ADVANCES IN
APPLIED MICROBIOLOGY

VOLUME 109

VOLUME ONE HUNDRED AND NINE

ADVANCES IN APPLIED MICROBIOLOGY

Edited by

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Dundee, Scotland, United Kingdom

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Wilmington, Delaware, United States

ACADEMIC PRESS
An imprint of Elsevier

Contents

CHAPTER ONE

Microalgae for biofuel production

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Abstract

Microalgae have been used commercially since the 1950s and 1960s, particularly in the Far East for human health foods and in the United States for wastewater treatment. Initial attempts to produce bulk chemicals such as biofuels from microalgae were not successful, despite commercially favorable conditions during the 1970s oil crisis. However, research initiatives at this time, many using extremophilic microalgae and cyanobacteria (e.g., *Dunaliella* and *Spirulina*), did solve many problems and clearly identified biomass productivity and harvesting as the two main constraints stopping microalgae producing bulk chemicals, such as biofuels, on a large scale. In response to the growing unease

Advances in Applied Microbiology, Volume 109
ISSN 0065-2164
https://doi.org/10.1016/bs.aambs.2019.10.001

around global warming, induced by anthropogenic CO_2 emissions, microalgae were again suggested as a carbon neutral process to produce biofuels. This recent phase of microalgae biofuels research can be thought to have started around 2007, when a very highly cited review by Chisti was published. Since 2007, a large body of scientific publications have appeared on all aspects of microalgae biotechnology, but with a clear emphasis on neutral lipid (triacylglycerol) synthesis and the use of neutral lipids as precursors for biodiesel production. In this review, the key research on microalgal biotechnology that took place prior to 2007 will be summarized and then the research trends post 2007 will be examined emphasizing the research into producing biodiesel from microalgae.

1. Introduction

One of the major themes of the 21st century to date is the need to replace fossil fuels with fuels based on renewable energy to mitigate the rise in atmospheric CO_2, which is a major component in anthropogenic global warming. Renewable energy requires the use of biomass produced from recently fixed CO_2. The term "fixed CO_2" refers to CO_2 absorbed by a photosynthetic organism (for oxygenic photosynthesis this is a plant, alga or cyanobacterium) and converted into sugar with oxygen as a by-product. The CO_2 fixation reaction is shown below:

$$6CO_2 + 12\,NADPH + H^+ + 18ATP + 18H_2O \rightarrow C_6H_{12}O_6 + 6H_2O + 12\,NADP^+ + 18\,ADP + P_i \quad (1)$$

ATP and NADPH production is via electron transport driven by light energy absorbed by photosystems 1 and 2. The fixation of CO_2 to sugars ($C_6H_{12}O_6$) takes place in the Calvin cycle with the key CO_2 fixation reaction catalyzed by ribulose bisphosphate carboxylase (Rubisco). Utilizing light energy to fix CO_2 into biomass and then using the biomass (or components of the biomass) as a fuel can potentially lead to a carbon neutral fuel. The term "recently" indicates that the biomass has been grown in the recent past and this is to distinguish it from fossil fuel, in which the biomass was produced several 100 million years ago.

First-generation biofuels utilized crop plants as very well-established sources of recently produced biomass. The Brazilian model, set up originally in the 1970s, uses sugar cane waste as the source of its feedstock to produce bioethanol (Goldemberg, 2007). More recently, both the United States and Europe attempted to copy the Brazilian model, but using crops such as wheat as the biomass source. This led to a "food vs fuel" debate and claims

that turning arable land to fuel production was increasing food prices (Rosillocalle & Hall, 1987). In response, second-generation biofuels utilize lignocellulose (inedible to humans) waste from agricultural crops or use non-crop plants grown specifically for lignocellulose such as switchgrass or *Miscanthus*. In either case, there is no direct competition with food crops, but to produce fuel from the chemically recalcitrant lignocellulose is an expensive process due to heating the biomass in the presence of acids—costly and environmentally unfriendly (Himmel et al., 2007). This brings us to third-generation biofuels based on microalgal biomass and the subject of this review. Microalgae do not compete for agricultural land and their simple morphology (single cells or filaments) makes extraction of fuel precursors easier and more environmentally friendly than lignocellulose. Microalgae in the oceans are responsible for over 45% of global CO_2 fixation (Falkowski et al., 2004) and this makes them very good candidates to produce biofuels that are carbon neutral.

During the oil crisis of the 1970s, which kick started the Brazilian first-generation biofuel industry, attempts were made to utilize microalgae for biofuel production. However, the modern era of microalgal biofuels began with the review by Chisti published in 2007 in Biotechnology Advances. This highly cited review article (5081 citations on Web of Science at 3rd September 2019) stated the case for using microalgae as a source of biodiesel that could replace fossil fuel diesel (Chisti, 2007). As noted above, this was not a new idea, but the Chisti review was comprehensive and was published at a time when global environmental concerns about greenhouse gases were recognized by the intergovernmental panel on climate change (Metz, Davidson, Bosch, Dave, & Meyer, 2007). The organization of this review will be to treat the Chisti review as a "before and after" marker. The first section will examine the literature prior to 2007 and then the following section will look at the progress made since 2007. Section 4 will complete the review by examining the future prospects for microalgal biofuels.

2. Microalgal biofuels prior to 2007

2.1 History

Interest in microalgal biotechnology can be traced back to the 1940s and 1950s, when in the years after the second world war, algae were cultivated as a potential food source (Burlew, 1953). The green agriculture revolution based on the development of high yielding varieties of crop plants and the associated use of fertilizers dramatically increased crop yields from the 1950s

onwards (Evenson & Gollin, 2003). Therefore, mass cultivation of microalgae became limited to parts of the Far East, in particular Japan (Tamiya, 1957). The major reason for the early interest in microalgal biotechnology was due to the plant-like characteristics of many microalgae, i.e., their ability to use CO_2 as the sole source of carbon and sunlight as the only energy source. In Japan, the commercial microalgae industry grew up around the green algal genus *Chlorella* and exploited mixotrophic (CO_2 plus an organic carbon source) and heterotrophic growth to greatly increase the biomass yields (Yamaguchi, 1996). This allowed the establishment of a microalgal industry that still survives to this day in Japan and elsewhere in the Far East.

The other strand of microalgal biotechnology, dating back to the early 1960s, was the use of algae in wastewater treatment (Oswald, 2003). The work by Oswald and Benemann and their co-workers greatly influenced the design of open (raceway) ponds for microalgal growth with a view to mitigate pollutants and to produce algal biomass for animal feed (Oswald, 1988a, 1988b). The most common design of a raceway pond consists of an elongated oval with baffles at both ends to create turbulence. The baffles avoid dead zones at the ends of the pond, which lead to a build-up of algal biomass that disturbs the smooth running of the ponds. Mixing of the pond is due to a simple paddle wheel device, which can be powered using solar energy, to drive the algal suspension around the pond. Injection of CO_2 at the sump allows good mixing of the CO_2 into the pond. The pond is essentially a concrete bowl with a plastic lining to prevent the algae seeping into the concrete. One of the most expensive jobs required to maintain good functioning of the pond is to replace the lining on a regular basis.

2.2 Extremophilic microalgae

Another approach to establish microalgal biotechnology was to grow extremophilic microalgae in low cost outdoor ponds. It was recognized that extremophilic microalgae would be much less liable to contamination in the open ponds and the environmental stress could be used to maximize the production of the desired biochemical. This approach is exemplified by the use of the salt tolerant green alga *Dunaliella* (Benamotz & Avron, 1990). *Dunaliella* is one of the main genera of green microalgae that have been exploited successfully for biotechnology. *Dunaliella* genus contains a number of species, some of which are halotolerant (e.g., *D. tertiolecta* and *D. primolecta*) and some that are extremely halotolerant and possibly even halophilic (*D. parva* and *D. salina*). Initially, in the 1970s, glycerol

was projected to be the main commercial product from *Dunaliella* because there is a direct relationship between external salinity and internal concentration of glycerol, which can reach 80% of the overall dry biomass (Benamotz & Avron, 1973). However, glycerol production has never become commercially viable using *Dunaliella*, due to competition from oil industry in 1980s and 1990s and then to competition from renewable biodiesel production in the 21st century. The transesterification process to produce biodiesel leads to glycerol synthesis as a by-product keeping the price of glycerol very low. However, in addition to glycerol, *Dunaliella* also produce β-carotene up to about 10% of their dry weight. Beta-carotene is a valuable compound and is marketed under three categories: (a) beta-carotene extracts in vegetable oil, also contains other carotenoids—used in food products as a coloring agent, (b) *Dunaliella* powder for human use—as health food/food supplement—with possible but controversial anti-cancer properties and (c) dried *Dunaliella* biomass for animal feed and aquaculture (Benamotz & Avron, 1990).

The other extremophile exploited was the cyanobacterium *Spirulina* (*Arthrospira platensis*), which has been used as food by humans for centuries (Ciferri, 1983). *Spirulina* grows optimally at pH 10 and *Spirulina* biomass is a healthy foodstuff, which has FDA approval in the United States. It is a good source of the polyunsaturated fatty acid γ-linolenic acid, which is important for a healthy diet in humans. The second product from *Spirulina* is the water-soluble accessory pigment phycocyanin, which is marketed as Lina Blue in Japan, and is used in the food and cosmetics industries (Ciferri, 1983). Pigments such as β-carotene and phycocyanin are valuable products used as food colorants and as healthy additives to food. The high value pigments and using microalgae as feedstocks for aquaculture were the main products of algal biotechnology during the 1970s and 1980s, but the market sizes were relatively modest around US$10 million per annum (Benemann, Tillett, & Weissman, 1987).

2.3 Open ponds vs photobioreactors

Nevertheless, the idea of using microalgae to produce lower value products that have very large market sizes was never totally abandoned and in 1995 a comprehensive review on alternative fuels contained a section on lipid production from microalgae and its feasibility for biodiesel production (Kosaric & Velikonja, 1995). Producing biofuels from algae falls into this category of a very large market size, but low value with an

already very well-established fossil fuel driven industry in place. The necessity to produce microalgae on a very large scale led to investigations into the technology required for bulk production of microalgae. This built on the work of Oswald and Benemann mentioned above to produce large-scale outdoor raceway ponds driven by simple paddle wheel systems (Borowitzka, 1999).

As noted by Borowitzka in this article (Borowitzka, 1999) there are a number of enclosed bioreactor systems that can offer several advantages over the open ponds. The argument between the use of open ponds and enclosed bioreactor systems has been a long running thread throughout algal biotechnology history (Carvalho, Meireles, & Malcata, 2006). There are clear advantages on both sides of the argument (see Table 1), but in terms of bulk products such as biofuels it is essential that production takes place outdoors, but this does not completely rule out a large enclosed outdoor type of bioreactor (Gilmour & Zimmerman, 2012). However, it is difficult to conceive how any enclosed bioreactor can be scaled up sufficiently to meet the demand for a biofuel (Benemann, 2013). Nevertheless, the much greater level of control that can be applied to the culture using a photobioreactor system can produce very high quality products in an axenic environment. An example of the type of high quality products that can be produced in this way are ^{13}C labeled chemicals (Berthold et al., 1991).

2.4 Potential biofuels from microalgae

Third-generation biofuels that can be produced from microalgae biomass include (a) biodiesel from lipids, (b) bioethanol from starch, (c) photosynthetically generated biohydrogen and (d) anaerobic fermentation of algal

Table 1 Comparison of raceway ponds and photobioreactors.

Open raceway ponds	Enclosed photobioreactors
Simple design using mixing by paddle wheel	Variety of designs, e.g., flat plate, helical, etc.
Environmental conditions not optimized	Controlled environmental conditions
Low operation costs	High operation costs
High levels of contaminating species—need extremophile algae	Low levels of contaminating species
CO_2 can escape to the atmosphere	O_2 can build-up if mixing insufficient
Relatively easy to scale up if land area available	More difficult to scale up for most formats

biomass to produce biogas (mainly methane). The most promising and most widely researched option is the biodiesel production from neutral storage lipids, mainly consisting of triacylglycerol (TAGs) (Chisti, 2007). The importance of TAG is that it can easily be converted into fatty acid methyl esters (FAMEs) via transesterification in the presence of an alkali metal hydroxide catalyst or an alkoxide catalyst (e.g., sodium methoxide). An excess of methanol is used to drive the reaction in the desired direction (see Eq. 2). Transesterification reaction is a continuous process that takes place in stirred tanks at around 60 °C and the glycerol by-product is removed by continuous centrifugation. Transesterification is a highly efficient process and can reach values of 99% efficiency.

$$\text{Triacylglycerol} + 3 \times \text{Methanol} \xrightarrow{\text{Sodium methoxide}} \text{Glycerol} + \text{Fatty Acid Methyl Esters} \quad (2)$$

FAMEs are biodiesel and can be utilized in diesel engines (Knothe, 2005). The original diesel engine dating back to the late 19th century ran on vegetable oil and continued to do so into the 1920s before petroleum-based diesel took over this role (Subramaniam, Dufreche, Zappi, & Bajpai, 2010). The diesel engine is robust and in addition to vegetable oil, a number of different fuel sources were originally proposed including finely powdered coal (Shay, 1993). This historical observation led to the idea of directly using microalgal cells without the need to extract the high-energy lipids and thus save considerably on processing costs. For this to be feasible, the calorific value of the algal cells would need to approach the calorific value of biodiesel ($43\,kJ\,g^{-1}$). For *Chlorella* species grown on normal Watanabe medium, the calorific value of the biomass is between 18 and $21\,kJ\,g^{-1}$ (Illman, Scragg, & Shales, 2000). By growing the cells under nitrogen limitation to increase the proportion of lipids, the calorific value increased to $29\,kJ\,g^{-1}$. This equates to a total lipid content of 63% (w/w) (Illman et al., 2000). A liquid fuel was produced that consisted of an algae (*Chlorella*) slurry (partly dried algal biomass) mixed with esters of rapeseed oil that worked successfully in a test diesel engine (Scragg, Morrison, & Shales, 2003). This work confirmed the versatility of the diesel engine and but it also demonstrated clearly that extraction of high-energy neutral lipid from algal cells is required to approach the calorific content of petroleum diesel. The calorific value of FAMEs from microalgae is about $38.5\,kJ\,g^{-1}$, i.e., approximately 80% of the average energy in petroleum oil (Chisti, 2007).

2.5 Lipid synthesis in microalgae

Neutral lipid (TAG) is not the only lipid type produced by microalgae cells. Polar (membrane) phospholipids, sulpholipids and structural galactolipids are key components of living cells and during active growth are prioritized over TAG, which has a storage role (Thompson, 1996). TAGs are only accumulated under stress conditions as energy storage lipids. Actively growing green algae mainly use starch as an energy store and large deposits of starch can be found in the chloroplasts of green algae (Thompson, 1996). Lipid synthesis for energy storage only occurs under highly stressful conditions that stop or very largely inhibit growth. As mentioned in the previous section, TAG are the most useful lipids for biodiesel production using transesterification (Chisti, 2007) and so it is desirable to favor TAG synthesis over other lipid synthesis pathways. To slow down or stop growth of the microalgal cells, an environmental stress (e.g., high salinity) or nutrient limitation (e.g., nitrogen or phosphorus) are mainly used. For diatoms, silicon limitation can be used to switch on TAG synthesis (Roessler, 1988). Many, but not all, microalgae will respond by accumulating TAG. The physiological reason for TAG accumulation is probably to sequester ATP and NADPH that would normally be employed in underpinning cell growth (Thompson, 1996).

2.5.1 Lipid synthesis pathways

Glycerol-3-phosphate and acetyl CoA are the precursor molecules for lipid biosynthesis. Glycerol-3-phosphate (G-3-P) is synthesized from dihydroxyacetone phosphate (a central metabolite in the glycolysis pathway) by the enzyme glycerol-3-phosphate dehydrogenase. As shown in Fig. 1, four enzyme catalyzed reactions lead from G-3-P to TAG. The final enzyme, diacylglycerol acyl transferase (DAGAT), is unique to TAG synthesis and is an obvious target for upregulation of the gene(s) involved in DAGAT synthesis. Very little TAG is synthesized in actively growing microalgae, most of the phosphatidic acid (PA) and diacylglycerol (DAG) are used to produce phospholipids and polar lipids/galactolipids.

The other part of the pathway for producing neutral lipids (TAG) is the generation of the fatty acid (acyl) chains. The first reaction is catalyzed by acetyl CoA carboxylase (ACCase) as shown below in Eq. (3).

$$\text{Acetyl CoA} + HCO_3 + \text{ATP} \rightarrow \text{Malonyl CoA} + \text{ADP} + P_i + H^+ \quad (3)$$

A repeating sequence of reactions catalyzed by keto-acyl synthases builds up the fatty acid chain to a length of 18 carbons for saturated and

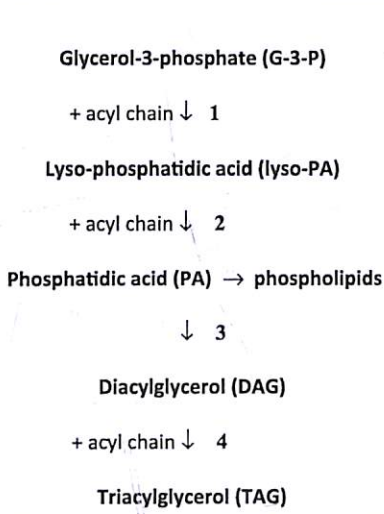

Fig. 1 TAG biosynthesis from glycerol-3-phosphate and fatty acid (acyl) chains produced from acetyl CoA. 1 = glycerol-3-phosphate acyltransferase. 2 = acylglycerol-3-phosphate acyltransferase. 3 = phosphatidic acid phosphatase. 4 = diacylglycerol acyltransferase.

Table 2 Key saturated and monounsaturated fatty acids found commonly in microalgae.

Common name	Systematic name	Carbon number:double bond
Palmitic acid	Hexadecanoic acid	C16:0
Palmitoleic acid	Hexadeca-9-enoic acid	C16:1
Stearic acid	Octadecanoic acid	C18:0
Oleic acid	Octadeca-9-enoic acid	C18:1
Linoleic acid	Octadeca-9,12-dienoic acid	C18:2
α-Linolenic acid	Octadeca-9,12,15-trienoic acid	C18:3
γ-Linolenic acid	Octadeca-6,9,12-trienoic acid	C18:3

monounsaturated fatty acids (see Table 2). Desaturases and elongases produce polyunsaturated and longer chain fatty acids, respectively.

The growth phase affects the lipid content of cells with a higher percentage of fatty acids found in the form of TAG in stationary phase—this may well be due to nutrient depletion in the medium. Physiological role of TAG biosynthesis is partly energy and carbon storage, but TAG synthesis is also a part of the system (with carotenoids) that reduces the level of free radicals within cells (Goodenough et al., 2014; Pick, Zarka, Boussiba, & Davidi, 2019).

The observation that limiting nitrogen induces many eukaryotic microalgae to produce TAG indicated a good way forward to produce biodiesel from microalgae. The fluorescent dye Nile red has proved to be a good probe to screen for TAG production as demonstrated by Cooksey and co-workers in the 1980s (Cooksey, Guckert, Williams, & Callis, 1987). Work on the diatom *Chaetoceros muelleri* showed a five to seven times increase in neutral lipid (TAG) when nitrogen was removed from the medium (McGinnis, Dempster, & Sommerfeld, 1997). However, the growth rate of the alga was also drastically decreased under nitrogen deprivation. This "trade off" between TAG accumulation and reduced growth is a major obstruction to efficient production of TAG by microalgae. To understand why this is a major problem, consider the extreme case of "obese" *Chlamydomonas* mutants which have very high values of TAG per cell, but have stopped growing completely (Goodenough et al., 2014). In this case, TAG production per cell is high, but volumetric TAG production per liter would be extremely small.

2.5.2 Fatty acid composition

The composition of fatty acids (see Table 2) will determine the quality of biodiesel that can be produced via the transesterification process (Knothe, 2005). Fatty acids with chain lengths from C16 to C18 are ideal and the degree of unsaturation (number of double bonds) is crucial. If there is too high a proportion of saturated fatty acids (e.g., palmitic acid, C16:0) or too high a proportion of polyunsaturated fatty acids (PUFAs) with two or more double bonds (e.g., EPA C22:5), then the fuel properties will not meet the standards required. Standards are applied to diesel fuel to make sure that it is suitable for use in diesel engines. Two commonly used standards are based on European Standard (EN14214) the American Society for Testing and Materials (ASTM D6751) standard. Diesel standards include a number of criteria such as cetane number (for good ignition characteristics), heat of combustion, cold flow, oxidative stability, viscosity and lubricity (Islam et al., 2013). A mixture of mainly saturated and monounsaturated (e.g., oleic acid, C18:1) is close to the optimum (Knothe, 2005). Fatty acid methyl ester (FAME) profiles were measured for nine microalgae to allow the estimation of their biodiesel characteristics with regard to the EN14214 and ASTM standards. The best biodiesel properties were found for *Chlorella vulgaris* lipid followed by *Nannochloropsis oculata* lipid (Islam et al., 2013). If the *N. oculata* biomass was harvested in late logarithmic phase or early stationary phase

(depending on the growth medium used), when PUFAs were low, then the biodiesel extracted would be superior to that from *C. vulgaris*. This very interesting study shows how carefully the growth of biomass and extraction of lipids needs to be carried out to produce a suitable biodiesel (Islam et al., 2013).

2.6 Harvesting

Arguably, the biggest problem associated with microalgae biotechnology from the start of commercialization is harvesting the cells. It was quickly realized by the pioneers of microalgae mass cultures that light becomes limiting very quickly in photoautotrophic conditions in open ponds or bioreactors. This means that the highest biomass reached is only about $1\,gL^{-1}$ due to light limitation in densely green cultures. In most mature industrial processes, $100\,gL^{-1}$ would be the minimum biomass expected to yield a good return. Therefore, algal biomass is around 100 times more dilute and this is a serious challenge to any cost effective harvesting method. A number of studies have shown that harvesting can account for almost 30% of the total production cost for microalgal products (Grima, Belarbi, Fernandez, Medina, & Chisti, 2003). Continuous centrifugation is the established method for harvesting high value algal products from large volumes of culture with 90% of biomass harvested from a 4% to 5% algal slurry. However, the cost of the equipment, and particularly the running costs, mean that this method is not suitable for bulk chemical production such as biofuels. Wastewater treatment in sewage works uses simple gravity sedimentation and can process large volumes cheaply in terms of money and energy. However, the small cell size of microalgae (often less than 10 μm in diameter) means that settling rates are too slow for commercial purposes. Filtration is the other classic separation method, but it is only suitable for large filamentous species such as *Spirulina*. The most suitable methods for harvesting microalgae are flocculation and flotation (Grima et al., 2003). Flocculants are multivalent cations or cationic polymers that neutralize the negative charges on the surface of microalgal cells, thus allowing them to clump more easily. The cationic polymers also physically bind the cells together, e.g., polyferric sulfate and chitosan. The choice of flocculant depends on the product, e.g., flocculants containing heavy metals would not be suitable for food and feed products. Chitosan is an edible flocculant, but it is not effective at seawater salinities and above (Bilanovic, Shelef, & Sukenik, 1988). Flotation methods used in conjunction with flocculants, employ dissolved or dispersed air to

produce bubbles that lift the algal cells to the surface for harvesting. Traditional air flotation used in sewage works is very energy intensive and again adds to harvesting costs if used with microalgae.

2.7 Efficiency of microalgae photosynthesis

Efficiency of photosynthesis is based on the following calculations: (a) about 44% of sunlight is available for absorption by microalgal pigments, i.e., wavelengths 400–700 nm and (b) efficiency of converting photon energy to fixed carbon is around 27%. Therefore, the theoretical maximum for algal photosynthesis is $0.44 \times 0.27 = 0.119 = 11.9\%$. Unavoidable losses due to respiration bring this figure down to about 10%. Any claims for a photosynthetic efficiency above 10% should be examined critically and may depend on very specific conditions that are not applicable to large-scale growth of microalgae. Also, this theoretical figure of 10% has never been achieved by any large-scale algal culture and 3–5% is a more realistic goal. This still compares favorably with 1–2% for crop plant photosynthetic efficiency (Chisti, 2007). As already mentioned, light absorption in dense cultures of microalgae is a major limiting factor for biomass productivity. Ways to overcome light limitation will be discussed later in this review.

2.8 Summary

There are many positive attributes to make microalgae potential candidates for biofuel (biodiesel) production. In particular, the know-how accumulated by companies growing microalgae for high value products (pigments, aquaculture feed) can be applied to growth of microalgae for biofuel production. The downstream processing to produce biodiesel using transesterification is well-established from first-generation biofuels, but initial harvesting is a problem from low density algal cultures. Overall biomass productivity needs to be optimized to provide a high volumetric yield of precursors (TAG) for biodiesel production.

3. Microalgal biofuels post 2007

As outlined in Chisti's, 2007 review and emphasized by his later review in 2013 there are a number of hurdles to overcome before biodiesel can be made commercially from microalgae (Chisti, 2007, 2013). In his later review, Chisti suggested that the following were the major constraints on producing biodiesel from microalgae. First, the availability of industrial

point sources of CO_2, many pilot plant demonstration facilities do not use industrially sourced CO_2, which is unrealistic in commercial terms. Second, it is essential to build in the recycle of key nutrients (principally N and P) to any large-scale algal growth facility. Third, although often suggested as an ideal medium, wastewater can only support a tiny fraction of biofuel requirements, e.g., wastewater produced from a large city of 10 million inhabitants would only support less than 5% of the annual fuel consumption of the city. Finally, a very useful measure of the efficiency of a new fuel is the energy ratio, i.e., the ratio of energy contained in the new fuel compared to the amount of fossil fuel energy used to make the new fuel. In comparison with oil industry norms, an energy ratio of at least seven would be desirable, at the moment algal biodiesel energy ratios have been estimated to be between 0.5 and 1.4 (Chisti, 2013). Some of the current ideas and proposals to overcome these constraints will be discussed in this part of the review.

First, to get an idea of the major research areas worked on between 2007 and 2019, Chen et al. produced metric data on algal biofuel and algal high value products research (Chen, Li, & Wang, 2019). The most studied algal (including cyanobacteria) genus during this period was *Chlorella* followed by *Chlamydomonas*. A heat map of keywords produced by Chen et al. showed biofuel(s) and lipid to be most common after microalgae itself. *Nannochloropsis* (eustigmatophyte), *Scenedesmus* and (perhaps surprisingly since it does not produce high lipid levels) the cyanobacterium *Spirulina* were the next most common genera studied for biofuel production after *Chlorella* and *Chlamydomonas* (Chen, Li, et al., 2019). The most interesting finding from this metric research was that only 15 algal genera in total commonly appeared in the different research areas categorized in the work—the other 10 genera being 6 from the eukaryotic algae (*Phaeodactylum* [diatom], *Botryococcus, Haematococcus, Dunaliella, Isochrysis* and *Chaetoceros*) and three prokaryotic genera (*Anabaena, Synechocystis* and *Synechococcus*). The main conclusion from this analysis is that there are many genera of eukaryotic microalgae and prokaryotic cyanobacteria that have not been studied in detail for their commercial potential.

3.1 Improving efficiency of microalgal photosynthesis

One of the reasons that the group of microalgae is put forward as containing ideal organisms for biofuel production is due to the high efficiency of their photosynthetic reactions in comparison to higher plants. As already noted, up to 10% of solar energy can be fixed into biomass by microalgae, which

would equate to 280 tons of dry biomass per hectare per year (Benedetti, Vecchi, Barera, & Dall'osto, 2018). One problem is that evolution selects for cells with large arrays of antenna (light harvesting) chlorophyll molecules to maximize light absorption and deprive competitor cells of light (Ort, Zhu, & Melis, 2011). In a dense algal culture, the first few layers of cells absorb most of the light, often becoming photoinhibited. In contrast, cells beneath the surface layers are light limited. Rapid mixing of the culture helps to cycle the cells quickly between high light and dark, but nevertheless the photosynthetic efficiency of such a high density culture is far below the theoretical maximum and may not even reach 3% efficiency.

For commercial growth of microalgae, reduced photosynthetic efficiency is a highly significant limiting factor, because up to 80% of photons from sunlight are wasted. Melis, in 2009, estimated that photosynthetic productivity could be increased by 300%, if this light mismatch could be addressed (Melis, 2009). The suggested way forward is to select for algal cells that have smaller antenna sizes (Lee, Mets, & Greenbaum, 2002) and this has been successfully demonstrated for *Dunaliella salina* cells (Melis, Neidhardt, & Benemann, 1998). To apply this to algal biotechnology, stable mutants are required, since cells will revert to larger antenna sizes once the selective pressure of high light is removed. One advantage of mutant microalgal cells with small antenna sizes is that they cannot compete with wild type algae in natural environments and cannot "escape" into the wild (Ort et al., 2011). The downside is that the same mutant cells would be susceptible to take over by wild type strains invading an outdoor pond culture.

A very different approach was recently taken by Burak et al., who examined using wavelength shifting films that can be applied to the walls of a photobioreactor (Burak, Dunbar, & Gilmour, 2019). The films were produced by dissolving the appropriate dyes (e.g., Coumarin or Orange 2G) in tetrahydrofuran and then mixing the dyes with polydimethylsiloxane (PDMS) before being poured into a mold to produce a rubbery film. Using a Coumarin PDMS film, UV light is shifted into the blue light part of the spectrum and became available to drive photosynthesis. Application of the Coumarin PDMS film to a bioreactor containing the green alga *D. salina* led to a 36.9% increase in final biomass (as determined by optical density) after approximately 3 weeks growth (Burak et al., 2019). A similar experiment using an Orange 2G PDMS film (shifts green light to red light) led to a smaller increase in biomass (18.8%). This builds on earlier work by

Wondraczek et al. (2013) and Amrei, Nasernejad, Ranjbar, and Rastegar (2014) showing enhanced growth and photosynthetic activity in response to wavelength shifting dyes. This type of engineering approach can potentially increase significantly the productivity of microalgae grown in photobioreactors.

Another approach to increasing photosynthetic efficiency is the use of flashing light. Algal cells in a well-mixed bioreactor or algal raceway pond, will experience a flashing light effect as the move in and out of the illuminated area at the wall of the bioreactor or the surface of the pond (Abu-Ghosh, Fixler, Dubinsky, & Iluz, 2016). Controlling the use of flashing light is more applicable to photobioreactors and flashing light can be utilized more efficiently by dense algal cultures (Carvalho et al., 2006; Kim, Kim, Lee, & Lee, 2006). A number of possible mechanisms, which are not mutually exclusive, to explain the flashing light effect include less photoinhibition, lower loss of light energy by heat dissipation and more efficient use of the Calvin cycle (Abu-Ghosh et al., 2016).

Optimum light utilization will involve the interplay between the circadian light dark cycle and the frequency of the flashing light. Using light emitting diodes (LEDs) allow a very high light intensity to be generated during the "flash," which improves the energy efficiency of the system. Higher algal growth can be achieved under flashing light then under the same overall amount of light energy supplied continuously (Schulze, Guerra, Pereira, Schuler, & Varela, 2017). Although flashing light technology can be carefully optimized for a photobioreactor set up by controlling the air flow and bubbling rate, it should also be possible to use a cruder version of the flashing light effect in open ponds by controlling the mixing velocity induced by the paddle wheel. It is also becoming evident that different flashing light regimes can influence the pigment composition of algal cells and also potentially other cell constituents such as lipids.

3.2 Heterotrophy

The previous section examined the possibilities of increasing photosynthetic efficiency, but an alternative route is to add organic compounds to the algal growth media to induce heterotrophic (in the dark) or mixotrophic (in the light) growth. Heterotrophic growth of microalgae may seem to have limited possibilities for algal biotechnology, since the cells are grown in conventional stirred tank bioreactors in the dark. Advantages of using bioreactors

were noted above (lack of contamination and much better control of the growth process), but if glucose is used to replace CO_2 fixation, then it makes up 80% of the cost of the medium (Deng et al., 2019; Li, Xu, & Wu, 2007). Therefore, it is essential to use a cheap source of carbon, such as molasses (waste product from sugar refineries) rather than pure sugars. A thermotolerant species of *Micractinium* grown heterotrophically on molasses produced mainly palmitic (C16:0) and linoleic acids (C18:2) (Engin, Cekmecelioglu, Yucel, & Oktem, 2018). This is a generally good mix of fatty acids for biodiesel production but the carbon neutral status of using heterotrophy to produce biodiesel will need to be determined by careful life-cycle analysis of the whole process, including the source of organic carbon.

3.3 Mixotrophy

Mixotrophic growth, where light and CO_2 are still utilized in addition to the organic carbon compound, may offer more substantial advantages for biofuel production and contribute to CO_2 mitigation (Deng et al., 2019; Krzeminska & Oleszek, 2016). The advantages of mixotrophic growth include the simultaneous use of the Calvin cycle and pentose phosphate pathways, biomass loss in the dark period of a light/dark diurnal cycle is much reduced and oxygen accumulation leading to oxidative stress is largely eliminated (Patel, Joun, Hong, & Sim, 2019). Disadvantages of mixotrophic growth are the downregulation of chlorophyll biosynthesis and reduced activity of the Calvin and TCA cycles (Zhang et al., 2017). It is also often forgotten that supplying high levels of CO_2 at a scale that is required for biofuel production can be a significant challenge, because industrial sources of CO_2 may be geographically remote from the algal ponds and the cost of transporting the CO_2 would be prohibitive (Pate, Klise, & Wu, 2011). Mixotrophic growth can alleviate this problem by using the CO_2 from algal respiration in photosynthesis and the O_2 from photosynthesis can be used to drive respiration (Smith, Bangert, Wilkinson, & Gilmour, 2015). A number of studies have looked at mixotrophic growth of green microalgae and in some cases photosynthesis and respiration work non-competitively, i.e., heterotrophic and autotrophic growth rates added together equal the mixotrophic growth rate (Endo, Sansawa, & Nakajima, 1977; Kobayashi, Kakizono, Yamaguchi, Nishio, & Nagai, 1992; Martinez & Orus, 1991). However, under non-aerated conditions, a synergistic effect can be seen where the growth rate of mixotrophically-grown cells can exceed the combined growth rate of autotrophically- and heterotrophically-grown cells.

Smith et al. demonstrated a 1.74 times (acetate) and 1.34 times (glucose) increase in mixotrophic growth over combined autotrophic/heterotrophic growth rate for the green alga *Micractinium inermum* (Smith et al., 2015). This type of synergistic effect can reduce the costs of supplying CO_2 to the cultures and avoid the accumulation of oxygen detrimental to microalgal growth. More recently, the synergistic effect of mixotrophy was clearly demonstrated in *Chlorella zofingiensis*, but the authors present an alternative mechanism to explain the synergy. Instead of gas exchange between photosynthesis and respiration their hypothesis suggests that photosynthetically generated ATP and NADPH can preferentially be channeled to general cell metabolism rather than supporting Rubisco catalyzed CO_2 fixation in the Calvin cycle (Zhang et al., 2017).

The choice of organic carbon source to add is very important and the most commonly used carbon sources are glucose or acetate. As mentioned above, it is not feasible to use pure sugars and waste products such as molasses are an alternative. Glycerol is a very cheap carbon source because it is a by-product of first-generation biodiesel production during the transesterification process (see Eq. 2). However, both molasses and waste glycerol are highly colored and will inhibit photosynthesis during mixotrophic growth due to light attenuation. The waste glycerol will also contain methanol from the transesterification reaction, which may be harmful to microalgal growth. Therefore, it is not trivial to source suitable carbon containing waste products to support mixotrophy. It is likely that nutrient rich domestic or agricultural waste water will be a more suitable source of carbon for mixotrophy and in addition, the microalgae can remove nitrogen and phosphorous from these waste streams as happens in water treatment plants (Pang et al., 2019).

An important part of the studies on mixotrophic growth of microalgae for biofuel production is to examine the effect on their fatty acid composition. As already stated, saturated and monounsaturated fatty acids are most favorable for biodiesel production and polyunsaturated fatty acids (PUFAs) are much less favorable due to oxidative instability (Knothe, 2005). For *Auxenochlorella protothecoides*, addition of $5\,g\,L^{-1}$ glucose to the medium increased the amount of oleic acid (C18:1) and decreased the amount of linolenic acd (C18:3) which improves the fatty acid profile for biodiesel production. In contrast, addition of glucose to *Chlorella kessleri* cultures significantly increased the percentage of PUFAs, which would be detrimental to biodiesel production (Deng et al., 2019). Clearly, the effect of mixotrophic growth on fatty acid composition is another variable that needs to be monitored.

As well as varying the type and source of the organic carbon, the light intensity and quality can also be changed to see the effects on mixotrophic growth (Patel, Joun, et al., 2019). Many algal species (e.g., most strains of *Dunaliella*) will not grow mixotrophically nor heterotrophically. Other algal species require light to take up exogenous glucose, e.g., a strain of *C. vulgaris* showed negligible growth in the dark despite good mixotrophic growth (Subramanian, Yadav, & Sen, 2016). Using *Chlorella protothecoides* (UTEX-256), Patel et al. showed that mixotrophic growth on glucose and acetate was optimized with different light regimes. Growth on acetate was best under continuous high light ($150\,\mu mol\,m^{-2}\,s^{-1}$) conditions, whereas growth on glucose was equally good under both high light and light limiting conditions ($35\,\mu mol\,m^{-2}\,s^{-1}$) and was similar under continuous light or 16 h light: 8 h dark (Patel, Joun, et al., 2019). Glycerol was a poor substrate for mixotrophic growth of *C. protothecoides* under any of the light regimes. Quality and photoperiod of light used can be important for mixotrophic cultivation of microalgae. Lutein pigment production was investigated in the green microalga *Scenedesmus obliquus* supplemented with 2% (w/v) glucose and a 12 h light:8 h dark photoperiod and two stage process of first blue light (420–450 nm) and then red light (660–700 nm). This work demonstrates the complex relationship between light quality and mixotrophic growth (Chen, Hsu, et al., 2019). Using the model green alga, *Chlamydomonas reinhardtii*, Smith and Gilmour examined the influence of mixotrophic growth (using acetate), photoperiod (continuous light vs 12 h light:12 h dark) and nitrogen deprivation on the carbon and lipid metabolism (Smith & Gilmour, 2018). Both photoperiod and the addition of acetate had a significant effect on lipid synthesis in *C. reinhardtii*. Acetate uptake leads to a metabolism dominated by respiration. Only after starch storage had reached capacity did neutral lipid (TAG) begin to accumulate. This is in agreement with work on *Dunaliella* by Pick and Avidan discussed below.

3.4 Inducing efficient synthesis of neutral lipids (TAG)

As noted in Section 2.5.1, nitrogen limitation induces TAG synthesis in many microalgal species. However, particularly in green microalgae, nitrogen limitation also induces starch synthesis (Razeghifard, 2013; Slocombe et al., 2015). The relationship between starch synthesis and TAG synthesis is not clear, when both are used as energy and carbon stores under nitrogen limitation. Work by Pick and Avidan using *Dunaliella*

tertiolecta showed that starch synthesis occurred first after the onset of nitrogen deprivation and then TAG synthesis occurred later in the process (Pick & Avidan, 2017). Based on pulse labeling experiments, it was estimated that around 66% of total TAG accumulated was made from the breakdown of the previously formed starch and most of the rest of the TAG was produced de novo from phospholipids (Avidan & Pick, 2015). The requirement for preformation of starch before the formation of TAG is an important piece of information in the quest to understand TAG biosynthesis in green algae.

Synthesis of TAG is generally recognized to depend on increasing the flow of carbon toward acetyl CoA, e.g., the upregulation of plastidic (chloroplast-based) pyruvate dehydrogenase (PDH) in the high lipid producing alga *Chlorella desiccata* led to increased amounts of acetyl CoA and therefore increasing amounts of TAG (Avidan, Brandis, Rogachev, & Pick, 2015). Further work from the same lab showed that a PDH-bypass involving three enzymes (pyruvate decarboxylase, acetyl CoA synthase and alcohol dehydrogenase) is crucial to maintaining TAG production in *C. desiccata* during nitrogen deprivation. The most likely explanation for the need for the PDH-bypass is to maintain activity of both plastidic and mitochondrial pyruvate dehydrogenase (Avidan & Pick, 2015). This work illustrates the complexity of the biochemical mechanisms leading to TAG biosynthesis in oleaginous microalga.

All of the work described so far in this section relates to green microalgae, but non-green species are also potentially very good TAG producers. In the study by Huete-Ortega et al., the diatom *Phaeodactylum tricornutum* and the eustigmatophyte *Nannochloropsis oceanica* were compared to see the effect on lipid production of super-saturating light intensities ($1000\,\mu mol\,m^{-2}\,s^{-1}$) and using ammonium as the sole source of nitrogen (Huete-Ortega et al., 2018). Interestingly, the response of the two algae was very different, for *P. tricornutum* very high light intensity and ammonium maximized lipid productivity, whereas exactly the opposite was found for *N. oceanica*.

3.5 Biorefinery concept: Co-production of biofuels and high value products

The idea of a biorefinery is to duplicate the efficiency of an oil refinery by utilizing all of the algal biomass and not just lipids for biodiesel. Algal cells will be used as "cell factories" to produce a range of valuable co-products to potentially offset the cost of biofuel production

(Coh et al., 2019; Ferreira, Rios Pinto, Maciel Filho, & Fregolente, 2019; Suganya, Varman, Masjuki, & Renganathan, 2016). In addition to biodiesel, algal species can be utilized to produce other alternative fuels such as ethanol, 1-butanol and isobutanol (Wijffels, Kruse, & Hellingwerf, 2013). The majority of research into producing ethanol from microalgae has taken place in cyanobacteria because their prokaryotic characteristics make them amenable to genetic engineering and synthetic biology approaches (Atsumi, Higashide, & Liao, 2009; Gao, Zhao, Li, Tan, & Lu, 2012).

In their review, Wijffels et al. make the interesting point that the small prokaryotic cyanobacterial cells are best for the synthesis of small molecules (such as ethanol) that can be secreted from the cells into the medium, whereas the larger celled eukaryotic algae are better producers of storage products such as lipids and starch (Wijffels et al., 2013). A range of carbohydrates can be produced by eukaryotic microalgae including sulfated polysaccharides from *Haematococcus lacustris* (Park et al., 2011). A number of polysaccharide mixtures from a range of microalgae (green, red and diatoms) were shown to have pharmacological properties such as anti-inflammatory and immunostimulating (Park et al., 2011; Yen et al., 2013). As noted earlier, pigments from algae are another valuable product extracted in the biorefinery process. In addition to the well-established commercial products, such as β-carotene from *Dunaliella,* astaxanthin from *Haematococcus* and phycocyanin from *Spirulina*, chlorophyll itself can be a valuable co-product, e.g., pheophorbide (chlorophyll with the magnesium and phytol removed) can have medicinal uses as a photosensitiser in some cancer treatments (Busch, Cengel, & Finlay, 2009).

A very good example of a microalgal biorefinery based on using brewery effluent was described by Ferreira, Ribeiro, et al. (2019). The organism utilized was *S. obliquus* and the authors aim was to produce a circular bioeconomy, which is probably most relevant in the short-term to small/medium sized enterprises such as breweries. This was a proof of concept lab-based study, which demonstrated high removal rates of nitrogen and phosphorus and lowering of the COD. The alga *S. obliquus* produced a generally favorable mix of fatty acids (C16:0, C18:1 and C18:2) when grown on brewery waste, although the overall proportion of unsaturated fatty acids was rather high at 65% (Ferreira, Ribeiro, et al., 2019). An interesting part of this study examined the effect of the algal biomass on the germination of barley and wheat seeds. A positive effect on the germination and growth of barley seedlings suggests that the *S. obliquus* biomass could be used as biofertilizer supporting the circular bioeconomy concept.

3.6 Microalgae consortia

Using microalgae in consortia with other microorganisms, such as yeast and bacteria, has potential to increase biomass production by microalgae (Donohue & Cogdell, 2006; Yao et al., 2019). In natural consortia, microorganisms display a number of interactions including symbiosis, mutualism and predator/prey. In many cases, the product of metabolism of one organism can serve as a substrate for a second organism (Padmaperuma, Kapoore, Gilmour, & Vaidyanathan, 2018). In terms of algal productivity, a yeast co-culture can produce CO_2 for photosynthetic carbon fixation in the Calvin cycle and the algae can provide the yeast with O_2. If the alga and the yeast species are both oleaginous (oil/lipid producing) then increased biomass of both species will lead to an enhanced production of lipid for biodiesel. A specific example of an algal/fungal consortium involves the lipid producing marine alga *N. oceanica* and the mycelial oleaginous soil fungus *Mortierella elongata* (Du et al., 2018). In this case, the two organisms grown separately under lipid inducing conditions (low levels of nitrogen for *N. oceanica* and seawater salinity for *M. elongata*) were then combined together to allow the fungus to capture the very small *N. oceanica* cells. The fungal/algal interaction greatly assists in the harvesting of the biomass—a very unusual type of bioflocculation (Du et al., 2018). A bioprospecting project (using freshwater from South Florida, USA in conjunction with culture collection strains) identified a species of the green algal genus *Characium* as having a high lipid productivity (Berthold, Shetty, Jayachandran, Laughinghouse, & Gantar, 2019). The authors then produced co-cultures with 39 strains of freshwater bacteria and observed that 11 bacterial species stimulated the growth of *Characium*. However, only one bacterial strain stimulated both algal biomass and lipid productivity—this bacterium was identified as *Pseudomonas composti* using 16S ribosomal DNA sequencing (Berthold et al., 2019). Utilization of O_2 by *P. composti* may well account for some of the positive effects on growth of *Characium*, but interestingly cell-free extracts of *P. composti* also had a beneficial effect on algal growth.

3.7 Harvesting

As mentioned in the pre-2007 section, harvesting has been a major problem throughout the development of microalgal biotechnology due to the small size of the cells and the relatively low maximum biomass levels reached during photoautotrophic growth (Grima et al., 2003; Vandamme, Foubert, & Muylaert, 2013). More recent work built on the methods of flotation and

flocculation to improve harvesting efficiency of dilute microalgae cultures. Coward et al. developed a novel foam flotation system, which utilized cetyl trimethylammonium bromide (CTAB) as a surfactant to enhance the production of foam (Coward, Lee, & Caldwell, 2013). The characteristics of the foam produced altered depending on the concentration of surfactant used. The authors also designed a foam column leading from the top of the bioreactor to a collection vessel and the length of this column was crucial for effective collection of the biomass in the foam. These parameters plus air flow rate, feed flow rate and sparger type were optimized in a follow-up paper, which describes a continuous harvesting version of the foam flotation system (Alkarawi, Caldwell, & Lee, 2018). The system was tested on both freshwater (*C. vulgaris*) and marine (*Isochrysis galbana* and *Tetraselmis suecica*) species of algae. The major reasons for using this system are the high efficiency of harvesting (both in terms of recovery efficiency and concentration factor) and the low level of power consumption, the latter is essential for harvesting algal biomass for biofuels in a cost effective way.

Recent work by Augustine et al. using *Parachlorella* demonstrated that magnetic particles coated with either chitosan or $FeCl_3$ were superior to the flocculants only and that as well as rapid harvesting, the remaining cell-free medium was suitable for further growth of microalgae (Augustine, Tanwar, Tremblay, & Kumar, 2019). The ability to recycle spent medium in a form that can be reused for the next batch of algal growth is another important aspect of harvesting economics.

3.8 Genetic manipulation of microalgae

Two general approaches are (a) random mutagenesis using UV or chemical mutagens to generate mutant strains that are then selected for desirable traits and (b) genetic engineering where "foreign" genes are introduced to provide a new trait or by using a gene knockout system (such as CRISPR) to delete a competing pathway. The latter option normally requires knowledge of the genome which is often not available for microalgae. The advantage of random mutagenesis is that the resulting mutants are not genetically engineered and will be more likely to be accepted as suitable organisms to grow in outdoor algal ponds. The disadvantage of random mutagenesis is that you may isolate and purify a high lipid producing mutant, but you may not know what the basis of the overproduction is without considerable extra work to identify the genes involved using, for example, RNA Seq.

A recent example of random mutagenesis that did lead to a partly characterized mutant was the study by Anthony et al. using *C. vulgaris* (Anthony et al., 2015). In this study, UV light and the chemical mutagen 5′fluorodeoxyuridine were used and several mutants were produced that had slightly decreased biomass levels, but significantly increased lipid production. In addition, for the UV induced mutants, membrane lipids (phospholipids and galactolipids) were much reduced due to degradation of the thylakoid membranes. This increase in neutral over polar lipids is beneficial for biofuel production. Nitrogen starvation increased neutral lipid production in *C. vulgaris* wild type cells, but not in the mutant cells. The degree of fatty acid unsaturation was also affected in the UV mutants, the level of polyunsaturated fatty acids is decreased, again favoring biodiesel production. The final key observations by Anthony et al. were the upregulation of the acetyl CoA carboxylase gene and downregulation of a fatty acid desaturase gene (omega-6) which appears to be a key enzyme in controlling the saturation/desaturation ratio for *C. vulgaris* fatty acids (Anthony et al., 2015).

One bottleneck in producing mutants with microalgae is the selection process. The previous study (Anthony et al., 2015) used high levels of antibiotics to select for mutants with a subsequent further selection for high lipid using the fluorescent dye Nile red. It is possible to combine these two steps using the flow cytometry technique of fluorescence activated cell sorting (FACS) in combination with Nile red to select for high neutral lipid producing mutants (Lim, Schuhmann, Sharma, & Schenk, 2015; Mendoza et al., 2008). The combination of UV or chemical mutagenesis, coupled to FACS with Nile red, is a powerful technique to isolate mutant strains synthesizing high levels of TAG (neutral lipid). A similar study used the green microalga *Chlorococcum littorale* and the high lipid producing cells were detected using FACS, but with the fluorescent dye Bodipy rather than Nile red (Cabanelas, Van Der Zwart, Kleinegris, Wijffels, & Barbosa, 2016). To optimize the FACS process, the cells were sorted five times, each time after a period of nitrogen starvation. At the end of this selection process, the TAG productivity of the selected strain (S5) was 1.9 times that of the wild type. A subsequent study using the *C. littorale* S5 mutant strain demonstrated its capacity to produce biomass and lipid on a large scale with a mixture of lab-scale experiments and indoor/outdoor simulations based on four locations around the world (Cabanelas et al., 2017). The use of modeling and validated simulations are an important step forward in assessing the viability of microalgae strains for biofuel production.

Most genetic analysis of eukaryotic algae has been carried out on the green algae genera of *Chlamydomonas* and *Chlorella* (Merchant, Kropat, Liu, Shaw, & Warakanont, 2012). However, increasing interest is evident for the eustigmatophyte algal genus *Nannochloropsis*, which contains species known to produce high levels of neutral lipids (TAG). The genomes of six species of *Nannochloropsis* were compared and only 2700 genes were shared by all 6 species, but their pan-genome contained over 38,000 genes (Wang et al., 2014). This large pan-genome may suggest that significant species-specific loss of genes has occurred within the *Nannochloropsis* genus. It is also apparent from the work of Wang et al. that the basis of high TAG production in *Nannochloropsis* depends on genes that have originated in green and red algal species that were engulfed in secondary endosymbiont events that produced eustigmatophyte algae like *Nannochloropsis*. This study is a fascinating insight into the evolution of the *Nannochloropsis* genome and will have important consequences for any attempts to genetically engineer *Nannochloropsis* species (Wang et al., 2014).

For cyanobacteria, ethanol production was significantly increased by genetically engineering *Synechocystis* by disrupting the polyhydroxybutyrate synthesis pathway to divert acetyl CoA toward ethanol production. Additionally, pyruvate decarboxylase was introduced from *Zymomonas mobilis* and an endogenous alcohol dehydrogenase was overexpressed leading to the significant increase in ethanol production (Gao et al., 2012). Using *Synechococcus elongatus,* Rubisco was overexpressed and then the genes for the four enzymes that convert pyruvate to isobutyraldehyde were genetically engineered into *S. elongatus*. Isobutyraldehyde is a precursor for isobutanol, which is a better biofuel than ethanol in terms of energy density and stability. In addition, due to a low boiling point and high vapor pressure, isobutyradehyde is much easier to harvest from the medium during growth of the cyanobacterium (Atsumi et al., 2009).

4. Future prospects

4.1 Metabolomics and synthetic biology

Genetic and/or metabolic engineering may help to overcome many of the limitations of using microalgae to produce biofuels. The previous section demonstrated some of the methods already used and in this final section very recent work will be summarized to show what may be possible in the near future. The first limitation to metabolic engineering is that a single gene modification will not normally lead to the desired increase in product

(Sun, Ren, Zhao, Ji, & Huang, 2019). There are exceptions to this rule, e.g., targeting DGAT the final enzyme in the TAG biosynthesis pathway (see Fig. 1) and overexpressing the *DGAT2* gene in *Nannochloropsis* did result in a significant increase in TAG accumulation (Li et al., 2016). This is presumably because DGAT is the rate-limiting step for TAG synthesis in *Nannochloropsis*. Knocking out a gene to block a competing pathway is another single gene event that can be effective, e.g., a knock-down of *PEPC* gene decreased the synthesis of oxaloacetate from phosphoenolpyruvate and increased the flow of carbon toward fatty acid synthesis (Sun et al., 2019). However, an exciting way forward is to use transcription factor engineering since this allows multiple parts of a pathway to be up- or down-regulated simultaneously. Currently, knowledge of microalgal transcription factors is fairly poor, but this is changing at a rapid rate (Sun et al., 2019).

Gene editing using CRISPR (clustered regularly interspaced short palindromic repeats) has been shown to increase the efficiency of editing in a range of biological systems. CRISPR systems are now being developed for cyanobacteria, e.g., *Anabaena* (Niu et al., 2019) and for eukaryotic microalgae, e.g., *P. tricornutum* (Lin, Tan, Hsiang, Sung, & Ng, 2019; Patel, Soni, et al., 2019). It can be anticipated that in the near future, gene editing using CRISPR will have a very beneficial impact on the commercial viability of producing biofuels from microalgae.

Acknowledgements

I would like to thank Tom Burns for his critical reading of the manuscript.

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