Chapter

Physiological Limitations and Solutions to Various Applications of Microalgae

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Abstract

Despite over a century of research, the various applications of microalgae have only been realized only since the 1940s. With a repertoire of valuable products like biodiesel, astaxanthin, canthaxanthin, lutein, β-carotene, phycocyanin, chlorophyll a, polyunsaturated fatty acids, exopolymeric substance (EPS), and biohydrogen, the commercial importance of microalgae and demand for its product is gaining increasing attention. However, successful transition of the synthesis of microalgal products from laboratory to industries has yet to be realized, even after over 70 years of extensive research. This failure of commercial success of microalgal products can be attributed to the lack of understanding of the physiological role of the products and biological constraint placed by the bioenergetics and physiology, which has been largely ignored. This chapter focuses on the physiological limitations behind synthesis of microalgal products, highlights the crucial unknowns behind the role and synthesis of these products, and hints strategies to overcome the limitations to realize the commercial dream of microalgal products.

Keywords: microalgae, biofuels, PUFA, EPS, hydrogen

1. Introduction

The biological reaction of photosynthesis has played a significant role in shaping the course of evolution through the great oxygenation event converting atmospheric CO_2 into organic form that is being currently extracted as modern day fossil fuels and finally balancing the elemental cycles (C, N, P, and Si) on Earth. The magnitude of the impact photosynthesis reaction has on humans has encouraged scientists, engineers, and others to wonder about exploiting the potential of this reaction in different ways. Factors such as simplicity, metabolic elasticity, diversity, shorter life cycles, and the range of commercially important products synthesized make microalgae the most attractive and practical option of all the other photosynthesizing life forms. Some of the commercially important products produced by microalgae include biofuels (i.e., biodiesel, biohydrogen), high value products (i.e., astaxanthin, canthaxanthin, β-carotene), polyunsaturated fatty acids (i.e., omega-3-fatty acids), farming products (i.e., animal and fish feeds), fertilizer, single-cell proteins, and miscellaneous products such as exopolymeric substances (EPS) that have various applications. Despite the potential of microalgae in producing such a variety of commercially important products, the idea of microalgae mega factories has yet to become a reality. Several factors impede the commercial success

of microalgal products. This includes existing sources (fossil fuels), technological development, and physiological limitation. In this chapter, we will describe the various known physiological limitations associated with productions of various microalgal products in a commercial scale and list possible solutions.

2. Biodiesel from microalgae

Spoehr and Milner in 1949 demonstrated that the lipid content increased in the green microalga *Chlorella* under nitrogen starvation [1]. However, it was not until the early 1970s, with the oil embargo and the increase in energy prices, that the concept of lipids from microalgae for biofuel production gained attention [2]. Despite decades of research, biodiesel from microalgae has yet to be introduced to the gas station near you. It has been reported that microalgae can produce around 20–50% of their dry weight as lipids [3, 4], with only one research article suggesting as high as 80% [5]. The amount of lipid produced by a variety of species under optimum growth conditions is summarized in **Table 1**. The ability to synthesize a higher percentage of their dry weight as lipids makes microalgae the highest producer of

Microalga	Taxa	%Lipid	%Protein	%CHO	Growth rate d^{-1}	Source
Amphora sp.	Bacillariophyceae	13.6	17.3	74.9	5.1	[6]
Amphiprora hyaline (ENTOM3)	Bacillariophyceae	22–37			2.3	[6]
Anabaena cylindrical	Cyanophyceae	4–7	43–56	25–30		[7]
Ankistrodesmus falcatus	Chlorophyceae	40.3	14.3	18.3	2.89	[6]
Boekelovia sp.	Chrysophyceae	20.7			3.43	[6]
Botryococcus braunii	Chlorophyceae	54.2	20.6	14.3	1.8	[6]
Chaetoceros sp.	Bacillariophyceae	22.2	31.9	43	4.3	[6]
Chaetoceros calcitrans	Bacillariophyceae	16	34	6		[8]
Chaetoceros gracilis	Bacillariophyceae	7	12	5		[8]
Chaetoceros muelleri	Bacillariophyceae	31	59	10		[9]
Chlamydomonas reinhardtii	Chlorophyceae	21	48	17		[7]
Chlorella sp.	Chlorophyceae	34–48	19–31		1.33	[6]
Chlorella ellipsoidea	Chlorophyceae	8.9	26.1	26.3	5.3	[6]
Chlorella protothecoides	Chlorophyceae	15–56				[6]
Chlorella pyrenoidosa	Chlorophyceae	2	57	26		[7]
Chlorella vulgaris	Chlorophyceae	14–22	51–58	12–17		[7]
Cyclotella sp.	Bacillariophyceae	42.1	16.4	10.2	5.1	[6]
Dunaliella bioculata	Chlorophyceae	8	49	4		[7]
Dunaliella salina	Chlorophyceae	6	57	32		[7]
Dunaliella tertiolecta	Chlorophyceae	15–43			2.58	[6]
Euglena gracilis	Chlorophyceae	14–20	39–61	14–18		[7]
Isochrysis aff. Galbana	Prymnesiophyceae	26	23.3	20.5	2.83	[6]
Monoraphidium sp.	Chlorophyceae	17.9–24.4		25.5	3.1	[6]
Nannochloris	Chlorophyceae	31–63			3.19	[6]

Microalga	Taxa	%Lipid	%Protein	%CHO	Growth rate d^{-1}	Source
Nannochloropsis	Chlorophyceae	46–68			1.05	[6]
Nannochloropsis granulata	Chlorophyceae	24–28	18–34	27–36		[10]
Nannochloropsis oculata	Chlorophyceae	17.8–39.9				[6]
Nannochloropsis salina	Chlorophyceae	59.8	24.3	15.9	1.05	[6]
Nannochloropsis sp.	Chlorophyceae	64			1.04	[6]
Navicula acceptala	Bacillariophyceae	32–48			3.8	[6]
Nitzschia sp.	Bacillariophyceae	27	36	16		[6]
Nitzschia dissipata	Bacillariophyceae	66	12.6	9.3	1.32	[6]
Nitzschia closterium	Bacillariophyceae	13	26	10		[8]
Oocystis pusilla	Chlorophyceae	10.5	39	37		[6]
Phaeodactylum tricornutum	Bacillariophyceae	16–50	31–35	11–17	1.96	[6, 8, 10]
Porphyridium cruentum	Rhodophyceae	9–14	28–39	40–57		[7]
Prymnesium parvum	Prymnesiophyceae	22–38	28–45	25–33		[7]
Scenedesmus obliquus	Chlorophyceae	12–14	50–56	10–17		[7]
Scenedesmus quadricauda	Chlorophyceae	1.9	47			[7]
Scenedesmus dimorphus	Chlorophyceae	16–40	8–18	21–52		[7]
Skeletonema costatum	Bacillariophyceae	10	25	5		[8]
Spirogyra sp.	Chlorophyceae	11–21	6–20	33–64		[7]
Spirulina maxima	Cyanophyceae	6–7	60–71	13–16		[7]
Spirulina platensis	Cyanophyceae	4–9	46–63	8–14		[7]
Synechococcus sp.	Cyanophyceae	11	63	15		[7]
Tetraselmis sp.	Chlorophyceae	18	46	36	2.1	[6]
Tetraselmis chuii	Chlorophyceae	20			2.1	[6]
Tetraselmis maculate	Chlorophyceae	3	52	15		[7]
Tetraselmis succia	Chlorophyceae	15–33			2.1	[6]
Thalassiosira pseudonana	Bacillariophyceae	19	34	9		[8]

Table 1.
Summary of macromolecular composition and growth rate of microalgae belonging to various taxa.

lipid per unit mass and arguably the most practical choice for the production of biodiesel. Theoretical estimates of maximum biomass production vary from 24 to 65 g m^{-2} • day^{-1} [7]. Benedetti et al. have reported a theoretical maximum up to 280 tons ha^{-1} $year^{-1}$ of biomass but also noted that the actual cultivation record never exceeded 100 tons ha^{-1} $year^{-1}$ [11]. Even assuming the theoretical maximum estimates of biomass and 80% of the biomass composition as lipid, the maximum amount of 224 tons ha^{-1} $year^{-1}$ of lipids is what can theoretically be produced. However, the lipid produced has to be transesterified to fatty acid methyl esters, which then can be used as biodiesel, which can result in some loss. However, earlier studies have reported up to 96% recovery of lipids through direct transesterification [12]. With 4% loss by transesterification, a maximum of 215 tons ha^{-1} $year^{-1}$ of biodiesel can be produced. It is important to note that a recent study on *Scenedesmus accuminatus* using open and polyhouse raceway ponds estimated around 2.1 tons

ha^{-1} $year^{-1}$, which is 100 times lower than the theoretical maximum [13]. Another study with *Chlorella* sp. L1 and *Monoraphidium dybowskii* Y2 using batch and semicontinuous mode in a raceway pond resulted in lipid productivities of 13.91 and 14.45 ton ha^{-1} $year^{-1}$ in semicontinuous mode [14], tenfold lower than the theoretical estimates. This discrepancy between theoretical estimates and laboratory and field tests are primarily due to physiological limitations, especially carbon fixation and light absorption [15, 16].

The current demand of fossil fuel is approximately 100 million barrels per day (~11,563 million liters per day), which is only expected to increase with time (105 million barrels per day by 2021) [17]. To meet this magnitude of global demand, assuming 15 ton ha^{-1} $year^{-1}$ lipid productivity from microalgae, around 276 million hectares of land would be required for microalgal cultivation every day. This huge demand can be reduced by increasing the lipid productivity of microalgae by minimizing energy and carbon wasting physiological processes in the cell and redirecting it towards lipid synthesis. RUBISCO, the enzyme catalyzing the dark reaction of photosynthesis, wherein the atmospheric or dissolved CO_2 is converted into organic triose phosphate, has a lower affinity to CO_2. In addition, oxygen is a competitive substrate for RUBISCO, catalyzing the reaction of photorespiration wherein energy and NADPH are utilized and fixed CO_2 is released. Therefore, during CO_2 limitation, some species of microalgae utilize a carbon concentration mechanism (CCM) that increases the concentration of CO_2 at the site of RUBISCO, leading to the catalysis of the dark reaction of photosynthesis. Although the CCM is an ingenious mechanism evolved to address the lower CO_2 affinity of RUBISCO and the competition by oxygen, the process consumes one to two molecules of ATP [18]. This energy can instead be redirected towards lipid synthesis by simply bubbling CO_2 into the cultures [19] or by selection CCM lacking microalgae species.

Finkel et al. found that the median macromolecular composition of nutrient-sufficient exponentially growing microalgae is 17.3% lipid, 32.2% protein, and 15% carbohydrates [20]. For biofuels, lipids are the most important cellular fraction. *Botryococcus braunii* has the highest lipid as percent dry weight, 43%, while *Tetraselmis suecica* and *Dunaliella tertiolecta* have the least (<10%). Also in species-specific differences (**Table 1**), Finkel et al. showed that there are some phylogenetic differences [20]. Cyanobacteria have the lowest lipid content (11.7%; dry wt), while the Bacillariophyta (diatoms) had the highest (18.6–21.3%; dry wt). In fact, in most cases, these median values are all significantly lower than those values reported in **Table 1**, which are up to 60% (w/w) for lipid, protein, and carbohydrate. The values at the higher end of the spectrum are typically induced after some kind of stress (e.g., light, nutrient) is placed on the microalgae. Nutrient stress is most often used to induce lipid production, particularly nitrogen [5, 21, 22]. These large lipid stores are thought to provide a growth advantage under variable resource supplies [23].

Light harvesting systems of microalgae are usually capable of absorbing between 350 and 700 nm; however much of the radiation extending out to 1100 nm remains unused. In addition, Raven et al. calculated a 22% loss of energy even within the spectrum of 350–700 nm [24]. However, anoxygenic photosynthetic bacteria possess bacteriochlorophylls capable of absorbing light up to 1050 nm and perform photochemistry [19]. These bacteriochlorophyll-based photosystem can be engineered into microalgae. By replacing photosystem I, it would extend the range of absorbed photons that can be used for photochemistry [25], therefore the energy available for lipid synthesis. It has been proposed that smaller cells can photosynthetically perform better larger cells [26, 27], primarily attributing to the "package effect." Package effect refers to the inverse relationship between light harvesting ability of the cells and the cell volume. However, studies have shown that larger cells tend to decrease the antennae size, thereby counteracting the negative impacts of

package effect [28, 29]. Moreover, several studies have shown that decreasing the antennae size significantly increased the photosynthetic efficiencies [30, 31]. This could be due to decreased photoinhibition and decreased allocation of resources towards light harvesting systems [19]. However, the hypothesis that larger cells would be less photosynthetically efficient than smaller cells due to package effect was recently disproved by a recent study by Malerba et al. [32], which showed that larger cells developed compensatory mechanisms by reducing the antenna size, increasing the connectivity between the photosynthetic units, and decreasing the levels of photo-protective pigment β-carotene. This in turn minimized the negative significances of larger cell volume-induced package effects. Exposure to extreme conditions such as photoinhibitory light levels, UVA and UVB radiation, and nutrient and temperature stress could result in oxidative damage to cellular components especially proteins. These kinds of damage result in breakdown of the damage component and re-synthesis of these components or adaptive changes to the cellular components. These changes however have a cost in terms of energy (i.e., ATP, NADPH) and resources which could have been directed towards biomass and lipid synthesis. Protein turnover during scotophase has been shown to consume about one-third of the total respiratory ATP production [33]. Exposure to nitrogen-limiting conditions could result in upregulation of nitrogenase in diazotrophic cyanobacteria, which in turn could result in 10% decrease in growth rates [34, 35]. These are avoidable energy and resource expenditure, which can be easily prevented by providing optimal light, nutrient, and temperature during mass cultivation.

Lipids are not secreted/excreted actively by all microalgae, with exception of *Botryococcus* sp. [36]. This constrains the maximum amount of lipid that is synthesized and stored in a microalgal cells, as the largest reported cell volume for a microalgae is for species *Noctiluca scintillans* belonging to the class Dinophyceae (83,700,000 μm^3) [37]. The most commonly used microalgal species in biotechnology belong to the class Chlorophyceae such as *Chlorella* sp., *Haematococcus* sp., and *Scenedesmus* sp., which have cell volumes of 34.5, 48, and 26,200 μm^3, respectively [38], and the species belonging to class Bacillariophyceae such as *Phaeodactylum tricornutum* and *Thalassiosira pseudonana* which have cell volumes of ~60–100 μm^3 and 28–200 μm^3 [37, 39]. On the other hand, the maximum cellular density has an inverse relationship with the volume of a cell, by a factor of 3/4 [38]. This further constrains the maximum amount of lipids that can be synthesized in a microalgal mass culture. However, if the lipids were secreted in a manner similar to EPS, physiological limitations such as cell volume or maximum density would not play a constraining role on the maximum amount of lipid that can be synthesized in an algal mass culture. Microalgal species like *Botryococcus* sp. has already been shown to actively secrete lipids that can be converted into fuel-grade biodiesel. However, their slower growth rates limit the maximum lipids that can be synthesized. Microalgal species like *Scenedesmus* has been shown to grow as fast as 1.53 per day [40]. Therefore, genetic engineering techniques to integrate the lipid-secreting trait into the fast-growing *Scenedesmus* sp. could be a possible solution.

Phototrophic modes of cultivation depend on two critical factors, namely, light and carbon dioxide [16]. Therefore, microalgae grown phototrophically can either be limited in light or carbon dioxide or both, limiting the maximum cellular density one can achieve using this mode of cultivation. In addition, various inefficiencies described above with light-harvesting abilities of microalgae, lower affinity for CO_2 of RUBISCO, cost associated with protein turnover, and photoinhibition can be simply avoided by growing microalgae heterotrophically and mixotrophically. Extensive amount of research suggests enhanced biomass production under heterotrophic and mixotrophic modes of cultivation, increasing the cellular density to as much as 4 to 5 times [41, 42]. Further modification in these modes of cultivation such as using

fed-batch mode has resulted in further increase in biomass as much as two- to five-fold [43, 44]. Therefore, by growing a hybrid strain of lipid-secreting *Botryococcus* sp. and fast-growing *Scenedesmus* sp., in a fed-batch heterotrophic or mixotrophic cultivation system, one can possibly overcome the physiological limitations of the maximum amount of lipids that can be synthesized in a microalgal system.

3. High valued products

Apart from lipids for biodiesel, microalgae synthesize other products of value such as astaxanthin, canthaxanthin, lutein, β-carotene, phycocyanin, chlorophyll *a*, and polyunsaturated fatty acids (PUFAs) such as γ-linolenic acid, docosahexaenoic acid, and eicosapentaenoic acid. A subset of these, astaxanthin, canthaxanthin, lutein, and β-carotene, have antioxidant properties and are commercially high valuable pharmaceutical products. Phycocyanin is a high value natural food dye, and products such as γ-linolenic acid and eicosapentaenoic acid are considered animal-free based sources of essential fatty acids. Some of these products are also considered to boost the immune system. **Table 2** summarizes the common high value products, their use, the species of microalgae most commonly grown to harvest the products, and the global market values.

Product	Use	Species (dominant)	Global market value*
Carotenoids			Total US$1.2 billion in 2010
β-carotene	*Antioxidant, anti-inflammatory*	*Dunaliella salina, Dunaliella bardawil*	US$ 300–1500 kg^{-1}
Astaxanthin	Feed additive for farmed fish; pigmenter of the fish flesh	*Haematococcus pluvialis*	US$ 2500–7000 kg^{-1}
Phycobilins	Food coloring, cosmetics coloring		US$ 60 million
C-Phycocyanin	*Antioxidant, anti-inflammatory*	*Spirulina*, *Porphyridium*, *Rhodella, Galdieria*	US$ 500 to 100,000 kg^{-1}
Fatty acids *DHA* *EPA* *PUFA*	*Health food supplements*	*Nannochloropsis, Tetraselmis, Isochrysis, Thalassiosira, and Chaetoceros*	
Docosahexaenoic acid		*Crypthecodinium cohnii*	US$140 kg^{-1}
Omega-3 oils			US$1.5 billion or US$80–160 kg^{-1}
Sterols	Pharmaceutical applications or in functional foods		US$ 300 million
Polyhydroxyalkanoates	Production of biodegradable plastics	*Spirulina, Synechocystis*	

**(from Borowitzka - 2013, Chew et al. 2017) [45, 46].*

Table 2.
Summary of the main high value products derived from microalgae.

The major fraction of studies on microalgal PUFA have primarily focused on screening for microalgal species and optimization of conditions that lead to enhanced PUFA products. However, we have no clear understanding on the physiological role of PUFAs in microalgae. It is hypothesized that PUFAs might play a role in homeoviscous adaptation [47], i.e., the enhanced fluidity provided by increased unsaturation membrane fatty acids. Additionally, the fatty acid composition and therefore the PUFA concentrations appear to have some level species specificity, suggesting varying roles [48]. However, the physiological roles of PUFAs are not yet confirmed in microalgae, and therefore strategic optimization of their synthesis in microalgae has not been realized. Nevertheless, PUFAs make a significant portion of the neutral lipid content synthesized in microalgae; therefore optimization strategies discussed above to enhance biomass and lipid synthesis should also increase the PUFA content in microalgae.

Astaxanthin is another promising product synthesized by microalgae that has already achieved a profit of $200 million per year [49]. Astaxanthin is a red ketocarotenoid pigment ubiquitous in nature and has antioxidative, anti-inflammatory, and anti-apoptotic properties. It is also proposed as a potential therapeutic agent for cardiovascular and neurological diseases [50]. Apart from its pharmaceutical use, this product is also used as a pigment source in aquaculture of salmon and trout [51]. Despite the relatively vast amount of research into its properties and applications, the mass production of astaxanthin is still unable to meet its huge market demand [49]. This problem can be primarily attributed to the lack of knowledge on the underlying mechanisms of why these algae accumulate astaxanthin similar to PUFAs and hence the lack of better strategies to optimally produce the pigment. The most prominent hypothesis regarding its production includes a multifunctional photoprotective response to stress induced by exposure to unfavorable conditions (excess light, UV-B radiation, nutrient deprivation) leading to ROS formation [52–54]. However, this hypothesis does not explain the synthesis of astaxanthin under heterotrophic conditions [55, 56]. Therefore, more studies are needed to have a better understanding of the physiological roles of astaxanthin.

Nutrient limitation such as nitrogen, phosphorus or sulfur are widely used strategies for inducing astaxanthin accumulation in *Haematococcus pluvialis* [57, 58]. However, nutrient limitation reduces the maximum amount of biomass one can achieve, thereby reducing the total amount of astaxanthin that can be produced. Additional strategies for inducing astaxanthin production include high salt stress and high light exposure, but these external stressors also lower the biomass yield and therefore the associated pigment production. Using stress as a mechanism to induce astaxanthin synthesis has the fundamental problem of stopping cell cycle and therefore reducing biomass and astaxanthin production. Hence, new strategies to boost astaxanthin and biomass production with the use of stressors need to be discovered.

Phycocyanin is a phycobiliprotein exclusively produced by cyanobacteria and commercially important as a high value natural blue coloring agent for food. Being a part of light-harvesting pigment-protein complex in cyanobacteria, phycocyanin is very critical in light capture and therefore is an indispensable element in the growth and survival of cyanobacteria. Phycocyanin has been shown to constitute up to 60% of the total cellular protein content [59]. As it is involved in light capture, its synthesis is tightly regulated by the wavelength of light the cells are exposed to. Green light has been shown to stimulate the synthesis of phycocyanin, whereas red light has the opposite effect [60]. In addition, low light levels have been also shown to induce accumulation of phycocyanin [61, 62]. Other factors such as glucose and salt have also been shown to enhance the synthesis of phycocyanin [61]. Despite knowing their physiological roles, the commercial success of phycocyanin from

microalgae is limited as their production is strictly dependent on the maximum amount of biomass that can be generated. A suggested final concentration of higher than 10% of cell dry weight of phycocyanin is required to make a profit over the cost of pigment downstream separation [63]. With factors such as blue light and low light levels required to induce maximum cellular synthesis of phycocyanin, the growth is significantly attenuated under these low energy light conditions, thereby limiting the maximum biomass and hence the maximum quantity of phycocyanin that can be synthesized. Therefore, a dual phase production approach to maximize the biomass production under mixotrophic conditions with cheaper organic carbon source such as molasses [41] followed by the second phase of low levels of blue light to stimulate the synthesis phycocyanin can significantly maximize the total amount of phycocyanin that be produced.

4. Exopolymeric substances from microalgae

Studies show that microalgae actively release from 3 to 40% of the fixed carbon into the surrounding environment as exopolymeric substances, mostly polysaccharides and proteins but also nucleic acids, DNA, RNA, and other macromolecules [64]. Although initially presumed as experimental artifact or a product of dead and decaying phytoplankton [65], EPS is now universally accepted as a product that is actively secreted by microalgae. The relatively higher percentage of fixed carbon released extracellularly has led the physiologist to question the reasons behind this phenomena. Several hypotheses have been put forward, including carbon overflow, photoprotection of the over-reduced photosystems, motility, self-defense mechanisms, active selection of phycosphere residents, and passive excretion due to osmosis and permeability. The hypothesis of carbon overflow and photoprotection has been discredited due to the presence of proteins, amino acids, and vitamins in the released substances and due to the secretion of EPS during the night [66]. The hypothesis of EPS secretion as a self-defense mechanism, motility, active selection of phycosphere residents, and passive excretion due to osmosis and permeability needs to be experimentally tested.

Experimental studies have shown contrasting results in the secretion of EPS in response to environmental factors such as temperature, nutrient (N, P, and S) limitation, salinity, and heterotrophy/mixotrophy [67]. The results vary depending on the species of microalgae being tested. In addition, EPS secretion during various phases of growth was species dependent, with some showing an increased secretion during stationary phase and others in exponential phase. Overall, with no universal explanation behind the mechanisms of EPS secretion by phytoplankton, and multiple hypotheses explaining the phenomena, strategic means to regulate the production and composition of EPS release by microalgae is clearly lacking. Although more research is needed, EPS are usually composed of carbohydrates, nitrogenous compounds, lipids, and organic acids [68]. Polysaccharides usually could account for 80–90% of the EPS composition even under healthy conditions [69]. Nitrogenous compounds, such as amino acids and proteins, on the other hand, only make up to 4–7% of the total EPS secreted [70, 71]. These protein fractions can include exoenzymes like phosphatase, β-glucosidase [72], and siderophores such as ISIP2a [73]. Characterization of EPS involves quantification of organic matter released as polysaccharides, proteins, lipids, neutral sugars, and/or uronic acids. EPS characterization of these macromolecules is often performed under the assumption these are the dominant molecules, however, possibilities of the same molecules possessing both sugar chain and a protein moiety are quite certain. Moreover, rarely are the monomers that make up these polymers investigated

(e.g., sugars that make up these polysaccharides), and therefore the polymeric composition and the structure and physical properties remain unknown. Therefore, more in-depth characterization of EPS from microalgal species of potential and under different growth conditions and growth phase are needed to integrate EPS from microalgae into the algal biotechnology market. Needless to say, these characterization studies of EPS have to be performed with appropriate controls within the context of its application, whether as a surfactant, lubricant, antioxidant, anti-inflammatory, antivirus, antibacterial, antifungal, or anticoagulant.

Xiao et al. [74] in their review compared the emulsifying activity of EPS from *Dunaliella salina* (88% retention) reported by Mishra et al. [75] to commercially available surfactants Tween 20 (65% retention), Tween 80 (60% retention), and Triton X-100 (65% retention). Furthermore, being extracellularly secreted, EPS is not constrained by physiological feedback inhibition mechanisms, or retention capacity dictated by the maximum cell volume, or the need of expensive procedures to lyse the cell to release the products from the cell, unlike other microalgal products (such as lipid for biodiesel, PUFAs, and high valued pigments). Therefore, with better-focused research, EPS has a relatively greater potential of becoming a commercial reality as a microalgal product.

5. Hydrogen from microalgae

With the raising awareness of cleaner and sustainable fuel, development of hydrogen fuel cell-powered cars, and the high cost and greenhouse gas emission associated with thermochemical hydrogen production, microalgae is increasingly becoming an attractive source for the fuel. First observed by Hans Gaffron in 1939 [76], this phenomenon of hydrogen production has been extensively studied since then. The production of hydrogen by microalgae only occurs during anaerobic conditions, due to the sensitivity of hydrogenase (the enzyme catalyzing the reversible reaction of hydrogen production) to oxygen. Three major enzymes that lead to the production of hydrogen in microalgae include (1) reversible/classical hydrogenases, (2) membrane-bound uptake hydrogenases, and (3) nitrogenase enzyme [77, 78]. Of all three, the reversible/classical hydrogenase is the most studied enzyme. Located in the chloroplast, the primary electron donor for this enzyme is photosystem I (PS I). However, the generation of molecular oxygen through photolysis by photosystem II (PS II) inhibits the activity of hydrogenase. Therefore, this enzyme only functions when the rate of photosynthesis is below the compensation point (rate of photosynthesis = rate of respiration). Past studies have achieved this condition by either flushing the system with argon or nitrogen [79] or using PS II lacking mutants [80], or selective excitation of PS I through far red light (>710 nm) [81], or more commonly through sulfur deprivation [82]. During a combination of anaerobiosis and below compensation point conditions, supply of electron from PSI to hydrogenase has been shown to either come from the excitation of PS I, and/or through the photolysis of water, and/or through non-photochemical reduction of the plastoquinone pool through type II NAD(P)H dehydrogenase that mediates the transfer of electron derived from anaerobic catabolism of cellular carbon reserve, primarily thought to be proteins at this point [83, 84].

It is proposed that under ideal conditions, one should expect a generation of 2:1 H_2/O_2 per 8 photons [85]. However, only around 20% of this efficiency is practically achieved [86]. The discrepancy between theory and practical estimations could be due to several physiological reasons unaccounted for in the theoretical estimation. (1) The physiological role of hydrogenase was although a mystery for the most part [86], discuss it's to act as an electron sink and hence oxidation of reducing

equivalents derived through anaerobic catabolism of cellular carbon reserve. Therefore, all the physiological and thermodynamic limitations that apply to anaerobic catabolism of cellular would in turn limit the supply of electrons and protons to hydrogenase. (2) Hydrogenase catalyzes a reversible reaction, and Kessler [84] has shown a "photoreduction" process of uptake of hydrogen gas to reduce molecular oxygen or CO_2 similar to the photosynthate derived under aerobic conditions. Whether the reduction of CO_2 through uptake of hydrogen gas by hydrogenase under anaerobic conditions involves the Calvin cycle remains to be demonstrated. Regardless, the uptake of hydrogen by hydrogenase should contribute to the reduced efficiency of the hydrogenase. (3) Direct oxidation of photosysnthetically derived reducing equivalents would mean a net production of 3.1:0 ATP/NADPH per 8 photons (4 molecules of H_2O) and generation of 2 H_2. This would provide no anabolic advantage to the cells producing hydrogen, as this would lead to a big imbalance in the ATP/NADPH levels in the cells. (4) Often the yields of hydrogen derived under sulfur deprivation are compared to the theoretical yield of 2:1 H_2/O_2 per 8 photons; however, during sulfur deprivation, the anaerobiosis is created by the failure to regenerate the high sulfur photo-damaged D1 protein; therefore the hydrogen gas derived is not directly through photolysis of water but instead through catabolism of carbon reserves [84, 87]. These carbon reserve can be starch or proteins [82], and every molecule of glucose derived from the breakdown of starch, is then catabolized to pyruvate through glycolysis yielding 2 NADH and ATP, based on the observation by Melis et al. [82] of increase in acetate levels beyond 120 hour incubation in sulfur deprived anaerobic condition in the presence of light and no ethanol of formate secretion, hints towards catabolism of pyruvate to acetate possibly via phosphotransacetylase and acetate kinase, which would generate an additional molecule of NADH and ATP. Atteia et al. [88] have detected phosphotransacetylase and acetate kinase in the species *Chlamydomonas reinhardtii* that was used in Melis et al. (2000) study [82]. Overall, this process of sulfur deprivation-induced anaerobiosis leads to a total of 3 NADH and 3 ATP, and 2 molecules of acetate from 1 molecule of glucose; therefore a net loss of 8 protons and hence $4H_2$ in the form of 2 acetate molecules and formation of $6H_2$ occurs in this biochemical pathway. This, when expressed per photon cost, would result in $6H_2$ per 32 photons (the ideal required amount to make a molecule of glucose), which would result in 75% efficiency compared to the ideal generation of $2H_2$ per 8 photons directly through photolysis of water via photosynthesis. The discrepancy of the observed 20% efficiency vs. calculated 75% via fermentation can be due to the existence of other pathways competing for the same NADH. Oxidative pentose phosphate pathway has been shown to be upregulated under sulfur deprivation-induced anaerobiosis [89, 90]; however, whether reductive pentose phosphate pathway is upregulated remains unknown. This is probably due to the cellular demand for ribulose 5-phosphate to synthesize nucleic acids and NADP and NAD to maintain the integrity/protect the DNA and cellular metabolism. Nevertheless, if the glucose was catabolized only via oxidative pentose phosphate pathway, only 2 NADPH would be synthesized, which would yield just two molecules of H_2, which would match the observed 20% efficiency of H_2 generation. However, despite nucleic acid synthesis, further catabolism of ribulose 5-phosphate is inevitable and should lead to generation of more NADH. Furthermore, catabolism via oxidative pentose phosphate pathway solely would not explain the increase in acetate levels observed by Melis et al. [82]. On the other hand, if proteins served as carbon source during sulfur-deprived anaerobiosis, we hypothesize only amino acid with three or more carbon chains would be utilized, based on the absence of acetate consumption during sulfur-deprived anaerobiosis observed by Melis et al. [82] and the absence of H_2 production and methyl viologen

reduction when supplied with α-ketoglutarate during anaerobiosis as observed by Noth et al. [91]. Both these studies indicate that TCA cycle was not active during anaerobiosis, which explains the absence of acetate and α-ketoglutarate uptake under such conditions, therefore making catabolism of amino acid smaller than the three-carbon chain (glycine) and serving as a source for reducing equivalents for impractical H_2 production. However, Noth et al. [91] did observe H_2 production and methyl viologen reduction when *Chlamydomonas reinhardtii* was grown on oxaloacetate, which suggest glyoxylate pathway might be still active under anaerobiosis. It is important to note the absence of acetate uptake in sulfur deprivation-induced anaerobiosis as seen in Melis et al. [82] study and the opposite phenomena observed by Gibbs et al. [87] where acetate was readily uptaken when anaerobiosis was established via flushing with N_2. This suggests that glyoxylate pathway is inhibited under sulfur deprivation-induced anaerobiosis, probably due to the iron–sulfur (Fe—S) cluster of aconitase [92], thereby limiting the ability of cells to use external acetate as a carbon source. Therefore, sulfur deprivation-induced anaerobiosis would lead to relatively lower H_2 production than N_2 flushing-induced anaerobiosis, which is due to the extra one NADH and FADH produced via glyoxylate pathway that could potentially yield an additional two molecules of H_2 per molecule of glucose and the two molecules of H_2 per molecule of external acetate metabolized. Nevertheless, calculation of H_2 per photon derived through protein catabolism is complicated by the presence of three to five carbon substrates and the various pathways through which they can be broken down to acetate. Isotope labelling studies will definitely help shed light into which amino acids are preferentially degraded during anaerobiosis and allow for a more accurate determination of H_2 per photon. Studies have suggested that using genetic engineering to develop mutants lacking the ability to carry out state transitioning, cyclic electron transport, and mutants that have a smaller antennae size produced more hydrogen [93, 94].

Hydrogen is also produced in the dark and therefore in the absence of excitation and transfer of electrons by PS I. Noth et al. [91] under anaerobic conditions generated by N_2 flushing observed around 0.422 H_2 μg Chl^{-1} in the dark compared to 3.128 H_2 μg Chl^{-1} in *Chlamydomonas reinhardtii*. Along with the H_2, fermentative metabolism in the dark also leads to the production of formate, acetate, and ethanol [87]. Atteia et al. [88] revealed the presence of pyruvate-formate lyase in *Chlamydomonas reinhardtii*, suggesting mixed fermentative metabolism via pyruvate-formate lyase, aldehyde-alcohol dehydrogenase, phosphotransacetylase, and acetate kinase, leading to the formation of formate, ethanol, and acetate [91]. Furthermore, Noth et al. [91] suggested pyruvate ferrodoxin oxidoreductase and not hydrogenase being responsible for H_2 production in the dark. The combination of mixed acid fermentation and pyruvate ferrodoxin oxidoreductase suggests the operation of a different set of pathways in dark anaerobic conditions compared to light. More studies are needed to study to confirm this hypothesis and also to test whether these pathways are mutually exclusive. Even though the hydrogen produced were around 87% lower than that observed in the light, the absence of photosystem involvement eliminates the complication of oxygen-induced inhibition of hydrogenases and competition for NADPH by photorespiration. In addition, the complete heterotrophic nature of this production and the valuable co-products such as ethanol and acetate allows for further optimization and scaling up the fermentative reactions, leading up to hydrogen production. Overall, the process of hydrogen production from microalgae via anaerobiosis whether in the light and/or dark clearly has a strong potential, especially with the development of hydrogen fuel cell-powered cars. Given the advantage of being a cleaner source of hydrogen than thermochemical production, hydrogen production from microalgae can definitely benefit more from more research.

6. Conclusions

Overall, the successful transition of microalgal products from laboratory to industry largely depends on addressing various physiological limitations of microalgae. Biodiesel production from microalgae, although requires further research, can achieve commercial success by simple modifications such as heterotrophic and mixotrophic cultivation of microalgae in combination with genetic engineering to gain properties such as fast-growing and high lipid-secreting ability in microalgae. High valued products from microalgae can also benefit from similar modification such as heterotrophic and mixotrophic cultivation; however, the lack of knowledge on the physiological role and the biochemical pathway regulating the synthesis of these products demands further research to strategically optimize the production to its maximal potential. EPS production by microalgae, on the other hand, has a benefit of the product being naturally secreted by microalgae; however, its commercial success is hindered by the lack of knowledge of its physiological role and the nebulous nature of its applications. Hydrogen production from microalgae is a promising candidate for being a cleaner source of energy over other alternatives; however, its failure to gain commercial attention is primarily due to the limited research invested.

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Conflict of interest

The authors declare no conflict of interest.

Acronyms and abbreviations

CO_2	Carbon dioxide
H_2	hydrogen
O_2	oxygen
N_2	nitrogen
H_2O	water
Fe-S	iron–sulfur
C	carbon
N	nitrogen
P	phosphorus
S	sulfur
Si	silica
EPS	exopolymeric substances
RUBISCO	ribulose bisphosphate carboxylase oxidase
NAD	nicotinamide adenine dinucleotide
NADH	(reduced) nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	(reduced) nicotinamide adenine dinucleotide phosphate
FAD	flavin adenine dinucleotide

FADH	(reduced) flavin adenine dinucleotide
CCM	carbon concentration mechanism
ATP	adenosine triphosphate
ADP	adenosine diphosphate
UVA	ultraviolet rays A
UVB	ultraviolet rays B
PUFAs	polyunsaturated fatty acids
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
PS-I	photosystem I
PS-II	photosystem II

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