, the victim might have inhaled less amount of water or the death may be sudden due to some disease or the person may be under the influence of any drug or alcohol intoxication which becomes the cause of death rather not drowning.

A "criteria of concordance" was set by Pollanen (1998a) which states that a remarkable amount of diatoms present inside the organs should match with those present in the putative drowning medium and it resolves many controversial queries of the critics. Ludes (2013) concluded approximate number of complete diatoms in different organs to infer the test positive for drowning (20 diatoms from 100 µL of sediment from 2 g of lung sample and more than 5 from the 100 µL of sediment from 2 g of other distant organs). Consequently, all the opinions concluded by the critics were resolved and the diatom tests are still considered as a Golden standard in forensics.

16.8 Conclusion

Diatoms provide good corroborative evidence in drowning to determine the cause of death as well as the site of crime. These small sized cosmopolitan species are widely present in freshwater and marine water bodies and have a special feature of silicified frustules. Due to their small size, they easily enter inside the body of a drowned victim through respiration and then get circulated to various organs until the circulation ceases. But in case where dead body is dumped in water in a manner to manipulate the crime scene, the entry of diatoms inside the body becomes limited due to no respiration or circulation. Very few diatoms penetrate inside the lungs through hydrostatic pressure of water; thereby in *antemortem* drowning, the diatoms are extremely rich in the lungs and can be seen easily in the distant body organs (liver, kidney, spleen, bone marrow, and brain) whereas in *postmortem* immersion, they are found mostly inside the lungs with very poor amount as also in other organs. Moreover, the quantitative and qualitative analysis with the above discussed methods makes them suitable in determining the site of drowning. It has been concluded that the efficiency of diatom test depends on certain criteria like contamination from various sources, quantity of diatoms in the drowning medium, amount of diatoms recovered from the organs, and the suspected drowning medium. Apart from certain controversies linked to the validity of diatom test, they are still considered as a major effective tool in solving the mystery of drowning cases. Thus, from the above account, the fact of diatoms being the golden standards in the field of forensics cannot be denied. Under the proper surveillance, the diatom test proves authentic in the study of drowning related cases. In future, the molecular approaches like PCR, diatomological mapping, automatic diatom identification and classification (ADIAC), and the use of artificial intelligence with the neural networks can add on the authenticity for the diatom analysis information.

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