Microalgae as a Feedstock for Biofuel Precursors and Value-Added Products: Green Fuels and Golden Opportunities

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The prospects of biofuel production from microalgal carbohydrates and lipids coupled with greenhouse gas mitigation due to photosynthetic assimilation of CO_2 have ushered in a renewed interest in algal feedstock. Furthermore, microalgae (including cyanobacteria) have become established as commercial sources of value-added biochemicals such as polyunsaturated fatty acids and carotenoid pigments used as antioxidants in nutritional supplements and cosmetics. This article presents a comprehensive synopsis of the metabolic basis for accumulating lipids as well as applicable methods of lipid and cellulose bioconversion and final applications of these natural or refined products from microalgal biomass. For lipids, one-step *in situ* transesterification offers a new and more accurate approach to quantify oil content. As a complement to microalgal oil fractions, the utilization of cellulosic biomass from microalgae to produce bioethanol by fermentation, biogas by anaerobic digestion, and bio-oil by hydrothermal liquefaction are discussed. Collectively, a compendium of information spanning green renewable fuels and value-added nutritional compounds is provided.

Keywords: Microalgae; Biofuels; Biochemicals; Lipid Profiles; Algal Strain Development

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INTRODUCTION

Increased carbon dioxide release coupled with declines in non-renewable crude oil resources has resulted in the consideration of liquid biofuels derived from plant materials as a potential valuable alternative source of energy. Moreover, liquid biofuels can be regarded as a form of solar energy stored in plant organisms, in contrast to other renewable energy sources such as tidal and wind, and they are also compatible with existing engines within the transportation infrastructure. Currently, first generation bioethanol feedstocks such as sugar-based crops of sugarcane (Singh *et al.* 2013), sugar beet (Balcerek *et al.* 2011), starch-based crop of cassava (Osei *et al.* 2013), and biodiesel feedstocks such as rapeseed (Iriarte *et al.* 2011), soybeans (Alcantara *et al.* 2000), palm oil (Kansedo *et al.* 2009) as well as sunflower (Buratti *et al.* 2012) have been criticized on account of their direct competition with food resources. To make the situation worse, the growth of oil crops for transportation fuel production may occupy arable land that can instead be used for food crops cultivation (Ahmad *et al.* 2011). To eliminate those

drawbacks, a second-generation of non-food crops has been developed for bioenergy production. These non-food energy crops are reed (Kuhlman *et al.* 2013), switchgrass (Bansal *et al.* 2013), corn stover (Leboreiro and Hilaly 2013), giant *Miscanthus* (Lewandowski *et al.* 2003) for bioethanol along with jatropha (Hailegiorgis *et al.* 2013; Pramanik 2003), *Euphorbia antisyphilitica* (Padmaja *et al.* 2009), and waste cooking oils (Canakci 2007). However, the technology for conversion of these crops to biofuels still needs to be improved for profitable commercial utilization (FAO 2008). A recent report indicated that production costs of the second biofuels are estimated to be 17-26 $/GJ in 2020 and 14-23 $/GJ in 2030, compared to 20–30 $/GJ for fossil fuels. The conversion process, supply chain logistic, local labor costs, and agricultural efficiency are key factors in economic analysis (van Eijck *et al.* 2014).

Microalgae are a class of third-generation biofuel feedstocks that, compared to conventional carbohydrate or oil crops, present a number of unique advantages. While whole algal biomass may serve as feedstock for biofuel production, the energy-dense algal reserves such as polysaccharides and especially lipids (about 17 and 38 kJ g^{-1}, respectively (Berg *et al.* 2002) are especially appealing. Generation of biomass with high-level of energy reserves, which is a prerequisite for efficient biofuel production, requires minimizing of energy-intensive cellular processes while maximizing the energy and carbon flow into biofuel precursors.

In addition to serving as an alternative fuel source, microalgae are also an important source of high-value chemicals, including carotenoids (Borowitzka 2010), omega-3 polyunsaturated fatty acids, such as eicosapentaenoic (EPA) and docosahexaenoic acid (DHA) (Mendes *et al.* 2009), phycobilins (Singh *et al.* 2005), and astaxanthin (Liu *et al.* 2013). Thus, microalgae can serve as feedstock for value-added biochemicals. This paper summarizes the chemical products derived from microalgae considered to have the greatest potential. It aims to provide a comprehensive synopsis of the metabolic basis for accumulating these valuable biomolecules, applicable methods of bioconversion to generate biofuel, and applications of the natural or refined products from microalgae, ranging from green renewable fuels to value-added nutritional compounds.

CARBOHYDRATES

The microalgal carbohydrate content varies significantly from as low as 5% to as high as 80% and consists of soluble monosaccharides or disaccharides (usually intermediate products), energy storage polymeric carbohydrates, cell envelope structural polysaccharides, and extracellular polysaccharides. While the carbohydrate metabolism in microalgae is still not revealed completely, the energy storage carbohydrates (*e.g.* starch) are typically synthesized in either chloroplast or cytosol (Klein 1987; Zeeman *et al.* 2010) and may represent up to 40-55% of the cell's dry mass (Gonzalez-Fernandez and Ballesteros 2012; John *et al.* 2011). In contrast, several studies showed that the cell wall structural polysaccharides are synthesized by plasma membrane complexes (cellulose) and by Golgi-localized enzymes (*e.g.* hemicellulose and pectin) (Lerouxel *et al.* 2006; Popper *et al.* 2011; Driouich *et al.* 2012). While the lack of lignin and a relatively high fraction of structural polysaccharides in cell weight make microalgae a potentially attractive feedstock for biofuels, the starch represents an even more advantageous substrate. Starch does not require costly pretreatment and could be easily

processed using the existing bioethanol infrastructure developed for conversion of starches produced by terrestrial plants (*e.g.* corn). Nevertheless, the carbohydrate and particularly starch productivity has to be improved in order to make algal biofuel production economically efficient and sustainable.

Effect of Environmental Factors on Synthesis and Accumulation of Carbohydrates in Microalgae

Energy (light) and carbon (CO_2) abundance, nutrient availability, environmental parameters (temperature, pH, salinity, and inhibiting compounds), and the presence or absence of other organisms may have an impact on algal metabolism and therefore on carbohydrates synthesis and accumulation. Additionally, the recent advances in algal biotechnology allow application of genetic engineering techniques for redirection of the carbon flow towards the desired products (Radakovits *et al.* 2010; Aikawa *et al.* 2015). The beneficial effects on algal starch content and productivity through manipulation by some of the factors mentioned above are described below and summarized in Table 1.

Inorganic and organic carbon

Carbon dioxide serves as the only carbon source for photoautotrophically grown microalgae. Several studies report improvements of both biomass growth rate and enhancing of starch accumulation as result of shifting from the ambient level (0.04%) to 2% CO_2 in enriched air (Cheng *et al.* 2015; Tanadul *et al.* 2014) or increasing the dissolved CO_2 concentration from 3 to 190 μmol L^{-1} in an aqueous medium by pH adjustment (Xia and Gao 2005). However, the algal response to high CO_2 content has been found to be species-specific. For example, Cheng *et al.* (2015) found that starch content was nearly two times higher in *Chlorella vulgaris* and *C. variabilis* when cultured with 2% CO_2, but it did not increase in *C. sorokiniana* and *C. minutissima*. Similarly, the activities of carbonic anhydrase and nitrate reductase enzymes in *C. pyrenoidosa* and *Chlamydomonas reinhardtii* exhibited different responses to elevation of dissolved CO_2 content (Xia and Gao 2005). Moreover, other studies demonstrated that increasing CO_2 content from ambient to 2-5% improves biomass growth but has either negative (up to 2-3 fold reduction) or no effect on starch accumulation when applied without the cell arrest (Thyssen *et al.* 2001; Izumo *et al.* 2007; Li *et al.* 2013). Lowering CO_2 concentration can induce carbon dioxide concentrating mechanisms (CCM) that may enhance CO_2 supply to ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), and thus improve photosynthesis efficiency and carbon storage (Raven 2010).

A number of microalgae are able to utilize organic carbon substrates (*e.g.* glucose, acetate, or glycerol) as the only source of carbon and energy (heterotrophy) or together with inorganic carbon and light (mixotrophy). Supplementation with organic carbon is mostly investigated and applied to boost algal lipid content. But a few studies documented enhancement of carbohydrate or starch content in *Chlorella* species under mixotrophy or heterotrophy (Abreu *et al.* 2012; Yeh and Chang 2012; Li *et al.* 2015a). In contrast, switching from photoautotrophy did not benefit starch accumulation in *C. reinhardtii* without stressing the cells (Ball *et al.* 1990). Importantly, the application of waste-derived organic carbon for mixotrophic or heterotrophic production of algal biomass, demonstrated by Abreu *et al.* (2012), is likely to be the only economical substrate source for biofuel production. In fact, a number of species from *Chlorella* (*C. vulgaris* (Perez-Garcia *et al.* 2010; Heredia-Arroyo *et al.* 2011; Abreu *et al.* 2012; Mitra *et al.* 2012; Farooq *et al.* 2013), *C. sorokiniana*, *C. pyrenoidosa* (Hongyang *et al.* 2011;

Wang *et al.* 2012), *C. protothecoides, C. minutissima, C. kessleri)*, *Scenedesmus* (*S. obliquus* (Hodaifa *et al.* 2009; Zhang *et al.* 2013), *S. dimorphus, S. quadricauda, S. bijuga*), *Chlamydomonas* (*C. debaryana, C. globosa*), and *Micractinium* genera have been reported to grow either mixotrophically or heterotrophically in various types of wastewater including municipal, sludge anaerobic digestion effluent, soybean or starch processing, brewery, ethanol thin stillage, piggery, dairy, and poultry media (Bhatnagar *et al.* 2011; Park *et al.* 2012; Bohutskyi *et al.* 2015c). More specifically, the increase of carbohydrate was observed when *Scenedesmus obliquus* was mixotrophically cultivated in municipal wastewater supplemented with CO_2 and food wastewater (Ji *et al.* 2015). The carbohydrate content of *Chlorella vulgaris* JSC-6 using 5-fold dilution of swine wastewater was 58.3 and 54.0% of dry weight under mixotrophic and heterotrophic growth respectively (Wang *et al.* 2015). It has been noted that microalgae show the ability to accumulate carbohydrate in wastewater and the production of carbohydrate from microalgae grown in wastewater could be viable with the advances in cultivation technology. Microalgae in wastewater could be the best source to generate not only biodiesel, the one with high carbohydrate content have the potential to produce the other products such as bioethanol and bio-oil (Abinandan and Shanthakumar 2015).

Light intensity

Photoautotrophic metabolism utilizes light as the only energy source for generation of new cells as well as energy-storage molecules such as starch. Also, several enzymes involved in carbon fixation (*e.g.* Calvin-Benson cycle) and therefore starch synthesis are light-activated through the ferredoxin/thioredoxin system (Buchanan 1984; Michalska *et al.* 2009).

In general, low light intensity is not favorable for high-rate accumulation of starch since all energy is utilized to maintain homeostasis and for cell division. Several studies reported that elevation of light intensity from 60-75 to 300-400 μmol m^{-2} s^{-1} boosted carbohydrate/starch content from 5-15 to 30-40% (Friedman *et al.* 1991; De Philippis *et al.* 1992; Ho *et al.* 2012).

Similarly, the mean light intensity above 265 μmol m^{-2} s^{-1} was found to be required for starch accumulation in *C. vulgaris* (Brányiková *et al.* 2011). In contrast, Carvalho *et al.* (2009) reported absence of strong relation between light intensity and carbohydrate or starch accumulation, suggesting either carbon limitation or elimination of other carbon sinks such as protein synthesis or cell division.

However, oversaturation with light may also cause a decrease in algal growth rate due to formation of harmful reactive oxygen species (Leverenz *et al.* 1990; Zhu *et al.* 2008; Stephenson *et al.* 2011). In addition, irradiation is not applicable for starch induction for outdoor facilities due to the lack of control over the sunlight, which fluctuates along with season and weather conditions impacting algal carbohydrate content (Tredici *et al.* 1991).

Temperature

The growth rate and therefore productivity of all cell components including carbohydrates increases until it reaches the optimal temperature of around 25 to 30 °C due to increasing activities of carbon-fixing enzymes (*e.g.* RuBisCO; see Nakamura and Miyachi 1982b). Although the detailed effect of temperature on carbohydrate content remains unclear, a few authors demonstrated a stimulation of carbohydrate synthesis

when the temperature was increased to an optimal range (Oliveira *et al.* 1999; Gigova *et al.* 2012). On the other hand, Renaud *et al.* (2002) reported a lack of relation between temperature and carbohydrate contents for *Isochrysis* sp., *Rhodomonas* sp., *Cryptomonas* sp., *Chaetoceros* sp., and Prymnesiophyte alga. Finally, Nakamura and Miyachi (1982a) observed a maximum starch content in *C. vulgaris* around 20 to 24 °C and rapid loss of starch along with further elevation of temperature to 38 °C. This reduction may be result of a stronger activation of the starch-degrading enzymes (*e.g.* α-glucan phosphorylase) than the enzymes responsible for carbon fixation (*e.g.* RuBisCO) at higher temperatures (Nakamura and Miyachi 1982b). A distinct effect on different enzymes is confirmed by the reduction of amylopectin and increase of amylose fractions in starch with the increases in the temperature from 10 to 38 °C (Nakamura and Imamura 1983).

pH

The pH affects all organisms due to its impact on the chemistry of most inorganic and organic molecules and therefore on kinetics of metabolic reactions. However, pH has an additional influence on photoautotrophs through its relation with CO_2 solubility and the carbonic acid protonation state (Eq. 1).

$$CO_2(g) + H_2O(l) \leftrightarrow H_2CO_3(aq) \leftrightarrow HCO_3^- + H^+ \leftrightarrow CO_3^{2-} + 2H^+ \quad (1)$$

While the optimal pH is species-specific, it is in the range from 7.0 to 9 for many freshwater and marine microalgae. However, since the pH generally increases along with photosynthetic activity and CO_2 consumption, many green microalgae and cyanobacteria are able to grow at pH values as high as 10.5 to 11. The change in carbohydrate content in response to pH change is species specific as well. The maximum accumulation of carbohydrate was reported for *Skeletonema costatum* at pH 7 (Taraldsvik and Myklestad 2000), for *Dunaliella bardawil* at pH 7.5 and *Chlorella ellipsoidea* at pH 9 (Khalil *et al.* 2009).

Enhancing Synthesis and Accumulation of Algal Carbohydrates

As discussed above, while providing a sufficient amount of light and carbon for optimal algal growth is important, it does not guarantee accumulation of energy reserves in large quantity due to the tremendous expenditure of energy and carbon for cell duplication. Therefore, arresting of cell cycle is a typical method for maximizing carbon flow into energy storage molecules.

Application of chemical inhibitors for arresting the cell cycle

Various algal cell processes can be targeted to arrest cell division. For example, Zachleder demonstrated the inhibition of nuclear DNA synthesis in *Scenedesmus quadricauda* by application of FdUrd (5-fluorodeoxyuridine), specifically inhibiting thymidylate synthase (Zachleder 1994, 1995). While FdUrd-treated cells accumulated higher starch content than the untreated control, the difference was not dramatic because FdUrd did not inhibit biosynthesis of other biomolecules, including proteins and RNA. More promising results were achieved by applying the antibiotic cycloheximide, which boosted the starch content in *C. vulgaris* to nearly 60% by specifically inhibiting biosynthesis of proteins in cytoplasm (Brányiková *et al.* 2011). However, industrial application of chemical inhibitors like that may have a detrimental impact on the environment as well.

Table 1. Comparison of Starch Induction Techniques Reported in the Literature

Organism	Irradiance (μmol m^{-2} s^{-1})	Starch (wt%) [A]	Induction method (cultivation time, days / CO_2 content in supplied air, %)	Biomass density [A], g L^{-1}	Starch productivity [A] (g L^{-1} d^{-1})	References
Nutrient limitation						
Chlorella vulgaris	300	55 (20)	N limitation (3 d / 2% CO_2)	2.25 (+60%)	0.27	(Behrens *et al.* 1989)
Chlorella vulgaris P12	70	41 (5.4)	N limitation (3 d / 2% CO_2)	n/a	n/a	(Dragone *et al.* 2011)
C. zofingiensis CCALA 924	150	43 (6.8)	N limitation (1 d / 1% CO_2)	0.47 (+75)	0.18	(Zhu *et al.* 2014)
Tetraselmis subcordiformis FACHB-175	200	54 (4)	N limitation (2 d / 3% CO_2)	1.3 (+310%)	0.49	(Yao *et al.* 2012)
Chlorella vulgaris CCALA 924	780	60 (45)	N limitation (0.5 d / 2% CO_2)	0.25 (+150%)	0.22 (0.88)	(Brányiková *et al.* 2011)
	780	55 (45)	P limitation (0.5 d / 2% CO_2)	0.52 (+420%)	0.53 (0.88)	
	780	60 (45)	S limitation (0.5 d / 2% CO_2)	0.9 (+800)	0.98 (0.88)	
Tetraselmis subcordiformis FACHB-175	200	62 (9)	S limitation (2 d / 3% CO_2)	2.0 (+400%)	0.62	(Yao *et al.* 2012)
	200	41 (15)	P limitation (2 d / 3% CO_2)	1.55 (+290)	0.29	(Yao *et al.* 2013a)
Scenedesmus obliquus CNW-N	140	52(38)	N limitation (3d / 2.5% CO_2)	2.2	0.38	(Ho *et al.* 2012)
Neochloris oleoabundans HK-129	100	44(30)	N limitation (2d / 4% CO_2)	0.42	0.091	(Sun *et al.* 2014a)
Elevation of carbon and energy supply						
Chlorella vulgaris UTEX 259	200	21 (11)	CO_2 elevation to 2% (6.25 d)	0.52 (n/a)	n.a.	(Cheng *et al.* 2015)
Chlorella variabilis NC64A	200	5.3 (2.8)	CO_2 elevation to 2%(6.25 d)	0.54 (n/a)	n.a.	
Chlorella sorokiniana UTEX 2805	185[A]	27 (20)	CO_2 elevation to 2% (2 d)	0.9 (+140)	0.19 (0.06)	(Tanadul *et al.* 2014)

Chlorella vulgaris CCALA 924	780	19.5 (8)	photo-induction, initial mean light intensity increased from 290 to 435 μmol m^{-2} s^{-1}, (0.5 d / 2% CO_2)	1.45 (+435)	0.84 (0.33)	(Brányiková *et al.* 2011)
Chlorella protothecoides	300	42[B] (7)	photo-induction of heterotrophic cells (0.5 d /)	9.0 (-27%)	5.9	(Li *et al.* 2015b)
Chlorella sorokiniana UTEX 1602	100	27 (2)	mixotrophy, glucose (2 d / 1% CO_2)	3.65 (+7,200)	0.49 (0.03)	(Li *et al.* 2015a)
	0	14 (2)	same, heterotrophy	1.65 (+3,200)	0.12 (0.03)	
Combined and other methods						
Tetraselmis subcordiformis FACHB-175	150	58 (49)	0.2x salt level and N limitation (1 d / 3% CO_2)	1.2 (+100%)	0.62 (0.51)	(Yao *et al.* 2013b)
Tetraselmis subcordiformis FACHB-175	150	24 (10)	2.5x salt level as NaCl (1 d / 3% CO_2)	0.85 (+70%)	0.16 ± 0.05	(Yao *et al.* 2013b)
Chlorella sorokiniana UTEX 1602	100	15 (2)	mixotrophy, glucose and N limitation (0.5 d / 1% CO_2)	n.a.	n.a.	(Li *et al.* 2015a)
	0	6 (2)	same, heterotrophy	n.a.	n.a.	
Chlorella vulgaris NIES-2173	70	22 (14)	mixotrophy, acetate and S limitation (7 d / ambient)	0.4 (+1,900)	0.012 (0.01)	(Mizuno *et al.* 2013)
Chlorella lobophora SAG 37.88	70	23 (14)	same, (1 d / ambient)	0.05 (+400)	0.011 (0.006)	
Chlorella sorokiniana NIES-2169	70	24 (8.9)	same, (7 d / ambient)	0.42 (+950)	0.014 (0.009)	
Parachlorella kessleri CCAP 211-11h	70	51 (21)	same, (7 d / ambient)	0.55 (+1,000)	0.039 (0.022)	
Chlorella vulgaris CCALA 924	780	54 (30)	cell cycle arrest, cycloheximide 1 mg/L (0.5 d / 2% CO_2)	2.23	2.27 (0.46)	(Brányiková *et al.* 2011)
Neochloris oleoabundans HK-129	50	39	2-day N-starvation and 0.0185 mM Fe^{3+} concentration.	0.43	0.082	(Sun *et al.* 2014a)
	200	44	same	0.41	0.089	
Scenedesmus sp. CCNM 1077	60	33(19)	400 mM of NaCl (9 d)	0.27	n.a.	(Pancha *et al.* 2015)

[A] the values in the brackets represent the starch content, biomass density or starch productivity values for control untreated control sample; [B] photon flux density was estimated as 0.0185 μmol photons m^{-2} s^{-1} per 1 Lux

Nutrient limitation

Cultivation of microalgae in nutrient-depleted conditions is a widely applied method to stop the generation of new cells since nutrients are required for the synthesis and function of DNA, RNA, proteins, pigments, and co-enzymes. It is important to keep in mind that elevation of starch content does not automatically mean enhancing its productivity, which is a fraction of both biomass productivity and starch content in biomass. Hence, optimal processing requires providing the optimal supply of carbon and light resources and ensuring the generation of an adequate amount of "lean" algal biomass prior to nutrient depletion.

While the limitation in nitrogen is the most widely studied method for induction of starch synthesis, depletion in other nutrients such as phosphorus or sulfur may be successfully applied as well (Table 1). Importantly, a combination of nitrogen limitations together with sufficient amounts of light (under 50 μmol m^{-2} s^{-1}) and carbon (2% CO_2) were found to be the most efficient way to boost starch content up to 55% of the dry weight in *Chlorella vulgaris* (Behrens *et al.* 1989). Starch can also be enhanced up to 50-60% under sulfur deprivation together with sufficient amounts of light during relatively short periods of time (0 to 3 days) in other species (Brányiková *et al.* 2011; Yao *et al.* 2012).

LIPIDS

The lipid content of algae often ranges from 20 to 50% under different cultivation conditions, especially in stressed environments. Nitrogen limitations or other stresses, enhance lipid accumulation for almost all microalgal species (Araujo *et al.* 2013). For fuel production, the lipid content (% lipid per dry weight of biomass) and the lipid productivity (volumetric g L^{-2} d^{-1} or areal g m^{-2} d^{-1}) are pivotal considerations to evaluate the potential of algal strains to produce biofuel. In addition, long-chain omega-3 polyunsaturated fatty acids (PUFAs) are valuable lipids from microalgae. They cannot be synthesized by higher plants or animals and are widely used as nutrition supplements. EPA and DHA have attracted much attention due to their prominent bioactivities. DHA production from microalgae has already been commercially exploited (Spolaore *et al.* 2006), and the production of EPA from some microalgae may be maximized when the microalgae are heterotrophically cultivated in low-cost medium (Wen and Chen 2003).

Maximizing Neutral Lipid Production in Microalgae

The biomass and the oil content in each cell are two key factors that account for the overall yield of microalgal biodiesel. Neutral lipids, commonly stored as triacylglycerol (TAG), are the principal source of biodiesel. A schematic of the biochemical pathways for TAGs is provided in Fig. 1.

There are several ways to maximize the rate of production of TAGs. Manipulations of the nutrient supply, different light intensity, light wavelength, and temperature have already been established to be associated with the enhancement of TAGs in microalgae. For example, Klok *et al.* (2013) found that the excess light absorption combined with limitation of nitrogen nutrition will enhance the yield of TAGs in *Neochloris oleoabundans* from 1.5% to 12.4% (w/w). Guo *et al.* (2015) observed that a 30% enhancement of lipids of *Chlorella* sp. was obtained under the light intensity of 320 μmol m^{-2} s^{-1}, which was higher than at 80, 160 and 240 μmol m^{-2} s^{-1}.

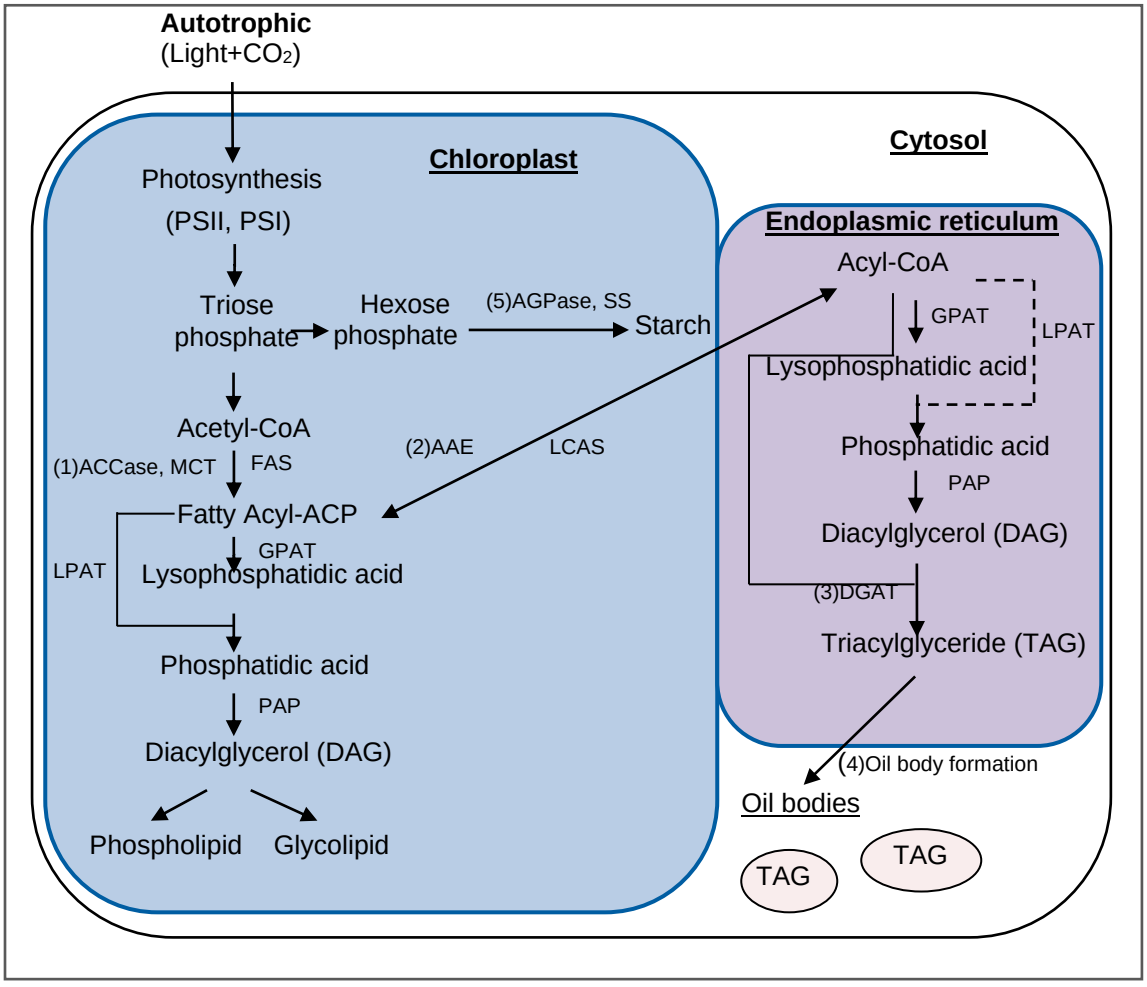

Fig. 1. Basic overview of the pathways of carbon capture and lipid biosynthesis (Klok *et al.* 2014; Scott *et al.* 2010). Enzyme abbreviations: ACCase, acetyl-CoA carboxylase; MCT, malonyl-CoA:ACP transacylase; FAS, fatty acid synthase; AAE, acyl-ACP esterase; LCAS, long-chain acyl-CoA synthase; GPAT, glycerol-3-phosphate acyltransferase; LPAT, lysophosphatidic acid acyltransferase; DGAT, diacylglycerol acyltransferase; PAP, phosphatidic acid phosphatase; AGPase, ADP-glucose pyrophosphorylase; SS, starch synthase

Kim *et al.* (2014) stated that FAME yield of *Nannochloropsis gaditana* under red light was 21.1% of dry weight, two fold higher than that under white light. The enhanced TAG content was from 18.59 to 31.71% of dry weight in *Isochrysis galbana* under combined optimal temperature (30 °C) and light intensity (300 μmol m^{-2} s^{-1}) (Kurpan Nogueira *et al.* 2015).

Manipulation of lipid biosynthesis in algae could also be achieved through metabolic engineering, especially the overexpression of enzymes (Rosenberg *et al.* 2015). Combining an improved understanding of the metabolic pathways together with their regulation and interdependence can potentially contribute to improving the oil content (Rosenberg *et al.* 2008). A number of research studies addressing these challenges are summarized below.

Evaluation of lipid synthesis enzymes influencing TAG production

The production of fatty acids that control the lipid biosynthesis may be regulated by acetyl CoA carboxylase (ACCase) (Bao and Ohlrogge 1999). Different effects on oil content have been observed in microalgae and higher plants with overexpression of acetyl-CoA carboxylase (Step 1). Increasing the activity of ACCase resulted in higher lipid content, which has been demonstrated in higher plants such as rapeseed (Roesler *et al.* 1997). Unfortunately, a two to three-fold higher ACCase activity in the transformed *Cyclotella cryptica* and *Navicula saprophila* did not affect the TAG level (Dunahay *et al.* 1996). The effects of overexpression of other enzymes, which are related to the synthesis of TAGs, have been explored in several studies. Overexpression of acyl-ACP esterase in *Phaeodactylum tricornutum* changed the fatty acid profile with cellular lipid content increasing two-fold, demonstrating that biofuel production can be enhanced by the overexpression of thioesterases (Step 2) (Radakovits *et al.* 2011).

Understanding the regulation of lipid body metabolism in algae, especially how the nitrate depletion induces the lipid accumulation, may contribute to increasing the oil content in the future. Previous studies have provided insights regarding this underlying mechanism. Msanne *et al.* (2012) reported that increasing transcript abundance was indicated for several enzymes for diacylglycerol:acyl-CoA acyltransferases when the microalga *Chlamydomonas* was photoautotrophically cultivated under deprivation of nitrogen, while genes encoding enzymes for *de novo* fatty acid synthesis, such as 3-ketoacyl-ACP synthase I were decreased (Step 3). Li *et al.* (2012) found that the transcript levels of genes concerning pyruvate and acetyl-CoA synthesis increase dramatically under nitrate depletion condition. It is likely that pyruvate and acetyl-CoA are important to TAG synthesis. A major integral protein was found in *Chlorella* oil bodies and may play an important role in supporting the stability and structure of lipid storage organelles (Step 4) (Lin *et al.* 2012).

Elimination of 'redundant' pathways to make precursor metabolites available for biofuel production

In order to address metabolic bottlenecks to lipid production, Wang *et al.* (2009) observed that a deficiency of ADP-glucose pyrophosphorylase, an enzyme essential for production of starch (Fig. 1, step 5), contributes to the accumulation of TAG. Li *et al.* (2010) reported that the neutral lipid content in the *Chlamydomonas reinhardtii* starchless mutant that were cultivated under high light and nitrate depletion was dramatically increased when the starch production pathway was blocked. It was found that the neutral and total lipids rose to 32.6% and 46.4% of dry mass, respectively, in the mutant BAFJ5 lacking ADP-glucose pyrophosphorylase (Step 5).

ALGAL BIOMASS CONVERSION INTO BIOFUELS

Production of biodiesel from microalgal lipids provides opportunities to generate biofuel. In addition, microalgae can be used as a cellulosic biomass to produce bioethanol by fermentation, biogas by anaerobic digestion, and bio-oil by hydrothermal liquefaction. Furthermore, these technologies can also use microalgal residues after lipid extraction, and the combined productions of biofuel from microalgae could maximize their overall value.

Bioethanol Production from Microalgal Carbohydrates

Sugar/starch and lignocellulosic biomass are generally used as feedstocks to produce bioethanol. Although sugar is easier to be fermented than starchy or cellulosic materials, it usually comes from agricultural crops and thus is not cheap. In addition, sugar crops are also used as human and animal food (Walker 2011). Starch or lignocellulosic feedstocks are cheaper than sugar, but pretreatment prior to fermentation that converts the starch or lignocellulosic feedstock to fermentable sugars is required. However, lignin, existing in lignocellulosic feedstock, is very hard to degrade and raises the cost of pretreatment (Harun *et al.* 2010).

Microalgae capable of accumulating high amounts of polysaccharides can also serve as a feedstock for bioethanol production. Although in most circumstances, the polysaccharides are entrapped in the microalgal cell wall, pre-treatment can break the cell wall and convert these complex polysaccharides into simple sugars prior to bioethanol fermentation of the starchy or cellulosic materials. The absence of lignin in microalgae may make the pretreatment and hydrolysis step easier.

Acid pretreatment is widely used. After using 3% (v/v) of sulfuric acid to treat *Chlorococcum humicola* at 160 °C for 15 min, a 52 wt% (ethanol/microalgae, g/g) maximum production was achieved (Harun and Danquah 2011). Ho *et al.* (2013) compared enzymatic and acid hydrolysis of *Chlorella vulgaris* biomass, a strain containing up to 51% carbohydrate and found the glucose yield was 90.4% and 93.6% of microalgal carbohydrates, respectively. These results indicate that the exploitation of carbohydrate-accumulating microalgae as a raw material for bioethanol fermentation is economically viable. The other promising option is alkaline pretreatment. Harun *et al.* (2011) stated that the bioethanol yield obtained was 26% of algal biomass by using 0.75% (w/v) of NaOH to pretreat *Chlorococcum infusionum.* The cost of the pretreatment step for ethanol production can be lowered by exploiting the carbohydrate-rich microalgae's existing enzymatic or anaerobic digestion systems (John *et al.* 2011).

In addition to using the microalgal biomass to produce bioethanol directly, research has shown that 60% higher ethanol yields can be produced from *Chlorococum* sp. biomass in which lipids were extracted prior to fermentation compared to the undamaged cells (Harun *et al.* 2010). John *et al.* (2011) summarized the algae strains that can be used for bioethanol fermentation after oil extraction. The starch or fermentable biomass in *Saccharina latissima, Laminaria hyperborea, Chlamydomonas reinhardtii*, and *Spirulina fusiformis* can present up to 50% of biomass after oil extraction and they are useful as feedstock for producing bioethanol (John *et al.* 2011). Bioethanol can be produced either before or after lipids extraction and offers an alternative bioenergy end product, and the combination of biodiesel and bioethanol production from the same microalgal biomass can improve their overall value.

Biodiesel Production from Microalgal Lipids

Lipids extraction for biodiesel

Extraction of microalgal oil can be achieved by physical, chemical, or biological procedures as well as a combination of each. The existing methods include mechanical pressing, solvent extraction, solvent extraction in combination with enzymatic hydrolysis, supercritical fluid extraction, microwave-assisted extraction, ultrasonic-assisted extraction, and osmotic shock (Mercer and Armenta 2011). Table 2 shows results from some recent extraction studies and the lipid yields, especially more valuable fatty acids.

Table 2. Common Lipid Extraction Methods and their Efficiency

Extraction method	Organism	Lipid yield (wt%)	Fatty acid (% in recovered oil)	References
SC-CO_2	*Botryococcus braunii*	17.6	Myristic – 27.8 Palmitic – 30.1 DHA – 24.8	(Santana *et al.* 2012)
SC-CO_2	*Schizochytrium limacinum*	33.9	DHA - 27.5	(Tang *et al.* 2011)
SC-CO_2	*Crypthecodinium cohnii*	8.6	DHA – 42.7 Palmitic – 25.3	(Couto *et al.* 2010)
The Bligh and Dyer method assisted by ultrasound	*Chlorella vulgaris*	52.5	Palmitic-39 Oleic – 23 Linolenic – 3	(Araujo *et al.* 2013)
Ultrasonic assisted extraction Soxhlet	*Crypthecodinium cohnii*	25.9 4.8	DHA – 39.3 Palmitic – 37.9 DHA – 39.5 Palmitic – 38.0	(Cravotto *et al.* 2008)
Bead-beating Microwaves Sonication Osmotic shock	*Scenedesmus* sp.	9 10 8.5 8.5	Oleic acid (C18:1) and linoleic acid (C18:2) are dominant components	(Lee *et al.* 2010)
Sonication followed by vortex mixing and n-hexane as solvent	*C. minutissima* *T. fluviatilis* *T. pseudonana*	15.5 40.3 39.5	N/A	(Neto *et al.* 2013)
Solvent(Ethyl acetate/methanol); Solvent(n-Hexane) with bead-beating pretreatment; SC-CO_2 with Bead-beating pretreatment	*Pavlova* sp.	44.7 15.3 17.9	N/A	(Cheng *et al.* 2011)

SC-CO_2 = supercritical carbon dioxide extraction

As shown in Table 2, the effectiveness in recovering lipids and the lipid compositions vary across different species. The extraction methods also have an impact on lipid yields from algae.

Lipid extraction is a vital downstream step for biodiesel production. Effective lipid extraction procedures that are low energy-intensive and eliminate the negative effects on the environment such as avoiding the usage of large amounts of solvent, have attracted much attention. In most cases, solvent extraction in combination with cell disruption resulted in more effective lipid removal than solvent extraction alone. Moreover, the purity of the extracted oil is important, and other cellular components such as DNA and chlorophyll should be eliminated in case of the contamination. Recently, Goettel *et al.* (2013) found that prior to lipid extraction, pulse electric field (PEF) treatment for cell wall disruption can enhance the effectiveness of extraction. In a first step, PEF treatment facilitates separation of water-soluble intracellular substances. In a subsequent step, solvent extraction resulted in a higher lipid yield. This selection process might provide the chance to obtain microalgal-based biorefinery by adopting the PEF technology.

Another approach for accessing lipids is to adopt biological methodologies such as using enzymes to decompose the thick cell wall of microalgae. Cellulases have proven to be effective in hydrolyzing the cell wall of microalgae. For example, Fu *et al.* (2010) used an electrospun polyacrylonitrile (PAN) nanofibrous material to immobilize cellulases. The hydrolyzing conversion rate was 62% during first time use and kept at 40% after reusing it five times. Yin *et al.* (2010) found the yields of protein, chlorophyll, and peptides were increased after the *Chlorella* suspensions were treated by cellulases from *Cellulomonas* sp. YJ5. Cho *et al.* (2013) also found that compared to no enzymatic hydrolysis process, the lipid extraction yield and the total fatty acid methyl ester (FAME) productivity of *Chlorella vulgaris* were improved substantially with the treatment of cellulose hydrolysis prior to solvent extraction.

In-situ transesterification

Finally, after extraction, the crude lipids are converted to biodiesel by transesterification. However, this conventional two-step approach to biodiesel production suffers from compounding inefficiencies. Moreover, Laurens *et al.* (2012b) pointed out that it is hard to standardize the analytical methods that are used for calculation of algal biomass components. Eight researchers at three institutions collected the algal biomass data and found that many factors would affect the measurements of lipid and protein. As such, more simple and accurate approaches to quantify cellular oil content in algae should be developed. To address this, a single-step *in-situ* transesterification process, particularly for calculation of algal FAMEs, was developed. *In-situ* transesterification refers to the direct conversion of whole algal biomass into fatty acids using either acid or base catalysts, or other catalysts. No prior lipid extraction procedure is needed (Carrapiso and García 2000). Table 3 contains a compilation of recent results using *in-situ* transesterification for the production of biodiesel from algae.

Collectively, the results in Table 3 indicate that *in-situ* transesterification could be simpler and may also achieve high efficiency. Furthermore, gravimetric methods, which calculate crude lipid yield after extraction, have many limitations, which are highly affected by the type of solvent and the components of lipids in algae. For example, some cellular components such as pigments, waxes, proteins, and sterols that cannot be converted to FAME will be co-extracted during the tradition extraction process and thus may lead to overestimates of the lipid yield (Palmquist and Jenkins 2003). Therefore, gravimetric weights can include non-saponifiable lipids, some of which may not be appropriate feedstocks for biodiesel production. Alternatively, *in-situ* methods provide more reliable and accurate data for the selection of microalgal fuel potential by calculating the FAME yield.

In addition, the FAME profiles have a significant effect on the quality of biodiesel. Microalgae with predominant FAME profiles of C:16 and C:18 are best suited for biodiesel production, which has been investigated previously. For example, *Scenedesmus obliquus* is quite suitable for generating high quality biodiesel with a high content of C16:0 (22.32-26.39% of total fatty acids) and C18:1 (57.29 to 60.59% of total fatty acids), thus possessing higher a cetane number (CN), lower iodine value (IV), and higher oxidative stability (Wu and Miao 2014). Generally, FAME with higher degrees of saturation and longer carbon chains possess higher CN; therefore better ignition properties and better engine performance can be achieved (Ramos *et al.* 2009). The higher level of C18:1 and C16:0 over C18:0 in *Chlorella vulgaris* and *Botryococcus braunii* tended to balance the effects of carbon chain number and saturation on the

ignition properties and cold filter plugging point (CFPP). These two species could also be explored to obtain high-quality biodiesel (Nascimento *et al.* 2015). Conversely, *Chlorella pyrenoidosa* cultivated with nitrate results in high level of PUFA (*e.g.*, C18:2 and C18:3), resulting in poor ignition properties and oxidation stability, and this species failed to meet the biodiesel standard (Wu and Miao 2014). Similarly, certain strains of *Chlorella sorokiniana* produce more 18:2 than 18:1 and PUFA (*e.g.*, C18:2 and C18:3), which can be further enhanced under nitrogen deprivation, as investigated previously (Rosenberg *et al.* 2014). Thus, *Chlorella sorokiniana* could serve as a feedstock for nutritional oils as a complement to biodiesel.

Table 3. *In-situ* Transesterification for the Production of Biodiesel from Algae

Algal feedstock	Pretreatment and oil extraction	Catalyst; conditions	Acyl donor; co-solvent	FAME yield (% of dry biomass)	Reference
Chlorella sorokiniana	Two step *In-situ* process	Pre-esterification: Amberlyst-15 90 °C, 60 min; base-transesterification: KOH; 90 °C, 10 min	Methanol; none	94.87 ± 0.86 FAME recovery (%)	(Dong *et al.* 2013)
Chlorella vulgaris	*In-situ* process	NaOH; 60 °C for 75 min	Methanol; none	96.8±6.3 FAME recovery (%)	(Velasquez-Orta *et al.* 2012)
Chlorella	*In-situ* process with mechanically stirred	H_2SO_4; 60 °C for 8 h	Methanol; Diethyl-ether	29.7 ± 1.1	(Ehimen *et al.* 2012)
Chlorella vulgaris (cultivated in N deficient condition)	*In-situ* from wet algal biomass (water content, 80 wt%)	HCl; 85 °C for 1 h	Methanol; none	56.54±0.7	(Laurens *et al.* 2012a)
Chlorella pyrenoidosa	*In-situ* from wet algal biomass (water content, 80 wt%)	H_2SO_4; 60 °C for 30 min; heating via microwave irradiation	Methanol: chloroform (1:1); none	10.51 ± 1.37	(Cheng *et al.* 2013)
Chlorella vulgaris	*In-situ* from wet algal biomass (water content, 80 wt%)	Using subcritical water as catalyst; 175 °C for 4h	Methanol; none	29	(Tsigie *et al.* 2012)

Biogas Production from Whole Algal Cells or Lipid-Extracted Algal Residues through Anaerobic Digestion

Anaerobic digestion (AD) represents another approach for the bioconversion of algal biomass to energy in the form of methane-rich biogas (Bohutskyi and Bouwer 2013). Importantly, the amount of energy produced through AD does not depend strictly on the biomass biochemical composition if compared to biodiesel or bioethanol processes since AD is able to convert most cell fractions into biogas. This advantage makes possible utilization of low-quality algal biomass cultivated in wastewater (Bohutskyi *et al.* 2015c) or even low-quality algae collected from contaminated lakes and ponds, improving sustainability of coastal ecosystems (Yuan *et al.* 2011).

In addition, biogas and biodiesel production can be integrated into other bioprocesses in which lipids are extracted first for biodiesel and lipid-extracted algal residues (LEA) utilized through AD, enhancing the energy produced by 30 to 50% (Bohutskyi *et al.* 2014b). Finally, implementation of AD in the algal conversion process allows recovering some essential nutrients from LEA and their subsequent recycling back for the further cultivation of microalgae. Nearly 50% of the nitrogen, phosphorus, and sulfur, as well as part of trace elements, may be recycled, replacing costly chemical fertilizers and enabling a more sustainable scale-up algal biofuel processing (Bohutskyi *et al.* 2015a). However, efficient conversion of algal biomass into biogas may require, preliminary pretreatment (*e.g.* thermal, thermochemical, or enzymatic) because certain algal biochemical constituents including the cell wall may be recalcitrant to biodegradation and limit biogas yields (Bohutskyi *et al.* 2014a; 2015a). Although biogas production from microalgae still has a number of hurdles, progress in this area in recent years demonstrates its great potential for enhancing the utilization of complete algal biomass for biofuels and bioenergy.

Hydrothermal Liquefaction or Gasification of Microalgal Biomass

Hydrothermal liquefaction is another option to produce bio-oil, along with productions of gaseous, aqueous, and solid by-products (López Barreiro *et al.* 2013). This HTL-produced bio-oil represents a mix of aromatic hydrocarbons, heterocyclic, phenol, amine, amide, indole, alkane, and nitrile (Chaiwong *et al.* 2013). Previous research has focused on strain selection in terms of the use of microalgae as a source of whole biomass for hydrothermal conversion. In fact, either the whole microalgal biomass or the microalgal residues after extraction of value-added products including lipids, pigments, polysaccharide or protein, *etc*., can be utilized for thermochemical conversion (López Barreiro *et al.* 2013). The combination of value-added constituent extraction and thermochemical conversion may be beneficial to make the process of utilizing microalgae economically viable.

The effect of microalgae biochemical compositions on the bio-oil yield from microalgae is in debate. Biller and Ross (2011) noted that strains with higher lipid content achieved higher conversion efficiency, while other research indicates that high bio-oil yield can also be obtained from microalgae with low lipid content (Ross *et al.* 2010; Yu *et al.* 2011). Either way the requirements for raw materials for thermochemical conversion are not so strict compared to biodiesel or bioethanol processes that depend strongly on lipid and carbohydrate algal contents. Overall, the strain that possesses high biomass productivity and can also be used for value-added products may be the best option. For example, *Nannochloropsis* sp. appears to be a promising raw material for combining biodiesel and bio-oil production. *Nannochloropsis* sp. has a high lipid content and is a good source of polyunsaturated acid, EPA (Hu and Gao 2006). Research has shown that either bio-oil or gas products can be retrieved from up to 90% of the chemical energy initially existing in the *Nannochloropsis* sp. (Brown *et al.* 2010). Furthermore, different heterogeneous catalysts can also affect the efficiency of hydrothermal liquefaction of *Nannochloropsis* sp. as well (Duan and Savage 2010). Thus, further research on hydrothermal conversion of *Nannochloropsis* sp. after extraction of value-added contents should be investigated further and it could expand the overall economic balance sheet for algal conversion processes generating oils.

ADDED-VALUE ALGAL PRODUCTS

Polyunsaturated Fatty Acids (PUFAs)

Polyunsaturated fatty acids (PUFAs) play an important role in human health and physiology, and PUFAs have been shown to reduce the risk of cardiovascular disease (Mozaffarian and Wu 2011).

Currently, PUFAs are mainly extracted from fish and fish oils that are likely to become even more prevalent, including cod liver oil (30% EPA + DHA), tuna oils (20 to 24% EPA + DHA), and salmon oils (15 to 20% EPA + DHA). Krill and squid oils may also be used eventually, but in krill, the EPA and DHA are bound to the phospholipids, and thus, further processing is required (Borowitzka 2013). However, the consumption of fish oil has many limitations due to the possible accumulation of toxins, fish odor, and the presence of mixed fatty acids (Pulz and Gross 2004). Furthermore, these oils are not suitable for vegetarian diets.

Microalgae have historically been used to produce long-chain polyunsaturated fatty acids (PUFA) such as γ-linolenic acid, arachidonic acid, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) (Ratledge 2010). The productivity and yield figures shown in Tables 4 demonstrate that microalgae could potentially replace fish sources to produce EPA and DHA.

Carotenoids

Carotenoids are a class of photosynthetic pigments found in microalgae in addition to chlorophyll. For example, lutein, zeaxanthin, beta-carotene, and astaxanthin represent the major carotenoids observed in plants.

Astaxanthin

Astaxanthin is a keto-carotenoid that possesses strong antioxidant activity. The antioxidant capacity of astaxanthin is observed to be 10-times greater than beta-carotene and 500-times greater than alpha-tocopherol (Dufossé 2007). As a result, astaxanthin can be used for treatment of a number of diseases including cancer, diabetes, cardiovascular, as well as liver, neurodegenerative, and gastrointestinal diseases, *etc.* (Ambati *et al.* 2014). It has also been widely used in health supplements, cosmetic products, and in the food industries because of its powerful antioxidant activity and strong pigmentation (Ambati *et al.* 2014; Guerin *et al.* 2003).

The main natural sources of astaxanthin are microalgae, yeast (*Xanthophyllomyces dendrorhous)*, salmon, trout, Pacific krill (*Euphausia pacifica)*, Antarctic krill *(Euphausia superba)*, shrimp (*Pandalus borealis*), and crayfish (Ambati *et al.* 2014; Fassett and Coombes 2011). *Haematococcus pluvialis* and *Chlorella zofingiensis* are two prominent strains of microalgae known to produce astaxanthin. *Haematococcus pluvialis* has been a commercial source of the antioxidant (Cysewski 2004; Rao *et al.* 2007). The antioxidant activity of *H. pluvialis* is associated with its carotenoid content, and the antioxidant capacity of astaxanthin di-esters has been observed to be 60% higher than the astaxanthin monoester and twice than free astaxanthin (Cerón *et al.* 2007). The remarkable accumulation of astaxanthin in *Haematococcus pluvialis* and *Chlorella zofingiensis* can often be maximized under stress culture conditions, as shown in Fig. 2 (Ip and Chen 2005; Liu *et al.* 2014; Sun *et al.* 2008). Some stress factors that will alter the growth and accumulation of astaxanthin in *Haematococcus pluvialis* and *Chlorella zofingiensis* are listed in Table 5.

Table 4. DHA and EPA Production by Microalgae in the Literature

	Potential application (Spolaore *et al.* 2006)	DHA productivity (g L^{-1} day^{-1})/ EPA productivity (mg L^{-1} day^{-1})	DHA/EPA of biomass (%, w/w)	DHA/EPA (% of total fatty acid)	Reference
DHA producer	Infant formulas; Nutritional supplements; Aquaculture	-		-	-
Crypthecodinium cohnii		-	7.79	51.12	(Jiang *et al.* 1999)
Schizochytrium limacinum		0.52	14.82	-	(Ethier *et al.* 2011)
Schizochytrium sp.		3	-	40	(Ganuza *et al.* 2008)
Schizochytrium sp.		2.86	-	49	(Ren *et al.* 2010)
Schizochytrium sp.		-	-	25	(Ratledge 2004)
Schizochytrium limacinum		-	-	27.5	(Tang *et al.* 2011)
Schizochytrium limacinum		0.51	17.05	-	(Chi *et al.* 2007)
Aurantiochytrium limacinum		3.7	-	23.9	(Rosa *et al.* 2010)
Aurantiochytrium sp.		-	14.10	25.67	(Kim *et al.* 2013)
Aurantiochytrium		-	7	64.1	(Manikan *et al.* 2015)
EPA producer	Nutritional supplements; Aquaculture	-		-	-
Monodus subterraneus		56	-	31.8	(Lu *et al.* 2001)
Phaeodactylum tricornutum		-	-	28.4	(Zhukova and Aizdaicher 1995)
Nannochloropsis sp.		-	-	35	(Sukenik 1991)
Nannochloropsis gaditana		13.86	-	37.83	(Mitra *et al.* 2015)
Nannochloropsis oculata		-	3.7	21–23	(Pieber *et al.* 2012)
Nitzschia laevis		175	-	22.4	(Wen and Chen 2001)
Nitzschia laevis		49.7	3.14	16.02	(Wen *et al.* 2002)
Nitzschia frustulum		-	-	23.1	(Renaud *et al.* 1994)

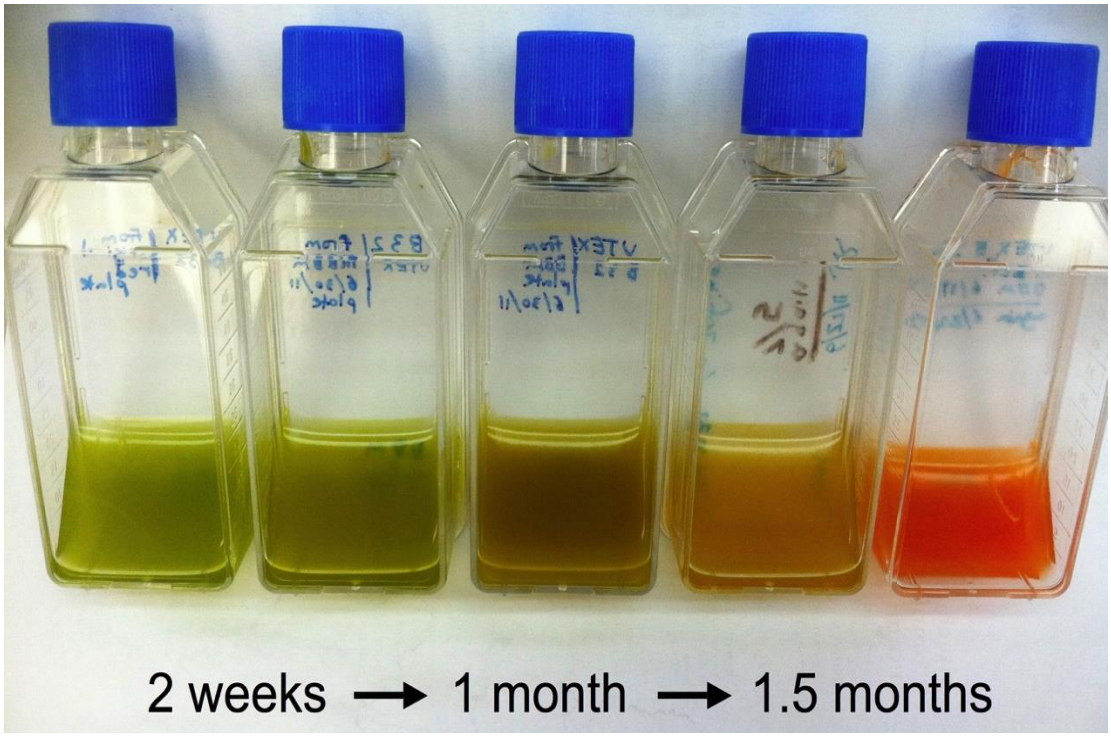

Fig. 2. Natural induction of carotenoid accumulation in *Chlorella zofingiensis* due to nutrient deprivation in small-scale batch culture. Over the course of weeks to months, a striking change in pigmentation can be observed in liquid culture. This shift from chlorophyll to carotenoid biosynthesis can be stimulated by a variety of stressors including nitrogen deprivation and high light intensity.

Beta-carotene

Like astaxanthin, beta-carotene is widely used in the food, pharmaceutical, and cosmetics industries. The cultivation of the microalgae *Dunaliella bardawil* for the commercially production of astaxanthin dates back to the 1980s (Borowitzka 2013). Numerous studies have been carried out on the extraction and isolation of beta-carotene from *D. salina*.

Supercritical fluid extraction (SFE) may be the most widely used technique for beta-carotene extraction. Mendes *et al.* (2003) found that cis-isomers of synthetic and natural beta-carotene dissolved better than all-trans isomers in supercritical CO_2 and resulted in higher yields.

A recent study indicated that efficient extraction of beta-carotene from *D. salina* yield was achieved through centrifugal partition extraction (CPE). In this case, 65% beta-carotene recovery was achieved using ethyl oleate with 5% dichloromethane as an extraction solvent. The least amount of cells were damaged and the measurement of photosynthetic activity showed that more than 65% of the cells were kept viable. Sustainable growth was achieved by applying this "biocompatible" extraction after dichloromethane evaporation. This approach allows for continuous cultivation of *D. salina* through application of CPE process coupled with photobioreactor (Marchal *et al.* 2013).

Table 5. Some Factors Affecting Growth and Astaxanthin Production of *Haematococcus pluvialis* and *Chlorella*

Strain	Factors	Cultural type	Results	Reference
Haematococcus pluvialis	Effects of daytime and light temperature	Autotrophic	Raising the daytime or night temperature could stimulate night accumulation of astaxanthin for temperatures up to 28 °C; Higher biomass and astaxanthin content were observed under the daytime temperature of 28 °C and the night temperature below 28 °C.	(Wan *et al.* 2014)
Haematococcus pluvialis	Temperature	Mixotrophic	The astaxanthin content was 1.4-fold higher below 27 °C than under 20 °C.	(Giannelli *et al.* 2015)
Haematococcus pluvialis	Flashing light	Autotrophic	Astaxanthin concentration was higher under higher illumination by flashing light than continuous light.	(Katsuda *et al.* 2006)
Chlorella zofingiensis	Glucose and nitrate	Mixotrophic	Substrates with high concentration of glucose (30 g L^{-1}) and low nitrate supply (0.55 g L^{-1}) were optimal for accumulation of astaxanthin (12.5 mg L^{-1}).	(Ip *et al.* 2004)
Chlorella zofingiensis	Iron concentration	Mixotrophic	0.2 mM Fe^{2+} with the corresponding values of 2.2 mg g^{-1} (*i.e.*, 25.8 mg L^{-1}) of astaxanthin and 41.8 % dry weight (*i.e.*, 5 g L^{-1}) of total fatty acids.	(Wang *et al.* 2013)
Chlorella zofingiensis	Glucose	Heterotrophic	Maximum specific growth rate 0.031 h^{-1} at glucose concentrations of 20 g L^{-1}; Astaxanthin yield at 10.3 mg L^{-1} at glucose concentrations of 50 g L^{-1}	(Ip and Chen 2005)
Chlorella zofingiensis	Different sugar sources: glucose, mannose, fructose, sucrose, galactose and lactose	Heterotrophic	Glucose, mannose were the best carbon sources for the algal growth; High cell dry weight concentration (*ca*. 53 g L^{-1}) and high astaxanthin production (*ca*. 32 mg L^{-1}) under glucose concentration of 5 to 20 g L^{-1}.	(Sun *et al.* 2008)
Chlorella zofingiensis	Glucose	Heterotrophic	Lipid yield was 9-fold higher with substrate of 30 g L^{-1} of glucose compared to phototrophic culture.	(Liu *et al.* 2011)
Chlorella zofingiensis	Light	Autotrophic	A high growth rate in strong light of 1000 µmol m^{-2} s^{-1}	(Imaizumi *et al.* 2014)

Phycobilins

Phycobilins are water-soluble pigments that, once purified, can be used as cosmetics, colorants in food, and as fluorescent labeling reagents in different analytical techniques. Phycobilins consist of four pyrrole rings connected by a single carbon bridge. Water-solubale phycobiliprotein is formed when phycobilins are connected to polypeptides. According to the absorption spectra, phycobilins can be classified into three types: phycoerythrins (PEs) or phycoerythrocyanins (PECs) (480 to 580 nm); phycocyanins (PCs), (600 to 640 nm); and allophycocyanins (APCs, 620 to 660 nm) (Yen *et al.* 2013).

Spirulina platensis is an excellent source of phycobiliprotein [particularly, allophycocyanin (APC) and c-phycocyanin (CPC)] (Eriksen 2008; Patil *et al.* 2008). Previous research reported that C-phycocyanin and allophycocyanin can be separated and purified simultaneously by aqueous two-phase extraction (ATPE). Moreover, a superior separation of phase forming components from the products along with an increase in APC purity was achieved through integration of membrane processing in combination with ATPE (Patil *et al.* 2008).

High-value Polysaccharides

Polysaccharides represent yet another category of high-value products available from microalgae. They have been shown to exhibit and number of health benefits and interesting structural characteristics (Arad and Levy-Ontman 2010). Sulphated exopolysaccharides in particular, which can be released into the medium, have been utilized in health care and food (Raposo *et al.* 2013). They also display remarkable pharmacological activities and can be used as antioxidant and anti-inflammatory agents (Chen *et al.* 2010; Matsui *et al.* 2003). The algal strains that are good sources of sulphated exopolysaccharides include *Cylindrotheca closterium* (Staats *et al.* 1999), *Chlorella stigmatophora* (Guzmán *et al.* 2003), *Cochlodinium polykrikoides* (Hasui *et al.* 1995), *Gyrodinium impudicum* (Yim *et al.* 2007), and *Isochrysis galbana* (Sun *et al.* 2014b), among others. Arad and Levy-Ontman (2010) found that the cell-wall sulfated polysaccharides in red algae also possess unique structures, composition, fluid dynamics, and high stability. When combined with their useful bioactivities, these structural properties can be useful for various potential biotechnology applications.

CONCLUSIONS

The biochemical composition of microalgae offers a wide diversity of products with a range of biotechnology applications. The application of microalgal carbohydrates for bioethanol production can be an environmentally friendly approach relative to many other options. Biodiesel production from microalgal biomass with high lipid content is even more promising due to an even higher energy density of lipids. Moreover, the carbohydrate and lipid contents may be enhanced in the future through directed engineering efforts. In terms of lipid quantification, *in-situ* methods may prove to be simpler and more accurate compared to conventional extraction and transesterification methods, thereby aiding in the selection and screening of algal species for desirable biofuel properties. A combination of lipids extraction and the utilization of microalgal polysaccharides may offer a more feasible pathway due to more complete utilization of biomass. However, breakthrough technological innovations are needed to make algal

biofuels economically efficient. Further research is required on selection of strains rich in fuel precursors and on genetic engineering methods for expression of enzymes responsible for their biosynthesis. Screening for robust and biodegradable algal strains that can be cultured in wastewater for anaerobic digestion to generate biogas has potential applications. Also a deeper understanding of the effect of the cell composition on the hydrothermal liquefaction or gasification process yields will be useful. All in all, much has been achieved but more research needed to be undertaken in order to make cellulosic microalgal biomass as a successful large-scale and commercialized product.

Microalgae also have a long history and even greater potential as sources of fatty acids, especially the long-chain polyunsaturated fatty acids, such as γ-linolenic acid, arachidonic acid, EPA, and DHA, which may serve as viable sources of precious PUFAs to replace fishmeal. Other valuable biochemical constituents of microalgae offer nutraceutical and pharmaceutical applications including astaxanthin and beta-carotene as antioxidants and food, phycobilins as cosmetics and colorants, and bioactive polysaccharides as anti-inflammatory drugs. In light of these advantageous traits, more in depth and sustained investigations of microalgal biorefinery processes are warranted. Moreover, using microalgae as a feedstock for biofuel in combination with its application as a value-added product will make processes commercially viable sooner and help to realize the great opportunities present in microalgae.

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