

HARNESSING PLANT BIOMASS FOR BIOFUELS AND BIOMATERIALS

Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances

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Summary

Microalgae represent an exceptionally diverse but highly specialized group of micro-organisms adapted to various ecological habitats. Many microalgae have the ability to produce substantial amounts (e.g. 20–50% dry cell weight) of triacylglycerols (TAG) as a storage lipid under photo-oxidative stress or other adverse environmental conditions. Fatty acids, the building blocks for TAGs and all other cellular lipids, are synthesized in the chloroplast using a single set of enzymes, of which acetyl CoA carboxylase (ACCase) is key in regulating fatty acid synthesis rates. However, the expression of genes involved in fatty acid synthesis is poorly understood in microalgae. Synthesis and sequestration of TAG into cytosolic lipid bodies appear to be a protective mechanism by which algal cells cope with stress conditions, but little is known about regulation of TAG formation at the molecular and cellular level. While the concept of using microalgae as an alternative and renewable source of lipid-rich biomass feedstock for biofuels has been explored over the past few decades, a scalable, commercially viable system has yet to emerge. Today, the production of algal oil is primarily confined to high-value specialty oils with nutritional value, rather than commodity oils for biofuel. This review provides a brief summary of the current knowledge on oleaginous algae and their fatty acid and TAG biosynthesis, algal model systems and genomic approaches to a better understanding of TAG production, and a historical perspective and path forward for microalgae-based biofuel research and commercialization.

Keywords: microalgae, lipids, triacylglycerol, fatty acids, biofuels, *Chlamydomonas reinhardtii*.

Introduction

Oxygenic photosynthetic microalgae and cyanobacteria (for simplicity, algae) represent an extremely diverse, yet highly specialized group of micro-organisms that live in diverse ecological habitats such as freshwater, brackish, marine and hyper-saline, with a range of temperatures and pH, and unique nutrient availabilities (Falkowski and Raven, 1997). With over 40 000 species already identified and with many more yet to be identified, algae are classified in multiple major groupings as follows: cyanobacteria (Cyanophyceae), green algae (Chlorophyceae), diatoms (Bacillariophyceae), yellow-green algae (Xanthophyceae), golden algae (Chrysophyceae), red algae (Rhodophyceae), brown algae (Phaeophyceae), dinoflagellates (Dinophyceae) and 'pico-plankton' (Prasinophyceae and Eustigmatophyceae).

Several additional divisions and classes of unicellular algae have been described, and details of their structure and biology are available (Van den Hoek *et al.*, 1995). Thousands of species and strains of these algal taxa are currently maintained in culture collections throughout the world (<http://www.utex.org>; <http://ccmp.bigelow.org>; <http://www.ccap.ac.uk>; <http://www.marine.csiro.au/microalgae>; <http://wdcm.nig.ac.jp/hpcc.html>).

The ability of algae to survive or proliferate over a wide range of environmental conditions is, to a large extent, reflected in the tremendous diversity and sometimes unusual pattern of cellular lipids as well as the ability to modify lipid metabolism efficiently in response to changes in environmental conditions (Guschina and Harwood, 2006;

Thompson, 1996; Wada and Murata, 1998). The lipids may include, but are not limited to, neutral lipids, polar lipids, wax esters, sterols and hydrocarbons, as well as prenyl derivatives such as tocopherols, carotenoids, terpenes, quinones and phytolated pyrrole derivatives such as the chlorophylls.

Under optimal conditions of growth, algae synthesize fatty acids principally for esterification into glycerol-based membrane lipids, which constitute about 5–20% of their dry cell weight (DCW). Fatty acids include medium-chain (C10–C14), long-chain (C16–18) and very-long-chain (\geq C20) species and fatty acid derivatives. The major membrane lipids are the glycosylglycerides (e.g. monogalactosyldiacylglycerol, digalactosyldiacylglycerol and sulfoquinovosyldiacylglycerol), which are enriched in the chloroplast, together with significant amounts of phosphoglycerides (e.g. phosphatidylethanolamine, PE, and phosphatidylglycerol, PG), which mainly reside in the plasma membrane and many endoplasmic membrane systems (Guckert and Cooksey, 1990; Harwood, 1998; Pohl and Zurheide, 1979a,b; Wada and Murata, 1998). The major constituents of the membrane glycerolipids are various kinds of fatty acids that are polyunsaturated and derived through aerobic desaturation and chain elongation from the 'precursor' fatty acids palmitic (16:0) and oleic (18:1 ω 9) acids (Erwin, 1973).

Under unfavorable environmental or stress conditions for growth, however, many algae alter their lipid biosynthetic pathways towards the formation and accumulation of neutral lipids (20–50% DCW), mainly in the form of triacylglycerol (TAG). Unlike the glycerolipids found in membranes, TAGs do not perform a structural role but instead serve primarily as a storage form of carbon and energy. However, there is some evidence suggesting that, in algae, the TAG biosynthesis pathway may play a more active role in the stress response, in addition to functioning as carbon and energy storage under environmental stress conditions. Unlike higher plants where individual classes of lipid may be synthesized and localized in a specific cell, tissue or organ, many of these different types of lipids occur in a single algal cell. After being synthesized, TAGs are deposited in densely packed lipid bodies located in the cytoplasm of the algal cell, although formation and accumulation of lipid bodies also occur in the inter-thylakoid space of the chloroplast in certain green algae, such as *Dunaliella bardawil* (Ben-Amotz et al., 1989). In the latter case, the chloroplastic lipid bodies are referred to as plastoglobuli.

Hydrocarbons are another type of neutral lipid that can be found in algae at quantities generally <5% DCW (Lee and Loeblich, 1971). Only the colonial green alga, *Botryococcus braunii*, has been shown to produce, under adverse environmental conditions, large quantities (up to 80% DCW) of very-long-chain (C23–C40) hydrocarbons, similar to those found in petroleum, and thus has been explored over the decades as a feedstock for biofuels and biomaterials. A discussion of hydrocarbons in this alga is beyond the

scope of this review, but other reviews of this topic have been published recently (Banerjee et al., 2002; Metzger and Largeau, 2005).

As many algal species have been found to grow rapidly and produce substantial amounts of TAG or oil, and are thus referred to as oleaginous algae, it has long been postulated that algae could be employed as a cell factories to produce oils and other lipids for biofuels and other biomaterials (Benemann et al., 1982; Borowitzka, 1988; Burlew, 1953; Hill et al., 1984; Meier, 1955; Sheehan et al., 1998). The potential advantages of algae as feedstocks for biofuels and biomaterials include their ability to:

- (i) synthesize and accumulate large quantities of neutral lipids/oil (20–50% DCW),
- (ii) grow at high rates (e.g. 1–3 doublings per day),
- (iii) thrive in saline/brackish water/coastal seawater for which there are few competing demands,
- (iv) tolerate marginal lands (e.g. desert, arid- and semi-arid lands) that are not suitable for conventional agriculture,
- (v) utilize growth nutrients such as nitrogen and phosphorus from a variety of wastewater sources (e.g. agricultural run-off, concentrated animal feed operations, and industrial and municipal wastewaters), providing the additional benefit of wastewater bio-remediation,
- (vi) sequester carbon dioxide from flue gases emitted from fossil fuel-fired power plants and other sources, thereby reducing emissions of a major greenhouse gas,
- (vii) produce value-added co-products or by-products (e.g. biopolymers, proteins, polysaccharides, pigments, animal feed, fertilizer and H₂),
- (viii) grow in suitable culture vessels (photo-bioreactors) throughout the year with an annual biomass productivity, on an area basis, exceeding that of terrestrial plants by approximately tenfold.

Based upon the photosynthetic efficiency and growth potential of algae, theoretical calculations indicate that annual oil production of >30 000 l or about 200 barrels of algal oil per hectare of land may be achievable in mass culture of oleaginous algae, which is 100-fold greater than that of soybeans, a major feedstock currently being used for biodiesel in the USA. While the 'algae-for-fuel' concept has been explored in the USA and some other countries, with interest and funding growing and waning according to the fluctuations of the world petroleum oil market over the past few decades, no efforts in algae-based biofuel production have proceeded beyond rather small laboratory or field testing stages. The lipid yields obtained from algal mass culture efforts performed to date fall short of the theoretical maximum (at least 10–20 times lower), and have historically made algal oil production technology prohibitively expensive (Hu et al., 2006; Sheehan et al., 1998).

Recent soaring oil prices, diminishing world oil reserves, and the environmental deterioration associated with fossil

fuel consumption have generated renewed interest in using algae as an alternative and renewable feedstock for fuel production. However, before this concept can become a commercial reality, many fundamental biological questions relating to the biosynthesis and regulation of fatty acids and TAG in algae need to be answered. Clearly, physiological and genetic manipulations of growth and lipid metabolism must be readily implementable, and critical engineering breakthroughs related to algal mass culture and downstream processing are necessary.

The purpose of this review is to provide an overview of the current status of research on oleaginous algae, including the biochemistry and molecular biology of fatty acid and lipid biosynthetic pathways leading to the formation and accumulation of TAG, and an understanding of how these pathways could be utilized for the commercial production of algal feedstock for biofuels. This review provides a summary of the oleaginous algae isolated and evaluated for their lipid content over the past 60 years, and of the currently described fatty acid and TAG biosynthetic pathways and how the pathways are affected by environmental and biological factors. In this context, the potential roles of algal model systems and new research approaches and methodologies (such as functional genomics, proteomics and metabolomics) in algal lipid research will be discussed. A short historical perspective is provided, which links current efforts to the US Department of Energy-funded 'Aquatic Species Program' on algal oil production from 1978 to 1996. Finally, the path forward for algal feedstock-based biofuels, with respect to both challenges and opportunities, will be discussed.

Content and fatty acid composition of algae

Lipid and triacylglycerol content

The majority of photosynthetic micro-organisms routinely used in the laboratory (e.g. *Chlamydomonas reinhardtii*) were selected because of ease of cultivation, or as genetic model systems for studying photosynthesis (Grossman *et al.*, 2007; Merchant *et al.*, 2007). These few organisms were not selected for optimal lipid production. Therefore, examination of lipid synthesis and accumulation in diverse organisms has the potential for novel insights into new mechanisms to enhance lipid production. Over the past few decades, several thousand algae, and cyanobacterial species, have been screened for high lipid content, of which several hundred oleaginous species have been isolated and characterized under laboratory and/or outdoor culture conditions. Oleaginous algae can be found among diverse taxonomic groups, and the total lipid content may vary noticeably among individual species or strains within and between taxonomic groups. Of the strains examined, green algae represent the largest taxonomic group from which

oleaginous candidates have been identified. This may not be because green algae naturally contain considerably more lipids than other algal taxa, but rather because many green algae are ubiquitous in diverse natural habitats, can easily be isolated, and generally grow faster than species from other taxonomic groups under laboratory conditions. Figure 1(a) summarizes the total lipid contents of oleaginous green algae reported in the literature. Each data point represents the total lipid of an individual species or strain grown under optimal culture conditions. Oleaginous green algae show an average total lipid content of 25.5% DCW. The lipid content increases considerably (doubles or triples) when the cells are subjected to unfavorable culture conditions, such as photo-oxidative stress or nutrient starvation. On average, an increase in total lipids to 45.7% DCW was obtained from an oleaginous green algae grown under stress conditions. An effort was made to determine whether green algae at the genus level exhibit different capacities to synthesize and accumulate lipids. Statistical analysis of various oleaginous green algae indicated no significant differences. The intrinsic ability to produce large quantities of lipid and oil is species/strain-specific, rather than genus-specific (Hu *et al.*, 2006).

Figure 1(b) illustrates the lipid content of oleaginous diatoms of freshwater and marine origin grown under normal and stress culture conditions (Hu *et al.*, 2006). Statistical analysis indicated that the average lipid content of an oleaginous diatom was 22.7% DCW when maintained under normal growth conditions, whereas a total lipid content of 44.6% DCW was achievable under stress conditions.

Figure 1(c) shows the lipid content of oleaginous algae identified as chrysophytes, haptophytes, eustigmatophytes, dinophytes, xanthophytes or rhodophytes (Hu *et al.*, 2006). Similar to oleaginous green algae and diatoms, these species/strains show average total lipid contents of 27.1% and 44.6% DCW under normal and stress culture conditions, respectively.

The increase in total lipids in aging algal cells or cells maintained under various stress conditions consisted primarily of neutral lipids, mainly TAGs. This was due to the shift in lipid metabolism from membrane lipid synthesis to the storage of neutral lipids. *De novo* biosynthesis and conversion of certain existing membrane polar lipids into triacylglycerols may contribute to the overall increase in TAG. As a result, TAGs may account for as much as 80% of the total lipid content in the cell (Kathen, 1949; Klyachko-Gurvich, 1974; Suen *et al.*, 1987; Tonon *et al.*, 2002; Tornabene *et al.*, 1983).

Cyanobacteria have also been subjected to screening for lipid production (Basova, 2005; Cobelas and Lechado, 1989). Unfortunately, considerable amounts of total lipids have not been found in cyanophycean organisms examined in the laboratory (Figure 1d), and the accumulation of neutral

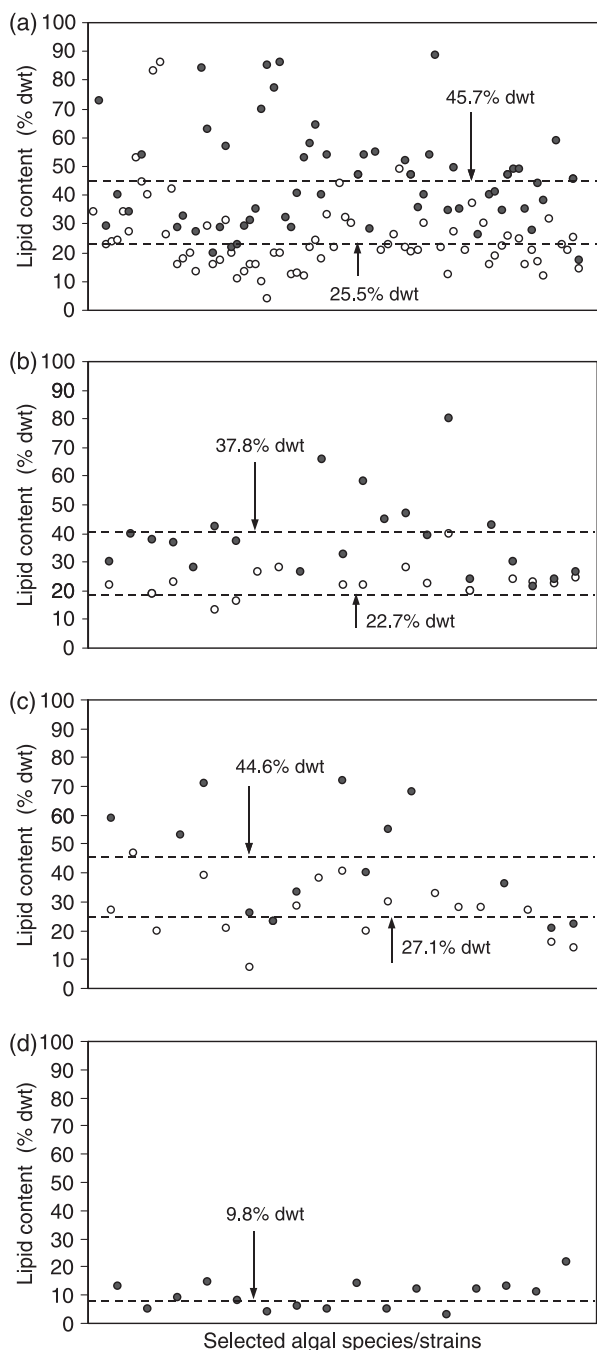


Figure 1. Cellular lipid content in various classes of microalgae and cyanobacteria under normal growth (NG) and stress conditions (SC). (a) Green microalgae; (b) diatoms; (c) oleaginous species/strains from other eukaryotic algal taxa; (d) cyanobacteria. Open circles: cellular lipid contents obtained under normal growth or nitrogen-replete conditions. Closed circles: cellular lipid contents obtained under nitrogen-depleted or other stress conditions. The differences in cellular lipid content between cultures under normal growth and stress growth conditions were statistically significant for all three groups (a, b and c) of algae examined using Duncan's multiple range test with the ANOVA procedure.

lipid triacylglycerols has not been observed in naturally occurring cyanobacteria.

Fatty acid composition

Algae synthesize fatty acids as building blocks for the formation of various types of lipids. The most commonly synthesized fatty acids have chain lengths that range from C16 to C18 (Table 1), similar to those of higher plants (Ohlrogge and Browse, 1995). Fatty acids are either saturated or unsaturated, and unsaturated fatty acids may vary in the number and position of double bonds on the carbon chain backbone. In general, saturated and mono-unsaturated fatty acids are predominant in most algae examined (Borowitzka, 1988). Specifically, the major fatty acids are C16:0 and C16:1 in the Bacillariophyceae, C16:0 and C18:1 in the Chlorophyceae, C16:0 and C18:1 in the Euglenophyceae, C16:0, C16:1 and C18:1 in the Chrysophyceae, C16:0 and C20:1 in the Cryptophyceae, C16:0 and C18:1 in the Eustigmatophyceae, C16:0 and C18:1 in the Prasinophyceae, C16:0 in the Dinophyceae, C16:0, C16:1 and C18:1 in the Prymnesiophyceae, C16:0 in the Rhodophyceae, C14:0, C16:0 and C16:1 in the Xanthophyceae, and C16:0, C16:1 and C18:1 in cyanobacteria (Cobelas and Lechado, 1989).

Polyunsaturated fatty acids (PUFAs) contain two or more double bonds. Based on the number of double bonds, individual fatty acids are named dienoic, trienoic, tetraenoic, pentaenoic and hexaenoic fatty acids. Also, depending on the position of the first double bond from the terminal methyl end (ω) of the carbon chain, a fatty acid may be either an ω 3 PUFA (i.e. the third carbon from the end of the fatty acid) or an ω 6 PUFAs (i.e. the sixth carbon from the end of the fatty acid). The major PUFAs are C20:5 ω 3 and C22:6 ω 3 in Bacillariophyceae, C18:2 and C18:3 ω 3 in green algae, C18:2 and C18:3 ω 3 in Euglenophyceae, C20:5, C22:5 and C22:6 in Chrysophyceae, C18:3 ω 3, 18:4 and C20:5 in Cryptophyceae, C20:3 and C20:4 ω 3 in Eustigmatophyceae, C18:3 ω 3 and C20:5 in Prasinophyceae, C18:5 ω 3 and C22:6 ω 3 in Dinophyceae, C18:2, C18:3 ω 3 and C22:6 ω 3 in Prymnesiophyceae, C18:2 and C20:5 in Rhodophyceae, C16:3 and C20:5 in Xanthophyceae, and C16:0, C18:2 and C18:3 ω 3 in cyanobacteria (Basova, 2005; Cobelas and Lechado, 1989).

In contrast to higher plants, greater variation in fatty acid composition is found in algal taxa. Some algae and cyanobacteria possess the ability to synthesize medium-chain fatty acids (e.g. C10, C12 and C14) as predominant species, whereas others produce very-long-chain fatty acids (>C20). For instance, a C10 fatty acid comprising 27–50% of the total fatty acids was found in the filamentous cyanobacterium *Trichodesmium erythraeum* (Parker *et al.*, 1967), and a C14 fatty acid makes up nearly 70% of the total fatty acids in the golden alga *Prymnesium parvum* (Lee and Loeblich, 1971). Another distinguishing feature of some algae is the large amounts of very-long-chain PUFAs. For example, in the

Table 1 Fatty acid composition of some algae and cyanobacteria (percentage of total fatty acids)

Fatty acid	Bacillariophyta		Euglenozoa		Chlorophyta		Haptophyta		Pinguiculate		Cyanophyta		Cryptophyta		Dinophyta		
	Ba	C.sp	N.sp	M.s	C.s	C.v	P.i	E.h	I.g	P.p	G.c	A.sp	T.e	H.b	R.l	G.s	S.sp
C10:0																	
C11:0																	
C12:0																	
C14:0	32.0	23.6	6.9	2.3	35.1	23.1	18.7	22.0	29–34	7.21	2.0	18.0	6.5	3.2			
C14:1																	
C14:2																	
C15:0	5.0	9.2	19.9	20.2	2.2	1.1	3.7	4.4	9–13	1–2	0.2	0.5					
C16:0																	
C16:1 _ω 5																	
C16:1 _ω 7	27.0	36.5	27.4	26.9	4.0	5.0	0.7	4.0	5–7	4–7	13.0	5.0	2.6	0.7			
C16:1 _ω 9																	
C16:2 _ω 4																	
C16:2 _ω 7	2.0	0.9			11.0	12.0		1.0									
C16:3	8.0	2.6			17.0	2.1											
C17:0																	
C18:0																	
C18:1 _ω 7																	
C18:1 _ω 9	3.0	1.7	4.5	5.0	9.2	14.3	13.0	6.6	1–2	3–7	2.0	10.1	11.8	1.2			
C18:1 _ω 13																	
C18:2 _ω 6																	
C18:3 _ω 3																	
C18:3 _ω 6	0.5	0.7			23.0	10.0		7.0		6–19	11.0	16.0	0.3	0.2			
C18:4 _ω 3	0.9				0.6			1.1					0.1	5.2			
C18:5 _ω 3	0.6	4.2			1.2	8.0	10.0						3.7	10.6			
C20:0																	
C20:1																	
C20:4 _ω 6	4.1				58.9												
C20:5 _ω 3	26.0	8.0	34.9	37.1				5.0	5.5				0.5				
C22:5 _ω 3									39.2				14.1	1.8			
C22:6 _ω 3									13.3				24.2	18.8			
C24:0																	

Abbreviation of algal species: B.a., *Biddulphia aurica* (Orcaut and Patterson, 1975); C.sp., *Chaetoceros* sp. (Renaud et al., 2002); N.sp., *Nannochloropsis* sp. (Sukienik, 1999); M.s., *Monodus subterraneus* (Cohen, 1999); C.s., *Chlorella sorokiniana* (Patterson, 1970); C.v., *Chlorella vulgaris* (Harris et al., 1965); P.i., *Parietochloris incise* (Khozin-Goldberg et al., 2002); E.h., *Emiliania huxleyi* (Volkman et al., 1981); I.g., *Isochrysis galbana* (Volkman et al., 1981); P.p., *Phaeomonas parva* (Kawachi et al., 2002); G.c., *Glossomastix chrysoiplasta* (Kawachi et al., 2002); A.sp., *Aphanocapsa* sp. (Kenyon, 1972); S.p., *Spirulina platensis* (Mühling et al., 2005); T.e., *Trichodesmium erythraeum* (Parker et al., 1967); H.b., *Hemiselmiss brunesceus* (Chuecas and Riley, 1969); R.l., *Rhodomonas lens* (Beach et al., 1970); G.s., *Gymnodinium sanguineum*; S.sp., *Scrippsiella* sp. (Mansour et al., 1999).

green alga *Parietochloris incise* (Bigogno *et al.*, 2002), the diatom *Phaeodactylum tricornutum* and the dinoflagellate *Cryptothecodinium cohnii* (De Swaaf *et al.*, 1999), the very-long-chain fatty acids arachidonic acid (C20:4 ω 6), eicosapentaenoic acid (C20:5 ω 3) or docosahexaenoic acid (C22:6 ω 3) are the major fatty acid species, accounting for 33.6–42.5%, approximately 30% and 30–50% of the total fatty acid content of the three species, respectively.

It should be noted that much of the data provided previously comes from the limited number of species of algae that have been examined to date, and most of the analyses of fatty acid composition from algae have used total lipid extracts rather than examining individual lipid classes. Therefore, these data represent generalities, and deviations should be expected. This may explain why some fatty acids seem to occur almost exclusively in an individual algal taxon. In addition, the fatty acid composition of algae can vary both quantitatively and qualitatively with their physiological status and culture conditions, as described in more detail later.

Biodiesel, produced by the trans-esterification of triglycerides with methanol, yielding the corresponding mono-alkyl fatty acid esters, is an alternative to petroleum-based diesel fuel (Durrett *et al.*, 2008). The properties of biodiesel are largely determined by the structure of its component fatty acid esters (Knothe, 2005). The most important characteristics include ignition quality (i.e. cetane number), cold-flow properties and oxidative stability. While saturation and fatty acid profile do not appear to have much of an impact on the production of biodiesel by the trans-esterification process, they do affect the properties of the fuel product. For example, saturated fats produce a biodiesel with superior oxidative stability and a higher cetane number, but rather

poor low-temperature properties. Biodiesels produced using these saturated fats are more likely to gel at ambient temperatures. Biodiesel produced from feedstocks that are high in PUFAs, on the other hand, has good cold-flow properties. However, these fatty acids are particularly susceptible to oxidation. Therefore, biodiesel produced from feedstocks enriched with these fatty acid species tends to have instability problems during prolonged storage.

Biosynthesis of fatty acids and triacylglycerols

Lipid metabolism, particularly the biosynthetic pathways of fatty acids and TAG, has been poorly studied in algae in comparison to higher plants. Based upon the sequence homology and some shared biochemical characteristics of a number of genes and/or enzymes isolated from algae and higher plants that are involved in lipid metabolism, it is generally believed that the basic pathways of fatty acid and TAG biosynthesis in algae are directly analogous to those demonstrated in higher plants. It should be noted that because the evidence obtained from algal lipid research is still fragmentary, some broad generalizations are made in this section based on limited experimental data.

Fatty acid biosynthesis

In algae, the *de novo* synthesis of fatty acids occurs primarily in the chloroplast. A generalized scheme for fatty acid biosynthesis is shown in Figure 2. Overall, the pathway produces a 16- or 18-carbon fatty acid or both. These are then used as the precursors for the synthesis of chloroplast and other cellular membranes as well as for the synthesis of

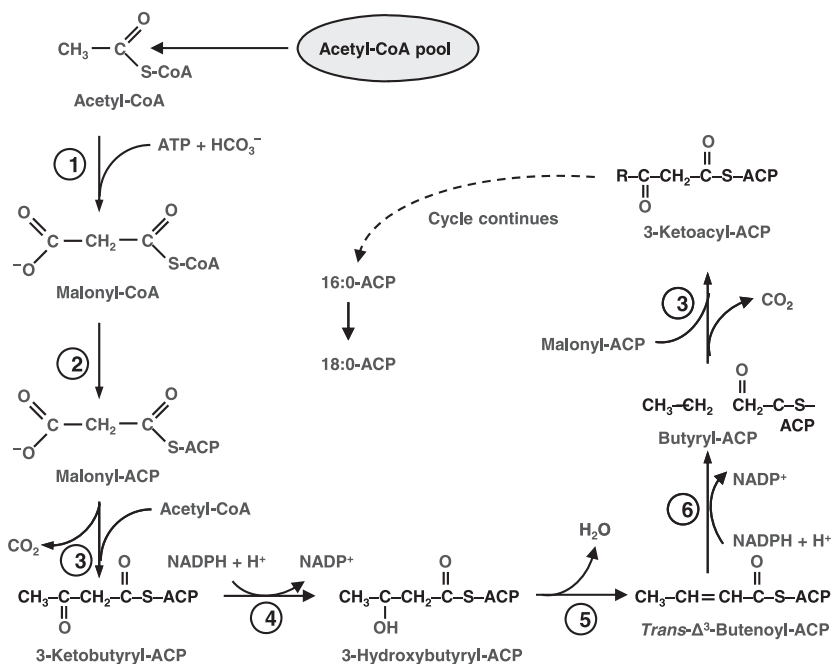


Figure 2. Fatty acid *de novo* synthesis pathway in chloroplasts.

Acetyl CoA enters the pathway as a substrate for acetyl CoA carboxylase (Reaction 1) as well as a substrate for the initial condensation reaction (Reaction 3). Reaction 2, which is catalyzed by malonyl CoA:ACP transferase and transfers malonyl from CoA to form malonyl ACP. Malonyl ACP is the carbon donor for subsequent elongation reactions. After subsequent condensations, the 3-ketoacyl ACP product is reduced (Reaction 4), dehydrated (Reaction 5) and reduced again (Reaction 6), by 3-ketoacyl ACP reductase, 3-hydroxyacyl ACP dehydrase and enoyl ACP reductase, respectively (adapted and modified from Ohlrogge and Browse, 1995).

neutral storage lipids, mainly TAGs, which can accumulate under adverse environmental or sub-optimal growth conditions.

The committed step in fatty acid synthesis is the conversion of acetyl CoA to malonyl CoA, catalyzed by acetyl CoA carboxylase (ACCase). In the chloroplast, photosynthesis provides an endogenous source of acetyl CoA, and more than one pathway may contribute to maintaining the acetyl CoA pool. In oil seed plants, a major route of carbon flux to fatty acid synthesis may involve cytosolic glycolysis to phosphoenolpyruvate (PEP), which is then preferentially transported from the cytosol to the plastid, where it is converted to pyruvate and consequently to acetyl CoA (Baud *et al.*, 2007; Ruuska *et al.*, 2002; Schwender and Ohlrogge, 2002). In green algae, as glycolysis and pyruvate kinase (PK), which catalyzes the irreversible synthesis of pyruvate from PEP, occur in the chloroplast in addition to the cytosol (Andre *et al.*, 2007), it is possible that glycolysis-derived pyruvate is the major photosynthate to be converted to acetyl CoA for *de novo* fatty acid synthesis. An ACCase is generally considered to catalyze the first reaction of the fatty acid biosynthetic pathway – the formation of malonyl CoA from acetyl CoA and CO₂. This reaction takes place in two steps and is catalyzed by a single enzyme complex. In the first step, which is ATP-dependent, CO₂ (from HCO₃⁻) is transferred by the biotin carboxylase prosthetic group of ACCase to a nitrogen of a biotin prosthetic group attached to the ε-amino group of a lysine residue. In the second step, catalyzed by carboxyltransferase, the activated CO₂ is transferred from biotin to acetyl CoA to form malonyl CoA (Ohlrogge and Browse, 1995).

According to Ohlrogge and Browse (1995), malonyl CoA, the product of the carboxylation reaction, is the central carbon donor for fatty acid synthesis. The malonyl group is transferred from CoA to a protein co-factor on the acyl carrier protein (ACP; Figure 2). All subsequent reactions of the pathway involve ACP until the finished products are ready for transfer to glycerolipids or export from the chloroplast. The malonyl group of malonyl ACP participates in a series of condensation reactions with acyl ACP (or acetyl CoA) acceptors. The first condensation reaction forms a four-carbon product, and is catalyzed by the condensing enzyme, 3-ketoacyl ACP synthase III (KAS III) (Jaworski *et al.*, 1989). Another condensing enzyme, KAS I, is responsible for producing varying chain lengths (6–16 carbons). Three additional reactions occur after each condensation. To form a saturated fatty acid the 3-ketoacyl ACP product is reduced by the enzyme 3-ketoacyl ACP reductase, dehydrated by hydroxyacyl ACP dehydratase and then reduced by the enzyme enoyl ACP reductase (Figure 2). These four reactions lead to a lengthening of the precursor fatty acid by two carbons. The fatty acid biosynthesis pathway produces saturated 16:0- and 18:0-ACP. To produce an unsaturated fatty acid, a double bond is introduced by the soluble

enzyme stearoyl ACP desaturase. The elongation of fatty acids is terminated either when the acyl group is removed from ACP by an acyl-ACP thioesterase that hydrolyzes the acyl ACP and releases free fatty acid or acyltransferases in the chloroplast transfer the fatty acid directly from ACP to glycerol-3-phosphate or monoacylglycerol-3-phosphate (Ohlrogge and Browse, 1995). The final fatty acid composition of individual algae is determined by the activities of enzymes that use these acyl ACPs at the termination phase of fatty acid synthesis.

ACCases have been purified and kinetically characterized from two unicellular algae, the diatom *Cyclotella cryptica* (Roessler, 1990a) and the prymnesiophyte *Isochrysis galbana* (Livne and Sukenik, 1990). Native ACCase isolated from *Cyclotella cryptica* has a molecular mass of approximately 740 kDa and appears to be composed of four identical biotin-containing subunits. The molecular mass of the native ACCase from *I. galbana* was estimated at 700 kDa. This suggests that ACCases from algae and the majority of ACCases from higher plants are similar in that they are composed of multiple identical subunits, each of which are multi-functional peptides containing domains responsible for both biotin carboxylation and subsequent carboxyl transfer to acetyl CoA (Roessler, 1990a).

Roessler (1988) investigated changes in the activities of various lipid and carbohydrate biosynthetic enzymes in the diatom *Cyclotella cryptica* in response to silicon deficiency. The activity of ACCase increased approximately two- and fourfold after 4 and 15 h of silicon-deficient growth, respectively, suggesting that the higher enzymatic activity may partially result from a covalent modification of the enzyme. As the increase in enzymatic activity can be blocked by the addition of protein synthesis inhibitors, it was suggested that the enhanced ACCase activity could also be the result of an increase in the rate of enzyme synthesis (Roessler, 1988; Roessler *et al.*, 1994). The gene that encodes ACCase in *Cyclotella cryptica* has been isolated and cloned (Roessler and Ohlrogge, 1993). The gene was shown to encode a polypeptide composed of 2089 amino acids, with a molecular mass of 230 kDa. The deduced amino acid sequence exhibited strong similarity to the sequences of animal and yeast ACCases in the biotin carboxylase and carboxyltransferase domains. Less sequence similarity was observed in the biotin carboxyl carrier protein domain, although the highly conserved Met-Lys-Met sequence of the biotin binding site was present. The N-terminus of the predicted ACCase sequence has characteristics of a signal sequence, indicating that the enzyme may be imported into chloroplasts via the endoplasmic reticulum.

Triacylglycerol biosynthesis

Triacylglycerol biosynthesis in algae has been proposed to occur via the direct glycerol pathway (Figure 3) (Ratl-

edge, 1988). Fatty acids produced in the chloroplast are sequentially transferred from CoA to positions 1 and 2 of glycerol-3-phosphate, resulting in formation of the central metabolite phosphatidic acid (PA) (Ohlrogge and Browse, 1995). Dephosphorylation of PA catalyzed by a specific phosphatase releases diacylglycerol (DAG). In the final step of TAG synthesis, a third fatty acid is transferred to the vacant position 3 of DAG, and this reaction is catalyzed by diacylglycerol acyltransferase, an enzymatic reaction that is unique to TAG biosynthesis. PA and DAG can also be used directly as a substrate for synthesis of polar lipids, such as phosphatidylcholine (PC) and galactolipids. The acyltransferases involved in TAG synthesis may exhibit preferences for specific acyl CoA molecules, and thus may play an important role in determining the final acyl composition of TAG. For example, Roessler *et al.* (1994) reported that, in *Nannochloropsis* cells, the lyso-PA acyltransferase that acylates the second position (*sn*-2) of the glycerol backbone has a high substrate specificity, whereas glycerol-3-phosphate acyltransferase and DAG acyltransferase are less discriminating. It was also determined that lyso-PC acyltransferase prefers 18:1-CoA over 16:0-CoA.

Although the three sequential acyl transfers from acyl CoA to a glycerol backbone described above are believed to be the main pathway for TAG synthesis, Dahlqvist *et al.* (2000) reported an acyl CoA-independent mechanism for TAG synthesis in some plants and yeast. This pathway uses phospholipids as acyl donors and DAG as the acceptor, and the reaction is catalyzed by the enzyme phospholipid:diacylglycerol acyltransferase (PDAT). In an *in vitro* reaction system, the PDAT enzyme exhibited high substrate specificity for the ricinoleoyl or the vernoloyl group of PC, and it was suggested that PDAT could play an important role in the specific channeling of bilayer-disturbing fatty acids, such as ricinoleic and vernolic acids, from PC into the TAG pool (Dahlqvist *et al.*, 2000). Under various stress conditions, algae usually undergo rapid degradation of the photosynthetic membrane with concomitant occurrence and accumulation of cytosolic TAG-enriched lipid bodies. If a PDAT

orthologue were identified in an algal cell, especially in the chloroplast, then it is conceivable that that orthologue could use PC, PE or even galactolipids derived from the photosynthetic membrane as acyl donors in the synthesis of TAG. As such, the acyl CoA-independent synthesis of TAG could play an important role in the regulation of membrane lipid composition in response to various environmental and growth conditions, not only in plants and yeasts but also in algae.

In most of the algal species/strains examined, TAGs are composed primarily of C14–C18 fatty acids that are saturated or mono-unsaturated (Harwood, 1998; Roessler, 1990b). As exceptions, very-long-chain (>C20) PUFA synthesis and partitioning of such fatty acids into TAGs have been observed in the green alga *Parietochloris incise* (Trebouxiophyceae) (Bigogno *et al.*, 2002), the freshwater red microalga *Porphyridium cruentum* (Cohen *et al.*, 2000), marine microalgae *Nannochloropsis oculata* (Eustigmatophyceae), *P. tricornutum* and *Thalassiosira pseudonana* (Bacillariophyceae), and the thraustochytrid *Thraustochytrium aureum* (Iida *et al.*, 1996). A strong positional preference of C22:6 in TAG for the *sn*-1 and *sn*-3 positions of the glycerol backbone was reported in the marine microalga *Cryptocodinium cohnii* (Kyle *et al.*, 1992). It has been proposed that very long PUFA-rich TAGs may occur as the result of 'acyl shuttle' between diacyl glycerol and/or TAG and phospholipid in situations where PUFAs are formed (Kamisaka *et al.*, 1999). The biosynthesis of very long PUFAs is beyond the scope of the current review, but has been reviewed in detail elsewhere (Certik and Shimizu, 1999; Guschina and Harwood, 2006).

Comparison of lipid metabolism in algae and higher plants

Although algae generally share similar fatty acid and TAG synthetic pathways with higher plants, there is some evidence that differences in lipid metabolism do occur. In algae, for example, the complete pathway from carbon dioxide fixation to TAG synthesis and sequestration takes place within a single cell, whereas the synthesis and accumulation

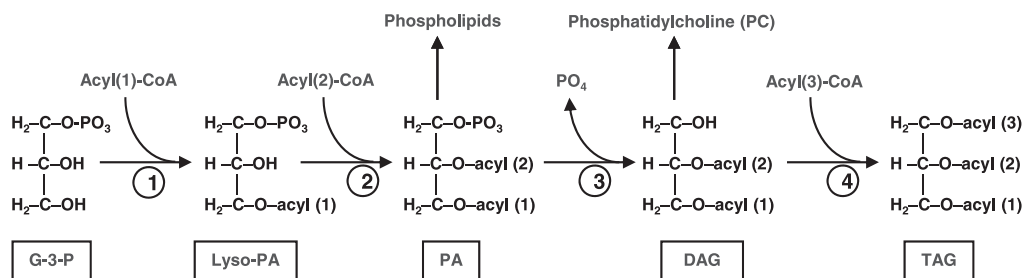


Figure 3. Simplified schematic showing the triacylglycerol biosynthesis pathway in algae.

(1) Cytosolic glycerol-3-phosphate acyl transferase, (2) lyso-phosphatidic acid acyl transferase, (3) phosphatidic acid phosphatase, and (4) diacylglycerol acyl transferase.

Adapted from Roessler *et al.*, 1994b.

of TAG only occur in special tissues or organs (e.g. seeds or fruits) of oil crop plants. In addition, very long PUFAs above C18 cannot be synthesized in significant amounts by naturally occurring higher plants, whereas many algae (especially marine species) have the ability to synthesize and accumulate large quantities of very long PUFAs, such as eicosapentaenoic acid (C20:5 ω 3), docosahexaenoic acid (C22:6 ω 3) and arachidonic acid (C20:4 ω 6). Recently, annotation of the genes involved in lipid metabolism in the green alga *C. reinhardtii* has revealed that algal lipid metabolism may be less complex than in *Arabidopsis*, and this is reflected in the presence and/or absence of certain pathways and the apparent sizes of the gene families that represent the various activities (Riekhof *et al.*, 2005).

Factors affecting triacylglycerol accumulation and fatty acid composition

Although the occurrence and the extent to which TAG is produced appear to be species/strain-specific, and are ultimately controlled by the genetic make-up of individual organisms, oleaginous algae produce only small quantities of TAG under optimal growth or favorable environmental conditions (Hu, 2004). Synthesis and accumulation of large amounts of TAG accompanied by considerable alterations in lipid and fatty acid composition occur in the cell when oleaginous algae are placed under stress conditions imposed by chemical or physical environmental stimuli, either acting individually or in combination. The major chemical stimuli are nutrient starvation, salinity and growth-medium pH. The major physical stimuli are temperature and light intensity. In addition to chemical and physical factors, growth phase and/or aging of the culture also affects TAG content and fatty acid composition.

Nutrients

Of all the nutrients evaluated, nitrogen limitation is the single most critical nutrient affecting lipid metabolism in algae. A general trend towards accumulation of lipids, particularly TAG, in response to nitrogen deficiency has been observed in numerous species or strains of various algal taxa, as shown in Figure 1 (Basova, 2005; Beijerinck, 1904; Cobelas and Lechado, 1989; Merzlyak *et al.*, 2007; Roessler, 1990b; Shifrin and Chisholm, 1981; Spoehr and Milner, 1949; Thompson, 1996).

In diatoms, silicon is an equally important nutrient that affects cellular lipid metabolism. For example, silicon-deficient *Cyclotella cryptica* cells had higher levels of neutral lipids (primarily TAG) and higher proportions of saturated and mono-unsaturated fatty acids than silicon-replete cells (Roessler, 1988).

Other types of nutrient deficiency that promote lipid accumulation include phosphate limitation and sulfate

limitation. Phosphorus limitation resulted in increased lipid content, mainly TAG, in *Monodus subterraneus* (Eustigmatophyceae) (Khozin-Goldberg and Cohen, 2006), *P. tricorntum* and *Chaetoceros* sp. (Bacillariophyceae), and *I. galbana* and *Pavlova lutheri* (Prymnesiophyceae), but decreased lipid content in *Nannochloris atomus* (Chlorophyceae) and *Tetraselmis* sp. (Prasinophyceae) (Reitan *et al.*, 1994). Of marine species examined (Reitan *et al.*, 1994), increasing phosphorus deprivation was found to result in a higher relative content of 16:0 and 18:1 and a lower relative content of 18:4 ω 3, 20:5 ω 3 and 22:6 ω 3. Studies have also shown that sulfur deprivation enhanced the total lipid content in the green algae *Chlorella* sp. (Otsuka, 1961) and *C. reinhardtii* (Sato *et al.*, 2000).

Cyanobacteria appear to react to nutrient deficiency differently to eukaryotic algae. Piorreck and Pohl (1984) investigated the effects of nitrogen deprivation on the lipid metabolism of the cyanobacteria *Anacystis nidulans*, *Microcystis aeruginosa*, *Oscillatoria rubescens* and *Spirulina platensis*, and reported that either lipid content or fatty acid composition of these organisms was changed significantly under nitrogen-deprivation conditions. When changes in fatty acid composition occur in an individual species or strain in response to nutrient deficiency, the C18:2 fatty acid levels decreased, whereas those of both C16:0 and C18:1 fatty acids increased, similar to what occurs in eukaryotic algae (Olson and Ingram, 1975). In some cases, nitrogen starvation resulted in reduced synthesis of lipids and fatty acids (Saha *et al.*, 2003).

Temperature

Temperature has been found to have a major effect on the fatty acid composition of algae. A general trend towards increasing fatty acid unsaturation with decreasing temperature and increasing saturated fatty acids with increasing temperature has been observed in many algae and cyanobacteria (Lynch and Thompson, 1982; Murata *et al.*, 1975; Raison, 1986; Renaud *et al.*, 2002; Sato and Murata, 1980). It has been generally speculated that the ability of algae to alter the physical properties and thermal responses of membrane lipids represents a strategy for enhancing physiological acclimatization over a range of temperatures, although the underlying regulatory mechanism is unknown (Somerville, 1995). Temperature also affects the total lipid content in algae. For example, the lipid content in the chrysophytan *Ochromonas danica* (Aaronson, 1973) and the eustigmatophyte *Nannochloropsis salina* (Boussiba *et al.*, 1987) increases with increasing temperature. In contrast, no significant change in the lipid content was observed in *Chlorella sorokiniana* grown at various temperatures (Patterson, 1970). As only a limited amount of information is available on this subject, a general trend cannot be established.

Light intensity

Algae grown at various light intensities exhibit remarkable changes in their gross chemical composition, pigment content and photosynthetic activity (Falkowski and Owens, 1980; Post *et al.*, 1985; Richardson *et al.*, 1983; Sukenik *et al.*, 1987). Typically, low light intensity induces the formation of polar lipids, particularly the membrane polar lipids associated with the chloroplast, whereas high light intensity decreases total polar lipid content with a concomitant increase in the amount of neutral storage lipids, mainly TAGs (Brown *et al.*, 1996; Khotimchenko and Yakovleva, 2005; Napolitano, 1994; Orcutt and Patterson, 1974; Spoehr and Milner, 1949; Sukenik *et al.*, 1989).

The degree of fatty acid saturation can also be altered by light intensity. In *Nannochloropsis* sp., for example, the percentage of the major PUFA C20:5 ω 3 remained fairly stable (approximately 35% of the total fatty acids) under light-limited conditions. However, it decreased approximately threefold under light-saturated conditions, concomitant with an increase in the proportion of saturated and mono-unsaturated fatty acids (i.e. C14, C16:0 and C16:1 ω 7) (Fabregas *et al.*, 2004). Based upon the algal species/strains examined (Orcutt and Patterson, 1974; Sukenik *et al.*, 1993), it appears, with a few exceptions, that low light favors the formation of PUFAs, which in turn are incorporated into membrane structures. On the other hand, high light alters fatty acid synthesis to produce more of the saturated and mono-unsaturated fatty acids that mainly make up neutral lipids.

Growth phase and physiological status

Lipid content and fatty acid composition are also subject to variability during the growth cycle. In many algal species examined, an increase in TAGs is often observed during stationary phase. For example, in the chlorophyte *Parietochloris incise*, TAGs increased from 43% (total fatty acids) in the logarithmic phase to 77% in the stationary phase (Bigogno *et al.*, 2002), and in the marine dinoflagellate *Gymnodinium* sp., the proportion of TAGs increased from 8% during the logarithmic growth phase to 30% during the stationary phase (Mansour *et al.*, 2003). Coincident increases in the relative proportions of both saturated and mono-unsaturated 16:0 and 18:1 fatty acids and decreases in the proportion of PUFAs in total lipid were also associated with growth-phase transition from the logarithmic to the stationary phase. In contrast to these decreases in PUFAs, however, the PUFA arachidonic acid (C20:4 ω 6) is the major constituent of TAG produced in *Parietochloris incise* cells (Bigogno *et al.*, 2002), while docosahexaenoic acid (22:6 ω 3) and eicosapentaenoic acid (20:5 ω 3) are partitioned to TAG in the Eustigmatophyceae *N. oculata*, the diatoms *P. tricornutum* and *T. pseudonana*, and the haptophyte *Pavlova lutheri* (Tonon *et al.*, 2002).

Culture aging or senescence also affects lipid and fatty acid content and composition. The total lipid content of cells increased with age in the green alga *Chlorococcum macrostigma* (Collins and Kalnins, 1969), and the diatoms *Nitzschia palea* (von Denffer, 1949), *Thalassiosira fluviatillii* (Conover, 1975) and *Coscinodiscus eccentricus* (Pugh, 1971). An exception to this was reported in the diatom *P. tricornutum*, where culture age had almost no influence on the total fatty acid content, although TAGs were accumulated and the polar lipid content was reduced (Alonso *et al.*, 2000). Analysis of fatty acid composition in the diatoms *P. tricornutum* and *Chaetoceros muelleri* revealed a marked increase in the levels of saturated and mono-unsaturated fatty acids (e.g. 16:0, 16:1 ω 7 and 18:1 ω 9), with a concomitant decrease in the levels of PUFAs (e.g. 16:3 ω 4 and 20:5 ω 3) with increasing culture age (Liang *et al.*, 2006). Most studies on algal lipid metabolism have been carried out in a batch culture mode. Therefore, the age of a given culture may or may not be associated with nutrient depletion, making it difficult to separate true aging effects from nutrient deficiency-induced effects on lipid metabolism.

Physiological roles of triacylglycerol accumulation

Synthesis of TAG and deposition of TAG into cytosolic lipid bodies may be, with few exceptions, the default pathway in algae under environmental stress conditions. In addition to the obvious physiological role of TAG serving as carbon and energy storage, particularly in aged algal cells or under stress, the TAG synthesis pathway may play more active and diverse roles in the stress response. The *de novo* TAG synthesis pathway serves as an electron sink under photo-oxidative stress. Under stress, excess electrons that accumulate in the photosynthetic electron transport chain may induce over-production of reactive oxygen species, which may in turn cause inhibition of photosynthesis and damage to membrane lipids, proteins and other macromolecules. The formation of a C18 fatty acid consumes approximately 24 NADPH derived from the electron transport chain, which is twice that required for synthesis of a carbohydrate or protein molecule of the same mass, and thus relaxes the over-reduced electron transport chain under high light or other stress conditions. The TAG synthesis pathway is usually coordinated with secondary carotenoid synthesis in algae (Rabbani *et al.*, 1998; Zhekisheva *et al.*, 2002). The molecules (e.g. β -carotene, lutein or astaxanthin) produced in the carotenoid pathway are esterified with TAG and sequestered into cytosolic lipid bodies. The peripheral distribution of carotenoid-rich lipid bodies serve as a 'sunscreen' to prevent or reduce excess light striking the chloroplast under stress. TAG synthesis may also utilize PC, PE and galactolipids or toxic fatty acids excluded from the membrane system as acyl donors, thereby serving as a mechanism to

detoxify membrane lipids and deposit them in the form of TAG.

Role of algal genomics and model systems in biofuel production

Because of the potential for photosynthetic micro-organisms to produce 8–24 times more lipids per unit area for biofuel production than the best land plants (Sheehan *et al.*, 1998), these microbes are in the forefront as future biodiesel producers. While cyanobacteria, for which over 20 completed genome sequences are available (http://genome.jgi-psf.org/mic_cur1.html) (over 30 are in progress), produce some lipids, the nuclear genomes of only eight microalgae, some of which can produce significant quantities of storage lipids, have been sequenced to date (http://genome.jgi-psf.org/euk_cur1.html). These eukaryotes include *C. reinhardtii* and *Volvox carteri* (green alga), *Cyanidioschizon merolae* (red alga), *Osteococcus lucimarinus* and *Osteococcus tauris* (marine pico-eukaryotes), *Aureococcus anophagefferens* (a harmful algal bloom component), *P. tricornutum* and *T. pseudonana* (diatoms). The only organism among the latter for which extensive genomic, biological and physiological data exist is *C. reinhardtii*, a unicellular, water-oxidizing green alga (Grossman, 2005; Merchant *et al.*, 2007; Mus *et al.*, 2007). For these reasons, *Chlamydomonas* has emerged recently as a model eukaryote microbe for the study of many processes, including photosynthesis, phototaxis, flagellar function, nutrient acquisition, and the biosynthesis and functions of lipids.

The recent availability of the *Chlamydomonas* genome sequence and biochemical studies indicate that this versatile, genetically malleable eukaryote has an extensive network of diverse metabolic pathways that are unprecedented in other eukaryotes for which whole-genome sequence information is available. The flexibility of *Chlamydomonas* metabolism to deal with various environmental conditions is a source of interest to a large number of research groups working in renewable energy. *Chlamydomonas* is of particular interest to renewable energy efforts because its metabolism can be manipulated by nutrient stress to accumulate various energy-yielding reduced compounds.

The advantage of *C. reinhardtii* as a model for oxygenic photosynthesis derives mainly from its ability to grow either photo-, mixo- or heterotrophically (in the dark and in the presence of acetate) while maintaining an intact, functional photosynthetic apparatus. This property has allowed researchers to study photosynthetic mutations that are lethal in other organisms. Moreover, *C. reinhardtii* spends most of its life cycle as a haploid organism of either mating type + or – (Harris, 1989). Gametogenesis is triggered by environmental stresses, particularly nitrogen deprivation (Sager and Granick, 1954), and its occurrence can be synchronized by light/dark periods of growth (Kates and

Jones, 1964). During its haploid stage, *C. reinhardtii* can be genetically engineered and single genotypes easily generated. Additionally, different phenotypes can be obtained by crossing two haploid mutants of different mating types carrying different genotypes. Conversely, single-mutant genotypes can be unveiled by back-crossing mutants carrying multiple mutations with the wild-type strain of the opposite mating type.

Chlamydomonas reinhardtii can also be used as a model organism for fermentation, given the number of pathways identified under anaerobic conditions biochemically (Gfeller and Gibbs, 1984; Ohta *et al.*, 1987) or by microarray analysis (Mus *et al.*, 2007). The results, summarized in Figure 4, suggest that both the pyruvate formate lyase (PFL) and the pyruvate ferredoxin oxidoreductase (PFR) pathways are functional in *C. reinhardtii* under anaerobiosis, as well as the pyruvate decarboxylase (PDC) pathway. The former two pathways generate acetyl CoA (a precursor for lipid metabolism) and either formate (PFL) or H₂ (PFR), and the latter can generate ethanol through the alcohol dehydrogenase (ADH)-catalyzed reduction of acetaldehyde. Finally, acetyl CoA can be further metabolized by *C. reinhardtii* to ethanol, through the alcohol/aldehyde bifunctional dehydrogenase (ADHE) activity, or to acetate, through the sequential activity of two enzymes, phosphotransacetylase (PAT) and acetate kinase (ACK). The last reaction releases ATP. Mus *et al.* (2007) and Hemschemeier and Happe (2005) proposed that the unprecedented presence of all these pathways endows *C. reinhardtii* with a higher flexibility to adapt to environmental conditions. Finally, fermentative lactate production has been detected under certain conditions (Kreuzberg, 1984).

Although pathways for fatty acid biosynthesis are present in *C. reinhardtii* (Figure 5), they are not known to be over-expressed under normal photo-autotrophic or mixotrophic growth (Harris, 1989). However, these pathways could be artificially over-expressed in *C. reinhardtii* in the future, underscoring the potential of this organism as a model for biofuel production as well.

Global expression profiling of *Chlamydomonas* under conditions that produce biofuels (H₂ in this case) (Mus *et al.*, 2007) has been reported using second-generation microarrays with 10 000 genes of the over 15 000 genes predicted (Eberhard *et al.*, 2006; Merchant *et al.*, 2007). Much of the information that was reported involves fermentative metabolism, as discussed above. No concerted effort to characterize up- and downregulation of genes associated with lipid metabolism when *Chlamydomonas* is exposed to nutrient stress has yet been reported. Nevertheless, N-deprived *C. reinhardtii* will over-accumulate starch and lipids that can be used for formate, alcohol and biodiesel production (Mus *et al.*, 2007; Riekhof *et al.*, 2005). Future biofuel-related studies may recognize limitations to the information that can be provided by high-density microarrays (the low signal-to-noise level limits identification of genes whose mRNA copy

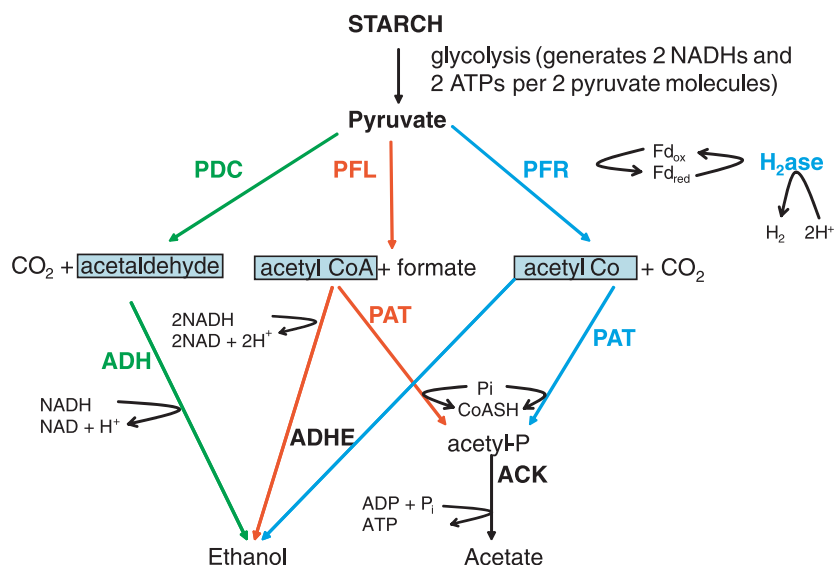


Figure 4. Fermentative pathways identified in *Chlamydomonas reinhardtii* following anaerobic incubation (adapted and modified from Mus *et al.*, 2007).

Under aerobic conditions, pyruvate is metabolized predominantly by the pyruvate dehydrogenase complex to produce NADH and acetyl CoA, the latter of which ties into lipid metabolism (see Figure 5). ACK, acetate kinase; ADH, alcohol dehydrogenase; ADHE, alcohol aldehyde bifunctional dehydrogenase; H₂ase, hydrogenase; PAT, phosphotransacetylase; PDC, pyruvate decarboxylase; PFL, pyruvate formate lyase; PFR, pyruvate ferredoxin oxidoreductase.

numbers are low, and the dynamic range of the signal is quite compressed). However, new tag-sequencing methodologies such as 454 and Solexa, which can give an accurate whole-genome picture of expression data, have the potential to provide a quantitative picture of the mRNAs in algal samples.

Procedures for metabolite profiling of *C. reinhardtii* CC-125 cells, which quickly inactivate enzymatic activity, optimize extraction capacity, and are amenable to large sample sizes, were reported recently (Bölling and Fiehn, 2005). The study is particularly relevant to this review, because it explored profiles of Tris-acetate/phosphate-grown cells as well as cells that were deprived of sulfate. Nitrogen-, phosphate- and iron-deprivation profiles were also examined, and each metabolic profile was different. Sulfur depletion leads to the anaerobic conditions required for induction of the hydrogenase enzyme and H₂ production (Ghirardi *et al.*, 2007; Hemschemeier *et al.*, 2008). Rapidly sampled cells (cell leakage controls were determined by ¹⁴C-labeling techniques) were analyzed by gas chromatography coupled to time-of-flight mass spectrometry, and more than 100 metabolites (e.g. amino acids, carbohydrates, phosphorylated intermediates, nucleotides and organic acids) out of about 800 detected could be identified. The concentrations of a number of phosphorylated glycolysis intermediates increase significantly during sulfur stress (Bölling and Fiehn, 2005), consistent with the upregulation of many genes associated with starch degradation and fermentation observed in anaerobic *Chlamydomonas* cells (Mus *et al.*, 2007). Unfortunately lipid metabolism was not studied. Finally, researchers are starting to ask whether *Chlamydomonas* and other green algae have the required metabolic pathways to produce other energy-rich products such as butanol.

Chlamydomonas proteomics is in its infancy, but there have been a number of relevant studies, as reviewed by

Stauber and Hippler (2004). However, to our knowledge, no proteomics research has yet been reported in algae under biofuel-producing conditions.

Historical perspective and recent advances

Early work: pre-Aquatic Species Program

Mass culture of microalgae. Prior to the establishment of the US Department of Energy's (DOE) Aquatic Species Program, very little work had been conducted on biofuel production from lipid-accumulating algae. While the general idea of using algae for energy production has been around for over 50 years (Meier, 1955), the concept of using lipids derived from algal cells to produce liquid fuels arose more recently. Historically, algae have been seen as a promising source of protein and have been actively cultured by man for centuries, mainly for food. Growing algae as a source of protein on a large scale in open ponds was first conceived by German scientists during World War II (Soeder, 1986). The first attempt in the USA to translate the biological requirements for algal growth into engineering specifications for a large-scale plant was made at the Stanford Research Institute (1948–1950). During 1951, Arthur D. Little made a further advance through the construction and operation of a *Chlorella* pilot plant for the Carnegie Institute (Burlew, 1953). These studies eventually provided some of the most comprehensive early information on the growth, physiology and biochemistry of algae. Therefore, the concept of using mass-cultured algae for fuel production could be traced directly back to these early efforts on using algae for food production.

Microalgae as a source of energy. The concept of using algae as a fuel was first proposed by Meier (1955) for the production of methane gas from the carbohydrate fraction of

community through the Hawaii Culture Collection (<http://prcmb.hawaii.edu/research/HICC/index.html>).

Microalgal physiology and biochemistry. Studies on algal physiology under the ASP centered on the ability of many species to induce lipid biosynthesis under conditions of nutrient stress (Dempster and Sommerfeld, 1998; McGinnis et al., 1997). Focusing on the diatom *Cyclotella cryptica*, biochemical studies indicated that silicon deficiency led to increased activity of the enzyme ACCase, which catalyzes the conversion of acetyl CoA to malonyl CoA, the substrate for fatty acid synthase (Roessler, 1988). The ACCase enzyme was extensively characterized (Roessler, 1990a). Additional studies focused on the pathway for production of the storage carbohydrate chrysolaminarin, which is hypothesized to compete with the lipid pathway for fixed carbon. UDP-glucose pyrophosphorylase (UGPase) and chrysolaminarin synthase activities from *Cyclotella cryptica* were also characterized (Roessler, 1987, 1988).

Microalgal molecular biology and genetic engineering. In the latter years of the ASP, the research at the National Renewable Research Laboratory focused on the genetic engineering of green algae and diatoms for enhanced lipid production. Genetic transformation of microalgae was a major barrier to overcome. The first successful transformation of a microalgal strain with potential for biodiesel production was achieved in 1994, with successful transformation of the diatoms *Cyclotella cryptica* and *Navi-cula saprophila* (Dunahay et al., 1995). The technique utilized particle bombardment and an antibiotic resistance selectable marker under the control of the ACCase promoter and terminator elements. The second major accomplishment was the isolation and characterization of genes from *Cyclotella cryptica* that encoded the ACCase and UGPase enzymes (Jarvis and Roessler, 1999; Roessler and Ohlogge, 1993). Attempts to alter the expression level of the ACCase and UGPase genes in *Cyclotella cryptica* using this transformation system met with some success, but effects on lipid production were not observed in these preliminary experiments (Sheehan et al., 1998).

Process development. During the course of the ASP research, it became apparent that cost-effective solutions to algal de-watering, lipid extraction and purification, and conversion to fuel are critical to successful commercialization of the technology. Of the various studies, de-watering via flocculation was deemed encouraging (Sheehan et al., 1998), and various solvents were tested for their ability to extract lipid from algal biomass (Nagle and Lemke, 1989).

Outdoor testing. Several demonstrations of large-scale outdoor microalgal cultivation were conducted under the ASP (Sheehan et al., 1998). Of particular note was the out-

door test facility in Roswell, New Mexico, operated under sub-contract to Microbial Products Inc. (Weissman et al., 1989). Utilizing two 1000 m² raceway ponds, long-term, stable production of algal biomass was demonstrated. The efficiency of sparged CO₂ utilization was shown to be over 90%, and occasional biomass productivities of up to 50 g m⁻² day⁻¹ were observed. However, overall productivity was limited by low temperatures and averaged closer to 10 g m⁻² day⁻¹.

Analysis. Resource assessments conducted under the ASP demonstrated the availability of ample land, saline water and CO₂ resources in the desert in the south-western USA for this technology, with the potential for many billions of gallons of fuel production. Cost analyses underlined the necessity of low-cost culture systems such as open ponds, and the fact that biological productivity has the single largest influence on fuel cost. Even with optimistic assumptions about CO₂ credits and how far productivity could be improved, estimated fuel costs were determined to range from \$1.40 to \$4.40 per gallon in 1995 (Sheehan et al., 1998). While costs for the technology were deemed as never being competitive with the projected cost of petroleum diesel, the landscape has clearly changed in the intervening decade.

Overall, the ASP demonstrated the feasibility of algal culture as a source of oil, and resulted in important advances in the technology. However, the need to understand algal lipid accumulation mechanisms and to develop highly productive strains became apparent in order to make the technology commercially feasible.

Path forward for algal feedstock-based biofuels

This review highlights the need for concentrated research on the biosynthesis of algal lipids, especially TAGs, if we are to better understand and manipulate algae for the production of biofuels. While algae appear to provide the natural raw material in the form of a lipid-rich feedstock, our understanding of the details of lipid metabolism to enable manipulation of the process physiologically and genetically is lacking. Furthermore, the lack of resources to develop pilot or demonstration-scale production facilities at suitable locations, as well as the intermittent and short-term support for related research and development activities, has historically impeded the development of an algae-based biofuel technology. Over 20 years ago, the ASP illustrated the potential of algae to provide liquid energy. To reap the benefits of that potential will not only require critical engineering innovations and breakthroughs related to algal mass culture and downstream processing, but also focused research on fundamental biological questions related to regulation of lipid metabolism. Several biological challenges and opportunities lie ahead.

(1) At the biochemical level, available information about fatty acid and TAG synthetic pathways in algae is still fragmentary. We lack, for example, critical knowledge regarding both the regulatory and structural genes involved in these pathways and the potential interactions between pathways. Because fatty acids are common precursors for the synthesis of both membrane lipids and TAG, how the algal cell coordinates the distribution of the precursors to the two distinct destinations or inter-conversion between the two types of lipids needs to be elucidated. Assuming that the ability to control the fate of fatty acids varies among algal taxonomic groups or even between isolates or strains of the same species, the basal lipid/TAG content may, in effect, represent an intrinsic property of individual species or strains.

(2) Beyond the level of lipid metabolism, how algal cells control the flux of photosynthetically fixed carbon and its partitioning into various groups of major macromolecules (i.e. carbohydrates, proteins and lipids) is another critical area of research. A fundamental understanding of 'global' regulatory networks that control the partitioning of carbon between lipids and other alternative storage products will be absolutely essential for metabolic engineering of algal cells for over-production of lipids.

(3) A critical evaluation of the relationship between the cell cycle and TAG production is needed. Understanding the control mechanisms underlying this phenomenon will enable genetic manipulation of selected algal strains that exhibit rapid growth and TAG accumulation simultaneously to ensure maximum sustainable oil production.

Addressing the above challenges will require a battery of new approaches and tools. The green alga *C. reinhardtii* is a model organism for this purpose. When coupled with functional genomics, proteomics and metabolomics tools, the model system will undoubtedly provide new insights into the regulation and dynamics of lipid metabolism and the control over oil production in algae.

(4) The isolation and characterization of algae from unique aquatic environments should be a continuing effort. The several thousand algal strains examined for lipids/oils so far represent only a small portion of the over 40 000 identified species available in nature. The surprising variation in fatty acid composition and content found in the algal isolates should be an encouragement to continue this effort. Research on additional organisms will surely provide novel insights into the unique mechanisms that algae possess for more efficient lipid/oil production.

(5) Metabolic engineering through genetic manipulation represents yet another promising strategy for the over-production of algal oils. The available approaches may include random and targeted mutagenesis and gene transformation. Cloning and transforming genes that influence the synthesis of lipids or improve robustness in growth performance in selected algal strains proven to be amenable

to mass culture will enhance the overall performance and sustainable production of TAG or other lipids.

(6) Developing innovative large-scale culture systems that will enable selected algal strains to achieve high and sustained growth rates and oil yields is essential to developing an algal-based biofuel industry. A comprehensive literature review (Hu *et al.*, 2006) indicates that open raceway ponds can be cost-effective for a limited number of algal species in producing protein- or carotenoid-rich biomass, but are less effective or sustainable for production of oil-rich biomass due to the inherent technical constraints of current pond designs. Although substantial increases in biomass and oil production have been achieved in closed photobioreactors of various configurations, high capital and operational costs associated with the tested closed photobioreactors may prevent their application as a commercial production system. Innovative concepts for algal culture systems that will increase the production of oil-rich biomass while at the same time reducing the cost are needed. A hybrid culture system concept that integrates an open raceway system and a closed photobioreactor in various configurations may provide a path forward to commercialization of an industry based on algal lipid biosynthesis. Further research efforts should also be focused on the reduction of costs and energy consumption associated with the downstream processing of algal biomass, including developing more efficient processes for harvesting and de-watering. Lipid extraction from algal biomass represents another major task that will also require either modified or new approaches as processing algal biomass for oil introduces challenges that have not been previously encountered with oil seeds.

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