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Microalgae lipid research, past, present: A critical review for biodiesel production, in the future

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Abstract

Biodiesel has received much attention in recent years. The motivation for renewable fuel is not just energy independence but also mitigation of global climate disruption, which has been called "the deficiency challenge of our era" Energy, is essential and vital for development, and the global economy literally runs on energy. Although numerous reports are available on the production of biodiesel from vegetable oils of terrestrial oil-plants, such as soybean, sunflower and palm oils the use of fossil fuels as energy now widely accepted as unsustainable due to depleting resources and also due to the accumulation of green house gases in the environment. Biodiesel demand is constantly increasing as the reservoir of fossil fuel are depleting. Unfortunately biodiesel production from oil crop, waste cooking oil and animal fats are not able to replace fossil fuel. The viability of the first generation biofuel production is however questionable because of the conflict with food supply. The manuscript is an attempt to review the potential of microalgae in lipid production and provides a brief summary of genes which enhances the lipid synthesis and biomass productivity of different species of micro algae.

Keywords: Biodiesel, Fossile fuel, Lipid, Microalgae, Triacylglycerols

INTRODUCTION

Production of biodiesel from micro algae is a newly emerging field and appears to be a potential alternative. Microalgae represent an exceptionally diverse but highly specialized group of micro organisms adapted to various ecological habitats. Micro algal biotechnology appears to possess high potential for biodiesel production because a significant increase in lipid content of microalgae is now possible through genetic engineering approaches. Microalgae are photosynthetic microorganism which converts sunlight, water and Co_2 to sugars, from which macromolecules such as lipids and triacylglycerides (TAGs) can be obtained. The TAGs are the promising and sustainable feedstock for biodiesel production. Many micro algae have the ability to produce substantial amounts (20-50%) of triacylglycerols (TAGs) as a storage lipid under photo-oxidative stress or other adverse environmental conditions. However, the expression of genes involved in fatty acid synthesis is poorly understood in micro algae.

TRENDS: BIOMASS TO BIODIESEL

Bio-diesel, primarily rapeseed methyl ester, has been in commercial use as an alternative fuel since 1988 in many European countries, including France, Austria, Germany, Sweden, Italy, Belgium, Hungary and Czech Republic (Connemann, 1977; Staat and Vallet, 1994; Ahn *et al.* 1995; Cvengros and Povazanec, 1996). German bio-diesel standard DIN V 51606 requires a rapeseed ester to have density at 15˚C between 0.875 and 0.900 g/ml, viscosity at 15˚C between 0.875 and 0.900 g/ml, viscosity at 15˚C between 3.5 and 5.0 mm^2/s (cSt), acid number below 0.5 mg KOH/g and iodine value less than 115g iodine/100g, among many other specifications. A 90% tax deduction proposed by the European Community for the use of bio-diesel enables it to be sold in Europe at prices competitive to conventional diesel fuel. The total European production of bio-diesel for 1997 was estimated at 660,000 tons. In the United States, Bio-diesel, primarily made from soybean oils, also is being commercialized. Several demonstration programs use bio-diesel to fuel municipal buses, trucks and construction equipment (Tickell and Tickell, 1999). In Canada, the Canadian Renewable Fuels Association (CRFA), whose members including the Saskatchewan Canola Development Commission and the Ontario Soyabean Growers Marketing Board, promotes renewable bio-fuels (ethanol, bio-diesel) for automotive transportation through consumer awareness and government liaison activities. However, the suggested 20% bio-diesel blended diesel fuel currently costs 10cents more per blended liter than the conventional diesel due to high feed cost of vegetable oil, thereby preventing its large-scale use in North America.

Much work has been done in the past two decades on the production and real-world market performance of bio-diesel, which has been reviewed recently by Knothe *et al.* (1997). The most commonly used alcohol for transesterification is methanol because of its lower price than that of other lower alkanols. Rapeseed, canola, sunflower, soybean oils, beef tallow and many other oils have been used for the production of bio-diesel esters (Freedman and Pryde, 1982; Hassett and Hasan, 1982; Kusy, 1982; Freedman *et al.* 1986; Shay, 1993; Peterson *et al.* 1996, 1997; Ma and Hanna, 1999). Peterson *et al.* (1997) reported detailed batch transesterificaion

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procedures for methyl and esters of four lipids using KOH catalyst at room temperature. Use of absolute ethanol was found essential to produce ethyl esters successfully, which was characterized by the production of two liquid phases: ester and denser glycerol phases at the end of the reaction. The specific gravities of the eight bio-diesel esters were 0.87-0.88, while the viscosities were 3.9-6.2 cSt at 40˚C. Ahn *et al.* (1995) carried out methyl ester transesterification of rapessed oil in two steps and achieved 99% yield. During the soybean methyl ester preparation, Boocock *et al.* (1996, 1998) added tetrahydrofuran as a co-solvent. The co-solvent transformed the soybean oil/methanol two-phase system into a one-phase system. As a result of improved mass transfer between the reactants, the methanolysis process was rapid and was completed in a few minutes. Lee *et al.* (1995) prepared esters of vegetable oils and animal fats with C_3 - C_5 branched-chain alcohols, and examined their low temperature properties by using differential scanning calorimetry. Compared with the common methyl esters, branched-chain esters greatly reduced the crystallization onset temperature of neat esters and ester diesel fuel blends. They found that 2-prophy and 2-butyl esters of soybean oil crystallized 7-11˚C and 2-14˚C lower, respectively, than the soy methyl esters. Sodium alkoxides, which were prepared by dissolving metal sodium in anhydrous alcohol, acted as the catalytic species for the transesterification with branched-chain alcohol. A considerably high alcohol-to-oil ratio of 66:1 was used for the preparation of 2-propyl and 2-butyl esters.

Often, the vegetable oils of choice for bio-diesel production are those which occur abundantly in the region of testing. Therefore, rapeseed oil is the primary bio-diesel source in Europe whiles in the United States both rapeseed and soybean oils are used.

Rapid Global market growth

The global market for biodiesel is has shown an insatiable appetite in the recent past (Fig. 1) and poised for explosive growth in the next ten years. Although Europe currently represents 80% of global biodiesel consumption and production, the U.S. is now ramping up production at a faster rate than Europe, and Brazil is expected to surpass U.S. and European biodiesel production by the year 2015. It is possible that Biodiesel could represent as much as 20% of all on-road diesel used in Brazil, Europe, China and India by the year 2020 with the pursuit of second generation, non-food feedstocks. Biodiesel demand and over-capacity in Europe, the US and Asia is driving investment in the global trade of alternative feedstocks.

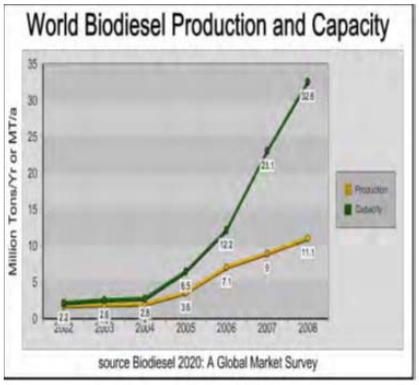


Fig:1 Biodiesel 2020: Global market survey, feed stock trends and forecasts, multi-client study, [Susanna Retka Schi, (2008)].

Microalgae taxonomic characterictics and cultivation

Taxonomic characteristics of Biodiesel Producing Microalgae:

Dunaliella: Domain: Eukaryota, Kingdom: Viridiplantae, Phylum: Chlorophyta, Class: Chlorophyceae, Order: Chlamydomonadales, Family: Dunaliellaceae, Genus: ***Dunaliella,*** species: *D.acidophila, D.bardawil, D.bioculata, D.lateralis, D.maritima, D.tertiolecta* is a marine green flagellate with a cell size of 10-12 µm. This strain is reported to have oil yield of about 37% (organic basis). *D. tertiolecta* is a fast growing strain and that means it has a high CO_2 sequestration rate as well.

Nannochloropsis: Domain: Eukaryota, Kingdom: Chromalveolata, Phylum: Heterokontophyta, Class: Eustig matophyceae, Genus: ***Nannochloropsis,*** *Nannochloropsis* is a genus of alga comprising approximately 6 species, *N. gaditana*, *N. granulate*, *N. limnetica*, *N. oceanic*, *N. oculata*, *N. salina*. The genus in the current taxonomic classification was first termed by Hibberd (1981). The species have mostly been known from the marine environment but also occur in fresh and brackish water. All of the species are small, nonmotile spheres which do not express any distinct morphological features, and cannot be distinguished by either light or electron microscopy. The characterization is mostly done by rbcL gene and 18S rDNA sequence analysis. It is different from others related microalgae in that they lacks chlorophyll b and c. *Nanochloropsis* is able to build up a high concentration of a range of pigment such as taxanthin, zeaxanthin and canthaxanthin. The algae have a very simple ultrastructure, compared to neighbouring taxa. They have a diameter of about 2 micrometers. It is considered a promising alga for industrial applications because of its ability to accumulate high levels of polyunsaturated fatty acids. It is mainly used as an energy-rich food source for fish larvae and rotifers.

Botryococcus braunii: Kingdom: Plantae, Division: Chlorophyta, Class: Chlorophyceae, Order: Chlorococcales, Family: Dictyosphaeriaceae, Genus: Botryococcus, Species: ***Botryococcus braunii***, *Botryococcus braunii* (Bb) is a green, pyramid shaped planktonic microalga of the order Chlorococcales (class Chlorophyceae) that is of potentially great importance in the field of biotechnology. Colonies held together by a lipid biofilm matrix can be found in temperate or tropical oligotrophic lakes and estuaries, and will bloom when in the presence of elevated levels of dissolved inorganic phosphorus. The species is notable for its ability to produce high amounts of hydrocarbons, especially oils in the form of Triterpenes, that are typically around 30-40 percent of their dry weight. Compared to other green algae species it has a relatively thick cell wall that is accumulated from previous cellular divisions; making extraction of cytoplasmic components rather difficult. Fortunately, much of the useful hydrocarbon oil is outside of the cell.

Dunaliella salina: Kingdom: Plantae, Phylum: Chlorophyta, Class: Chlorophyceae, Order: Volvocales, Family: Dunaliellaceae, Genus: Dunaliella, Species: ***Dunaliella salina***, *Dunaliella salina* is a type of halophile pink micro-algae especially found in sea salt fields. Known for its anti-oxidant activity because of its ability to create large amount of carotenoids, it is used in cosmetics and dietary supplements. Few organisms can survive in such highly saline conditions as salt evaporation ponds. To survive, these organisms have high concentrations of β-carotene to protect against the intense light, and high concentrations of glycerol to provide protection against osmotic pressure. This offers an opportunity for commercial biological production of these substances.

Isochrysis: Kingdom: Chromalyeolata, Phylum: Haptophyta, Class: Prymnesioophyceae, Order: Isochrysidales, Family:

Isochrysidaceae, Genus: Isochrysis, Species: *I. galbana*. *Isochrysis galbana* is a microalgae. It is the type species of the genus Isochrysis. It is an outstanding food for various bivalve larvae and is now widely cultured for use in the bivalve aquaculture industry.

Phaeodactylum: Kingdom: Chromalveolata, Phylum: Heterokontophyta, Class: Bacillariophyceae, Order: Naviculales, Family: Phaeodactylaceae, Genus: *Phaeodactylum,* Species: *P. tricornutum.* It is a diatom. It is the only species in the genus Phaeodactylum. Unlike other diatoms *P. tricornutum* can exist in different morphotypes (fusiform, triradiate, and oval), and changes in cell shape can be stimulated by environmental conditions. This feature can be used to explore the molecular basis of cell shape control and morphogenesis. Furthermore *P. tricornutum* can grow in the absence of silicon, and the biogenesis of silicified frustules is facultative, thereby providing opportunities for experimental exploration of silicon-based nanofabrication in diatoms.

Cultivation:

Heterotrophic cultivation of microalgae offers several advantages over phototrophic cultivation including elimination of light requirement, good control of the cultivation process, and low-cost for harvesting the biomass because of higher cell density obtained in heterotrophic culture of microalgae Chen Johns (1991). In heterotrophic culture, both cell growth and biosynthesis of products are significantly influenced by medium nutrients and environmental factors. Carbon sources are the most important element for heterotrophic culture of microalgae in the production of lipids. For example, although the green microalgae *C. protethecoides* can grow photoautotrophically or heterotrophically. Heterotrophic growth of *C. protothecoides* using acetate, glucose, or other organic compounds as carbon source results in much higher biomass as well as lipid content in cells Wu Qy *et al.* (1994). Liu *et al.* (1999) compared several carbon sources and concluded that glucose was preferred. In order to lower the production cost of microalgal oils as biodiesel, cheaper carbon sources should be considered. For instance, corn powder hudrolysate (CPH) or molasses instead of glucose may be used as organic carbon source in heterotrophic culture. It was reported occasionally CPH was superior to glucose solution, because CPH contained some beneficial components to *Chlorella*, and as a result, *C. protothecoides* produced 55.2% crude lipids in the cells with a cell dry weight concentration of 15.5gL-1 Xu *et al.* (2006).

Salinity, pH, and dissolved O2 are also important factors affecting the heterotrophic cultivation of microalgae Jiang , Chen (2000) Chen (1991). Besides, it has been demonstrated that different cultivation modes greatly affect the lipid accumulation in microalgae. Heterotrophically grown microalgae usually accumulate more lipids than those cultivated photoautotrophically Miao, Wu (2006). Some phototrophic microorganisms could also be grown on cheap organic substrates heterotrophically Chen (1996). Evidences has shown that the “dark metabolism” of photosynthetic plants and microalgae is similar to that of non-photosynthetic organisms such as yeasts.

Two microalgae speicies (*Scenedesmus obliquus* and *Neochloris oleobundans*) were cultivated in closed sleeve photobioreactors in order to select the best oil producer for further large-scale open raceway pond cultivations, aiming at biofuel production. *Scenedesmus obliquus* reached a higher maximum biomass concentration (1.41 g 1-1) with a lower lipid content (12.8% w/w), as compared to *N. oleabundans* [maximum biomass concentration of 0.92 g 1-1 with 16.5% (w/w) lipid content]. Both microalgae showed adequate fatty acid composition and iodine values as substitutes for diesel fuel. Based on these results, *N. oleoabundans* was selected for further open raceway pond cultivations. Under these conditions, *N. oleobundans* reached a maximum biomass concentration of 2.8 g 1-1 with 11% (w/w) of lipid content. A high correlation between the Nile Read fluorescence intensity measured by flow cytometry and total lipid content assayed by the traditional gravimetric lipid analysis was found for both microalgae, making this method a suitable and quick technique for the screening of microalgae strains for lipid production and optimization of biofuel production bioprocesses. Medium growth optimization for enhancement of microalgal oil production is now in progress by Silva *et al.* (2002).

Nutrition and Nutrients

Heterotrophic cultivation of microalgae for lipids production:

Although microalgae can utilize light efficiently, phototropic growth of microalgae is often of because of light limitation at high cell densities on a large scale Wen Zy *et al.* (2003) or “photoinhibition” due to excessive light, especially in sunny days Myres, Buurr Go (1940). In view of these disadvantages associated with photoautotrophic cultivation, heterotrophic growth of microalgae in conventional fermentors should be favorably considered Chen (1996).

Microalgal lipids are the oils of future for sustainable biodiesel production. However, relatively high production cost due to low lipid productivity have been one of the major obstacles impeding their commercial production. They studied the effects of nitrogen sources and their concentrations on cell growth and lipid accumulation of *Neochloris oleoabundans*, one of the most promising oil-rich microalgal species. While the highest lipid cell content of 0.40 g/g was obtained at the lowest sodium nitrate concentration (3 mM), a remarkable lipid productivity of 0.133 g1^{-1} day^{-1} was achieved at 5 mM with a lipid cell content of 0.34 g/g and a biomass productivity of 0.40 g 1^{-1} day^{-1}. The highest biomass productivity was obtained at 10 mM sodium nitrate, with a biomass concentration of 3.2 g/l and a biomass productivity of 0.63 g1^{-1} day^{-1}. It was observed that cell growth continued after the exhaustion of external nitrogen pool, hypothetically supported by the consumption of intracellular nitrogen pools such as chlorophyll molecules. The relationship among nitrate depletion, cell growth, lipid cell content, and cell chlorophyll content by Yanqun Li *et al.* (2008).

It was also reported that *Prophyridium cruentum* might double its total lipid content (mainly neutral lipids) under nitrogen starvation Becker (1994). Nevertheless, nitrogen starvation might not always result in an increase in total lipid content in microalgae but a change in lipid composition. Zhila *et al.* (2005) reported that the algae *Botryococcus braunii* contained high content (28.4-38.4%) of oleic acid under nitrogen limitation, but the content of total lipids and triacylglycerols did not change. It is also noticeable that the overall rates of oil production might be lower in the case of nutrient deficiency due to the overall lower biomass achieved. Therefore factors other than nitrogen should be considered altogether. Initial carbon to nitrogen (C/N) ratio in the medium has significant impact on the biosynthesis of lipids in microalgae. With the addition of glucose as organic carbon source to the medium and the tremendous decrease of nitrogen source in the medium, a crude lipid content up to 55.2% was achieved in heterotrophic *C. protothecoides* Miao, Wu (2004). Chen and Johns (1991) also investigated the effect of C/N ratio and aeration on the fatty acid composition of

heterotrophic *Chlorella sorokiniana*. When C/N ratio of approximately 20, cell lipid content was at a minimum and increased at both higher and lower C/N values. Environment factors also cannot be neglected in the growth of microalgae and the formation of fatty acids. The high PUFAs content at low temperature might be explained by the fact that the algae need to produce more PUFAs to maintain cell membrane fluidity. Another reason might be that low temperature could lead to high level of intracellular molecular oxygen and hence improves the activities of the desaturase and elongase involved in the biosynthesis of PUFAs Jiang, Chen (2000). However, the effect of temperature on cell growth and PUFAs production may not be always the same as mentioned above Wen *et al.*

The effects of silicon deficiency on the metabolism and composition of lipids in *Cyclotella cyptica* T13L were examined and showed that silicon-de-ficient cells had higher levels of neutral lipids (primarily triacylglycerols) and higher proportions of saturated and monounsaturated fatty acids than silicon-replete cells. After 4h of silicon-de-ficiency, the percentage of newly assimilated NaH14Co3 partiotioned into lipids increased from 27.6% to 54.1%, whereas the percentage partiotioned into chrysolaminarin decreased from 21.6% to 10.6%. In addition, pulse-chase experiments with NaH14CO3 indicated that the amount of 14ºC in the total cellular lipid fraction increased by 32% after 12h of silicon deficiency despite the absence of additional photoassimilable 14ºC. Therefore, the accumulation of lipids in response to silicon deficiency appears to be due to two distinct processes: (1) an increase in the proportion of newly assimilated carbon partitioned into lipids, and (2) a slow conversion of previously assimilated carbon from non-lipid compounds into lipids by Roessler *et al.* (1988).

The growth, dry weight, fatty acid weight and fatty acid composition of two clones of *Thalassiosira pseudonana* were measured under several growth conditions. Determinations of total cellular fatty acids were made using chemical ionization mass spectrometry. Both clones had the same fatty acids, dominated by C14:0, C16:0, C16:1, C16:3, C16:4, C18:4 and C20:5, though sented 5-10% the dry weight of a cell during log phase growth and up to 22% during stationary phase. The C16 fatty acids of both clones changed as the cultures aged, though much more markedly in the Sargasso Sea clone (13.1) than in the estuarine one (3H). The C16:0 and C16:1 acids of both clones declined sharply in the dark and were replenished in the light. Cells maintained in constant illumination, but with no cell division, produced large quantities of these acids. Cells of clone 13-1 treated with polychlorinated biphenyl (PCB) initially grew more slowly than control cells, weighed more, and had higher relative amounts of C16:0 and C16:1. Fatty acid studies may provide useful indicators of ecologically important energy reserves and membrane adaptations in the algae by Nicholas Fisher *et al.* (1978).

Photobioreactors and Microalgal Cultures

Microalge can transform carbon dioxide from the air and light energy through photosynthesis to various forms of chemical energies such as polysaccharides, proteins, lipids and hydrocarbons. Compared to higher plants, microalgae have a number of advantages including higher photosynthetic efficiency and growth rate Christ (2007). In phototrophic culture, usually microalgae can be grown in two systems such as open ponds and enclosed photobiorectors. Enclosed photobioreactor systems is more suitable for some micro algae which are readily contaminated by other microbes, except for some special microalgae which can survive well in extreme environments such as high pH (e.g., *Spirulina*) and high salinity (e.g., *Dunaliella*) or can grow very rapidly (e.g., Chlorella) in the open pond. Because of better environmental control, enclosed photobioreactor system has been suggested for the production of high-value long-chain fatty acids (e.g., DHA, EPA). Nevertheless, due to the high cost in terms of operation and capital investment and the small scale due to the complexity of bioreactor design compared to open pond system, it might not be economical to produce biodiesel on a large scale by enclosed photoreactors. Open pond system is perhaps more suitable for cultivating microalgae for biodiesel because of its relatively cheap operating cost compared to the enclosed photobioreactors. The basic requirements for microalgal phototrophic growth should include carbon dioxide, other macro-and micro-nutrients, as well as light by Guanhua Huang *et al.* (2010).

While many microalgae strains naturally have high lipid content (ca. 20–50% dry weight), it is possible to increase the concentration by optimizing the growth determining factors such as the control of nitrogen level Hu *et al.* (2008) Widjaja *et al.* (2009), light intensity Qin *et al.* (2005), temperature Qin (2005), salinity Wu, Hsieh (2008), CO2 concentration *Chiu et al.* (2009) and harvesting procedure Widjaja *et al.* (2009). However, increasing lipid accumulation will not result in increased lipid productivity as biomass productivity and lipid accumulation are not necessarily correlated Rodolfi *et al.* (2008). Lipid accumulation refers to increased concentration of lipids within the micro algae cells without consideration of the overall biomass production. Lipid productivity takes into account both the lipid concentration with in cells and the biomass produced by these cells and is therefore a more useful indicator of the potential costs of liquid biofuel production.

High-density fermentation of microalga *Chlorella protethecoides* in bioreactor for bio-diesel production reported by Wei Xiong, *et al.* (2008) Algal-fermentation-based bio-diesel production was realized through high-cell density fermentation of *Chlorella protothecoides* and efficient transesterification process. Cell density achieved was 16.8 g1^{-1} in 184 h and 51.2 g1^{-1} in 167 h in a 5-1 bioreactor by performing preliminary and improved fed-batch culture strategy, respectively. The lipid content was 57.8, 55.2, and 50.3% of cell dry weight from batch culture strategy, respectively. The lipid content was 57.8, 55.2, and 50.3% of cell dry weight from batch, primary, and improved fed-batch culture in 5-1 bioreactor. Transesterification was catalyzed by immobilized lipase, and the conversion rate reached up to 98%. The properties of biodiesel from *Chlorella* were comparable to conventional diesel fuel and comply with US standard for Biodiesel. In a word, the approach including high-density fermentation of *Chlorella* and enzymatic transesterification process were set up and proved to be a promising alternative for biodiesel production by Liam Brennan *et al.*

Microalga *Scenedesmus obliquus* as a potential source for biodiesel production reported by Shoveon Mandal *et al.* (1992). Biodiesel form microalgae seem to be the only renewable biofuel that has the potential to completely replace the petroleum-derived transport fuels. Therfore, improving lipid content of completely replace the petroleum-derived transport fuels. Therefore, improving lipid content of microalgal strains could be a cost-effective second generation feedstock for biodiesel production.

Lipid accumulation in *Scenedesmus obliquus* was studied under various culture conditions. The most significant increase in lipid reached 43% of dry cell weight (dcw), which was recorded under N-deficiency (against 12.7% under control condition). Under P-deficiency and thiosulphate supplementation the lipid content also increased up to 30% (dcw). Application of response surface

methodology in combination with central composite rotary design (CCRD) resulted in a lipid yield of 61.3% (against 58.3% obtained experimentally) at 0.04, 0.03, and 1.0 g1-1 of nitrate, phosphate, and sodium thiosulphate, respectively for time culture of 8 days. *Scenedesmus* cells pre-growth in glucose (1.5%)-supplemented N 11 medium when subjected to the above optimized condition, the lipid accumulation was boosted up to 2.16 g 1-1, the value ≈ 40-fold higher with respect to the control condition. The presence of palmitate and palmitate and oleate as the major constituents makes *S. obliquus* biomass a suitable feedstock for biodiesel production.

Microalgae are a diverse group of prokaryotic and eukaryotic photosynthetic microorganisms that grow rapidly due to their simple structure. They can potentially be employed for the production of biofuels in an economically effective and environmentally sustainable manner. Microalgae have been investigated for the production of a number of different biofuels including biodiesel, bio-oil, bio-syngas, and bio-hydrogen. The production of these biofuels can be coupled with flue gas co2 mitigation, wastewater treatment, and the production of high-value chemicals. Microalgal farming can also be carried out with seawater using marine processing (e.g., harvesting, drying, and thermochemcial processing) are expected to further enhance the cost effectiveness of the biofuel from microalgae strategy by Yanqun Li (2008).

Biochemistry of Important Biodiesel Producing Microalgae

The Biochemical here refers to the strategy of enhancing lipid production of microalgae by controlling the nutritional or cultivation conditions (e.g., temperature, pH, and salinity) to channel metabolic flux generated in photobiosynthesis into lipid biosynthesis of microalgae. Nitrogen-starvation has been observed to lead to lipid accumulation in a number of microalgal species. For instance, *Chlorella* usually accumulates starch as storage material. However, it was observed by Illman *et al.* (2000) that *C. emersonii, C. minutissima, C. vulgaris* and *C. pyrenoidosa* could accumulate lipids of upto 63%, 57%, 40%, and 23% of their cells on a dry weight basis, respectively, in low-N medium. Cells started to accumulate lipid when light had good penetration (at low cell density), when individual cells were exposed to a large quantity of light energy, resulting in more metabolic flux generated from photosynthesis to be channeled to lipid accumulation on an unit biomass basis. In addition to nutrient-starvation, other stress conditions may also cause enhanced accumulation of lipids in microalgae. An inherited disadvantage of the biochemical is however, nutrient-starvation or the physiological stress required for accumulating high lipid content in cells is associated with reduced cell division (Ratledge, 2002). The overall lipid productivity is the product of cell lipid content multiplied by biomass productivity.

Algae vary from small and single-celled to complex multicellular species. Algae that can only be seen by microscope is called microalgae. Algae contains, 60~70% Protein, 3 times higher than meat/fish. A complete protein contains all essential amino acid. High content of linoleic acid, a member of an essential fatty acid called omega-6-fatty acids. High cellulose level, 8~12%. All essential vitamins, especially beta-carotene (2 times higher than carrot!) and vitamin B complex. Iron content is 50 times as spinach. Calcium content is 5 times as milk. Also contains trace elements K, Mg, Mn, and I. Pigments such as chlorophyll and phycocyanin. Extract contains more than 15 kinds of Carotenoids and vitamin E.

Fatty acids composition of 10 species of microalgae was determined at the exponential phase and the stationary phase. The microalgae consist of two species of diatoms, *Bacillariophyceae,* (*Nitzschia cf. ovalis, Thalassiosira sp.*) five species of green microalgae, Prasinophyceae (*Tetraselmis sp.*) and Chlorphyceae, (*Dictyosphaerium pulchellum, Stichococcus sp., Chlorella sp., Scenedesmus falcatus*) and three species of blue green microalgae, Cyanophyceae (*Anacystis sp., Synechococcus sp., Synechocystis sp.*). G-11 media was used for Cyanophyceae by Jarunan pratoomyot *et al.*

The microalgae were cultured beneath light intensity 143 μE$m^{-2}s^{-1}$, light: dark illustration 12:12 hrs., temperature 28°C, and salinities 8-30 psu. The microalgae were harvested for analyzing fatty acid by centrifugal machine at 3500 rpm for 5 min at temperature 20°C and stored at -80°C prior to analysis. Fatty acids composition of microalgae differed from species to species. The majority fatty acids composition of diatoms at the exponential phase and the stationary phase were C16:1n-7 (17.12-31.47% and 28.22-42.02%), C16:0 (13.25-19.61% and 18.83-20.67%), C20:5 n-3 (16.65-26.67% and 11.32-23.68%) respectively. The principle fatty acids composition of green microalgae, Prasiophyceae, *Tetraselmis sp.* were C18:3n-3 (16.17-16.67%), C16:0 (15.33-17.45%), C18:1n-9 (12.25-15.43%), C18:2n-6 (9.66-19.97%). The fatty acids composition of green microalgae Chlorphyceae, were C18:3 n-3 (20.02-26.49% and 15.35-30.63%), C16:0 (5.76-17.61% and 11.41-20.03%), C18:2n-6 (4.67-17.54% and 7.48-20.61%) respectively. The major amounts of fatty acids content of blue green microalgae were C16:1n-7 (9.28-34.91% and 34.48-35.04%), C14:0 (13.34-25.96% and 26.69-28.24%), C16:0 (5.89-29.15% and 5.70-16.81%) except for *Anacystis sp.* which had high amount of C18:3 n-3 (23.18-27.98%) but low amount of C14:0 (3.66-4.98%). Bacillariopyceae contained the highest amount of highest amount of highly unsaturated fatty acids (HUFAs) at both growth phases. Prasinophyceae had a small amount while in Chlorophyceae and Cyanophyceae they were not detected. *Nitzschia cf. ovalis* and *Thalassiosira sp.* Had amount of C20:4n-6 (0.084-40%), C20:5n-3 (11.32-26.67%) and C22:6 n-3 (0.80-4.20%) respectively. *Tetraselmis sp.* Had amounts of C20:4n-6 and C20:5 n-3 ranging from 0.99-1.13% and 4.18-4.70% respectively. In conclusion, *Nitzschia cf. ovalis* and *Thalassiosira sp.* would serve as good nutritional sources of HUFAs for aquaculture animals by Siti Aleha Hamid *et al.* (2008).

The biochemical composition and fatty acid content of twelve strains of filamentous, heterocystous, nitrogen-fixing cyanobacteria have been determined. When grown under dizotrophic conditions, protein, carbohydrate, lipid, and nuclic acids comprised 37-52%, 16-38%, 8-13%, and 8-11% of the dry weight, respectively. The presence of a combined nitrogen source resulted in an increase in the protein content of the cells and a decrease in the levels of lipids and carbohydrates, although biomass productivity was not affected significantly. Biochemical composition also changed during culture growth, with the highest levels of proteins and lipids occurring as the culture entered stationary phase, whereas the highest levels of carbohydrate and nucleic acids were found during the exponential phase. Total fatty acids levels in the strains assayed ranged between 3 and 5.7 % of the dry weight. With regard to fatty acid composition, all strains showed high levels of ployunsaturated fatty acuds (PUFAs) and saturated fatty acids (SAFAs), with values of 24-45% and 31-52% of total fatty acids (MUFAs) were in general lower (11-32%). Palmitic acid (16:0) was the most prevalent SAFA, whereas palmitoleic (16:1n-7) and oleic acid (18:1n-9) were the most abundant MUFAs in all the strains. Among PUFAs, £-linolenic acid

(GLA, 18:3n-6) was present at high levels (18% of total fatty acids) in Nostoc sp. (Chile) and at lower levels (3.6% of total fatty acids) in Anabaenopsis sp. The presence of GLA has not been previously reported in these genera of cyanobacteria. The rest of the strains exhibited high levels (12-35% of total fatty acids) of α-linolenic acid (ALA, 18:3n-3). Linoleic acid (18:2n-6) was also present at a substantial level in most of the strains. Eicosapentaenoic acid (EPA, 20:5n-3) was also detected in *Nostoc* sp. (Albufera). Some filamentous nitrogen-fixing cyanobacteria therefore represent potential sources of commercially interesting fatty acids by Vargas *et al.* (1998).

Lipid profiles of microalgae

A comparative study of lipid content and composition in three microalgae viz., *Anabaena doliolum, Anacystics nidulans* and *Chlorella vulgaris* has been made. Lipid content was highest in the alga *C. vulgaris* and lesser in blue-green algae *A. nidulans* and *A. doliolum*. The chief lipids of the blue-green were glycolipids whereas those of *C. vulgaris* comprised glycolipids as well as significant amounts of neutral and phospholipids. Monogalatosyl diglyceride (MGDG) is the principal class of lipid in the three algae. Digalactosyl diglyceride (DGDG) content of *C. vulgaris* was higher than in blue-green,where as the sulfoquinovosyl diglyceride (SQDG) content of the blue -green algae were higher than in the green alga. Phosphatidyl glycerol (PG) was highest in *A. nidulans*, followed by *A. doliolum* and *C. vulgaris*. The fatty acids of the three alge showed characteristic differences. The ratio of unsaturated to saturated fatty acids increased from unicellular to filamentous blue-green and the green alga. Fatty acid composition of Antarctic cyanobacteria reported by Benjamin Pushparaj, et.al., (2008) The 31 Antarctic cyanobacterial strains, object of this study, were isolated from south polar sediments, soil and lichen associations and belong to the genera of *Cyanothece, Chondrocystis, Leptolyngbya, Pseudophormidium, Phormidium, Hormosicilla* and *Nostoc*. They are maintained in the Culture Collection of the Istituto per lo Studio degli Ecosistemi (ISE-CNR, Florence) by Rathore *et al.* (1993).

The fatty acid composition varied among the strains, with relatively high quantities of polyunsaturated fatty acids. Arachidonic acid was found in two strains of *Phormidium pseudopristleyi* at 24% and 32% of the total fatty acid content. The total lipid content and the C/N ratio varied among strains from 13% to 9% and 3.7 to 11.2. The diversity of saturated and unsaturated fatty acid composition among the Antarctic strains and their possible application as nutrient supplements. The monocarboxyclic fatty acids and hydroxyl fatty acids of three species of freshwater microalgae-*Vischeria punctata Vischer, Vischeria Helvetica (Vischer* et Pascher) Taylor, and Eustigmatos vischeri (Hulbert) Taylor, all from the class Eustigmatophyceae-were examined. Each species displayed a very similar distribution of fatty acids, the most abundatnt of which were 20:5n-3, 16:0, and 16;1n-7; C18 polyunsaturated fatty acids were minor components. These fatty acids were minor components. These fatty acid distributions closely resemble those found in marine eustigmatophytes but are quite distinct from those found in most other algal classes. These microalgae also contain long-chain saturated and unsaturated monohydroxy fatty acids. Two distinct types of hydroxyl fatty acids were found: a series of saturated α-hydroxy acids ranging from C24 to C30 with a shorter series of monounsaturated α-hydroxy acids ranging from C26 to C30 together with a series of saturated ß-hydroxy acids ranging from C26 to C30. The latter have not previously been reported in either marine or freshwater microalgae, although C30 to C34 midchain (*w*-18)-hydroxy fatty acids have been identified in hydrolyzed extracts from marine eustigmatophytes of the genus *Nannochlorposis,* and C22 to C26 saturated and monounsaturated α-hydroxy fatty acids have been found in three marine chlorophytes. These findings have provided a more complete picture of the lipid distributions within this little studied group of microalgae as well as a range of unusual compounds that might prove useful chemotaxonomic markers. The functions of the hydroxyl fatty acids are not known, but a link to the formation of the lipids precursors of highly aliphatic biopolymers is suggested.

They studied to characterize the fatty acid profiles of some planktonic and benthic marine dinoflagellates from Malaysian waters. Clonal batch cultures of Alexandrium affine, *A. leei, A. minum, A. tamarense, A.tamiydvanichii,Coolia monotis, Prorocentrum emarginatum, P.mexicanum, Ostreopisis ovata* and *Amphidinium sp.* They have harvested at late exponential phase, and total lipid was extracted. Samples were derivatized to produce fatty acid methyl esters (FAMEs). FAMEs were analyzed on a gas chromatograph with frame ionization detection. The total number of fatty acids detected in the clones ranged from 10 in the *A. tamarense* AtPA04 clone to 22 in the *C.monotis* CmPL01 clone. Fatty acids found in all clones were myristic acid (14.0), palmitic acid (16:0), stearic acid (18.0), linoleic acid (18:2*w*9c) and oleic acid (18:1*w*9c). In all clones only a few fatty acids were dominant. In the *Alexandrium* clones the dominant fatty acids were 16:0, 18:0, cis-13, 16-docosadienoic acid (22:2), 18:2*w*6c and 18:1*w*9c. There was almost complete absence of omega-3 polyunsaturated fatty acids (PUFA) in the *Alexandrium* clones. In the benthic species the major fatty acids were 16:0, 18:0, cis-13, 16-docosadienoic acid (22:2), 18:1*w*9c. There was almost complete absence of omega-3 polyunsaturated fatty acids (PUFA) in the *Alexandrium* clones. In the benthic species the major fatty acids were 16:0, eicosapentaenoic acid (EPA, 20:5*w*3), docosahexaenoic acid (22:6*w*3), 18:2*w*6c and 18:1*w*9c. In the prorocentrum clones the major fatty acids were 14:0, 16:0, palmitoleic acid (16:1) and EPA. Total PUFA content in the benthic species were 37%-56%, while in the planktonic species the content was 19%-44%. The fatty acid profiles could not differentiate between species. However, cluster analysis and principal components analysis were able to clearly discriminate between the *Alexandrium* group, *Prorocentrum* group and benthic species group by Siti Aleha Hamid *et al.* (2008).

The lipid composition of a planktonic prokaryote, *Prochlorothrix hollandica* Burger-Wiersma, isolated from Lake Loosdrecht (The Netherlands) has been determined. This species is only the second prokaryote that has been found to contain chlorophylls a and b. Its lipid composition is similar to that of another prochlorphyte, *Prochloron didemni* Lewin, as well as to some cyano bacteria and bacteria, but there are also some unique features. Major fatty acids were 14:0, 14:11w5, 16:0,16:1w7 and two novel fatty acids 16:1w12 (hexadec-4-enoic acid) and a new 16:2 isomer. Double bond positions in monounsaturated fatty acids and alkenes were determined by derivation with dimethyl disulfide followed by analysis of the products by gas chromatography-mass spectrometry. Hydrocarbons consisted mainly of the pentacyclic triterpene hop-22(29)-ene andstraight-chain n-heptadecane and n-heptadec-5-ene. The presence of hopanoids, low abundance of triacylglycerols and absence of sterols clearly show that the lipid biochemistry of this organism is more closely related to that of prokaryotes than to eukaryotes even though it contains chlorophyll b which is more typical of green algae by John Volkman *et al.* (1988).

The relationship of lipids with light and chlorophyll measurements in freshwater algae and periphyton observed by Guillermo Napoliano (2004). Lipid content and lipid class composition were determined in stream periphyton and filamentous green algae *Cladophora sp*. and *Spirogyra sp*. Sterols and phospholipids were compared to chlorophyll a (chl a) as predictors of biomass for stream periphyton and algae. Sterols can be quantified rapidly from a few milligrams of algae and appear to be a useful predictor of eukaryote biomass, whereas cellular levels of chl a vary substantially with light conditions. Phospholipds (or phospholipids fatty acids) are considered to be reliable measure of viable microbial biomass. Nevertheless, phospholipids content varied substantially and unpredictably among algae and periphyton under different light regimes. Irradiance also had a significant effect on storage lipids: HL(High Light) *Cladophora* and HL *periphyton* had 2 × and 5 × greater concentrations of triacylglycerols, respectively, compared to their LL (Low Light) forms. HL and LL algae also differed in the concentration of several major fatty acids. These light-induced changes in algal lipids and fatty acids have important implications for grazers. Regulation of fatty acid composition by irradiance level in the eustigmatophyte *Nannochloropsis sp*. reported by Assaf Sukenik *et al.* (1989).

Chemical composition and quantitative cytological measurements were determined for the eustigmatophyte *Nannochloropsis sp*. Cultures were grown in turbidostats at three irradiance levels, growth-limited light, growth-satphyll a content decreased as irradiance level increased, concomitant with a disproportionate reduction in carotenoid content. *Nannochloropsis sp*. Grown in saturating light was characterized by a high content of lipid, fatty acids and carbohydrate compared with cells grown in light-limiting conditions. The increase in cellular lipid content coincided with a reduction in the percentage of eicosapentaenoic acid (C20:5) and arachidonic acid (C20:4), fatty acids that are mainly associated with galactolipids, and with an increase in the relative abundance of palmitic acid (C16:0) and palmitic acid (C16:0) and palmitoleic acid (C:16:1). At growth-limiting light conditions, *Nannochloropisis sp.* preferentially synthesized galactolipids; however, as growth became light saturated, relatively more neutral lipids, mainly triacylglycerols, were synthesized. Changes in lipid content and composition were qualitatively related to changes in cell morphology. Cells grown under low light conditions were characterized by a large relative volume of chloroplast, high surface density of thylakoid membranes and low relative volume of lipid storage bodies. The physiological implications of the change in cellular lipid composition and ultrastructure are discussed in relation to light/shade adaptation.

Carotenoid composition and spectroscopic characteristics were analyzed for *Pterosperma cristatum* of green algae. This alga contained a substantial amount of carotenoid esters, siphonaxanthin C14:1 trans-2 ester and 6'-OH siphonaxanthin C14:1 trans-2 ester, but lacked lutein. This is the first report of carotenoid C14:1 trans-2 esters from phototropic organisms. In vivo absorption spectra and excitation spectra of the cells revealed that these carotenoids absorbed blue-green light and could transfer energy to chl a. These carotenoids were concluded to function as antenna pigments in *P. cristatum* by Yukie yoshii *et al.* (2002).

The chlorarachniophyceae are unicellular eukaryotic algae characterized by an amoeboid morphology that may be the result of secondary endo symbiosis of a green alga by a nonphotosynthetic amoeba or amoeboflagellate. Whereas much is known about the phylogeny of chlorarachniophytes, little is known about their physiology, particularly that of their lipids. In an initial effort to characterize the lipids of this algal class, four organisms from three genera were examined for their fatty acid and sterol composition by Leblond *et al.* (2009).

Fatty acids from lipid fractions containing chloroplast-associated glycolipids, storage triglycerides, and cytoplasmic membrane-associated polar lipids were characterized. Glycolipid-associated fatty acids were of limited composition, principally eicospentaenoic acid [20:5(n-3)] and hexadecanoic acid (16.0). Triglyceride-associated fatty acids, although minor, were found to be similar in composition. The polar lipid fraction was dominated by lipids that did not contain phosphorus and had a more variable fatty acid composition with 16:0 and docosapentaenoic acid [22:5 (n-3)] dominant along with a number of minor C18 and C20 fatty acids. Crinosterol and one of the epimeric pair poriferasterol/stigmasterol were the sole sterols. Several genes required for synthesis of these sterols were computationally identified in *Bigelowiella natans* Moestrup. One sterol biosynthesis gene showed the greatest similarity to Smt1 of the green alga, *Chlamydomonas reinhardtii*. However, homologues to other species, mostly green plant species, were also found. Further, they discussed the method used for identification suggested that the sequences were transferred to a genetic compartment other than the likely original location, the nucleomorph nucleus.

The internal lipid,carotenoid, and toxin concentrations of Karenia brevis *et al.* (2009) are influence by its ability to use ambient light and nutrients for growth and reproduction. They reported the changes in *K. brevis* toxicity, lipid class, and carotenoid concentrations in low-light, nitrate-replete (250 µmol quanta m-2. s-1 , 8oµM No3); and high-light, nitrate-reduced (960 µmol quanta m-2. s-1,<5 µM No3) mesocosms. Reverse-phase HPLC quantified the expoxidation state (EPS) of the xanthophylls-cycle pigments diadinoxanthin and diatoxanthin, and a chromarodiatroscan thin layer chromatography/flame ionization detection (TLC/FID) system quantified changes in lipid class concentrations. EPS did not exceed 020 in the low-light mesocosm, but increased to 0.65 in the high-light mesocosms. Triacylglycerol and monogalactosyldiacylglycerol (MGDC) were the largest lipid classes consisting of 9.3% to 48.7% and 37.3% to 69.7% of total lipid, respectively. Both lipid classes also experienced the greatest concentration changes in high-light experiments. *K. brevis* increased EPS and toxin concentrations while decreasing its lipid concentrations under high light. *K. brevis* may mobilize its toxins into the surrounding environment by reducing lipid concentrations environment by reducing lipid concentrations, such as sterols, limited competition, or toxins, such as sterols limiting are decreased in high light, reducing any protective mechanism against their own toxins by Blake Schaeffer *et al.* (2009).

The lipids of Cryptomonas rufescens (Skuja) cells have been analyzed. Quantitative changes of polar and neutral lipids were observed during cell encystment, induced by cultures in a nitrogen-deficient medium. During encystment, thylakoids disappeared while unsaturated galactolipids, characteristics of chloroplast membranes, decreased and neutral lipids accumulated in the cytoplasm. When excystment was induced, the reversal of the phenomenon was observed while thylakoids containing galactolipids were formed by Lichtle *et al.* (1984).

The fatty acid, sterol and chlorophyll composition of the calcified, unicellular alga *Thoracosphaera himii* (Lohmann) Kamptner are reported. The presence of 4, 23, 24-trimethyl-5α-cholest-22E-en-3β-ol (dinosterol), 4,23,24-trimethyl-5α-cholest-22E-en-3-one

(dinosterone) and the predominance of C18, C20 and C22 unsaturated fatty acids, including the acid 18:5*w*3,indicates that *T. heimii* is a dinoflagellate. The fatty acid:sterol ratio (1.3), is typical of dinoflagellates. The geochemical significance of dinosterone, the high relative concentration of 4-desmethyl-5α-stanols and the role of 23-methyl-5α-cholest-22E-en-3β-ol in the biosynthesis of dinosterol in *T. heimii* by Gary Jones *et al.* (1983).

The fatty acid spectra of 6 periphyton communities developed in laboratory streams at different combinations of light intensity and current velocity were determined by gas-liquid chromatography and silver nitrate thin-layer chromatography. Differences in species composition of the communities apparently had no striking effect on proportions of palmitic and stearic acids, whereas concentrations of myristic, and a C20:5 acid were more closely relatd to taxonomic differences. In general, communities dominated by blue-green algae exhibited relatively high proportions of oleic, linoleic, and linolenic acids and low proportions of palmitoleic acid and in C20:5 acid, as compared to communities consisting primarily of diatoms. The data also indicated an inverse relationship between between fatty acid redundancy and species diversity by Mcintire *et al.* (1969).

They described the use of lipases for the production of a lipid fraction rich in docosahexaenoic acid (DH) using phospholipids from the microalga *Isochrysis galbana* as raw materials. Fatty acid compositions of total and lipid classes of *I. galbana* were studied. DHA accounted for 13.5% of total fatty acids. However, the DHA content of the phospholipid fraction was 50% and 75% of total DHA was carried by phospholipids, mainly at the sn-2 position. These phospholipids contained neither eicosapentaenoic acid nor arachidonic acid Selective phospholipid. Five lipases showed discrimination between DHA and other fatty acids. A lipid fraction containing 80% of DHA was obtained with two lipases. Even though both preparations were equivalent in terms of DHA enrichment (1.5-1.6), lipase from *Rhizopus oryzae* gave better results as far as DHA recovery (85%) is Concerned. Lipase from Mucor javanicus gave a slightly lower recovery yield (71%) for the same reaction time (3h) by Devos *et al.* (2001). *Cynobacterium Mastigocladus laminosus* Cohn., was studied for total lipid content and lipid composition. It was observed that at 26˚C the lipids were observed on T.L.C plate up of which one spot with RF value 0.71 appeared to be specific to thermophilic cynobacterium as it was not observesed earlier in other heterocystous mesophilic cyanobacteria by Singh *et al.* (1991).

Carboxylation of acetyl-CoA to form malonyl-CoA, the committing step of fatty acid biosynthesis

Lipid biosynthesis starts with the acetyl-CoA carboxylase (ACC), which catalyzes the important committing step of the fatty acid synthetic pathway, the biotin-dependent carboxylation of acetyl-CoA to form malonyl-CoA (Davis *et al.* 2000). The *Escherichai coli,* ACC is a protein containing four subunits, which are encoded by genes accA, accB, accC and accD that are located at different positions on the chromosome. It is a trifunctional enzyme with a biotin carboxyl carrier protein, a biotin-carboxylase sub-unit and a carboxyl-transferase subunit joined together into a heterotrimeric complex. In contrast, eukaryotic cells encode a multi-domin single polypeptide, which is responsible for all the functions of the ACC (Sasaki *et al.* 2004). In animal cells, Acc is located in the cytoplasm and thus has to use cytosolic acetyl-CoA for malonyl-CoA formation and acyl chain elongation. Yeasts have both cytosolic and mitochondrial ACC, but it has been demonstrated to be able to survive with a non- functional mitochondrial enzyme. In plants, fatty acid synthesis occurs entirely in plastids of developing seeds, and ACC uses the acetyl-CoA that is found in this organelle. The plastids ACC has a different structure than the cytosolic ACC. It is a multi-subunit prokaryotic type enzyme, as opposed to the multifunctional eukaryotic type located in the cytoplasm.

Acyl chain elongation

Once malonyl-CoA is synthesized, it is transferred by malonyl CoA:ACP transacetylase to the acyl-carrier protein (ACP) of the fatty acid synthase (FAS) multi-enzymatic complex (Subramanyam *et al.* 1998). Bacteria and plants have type II FAS, which is a multi-subunit protein in which each individual peptide is dissociated and can catalyse an enzymatic reaction, as opposed to the type I FAS found in yeast and vertebrates, which is a multifunctional protein.FAS catalyzes fatty acid elongation by condensing malonyl-CoA molecules and acetyl-CoA. ACP, one of the FAS subunits, contains a thiol group that can form malonyl-ACP via forming thioesters with malonyl-CoA, and afterwards with the growing acyl chain in order to assure its transport. ACP can also fix acetyl-ACP. Then, the acetyl-group is transferred to other subunits of the FAS, the ketoacyl-ACP synthase (KAS), which catalyzes the condensation of malonyl-ACP or the growing acyl chain to form ketobutyryl-ACP or ketoacetyl-ACP. This resulting compound is first transformed via three successive reaction, i.e., reduction, dehydration and reduction, and then condensed with another malonyl-CoA. This cycle is repeated until the saturated chain of a palmitic (16:0) or a stearic acid (18:0) is formed. At last, ACP-thioesterase cleaves the acyl chain and liberates the fatty acid.

To obtain longer or unsaturated chains, elongases and destaturases are required, which act on palmitate or stearate. These enzymes are located in endoplasmic reticulum membrane and mitochondria. They can produce long chain fatty acids, as well as unsaturated acyl chains. They then act on the composition of the fatty acid pool but on their accumulation level. Many experiments have been carried out to modify the lipid content in transgenic plants using these enzymes, such as the increase of omega-3 production by Budziszewski *et al.* (2007).

Triacylglycerol formation (TAG)

For eukaryotes, TAG formation takes place in specialized organelles, i.e., the mitochondria or/and plastid (plants only) located in the endoplasmic reticulum. In contrast, the TAG synthesis takes place in the cytoplasm of prokaryotic cells. This process yields neutral lipids, a way to store fatty acids. The first step of TAG synthesis is the condensation (acylation) of glycerol-3-phosphate (G3P) with an acyl-CoA to form lysophosphatidate (LPA) which is catalysed by acyl-CoA:-sn-3-phosphate acyl-transferase (GPAT): this enzyme exnibits the lowest specific activity of the TAG synthesis pathyway, and was suggested to be potentially the rate limiting step (Cao *et al.* 2006; Coleman *et al.* 2004). It is subjected to many regulatory controls at the post-trascriptional level phorylation and by allostery. The LPA is then further condensed, catalysed by condensed, catalyst by acyl-CoA; acylglycerol s 1glycerol-in-3 phosphate acyl-transferase (GPAT), with another acyl-CoA to produce phosphatidate (PA) (Athenstaedt *et al.* 1999). Afterwards, PA can be dephosphorylated by phosphatidic acid phosphates (PAP) to produce diacylglycerol. At last synthesis of TAG is catalyzed by acyl-CoA; Fig:3 The Fatty Acid and TAG Biosynthesis (A Scott *et al.* 2010) diacyglycerol acyl-transferase [DGAT], which incorporates the third acyl-CoA into the diacylglycerol molecule. This enzyme is also known as an important regulator for this pathway (Oelkers *et al.*

2002). TAGs can then be stored in oil bodies by Murphy (2001).

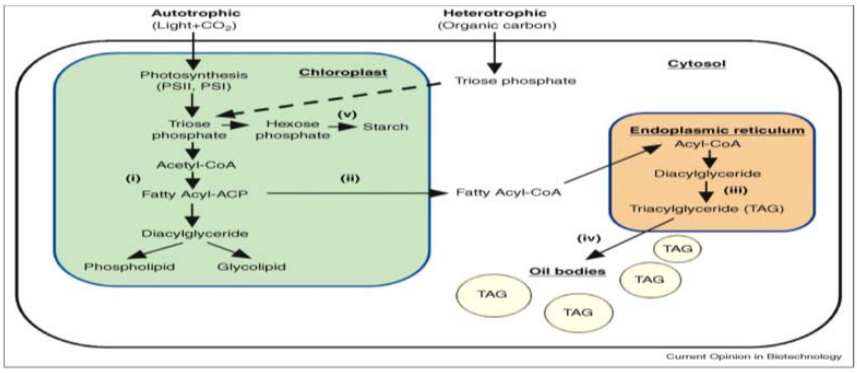


Fig:2 Basic over view of the pathway of carbon capture and lipid biosynthesis. Only the major steps are indicated for clarity. Precursor fatty acids are are synthesized de novo in the chloroplast, using either carbon fixed during photosynthesis, or from an exogenous supply of organic carbon; the exact nature of what enters the chloroplast is unknown in algae (dashed line). Free fatty acids are exported from the chloroplast and then converted to TAGs in the endoplasmic reticulum (ER), where they bud off into oil bodies in the cytosol. Key: (i) = acetyl-CoA carboxylase (ACCase) and fatty acid sunthase (FAS); (ii) = Fatty acid thioesterases and acyl-CoA synthetases; (iii) = TAG biosynthesis enzymes, including acyl-CoA:diacylglycerol acyltransferase (DGAT); (iv) = oil body formation; and (v) = ADP-glucose pyrophosphorylase and starch synthase [A Scott *et al.* (2010)]

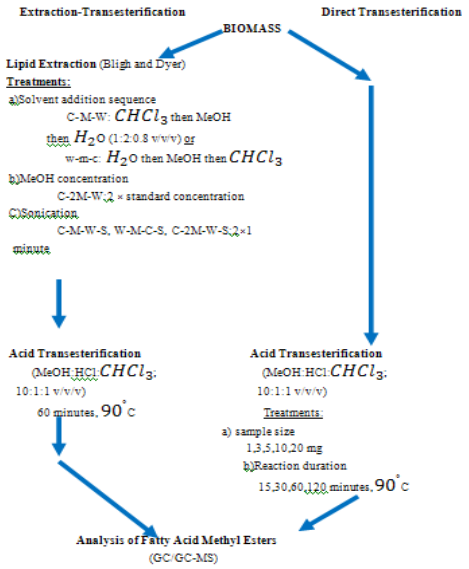


Fig: 3 Flow diagram of Lipid Extraction: Lewis *et al.* (2000).

Algae as a Source of Polyunsaturated Fatty Acids

The production of polyunsaturated fatty acids (PUFA) by marine and freshwater microalgae is the subject of intensive research and increasing commercial attention. Heterotrophic production of eicosapentaenoic acid by microalgae. Biotechnology production and application of the w-3 polyunsaturated fatty acid docosahexaenoic acid. Some species of freshwater and marine algae contain large amounts of high-quality PUFs and are widely used at the moment to produced PUFAs for aquaculture operations. They can grow heterotrophically on cheap organic substrates, without light, under well-controlled cultivation conditions. The following strategies are considered important in order to increase the use of algae for commercial production of PUFAs in the near future: the further selection and screening and screening of oleaginous species, improvement of strains by genetic manipulation, optimization of culture conditions and development of efficient cultivation systems. Moreover, it is often important to know whether PUFAs are present within membrane lipids, e.g., phosphor- or glycolipids, or if they are present in the cytosol as part of triacylglycerols.

Lipid Extraction Methods

Freeze-dried cells were weighted in 10mL screw-top test tubes, to which a fresh solution of the transesterification reaction mix (methanol / hydrochloric acid, chloroform, 10:1:1 v/v/v) was added. Cells were suspended in this solution by vortex mixing and immediately placed at 90°C for 60min for transesterification. Transesterification reaction tubes were removed from the heater and cooled to room temperature. One milliliter of water was then added to each tube and the fatty acids methyl esters extracted (hexane/chloroform, 4:1 v/v and 3×2 mL). Samples were diluted with chloroform containing a known concentration of 19:0 (nonadecanoic acid) as the internal injection standard and injected into the chromatograph by Tom Lewis *et al*. (2000).

Cells were lyophilized in 2mL screw-top vials containing 10µg nonadecanoic acid (Sigma) as an internal standard. To each vial, 0.5 mL 1 N HCL in methanol and 0.2 mL hexane was added. The vials

were heated at 85°C for 2h and cooled to room temperature. The hexane phase containing the fatty acid methy esters was partitioned from the aqueous phase by the addition of 0.25 mL 0.9% KCL. The hexane phase was transferred to Teflon-capped vials and analyzed by gas chromatography with flame ionization detection by Larson *et al.* (2001).

Samples were dissolved in 2 mL of a freshly prepared mixture of acetyl chloride and methanol, at a ratio of 5:100 (v/v), together with 1 mg of tricosanoic acid as an internal standard. The reagents were placed in Teflon-capped Pyrex tubes, and the reaction continued at 100°C for 1 h under pure nitrogen and darkness. After cooling to 30~40°C, 1 mL of extracting solvent (hexane or isooctane) was added and the FAME solvent solution was mixed in the vortex for a specific period of time (5 to 30s). Purification of the solution was achieved either by saltingout(using1mL of saturated sodium chlorid solution) or washing (using 1 mL of saturated sodium chloride solution) or washing (using 1 mL of water), causing the formation of two immiscible phases, which were then allowed to separate. The upper extracted solvent phase was recovered, dried over anhydrous Na_2SO_4, and analyzed using a gas chromatograph [Lepage and Roy, modified by Cohen *et al.* 1988].

Samples and 5µL 19:0 solution were placed in test tubes. One milliliter of freshly prepared tranesterification reagent (methanol/acetyl chloride, 20:1 v/v) was added to each tube. The tubes were heated at 100°C for 1h for transmethylation, and shaken every 10~15 min. The mixture was cooled to room temperature, and 1 mL each of water and hexane were added. The tubes were then shaken and centrifuged. Two phases were produced; the upper phase was produced; the upper phase (hexane) was transferred to another tube. This operation was repeated twice, to optimize sample lipid extraction. The hexanic phase was dried under N_2 atmosphere and FAMEs were suspended in 0.5 mL of hexane and injected into the gas chromatograph by Lepage *et al.* (1998).

The addition of 2 mL of chloroform-methanol 2:1 (v/v) was added to each sample. The mixture was mechanically shaken for 10min. After centrification-methanol 2:1 (v/v) was added to the precipitate and the same procedure was repeated. The lower phase was pooled and 145 mM NaCl was added in order to separate the methanol and chloroform phase. Following centrifugation, the lower phase containing the lipids was evaporated to dryness at room temperature under a gentle stream of nitrogen. The residue was solubilized in a mixture of 1 mL of methanol-benzene 3:2 (v/v). The mixture was then subjected to methanolysis at 100°C for 1 h. cooled to room temperature and an internal standard was added. The specimens were then processed and injected into the gas chromatograph by Folch *et al.* (1957).

Lipid Extraction – Solvent

Neutral lipids or generally storage lipids are extracted with relatively non-polar solvents such as diethyl ether or chloroform but membrane-associated lipids are more polar and require polar solvents such as ethanol or methanol to disrupt hydrogen bondings or electrostatic forces. To avoid peroxidation of the extracted lipids, during the procedure or later, all solvents should be used peroxide-free. The most convenient is to use "pesticide grade" or HPLC grade" and to buy small quantities at once.

Elumalai *et al* (2011) proved that biodiesel can be produced from Freshwater (Temple tanks, Forest lagoons, Inland lakes, Rock ponds) microalgae (*Chlorella vulgaris*) had the percentage of 49% lipid content during n-Hexane soaking period of about 210min. A gradual increase in lipid content was observed as the soaking period in n – Hexane was increased, which could be due to softening of the dried algal biomass by the solvent action.

Furthermore, to avoid contamination of extracted lipids by non-volatile material contained in the large volume of solvent used in the first step of extraction, it is important to use organic solvent containing less than 5 mg dry matter per liter, 1 mg/l is currently proposed. Diethyl ether, dioxan, isopropyl ether and tetrahydrofuran form peroxides on storage and must be kept in dark bottles or in dark areas. These solvents should be routinely tested to prove that peroxide levels are kept low before use. It must be emphasized that peroxides, even at low levels, may present an explosion hazard after concentration of solvents. Because of its low flammability and its natural origin, ethyl lactate was proposed as a substitute for ethyl acetate in exctracting carotenoids from foods. Alcohols are good solvents for most lipids, methanol and ethanol being the most popular. Ethanol can be contaminated on storage by aldehydes. Chloroform is a popular solvent, particularly for lipids of intermediate polarity and when mixed with methanol it becomes a general extraction solvent. Chloroform and dichloromethane are currently stabilized by addition of ethanol (up to 1%) or methyl-2-butene-2 (about 20 mg/l). Among hydrocarbons, hexane is the most popular but is a good solvent only for lipids of low polarity. Its main use is to extract neutral lipids from mixtures of water with alcohols. A mixture of isomers, called "hexanes", can be used for the same purpose. Hexane can be replaced by petroleum ether which is a mixture of various hydrocarbons with 5 to 8 carbon atoms. Cyclohexane is sometimes used to store lipid extracts in the cold without danger of evaporation since it freezes at about 6°C. Benzene is no more used since it is now considered as a potent carcinogenic substance; it may be replaced by toluene which, nevertheless, is more difficult to evaporate. A simple test for peroxides in water-immiscible solvents: shake 2 ml of solvent with 1 ml of a freshly prepared KI solution (10% in water) and a drop of starch indicator solution, a blue color is rapidly developing if peroxides are present (a faint yellow/brown color after addition of KI indicates low levels of peroxides) Ishida *et al.* (2009).

Transesterification

Transesterification of *Nannochloropsis oculata* microalga's lipid to biodiesel on Al2O3 supported CaO and MgO catalysts described by Umdu *et al.* (2009) The activities of Al2O3 supported CaO and MgO catalysts in the transesterification of lipid of yellow green microalgae, *Nannochlorpsis oculata*, as a function of methanol amount and the CaO and MgO loadings at 50°C. It was found that pure CaO and MgO were not active and CaO/ Al2O3 catalyst among all the mixed oxide catalysts showed the highest activity. Not only the basic site density but also the basic strength is important to achieve the high biodiesel yield. Biodiesel yield over 80 wt.% CaO/ Al2O3 catalyst increased to 97.5% from 23% when methanol/lipid molar ratio was 30.

The viscosities of vegetable oils and microalgal oils are usually higher than that of diesel oils. Hence, they cannot be applied to engines directly. The transesterification of microalgal oils will greatly reduce the original viscosity and increase the fluidity. Few reports on the production of biodiesel from microalgal oils are available, Chisti (2007). Nevertheless the technologies of the biodiesel production for vegetable oils can be applied to the biodiesel production of microalgal oils because of the similar physical and chemical properties. In the process of transesterification, alcohols are key substrates in transesterification. The commonly used alcohol but

methanol is applied more widely because of its low-cost and physical advantages. Alkali, acid, or enzyme catalyzed processes may be applied in transesterification. The use of acid catalyst has found to be useful for the conversions of high free fatty acid feedstocks to esters are too slow. Alkali catalysts have higher reaction rate and converstion than acid catalysts for the transesterification of triglyceride. Alkali-catalyzed transesterification is about 4000 times faster than the acid catalyzed reaction. So, alkali-catalyzed transesterification is most frequently used commercially. The free fatty acid (FFA), however, may react with the alkali catalyst to form soap and water which results in the loss of alkali catalysts in the process of reaction. There-fore, additional catalysts must be added to compensate for the catalyst loss to soap. When the FFA level is above 5%, the soap will inhibit separation of the methyl esters and glycerol and causes emulsion formation during the water washing. Therefore, it is necessary to first convert FFAs to methyl eaters in order to reduce the contents of FFAs, and the low FFAs pretreated oil is transesterified with an alkali catalyst to convert triglycerides to methyl esters. In contrast, enzymes exhibit good tolerance to the FFA level of the feedstock, but the enzymes are expensive and may not be able to provide the degree of reaction required to meet the ASTM fuel specification. Immobilization of the enzyme and multiple enzymes may provide more choices in the future.

Besides, the effects of molar ratio of glycerides to alcohol; catalysts, reaction temperature and time, the contents of FFAs and water in oils and fats have been reported in some recent reports. It was reported that biodiesel could be produced by acidic transesterification with 56:1 M ratio of methanol to microalgal oil at temperature at 30˚C and 100% catalyst quantity (based on oil weight) was achieved. The most abundant composition of microalgal oil transesterified with methanol is $C_{19}H_{36}O_2$, which is suggested to accord with the standard of biodiesel. In recent years, there have been many reports about the applications of transesterified technologies for biodiesel by Stavarache *et al.* (2007).

Lipid Staining

Nile red staining:

Nile red (9-diethylamino-5H-benzo [α] phenoxa-] phenoxazine-5-one) was dissolved in 0.1 mg/ml with acetone. Fluorescence spectra were measured with the spectro fluorometer FP-750 with PC control equipment (JASCO, Tokyo, Japan). The acquiring and processing of the data were done using the PC software (JASCO). In order to eliminate the effect of auto-fluorescence of the cells and scattering of the cell suspension, the fluorescence spectra were obtained as follows. A 100-µl aliquot of the culture broth of was mixed with 2ml of 10mM potassium phosphate buffer with 0.15M KC1 (pH 7.0; PBS) in a 10-mm acryl cuvette. The spectrum in a wavelength region of 400 to 700nm for the cell suspension without Nile red was recorded. The 10-µl Nile red solution was then added and mixed well. Five minutes later, the spectrum in the same wavelength region was recorded again. The cell suspension in a cuvette was mixed well by an upside-down inversion just before measurement in order to avoid cell sedimentation. Spectra were corrected by subtracting the spectra before and after the Nile red addition.

Chlorella vulgaris and Diatoms were cultivated and maintained in different stress system like high light intensity (18:6, Light: Dark) and N_2 limited condition. After the stationary phase, the cells were stained by Nile red and Bodipy. The slides were examined under fluorescence in situ hybridization microscopy, it showed the *Chlorella vulgaris* cultivated under N_2 limited system yield high lipid bodies than light induced system and Stressed Diatoms. While Bodipy is a best tool for staining algal lipid bodies than Nile Red stain (Elumalai *et al* 2011).

Elumalai *et al* (2011) evaluated 10 different Microalgal lipid contents by Nile red and Bodipy staining and the ultra structural studies carried out by Scanning Electron Microscope (SEM). The micro algal samples were collected from province of Tamil Nadu, located in Southern India. Compare to Nile Red stain, Bodipy is a specific stain for to detect lipid content present in the living algal cells. *Chlorelvulgaris* stained by Bodipy have shown more lipid content than other microalgae collected from various locations.

Elumalai *et al* (2011) surveyed the population, identification, morphological, and examined the Nile red method with microalgae genus of various classes from Chennai, Mahapaliburam and Kovallam. Physical and chemical treatments were applied to the existing Nile red method to improve the effectiveness and efficiency. The following algae were present *Chlorella vulgaris, Nannochloropsis, Dunaliella tertiolecta, Tetraselmis suecica, Chlorococcum humicolo, Scenedesmus acuminatus, Amphora coffeaformis,* and *Nitzschia longissima*.

Lipid analysis: Gravimetric determination of neutral lipids: Lipids were extracted in a chloroform–methanol–water system according to Bligh and Dyer (1959). The extract was evaporated in a water bath (30 °C) using a rotary evaporator (Büchi, Switzerland) to remove solvents. Crude lipids were further separated by column chromatography using silicon gel (60–200 mesh) (Merck Corp., Germany) according to Alonzo and Mayzaud (1999) using the following solvent sequence: 6 volumes of chloroform to collect the neutral lipid class and 6 volumes of methanol to collect the polar lipids. Each lipid fraction was transferred into a pre-weighed vial, initially evaporated in a water bath (30 °C) using a rotary evaporator and then dried under high vacuum. The dried residuals were placed under nitrogen and then weighed.

Nile red fluorescence determination of neutral lipid: After the algal cell suspensions were stained with Nile red, fluorescence was measured on a Varian 96-well plate spectro fluorometer using medium scan control and high PMT detector voltage mode. According to the pre-scan of excitation and emission characteristics of neutral lipid standards, the excitation and emission wavelengths of 530 nm and 575 nm were selected by Chen *et al.* (2009).

Optimization of the modified Nile red fluorescence method: DMSO concentration: 5 µL algal samples of known cell concentration were introduced into individual wells of a 96-microplate containing 3 µL of a 50 µgMl−1 Nile red solution and 292 µL DMSO aqueous solutions with the concentrations ranging from 1% (v/v) to 40%. The 96-well plate was vortexed (120 rpm) and incubated at 40 °C for 10 min. After algal cells were stained, fluorescence emissions were recorded with a Varian spectrophotometer equipped with a 96-well plate reading mode. Unless stated otherwise, six replicates of each treatment were analyzed. Nile red dye concentration: Nile red dye, at different concentrations, ranging from 0.1 to 10 µg /ml was used following the experimental procedure detailed above to optimize the dye concentration. In this experiment, 25% DMSO (v/v) was used in the staining solutions. Staining time: For the optimization of staining time, algal suspensions of defined cell concentrations were stained with Nile red in 25% DMSO aqueous solutions. Staining times of 5 min, 10 min, 20 min, 30 min, 60 min and 100 min were evaluated by Chen *et al.* (2009). Staining temperature: Staining temperatures, ranging from 20 °C to 80 °C were investigated following the staining procedure described above. Algal cell concentration: To optimize cell

concentrations for determination of cellular neutral lipid, several cell concentrations, ranging from 10 to 8×105 cells mL−1 were evaluated using the above procedures for cell staining. Comparison of lipid content by Nile red fluorescence method and conventional gravimetric method: To verify that the modified Nile red fluorescence method was effective in determining neutral lipid content, the modified method and conventional gravimetric method were compared for the green alga *C. vulgaris*. The cells used were from a 12-day liquid culture. For fluorescence determination, six replicates were used and for gravimetric determination, 3 replicates were used by Chen *et al.* (2000).

Bodipy Lipid Staining:

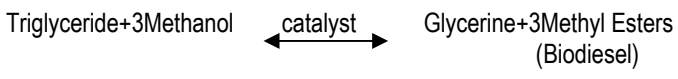
The intensely fluorescent Bodipy 4,4-difluoro-3a,4adiaza-s-indacene) fluorophore is intrinsically lipophilic, unlikemost other long-wavelength dyes. Consequently, probes incorporating this fluorophore are more likely to mimic the properties of natural lipids. Molecular Probes prepares bodipy fatty acid, phospholipid, cholesteryl ester and sphingolipid analogs that undergo native-like transport and metabolism in cells.1 They are therefore effective tracers of lipid trafficking, as well as being useful general-purpose membrane probes. Several nonpolar bodipy dyes for staining neutral lipids, oils and polymers are available. The spectroscopic properties of bodipy lipid probes are summarized in Table.4

Fatty Acids

Biosynthetic incorporation of Bodipy fatty acids into baby hamster kidney cells (BHK) cells has been characterized by HPLC analysis.3 Incorporation levels into glycerophosphocholine were found to be greater than 90% for Bodipy 500/510 dodecanoic acid (D-3823). Microscopic examination of biosynthetically labeled cells revealed localized areas of red-shifted fluorescence. This long-wavelength fluorescence results from the accumulation of Bodipy dye–labeled neutral lipids in cytoplasmic droplets at concentrations sufficient to induce the formation of BODIPY excimers. Absorption of Bodipy fl and Bodipy 500/510 is well matched to argon-ion laser excitation at 488nm, allowing analysis of cellular fatty acid uptake by flow cytometry and confocal microscopy. Bodipy fl dodecanoic acid (D-3822) has been employed to examine the co-transfer of lipids and membrane proteins from human neutrophils to the parasite *Schistosoma mansoni*. Researchers have also used BODIPY fatty acids and phospholipids to visualize compartmentalization of specific lipid classes in this parasite. Binding of BODIPY fatty acids to bovine serum albumin can be monitored by the accompanying fluorescence quenching caused by charge-transfer interactions with aromatic amino acid residues. BODIPY 581/591 C11 (D-3861) can be used to measure antioxidant activity in lipid environments by exploiting its loss of fluorescence upon interaction with peroxyl radicals.

Biodiesel production:

Biodiesel is a biofuel consisting of monoalkyl esters that are derived from organic oils, plant or animal, through the process of tranesterification (Demirbas, 2007). The biodiesel transesterification reaction is as follows:



This is an equilibrium reaction where an organic oil, or triglyceride, can be processed into biodiesel, usually in the presence of a catalyst, and alkali such as potassium hydroxide (Christi, 2007; Demirbas, 2007). A excess of methanol is used to force the reaction to favour the right side of the equation. The excess methanol is later recovered and reused. At 60˚C, the reaction can complete in 90 minutes. The triglyceride is a complex molecule that plants and animals use for storing food energy; in more simple terms, it is fat. The process of making biodiesel occurs as follows: A) the triglycerides, methanol, and catalyst are placed in a controlled reaction chamber to undergo transesterification, B) the initial product is placed in a separator to remove the glycerine by-product, C) the excess methanol is recovered from the methyl esters through evaporation, and D), the final biodiesel is rinsed with water, pH neutralized, and dried by Xu *et al.* (2006).

The utilization of corn powder hydrolysate instead of glucose in heterotrophic culture greatly reduced the cost of production, which is important for the biodiesel production by microalgae in terms of economical significance. Nitrogen is also an essential macronutrient in lipids production. Complex nitrogen source might be superior to simple nitrogen source in heterotrophic culture of microalgae, because it might provide amino acids, vitamins and growth factors simultaneously. Industrial wastewater rich in nitrogen also can be considered for the cultivation of microalgae. Monosodium glutamate waste after diluted was well treated as a cheap fermentation medium for *Rhodotorula glutinis* to biosynthesize lipids as the raw material for the production of biodiesel Xue , *et al.* (2006). Many microalgae growing under nitrogen limitation show enhanced lipid content. In the late 1940s, it was noted that nitrogen starvation is most influential on lipid storage and lipid fractions, and as a result of nitrogen starvation, the lipid content as high as 70.85% of dry weight was reported Becker (1994).

Initial research focused on the isolation of high lipid content microalgae that could be cultivated in large-scale open pond cultivation for biodiesel production Weissman , (1992), and capturing CO2 from coal-fired power plants as biological emission control process Chelf (1991). The primary findings of the outlined research were: (1) increment in oil accumulation in algal cells due to nitrogen-deficiency is inversely proportional to oil productivity of entire cultures due to lower total productivity resulting from lower nutrient levels; (2) open pond production is most appropriate for large-scale microalgae production due to low costs; (3) maintenance of uncontaminated mono-specific microalgae cultures in open ponds for sustainable high production is exceedingly difficult.

The most effective method of improving microalgae lipid accumulation is nitrog enlimitation, which not only results in the accumulation of lipids, but also results in a gradual change of lipid composition from free fattyacids to triacylglycerol (TAG) Widjaja A (2009). TAGs are more useful for conversion to biodiesel Meng *et al.* (2009). Lipid accumulation in microalgae occurs when a nutrient (typically nitrogen, but can be silicate for diatoms) is exhausted from the medium or becomes the growth limiting factor. Cell proliferation is prevented but carbon is still assimilated by the cell and converted to TAG lipids that a restored with in existing cell there by increasing the concentration Meng *et al.* (2009).

Wu and Hsieh Wu, Hsieh C-H (2008) investigated the effects of salinity, nitrogen concentration and light intensity on lipid productivity, and recorded upto76% increase in production of lipids for specific growth condition when compared to more typical growth processes. Weldy and Huesemann Weldy *et al.* (2007) argued that for lipid production, the percentage lipid content of microalgae was less important than maximization of growth rates. For example, they recorded higher lipid productivity (0.46g 1^{-1} per day) under N-sufficient condition and high light intensity when compared with N-

deficient culture (0.12 $g1^{-1}$ per day). Chiuetal. Chiu *et al.* (2009) established that 2% (v/v) CO2 concentration was optimal for *Nannochloropsis oculata* to achieve maximum biomass and lipid productivity. They achieved 0.48 $g1^{-1}$ per day and 0.142 $g1^{-1}$ per day for biomass yield and lipid production, respectively.

Energy crises, global warming, and climatic changes call for technological and commercial advances in manufacturing high-quality transportation fuels from unconventional feedstocks. Microalgae is one of the most promising sources of biofuels due to the high yields attained per unit area and because it does not displace food crops. *Neochloris oleabundans* (Neo) microalga is an important promising microbial source of single-cell oil (SCO). Different experimental growth and lipid production conditions were evaluated and compared by using optical density (540 nm), dry-weight determination, and flow cytometry (FC). Best Neo average biomass productivity was obtained at 30°C under conditions of nitrogen-sufficiency and CO2 supplementation (N+/30°C/CO2), with an average doubling time of 1.4 days. The second and third highest productivities occurred with N-sufficient cultures without CO2 supplementation 26°C (N+/26°C) and at 30°C (N+/30°C), with doubling times of 1.7 and 2.2 days, respectively. Microbial lipid production was monitored by flow cytometry using Nile red (NR), a lipophilic fluorochrome that possesses several advantages characteristics for in situ screening near real time (at line). Results showed maximum lipid content (56%) after 6 days of nitrogen depletion under nitrogen starvation without CO2 supplementation (N-/30°C), followed by N-/30°C/CO2 and N-1 of N starvation, respectively. The adequate fatty acid profile and iodine value of Neo lipids reinforced these microalgae as a good source of SCO, in particular for use as biodiesel by Gouveia *et al.* (2009).

Thermo Gravimetric Analysis (TGA)

The Thermo gravimetric analysis (TGA) for the study of microalgal species has been proved by several authors. As an example, Pane *et al.* (2001), applied TGA in an air atmosphere to study the effects of temperature on a marine planktonic alga, reporting the existence of marked differences between the different phases of growth, related to the presence of different molecules produced during the algal growth and to the differences in the thermal properties of these intracellular molecules. These authors differentiate three steps in the weight loss TGA. The first stage of weight loss occurs in the 40-180°C range and corresponds to the loss of free water and water loosely bound to biomolecules. In this process, the cell structure is progressively destroyed, and phenomena such as alteration of lipid structures and proteic thermal unfolding occur. The second step occurs in the 180-400°C range, and involves the decomposition of proteins and carbohydrates. This step produces the main loss of weight. Finally, the third stop occurs in the 400-760°C range and corresponds to the complete oxidation of the organic matter. Peng *et al.* (2001) pyrolyzed two kinds of autotrophic microalgae in TGA equipment in order to investigate their pyrolytic characteristics. These authors also identify three stages of decomposition, i.e., dehydration, devolatilization and solid decomposition, and performed a kinetic study in order to the study of a composite of polyethylene-*chlorella*.

The on-line combination of TGA and Fourier Transform Infrared Spectrometry (FTIR) has been successfully applied for the study of the evolution with time of the volatile products evolved in the thermal and catalytic pyrolysis of polymers Marcilla *et al.* (2005), showing the ability of this technique to characterize the nature of the chemical compounds which are decomposed in the different degradation steps involved in the pyrolysis of ethylene-vinyl acetate copolymers. TGA has been applied for the study of the evolution with time of the volatile products evolved in the thermal pyrolysis of *Nannochloropsis* sp. for the characterization of the different decomposition steps of this microalgae specie. In this way, the microalgae cells have been treated in order to separate the lipid fraction, by breaking the cells and extracting the fraction soluble in hexane, and both fractions, i.e., the and the solid residue, have been also analyzed by TGA.

The TGA and the DTG corresponding to the microalgae show three main decomposition steps (I.e., <180°C corresponding to the dehydration, 180-540°C corresponding to devolatilization and >540°C Correponding to the slow decomposition of the solid residue resulting from the previous step), the results obtained in this work permit us to observe that the main decomposition step, which occurs in the 180-540°C range, is actually a very complex process, involving at least three overlapped steps, such as the shoulder and overlapped peaks observed in the corresponding DTG. According to, the temperature of maximum decomposition rate (i.e., the DTG-Peak temperatures) observed for the microalgae pyrolysis are:

First step (devolatilization): 125°C.

Second step (devolatilization): three overlapped peaks at 290,340 and 460°C.

Third step (solid residue decomposition): 740°C.

Stress Based Lipid Changes

Changes in plankton P forms with environmental stress. Many of these forms are important metabolically and structurally, such as DNA, sugar phosphates and phospholipids, and are organic compounds chemically linked to C. Thus, if C fixation is altered by environmental stress, then organic P forms could also be altered. The C:P ratio has been shown to remain close to the Redfield ratio under low-light conditions, but to increase under high light conditions (Sterner et al., 1997), but it is not known if this also produces changes in the distribution of organic P forms. Inorganic P forms, such as orthophosphate and polyphosphate, may also be altered by environmental stress. Indeed, in bacteria, polyphosphates and associate enzymes such as polyphosphate kinase are involved in a wide range of aspects ofmetabolism, and play an important role in responding to a variety of environmental stresses (Jahid et al., 2006; Manganelli, 2007; Brown and Kornberg, 2008). For algae, luxury accumulation of polyphosphate is well known (e.g. Droop, 1973; Stevenson and Stoermer, 1982; Sterner and Elser, 2002). With respect to stress, it appears that polyphosphate accumulation in *Dunaliella* salina can be altered by alkaline stress (Pick *et al.* 1990) and osmotic stress (Bental *et al.* 1991). However, there are few reports to indicate that changes in polyphosphate synthesis represent an environmental stress response in algae to the same extent as has been observed for bacteria.

Light stress:

There were no significant differences in total P, C, N or the C:N, C:P or N:P ratios between control cultures and those grown under low light conditions (Table 5). Cultures grown under high light conditions had significantly higher total P concentrations than control samples, but no differences for C or N, or the C:N, C:P or N:P ratios. Correlation analysis combining low light, high light and control samples shows that light intensity was positively correlated with total P (r=0.58; P=0.01), N (r=0.63; P=0.004), and C (r=0.47; P=0.04) and was negatively correlated with the C:N ratio (r=−0.54; P=0.02). No significant differences in any of the C spectral regions or modeled C

forms were observed for samples grown under low light conditions, relative to controls (Table 6). Algae grown under light conditions showed a significant increase in the O-Aromatic C (C VI) spectral region, but this did not produce any significant differences in modeled C forms. Correlation analysis combining low light, high light and control samples shows that light intensity was positively correlated with the C VI spectral region (r=0.43; P=0.06), and protein (r=0.46; P=0.05), and was negatively correlated with lipids (r=−0.41; P=0.08). Principal components analysis for the 13C CPMAS spectra did not show any significant grouping for samples grown under low or high light conditions, relative to controls. There was a significant decrease in the relative percent of P in the monoester 1 region in the low light samples relative to controls (Table 7), but the concentration of P in the monoester 1 region was not significantly different (Table 8). There were no other differences in P forms, in relative percent or concentration, for the low light treatment. For the high light treatment, there were no significant differences in the relative percentages of P forms compared with controls, but the concentration, for the low light treatment. For the high light treatment, there were no significant differences in the relative percentages of P forms compared with controls (Table 7). However, the concentration of orthophosphate, monoester 2 and organic P were significantly higher in the samples grown under high light conditions than the controls. Principal component analyses and correlation analyses combining low light, high light and control samples did not show any significant relationships for P forms or compound classes.

Under the influence of light and dark condition *C. vulgaris* change the fatty acid profiles. To get better yield of biofuel, the growth of the microalgal isolate was optimized with the addition of nutrients and salts under light and dark conditions. The lipid fractions were extracted from the biomass through solvent extractions and the fractions were analyzed for biodiesel under GC-MS. The percentage of lipids synthesized from *C. vulgaris* under light and dark conditions were analyzed and compared. The algae from dark sample shows rich in saturated fatty acid (capric acid, lauric acid & myristic acid) and considerable amount of PUFA (hexadecatrienoic acid, stearidonic acid, eicosapenaenoic acid, docosahexaenoic acid) when compare to algae grown under light. So the algae grown in dark condition is an excellent source for high yield of saturated fatty acids (Elumalai *et al* 2011).

Table 1: The Oil content of various microalgae, Luisa Gouveia *et al.* (2009)

Microalgae	Oil content (% dry wt)
Botryococcus braunii	25-75
Chlorella sp	28-32
Crypthecodinium cohnii	20
Cylindrotheca sp	16-37
Dunaliella primolecta	23
Isochrysis sp	25-33
Monallanthus salina	>20
Nanochloris sp	20-35
Nannochloropsis sp	31-68
Neochloris oseoabundans	35-54
Nitzschia sp	45-47
Phaeodactylum tricornutum	20-30
Schizochytrium sp	50-77
Tetraselmis suecia	15-23

Table 2: Fatty acid composition of microalgal oil, Ayhan Demirbas (2009).

Fatty acid	Chain length: no Of double bonds	Oil composition (w/total lipid)
Palmitic acid	16:0	12-1
Palmitoleic acid	16:1	55-7
Stearic acid	18:0	1-2
Oleic acid	18:1	58-60
Linoleic acid	18:2	4-20
Linolenic acid	18:3	4-30

Table:3 Spectral Properties of Bodipy lipid probes

Wavelength Range	Fluorescence excitation maxima from 500 nm to ~650 nm. Emission maxima from 510 nm to ~665 nm.
Spectral Bandwidth	Narrow
Fluorescence Stokes Shift	Small. Spectral overlap results in Förster transfer radius (Ro) = 57 Å for BODIPY FL.
Fluorescence Quantum Yield	High. Typically 0.9 in fluid phase lipid bilayers.
Molar Absorptivity	High. max typically >80,000 cm-1M-1.
Sensitivity to Environment	Generally low. Fluorescence quantum yields of fatty acids are not diminished by collisional interactions with aromatic amino acids.
Concentration Dependence	Long-wavelength excimer emission detectable at incorporation levels of about 1:10 mole: mole with respect to unlabled phospholipid in lipid bilayers.

Data in this table are compiled, in part, from J Am Chem Soc 116, 7801 (1994) and Anal Biochem 198, 228 (1991).

Table:4 Biomass productivity, lipid content and lipid productivity of 30 microalgal strains cultivated in 250-mL flasks

Algal Group	Microalgae strains	Habitat	Biomass Productivity (g/L/day)	Lipid content (% biomass)	Lipid productivity (mg/L/day)
Diatoms	*Chaetoceros muelleri F&M-M43*	Marine	0.07	33.6	21.8
	Chaetoceros calcitrans CS 178	Marine	0.04	39.8	17.6
	P. tricornutum F&M-M 40	Marine	0.24	18.7	44.8
	Skeletonoma costatum CS 181	Marine	0.08	21.0	17.4
	Skeletonoma sp. CS 252	Marine	0.09	31.8	27.3
	Thalassioria pseudonana CS 173	Marine	0.08	20.6	17.4
	Chlorella sp. F&M-M48	Freshwater	0.23	18.7	42.1
	Chlorella sorokiniana IAM-212	Freshwater	0.23	19.3	44.7
	Chlorella vulgaris CCAP 211/11b	Freshwater	0.17	19.2	32.6
	C. vulgaris F&M-M49	Freshwater	0.20	18.4	36.9
Green Algaea	*Chlorococcum sp. UMACC 112*	Freshwater	0.28	19.3	53.7
	Scenedemus quadricauda	Freshwater	0.19	18.4	35.1
	Scenedemus F&M-M19	Freshwater	0.21	19.6	40.8
	Scenedemus sp. DM	Freshwater	0.26	21.1	53.9
	T. suecica F&M-M33	Marine	0.32	8.5	27.0
	Tetraselmis sp. F&M-M34	Marine	0.30	14.7	43.4
	T. suecica F&M-M35	Marine	0.28	12.9	36.4
	Ellipsoidion sp. F&M-M31	Marine	0.17	27.4	47.3
	Monodus subterraneus UTEX 151	Freshwater	0.19	16.1	30.4
	Nannochloropsis sp. CS 246	Marine	0.17	29.2	49.7
Eustigma--tophytes	*Nannochloropsis sp. F&M-M26*	Marine	0.21	29.6	61.0
	Nannochloropsis sp. F&M-M27	Marine	0.20	24.4	48.2
	Nannochloropsis sp. F&M-M24	Marine	0.18	30.9	54.8
	Nannochloropsis sp. F&M-M29	Marine	0.17	21.6	37.6
	Nannochloropsis sp. F&M-M28	Marine	0.17	35.7	60.9
	Isochrysis sp. (T-ISO) CS 177	Marine	0.17	22.4	37.7
	Isochrysis sp. F&M-M37	Marine	0.14	27.4	37.8
Prymnes--iophytes	*Pavlova salina CS 49*	Marine	0.16	30.9	49.4
	Pavlova lutheri CS 182	Marine	0.14	35.5	50.2
Red algae	*Porphyridium cruentum*	Marine	0.37	9.5	34.8

Table 5: Total p, total C, total N and the C:N, C:P and N:P ratios for algal cultures grown under control conditions and under light, temperature, and p nutrition stress. Results shown are averages and (standard deviation) [Barbara J. Cade-Menun *et al.* (2010)]

	Total P $\mu mol\, g^{-1}$	Total C mol g^{-1}	Total N mol g^{-1}	C:N	C:P	N:P
Low It	288.1 (102.6)	22.8(6.71)	3.44(1.01)	6.75(1.26)	89.0(47.8)	13.3(5.77)
Control	278.3(102.9)	23.0(7.18)	3.44(0.95)	6.83(1.41)	90.6(33.8)	14.0(6.49)
High It.	4522(111.3)*	37.0(23.3)	8.56(6.05)	4.82(1.57)	77.7(31.2)	17.9(7.99)
Control	217.5(96.2)*	18.3(3.29)	3.26(1.19)	6.03(1.35)	97.9(45.0)	16.8(7.66)
Low tmp.	296.1(253.8)	16.9(3.31)	2.56(0.83)	6.96(2.41)	91.0(26.6)	13.1(9.23)
Control	203.6(49.1)	11.4(5.9)	1.44(0.78)	8.01(0.22)	61.4(44.1)	7.74(5.71)
High tmp.	490.8(282.2)	21.4(11.8)	3.50(1.78)	6.04(0.47)	45.7(7.39)	7.55(1.17)
Control	651.5(336.0)	29.1(33.3)	4.67(0.38)	6.23(0.51)	54.8(29.7)	8.65(4.38)
Low P	250.2(263.4)	27.7(4.46)	3.42(1.53)	8.99(2.71)	275.4(203.2)*	22.0(19.3)*
Control	556.1(286.8)	26.9(5.94)	3.80(0.75)	7.11(1.14)	59.5(30.3)*	7.89(3.14)*
High P	2837.5(2062.6)	19.0(10.0)	3.16(1.96)	6.30(0.65)	12.2(12.0)*	2.03(2.10)*
Control	881.0(1328.0)	16.0(8.00)	2.35(1.19)	6.73(1.51)	79.8(64.3)*	11.3(9.15)*

Table 6: 13C CPMASS Spectral regions and modeled C forms for algal cultures grown under control conditions and under temperature (temp.). P nutrition and light stress. Carb, Carbohydrate; Prot, Protein; Arom, aromatics; Cbnl, carbonyl; Cr;Pr, carbohydrate: Protein ratio. Results shown are average and (standard deviation) [Barbara J. Cade-Menun *et al.* (2010)]

	CI	CII	CIII	CIV	CV	CVI	CVII	Carb	Prot	Arom	Lipid	Cbnl	Cr:Pr
Low light	32.9 (7.7)	12.9 (1.2)	23.2(5.7)	3.2 (1.2)	9.0 (1.5)	2.8 (1.6)	16.1 (2.8)	32.3 (7.8)	54.9 (11.5)	4.3 (2.0)	8.3 (10.4)	0.3 (0.6)	0.63 (0.27)
Control	36.5 (7.7)	13.0 (1.3)	21.9(6.2)	2.9 (1.8)	8.4 (1.0)	2.2 (0.9)	15.2 (2.0)	25.8 (14.0)	56.8 (20.8)	4.6 (4.1)	11.1 (9.6)	1.6 (2.2)	0.55 (0.35)
High light	27.1 (4.9)	13.8 (0.6)	24.9 (1.5)	4.2 (0.3)	9.6 (1.1)	3.9* (0.6)	16.7 (3.6)	20.9 (16.8)	76.0 (20.4)	2.0 (4.1)	0.0 (0.0)	1.1 (1.4)	0.34 (0.30)
Control	33.3 (8.7)	12.9 (1.8)	23.7 (2.2)	3.3 (1.2)	8.4 (0.8)	2.7* (0.8)	15.9 (17.8)	24.2 (24.9)	65.6 (5.5)	4.3 (7.2)	4.9 (2.2	1.1 (0.43	0.48 (6.1)
Low temp.	33.7 (13.1)	12.6 (1.4)	20.5 (2.2)	3.7 (3.4)	112.1 (1.8)	4.7 (7.6)	12.5 (7.6)	27.1 (2.7)	54.0 (14.3)	7.5 (3.8)	11.0 (13.4)	0.4 (0.8)	0.53 (0.14)
Control	37.4 (1.1)	12.7 (1.1)	19.6 (0.1)	3.1 (0.2)	10.9 (0.2)	2.6 (0.1)	14.0 (0.5)	26.7 (0.3)	44.5 (1.0)	12.7 (0.6)	15.9 (1.7)	0.3 (0.4)	0.60 (0.02)
High temp.	33.3 (4.1)	12.1 (0.3)	22.8 (1.3)	2.5 (1.7)	2.6 (2.3)	2.1 (0.4)	19.7* (0.6)	30.2 (2.1)	56.1 (5.1)	3.0 (2.8)	6.0* (3.1)	4.7 (2.5)	0.54 (05)
Control	39.7 (1.6)	12.1 (1.0)	21.1 (2.6)	2.1 (1.1)	7.9 (1.6)	1.8 (1.1)	15.5* (1.6)	29.0 (3.2)	54.3 (1.0)	2.4 (3.4)	13.8* (0.0)	0.6 (0.8)	0.53 (0.05)
Low P	33.6 (5.0)	11.9* (0.9)	27.6 (4.8)	3.9 (1.8)	7.8 (1.1)	1.5 (0.4)	13.9 (3.3)	38.6 (7.0)	40.8 (14.4)	5.6 (3.9)	13.1 (9.1)	2.0 (2.3)	1.04 (0.44)
Control	38.2 (6.3)	13.4* (0.6)	22.7 (8.3)	2.8 (1.3)	7.5 (1.9)	1.3 (0.5)	14.2 (2.4)	31.9 (11.4)	47.1 (5.0)	3.8 (3.0)	15.5 (8.6)	1.8 (2.4)	0.68 (0.24)
High P	31.4 (8.3)	12.9 (2.0)	22.6 (2.5)	4.1 (1.3)	9.5 (2.8)	3.6 (1.8)	15.9 (3.3)	30.2 (2.9)	54.8 (6.2)	6.4 (4.7)	6.4 (6.7)	2.2 (4.9)	0.55 (0.05)
Control	34.7 (2.8)	13.4 (0.6)	22.5 (5.5)	3.0 (0.6)	9.0 (2.4)	3.2 (1.6)	14.4 (1.4)	30.2 (7.9)	53.3 (11.6)	6.0 (6.8)	8.8 (5.7)	1.7 (3.5)	0.58 (0.16)

Table 7: Phosphorus forms, in relative percent (%), determined by 31P NMR spectroscopy for algal cultures grown under control conditions and under temperature (temp.), P nutrition (low P, high P) and light stress. Orth, orthophosphate; pyro, pyrophosphate; poly, polyphosphate; mono1, orthophosphate monoester 1 region; mono2, orthophosphate monoester 2 region; OthDi, orthophosphate diesters other than DNA; Porg, organic P calculated from 31P NMR spectra. See text for details on these regions. Results shown are averages and (standard deviation) [Barbara J. Cade-Menun *et al.* (2010)]

	Orth	Pyro	Poly	Mono1	Mono2	DNA	OthDi	Porg
Low light	41.3 (11.6)	13.5 (9.0)	0.7 (0.9)	5.5* (3.0)	32.3 (13.0)	5.8 (3.0)	1.0 (0.6)	44.5 (16.6)
Control	33.1 (11.8)	16.2 (13.2)	0.5 (0.7)	10.7* (6.3)	31.4 (17.3)	7.1 (7.8)	1.0 (1.3)	50.3 (19.4)
High light	46.0 (7.6)	9.4 (6.4)	0.0 (0.0)	6.5 (2.5)	33.9 (7.5)	3.7 (2.7)	0.4 (0.5)	44.6 (9.7)
Control	33.5 (12.3)	6.6 (3.1)	0.0 (0.0)	8.9 (3.1)	42.7 (5.7)	7.2 (8.1)	1.1 (1.2)	59.9 (14.9)
Low temp.	70.8* (3.9)	10.6 (11.0)	0.0 (0.0)	3.3 (1.1)	12.0 (6.4)	2.8 (1.8)	0.7 (0.6)	18.8 (8.7)
Control	79.3* (0.5)	1.5 (0.9)	0.4 (0.5)	3.1 (1.4)	13.2 (0.3)	1.9 (0.7)	0.8 (0.1)	19.0 (1.9)
High temp.	54.0 (6.5)	18.7 (6.3)	0.3 (0.5)	3.8 (1.2)	19.8 (7.2)	2.3 (1.4)	1.1 (0.6)	27.1 (7.4)
Control	59.4 (10.7)	11.9 (9.6)	0.3 (0.5)	3.8 (1.2)	21.0 (14.1)	1.8 (0.9)	0.3 (0.5)	28.5 (17.4)
Low P	37.8 (24.4)	9.0 (10.0)	0.3 (0.7)	9.1 (3.6)	27.2 (17.0)	13.1 (13.7)	3.4 (5.6)	52.9 (27.3)
Control	50.8 (27.5)	20.2 (13.8)	0.7 (0.7)	6.1 (6.8)	16.9 (9.7)	4.5 (7.4)	0.9 (1.1)	28.4 (18.7)
High P	54.1* (11.6)	37.2* (18.2)	0.0 (0.0)	2.1 (1.8)	5.2 (5.0)	1.2 (0.4)	0.2 (0.4)	8.7 (7.4)
Control	79.3* (10.1)	2.4* (0.6)	0.1 (0.3)	2.9 (1.6)	11.8 (6.5)	2.3 (1.8)	1.2 (1.3)	18.2 (10.5)

Table 8: Phosphorus forms, in μmol g−1, determined by 31P NMR spectroscopy for algal cultures grown under control conditions and under temperature (temp.), P nutrition (low P, high P) and light stress. Orth, orthophosphate; pyro, pyrophosphate; poly, polyphosphate; mono1, orthophosphate monoester 1 region; mono2, orthophosphate monoester 2 region; OthDi, orthophosphate diesters other than DNA; Porg, organic P calculated from 31P NMR spectra. See text for details on these regions. Results shown are averages and (standard deviation) [Barbara J. Cade-Menun *et al.* (2010)]

	Orth	Pyro	Poly	Mono1	Mono2	DNA	OthDi	Porg
Low light	124.2 (67.5)	44.8 (36.0)	2.6 (03.4)	14.8 (8.4)	84.2 (31.1)	15.2 (8.9)	2.3 (1.1)	116.5 (38.8)
Control	96.3 (49.7)	53.5 (49.4)	1.8 (2.3)	31.0 (26.7)	75.2 (43.0)	18.0 (22.9)	2.5 (3.5)	126.6 (47.6)
High light	212.0* (60.9)	38.3 (20.2)	0.0 (0.0)	27.9 (8.6)	156.1* (60.4)	16.1 (12.7)	1.8 (2.1)	201.8* (62.6)
Control	80.6* (48.8)	16.5 (12.7)	0.0 (0.0)	18.3 (8.5)	88.8* (31.6)	11.4 (6.8)	1.8 (1.3)	120.3* (35.7)
Low temp.	203.9 (165.2)	49.4 (74.6)	0.0 (0.0)	7.9 (3.4)	25.3 (5.4)	7.2 (4.8)	2.5 (3.1)	42.9 (15.9)
Control	161.2 (37.9)	2.7 (1.2)	0.6 (0.6)	6.7 (4.4)	26.8 (5.9)	4.0 (2.4)	1.5 (0.5)	39.0 (13.2)
High temp.	149.4 (109.6)	62.5 (66.2)	0.4 (0.7)	12.4* (12.4)	58.4 (51.3)	8.7 (10.9)	4.3 (5.4)	83.8 (79.5)
Control	411.0 (269.3)	85.8 (68.8)	1.7 (3.0)	29.4* (12.4)	112.8 (41.8)	153.0 (39.7)	10.0 (4.3)	153.0 (39.7)
Low P	137.2 (231.1)	15.3* (12.2)	0.2* (0.4)	28.4 (38.9)	39.9 (43.6)	25.4 (29.5)	3.9 (3.4)	97.6 (71.6)
Control	332.1 (297.5)	87.5* (40.0)	2.6* (2.4)	27.0 (22.1)	84.4 (47.1)	18.8 (27.8)	3.7 (4.2)	134.0 (64.6)
High P	1366.1 (854.8)	1293.0* (1196.2)	0.0 (0.0)	38.1* (8.4)	89.0* (30.6)	29.9* (16.1)	1.4 (3.1)	158.3* (47.8)
Control	782.1 (1229.6)	22.8* (33.5)	0.2 (0.5)	16.9* (21.4)	46.3* (35.0)	10.0* (10.8)	2.7 (2.6)	75.8* (65.6)

Table: 9 Lipid synthesis enhancement genes (enzymes) [Christopher Q. Lan *et al.* (2009)]

Gene (enzyme)	Source-species	Receiver-species	Note	Refs
accA, accB, accC, accD, (ACC), teas (thioesterase I)	*E. coli* (BL21) (bacteria)	*E. coli* (BL21) (bacteria)	6× fatty acid Synthesis	Davis *et al*. (2000)
Acc1 (cytosolic ACC)	*Arabidopsis* (plant)	*Brassica napus* (plant)	1–2× plastid ACC + 6% fatty acid content	Roesler *et al.* (1997)
Acc1 (ACC)	*Arabidopsis* (plant)	*Solanum tuberosum* (plant)	5× TAG content	Klaus *et al.* (2004)
Acc1 (ACC)	*Cyclotella cryptic* (alga)	*Cyclotella cryptica* (alga)	2–3× ACC activity, no change in lipid content	Dunahay *et al.* (1995) and Dunahay *et al.* (1996)
Acc1 (ACC)	*Cyclotella cryptic* (alga)	*Navicula saprophila* (alga)	2–3× ACC activity, no change in lipid content	Dunahay *et al.* (1995) and Dunahay et al. (1996)
fabF (KAS II)	*E. coli* (bacteria)	*E. coli* (bacteria)	Toxic (CoA pool from 0.5–40% in malonyl-CoA)	Subrahmanyam and Cronan (1998)
fabH (KAS III)	*E. coli* (bacteria)	*Brassica napu* (plant)	Stress, arrest of the cell Growth	Verwoert *et al.* (1995)
KAS III	*Spinacia oleracea* (plant)	*Nicotiana tabacum* (plant)	16:0 accumulation lower oil Content	Dehesh *et al.* (2001)
KAS III	*Spinacia oleracea* (plant)	*Arabidopsis* (plant)	16:0 accumulation lower oil Content	Dehesh et al. (2001)
KAS III	*Spinacia oleracea* (plant)	*Brassica napus* (plant)	16:0 accumulation lower oil Content	Dehesh *et al.* (2001)
LPAT	*Saccharomyces cerevisiae* (yeast)	*Brassica napus* (plant)	6× oil content	Zou *et al*. (1997)
are1 and are2 (DGAT)	*Arabidopsis thaliana* (plant)	Yeast	3–9× TAG content	Bouvier-Nave *et al*. (2000)
are1 and are2 (DGAT)	*Arabidopsis thaliana* (plant)	*Nicotiana tabacum* (plant)	7× TAG content	Bouvier-Nave *et al*. (2000)
DGAT	*Arabidopsis* (plant)	*Arabidopsis* (plant)	+10–70% oil Content	Jako *et al*. (2001)
acs (ACS)	*E.coli* (MG1655) (bacteria)	*E.coli* (MG1655) (bacteria)	9× ACS activity, increased acetate assimilation	Lin *et al.* (2006)
malEMt and malEMc (ME)	*Mortierella alpina* and *Mucor circinelloides*(fungi)	*Mucor Circinelloides* (fungi)	2.5× lipid accumulation	Zhang *et al.* (2007)
ACL	Rat	Tobacco	+16% lipid content	Rangasamy and Ratledge (2000)
Antisens PEP gene	*Agrobacterium tumefaciens*	*Brassica napus*	+6.4–18% oil Content	Chen *et al.* (1999)

Temperature Stress:

Growth under low and high temperature did not produce any significant differences relative to control cultures for total P, C, and N, or the C:N; C:P and N:P ratios (Table 5). None of these parameters were significantly correlated with temperature in correlation analyses combining low temperature, high temperature and control samples. There were no significant differences in C spectral regions or modeled C forms for low-temperature cultures relative to controls (Table 6). The high-temperature cultures, however, had a significant increase in the carbonyl C (C VII) spectral region, and a significant decrease in lipids relative to controls (Table 6). Correlation analyses combining low temperature, high temperature and control samples showed negative correlations to the C V (r=−0.72; P=0.02) and C VI regions (r=−0.62; P=0.06), and positive correlations to the C VII region (r=0.61; P=0.6) and to carbonyl (r=0.71; P=0.02).Principal components analysis also showed a significant difference in 13C CPMAS spectra for high temperature cultures relative to controls, but no significant differences for low-temperature samples. Low-temperature stress produced a significant decrease in the relative percent of orthophosphate (Table 7), but no changes in the relative percentages of other P forms (Table 8). There were no significant differences in concentrations of any P forms in the low temperature samples compared with the controls. The high-temperature cultures were not significant different from controls in any P forms, in relative percent or concentration. Principal components analyses and correlation analyses combining low temperature, high temperature and control samples did not show any significant relationships for P forms or compound classes.

Phosphorus stress:

Growth under low-P conditions did not produce significant differences in total P, N or C, or the C:N ratio, relative to controls (Table 5). However, it did produce significantly higher C:P and N:P ratios. Growth under high-P conditions also did not produce significant differences in or P, N and 2C, or the C:N ratio, relative to controls, but the C:P and N:P ratios were significantly lower than the controls. Correlation analysis combining high and low-P treatments with controls showed a positive correlation with total P (r=0.67; P=0.001) and negative correlations of P nutrition with the C:P (r=−0.45; P=0.05) and N:P (r=−0.46; P=0.04) ratios. Low-P nutrition resulted in a significant decrease in the N-alkyl and methoxyl C (C II) spectral region, but no significant differences in modeled C forms or the carbohydrate:protein ratio (Table 6). High-P nutrition did not produce any significant differences in C spectral regions or modeled C forms. Correlation analysis combining high and low-P treatments with controls showed P nutrition to be positively correlated with the O-aromatic C (C VI) spectral region (r=0.47; P=0.05). There were no significance differences in the 13C CPMAS spectra from PCA. Low-P nutrition did not produce any significant differences in P forms, in relative percent or concentration (Tables 7 and 8), and did not show any differences with PCA of the 31P NMR spectra. High-P nutrition significantly decreased the relative percent of orthophosphate and increased the relative percent of pyrophosphate (Table 7). It also significantly increased the concentrations of pyrophosphate,monoester 1, monoester 2, DNA and organic P (Table 8), and produced significant differences in the 31P NMRspectra, as shown by PCA. Correlation analyses combining low-P, high-P and control samples showed that P nutrition was positively correlated with pyrophosphate (r=0.67; P=0.001) and negatively correlated with monoester 1 (r=−0.41; P=0.07), monoester 2 (r=−0.50; P=0.02) and organic P (r=−0.51; P=0.02).

Nitrogen stress:

Using the same microalga, an additional investigation was carried out on the effect resulting from a reduction of the nitrogen concentration in the medium. Nitrogen limiting conditions were in fact reported to significantly increase the lipid fraction of many microalgae [Illman *et al.* (2000)]. For this purpose, the concentration of nitrate in both media for *N. oculata* and *C. vulgaris* batch growth was reduced to half and quarter of the standard media described in Section 2 while the light intensity and the air fluxwere kept the same trough the experiments. Whereas its specific growth rate was not significantly affected, a threefold increase in lipid content took place. In contrast, *N. oculata* showed a gradual decrease in the growth rate accompanied by almost a duplication of the lipid content. These results as a whole suggest that, to perform an effective biodiesel production from microalgae, the optimum compromise between a slowdown in growth and the increase in the lipid fraction should be achieved.

Effect of pH, Light, Temperature on Lipid Production

Effect of pH:

The microalgae are sensitive to pH changes, being its control essential for keeping high growth rates. As microalgae are able to metabolise the inorganic carbon CO_2, there is an equilibrium trend for the pH to increase. Its control can be done by using buffers or inorganic acids. pH control is not very difficult to achieve and it can be also help to pressure a pure culture.

The effect of pH on growth of the alga and hydrocarbon yields was studied using Chu 13 media in the pH range of 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5. The experiment was carried out in Erlenmeyer flasks (150ml) containing 40ml of Chu 13 modified medium and the pH of the medium was adjusted before autoclaving. All the flasks were inoculated uniformly at 25% (v/v) inoculums of 2 weeks old microalgae (Botryococcus braunii) culture. The culture flasks were inoculated for 3 weeks at 25 ± 1˚C temperature with 1.2 ± 0.2 klux and 16:8 hrs light dark cycle, and the culture was harvested and analysed for biomass and hydrocarbon yields.

Effect of Light:

A considerable amount of work has been done on the effect of high-frequency light:dark fluctuations (>100 Hz) on the specific-growth rate or oxygen production rate. The chlorophyt *Chlorella* is able to use light flashes of 1 ms or less as efficiently as continuous light of the same time-averaged photon flux density (PFD). These light flashes were followed by dark periods 5–40 times as long. This effect is called the flashing-light effect (Kok, 1953; Phillips and Myers, 1954; Matthijs *et al.* 1996; Nedbal *et al.* 1996) A turbidostat-type reactor was used for cultivation of *C. reinhardtii* for 1 or 2 weeks under a certain light regime. The turbidostat in the suspension volume was 70 ml and the height of the suspension was 3 cm. Illumination of the turbidostat was provided by a halogen lamp (20, 35 or 50 W). The lamp could be dimmed with only minor effects on the spectral distribution. The lamp was connected to a timer to apply a 16:8 h day:night cycle. Two other timers, determining on- and off-time, were used to impose different light:dark cycles during the 16 h day period. The optical density in the turbidostat was monitored by means of a 2-p PAR-sensor (IMAGDLO, The Netherlands) placed

under the bottom of the reactor to measure the out-going PFD. This PFD was continuously compared to a setpoint and when it was lower the suspension was temporarily diluted. In this way the optical density at 680 nm (OD680) of the algal culture was maintained between 0.17 and 0.25. The turbidostat was also equipped with a water jacket connected to a temperature-controlled water bath, 24–26.5°C. The suspension was gently bubbled with an air:carbon dioxide mixture at 11 l h^{-1}, and mixed with a magnetic stirring bar. The pH of the culture was maintained between 6.6 and 6.9 by setting the carbon dioxide concentration to 3–10% v/v. Carbon dioxide was never limiting under these conditions. The turbidostat was operated under non-aseptic conditions. The cultures were checked regularly under a microscope but contamination was never observed.

Effect of Temperature:

Chlorella vulgaris growth appeared to be affected at temperatures above 30˚C. Further increase in temperature (38˚C) led to an abrupt interruption of microalgal growth, and later the cells dead. This result was easily visible because of color change of the cells from green to brown, and consequently the microalgal growth rate was even negative by Brown *et al.* As far as *N. oculata* is concerned, changes from the optimal conditions of growth (20 ◦C) also resulted in significant changes in the microalgae growth rate. For temperatures below this threshold, growth rate more than halved, falling from 0.13 to 0.06 day−1. A sharp drop in microalgae growth ratewas also noticed at higher temperature (25 ◦C), as reported in the literature Brown *et al.* (1998).

Low-temperature-induced ß-carotene and fatty acid synthesis, and ultrastructural reorganization of the chloroplast in *Dunaliella salina* (Chlorphyta) observed by Mendoza *et al.* (1996) The effect of suboptimal growth temperature on ß-carotene and fatty acid biosynthesis, and on the ultrastructural reorganization of the chloroplast, in the green unicellular alga *Dunaliella salina* has been studied. A decrease from the optimal temperature for growth (30ºC) to a suboptimal (18ºC) temperature induced ß-carotene synthesis and increased lipid content in *D.salina* cells, therby promoting the formation of lipid-carotene globules in the chloroplast periphery. The content of polyunsaturated fatty acid was higher in cells cultured at low temperature. Their results suggested that the induction of carotenogenesis and accumulation of polynunsaturated fatty acids are mechanisms of acclimation to unfavourable environmental contains for growth.

Effects of lowering temperature during culture on the production of polyunsaturated fatty acids in the marine diatom *Phaeodactylum tricornutum* (Bacillariophyceae) recorded by Hanming Jiang and Kunshan Gao (2004) The composition of fatty acids and contents of eicosapentaenoic acid (EPA) and polyunsaturated fatty acids (PUFAs) of the economically important marine diatom, *Phaeodactylum tricornutum* (Bohlin), were investigated to se whether reducing the culture temperature enhances the production of EPA and PUFAs. The contents of EPA and PUFAs of *P. tircornutum* were found to be higher at lower temperature when cultured at 10, 15, 20, or 25'C. When the cells grown at 25'C were shifted to 20, 15, or 10'C, the contents per dry mass of PUFAs and EPA increased to the maximum values in 48, 24, and 12h, respectively. The highest yields of PUFAs and EPA per unit dry mass (per unit volume of culture) were 4.9% and 2.6% (12.4 and 6.6 mg.L) respectively, when temperature was shifted from 25 to 10'C for 12h, both being raised by 120% compared with the control. The representative fatty acids in the total fatty acids, when temperature was lowered from 25 to 10'C decreased proportionally by about 30% in C16.0 and 20% in C16:1 (n-7) but increased about 85% in EPA. It was concluded that lowering culture temperature of *P. tricornutum* could significantly raise the yields of EPA and PUFAs by Hanming Jiang and Kunshan Gao (2004).

Temporal variation in fatty acid composition of *Ulothrix zonata* (Chlorophyta) from ice and benthic communities of Lake Baikal by Svetlana Osipova *et al.* (2009) Fatty acid (FA) composition of total lipids was compared between populations of the freshwater alga *Ulothrix zonata* growing in the interstitial water of the ultrapure ice cover of Lake Baikal in early spring (ice population). FA content was analysed by chromatographic mass spectrometry. Their observations over two years indicated that the FA profiles of both Baikalian ice and benthic populations of *U. zonata*, as are typical for other green algae, showed a high level of polyunsaturated FA (50-70% depending on season) related to the adaptation of the alga to low temperature. Rising temperature in the algal habitat entailed a prominent drop in the prominent drop in the proportion of monoenoic cis-7-hexadecenoic acid (from 0.8% to 3.5% in 2006). These results favour the hypothesis that monoenoic. FAs play a principal role in maintaining the cellular membrane fluidity of *U. zonata* Osipova *et al.* (2009).

Genetic Engineering approaches

Biotechnology and Genetic Engineering:

Although biotechnological processes based on transgenic microalgae are still in their infancy, researchers and companies are considering the potential of microalgae as green cell-factories to produce value-added metabolites and heterologous proteins for Pharmaceutical applications, Leon-Banares *et al.* (2004). The commercial application of algal transgenicsis beginning to be-realized and algal biotechnology companies is being established. It was predicted that microalgae, due to the numerous advantages they present, could offer a powerful tool for the production of commercial molecules in a near future (Cadoretetal, 2008). The fast growing interests in the use of transgenic microalgae for industrial applications is powered by the rapid developments in microalgal biotechnology. Complete genome sequences from the Red alga *Cyanidioschyzon merolae* (Nozaki *et al.* 2007), the diatoms *Thalassiosira pseudonana* (Armbrust *et al.* 2004) and *Phaeodactylum tricornutum* (Bowler *et al.* 2008) and the unicellular green alga *Ostreococcustauri* (Derelle *et al.* 2006) have been completed. Nuclear transformation of various microalgal species is now a routine, chloroplast transformation has been achieved for green, red, and euglenoid algae,and further success in organelle transformation is likely as the number of sequenced plastid, mitochondrial, and nucleo morph genomes continues to grow (Walker *et al.* 2005). Various genetic transformation systems have been developed in green algae such as *Chlamydomonas reinhardtti* and *Volvox carteri* (Walker *et al.* 2005). The fast developments of microalgal biotechnology permit the Isolation and use of key genes for genetic transformation. Of particular relevance, acetyl-CoA carboxylase (*ACC*) was first isolated from the microalga *Cyclotella cryptica* in 1990 by Roessler (1990) and then successfully transformed by Dunahay et al. (Dunahay *et al.* 1995, 1996; Sheehan *et al.* 1998) into the diatoms *C. cryptic* and *Navicula saprophila.* The *ACC* gene, acc1, was overexpressed with the enzyme activity enhanced to 2–3-folds. These experiments demonstrated that *ACC* could be transformed efficiently into microalgae although no significant increase of lipid accumulation was observed in the transgenic diatoms (Dunahay et al., 1995, 1996). It also suggests that over expression of ACC enzyme alone might not be sufficient to

enhance the whole lipid biosynthesis pathway (Sheehan *et al.* 1998). Even though there is no success story with respect to lipid over production of microalgae using the genetic engineering (GE) approach up to now, a solid understanding towards the global TAG biosynthesis path-way, which is generally accepted to be identical throughout all species except the differences in the location of reactions and the structure of some key enzymes, has been established. Extensive studies have also been carried out regarding the enhancement of lipid production using the GE approach in different species. This review will provide a valuable back ground for future studies with microalgae by Sheehan *et al.* (1998).

Genetic engineering for lipids production:

In 1960s, cyanobacteria were chosen as ideal material for academic research by scientists. Since the genome of *Anabaena* PCC7120 was successfully cloned, the number of cloned functional genes in cyanobacteria has increased to over 130 Qin (1999). *Acc1* is a kind of restriction enzyme which was cloned from oceanic diatom *Cylclotella cryptic* had been efficiently expressed in *C. cryptic* for the production of bio-fuel Roessler (1988). A new method for biodiesel production from microalgal oils has been developed by the application of genetic engineering recently. National Renewable Energy laboratory in the USA (NREL) has established engineered microalgae which belong mostly to diatom species. The lipid content of the engineered microalgae increased to above 60% in laboratory conditions and above 40% in outdoors cultivation, whereas the lipids content in microalgae is 5-20% in common natural conditions.

The improvement of lipid content in engineered microalgal cells is mainly due to the high expression of acetyl-CoA carboxylase gene, which plays an important role in the control of the level of lipid accumulation. At present, the research has focused on choosing a proper molecular carrier, making *ACC* gee full expression in bacteria, yeast, and plant. Furthermore, the modified *ACC* gene is being introduced into microalgae to obtain more efficient expression. The utilization of engineered microalgae for the production of biodiesel has important economic and environmental benefits. Its superiorities include high yield of microalgae; saving agricultural resources by using seawater as natural medium; the cellular content of lipids in microalgae is several times higher than that in terrestrial plants.

Enhancement of lipid production using biochemical, genetic and transcription factor engineering approaches was described by Courchesne *et al.* (2009) Three possible strategies for enhanced lipid overproduction in microalgae include : the bio-chemical engineering (BE) approaches, the genetic engineering (GE) approaches, and the transcription factor engineering (TFE) approaches. The BE strategy relies on creating a physiological stress such as nutrient-starvation or high salinity to channel metabolic fluxes to lipid accumulation. The GE strategy exploits our understanding to the lipid metabolic pathway, especially the rate-limiting enzymes, to create a channeling of metabolites to lipid biosynthesis by overexpressing one or more key enzymes in recombinant microalgal strains. The TFE strategy is an emerging technology aiming at enhancing the production of a particular metabolite by means of overexpressing TFs regulating the metabolic pathways involved in the accumulation of target metabolities. Currently, BE approaches are the most established in microalgal lipid production. The TFE is a very promising strategy because it may avoid the inhibitive effects of the BE approaches and the limitation of "secondary bottlenecks" as commonly observed in the GE approaches. However, it is still a novel concept to be investigated systematically.

Research on - fatty acid desaturases from the microalgae Thalassiosira pseudonana was reported by Thierry Tonon, et.al., (2005) Analysis of a draft nuclear genome sequence of the diatom *Thalassiosira pseudonana* revealed the presence of 11 open reading frames showing significant similarity to functionally characterized fattyacid front-end desaturases. The corresponding genes occupy discrete chromosomal locations as determined by comparision with the recently published genome sequence. Phylogenetic analysis showed that two of the *T. pseudonana* desaturase (*Tpdes*) sequences grouped with proteobacterial desaturases that lack a fused cytochrome b5 domain. Among the nine remaining gene sequences, temporal expression analysis revealed that seven were expressed in *T. pseudonana* cells. One of these, *TpdesN*, was previously characterized as encoding a Δ11-desaturase active on palmitic acid. From the six remaining putative desaturase genes, we report here that three, *TpdesI, TpdesO* and *TpdesK,* respectively encode Δ6-, Δ5-and Δ4-desaturases involved in production of the health beneficial polyunsaturated fatty acid DHA (docosahexaenoicacid).

CONCLUSION

This review focused research on biosynthesis of TAG, from microalgae. A critical evaluation of the relationship between the cell cycle and TAG production is needed. Understanding of this mechanism will enable genetic manipulation of specific algal strains that show rapid growth and high lipid accumulation for increased biofuel production. Biofuel production from microalgae needs more research. Research in lipid metabolism and lipid producing genes will help to improve biofuel production to the required level. Microalgae are a natural resource which can act as high lipid feedstock and further genetic manipulation is need for producing potential fuel. Cultivation and harvesting of microalgal biomass is problematic still. So further improved techniques are needed to resolve these problems. Use of a biorefinery concept and advances in raceway ponds engineering will help to reduce the cost of production. Further, research in genetic engineering, biomass production and downstream process will develop enormous biofuel in future.

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