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Edited by Eduardo Jacob-Lopes, Leila Queiroz Zepka and Maria Isabel Queiroz

MICROALGAL BIOTECHNOLOGY

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http://dx.doi.org/10.5772/intechopen.71228
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First published in London, United Kingdom, 2018 by IntechOpen
eBook (PDF) Published by IntechOpen, 2019
IntechOpen is the global imprint of INTECHOPEN LIMITED, registered in England and Wales, registration number: 11086078, The Shard, 25th floor, 32 London Bridge Street
London, SE19SG – United Kingdom
Printed in Croatia

British Library Cataloguing-in-Publication Data
A catalogue record for this book is available from the British Library

Additional hard and PDF copies can be obtained from orders@intechopen.com

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p. cm.

Print ISBN 978-1-78923-332-2

Online ISBN 978-1-78923-333-9

eBook (PDF) ISBN 978-1-83881-417-5

Meet the editors

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Contents

Preface

This book shows how microalgae can be used biotechnologically to solve industrial and agricultural problems, besides producing consumables in a sustainable way. Divided into 10 chapters, the book explores some potential technological routes of the microalgae-based processes and products. The chapters presented in this book are intended to help provide a deeper understanding and insight into promises and challenges for microalgal biotechnology as a substantial contributor to the future of food, feed, chemicals, pharmaceuticals, energy and fertilizer supplies. Given the book's breadth of coverage and extensive bibliography, it offers an essential resource for researchers and industry professionals working in applied phycology.

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Chapter 1

Introductory Chapter: Microalgae Biotechnology. A Brief Introduction

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.73250

1. A general overview on microalgal biotechnology

The diversity of beings that make up the biological universe that comprehends the planet is simply dazzling. Not less dazzling than the whole is, with no doubt, each unit in special, even though being able to present itself in some similar aspects morphologically and physiologically to the others. Regardless of the specific pattern of the protein synthesis of each organism, they all perform chemical transformations, however, with due metabolic differences, which stimulate the potential biotechnological interest.

From the multiple metabolic diversities, it has been highlighted under the biotechnological point of view, the microalgae. This terminology comprehends a variety of prokaryotic or eukaryotic organisms, autotrophs and many capable of development themselves heterotrophically. The microalgae have been considered promisors organisms to various biotechnological applications in function of its potential of use to diverse renewable forms of useful substances and sustainable processes.

In morphologic, physiologic and structural characterization terms, the microalgae are organisms beings of extraordinary adaptive capacity susceptible to survive in an environmental diversity. The morphologic term aims to describe the shape, the size and the growth of an organism. The microalgae are highly adaptable not only in the physiologic aspect, but also are carrier of a quite diverse morphology. Mainly with respect to the size of these organisms, the physiologic properties are determinant factors and are directly connected to their cellular structure.

The microalgae term is devoid of taxonomic value. Once they constitute a polyphyletic and highly diversified group of microscopic beings present in aquatic systems, on its great majority photosynthesizes and present a vegetative structure known as thallus, whose cellular differentiation is characteristically small or null [1].

The principal standards to classify these microorganisms are pigmentation, chemical nature of the reserve products, and basis cellular structure. According to Mata et al. [2], under the denomination of microalgae are included organisms with two types of cellular structure: prokaryotic structure, with representatives in the groups *Cyanophyta* and *Prochlorophyta*; eukaryotic structure, with representatives in the groups *Glaucophyta*, *Rhodophyta*, *Ochrophyta*, *Haptophyta*, *Cryptophytes*, *Dinophyta*, *Euglenophyta*, *Chlorarachniophyte* and *Chlorophyta*. However, it stands out under the biotechnological exploitation aspect the groups: cyanobacteria (*Cyanophyta*), Chlorophyceae (*Chlorophyta*) and diatoms (*Ochrophyta*).

The cyanobacteria are classified in the eubacteria kingdom. It has never presented flagella, and by having its cellular organization of the prokaryotic type, it does not possess nucleus or organelles. They present in their structure the chlorophyll and the photosystems I e II, in contrasts to others photosynthetic bacteria, which allow them to realize the photosynthesis in the presence of oxygen. Some species are strictly phototrophic, while others act in, optional mode, being able to grow heterotrophically [3].

On the other hand, the Chlorophyceae present a great variety in the levels of organization, from unicellular, a flagellated microalgae or not, until the morphologically complex thallus. As well as cyanobacteria, the Chlorophyceae can be found in almost all environments, however, about 90% of the total of species (mainly the microscopy forms) occur in fresh water [4].

In relation with diatoms, they have been characterized by a cellular wall denominated frustula, and beyond the chlorophyll a and c, the plastids contain fucoxanthin and other xanthophylls, such as neofucoxanthin, diadinoxanthin and diatoxanthin. The main reserve substance of such organisms is chrysolaminarin, but the cells can also accumulate lipids and in general, are deprived of flagella and almost always heterotrophic. The diatoms inhabit the photic zone of oceans (up to about 200 m deep), seas, lakes, and rivers, presenting benthic and planktonic form [4].

The peculiar properties of each group made these microorganisms beings metabolically differentiated. Although the pigmentary profile of microalgae makes the photosynthesis the principal metabolic model, these organisms stand out by notable versatility to provide the necessary energy to the growth and maintenance, which identify them as a potential source of resources to be exploited biotechnology.

As previously mentioned, the microalgae possess a versatility in relation to the maintenance of their structures, utilizing different energy metabolisms as photosynthesis, respiration, and the nitrogen fixation, its utilization initially depends on the evaluative origin and also of the environment conditions or of the cultivation conditions [5].

From the use of the carbon source, we can differentiate two basic types of nutrition from microorganisms: autotrophic or heterotrophic. Between the autotrophic exist the photosynthetics,

whose obtain energy for the metabolism of light, and the heterotrophic, whose obtain energy to the metabolism originated from inorganic compounds or of ions and the nitrogen fixation [5]. The photosynthetic cultivation involves the utilization of light as the only energy source, which is converted in chemical energy by the photosynthesis process.

On this cultivation conditions, there is a direct relation between the photosynthetic activity and the microorganism's growth, since the light is the substrate and its intensity influences in the specific rate of growth, being capable to be identified or not by the photo-inhibition [6]. The reactions of light capture on upper plants and eukaryotic algae occur in the internal membranes of chloroplasts, in thylakoids and in the plasma membrane ramification, where are found the photosynthetic pigments (chlorophylls, carotenoids, and phycobiliproteins) and the enzymes necessary for the use of light and conversion of carbon dioxide. The pigments are located in highly organized structures called photosystem I (PSI), or reaction center (P700), and photosystem II (PSII) or reaction center (P680), whose are interconnected through an electron transport chain. The photosystems are enzymatic complexes capable of utilize the light as a reducing element, producing the driving force for the transport of electrons. On prokaryotic microalgae, these structures are in the thylakoids. When the light is absorbed, a series of oxidation reactions are started [7, 8].

In the presence of organic molecules as sugars, organic acids and acetate, some microalgae and cyanobacteria are capable of using the heterotrophic metabolism in the dark. Thus, the metabolism consists in the substitution of atmospheric CO_2 of the photosynthetic cultures by exogenous carbon sources, which makes it possible the increase of the concentration of biomass and of the productivity. Furthermore, the microalgae are capable of metabolize different forms of nitrogen compounds to endure the growth and the cellular manutention. Nitrogenous organic sources, such as urea and amino acids, have access to the interior of the cell through active transport. Some amino acids have been used as source of carbon and nitrogen to support the microalgal growth in the dark, but the most widespread form as a source of nitrogen is urea, which is hydrolyzed in NH_3 and CO_2, being able the two generated compounds to being utilized to the cellular growth [9].

Beyond the mentioned metabolisms, it is common to observe another metabolic process, the mixotrophic. This one is equivalent to the autotrophic and the heterotrophic, where the organic compounds and CO_2 are necessary to the growth, and where they are operated simultaneously the respiratory and the photosynthetic system, although the realization of photosynthesis is its principal energy source. However, the mixotrophic organisms assimilate organic compounds as a carbon source, while using inorganic compounds as electrons donors [10].

Particularly, in relation to photosynthetic cultivation, this presents a unique demand in industrial biotechnology, which is the contribution of light energy to the cells. This feature substantially modifies the bioreactor configuring for the microalgal cultivation. In this sense, the choice of the ideal photobioreactor is a crucial factor to the good performance of any microalgae culture system, since this is one function of the environment conditions and of the cultivation. Without taking into consideration any economic aspect, the photobioreactor must present some basic design requirements: efficient supply of light energy and CO_2; controlled temperature; suitable mixing system; availability of nutrients; facility in the control of reaction conditions; and facility in the scale-up [11].

Both the quantity and quality of the light source affect the cellular growth rate. When the intensity of the light is low, the growth rate is proportional to the light intensity. However, when the light intensity is much higher than the value of the saturation constant, it occurs the photoinhibition of the growth, which generally is caused by the reversible damage to the photosynthetic apparatus. The natural or artificial illumination can be utilized in function of the required characteristics in the cultivation system [6, 11].

In relation to the nutrition, despite of the different between the species, to a good growth, the culture medium must provide all the macronutrients and micronutrients demanded. In the group of the macronutrients are C, N, O, H and P, whose are considered as essential, and also Ca Mg, S, and K. In case of the macronutrients it has the Fe, Mn, Cu, Mo and Co, some species also need low concentrations of vitamins [12].

The carbon source most utilized in photosynthetic cultivations is the carbon dioxide, which can be in its normal or dissociate form (HCO_3–) in the cultivation medium. The ideal concentration of CO_2 in the medium still is not well elucidate and it varies according to the specie of microalgae, however, generally are used concentrations between 3 and 15%. Considering the low solubility of CO_2 in liquids, there must be an efficient transference of CO_2 to the medium, in order to raise the volumetric mass transfer coefficients (Kla) to guarantee a suitable cellular growth [13].

The control of temperature also is indispensable in order to assurance the stability of the culture. In general, the ideal temperature of the cultivation occurs in the mesophyll region (25–35°C), although some thermophilic strains resist to temperatures in the range of 60°. The majority of the cultivation systems assume the variation of temperature as a result of environment variation, though the use of heating mantle, serpentines, and external heat exchangers can be installed for the control of the temperature of microalgae bioreactors [12, 13].

Finally, agitation is a necessary operation in the cultivation of these microorganisms, since it ensures the spatial uniformity of reaction vessels, favoring the exposure of cells to light, the heat transfer, and the thermal stratification, as well as improving gas exchange. A suitable mixture minimizes yet the formation of cellular aggregates that increase the global inefficiency of the bioreactor. Although fundamental to the suitable development of the process, the operation of the mixture is related to hydrodynamic stresses associated to the cellular shear, which damages and inhibit the microalgal growth. The microalgae bioreactors are normally equipped with pneumatic aeration systems and mechanical agitation, or yet with a combination between these systems [14].

The cultivation of microalgae in large scale have started before the middle of the twentieth century, since then it has already been reported a wide range of cultivation systems. The differentiation of these cultivation systems depends principally of the cost, the type of product desired, the source of nutrients and the CO_2 capture. The culture systems are generally classified according with its conditions of project as open or closed systems [15].

Traditionally, the open systems have been widely used to the cultivation in large scale, due to its simplicity and low cost. These systems of cultivation present two principal configurations: circular and raceway ponds, that consist in a shallow tank (20–30 cm deep) of circular or oval

geometry, equipped with mechanical agitation systems, which expose the culture medium to the air by bubbling. Unfortunately, these photobioreactors allow only a limited control of the operation conditions. Besides that, the productivity is low, due the low absorption of light in the tank bottom and the major probability of contamination. Other limitations of these type of cultivation include a major necessity of space of land to the cultivation, losses by evaporation, high temperatures and, consequently, low efficiency of mass transfer [16].

An alternative to the open photobioreactors are the closed systems, which enable a great variety of configuration and significantly increase the performance of the cultivation. Three main configurations dominate the arrangements of closed photobioreactors are the tubular systems, the flat plates and the vertical columns. These systems are characterized by high photosynthetic efficiencies associated to a greater precision and control of the operational variables, lower risk of contamination and minimization of water losses by evaporation. Although, are severely limited by capital costs and scale-up [15, 17].

Futhermore, microalgae are important bioresources that have a wide range of biotechnological applications. The metabolic characteristics of the microalgae make these microorganisms an important source of resources to be explored. Associated with photosynthetic metabolism, the respiration and nitrogen fixation constitute important metabolic routes, passable of being biotechnologically explored for diverse purposes [18, 19].

The utilization of microalgae for the treatment of wasterwater is particularity attractive, due to its abilities in assimilating nutrients as organic matter, NO_3^-, PO_4^{3-}, NH_4^+, CO_2 and heavy metals [20]. The biological treatment of wastewaters occurs in heterotrophic bioreactors, where the organic matter and the inorganic nutrients are simultaneously converted in biomass in the absence of light. These processes are considered a cheap alternative to the conventional forms of treatment of secondary and tertiary effluents [21]. On the other hand, the photosynthetic cultivations of microalgae demonstrate to be one of the mitigation technologies of CO_2 most promisor, since that these microorganisms present high photosynthetic rates when compared to other upper plants, besides having a high resistance to high concentrations of carbon dioxide [8].

In this sense, the microalgae present versality to associate the treatment processes of wastes with the parallel production of inputs. The main biomolecules of commercial interest are the intracellular substances (pigments, fatty acids, proteins, and carbohydrates), the extracellular substances (carbohydrates and volatile compounds) [22–24].

The crescent interest in the natural and organic production has pressed the development environmentally correct technologies to a sustainable agriculture. The reduction in the fertility of the soils, the low efficiency in the use of chemical fertilizers, the increase of the environment pollution, and the decline of the productivity of important of agricultural crops are associated to the need to develop and implement biofertilization techniques that promote a reduction in the use of chemical fertilization in parallel to the increase of the efficiency of the use of these nutrients. In this way, the use of microalgae, preferentially the cyanobacteria, whether of free-living or symbiotically associated to other organisms, is considered an alternative in potential to supply the practices of organic agriculture. Independent of the metabolic pathway adopted in the cultivation, photosynthetic or heterotrophic, the microalgal biomass present mineral

substantial composition, that can reach 25% of the biomass dry weight. Furthermore, the presence of mineral elements of commercial importance as nitrogen (N), phosphorus (P) and potassium (K) potentiate the use of these in biosolids in the formulation of organic fertilizers [25].

In additional, when processed through chemical or biological reactions, the microalgae can provide different types of renewable biofuels that are called of biofuels of third generation. These include biodiesel, biohydrogen and bioethanol. The biodiesel is a mixture of alkyl esters and fatty acids obtained by transesterification. The transesterification is a reaction of multiple phases, where triglycerides are converted into diglycerides, subsequently these are converted into monoglycerides, which are then converted into esters (biodiesel) and glycerol (co-product) [2]. The bioethanol is obtained by biochemical processes through the fermentation of sugars (cellulose, xylose, galactose, arabinose, glucose and mannose) of the biomass and subsequent hydrolysis of the starch and cellulose content by a thermochemical process. On the other hand, the biohydrogen can be produced through two enzymatic pathways: direct photolysis or indirect photolysis [23, 26].

The commercial exploration in large scale of the microalgal intracellular content has started in de decade of 1950, motivated by the elevated content of proteins of biomass to utilization as an alternative food resource [27]. Since then, it has opened a wide range of passable products to be explored. Between them are found the pigments, classified in carotenoids, phycobiliproteins and chlorophylls that are responsible for the colors yellow/orange, red/blue and green, respectively [24]. The cyanobacteria in particular synthesize high levels of phycobiliproteins, with percentages that reach up to 8% of its dry weight. These pigments have been used as non-radioactive fluorescence markers when covalently bound to antibodies, biotin, lecithin and hormones. Beyond these applications, the phycobiliproteins present important antioxidant and anti-inflammatory activity. In function of the stability of molecules, the phycocyanin is used in the formulation of cosmetics as perfumes and makeup to the eyes. The carotenoids are other important class of pigments abundantly found in microalgae. It is well known to the pro-vitamin A activity of β-carotene and in its effects on the vision and in the immune system. Beyond this, the antioxidant activity of the carotenoids is associated to the prevention of cancer, atherosclerosis, degenerative diseases, and aging [28]. In consequence of these properties, innumerous carotenoids have been approved by the regulatory agencies in diverse countries as natural dyes of food and feed, with special emphasis to the astaxanthin produced by the chlorophyceae *Haematococcus pluvialis*, which represents the largest natural source of this carotenoid. This pigment has been extensively utilized in the feeding of salmon and trout as a coloring agent. Finally, it must be considered some keto-carotenoids and glycosylated carotenoids found exclusively in microalgal cells such as myxoxanthophyll, equinenone and canthaxanthin [24].

Some proteins, peptides and amino acids present biological functions associated to nutritional benefits and the human health. Thus, as the majority of species of microalgae present contents above 50% of protein in dry weight, these biopolymers can be uses as nutraceuticals or included in formulation of functional foods. Beyond of the hypolipidemic and hypoglycemic properties, the ingestion of unicellular proteins is associated to the reduction of the cholesterol and the triglyceride levels. Finally, some proteins of microalgal origin

are associated to the stimulation of the production of the hormone cholecystokinin that regulates the appetite suppression and therefore, has been considered in the formulation of functional foods against the obesity.

Beyond the cholesterol, some microalgae species produce unconventional sterols such as brassicasterol, campesterol, stigmasterol, and sitosterol. In function of the high levels of sterols, these species have been used in the formulation of rations for the growth of juveniles, especially oysters [29]. In addition, the solar blocking compounds derived from microalgae have emerged as an alternative to the synthetic molecules and/or molecules of botanical origin. The compounds with photoprotective action include two main classes: the amino acids of mycosporine type (MAAs), and the scytonemin. These compounds present high blocking efficiency, photostability and low toxicity. The MAAs are compounds derived from iminocarbonyl of the cyclohexenone chromophore from mycosporins, that possess a conjugated nitrogenous substituent ring (an amino acid or an aminoalcohol). These compounds are soluble in water and present UVB action. They are found in both eukaryotic and prokaryotic microalgae. The scytonemin is an alkaloid indole, liposoluble, found exclusively in cyanobacteria. Not included in any of the previous chemical groups, compounds such as ciguatoxin, karatungiol, okadaic acid, and gamma-aminobutyric acid have been identified in microalgal extracts. Some marine dinoflagellates, belonging to the Dinophyta division, synthesize ciguatoxin and okadaic acid that present antifungal action. The okadaic acid has also been associated to the promotion of the secretion of the nerve growth factor (NGF). The karatungiol is another antibiotic molecule produced by marine dinoflagellates with antifungal and antiprotozoal activity. Finally, the gamma-aminobutyric acid is an amino acid that has a stimulating and regulating action of the brain development. It is associated to the neuronal excitability and the muscle tone.

Another class of compounds with positive effect tin the human health are the fatty acids. Inside this group, to the majority of species, the polyunsaturated fatty acids, of the families ω3 and ω6, correspond to the largest fraction, being capable to get to 60% of the total lipids. On the other hand, microalgae also are classified as a good source of mineral salts such as phosphorus, iron, manganese, copper, zinc, magnesium and calcium. This composition makes the biomass a passable source of being utilized as food supplement in aquiculture and also as fertilizing. Carbohydrates can also be produced intracellularly. They are found in the form of starch or simple sugars such as arabinose, xylose, mannose, galactose and glucose, as well as less common sugars such as rhamnose, fucose and uronic acids [30].

In addition, microalgae present the capacity to accumulate extracellular polymeric substances (EPS) on the surface of the cell as a form of protection for them. EPS's are heterogeneous matrices of polysaccharide polymers, proteins, nucleic acids and phospholipids. The microalgal exopolymer have multiple industrial applications. In this sense, they can be applied in the food industry as thickeners and gelling agents. In the pharmaceutical industry they can be used as a hydrophilic matrix for controlled release of medicines, in the development of bacterial vaccines and to increase non-specific immunity. In addition, some EPS's possess biosurfactant characteristics and are being used in bioremediation of waters and soils [31].

Thus, based on issues above summarized, it's possible to conclude that the diversity of important applications of microalgae in innumerable technological routes of production makes these microorganisms become biocatalysts with a wide potential of agricultural and mainly industrial exploration. Regardless of these potentialities, the competition with consolidated technological routes based, for example, on non-renewable fossil inputs, often makes economically unfeasible in the present scenario the microalgae-based processes and products. Therefore, new industrial approaches have been proposed and implemented in order to effectively enable the technical and economic success of these technologies. The integration and intensification of processes associated to the concept of biorefinery have been considered as the main engineering strategies that will enable a large commercial exploration of the microalgae-based processes. These new technological routes are orientated to the effective use of the industrial resources based on equipment, materials and processing techniques. These three approaches of engineering of process will allow in mid-term the consolidation of microalgae-based processes as effective enhancers of the industrial sustainability, balancing the vectors of the environment, economy and the society.

The chapters presented in this book are intended to help provide a deeper understanding and insight into promises and challenges for microalgal biotechnology to be a substantial contributor to future of food, feed, chemicals, pharmaceuticals, energy, and fertilizer supplies.

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Chapter 2

Checklist, Qualitative and Quantitative Analysis of Marine Microalgae from Offshore Visakhapatnam, Bay of Bengal, India for Biofuel Potential

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.75549

Abstract

Observation on the productivity parameters in relation to micro algal biodiversity helps to know the population in particular season and spatial. The study investigates in detail the seasonal and spatial variation of microalgae with special emphasis on their interrelationship of chlorophyll concentration. In order to obtain the information on distribution and abundance of Visakhapatnam Coast microalgae for isolation, fortnightly intervals samplings was carried out. Investigation has been made on the microalgae with special reference to the phylum Ochrophyta, Dinophyta, Chlorophyta, Euglenozoa, Haptophyta and Cyanophyta. Abundance of species under different season of pattern was Pre-monsoon>Post monsoon>Monsoon. The data evaluated from this study was used to prepare the checklist for marine microalgal diversity of Visakhapatnam offshore region.

Keywords: chlorophyll, microalgal abundance, checklist of marine algae, phytoplankton, Vizag coast, Bay of Bengal

1. Introduction

Andhra Pradesh is one of the six States/U.Ts of India adjoining the Bay of Bengal with a coastline of 974 km and the continental shelf area of 33, 227 sq. km. East coast India, surface currents skirting the coast move in a northerly direction during part of the year, and the opposite direction during the rest of the year [1]. Influx of untreated wastewaters into the aquatic bodies that are challenging the stability of nations [2]. Since in the middle of 19th century East coast of India, Visakhapatnam coastal waters pollution caused by the effluents from nearby industries, like steel

plant, petroleum refinery, fertilizer plant and a lead and zinc smelter are discharged into a North-Western arm of the inner harbor through surface drain known as Meghadri gedda and city's domestic sewage drains directly into the Northern arm of the inner harbor [3]. Microalgal abundance and distribution critically depending on various physical, chemical and biological factors [4] and their ability to assimilate sufficient carbon, nitrogen and phosphorous, as well as minor nutrients, to ensure replication. Alterations in species richness are mainly due to the variability of abiotic factors, such as short-term climatic variations [5].

Multi-population microalgae and some native isolates i.e., *Tetraselmis* sp., *Chlorella* sp. were cultivated in open air pond in East cost of India proved the potential for biofuel production. Exploring microalgae diversity to find out the suitable season and spatial for microalgae isolation in Visakhapatnam coast since, the microalgae having wide application in biofuel and pharmaceutical. The research work from Andhra University, India revealed that Visakhapatnam coast is one of the potential sources for microalgae. Two years field work data from this study shows the relationship between the spatial and seasonal variations in Bay of Bengal, East Coast of India, and Visakhapatnam. Qualitative and quantitative analysis showed the feasibility to isolate the potential candidate strains for biofuels from the coastal water of Visakhapatnam.

2. Materials and methods

2.1. Description of the study area and physiography of sampling sites

Bay of Bengal, a semi-enclosed tropical basin, is a part of the northern Indian Ocean and experiences seasonal changes in circulation and climate due to the monsoons. Visakhapatnam is a coastal city located on the eastern seaboard of India between Chennai and Calcutta (latitude 17°38′N and 17°45′N and longitude 83°16′E and 83°21′E) surrounded on three sides by the overlapping mountain ranges, and the South-eastern city is safeguarded by the Bay of Bengal. The South-west monsoon starts late in June and lasts till early October. North-east monsoon closely follows the South-west monsoon and extends till December. Fourteen sampling stations were selected along the Visakhapatnam offshore line and surface water samples were collected at the 30–40 M depth line of the sampling point. Sampling sites and global positioning systems of sampling locations was represented in **Figure 1**. The sampling stations between Bheemunipatnam (station 1) and Coastal Battery (station 7) cover the area like waste water of shrimp culture ponds, seafood processing centers, shrimp hatcheries, fish cages cultures and sewage outfalls enter into the coastline. The sampling stations between Harbor (station 8) and Pudimadaka (station 14) cover the areas where the effluents from the major power plants such as Nuclear Power Corporation of India limited and East Coast Energy limited enter on the coastal line.

2.2. Sample collection and estimation of chlorophyll

A mechanized boat was used to get into the sea for sample collection. Physico-chemical examination of sea surface waters in relation to microalgal abundance from Bheemunipatnam (approximately 23 km northward from the Visakhapatnam port) to Pudimadaka (approximately

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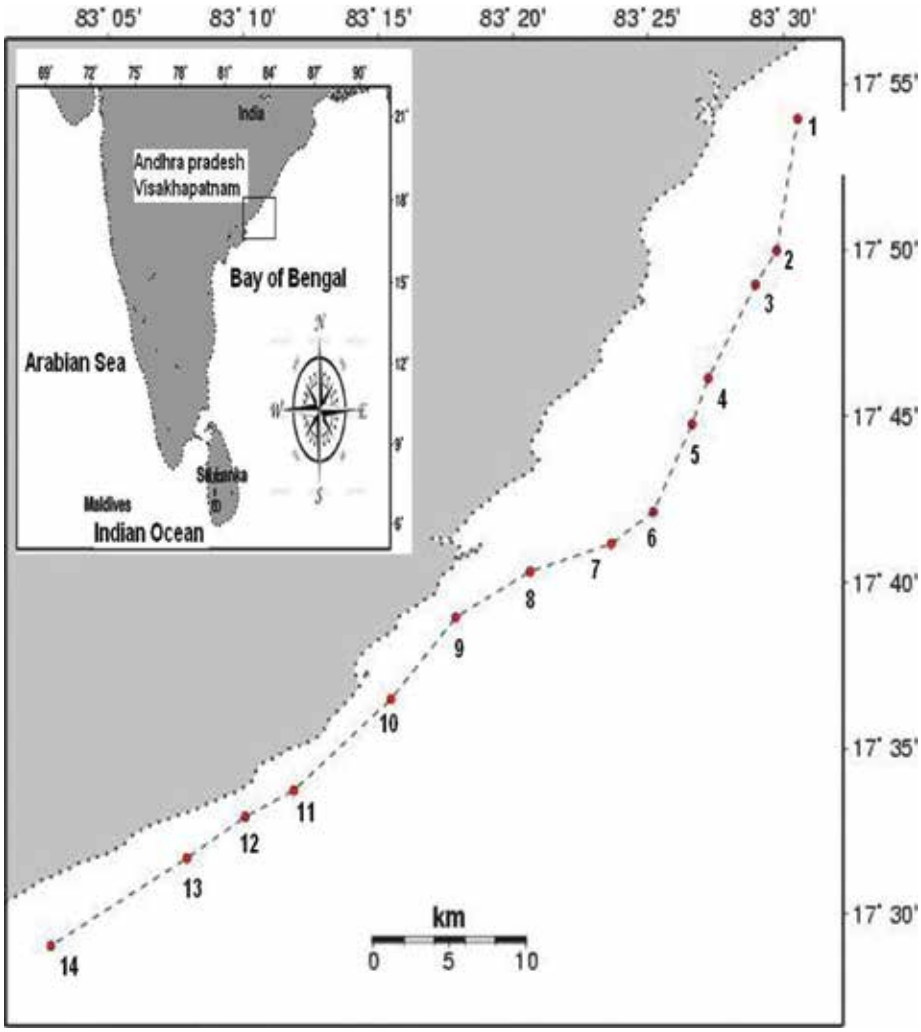

Figure 1. Map showing the sampling stations (1–14).

40 km southward from the Visakhapatnam port), on the East Coast of India, was carried out at fortnightly intervals for a period of 2 years (February 2011 to January 2013) at 14 selected stations, along the Visakhapatnam offshore region. Samples were collected using clean plastic buckets from the surface sea water and transferred into 1 L amber color plastic bottles and stored in refrigerator until further analysis. The water sample free of zooplankton is filtered through Millipore (0.45 μm, 47 mm) filter paper and the pigments chlorophyll 'a', 'b' and 'c' are extracted from the phytoplankton by using 90% acetone.

2.3. Collection of microalgae and identification

One hundred liters of sea surface water at each station was collected and filtered through cone-shaped phytoplankton net of 20-μm mesh size, made by bolting silk and concentrated to

500 ml was transferred into pre-cleaned polyethylene bottles. The filtrate was preserved in 3% neutralized Lugol's iodine solution. All the water samples were filtered with 60-μm size zooplankton net in order to remove the zooplankton and debris. The filtrate was collected into five liters capacity Polyethylene Terephthalate (PET) jar and kept undisturbed for 2 days to achieve complete sedimentation. After sedimentation of phytoplankton, the supernatant solution was siphoned out to concentrate the volume to accurately about 100–200 ml. Concentrated samples were examined under bright field, dark ground illumination and phase contrast at ×200, ×400 and ×1000 magnifications with the help of Axio scope A1 and Primo Vert (Carl Zeiss, Germany) microscopes. Identification of microalgae was done using an inverted research microscope based on standard keys [6–15] and current taxonomical details were been updated according to World Register of Marine Species and Algaebase (2015).

3. Results

3.1. Chlorophyll 'a'

In the year 2011–2012, the highest and lowest mean chlorophyll 'a' recorded in station 13 (PRM) and station 9 (MON) were 4.81 ± 2.86 and 0.68 ± 0.36 μg l^{-1}, respectively. During the post monsoon period, the maximum and minimum chlorophyll 'a' recorded was 1.82 ± 1.53 and 0.85 ± 0.55 μg l^{-1} respectively at station 9 and station 7 (**Figure** 2**a**). In the year 2012–2013, the highest and lowest mean chlorophyll 'a' recorded in station 9 (MON) and station 3 (PRM) were 3.76 ± 1.92 and 1.54 ± 1.04 μg l^{-1}, respectively. During the post monsoon period, the maximum and minimum chlorophyll 'a' recorded was 2.66 ± 1.79 and 1.92 ± 1.34 μg l^{-1} respectively at station 7 and station 2 (**Figure** 2**b**). In the both sampling years, two way ANOVA showed significant differences between the season ($p < 0.001$) but not between the stations.

3.2. Chlorophyll 'b'

In the year 2011–2012, the highest and lowest mean chlorophyll 'b' recorded in station 5 (PRM) and station 7 (POM) were 3.15 ± 2.28 and 0.70 ± 0.51 μg l^{-1}, respectively. During the monsoon period, the maximum and minimum Chlorophyll 'b' recorded was 1.99 ± 1.58 and 0.99 ± 0.48 μg l^{-1} respectively at station 4 and station 8 (**Figure** 3**a**). In the year 2012–2013, the highest and lowest mean chlorophyll 'b' recorded in station 6 (MON) and station 11 (PRM) were 4.14 ± 2.73 and 1.50 ± 1.35 μg l^{-1}, respectively. During the post monsoon period, the maximum and minimum chlorophyll 'b' recorded was 2.51 ± 1.79 and 1.69 ± 0.73 μg l^{-1} respectively at station 7 and station 13 (**Figure** 3**b**). In the both sampling years, two way ANOVA showed significant differences between the season ($p < 0.001$) but not between the stations.

3.3. Chlorophyll 'c'

In the year 2011–2012, the highest and lowest mean chlorophyll 'c' recorded in station 6 (PRM) and station 12 (POM) were 3.52 ± 2.3 and 0.96 ± 0.61 μg l^{-1}, respectively. During the monsoon

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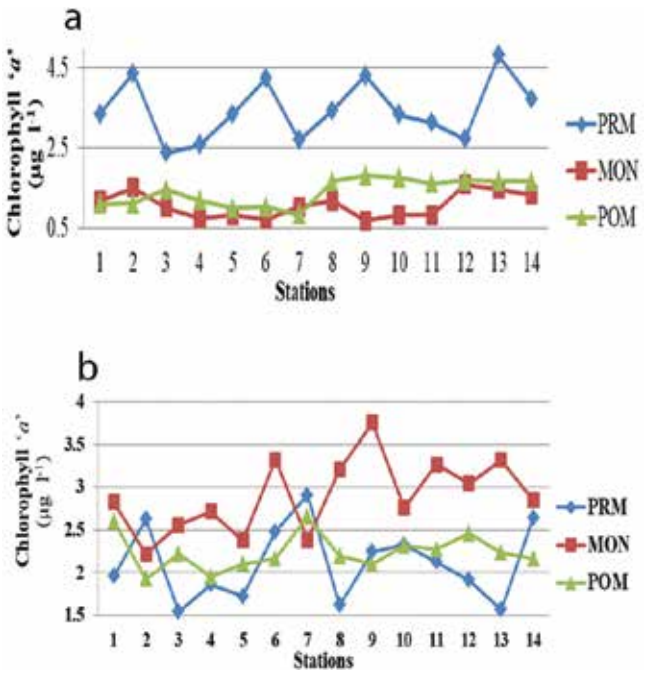

Figure 2. Seasonal and spatial variations in chlorophyll '*a*' (a) for the sampling year 2011–2012 and (b) for the sampling year 2012–2013.

period, the maximum and minimum chlorophyll 'c' recorded was 2.81 ± 2.13 and 1.49 ± 0.83 μg l^{-1} respectively at station 13 and station 6 (**Figure** 4**a**). In the year 2012–2013, the highest and lowest mean chlorophyll 'c' recorded in station 6 (MON) and station 4 (PRM) were 5.34 ± 1.31 and 1.52 ± 1.11 μg l^{-1}, respectively. During the post monsoon period, the maximum and minimum chlorophyll 'c' recorded was 3.91 ± 2.09 and 2.91 ± 1.31 μg l^{-1} respectively at station 7 and station 13 (**Figure** 4**b**). In the both sampling years, two way ANOVA showed significant differences between the season ($p < 0.001$) but not between the stations.

3.4. Seasonal mean value chlorophyll 'a, b, c'

The seasonal mean value for chlorophyll 'a, b, c' for the both sampling year was represented in **Table 1**. The seasonal mean value for chlorophyll 'a' varied between 1.07 ± 0.86 μg l^{-1} (MON) and 3.46 ± 2.05 μg l^{-1} (PRM) in the year 2011–2012. In the year 2012–2013, seasonal mean value fluctuated between 2.11 ± 1.83 μg l^{-1} (PRM) and 2.9 ± 1.47 μg l^{-1} (MON). The highest annual mean value 2.42 ± 1.46 μg l^{-1} was recorded in 2012–2013 and lowest annual mean value 1.98 ± 1.35 μg l^{-1} was recorded in the sampling year 2011–2012. In the year 2011–2012, the highest and lowest seasonal mean chlorophyll 'b' recorded in pre-monsoon and post monsoon was 2.76 ± 1.2 and 1.13 ± 1.07 μg l^{-1}, respectively. In the year 2012–2013, the highest and lowest mean chlorophyll 'b' recorded in monsoon and post monsoon was 3.07 ± 1.33 and 2.07 ± 0.96 μg l^{-1}, respectively. The highest and lowest annual average of chlorophyll 'b' was

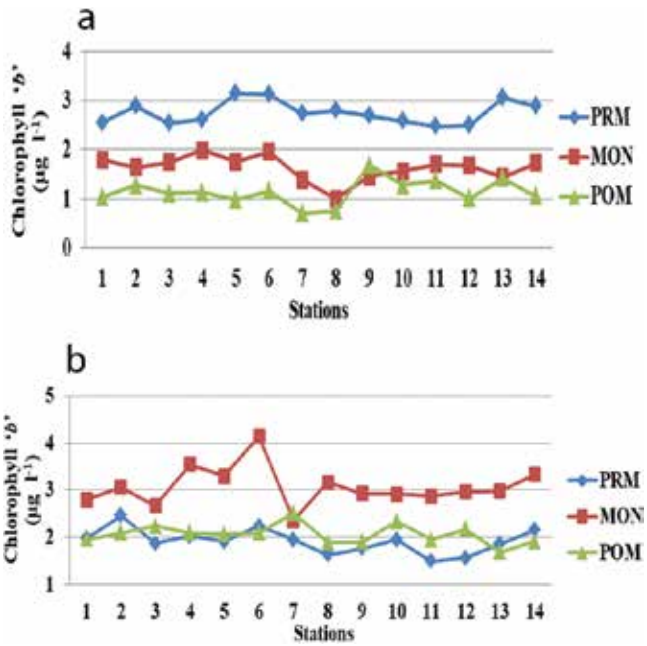

Figure 3. Seasonal and spatial variations in chlorophyll '*b*' (a) for the sampling year 2011–2012 and (b) for the sampling year 2012–2013.

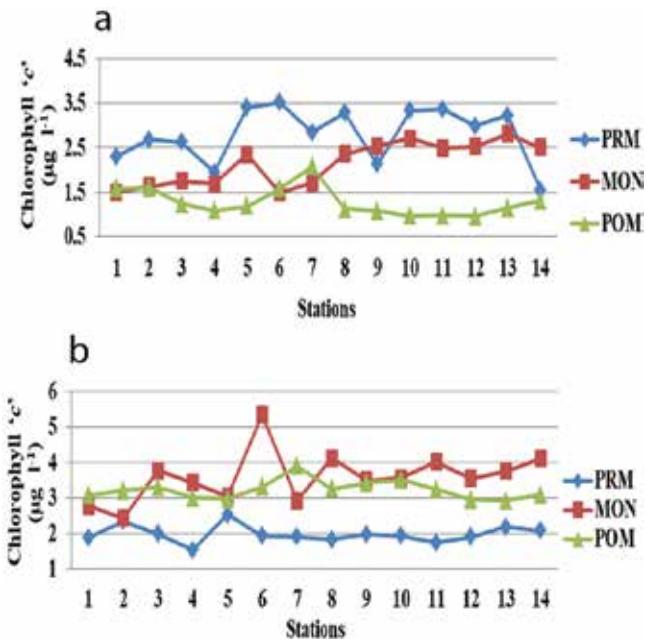

Figure 4. Seasonal and spatial variations in chlorophyll '*c*' (a) for the sampling year 2011–2012 and (b) for the sampling year 2012–2013.

Chlorophyll	Pre-monsoon (n = 112)		Monsoon (n = 112)		Post monsoon (n = 112)		Annual (n = 336)	
	2011–2012	2012–2013	2011–2012	2012–2013	2011–2012	2012–2013	2011–2012	2012–2013
Chlorophyll '*a*' ($\mu g\ l^{-1}$)	3.46 ± 2.056	2.11 ± 1.83	1.07 ± 0.86	2.9 ± 1.47	1.41 ± 1.11	2.24 ± 1.07	1.98 ± 1.35	2.42 ± 1.46
Chlorophyll '*b*' ($\mu g\ l^{-1}$)	2.76 ± 1.2	1.92 ± 1.14	1.63 ± 1.28	3.07 ± 1.33	1.13 ± 1.07	2.07 ± 0.96	1.84 ± 1.6	2.35 ± 1.14
Chlorophyll '*c*' ($\mu g\ l^{-1}$)	2.80 ± 1.619	1.98 ± 1.21	2.15 ± 1.75	3.59 ± 1.81	1.28 ± 1.02	3.22 ± 1.18	2.08 ± 2.81	2.93 ± 1.41

Table 1. Analytical mean values (mean ± standard deviation) of chlorophyll a b c for the sampling years 2011–2012 and 2012–2013.

2.35 ± 1.14 and 1.84 ± 1.6 $\mu g\ l^{-1}$ in 2012–2013 and 2011–2012, respectively. In the year 2011–2012, the highest and lowest seasonal mean chlorophyll 'c' recorded in pre-monsoon and post monsoon was 2.80 ± 1.62 and 1.28 ± 1.02 $\mu g\ l^{-1}$, respectively. In the year 2012–2013, the highest and lowest mean chlorophyll 'c' recorded in monsoon and pre monsoon was 3.59 ± 1.81 and 1.98 ± 1.21 $\mu g\ l^{-1}$, respectively. The highest and lowest annual mean value of chlorophyll 'c' was 2.93 ± 1.41 and 2.08 ± 2.81 $\mu g\ l^{-1}$ in 2012–2013 and 2011–2012, respectively.

3.5. Microalgal diversity

Microalgae characteristics were given in **Table 2**. A total of 191 species of microalgae were identified from the 14 study sites along the Visakhapatnam offshore region, Bay of Bengal. Of these, 131 species were recorded under Ochrophyta division (68.58%) (127 species are diatoms (40.84% Centrales, 25.65% Pennales), 3 species belong to silicoflagellates 1.57% and 1 species in Eustigmatophyceae 0.52%), 35 species (18.3%) belong to dinophyta, 11 species (5.76%) belong

Division	Characteristics				
	Class	Family	Genus	Species	% of species
1. Ochrophyta	4	38	66	131	68.5
2. Dinophyta	1	7	10	35	18.3
3. Cyanophyta	1	6	8	11	5.76
4. Chloropyta	3	5	5	7	3.66
5. Euglenozoa	1	2	2	4	2.09
6. Haptophyta	2	2	3	3	1.57
Total	12	59	91	191	100
Centrales	1	21	33	78	40.84%
Pennales	1	15	30	49	25.65%
Dictyochaceae	1	1	2	3	1.57%
Monodopsidaceae	1	1	1	1	0.52

Table 2. Characteristics of microalgae for the both sampling years.

to cyanophyta, 7 species (3.66%) belong to chlorophyta, 4 species (2.09%) belong to euglenozoa and 3 species (1.57%) belong to haptophyta. The community composition of phytoplankton was dominated by Centrales, which represented by 21 families with 33 genera. *Chaetoceros* (15 species), *Bacteriastrum* (5 species), *Coscinodiscus* and *Thalassiosira* (6 species) genus were encountered with more than five species and *Rhizosolenia* and *Triceratium* genus were recorded with four species under centric diatoms. Pennate diatoms were recorded with 30 genera and 49 species. In pennate diatoms, maximum four species were recorded under the same genus of *Pleurosigma* and three species were encountered in each genus of *Nitzschia, Amphora*, *Diploneis* and *Fragilariopsis*. In Dinophyta, 7 families and 10 genera were recorded, maximum number of species found in the genus were *Neoceratium* (8), *Protoperidinium* (8) *Prorocentrum* (3) and *Dinophysis* (4). In Cyanophyta, six families and eight genera were recorded. *Trichodesmium*, *Lyngbya* and *Schizothrix* genus were encountered with two species and remaining genera was observed with single species. In the division of Chlorophyta, 3 classes, 5 families and 5 genera were recorded, in this division species two species was encountered with *Tetraselmis* and *Dunaliella* genus. The division Haptophyta was observed with 3 species and 3 genera. In the Euglenozoa division, 2 genera with 4 species were noted and Silicoflagellates was observed with 2 genera and 3 species. During the course of study phytoplankton showed distribution pattern as: Centric diatoms>PennateDiatoms>Dinophyta>Cynophyta>Chloropyta>Euglenozoa>Haptophyta.

3.5.1. Seasonal variations in qualitative abundance of microalgae

Seasonal variation in microalgae qualitative abundance was given in **Table 3**. Seasonal fluctuations of species composition in both sampling years varied from 150 (MON) to 161 (PRM). The species from phylum Ochrophyta fluctuated between 103 (PRM 2012–2013) and 115 (PRM 2011–2012). Centric diatoms varied between 66 (POM) and 70 (PRM) in the total of 78. Pennate

Division	**2011–2012**			**2012–2013**		
	PRM	**MON**	**POM**	**PRM**	**MON**	**POM**
1. Ochrophyta	115	106	107	110	103	107
2. Dinophyta	23	27	26	26	28	28
3. Cyanophyta	11	9	9	10	9	8
4. Chlorophyta	6	7	6	7	4	5
5. Euglenozoa	4	3	2	3	3	3
6. Haptophyta	2	3	2	2	3	2
Total	161	155	154	158	150	153
Centrales	69	68	68	70	67	66
Pennales	42	36	38	37	33	38
Dictyochaceae	3	1	2	2	2	2
Monodopsidaceae	1	1	1	1	1	1

Table 3. Quality abundance of microalgae.

diatoms ranged from 33 (MON) to 42 (PRM) from the total of 46. Dinoflagellate showed the variation between 23 (PRM) and 28 (MON) species in the total of 35. Cyanobacteria found to be low in post monsoon (8) and high during pre-monsoon (11) in the total of 11. In the division of haptophyta, species abundance fluctuated between 2 and 3 during pre-monsoon and monsoon, respectively and chlorophyta species ranged from 4 to 7 during monsoon and pre-monsoon, respectively.

3.5.2. Seasonal variations in qualitative abundance of microalgae

Checklists (species composition) of microalgae on the Visakhapatnam offshore region were summarized in Appendix A. The samples of all station were pooled for seasonal distribution analysis into samples of three season (pre-monsoon, monsoon and post monsoon) which helped in obtaining information about the distribution and species composition (or) diversity of the sea surface water. Abundance of phytoplankton during the study period (191 species) was reported along the Visakhapatnam Coast throughout the sampling years. Six divisions of microalgae Ochrophyta, Dinophyta, Cyanophyta, Chlorophyta, Euglenozoa and Haptophyta were recorded. The class Bacillariophyceae and Coscinodiscophyceae comprised of 36 families, 63 genera and 124 species. Altogether 191 species of microalgae belonging to the classes of Bacillariophyceae, Coscinodiscophyceae, Dinophyceae, Euglenophyceae, Chlorodendrophyceae, Chlorophyceae, Eustigmatophyceae, Dictyophyceae, Prymnesiophyceae, Pavlovophyceae, Trebouxiophyceae and Cyanophyceae were identified. Of these, *Tetraselmis gracilis, Dicrateria inornata, Thalassiosira subtilis, Chaetoceros muelleri, Chaetoceros diversus, Skeletonema costatum, Thalassiosira subtilis* and *Asterionella inflata* were considered important species based on the order of their abundance and frequency of occurrence. Present study indicates that the diatoms are the dominant group followed by the dinoflagellates and others.

4. Discussion

Microalgal diversity is extremely important to analyze the status of an ecosystem. Local microalgae species have a competitive advantage under the local geographical, climatic and ecological conditions [16]. The levels of chlorophyll are the proof of photosynthetic activities and there was a distinct seasonality observed in the levels of phytoplankton biomass at the study sites. Total phytoplankton representing the maximum concentration of chlorophyll '*a*' was recorded along the Visakhapatnam Coast during pre-monsoon (2011–2012) and monsoon (2012–2013). Surface phytoplankton abundance (as chlorophyll '*a*' concentration) levels, reached up to 4.81 μg l^{-1}, occurred in pre-monsoon, with the further smaller peaks in post monsoon and monsoon periods of the sampling year 2011–2012. In the year 2012–2013, peak chlorophyll levels reached up to 3.76 μg l^{-1}, in monsoon. Highest chlorophyll '*a*' concentration was reported during pre-monsoon (2011–2012) coincided with the period of upwelling and in the year 2012–2013, and the monsoon coincided with large scale mixing between surface river waters and deeper nutrient rich bottom waters [17]. The annual average (13.4 μg l^{-1}) chlorophyll '*a*' was reported for the entire euphotic zone of EEZ of Arabian Sea and it ranged from 0.1 to 96.4 μg l^{-1} [18]. Chlorophyll '*a*' was found between 3.31 and 99.12 μg l^{-1} in surface

water off Gopalpur, East coast of India, Bay of Bengal [19] and varied between 0.21 and 30.82 μg l^{-1} off Mangalore, West Coast of India [20] and the highest value 8.28 μg l^{-1} was observed during the post monsoon off Cape Comorin [21]. In the present work, the higher concentration of chlorophyll '*b*' was recorded during monsoon, 2012–2013 and the West coast also recorded the highest concentration of chlorophyll '*b*' (20.41 μg l^{-1}) during the monsoon [22]. Relatively high chlorophyll '*b*' indicates the presence of ultra or nano-planktonic microalgae coming under the class Chlorophyceae/Euglenophyceae/Prochlorophyceae [23].

Chlorophyll '*c*' was recorded in lower concentrations during the pre and post monsoon season (2012–2013) and the same pattern was also observed in West Coast of India [22]. Lowest chlorophyll and DO concentrations were recorded during pre-monsoon period (2012–2013) has clearly indicated that plankton growth during pre-monsoon was reduced because of oxygen demand by the organic matter in the period of May and June [24]. The highest seasonal average of chlorophyll '*a*', '*b*' and '*c*' in all stations were recorded during the monsoon of 2012–2013 due to the nutrient rich land runoff water in to the coastal areas. Monsoon rains and associated land runoff and nutrient loading determines the balance of organic to inorganic loadings which act as major factors controlling community responses of microalgae [25].

Earlier studies have reported that the nutrient supply could have significant effect on community composition of phytoplankton [26]. The nitrogen limitation is known to have a significant effect on phytoplankton composition. To determine the growth of phytoplankton, nutrients are the primary factors. The highly seasonal nature of monsoon rains might have increased the concentration nutrients (nitrite, nitrate and silicate) in monsoon period. The recorded low values during pre- and post-monsoon period may be due to its utilization by phytoplankton as evidenced by high photosynthetic activity and the dominance of neritic seawater having a negligible amount of nitrate [27]. In East Coast of India, Bay of Bengal a total number 249 species of phytoplankton comprising of 131 species of dinoflagellates, 111 species of diatoms and 7 species of cyanobacteria were recorded during 2004 [28]. In 2012, EEZ micro algae distributed studies reported 71 species, 30 genera with 22 families under Bacillariophyceae and 88 species of dinoflagellates encountered with 22 genera and 18 families [29]. In Cyanophyceae, 3 genera with 4 species and in Dictochophyceae one species were also reported in their studies.

Microalgal population (Diatoms>Dinoflagellates>Cyanophyta>Chlorophyta) pattern of this record was similar to that reported from Coastal waters off Rushikulya estuary, East Coast of India [30, 31]. Eurythermal and euryhaline nature of diatoms in all the three season leads to their dominance [32] and have been observed to bloom regularly along the Indian Coast during June to October [33, 34]. Dominance of diatom over dinoflagellates coincides to the report from Indian coastal water [35–37] and world oceans [38, 39]. A cosmopolitan genus such as *Chaetoceros* was dominant with 15 species followed by other major genera such as *Coscinodiscus* (6 species), *Nitzschia* (3 species) and *Rhizosolenia* (4 species) in this study. *Oscillatoriaceae* and *Phormidiaceae* were the dominant family in Cyanophyceae class with 3 species and followed by *Schizotrichaceae* (2 species) and the trend was similar to the studies in the same coast [40, 41]. Epiphytic cyanobacteria, *Dactylococcopsis* and *Synechococcus* and 17 genera belonging to chlorophyceae including *Oocystis, Chlorella vulgaris* was recorded in Palk Strait [42] and a total 44 species of Cyanobacteria from Tamilnadu [43] and Kerala [44].

Seasonal succession of phytoplankton population indicated that dinoflagellates mainly *Neoceratium* and *protoperidinium* as well as silicoflagellates; *Dictyocha* were dominant population in pre-monsoon periods. In diatoms *Rhizosolenia, Guinardia, Thalassiosira, Chaetoceros and Asterionella* genus were relatively more dominantly throughout the sampling years. The same sequences in the phytoplankton abundance were reported at East Coast India [45] and from Pakistan 15 species in *Navicula* was reported [46]. Generally, *Skeletonema costatum* found to be dominant in this Coast coincides was agree with earlier studies [45, 47, 48]. *Chaetoceros* species have contributed high in total population of centric diatoms and it is coincided by the findings during pre-monsoon periods [45, 49]. In Sundarbans also diatom reported as dominant group in the overall phytoplankton group and *Skeletonema* and *Chaetoceros* are more abundant species East Coast of India [50].

In the current study, genus *Asterionella* and *Fragilariopsis* were observed throughout the year but the highest numbers were observed in the months of April and June 2013. *Asterionella japonica* bloom and discoloration Off Waltair, East coast [51] and north western Bay of Bengal [52] strongly supporting our findings. *Pleurosigma* species with five different classes was reported as dominant species in Nizampatnam, East Coast of India [53]. The maximum numbers of diatoms recorded in post monsoon period was only five numbers more than that of pre-monsoon. In waters off Visakhapatnam Coast, some genera of green algae under the division of Chlorophyta i.e. *Chlorella*, *Tetraselmis*, and *Dunaliella* were recorded throughout in both samplings years. Prasinophyceae and Trebouxiophyceae classes were found predominantly along with some prokaryotic and eukaryotic picoplankton species in the same Coast [54]. Highest number of species under chlorophyta was recorded during pre-monsoon periods like cyanophyceae. In contrary, Southwest Coast of India had recorded that the cyanophycean and chlorophycean species distributed during the monsoon period of the years 2006–2008 [22]. As like as centric diatoms, cyanophyta also showed a maximum number of species during pre-monsoon period, however, the maximum values were obtained for dinoflagellates during monsoon period. The annual mean water temperature 29°C for both sampling years supported for the abundance of flagellates throughout the year. The abundance of flagellate species was commonly occurring at higher water temperatures [55]. In the Visakhapatnam coastal waters, Haptophyceae and Prasinophyceae classes were most abundant [56] and 17 species of flagellates represented by 6 diverse groups in the same coastal waters [57]. *Dictyocha fibula* was recorded during monsoon periods of both the sampling years except during post monsoon (2011–2012) and the same sequence were reported the lower abundance of oceanic species *Dictyocha fibula* during pre- and post-monsoon [58].

5. Conclusion

Chlorophyll concentrations and diversity of microalgae in Visakhapatnam offshore region studied in detail for a period of 2 year (2011–2012 and 2012–2013). Our results revealed that the diatoms were found to be dominant with number of species in Visakhapatnam offshore waters, Bay of Bengal. From this study, we had found the suitable spatial and season to get sea water to isolate particular species of microalgae and which is use full for shrimp hatchery in

that coastal zone. And another advantages of this study was pin point spatial of this coastal area may help to isolate microalgae can be grown in open pond without any major contamination to produce biomass for biodiesel production.

Acknowledgements

The authors are grateful to the Council for Scientific and Industrial Research (CSIR), Government of India, New Delhi, India. This study was undertaken as a part of CSIR-NMITLI project (grant no.: 5/258/64/2009 — NMITLI; date: 19-04-2010) on "Biofuel from marine microalgae" co-funded by the Ministry of Earth Sciences (MoES).

A. Species composition of microalgae along the Visakhapatnam offshore region

	CENTRIC DIATIOMS Phylum: Ochrophyta (Cavalier-Smith in Cavalier-Smith & Chao, 1996)		**Class: Coscinodiscophyceae (Round & Crawford in Round, Crawford & Mann, 1990)**	**2011–2012**			**2012–2013**		
S. No	**Family**	**S. No**	**Species**	**PRM**	**MON**	**POM**	**PRM**	**MON**	**POM**
1	*Bellerocheaceae* (Round and Crawford in Round *et al*. 1990)	*1*	*Bellerochea malleus* (Brightwell) Van Heurck 1885	+	+	+	+	+	+
2	*Biddulphiaceae* (Kutzing, 1844)	*2*	*Biddulphia biddulphiana* (Smith) Boyer, 1900	+	+	—	+	+	+
3	*Chaetocerotaceae* (Ralf in Pritchard, 1861)	*3*	*Bacteriastrum comosum* (Pavillard, 1916)	+	+	+	+	+	+
		4	*Bacteriastrum delicatulum* (Cleve, 1897)	+	+	+	+	+	+
		5	*Bacteriastrum furcatum* (Shadbolt, 1854)	+	+	+	+	+	+
		6	*Bacteriastrum hyalinum* (Lauder, 1864)	—	+	+	—	+	—
		7	*Bacteriastrum elongatum* (Cleve, 1897)	+	+	+	+	+	+
		8	*Chaetoceros affinis* (Lauder, 1864)	+	+	+	+	+	+
		9	*Chaetoceros atlanticus* (Cleve, 1873)	+	+	+	+	+	+
		10	*Chaetoceros lauderi* (Ralfs in Lauder, 1864)	+	+	+	+	+	+
		11	*Chaetoceros compressus* (Lauder, 1864)	—	+	—	—	+	—
		12	*Chaetoceros muelleri* (Lemmermann, 1898)	+	+	+	+	+	+

	CENTRIC DIATIOMS Phylum: Ochrophyta (Cavalier-Smith in Cavalier-Smith & Chao, 1996)	**Class: Coscinodiscophyceae (Round & Crawford in Round, Crawford & Mann, 1990)**		**2011–2012**			**2012–2013**		
S. No	**Family**	**S. No**	**Species**	**PRM**	**MON**	**POM**	**PRM**	**MON**	**POM**
		13	*Chaetoceros tortissimus* (Gran, 1900)	—	+	+	+	—	+
		14	*Chaetoceros curvisetus* (Cleve, 1889)	+	+	+	+	+	+
		15	*Chaetoceros decipiens* (Cleve, 1873)	+	+	+	+	+	+
		16	*Chaetoceros diadema* (Ehrenberg) Gran, 1897	—	—	+	—	+	—
		17	*Chaetoceros diversus* (Cleve, 1873)	+	+	+	+	+	+
		18	*Chaetoceros didymus* (Ehrenberg, 1845)	+	—	—	+	—	—
		19	*Chaetoceros laevis* (Leuduger-fortmoral, *1892)*	+	+	+	+	+	+
		20	*Chaetoceros lorenzianus* (Grunow, 1863)	—	+	+	+	+	+
		21	*Chaetoceros messanense* (Castracane, 1875)	+	+	—	+	+	—
		22	*Chaetoceros paradoxus Var. eibenii* (Grounow) Grounow, 1896	+	+	—	+	—	—
4	*Coscinodiscaceae* (Kutzing, 1844)	*23*	*Coscinodiscus curvatulus* (Grunow in Schmidt, 1878)	+	+	+	+	+	+
		24	*Coscinodiscus granii* (Gough, 1905)	+	+	—	+	+	—
		25	*Coscinodiscus radiatus* **(**Ehrenberg, 1840)	+	+	—	+	—	—
		26	*Coscinodiscus centralis* (Ehrenberg, 1844)	+	+	+	+	+	+
		27	*Coscinodiscus jonesianus* (Greville) Ostenfeld	—	—	+	—	+	+
		28	*Coscinodiscus perforatus* (Ehrenberg, 1844)	—	—	+	—	+	+
5	*Corethraceae* (Lebour, 1930)	*29*	*Corethron hystrix* (Hensen, 1887)	+	+	+	+	+	+
		30	*Corethron inerme* (Karsten, 1905)	+	+	+	+	+	+
6	*Gossleriellaceae* (Round in Round *et al*. 1990)	*31*	*Gosleriella tropica* (Schutt 1892)	+	—	—	+	—	—
7	*Heliopeltaceae* (Smith, 1872)	*32*	*Actinoptychus campanulifer* (Schmidt, 1875)	+	+	+	—	+	+
8	*Hemiaulaceae* (Heiberg, 1863)	*33*	*Hemiaulus hauckki* (Grunow) ex van Heurck, 1882	+	+	+	+	+	+
		34	*Hemiaulus membranaceus* (Cleve, 1873)	+	—	—	+	+	—

	CENTRIC DIATIOMS Phylum: Ochrophyta (Cavalier-Smith in Cavalier-Smith & Chao, 1996)	**Class: Coscinodiscophyceae (Round & Crawford in Round, Crawford & Mann, 1990)**		**2011–2012**			**2012–2013**		
S. No	**Family**	**S. No**	**Species**	**PRM**	**MON**	**POM**	**PRM**	**MON**	**POM**
		35	*Hemiaulus sinensis* (Greville, 1865)	+	+	+	—	+	+
		36	*Eucampia cornuta* (Cleve) Grunow, 1883	+	+	+	+	+	+
		37	*Eucampia zodiacus* (Ehrenberg, 1839)	+	+	+	+	+	+
		38	*Cerataulina pelagic* (Cleve) Hendey, 1937	+	+	+	+	+	+
9	*Hemidiscaceae* (Hendey, 1937)	*39*	*Actinocyclus octonarius* var. crassus (Smith) Hendey, 1954	+	+	—	+	+	—
		40	*Actinocyclus ehrenbergii* (Ralfs in Pritchard, 1861)	+	—	+	+	—	+
		41	*Hemidiscus cuneiformis* (Wallich, 1860)	+	+	+	+	+	+
		42	*Palmeria hardmaniana* (Greville, 1865)	+	+	+	+	+	+
10	*Hyalodiscaceae* (Crawford in Round *et al*. 1990)	*43*	*Podosira stelliger* (Bailey) Mann, 1907	+	+	+	+	+	+
11	*Leptocylindraceae* (Lebour, 1930)	*44*	*Leptocylindrus danicus* (Cleve, 1889)	+	+	+	+	+	+
		45	*Leptocylindrus minimus* (Gran, 1915)	+	+	+	+	+	+
12	*Lithodesmiaceae* (Round in Round *et al*. 1990)	*46*	*Lithodesmium undulatum* (Ehrenberg, 1839)	—	+	+	—	+	+
		47	*Ditylum brightwellii* (West) Grunow, 1885	+	+	+	+	+	+
		48	*Ditylum sol Grunow* (Grunow) De Toni, 1984	+	+	+	+	+	+
		49	*Paralia sulcata* (Ehrenberg) Cleve, 1873	+	—	+	+	—	+
13	*Melosiraceae* (Kutzing 1844)	*50*	*Melosira moniliformis* (Muller) Agardh, 1824	+	+	+	+	+	+
		51	*Melosira nummuloides* (Agardh, 1824)	+	+	+	+	+	+
14	*Rhizosolenia* (De Toni, 1890)	*52*	*Rhizosolenia castracanei* (Peragallo, 1888)	+	+	+	+	+	+
		53	*Rhizosolenia crassa* (Schimper, 1905)	+	+	+	+	+	+
		54	*Rhizosolenia imbricate* (Brightwell, 1858)	+	+	+	+	+	+
		55	*Rhizosolenia formosa* (Peragallo, 1888)	+	+	+	+	+	+
		56	*Proboscia alata* (Brightwell) Sundstrom, 1986	—	+	+	—	—	+

	CENTRIC DIATIOMS Phylum: Ochrophyta (Cavalier-Smith in Cavalier-Smith & Chao, 1996)		**Class: Coscinodiscophyceae (Round & Crawford in Round, Crawford & Mann, 1990)**	**2011–2012**			**2012–2013**		
S. No	**Family**	**S. No**	**Species**	**PRM**	**MON**	**POM**	**PRM**	**MON**	**POM**
		57	*Guinardia flaccid* (Castracane) Peragallo, 1892	+	+	+	+	+	+
		58	*Guinardia striata* (Stolferfoth) Hasle, 1996	+	+	+	+	+	+
		59	*Dactyliosolen fragilissimus* (Bergon) Hasle, 1966	+	+	+	+	+	+
15	*Skeletonemaceae* (Lebour, 1930)	60	*Skeletonema costatum* (Greville) Cleve, 1878	+	+	+	Contd. +	+	+
16	*Stephanodiscaceae* (Glezer and Makarova, 1986)	61	*Cyclotella striata* (Kutzing) Grunow, 1880	+	+	+	+	+	+
17	*Stephanopyxidaceae* (Nikole in Round *et al*.1990)	62	*Stephanopyxis palmeriana* (Greville) Grunow, 1884	+	+	+	+	+	+
18	*Streptothecaceae* (Crawford,1990)	63	*Helicotheca tamesis* (Shrubsole) Richard, 1890	+	—	+	+	—	+
19	*Thalassiosiraceae* (Lebour 1930)	64	*Thalassiosira longissima* Baltica (Grunow) Ostenfeld 1901	+	+	+	+	+	+
	Thalassiosiraceae (Hasle, 1973)	65	*Thalassiosira eccentric* (Ehrenberg) Cleve, 1903	+	+	+	+	+	+
		66	*Thalassiosira subtilis* (Ostenfeld) Gran 1900	+	+	+	+	+	+
		67	*Thalassiosira leptopus* (Grunow ex Van Heurck) Hasle & Fryxell, 1977	+	+	+	+	+	+
		68	*Thalassiosira anguste-lineata* (Schmidt) Fryxell & Hasle, 1977	+	+	+	+	+	+
		69	*Thalassiosira oceanica* (Halse, 1983)	+	+	+	+	+	+
		70	*Planktoniella sol* (Wallich) Schutt, 1892	+	+	+	+	—	+
20	*Lauderiaceae* (Schutt) Lemmermann, 1899	71	*Lauderia annulata* (Cleve, 1873)	+	+	+	+	+	+
21	*Triceratiaceae* (Schutt) Lemmermann, 1899	72	*Triceratium favus* (Ehrenberg, 1839)	+	+	+	+	+	+
		73	*Triceratium robertsianum* (Graville, 1863)	+	+	+	+	+	+
		74	*Triceratium reticulum* (Ehrenberg, 1844)	+	—	+	+	—	+
		75	*Triceratium alternans* (Bailey) Mann, 1907	+	+	+	+	+	+

	CENTRIC DIATIOMS Phylum: Ochrophyta (Cavalier-Smith in Cavalier-Smith & Chao, 1996)		**Class: Coscinodiscophyceae (Round & Crawford in Round, Crawford & Mann, 1990)**	**2011–2012**			**2012–2013**		
S. No	**Family**	**S. No**	**Species**	**PRM**	**MON**	**POM**	**PRM**	**MON**	**POM**
		76	*Odontella longicruris* (Greville) Hoben, 1983	+	+	+	+	+	+
		77	*Odontella mobiliensis* (Bailey) Grunow, 1884	+	+	+	+	—	—
		78	*Odontella sinensis* (Greville) Grunow, 1884	+	+	+	+	+	+
			Class 1, Families 21, Genera 33 and Species 78.	69	68	68	70	67	66

	Pennate Diatoms Phylum: Ochrophyta (Cavalier-Smith in Cavalier-Smith & Chao, 1996)		**Class: *Bacillariophyceae* (Haeckel, 1878)**	**2011–2012**		**2012–2013**			
S.No	**Family**	**S.N**	**Species**	**PRM**	**MON**	**PRM**	**MON**	**PRM**	**MON**
1	*Amphipleuraceae* (Grunow, 1862)	*1*	*Amphiprora gigantea* (Grunow, 1860)	+	+	+	+	+	+
		2	*Amphiprora alata* (Ehrenberg),1845	+	+	—	+	—	+
		3	*Frustulia specula* (Amosse, 1932)	+	+	—	—	+	—
2	*Bacillariaceae* (Ehrenberg, 1831)	*4*	*Nitzschia longissima* (Brebisson, in Kutzing) Ralfs, 1861	+	+	+	+	+	+
		5	*Nitzschia macilenta* (Gregory, 1857)	+	+	+	+	+	+
		6	*Nitzschia sigmoidea* (Nitzsch) W. Smith 1853	+	+	+	+	+	+
		7	*Ceratoneis closterium* (Ehrenberg, 1839)	+	+	+	+	+	+
		8	*Bacillaria paxillifera* (Muller.) Hendey, 1951	—	—	+	—	+	+
		9	*Pseudonitzschia australis* (Frenguelli, 1939)	+	+	+	+	+	+
		10	*Pseudonitzschia pungens* (Grunow ex cleve) Hasle, 1993	+	+	+	+	+	+
		11	*Tryblionella compressa* (Bailey) Poulin, 1990	+	+	+	+	+	+
3	*Catenulaceae* (Mereschkowsky, 1902)	*12*	*Amphora laevis* (Gregory, 1857)	—	+	+	—	+	+
		13	*Amphora delphineiformis* (Levkov, 2009)	+	+	+	+	+	—
		14	*Amphora obtusa* (Gregory, 1857)	+	+	+	+	+	+
4	*Cocconeidaceae* (Kutzing, 1844)	*15*	*Cocconeis placentula var. euglypta* (Ehrenberg) Grunow, 1884	+	+	—	+	—	—

	Pennate Diatoms Phylum: Ochrophyta (Cavalier-Smith in Cavalier-Smith & Chao, 1996)		**Class: *Bacillariophyceae* (Haeckel, 1878)**	**2011–2012**			**2012–2013**		
S.No	**Family**	**S.N**	**Species**	**PRM**	**MON**	**PRM**	**MON**	**PRM**	**MON**
5	*Cymbellaceae* (Greville, 1833)	*16*	*Cymbella cymbiformis* (Agardh, 1830)	+	+	+	—	+	+
		17	*Cymbella cistula* (Ehrenberg) Kirchner, 1878	+	+	—	+	—	—
6	*Diploneidaceae* (Mann, 1990)	*18*	*Diploneis splendida* (Cleve, 1894)	+	+	+	+	+	+
		19	*Diploneis bombus* (Ehrenberg, 1953)	+	—	+	+	+	+
		20	*Diploneis littoralis* (Donkin) Cleve, 1894	+	—	+	+	+	+
7	*Rhaphoneidaceae* (Forti, 1912)	*21*	*Delphineis surirella* (Ehrenberg) Andrews 1981	+	+	+	+	+	+
		22	*Delphineis surirelloides* (Simonsenii) Andrews, 1977	+	+	+	+	+	+
8	*Fragilariaceae* (Greville, 1833)	*23*	*Tabularia fasciculata* (Agardh) Williams & Round, 1986	+	+	—	+	—	—
		24	*Synedra ulna* (Nitzsch) Ehernberg, 1832	—	—	+	+	—	+
		25	*Asterionellopsis glacialis* (Castracane) Round, 1990	+	—	+	+	—	+
		26	*Asterionella inflata* (Heib, 1863)	+	—	+	+	+	+
		27	*Fragilaria crotonensis* (Kitton, 1869)	+	+	+	+	+	+
		28	*Fragilariopsis oceanica* (Cleve) Hasle, 1965	+	+	+	+	+	+
		29	*Fragilariopsis doliolus* (Wallich) Medlin & Sims, 1993	+	+	+	+	+	+
		30	*Fragilariopsis kerguelensis* (O'meara) Husbedt, 1952	+	+	+	+	+	+
9	*Licmophoraceae* (Kutzing, 1844)	*31*	*Licmophora abbreviate* (Agarth, 1831)	+	—	+	—	—	+
10	*Lyrellaceae* (Mann, 1990)	*32*	*Lyrella hennedyi* (Smith) Stickle & Mann, 1990	+	+	+	—	+	+
		33	*Lyrella lyra* (Ehrenberg) Karajeva, 1978	+	+	—	+	—	+
11	*Naviculacaea* (Kutzing, 1844)	*34*	*Navicula semen* (Ehrenberg) 1843	+	+	+	+	+	+
		35	*Navicula peticolasii* (Peragallo, 1909)	+	—	+	+	+	—
		36	*Trachyneis aspera* (Ehrenberg) Cleve, 1894	—	—	+	—	+	+
		37	*Meuniera membranacea* (Cleve) Silva, 1996	+	+	+	+	—	+
		38	*Pleurosigma directum* (Grunow, 1880)	+	+	+	+	+	+
		39	*Pleurosigma elongatum* (Smith, 1852)	+	+	+	+	+	+

	Pennate Diatoms Phylum: Ochrophyta (Cavalier-Smith in Cavalier-Smith & Chao, 1996)		Class: *Bacillariophyceae* (Haeckel, 1878)	2011–2012			2012–2013		
S.No	**Family**	**S.N**	**Species**	**PRM**	**MON**	**PRM**	**MON**	**PRM**	**MON**
		40	*Pleurosigma normanii* (Ralfs in Pritchard 1861)	+	+	+	+	+	+
		41	*Gyrosigma balticum* (Ehrenberg) Robenhorst, 1853	+	+	+	+	—	+
12	*Phaeodactylaceae* (Lewin, 1958)	*42*	*Phaeodactylum tricornutum* (Bohlin, 1897)	+	+	+	+	+	+
13	*Striatellaceae* (Kutzing, 1844)	*43*	*Grammatophora marina* (Lyngbye) Kutzing, 1844	+	+	—	+	—	+
14	*Surirellaceae* (Kutzing, 1844)	*44*	*Surirella patella* (Kutzing, 1844)	+	—	+	—	—	+
15	*Thalassionemataceae* (Round and Crawford in Round *et al.* 1990)	*45*	*Thalassionema bacillare* (Heiden) Kolbe, 1955	+	+	+	+	+	+
		46	*Thalassionema nitzschioides* (Grunow) Mereschkowsky, 1902	+	+	+	+	+	—
		47	*Thalassionema frauenfeldii* (Grunow) Tempere & Peragallo, 1910	+	+	+	+	+	+
		48	*Thalassiothrix longissima* (Cleve & Grunow 1880)	+	+	+	+	—	+
		49	*Thalassiothrix heteromorpha* (Karsten, 1907)	+	+	—	—	+	—
	Class 1, Families 15, Genera 30 and Species 49			42	36	38	37	33	38

Phylum: Ochrophyta		**S. No**	**Species**	**2011–2012**			**2012–2013**		
				PRM	**MON**	**POM**	**PRM**	**MON**	**POM**
Family			**Class 1: Dictyochophyceae (Silva, 1980)**						
1	Dictyochaceae (Lemmermann, 1901)	1	*Dictyocha fibula* (Ehernberg, 1839)	+	+	—	+	+	+
		2	*Dictyocha staurodon* (Ehrenberg, 1844)	+	—	+	+	—	—
		3	*Octactis octonaria* Ehernberg Hovasse, 1946	+	—	+	—	+	+
2	Monodopsidaceae (Hibberd, 1981)		Class 2: Eustigmatophyceae (Hibberd & Leedale, 1971)						
		4	*Nannochloropsis gaditana* (Lubian, 1982)	+	+	+	+	+	+
	Families 2, Class 2, Genera 3 and Species 4			4	2	3	3	3	3

http://dx.doi.org/10.5772/intechopen.75549

	Phylum: Dinophyta (Round, 1973)		Class: Dinophyceae (Fritsch in West & Fritsch, 1927)	2011–2012			2012–2013		
	Family		**Species**	**PRM**	**MON**	**POM**	**PRM**	**MON**	**POM**
1	Ceratiaceae (Lindeman, 1928)	1	*Neoceratium breve* (Ostenfeld & Schmidt) Gomez, Moreira & Lopez-Garcia, 2010	+	+	+	+	+	+
		2	*Neoceratium furca* (Ehrenberg) Gomez, Moreira & Lopez-Garcia, 2010	—	+	+	+	+	+
		3	*Neoceratium karsteni* (Pavillard, 1907) Gomez, Moreira & Lopez-Garcia, 2010	—	+	+	—	+	+
		4	*Neoceratium macroceros* (Ehrenberg) Gomez, Moreira & Lopez-Garcia, 2010	—	+	—	+	+	—
		5	*Neoceratium teres* (Kofoid) Gomez, Moreira & Lopez-Garcia, 2010	+	—	—	+	+	+
		6	*Neoceratium tripos* (Muller) Gomez, Moreira & Lopez-Garcia, 2010	—	+	+	+	—	+
		7	*Neoceratium symmetricum* (Pavillard) Gomez, Moreira & Lopez-Garcia, 2010	—	+	+	+	+	+
		8	*Neoceratium horridum* (Gran) Gomez, Moreira & Lopez-Garcia, 2010	—	—	+	—	+	+
		9	*Ceratium seta* (Ehrenberg) Kent, 1881	+	+	+	—	—	+
		10	*Ceratium pacificum* (Wood, 1963)	+	—	+	+	+	—
		11	*Ceratium uteri* (Campbell, 1934)	—	+	+	+	+	+
2	Dinophysaceae (Butschli, 1885)	12	*Dinophysis caudata* (Saville-Kent, 1881)	+	+	+	+	—	+
		13	*Dinophysis dens* (Pavillard, 1915)	+	+	—	+	—	+
		14	*Dinophysis fortii* (Pavillard, 1923)	+	+	—	+	+	+
		15	*Dinophysis miles* (Cleve, 1900)	+	+	+	+	+	+
		16	*Ornithocercus magnificus* (Stein, 1883)	+	+	+	—	—	+
		17	*Ornithocercus thumii* (Schmidt) Kofoid & Skoggberg, 1928	+	—	+	+	+	+
3	Gymnodiniaceae (Lankester, 1885)	18	*Karenia brevis* (Davis) Hansen & Moestrup, 2000	+	+	+	+	+	+
		19	*Gymnodinium danicans* (Cambell, 1973)	+	+	—	+	—	+
		20	*Gymnodinium dentatum* (Larsen, 1994)	+	—	+	+	+	—
		21	*Akashiwo sanguine* (Hirasaka) Hansen & Moestrup, 2000	—	+	+	+	+	+
4	Protoperidiniaceae (Taylor, 1987)	22	*Protoperidinium thorianum* (Paulsen) Balech, 1974	+	+	+	+	+	+
		23	*Protoperidinium depressum* (Bailey) Balech, 1974	+	+	—	+	—	—
		24	*Protoperidinium elegans* (Cleve) Balech, 1974	+	+	+	+	+	—
		25	*Protoperidinium oceanicum* (VanHoffen) Balech, 1974	+	—	+	—	+	+

	Phylum: Dinophyta (Round, 1973)		Class: Dinophyceae (Fritsch in West & Fritsch, 1927)	2011–2012			2012–2013		
	Family		**Species**	**PRM**	**MON**	**POM**	**PRM**	**MON**	**POM**
		26	*Protoperidinium pellucidum* Bergh, 1881	—	+	+	—	+	—
		27	*Protoperidinium subinerme* (Paulsen) Loeblich III, 1970	—	+	—	+	+	—
		28	*Protoperidinium pallidum* (Ostenfeld) Balech, 1973	—	+	+	+	+	—
		29	*Protoperidinium pentagonum* (Gran) Balech, 1974	+	+	—	—	+	+
5	Phyrophacaceae (Lindemann, 1928)	30	*Pyrophacus steinii* (Schiller) Wall & Dale 1971	+	+	+	+	+	+
		31	*Pyrophacus horologium* (Stein, 1883)	+	+	+	—	+	+
6	Prorocentraceae (Stein, 1883)	32	*Prorocentrum lima* (Ehrenberg) Stein, 1878	—	+	+	+	+	+
		33	*Prorocentrum gracile* (Schutt, 1895)	+	+	+	+	+	+
		34	*Prorocentrum micans* (Ehrenberg, 1834)	+	—	+	—	+	+
7	Noctilucaceae (Kent, 1881)	35	*Noctiluca scintillans* (Macartney) Kofoid & Swezy, 1921	+	—	—	+	—	+
Class 1, Families 7, Genera 11, Species 35				23	27	26	26	28	28

	Phylum: Cyanobacteria (Stanier ex Cavalier-Smith, 2002)		Class: Cyanophyceae (Schaffner, 1909)	2011–2012			2012–2013		
S. No	**Family**			**PRM**	**MON**	**POM**	**PRM**	**MON**	**POM**
1	Oscillatoriaceae (Engler, 1898)	1	*Lyngbya majuscule* (Harvey ex Gomont, 1892)	+	—	+	+	—	+
		2	*Lyngbya confervoides* (Agardh ex Gomont, 1893)	+	+	+	+	+	+
		3	*Oscillatoria princeps* (Vaucher ex Gomont, 1892)	+	+	+	+	+	—
2	Phormidiaceae (Anagnostidis & Komarek, 1988)	4	*Phormidium nigroviride* (Thwaites ex Gomont) Anagnostidis & Komarek, 1988	+	+	—	+	+	—
		5	*Trichodesmium thiebautii* (Gomont, 1892)	+	+	+	+	+	+
		6	*Trichodesmium erythraeum* (Ehrenberg ex Gomont, 1893)	+	+	+	+	+	+
3	Nostocaceae (Eichler, 1886)	7	*Anabaenopsis elenkinii* (Miller, 1923)	+	—	+	+	+	+
4	Schizotrichaceae (Elenkin, 1949)	8	*Schizothrix calcicola* (Agardh) Gomont ex Gomont, 1892	+	+	+	+	—	+

	Phylum: Cyanobacteria (Stanier ex Cavalier-Smith, 2002)		**Class: Cyanophyceae (Schaffner, 1909)**	**2011–2012**			**2012–2013**		
S. No	**Family**			**PRM**	**MON**	**POM**	**PRM**	**MON**	**POM**
		9	*Schizothrix fuscescens* (Kutzing, 1843)	+	+	+	+	+	+
5	Synechococcaceae (Nageli, 1849)	*10*	*Synechococcus* sp. (Nageli, 1849)	+	+	—	—	+	+
6	Chroococcaceae (Hansgirg, 1888)	*11*	*Dactylococcopsis* sp. (Hansgirg, 1888)	+	+	+	+	+	—
Class 1, Families 6, Genera 8 and Species 11.				11	9	9	10	9	8

Phylum: Euglenozoa (Cavalier-Smith, 1981)		**Species**		**2011–2012**			**2012–2013**		
Family				PRM	MON	POM	PRM	MON	POM
	Class 1: Euglenophyceae (Schoenichen, 1925)								
1	Eutreptiaceae (Hollande, 1942)	1	*Eutreptia lanowii* (Steuer, 1904)	+	+	—	—	+	+
		2	*Eutreptia viridis* (Perty, 1852)	+	+	—	+	—	+
2	Euglenaceae (Dujardin, 1841)	3	*Euglena proxima (*Dangeard, 1901)	+	—	+	+	+	—
		4	*Euglena ascusformis* (Schiller, 1925)	+	+	+	+	+	+
Class 1, Families 2, Genera 2, Species 4				4	3	2	3	3	3

Phylum: Chlorophyta (Pascher, 1914)									
Family	Class 1: Chlorodendrophyceae (Massjuk, 2006)								
1	Chlorodendraceae (Oltmanns, 1904)	1	*Tetraselmis gracilis* (Kylin) Butcher, 1959	+	+	+	+	—	+
		2	*Tetraselmis chui* (Butcher, 1959)	+	+	+	+	+	—
	Class 2: Chlorophyceae (Wille in Warming, 1884)								
2	Dunaliellaceae (Christensen, 1967)	3	*Dunaliella tertiolecta* (Butcher, 1959)	+	+	+	+	+	+
		4	*Dunaliella salina* (Dunal) Teodoresco, 1905	+	+	+	+	+	+
3	*Chlamydomonadaceae* (Stein, 1878)	5	*Chlamydomonas reinhardtii* (Dangeard, 1888)	—	+	+	+	—	+
	Class 3: Trebouxiophyceae (Friedl, 1995)								
4	*Chlorellaceae* (Brunnthaler, 1913)	6	*Chlorella vulgaris* (Beijerinck, 1890)	+	+	+	+	+	+
5	Oocystaceae (Bohlin, 1901)	7	*Oocystis solitaria* (Wittrock, 1879)	+	+	—	+	—	—
Class 3, Families 5, Genera 5 and Species 7.				6	7	6	7	4	4

Phylum: Haptophyta (Cavalier-Smith, 1986)	Class: Prymnesiophyceae (Hibberd, 1976)						
1 Prymnesiaceae (Conrad ex O.C.Schmidt, 1931)	1 *Dicrateria inornata* (Parke, 1949)	+	+	+	+	+	+
	2 *Isochrysis galbana* (Parke, 1949)	+	+	—	+	+	+
	Class: Pavlovophyceae (Cavalier-Smith) Green & Medlin in Edvardsen *et al.*, 2000						
2 Family: Pavlovaceae (Green, 1976)	3 *Diacronema lutheri* (Droop) Bendif & Veron, 2011	—	+	+	—	+	—
2 Family, 2 Class, 3 Genera and 3 Species		2	3	2	2	3	2

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Chapter 3

Effect of Hydrodynamic Conditions of Photobioreactors on Lipids Productivity in Microalgae

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.74134

Abstract

This research presents the effect of hydrodynamic conditions at different rates of aeration (1.4, 1.8, and 2.3 vvm) and the geometry of two photobioreactors with internal lighting on lipid productivity and other parameters of *Chlorella vulgaris*. A two-step nitrogen-reduction cultivation mode was applied for promoting lipid accumulation. The inoculum was cultivated initially at 90 mg L^{-1} $N\text{-}NH_4^+$, and at the end of the exponential phase, it was fed to 11 L photobioreactor at 20 mg L^{-1} of $N\text{-}NH_4^+$. The results showed that with similar aeration rates, the hydrodynamic regime in both photobioreactors was different. However, the increase in shear rate and agitation did not cause cell damage or photoinhibition. The maximum cell growth was 12×10^6 cells mL^{-1}. The highest consumption of nitrogen was 19% and shear rates were of 120-340 s^{-1}. The highest lipid productivity was reached in bubble column at 1.8 vvm with 0.650 mg·L^{-1} d^{-1}.

Keywords: shear rate, aeration rate, photobioreactors, *Chlorella vulgaris*, nitrogen limitation

1. Introduction

Several reports have demonstrated that certain species of microalgae can store large amounts of triacylglycerol (TAG), which are the raw materials for biodiesel production. The mixture of saturated and unsaturated fatty acid chains (C_{12}–C_{22}) present in many microalgae favors the production of biodiesel [1, 2]. Certain species of microalgae tend to reach a high lipid content

(20–50% dry cell weight) and may increase it by controlling various biotic and abiotic factors of the crop, such as light intensity, photoperiod, temperature, nutrients, mode, and the intensity of agitation [3]. The total yield of lipids from microalgae depends not only on the concentration of biomass reached but also on the cellular oil content. It should be noted that understress conditions by nutrient limitation, cell growth tends to decrease, while lipid content increases [4, 5]; therefore, the most important variable to maximize biodiesel production from microalgae cultures is lipid productivity considered in grams of lipids per liter of culture per day [5, 6]. The cultivation of microalgae to industrial scale can be performed in open systems such as ponds (*raceways*) and closed systems called photobioreactors (PBR). In both systems, the source and intensity of light are critical factors affecting phototrophic growth performance of microalgae [1]. The open systems usually are less expensive to build and operate; they are more durable than PBR and have greater production capacity. However, they require more land extension, more susceptible to weather conditions without temperature control and lighting prone to contamination and self-shadowing, which can lead the culture to total collapse [7]. The PBRs have certain advantages such as better control over culture and growth conditions, prevention of evaporation, loss reduction of CO_2, higher cell densities, volumetric productivities, greater safety and protection of the environment, and less invasion by microorganisms. Similarly, these equipments show some disadvantages such as overheating, oxygen accumulation, difficulty in scaling, high cost of construction and operation, possible cellular stress damage by shear, and deterioration of the material used in the photo-step [4, 8]. These disadvantages can be solved by an adequate reactor design. Mixing is an important variable, since it ensures that the cells within the equipment can access the light and prevent the accumulation of oxygen in the culture medium, preventing the precipitation of the cells or their adhesion to the walls of the equipment. For any type of PBR used in the algal culture, efficient mixing is required in order to produce a uniform dispersion of the microalgae in the culture medium, thus eliminating concentration, light, nutrient, and temperature gradients. However, high speeds often are not practical because the shear rates that often damage cells are increased [9]. It has been documented that excessive mechanical agitation creates turbulence, which can cause permanent damage to the cellular structure affecting the growth and production of metabolites; conversely, a poor agitation can cause sedimentation and cell death [4, 7, 8]. Within the vertical column PBR, two configurations can be mentioned: airlift type and conventional bubble column. In comparison with the horizontal type, these present a better degassing, preventing the accumulation of oxygen and not inhibiting algal growth. The bubble column is a simple container in which the gas is injected from the bottom and random mixing is produced by rising bubbles. An airlift reactor consists of two flow regions, downcomer and riser, which can be arranged concentrically or connected cyclically. The continuous movement of the liquid and its consequent mixing capacity is due to the constant addition of a gas stream in the ascending zone, generating a forced convection for the liquid [8, 10, 11]. The hydrodynamic differences in these equipments can affect the physical and biochemical properties of microalgal cells during the culture process. Due to these differences, it can be mentioned that at the same aeration rate, the airlift configuration can cause greater turbulence and poor cell growth due to the phenomenon of photoinhibition due to excess light by the number of times that cells access the light source and other negative aspects such as hydrodynamic shear stress [8]. The interest of this study was to evaluate the effect of hydrodynamic

conditions at different aeration rates on lipid productivity and other parameters of *Chlorella vulgaris* in cultures with nitrogen limitation using two PBRs (bubble column and airlift).

2. Materials and methods

2.1. Selection of strain and culture medium

The *Chlorella vulgaris* microalga was obtained from the Cepario of the Center for Scientific Research and Higher Education of Ensenada (CICESE), Mexico. *C. vulgaris* was selected because of its high potential for the production of biodiesel, from its high productivity and fatty acid profile [6], as well as the capacity to develop in urban wastewater, commercial media, and nitrogen limitation conditions [12–15]. For acclimation, *C. vulgaris* was cultivated in culture medium at pH = 7, with a composition similar to the effluent from the primary treatment of an urban wastewater treatment plant as follows [16]: 7 mg NaCl, 4 mg $CaCl_2$, 2 mg $MgSO_4 \cdot 7H_2O$, 15 mg KH_2PO_4, and 115.6 mg NH_4Cl, all dissolved in 1 L of distilled water. Trace metals and vitamins were aggregated according to medium f/2 of Guillard and Ryther [17]. During acclimation (1 month), the microalgae was transferred to fresh culture medium every 7 days at 28 ± 1°C and a light intensity of 100 μE m^{-2} s^{-1}.

2.2. Cultivation process

When starting the experiments, *C. vulgaris* was cultivated in an enriched medium at 90 mg L^{-1} nitrogen; subsequently, the concentration of the culture was reduced to 20 mg L^{-1}, similarly to that described by Robles-Heredia et al. [3]. Of the stock culture, a fraction was taken and transferred to the four bubble column seedlings, adding 200 mL each to one cell concentration of 15×10^4 cells mL^{-1} (Section 2.1). Fresh culture medium was added, starting the culture to a concentration of 90 mg L^{-1} of N and volume of operation of 2.5 L; continuous aeration of 0.4 vvm (volumetric flow of air per minute per unit volume of medium) and external white light illumination at a light intensity of 225 μE m^{-2} s^{-1} were supplied. Cell growth was monitored by cell counting in the Neubauer chamber using an optical microscope with a 40× lens. During the exponential growth phase (5 days), the volume of the four seedbeds was diluted (40–50%) to inoculate two 11 L PBRs, so that when the fresh medium was added, the initial concentration of $N\text{-}NH_4^+$ in the medium was 20 mg L^{-1}. The cultures were maintained and monitored for 5 days, during which 100 mL of each reactor, dry biomass, $N\text{-}NH_4^+$ consumption, and lipid productivity every 24 h were sampled to determine cell counts in the Neubauer chamber every 12 h.

2.3. Photobioreactor test

The culture was realized at the same time in two PBRs, airlift (RAF) and bubble column (COB), both with an operating volume of 11 L and a height of 95 cm. The COB consists of two vertical concentric glass tubes; the light source is a fluorescent white light lamp located inside the inner tube, with an intensity of 300 μE m^{-2} s^{-1}. The radial light path (distance between the outside of the inner tube and the inside of the outer tube) is 5 cm so as not to favor self-shadowing.

The air was injected through four cylindrical diffusers made of plastic material and porous structure distributed radially in the base. In the COB reactor, the flow of injected air drives the culture medium to the top, and, by gravity, the cells descend; this mixing process is continuous for the duration of the culture period. As for the RAF reactor, the construction is the same as the COB reactor, but with the addition of a transparent acrylic tube or draft tube between the inner lighting tube and the outer tube, so that mixing is carried out by airlift effect. The air is injected into the section formed between the baffle and the inner tubes, where the riser is made and in the upper part of the baffle the fluid descends (downcomer) between the baffle and the external glass tubes. Design parameters correspond to a working volume of 11 L (0.011 m^3) of culture medium with rate L/D and illuminated surface/volume medium in m^{-1} (3.6 and 10.9) for COB, whereas in RAF it was 4.0 and 12.1 m^{-1}, respectively. Other parameters required are the following: h_L in m is the height of the liquid at rest without gas flow (liquid holdup), for COB it is 0.8, while in RAF it was 0.86; h_G is the height of the column including gas retention; h_B is the distance between the base and the deflector (0.05 m); d_0 is the inner diameter of the outer tube (0.144 m); and d_{bi} = 0.0953 m and d_{bo} = 0.1016 m are the internal and external diameters of the deflector, respectively. Sectional area in the PBRs is A_c = 0.0140 m in COB, and A_r = 0.0048 m and A_d = 0.0082 m are the sectional areas in the riser and downcomer of the RAF, respectively. Letters A, B, C, E, F, G, and H in that order consider the common parts of both PBRs, degassing valve, top cover, top cover flange, outer tube, central tube for lighting, lower cover flange, and diffusers-silencers.

The main dimensions of both PBRs are shown in **Figure 1a** and **b**.

The shaded area in RAF is the so-called free area between the riser and the downcomer h_B, *Cr* is a clamping ring, and D is a Baffle tube. Section A_b is the free area between the riser and downcomer in the RAF and corresponds to the surface of an imaginary cylinder of diameter d_{bo} and height h_B (see **Figure 1a** and **b**).

2.4. Experimental design

The effect of different operating conditions on lipid productivity was estimated using a 2 × 3 factorial design with two replicates, with lipid productivity as a variable response, considering the factors such as PBR geometry in two levels (RAF and COB) and aeration rate in three levels (1.4, 1.8, and 2.3 vvm) and using aeration as inferior limit with 0.91 vvm in RAF and as upper limit 3.4 vvm in COB. The geometry of the PBR is related to the shear rate that predominates in the same, being expected a higher shear rate in the RAF riser than in the COB. The results of the experimental design were analyzed using a complete factorial analysis of variance (ANOVA) (α: 0.05) using STATISTICA V7 program [18].

2.5. Shear rate

To relate the geometry of the PBR (bubble column or airlift) with the prevailing shear rate within the same, it is recognized that the characteristic shear rate (γ) in the PBR is a function of the surface velocity of the U_g gas, so that Eq. (1)

$$\gamma = 1000\, U_g^{\,2} \tag{1}$$

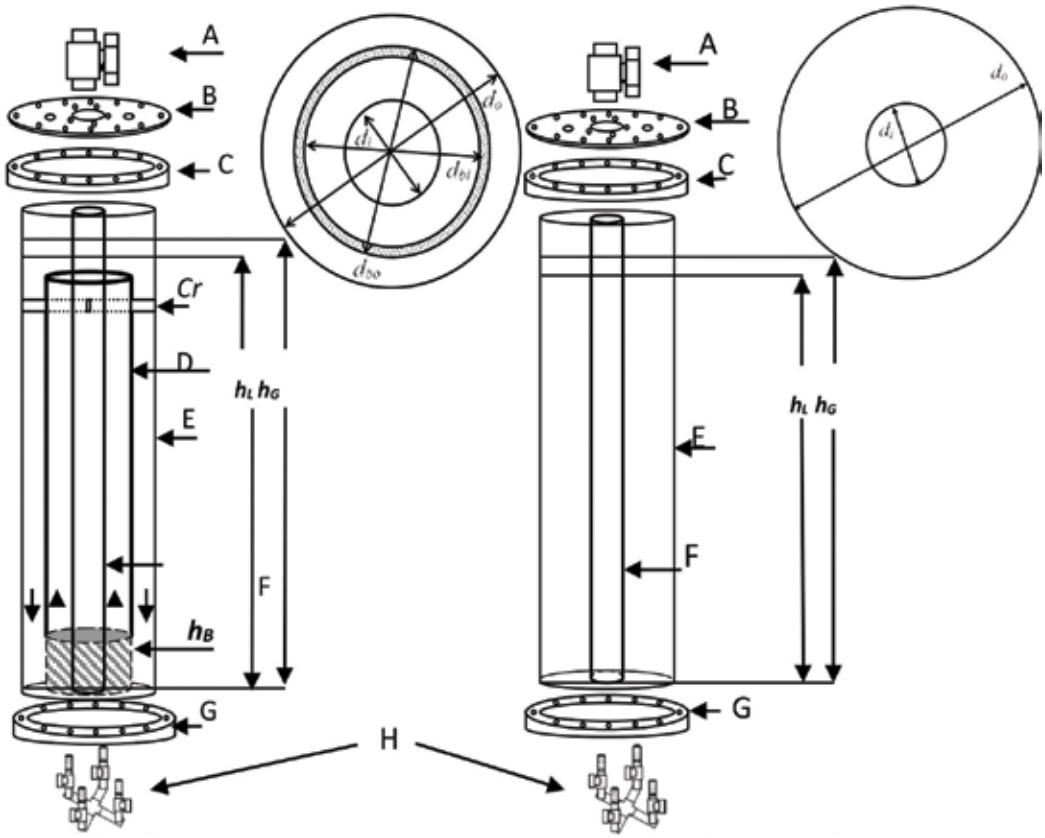

Figure 1. (a) Photobioreactor RAF and (b) photobioreactor COB.

Equation (1) is valid in the range of $0.008 < Ug < 0.09$ m s^{-1} and 1000 m^{-1} for airlift column [19]. For the case of the airlift, the characteristic shear rate exists in the riser, and the shear rate is calculated with the same Eq. (1) but using the speed of the gas in the riser U_{gr}. The pneumatic power (PG/V_L) given in Wm^{-3} is considered as the energy or power generated by the inflow of gas or air injected to the equipment to be exerted by expansion of the movement of agitation of the fluid inside the photobioreactor. For a bubble column, pneumatic power is calculated with Eq. (2) [20]:

$$\frac{PG}{V_L} = \rho.L_g.U_g \tag{2}$$

The pneumatic power in an airlift reactor [21] is evaluated by means of Eq. (3):

$$\frac{PG}{V_L} = \rho.L_g.U_{gr}\left(\frac{A_r}{A_r + A_d}\right) \tag{3}$$

where U_g is calculated with Eq. (4)

$$U_g = U_{gr}\left(\frac{A_r}{A_r + A_d}\right) \tag{4}$$

As can be seen, the pneumatic power in both PBRs is the same for each value of the aeration rate.

2.6. Lipid productivity

After 5 days of the cultivation stage in both PBRs, algal biomass was obtained by means of a flocculation process using a chitosan solution according to the technique of Romero and Ferrán [22], modified for this job, it was considered to chitosan was used for its properties of cell immobilization and avoid dispersion [23]. Subsequently, the harvested biomass was lyophilized for 48 h, at a temperature of −40°C and a pressure of 0.133 mbar. The determination of total lipids was performed by the method reported by Bligh and Dyer [24], which is modified as follows: a 10 mg sample of lyophilized biomass was placed in each tube and mixed with 4 mL of methanol, 2 mL of chloroform, and 0.5 mL of distilled water. The mixture was sonificated for 15 min and subsequently to centrifugation at 4000 rpm for 15 min; the supernatant was removed, to which 2 mL of distilled water was added and vigorously stirred. It was centrifuged again at 4000 rpm for 15 min to observe the biphase. With Pasteur pipette, the upper aqueous phase was removed, and the lower lipid-chloroform phase was dried with nitrogen gas to remove the chloroform and concentrate lipids. Subsequently to this concentrate, 3 mL of 2% potassium dichromate was added in each tube and placed in a water bath at 100°C for 15 min. After cooling in a water bath, 4.5 mL of distilled water was added, vigorously stirred, and cooled to room temperature to read absorbance in the spectrophotometer at 590 nm. It is important to indicate that the same preparation sequence was followed in two tubes but without lyophilized biomass in the calibration of the spectrophotometer. Previously, the absorbance value A correlated with the C_L lipid concentration (in mg L^{-1}), using a tripalmitin standard, resulting in the equation (5):

$$C_L = 0.5874A - 0.036 \quad with \ r^2 = 0.99 \tag{5}$$

The lipid content of the sample is considered as the lipid composition in (% ww^{-1}) on dry basis w, and this in turn results in a P_L lipid productivity (in mg L^{-1} d^{-1}) with the following equation (6):

$$P_L = \frac{w_1 X_1 - w_2 X_2}{t_1 - t_2} \tag{6}$$

where X_i is the mass concentration of dry biomass in the medium at time t_i.

3. Results and discussions

3.1. Effect of the aeration rate on cell growth and nitrogen consumption

Figure 2 shows the cell growth curves in the seedbed and in the PBR at the aeration rates studied. This mode of cultivation is called two-stage cultivation, where in the first stage (the seedbed) the cells grows under nitrogen sufficiency (90 mg L^{-1}) and in the second stage (the PBR), the concentration of nitrogen is reduced considerably (to 20 mg L^{-1}).

The idea of the two-stage cultivation mode is to stimulate the overproduction of intracellular lipids in the microalgae, at the expense of reduced cell growth [3].

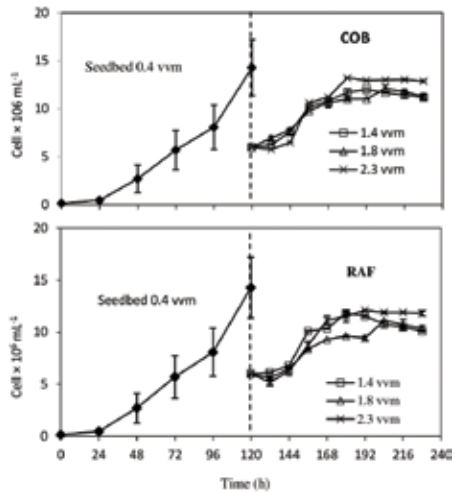

Figure 2. Cell growth curves in the seedbed and in both PBRs.

Table 1 shows the growth data (maximum value of cell density, specific growth rate μ, and consumption of $N\text{-}NH_4^+$) for all experimental treatments.

At sufficient nitrogen conditions, the seed reactors reached high values in the cell density of 14.3×10^6 cells mL^{-1} and in the specific growth rate ($\mu = 0.93\ d^{-1}$). While there is no inhibition of cell growth due to nutrient limitation, the rate of aeration is a factor that favors the contact of cells and light, reducing the effect of self-shading. This can be observed according to the results obtained (**Table 1**) where, with the increase of the aeration rate to values of 2.3 vvm, in both PBRs (COB and RAF) statistically significant changes were obtained in the growth parameters in comparison with the other treatments. The consumption curves of $N\text{-}NH_4^+$ are presented in **Figure 3** superimposed on the growth curves in both PBRs and at different aeration rates. Nitrogen consumption remained constant in all runs, between 30 and 38% of available nitrogen, without showing significant differences (**Table 1**). These nitrogen uptake values were low compared to Mata et al. [1] and Gouveia and Oliveira [13]. In **Figure 3**, it is observed that the consumption of nitrogen, although it occurs slowly, continues even after

	RAF			**COB**		
	Maximum cell density (cell×10⁶ mL⁻¹)	**μ (d^{-1})**	**Uptake $N\text{-}NH_4^+$ (%)**	**Maximum cell density (cell×10⁶ mL⁻¹)**	**μ (d^{-1})**	**Uptake $N\text{-}NH_4^+$ (%)**
1.4 vvm	11.8 ± 0.11^a	0.31 ± 0.002^a	32.6 ± 1.0^a	12.0 ± 0.03^a	0.28 ± 0.014^a	32.2 ± 1.0^a
1.8 vvm	11.1 ± 0.02^a	0.33 ± 0.021^a	30.3 ± 1.7^a	12.1 ± 0.34^a	0.28 ± 0.021^a	30.3 ± 0.3^a
2.3 vvm	12.1 ± 0.00^b	0.50 ± 0.014^b	34.2 ± 0.4^a	13.0 ± 0.03^b	0.43 ± 0.002^b	38.8 ± 0.2^a

Different letters in the same column indicate significant differences according to Tukey's test ($p \geq 0.05$); (±standard deviation). Data taken from doctoral thesis [34].

Table 1. Average data of maximum cell density, specific growth rates (μ), and nitrogen uptake of ***C. vulgaris*** in both PBRs at the proposed aeration rates.

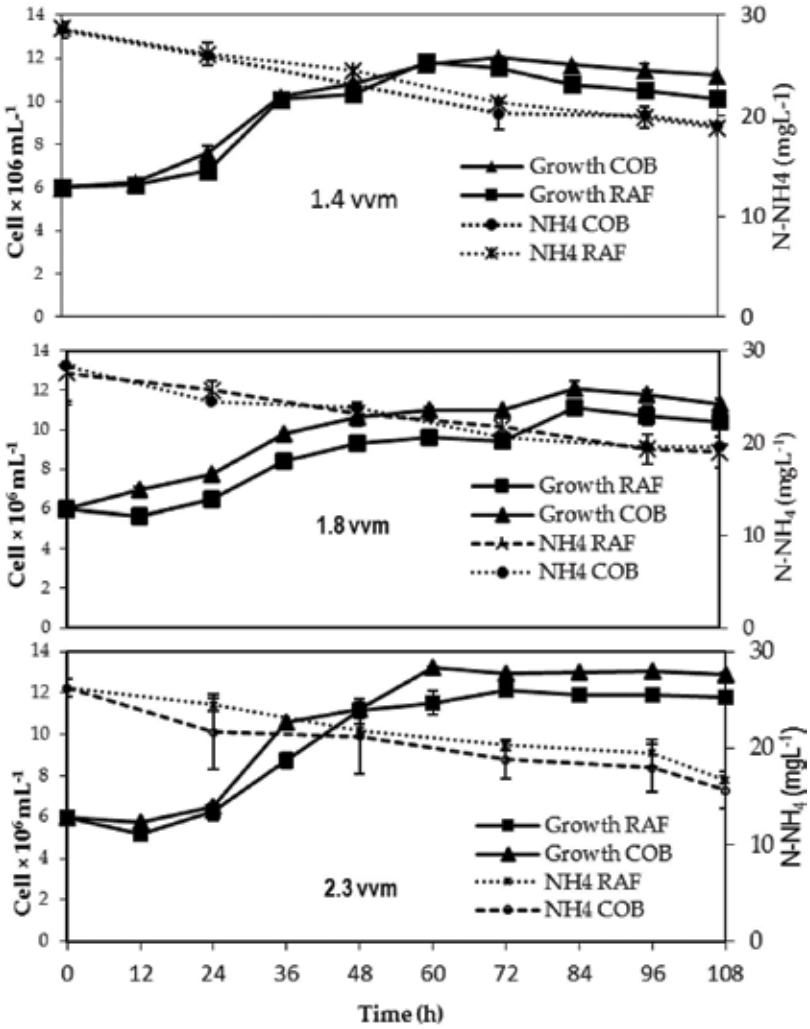

Figure 3. Growth curves and nitrogen uptake of C. vulgaris at the different aeration rates in the PBR.

the exponential phase of growth has ended. This indicates that although there was a change in the algal metabolism when going from the seed reactor to the PBR, redirecting the use of available nitrogen to storage metabolites, the limitation of growth was not exclusively due to the scarcity of nitrogen in the medium. A possible explanation for this behavior is that the cells adapted to the conditions of the culture medium to continue growing without resenting the scarcity of nitrogen. That is to say, a stress for nutrients did not occur, maintaining the metabolism acquired in the seedling stage, unlike of what is reported by [13, 15, 25–27], where nitrogen insufficiency and the phenomenon of nutrient stress were present. In addition, upon reaching the stationary phase and cell growth, microalgae would have greater difficulty in accessing light to perform the process of photosynthesis and consume nitrogen only to maintain their cellular functions and produce storage metabolites [13, 28, 29].

The values reached of μ in both PBRs (shown in **Table 1**) were statistically similar to the flows of 1.4 and 1.8 vvm; in the same way, the values of maximum average cell density in both equipments (RAF and COB) did not show significant differences between them (**Table 1**). From **Figure 3** and **Table 1**, it is also possible to indicate that the specific growth rates increased in a similar way as the aeration rate in each treatment increased, showing significant differences only with respect to an aeration rate of 2.3 vvm, where a higher μ and cell density were reached for both reactors. Taking into consideration of the above and according to the increments of cell growth and specific growth rate (μ) reached at the aeration rates of 1.8 and 2.3 vvm, it can be mentioned that there were no aspects related to photoinhibition, or stress by shear or sedimentation, since there were suitable agitation and mixing conditions. Maximum cell densities between 60 and 72 h were reached at the aeration rate of 2.3 vvm, both in airlift and in the bubble column. The removal values of N were not as expected, which implies that conditions of N limitation were not reached, since the cells adapted to continue cell development without resenting the nitrogen shortage, maintaining the metabolism that they had in the seedling stage. It should be noted that high nitrogen consumption (85–90%) was not obtained in the seed reactor, so that by decreasing the concentration of nitrogen at the end of the first stage (seedbed) from 60 to 20 mg L^{-1} in the PBR, the microalgae did not suffer the decrease of nitrogen in the medium. The growth parameters observed can be related to the calculated hydrodynamic data; the data of the pumping power and characteristic cutoff rate according to each of the aeration rates used are indicated in **Table 2**. The biphasic flow regimes were different in each equipment, according to the gas superficial velocities applied in each PBR. To characterize the biphasic air/culture medium flow within the PBR, a classification of flow patterns is commonly used as homogeneous bubbling, slug (plug), churn (heterogeneous turbulent), and annular [30].

According to the data obtained, it was observed that the flow in the RAF reactor was maintained in the slug regime, while in the COB reactor, it presented homogeneous bubbling type. These regimes have important differences in the degree, type of agitation, and mixing. At the same pumping power, the surface velocity of the gas in the riser of the RAF is much higher than in the COB because the flow section is smaller. Consequently, the flow rate slug is considerably higher than in homogeneous bubbling, due to the greater turbulence generated between the two phases. **Figure 4** compares the specific growth rate measured against the characteristic shear rate calculated for each geometry. It is observed that in spite of increasing almost twice

PBR	Aeration rate (vvm)	*PG/VL* (Wm^{-3})	*Ug ó Ugr* (ms^{-1})	γ (s^{-1})
COB	1.4	176	0.018	134
	1.8	235	0.024	155
	2.3	294	0.030	173
RAF	1.4	176	0.052	228
	1.8	235	0.070	264
	2.3	294	0.087	295

Table 2. Hydrodynamic data calculated at the aeration rates studied in both PBRs.

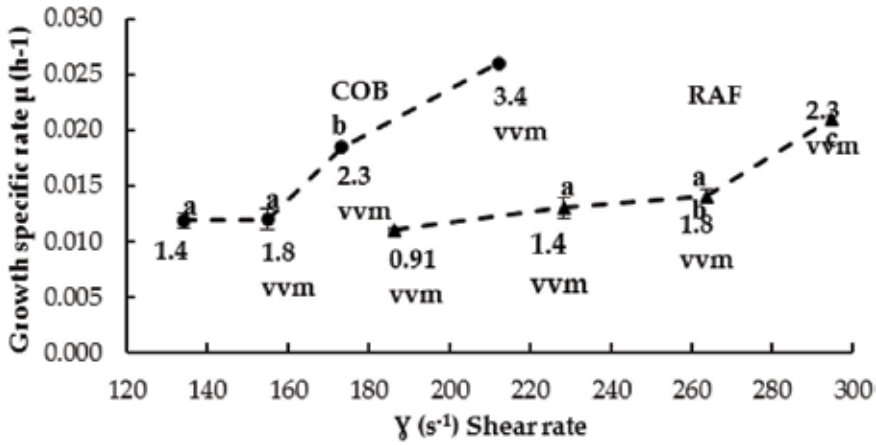

Figure 4. Maximum growth rate vs. shear rate at the airflows indicated on both equipments. Different letters indicate significant differences of shear rate at the proposed aeration rates according to Tukey's test (p≥0.05).

the shear rate in the same PBR, a negative effect was not obtained in the growth rates, as has been reported in reactors with high values of shear rate [11]. It is ruled out that in this experimental range there is a sublethal damage in the cells due to shear stress. On the contrary, at higher aeration rates, the effect of agitation and mixing dominated the shear effort, improving the gas exchange and the accessibility of light, achieving in turn specific higher growth rates.

On the other hand, **Figure 5** relates the maximum cell concentration X_{max} and the calculated shear rate. An important trend of an increase of the X_{max} with the aeration rate (and the shear rate) is observed until reaching a critical value in which the increase in the shear rate does not influence X_{max} more.

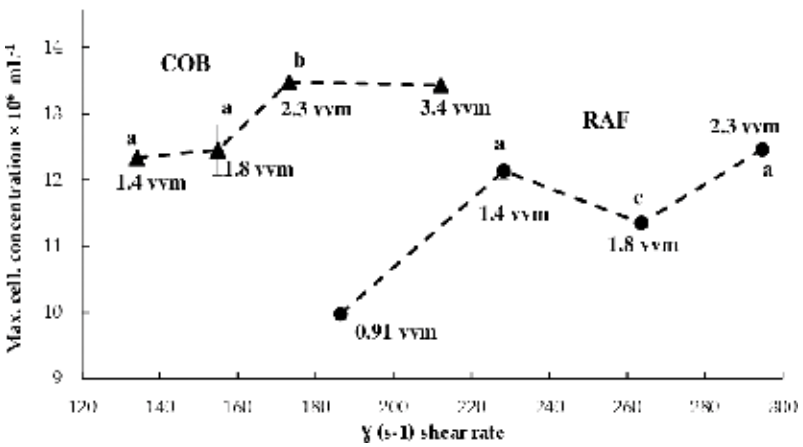

Figure 5. Maximum cell concentration vs. shear rate at the airflows indicated on both equipments. Different letters indicate significant differences of shear rate at the proposed aeration rates according to Tukey's test (p≥0.05).

These maximum values were found in 2.3 vvm for COB and 1.4 vvm for RAF. It is thus identified that the overall effect of the aeration rate on cell growth is to accelerate the growth of *C. vulgaris* due to better mixing, until reaching the maximum value of cell density that allows mass transfer (more likely CO_2 from the gas to the cell) or the available lighting for photosynthesis. **Figures 6** and **7** also include two aeration rates that were tested outside the experimental design (0.91 vvm for RAF and 3.4 vvm for COB). These two values were only made to confirm the trends in the shear rates already discussed.

Due to the supplied aeration rate and configuration of both equipments, it is possible that the cells have changes in their metabolism and growth. In order to identify if there is any type of cellular damage by shear stress in both PBRs, the values of the shear rate were compared, proportional to the shear stress or also known as shear effect, calculated at the different aeration rates proposed in the experimental design (1.4, 1.8, and 2.3 vvm). Due to the aeration rate and configuration of both equipments, it is possible that cells show changes in growth and metabolism. In order to identify if there is any type of cellular damage by shear stress

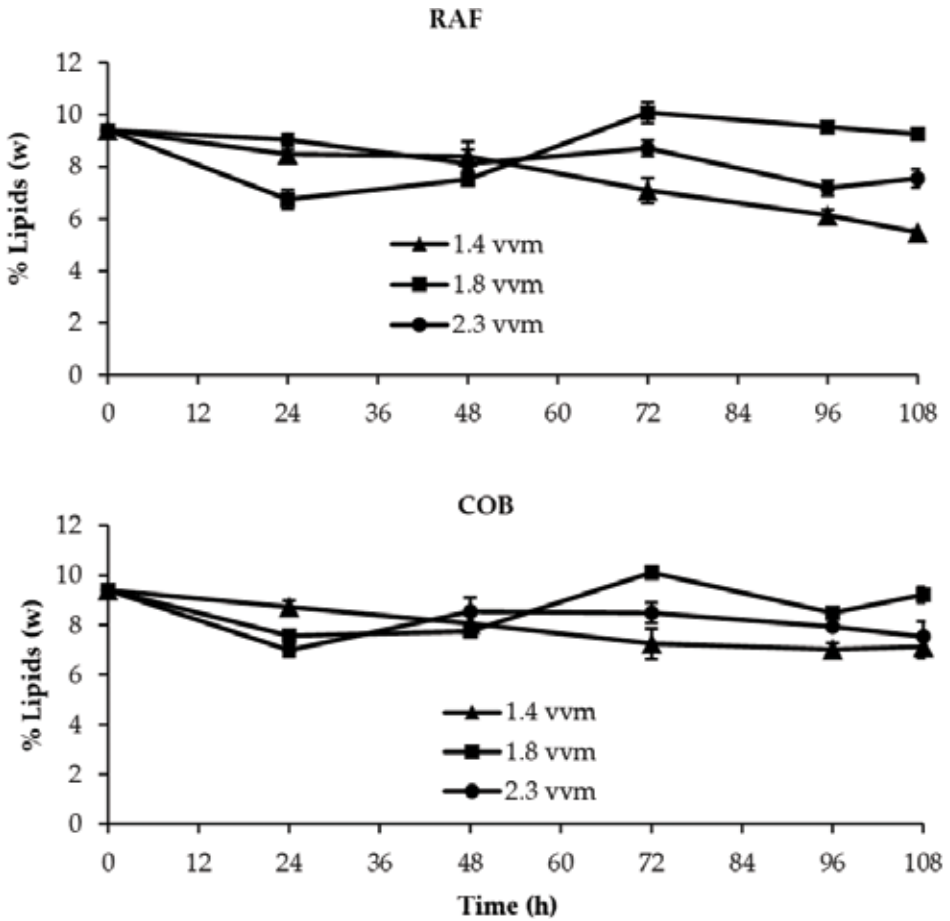

Figure 6. Kinetics of the percentage (%) of lipids (dry basis) in both PBRs at the aeration rate studies.

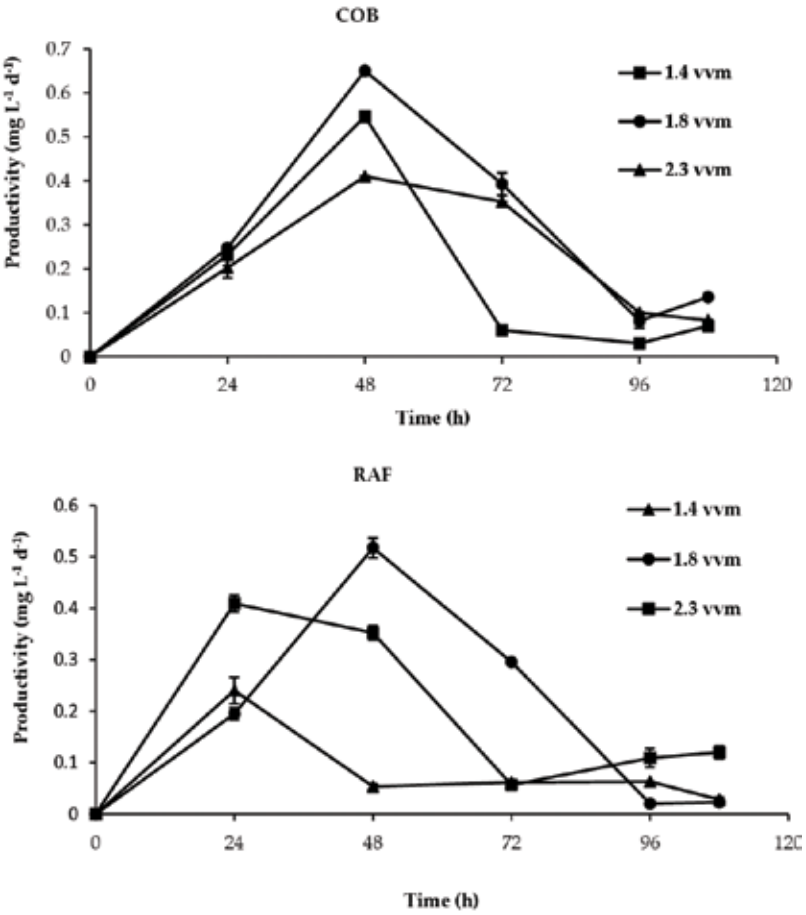

Figure 7. Kinetics of the lipid productivity in both PBRs at the aeration rate studies.

in both PBRs, values of the shear rate that is proportional to the shear stress or also known as shear effect were compared. These were calculated at the proposed aeration rates in the experimental design (1.4, 1.8, and 2.3 vvm). In the RAF reactor, the flow and configuration regime caused the gas and liquid surface velocities to have higher values than in COB, which is related to higher shear rates as aeration rates increased. These differences are related to the configuration of each reactor and in some cases can cause cell damage; however, some species can be adapted to high shear stress conditions. According to **Figure 5**, it can be indicated that although the RAF equipment presented values of cutoff rates about 1.5 times higher than in the COB equipment, with similar aeration flows, there was no negative effect on the specific growth rates, eliminating the possibility of sublethal damage to cells due to shear stress, in this experimental range. On the contrary, it can be pointed out that at higher aeration rates, the effect of agitation and mixing dominated the shear effort, producing higher specific rates of growth. According to the previous result, it can be demonstrated that by means of a slight modification to the geometry of the PBR it is possible to substantially change

the characteristic agitation of the system and reduce the culture time necessary to reach the maximum cell density. However, it should be noted that the configuration of the equipment and the rate of aeration supplied could cause irregularities in the properties of microalgal cells during cultivation [31, 32]. For this reason, it must be verified if this regime change does not cause effects on other important parameters of the crop, such as the lipid content and the weight of dry biomass, among others. In the same way, in **Figure 4** it can be observed that the maximum aeration rate was 1.8 vvm, since by increasing the aeration flow, an increase in the specific rate of growth is manifested. This may indicate that there is a critical point around this value (1.8 vvm) in which *C. vulgaris* adapts its metabolism and reacts to greater turbulence to increase its growth. Taking this into account, it can be assumed that at low aeration rates, the cells would have greater difficulty to access the light source and perform photosynthesis, presenting themselves with self-shading and/or less agitation, consuming the necessary nitrogen, just to maintain their functions and produce metabolites and other storage products [13, 29, 30].

3.2. Effect of the rate of aeration on lipid production

The lipid content in dry base w and the concentration of dry biomass X were monitored every 24 h in the PBR, with the objective of performing a kinetics of the productivity of lipids (P_L) for each experiment, calculated with Eq. (6). The kinetics obtained are presented in **Figures 6** and **7**.

In **Figure 6** it can be seen that in general the lipid content was low, between 8 and 10%, lower in a range between 22 and 30% than in other studies [12, 13, 30, 33]. This is probably related to the fact that there was no effect of nitrogen limitation in the second stage of culture of the PBR, which caused a low lipid content. According to this condition, it can be noted that the concentration of nitrogen at the end of the first stage of the seedbed was higher than expected (45 mg L^{-1}). Therefore, when performing the dilution at 20 mg L^{-1} in the PBRs, the volume of inoculum in relation to the number of cells did not show the effect of nitrogen limitation from the beginning, despite the fact that cell growth during the culture process in the equipment doubled. This situation suggests that the microalgae adapted to continue with their growth metabolism and by not resenting the change due to the reduction or limitation of nutrients in the culture medium in the PBR; the cells grew in number, without achieving a greater amount of lipids for storage. This situation of low lipid performance was related to the results of low nitrogen consumption in all treatments. In the RAF experiments, the lipid content decreased throughout the crop, with the exception of the culture at 1.8 vvm, where it decreased during the first 48 h, but a maximum peak was observed at 72 h. This value (10.3%) was also the maximum observed in all the curves. The same behavior is observed for the COB. The maximum lipid productivity values occur after 48 h of culture in RAF and 24 h in COB (see **Figure 7**), with the notable exception in the latter of the experiment at 1.8 vvm that occurred after 48 h. The behavior of productivity kinetics is due to the fact that cell density reaches a maximum of around 48 h and the lipid content remains relatively constant, so as time goes on increasing, productivity decreases, according to Eq. (6).

Table 3 shows the results related to the production of lipids in both PBRs at the different rates of aeration. It is observed that the variations in lipid content are only significantly different

PBR	Aeration rate (vvm)	X (g L^{-1})	w_{max} (%ww^{-1})	Maximum P_L (mg L^{-1} d^{-1})	Increase in P_L [†] (%)
RAF	1.4	0.13 ± 0.042[a]	8.36 ± 0.01[a]	0.240 ± 0.012[a]	
	1.8	0.27 ± 0.042[b]	10.37 ± 0.00[b]	0.528 ± 0.002[b]	120
	2.3	0.16 ± 0.021[a]	9.12 ± 0.01[a]	0.408 ± 0.025[c]	70
CBA	1.4	0.22 ± 0.056[a]	8.54 ± 0.00[a]	0.552 ± 0.026[b]	
	1.8	0.30 ± 0.010[b]	10.28 ± 0.00[b]	0.650 ± 0.019[d]	17
	2.3	0.24 ± 0.041[a]	8.11 ± 0.00[a]	0.410 ± 0.016[c]	−26

[*]Different letters in the same column indicate significant differences according to Tukey's test ($p \geq 0.05$); (±standard deviation). Data taken from doctoral thesis [34].

[†]Increase in lipid productivity with respect to 1.4 vvm

Table 3. Dry biomass X, lipid content w, and maximum P_L lipid productivity in both PBRs at the proposed aeration rates.

from the 1.8 vvm aeration rate for both PBRs. The observed changes in lipid productivity are mainly affected by the lipid content w, not so much for the concentration of dry biomass X, since statistically no significant differences are observed in the data obtained from the biomass in both PBRs at the rate of aeration (see **Table 3**).

Although the highest percentage of the increase in productivities occurred in the RAF (120%), in COB the highest lipid productivity observed was 0.65 mg $L^{-1}d^{-1}$. Since the values of lipid content were not very different between geometries, the difference in productivity can be attributed to the fact that in COB higher cell growths were achieved. According to the values reached in **Figure 7** and **Table 3**, it is observed that in COB the highest value of lipid productivity was obtained at 48 h with respect to all aeration rates with 0.65 mg L^{-1} d^{-1}; in addition, as already mentioned, cell growth has a greater impact in relation to the reached value of lipid productivities. In relation to the RAF reactor, the highest productivity (0.528 mg L^{-1} d^{-1}) was obtained at 48 h at the 1.8 vvm aeration rate; later, a decay was observed until the end of the experiment, and this was due to a reactivation by agitation, affecting cell growth, generating a cellular increase. The results of lipid productivities were not the expected ones, since they were in the range of 0.2–0.650 mg L^{-1} d^{-1}, that is, 40% lower, compared with other studies that used similar conditions in the cultivation process with microalgae, [5, 13, 26]. This situation would confirm in a certain way what had been supposed previously.

4. Conclusions

The interest of this work was to evaluate the effect of hydrodynamic conditions at different aeration rates, on lipid productivity and other parameters such as cell growth, specific growth rate, nitrogen removal, and lipid accumulation of *Chlorella vulgaris* in cultures with nitrogen limitation using two geometries of PBRs (bubble column and airlift). In relation to cell growth, the values of μ in both PBRs were statistically similar with 0.312 d^{-1} at an aeration flow of 1.4 vvm. While at the aeration rate of 1.8 vvm, μ was 0.336 d^{-1} for the reactor RAF, while in COB the

value of μ was 0.288 d^{-1} for the same aeration rates (1.4 and 1.8 vvm). Similarly, the values of maximum average cell density in both equipments did not show significant differences between them (11.82 and 11.11) × 10^6 cells mL^{-1} to (1.4 and 1.8 vvm) for the reactor RAF; while in reactor COB the average cell density was (12.00 and 12.11) × 10^6 cells mL^{-1} to (1.4 and 1.8 vvm). However, specific growth rates increased (0.504 and 0.432 d^{-1}) in RAF and COB, respectively, as long as it increases the aeration rate in each treatment, showing significant differences only with respect to an aeration rate of 2.3 vvm for both PBRs. According to the increments reached of cell growth and specific growth rate (μ) at the aeration rates of 1.8 and 2.3 vvm, it can be mentioned that there were no related to photoinhibition nor stress by shear or sedimentation, since the conditions of agitation mixing were sufficient. Both equipments presented different agitation flows according to the proposed aeration rates. It can be mentioned that the biphasic flow regimes were different in each equipment, due to the differences in the values of the superficial gas velocities calculated at the proposed aeration rates. According to the values obtained from the gas velocity, it is observed that in the airlift the working regime was in the Slug type flow range, while in the bubbling column it was developed as a homogeneous bubbling type. This speaks to the degree of agitation and mixing that existed in each team. N limiting conditions were not reached; this is probably because the cells achieved an early adaptation to low concentrations of nitrogen, supporting their metabolism and growth from the seed reactor stage, where high nitrogen consumption was not obtained (85–90%). So that by decreasing the concentration of nitrogen (45 mg L^{-1}) at the end of the first stage (seedbed) to 20 mg L^{-1} in the PBR, the microalgae did not suffer the decrease of nitrogen in the medium. Although it was observed that increasing the aeration rate also increased cell growth, the highest possible lipid production was not reached in these bioreactor geometries. In general, the lipid content was low, between 8 and 10%, lower in a range between 22 and 30% than in other studies. In the COB reactor, the highest lipid productivity value (0.650 mg L^{-1} d^{-1}) was obtained at 48 h with respect to all aeration rates. In relation to the RAF reactor, the highest productivity (0.528 mg L^{-1} d^{-1}) was obtained at 48 h at the 1.8 vvm aeration rate; later, a decay was observed until the end of the experiment, and this was due to a reactivation by agitation, affecting cell growth. The achieved results of lipid productivities were not as expected since they were in the range of 0.2–0.650 mg L^{-1} d^{-1}, that is, 40% lower, compared with other studies that used similar conditions. Although the highest percentage of the increase in productivities occurred in the RAF reactor (120%), in the COB reactor, the highest lipid productivity (0.65 mg $L^{-1}d^{-1}$) was observed. Since the values of lipid content were not very different between both geometries, mainly at the aeration rate of 1.8 vvm (10.28 and 10.37) % ww^{-1}, the difference in productivity can be attributed to the fact that the COB reached higher values of cell growths. The results obtained are probably related to the fact that there was no effect of limiting nitrogen in the second stage of cultivation of the PBR, which caused a low lipid content, as already mentioned.

Acknowledgements

The National Council of Science and Technology (CONACyT) funded this research through projects 145521 and Basic Science 179857.

Conflict of interest

The authors indicate that there is no conflict of interest for the publication of this manuscript.

Nomenclature

A	Absorbance
RAF	Airlift reactor
Ab	Free area between the riser and the downcomer in m^2
COB	Bubble column
cells mL^{-1}	Cells per milliliter
d_{bi}	Internal diameter of the deflector (m)
d_{bo}	External diameter of the deflector (m)
d_h	Hydraulic diameter
d_i	Diameter of the inner tube where the lighting is performed (m)
d.i.	Internal diameter
d_o	External diameter of the external tube (m)
PBR	Photobioreactor
Fg	Air intake flow in m^{-3} s^{-1}
g	Acceleration of gravity in m s^{-2}
h_B	Distance between base and deflector (m)
h_G	Height of the column including gas retention (m)
h_L	Height of liquid at rest without gas inlet (m)
kB	Coefficient of friction loss
mg $L^{-1}d^{-1}$	Milligrams per liter per day
n.d.	Not detectable
P_L	Lipid productivity
rpm	Revolutions per minute
Ton	Ton
U_g	Superficial gas velocity in ms^{-1}

U_L	Liquid superficial velocity in ms^{-1}
vvm	Volumetric airflow (per minute) per unit volume of medium in min^{-1}
$\mu E\ m^{-2}\ s^{-1}$	Microeinstein per square meter per second

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Chapter 4

The Influence of Microalgae Addition as Co-Substrate in Anaerobic Digestion Processes

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.75914

Abstract

Growth microalgae could be used as co-substrates in anaerobic digestion processes to produce biogas of a high-calorific value, which could be expended as heat or electricity in cogeneration engines. Lignocellulosic and high-carbon content wastes, due to their characteristics, hinder anaerobic digestion processes. The use of microalgae as a co-substrate with high-carbon content residues can adjust the C/N ratio and thereby obtain, in some cases, a higher biogas production and greater biodegradability of wastes during anaerobic digestion than without co-digestion options. In addition, microalgae and cyanobacteria are photosynthetic microorganisms that can produce oxygen and oxidize the organic matter and NH_4^+ contained in wastewaters. The growth of microalgae in industrial effluents and wastewaters can considerably reduce the organic matter contained in them and their pollutant load. This growth can take advantage of the nutrients that still remain in industrial effluents, avoiding the use of clean water for the growth of biomass. The chapter will focus on an overview of microalgae anaerobic co-digestion with different wastes and the benefits of this option.

Keywords: microalgae, wastewaters, biogas, anaerobic digestion, microalgae growth

1. Introduction

One of the main challenges that society will face in the near future is the potential lack of traditional energy sources. The rising price of fossil-based fuels and their negative environmental impact combined with increasing energy consumption make the demand for renewable energy sources greater. For this reason, a wide variety of biomass has been investigated

in order to evaluate its potential as a proper feedstock for the production of different biofuels, such as biodiesel, bio-methanol, bio-hydrogen, bio-oil, and biogas [1]. Nevertheless, the increasing world population will need an adequate food supply, which could be a problem if cultivated land is destined to biofuels and not to human or animal feed. Thus, non-edible biomass, which does not require usable land, would be a promising alternative. In this regard, the attention of the scientific community has been focused on oleaginous microorganisms, such as microalgae and cyanobacteria, in recent years. Microalgae do not need agricultural land for growing, and they improve air quality through CO_2 removal and require minimal use of fresh water resources [2].

The main properties that make some microalgae and cyanobacteria good alternatives as biomass for biofuel production include their highly efficient photosynthetic mechanisms [3]; their elevated biomass production of up to 5–10% vs. 0.5–3% in plants [4]; their growth rates, which are 5–10 times faster than land-based feedstock [5]; and their accumulation of lipids and carbohydrates [1, 6]. However, the main nutrients required for the growth of microalgae and cyanobacteria are inorganic carbon (some microalgae species are able to utilize organic carbon), inorganic nitrogen (ammonium or nitrate), and phosphorous. These requirements can make their growth expensive in some cases. For example, generating 1 kg of biodiesel in fresh water requires 3,726 kg of water, 0.33 kg of nitrogen, and 0.71 kg of phosphate [7]. However, it is now known that microalgae can be grown using nutrient-rich wastewaters like digestates from anaerobic digestion processes such as liquid supernatants rich in nitrogen and phosphorous, animal manure or textile wastewater [8], food wastewater [9], and aquaculture wastewater [10], among others. Even in saline waters, which are usually rich in nitrogen [11], this disadvantage to the water quality for growth is easily overcome. In the same way, recycling harvest water reduces the water and nutrient requirements by 84 and 55%, respectively [7]. The use of wastewater for microalgae and cyanobacteria growth presents the advantage of reducing the cost and environmental impact of the system by reducing the use of clean water and mineral nutrients while biomass productivity is comparable to that obtained from a synthetic medium [12].

Microalgae also uptake carbon by the photosynthetic process during growth, reducing CO_2 emissions 10 times more efficiently than those reduced in a forest [6, 13], by transforming CO_2 into new biomass. Microalgae culture can contribute simultaneously to both CO_2 fixation and wastewater treatment [14]. Hirata et al. [15] found that a batch culture of *Chlorella* sp. UK001, using sunlight as a light source and growing at a mesophilic temperature with pH between 5.5 and 6.0, achieved a mean rate of CO_2 fixation during the culture of 0.0318 g CO_2/L·d. Maeda et al. [16] found that another strain of *Chlorella*, strain *Chlorella* sp. T-1, was an ideal candidate for the biological fixation of CO_2 exhausted by a coal-fired thermal power plant. Other authors demonstrated that the strain *Chlorella* sp. MTF-15 could efficiently utilize CO_2, NO_x, and SO_2 from the different flue gases obtained in a steel plant: flue gas from a coke oven, flue gas from a hot stove, and flue gas from a power plant for cultivation [17].

Furthermore, the growth of microalgae in wastewaters aids in the treatment of pollutant wastewaters and could be introduced as a tertiary treatment [18–22]. In addition, the capacity of microalgae for synthesizing and accumulating different compounds, which could be

considered for pharmaceutical and nutraceutical purposes, is an added value [23]. The different metabolic pathways of fresh and marine water algae provide promising sources of fatty acids, steroids, carotenoids, polysaccharides, lectins, and halogenated compounds, among others [24]. Microalgae are the most promising sources of pigments and natural carotenoids of commercial interest, including β-carotene, lutein, and astaxanthin [25, 26]. Furthermore, the carotenoids produced by microalgae are devoid of the toxic effects associated with synthetic derivatives [26]. Microalgae are also used as nutritional supplements for animals and humans because of the quality of proteins that they produce. *Spirulina, Chlorella, Dunaliella,* and *Nostoc* are microalgae and cyanobacteria grown for human consumption [25].

The most common systems for the cultivation of microalgae used for biogas production are open pond reactors (OPRs), photobioreactors (PBRs), and hybrid systems. OPRs are relatively low-cost systems, although the biomass yield is lower and contamination is quite common. PBR systems permit a higher control over microalgae growth and its optimization; nevertheless, the cost of these systems is much higher than OPRs [6].

Different approaches to microalgae as biomass for biofuel extraction have been studied, but not all of them with the same success. Regarding lipid accumulation for biodiesel production, algae grown in wastewater typically showed lipid mass fractions in the volatile suspended solids (VSSs) in the range of 4.9–11.3%. This fraction is much lower than that recommended for economical biodiesel production [27]. In order to enhance the energy potential of microalgae and cyanobacteria, anaerobic digestion has been studied [1, 6] as another alternative. Anaerobic digestion is a complex biological process in which organic raw materials are converted to biogas through the action of a consortium of microorganisms that are sensitive to or completely inhibited by oxygen. Biogas is a mixture of methane (60–70%) and carbon dioxide (30–40%) and traces of other constituents (hydrogen, hydrogen sulfide, etc.) of high-energetic value from 20 to 25 MJ/m^3 [28]. Around 31 m^3 of methane per 100 kg of chemical oxygen demand (COD) fed into an anaerobic reactor can be produced, with a maximum energetic value of 108 kWh as electric energy or 308 kWh as heat. It has been reported in the literature that microalgae and cyanobacteria can be potentially used for energy recovery through anaerobic digestion, although the yields obtained depend highly on the species and the operational conditions of growth [1, 29]. The initial studies in the 1950s [30] obtained values of methane yields of 0.17–0.32 L CH_4/g SV_{added} for *Chlorella* and *Scenedesmus* in batch processes, although other authors found higher values of methane yield at 0.587 L CH_4/g SV_{added} and 0.505 L CH_4/g SV_{added} for *Chlamydomonas reinhardtii* and *Dunaliella salina*, respectively [29].

Growth conditions could affect the morphology and intracellular substances in microalgae. The thickness of the cell walls in microalgae could be increased due to stressed growing conditions, which could be a disadvantage during anaerobic digestion [27]. In addition, microalgae and cyanobacteria present a low C/N ratio, which could lead to an ammonium accumulation and result in an inhibition of the digestion process. Samson and LeDuy [31] reported concentrations of ammonia of up to 7000 mg/L for the anaerobic digestion of the protein-rich cyanobacteria *Spirulina maxima*. However, the use of microalgae as co-substrate with other substrates or feedstocks in anaerobic co-digestion processes can improve these limitations and bring certain benefits. Anaerobic co-digestion is the simultaneous anaerobic

digestion of two or more substrates, and it is a proven approach to overcome the drawbacks of single digestion [32]. Mata-Alvarez et al. [33] in the year 2000 already wrote: "The use of a co-substrate, in most cases improves biogas yield due to positive synergisms established in the digestion medium and the supply of missing nutrients by the co-substrates." Co-digestion has several advantages as follows: adjusting the C/N ratio, improving the nutrients, and diluting the inhibitor compounds [34]. The co-digestion of microalgae with high-carbon biomass leads to a better balanced substrate for anaerobic digestion [12, 13, 27]. Nevertheless, there are some problems that must be solved, such as the breakage of the thick cellular walls in some microalgae and cyanobacteria. Prospective methods could be different kinds of pretreatments before anaerobic digestion in some particular cases.

Nonetheless, due to the high variety of microalgae and cyanobacteria and the wide range of different uses, it is not clear yet what the most effective process for biofuel production is. Although to this respect, some authors suggest that the direct use of microalgae or cyanobacteria in an anaerobic co-digestion process is the best choice, while other researchers propose that the best choice is to produce biofuel as a first step followed by an anaerobic digestion of the residual by-products [1]. This chapter aims at providing a current perspective of microalgae exploitation as biomass in anaerobic digestion and co-digestion processes and shows the advantages of their growth in wastewater and anaerobic digestates.

2. Microalgae growth in wastewaters

The cultivation of microalgae in wastewater has long been recognized as a viable option for sustainable biomass production and wastewater treatment [18–21]. The main nutritional requirements for microalgae growth include nitrogen, phosphorus, and micronutrients such as iron, magnesium, and calcium, which are present in wastewater. Recent developments in microalgal research have demonstrated that microalgae have the required metabolic potential to effectively reduce high concentrations of nutrients such as carbon, phosphorous, and nitrogen present in different wastewater streams [21]. Some species of microalgae have the ability to take up other pollutants, such as heavy metals and harmful chemicals [20]. Therefore, microalgae can be used to serve a dual purpose for the treatment of wastewater as well as generating biomass for various applications because microalgae are rich in carbohydrates, proteins, and lipids.

Various wastewater streams including municipal, industrial, agricultural wastewater, as well as primary and secondary effluent, centrate, and anaerobic digestion effluent were exploited as suitable nutrient media for microalgae cultivation. Each wastewater stream has its own characteristics and challenges such as nutrient variability and the presence of potential inhibitors that could impact microalgal growth. Recently, many investigations have been developed to overcome challenges such as low nutrients, high turbidity, bacterial contamination, and specific toxic materials associated with different wastewaters.

The types of wastewater utilized for algae cultivation also affect the scope of biomass for various applications [21].

An alternative for recovering energy from microalgae is based on the application of anaerobic digestion processes [35]. In such processes, all organic matters (proteins, carbohydrates, and lipids) present in microalgae biomass would be converted into methane and carbon dioxide (biogas). Several advantages are recognized when energy production from whole microalgae through biogas generation is considered: biogas production involves high-energy yields; biogas production would not require microalgae biomass drying (it involves wet fermentation); biogas can be used to produce heat and electricity through co-generation; microalgae cultures can be used for biogas upgrading (i.e. CO_2 biosequestration); and so on. However, some microalgae have a very low C/N ratio, which hinders and inhibits a further anaerobic digestion. Ammonia toxicity and recalcitrant cell walls are commonly cited causes of the low-methane yields found in the anaerobic digestion of some microalgae [36]. Moreover, anaerobic co-digestion of microalgae with other types of biomass such as solid and liquid wastes is quite feasible [35]. The benefits of co-digestion lie in balancing the C/N ratio in the co-substrate mixture, as well as macro and micronutrients, pH, inhibitor/toxic compounds, and dry matter [37].

The main phyla (and species) of microalgae that are being used for biogas production through anaerobic digestion and co-digestion processes are as follows [20, 21, 38]:

- **Chlorophytes**, such as *Chlorella* sp./*vulgaris*/*sorokiniana*; *Scenedesmus* sp./*quadricauda*/*obliquus*; *D. salina*; *Nannochloropsis salina*; *Botryococcus braunii*; *Micractinium* sp.; and *Selenastrum capricornutum*.
- **Haptophytes,** such as *Isochrysis galbana*.
- **Cyanobacteria,** such as *Arthrospira platensis* and *Oscillatoria tenuis*.
- **Binary and mixed culture systems:** In mixed culture systems, different microorganisms develop a synergetic relationship and live together by benefiting each other. For instance, in a binary system, a photosynthetic microalga is grown with a heterotrophic microalga or bacteria. In this matrix, microalgae produce oxygen and organic compounds, which are utilized by co-existing heterotrophic microorganisms.

2.1. Chlorophytes

2.1.1. Chlorella genus

The growth of the green algae *Chlorella* sp. in wastewater after primary settling of a local municipal wastewater treatment plant was evaluated by Wang et al. [39]. They observed a growth rate of 0.429 d^{-1} with excellent removal of ammonium (NH_4^+-N) (74.7%), P (90.6%), and COD (56.5%). These authors also investigated the growth of *Chlorella* sp. using different phases (raw, secondary, and centrate) and demonstrated that the growth rate of microalgae and nutrient removal efficiencies was proportional to the nutrient concentration of the wastewater selected for its cultivation with the highest growth in centrate followed by raw wastewater. Osundeko and Pittman [40] reported a high-sodium concentration of 400 mg/L in sludge liquor/centrate, which can be toxic to freshwater microalgal species, though some

Chlorella sp. are tolerant to salinity. More recently, Lu et al. [41] evaluated the biomass productivity and nutrient removal capacity of *Chlorella* sp. in raw dairy wastewater using both indoor bench-scale and outdoor pilot-scale photobioreactors. Results from this study have shown a higher biomass productivity of 260 mg/(L·d) and high nutrient (N and P) removal (83.3 and 38.3 mg/(L·d), respectively) in indoor bench-scale cultures when compared to outdoor pilot-scale cultures with biomass of 110 mg/(L·d) and nutrient removal of 41.3 mg/(L·d) for N and 6.5 mg/(L·d) for P. These differences could have resulted due to the uncontrolled environmental and operational factors that might have affected the microalgae growth during outdoor cultivation.

Nutrient limitation is one of the key challenges for microalgal cultivation in secondary/tertiary wastewater. The supplementation of nutrients is proposed as an alternative method to overcome the nutrient limitations in wastewater. In this sense, Cabanelas et al. [42] identified the potential of coupling a wastewater treatment plant effluent with glycerol for supporting the mixotrophic production of *Chlorella vulgaris* and *Belippo terribilis*. The cultivation of *C. vulgaris* in mixotrophic mode was also studied in a mixture of primary and secondary wastewaters with different ratios (25, 50, and 75 vol.% of the primary wastewater). It was observed that using 25% of the primary wastewater and 75% of secondary wastewater resulted in 100% of COD removal, 100% of ammonium removal, and 82% of nitrate removal [43].

Recently, Ansari et al. [44] studied the cultivation of *Chlorella sorokiniana* in aquaculture wastewater with sodium nitrate supplementation and observed comparable biomass yields to the synthetic medium. In their study, they also observed high ammonia, nitrate, COD, and phosphate removal and proposed that treated water can be redirected toward aquaculture. The biomass obtained in this study showed sufficient lipid, carbohydrate, and protein concentrations to be used as feed supplement. Ramanna et al. [45] supplemented 1.5 g/L urea as a cheap N source for the cultivation of *C. sorokiniana* and achieved a biomass production of 0.218 g/L. A supplementation strategy can yield high-biomass productivities; however, it depends on the nutrient composition of the wastewater used and the requirements of the selected microalgal strain.

For the realization of microalgal CO_2 capture and utilization, the selection of microalgal species tolerant to CO_2 from various environments and the characterization of growth influencing environmental factors are required [46]. The proper selection of species and optimized cultivation conditions, i.e., light intensity, temperature, nutrient availability, and pH, can maximize CO_2 sequestration. *Chlorella* sp. has been widely reported to possess good carbon sequestration potential. Previous studies have obtained hydrocarbons from microbial lipids for their conversion into sustainable fuels as a substitute for fossil hydrocarbons. Furthermore, microalgae have significant applications in the production of valuable materials in the food and pharmaceutical industries, resulting in a high value-added process in the biosequestration of CO_2 [46].

Microalgae with a lipid content of lower than 40% of their dry weight make the anaerobic digestion route more feasible than biodiesel in terms of energy recovery. Ras et al. [47] proposed coupling the process of microalgal biomass production and anaerobic digestion. In this process, *C. vulgaris* was cultivated using the nutrient-rich digestate from an anaerobic

digester; the microalgal biomass was then anaerobically digested to produce methane. In a later study, with hydraulic retention time (HRT) of 28 days, 51% COD removal and methane production of 240 mL/g VSS were achieved. The use of microalgae as a feedstock for bioethanol production is considered to be a sustainable approach to bioethanol production. Microalgal species such as *Chlorella* store energy in the form of starch [48]. The starch accumulated in the microalgae can be easily hydrolyzed to glucose using chemical or enzymatic method. The sugar produced can be subsequently fermented to ethanol. Ho et al. [48] investigated the potential of *C. vulgaris* PS-E as the bioethanol feedstock. This species contains 51% of carbohydrates, which were hydrolyzed through an enzymatic process to give a glucose yield of 0.461 g glucose/g dry biomass. The ethanol yield obtained in their study was 11.7 g/L.

C. vulgaris was also reported to be a successful bioremediation agent of palm oil mill effluent (POME), with reductions of ammonia-nitrogen, phosphorus, COD, and biochemical oxygen demand (BOD) of 61, 84, 50.5, and 61.6%, respectively [49]. Bich et al. [50] reported that *C. vulgaris* was used in the treatment of rubber latex concentrate processing wastewater and that this microalga reduced the COD and total Kjeldahl nitrogen (TKN) by 93.4 and 79.3%, respectively. Another study carried out by Nordin et al. [51] used high-rate algal ponds (HRAPs) to treat rubber effluent from an anaerobic digester, and the reductions in COD, BOD, NH_3-N, and phosphorous reached 69.1, 87.4, 62.2, and 21.3%, respectively. In the HRAP, *Chlorella* was the predominant genus [51].

Moderately polluted textile wastewater was previously reported to be treated using the microalga *C. vulgaris*, with color and COD reductions of up to 69.9 and 75.7%, respectively [52]. Another study found that this species could degrade 63–69% mono-azo dyes into simple aromatic compounds [53]. Lim et al. [54] investigated the treatment of textile wastewater using 10 different strains of microalgae and found that *C. vulgaris* was able to remove color from the wastewater. When cultured in a HRAP, color removal reached 50% along with high reductions in COD, PO_4^{3-}-P, and NH_4^+-N [54].

Two wild-type green algae such as *Micractinium* sp. and *Chlorella* sp. can also be grown in high-nitrogen wastewater (mixture of sludge centrate and primary effluent wastewater). The extraction and analysis of extracellular polymeric substances (EPSs) in both algal species during cultivation showed that *Micractinium* generated a higher amount of EPS proteins than *Chlorella* [27]. This fact affects the anaerobic biodegradability and methane yield when these algae are anaerobically co-digested with waste-activated sludge (WAS).

2.1.2. Scenedesmus genus

Food wastewater (FW), rich in nutrients including N, P, Ca, Fe, Al, and total organic carbon (TOC), was also effectively used for microalgal cultivation [9]. The effect of FW supplementation on the biomass and lipid productivity of *Scenedesmus obliquus* cultivated in Bold's Basal Medium (BBM) was recently investigated by Ji et al. [9]. They reported a substantial increase in growth and lipid productivity with supplementation of 1% FW to BBM. Furthermore, the fatty acid methyl ester (FAME) analysis revealed that the palmitic and oleic acid contents increased by up to 8% with the addition of FW. They also noted that FW promoted algal autoflocculation due to the formation of inorganic precipitates at an alkaline pH [9]. Similarly, the

biomass, lipid productivity, and nutrient removal efficiency of *S. obliquus* cultivated under mixotrophic conditions in municipal wastewater were reportedly enhanced when supplemented with FW and flue gas CO_2 [55].

Shanab et al. [56] demonstrated that out of three fresh water microalgal isolates selected for heavy metal tolerance studies, *Scenedesmus quadricauda* showed tolerance to heavy metals such as Hg^{2+}, Pb^{2+}, and Cd^{2+} in up to 100 mg/L concentrations. Research on the applications of immobilized microalgal cells indicated that immobilized algal cells are more tolerant to heavy metal stress when compared to free living cells [56].

Scenedesmus sp. has also been widely reported with good carbon sequestration potential [57]. These studies obtained hydrocarbons from microalgal lipids for their conversion into sustainable biofuels as a substitute for fossil hydrocarbons. Furthermore, microalgae have significant applications in the production of valuable materials in the food and pharmaceutical industries, producing a high value-added process in the biosequestration of CO_2 [57].

Similar to bioconversion, some microalgae can also carry out the biosorption of textile wastewater. For instance, *S. quadricauda* has been successfully employed as biosorbent to remove remazol brilliant blue R (RBBR) [58, 59].

In a very recent study, microalgae digestate and secondary effluent were used to grow *Scenedesmus* sp. in a tertiary treatment using a 30 L closed photobioreactor for cultivation. The microalgae biomass, composed of *Scenedesmus* sp., reached and maintained a concentration of 1.1 g TSS/L during 30 days [22]. A complete removal of $N\text{-}NH_4^+$ and $P\text{-}PO_4^{3-}$ and high nitrate and organic matter removals were achieved (58% $N\text{-}NO_3^-$ and 70% COD) with 8 days of HRT [22].

2.1.3. Dunaliella salina

A very recent study assessed the feasibility of the cultivation of *D. salina* in controlled environment tertiary-treated municipal wastewater [60]. *D. salina* was selected for its high β-carotene generation capacity and for being a halophilic species to protect our fresh water resources further through wastewater remediation. Nutrient analyses indicated that *D. salina* can significantly remove nitrate, ammonia, and phosphorus from municipal wastewater in the range of 45–88%. Among all combinations studied, optimal algal growth was observed at 30 ppt salinity level, with a 75% wastewater concentration (3:1 ratio of wastewater and saline water mixture, which is the growth medium). These findings concluded that *D. salina* has great capacity for nutrient uptake while providing high-value bioproducts [60].

Another study assessed the production rates of some native microalgae growing in media supplemented with algal digestate, urban wastewater, or digested sludge. Very low production rates, or no growth, were measured when microalgae isolated from high-salinity waters (*D. salina*) were used, suggesting that populations well adapted to extreme environmental conditions are not suitable candidates for growing in wastewater or anaerobic digestate [61].

2.1.4. Nannochloropsis salina

The potential for *N. salina* to be integrated with contaminated water sources was assessed for the concurrent production of a biofuel feedstock while providing an environmental service

through bioremediation [62]. Individual contaminants (As, Cd, Cr, Co, Cu, Pb, Ni, Hg, Se, and Zn) at various concentrations ranging from a low concentration (1X) to higher concentrations (10X and 40X) found in contaminated systems (mine tailings, wastewater treatment plants, produced water) were introduced into growth media. Biological growth experimentation was performed in triplicate at the various contaminant concentrations and at three different light intensities. Results showed that baseline concentrations of each contaminant slightly decreased biomass growth between 89 and 99% of the control with the exception of Ni, which dramatically reduced growth. Increased contaminant concentrations resulted in progressively lower growth rates for all the contaminants tested. Lipid analysis showed that most baseline contaminant concentrations slightly decreased or had minimal effects on lipid content at all light levels. Trace contaminant analysis on the biomass showed that Cd, Co, Cu, Pb, and Zn were sorbed by the microalgae with minimal contaminants remaining in the growth media, which illustrated the effectiveness of microalgae to bioremediate these contaminants when levels are sufficiently low and to not detrimentally impact productivity. The microalgae biomass was less efficient in the sorption of As, Cr, Ni, and Se [62].

Another study revealed that metal levels in municipal wastewaters were unlikely to inhibit algal growth and lipid production at least by metals, which are tolerant to microalgae like *N. salina*. Cells grew without inhibition in treated municipal wastewater or centrate derived from wastewater treatment with the addition of up to 75% v/v in their normal growth medium minus nitrogen and phosphorus [63].

2.1.5. Botryococcus braunii

B. braunii is a microalga, which is regarded as a potential source of renewable fuel because of its ability to produce large amounts of lipids that can be converted into biodiesel. Agro-industrial by-products and wastes are of great interest as cultivation medium for microorganisms because of their low cost, renewable nature, and abundance. Two strategies for the low-cost production of *B. braunii* biomass with high-lipid content were performed: (i) mixotrophic cultivation using molasses, a cheap by-product from the sugar cane plant as a carbon source, and (ii) photoautotrophic cultivation using nitrate-rich wastewater supplemented with CO_2 as a carbon source. Mixotrophic cultivation added with 15 g/L molasses produced a high amount of biomass at 3.05 g/L with a high-lipid content of 36.9%. The photoautotrophic cultivation in nitrate-rich wastewater supplemented with 2.0% CO_2 produced a biomass of 2.26 g/L and a lipid content of 30.3%. The benefits of this photoautotrophic cultivation are that this cultivation would help to reduce the accumulation of atmospheric carbon dioxide and more than 90% of the nitrate could be removed from the wastewater. When this cultivation was scaled up in a stirred tank photobioreactor and run with the semi-continuous cultivation regime, the highest microalgal biomass of 5.16 g/L with a comparable lipid content of 32.2% was achieved [64].

To understand the potential of using swine lagoon wastewater to cultivate *B. braunii* for biofuel production, the growth characteristics of *B. braunii* 765 cultivated in aerated swine lagoon wastewater (ASLW) without sterilization and pH adjustment were investigated. The results showed that the alga strain could maintain a competitive advantage over the 26-day cultivation. The highest dry biomass of alga grown in ASLW was 0.94 mg/L at Day 24, which was 1.73 times that

grown in a BG 11 medium, an artificial medium normally used for *B. braunii* cultivation. And the algal hydrocarbon content was 23.8%, which was more than twice that in the BG 11 medium. Additionally, after the 26-day cultivation period, about 40.8% of TN and 93.3% of TP in ASLW were removed, also indicating good environmental benefits of algal bioremediation [65].

A study was conducted to evaluate the possibility of using wastewater from a soybean curd manufacturing plant as a growth promoter of *B. braunii* strain BOT-22. Soybean curd wastewater (SCW) was added to a AF-6 medium to set final concentrations at 0 (control), 1, 2, 5, and 10% (v/v). The growth and hydrocarbon production observed in the cultures with 1 and 2% SCW were significantly higher than that observed in the control. It was postulated that proteins and/or reducing sugars in SCW could enhance the growth [66].

2.1.6. Micractinium genus

The strain *Micractinium* sp. IC-76 was grown in municipal wastewater and showed a biomass productivity of 37.1 ± 4.1 mg/(L d) and a lipid content of 36.2 ± 0.1%, with a total content of saturated and monounsaturated fatty acids of 71.9%. The efficiency of nitrogen ($N\text{-}NH_4^+$) and phosphorus ($P\text{-}PO_4^{3-}$) removal was 96.4 ± 0.7 and 77.8 ± 5.6%, respectively. The strain *Micractinium* sp. IC-76 in the stationary phase of growth showed a significant difference in carbohydrate metabolism, especially sucrose concentration. High-lipid induction during cultivation in wastewater was also driven by changes in the biosynthesis of amino acids, fatty acids, and the tricarboxylic acid cycle [67].

Micractinium sp. Embrapa|LBA32 presented vigorous growth in a light-dependent manner in undiluted vinasse under non-axenic conditions. Microalgae strains presented higher biomass productivity in vinasse-based media when compared to standard BBM in cultures performed using 15 L airlift flat plate photobioreactors. Chemical composition analyses showed that proteins and carbohydrates comprise the major fractions of algal biomass. Glucose was the main monosaccharide detected, ranging from 46 to 76% of the total carbohydrate contents according to the culture media used [68].

2.2. Haptophytes: *Isochrysis galbana*

A recent study investigates the capacity of *I. galbana* in the bioremediation of aquaculture wastewater from a gray mullet *Mugil cephalus*. The experiment was conducted in batch conditions for 7 days using completely mixed bubble column photobioreactors. After 2 days, *I. galbana* removed 32 and 79% of dissolved inorganic nitrogen and dissolved inorganic phosphorus, respectively [10].

It has been also reported that *I. galbana* cultured in open ponds has fatty acids and a high-protein content, which are suitable for animal nutrition [20].

2.3. Cyanobacteria

2.3.1. Arthrospira platensis

Phosphorus can be recycled from wastewater through microalgal cultivation and provided to crop plants in the form of microalgal biofertilizers. Guldhe et al. [21] reported filamentous

cyanobacteria *A. platensis* cultivated in aquaculture wastewater as algal biofertilizer for the leafy vegetables Arugula (*Eruca sativa*), Bayam Red (*Amaranthus gangeticus*) and Pak Choy (*Brassica rapa* ssp. *chinensis*). In their study, *A. platensis* biomass showed lower amounts of NPK, while amounts of iron, magnesium, calcium, and zinc were found to be higher in algal biomass when compared to chemical fertilizer (Triple Pro 15-15-15).

Microalgae are a rich source of proteins, pigments, and omega fatty acids and thus find application in human and animal feed production. *A. platensis* is one of the dominant species of microalgae used in the health food industry [69].The omega fatty acids from this microalga are used as human food and animal feed supplements. Phang et al. [70] found that the biomass composition of *Arthrospira* cultured in a high-rate algal pond for the treatment of sago starch processing wastewater can be used as high-quality animal feed, especially in the aquaculture industry. During the mentioned treatment of sago processing wastewater using *Spirulina*, COD, PO_4^{3-}-P, and NH_4^+-N reductions of 94, 93, and 99%, respectively, were achieved [70].

Zainal et al. [71] reported that *A. platensis* was able to treat wastewater containing heavy metals and removed manganese by 84.9%; chromium by 83.8%; arsenic by 71.4%; nickel by 61.9%; zinc by 55%; copper by 52.8%, and iron by 45.1%.

Similar to bioconversion, microalgae could also carry out the biosorption of textile wastewater. For instance, *A. platensis* was used as a biosorbent to remove reactive red 120 (RR-120) from its aqueous solution. It achieved the maximum biosorption capacity of 482.2 mg/g removing 97% RR-120 from the solution [72].

2.3.2. Oscillatoria tenuis

The performance of *O. tenuis* to remove nitrogen, phosphorus, and COD from secondary effluents of municipal domestic wastewater was investigated in batch experiments. *O. tenuis* had a biomass productivity of 150 mL/(L·d), a removal rate of NH_4^+-N of 96.1%, and total phosphorus and COD removal efficiencies of 82.9 and 92.6%, respectively, within 7 days at an aeration rate of 1.0 L/min [73].

At the same time, *O. tenuis* showed its capacity to remove reactive dyes from textile wastewater. This species degraded azo dyes into simple aromatic amines and decolorized dye wastewater [59].

2.4. Binary and mixed culture systems

Maintaining the uni-algal system requires a super clean environment, which can be attained under laboratory conditions only. In the outdoor cultivation of microalgae, it is almost impossible to maintain a uni-algal system. If so, it requires a lot of expertise and skills. Moreover, the biomass productivity of the uni-algal system is limited because of suppressed metabolic activity during night time or dark periods. Alternatively, heterotrophic microalgae are used, which are less sensitive to photoperiods, grow fast, and return high-biomass yields. However, a significant amount of CO_2 is produced during oxidative metabolism, which remains unused and is released into the environment. This CO_2 can be further utilized by employing autotrophic microalgae in the cultivation matrix. Therefore, the concept of a binary culture system arises [38]. Binary culture is considered superior to the uni-algal system in several

different ways: binary culture can use wastewater as a nutrients source without sterilization unlike in single systems; microalgae observe a low level of contamination in binary culture because bacteria protect those invading pathogens; microalgae with increased growth rate would decrease the cultivation time and reduce the overall cost; binary culture also aids in bioflocculation and lipid induction; and so on [38].

Species selection is crucial for the success of microalgae cultivation in wastewater. Combining different species with varying metabolic potential would provide robustness to fluctuations in environmental factors and wastewater compositions, thereby giving more stability to the system. For instance, the potential application of microalgae consortia (*Chlorella* sp., *Scenedesmus* sp., and *C. zofingiensis*) compared to monoculture (*Chlorella* sp.) for the treatment of dairy wastewater was evaluated by Qin et al. [74]. They reported a significantly higher COD removal (57–62%) and phosphorous removal (91–96%) by microalgae consortia when compared to the monoculture of *Chlorella* sp. Furthermore, FAME profiles indicated that lipids produced from the microalgae consortia cultivation system were more suitable for biodiesel production [74].

In a very recent study [8], a mixed microalgae consortium (highly dominated by *Chlorella* species and small portions of *Scenedesmus* sp.) was cultivated using digestate (D), animal manure (AM), and textile wastewater (TW) as growth medium providing mainly N (nitrogen) and P (phosphorous) sources without any extra nutrient addition. After a cultivation period of 13 days, P was completely removed (100%); however, N was still remaining, and the removal rates of 70.1, 72.3, and 16.7% for TW, AM, and D, respectively, were achieved. The peak growth rate and biomass production of 0.419 d^{-1} and 0.4 g/L (in terms of volatile solids, VSs) were achieved using TW as growth medium [8].

3. Use of microalgae for biogas production through anaerobic digestion

Anaerobic digestion is a series of biological processes in which microorganisms break down biodegradable material in the absence of oxygen. The end-products of anaerobic digestion are biogas and a digestate. Recently, algal biomass has been identified and developed as a renewable fuel source, and the growth of algal biomass for methane production has been increased.

The first study concerning the anaerobic digestion of microalga was carried out by Goluke et al. [30]. *Scenedesmus* sp. and *Chlorella* sp. were used as substrates for anaerobic digestion under different conditions. The authors finally concluded that microalgae have a relatively low digestibility due to the slowly biodegradable cell wall. Recently, one of the first studies about using algal biomass in anaerobic digestion was carried out by De Schamphelaire et al. [75]. This work consisted of designing a closed loop where algal biomass was used to obtain biogas. The maximum methane yield reached was 65 mL/day. More recently, in 2013, Torres et al. [35] defined the ideal microalgae for anaerobic digestion as a large cell microalga with a very thin cell wall or lacking it, with a high-growth rate in non-sterile medium and great resistance against natural pollutants. In one of the latest studies on the anaerobic digestion of microalgae, the authors pointed out the main limitations during the anaerobic digestion of

microalgae, noting the low degradability of the cell wall, ammonium toxicity, and salinity as the main inhibitors of anaerobic digestion [76].

However, the use of microalgae as co-substrate is an approach to dilute complex compounds and balance the C/N ratio. Co-digestion has several advantages such as adjusting the C/N ratio, nutrients, and inhibitor compounds [34]. Ajeej et al. [77] also reported the increased activity of methanogenic microorganisms, a decreased anaerobic digestion inhibition by ammonium, and even increased cellulose activity when carbon-rich materials were added. Taking into account that the C/N ratio of the microalgal biomass is around 10:1 [78], the microalgae biomass can be considered as a suitable feedstock for carbon-rich substrates [79].

The main microalgae used for co-digestion have been described in the following paragraphs.

3.1. Chlorophytes

3.1.1. Chlorella genus

Ehimen et al. [80] added lipid-extracted *Chlorella* biomass resulting from microalga diesel production to glycerol (main by-product formed during the transesterification process) and observed an increase in the methane yield of 50% when compared to the digestion of residual biomass alone.

Wang et al. [81] used the biomass of microalga *Chlorella* sp. grown in laboratory culture for co-digestion with WAS. The batch experiments were carried out under mesophilic conditions with a working volume of 100 mL. Different volumes of algae and WAS were added to the digester. They experimentally proved that the addition of WAS improved the anaerobic digestion of the microalga *Chlorella*, producing 73–79% more methane than single microalga digestion. Similar results were obtained by Li et al. [82], who co-digested *Chlorella* sp. with chicken manure in batch experiments. The co-digestion enhanced the methane production obtained during the single digestion of chicken manure and *Chlorella* sp. by 14.20 and 76.86%, respectively. By contrast, Retfalvi et al. [83], using the same C/N ratio, but pretreating the microalga, did not observe any positive effects on methane production.

Beltran et al. [84] assessed the co-digestion of *C. sorokiniana* with WAS. Different co-digestion mixtures were tested in biochemical methane potential (BMP) tests under mesophilic conditions. The highest methane yield obtained was 442 mL CH_4/g VS for the mixture 75% WAS and 25% microalga. This value was 22 and 39% higher than that obtained in the anaerobic digestion of the sole substrates, WAS and microalga, respectively. This mixture clearly improved anaerobic digestion by ensuring its viability, suitability, and efficiency.

Rusten and Sahu [85] co-digested *Chlorella* sp. biomass and wastewater sludge (pretreated sludge liquor).The specific methane gas production (mL CH_4/g VS_{fed}) was not increased when compared to the anaerobic digestion of wastewater sludge alone. The co-digestion process achieved between 65 and 90% of specific methane gas production for sludge liquor depending on the HRT, temperature of incubation, and pretreatment of algae biomass. However, this study indicated that tested microalga could be cultivated in reject water to remove nitrogen and phosphorus from the sludge liquor.

In a recent study, Mahdy et al. [86] investigated the anaerobic co-digestion of *C. vulgaris* and manure. They used five different mixtures in a batch mesophilic experiment. The percentage 80:20 microalga:manure produced 431 mL CH_4/g VS, while the methane yield of the single microalga produced 415 mL CH_4/g VS. Despite the high-ammonium levels (3.7–4.2 g NH_4^+-N/L), using ammonia tolerant inoculums resulted in a relatively high-methane yield.

According to Li et al. [82], *Chlorella* 1067 was cultivated in a chicken manure-based digestate, and the resulting algae biomass was used as co-substrate with chicken manure in anaerobic co-digestion. The growth of microalga in manure-based digestate recycled about 91% of the total nitrogen and 86% of the soluble organic phosphorous. During co-digestion, the highest methane production was 238.71 mL CH_4/g VS, obtained at the mixing ratio of 8:2 (chicken manure to *Chlorella* 1067 according to the VS).

3.1.2. Scenedesmus genus

Ramos-Suarez et al. [87] described *Scenedesmus* sp. biomass as a non-suitable substrate for anaerobic digestion due to its low degradability and low methane production. In contrast, during their investigations, they used the biomass of microalga as co-substrate with *Opuntia maxima* cladodes. Bioreactors were used to grow *Scenedesmus* sp., and the biomass was co-digested with different percentages of cladodes of 1 or 2 years of age in order to avoid an increase in lignocelluloses. C/N ratios from 6.0 to 51.3 were used, proving that co-digestion improved methane yield and kinetics when compared to the mono-digestion of both substrates. The best mixture turned out to be the C/N ratio of 15.6. The methane yield for this mixture was 233.6 ± 16.4 mL CH_4/g VS and was increased by 66.4 and 63.9% when compared to *Scenedesmus* sp. biomass and *O. maxima*, respectively, when digested alone.

Astals et al. [88] assessed the co-digestion of pig manure and *Scenedesmus* sp. with and without the extraction of intracellular algal co-products. Proteins and/or lipids were extracted from *Scenedesmus* sp. This process increased methane yield by 29–37% when compared to raw microalga biomass. Co-digestion experiments showed a synergy effect between pig manure and raw microalga that increased raw algae methane yield from 163 to 245 mL CH_4/g VS. A similar synergy effect was not observed when algal residues were co-digested with pig manure.

Arias et al. [22] used microalga digestate and secondary effluent to grow microalga in a tertiary wastewater treatment, and then the microalga biomass was co-digested for biogas generation. The algal biomass was mainly composed of *Scenedesmus* sp. The algae biomass and the WAS were pretreated by autohydrolysis reaching 11.4 and 25.7% of solubilization, respectively. The solubilization of *Scenedesmus* biomass was lower than the solubilization of WAS after pretreatment, and *Scenedesmus* has been reported to have a complex multilayer cell wall [89]. After pretreatment both substrates were co-digested in different proportions. The maximum methane yield obtained was 204 mL CH_4/g VS for the anaerobic digestion of 100% WAS. On the other hand, the methane yield of the anaerobic digestion of 100% microalga exhibited a 64% lower methane production and reached 134 mL CH_4/g VS. The mixture of 20% microalga and 80% WAS produced 187 mL CH_4/g VS, while the mixture of 50% microalgae and 50% WAS produced 162 mL CH_4/g VS, and the mixture of 80% microalga and 20% WAS produced

132 mL CH_4/g VS. The results showed neither positive nor negative synergies between substrates, meaning that co-digestion did not improve microalga anaerobic biodegradability [22].

3.1.3. Dunaliella salina

According to Fernández-Rodríguez et al. [36], the addition of olive mill solid waste (OMSW) to *D. salina* biomass resulted in the improvement in methane yield and biodegradability of OMSW when compared to the anaerobic digestion of the sole substrates. The experiment was carried out in batch, and different percentages of OMSW and *D. salina* biomass were tested. The highest biodegradability was found for the co-digestion mixture of 50% OMSW and 50% *D. salina*. Nevertheless, the maximum methane production, 330 mL CH_4/g VS, and the highest methane production rate were obtained for the co-digestion mixture of 75% OMSW and 25% *D. salina*, keeping a C/N ratio close to 26.7.

3.1.4. Nannochloropsis salina

Another approach to enhance biogas production from microalga through co-digestion was assessed by Schwede et al. [90]. Corn silage is one is the most common waste products produced around all over the world. Corn silage is characterized as being a lignocellulosic residue and very difficult to digest by anaerobic digestion [91]. The experiment carried out by Schwede et al. [90] reached a high-methane yield using *N. salina* as a co-substrate of corn silage. The mixture balanced the nutrient composition due to the corn silage providing mainly carbon and the microalga providing nitrogen, which helped to balance the C/N ratio from 65 (*N. salina*) or 32.6 (corn silage) to 21.2 (Mixture *N. salina*/corn silage). This mixture, C/N = 21.2, reached 9% more methane than that obtained in the anaerobic digestion of the corn silage alone.

3.1.5. Botryococcus braunii

Neumann et al. [92] reported that the anaerobic co-digestion of lipid-spent *B. braunii* (LSBB) with WAS and glycerol showed no significant increase in BMP when mixing these substrates. However, the kinetic constant of the mixture 25% WAS-75% LSBB was much higher than those obtained for WAS and LSBB alone. The mixture of 10% glycerol and 90% LSBB did not show a higher kinetic constant or methane production. The authors concluded that the application of different cultivation procedures, lipid extraction methods, and anaerobic conditions will result in different microalga biomass compositions and characteristics, which affect the productivity of microalgal methane.

3.1.6. Micractinium genus

Wang et al. [27] applied WAS to the digestion of microalga biomass consisting of *Micractinium* sp. The algae biomass was grown in high-nitrogen wastewater (mixture of sludge centrate and primary effluent wastewater). The microalga showed a good ability for nutrient removal throughout the growth. The co-digestion of microalga biomass and WAS improved the solubilization efficiency and the biodegradability of the microalgae. The methane yield obtained

for the microalga was 209 mL/g VS. The co-digestion of algae with WAS improved the volatile solid reduction, the solubilization efficiency of the algae, and their biogas yield. However, the methane production of the WAS alone showed no improvement.

3.1.7. Selenastrum capricornutum (Chlorophyta) and Isochrysis galbana (Haptophyta)

I. galbana and *S. capricornutum* were co-digested with sewage sludge under mesophilic (33°C) and thermophilic (55°C) conditions [93]. Under mesophilic conditions, the anaerobic digestion of sewage sludge produced 451 ± 12 mL biogas/g VS. The microalga *I. galbana* produced 439 mL biogas/g VS, and *S. capricornutum* produced 271 mL biogas/g VS. When a substrate mixture was fed, biogas production showed quite similar values for all experiments, regardless of the sludge to microalga ratio in the mixture. The average biogas production was 440 ± 25 mL biogas/g VS. So, microalga and sewage sludge co-digestion did not improve biogas yield in comparison with individual digestions of both substrates under mesophilic conditions. Under thermophilic conditions, the biogas production of *I. galbana* was 261 ± 11 mL biogas/g VS, and the production of *S. capricornutum* was 185 ± 7 mL biogas/g VS. The amount of methane decreased by 40.5 and 31.7% for *I. galbana* and *S. capricornutum*, respectively, when compared to their biogas production at 33°C. The increase in temperature had a negative influence on microalga digestion. However, temperature had a huge beneficial effect on sewage sludge. The production of biogas reached 566 ± 5 mL biogas/g VS, indicating that 25.5% more biogas was produced by increasing temperature. The experiment presented similar tendencies, the higher the volatile solid, the lower the biogas production.

3.2. Cyanobacteria

3.2.1. Arthrospira platensis

A. platensis was characterized as having a high level of protein and, therefore, a high-nitrogen content [94]. Biomass with a high-nitrogen content could be used as co-substrate with high-carbon content substrates [95]. This study investigated the co-digestion of *A. platensis* with barley straw, beet silage, and brown seaweed at a C/N ratio of 25, the optimal ratio for anaerobic digestion [96]. The experiments were carried out in batch and semi-continuous systems. The C/N ratios of the substrates were 4.3, 145.5, 41.7, and 28.7 for *A. platensis*, barley straw, beet silage, and seaweed *Laminaria digitata*, respectively. The methane productions during the batch experiments were 357.1, 196.8, 393.5, and 306.5 mL_N/gVS for *A. platensis*, barley straw, beet silage, and seaweed *L. digitata*, respectively. The co-digestion of 45% *A. platensis* and 55% beet silage produced 360.9 mL_N/gVS. The co-digestion of 85% *A. platensis* and 15% barley straw produced 347.8mL_N/gVS, and the best co-digestion mixture of *A. platensis* and *L. digitata* (15–85%) produced 311.5 mL_N/gVS. Mono-digestion of *A. platensis* led to high-methane yields in the semi-continuous mode but only at low-organic rates of 1.0 g VS/L·d. Co-digestion with carbon-rich substrates had a positive effect on process stability. The highest biogas production occurred during co-digestion of microalga with beet silage. The best process stability was found at an organic loading of 4.0 g VS/L·d during co-digestion with the seaweed *L. digitata* [95].

A. platensis was co-digested with WAS in batch and in semi-continuous systems [97]. During the batch tests, the system reached 89–93% volatile solid reduction. The biogas production was between 210 and 260 mL CH_4/g VS. In the continuous studies, a two-phase anaerobic digestion system was investigated. The system achieved 60% of volatile solid reduction with 525 mL biogas/gVS·d. The co-digestion of *A. platensis* and sewage sludge improved biogas production and volatile solid reduction. The best mixture was 66.6% WAS and 33.3% *A. platensis* based on volatile solids. The maximum methane production was 640 mL biogas/g VS·d with a 62.5% reduction in volatile solids. The methane content in the biogas was 77%.

3.2.2. Oscillatoria tenuis

Cheng et al. [73] carried out batch experiments to investigate the performance of *O. tenuis* to remove nitrogen, phosphorus, and COD and from the secondary effluents of municipal domestic wastewater. The potential of biogas production was also investigated by applying the co-digestion of *O. tenuis* with pig manure. *O. tenuis* had a good biomass productivity, which ranged from 104 to 150 mg/L·d, and was beneficial for the subsequent anaerobic digestion. A maximum methane yield of 191 mL CH_4/g VS was achieved through co-digestion of this microalga with pig manure at a mixing ratio of 2.0.

3.3. Binary culture system

3.3.1. Scenedesmus genus and Chlorella genus

Zhen et al. [98] used a mixed microalgae culture of *Scenedesmus* sp. and *Chlorella* sp., which were co-digested with food waste in a batch system under mesophilic conditions. The results showed that supplementing food waste with microalga significantly improved the performance of microalga digestion. The highest methane yield achieved was 639.8 ± 1.3 mL/g VS, which was reached at a microalga/food waste ratio of 0.2:0.8, obtaining a 4.99-fold increase with respect to microalgae alone (106.9 ± 3.2 mL/g VS).

3.3.2. Microalgae and bacteria

Solé-Bundó et al. [99] grew microalgae biomass in wastewater, and subsequently, the algae-bacteria biomass was co-digested with wheat straw. Batch systems were used for testing different substrate percentages (20–80%, 50–50% and 80–20%, microalgae and wheat straw, respectively, on a volatile solid basis). The highest synergies in degradation rates were observed by adding at least 50% wheat straw. Therefore, the co-digestion of 50% microalgae biomass and 50% wheat straw was further investigated in mesophilic semi-continuous lab-scale reactors. The results showed that the methane yield was increased by 77% in the co-digestion when compared to microalgae biomass mono-digestion.

Table 1 summarizes the different microalgae and co-substrates tested in anaerobic co-digestion processes including the improvement in the methane yields observed.

Microalga	Co-substrate	Conditions	Improvement in methane yield (%)	Reference
Lipid-extracted *Chlorella* biomass	Glycerol C/N = 12.44	Laboratory scale, continuously stirred tank reactor, at mesophilic temperature	>50 (compared to microalga)	[80]
Chlorella sp. (4%)	WAS (96%)	Batch at mesophilic temperature	73–79 (compared to microalga)	[81]
Chlorella 1067 (20%)	Chicken manure (80%)	Batch experiments	77 (compared to microalga)	[82]
Pretreated *Chlorella* sp. (80%)	Chicken manure (20%)	Batch experiments	No positive effect	[83]
C. sorokiniana (25%)	WAS (75%)	Batch at mesophilic temperature	39 (compared to microalga)	[84]
Chlorella sp. (12%)	Wastewater sludge (88%)	Batch at mesophilic temperature	12 (compared to single substrate)	[85]
C. vulgaris (80%)	Manure (20%)	Batch at mesophilic temperature	3.8 (compared to microalga)	[86]
Scenedesmus sp. (25%)	*O. maxima* cladodes (75%)	Batch at mesophilic temperature	66.4 (compared to microalga)	[87]
Scenedesmus sp. (15%)	Pig manure (85%)	Batch at mesophilic temperature	50.3 (compared to microalga)	[88]
Scenedesmus sp. (20%)	WAS (80%)	Batch at mesophilic temperature	39.5 (compared to microalga)	[22]
D. salina (25%)	OMSW (75%)	Batch at mesophilic temperature	3 (compared to single substrate)	[36]
N. salina (16.6%)	Corn silage (83.4%)	Batch at mesophilic temperature	6 (compared to microalga)	[90]
Lipid-spent *B. braunii*	WAS and glycerol	Batch at mesophilic temperature	No positive effect	[92]
Micractinium sp. (79%)	WAS (21%)	Batch at mesophilic temperature	10 (compared to microalga)	[27]
I. galbana and *S. capricornutum*	Sewage sludge	Batch at mesophilic and thermophilic temperature	No positive effect	[93]
A. platensis (85%)	Barley straw (15%)	Batch at mesophilic temperature	76.7 (compared to single substrate)	[95]
A. platensis (45%)	Beet silage (55%)	Batch at mesophilic temperature	1.1 (compared to microalga)	[95]
A. platensis (15%)	*L. digitata* (85%)	Batch at mesophilic temperature	1.6 (compared to single substrate)	[95]
A. platensis (33.3%)	WAS (66.6%)	Two stages semi-continuous	32.5 (compared to microalga)	[97]
O. tenuis (66.6%)	Pig manure (33.3%)	Batch at mesophilic temperature	*	[73]

Microalga	Co-substrate	Conditions	Improvement in methane yield (%)	Reference
Scenedesmus genus + *Chlorella* genus (20%)	Food waste (80%)	Batch at mesophilic temperature	498.5 (compared to microalga)	[98]
Chlorella sp. + some *Monoraphidium* sp. (50%)	Wheat straw (50%)	Batch at mesophilic temperature	77 (compared to microalga)	[99]

C, carbon; N, nitrogen; WAS, waste-activated sludge; OMSW, olive mill solid waste; *, not available.

Table 1. Improvement of methane yields after anaerobic co-digestion processes of microalgae with different substrates.

4. Microalgae growth in anaerobic digestates

4.1. Physico-chemical characterization of digestates

The anaerobic digestate studied by Solé-bundó et al. [100] presented low dry matter content (~3%), and these digestates can therefore be treated as liquids that could be directly spread onto soil as fertilizer. A problem arises when transportation is required and moisture reduction could be necessary. Anaerobic digestate from microalgae co-digestion was observed to present better water release than the digestate from single microalga digestion.

Other parameters that could have a negative impact on soil (pH, electrical conductivity, and volatile fatty acids) were lower in the co-digestion digestates, indicating that microalgae co-digestion resulted in a more stable digestate.

In general, among the bibliography, anaerobic digestates from agro-food industries presented higher organic contents than those from microalgae digestion [101], which could be explained due to organic matter mineralization during anaerobic digestion processes. The use of microalgae as co-substrate in the digester reduces the VS/TS ratio when compared to microalga alone (from 53 to 54–47%) due to the better biodegradability of the organic compounds of the co-substrate.

In order to evaluate the feasibility of these anaerobic digestates as fertilizers, some elemental nutrients were evaluated. The total nitrogen content was higher in the non-co-digested microalgae (80 g/kg TS and 56 g/kg TS), although the $N\text{-}NH_4^+$/TKN ratio, which represents the soluble mineral nitrogen fraction, only varied from 30.9 to 33.8% among all digestates. Moreover, the C/N ratio was low across the board, which means that in each case the nitrogen content is too high for its use as fertilizers, although it could be used as soil amendment. This problem could be sorted out by using a high-carbon content co-substrate like OMSW or corn silage. Phosphorous and potassium were found slightly higher in the digestates from non-co-digestion, although in each case the content was relatively low and similar to other anaerobic digestates reported in the literature. Calcium, magnesium, and sodium were also analyzed, and no difference was observed among the different digestates [100].

On the whole, the anaerobic digestate from microalgae co-digestion presented better suitability for nutrient supply in soil due to its low C/N ratio, which could be enhanced by using a co-substrate with a higher carbon content.

4.2. Microalgae growth in digestates

The anaerobic digestion of biomass produces a high-nutrient digestate, which is usually used as crop fertilizer, and also could be used as a nutrient supply for microalgae growth in order to reduce the use of external sources of nitrogen and phosphorous [102]. Moreover, wastewaters and other biomass present a reduction in suspended solids and color, better degradability, a more stable pH, and a reduction in pathogens after the anaerobic digestion process, which could enhance microalgae growth when compared to the non-digested biomass.

The main factors that could affect the microalgae growth in anaerobic digestates are the nitrogen and phosphorous contents as well as the pH profile. pH could be increased due to active photosynthesis or insufficient CO_2 supply, which could provoke a $N\text{-}NH_4^+$ disappearance through gas stripping and a $P\text{-}PO_4^{3-}$ precipitation when the medium presents a high concentration of Ca^{2+} [103]. Thus, when the pH of the medium is increased due to microalgae activity, nitrogen and phosphorous depletion do not necessarily mean an increase in biomass. Moreover, it has been reported that an ammonia concentration higher than 2 mM, when pH exceeds 8.1, presented a toxic effect on algae growth [104]. Regarding phosphorous content, it has been reported that 5 mg P/L was sufficient for adequate algae growth when the N/P ratio was around 15, although other studies suggested that N should be the limiting factor [103].

On the other hand, the organic load in these anaerobic digestates is reduced after microalgae cultivation. Nitrogen and phosphorous could be completely removed when the conditions are optimum and COD reduction could reach 44–85% depending on culture conditions and microalgae species [103].

4.2.1. Chlorophytes

4.2.1.1. Chlorella genus

An early study used different microalgae cultivated in swine manure anaerobic digestate diluted with tap water (0.6–3.0%) in order to evaluate its effect on microalgae growth. *Chlorella* sp. was the only species that presented pH stability (pH = 8.5 during 8 days), which indicated that the nitrogen removal was directly related to biomass production. Regarding temperature conditions, *Chlorella* sp. did not show any difference in biomass yield when the temperature was raised from 10 to 20°C. COD reduction in the anaerobic digestate reached 60%. The best conditions for the highest concentration (41 mg dry wt/L·d) were 20°C and a manure concentration of 2% [103].

4.2.1.2. Parachlorella kessleri

P. kessleri was cultivated (12 days; 25°C; air flow: 0.5–1 L/min; illumination: 200 $\mu mol/m^2 \cdot s$) in the anaerobic digestate derived from the co-digestion of end-of-life dairy products with a given mixture of agro-industrial wastes [107]. Prior to the growth of algae, the anaerobic digestate was filtered, diluted (2–10%), and then split into two different samples, one sterilized and the other not. Under the best conditions (2% dilution), *P. kessleri* presented a biomass yield of 270 mg/L, regardless of the use of sterilized or non-sterilized anaerobic digestate.

Moreover, according to the nutrient removal, the nitrogen depletion (up to 100%) and the phosphorous reduction (93.4%) were higher when the anaerobic digestate was sterilized and diluted by up to 2%. Nevertheless, the maximum COD removal (33.3%) was achieved with the non-sterilized anaerobic digestate and a higher dilution (10%). Regarding the fatty acid accumulation, after 25 days of growth, the concentration observed (31.1% dry weight) was higher than in the control essay (19.6% dry weight).

4.2.1.3. Scenedesmus genus

De la Noüe et al. [103] studied the growth of different microalgae in swine manure anaerobic digestate diluted with tap water (0.6–3.0%). The results showed that *Scenedesmus obliquus* presented a response to high temperature, which could be a problem for outdoor work. This microalga was able to reduce the COD content of the anaerobic digestate by up to 85% with a microalga concentration of 57 mg dry wt/L·d at 20°C and with a manure concentration of 2% after 15 days.

In a different study, *S. obliquus* was cultivated in the abovementioned conditions [107]. Under the best conditions (2% dilution), *S. obliquus* presented a biomass yield of 231 mg/L, regardless of the use of sterilized or non-sterilized anaerobic digestate. Moreover, according to the nutrient removal, the nitrogen depletion was higher (up to 100%) when the anaerobic digestate was sterilized and diluted by up to 2%. Nevertheless, the phosphorous reduction was higher (92.5%) when the anaerobic digestate was not sterilized, and the maximum COD removal (53.7%) was achieved with the non-sterilized anaerobic digestate and a higher dilution (10%). The fatty acid accumulation (26.6% dry weight) was higher after 25 days of growth than in the control essay (24.5% dry weight).

Different anaerobic digestates from microalgae biomass co-digestion with swine and cow manure and vegetable wastes were selected for the growth of *Scenedesmus* sp. AMDD at 22°C [102]. Nitrogen was adjusted to 1.5 mM (NH_3-N) with deionized water, and different phosphorous concentrations were evaluated. Moreover, digestates were filtered to reduce the bacterial load. This study showed that the use of an anaerobic digestate from the co-digestion of microalgae biomass presented a good microalga growth rate. Animal manure digestate without co-digestion did not produce a complete nitrogen removal, which was improved when Mg^{+2} was added in the media growth. This element was indicated as a key nutrient for microalgae growth, and it was concluded that 0.03 ± 0.02 mM was adequate for optimal growth.

4.2.1.4. Micractinium pusillum

M. pusillum was grown in a cheese factory anaerobic digestate at 20°C and proven to present a satisfactory microalga growth rate. After 4 days, the pH reached 8.5, and the ammonia depletion was complete, although, according to the high pH, it could be due to the stripping of ammonia or bacterial activity. P-PO_4^{3-} removal reached 33%, and the biomass yield was 137 ± 21 mg dry wt/L·d. Moreover, it was observed that the presence of suspended organic matter caused cell clogging and the adhesion of *M. pusillum* to the walls of the culture vessels [105].

4.2.2. Cyanobacteria

4.2.2.1. Phormidium bohneri

De la Noüe et al. [103] also studied the growth of *P. bohneri.* The nitrogen toxic effect for *P. bohneri* was observed at 3.2 mM N-NH_4^+, which indicated that *P. bohneri* presented a higher nitrogen resistance than other common cyanobacteria. Moreover, an increase in temperature (from 10 to 35°C) produced an increase in biomass production. It was observed that a concentration of 0.1–0.5 mg Cu^{2+}/L showed a toxic effect on *P. bohneri.* Seventy-five percent of COD removal from the anaerobic digestate was achieved. The higher concentration of *P. bohneri* (32 mg dry wt/L·d) was reached with a 2% swine manure dilution at 20°C.

When *P. bohneri* was cultivated in a cheese factory anaerobic digestate at 20°C, a rapid increase in pH was observed after 4 days (from 8.4 to 10.9). No significant amount of NH_4^+ was observed after the process, although, according to the high pH, it could be due to the stripping of ammonia or bacterial activity. P-PO_4^{3-} removal reached 69% with a biomass yield of 329 ± 24 mg dry wt/L·d [105].

4.2.2.2. Spirulina maxima

In an early study, *S. maxima* was observed to need a high concentration of bicarbonate ions for optimal growth [106]. When it was cultivated in swine manure anaerobic digestate diluted with seawater, an increase in the microalga growth rate was observed with CO_2 supplementation. After 15 days, the anaerobic digestate presented a complete N-NH_4^+ reduction, phosphate removal of 99.3%, nitrogen depletion of 76%, and a reduction in volatile solids of 28%.

5. Conclusions

Microalgae are renowned as a powerful biotechnology platform for the production of a wide range of value-added products. These include biofuels, animal and aquaculture feeds, and high-value commercial products, such as pigments, polysaccharides, bioplastics, and other organic compounds. Microalgae have also been proposed for a biorefinery model where multiple compounds can be produced simultaneously from harvested microalgal biomass grown in wastewaters and in anaerobic digestion digestates. The growth of the biomass in industrial wastewater and/ or anaerobic digestates has been proven to be a feasible alternative to synthetic mediums.

Regarding the anaerobic digestion of microalgae and cyanobacteria biomass, co-digestion allows to improve the low C/N ratio of microalgae and cyanobacteria, balance the nutrients, and avoid the possible inhibitions in many cases. Furthermore, the produced digestate after the anaerobic digestion process presented better stability when a high-carbon biomass is co-digested with microalgae or cyanobacteria biomass.

However, the wide variety of microalgae and cyanobacteria and the different types of high-carbon biomass make it difficult to ascertain a general assessment about the enhancement of methane production when these two biomasses are co-digested. In this respect, it seems that the use of microalgae/bacteria consortium could reduce drawbacks from working with

pure species by favoring positive synergetic effects. Further studies will be needed in order to obtain a proper mixture culture.

Acknowledgements

The authors wish to express their gratitude to the regional government of Andalucía "Junta de Andalucía" (project RNM-1970) for providing financial support. Dr. Rincón wishes to thank the "Ramón y Cajal" Program (RYC-2011-08783 contract) from the Spanish Ministry of Economy and Competitiveness for providing financial support.

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Chapter 5

Algal Fuel Cell

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.74285

Abstract

Algal Fuel Cells (AFC) are bioelectric devices that use photosynthetic organisms to turn light and biochemical energy into electrical energy. The potential of a fully biotic AFC still remains an unexplored area of research and hence it has led to rethink the prospective use of plant-based bioelectricity. AFC consists of an anode and a cathode connected by an external electric circuit and separated internally by a membrane/no membrane in which the growth of algae is assessed. The key parameters for evaluating the performance of AFC are electrodes, separators, oxygen supplement, nutrients and its configurations. By controlling these parameters, the electric power production can be optimized. This chapter discusses the recent trends examined by a number of researchers and are interpreted to gain a better understanding. It is stressed that a greater focus must be given for a complete comprehension of the algal processes required for the development of AFC applications. Thus, it can be concluded that a further development of AFC technology with reduced costs and improved performance is required for sustainable development.

Keywords: algae, algal fuel cell, photosynthetic electrode, photo bio-reactor, renewable energy

1. Introduction

Almost 80% of world energy consumption is from the combustion of fossil fuels. The depletion of these fossil fuels necessitates the importance of renewable energy synchronization. Fossil

fuels on combustion pollute the environment by emitting huge amount of CO_2 to the atmosphere resulting in global climate change. The risks of over-dependence on fossil fuels can be avoided by using renewable and carbon-neutral energy sources in a large amount. The concern and awareness of the harmful impact of mineral-based fuels on the environment have pushed the research towards the production of eco-friendly energy from renewable sources. Renewable energy, which can be harvested from the sun either by photovoltaic energy or in the form of biomass energy as solar energy is considered as the mother of all energy, will play a predominant role in future. Globally, carbon neutral energy has been receiving the attention of extensive researches during last decades.

During the eighteenth century, the novel idea of generating electric energy from biological route emerged. The potential of using microorganisms that convert organic or inorganic compounds into electrical power was studied [1]. This process occurs through metabolic activity of microorganisms at ambient pressure and temperature [2]. Microbial fuel cells are the devices capable of producing bioelectricity from different sources of substrates [3, 4]. The substrate is regarded as one of the essential biochemical factors affecting power generation in microbial fuel cells. The consideration of microbial fuel cells as a marginal scientific issue has been catching up with other bioconversion concepts in recent years.

New designs have evolved and the operation has moved towards AFC for generating bioelectricity through the photosynthesis reaction by microalgae. Microalgae are considered as eco-friendly organisms having high photosynthetic efficiency and rapid reproduction and are also a good source of fuel with their neutral lipid content. Algae use energy from sunlight in the photosynthetic reaction in which they consume carbon dioxide to produce oxygen. The first creations of algae were cyanobacteria, the small sized blue green algae responsible for the early transformation of the earth's atmosphere. Algae play a significant role in the production of oxygen. In the current situation, there is need to reduce carbon dioxide and in this way algae convert carbon dioxide to oxygen where lights stimulate the CO_2 fixation by Calvin cycle. The photosynthesis reaction is considered as one of the complex biological redox reactions happening naturally and carried out by algae and plants in which they are able to use energy from the sun to produce carbohydrates and oxygen through multiple redox reactions. They also produce additional compounds during the process which may be utilized for energy or employed in the synthesis of other molecules [5, 6].

AFC is a promising technology which can capture CO_2 inexpensively with the help of algae. Generally, microalgae grow in a bioreactor or open pond where they can use the sunlight, CO_2 and nutrient. Therefore, new designs were employed for enabling the microalgae in a microbial fuel cell to generate electricity with different electrode materials.

2. Algal fuel cell configuration

AFCs are electro biochemical devices which have anode and cathode compartments enclosed with a photosynthetic microorganism. Here photosynthesis is carried out and they act as electron donors producing organic metabolites. The main objective of configuring AFC is to

increases the power density and achieving high performance in order to create a cost effective system. Major configurations of AFC are a single chamber, two chambers, three chambers, coupled and sediment types.

In single chamber AFC, bacteria and microalgae are grown together in one chamber which is membrane less wherein micro-algae forms a biofilm on the anode and some are usually configured with an air cathode [7, 8]. Carbon dioxide generated by autotrophic and heterotrophic organisms are consumed simultaneously by algae in the same chamber. In single chamber AFC, bacterial co-cultures are grown synergistically with algal co-cultures [9]. Single chambers are easy to manage in lab when compared to other configurations. Single chamber AFC are easy to operate, cost effective in scaling up and can be used commercially.

Dual or two chambered AFC consists of two separate chambers in which microalgae and bacteria are separated by a membrane [10, 11]. In two chambered configuration, cathodic compartment contains microalgae that are illuminated for photosynthesis reaction. The anodic compartment is also illuminated making the algae to cover the bacterial compartment in most of the studies. Highly relative internal resistance and crossover of the membrane are some issues are associated with this system.

Three chambered MFCs are an additional chamber containing salt water that gives stress to the production of power. The third chamber is in between the cathode and anode chambers. Partial desalination is observed in the middle chamber where cations move towards cathode and anion towards anode [7].

In sediment AFC, an anode is buried in sediment and a cathode is on the top of sediment immersed in the water having microalgae. The differences in existing electro-potentials generate energy [12, 13]. During this process the released electrons are captured by the anode and current is generated in an electrical circuit. In this configuration, cathode compartment was changed to biogenic one.

3. Bio-active organisms

Microalgae are one of the best bioactive metabolites for a microbial fuel cell which can mitigate CO_2. The mechanism of donating an electron and accepting it is still uncertain. The understanding of the mechanism is important for improving the performances of AFCs. Some studies predicted that dumping of cells in a certain environment causes the reduction of power against oxidative stress. Researchers have explained these mechanisms by using specific inhibitors of electron transport chain in microalgae [14, 15]. Another prediction has reported that microorganisms use electrical signal for communicating and this is explained in many complex communities containing autotrophic and heterotrophic, eukaryotic and prokaryotic organisms where electrogenic microorganisms exist [16]. Many researchers have reviewed and recommended microbial fuel cells using microalgae for the right selection of the type of algal strain to maximize power production [17, 18]. The study to determine the method of screening the right strain is few in number. A recent study has made an effort for

cost-effective photosynthetic microbial fuel cell design with highly reproducible electrochemical characteristics that can be used to screen algae and cyanobacteria for photosynthetic electrogenic activity. *Paulschulzia pseudovolvox* (*Chlorophyceae*) is identified as good electrogenic qualities among several cyanobacteria [19].

4. Interactions between algae and electrodes

4.1. Anolyte

The anolyte used in AFC is rich in carbon source such as glucose formate and acetate being similar to other prepared sources like LB medium, *Scenedesmus* algae in powder form, fruit industry liquid waste and wastewater [9, 11, 20–24]. The factors affecting the generation of power depends on the types of anolytes used and their internal resistance. The efficiency and power production of AFC depend on the resistance of membrane on anolyte, high ion generation in the anodic chamber and oxygen crossover through the membrane. Some of the problems faced by AFC are membrane fouling, high COD and low pH of anolyte. To overcome these problems, membrane pretreatment and continuous monitoring of the internal conditions of the anodic chamber is necessary.

4.2. Catholyte

The commonly used catholyte in AFC is microalgae. Microalgae in cathode help in reducing the CO_2 emitted from bacterial metabolism, respiration providing economic and environmental sustainability. Blue green algae, *Chlorella vulgaris*, *Desmodesmus sp.*, etc. are some of the microalgae used in the cathode compartment of AFC. *Chlorella vulgaris* is one of the common microalgae which have been studied extensively as a catholyte by many researchers. It is influenced by several factors such as electron consumption by methanogenesis, aerobic respiration by the cathodic biofilm and oxygen crossover which is hindered during COD removal [25]. Moreover, algal biofilms can limit the diffusion of oxygen affecting the performance of AFC [26]. Researchers have reported 92% of COD removal and 90–80% removal of inorganic components with 2.2 mW^{-3} of power density [27]. The yielded biomass from AFC can be used as animal feed or for energy and bio-product generation [28].

4.3. Electrode material

Electrode materials play a vital role in AFC because of its overall cost effectiveness and the performance in power generation. Properties such as good electrical conductivity and low resistance, strong biocompatibility, chemical stability and anti-corrosion, large surface area and appropriate mechanical strength and toughness are to be considered in the selection of an electrode material. Commonly used anode materials are graphite plates and rods, carbon fiber brushes, carbon cloth, carbon paper, carbon felt, carbon nanotubes and granulated graphite [17]. Carbon electrode is used extensively due to its low cost when compared to other electrodes. Biofilm helps in trapping the electron with the help of electrode and algal substrate. Therefore, cathode graphite felt coated with platinum, 10% Teflon coated on carbon paper, etc. are preferred to increase biofilm formation on the cathode.

http://dx.doi.org/10.5772/intechopen.74285

5. Membrane

The membrane is the heart of this system which is highly expensive. This results in the increase of the overall cost of AFC. Membranes act as a separator for the anode and cathode compartments. The substrate that is used in this system tends to produce electrons and protons which are passed through the membrane for the separation of specific ions. Though the membranes are used as a barrier, it has some issues. The motion of ions from the anode to cathode chamber slowly increases the protons in the anode chamber and the negatively charged ions in the cathode chamber. This results in low and high pH in anode and cathode.

The overall performance of AFC can improve by a membrane separator having micellar porous structure separating the specific ions from anode chamber to cathode chamber. Proton exchange membrane and electron exchange membrane are the most preferred membranes due to their superior conductivity properties. But these are unsuitable for high power scale application due to their need for hydration and high cost. Some of the studies have explored the use of alternative membranes of low cost which are: cation exchange membranes such as sulfonated polyether ether ketone, sulfonated polystyrene-ethylene-butylene-polystyrene, CMI-7000 and Hyflon ion, anion exchange membranes such as AMI-7000, salt bridges and porous materials such as J-Cloth, glass fiber filters, nylon, nonwoven cloth, earthenware pot, ceramic, terracotta, compostable bags and latex glove. The use of these inexpensive membranes occasionally causes difficulties like high internal resistance.

6. Influence of carbon dioxide

The healthy growth of algae in AFC is essential for efficient power production which is influenced by growth media, nutrient supplement and CO_2. The optimal growth of microalgae is achieved when the cathodic chamber is bubbled with CO_2 or by diverting CO_2 produced in the anodic chamber which concludes that the microalgae is able to fix CO_2 by consuming the inorganic carbon in cathodic chamber and CO_2 produced in the anionic chamber which permeates through the membrane [23, 29]. The micro-algae also prefer to use CO_2 in the presence of light and organic carbon the result of which is the production of daytime electricity depending on the organic loading rate and light irradiation. In some cases, a higher concentration of CO_2 causes adverse effect on algae during the early stages of growth. The dissolved CO_2 eventually decreases the pH of the electrolyte and this pH of the algal inoculums must be high initially to overcome. Apart from this, CO_2 concentration also affects the lipid content of microalgae. The cells produce polyunsaturated fatty acids under high CO_2 concentrations. A 6% lipid content increase was observed accompanied by a 10–15% increase in CO_2 supply [30].

7. Influence of light source

Algae and higher plants contain two major photosynthetic systems in thylakoid membrane. They are classified as photosystem I and photosystem II containing chlorophylls and carotenoid

pigments respectively for light energy absorption [31, 32]. The chlorophyll pigment adsorbs wavelength between 650 and 750 nm in the red region while carotenoids pigment absorbs wavelength between 450 and 500 nm in the blue region. This mechanism of transferring excitation energy by both chlorophyll and carotenoids results in higher efficiency of photosynthesis over a wide range of wavelengths [32]. However, the absorption of wavelength by the pigments depends entirely on the type and history of microalgae [33].

During photosynthesis, light energy absorbed by chlorophyll induces the transfer of electrons and hydrogen ions from water to an acceptor called NADP+ where they are temporarily stored. The light reactions use solar power to reduce NADP+ to NADPH by adding a pair of electrons along with a proton from which electrical power may be generated [34].

Photosynthesis rate can be increased by proper light source and light intensities leading to higher cell growth and generation of electrons. As a result, higher bioelectricities might be observed with an optimized light source installed in photo microbial fuel cells. However, only a few studies focusing on the influence of specific light supply or intensities upon power generation and cell growth of photosynthetic microorganisms have been carried out. Xing et al. [35] found that the exposure of AFC to incandescent light increased power densities by 8–10% for glucose fed reactors and 34% for acetate fed reactors when compared to the reactors operated under dark condition. But, Fu et al. [36] obtained a higher power density and open-circuit voltage when AFC was operated under dark condition by using *Spirulina platensis* as biocatalyst. Yeh et al. [37] had investigated the effect of the type and light intensity of artificial light sources on the cell growth of microalgae *Chlorella vulgaris*. They found that fluorescent light source was effective in indoor cultivation of these microalgae with an overall productivity of 0.029 g dry cell weight $L^{-1}d^{-1}$ and it was obtained by using light source having a light intensity of 9 W m^{-2}. Similarly, *S. platensis* and *H. pluvialis* cultivated under red LED light condition showed better growth profile [35, 38, 39]. On the other hand, *Nannochloropsis sp.* showed a maximum specific growth rate of 0.64, 0.51, 0.54 and 0.58 d^{-1} when exposed to blue, red, green and white light respectively [40].

8. Influence of fouling

8.1. Membrane fouling

The most important component of AFC is a membrane which acts as a physical separator and ion selective in passing protons. Moreover, it also hinders the passage of other materials and prevents the crossover of oxygen from the cathode to the anode. Microbes grow on the surface of the membrane causing membrane fouling when AFC is operated for a long term. Membrane fouling occurs when organic foulants such as extracellular polymeric substances aggregate on the surface of the membrane. The negatively charged sulfonate groups in the membrane are prone to this type of fouling especially at low pH [41]. This bond eventually contributes to the formation of a strong biofouling layer on the membrane.

8.2. Biofouling

Oxygen reduction reaction occurs on the exposed area of catalyst and its framework present in three-phase boundaries. Over potential of this is efficiently reduced by commonly used

expensive catalyst. The latest development in low cost catalyst like carbon based cathode delivers equivalent performance due to abundant pores and larger specific area. However, the main drawbacks of this porous structure are their low resistance to biological fouling. Therefore, ionic membranes and separators are used in AFC to reduce this effect on proton exchange layers.

Biofouling is caused by the bacteria attached to the surface of catalyst layer that releases extracellular polymeric substances. Biofouling on catalyst layer is similar to biofilm on membranes and separators. It is a thick layer developed on carbon based cathode that increases the diffusion resistance responsible for the declined performance during the long term. Further, it also decreases the activity of dopants on the surface of catalyst layer by the combined effect of biofilms with salt deposition. This was evident from the research of Zhang et al. [42] in which improved power density of cathode increase up to 29% was observed after removing the fouling by weak hydrochloric acid. But there are not clear and sufficient demonstrations regarding the individual effect of biofouling located on the surface of the catalyst layer and inside the layer.

9. Energy analysis

Economic success of AFC is directly related to power generation, algal biomass production in combination with other application in a fully biotic cell. Even though there is enormous progress in the research in this area, there are still difficulties in practical applications. The overall power output of the system decreases with the increase in the dimension of AFC. This is mainly due to poor mixing and deprived configuration of electrodes. Laboratory scale reactors having a capacity less than 50 mL relatively generate high power densities greater than 500 Wm^{-3} whereas configurations having larger than 2 L normally produce a power density less than 30 Wm^{-3}. The energy data of AFC are generally expressed in normalized energy recovery expressed in kilowatt hours per cubic meter based on the volume of reactor. Simple anode substrate produces more electricity than complex substrate due to easy degradation pathways. For instance, acetate produce much higher power densities than glucose (<0.03), sucrose (−< 0.01) and wastewaters (<0.01) which are complex. Similarly, average normalized energy recovery with acetate, glucose and wastewater are 0.25, 0.18 and 0.04 kWh m^{-3} respectively [43].

A good separation between the electrodes is necessary to prevent interaction between oxygen diffusion, anolyte, catholyte and other materials. This is facilitated by a solid electrolyte or an oxygen gradient. The commonly used solid electrolytes include cation exchange membrane, anion exchange membrane, proton exchange membrane and other materials like textiles, woven fabrics, eggshell, papers, glass wool, etc. [44]. These materials greatly affect energy recovery, performance and capital cost of AFC. Some of the researches show that ion exchange membranes have a lower normalized energy recovery 0.14 ± 0.40 kWh m^{-3} when compared to the membrane-less system which has 0.23 ± 0.46 kWh m^{-3} ($p < 0.05$) [43].

Stacking AFC in parallel or series configuration helps to achieve preferred voltage and current output [45]. This shows some encouraging results for the technical feasibility of operating multiple AFCs. It is proven that a stack consisting of 40 identical 20 mL units can achieve an open-circuit voltage of 13.03 V [46]. Similarly, by shuffling the parallel and serial electric connections in a stack an external power management system can extract a power of ~200 mW which can drive a 60-W DC motor [47].

The information on energy recovery helps to establish an overall energy balance. The improvement of energy recovery through optimizing configuration, operation, microbiology and materials will make AFCs more attractive. On the other hand, adopting proper strategies to reduce the energy requirement of operation may compensate for low energy recovery. Incorporating other energy producing processes such as biogas production, algal biomass harvesting, biohydrogen etc., will increase the energy independency. Further, modifying the process for desalination, nutrient recovery and production of valuable chemicals will also maximize the benefits of AFC.

10. Application and adaptability

AFC has attractive properties that ensure further development and applications of this technology. It can be easily combined with green roofs to create electricity where photosynthetic and electrochemical reactions are carried out by a continuously growing population of microorganisms in living solar cells. This makes the system capable of self-repair, giving long lifetime and low maintenance. Moreover, using these reproducing organisms living in solar cells does not require any special catalysts that in solar cells are costly and toxic. Therefore, it can be used in natural surroundings with no risk of pollution. AFC also has organic material as intermediate energy carriers between the photosynthetic and the electrochemical portions of the cell which help them in generating electricity at night [48]. Closed loop AFC systems can preserve nutrients for the organisms which enable long-term, low-maintenance power production. Integrated AFC will add value to other applications such as food, agriculture, biomass for bio-energy production etc. [49, 50]. Similarly, it can be coupled with wastewater and surface water treatment to supply extra organic matter for energy production and in turns providing treated water [51].

11. Challenges

Algae fuel cells are not without limitations. They need high cost infrastructure and energy for harvesting and growth. Another problem associated with microbial fuel cells is the pH membrane gradient which reduces cell voltage and power output. This problem is caused by acid production at the anode, alkaline production at the cathode and the nonspecific proton exchange through the membrane. The high cost of membrane commonly used in laboratories as a proton-permeable membrane would also limit the applications [52]. In addition, the slow rate of oxygen reduction at cathode electrodes is also a major limiting factor for power generation.

Need for improved engineering on downstream algae biofuel processing from AFC for sustainable energy production is another challenge. It includes effective strategies for nutrient circulation and light exposure in designing photo-bioreactors that are reasonably cheap for large-scale deployment in low-cost systems. The secondary challenge related to this is the extraction of crude algae oil which is mostly addressed from the engineering side. The extraction technologies which are successfully demonstrated are relatively expensive. On the other hand, challenges associated with the management of algae bio-oil conversion to usable liquid fuels need improved catalysts similar to petroleum crude.

12. Conclusion

AFC is a developing technology with a huge potential to capture solar energy and convert it to electricity. Similarly, the regenerated biomass during the process can be converted into secondary biofuels like solid biomass, bioethanol, biogas, etc. which is an added advantage. This technology also remediates wastewater, removes heavy metals, dye decolorizes, etc. Even though various studies have focused on increasing the performance parameters, physical and catalytic parameter variations, improvement of power generation, cost effective electrode materials, selection of bioactive organisms and finding out an alternative membrane to give cost effective solution need to be addressed. In near future, algae will become a sustainable technology and development in this research area. The possibility of using bioengineering, molecular biology, biotechnology and electrical engineering together to improve the efficiency of AFC is not a farfetched idea. Some studies like life cycle analysis based on commercial-scale, increasing power density, optimization technological methods on AFC configuration need special attention and investigation.

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Chapter 6

Cyanobacteria and Microalgae in the Production of Valuable Bioactive Compounds

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.74043

Abstract

In the last decades, an increasing attention has been directed toward the possibilities of growing algae commercially. This interest has been partially due to the fact that some strains of microalgae and cyanobacteria have demonstrated the ability to produce a variety of bioactive products. Both, primary and secondary metabolism of these microorganisms has been demonstrated to play a key role in the production of special chemicals. Antioxidants, for instance, can be produced by some algal strains to protect photosynthetic cells from oxidative stress. Microalgae can produce a variety of polyunsaturated and monounsaturated fatty acids with clear health benefits for human nutrition. Potential products obtained from cyanobacteria and microalgae exhibiting interesting medical properties include polysaccharides, glycerol, glycoproteins, and antibiotics. From the aforementioned products, especially relevant has become the search of new antibiotics. The potential spread of bacterial resistance and the foreseen decrease on efficiency on antibiotics, has largely stimulated the research on novel antibiotics sources. Among these sources, cyanobacteria and microalgae have demonstrated a vast and just barely explored potential.

Keywords: bioactive products, pharmaceuticals, primary and secondary metabolism, microalgae, cyanobacteria, antibiotics

1. Introduction

Cyanobacteria (prokaryotic green-blue algae) and microalgae (eukaryotic microalgae) are regularly found in water bodies, desert crusts, or even in symbiosis with other animals. They can live in large varieties of environmental conditions, including low or high temperatures, high-light intensities, pH and salinity [1]. In the last decades, increasing attention has been paid to

the potential of growing these kinds of organisms with commercial purposes. Part of the added value of this type of biomass is based on the fact that it can be used in human and animal nutrition (i.e. fish feed in aquaculture facilities). Moreover, some extracts from microalgae can be used to produce cosmetics and a variety of different bioactive products, such as pharmaceutical compounds [2–4]. The diversity of cyanobacteria and microalgae is immense, with species, genera, or even classes being discovered every year. On the estimated millions of exiting species, about 30,000 have been described; but nowadays, not more than a dozen is regularly cultivated and exploited in large scale for commercial biotechnological purposes. On top of that, research on how the culture conditions affect the production of important bioactive substances remains nowadays very scarce. Some authors, such as Spoehr and Milner [5], proved that manipulating microalgae or cyanobacteria growth conditions, for instance, by applying different forms of stress to the cells, could promote the production of biomass with valuable secondary metabolites, some of which presents pharmaceutical and/or industrial values. In most of the cases, the production of valuable metabolic products by cyanobacteria and microalgae is a two-step process. In the first step, the microorganisms are grown under optimal conditions to maximize the production of biomass. This process is followed by a second step where stress factors, such as high light intensity or nutrients deprivation, are applied to the culture to induce the production of valuable secondary metabolites with the pursued pharmaceutical [6, 7] or antioxidant properties. In this chapter, the production of a variety of bioactive compounds by cyanobacteria and microalgae has been reviewed.

2. Valuable bioactive products from cyanobacteria and microalgae

Variations in temperature, light, pH, salinity and nutrient availability have been extensively investigated to study their impact on microalgae growth and their primary and secondary metabolic products. Primary metabolites are those directly involved in normal growth development, reproduction, cell division, or metabolism. They include for instance the production of lipid, such as polyunsaturated fatty acids (PUFA) [8–11], antioxidants such as carotenoids, and some types of proteins (**Figure 1**). Secondary metabolites are those compounds that are not used by organisms for their primary needs and include compounds that act as hormones, antibiotics, or toxins, among others [12]. The production of secondary metabolites appears to be specie and strain specific [13], and is possibly associated to the exposure of the microorganism to specific environmental conditions [6, 14] caused, for instance, by stress factors. In a study carried out by Lustigaman in 1988, the production of antibiotic activity by *Dunaliella* spp. was investigated. The study was based on isolating extracts of these microalgae from two different environmental scenarios; one clean and one polluted water system. The study demonstrated that nonproteinous substances inhibiting the activity of the bacteria *Escherichia coli* were only produced by the microalgae *Dunaliella* spp. under exposure to the polluted water. It was, therefore, suggested that microalgae growing in adverse conditions are more likely to produce secondary metabolites with antibacterial activity [15].

Nowadays, the major products obtained from microalgae with industrial use are carotenoids and algal biomass, which are mainly used for human and animal feed and for aquaculture.

http://dx.doi.org/10.5772/intechopen.74043

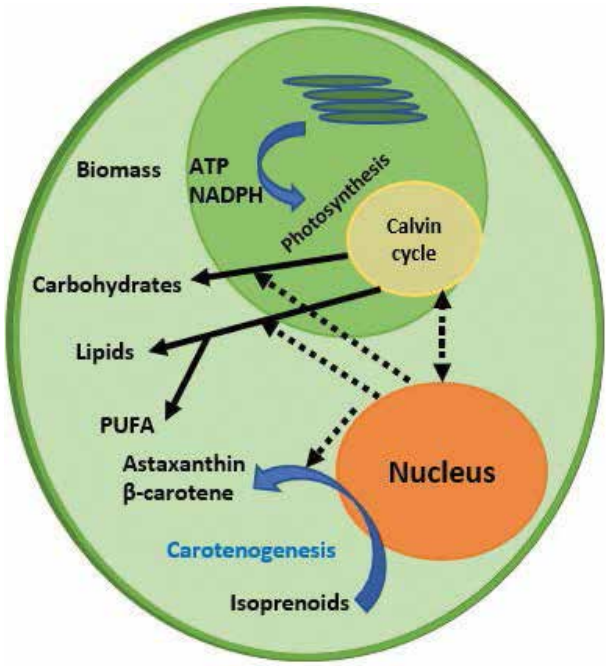


Figure 1. Example of some primary metabolic routes and their products in microalgae. Adapted from Rosenberg et al. [16].

Microalgae can also produce other antioxidants, such as vitamins C and E, and even butylated hydroxytoluene (BHT). Fatty acids are also produced as primary metabolic products, playing an important role protecting the cells against oxidative stress. Other metabolic products obtained from microalgae and exhibiting medical properties are special polysaccharides, glycerol and myscosporine-like amino acids (MAA). In addition to the aforementioned compound families, glycoproteins, antifreeze proteins and antibiotics can also be produced by these microorganisms. Some of these substances have demonstrated a set of interesting bioactivities [11]. An overview of the potential bioactive metabolites is presented in **Figure 2**.

2.1. Antioxidants

Eukaryotic microalgae and cyanobacteria are often exposed to high oxygen levels and high irradiance conditions. As a response to this potential oxidative stress, these organisms have developed defense systems based on the production of different antioxidants. The main goal of these substances is to preserve cells from oxidative stress, which may otherwise cause damage to essential biological structures, such as DNA, proteins and lipids. Oxidative stress in humans and animals can also lead to severe health problems, such as atherogenesis, cancer, neurodegenerative diseases, infant retinopathy, muscular degeneration and renal failure, along with other problems [17–19]. Dietary intake of antioxidants from these organisms has shown the ability to limit or prevent certain health issues. For instance, many substances found in algae,

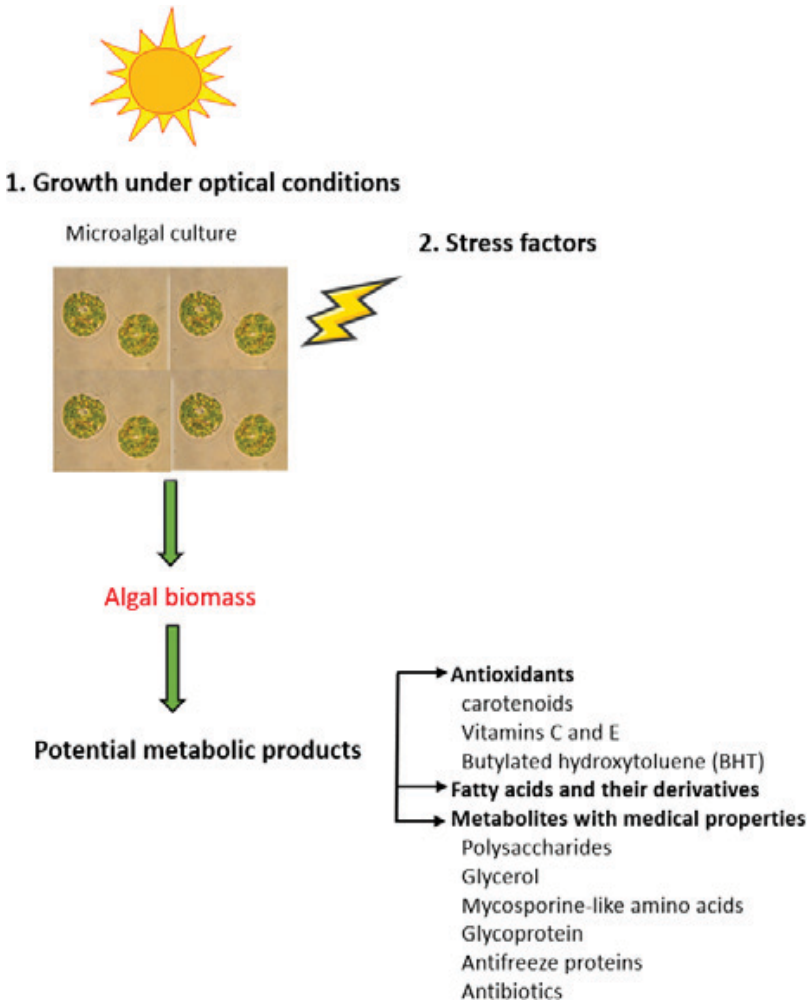


Figure 2. Overview of the potential bioactive metabolites produced by microalgae and cyanobacteria. Adapted from Skjånes et al. [11].

such as carotenoids, vitamins C and E or butylated hydroxytoluene (BHT) have such antioxidant effects [11]. Carotenoids have been largely used as supplement in human nutrition as well as in food and animal feed, poultry and fish. Vitamin C may be found in tablets for human consumption as well as in meat, where it has been largely used to prevent oxidation processes and the discoloration of the product during storage. Vitamin E can be commonly found in supplements for human health and has been largely used in food industry. Another largely used antioxidant is astaxanthine. This substance has become very popular recently as supplement for human nutrition and is commonly found in, i.e., salmon food formulations to intensify the pigmentation of the fish growth in aquaculture facilities. **Table 1** gathers some examples of microalgae and the type of antioxidant substance that they produce.

Specie of microalgae	Type of antioxidant substance	Ref.
Botryococcus braunii	β-Carotene BHT	[20, 21]
Chlamydocapsa nivalis	Phenolic antioxidants	[22]
Chlorella pyrenoidosa	Vitamins E	[23]
Chlorella spp.	Vitamin C	[24]
Chlorella vulgaris	Vitamins C and E Lutein (carotenoid)	[20] [25]
Scenedesmus obliquus	Vitamins C and E	[20]
Scenedesmus quadricauda	Vitamins C and E	[23]
Chlamydocapsa spp.	Lutein, canthaxanthin and astaxanthin (carotenoids)	[26]

Table 1. Antioxidant substances produced by microalgae.

2.2. Fatty acids and their derivatives

Fatty acids are essential components of the diet. They can occur in the cells as glycolipids and phospholipids forming the cellular membranes, or as storage products for energy and carbon in the form of triglycerides [27]. In some cases, triglycerides may also have a role protecting against oxidative stress, and the lack of these nutrients can cause severe damage to the organism. Fatty acids can be produced by eukaryotic microalgae and cyanobacteria, and in some cases they can produce them in large amounts [28]. The truly essential fatty acids are omega-3 fatty acids, such as linoleic acid and α-linoleic acid. Both humans and animals are dependent on obtaining them from the diet, because they are used as starting points for building longer chains of fatty acids. Food supplements of omega-3 are known to have beneficial health effects in the prevention of coronary heart disease, hypertension, type 2 diabetes, renal disease and chronic obstructive pulmonary disease, among others [29]. A summary including the production of fatty acids produced by microalgae is presented in **Table 2**. Some of the industrial applications of fatty acids include cosmetic formulations, food, personal care, and pharmaceutical products.

2.3. Polysaccharides

Certain polysaccharides from microalgae have been shown to have remarkable biomedical properties. Several studies have demonstrated that microalgae, such as *Chlorella vulgaris* and *Scenedesmus quadricauda* are able to presumably produce sulfated polysaccharides that function as protection against microcystin oxidative stress [35]. Crude polysaccharide extracts obtained from *Chlorella stigmatophora* and *Phaeodactylum tricornutum* showed anti-inflammatory activity in the carrageenan-induced paw edema test [36]. Moreover, other crude polysaccharide extracts from *Chlorella pyrenoidosa* presented antitumoral activity against A549 (cell human lung carcinoma) *in vitro* [37]. Furthermore, polysaccharides can also present other

Specie of microalgae	Type of fatty acid	Ref.
Ankistrodesmus sp.	α-linolenic acid	[30]
Botryococcus braunii	Linoleic acid	[31]
Botryococcus spp.	α-linolenic acid	[31]
Chamydomonas spp.	α-linolenic acid	[32]
Chlorella minutissima	Eicosapentaenoic acid	[33]
Scenedesmus obliquus	α-linoleic acid Linoleic acid	[23]
Scenedesmus quadricauda	α-linoleic acid	[34]

Table 2. Fatty acids produced by microalgae.

health-promoting effects on, for instance, gastric ulcers, wounds and constipations [38, 39]. However, their exact function in the algae cells remains still unknown.

2.4. Glycerol

Glycerol can function as osmoregulator and osmoprotector of enzymes. This substance has been accumulated in substantial amounts in halotolerant species during salt stress conditions. The production of glycerol in algae is regulated by external water activity, but high light intensities may inhibit its production [40]. In some cases, the algae can also excrete glycerol as a response to high concentrations of CO_2 rather than salt stress condition [41]. Glycerol is widely used in cosmetics, pharmaceuticals, paint, food, tobacco, pulp and paper, or in the production of a large variety of chemicals [42]. Some examples of microalgae producing glycerol are *Brachiomonas submarina* [43], *Chlamydomonas* spp. [41] and *Dumaliella salina* [44]. Glycerol can be found in a large variety of commercial products and applications, such as cosmetics and food products, drugs and pharmaceuticals.

2.5. Lectins

Lectins are carbohydrate-binding proteins that are located within protein bodies in the cell. Lectins from algae have high specificity for complex oligosaccharides, glycoproteins, or glycolipids. They are useful in medical science, for instance, for the detection of disease-related alterations of glycan synthesis, and for cell markers for diagnosis purposes including infectious agents, i.e., viruses, bacteria, fungi and/or parasites. Different strains of *Chlorella*, such as *Chlorella minutissima* [45], *Chlorella pyrenoidosa* [46, 47] and *Chlorella* spp. [45] produce metabolites with antimicrobial activity and this activity has been preliminary hypothesized to be due to lectins [47]. Studies conducted with other algae strains, such as *Desmococcus olivaceus* [45], *Scenedesmus quadricauda* [46] and *Scenedesmus* sp. [45, 48], have reported that the production of

these lectins can be induced by growth-limiting conditions like nutrient deprivation and/or light stress conditions [49].

Some companies, for instance, Lectin Labs Ltd., have developed lectin formulations, and claim that these lectins interfere or destroy the development of the disease-causing processes, even in cases where antibiotics are ineffective.

2.6. Mycosporine-like amino acids

Mycosporine-like amino acids (MAA) are a group of molecules consisting of an amino acid bound to a chromophore molecule that absorbs light. These amino acids are involved in protecting the organism against UV radiation and are produced in significant amounts by, for example, the high UV-tolerant snow algae *Chlamydomonas nivalis* and other green algae species. The production of MAA is induced by exposing the microalgae to UV-light and the resulting irradiance stress reactions. Nevertheless, there are indicators pointing out that a decrease in nitrogen levels leads to a decrease in the production of MAA [50, 51]. MAAs from algae have been explored for commercial purposes which have resulted, for instance, in commercial skin-care products for UV protection [52]. Some examples of microalgae that produce MAA are *Ankistrodesmus spiralis*, *Chlorella minutissima*, *Scenedesmus* sp. and *Scotiella nivalis* [51].

2.7. Glycoproteins

Glycoproteins are relevant biological structures formed by a protein covalently linked to one or more carbohydrate units. These structures have a large set of biological functionalities and some microalgae have demonstrated to be a potential source of them. For instance, a glycoprotein obtained from *Chlorella vulgaris* was found to exhibit anticancer activity through antimetastatic immunopotentiation [53, 54]. Other microalgae presenting anticancer activity are *Desmococcus olivaceus* [45], *Scenedesmus* sp. [45, 48], *Dunaliella bardawil* [55] and *Dunaliella salina* [44], among others. However, little has been done to identify similar compounds with activity from other algal species, nor to consider possibilities for optimization of the production of these glycoproteins by manipulating growth conditions [11].

2.8. Antifreeze proteins

Cold adapted strains of green algae, such as those living in polar environments, are often producers of antifreeze proteins (AFPs), also designated as ice structuring proteins (ISPs). These proteins are key elements for the survival of some organisms, since they prevent damages occurring as a result of very low temperatures. They exhibit unique properties because they are able to bind to ice crystals, prevent recrystallization and protect other proteins from damage. AFPs extracted from algae or other microorganisms can be used for cryopreservation, frozen food preservation, transgenic crops and even weather modification [56–58]. There are some microalgae such as *Chlorella pyrenoidosa* that can produce AFPs that additionally exhibit

antifungal properties [46, 47]. AFPs are currently being explored in some formulations to reduce cold-induced damage in medical, food and cosmetic products with the target of lengthening shelf life of the frozen gods. The extraordinary properties of AFPs allow hypothesizing a growing number of businesses including AFPs in their future formulation of products.

2.9. Antibiotic activity

Some strains of microalgae can produce metabolites with antibiotic activity aimed at killing or inhibiting bacterial growth. In some cases, this activity has only been identified in general extracts from the algal culture, without properly determining the chemical identity of the active compound/s [45, 47]. There are indications that antibiotics are more likely to occur in strains isolated from environments polluted by bacteria than in strains isolated from cleaner environments [59]. For instance, the methanolic extracts of *Tetraspora cylindrica* present antibacterial activity against *Corynebacterium diphtheria*, *Klebsiella pneumoniae* and *Shigella boydii*, among others. These extracts also present antifungal activity against: *Curvularia lunata*, *Fusarium sporotrichoids*, *Macrophomina phaseolina*, *Rhizoctonis solani*, *Sclerotium rolfsii* and *Trichoderma harzianum* [60].

In the last decade, the screening and bioprospecting of microalgae and cyanobacteria for antibiotics and pharmacologically active compounds has received a lot of attention. This is because a large number of antibiotic compounds, many of them with unusual and novel structures, have been isolated and characterized from extracts of microalgae [15]. Similarly, many cyanobacteria have been shown to produce antiviral and antineoplastic compounds. A range of pharmacological activities have also been observed in some extracts of microalgae which active principles, in most of the cases, are still unknown. Several of these bioactive compounds found in microalgae extracts may find application in human or veterinary medicine and agriculture. Others could be used, for instance, as research tools or as structural models for the development of new drugs [15]. Microalgae are particularly attractive as natural sources of bioactive molecules because they have the potential to produce these compounds in culture. This enables the production of structurally complex molecules which are difficult or impossible to produce by chemical synthesis [61].

Many of the antibiotics and pharmaceuticals in current use have their origins in nature and are the product of systematic screening of terrestrial organisms, such as higher plants and soil microbes. For instance, of approximately 13500 known naturally occurring antibiotics, 5500 are produced by actinomycetes, while approximately 3300 are produced by higher plants and, of these, about 90 are in current medical use [62]. Much of the work concerned with the isolation, screening, and physiology of antibiotic-producing microorganisms has been focused on heterotrophs. However, very little attention has been paid to other groups, such as microalgae which are able to grow under diverse nutritional conditions: photo-autotrophically or chemoheterotrophically [63].

Moreover, many marine algae produce antibiotics substances that are capable of inhibiting bacteria, viruses, fungi and other epibionts. It also appears that the antibiotic characteristic is dependent on many factors, i.e., the algae strain, the microorganisms, the season and the growth condition [64–67]. Several extractable compounds, for instance, cyclic polysulfides and halogenated compounds, are toxic to microorganisms and, therefore, responsible for the antibiotic activity of some marine algae [68–70].

2.9.1. Antibacterial activity of cyanobacteria

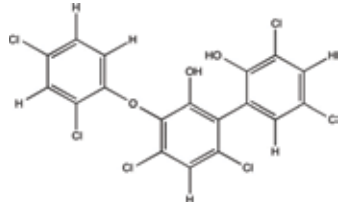
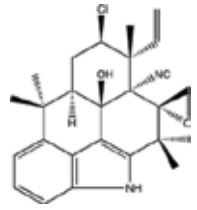
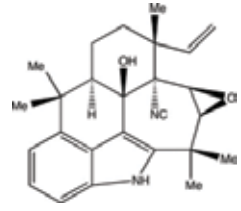
Cyanobacteria are phototrophic organisms with a classic prokaryotic cell organization, but similar to eukaryotes they conduct photosynthesis and respiration in their active membrane system [71]. Microalgae grow ubiquitously and produce, in addition to toxins, a wide range of bioactive metabolites with potential application in biotechnology [72]. These characteristics have made them the focus of intense examination in the last decade [73, 74].

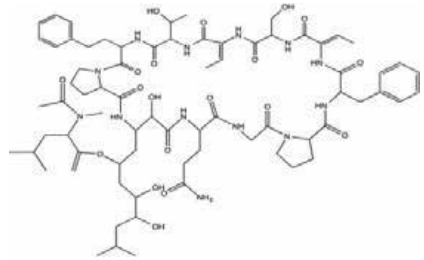
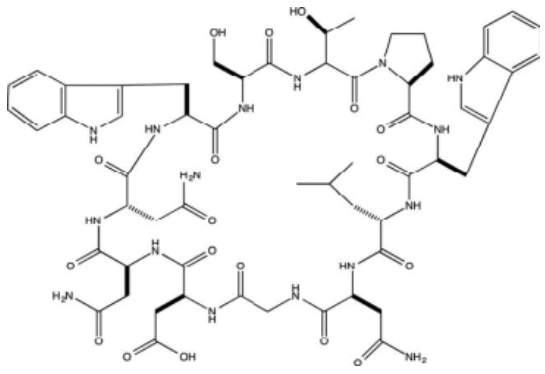
To date, only a few compounds have been extracted and commercialized, including nutraceuticals, cosmetic products and other high-value molecules [39, 75]. Some purified compounds have promising commercial applications as bioplastics, biofertilizers, antiviral, antifungal, anticancer and antibacterial drugs [76–78]. **Table 3** illustrates some examples of antibacterial, antifungal and antimycobacterial compounds extracted from cyanobacteria.

2.9.2. Antibacterial activity of microalgae

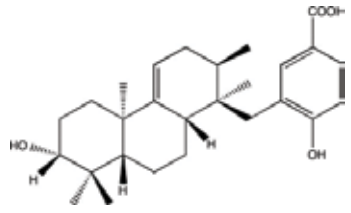
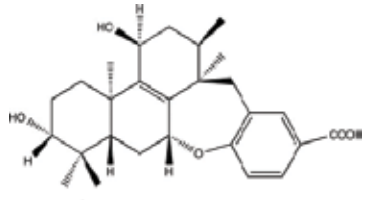
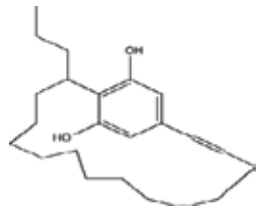
The production of bioactive compounds from cyanobacteria has received more attention than from eukaryotic microalgae. The reason may be probably based on the simpler culture methods available for cyanobacteria growth, and also to their greater resistance to bacterial contamination [89]. Nevertheless, more and more studies have recently focused on the synthesis of bioactive compounds, such as isoprenoids, polyketides, no ribosomal peptides, polyunsaturated fatty acids and alkaloids, by eukaryotic microalgae [90], used to inhibit bacterial activity [6, 91, 92]. In addition, further studies have identified fatty acids, terpenes, carbohydrates, glycolipids, lipoproteins, bromophenols and tannins, among other, as compounds that exhibit antibacterial activity against human pathogens [93, 94].

Microalgae accumulate cell-associated antibacterial substances [95, 96], and some studies have shown different levels of antibacterial activity in different microalgae cultures [95, 97–99]. Moreover, crude extracts from different species of eukaryotic microalgae have shown effectiveness against both Gram positive (Gram+) and Gram negative (Gram−) bacteria, as well as *Mycobacterium tuberculosis* [100–104]. This could suggest, therefore, the potential of microalgae for the production of compounds with a broad-spectrum activity, which is highly desired for the production of new antibiotics. However, many compounds extracted from these organisms are likely to be impractical as antibiotics for medical uses as a result of, for instance, its toxicity or inactivity *in vivo* [61]. **Table 4** presents a summary of the eukaryotic microalgae with the highest antibacterial activity or the widest spectrum of activity of large screening programs to date.

Compound	Species	Chemical structure	Molecular formula	Activity [79]
Ambigol A [80]	*Fischerella ambigua* [80]	 [80]	$C_{18}H_8Cl_6O_3$	Antibacterial Antifungal
Fischambiguine B [81]	*Fischerella ambigua* [81]	 [81]	$C_{26}H_{29}ClN_2O_2$	Antimycobacterial
Ambiguine I isonitrile [82, 83]	*Fischerella* sp. and *ambigua* [82, 83]	 [82, 83]	$C_{26}H_{30}N_2O_2$	Antibacterial Antimycobacterial
Pahayokolide A [84]	*Lyngbya* sp. [84]		$C_{72}H_{1\ 05}N_{13}O_{20}$	Antibacterial

Compound	Species	Chemical structure	Molecular formula	Activity [79]
		 [84]		
Kawaguchi peptin B [85]	*Microcystis aeruginosa* [85]	 [85]	$C_{58}H_{76}N_{16}O_{18}$	Antibacterial
Noscomin [86]	*Nostoc commune* [86]		$C_{27}H_{38}O_4$	Antibacterial Antifungal

http://dx.doi.org/10.5772/intechopen.74043

Compound	Species	Chemical structure	Molecular formula	Activity [79]
		 [86]		
Diterpenoid [87]	*Nostoc commune* [87]	 [87]	$C_{22}H_7O_5$	Antibacterial
Nostocycline A [88]	*Nostoc* sp. [88]	 [88]	$C_{23}H_{34}O_2$	Antibacterial

Adapted from Senhorinho et al. [15].

Table 3. Antibacterial compounds extracted from microalgae.

Microalgae specie	Antibacterial compound/Fraction	Gram+ inhibition	Gram− inhibition	Ref.
Green algae				
Chlamydomonas reinhardtii	Aqueous or methanolic and hexanolic extracts	*Bacillus subtilis* *Staphylococcus aureus* *Staphylococcus epidermidis*	*Escherichia coli* *Pseudomonas aeruginosa* *Salmonella typhi*	[106]
Chlorella minutissima	Ethanolic extracts	*S. aureus*	*E. coli* *P. aeruginosa*	[45]
Chlorella pyrenoidosa	Various organic solvent extracts: ethanol, acetone, diethyl ether, and methanol	*B. subtilis* *S. aureus*	*E. coli* *P. aeruginosa*	[46]
Chlorella vulgaris	Chlorellin	*B. subtilis* *S. aureus* *Streptococcus pyogenes*	*E. coli* *P. aeruginosa*	[97]
Chlorella vulgaris	Aqueous or methanolic and hexanolic extracts	*B. subtilis* *S. aureus* *S. epidermidis*	*E. coli* *P. aeruginosa* *S. typhi*	[106]
Chlorococcum HS-101	α-linolenic acid	*B. subtilis* *Bacillus cereus* *S. aureus* *MRSA*	*Enterobacter aerogenes*	[107–109]
Chlorococcum humicola	Various organic solvent extracts: acetone, benzene, chloroform, diethyl ether, ethyl acetate, ethanol, hexane, and methanol Purified pigments: carotenoid and chlorophyll	*B. subtilis* *S. aureus*	*E. coli* *P. aeruginosa* *Salmonella typhimurium* *Klebsiella pnemoniae* *Vibrio cholerae*	[110]
Desmococcus olivaceus	Ethanolic extracts	*S. aureus*	*E. coli* *P. aeruginosa*	[45]
Dunaliella primolecta	Polyunsaturated fatty acids: α-linolenic acid	*B. cereus* *B. subtilis* *S. aureus* *MRSA*	*E. aerogenes*	[107, 109]
Dunaliella salina	Indolic derivative Polyunsaturated fatty acids β-ionone and neophytadiene	*S. aureus*	*E. coli* *P. aeruginosa*	[111–113]
Dunaliella sp.	Lysed cells	*S. epidermidis* *Micrococcus luteus*	*Proteus vulgaris*	[59]
Haemotococcus pluvialis	Short-chain fatty acids	*S. aureus*	*E. coli*	[114, 115]
Klebsormidium sp.	Pellet	*B. Subtilis*	No effect	[116]
Pseudokirchneriella subcapitata	Methanolic extracts	*S. aureus*	*P. aeruginosa*	[111]
Scenedesmus obliquus	Long-chain fatty acid	*S. aureus*	*E. coli* *P. aeruginosa* *Salmonella* sp.	[117]
Scenedesmus quadricauda	Various organic solvent extracts: ethanol, acetone, diethyl ether, and methanol	*B. subtilis* *S. aureus*	*E. coli* *P. aeruginosa*	[46]

Microalgae specie	Antibacterial compound/Fraction	Gram+ inhibition	Gram− inhibition	Ref.
Scenedesmus sp.	Ethanolic extracts	*S. aureus*	*E. coli* *P. aeruginosa*	[45]
Red algae				
Porphyridium aerugineum	Phycobiliproteins	*S. aureus* *S. pyogenes*	Not tested	[118]
Porphyridium sordidum	Pellet	*B. subtilis*	*E. coli* *Pseudomonas fluorescens*	[116]
Porphyridium purpureum	Methanolic extracts	*B. subtilis*	*E. coli* *Pseudomonas fluorescens*	[116]
Rhodella reticulate	Exopolysaccharides	*S. aureus* *B. cereus* *S. pyogenes*	No effect	[118]
Diatoms				
Asterionella glacialis	Whole cell	*S. aureus* *S. epidermidis* *M. luteus* *Sarcina* sp.	*E. coli*	[119]
Attheya longicornis	Methanolic extracts	*S. aureus* *MRSA*	No effect	[120]
Chaetoceros mulleri	Unsaturated fatty acid-containing lepidic fractions (triglycerides and docosa-pentaenoic acid (DPA))	*B. subtilis* *S. aureus*	*E. coli*	[121, 122]
Navicula delognei	Transphytol ester Hexadecatetraenoic and octadecatetraenoic acids	*S. aureus* *S. epidermidis*	*S. typhimurium* *P. vulgaris*	[123]
Phaeodactylum tricornutum	Eicosapentaenoic acid [124] Palmitoleic and hexadecatrienoic acids (HTA)	*B. cereus* *Bacillus Weihenstephanensis* *S. aureus* *S. epidermidis* *MRSA*	No effect	[125]
Rhizosolenia alata	Various organic solvent extracts: acetone, chloroform, chloroform: methanol (1:1), methanol: distilled water (4:1) and distilled water.	*B. subtilis*, *S. aureus*	*E. coli* *P. aeruginosa* *P. vulgaris* *S. typhi* *V. cholerae*	[126]
Skeletonema costatum	Aqueous and organic extracts: chloroform: methanol (2:1).	*B. subtilis* *S. aureus*	*P. aeruginosa*	[95]
Hapotophytes				
Isochrysis galbana	Chlorophyll a derivative: Pheophytin a and chlorophyllide a	*S. aureus* *Streptococcus faecalis* *S. pyogenes* *Micrococcus* sp.	Not tested	[127, 128]

Adapted from Falaise et al. [105].

Table 4. Antibacterial activity observed in different extracts from microalgae against human pathogens.

http://dx.doi.org/10.5772/intechopen.74043

3. Conclusions

Cyanobacteria and microalgae have demonstrated a large potential as innovative sources of a large variety of bioactive compounds, such as fatty acids, antioxidants, antifreeze proteins and even antibiotics. While the characterization of substances as fatty acids is relatively well-established and straightforward, an information gap still remains in the elucidation of structures of antibiotics. Despite the fact that a variety of extracts obtained from microalgae biomass have demonstrated a clear antibiotic capacity, the structure of the molecules involved in the observed activity still remains unclear. There is a clear and almost unrevealed potential in the development of innovative nutraceutical and pharmaceutical industries based on cultivation of microalgae and cyanobacteria and their exploitation in the production of bioactive substances. Cyanobacteria and microalgae adapted to extreme environments for sure have an enormous potential that thorough bioprospecting approaches can help to unveil.

Acknowledgements

This research was financially supported through NordForsk NCoE Programme "NordAqua" (Project # 82845).

Conflict of Interest

The authors certify that they have no conflict of interest.

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Chapter 7

Spirulina Phycobiliproteins as Food Components and Complements

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.73791

Abstract

Spirulina has a documented history of use as a food for more than 1000 years, and has been in production as a dietary supplement for 40 years. Among many of *Spirulina* bioactive components, blue protein C-phycocyanin and its linear tetrapyrrole chromophore phycocyanobilin occupy a special place due to broad possibilities for application in various areas of food technology. The subject of this chapter is up-to-date food applications of these *Spirulina* components, with a focus on their use as food colorants, additives, nutriceuticals, and dietary supplements. Their other actual and future food application possibilities will also be briefly presented and discussed.

Keywords: *Spirulina*, C-phycocyanin, phycocyanobilin, food components, food complements

1. Introduction: *Spirulina* as a superfood

The blue-green microalgae *Spirulina* has been used in human nutrition for centuries. It is still used as food in some places, such as the Lake Chad area, where it is sold as dried bread called "dihe" [1]. For human consumption, the commercial production of *Spirulina* dates back to the 1970s. *Spirulina* used in human nutrition is the dried commercial biomass of two cyanobacteria species traditionally called *Spirulina platensis* and *Spirulina maxima*, which belong to the genus *Arthrospira* spp. Taxonomically, these organisms are classified in kingdom Bacteria; phylum Cyanobacteria; order Oscillatoriales; family Phormidiaceae [2].

Spirulina is filamentous, helical, photosynthetic cyanobacteria naturally inhabiting alkaline brackish and saline waters in tropical and subtropical regions. Biochemical analysis has revealed its exceptional nutritive properties, so it is referred in the literature as "super food" or "food of the future" [2]. *Spirulina* is one of the richest natural sources of proteins and essential amino acids, as well as an excellent source of vitamins (primarily A, K, and vitamin B complex), macro- and micro-elements (calcium, potassium, magnesium, iron, iodine, selenium, chromium, zinc, and manganese), essential fatty acids, including γ-linoleic acid (GLA), glycolipids, lipopolysaccharides, and sulfolipids [3]. *Spirulina* is especially rich in a variety of pigments, such as chlorophylls, β-carotene, xanthophylls, and phycobilins (phycobiliproteins) (**Table 1**).

A huge number of *in vitro* and *in vivo* studies, published in the last few decades, have revealed potentially beneficial effects of *Spirulina* on human health. Health benefits mainly arise from the antioxidant effect of algae as a whole, or from its individual ingredients, such as phycobiliproteins (Section 2). Moreover, the presence of significant amounts of GLA, sulfated polysaccharide (calcium spirulin), and sulfolipids additionally contribute to health-promoting activities of *Spirulina* [3].

Several dried biomass products of *Spirulina* have categorized as "generally recognized as safe" (GRAS) by the Food and Drug Administration (FDA) of USA. A recommended dosage for adults is usually in the range of 3–10 g of *Spirulina* per day, while maximum daily intake should not exceed 30 g [3]. Extensive safety studies of *Spirulina* did not show the presence of cyanobacterial toxins [1]. *Spirulina* production requires the use of high quality nutrients and accurate determination of heavy metals in the culture medium, as well as in the biomass. Heavy metal analysis of commercial *Spirulina* products did not found to exceed the regulatory levels [2]. Nevertheless, it should be paid much attention during *Spirulina* cultivation to prevent contamination with heavy metals or the other cyanobacteria, capable to produce toxins.

Substance	Quantity/activity per serving (3 g*)	% DV**	Substance	Quantity/activity per serving (3 g*)	% DV**
Total carbohydrates	<1 g	<1	Chromium	50 μg	41
Proteins	2 g	4	Sodium	35 mg	<2
Vitamin A (as β-carotene)	11,250 IU	230	Potassium	60 mg	2
Vitamin K	75 μg	94	C-phycocyanin	240 mg	–
Vitamin B12	9 μg	150	GLA	32 mg	–
Iron	7 mg	39	Chlorophyla a	30 mg	–
Magnesium	15 mg	4	Total carotenoids	15 mg	–
Manganese	0.4 mg	20	Superoxid dismutase	2500 U	–

*Recommended daily value of *Spirulina* powder.

**Percent daily values (DV) are based on a 2000 calories diet.

Table 1. Nutritional profile of commercial Spirulina powder (Nutrex, Hawaii, USA).

http://dx.doi.org/10.5772/intechopen.73791

2. Phycobiliproteins

Phycobiliproteins are photosynthetic antenna pigments in the cyanobacteria, red and cryptophyte algae, that efficiently harvest light energy, which is subsequently transferred to chlorophylls during photosynthesis. Therefore, phycobiliproteins significantly contribute to the global photosynthesis. Phycobiliproteins are deeply colored, highly fluorescent, and water-soluble proteins with high propensity to form oligomers (hexamers) that constitute the building blocks of the extra-membranous antenna complex, phycobilisomes. Its intensive color arises from covalently attached linear tetrapyrrole chromophores (phycobilins) *via* thioether bonds to the cysteine residues [4].

Phycobilins are produced by heme metabolism. Heme is synthesized from protoheme IX by ferrochelatase. Then, heme oxygenase cleaves heme and biliverdin IXα is obtained. Biliverdin IXα is reduced by ferredoxin-dependent bilin reductases to obtain phycobilins. Final step in phycobiliproteins biosynthesis is the covalent attachment of bilin chromophores to the apoproteins, catalyzed by phycobiliprotein lyases. Slow spontaneous *in vitro* attachment of tetrapyrrole chromophores to the apoproteins has low fidelity and mixture of oxidation products is obtained [5].

Spirulina produces two phycobiliproteins: C-phycocyanin (C-PC) as the major pigment and allophycocyanin (APC), which is present in much smaller quantities, approximately at an 10:1 ratio [3]. C-phycocyanin level varies based on growing conditions, and may constitute up to 20% of the dry weight of *Spirulina* [6]. C-phycocyanin and APC are homologous proteins and both bind phycocyanobilin (PCB) chromophore [7, 8]. The presence of the third phycobiliprotein, red phycoerythrin, in *Arthrospira platensis* is the subject of debate. While some studies have found that *Spirulina* produces small amounts of phycoerythrin, the other ones did not detect phycoerythrin in *Spirulina* [9].

2.1. Structure and physicochemical properties of C-phycocyanin and phycocyanobilin

C-phycocyanin (CAS registry number 11016-15-2) is water-soluble, intensive blue protein with strong fluorescence. It is the heterodimer consisting of α- (~18 kDa) and β-subunits (~19 kDa), which form $\alpha\beta$ monomers, further aggregating to trimmers $(\alpha\beta)_3$ and hexamers $(\alpha\beta)_6$. Hexamer form represents a functional unit of phycobilisomes. C-phycocyanin is α-helicoidal protein, with one well-defined domain (similar to the globins) observed within the 3D structure of both chains. Color and intensive fluorescence of C-PC arises from PCB, covalently attached to Cys-84 of α-subunits, while β-subunit binds two PCB molecules *via* Cys-82 and Cys-153 residues [8]. Allophycocyanin has similar structure and physicochemical properties as C-PC. Unlike C-PC, β-subunit of APC binds only one PCB molecule (at Cys-84) [7–8]. Amino acid variation of phycocyanins between cyanobacteria and red algae species are very minor [10].

The VIS absorption spectrum of the native C-PC has pronounced specific peak at 620 nm, arising from bound PCB. Phycocyanobilin has a molecular weight of 586.7 g/mol and characteristic fluorescence spectrum with an emission peak at 640 nm. Spectra of free PCB differ from

spectra of native protein, in sense of intensity and shape of absorption and emission bands [11]. Bilin chromophore is a very sensitive indicator of the conformational state of the protein, enabling monitoring of C-PC denaturation/renaturation by standard spectroscopic methods. Thermal denaturation of C-PC induces shift of absorption maximum from 620 to 600 nm with significant decrease in protein absorbance (color intensity) and fluorescence [12]. Changes of PCB conformation upon denaturation induce these phenomena: chromophore in native protein has stretched conformation, while denaturation changes PCB conformation to the cyclic, similar to the free chromophore [13].

2.1.1. Production, isolation, and purification of C-phycocyanin and phycocyanobilin

Thanks to the high protein (C-PC) content, as well as large availability, *Arthrospira platensis* is culture of choice for C-PC production. *Spirulina* growth requires dry, hot, and sunny climatic conditions [14]. Photoautotrophic *Spirulina* production is outdoor method, used for commercial production of C-PC at tropical and subtropicals regions, in open ponds and raceways. In the mixotrophic production, *Spirulina* cultivation is performed in an enclosed reactor with the addition of glucose, yielding a higher amount of C-PC. *Spirulina* can grow even heterotrophically, but in this case small yield of pigments is obtained [10]. Presence of covalently attached chromophore makes recombinant production of C-PC more complicated in comparison to other proteins. Complete synthesis of C-PC depends not only on co-expression of α- and β-chains, but also on parallel synthesis of PCB and its covalent attachment to protein [15].

Crucial parameters for C-PC production are lighting conditions (light spectrum, quality, intensity, and cycle), climatic conditions (pH and temperature), and media type. Their optimization strategies are reviewed in [16], with higher productivity in closed bioreactor systems than open ponds. Utilization of agricultural waste to replace the synthetic chemicals in algae cultivation media could also have enviro-economical impact.

Isolation of C-PC in high yield requires efficient extraction process. There are several effective approaches used for C-PC extraction: freezing and thawing, homogenization with mortar and pestle, sonication, high pressure homogenization, osmotic shock (using distilled water), acid treatment, enzymatic treatment (by lysozyme), organic solvent extraction, etc. [17]. Potential applications of C-PC in medicine or for research purposes (as fluorescent tag) require its high purity. The purity of C-PC is evaluated using ratio between absorbance at 620 and 280 nm (A_{620}/A_{280}). C-PC preparations with A_{620}/A_{280} greater than 0.7 is considered as food grade, while preparations with A_{620}/A_{280} more than 3.9 and 4 have reactive and analytical grade of purity, respectively [14]. C-phycocyanin price strongly depends on its purity, ranging from $200 to $2.2 million per kilogram. Numerous different procedures for C-PC purification (usually after protein precipitation with ammonium sulfate) use one or more chromatographic steps (ion-exchange chromatography, hydrophobic chromatography, gel filtration, hydroxyapatite chromatography, and expanded bed adsorption chromatography) or two-phase aqueous extraction [10]. Changing light conditions during cultivation of *Spirulina* (blue and red light *vs*. normal) could increase yield and purity of C-PC [18].

Phycocyanobilin (CAS 20298-86-6) isolation requires cleavage of thioether bond between apoprotein and bilin chromophore, by acid hydrolysis, enzymatic cleavage, or alcohol reflux. The most common procedure for the cleavage of PCB from C-PC is still conventional reflux in methanol,

lasting up to 16 hours [10]. Performing ethanolysis in the sealed vessel at 120°C decreases reaction time to 30 minutes, and obtained PCB has higher purity in comparison to conventional reflux method [17]. Phycocyanobilin can be produced in mammalian cells by metabolic engineering, introducing genes for heme oxygenase-1 and PCB:ferrodoxin oxidoreductase, with simultaneous knock-down of biliverdin reductase A to prevent PCB reduction to phycocyanorubin [19].

3. Food applications of C-phycocyanin and phycocyanobilin

3.1. Stability and technologies to improve stability

Natural food colorants are often sensitive to heat, light, oxygen, acidic conditions, and exposure to oxidants, such as ascorbic acid and trace metal ions. Generally speaking, natural C-PC is not a particularly stable protein. It was found to be unstable to heat and light in aqueous solution. The presence of photosensitive PCB makes C-PC sensitive to light and prone to free-radical oxidation [20]. The optimum pH range for C-PC was found to be 5.0–6.0 [21] and it is insoluble in acidic solution (pH 3) [22]. The critical temperature for C-PC stability is 47°C, with a sharp drop in the protein half-life values above this temperature. At 50°C, the C-PC solution showed maximum stability at pH 6.0, while at 60°C the maximum protein stability was at pH 5.5 [23]. Exposure to light of 3×10^5 lux for 24 hours in aqueous solution at pH 5 and 7 caused ~80% of its degradation [22]. Therefore, although C-PC has high potential for applications in food industry, biotechnology, and medicine, stability issue is one of the limiting factors for its successful application.

There are an increasing number of studies dealing with development of methods to increase C-PC/PCB stability and expand their application to different food systems. Addition of 20% glucose, 20% sucrose, or 2.5% sodium chloride was considered suitable for prolonging the stability of the C-PC extract [23]. The natural protein cross-linker methylglyoxal does not significantly stabilize C-PC, whereas addition of honey or high concentration of sugars greatly diminishes thermal degradation of protein. After sterilization (80 and 100°C) of fructose syrups with mixture of C-PC and yellow pigment of *Carthamus tinctorius*, the syrups remain clear, with only partial blue color degradation even after 2 months of storage [6]. The rate of C-PC thermal degradation was decreased in the presence of benzoic acid, followed by citric acid and sucrose, while calcium chloride and ascorbic acid supported the least protein stability in comparison to the other food preservatives studied [24]. After solubilization into reverse micelles, C-PC embedded into the structured interfacial water layer was protected from the bleaching processes, reflecting in stable protein spectral parameters as long as the microemulsion was stable [25]. Incorporation of C-PC into polyethylene oxide nanofibers, or addition of sorbitol (50%) and glucose (20%), increased protein thermostability, considering its almost twice extended half-life [26]. C-phycocyanin incorporated into polysaccharide beads such as alginate/chitosan microcapsules and alginate microspheres, showed greater antioxidant activity and thermal stability. These beads are resistant in simulated gastric fluid, while rapidly release C-PC in simulated intestinal fluid [27]. The addition of anionic and ferulated beet pectin enhanced the color stability of the C-PC extract upon heating (65°C) and slowed down its degradation and color lost by proteases, such as Alcalase 2.4 L, papain, and bromelain [28]. C-phycocyanin stabilized by cross-linking of its subunits with formaldehyde exhibited

similar spectroscopic (absorption/fluorescence) properties as native protein, and showed adequate energy coupling after glutaraldehyde-mediated conjugation with R-phycoerythrin [29].

3.2. Safety and bioavailability

Numerous toxicological studies, such as acute, sub-chronic, chronic, mutagenic, teratogenic/developmental toxicity, carcinogenic, and multiple generational/reproduction tests, have confirmed excellent safety profile of *Arthrospira platensis* and *Arthrospira maxima* (Class A rating by the dietary supplements information expert committee of the US pharmacopeial convention). They were of paramount importance in the determination that water extracts of *Spirulina* or C-PC are safe as well. Only very rare, single-case events of adverse incidences associated with consumption of *Spirulina* have been reported [30].

Desert Lake Technologies, LLC got GRAS notification in 2012 for its CyaninPlus™ product, consistent with section 201(s) of the Federal Food, Drug and Cosmetic Act. It is a water extract of the *Spirulina platensis* or *Spirulina maxima* intended for use as an ingredient in food at levels of up to 250 mg per serving, equivalent to approximately 125 mg of C-PC.

In animal models, C-PC possesses low toxicity and lack of adverse effects. For example, in acute oral toxicity study, the measured LD_{50} values were estimated to be greater than 3 g/kg for rats and mice, without mortality even at the highest dose of C-PC from *Arthrospira maxima* tested (3 g/kg o.p.). No changes in behavior or histopathology, or effect on body weight were observed [31]. Furthermore, acute and sub-chronic oral toxicity study revealed that C-PC (isolated from *Spirulina platensis*) at high concentrations [0.25–5.0 g/kg body weight (w/w)] did not induce any symptoms of toxicity nor mortality of the albino rats [32]. In human randomized, double-blind, placebo-controlled study, high dose of C-PC-enriched aqueous extract from *Spirulina platensis*, equivalent to ~1 g phycocyanin per day (the highest dose generally recognized as safe by the US FDA), after 2 weeks showed safety regarding anticoagulant activity and platelet activation status markers, but reduced levels of aspartate transaminase and alanine transaminase in conjunction with rapid and robust relief of chronic pain [33]. Unlike cancer cells, C-PC is non-toxic to normal cells, for example, platelets and erythrocytes [34]. Although *Spirulina* is not regarded as source of allergens, there is one case report describing anaphylaxis caused by C-PC [35]. To conclude, animal and clinical scientific studies support that *Spirulina* and C-PC, its most abundant organic component, are safe for human consumption, in agreement by their more than 1000 years use in diet.

Bioavailability is the term used to describe how much of the nutrient are easily absorbed into the body and so is able to have an active effect. *Spirulina* is extremely digestible, high energy but low calorie and low fat natural food. Many studies demonstrated *in vivo* effects of orally administered *Spirulina* or C-PC [1–3]. Our research group has shown that C-PC is rapidly digested by pepsin in simulated gastric fluid, releasing chromopeptides varying in size 2–13 amino acid residues. Released chromopeptides had significant antioxidant activity and metal-chelating property, with cytotoxic effect on cancer cell lines positively correlating their antioxidative capacity, with chromophore portion being most responsible for these effects [36]. There is no literature data related to transport of PC-derived peptides or PCB from gastrointestinal tract to circulation. Our previous studies demonstrated that PCB binds to human serum albumin (HSA) with high affinity (2.2×10^6 M^{-1}) [37], and stabilizes protein structure

[38], suggesting that in circulation HSA most likely transports PCB to tissues, similar to other bioactive food-derived substances. Many studies observed *in vitro* effects of C-PC in cell culture, but the location of protein inside cells is controversial and it is still unknown whether C-PC requires a transport protein carrier to enter cells. For skin delivery and protection from oxidative stress damage, C-PC is characterized by a reduced bioavailability, due to its high molecular weight, and therefore it is encapsulated in hyalurosomes as carrier [39].

3.3. Interactions with food matrix components

In addition to their sensitivity to light, heat, and oxidants, natural food colors are prone to interact with other food ingredients, especially if they are carrying proteinous component, such as C-PC. Several studies have found that both C-PC and PCB binds to food matrix components, such as proteins, lectins, saccharides, lipids, and polyphenols [40–47].

C-phycocyanin non-covalently binds to bovine serum albumin (BSA), with binding constant 6.8×10^5 M^{-1} and n = 1.2, as determined by fluorescence quenching of BSA. FT-IR, and synchronous fluorescence spectroscopy confirmed the conformation of BSA has been affected the interaction with C-PC [40]. In the recent study, we found that PCB also interact with BSA, showing high affinity binding ($K_a = 2 \times 10^6$ M^{-1}), determined by protein fluorescence quenching and microscale thermophoresis. Two binding sites were detected on BSA, at the interdomain cleft and at subdomain IB, with stereo-selective binding of the *P* pigment conformer to the protein. Although complex formation partly masked the antioxidant properties of PCB and BSA, a mutually protective effect against free radical-induced oxidation was found [41]. Additionally, PCB binding to major whey protein β-lactoglobulin changes its secondary and tertiary structure, causing higher resistance to digestion by pepsin and pancreatin [42].

C-phycocyanin also interacts with food-derived lectins. Jacalin, tumor-specific lectin from cempedak, binds C-PC specifically in a carbohydrate-independent manner, and with affinities better than that for porphyrins. The binding pattern involves both ionic and hydrophobic interactions and more than one contact site [43]. Concanavalin A and peanut agglutinin can also interact with C-PC, although the nature of the interaction is distinctly different from that for jacalin. The legume lectins bind C-PC *via* two distinct sites, and the binding is weaker in the presence of their specific carbohydrate ligands. Therefore, lectins are proposed as useful carrier for targeted delivery of C-PC in photodynamic therapy [44].

Well-known cryoprotecting disaccharide trehalose interacts with C-PC and decreases the internal protein dynamics, slowing down molecular motions responsible for its unfolding and denaturation [45]. Although it was found that C-PC interacts with lipids at the air-water interface, the oxidation of monogalactosyldiacylglycerol could not be prevented by the introduction of C-PC molecules at the lipid-water interface [46]. Formation of complex between C-PC fragments and polyphenols, in order to obtain more stable blue color for application in food, feed, cosmetic, and pharmaceutical products, was recently patented [47].

3.4. Health-promoting effects

A good part of the bioactivity properties of *Spirulina* are assigned to the pronounced antioxidant capacity of C-PC, mainly attributed to its chromophore (PCB) moiety (see below). This

phycobiliprotein has proven (in)dependent therapeutic effects, such as anticancer, antiinflammatory, and antimicrobial effects, immune enhancement function, liver, and kidney protection, among others. These benefits were subject of many recent excellent review articles (e.g. [48]). As potential safe and non-toxic compounds, C-PC and PCB become a new hot spot in the medicine. The complex mechanisms of its pharmacological actions begin to be fully understood at the molecular level. C-phycocyanin is currently not in clinical use, because positive health-related reports are not integrated deeply and accurately enough, putting limitations to its application as a drug. Otherwise, C-PC-encapsulated chitosomes, capable of preserving the protein stability in the gastrointestinal tract and with enhancing efficacy are in development [49].

Phycocyanobilin is potent inhibitor of certain NADPH oxidase isoforms, likely because in mammalian cells it is rapidly reduced to phycocyanorubin, a close homolog of bilirubin. Over-activity of NADPH oxidase causes oxidative stress, and is known to mediate and/or exacerbate numerous pathological conditions [50]. *In vitro*, PCB is capable to modulate other important markers of oxidative stress and endothelial dysfunction, such as eNOS and/or VCAM-1, and to markedly up-regulate heme oxygenase-1, a key enzyme responsible for generation of a potent antioxidant bilirubin [51].

The suitable clinical dose of PCB remains to be defined. Without mass-produced pigment derived from commercially available PCB-enriched *Spirulina* extracts, bioengineered organisms, or chemically synthesized pigment, ingestion of whole *Spirulina* is still the least expensive way to benefit from this phytonutrient. A tablespoon of *Spirulina* powder (about 15 g) contains approximately 100 mg of PCB, daily dose that might be effective [50]. Interestingly, no relevant data about relative absorption and bioefficacy of free PCB or *Spirulina*-bound pigment exist for either rodents or humans.

3.4.1. Antioxidant properties

Proteins bearing colored prosthetic groups, such as a highly conjugated linear tetrapyrrole chromophore in C-PC, can be both the source and target of reactive species in biological systems. An extremely high antioxidant capacity of C-PC was unambiguously established, based on experiments carried out both in *vivo* and *in vitro*. It not only scavenges, for instance, peroxyl, hydroxyl, and superoxide radicals, but also inhibits the lipid peroxidation mediated by reactive oxygen species. The bilin group seems to be the main target, since the *in vitro* radical assisted bleaching of PCB color in protein clearly indicates its involvement in the scavenging of reactive species [52]. A key contribution of the structural components and various modulating factors on the antioxidant activity of C-PC will be briefly mentioned here, as they can influence protein utility as a food supplement and therapeutic agent.

C-phycocyanin is a more efficient peroxynitrite scavenger than free PCB due to (additional) interactions with tyrosine and tryptophan residues of the apoprotein [53]. Differences in the amino acid composition affect C-PC antioxidant capacity. Selenium-C-phycocyanin purified from Se-enriched *Spirulina platensis* exhibits stronger antioxidant free radicals scavenging activity than standard protein, attributed to the incorporation of selenoamino acids into the polypeptide chains of protein [54]. C-phycocyanin (from *Spirulina fusiformis*) exposed to blue light shows better *in vitro* antioxidant property than protein exposed to normal light, due to

marginal changes in the apoprotein cysteine content [55]. Interestingly, bilin group is not the main target of C-PC reaction with hypochlorous acid and singlet oxygen [56].

C-phycocyanin generates hydroxyl radicals in the light, while scavenging them in the dark. Radical generation ability disappears, but scavenging greatly increases in denaturated protein, confirming the role of phycobilin moiety in scavenging. Trypsin hydrolysis of C-PC demonstrated the apoprotein portion also made a significant contribution to the antioxidant activity [57]. The heat denatured (spray-dried) C-PC shows the same level of activity as the intact protein, finding important for preparation and utilization of C-PC [58]. C-phycocyanin can be cloned and expressed in *Escherichia coli*, to reduce the cost and time for protein production. Recombinant holoprotein (α-subunit) not only retained the spectroscopic characteristics of the native protein, but also its bioactive properties, including powerful radical scavenging activity [59]. Although less potent, recombinant apo-C-PC β-subunit acts as an antioxidant on human erythrocytes as well [60]. Other bioactivities of recombinant biliproteins should be further studied to provide additional health benefits.

3.5. Food colorants

In the last decades, consumers are becoming more educated and aware of what they eat, demanding for clean labeling of the food/beverage products and making the pressure to food industry to switch from artificial to natural ingredients and additives. The main consumers of vividly colored food products are children. Due to their low weight, they are at constant risk to exceed recommended daily intake (mg/kg weight) of artificial colorants. Nowadays, the leading confectioners switched to natural colors to avoid obligatory label warning for acceptable daily intake levels of the colorings. Consequently, market of natural food colors is in prominent expansion, expecting to reach $2.5 billion by 2025.

Compared with other natural pigments, natural blue pigments are rare, because a complex combination of molecular features (such as π-bond conjugation, aromatic ring systems, heteroatoms, and ionic charges) is required to absorb red light (~600 nm region) [61]. Anthocyanins are the primary source of blue color in plants, but their color is pH dependent. On the other hand, fungi and microorganisms produce many blue compounds in response to stress or predators and therefore their unpredictable biological activities make their safety for food use questionable. None of discovered natural blue pigments cannot reach shade, brilliance, vividness, molar absorptivity, and stability of Brilliant Blue FCF (Blue 1 or E133), the most used of approved synthetic blue food colorants, and concomitantly to be safe and cost-effective [61]. In this moment, the only permitted natural blue food colorants are gardenia blue (in Japan), blue anthocyanins and *Spirulina* color (composed mainly of C-PC). Although gardenia blue and blue anthocyanins have better stability to heat and light than C-PC [22], only C-PC can offer brightness, brilliance, and shade most similar to Brilliant Blue FCF, making this protein much more acceptable and ensuring seamless switches from artificial to natural food colors for existing food products. Trichotomine, indole alkaloid from kusagi berries (native in China and Japan), is the most promising natural bright blue colorant due to molar absorptivity (70,000 M^{-1} cm^{-1}) similar to that Brilliant Blue FCF (134,000 M^{-1} cm^{-1}). Limited supply of kusagi berries and the low concentration of pigment make this option economically unjustified [61].

In contrast, although PCB have relatively low molar absorptivity (37,900 M^{-1} cm^{-1}) [37], *Spirulina* can be sustainably produced in huge, almost unlimited amounts, and the high pigment concentration provide its extraction in cost-effective way.

Demand for C-PC as a natural blue food colorant has experienced exponential growth in the past 5 years, especially after FDA approval of *Spirulina* extract as a food colorant for gum and candy in 2013, with market estimated at more than $50 million. In 2014, its application was expanded to frosting, ice cream and frozen desserts, dessert coatings and toppings, dry beverage mixes and powders, yogurts, custards, puddings, cottage cheese, gelatin, breadcrumb, and ready-to-eat cereals. In 2015, coatings in dietary supplements and pharmaceuticals were also approved and, in this moment, C-PC is the only approved natural blue colorant in the US, Europe, and Asia. In US FDA Code of Federal Regulations, *Spirulina* extract is approved as color additive exempt from certification, prepared by the filtered aqueous extraction of the dried biomass of *Spirulina platensis* and containing phycocyanins as the principal coloring components [62].

Commercial powder formulations of C-PC, such as Linablue® (DIC Corporation, Japan), are declared as completely soluble in cold and warm water and <20% ethanol, making a homogeneous transparent solution, with stable color shade in the pH range 4.5–8.0 (except at C-PC pI value around pH 4.2), which can be improved by the presence of protein-containing ingredients; low thermal stability, which can be improved in high density sucrose solutions; with low light stability, which can be improved in the presence of antioxidant like ascorbate; and with no tongue dyeing effect. In combination of C-PC with red, yellow, and other natural colorants, it is possible to obtain vibrant green, purple, and other natural colors. As FDA is still considering the petition for copper chlorophyllin, natural green food color often involves C-PC or *Spirulina* extract mixed with safflower or turmeric extract (curcumin).

Due to its refreshing ice cool color, C-PC is also increasingly promoted as natural color for alcoholic beverages, such as FIRKIN Blue gin.

In comparison to artificial colors, natural colorings are less vivid, and interactions with food matrix components can result in further decrease in their vibrancy, or unwanted change in color and flavor. For example, our research group observed an instant clear color change from blue to green when PCB interacts with BSA [41]. Therefore, switching from artificial to natural colorings in existing food products can be challenging and complex.

3.6. Functional food additives, nutraceuticals, and dietary supplements

In last decade, there is an increase in chronic diseases and increasing costs of health care due to busy lifestyles and unhealthy nutrition. On the other hand, people are more health conscious and more interested in health-promoting products to improve their health quality. This imposed a demand for functional food ingredients and additives, nutraceuticals and dietary supplements of natural origin.

There are several studies dealing with incorporation of *Spirulina* or its proteins into different food, such as biscuits, pasta, milk-based products, various breads, and crisps, in order to create protein-enriched functional food products. In all these studies the food was fortified by whole *Spirulina* powder or biomass, except in the study where isolated C-PC was used [63]. *Spirulina* was incorporated into pasta (e.g. [64–66]), biscuits and cookies (e.g. [67–69]),

extruded products (e.g. [70, 71]), ice cream [72], yoghurt and acidophilus milk (e.g. [73–74], baby food formulas [75], and bread [76]. However, all these food products were thermally processed leading to destruction of C-PC and resulting in green-yellowish color of product due to partially retained carotenoids and chlorophyll. This fact was ignored in almost all studies, except in the study monitoring C-PC degradation at 615 nm [71].

Although protein component of C-PC added nutritive value to these products, bioactivity of sensitive PCB component cannot be exploited. The only way to take full advantage of health-promoting effects of bilin component, is addition of *Spirulina* biomass/powder, PCB-enriched *Spirulina* or C-PC alone, after all thermal food-processing steps. Similarly, although *Spirulina* fortification of milk have positive influence on viability of milk fermenting microbiota [74], their activity also decrease content of precious bilins. In the most of the studies dealing with *Spirulina* biomass-food enrichment, the limiting factor for quantity of added Spirulina was consumer acceptance due to sensory characteristics related to flavor and taste. The use of C-PC or *Spirulina* protein isolate/concentrate fraction would reduce undesirable fishy off-flavor of whole algae biomass and in that way notably improve consumer acceptance.

Possible advantages of joint administration of flavanol-rich cocoa powder and *Spirulina*, or PCB-enriched *Spirulina* extracts was proposed [77]. As inhibitor of NADPH oxidase, PCB would minimize NADPH oxidase-derived oxidative stress, while flavanols would promote vasodilation by up-regulation of •NO production. Cocoa-*Spirulina* powder blended with milk (cow's, soy, and rice) can yield a drink with a tasty rich chocolate flavor, as cocoa can mask the unpleasant flavor and odor of *Spirulina*/C-PC. These two nutraceuticals could complement each other actions in prevention of senile dementia by optimizing cerebrovascular perfusion, and by suppressing cerebral oxidant stress. Combined supplementation with PCB, citrulline, taurine, and supranutritional doses of folic acid and biotin could help in slowing the progression of diabetic complications, based on their complementary action on the oxidative stress and the associated loss of •NO bioactivity [78].

Besides being component of many dietary supplements, C-PC also becomes popular component of different wellness bioactive drinks, providing attractive blue color and nutraceutical properties [e.g. Ocean Mist by Allgalio Biotech, B Blue bioactive drink by B blue, Bloo tonic by Cidererie Nicol, Holy water by Juice Generation, Natura blue by Natura4Ever, Smart chimp by Smart chimp and many other drinks based on Blue Majik (C-PC-enriched organic extract of *Spirulina platensis* by E3Live) made by other producers].

Purified PCB is still not available as a nutraceutical supplement, but new research turned toward methods for efficient cleavage of PCB from the C-PC [17]. Further stabilization will enable commercially available PCB as food colorant and dietary supplement.

3.7. Future carriers of bioactive substances and food additives with promising techno-functional and food-preserving properties

In recent years, the food industry is in increasing search for new sources of inexpensive food protein having nutritional and techno-functional characteristics similar to high-cost animal proteins. In addition to plant one, proteins extracted from microalgae are becoming favorable alternative due to availability and sustainability of their production on one hand, and due to their extraordinary nutritional and bioactive properties, as well as suitable functional properties on the other hand.

In this moment, there are only few studies investigating the functional properties of *Spirulina* protein concentrate/isolate, with phycobilins being the main functional protein component. Proteins isolated from *Spirulina* are quite capable of reducing the interfacial tension at the aqueous-air interface at relatively lower bulk concentrations than common food proteins [79]. In comparison with soy protein isolates, *Spirulina* protein isolate (SPI) demonstrates lower water, but higher oil absorption capacity. SPI showed good emulsifying and foaming capacity, and ability to form protein films and gels [80]. This study demonstrated that emulsifying capacity, the emulsion aging stability, the emulsion microstructure and opacity as well as the foaming capacity and the foam stability were pH dependent. Also, emulsifying and foaming capacities have shown to be positively correlated to the protein solubility [80]. *Spirulina* protein isolate forms gels after heating (90°C) and cooling, showing fairly low minimum critical gelling concentrations (1.5 wt% in aqueous solution) compared to other food (soy) proteins [81]. *Spirulina* protein concentrate (SPC) have shown higher emulsifying and similar foaming capacity, when compared with soybean meal [82]. The rheological and textural parameters increased linearly with increased C-PC addition (0.25–1.25% w/w) in oil-in-water emulsions, suggesting C-PC emulsion stabilizing role [83]. From food technology point of view, these studies imply that C-PC or *Spirulina* proteins are promising food ingredients and additives, and that further studies are needed to fully exploit their most likely excellent functional properties.

In contrast to other natural food colors, but similar to other food-derived proteins, C-PC can be used to modify techno-functional properties of food matrices or as carrier of bioactive substances. As a biodegradable, biocompatible, and poor immunogenic protein molecule, C-PC is suitable as carrier for preparation of protein-based nanoparticles. Drug delivery *via* their loading into C-PC nanoparticles have shown to be more effective and safer [84, 85]. By analogy, C-PC-based nanoparticles can be used for food applications in the future as carrier for other active substances, acting together in synergistic manner and complementing mutual benefits. In order to fully utilize all benefits of valued bilin component, C-PC should be added only after all thermal pretreatments.

The natural food colorings are often associated with functional properties. C-Phycocyanin/PCB with their extraordinary antioxidative activities could have role in maintaining of the lipid oxidative stability, especially in food products with high lipid contents. Addition of C-PC was found to inhibit linoleic acid peroxidation and decrease TBARS value in liposome-meat system [86]. Some studies demonstrated that C-PC exhibited antibacterial and antifungal potential [87, 88], suggesting that C-PC/PCB can also can serve as antimicrobial agent. Silver nanoparticles-based antimicrobial packaging is a promising form of active food packaging, and C-PC was used for synthesis of bio-silver nanoparticles [89]. Incorporation of *Spirulina* powder in strudels can significantly retard lipid oxidation and reduced the number of yeast and mold resulting in prolonged shelf life [68]. Therefore, C-PC/PCB in addition to their role as food colors can contribute to food preservation and improvement of food shelf life and/or to the reduce addition of non-natural food preservatives.

4. Other applications of C-phycocyanin and phycocyanobilin

As we have seen, C-PC and PCB have excellent antioxidant properties. Irradiation of these molecules with visible light produces reactive oxygen species, making them good candidates for application in photodynamic therapy (PTD). Indeed, it was shown that anticancer activities of C-PC against breast cancer MCF-7 cells increases upon exposure to He-Ne laser (632.8 nm wavelength) [90]. C-phycocyanin has specific affinity for tumor-associated macrophages (TAM), which have been proposed to be a "target for cancer therapy". Formation of non-covalent conjugate between Zn-phthalocyanine and C-PC resulted in an enhanced photodynamic effect with selective accumulation in the tumor site, probably through the specific binding of C-PC to TAMs [91].

In comparison to other fluorophores, phycobiliproteins have a high molar extinction coefficient and fluorescence quantum yield, as well as a large Stokes shift. Therefore, C-PC could be a good candidate for applications as fluorescent marker. When C-PC is extracted by low ionic strength buffers monomers will be the dominant form, inducing decrease of protein fluorescence. Therefore, in order to obtain stabilized highly fluorescent oligomers cross-linking of C-PC is needed. Chemically stabilized C-PC, fused to the biospecific domains such as streptavidin, is used as a biospecific fluorescent assay. Further, C-PC fluorescence can be also used for *in vivo* monitoring of cyanobacterial growth and detection of toxic cyanobacteria in drinking water [15]. Furthermore, strong quenching of C-PC fluorescence Hg^{2+} ions implies its potential application as biosensor for heavy metals in aquatic systems [92]. Interestingly, PCB synthesized in mammalian cells through metabolic engineering could be useful optogenetic tool for regulation of cell processes by light [19].

Artificial photosynthesis is currently a hot topic in science and technology. Consequently, there are growing demands for designing photoelectron-chemical (PEC) cells, capable to perform artificial photosynthesis. In PEC devices, light-harvesting proteins (such as C-PC) are used to "sensitize" metal and semiconductor surfaces. BioPEC solar hydrogen generator with a hematite-phycocyanin hybrid photo anode was designed. In order to obtain PEC cells with higher performances, the stability of immobilized C-PC needs to be improved [93].

Beside the application for food and drink coloring, C-PC is also used as a cosmetics colorant in lipsticks, eyeliners, and eye shadows preparations [94].

5. Conclusion

The vast majority of studies regarding *Spirulina* bioactive components used whole algal (dried) mass or its aqueous extracts. Major *Spirulina* deep blue color protein C-phycocyanin and its bilin chromophore have remarkable potential for use in food technology, as safe food colorant, functional food additive, nutraceutical and/or dietary supplement, given their excellent health-related properties, and opportunity for sustainable and relatively inexpensive mass production.

Acknowledgements

This study was supported by the grant No. 172024 obtained from the Ministry of Education and Science of the Republic of Serbia.

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Chapter 8

The Potential for 'Next-Generation', Microalgae-Based Feed Ingredients for Salmonid Aquaculture in Context of the Blue Revolution

Sean Michael Tibbetts

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.73551

Abstract

Microalgae-based ingredients have potential to ensure continued growth of salmonid aquaculture for global sustainable food security in the blue economy. Algal biorefineries must valorize the entire crop to grow profitable microalgae-based economies. With massive growth and demand for novel sustainable ingredients, farmed salmonid feed sectors are highly promising areas to focus on. Microalgae-based ingredients for salmonid feeds may have market advantages in terms of lower input costs, aerial footprint, wastewater remediation benefits and carbon credits for industrial CO_2 conversion. A handful of microalgae-based ingredients have been proposed as candidates to supply well-balanced nutrients and immunostimulatory compounds. However, technical gaps exist and need addressing before the industry could economically incorporate microalgae-based ingredients into commercial feeds. Current knowledge on comprehensive biochemical composition is incomplete, highly heterogeneous, and information on their nutritional value is scattered and/or inconsistent. The aim of this chapter is to consolidate relatively fragmented data on biochemical composition and nutritional value of microalgae-based ingredients focusing on farmed salmonid feeds. Presented are discussions on the potential for such 'next-generation' ingredients, opportunities/challenges for their use and a compendium of studies evaluating their performance in feeds for economically relevant farmed salmonids, including rainbow trout (*Oncorhynchus mykiss*), Arctic charr (*Salvelinus alpinus*) and Atlantic salmon (*Salmo salar*).

Keywords: microalgae, salmonids, aquaculture feeds, composition, nutritional value

1. Importance of aquaculture in the blue revolution

Most developed countries are nearing their terrestrial agricultural output capacity. Terrestrial agriculture will be highly challenged to meet the demands for a growing human population. Food production requires an epic shift towards leveraging intrinsic competitive advantages from our aquatic environment. As such, we have now entered the blue revolution where dietary protein and essential nutrients are increasingly derived from aquatic environments. However, most traditional capture fisheries are depleted or harvested at their biological limits. As stated a half century ago by famous marine explorer and ecologist Jacques Cousteau "We must farm the sea" in order to foster strong global food security. This was reiterated in 2012 by former UN Secretary General Kofi Annan who stated "Aquaculture is crucial for supplying the world's food needs for the next 50 years". Recently, aquaculture has grown annually at 7.8%; far exceeding that of terrestrial farming systems like poultry (4.6%), pork (2.2%), dairy (1.4%), beef (1.0%) and grains (1.4%) [1]. As the appetite for seafoods outpaces what capture fisheries can supply, global farmed seafood supplies in 2009 matched wild-caught seafood and this proportion is projected to rise to 62% of all seafood supplies by 2030. This firmly secures aquaculture's position in the blue economy as the most efficient use of resources for global food production. Gentry et al. [2] reported that a small fraction of coastal ocean waters (0.015%), about the size of Lake Michigan, specifically selected for sustainable aquaculture (excluding areas that interfere with shipping lanes, ocean oil extraction or marine protected areas) is required to exceed current demand for seafood by 100-fold. For the first time in history, global aquaculture production exceeded beef production in 2011 and in 2014 farmed aquatic production was valued at $160 billion USD (74 million metric tons [mmt]) and will exceed $240 billion USD by 2022. Indeed, as global economist and Nobel Laureate Dr. Peter Drucker recently stated "Aquaculture, not the internet, represents the most promising investment opportunity of the 21st century".

2. Formulated compound aquaculture feeds

2.1. The aquafeeds dilemma

Of the 74 mmt of global farmed seafood produced annually, the majority (57 mmt or 77% of total) is from finfish and crustaceans, which are considered 'fed' aquaculture species. This means they require mass-produced formulated complete feeds (aquafeeds) and the production of aquafeeds will exceed 87 mmt by 2025. As a result, modern aquaculture is a major consumer of world fish meal and fish oil supplies, which has placed an unsustainable burden on traditional capture fisheries in South Pacific, South-East Asia and North Atlantic countries. This scenario represents a dramatic shift in use of these finite marine resources during the past half century. Regarding fish meal; feeds for terrestrial animals have traditionally demanded virtually all global supplies and aquafeeds consumed <1% of supply only a few decades ago, while today aquafeeds consume a staggering 73%. The situation is the same for fish oil where in 1960 virtually all supplies were used as hardened edible fats or refined industrial oils and aquafeeds

used <1% of supply, while today aquafeeds consume 71%. Aside from very real ecological issues, this tremendous demand has had a direct and highly consequential economic result of tripling the cost of fish meals and oils. While farmed salmonids represent a marginal contribution (3%) to total global farmed seafood supplies, they consume a disproportionate amount of these finite resources.

2.2. Industrial farming of salmonids

Farming of salmonids (e.g., salmon, trout, charr) uses feed inputs more efficiently than terrestrial animal protein production systems (e.g., beef, poultry and pork). Typical feed conversion ratio (FCR) for salmonids is 1.2 g feed g $gain^{-1}$ compared to 1.8–6.3 g feed g $gain^{-1}$ for livestock. This is due to higher dietary protein and energy retention efficiency in salmonid fish (23–31%) compared to terrestrial farm animals (5–21%). Also, since fish are poikilothermic and expend less energy maintaining their position in the water column, edible yields of farmed salmonids are higher (68%) than terrestrial livestock (38–52%). Salmonid farming occupies low carbon footprints and those farmed in Norway, Chile and Canada may, in fact, be the most ecologically sustainable meat products on the global food protein market. Greenhouse gas (GHG) emissions of 2.2 kg CO_2 eq. kg^{-1} of edible meat produced are reported in contrast to 2.7–30.0 kg CO_2 eq. kg^{-1} for chicken, pork and beef. However, it's important to note that salmonids are highly piscivorous and the industry remains greatly dependent upon global ocean resources; albeit to a far lower degree than previous decades. Most commercial salmonid feeds in 1995 contained ~53% fish meal, ~31% fish oil and ~16% alternative proteins and grains, while today most feeds contain ~27% fish meal, ~15% fish oil, ~43% alternative proteins and grains and ~15% alternative oils. In Norway, total dietary composition of wild marine-based ingredients has dropped from 90 to 30% between 1990 and 2013. Nevertheless, global demand for aquafeeds is less than 40 mmt but is expected to rise dramatically to 87 mmt which will continue to exacerbate the aquafeeds dilemma. Fish meal and fish oil obtained from reduction of wild-capture pelagic fish is beyond maximum sustainable limits, is becoming cost-prohibitive and could/should be better-used for direct human consumption. These wild populations may be even more pressured by global climate change and supplies will be insufficient to meet growing aquafeed demands and thus constrain aquaculture growth. This is particularly true in emerging economies like China where production accounts for 61% of global aquaculture and continues to grow rapidly.

2.3. Alternative feed ingredients—microalgae?

The aquafeeds dilemma is not new and herculean efforts were made over three decades to identify a broad range of new ingredients. This developed new commodity markets and resulted in significant industrial use of animal- and plant-based feed inputs. These include high-quality rendered animal by-products (e.g., poultry meals, hydrolyzed feather meals, meat and bone meals, blood meals, etc.) and plant-based meals and protein concentrates produced from oilseeds, grains, pulses and legumes as complete or partial replacements for fish meals. Similarly, terrestrial animal fats and plant-based oils (e.g., poultry fat, beef tallow, vegetable oils, etc.) have extensively replaced fish oil in farmed salmonid feeds. However,

these 'second-generation' ingredients are not without limitations. Most lack certain functional properties, palatability and nutritional profiles, and many have lower digestibility and may be limited by specific antinutritional factors (ANFs) which can impair feed intake, growth performance and fish health. Some may alter final product quality for the consumer and they are also becoming increasingly costly and ecologically unsustainable. Of critical importance is that increased use of these ingredients has forced farmed salmonid production to shift alignment to terrestrial agriculture which occupies large aerial footprints, is heavily dependent on fossil fuel-based fertilizers, chemical pesticides and freshwater irrigation. Additionally, these products are grown for our own consumption; so it is of key importance to reduce competition with human food resources for sustainable production of aquafeeds. Ecological and socioeconomic issues aside, the health benefits of consuming fatty fish like farmed salmonids have become serious concerns for human nutrition with the rising use of plant-based ingredients in salmonid feeds. Uncoupling of this scenario is desperately needed to effectively minimize environmental impacts and social inequities; however, it is not simple from technological, ecological or socioeconomic viewpoints and will require economic and political incentives from governments and substantial 'buy-in' from industry and private investors.

3. Microalgae-based products for salmonid feeds

3.1. Opportunities

To ensure continued growth of the sustainable salmonid aquaculture sector in ways that do not deplete important terrestrial and aquatic resources, a 'third-generation' of feed inputs is urgently needed and it is generally agreed that they must come from lower trophic levels. Microalgae such as *Chlorophyceae* (green algae), *Bacillariophyceae* (diatomaceous algae) and *Chrysophyceae* (golden algae) and prokaryotic microorganisms such as *Cyanophyceae* (blue-green cyanobacteria) are among the first lifeforms on earth; having appeared ~3.5 billion years ago. Many are amenable to cultivation under photoautotrophy (e.g., inorganic CO_2, nutrients and light), heterotrophy (e.g., organic carbon and nutrients) or mixotrophy (e.g., combined strategies) and cultivation technologies exist for growth in open or closed ponds, enclosed photobioreactors and fermenters. While microalgae as feedstocks for renewable bioenergies has driven technological advances recently, they remain far from economical viability and are uncompetitive with terrestrial oilseed crops and conventional fossil fuels. In the absence of high-value compounds, algal biorefineries should take a holistic approach that valorizes the entire algal crop as an attractive path towards a viable microalgae-based industry, and the feed sectors are promising areas to focus on. There is tremendous potential for microalgae cultivation (e.g., algaculture) to be co-located with industrial point-source emitters of waste 'outputs' (e.g., CO_2, nutrients, heat) which are essential 'inputs' for rapid microalgae growth and accumulation of nutrient-rich biomass. Microalgae-based ingredients produced for aquafeeds could have competitive market advantages over terrestrial crops in terms of input costs, lower aerial foot-print, and potential for wastewater remediation and carbon credits from CO_2 conversion. Recent search efforts for strains for bioenergy purposes has sparked great interest

from aquaculture nutritionists in terms of the biochemical composition of many microalgae and it is clear that some may be promising candidates for salmonid feeds based on their supply of well-balanced amino acids, essential omega-3 (n-3) long-chain polyunsaturated fatty acids (LC-PUFA), vitamins, minerals, carotenoids and bioactive compounds. While large-scale algaculture is a commercial reality in some parts of the world (e.g., Australia, China, Germany, India, Israel, Japan, Myanmar, Taiwan, United States), the sector is dominated by a handful of species with relatively insignificant annual production: *Arthrospira* (3,000 t), *Chlorella* (2,000 t), *Dunaliella* (1,200 t), *Nostoc* (600 t), *Aphanizomenon* (500 t), *Haematococcus* (300 t), *Crypthecodinium* (240 t) and *Schizochytrium* (10 t) and estimated dry biomass price is $8,000–300,000 USD per t. Most is presently destined for human health food markets but many producers have keen interest in penetrating the massive salmonid aquafeed sector if production tonnage can be increased and the price made more economical.

3.2. Challenges

As a cautionary note, some proponents of microalgae biotechnologies suggest that they are 'super-foods' and feeding microalgae to farmed salmonids makes perfect sense since that is what their wild counterparts would naturally consume. This thinking encourages development of lower-trophic, ecologically-sustainable salmonid feed ingredients but the notion is, unfortunately, flawed. While it's true many essential dietary nutrients for wild salmonids originate in aquatic phytoplankton (microalgae) and other single-celled organisms, they are delivered through 'indirect' passage of nutrients up the aquatic food chain and rarely via 'direct' intake; as salmonids do not actively seek to consume microalgae. The notion that wild, highly piscivorous salmonid fish derive nutrients from direct ingestion of microalgae is akin to the notion that wild, highly carnivorous lions derive nutrients from direct consumption of grass. On the contrary, higher trophic predators like salmonids evolved to rely on a progression of intermediary organisms (e.g., grazing phytoplankton, zooplankton, forage fish, etc.) to extract nutrients from complex food matrices that make up 'base-of-the-food chain' organisms (e.g., phytoplankton). This upward passage and trophic accumulation of essential nutrients, referred to as food-chain amplification, transforms them into forms that the relatively simple monogastric digestive system of salmonids can assimilate and use for productive purposes like protein synthesis, growth, tissue repair, metabolic energy and reproduction. The practical implication is that, in the absence of food-chain amplification, reliance on transformative intermediary organisms represents a nutritional barrier for direct feeding of microalgae to most monogastric animals, especially coldwater farmed salmonids. This is because their capacity to extract and utilize microalgal nutrients directly is limited by the highly recalcitrant cell walls of most microalgae, combined with the relatively short gastric (acidic) digestion phase in salmonid fishes. Some industrial downstream processing is almost certainly required in order for nutrient-rich microalgae to realize its potential as a much-needed next-generation ingredient. Like other ingredients once regarded as 'alternatives' but now established mainstream ingredients (e.g., corn, soy, wheat, canola, etc.), cost-effective processing technologies must be developed for microalgae to rupture cell walls, concentrate target nutrient levels, reduce/eliminate indigestible fibers, inactivate ANFs and increase nutrient digestibility for monogastric cold-water fish. With each processing step, nutritional value is increased but so is the cost of production and

ultimately the market price. To further attenuate this situation, unlike terrestrial crops, microalgae cultivations must begin with dewatering the highly dilute cells (typically by centrifugation) down to a dry biomass (typically by spray-drying) and usually some means of mechanical, chemical or enzymatic cell wall rupture is required, and all these processes are currently highly energy intensive and costly. Optimizing the balance between the types and extent of downstream processing and their associated costs to determine the 'point of diminishing returns' that yield algal ingredients of the highest nutritional value in a cost-effective manner for least-cost salmonid ration formulations will undoubtedly occur with innovation. However, very few microalgae-based salmonid feed ingredients have yet to reach the marketplace.

3.3. Nutrient composition of microalgae in relation to their use in salmonid feeds

Beyond high production costs and relatively high prices for microalgae for aquafeeds, several broad issues must be resolved before the salmonid aquaculture feed industry can adopt microalgae-based ingredients for routine use. First, microalgae are a widely diverse class of microorganisms and many complex issues exist around their highly variable nutrient composition. This chapter is a culmination of data collected from the literature on the relevant biochemical composition of ~50 genera of microalgae from the past century. Suffice to say that the sheer size of data tables and associated >150 references preclude inclusion within the confines of this chapter. For a relatively complete compendium of biochemical composition, readers are referred to Becker [3]. Generally, proximate composition of dry microalgae is extreme for ash (<1–53%), protein (2–73%), lipid (<1–83%), carbohydrate (1–64%) and energy (4–30 MJ kg^{-1}). This highly variable trend is predictably the same for genera that have been specifically evaluated for salmonid feeds (**Table 1**) for ash (1–53%), protein (3–73%), lipid (1–83%), carbohydrate (3–55%) and energy (6–30 MJ kg^{-1}). This variability is related to the extensive biological diversity of microalgae (e.g., >100,000 documented species) and the complexities associated with their use as biological factories, large variations in cultivation strategies, variable harvesting and downstream processing methods and under-developed and inconsistent nutrient characterization analytics. Also, in contrast to agricultural crop production, large-scale algaculture is still in its embryonic stage and production tonnage needs to dramatically rise to industrial levels to realize the benefits of economies of scale that will ensure reliable supply, consistent nutrient profile, high nutrient quality and cost-competitiveness that the massive salmonid aquafeed sector will require. Lessons could be learned from the relatively niche, poorly regulated natural health food market for microalgae such as *Chlorella*. Görs et al. [4] reported that quality control is poor for almost all *Chlorella*-based products on the global marketplace. For example, most are contaminated with bacteria, cyanobacteria and other unlisted algal species, contain highly variable levels of chlorophyll and/or its breakdown products and were greatly heterogeneous in biochemical and nutritional composition. It is also observed that *Chlorella* supplements are being marketed as 'super-foods' in part because they contain CGF (*Chlorella* Growth Factor). However, this is an ill-defined term and poorly understood consortium of various nitrogen-containing compounds that are not supported with scientific validation. This lack of quality control and nutritional 'proofing' cannot be tolerated in salmonid aquafeeds and quality assurance must be made a priority.

Genera	Ash (%)	Protein (%)	Lipid (%)	Carbohydrate (%)	Energy (MJ kg^{-1})
Arthrospira	3–13	42–73	2–16	8–25	6–23
Chlamydomonas	—	43–56	14–22	3–17	—
Chlorella	2–8	14–67	2–63	7–34	15–27
Crypthecodinium	4	15–23	20–56	—	29
Desmodesmus	16	21–27	1	—	17
Haematococcus	1–15	3–48	7–67	26–55	24
Isochrysis	13–31	20–45	16–53	13–18	—
Nanofrustulum	53	12	3	—	—
Nannochloropsis	7–23	18–48	2–68	8–36	19–27
Phaeodactylum	16–17	30–49	7–57	8–25	20
Schizochytrium	4–12	12–39	15–71	32–39	26
Scenedesmus	3–14	8–56	1–58	10–52	20–23
Tetraselmis	11–20	27–52	3–45	15–45	18–20
Thraustochytrium	8–11	12–21	8–83	39	18–30

Table 1. General proximate composition and energy content of various genera of microalgae evaluated for use in salmonid feeds (dry weight basis).

3.3.1. Protein and lipid composition

Contrary to popular belief, most industrialized microalgae species do not accumulate high-value essential n-3 LC-PUFA (e.g., those in the 20 and 22 carbon chain lengths). This essential lipid deficiency may relegate these species as poor nutritional value for use in salmonid feeds when, in fact, it's their potential for high protein accumulation that is of interest. While total protein content varies widely in the literature (often by several magnitudes) the essential amino acid (EAA) profile of that protein generally remains rather conserved among species, regardless of growth phase and/or cultivation conditions. **Table 2** shows the EAA composition of microalgae genera that have been evaluated for salmonid feeds. Leucine, arginine and lysine are generally predominant in microalgal protein (on average 7 g 100 g $protein^{-1}$), methionine, histidine and tryptophan are typically most limiting (on average 2 g 100 g $protein^{-1}$) and isoleucine, phenylalanine, threonine and valine are mid-range (on average 4 g 100 g $protein^{-1}$). An important factor when evaluating the protein quality of microalgae-based ingredients for nutrition is their concentrations of nucleic acids (RNA and DNA), which are sources of purines. It is known in primates that excessive consumption can elevate plasma uric acid, which may result in inflammatory arthritis (gout) and renal calculus (kidney stones) and this is related to the lack of digestive uricase enzyme in primates. Fortunately, farmed monogastric animals like swine, poultry and fish have different metabolic pathways which minimize accumulation of uric acid in the blood stream, such as excretion via allantoic acid, urea and ammonia. Additionally, microalgae typically contain lower levels of nucleic acids and purines (4–6%) than other single-cell proteins like yeast and bacteria (8–20%). Like other macronutrients, lipid content of microalgae varies widely and fatty acid (FA) composition is also highly

	Arthrospira	*Chlorella*	*Entomoneis*	*Haematococcus*	*Isochrysis*	*Nanofrustulum*	*Nannochloropsis*	*Phaeodactylum*	*Scenedesmus*	*Schizochytrium*	*Tetraselmis*	*Thraustochytrium*
Essential amino acid (g 100 g protein^{-1})												
Arginine	4–8	3–14	—	6–8	2–6	6	2–8	6	6–7	1–12	6–9	7
Histidine	1–5	1–6	—	<1–1	1–3	1	<1–3	2	2	<1–3	1–2	3
Isoleucine	<1–7	<1–4	—	2–5	1–5	4	<1–6	5	4–5	<1–3	3–4	4
Leucine	5–14	3–9	—	5–9	3–9	7	5–11	7	9	1–6	7	8
Lysine	3–8	2–10	—	4–6	2–6	7	3–8	6	5–6	<1–4	6–7	6
Methionine	1–5	<1–2	—	1	1–3	2	1–3	3	2	<1–10	2	3
Phenylalanine	3–7	2–8	—	2–5	2–6	4	2–6	5	5–7	<1–3	5	5
Threonine	3–7	<1–6	—	4–6	2–5	5	4–6	5	6	1–3	4–5	5
Tryptophan	<1–3	1–10	—	—	1–3	1	<1–4	3	<1–2	<1–2	1–2	1
Valine	3–7	2–7	—	3–5	2–6	5	3–7	5	6	<1–5	5	10
Fatty acid (% of total FAME)												
14:0	—	—	23	<1–1	17	7	1–8	4–7	—	1–4	2–4	1–12
16:0	26–45	14	17	12–29	12	26	11–43	11–32	15–16	16–38	14–25	14–46
18:0	2	1	<1	1–3	1	—	1–11	1–2	1	1–2	3	<1–9
16:1n-7	—	2	28	<1–1	3	38	2–31	19–43	2–3	<1	1–26	<1–13
17:1	—	4	—	<1–5	—	—	<1–10	—	4–5	—	—	—
18:1n-6	10–17	—	—	—	—	—	—	—	—	—	—	—
18:1n-9	—	45–47	1	5–44	7	—	1–12	3–9	24–30	<1–27	4–7	<1–43
18:1n-7	—	1	1	—	1	—	—	<1	—	<1	1–2	<1–10
16:2n-6	—	3–4	—	—	—	—	—	—	2	—	1	—
16:2n-7	—	—	—	—	2	—	—	1–2	—	—	—	—
16:3n-4	—	—	—	—	—	—	—	1–4	—	—	—	—
16:4n-3	—	<1	—	—	—	—	—	—	9–12	—	16–18	—
18:2n-6	11–12	21	1	20–33	<1–4	3	<1–19	1–6	13	<1–2	4–7	<1–10
18:3n-6	17–40	—	1	1–15	<1–1	—	<1–2	—	—	<1	—	<1–1
18:3n-3	—	6–7	<1	<1–40	1–6	1	<1–32	<1–3	18–23	<1	5–22	—

	Arthrospira	*Chlorella*	*Entomoneis*	*Haematococcus*	*Isochrysis*	*Nanofrustulum*	*Nannochloropsis*	*Phaeodactylum*	*Scenedesmus*	*Schizochytrium*	*Tetraselmis*	*Thraustochytrium*
18:4n-3	—	1	<1	1–6	4–19	—	<1–3	<1–1	2–3	<1	2–8	<1–1
20:4n-6	—	—	6	<1–7	<1	4	1–6	<1–1	—	1	<1–4	<1–15
20:5n-3	—	—	17	<1–1	<1–28	9	<1–28	8–35	—	1–16	2–8	1–20
22:5n-6	—	—	—	—	2	—	—	—	—	1–7	—	<1–21
22:6n-3	—	—	1	—	5–14	—	<1–3	<1–2	—	18–44	<1	3–68
Mineral (%)												
Calcium	0.1–1.4	<0.1–0.6	—	—	0.6	—	0.1	0.3	0.1–0.2	—	3.0	—
Magnesium	0.2–0.3	0.1–0.8	—	—	1.0	—	0.3	0.7	0.1–0.2	—	0.4	—
Phosphorous	0.1–1.3	0.3–1.8	—	—	<0.1–2.6	—	0.7	1.2	0.5–0.7	—	1.5	—
Potassium	0.6–2.6	<0.1–2.1	—	—	1.2	—	1.5	2.4	0.6–0.7	—	1.9	—
Sodium	0.4–2.2	<0.1–1.3	—	—	1.6	—	1.0	2.7	0.1	—	0.9	—
Sulfur	—	—	—	—	—	—	0.6	1.4	—	—	1.4	—
Trace element (mg kg^{-1})												
Copper	4	22–1900	—	—	—	—	18	55	15–25	—	102	—
Iron	539–1800	198–6800	—	—	15	—	1395	4773	1081–1777	—	1774	—
Manganese	19–37	20–4000	—	—	801	—	151	45	74–119	—	191	—
Selenium	2	1	—	—	—	—	<1	<1	<1–1	—	<1	—
Zinc	14–40	6–5500	—	—	19	—	32	50	38–63	—	64	—
Heavy metal (mg kg^{-1})												
Arsenic	<0.1–2.9	0.1–0.5	—	—	—	—	—	—	<0.1–2.4	—	—	—
Cadmium	<0.1–1.0	<0.1–0.1	—	—	—	—	—	—	<0.1–1.7	—	—	—
Mercury	<0.1–0.5	<0.1–0.1	—	—	—	—	—	—	<0.1–0.4	—	—	—
Lead	0.1–5.1	<0.1–2.0	—	—	—	—	—	—	0.6–6.0	—	—	—

Table 2. Biochemical composition of various genera of microalgae evaluated for use in salmonid feeds (dry weight basis).

heterogeneous. **Table 2** shows the FA composition of microalgae genera that have been evaluated for salmonid feeds. The only discreet trend is that the lipid fraction of most species is dominated by the saturated FA (SFA) palmitic acid (16:0) and the monounsaturated FA (MUFA) oleic acid (18:1n-9); which combined generally account for about 40% of total FAs. Many marine and freshwater species, particularly *Scenedesmus* and *Tetraselmis*, produce significant levels (~10% of total FAs) of the n-3 PUFA α-linolenic acid (18:3n-3; ALA) which, once in the body, can be desaturated and elongated as a metabolic precursor for endogenous cellular biosynthesis of the essential n-3 LC-PUFAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). However, while this is the case for most monogastric animals (including humans and salmonid fish), the extent to which this occurs in animals is limited and dependent upon activity levels of elongase and Δ5 and Δ6 desaturase enzymes in their tissue cells. In fact, this endogenous biosynthesis of n-3 LC-PUFA from ALA is rate-limiting in salmonids such as rainbow trout to a relatively low efficiency of 12–27% depending on various other dietary and farming conditions and thus essential n-3 LC-PUFA must still be added to salmonid diets. There are several, almost exclusively marine, photoautotrophic microalgae (reviewed by Colombo et al. [5]) that are good accumulators of EPA (up to 53% of total FAs), namely the marine genera *Chromophyte*, *Dunaliella*, *Isochrysis*, *Nannochloropsis*, *Pavlova*, *Phaeodactylum* and *Skeletonema*. However, the only ones evaluated in salmonid feeds are *Isochrysis* and *Nannochloropsis* (up to 28% of total FAs). The only known marine microalgae genera currently capable of industrial production of DHA at high levels are the *Thraustochytrids*, such as *Schizochytrium* and the dinoflagellates *Crypthecodinium*; all of which may accumulate up to 68% of total FAs as DHA. However, these species do not perform at high efficiency under photoautotrophic cultivation. As such, they are now cultivated heterotrophically in the absence of light in large-scale fermentation systems using organic carbon sources for industrial production of food-grade DHA and are available commercially in various processed forms (e.g., whole-cell lipid-rich powders, extracted oil emulsions, etc.). The aquaculture feed market is in desperate need of these DHA-rich oils as the Global Salmon Initiative (GSI) has indicated that its members could immediately take up 200,000 t annually of this novel alternative lipid source if it were available [6].

3.3.2. Elemental composition

There are limited data on elemental composition of microalgae and this is in contrast to macroalgae (seaweeds) where numerous species have been well characterized. This is not overly surprising as it is well-documented that most microalgae (excluding some diatoms) typically contain far less inorganic (ash) content (generally <20%) than seaweeds (22–64%). **Table 2** shows the mineral and trace element composition of microalgae genera that have been evaluated for salmonid feeds. With regard to the minerals most often required by farmed salmonids and therefore routinely supplemented in aquafeeds, calcium and magnesium levels in algal biomass are generally around 0.4% each while *Tetraselmis* appears to contain far higher levels of calcium (3%). Phosphorous levels in microalgae evaluated with salmonids are in the range from <0.1 to 2.6% but on average are around 1%. Potassium, sodium and sulfur levels are around 1–2% but appear more highly variable in the literature at <0.1–2.6%, <0.1–2.7% and 0.6–1.4%, respectively. For farmed salmonids, phosphorous (P) is the most limiting macromineral and is therefore

routinely supplemented in formulated feeds in various inorganic forms (e.g., calcium phosphates, sodium phosphates, potassium phosphates, ammonium phosphate and defluorinated rock phosphate) which are highly digestible by salmonid fish. One of the reasons for the high dietary demand for P by farmed salmonids is related to its critical role, along with calcium (Ca) and vitamin D, in the development and maintenance of the skeletal system and maintaining acid-base homeostasis in rapidly growing farmed fish. In salmonids, dietary P and body Ca pools become complexed together to form the principle component of their bone structure, known as hydroxyapatite ($Ca_5(PO_4)_3(OH)$). Fortunately, farmed salmonids are able to obtain the majority of their Ca needs from the surrounding water via direct absorption through their skin, scales and gills. However, fresh and marine culture water is generally low in P, so its requirement in the feed is highest of all macrominerals. Because of the importance of hydroxyapatite formation to healthy fish, it is not only the individual body pools of Ca and P that are important, but also their relative proportions to each other. As a result, the so-called Ca:P ratio is one of the most important considerations for mineral nutrition of farmed salmonids as it can influence their bioavailability, metabolism and physiological utilization and can also increase under-utilized P discharge into the aquatic environment and a ratio of 2:1 or less is recommended. A substantial imbalance in this ratio, especially if compounded by vitamin D deficiency, can result in poor growth performance, inferior feed conversion efficiency, anorexia and, in severe cases, skeletal deformations. The literature data for Ca:P ratio in microalgae that have been evaluated for salmonid feed applications is highly variable; ranging from 0.1:1 to 2:1. However, other common ingredients used in commercial salmonid feeds are also highly variable with lower ranges for terrestrial plant-based sources like conventional biofuel by-products (0.2–0.6:1), oilseeds (0.1–0.5:1) and grains (0.1–0.2:1) and far higher ranges for typical marine-based sources such as marine fish and crustacean by-products (1.3–9.1:1) and kelps (7.5:1). There are several likely reasons for the variations in Ca and P levels in microalgae including species differences, time of harvest and post-harvest downstream processing conditions. Historically, a large percentage of P in farmed salmonid feeds came from the mineral fractions of animal-based protein sources such as rendered animal by-products and fish meals, which are generally well digested (typically >50%) by salmonids. However, as these ingredients have become increasingly replaced by terrestrial plant-based protein sources in modern farmed salmonid feeds, the requirement for costly inorganic P supplementation has increased. This is because, unlike animal-based sources, total P levels in most plants are lower and, of that P, most is stored in the form of inositol polyphosphate, also known as phytic acid. This compound, when chelated with other minerals and trace elements such as divalent cations like Ca^{2+}, Mg^{2+} and Fe^{2+} in the feed, forms poorly digestible phytate. So, in addition to these plant-based ingredients supplying lower total levels of P to salmonid diets, phytate is also poorly digested; thus the availability of phytate-bound P is poor (generally <50%) for salmonids and can also act antagonistically to reduce the digestibility of protein and other essential minerals. In this regard, microalgae-based ingredients (although also plant-based) could potentially offer a great benefit for use in farmed salmonid feeds since it is believed that microalgae cells predominantly store inorganic P in vacuoles as polyphosphate granules, which may be more bioavailable for gastric liberation and intestinal digestion and absorption. Indeed, Tibbetts et al. [7] recently demonstrated in juvenile Atlantic salmon that dietary P digestibility was significantly higher in feeds containing more than 18% *Chlorella vulgaris* meals compared to an algae-free control diet based on fish meal and plant-based protein

ingredients, despite the fact that total dietary P levels were similar. Trace element composition of microalgae evaluated for use in salmonid feeds is highly heterogeneous for copper (4–1900 mg kg^{-1}), iron (15–6800 mg kg^{-1}), manganese (19–4000 mg kg^{-1}) and zinc (14–5500 mg kg^{-1}) while that of selenium is rather consistent (1 mg kg^{-1}). In general, the mineral and trace element composition of microalgae does not appear particularly unique relative to other common terrestrial plant-based salmonid feed ingredients, with the exception of iron (Fe). According to the literature, the Fe content of microalgae-based ingredients used in salmonid feed experiments is particularly rich at up to 0.7% of the biomass; which is high for a trace element. Fe is a key essential trace element required by salmonids and is associated with its critical role in cellular respiration, oxygen transport, acid-base balance and energy metabolism. As such, adequate Fe levels are required in the diet of salmonids as it forms a vital component of the red blood cells (erythrocytes) hemoglobin and plasma-transported circulatory system enzymes. Studies have shown that when dietary Fe is limited farmed salmonids generally become anemic so their feeds are typically supplemented with Fe at 30–60 mg kg^{-1} of diet. As companies producing salmonids feeds continue to search for natural sources of key nutrients to replace expensive chemically-synthesized feedstocks, these high levels of Fe may provide a unique and highly-marketable property for certain microalgae-based products. The high Fe content of many microalgae-based ingredients, relative to other common terrestrial plant-based salmonid feed ingredients, is likely due to the fact that most microalgae products generally contain the entire dried organism, including their chloroplast proteins responsible for photosynthesis, whereas other plant-based ingredients are produced from only the seeds which are non-photosynthetic. It is well documented that Fe is a principle component within the photosynthetically active cytochrome proteins (such as ferredoxin) in microalgal cells, responsible for electron transport to produce energy-rich components such as $NADPH_2$. Rather surprisingly, despite the fact that many phytoplankton are able to bioaccumulate environmental contaminants, there is a scarcity of information on the heavy metal contents of microalgae in the literature. **Table 2** shows the heavy metal composition of three microalgae genera that have been evaluated for salmonid feeds. Reported values for *Arthrospira* (formerly *Spirulina*), *Chlorella* and *Scenedesmus* for the key heavy metals of interest are arsenic (<0.1–2.9 mg kg^{-1}), cadmium (<0.1–1.7 mg kg^{-1}), mercury (<0.1–0.5 mg kg^{-1}) and lead (<0.1–6.0 mg kg^{-1}). Nearly all of these levels are several magnitudes lower than the proposed upper limits for safe consumption as animal feeds. However, most microalgae studied have been cultivated under pristine laboratory conditions using clean water, chemically-defined nutrient media and pure CO_2; whereas, industrial farming of microalgae is highly likely to utilize industrial flue-gas emissions and/or municipal or agro-industrial wastewaters as more cost-effective crop inputs. As such, safety and efficacy evaluation of microalgae-based ingredients for salmonids feeds must be made a priority consideration in the future, both by producers and regulatory bodies, as reviewed by Shah et al. [8]. As a starting point, several safety standards for microalgae consumption by humans was recently summarized by Matos [9]; including microbiological and insect contamination limits, and these standards could be reviewed and verified for their suitability for salmonid aquafeed applications.

3.3.3. Vitamin and carotenoid composition

Despite commercial claims of microalgae being vitamin-rich, there are minimal data in the literature on vitamin concentrations for a small number of species; namely *Arthorspira* (*Spirulina*),

Chlorella and *Scenedesmus*. Of the fat-soluble vitamins, values range widely for retinol (vitamin A; 8–84 mg 100 g^{-1}) and tocopherol (vitamin E; <1–2787 mg 100 g^{-1}) while menadione (vitamin K) concentrations are consistent (1 mg 100 g^{-1}). Reports for cholecalciferol (vitamin D) could not be found. Of the water-soluble vitamins, microalgae (based solely on *Chlorella*) appear richest in biotin (vitamin B_7; 192 mg 100 g^{-1}) but highly variable in both cobalamin (vitamin B_{12}; <1–126 mg 100 g^{-1}) and ascorbic acid (vitamin C; 8–100 mg 100 g^{-1}). Lower, and generally more consistent, concentrations are reported for thiamine (vitamin B_1; <1–5 mg 100 g^{-1}), riboflavin (vitamin B_2; 3–6 mg 100 g^{-1}) and pyridoxine (vitamin B_6; <1–5 mg 100 g^{-1}) while intermediate levels are reported for folic acid (vitamin B_9; <1–27 mg 100 g^{-1}), niacin (vitamin B_3; 11–32 mg 100 g^{-1}) and pantothenic acid (vitamin B_5; 1–22 mg 100 g^{-1}). Since many natural carotenoids display antioxidant-like properties in the body, there has been interest in their characterization in many microorganisms in recent years. In salmonid feeds, carotenoids generally represent high-value components when added either as dietary pigments (namely astaxanthin and/or canthaxanthin) or as biological antioxidants. However, in the former case, almost all commercial astaxanthin and canthaxanthin used in commercial salmonid feeds is synthetically produced and the industry is encouraged to replace these additives with more natural sources. Of the studies that have evaluated microalgae for salmonid feeds, very few reported their carotenoid composition. Based on limited data, chlorophyll content is in a fairly narrow range of 5–37 mg g^{-1} (average, 13 mg g^{-1}) and the samples appeared virtually devoid (generally <1 mg g^{-1}) of α-carotene, fucoxanthin, lycopene and zeaxanthin. Certain species may contain trace amounts of β-carotene (<12 mg g^{-1}) and lutein (<4 mg g^{-1}). While reported ranges are vast, some genera (e.g., *Chlorella* and *Haematococcus*) cultivated under optimized conditions have good potential for accumulation of astaxanthin (up to 550 mg g^{-1}) and canthaxanthin (up to 362 mg g^{-1}). Indeed, there are now commercially-available 'natural-source' astaxanthin products on the market for salmonid feeds that are produced from *Haematococcus* microalgae. However, the vast majority of natural-source astaxanthin used in salmonid feeds (mostly for organic certification) are produced from the bacteria *Paracoccus carotinfaciens* and the yeast *Phaffia rhodozyma*. Nonetheless, several companies globally are ramping up production of 'natural-source' astaxanthin from *Haematococcus* microalgae as the global salmonid feed sector continues to grow. Additionally, several workers are optimizing production of various strains of *Scenedesmus* for high accumulation of lutein, which is used as a high-value additive in poultry and fish feeds, cosmetics, drugs and health foods (~$300 million USD annually) and currently only comes from commercially farmed marigold petals.

3.4. Nutritional evaluation of microalgae for use in salmonid feeds

When evaluating the nutritional quality of potential novel ingredients for aquaculture feeds, nutritionists take a logical step-wise approach which generally involves: (1) comprehensive characterization of their major biochemical components, trace elements, possible anti-nutritional factors (ANFs) and contaminants; (2) assessment of the palatability of diets containing these novel ingredients to estimate their potential effects on feed consumption/feed refusal; (3) estimations of their nutrient digestibility through *in vitro* simulated enzymatic assays or measurement of nutrient digestibility using 'species-specific' digestive enzymes from the target animal species, which may be *in vitro* or *in vivo* (or a combination of both) and finally (4) validation of nutritional quality through *in vivo* studies with the target species to assess

various biological metrics (e.g., growth performance, nutrient utilization, expression of genes related to nutrient metabolism, intestinal and general animal health, product quality, etc.). Engle [10] appropriately points out other important logistical considerations that are often overlooked when evaluating new aquafeed ingredients, such as those based on microalgae. These include the importance of considering what impact(s) dietary inclusion of the novel ingredient might have on the functional and rheological properties of combined diet mixtures, finished pellet quality, product shelf-life and how it fits into established complex ingredient distribution and feed processing infrastructure within aquafeed production facilities. While there are estimates that up to 30% of the annual global microalgae supply is sold for animal feeds, the reality is that many of the aforementioned nutritional evaluation steps are incomplete or totally lacking for most microalgae-based aquafeed ingredients. Despite the encouraging trend towards microalgae-based ingredients for salmonid aquaculture, many of the nutritional claims lack scientific evidence because their required biochemical profiles, nutrient digestibility data, effect on the physical properties of compound aquafeeds and their effects on farmed salmonid performance are at best inadequate and typically non-existent. We can take *Chlorella* as an example, which are some of the most biotechnologically relevant microalgae for industrial applications. While these microalgae have long been proposed for large-scale cultivation for bioremediation, renewable energy feedstocks, health food supplements and sustainable animal and aquaculture feeds, there has never been a full and adequate strategic assessment of their nutritional quality as feed ingredients for salmonids; which are likely the most widely farmed coldwater fishes globally. This is also the typical case for virtually all other microalgae species under consideration for industrial mass algaculture for use in aquafeeds. While the present state of knowledge on the use of microalgae-based ingredients in salmonid feeds is still relatively scarce, the available literature has been summarized in this chapter (**Tables 3**–**6**) and discussed in the next sections.

3.4.1. Microalgae-based ingredients as protein sources

When evaluated as dietary protein sources for salmonid aquafeeds (**Tables 3**, **4**), studies have been conducted using various freshwater and marine microalgae genera with rainbow trout (*Arthrospira*, *Chlamydomonas*, *Nannochloropsis* and *Scenedesmus*), Artic charr (*Arthrospira*), Atlantic salmon (*Arthrospira*, *Chlorella*, *Desmodesmus*, *Entomoneis*, *Nannochloropsis*, *Nanofrustulum*, *Phaeodactylum* and *Tetraselmis*) and mink, *Mustela vison* (*Isochrysis*, *Nannochloropsis* and *Phaeodactylum*) as a proxy for Atlantic salmon. With rare exception, the microalgae cells tested were not cell-ruptured or their processing was left unspecified. This immediately puts into question the digestibility of these ingredients as most are known to possess highly recalcitrant cell walls and digestibility represents the first bottleneck for nutrient assimilation by an animal after consumption. Depending upon the microalgae species tested, salmonid species under investigation, the extent of downstream processing (e.g., drying, de-fatting, cell-rupture) and the methodologies applied, apparent digestibility coefficients (ADCs) for the various microalgae studied with salmonids are in a large, highly variable range of 32–85% (dry matter), 19–87% (protein), 55–94% (lipid), 51–83% (energy), 24–85% (carbohydrate), 27–99% (phosphorous), 81–102% (EAAs) and 59–93% (FAs). Based on feed intake, digestibility, growth performance, feed and nutrient utilization efficiency, whole-body and muscle composition,

Genera	Form	Inclusion levels	Main findings	Ref.
Arthrospira	Whole-cell meal	0–9%	Can be included at 7% for rainbow trout without adverse effects on growth and body composition.	[11]
Arthrospira	Whole-cell meal	0–10%	Rainbow trout fed diets with 10% *A. platensis* lost 50% less weight during a short-term fast.	[12]
Arthrospira	Whole-cell meal	0–10%	Rainbow trout fed up to 10% *A. platensis* had higher plasma red and white blood cell counts, plasma hemoglobin, serum protein, albumin and high-density lipoprotein cholesterol, reduced serum low-density lipoprotein, cholesterol, cortisol and glucose and levels were unchanged for hematocrit, serum total cholesterol, triglycerides and lactate.	[13]
Arthrospira	Whole-cell meal	0–30%	Digestibilities of *A. platensis* for Arctic charr were: organic matter (80%), dry matter (78%), protein (82%), energy (83%), phosphorous (99%) and EAAs (81–102%).	[14]
Nannochloropsis	Whole-cell meal	100%	Protein digestibilities of 79–87% were estimated for *N. granulata* by *in vitro* pH-Stat using rainbow trout stomach and pyloric caeca enzymes.	[15]
Scenedesmus / *Chlamydomonas*	Whole-cell meal	0–50%	*Scenedesmus* sp. / *Chlamydomonas* sp. blend can be included at 12.5% for rainbow trout without affecting growth and body composition.	[16]

Table 3. Present state of knowledge on dietary protein replacement with microalgae in farmed rainbow trout (*Oncorhynchus mykiss*) and Arctic charr (*Salvelinus alpinus*) feeds.

blood histochemistry, intestinal health and gene expression, it appears that salmonids can only tolerate low inclusion levels (<10% of the diet) of whole-cell *Arthrospira*, *Chlorella*, *Entomoneis*, *Isochrysis*, *Nannochloropsis*, *Phaeodactylum* and *Tetraselmis*. On the other hand, salmonids appear to tolerate higher inclusion levels (up to 20% of the diet) of whole-cell *Scenedesmus*/*Chlamydomonas* blend, de-fatted *Desmodesmus* and *Nanofrustulum* and cell-ruptured *Chlorella*. Commercially-produced microalgae-based ingredients presently available on the market are almost exclusively produced from *Arthrospira* (*Spirulina*), *Chlorella* and *Nannochloropsis*, while a few products are produced from *Isochrysis*, *Staurosira* and *Euglena*.

3.4.2. Microalgae-based ingredients as lipid sources

The dietary essential n-3 LC-PUFAs, EPA and DHA, required by farmed salmonids have traditionally been supplied by fish oil, which is manufactured from wild-caught pelagic fish deemed unsuitable for direct human consumption, and this practice is no longer ecologically or economically sustainable. Historically, consumption of fatty fish like salmonids was the best means at achieving the recommended daily intake of 500–1000 mg of EPA and DHA for support of cardiovascular and neuronal health. However, partial or total replacement of fish oils in farmed salmonid feeds with terrestrial lipid sources has started to diminish the content of these essential n-3 LC-PUFAs. While rendered animal fats and vegetable oils commonly used in modern salmonid feeds provide excellent sources of digestible energy (calories) for farmed fish, they lack essential n-3 LC-PUFA that are responsible for dietary health benefits

Genera	Form	Inclusion levels	Main findings	Ref.
Arthrospira	Whole-cell meal	0–30%	Digestibilities of *A. platensis* for Atlantic salmon were: organic matter (85%), dry matter (82%), protein (85%), energy (83%), phosphorous (27%) and EAAs (83–101%).	[14]
Arthrospira	Whole-cell meal	0–11%	*A. platensis* can be included at 11% for Atlantic salmon without affecting growth performance and feed utilization.	[17]
Chlorella	Whole-cell and cell-ruptured meals	0–30%	EAA indices are high (0.9) for *C. vulgaris*. Average digestibilities of whole-cell and cell-ruptured *C. vulgaris*, respectively, for Atlantic salmon were: protein (77 and 87%), EAAs (84 and 91%), carbohydrate (38 and 81%), starch (40 and 80%), energy (55 and 76%), lipid (67 and 85%), SFAs (61 and 62%), MUFAs (59 and 88%) and PUFAs (63 and 93%).	[7]
Chlorella	Cell-ruptured meal	0–20%	20% *C. vulgaris* combined with 20% soybean meal counteracted the negative effects of soybean meal induced enteropathy (SBMIE) in Atlantic salmon, however growth was reduced and digestibility was not measured.	[18]
Desmodesmus	Lipid-extracted meal	0–20%	Defatted *Desmodesmus* sp. can be included at 20% for Atlantic salmon without effects on growth, feed utilization, body/muscle composition and intestinal health and digestibilities were: protein (84%), lipid (94%) and energy (80%).	[19]
Desmodesmus	Lipid-extracted meal	0–30%	Digestibilities of *Desmodesmus* for Atlantic salmon were: dry matter (32–47%), protein (54–67%), ash (41–73%) and energy (51%) and extrusion processing can increase the digestibility compared to cold-pelleting.	[20]
Entomoneis	Whole-cell meal	0–5%	*Entomoneis* can be included at 5% for Atlantic salmon without affecting growth performance and body n-3 LC-PUFA was increased. Digestibilities were: dry matter (69–70%), protein (83–85%), lipid (87–88%) and nitrogen-free extract (24–31%).	[21]
Isochrysis	Whole-cell meal	0–24%	*I. galbana* cannot be included at any level without reducing digestibility in mink[1] (estimated protein digestibility was 19%).	[22]
Nannochloropsis	Lipid-extracted meal	0–30%	Digestibilities of *Nannochloropsis* for Atlantic salmon were: dry matter (48–63%), protein (72–73%), ash (36–80%) and energy (60%) and extrusion processing can increase digestibility compared to cold-pelleting.	[20]
Nannochloropsis	Whole-cell meal	0–24%	*N. oceanica* cannot be included at any level without reducing digestibility in mink[1] (estimated protein digestibility was 35%).	[22]
Nanofrustulum	Lipid-extracted meal	0–17%	Defatted *Nanofrustulum* can be included at 17% for Atlantic salmon without affecting growth, feed utilization, body and muscle composition.	[23]
Phaeodactylum	Whole-cell meal	0–12%	*P. tricornutum* can be included at 6% for Atlantic salmon without affecting digestibility, feed utilization and growth performance.	[24]
Phaeodactylum	Whole-cell meal	0–24%	*P. tricornutum* can be included at 6–12% without affecting digestibility in mink[1] (estimated protein digestibility was 80%).	[22]
Tetraselmis	Whole-cell meal	0–7%	*Tetraselmis* can be included at 7% for Atlantic salmon without affecting growth, feed utilization, body and muscle composition.	[23]

[1]As a proxy for Atlantic salmon.

Table 4. Present state of knowledge on dietary protein replacement with microalgae in farmed Atlantic salmon (*Salmo salar*) feeds.

Genera	Form	Inclusion levels	Main findings	Ref.
Crypthecodinium	Whole-cell meal	0–9%	*C. cohnii* can be included at 6% to restore muscle DHA levels of rainbow trout fed plant oil only diets.	[25]
Schizochytrium	Whole-cell meal	0–20%	*Schizochytrium* included at 20% for rainbow trout did not affect digestibilities of protein, EAAs or ash but levels above 13% reduced those for dry matter, energy, lipid and FAs.	[26]
Schizochytrium	Whole-cell meal	0–5%	*Schizochytrium* included at 5% for rainbow trout improved growth rates and condition factors (although not statistically) and distal intestinal 'global' microbiome was not negatively affected. Lactic acid bacterial (LAB) community (considered beneficial to healthy intestinal epithelium) were elevated.	[27]
Schizochytrium	Whole-cell meal	0–20%	When included at 13% for Atlantic salmon, *Schizochytrium* did not affect digestibilities of dry matter and protein but levels above 7% reduced those for lipid and most FAs.	[26]
Schizochytrium	Whole-cell meal	0–11%	*Schizochytrium* inclusion at 11% for Atlantic salmon effectively reduced harmful persistent organic pollutants in diets and muscle tissues, restored muscle DHA levels but muscle EPA levels were reduced. Growth performance was compromised above 5.5% inclusion.	[28]
Schizochytrium / Yeast extract	Whole-cell meal	0–15%	*Schizochytrium* / Yeast blend can be included at 6% for Atlantic salmon to partially replace fish oil without affecting growth, feed utilization, digestibility, product quality or intestinal health.	[29]
Schizochytrium	Whole-cell meal	0–10%	*Schizochytrium* can be included at 10% for Atlantic salmon without affecting growth performance, biological and biochemical parameters and immune response, however, after a disease challenge, cumulative fish mortality was higher than the control fish.	[30]

Table 5. Present state of knowledge on dietary lipid replacement with microalgae in farmed rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*) feeds.

associated with fatty seafood consumption. Terrestrial based oils and fats in salmonid feeds has come at the expense of EPA and DHA levels in the end product for the consumer. As a result, there is tremendous interest and forward momentum for the partial or total replacement of conventional fish and plant oils and animal fats in salmonid feeds with high n-3 LC-PUFA products of microalgal origin. The most suitable candidates are predominantly strains of *Schizochytrium* and *Crypthecodinium*. In fact, this area is presently the most advanced and first 'out-of-the-gate' in terms of making a real difference in salmonid feeds, with several products now on the market that are rapidly being added to the feedstock portfolios of global salmon aquafeed manufacturers. In addition to their ecological role in reducing pressures on wild stocks for reduction to fish meal and oil, there appear to be additional health benefits as well, which are currently being explored. Since heterotrophic cultivation of these strains is conducted under highly controlled fermentation conditions, the resulting ingredients are generally free of environmental contaminants like heavy metals, dioxins and PCBs; for which the conventional fish oil industry has received criticism. When evaluated as dietary lipid sources for salmonid aquafeeds (**Table 5**), studies have been conducted using these marine microalgae with rainbow trout (*Crypthecodinium* and *Schizochytrium*) and Atlantic salmon (*Schizochytrium*). In all cases, the ingredients tested were in a whole-cell (e.g., not cell-ruptured) dry powder form at levels of up to 20% of the diet and it was found that inclusion levels higher than

Genera	Form	Inclusion levels	Main findings	Ref.
Arthrospira	Whole-cell meal	0–10%	Inclusion of 7.5% *A. platensis* for rainbow trout resulted in suitable growth and skin/muscle carotenoid deposition and pigmentation.	[31]
Arthrospira	Whole-cell meal	0–10%	Inclusion of 10% *A. platensis* for rainbow trout resulted in high serum carotenoid levels which were positively correlated with growth, feed utilization, muscle carotenoid levels and muscle color. Serum carotenoid levels can be used to predict post-harvest fillet pigmentation levels.	[32]
Chlorella	Whole-cell meal	0–64 mg Ax^1/Cx^2 blend kg $diet^{-1}$	Muscle pigment levels of rainbow trout fed *C. vulgaris* were 1.5 times higher than those fed the control diet containing synthetic pigments; however, the control diet contained less than half of the dietary pigment; so the study was confounded.	[33]
Chlorella	Whole-cell meal	0–64 mg Ax/Cx blend kg $diet^{-1}$	Inclusion of *C. vulgaris* had no effects on feed intake or growth performance of rainbow trout but muscle pigment levels were reduced and carotenoid retention less efficient than synthetic pigments.	[34]
Haematococcus	Cell-ruptured meal	0–73 mg Ax kg $diet^{-1}$	All measured parameters were inferior when Ax was supplied by *H. pluvialis* in rainbow trout diets compared to synthetic Ax.	[35]
Haematococcus	Cell-ruptured meal	0–60 mg Ax kg $diet^{-1}$	*H. pluvialis* Ax is mostly (~88%) of the 3*S*,3′*S* optical stereoisomer, which was also reflected in rainbow trout muscle tissues and fillet color scores were the same as fish fed synthetic Ax. Coefficient of distance is useful to distinguish fish muscles tissues fed natural or synthetic Ax but is not sensitive enough to distinguish between various natural sources.	[36]
Haematococcus	Whole-cell meal	0–6% of diet (42 mg Ax / 44 mg Cx blend kg $diet^{-1}$)	Muscle carotenoid retention of rainbow trout fed 6% *H. pluvialis* was less than half that of those fed synthetic carotenoids and was attributed to the lack of cell-rupture and the small fish size used.	[37]
Haematococcus	Whole-cell meal	0–1%	Inclusion of 0.3% *H. pluvialis* for rainbow trout enhanced the antioxidant system and modulation of lipid and glucose metabolism, however, 1% raised serum aspartate aminotransferase (ASTA) activity indicating impaired liver function.	[38]
Haematococcus	Cell-ruptured meal	0–74 mg Ax kg $diet^{-1}$	Scalable high-pressure processing of *H. pluvialis* followed by spray-drying was effective at cell rupture without damaging carotenoid composition. *H. pluvialis* Ax optical isomer composition reflected that of rainbow trout muscle tissues but not skin. Growth and feed efficiency were not affected compared to those fed synthetic Ax but digestibility reduced.	[39]
Haematococcus	Cell-ruptured meal	0–50 mg Ax kg $diet^{-1}$	Serum Ax levels were reduced in rainbow trout fed *H. pluvialis* (esterified form) compared to synthetic (free form). Ax absorption is greater in the anterior intestine than the posterior, irregardless of form.	[40]
Haematococcus	Extracted oil	0–40 mg Ax kg $diet^{-1}$	Inclusion of Ax-rich oil extracts from *H. pluvialis* had no effects on rainbow trout growth. Natural esterified Ax is as efficiently utilized as synthetic free-form Ax.	[41]

Genera	Form	Inclusion levels	Main findings	Ref.
Haematococcus	Whole-cell meal	0–30 mg Ax kg diet^{-1}	Inclusion of *H. pluvialis* had no effects on female rainbow trout reproductive performance or egg protein and triglyceride content. Small (albeit inconsistent) improvements in egg lipid peroxidation and glutathione peroxidase activities noted.	[42]
Haematococcus	Whole-cell and cell-ruptured meals	0–40 mg Ax kg diet^{-1}	Inclusion of *H. pluvialis* in any form had no effect on growth performance of rainbow trout. Muscle and skin pigmentation was highest in fish fed synthetic Ax, followed by cell-ruptured *H. pluvialis* and the lowest was whole (intact) *H. pluvialis*. Unfortunately, the rate of cell wall breakage for cell-ruptured *H. pluvialis* was low (~60%).	[43]
Haematococcus	Cell-ruptured meal	0–80 mg Ax kg diet^{-1}	Weight gain of rainbow trout fed *H. pluvialis* equivalent to 40–80 mg Ax kg^{-1} was the same as those fed a diet with 80 mg Ax kg^{-1} synthetic Ax, however muscle and skin Ax deposition was less efficient than with synthetic Ax. As with the previous study, the rate of cell wall breakage for cell-ruptured *H. pluvialis* was low (~60%). Muscle tissues of fish fed diets with *H. Pluvialis* at any level contained significantly higher adonirubin, which may explain lower fillet color scores.	[44]

[1]Ax = astaxanthin.
[2]Cx = canthaxanthin.

Table 6. Present state of knowledge on dietary carotenoid replacement with microalgae in farmed rainbow trout (*Oncorhynchus mykiss*) feeds.

10–13% reduced nutrient digestibility for rainbow trout and Atlantic salmon. Moderately low dietary inclusion levels (5–7%) may enhance the beneficial microbiome of salmonids and reduce the concentrations of harmful persistent organic pollutants (POPs) in feeds and fish muscle tissues. Commercially-produced microalgae-based ingredients presently available on the market to supply n-3 LC-PUFA are almost exclusively produced from *Crypthecodinium* and *Schizochytrium* while a few products are produced from *Isochrysis*, *Nannochloropsis*, *Odentella*, *Tetraselmis* and *Ulkenia*.

3.4.3. Microalgae-based ingredients as carotenoid sources

In addition to microalgae as sources of essential nutrients, energy and LC-PUFAs, many also synthesize carotenoids and phycobiliproteins. Of particular interest is astaxanthin, which has become a rapidly growing area of study for the farmed salmonid aquafeed industry. The three predominant sources of commercially-available astaxanthin are chemical synthesis, yeast fermentation and algal induction. The cost of each are estimated at: synthetic (~$2,000 kg^{-1}) < *Phaffia* yeast (~$2,500 kg^{-1}) < *Haematococcus* microalgae (~$7,000 kg^{-1}), so it is clear that production costs must be greatly reduced before for the salmonid aquaculture industry is likely to shift to the wide use of astaxanthin derived from *Haematococcus* algae. However, the industry is feeling ever-growing pressure to reduce their reliance on synthetic astaxanthin, which is presently dominated by the commercial products Carophyll® Pink (DSM Nutritional Products)

and Lucantin® Pink (BASF Corporation). This represents an environmental and societal-driven opportunity for *Haematococcus*-based ingredients as 'natural-source' astaxanthin. In fact, the high oxygen radical-scavenging absorbance capacity (ORAC) antioxidant potential reported for *Haematococcus pluvialis*-derived astaxanthin and the fact that it is predominantly esterified (~94%), indicates its higher oxidative stability than synthetic astaxanthin, which is in a non-esterified (free) form. Additionally, *Haematococcus pluvialis*-derived astaxanthin has been certified as safe for human, animal and fish consumption, unlike synthetic astaxanthin. When evaluated as dietary carotenoid sources for salmonid aquafeeds (**Table 6**), studies have been conducted using various freshwater and marine microalgae genera with rainbow trout (*Arthrospira*, *Chlorella* and *Haematococcus*). The ingredients tested were inconsistent in their form, where some studies confirmed it to be a cell-ruptured dry powder while others used whole-cell (intact) powders, one study used an astaxanthin-rich oil emulsion and others did not specify its form or degree of processing. While *Chlorella vulgaris* has typically been evaluated as a protein source, some isolates cultivated under optimized conditions can accumulate natural astaxanthin and canthaxanthin. As such, a small number of studies were conducted with rainbow trout fed diets supplemented with *Chlorella vulgaris* to achieve dietary concentrations of 64 mg kg^{-1} of an astaxanthin/canthaxanthin blend. They showed that feed intake, growth performance and nutrient digestibilities were not affected, but they were inconsistent on flesh pigmentation efficiency. One study suggested that muscle carotenoid levels and overall pigmentation efficiency was lower than synthetic pigments while the other study observed muscle pigment levels 1.5 times higher than those fed synthetic pigments. However, it is important to note that since the control diet used in the latter study contained less than half the pigment than the *Chlorella vulgaris*-supplemented test diets, the imbalance confounds the study and makes the higher pigmentation efficiency questionable. In a similar manner, *Arthrospira platensis* (*Spirulina*) has typically been evaluated as a protein source for salmonids but it also synthesizes natural carotenoids. Two studies indicated that feeding rainbow trout on diets containing 5–10% *Spirulina* meal supported good growth and feed utilization and significantly increased serum, skin and muscle carotenoid deposition. This occurred despite the fact that the algal cells were not ruptured; providing further evidence of the less recalcitrant nature of the cell walls of cyanobacteria like *Arthrospira platensis* compared to chlorophytic microalgae like *Chlorella vulgaris*. By far, the most studied microalgae as a dietary carotenoid source for salmonid feeds is *Haematococcus pluvialis* with ~10 evaluations with rainbow trout. Of these studies, half used a cell-ruptured dry powder, one used an extracted astaxanthin-rich oil emulsion and the rest either used a whole-cell (un-ruptured) dry powder or did not specify the form. Studies using cell-ruptured *Haematococcus pluvialis* meal incorporated the ingredients at rates that achieved dietary astaxanthin concentrations of 40–73 mg kg^{-1} of diet and balanced those of the control diets containing the same astaxanthin concentration supplied in the synthetic form. A key finding from these studies was that natural-source astaxanthin from *Haematococcus pluvialis* was predominantly (~88%) made up of the 3*S*,3'*S* optical stereoisomer and that this was the same form subsequently incorporated into the muscle tissues of rainbow trout. Additionally, fillet color scores were the same as those fed an equivalent dietary concentration of synthetic astaxanthin (60 mg kg^{-1}). However, this latter finding contradicts other similar studies using cell-ruptured *Haematococcus pluvialis* meal in rainbow trout diets at similar astaxanthin levels (40–74 mg kg^{-1}) where pigmentation efficiency (measured as serum astaxanthin levels, muscle astaxanthin retention and fillet color) was inferior to synthetic astaxanthin. As might be expected, the use of whole-cell (intact) *Haematococcus pluvialis* meal in

rainbow trout diets generally reduced nutrient digestibility and pigmentation efficiency compared to synthetic astaxanthin and, in some cases, other negative effects were observed such as elevated levels of serum aspartate aminotransferase (ASAT) enzyme activity; an indication of possible liver damage. On the other hand, when an unspecified *Haematococcus pluvialis* meal was used at 30 mg kg^{-1} for rainbow trout broodstock diets, there appears to be a slight improvement in the lipid peroxidation status of fertilized eggs. However, overall reproductive performance of gravid female fish fed this diet was not significantly affected. The most encouraging results for the use of natural astaxanthin derived from *Haematococcus pluvialis* is when an extracted astaxanthin-rich oil emulsion was used in rainbow trout diets to provide 40 mg kg^{-1}. In this case, digestibility of the cell wall or broken cell wall fragments would not have been a concern and this was reflected in equal growth as fish fed the control diet. The study also found that, based on muscle and skin astaxanthin concentrations, diets containing the natural-source 'esterified' astaxanthin from *Haematococcus pluvialis* were equally as well utilized as those containing an equal supply of synthetic 'free-form' astaxanthin. Commercially-produced microalgae-based ingredients presently available on the market as sources of carotenoids are almost exclusively produced from *Haematococcus* while a small handful of products are produced from *Arthrospira* (*Spirulina*), *Dunaliella*, *Isochrysis*, *Nannochloropsis*, *Phaeodactylum* and *Tetraselmis*.

4. Concluding perspectives

While microalgae-based products have tremendous potential as 'next-generation' feed ingredients for sustainable salmonid aquaculture, few have yet to successfully be commercialized and reach the marketplace. Strains of *Schizochytrium* and *Crypthecodinium* as source ingredients for essential n-3 LC-PUFA and *Haematococcus* that effectively accumulates natural-source astaxanthin are promising high-value replacements for conventional fish oils and synthetic astaxanthin, respectively. As such, these products are rapidly becoming added to the feedstock portfolios of global salmonid aquafeed producers. However, substitution of protein-rich fish meals and terrestrial plant-based commodities presently used in salmonid feeds with protein-rich microalgae-based ingredients remains a challenge as a result of the fragmented and inconsistent information on their biochemical composition, inconsistent nutrient characterization analytics, variable digestibility related to recalcitrant cell walls and general scarcity of adequate nutritional investigations. More research is required to further evaluate the salmonid species-specific safety and efficacy of many microalgae-based products including their effects on growth performance, nutrient utilization, fish health and product quality. Further industrial research is needed to assess what effects they may have on the functional and rheological properties of combined feed mixtures, finished pellet quality, product shelf-life and how they fit into established feed ingredient distribution and feed processing infrastructure and value chains. For the further development and commercial adoption of microalgae-based ingredients for farmed salmonid feeds there is a need for additional technological advancements in the areas of industrial algaculture scale-up, standardization of cultivation strategies and down-stream processing methods to concentrate nutrient levels and increase their nutrient bioavailability. These advancements should enable the industry to provide nutrient-dense, highly digestible microalgae-based ingredients at cost-competitive prices.

Acknowledgements

The author greatly appreciates logistical support of Drs. Stephen O'Leary and Patrick McGinn and assistance of staff at the NRC-Marine Research Station. Thanks to Mr. Shane Patelakis and Drs. Stefanie Colombo and Stephen O'Leary for critical reviews of a draft of this manuscript and helpful suggestions to improve the quality of this chapter. Funding was provided by the National Research Council of Canada's Algal Carbon Conversion program. This is NRCC publication no. 56379.

Conflict of interest

The author declares no conflicts of interest.

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Chapter 9

Toward Future Engineering of the *N*-Glycosylation Pathways in Microalgae for Optimizing the Production of Biopharmaceuticals

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.73401

Abstract

Microalgae are eukaryotic and photosynthetic organisms which are commonly used in biotechnology to produce high added value molecules. Recently, biopharmaceuticals such as monoclonal antibodies have been successfully produced in microalgae such as *Chlamydomonas reinhardtii* and *Phaeodactylum tricornutum*. Most of these recombinant proteins are indeed glycosylated proteins, and it is well established that their glycan structures are essential for the bioactivity of the biopharmaceuticals. Therefore, prior to any commercial usage of such algae-made biopharmaceuticals, it is necessary to characterize their glycan structures and erase glycosylation differences that may occur in comparison with their human counterpart. In this context, the chapter summarizes successful attempts to produce biopharmaceuticals in microalgae and underlines current information regarding glycosylation pathways in microalgae. Finally, genome editing strategies that would be essential in the future to optimize the microalgae glycosylation pathways are highlighted.

Keywords: antibodies, biopharmaceuticals, genome editing tools, glycosylation, microalgae

1. Introduction

Microalgae are currently used for a broad spectrum of industrial applications including food and livestock feed industries, bioenergy, cosmetics, healthcare and environment [1–4]. Recently, due to their numerous advantages (high growth rate, easy cultivation, low production cost, etc.), microalgae have emerged as a solar-fueled green alternative cell factories for the production

of recombinant proteins [4–7]. Among different attempts to produce vaccines and biopharmaceuticals in microalgae, the production of monoclonal antibodies (mAbs) represents the most extensive work [7, 8]. Indeed, the first significant effort to produce recombinant mAb fragments was made in the green microalga *Chlamydomonas reinhardtii* with the synthesis and accumulation in its chloroplast of a human single chain antibody directed against the herpes simplex virus glycoprotein D (HSV8-lsc) [8]. Later, a full-length human IgG1 directed against anthrax was produced successfully in the chloroplast of *C. reinhardtii* [9]. The *Chlamydomonas*-made mAb was able to bind the anthrax protective antigen 83 (PA83) [9]. In another study, a series of complex chimeric proteins was expressed in the chloroplast of *C. reinhardtii.* Such chimeric proteins were composed of a single chain antibody fragment (*scFv*) targeting the B-cell surface antigen CD22, genetically fused either to the eukaryotic ribosome inactivating protein, gelonin, from *Gelonium multiflorm* [10] or to *Pseudomonas aeruginosa* exotoxin A domains 2 and 3 [11]. These molecules, termed immunotoxins, were encoded by a single gene that produces an antibody-toxin chimeric protein. Such algae-made immunotoxins are able to bind target B cells and efficiently kill them *in vitro* [11]. Full-length mAbs have also been expressed in the diatom *Phaeodactylum tricornutum* through nuclear transformation [12–14]. Those mAbs correspond respectively to a recombinant mAb directed against the nucleoprotein of Marburg virus, a close relative of Ebola virus [14] and to a human IgG1 directed against the Hepatitis B virus Antigen (HBsAg) [12, 13]. The latter has been biochemically characterized in order to check the quality of the diatom-made mAb as well as its *N*-glycosylation profile [15]. Moreover, it has been demonstrated that this glycosylated antibody is able to bind human Fcy receptors [16], thus suggesting that it could be efficient in human therapy.

When the production of biopharmaceuticals is considered, their *N*-glycosylation has to be investigated. Indeed, among the biopharmaceuticals that were approved in 2016 and 2017, 96% were glycosylated [17]. The glycosylation of the approved biopharmaceutical represents a critical quality attribute (CQA) that may affect its safety and biological activities [18–20]. In addition, introduction by the expression system of nonhuman epitopes on the recombinant protein may induce immune response after injection to patients [21]. Thus, the *N*-glycosylation of biopharmaceuticals is a real challenge for the commercial production of biopharmaceuticals. The glycosylation state of therapeutic proteins has to be accurately identified and characterized as per the World Health Organization and International Conference on Harmonization Q6B guidelines [17]. Therefore, in the context of developing the microalgae as alternative platforms for the production of biopharmaceuticals, the capability of these unicellular eukaryotic cells to introduce *N*-glycans on their endogenous proteins and on recombinant proteins, as well as their regulation, have to be considered and understood.

2. *N*-glycosylation in microalgae

2.1. General aspects

N-glycosylation is a major post-translational modification of proteins in eukaryotes. Protein *N*-glycosylation first starts by the synthesis of a lipid-linked oligosaccharide formed by transfer of monosaccharides on a dolichol pyrophosphate (PP-Dol) anchored in the membrane of

http://dx.doi.org/10.5772/intechopen.73401

the endoplasmic reticulum (ER) *via* the action of a set of enzymes named asparagine-linked glycosylation (ALG) [22, 23]. The final $Glc_3Man_9GlcNAc_2$ precursor is transferred *en bloc* by the oligosaccharyltransferase (OST) complex onto the asparagine residues of the consensus Asn-X-Ser/Thr sequences of a protein [22] (**Figure 1**). Alternative consensus sequences, such as Asn-X-Cys and Asn-X-Val, have also been found to be glycosylated in some proteins [24–26].

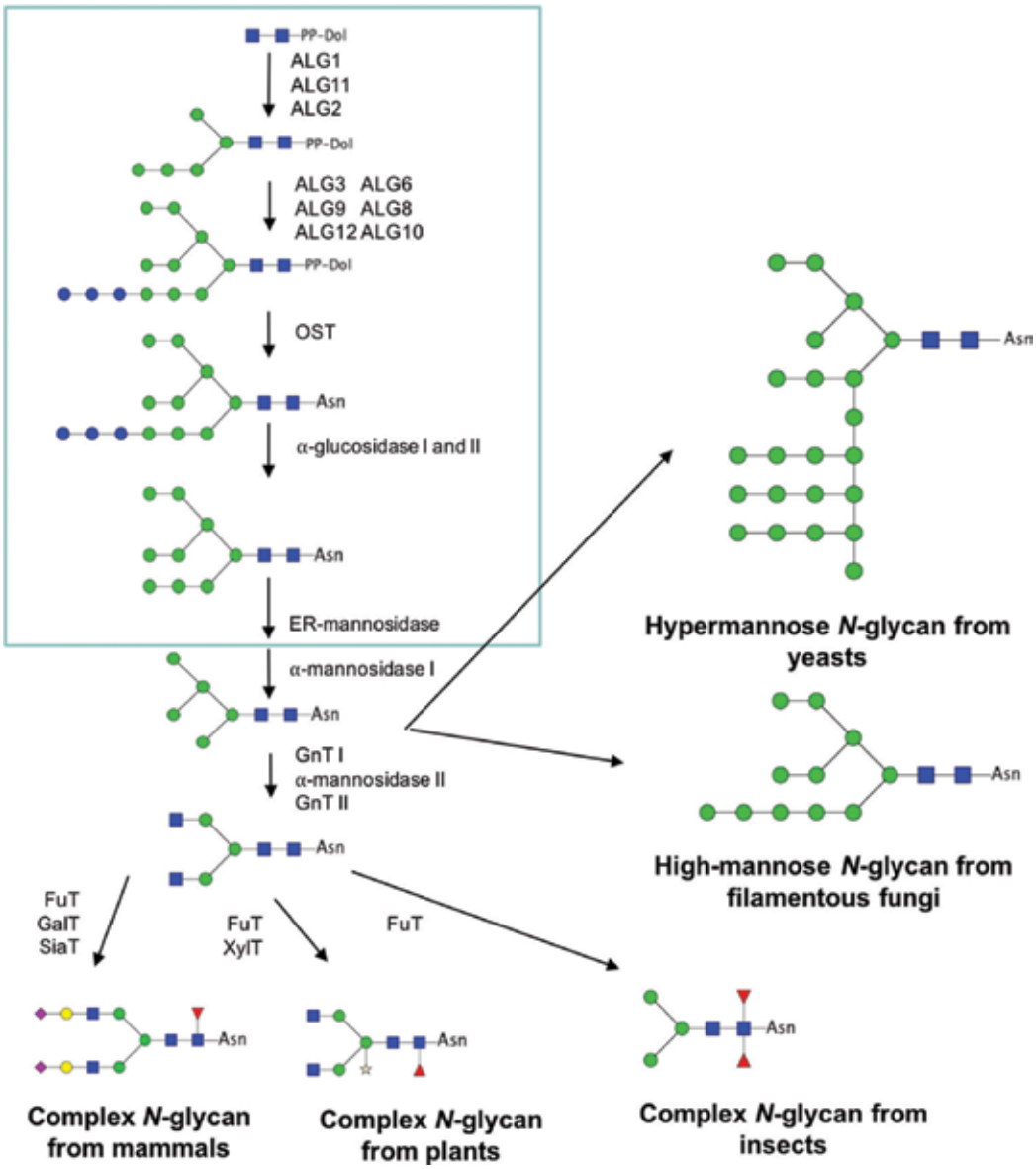

Figure 1. Comparison of protein *N*-glycosylation pathways in eukaryotes. Biosynthesis steps occurring in the ER are gathered in the box. Mature *N*-glycan structures observed in mammals, plants, insects, yeasts and filamentous fungi are drawn according to [33]. ■, N-acetylglucosamine; ☆, xylose; ●, mannose; ▲, fucose; ●, galactose; ◆, sialic acid; Asn, asparagine; PP-Dol, pyroPhosphate dolichol; FuT, fucosyltransferase; GalT, galactosyltransferase; SiaT, sialyltransferase; XylT, xylosyltransferase; ALG, asparagine-linked glycosylation; OST, oligosaccharyltransferase.

In the ER, neo-synthesized glycoproteins are then submitted to a quality control process through the deglucosylation by glucosidases and reglucosylation by an UDP-glucose: glycoprotein glucosyltransferase (UGGT) of the *N*-glycans. This allows the synthesis of monoglucosylated glycan intermediates that interact with ER-resident chaperones, thus ensuring proper folding of the glycoproteins [27]. When the glycoprotein is correctly folded, α-glucosidase II would finally remove the last glucose residue, and ER-mannosidase will eventually remove one mannose residue that leads to the formation of an oligomannoside $Man_{9/8}GlcNAc_2$. The quality control events are conserved in eukaryotes because they are crucial for the secretion of well-folded proteins [28]. As a consequence, whatever the expression system used, a recombinant therapeutic protein leaving the ER compartment exhibits a *N*-glycosylation similar to one of the reference proteins with unique oligomannoside $Man_{9/8}GlcNAc_2$ attached to the Asn residue of the *N*-glycosylation consensus site.

After transfer to the Golgi apparatus, oligomannosides resulting from the ER processing are modified by the action of specific mannosidases and glycosyltransferases [29]. These Golgi cell-specific repertoires give rise to various organism-specific oligosaccharides. In most eukaryotes, a *N*-acetylglucosaminyltransferase I (GnT I)-dependent *N*-glycan processing occurs (**Figure 1**). In this pathway, the α-mannosidase I converts $Man_{9/8}GlcNAc_2$ into the branched isomer of $Man_5GlcNAc_2$. Then, actions of GnT I, α-mannosidase II and GnT II, respectively, give rise to the core $GlcNAc_2Man_3GlcNAc_2$ that is common to most eukaryotes [27–31] (**Figure 1**). This core is then decorated by the action of specific glycosyltransferases that differ from one organism to another. This allows the protein to be decorated by organism-specific *N*-glycans that confer to the mature protein *in vivo* bioactivities [32]. It is worth noting that GnT I-independent *N*-glycan processing also occurs in some eukaryotes such as filamentous fungi and yeasts in which *N*-glycosylation in the Golgi apparatus results in the synthesis of high mannose and hypermannose *N*-glycans, respectively (**Figure 1**). As a consequence, in the context of the production of biopharmaceuticals by genetic engineering, such a diversity of mature *N*-linked glycans is a limitation because the expression system used may introduce inappropriate epitopes and heterogeneous glycosylation on the therapeutics and may also fail in introducing glycan sequences that are required for *in vivo* bioactivity of the biopharmaceuticals.

2.2. Protein *N*-glycosylation in microalgae

Overall, protein *N*-glycosylation in microalgae received little attention. Few studies, published in the 1990s have demonstrated that proteins secreted by green microalgae carry mainly oligomannosides or xylose-containing *N*-glycans based on affinodetection or enzymatic sequencing [34–36]. More recently, analysis by mass spectrometry of glycans *N*-linked to microalgae endogenous proteins has been reported. First, the 66 kDa cell wall glycoprotein from the red microalga *Porphyridium* sp. has been found to carry $Man_8GlcNAc_2$ and $Man_9GlcNAc_2$ oligomannosides containing 6-*O*-methyl mannose residues and substituted by one or two xylose residues [37, 38] (**Figure 2**). Investigation of *C. reinhardtii* has demonstrated that proteins in this green microalga carry oligomannosides ranging from $Man_2GlcNAc_2$ to $Man_5GlcNAc_2$ as well as $Man_4GlcNAc_2$ and $Man_5GlcNAc_2$ *N*-glycans containing 6-*O*-methyl mannoses and substituted by one or two xylose residues (**Figure 2**) [39]. Initially reported as branched

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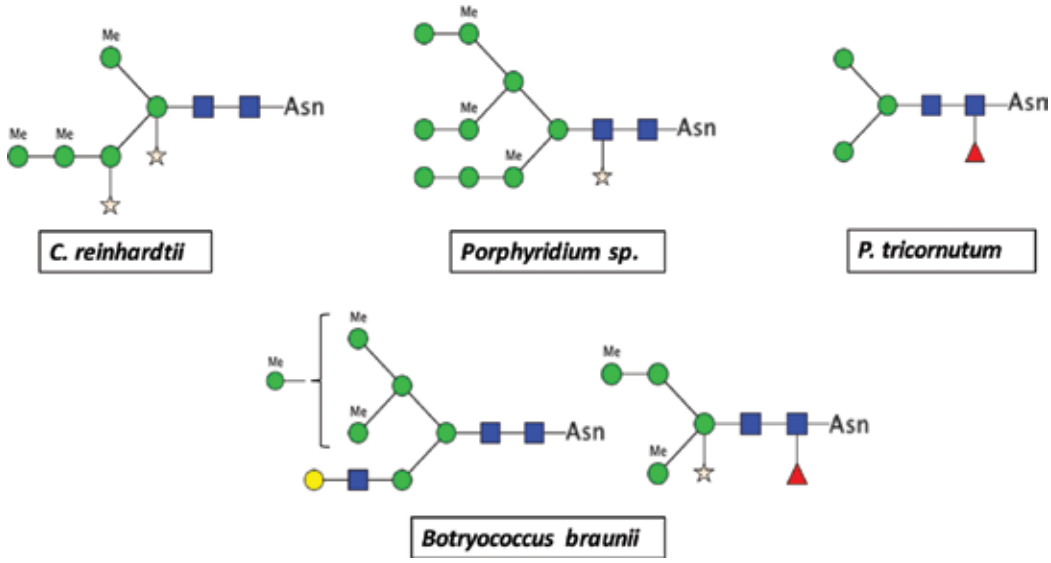

Figure 2. Major mature N-linked glycans from the green microalga *Chlamydomonas reinhardtii* and *Botryococcus braunii*, the red microalga *Porphyridium* sp. and the diatom *Phaeodactylum tricornutum*. N-glycan structures are drawn according to [33]. ■, N-acetylglucosamine; ☆, xylose; ●, mannose; ▲, fucose; ●, galactose; Asn, asparagine; Me, methyl.

oligomannosides, the structure of $Man_5GlcNAc_2$ was re-evaluated in 2017 as being linear sequences based on ESI-MS^n analyses [40]. Although mature *N*-glycans from *Porphyridium* sp. and *C. reinhardtii* share common structural features, the location of the xylose residues on the *N*-glycan differs between these two microalgae (**Figure 2**). As mature *N*-glycans do not exhibit any terminal GlcNAc residues, they were proposed to result from Golgi xylosylation and *O*-methylation of oligomannosides deriving from the precursor synthesized in the ER in a GnT I-independent processing, even if this needs to be completely elucidated and that methylation occurring in the ER cannot be ruled out yet [38].

N-glycan profile from *P. tricornutum* has been described to contain $Man_3GlcNAc_2$ to $Man_9GlcNAc_2$ oligomannosides and also minute amount of paucimannosidic fucosylated *N*-glycans (**Figure 2**) [41]. In contrast to *Porphyridium sp*. and *C. reinhardtii*, these *N*-glycans result from a GnT I-dependent pathway (**Figure 2**) [41]. As evidence, GnT I gene predicted in the *P. tricornutum* genome encodes an enzyme able to restore the maturation of complex-type *N*-glycans in the CHO Lec1 mutant that lacks endogenous GnT I activity [41]. *N*-glycans arising from a GnT I-dependent pathway have also been recently reported in the green microalga *Botryococcus braunii* through a glycoproteomic approach [42]. In contrast to *P. tricornutum*, these *N*-glycans harbor a GlcNAc residue at the nonreducing end as well as mono- and di-*O*-methylations of the core mannose residue. Moreover, this *N*-glycan bearing a terminal GlcNAc resulting from the GnT I activity could be further elongated with an additional hexose or methyl-hexose residue. In addition, proteins from this green microalga also exhibit methylated *N*-linked oligomannosides carrying core fucose and core xylose residues (**Figure 2**) [42].

In support to these biochemical data, protein *N*-glycosylation in microalgae can be drawn on the basis of public genomic databases. Microalgae genomes from different phyla are available

to date (https://genome.jgi.doe.gov/pages/tree-of-life.jsf) [4, 43]. Since protein *N*-glycosylation occurs in the ER and the Golgi apparatus, bioinformatics analyses of microalgae genomes must be investigated independently for the two compartments: search for gene encoding proteins involved in the precursor biosynthesis and the ER protein quality control on the one hand, and search for Golgi glycosidases and glycosyltransferases involved in the synthesis of mature *N*-glycans on the other hand.

Genes encoding subunits of OST, glucosidases, as well as ER-resident UGGT and chaperones are predicted in microalgae genomes suggesting that the process of ER quality control in these unicellular organisms is similar to the one described in other eukaryotic cells [41, 44, 45]. Among these putative ER candidates, only the activity of the α(1,3)-glucosidase, also called glucosidase II, from the red microalga *Porphyridium* sp. has been biochemically confirmed [44]. Most ALG genes are also predicted in microalgae genomes [39, 41, 44] suggesting that the synthesis of the oligosaccharide precursor is overall conserved. However, some of these ALG, that is ALG3, ALG9 and ALG12, are not predicted in *C. reinhardtii* [39, 45]. These ER enzymes are involved in the completion of the biosynthesis of the precursor $Man_9GlcNAc_2$-PP-Dol, prior to its glucosylation, by addition of mannose residues on the α(1,6)-mannose arm of the core (**Figure 1**). Reinvestigation in *C. reinhardtii* of the structure of oligomannosides and analysis of the ER *N*-glycan precursor [40] confirmed the absence of ALG3, ALG9 and ALG12 activities and the synthesis in this green microalga of linear oligomannoside sequences instead of branched isomer initially proposed in [39]. It is worth noting that in this truncated ER pathway, the presence of the triglucosyl extension is likely sufficient to ensure interaction of the *N*-glycan precursor with chaperones of the ER quality control process. In addition to the lack of the ALG3, ALG9 and ALG12 in *C. reinhardtii*, other microalgae genomes lack genes encoding ALG10 and GCS1, an α(1,2)-glucosidase [44]. Because ALG10 is the α(1, 2)-glucosyltransferase responsible for the addition of the α(1, 2)-glucose residue on the precursor *N*-glycan and GCS1 is responsible for trimming this residue, we hypothesize that the ER quality control in these microalgae involved only diglucosylated *N*-glycan intermediates.

With regard to Golgi *N*-glycosylation events, the presence of GnT I is predicted in some microalgae including haptophytes and cryptophytes, but not in *C. reinhardtii*, *Volvox* and *Ostreococcus* [41, 42]. As mentioned previously, *P. tricornutum* GnT I activity was confirmed by the complementation of CHO Lec 1 mutant cell line [41]. A recent study of *B. braunii* [42] confirmed the involvement of this transferase in this green microalga *N*-glycosylation pathway. Concerning other Golgi enzymes, α-mannosidases (CAZy GH 47) and α(1,3)-fucosyltransferases (CAZy GT10) are also predicted in microalgae genomes studied so far [41, 44, 45]. These enzymes are respectively involved in the trimming of mannose residue of oligomannosides and the transfer of fucose on the proximal GlcNAc. These sequences exhibit peptide motifs that were demonstrated to be required for activities of such Golgi enzymes, but, in contrast to GnT I, no biochemical data of their activity and specificity are available yet.

As depicted, protein *N*-glycosylation occurring in microalgae is specific and largely differs from the one described in mammals (**Figures 1** and **2**). Therefore, production in microalgae of biopharmaceuticals exhibiting *N*-glycans compatible with their use in human therapy

would be challenging and requiring metabolic engineering of the *N*-glycosylation pathway in microalgae. This will include the inactivation of enzymes that introduce nonhuman glycoepitopes onto *N*-linked glycans and complementation of microalgae with appropriate glycosyltransferases to introduce missing glycan sequences. These strategies have already been successfully carried out for the engineering of the *N*-glycan pathways in plants and yeasts [46, 47]. In addition, the success of the complementation with human glycosyltransferases requires the availability in the Golgi apparatus of appropriate nucleotide-activated sugars [48]. For instance, sialic acids that terminate bi-antennary *N*-glycans in mammals have not been reported in microalgae such as *P. tricornutum* and *Porphyridium* sp. [38, 41]. As well, there is no evidence for the import of GlcNAc in the Golgi apparatus in microalgae exhibiting a GnT I-independent *N*-glycan pathway, even if putative candidates for UDP-GlcNAc transporter have been identified in microalgae such as *C. reinhardtii* [49]. Indeed, the two GlcNAc of the chitobiose unit of *N*-linked glycans are transferred onto the PP-Dol lipid in the cytosolic face of the ER membrane. Currently, metabolic engineering strategies are now feasible due to the recent development of transgene expression and gene inactivation in microalgae as summarized in Section 3.

3. Genetic engineering tools now available to envision future *N*-glycosylation engineering in microalgae

3.1. Different tools to generate genome-modified organisms

Classical strategies of genetic engineering involve the modulation of gene expression including overexpression and inactivation by RNA interference [50–52]. The most used engineering methods are based on random insertional mutagenesis obtained by various processes such as conjugation, agitation with glass beads, electroporation, biolistic microparticle bombardment, agrobacterium-mediated transformation or multipulse electroporation. The transformation step is followed by phenotypic selection using antibiotics to generate genome-modified organisms [53]. Those processes present the advantage to be simple and reach a high level of transformed cells. For example, *P. tricornutum* transformation reached 1 per 10^6 cells with biolistic bombardment system [54]. However, cell-wall-less strains are required for almost all the classical methods quoted above [50, 55]. Furthermore, genetic stability of the mutagenesis obtained after transformation by random insertion depends on microalgae species [53]. For example, a high mutagenesis stability has been shown in *C. reinhardtii* [55]. Unlike, mutagenesis was unstable in *Thalassosiara weissflogii* [56]. More recently, new tools have been developed in order to knock in, knock out, modify, replace, or insert genes. These new genetic engineering tools consist of the action of nucleases effecting their molecular scissor activities in specific loci [52]. A break in the DNA causes activation of DNA repair mechanisms, which can be either the homologous-recombination (HR) or the non-homologous end-joining (NHEJ) [52]. The HR results in sequence modification in the target locus [57]. In the NHEJ process, the two ends of the broken chromosome are stuck together causing small deletions or small insertions [57]. These events confer several modifications of the target gene such as gene inactivations or

insertions. Very little is known about these mechanisms in microalgae due to their complexity as reported by Daboussi in 2017 [53].

Several researches have recently contributed to demonstrate that particular nucleases could be used for targeting stable modifications by acting like molecular scissors. Among these nucleases, we can quote meganucleases (MNs), zinc finger nucleases (ZFNs), transcriptor activator-like effector nucleases (TALENs) and finally, the famous clustered regularly interspaced short palindromic repeats (CRISPR)/nuclease Cas9 system. These four cited nucleases are described in the following paragraphs.

Meganuclease is an engineered endonuclease able to recognize and cleave a long specific DNA sequence from 18 to 30 base pairs. The meganuclease strategy requires to design a homing endonuclease from the LAGLIDADG family especially the I-*CreI* enzyme from *C. reinhardtii* implied in the targeting of interesting gene sequences that need to be modified [58]. This was tested for the first time in 2014 using *P. tricornutum* as a model [59]. In this study, two engineered meganucleases targeting genes involved in the lipid metabolism are allowed to obtain 29% of targeted mutagenesis [59]. Even successful, this strategy is time-consuming as compared to the other alternatives [52].

Zinc finger nucleases (ZFNs) are hybrid proteins composed of a restriction enzyme *FokI* with a designed zinc-finger DNA-binding domains [60]. These *FokI* enzymes are inactive in a homodimer conformation [61]. Therefore, cleavage of a typical DNA-target sequence requires to design two different ZFNs for binding to adjacent half-sites of a specific locus. Each designed ZFN is able to recognize a sequence of 9–12 nucleotides in the genome [52]. A set of zinc finger nucleases has been recently used to modify by insertion of template DNA, the *Cop3* gene locus encoding a light-activated channel in *C. reinhardtii* [62]. Moreover, in 2017, the genome editing was reliably performed using the ZFN strategy in order to inhibit and modify nuclear photoreceptor genes in this same microalga [63]. Despite these promising results, the ZFN system is barely used because of its low specificity. Indeed, cleavage of DNA requires both ZFN monomers to recognize a homologous target in the genome in the proper spatial orientation to assemble a functional ZFN [64]. Also, ZFN system is time-consuming implementation [64]. Nowadays, other designed nucleases like TALENs or CRISPR/cas9 are emerging in the scientific community to perform genome editing in microalgae.

Transcriptional activator-like effector nuclease (TALEN) system is similar to ZFN because it uses nucleases composed of a restriction enzyme domain fused to a DNA-binding domain (here the TAL effector domain) and a nonspecific DNA cleavage domain *FokI* [65]. TALEN proteins are characterized by a repeated 34-amino acid sequence that recognizes specific DNA sequences [66]. *P. tricornutum* lipid metabolism was recently modified using TALEN [59]. In this study, seven genes involved in this metabolism were modified. Each genome modification had a high frequency reaching up to 56% of colonies with targeted mutagenesis [59]. This genetic engineering allowed creation of a high lipid-producing strain by inactivating a key gene for carbohydrate energy storage [59]. Another team has inactivated successfully the urease gene in *P. tricornutum* with 24% of transformed colonies [67]. In addition, TALEN system has also been used in order to inactivate red/far-red light-sensing *phytochrome* gene of this diatom [68].

The clustered regularly interspaced short palindromic repeats (CRISPR)/cas9 system is the most famous engineered nuclease system of this decade because it is a powerful and precise tool applied in numerous eukaryotic organisms [69]. This system is based on the RNA-guided DNA cleavage defense system from archaea and many bacteria. Indeed, these organisms are able to store bacteriophage DNA fragments along a previous bacteriophage infection in the CRISPR locus, which is formed of DNA repeat sequences spaced by a unique DNA sequence. This system establishes the basis of a bacterial defense as a response to bacteriophage attacks [70]. This defense mechanism has been highlighted for the first time by Pr Emmanuelle Charpentier and her team in 2011 [70, 71]. The CRISPR/Cas9 system has been developed into a simple toolkit based on a custom single guide RNA (sgRNA) that contains a targeting sequence (crRNA sequence) and a cas9 nuclease-recruiting sequence (tracrRNA) [52]. In microalgae, CRISPR/cas9 has been used in *C. reinhardtii* [72]. However, the Cas9 nuclease production seemed to be toxic for the microalga limiting efficiency to obtain genome-modified strains [72]. Two years later, a new assay has been performed in this same microalga using another strategy avoiding toxicity [73]. Indeed, the authors succeeded to generate CRISPR/cas9-induced NHEJ-mediated knock-in mutant strains in three loci [73]. In the same year, CRISPR/cas9 gene knockout technology has been used in *P. tricornutum* to induce mutant for the *CpSRP43* gene, a member of the chloroplast signal recognition particle pathway. Using

	MN system	**ZFN system**	**TALEN system**	**CRISPR/cas9**
Actor(s) of gene targeting	Chimeric endonuclease	Chimeric endonuclease	Chimeric endonuclease	RNA guide and cas9 nuclease
Engineered protein origin	*Chlamydomonas reinhardtii* *Saccharomyces cerevisiae* [59, 77]	Xanthomonas [78]	Xanthomonas [78]	Bacteria and Archaea [70]
Nuclease specificity	Low	Moderate	Moderate	High
Mutagenesis frequency in microalgae	Up to 29% [59]	Not reported	Up to 56% [59]	Up to 63% [74]
Toxicity in cells	Low	Moderate[1]	Moderate[1]	Moderate [72]
Time investment	Very high [52]	Very high [64]	Moderate	Low
Possibility of multiple gene targeting	No	No	Yes	Yes
System cost[2]	Not reported	Expensive (4000–7000$)	Expensive (3000–5000$)	Cheap (500$)

[1]Source: https://www.news-medical.net/life-sciences/How-Does-CRISPR-Compare-to-Other-Gene-Editing-Techniques.aspx visited [Accessed: 2017-12-06].

[2]Source: http://www.biocompare.com/Editorial-Articles/144186-Genome-Editing-with-CRISPRs-TALENs-and-ZFNs/ [Accessed: 2017-12-06].

Table 1. Comparison of four specific genomic tools based on nuclease systems in order to generate genomic-modified species in microalgae.

this strategy, the authors obtained 31% of mutation efficiency [74]. This team targeted two other genes of the diatom using this technology and obtained from 25 to 63% of mutation level [74]. Adaptability of the CRISPR/Cas9 system has been demonstrated in other diatoms like *Thalassiosira pseudonana* [75] as well as in the heterokont, *Nannochloropsis oceanica* in order to knock out the nitrate reductase activity [76]. In conclusion, CRISPR/cas9 system is a promising technology to generate genome-modified organisms in microalgae. **Table 1** compares this system with the other nuclease systems cited above in terms of their technical characteristics and highlights their advantages and disadvantages.

3.2. Mutant libraries

The study of mutants impaired in a glycosidase or a glycosyltransferase implied in the *N*-glycan pathway is of great interest. Indeed, the synthesis of oligosaccharides is a sequential process. Inactivation of an enzyme usually results in the accumulation of its *N*-glycan substrate which enables the step-by-step dissection of the entire pathway. Moreover, mutant phenotyping of the glycosylation pathway allows to investigate to which extent the protein *N*-glycan processing is required for normal growth and development. An indexed and mapped mutant library has been created in *C. reinhardtii* by single random insertional mutagenesis of gene cassettes in 2016 [79]. This library already envisioned to study the function of genes encoding putative glycosyltransferases, glycosidases or even putative translocators in microalgae and to confirm their physiological role from reverse genetic studies.

4. Conclusion

The production of biopharmaceuticals in microalgae currently requires a better understanding of the *N*-glycosylation pathway mechanism and regulation. Such information can be gained by the use of mutant libraries like the one recently developed for *C. reinhardtii*. Indeed, characterization of each individual mutants will allow an understanding of a specific step of the *N*-glycan processing, and mutant cells could represent interesting cell lines for the production of biopharmaceuticals bearing a chosen *N*-glycan profiling.

Once these pathways would be completely deciphered in the microalgae model intended to be used for the production of biopharmaceuticals, the humanization of the *N*-glycosylation pathway could be initiated using designed engineered nucleases strategies recently developed in microalgae. We can now consider that transformed microalgae by these innovative new genomic tools will constitute in a near future one of the most suitable green cell factories for the production of humanized biopharmaceuticals.

Acknowledgements

The authors are indebted to all coworkers and students at the Glyco-MEV laboratory who are currently contributing to the microalgae research project or did so in the past. They are also

thankful to the University of Rouen Normandy, the region Normandie and the I.U.F. for their financial support.

Conflict of interest

The authors have declared no conflict of interest.

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Chapter 10

Bioeconomic Assessment of Microalgal Production

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.73702

Abstract

Today, microalgae play an important role for the worldwide biofuel demand, together with the production of high value-added products used in pharmaceutical, nutraceutical and cosmetic industries. In 2014, the European Union adopted a strategy for developing the bioeconomy, by utilizing microalgae which represent an emerging biological resource of great importance for its potential applications in different fields. Huge potential of tiny microalgae could support a microalgae-based biorefinery and microalgae-based bioeconomy opening up vast opportunities in the global algae business. Nevertheless, in spite of having been studied for over 50 years now, there are still only just a few corporations that are cultivating algae on a large or commercial scale due to operational and capital cost. Techno-economic modeling is a powerful tool for guiding research priorities and assessing the economics, environmental impact and sustainability of microalgal productions. In this chapter, microalgal productions are assessed within bioeconomical aspects and case-studies on microalgal biorefinery are discussed.

Keywords: microalgae, bioeconomy, microalgal biorefinery, bioproducts, biofuel, techno-economic analysis

1. Introduction

Increase of the human population has necessitated industrialization and manufacture since the industrial revolution. Experts estimate that the world population will reach about 9.5 billion by 2050 [1]. As a result, demand for natural resources such as food, feed, clean water, energy, housing and materials for clothing as well as demand for education and health services are increasing continuously. However, depletion of the natural resources, CO_2 emissions, and climate changes etc., decrease the quality of human life [2]. To solve

this problem, associations and governments are trying to put forward new approaches within the framework of sustainable development. The concept of sustainable development, which started to gain importance with the beginning of the twenty-first century, has accompanied the search for an appropriate economic model. At this point, the terms biotechnology and bioeconomics have gained more meaning and promise great hopes for the future [3]. The term of bioeconomic refers to an economic system in which biological resources are used instead of fossil resources in production processes. Therefore in bioeconomic strategies, economic growth is linked to environmental sustainability [1]. There are basically three factors in the emergence of bioeconomic strategies: limitation of fossil feedstocks, the negative effects of human activities on the environment and the innovative progresses in science and technology [4]. In this respect, bio-economic is central to all economic sectors for a higher standard of living. A bioeconomy involves three elements: biotechnological knowledge, renewable biomass, and integration across applications. The first element, biotechnological knowledge, is the principal of the bioeconomic model. Biotechnology offers technological solutions to health, natural resource and ecosystem sustainability issues and allows for increased productivity in different industries with new products and processes such as biopharmaceuticals, recombinant vaccines and industrial enzymes. R&D studies and innovation are essential for the development of biotechnology [1]. The second element is the use of renewable biomass. Renewable biomass covers a wide range from primary sources such as energy plants, trees and grasses; to agricultural and industrial wastes [5]. The third element is integration between knowledge and applications, based on generic knowledge and value-added chains that cross applications [4]. Due to the fact that most renewable biomass resources are also used in the food sector, a very important ethical question has arisen: Is it right to use food materials in different areas while many countries on earth have starvation problems? This problem is one of the most controversial issues today [6]. Researchers suggest the use of waste biomass for these discussions. However, there are some limitations on the use of wastes. For example, the production of chemicals for use in the pharmaceutical industry from wastes is not appropriate. Therefore, microalgae, which can be used in many different areas, are thought to be able to solve this problem [7]. Especially, developed biorefinery strategies and bioprocesses about microalgae are promising for the future in order to achieve economic sustainability. In biorefinery systems where microalgae are used as raw material, important biofuels such as biodiesel, bioethanol and biogas are produced and valuable chemical substances used in fields such as pharmaceutical, nutraceutical and cosmetic industries are produced. It is also possible to use microalgae as food and animal feed because of the high protein content [8]. Nevertheless, in spite of having been studied for over 50 years now, there are still only just a few corporations that are cultivating algae on a large or commercial scale. Because, algal investment is not economically feasible due to operational and capital cost. The rate of return is not short as it is expected. The operation cost is affecting the total cost significantly. The main part, which makes the process expensive due to operation and capital costs, are algae growth, harvesting, and dewatering. Although many innovations are performed in production of algal biomass day by day, in order to carry out sustainable and economical productions, algal biorefinery is the best choice for reducing production cost and obtaining various products with maximum efficiency [9]. In this chapter, definition of bioeconomy and its classification are described, techno-economic analysis of microalgal

productions are presented in detail and cost-effective approaches are evaluated case by case in basis. And all results were evaluated from a bioeconomic point of view.

2. Definition of bioeconomy

Although the term of bioeconomy has gathered much attention in recent years, it is existed since the development of the life sciences and biotechnology [10]. Usage of the term "bioeconomy" has become widespread due to the relationship between economy and biology in the world [11]. In order to reduce the effects of environmental problems and global warming, it is important to utilize bio-based products instead of fossil-based product [12]. A biomass based economy instead of fossil based product represents a significant shift in socio-economic, agricultural, energy and technical systems. The concept of a bioeconomy which is also called the "bio-based economy" in some reports, can be defined as an economy where the basic building blocks for materials, chemicals and energy are derived from renewable biological resources [13, 14]. The bioeconomy comprises the parts of using renewable biological resources from land and sea such as crops, forests, fish, animals and micro-organisms to produce food, materials and energy and also their use in a wide range of sciences such as, life sciences, agronomy, ecology, food, biotechnology, nanotechnology, and engineering [12, 15]. The bioeconomy entails the use of biotechnology on a large scale [16]. Biotechnology makes use of biological systems and processes to manufacture various products: such as industry (white biotechnology), medicine (red biotechnology), agriculture (green biotechnology), aquaculture (blue biotechnology), and pollution removal and bioremediation (gray biotechnology) [16]. Establishing an bioeconomy in Europe have a great potential, because economic growth and jobs in rural, coastal and industrial areas can be carried out, fossil fuel dependence can be reduced and the economic and environmental sustainability of primary production can be developed [17]. Biotechnology has various industrial applications including the manufacture of chemicals and biopharmaceuticals, bio-polymers and bio plastics, food, feed and biofuels [16]. White biotechnology or industrial biotechnology uses enzymes and micro-organisms to make bio-based products, including chemicals, food and feed, bioenergy, and textiles [10, 18–22]. Gray biotechnology is comprised from technological solutions created to protect the environment, like in the case of oil spills and purifying sewage water [23]. Green biotechnology is practiced to agricultural processes such as developing genetically modified crops or improve plant breeding techniques by using life science knowledge [24]. Blue biotechnology is a term that has been used to describe the marine and aquatic applications of biotechnology [19]. Red biotechnology relates to the health sector and production of pharmaceuticals [10, 25, 26].

3. Bioeconomy concept in Europe and the World

3.1. Bioeconomy in Europe

Europe has set a course for a resource-efficient and sustainable economy which is more innovative and promotes usage of renewable biological resources for industrial purposes, while ensuring biodiversity and environmental protection. In order to carry out this goal, the

European Commission has set a Bioeconomy Strategy and action plan [27]. This plan focuses on three key aspects as; developing new technologies and processes for the bioeconomy; developing markets and competitiveness in bioeconomy sectors; and pushing policymakers and stakeholders to work more closely together [27].

According to the reports, The German Bioeconomy Council had described that the share of produced or processed biomass, or in which biotechnological processes were used on bioeconomy innovation amounts to 4.9% of gross value added for and 6.3% of the working population was employed by these sectors in the EU-25 in 2005. Among the bio-industries, mostly food and wood industries are dominate the share of the bioeconomic gross value added in Germany as well as in the EU-25. The bioeconomy in Germany can be split into two parts: (1) "production and processing of biological resources" which holds 50% of value added and 40% of employment and (2) "trade and services related to biological resources" which captures the other half of value added and 60% of employment in the year 2010 [28].

As for France, in order to develop bioeconomy in France, studies are started to carry out in 2005. Industries and Agro-Resources (IAR) was focused on four strategic fields as; bio-based chemicals (bio lubricants, glues, building blocks, bio surfactants etc.), bio-based materials for the construction sector and transportation, bioenergy with advanced biofuels and biogas production, and ingredients for food and feed. In order to carry out this, IAR also takes into consideration life cycle analysis and environmental benefits as well as the production of sustainable renewable resources. These four topics are now in line with the definition of the Bioeconomy with the publication in February 2012 of the European bioeconomy roadmap. It is now widely recognized that the industrial biotechnologies are called to play an important role in the future of the bioeconomy in Europe and all around the world [29].

Spain sets its own strategy on bioeconomy in January 2016, which perform a sustainable and efficient production and utilization of biological resources. The targeted sectors are food, agriculture and forestry, conditioned by water availability. It also includes the production of industrial bioproducts and bioenergy obtained from the use and valorisation of wastes and residues and other non-conventional sources of biomass. The main focus of the bioeconomy in Spain is the use of biological resources to produce food and feed like as Germany [30].

According to the reports, almost 1.5 million jobs are related with the bioeconomy sector in Italy. Reports show that, Italy ranks 10th in the world as for exports of bio-based products, with a share of around 3%. It is stated that Italian Bioeconomy has great potential for growth which has stronger interactions between public and private stakeholders, different sectors and disciplines from the harvest to the various final products [31].

With having the sixth-largest economy in Europe, the Netherlands's industrial activity is consist of food processing, chemicals, petroleum refining, and electrical machinery. As for bioeconomy, their approaches and strategy are carry on slower than expected when it is compared to the other European countries. However, it is stated that the structure and strengths of its economy lend itself well for the transition to a bioeconomy. Another disadvantage with respect to the bioeconomy is that The Netherlands has no forestry biomass; the only potential

raw material is agricultural biomass. Since it does not have huge biomass potential, a large share of biomass will need to be imported [32].

3.2. Bioeconomy in USA and Canada

The US national bioeconomy strategy was drafted by the Office of Science and Technology Policy and the Executive Office of the President, under participation of different federal agencies. Individual persons and institutions from scientific and industrial areas were consulted for this strategy. The "National Bioeconomy Blueprint" which was the document of bioeconomy strategy of USA, is divided into two distinctive parts. The background and impact of the current bioeconomy is explained in the first part and the strategic objectives are described later. In USA, genetic engineering, DNA sequencing and automated high through-put manipulations of biomolecules, these three technologies are focused as the strategic fields for the bioeconomy. In the document, the possible contributions of government departments and funding agents were also reported. According to the document USA already has a bioeconomy strategy and some of the results which are aimed to achieve are listed. It was stated that, federal departments and agencies supporting biological research. As the focus of the strategy is biological research, the perspective is national with little outlook to the rest of the world [14, 34]. As for the Canada, "Canadian Blueprint: Beyond Moose and Mountains" was the equivalent of the USA's blueprint of bioeconomy strategy. However, there is no official strategic document for the development of a bioeconomy in Canada, nor any signs of one being prepared. Yet, the document present the requirement for actions and goals within the selected priority areas of capital, people and operational environment. In the bioeconomy strategy of Canada, the forest sector and agriculture, life science and clean technology play important role. Canada's largest producers of agricultural products is from Alberta and there are a lot of significant producer of forest products. Biomaterials, biochemical, and bioenergy are the areas which have taken marginal roles in Alberta's economy but are foreseen to grow [14].

3.3. Bioeconomy in Asia

According to the studies on bioeconomical approaches in Asia, four bio-industries as biopharmaceutical, biohydrogen, bioplastics and genetically modified crops come into prominence for bio-based economies through 2050. Provided forecasts reported that, development of the biohydrogen industry will be fastest in India, China and Malaysia, and China will be the largest supplier in 2050. The growth of the bio-pharmaceutical industry will be fastest in India, Malaysia, and China and India and Japan will be the two largest suppliers of biopharmaceutical products. Growth of the bio-plastic industry will be fastest in India, Malaysia and China; China will be the biggest supplier of bioplastics. Growth of GM crops will be fastest in Malaysia, India and China; India and China will be the two largest suppliers. In terms of the output values for the four bio-industries, the largest bioeconomies will be in India, China and Japan followed by Korea, Malaysia and Taiwan [33]. In these countries, bio-pharmaceuticals will be the most important bio-industry. Transitioning toward bioeconomy by developing industries based on biological processes will be fast if government should pay more efforts on R&D, biotechnology, human resources and its related infrastructure, industrial supply and sales chain [33].

4. Techno-economic assessment

Techno-economic assessment is a term which has been used since 2010 [35]. In this assessment, technical performance or potential and the economic feasibility of an innovative technology are evaluated [36]. This assessment can help making right choices during process development and the success rate of market introduction can be raised. It is important to perform a techno-economic assessment in an early development stage of an innovative technology. Therefore, the specific components which will be taken into account, should be considered carefully [35, 37]. Economic potential based on technical information and assumptions can be evaluated via techno-economic analysis. To design a commercial-scale industry or to make a decision for investment, the equipment information must be collected, and profits must be calculated [38]. For various industrial and biosystems evaluation, such as production of biofuel, and fine chemicals from biomass, techno-economic assessment is a useful method [39]. Engineering design, technical information, and costs and profits can be gathered with techno-economic assessment. It can provide support not only for a long-term business decision, but also for on-going process and improvement. In this assessment, system boundaries, flowcharts and assumptions are required, and main technical and economic parameters must be identified, respectively. By using these data, mass and energy balance are determined. According to the model, capital and operating costs are calculated, and profits are calculated to the economic potential [40, 41].

4.1. Techno-economic assessment of microalgae-based productions

Microalgae are microorganisms which have not very complex cell structures, can be single-celled or multicellular and can grow in aqueous media. It is estimated that more than 50,000 species of microalgae are presented in reports and studies. There are many studies on cultivation of algae. However, each algal species is worth studying separately, because algae species have different mechanism for adapting the cultivation medium and cultivation system. According to their structural properties, growth of each algae can show different growth pattern in these systems and medium. Microalgae species and production conditions should be determined according to the products [42]. Microalgae are produced in open (open ponds) and closed systems (photobioreactors). Considering productivity and obtaining special products such as nutraceuticals and pharmaceuticals, closed systems are more preferable than open systems. However, investment and operating costs of closed systems are higher than those of open systems [43]. Therefore, a very comprehensive economic analysis is required when establishing pilot scale systems. In the production of microalgae, biological factors, non-biological factors and operating parameters are influential. Biological factors include pathogens such as viruses and bacteria, and other algae species; non-biological factors include light, temperature, pH, salinity and nutrients; operating parameters comprises mixing, dilution rate and harvesting frequency [44]. In this section, techno-economic assessments of some microalgae based production systems in the literature have been examined and system costs (investment and operating costs) are shown in **Table 1**. As can be seen in **Table 1**, generally, techno-economic approaches have been carried out for biofuel production. Thomassen et al. [45] developed four different scenarios (basic, intermediate, advanced,

Species	Product	System	Investment cost	Operating cost	Ref.
D. salina	β-carotene	Open	66,020 €/tonnes	78,474 €/tonnes	[45]
D. salina	β-carotene	Open	63,226 €/tonnes	46,686 €/tonnes	[45]
D. salina	β-carotene	PBR	253,760 €/tonnes	77,977 €/tonnes	[45]
H. pluvialis	Astaxanthin	PBR	271,449 €/tonnes	80,782 €/tonnes	[45]
C. vulgaris	Bio-oil	Open	3.73 M $/tonnes	0.63 M $/tonnes	[46]
NA	Biofuel	Algal turf scrubber	339.64 $/tonnes	171 $/tonnes	[47]
NA	Biofuel	Open	351.2 $/tonnes	322.4 $/tonnes	[47]
N. salina	Biofuel*	PBR	327.74 MM $	$86.52 MM $	[48]
C. vulgaris	Biofuel**	PBR	5,352,657 $	1,977,831 $	[49]
NA	Biogas	Open	48,157 €/ha	7560 €/ha.yr	[50]
NA	Biodiesel	Open	390 MM $	37 MM $/yr	[51]
NA	Biodiesel***	PBR	990 MM $	55 MM $/yr	[51]

*For 10 million gallon of biofuel.

**Algae or fuel amount is not given.

***For 10 million gal/yr biodiesel.

Table 1. Investment and operating costs of microalgae based production.

alternative) to produce 170 tonnes (dry weight) microalgae per year in Belgium. They used open systems in basic and intermediate scenarios and photobioreactors (PBR) in advanced and alternative scenarios. As a result of the techno-economic assessment, it was seen that the investment costs of photobioreactors were about four times that of open systems and the most profits were in open ponds. It is also stated that this profit can be increased four times by recycling fractions. Juneja and Murthy [46] conducted plant design to produce *Chlorella vulgaris* using 227 million liters of wastewater per day and produce bio-oil from this microalgae. In this design, the bio-oil production process model is divided into five parts (growth, harvesting, hydrothermal liquefaction, bio-oil hydrotreating and co-product recovery). The investment cost of the plant, which will be set up for 28,111 tons of algae per year and 10 million liters of renewable diesel from these algae, is $ 105 MM; the operating costs would be $ 17.88 MM. They also stated that the total cost of open pond was $ 38,645/ha. In the study of Hoffman et al. [47], techno-economic analyzes of microalgae production in algal turf scrubber and open raceway pond systems was performed. As a result of the analysis, the total cost of algal turf scrubber and open raceway pond were $ 510/tonnes biomass and $ 673/tonnes biomass, respectively. It can be seen that capital costs are close for both systems; but operating costs are much higher than for open raceway ponds. Dutta et al. [52] conducted techno-economic analysis of algal biomass cultivation and biofuel production in two different regions (Portugal and USA). Biofuel production was designed as Case A (Portugal) which was carried out by solvent extraction, trans-esterification and product purification processes and as Case B (USA), it was performed by fermentation, distillation, and hydrodeoxygenation processes. Microalgae cultivation and dewatering (centrifuge and filtration) processes

are common for both cases. As a result of the analysis, the costs in Case A and Case B were calculated as $ 1279/tonnes and $ 430/tonnes respectively. The main reason for this difference is that the bioethanol and biogas produced in Case B reduce the energy input to the process. In the case study of Brownbridge et al. [53] techno-economic evaluation of biodiesel production from algae was carried out. The global sensitivity analysis revealed that the algal biodiesel production cost was sensitive to the following parameters: algae oil content > algae annual productivity per unit area > plant production capacity > carbon price increase rate. It is also estimated that for a large-scale plant (100,000 tonnes biodiesel per year), the production cost of biodiesel is 0.8–1.6 €/kg. Batan et al. [48] reviewed the technical and economic feasibility of a closed microalgae cultivation system (photobioreactor) for 10 million gallons of biofuel production per year. As a result of the techno-economic analysis, it is seen that 63% of the total cost is the operating cost, 30% is the investment cost and the remaining 7% is the land purchase. It was also found that the total investment cost was $ 327.74 MM and the operating cost was $ 86.52 MM/year. Barlow et al. [54] investigated the feasibility of producing renewable diesel by hydrothermal liquefaction of algal biomass produced in an algal biofilm reactor. Sensitivity analysis shows that the algal productivity is the most important parameter for fuel sales price. In addition, it has been stated that the use of wastewaters in microalgae cultivation has significantly reduced environmental problems. Xin et al. [49], have designed a pilot system for algal-based biofuel production. In the designed pilot scale system, microalgae production was carried out in photobioreactors and the total cost of production was calculated as $ 0.33/kg biomass. In this system, because of microalgae production in wastewater, the operation cost is reduced. Also chars produced as by-products in the system have been evaluated in the drying stage.

4.2. Case study for algal biorefinery

In our study, *Chlorella vulgaris* was chosen to produce β-carotene and biodiesel by presenting two scenarios. Production stages were illustrated in **Figure 1**. *Chlorella vulgaris* is highly used in the industrial field because of its high productivity (1.56 g/L.day), high rate of CO_2 fixation (1.99 g/L.day) and high tolerance to environmental conditions [55, 56]. One of the most important of application areas is biodiesel production (due to high lipid content). The lipid content of *Chlorella vulgaris* is approximately 15–25%; carbohydrate and protein contents of *Chlorella vulgaris* are 9 and 55%, respectively [45, 57]. Apart from these, *Chlorella vulgaris* contains high-grade carotenoids. This microalga contains approximately 75 μg/g dry mass of β-carotene [58]. The two scenarios each produce 100 tonnes of dry weight (DW) biomass per year. Each scenario assumes optimal growth conditions as found in the literature. All scenarios produce two products: β-carotene or biodiesel and a fertilizer, consisting of the residual biomass. In addition, glycerol as a by-product will be obtained in the production of biodiesel. The algal-based biorefinery is operated for 270 days per year. The other days cannot be used for cultivation because of inappropriate climate conditions and maintenance requirements.

PBR was selected as cultivation method for the production of the microalgae. *Chlorella* cultures were cultivated in BBM medium. The maximum biomass concentration was assumed to

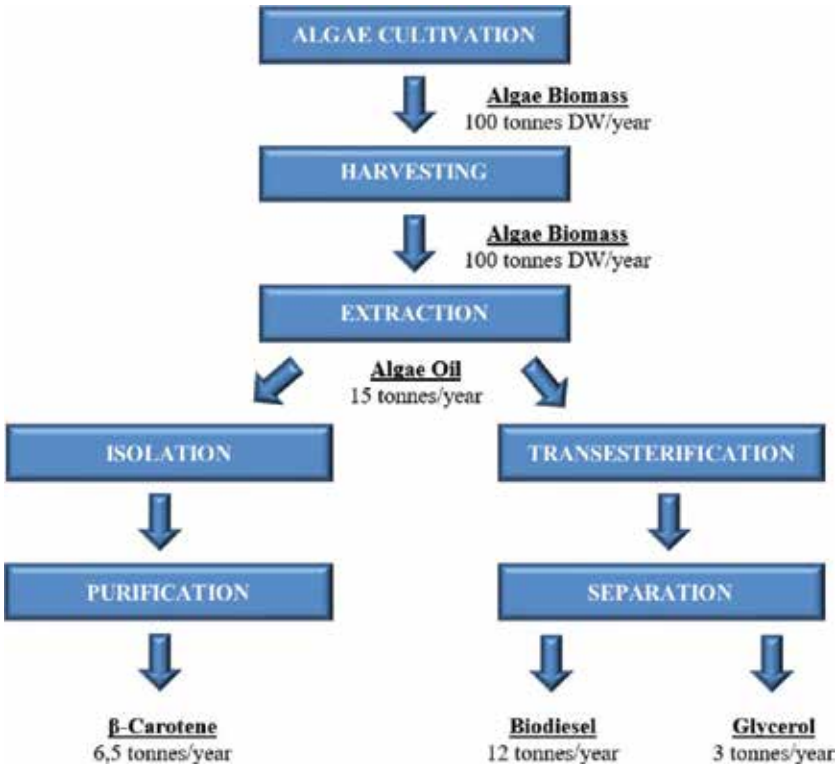

Figure 1. Illustration of the β-carotene and biodiesel production stages.

be 1.56 gr/L day [55]. The maximum specific growth rate was assumed to be 0.28 day^{-1}, based on the study of Yang et al. 2011. The reactor volume in cultivation stage was 300 m^3(R-101). A centrifuge (C-101) was used to harvest the microalgae (between streams 5 and 6). The centrifuge was assumed to have a biomass recovery rate of 97% and an energy consumption of 1.4 kWh/m^3 culture medium [59]. A drying step increased the solid concentration of the biomass flow was increased by drying step (between stream 6 and 7). The technological specifications for the drying step were based on the study of Leach et al. [60]. To calculate the total energy consumption of this spray dryer (S-101), a factor of 2.9 was used to account for the heat exchanger energy transition efficiency. The total energy consumption equaled 5.1 MJ per kg of removed water. Lipid extraction (R-102) was carried out with via using a ratio of 1:1 of hexane in (between streams 7 and 8). The filtration step separated the liquid fraction, which contained the lipids dissolved in the hexane, from the solid fraction, which contained the residual biomass (between streams 11 and 19). No energy consumption was required in this step. The solid fraction went to an evaporation (S-101) step to recycle the hexane. The remaining fraction was sold as fertilizer (stream 19). Hexane mixed with microalgae oil was distilled in a vacuum distillation to obtain a relatively pure stream of oil. The calculation of the energy consumption used the same heat transfer efficiency factor as the drying and evaporation step. In the first case, algae oil was used for biodiesel production where transesterification process (R-103) was carried out with 80% efficiency in

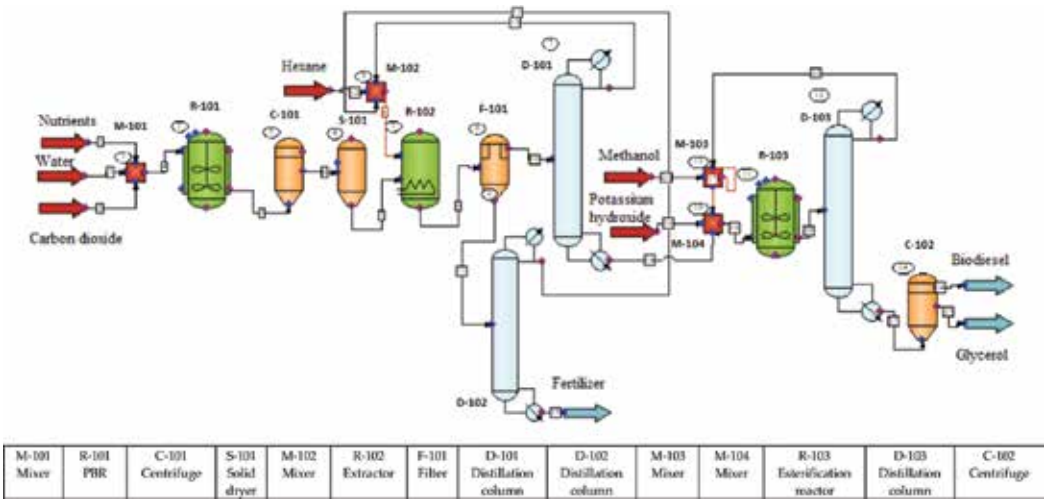

Figure 2. Process flow diagram of biodiesel production from microalgae.

Figure 2 which was created by Chemcad program (between streams 14 and 25). As for the second case, β-carotene production from microalgae after isolation and purification was assumed to be approximately 45%. Dry microalgae biomass was extracted (R-102) with acetone and β-carotene was obtained after sonication process in **Figure 3** (between stream 14 and 18). Inputs and outputs of β-carotene and biodiesel production from microalgae were given in **Table 2**.

Table 3 illustrates the main economic results for the two scenarios. When **Table 3** is examined, it is seen that the investment and operating costs are very close to each other in the two scenarios. The investment costs are the highest of all scenarios, due to the costs of the photobioreactor. The photobioreactor installation accounts for about 50% of the investment costs. Nutrients and chemicals account for about 30% of operating costs; and salaries constitute about 20% [49]. When revenues are examined, it is seen that there is a great difference. Because of this situation, β-carotene is a more valuable product than biodiesel. The average selling price of β-carotene is € 1370 per kg and the selling price of biodiesel is € 0.82/kg [45, 61, 62]. **Table 3** shows that this system is more suitable for β-carotene production. In order for biodiesel production to become economical, investment and operating costs must be reduced very seriously. In particular, the use of open ponds instead of photobioreactor will significantly reduce the investment cost. Furthermore, the use of an oil-rich microalga, production in wastewater and the use of recycled fractions will make biodiesel production more economical [45].

As mentioned in the introduction section, unlike the classical economy, bioeconomy includes the concepts of innovation, competition, knowledge based value added, and employment and sustainability. Within this approach, biological based productions or innovations are evaluated not only with techno-economic aspects, but with their systematic evaluation of the environmental effects of inputs and outputs at all stages in their life cycle. Life cycle involves modeling the life cycle of a product or production system. Life cycle analysis shows

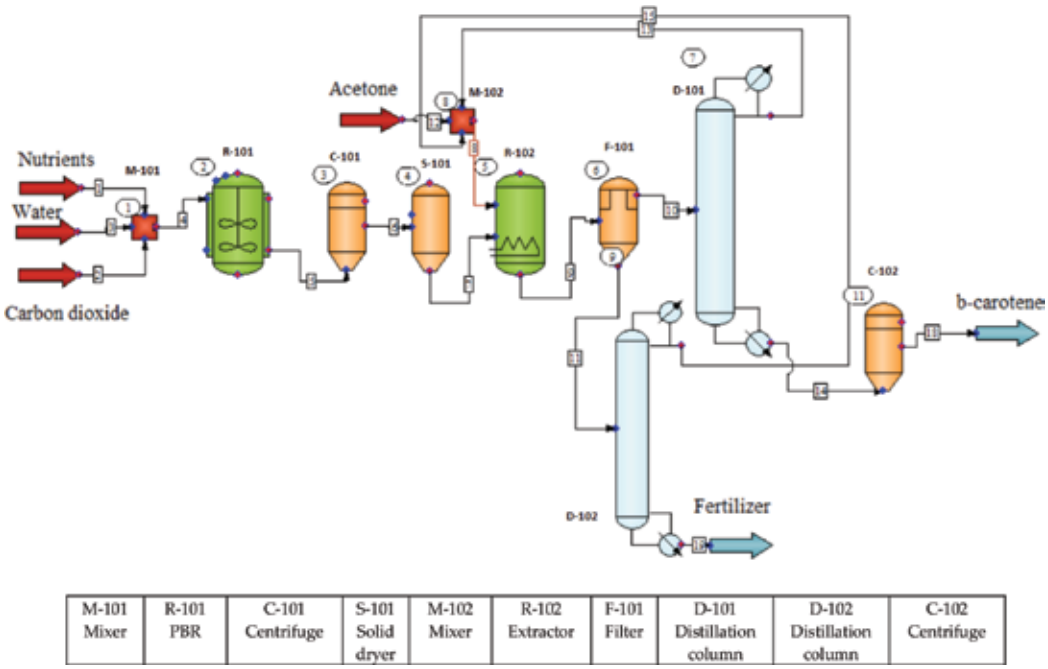

Figure 3. Process flow diagram of β-carotene production from microalgae.

	β-carotene	Biodiesel
Inputs		
Water (tonnes/yr)	81,000	81,000
CO_2 (tonnes/yr)	142,688	142,688
Nutrient (tonnes/yr)	9871	9871
Hexane (liter)	–	628.29
Acetone (liter)	4000	–
Electricity (GJ/yr)	10,675	9985
Heat (GJ/yr)	2231	2231
Land use (ha)	1.5	1.5
Outputs		
Product (tonnes/yr)	6.5	12
By-product* (tonnes/yr)	–	3*
Waste algae paste (tonnes/yr)	93.5	85

*Glycerol.

Table 2. Inputs and outputs of β-carotene and biodiesel production from microalgae.

	β-carotene	Biodiesel
Investment cost (€)	1,736,614	1,766,909
Operational costs (€/yr)	504,710	501,277
Revenues (€/yr)	4,270,500	11,698

Table 3. The economic results for the two scenarios.

all environmental impacts of an action; a system which comprises of evaluation of raw materials from the nature, and all the wastes that are returned to the nature. This assessment includes all the effects on the air, water and soil during the production, use and eventual destruction of the raw materials, including energy, as far as the product which is processed. This analysis is used both to identify and measure the effects directly (emissions produced during production and energy used etc.) as well as indirect (raw material disposal, product disposal, consumer use and disposal, etc.). These effects are directly connected with sustainability which is the ability to continuously process without consuming the basic resources of a society, an ecosystem or other similar interactive systems and without adversely affecting the environment. In this context, potential impact indicators are necessary for the selection and development of energy systems for the future. These indicators provide a common basis for comparing and evaluating different energy systems [63]. Bioethanol and biodiesel obtained from agricultural sources have lower global warming potentials, on the other hand, there are other environmental problems such as eutrophication, resource depletion and ecotoxicity that occur. Algal biotechnological production is a promising biotechnological area because of high photosynthesis efficiency, and low area requirement for cultivation of algae, and also nitrate and phosphate ions in wastewater can be a food source for algae. In addition to that, algae can utilize industrial CO_2 emissions directly as a carbon source [64]. In the recent life cycle analysis studies on algae systems show that sustainable productions seem to have increased. In these studies, it has been found that CO_2 emissions are effectively reduced in comparison of other production facilities [65]. Algae can recycle of pollutant nitrogen in wastewater. The use of a toxic substance such as urea by algae also shows the contribution of algae to the environment [66]. When all stages of the algal process are taken into consideration, it is seen that requirement of electricity occurs mostly during the cultivation of the algae. The energy requirements of all stages and global warming potentials are much lower than the growth phase. The energy requirement in the algal system and global warming potential depend on the oil productivity during growing, the circulation rate of algae during growing, and the industrial CO_2 gas concentration [67]. 40% of CO_2 emissions are generated from electricity generation, and 30% are from vehicle fuels. In 2013, global CO_2 emissions are 36 gigatonnes. Natural processes absorb half of this amount. Therefore, carbon dioxide shows a net increase of 18 gigatonnes per year in the atmosphere. One tonne of carbon is equivalent to $MW_{CO2}/MW_C = 44/12 = 3.7$ tonnes of carbon dioxide. In the equation, MW_{CO2} is the molecular weight of carbon dioxide, MW_C is the molecular weight of carbon, e_{CO2} is the carbon dioxide emission (kg_{CO2}/kWh), C_f is the carbon content in the fuel (kg_C/kg_{fuel}), and E_f is the energy content of the fuel (kWh/kg_{fuel}). Carbon dioxide emissions can be calculated from the following formula:

$$e_{CO2} = \left(C_f/E_f\right)\left(MW_{CO2}/MW_C\right) \quad (1)$$

In the case study of this chapter, carbon dioxide emission was found as 0.033 t_{CO2}/kWh which was lower than emissions of CO_2 from the combustion of the same amount of coal (anthracite) and natural gas. This indicates the advantage and positive contribution of the algal productions over fossil fuel sources. There is no global warming impact of the biodiesel process. Sander and Murthy [68], reported that; net CO_2 emissions are −20.9 and 135.7 kg/functional unit for a process utilizing a filter press and centrifuge in harvesting of algae. Furthermore, the −13.96 kg of total air emissions per functional unit, 18.6 kg of waterborne wastes, and 0.28 kg of solid waste are calculated as output. The largest energy input (89%) is in the natural gas drying of the algae. While net energy for filter press and centrifuge processes are −6670 and −3778 MJ/functional unit, CO_2 emissions are positive for the centrifuge process but they are negative for the filter press process. Moreover, 20.4 m^3 of wastewater is lost from the growth ponds during evaporation in the 4-day growth cycle. LCA has one major obstacle in algae technology: the need to efficiently process the algae into its usable components. LCA clearly shows a need for new technologies to make algae biofuels a sustainable, commercial reality. Another study reported that; when algal biofuel production modeled, substantial reductions in GHG emissions were achieved in the model due to the non-fossil treatment of the carbon in the biofuel and because substantial energy and nutrient recovery credits from processing of residuals were included. Fugitive emissions of methane and N_2O respectively totaled 14 and 23% of the whole pathway GHG emissions. Techno economic modeling must choose technologies that control these emissions. LCA requires superior data on fugitive emissions and must account for unrecovered nitrogen leading to N_2O. Nitrogen transported to fields to displace mineral fertilizers maybe has the potential to produce N_2O emissions. Nitrogen fraction, especially that which produces N_2O, a potent greenhouse gas with global warming potential 298 times that of CO_2. Agricultural techniques may be reduce capital costs substantially; however, these techniques need attentive evaluation with regard to fugitive emissions of N_2O. Lipid fraction and productivity are two strong drivers of economic viability. The large global warming potential for methane could make the costs for controlling methane emissions higher than the economic value returned and in that case, sustainability and economic drivers would be at odds [69]. Clarens et al. [66], reported that, the impacts associated with algae production were determined using a stochastic life cycle model and compared with switchgrass, canola, and corn farming. The results of this study indicate that these conventional crops have lower environmental impacts than algae in energy use, greenhouse gas emissions, and water regardless of cultivation location. The algae cultivation is driven dominantly by impacts, such as the demand for CO_2 and fertilizer. To reduce these impacts, flue gas, wastewater and novel biofuel production methods such as supercritical process, ultrasound and microwave assisted processes could be used to stabilize most of the environmental loads associated with algae [70]. To represent the benefits of algae production coupled with wastewater treatment, was expanded to include three different municipal wastewater as sources of nitrogen and phosphorus. The use of source-separated urine was found to make algae more environmentally beneficial than the terrestrial crops.

5. Conclusion

Algae have come into prominence as a future carbon-neutral biofuel feedstock because of their several advantages. Despite of having been studied for over 50 years now, there are still only just a few corporations that are cultivating algae for biofuel production on a large or commercial scale. The economics of producing algae for biofuel or bioproducts are not cost effective. For this reason, it is necessary to perform a techno-economic assessment and life cycle analysis before the pilot or large-scale microalgal productions to foreseen the pros and cons of the considered algal production system. In this chapter, before the evaluation of algal production with bioeconomical aspects, firstly, bioeconomy term has been described and its classification is given in detail. Also bioeconomy approaches of European countries and the world are presented to show the importance of microalgal production. Techno-economic assessment is explained and techno-economic assessment of microalgal productions are presented in detail and cost-effective approaches are evaluated case by case in basis. Also, two case studies are presented by us, to compare the economical inputs and outputs and environmental effect of the systems such as CO_2 emission and global warming potential has given. It is clear that, a biorefinery system which utilize wastewater or flue gas are economically viable. On the other hand, in the case of obtaining special products which will be utilize in pharmaceutical or food industry, genetic improvements, innovative and optimum design of cultivation systems which have different configuration or working principle, or various recycling systems should be considered to reduce the operational cost. And microalgae, which captured CO_2, should be used in many sectors, especially in biorefinery concept from the point of view of bioeconomy which comprises using renewable biological resources and sustainability.

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Edited by Eduardo Jacob-Lopes, Leila Queiroz Zepka and Maria Isabel Queiroz

Microalgal Biotechnology presents an authoritative and comprehensive overview of the microalgae-based processes and products. Divided into 10 discreet chapters, the book covers topics on applied technology of microalgae. *Microalgal Biotechnology* provides an insight into future developments in each field and extensive bibliography. It will be an essential resource for researchers and academic and industry professionals in the microalgae biotechnology field.

Published in London, UK

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ISBN 978-1-83881-417-5