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The Microalgae – A Future Source of Biodiesel

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ABSTRACT

High energy prices, rising energy imports, concerns about petroleum supplies and greater recognition of the environmental consequences of fossil fuels have driven interest in renewable transportation biofuels. To assuage the depleting fossil reserves, environmental concerns and the rising cost of fuels in the world market, there is a spurring demand to look for sustainable, greener fuels that are economically competitive with substantial environmental benefits. At this juncture, the positive attributes of biodiesel makes it a viable alternative to the conventional, petrodiesel. As the emphasis switched to production of natural oils for biodiesel, microalgae became the exclusive focus of the research. There is a need to develop and deploy microalgae technology as it provides an exciting option for the recycling of fossil fuel emissions. Algal biodiesel opens up a promising avenue for producing several "quads" of biodiesel because microalgae generally produce more of the right kind of high density natural oils needed for the production of biodiesel. Though, a nascent field today, microalgae seem to present the only bio-solution to replace fossil fuels completely. This paper reviews the production processes of biodiesel from microalgae, procedures involved in the microalgal propagation, harvesting and extraction of oil, microalgal strain improvement and importance of manipulation of microalgal lipid composition via metabolic engineering.

Keywords: fatty acids, isolation, lipases, microalgal oil, optimization, screening, transesterification, triacylglycerol

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INTRODUCTION

Over the past few decades the need to develop alternatives to finite fossil fuel resources have resulted in the development of fuel technologies focused on the production of biodegradable, renewable and non toxic fuel (Kalscheuer *et al.* 2006; Chisti 2007; Um and Kim 2009). Biodiesel is a cleaner alternative fuel with combustion properties very similar to petroleum diesel, most often used as an additive to improve the lubricity of pure ultra low sulfur petrodiesel fuel. Comparatively, it lowers the tailpipe emissions and is one of the most realistic candidates to replace the fossil fuels (Marchetti *et al.* 2007; Chisti 2008; Rodolfi *et al.* 2008; Rosenberg *et al.* 2008). It is a fuel comprised of mono-alkyl esters of long chain fatty acids derived from biologically produced oils or fats including vegetable oils, animal fats and microalgal oils (Song *et al.* 2008). It is a green fuel, does not contribute to the CO_2 saddle and produces drastically reduced engine emissions and contributes approximately 40 to 50% of the oxygen in the atmosphere. Lapinskiene *et al.* (2006) demonstrated that in non-adapted, aerated soil biodiesel had no toxic effect up to 12% (w/w) when compared to diesel which had toxic effects at above 3% (w/w). Biodiesel produced from agricultural crops and animal fats using existing methods cannot sustainably replace fossil-based transport fuels, but there is an alternative. Oil from microalgae is an alternative to popular feedstocks, like soybean, canola, jatropha, palm, animal fats etc. Biodiesel from agricultural crops cannot significantly contribute to replacing petroleum derived liquid fuels in the fore-

Received: 11 March, 2009. Accepted: 14 June, 2010.

Review

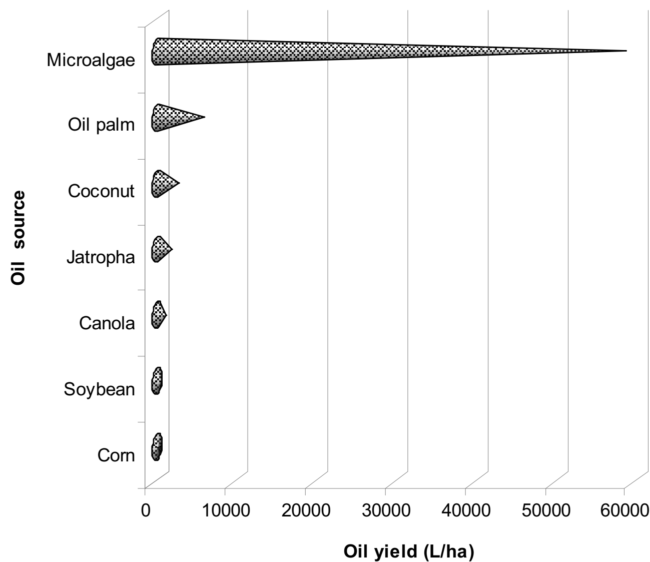

Fig. 1 Biodiesel yield in different sources.

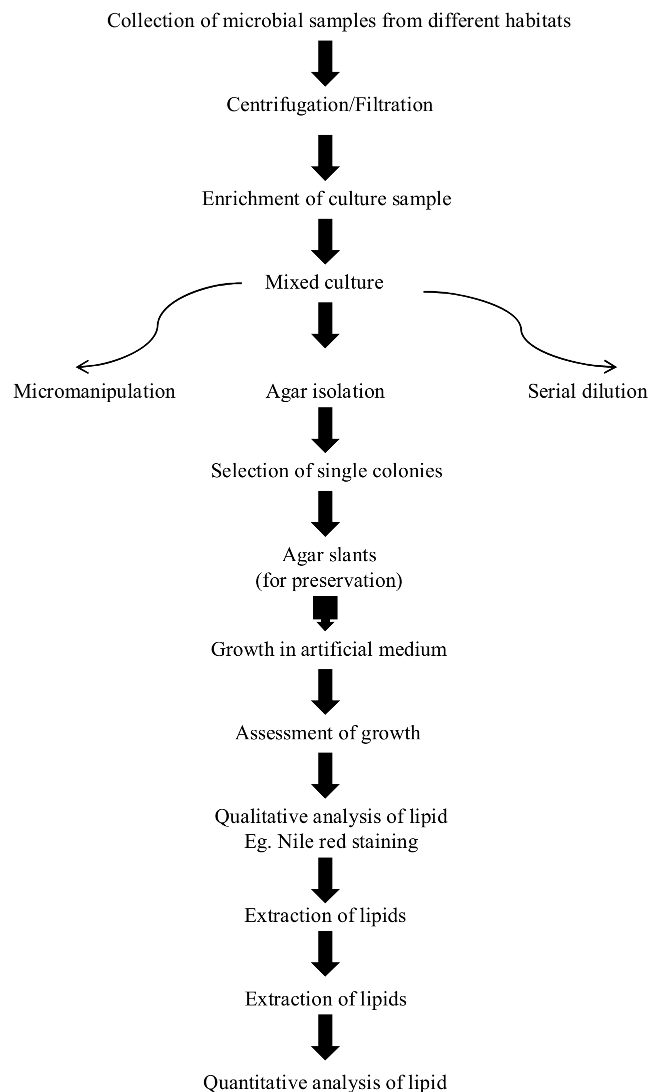

Fig. 2 Procedures involved in isolation and screening of microalgae.

seeable future. This scenario changes dramatically, if microalgae are used to produce biodiesel (**Fig. 1**). According to some estimates, the yield (per acre) of oil from microalgae is over 200 times the yield from the best-performing plant or vegetable oils and the biodiesel from algae in itself is not significantly different from biodiesel produced from vegetable or plant oils (Sheehan *et al.* 1998; Demirbas 2009). In addition, biodiesel from microalgae is better for the environment as it is made from renewable resources and has lower emissions compared to petroleum diesel (Chisti 2007). It is less toxic than table salt and biodegrades as fast as sugar. The reduced eco-toxicity and rapid biodegradation of biodiesel may be of importance in its utilization in environmentally sensitive areas; an example of its use could be that of marine fuel for boats using the Norfolk Boards, canals and waterways. As a renewable energy source to replace conventional fossil fuel, biodiesel has been becoming increasingly necessary for the global fuel market (Rodolfi *et al.* 2008; Song *et al.* 2008; Um and Kim 2009).

The global biodiesel industry has grown significantly over the past decade. Some of the main drivers behind this tremendous growth are reducing dependence on imported oil, using an environmentally friendly alternative to diesel and are reducing greenhouse gas emission. Microalgal biodiesel can be used in the existing diesel engines without (or slight) modifications and compatible with existing fuel distribution infrastructure (Du *et al.* 2008). Current economic modeling of algal biodiesel places the price of production in the range of 6.50-8.00 USD per gallon (Rosenberg *et al.* 2008). Biodiesel from microalgae seems to be the only renewable biofuel that has the potential to completely displace petroleum-derived transport fuels without adversely affecting supply of food as other crop products (Chisti 2008). A rapid expansion in microalgal biodiesel production capacity is being observed not only in developed countries such as Germany, United States, Italy and France but also in developing countries such as China, Brazil, India, Indonesia and Malaysia. Factors needed to lower algal biofuel production costs include maximizing the content of lipids and other biofuel precursors, increasing the rate of cell growth, identifying chemical inducers of these metabolic changes, and implementing multistage growth systems. The purpose of this article is to discuss the present understanding of biodiesel from microalgae in a manner that will stimulate interdisciplinary research with these microorganisms.

ISOLATION AND SCREENING OF MICROALGAE

In terms of biomass, microalgae form the world's largest group of primary producers and they occur as benthic, epithelic, symbiotic and pelagic forms (Matsunaga *et al.* 1999). The microalgal biomass contains all the essential amino acids, unsaturated fatty acids, carbohydrates, dietary fiber, and a whole range of vitamins and other bioactive compounds, so that it can be a highly suitable alternative in livestock feeding, human nutrition and perhaps also in biofuel industry (Radwan 1991; Spolaore *et al.* 2006; Del Campo *et al.* 2007; Chisti 2007). It is very important to screen microalgal strains before suitable strains can be selected for either application. Recently, microalgae have become targets for screening programmers in search of potential lipid yielders (Rodolfi *et al.* 2008). Ideally, primary screening should be rapid, inexpensive, predictive, specific, and effective for broad range lipids and applicable on a large scale. There are over 40000 species of algae already identified and many new species are yet to be identified (Hu *et al.* 2008). Among these very few were identified for their best lipid content in the cell (**Table 1**) and still more to be screened for their efficiency in lipid content.

For successful isolation of microalgae the natural environment conditions of the target species need to be determined and maintained under laboratory conditions (Matsunaga *et al.* 1999, 2007). Freshwater algae, marine algae and algae isolated from hyper-saline, thermophilic conditions are more difficult to culture due to their specific nutritional and environmental conditions, in addition terrestrial algal species are less sensitive to environmental conditions as they adapted to grow under harsh conditions (Jiménez *et al.* 2003). This makes isolating and selecting the right species for biodiesel production to be a complex process. There are

presently no established or proven technical protocols for the selection of microalgal species that would prove effective in outdoor algal mass cultures, with the goal of maximizing lipid content in the cell. **Fig. 2** illustrates a protocol for the isolation and screening of lipid producing microalgae.

Finding algae strains to grow is not too difficult but the cultivation of specific strains of algae for biodiesel could be however a bit more difficult, as they can require high maintenance and could get easily contaminated by undesirable species. It is important to have prior knowledge of the class of microalgae targeted for isolation as each class would have specific nutrient and environmental requirements that promote growth. Isolation of microalgae into culture by means of traditional methods is well established, beginning with the work of Beijerinck (1890). He was the first to isolate free-living *Chlorella* and *Scenedesmus* in allegedly pure cultures of other algae, including cyanobacteria (Beijerinck 1901) and diatoms (Lewis *et al* 1989). Many of the methods and basic culture medium concepts that are used today were developed in the early 1900s (Moore 1903; Chodat 1913; Pringsheim 1924; Bold 1942; Lewin 1959; Venkataraman 1969; Guillard 1975; Richmond 1986). So the isolation of microalgae from nature has a long history and traditional methods require skill, patience, and a good microscope. Not surprisingly, organisms from extreme envi-

Table 1 Lipid content of different microalgal species.

Microalgae	Oil content (% biomass)	Reference
Amphora coffeaformis	19.7	Renaud *et al*. 1999
Botrycoccus braunii	25-75	Chisti 2007
B. braunii	77.7	Yoo *et al*. 2010
Chaetoceros sp.	17.0	Renaud *et al*. 1999
Chaetoceros muelleri F&M-M43	33.6	Rodolfi *et al*. 2008
Chaetoceros calcitrans CS 178	39.8	Rodolfi *et al*. 2008
Chlorella sp.	28-32	Chisti 2007
Chlorella sp.	13.7	Wang *et al*. 2010
Chlorella protothecoides	57.9	Miao and Wu 2004
C. protothecoides	26.5	Wei *et al*. 2009
Chlorella sp. F&M-M48	18.7	Rodolfi *et al*. 2008
Chlorella sorokiniana IAM-212	19.3	Rodolfi *et al*. 2008
Chlorella vulgaris F&M-M49	18.4	Rodolfi *et al*. 2008
C. vulgaris CCAP 211/11b	19.2	Rodolfi *et al*. 2008
C. vulgaris	40	Illman *et al*. 2000
C. vulgaris	56.6	Liu *et al*. 2007
C. vulgaris	22.1	Sobczuk *et al*. 2008
Chlorococcum sp. UMACC 112	19.3	Rodolfi *et al*. 2008
Choricystis minor	24.4	Sobczuk *et al*. 2008
Crypthecodinium cohnii	20	Chisti 2007
Crytomonas sp.	22	Renaud *et al*. 1999
Cylindrotheca sp.	16-37	Chisti 2007
Dunaliella primolecta	23	Chisti 2007
Dunaliella	67	Takagi *et al*. 2006
Ellipsoidion sp. F&M-M31	27.4	Rodolfi *et al*. 2008
Gymnodium sp.	8-30	Mansour *et al*. 2003
Isochrysis sp.	25-33	Chisti 2007
Isochrysis sp. (NT14)	23.4	Renaud *et al*. 1999
Isochrysis sp. (T-ISO) CS 177	22.4	Rodolfi *et al*. 2008
Isochrysis sp. F&M-M37	27.4	Rodolfi *et al*. 2008
Monallanthus salina	>20	Chisti 2007
Monodus subterraneus UTEX151	16.1	Rodolfi *et al*. 2008
Nannochloris sp UTEX LB1999	34-50.9	Takagi *et al*. 2006
Nannochloropsis sp.	31-68	Chisti 2007
Nannochloropsis F&M-M26	29.6	Rodolfi *et al*. 2008
Nannochloropsis F&M-M27	24.4	Rodolfi *et al*. 2008
Nannochloropsis F&M-M24	30.9	Rodolfi *et al*. 2008
Nannochloropsis F&M-M29	21.6	Rodolfi *et al*. 2008
Nannochloropsis sp. F&M-M28	35.7	Rodolfi *et al*. 2008
Nannochloropsis CS246	29.2	Rodolfi *et al*. 2008
Neochloris oleoabundans	35-54	Chisti 2007
Nephroselmis sp.	10.5	Renaud *et al*. 1999
Nephroselmis sp.	13.8	Sobczuk *et al*. 2008
Nitzschina sp.	45-47	Chisti 2007
Nitzschia frustulum	***13.9***	Renaud *et al*. 1999
Parietochloris incise	43-77	Bigogno *et al*. 2002
Pavlova salina	50	Robert *et al*. 2009
Phaeodactylum tricornutum	20-30	Chisti 2007
Rhodomonas sp.	18.7	Renaud *et al*. 1999
Scenedesmus sp.	17.1	Yoo *et al*. 2010
Scenedesmus sp. LX1	53	Xin *et al*. 2010
Scenedesmus obliquus	61.3	Mandal and Mallick 2009
Schizochytrium sp.	50-77	Chisti 2007
Skeletonema costatum	13.5	Renaud *et al*. 1999
Skeletonema sp.	13.3	Renaud *et al*. 1999
Tetraselmis sueica	15-23	Chisti 2007
Tetraselmis sp.	12.6-13.8	Renaud *et al*. 1999

ronments and unusual habitats are less abundant in culture collections than are those from freshwater ponds, soils and coastal marine environments (Guillard 1995). Often, the first step toward successful isolation is the understanding and mimicking of the naturally occurring environmental conditions (Andersen and Kawachi 2005). For coastal marine algae, temperature and salinity are important, and for oceanic phytoplankters, water quality and metallic toxicity are additional concerns. Freshwater algae collected in non-winter months are frequently less sensitive to temperature, but pH or alkalinity may be important. Polar and snow algae are very sensitive to warmer temperatures, just as protists from hot springs or hydrothermal vents are sensitive to cooler temperatures. Algae from acid environments or hyper-saline environments require special culture media, but for terrestrial or soil microalgae, environment factors are less important. The second step toward successful isolation involves the elimination of contaminants, especially those that can out compete the target species. Techniques of dilution, single-cell isolation by micropipette, micromanipulation, and agar streaking are widely used for successful isolation.

A class of biomolecules synthesized by many species of microalgae is the neutral lipids, or triacylglycerols (TAGs) and their content in microalgae varies from organism to organism (**Table 1**). Some microalgae can produce more than 60% of their dry cell weight in the form of lipids under certain conditions (Chisti, 2007; Rodolfi *et al.* 2008; Demirbas 2009). Selecting the right starting microalgal species for high-level oil production would involve screening microalgal collections and species from nature for the best productivity characteristics which includes growth rate, oil content and fatty acid profile, robustness, resistance to invasion, and biofouling propensity, or metabolically engineering microalgal strains for enhanced lipid productivity by means of mutation and selection/screening, or by directed/rational approaches. Thus isolation, identification and screening of the best microalgal species are a crucial step in the process of biodiesel production and therefore require more attention. The dye nile red, 9-diethylamino-5H-benzo[α]phenoxazine-5-one, is an excellent vital stain for the detection of lipid droplets in microalgae by fluorescence microscopy and flow cytofluorometry (Elsey *et al.* 2007). With the help of Nile red staining we can easily screen the microalgae for the presence of lipid droplets in the cells. It is strongly fluorescent, but only in the presence of a hydrophobic environment (Greenspan 1985). Nile red can be applied to algal cells in an aqueous medium, and it does not dissolve the lipids.

INFLUENCE OF CULTURAL AND NUTRITIONAL PARAMETERS ON LIPID CONTENT OF MICROALGAE

The survival of microalgae in the laboratory, as well as in nature, depends on their ability to grow under certain chemical and physical conditions (Qin 2005). An understanding of these conditions enables us to characterize isolates and differentiate between different types of microalgae. Microalgae comprise a vast group of photosynthetic, heterotrophic organisms which have an extraordinary potential for cultivation as energy crops and the proximate chemical composition of microalgae is generally regarded as species specific and that it is usually regulated by environmental and nutritional factors (Preisig and Anderson 2005). The distribution of microalgal species depends not only on the selective action of the chemophysical environment but also on the organism's ability to colonize a particular environment (Watanabe 2002). Therefore, various culture media have been developed and used for isolation and cultivation of microalgae. Lipids and fatty acid composition in microalgae and the effects of environmental and nutritional factors requires more consideration with relation to light intensity, temperature, nitrate concentration and various other nutrients (Patil *et al.* 2008). Recently, Heredia-Arroyo *et al.* (2010) experimentally proved that different factors such as different carbon sources, carbon to nitrogen ratio, initial pH level, salinity, and rotational speed have affected the cell growth and the oil accumulation. Their experiments revealed that the heterotrophic and mixotrophic cultures of *Chlorella protothecoides* grew better than autotrophic cultures. Several stress factors were confirmed or discovered to significantly increase the lipid content of microalgae cells. The replacement of glycerol and acetate as carbon sources for microalgae cultivations provides potential for waste utilization: glycerol from biodiesel industry and acetate from biohydrogen production (Heredia-Arroyo *et al.* 2010). Culture conditions should resemble the microalgae's natural environment as far as possible; in reality many significant differences exist, most of which are deliberately imposed (Sheehan *et al.* 1998). Extensive measures must be taken to keep pure microalgal cultures chemically and biologically clean. Chemical contamination may have unquantifiable, often deleterious, and therefore undesirable effects on microalgal growth. Biological contamination of pure microalgal cultures by other eukaryotic and prokaryotic organisms in most cases invalidates experimental work, and may lead to the extinction of the desired microalgal species in culture throughout-competition or grazing. For an entirely autotrophic alga, all that is needed for growth is light, CO_2, water, nutrients and trace elements. By means of photosynthesis the microalgae will be able to synthesize all the biochemical compounds necessary for growth. Only a minority of microalgae seems, however, to be entirely autotrophic; many are unable to synthesize certain biochemical compounds and will require these to be present in the medium and this condition is known as auxotrophy (Sheehan *et al.* 1998).

Based on microalgal growth characteristics, two kinds of cultures can be defined; i) in limited volume (batch) cultures, resources are finite. When the resources present in the culture medium are abundant, growth occurs according to a sigmoid curve, but once the resources have been utilized by the cells, the cultures die unless supplied with new medium. In practice this is done by sub-culturing, i.e. transferring a small volume of existing culture to a large volume of fresh culture medium at regular intervals, ii) in continuous cultures, resources are potentially infinite: cultures are maintained at a chosen point on the growth curve by the regulated addition of fresh culture medium. In practice, a volume of fresh culture medium is added automatically at a rate proportional to the growth rate of the microalgae, while an equal volume of culture is removed.

Cultural parameters

Microalgae represent an immense range of genetic diversity and they are ubiquitously distributed throughout the biosphere and grow under the widest possible variety of conditions. They can be cultivated under aqueous conditions ranging from freshwater to situations of extreme salinity. They live in moist, black earth, in the desert sands and in all the conditions in between. Microalgae have been found living in clouds and are long known to be essential components of coral reefs. This wide span of ecological requirements plays a significant role in determining the range of metabolic products they produce. Therefore in the development of high yield lipid production process by microalgal cultures, optimization of medium components and environmental factors is vital because they can significantly affect oil yield and volumetric productivity.

Temperature

Temperature has long been known to be the primary determinant of species composition on a geographical scale, because the boundaries of biogeographical regions are associated with isotherms (Lüning 1990). The effects of temperature on the rates of biological processes are well known, but the importance of temperature in determining the occurrence of particular microalgal species is uncertain (Qin 2005). The cell yield was independent of temperature, but

the outcome of competition between species was highly dependent on temperature. The temperature at which cultures are maintained should ideally be as close as possible to the temperature at which the organisms were collected. An intermediate value of 18-20°C is most often employed. Temperature controlled incubators usually use constant temperature, although some models permit temperature cycling. In temperate regions ambient room temperature is generally acceptable for culturing purposes.

Light

The intensity, duration, and quality of light influence the dominance of algal species and the structure of algal communities (Bosence 1976; Qin 2005). Light condition, especially light intensity, is an important factor because the light energy drives photosynthesis. Typical light intensity requirements of microalgae are relatively low in comparison to higher plants (Qin 2005). Natural light is usually sufficient to maintain cultures in the laboratory. Cultures should never be exposed to direct sunlight, which may cause photopigment damage. Artificial lighting by fluorescent bulbs is often employed for culture maintenance and experimental purposes. Light intensity should range between 0.2-50% of full daylight (= 1660 $\mu E/s/m^2$), with 5-10% (*c.* 80-160 $\mu E/s/m^2$) most often employed (Qin 2005). Light intensity and quality can be manipulated with filters. Many microalgal species do not grow well under constant illumination, and hence a light/dark cycle is used.

Nutrients and other minerals

Media used for the cultivation of microalgae must supply all of the necessary nutrients required for cellular growth and maintenance of the organisms. A wide variety of culture media is employed by the phycologists for the isolation, growth and maintenance of pure cultures. A culture medium must supply suitable carbon, nitrogen and energy sources and other nutrients, sometimes including "growth factors". It is important to note that no one medium will support the growth of all microalgae. Accordingly, the elements required for the maintenance, growth and reproduction of all organisms will be used by different organisms in different ways.

Microalgal media are generally composed of three components: macronutrients, trace elements and vitamins. Macronutrients are generally considered to be nitrogen, phosphorus and silicon. However, silicon is required only for diatoms, silico flagellates and some chrysophytes (Harrison and Berges 2005). These macronutrients are generally required in a ratio of 16N:16Si:1P (Parsons *et al.* 1984; Brzezinski 1985). Most media do not balance the relative concentrations of macronutrients needed for algal growth. Nitrogen concentration of the cultural media has a strong affect on the fatty composition of microalgae (Piorreck *et al.* 1984; Li *et al.* 2005). Several popular media have nitrogen: phosphorus ratios >16:1, indicating that the phytoplankton would be phosphorus-limited in senescent phase (Berges *et al.* 2001). Unfortunately, experiments usually pay little attention to the nitrogen: phosphorus or nitrogen: silicon ratios in the medium that they are using, which will ultimately determine which nutrient limits growth and influences the chemical composition and physiological rates when the cells become senescent (Li *et al.* 2005). Similarly, carbon concentrations and carbon: nitrogen ratios are rarely considered. Inorganic (ortho) phosphate, the P form preferentially used by microalgae, is most often added to culture media, but organic (glycero) phosphate is sometimes used, particularly when precipitation of phosphate is anticipated. Most microalgae are capable of producing cell surface phosphatases which allow them to utilize this and other forms of organic phosphate as a phosphorus source.

The trace metals which are essential for microalgal growth are incorporated into essential organic molecules, particularly a variety of coenzyme factors which enter into photosynthetic reactions. Of these metals, the concentrations of Fe, Mn, Zn, Cu, Mo, Se and Co in natural waters may be limiting to algal growth. Little is known about the complex relationships between chemical speciation of metals and biological availability. It is thought that molecules which complex with metals (chelators) influence the availability of these elements. Chelators act as trace metal buffers, maintaining constant concentrations of free ionic metal. It is the free ionic metal, not the chelated metal, which influences microalgae, either as a nutrient or as a toxin. Without proper chelation some metals (such as Cu) are often present at toxic concentrations, and others (such as Fe) tend to precipitate and become unavailable to phytoplankton. In natural seawater, dissolved organic molecules (generally present at concentrations of 1-10 mg l^{-1}) act as chelators. The most widely used chelator in culture media additions is ethylenediaminetetraacetic acid (EDTA), which must be present at high concentrations since most complexes with Ca and Mg, present in large amounts in seawater. EDTA may have an additional benefit of reducing precipitation during autoclaving. High concentrations have, however, occasionally been reported to be toxic to microalgae. As an alternative the organic chelator citrate is sometimes utilised, having the advantage of being less influenced by Ca and Mg. Approximately half of all microalgal species tested have been shown to have a requirement for vitamin B_{12}, which appears to be important in transferring methyl groups and methylating toxic elements such as arsenic, mercury, tin, thallium, platinum, gold, and tellurium (Brand 1986), around 20% need thiamine, and less than 5% need biotin. No other vitamins have ever been demonstrated to be required by any photosynthetic microalgae (Harrison and Berges 2005). Heterotrophic fermentation of microalgae has been shown to accumulate high amounts of microalgal lipids, which are regarded as one of the most promising feedstocks for sustainable biodiesel production (Wei *et al.* 2009). This method has previously been used for efficient production of biomass and lipids (Muller-Feuga 2004). Under optimal conditions, microalgal populations are capable of doubling within hours and achieving high cell densities, corresponding to as much as 60 g of heterotrophic biomass per liter and 5 g of photoautotrophic biomass per liter (Muller-Feuga 2004). *Chlorella protothecoides* grown under heterotrophic conditions has shown to increase lipid production from 14.5% under autotrophic conditions to 55.2% (Miao and Wu 2006). For optimum lipid production, heterotrophic conditions may seem advisable as they produce larger quantities of lipids than microalgae grown under autotrophic conditions. Recent developments in heterotrophic cultivation and low-cost photobioreactors have provided additional economic advantages for the growth of microalgae (Chen and Chen 2006; Ugwu *et al.* 2008; Wei *et al.* 2009). Thus the lipid production potential of microalgae depends on the characteristics of the specific algal species and the cultivation strategies developed.

PRODUCTION OF MICROALGAL BIOMASS

The production of microalgal biodiesel requires large quantities of algal biomass. The only practicable methods of large-scale production of microalgae are raceway ponds (Terry and Raymond 1985; Molina Grima *et al.* 1999; Chisti 2007) and tubular photobioreactors (Molina Grima *et al.* 1999; Tredici 1999; Chisti 2007). Open pond culture is cheaper than culture in closed photobioreactors (Borowitzka 1999) but is limited to a relatively small number of algal species. Furthermore, commercial outdoor cultivation is generally restricted to tropical and subtropical zones in regions of low rainfall and low cloud cover. Although most algae require light and carbon dioxide, they are very diverse in their other environmental requirements (Borowotzka 2005). Each microalgal species has fairly specific requirements, and the various culturing systems and methods reflect this diversity (Borowotzka 2005). There is little literature on actual commercial culture systems because much of

the fine detail of culture process is commercially sensitive. The culture media used in the large-scale culture of microalgae are the same media used in the laboratory, with a few small modifications (Patil *et al.* 2008). The choice of medium used depends on several factors such as the growth requirements of the algae, how the constituents of the medium may affect lipid quality, quantity and cost.

Large-scale economical culture of microalgae in open ponds is very effective for a limited number of species. The success of such systems depends on a very good understanding of the physiology and ecology of the algae being cultured and the application of appropriate engineering principles to the design of the culture system. Although these systems have been in operation for more than 20 years, advances in the design and operation of these systems continue in light of experience gained and 'slime ranching', as it is affectionately known, continues to be a major way of producing valuable algal mass. Tridici (1999) has reviewed mass production of microalgae in photobioreactors. Many different designs of photobioreactor have been developed, but a tubular photobioreactor seems to be most satisfactory for producing microalgal biomass on the scale needed for biofuel production. Large quantities of algal biomass needed for the production of biodiesel could be grown in photobioreactors combined with photonics and biotechnologies. However, more precise economic assessments of production are necessary to establish with petroleum derived fuels. Closed, controlled, indoor algal photobioreactors driven by artificial light are already economical for special high-value products such as pharmaceuticals, which can be combined with production of biodiesel to reduce the cost.

LIPID EXTRACTION

Lipids are storage products of microalgae with high nutritional value, and their synthesis and accumulation by microalgae is a principle source of energy as they supply essential polyunsaturated fatty acids. Furthermore, microalgal lipids have been suggested as a potential diesel fuel substitute with an emphasis on the neutral lipids due to their lower degree of unsaturation and their accumulation in microalgal cells at the end of growth stage for biodiesel (Casadevall *et al.* 1985; McGinnis *et al.* 1997; Song *et al.* 2008). Extraction of lipids from microalgae is one of the more costly and debated processes involved in biodiesel production.

Very few reports are available in the lipid literature dealing with the topic of lipid extraction in detail presumably due to the fact that the methodology is tedious and lacks interest *per se*. Rapid determination of lipid content in microalgae is critical in order to find the feasibility of further using a specific microalgal strain for lipid production for biodiesel production (Chen *et al.* 2009). A wide range of lipid extraction methods are available and the choice of each method is based on efficiency, accuracy, cost-effectiveness, easy to carry out, high throughput capability, robustness and most importantly precision and reproducibility. Widely reported methods for the extraction of lipids from microalgal cells include the following; Folch method, gravimetric method, and Bligh and Dyer method. Two conventional methods that are frequently used by many lipid analysts involve solvent extraction and gravimetric determination (Chen *et al.* 2009). After separation of neutral lipids from the crude material, other analytical methods can be employed for quantification of the lipids and these include TLC, HPLC and GC. According to Chen *et al.* (2009), the method used for lipid analysis must ensure complete lipid extraction while avoiding decomposition and/ or oxidation of the lipid components. However, one major limitation of the conventional procedure is that it is time and labour intensive and therefore difficult to screen large number of algal samples. The main drawback of the conventional gravimetric method for lipid determination is that it involves several complicated steps such as biomass harvesting, lipid extraction, separation and concentration which can result in loss of some lipids (Elsey *et al.* 2007). Hence in order to get around this drawback, increasing attention is focused on *in situ* measurements of the lipid contents.

Nile red (NR) staining and time-domain nuclear magnetic resonance (TD-NMR) methods have been investigated for the quantification of lipid content in microalgae (Gao *et al.* 2008) since they are rapid, simple and feasible as compared to gravimetric methods. One major drawback of the NR staining method is that a number of green algae which produce lipids cannot be detected by this method because of the structure and composition of the thick and rigid cell walls which prevent the dye from penetrating the cell wall (Chen *et al.* 2009). Another disadvantage of the NR method is that it cannot detect neutral lipids in dead cells therefore restricting its use only to microorganisms that are alive. This can cause problems in the screening process whereby lipid producers in the *Chlorophyta* cannot be easily detected by the NR staining procedure. In confirming the effectiveness of the NR staining procedure for the determination of lipid content in algae from different taxonomic classes, Chen *et al.* (2009), reported that high fluorescence intensities in some algal species investigated is indicative of the neutral lipid content. These workers demonstrated that the green algal strains that showed weak fluorescence using the NR test were in fact high lipid producers. The conventional gravimetric methods was used and showed that the lipid content of these algal species ranged from 30.9 to 51.5% on a dry weight basis clearly showing the ineffectiveness of the NR method for some green algal species. However it has been demonstrated that pre-treating the green algal cells with DMSO (25% v/v) remarkably enhanced the detection of neutral lipids by the NR test.

Samorì *et al.* (2010) proposed a new procedure to extract hydrocarbons from dried and water-suspended samples of the microalga *Botryococcus braunii* by using switchable-polarity solvents (SPS) based on 1,8-diazabicyclo-[5.4.0]-undec-7-ene (DBU) and an alcohol. The high affinity of the non-ionic form of DBU/alcohol SPS towards non-polar compounds was exploited to extract hydrocarbons from algae, while the ionic character of the DBU-alkyl carbonate form, obtained by the addition of CO_2, was used to recover hydrocarbons from the SPS. DBU/octanol and DBU/ethanol SPS were tested for the extraction efficiency of lipids from freeze-dried *B. braunii* samples and compared with *n*-hexane and chloroform/methanol. The DBU/octanol system was further evaluated for the extraction of hydrocarbons directly from algal culture samples. DBU/octanol exhibited the highest yields of extracted hydrocarbons from both freeze-dried and liquid algal samples (16 and 8.2% respectively against 7.8 and 5.6% with *n*-hexane) (Samorì *et al.* 2010).

TRANSESTERIFICATION OF FATTY ACIDS (CHEMICAL CONVERSION OF BIODIESEL)

Biodiesel is produced through the transesterification of fats or oils which are subjected to a reaction with alcohol in the presence of a catalyst. The process removes roughly 10% of the original weight of the oil as glycerin which is the backbone of the triglyceride molecule (Meher *et al.* 2006). Triglycerides are the primary components of oil or fat. Different types of fats and oils may be used either alone or combined to produce biodiesel. This process has been used to reduce the high viscosity of triglycerides. The transesterification reaction is represented by the general equation as **Fig. 3**. It is one of the reversible reactions and proceeds essentially by mixing the reactants. However, the presence of a catalyst (a strong acid or base) accelerates the conversion. Normally, transesterification reaction will proceed either exceedingly slowly or not at all. Heat, as well as an acid or base are used to help the reaction proceed more quickly. It is important to note that the acid or base are not consumed by the transesterification reaction, thus they are not reactants but catalysts (Ma and Hanna 1999).

Transesterification of triglycerides produce fatty acid

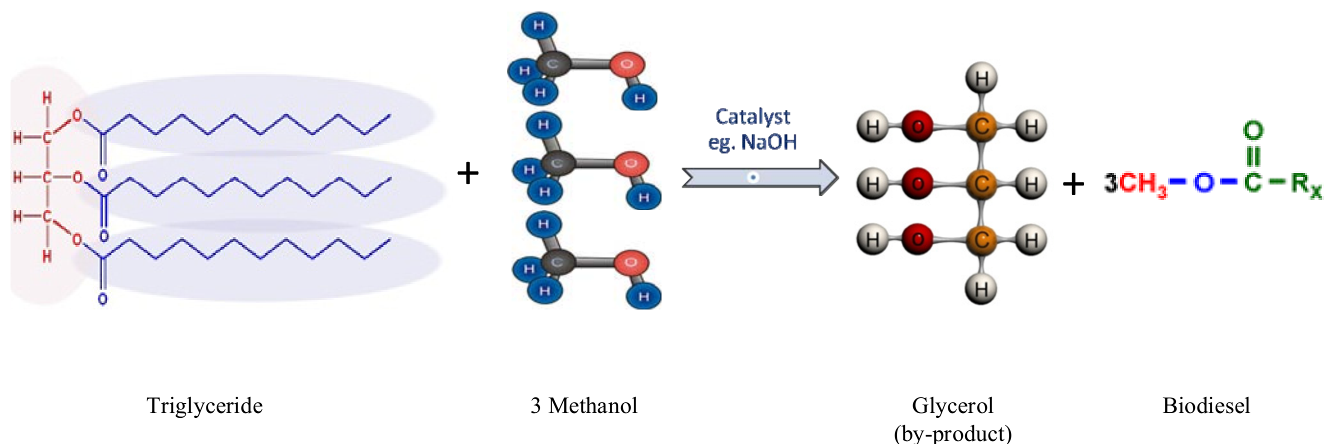

Fig. 3 Base-catalyzed transesterification of a triacylglyceride with alcohol.

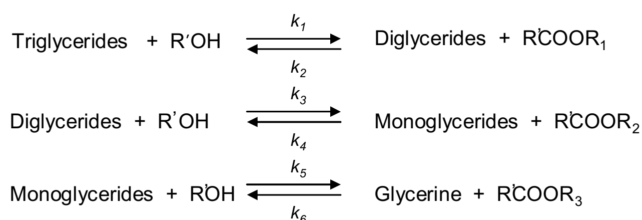

Fig. 4 Common equation for transesterification of triglycerides.

alkyl esters and glycerol. Diglycerides and monoglycerides are the intermediates in this process and the mechanism of the overall process is normally a sequence of three consecutive steps, which are reversible reactions and in all these reactions esters are produced (**Fig. 4**). The first step involves the attack of the alkoxide ion to the carbonyl carbon of the triglyceride molecule, which results in the formation of a tetrahedral intermediate. The reaction of this intermediate with an alcohol produces the alkoxide ion in the second step (**Fig. 4**). In the last step the rearrangement of the tetrahedral intermediate gives rise to an ester and a diglyceride (Ma and Hanna 1999). The process of transesterification is affected by various factors depending upon the reaction condition used (Meher *et al.* 2006). The free fatty acid and moisture content, catalyst type and concentration, molar ration of alcohol to oil and type of alcohol, reaction time and temperature, mixing intensity and organic co-solvents are key parameters for determining the viability of the oil transesterification process. As per the reported literature, most of the transesterification studies have been done on edible oils by using methanol and NaOH/KOH as catalyst (Meher *et al.* 2006). There are very few studies reported on oils which are produced by microalgae (Miao and Wu, 2006; Song *et al.* 2008). This transesterification process can be further improved to get good quality of biodiesel from the lipids of microalgae. Recently Umdu *et al.* (2009) present the activities of Al_2O_3 supported CaO and MgO catalysts in the transesterification of lipid of yellow green microalgae, *Nannochloropsis oculata*, as a function of methanol amount and the CaO and MgO loadings at 50°C. They found that pure CaO and MgO were not active and CaO/Al_2O_3 catalyst among all the mixed oxide catalysts showed the highest activity. They also proved that, not only the basic site density but also the basic strength is important to achieve the high biodiesel yield. In their study, biodiesel yield over 80 wt.% CaO/Al_2O_3 catalyst increased to 97.5 from 23% when the methanol/lipid molar ratio was 30. The quality of biodiesel is most important for engine part of view and various standards have been specified to check the quality. As per the analytical method reported in literature, high performance liquid chromatography (HPLC) is suitable to analyze the reaction intermediates and products of transesterification reaction. Recently, enzymatic approaches for biodiesel production have received much attention since these have many advantages over chemical methods: moderate reaction conditions, lower alcohol to oil ration, easier product recovery and environmental friendly (Shimada *et al.* 1999; Fukuda *et al.* 2001; Wardle 2003; Du *et al.* 2004, 2008).

ENZYMATIC PRODUCTION OF BIODIESEL

Enzymatic production of biodiesel has gained a lot of attention recently because of the feasibility of the production process and that the process is amenable to scale up to industrial level. In order to come out with a high quality biodiesel product, it is important to start off with substrates of high standard such as lipids from microalgae. The enzyme reaction conditions favouring a high yield of biodiesel need to be established through optimization experiments.

Biocatalysts for biodiesel synthesis

Lipases are enzymes that cleave the ester bonds of triacylglycerol to glycerol and fatty acids. However, lipases can also catalyse the reverse reaction in a low water environment (Sharma *et al.* 2001). Furthermore, lipases can also be used as biocatalysts both in aqueous and non-aqueous environments (Ma and Hanna 1999). Both hydrolysis and esterification can occur concurrently in a process termed interesterification (Sharma *et al.* 2001). Biodiesel production using lipases is possible but not very cost effective and a lot of research work is needed to find out optimal conditions for maximal yield of biodiesel. However this application is in its infancy and there are few reports on the use of lipases for biodiesel production (Du *et al.* 2008). The main raw materials for biodiesel production include plant oils, microbial oils and waste fats from various sources and these have been reviewed extensively elsewhere (Ma and Hanna 1999; Antczak 2009; Murugesan *et al.* 2009).

Reaction mechanism for biodiesel production

The production of biodiesel by transesterification employing alkali catalysts has been industrially accepted for its high conversion and reaction rates (Ranganathan *et al.* 2008). Depending on the substrates used, lipases can catalyze acidolysis (where an acyl moiety is displaced between an acyl glycerol and a carboxylic acid), alcoholysis (where an acyl moiety is displaced between an acyl glycerol and an alcohol), and transesterification (where two acyl moieties are exchanged between two acylglycerols) (Balcão *et al.* 1996). **Fig. 5** shows a schematic illustration of the transesterification process and downstream processing for the production of biodiesel.

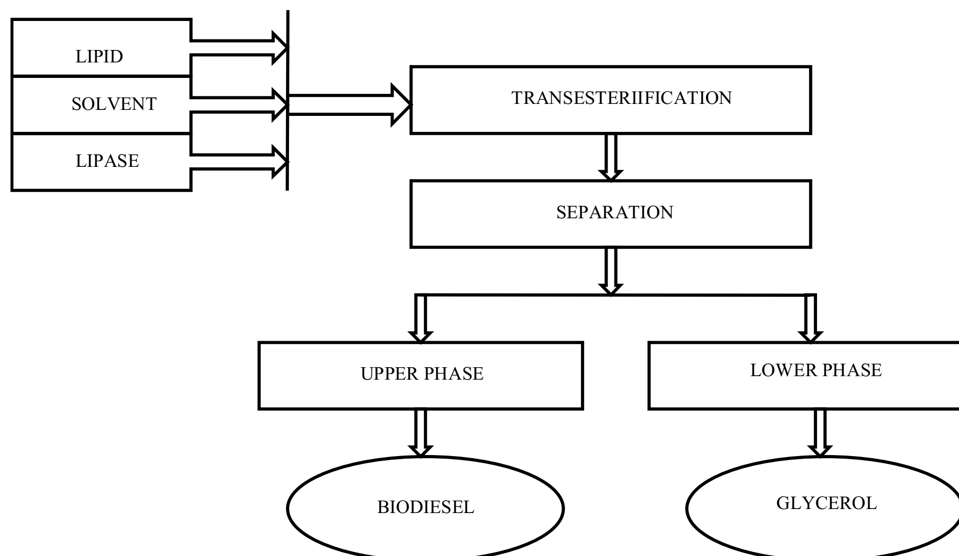

Fig. 5 A schematic representation of the sequence for the enzymatic production of biodiesel.

Parameters affecting biodiesel productivity

Enzymatic synthesis of biodiesel is affected by crucial parameters such as pH, enzyme choice and stability, temperature, water activity, choice of substrate, molar ratio of substrates, *inter alia* (Antczak *et al.* 2009). These authors give a detailed analysis and discussion of the interplay between lipase catalysed reactions carried out in non-aqueous systems and the yield of biodiesel.

Sources and distribution of lipases

Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) are produced by microorganisms (fungi and bacteria), animals and plants (Antczak *et al.* 2009). **Table 2** shows the main sources of lipases used for the commercial production of biodiesel. Commercial preparation of lipases is mainly by submerged fermentation of microbial cells under optimal conditions. The main advantage of the microbial source of lipases is due to their low cost of production and easy modification of properties (Antczak *et al.* 2009). Both extracellular and intracellular lipases have been used for biodiesel production but the extracellular enzymes have been reported to be widely used because of their easy preparation (Ranganathan *et al.* 2008). However, whole microbial cells can also be used for biodiesel production and they are reported to be cheaper and more robust and therefore more suitable for industrial biodiesel production (Antczak *et al.* 2009). Only a few lipases are reported for efficient biodiesel production both in organic solvent and solvent free systems (**Table 2**).

Costs and feasibility

The main advantage of using lipases in oleochemical processing is that it saves energy and minimizes thermal degradation during alcoholysis, acidolysis and glycerolysis (Sharma *et al.* 2001). Moreover, enzymatic transesterification has attracted much attention for biodiesel production recently because it produces high purity product and allows easy downstream processing and separation from the by-product, glycerol (Dizge and Keskinler 2008; Ranganathan *et al.* 2008). The main drawback for the large scale production of biodiesel is the cost of the enzyme and this can be solved by reusing the enzyme by immobilizing the biocatalyst on suitable biomass support particles and this has resulted in considerable increase in efficiency (Ranganathan *et al.* 2008). Another limitation of this process is that the activity of the immobilised enzyme is inhibited by methanol and glycerol which are present in the reaction mixture. In order to increase the cost effectiveness of the process, *tert*-butanol can be used as a solvent, continuous removal of glycerol and stepwise addition of methanol can significantly reduce the inhibitory effects.

GENETIC ENGINEERING

Throughout the past few decades obtaining large quantities of algal biomass has been achieved, but to obtain large amounts of lipids is no easy task. The key to unlocking the maximum oil producing capacity of algae may be through genetic and metabolic engineering and it has gained considerable importance in recent decades (Brown *et al.* 1993;

Table 2 Sources of lipases for biodiesel production in (A) organic solvents and (B) solvent free systems (B).

	References
(A) Sources of lipases for biodiesel production in organic solvents	
Pseudomonas fluorescens	Dossat *et al*. 1999; Iso *et al*. 2001; Soumanou *et al*. 2003
Pseudomonas cepacia	Nelson *et al*. 1996; Soumanou *et al*. 2003; Noureddini *et al*. 2005
Candida Antarctica, *Rhizopus delemar*, *Mucor miehei*, *Geotrichum candidum*, *Candida rugosa*	Nelson *et al*. 1996
Rhizopus oryzae	Ma *et al*. 2002; Ghamgui *et al*. 2004
(B) Sources of lipases that can be used for efficient biodiesel production in solvent free systems	
Pseudomonas fluorescens	Dossat *et al*. 1999; Iso *et al*. 2001
Candida antarctica	Watanabe *et al*. 2002; Nelson *et al*. 1996; Xu *et al.* 2003
Candida rugosa	Chowdary and Prapulla 2002; Shimada *et al*. 2002
Rhizopus oryzae	Ghamgui *et al*. 2004
Mucor miehei	Nelson *et al*. 1996
Rhizomucor miehei	Soumanou *et al*. 2003
Thermomyces lanuginosa	Iso *et al*. 2001; Du *et al*. 2003; Soumanou *et al*. 2003; Xu *et al*. 2003
Aspergillus niger	Haas *et al*. 2002

Chisti 2008; Meng *et al.* 2009). Although the application of genetic engineering to improve energy production phenotypes in eukaryotic microalgae is in its infancy, significant advances in the development of genetic manipulation tools have recently been achieved with microalgal model systems and are being used to manipulate central carbon metabolism in these organisms (Radakovits *et al.* 2010). It is likely that many of these advances can be extended to industrially relevant organisms. Considering the enormous biodiversity of microalgae and their importance in biodiesel production, genetic and metabolic engineering are becoming highly favored in the biodiesel research division. Before genetic engineering can occur, one needs to understand the functioning of the cellular pathways and the microalgal metabolism. Some of the microalgal genomes have been sequenced such as the diatoms *Thalassiosira pseudonana* and *Phaeodactylum tricornutum* and this aids in understanding the functional genes and the genome structure (Ambrust 2004). To date there are over 20 cyanobacterial genomes that have been sequenced, and 30 more are in progress (Hu *et al.* 2008); however, many species still need to be sequenced (especially those who can produce significant quantities of lipids for storage) which will allow for accurate gene manipulation and cloning.

Molecular studies focusing on the enzymatic change in the cell when exposed to chemical inducers will help to optimize growth conditions and hence maximize oil production by steering the synthesis to produce preferred substrates (Rosenberg *et al.* 2008). By understanding the key proteins in the cellular pathways the exact molecular responses that follow nitrogen starvation and the responses allowing cell growth may be the key to producing large oil supplies and large amounts of biodiesel. *Chlamydomonas* has been studied extensively regarding the biochemical pathways and the recent sequencing has shown that its ability to deal with various environmental conditions shows adjustment of the metabolism which has shown to be an interest for many research groups (Hu *et al.* 2008). *Chlamydomonas* metabolism has shown the ability to have its metabolism manipulated by being exposed to stressful conditions such as nutrient stress (Hu *et al.* 2008). Research on the change in protein levels in the biochemical pathways may show certain genes that have a functional role that has not been determined yet or not been associated with lipid storage or production. By focusing on these genes and genetically engineering the genome the algae of choice can be designed to produce a high oil yield.

Genetic and metabolic engineering involves the altering of genetic material to change its structure and characteristics. This is achieved by many processes such as the insertion of a transposon linked to various promoter and regulatory regions, insertion of recombinant DNA or site-directed mutagenesis (Meng *et al.* 2009). In algae, transformation can occur at the chloroplast and nuclear level. Stable recombination of the foreign DNA has proven to be problematic and by knowing the exact gene sequence, precise insertion of the recombinant DNA will be possible. This known insertion may help to eliminate the problems associated with genetic transformation such as little or no gene and protein expression and even gene silencing (nuclear transformation) of other genes due to unfavourable insertion of the transgene (León-Banares *et al.* 2004).

Some algae species have being successfully transformed with foreign DNA. The US Department of Energy's Aquatic Species programme (ASP) was the first to isolate the Acetyl Co-A Carboxylase (ACCase) enzyme and gene and to transform it into diatoms. Here it was over expressed to produce more of the protein. This protein catalyzes the first step in the synthesis of fatty acids and could therefore be advantageous once over expressed, resulting in more fatty acids being produced in a shorter time period (Sasaki and Nagano 2004). The construct, however, did not deliver a higher lipid production resulting in more research being needed (Sheehan *et al.* 1998). This transformation was the first to show the world that genetic engineering may prove to be a useful tool in engineering the 'perfect' algae and will be improved over time with more research.

Other genetic engineering techniques have proven useful in algae manipulation. To date, gene silencing has been successful in knocking out or silencing certain genes in diatoms (Kroth 2007). RNAi or antisense RNA expression knocks out the target mRNA and the respective gene therefore altering the phenotype. The Light Harvesting Antenna (LHA) protein complex has been down regulated via gene silencing (Mussgnug *et al.* 2007). This causes the thylakoids to be stacked less tightly and less solar energy to be absorbed by the cell. This reduces the reactive oxygen species which result when the photosynthesis system is overwhelmed by the light intensity and therefore allows for less stress on the algae. Because less solar energy is able to be absorbed, more solar energy is available to the same area and will have a greater light penetration in liquid culture (Rosenberg *et al.* 2008). Gene silencing can have a negative aspect too as it can occur when transgenes are inserted. The insertion position may interrupt the transcriptional regulators therefore hindering effective transcription (León-Banares *et al.* 2004).

This gene silencing technique was successful to down regulate the LHA complex producing the desired phenotypic effect, however, many other similar gene silencing processes could be achieved in many algal species if the genomic sequence was known and the metabolism was fully understood. By having the genome sequence, one can limit unwanted gene silencing from DNA insertion as the transcriptional regulatory elements will not be interrupted if the regulatory elements were identified in the sequence and were not spliced. This again illustrates the importance of algae genome sequencing.

Collectively, the progress in identifying relevant bioenergy genes and pathways in microalgae, and powerful genetic techniques have been developed to engineer some strains via the targeted disruption of endogenous genes and/or transgene expression has been realized in these areas is rapidly advancing our ability to genetically optimize the production of targeted biofuels (Beer *et al.* 2009).

By understanding the proteins and their interactions involved in the metabolic processes occurring in an algal cell and by having the genome sequence, one would be able to precisely manipulate the functional genes involved to optimize lipid production and indirectly optimize biodiesel production.

CONCLUSIONS AND FUTURE PROSPECTS

While microalgal oil certainly appears promising, it should be pointed out that a lot more input is needed to be analysed and further experimentations done before one can be sure of algal oil being a worthy large-scale substitute for petrodiesel. In theory, microalgae have the potential to be a major fuel resource. In practice, however, there are many questions to be answered and multiple issues to be resolved before biodiesel can be produced sustainably and affordably on a large-scale, from microalgae. In order for these questions to be answered and the issues resolved, we feel that a significant amount of research needs to be undertaken, possibly with a lot more attention from the major governments of the world. We might require a massive, focussed effort. There are no signs of such a massive amount of research being done in the field of oil from microalgae, even though one would imagine that the need for a suitable alternative energy source is a critical problem that needs to be faced. For sure, research is being conducted by some brilliant and devoted scientists, but we fear that these will not be enough and more research is needed to future biodiesel from microalgae.

ACKNOWLEDGEMENTS

The authors are thankful to the National Research Foundation (NRF), South Africa and Durban Ethekwini Municipality and Durban University of Technology for their kind encouragement and support.

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