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Screening and Identification of Everglades Algal Isolates for Biodiesel production

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FLORIDA INTERNATIONAL UNIVERSITY

Miami, Florida

SCREENING AND IDENTIFICATION OF EVERGLADES ALGAL ISOLATES FOR
BIODIESEL PRODUCTION

A thesis submitted in partial fulfillment of the

requirements for the degree of

MASTER OF SCIENCE

in

ENVIRONMENTAL STUDIES

by

Priyanka Narendar

2010

To: Dean Kenneth Furton
College of Arts and Sciences

This thesis, written by Priyanka Narendar, and entitled Screening and Identification of Everglades Algal Isolates for Biodiesel Production, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this thesis and recommend that it be approved.

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Florida International University, 2010

DEDICATION

I dedicate this thesis to my parents. They always believed in me and rendered their unconditional love and support that helped me complete this work.

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I am deeply indebted to my major advisor Dr. Krishnaswamy Jayachandran, for his constant guidance and motivation throughout my graduate career, without which I could not have accomplished this work. I also thank him for providing me with funding and all the resources necessary for the successful completion of this research.

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ABSTRACT OF THE THESIS
SCREENING AND IDENTIFICATION OF EVERGLADES ALGAL ISOLATES FOR
BIODIESEL PRODUCTION

by

Priyanka Narendar

Florida International University, 2010

Miami, Florida

Professor Krishnaswamy Jayachandran, Major Professor

This project investigates the potential of the 27 Everglades green algal strains for producing biodiesel. The five potential strains chosen by measuring the neutral lipid content using the Nile red method were *Coelastrum* 46-4, *Cocoid green* 64-12, *Dactylococcus* 64-10, *Stigeoclonium* 64-8 and *Coelastrum* 108-5. *Coelastrum* 108-5 and *Stigeoclonium* 64-8 yielded the same amount of lipids in both Gravimetric and Nile red method. A linear relationship between algal biomass and lipid accumulation was seen in *Coelastrum* 46-4, *Cocoid green* 64-12, *Stigeoclonium* 64-8 and *Coelastrum* 108-5 indicating that increase in algal biomass increased the lipid accumulation. Nitrogen and phosphorous stress conditions were also studied where higher lipid accumulation was observed significantly ($p < 0.05$) in 64-8 *Stigeoclonium* and 64-12 *Cocoid green*. Collectively, it could be summarized that *Coelastrum* 108-5, *Cocoid green* 64-12 and *Stigeoclonium* 64-8 were promising in some aspects and could be used for further studies.

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LIST OF ABBREVIATIONS

| | |
|--------------------|---|
| ASP | Aquatic Species Program |
| TAG | Triacylglycerides |
| DMSO | Dimethyl sulfoxide |
| PUFA | Polyunsaturated fatty acids |
| USEPA | United States Environmental Protection Agency |
| μm | micron meter |
| d | Day(s) |
| Exp | experiment |
| e | exponential |
| ml | milliliters |
| μg | micron gram |
| μl | micron litre |
| OD | Optical Density |
| nm | nano meter |
| $^{\circ}\text{C}$ | Degree centigrade |
| mg | milligram |
| g/L | grams per liter |
| % dw | percentage dry weight |

1. INTRODUCTION:

1.1 Need for Renewable Energy Resources:

The need for renewable sources of energy is increasing rapidly as a result of increase in global population and industrialization. Conventional sources of energy like natural gas, oil and coal have caused immense damage to the environment by increasing the carbon in the atmosphere causing various global climatic events, such as global warming (Sawayama et al., 1995). With 5% of the world's population, the United States consumes 25% of the world's petroleum, 43% of the gasoline and 25% of the natural gas. According to Oil and Gas Journal (O & GJ) estimates, at the beginning of 2004, the world wide reserves were 1.27 trillion barrels of oil and 6,100 trillion cubic feet of natural gas. Although these are proven recoverable reserves, today's consumption rate of about 85 million barrels per day of oil and 260 billion cubic feet per day of natural gas, suggest the reserves can last for the next 40 years for oil and 64 years for natural gas. According to the United States Environmental Protection Agency (US EPA), more than 90% of the greenhouse gas emissions are from the burning of fossil fuels. Combustion of fossil fuels produces harmful gases like nitrogen oxides, sulfur dioxide, volatile organic compounds and heavy metals. Therefore, the increase in the levels of these gases has contributed to environmental impacts such as global warming, acid rain and air quality deterioration. Also, the nation's dependence on nations of the Middle East for oil makes the country vulnerable to oil price spikes and supply disruptions. The rapid diminishing and increasing prices of fossil fuels, combined with increasing concerns of energy security and climate change have led to the research and development of renewable, cleaner energies such as, biomass derived fuels or biofuels (Chisty, 1980). Usage of biomass derived fuels in transportation can increase energy security, reduce green house gas emissions, enhance rural economies and protect ecosystems.

1.2 Biodiesel:

Biodiesel is one form of a biomass derived fuel. Biodiesels are made up of fatty acid methyl esters (FAMES) that are derived from triglycerides by the transesterification process. Biodiesel is currently receiving much attention because of its potential as a sustainable and environmental friendly alternative to the conventional fuels. There are several advantages of using biodiesel over conventional fuels. Biodiesel is safer than conventional petroleum based diesel for transportation uses because it contains low sulfur and polycyclic aromatic hydrocarbons (PAHs) compared to conventional fuels. Low sulfur is important in reducing the sulfur emissions, which are a significant source of acid rain. PAHs are a group of hydrocarbons found in heavy oil petroleum, which have complex ring structures that make them insoluble, slow to burn and carcinogenic (Wedel, 1999). Biodiesel blends have reduced amount of emissions of PAHs which would have reduced impact on health and environment. Moreover, biodiesel is oxygenated, making it a better lubricant than diesel fuel thus increasing the engine life. Oxygenation also means biodiesel is combusted more completely. Also, biodiesel has a higher cetane number which is a measure of fuel's ignition quality. Higher cetane number contributes to easy cold starting and low idle noise.

There are four primary ways to make biodiesel, direct use and blending, microemulsions, thermal cracking (pyrolysis) and transesterification (Ma and Hanna, 1999). In direct blending; blends at various concentrations can be used in the standard diesel engine without modification. Blends vary according to the geographical location and climate. Most commonly used blends vary from a blend of 2% biodiesel and 98% petroleum diesel, or "B2" up to a blend of 20% biodiesel and 80% petroleum diesel or "B20." Microemulsion diesel fuel technology uses a micro-emulsifier to compatibilize a diesel or biodiesel fuel and a water phase. The micro-emulsifier contains a surfactant and a compatibilizing agent (an oxygenate). Oxygenates used as micro-emulsifying agents include C₁₋₆ alcohols, benzyl alcohols and glycols (Griffith, 1990). Key

issues include oxygenate cost, flammability and impact on the stability of microemulsion fuel. The oxygenates must be cost effective to achieve economics, which are competitive with diesel fuels. Pyrolysis is a thermo-chemical conversion process used to produce energy from biomass. The organic compound is heated in the absence of oxygen to achieve decomposition. Transesterification is the most common way to manufacture biodiesel. The biodiesel can be used directly or blended with diesel fuel. (Zhang et al., 2003). Transesterification is processes in which an alcohol (e.g., methanol or ethanol) will be used in the presence of a catalyst (e.g., sodium hydroxide or potassium hydroxide) to chemically break the molecule of triglyceride into methyl or ethyl esters with glycerol as a byproduct (Peltiere, 2000). The majority of the fatty acids are in the form of Triacylglycerides (TAG), which is the suitable form of oil for biodiesel production. The short chain hydrocarbons are more ideal for biodiesel production (Pyle, Garcia et al. 2008). The most commonly synthesized fatty acids have chain lengths that range from C16 to C18 (C represents the number of carbon atoms in a fatty acid chain).

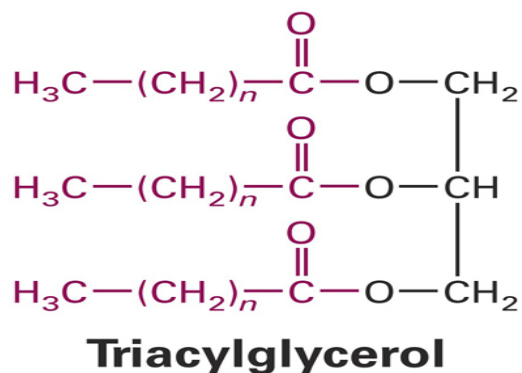


Figure 1: Molecular structure of Triacylglyceride

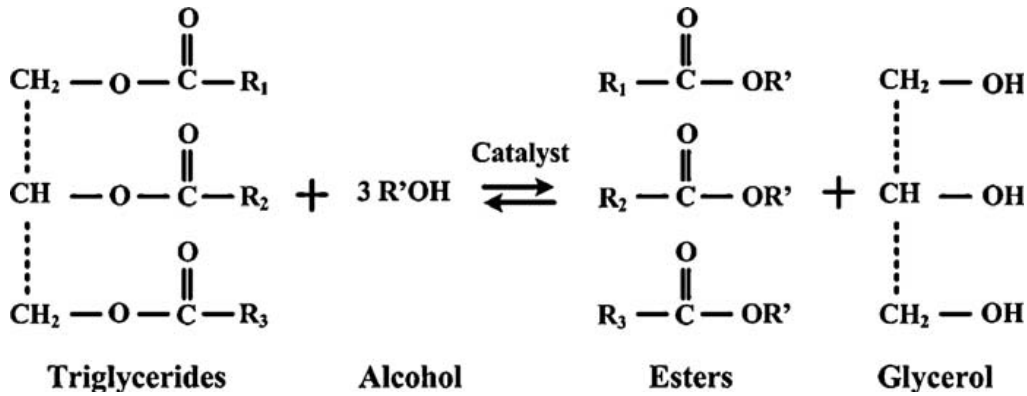


Figure 2: Transesterification reaction to produce biodiesel.

The structure of fatty acid esters determines the properties of biodiesel (Knothe, 2005). The most important characteristics include ignition quality, cold flow properties and oxidative stability. Although saturation and fatty acid profile do not have much influence on the production of biodiesel, they do affect the properties of the fuel produced. Biodiesel produced from feedstocks that are high in Polyunsaturated Fatty Acids (PUFA's) have good cold flow properties. PUFA's contain two or more double bonds. The individual fatty acids are named dioenoic, trienoic, tetraenoic, pentaenoic and hexaenoic fatty acids based on the number of double bonds and also based on the position of the first double bond from the terminal methyl end ω 3 PUFA (i.e., the third carbon from the end of the fatty acid) or ω 6 PUFA (i.e., the sixth carbon from the end of the fatty acid).

However, despite the benefits biodiesel commercialization can provide, the economic aspect of its production prevents its development and large-scale use, mainly because of the high-feed cost of vegetable oil (Antolin et al., 2002). The cost of biodiesel is over US \$0.5/L, compared to US \$0.35/L for normal diesel (Zhang et al., 2003). Exploring ways to reduce the high cost of biodiesel is of great interest in recent biodiesel research, especially for those methods concentrating on minimizing the raw material cost. Biodiesel has additional storage and management issues. Unlike petroleum based products, biodiesel tends to attract more moisture

which can lead to problems like fuel freezing, deposition of water in the fuel delivery system during cold weather and clogging of filters due to increased risk of microbial growth. Although, biodiesel is a reliable source of alternative energy resource but extensive research and development is needed to overcome the disadvantages of biodiesel and make them suitable for widespread commercial use.

1.2.1 Sources of producing Biodiesel:

The current sources of commercial biodiesel include soybean oil, oil palm, animal fat and waste cooking oil. These sources can be classified into three generations.

1.2.1.1 First generation fuels

The first generation of fuels is produced from feed stocks like soybean, rapeseed, oil palm and mustard. The disadvantages of using these sources are loss of biodiversity, excess utilization of water and increased greenhouse gas emissions. According to the US EPA, the burning of rapeseed can contribute nitrous oxide emissions, which can contaminate air. A report published by the United Nations Energy division in May 2007 states that biofuels from crop sources can increase greenhouse gas emissions as land would be converted from forests, wetlands and reserves to grow more soybean and mustard oil. Burning of forests would lead to an increased amount of carbon emissions that pollute the air.

First generation fuels also face serious moral issues, in the form of global food supply. If these fuels become lucrative for the farmers, they would start growing crops for fuel production instead of food production, which would lead to shortage of food, which in turn can lead to price hikes (food vs. fuel debate). The World Bank estimated that the food price had increased by 83% between 2005 and 2008. As a result, 100 million people have fallen into poverty and that 30% of that increase was a direct result of increased demand for biofuels (siteresources.worldbank.org/.../risingfoodprices_backgroundnote_apr08.pdf). In order to grow

crops for fuel, intensive farming techniques will be employed. These farming methods will have a wide range of negative impacts on the environment. Some of these impacts are soil erosion and excess use of pesticides and fertilizers. This increased use of pesticides and fertilizers leads to environmental problems like Eutrophication and water shortages. There are also issues with land availability with the first generation of biofuels. The amount of biomass required to replace a significant proportion of the fossil fuel used in the transportation sector runs into millions of tons. However, the crucial part is the biomass yield. Land use efficiency may be improved by choosing a crop with higher biomass yield. A downside of this approach is mono-cropping can lead to loss of soil fertility. Another concern with the first generation fuels is the low energy yield which makes it impractical for production on a large scale (Lang et al., 2001).

1.2.1.2 Second generation fuels

Second generation fuels are produced from lignocellulose. The goal of using second generation fuels is to produce fuel in a more sustainable fashion. Non-food crops such as Jatropha and Switch grass and residual parts of crops, such as stems, leaves and husks can be used for producing fuel. The advantage of using second generation fuels involves production of fuel on a sustainable basis. Lignocellulose ethanol can reduce the greenhouse gas emissions by 90% (Lang et al., 2001). However, the problem second generation fuels processes are addressing is to extract useful sugars that are locked inside by lignin and cellulose which are complex carbohydrates present in all plants. Lignocellulosic ethanol is made by freeing the sugar molecules from cellulose using enzymes. Enzymatic hydrolysis required to convert lignocellulose to ethanol is an expensive process and also poses a technical challenge. The following second generation fuels under current development are:

- a. Bio-Hydrogen is same as that of hydrogen except it is produced from a biomass feedstock. Biohydrogen can be used in fuel cells.

- b. BioDME, Fischer tropesch, Biohydrogen diesel are all used for the production of syngas. BioDME can also be produced from syngas using DME synthesis and also from Biomethanol using catalytic dehydration.
- c. Biomethanol is the same as methanol but it is produced from biomass. Biomethanol can be blended with petrol up to 10-20% without any infrastructure changes (Oliver, 2008)
- d. Butanol and Isobutanol are produced through the recombinant pathways expressed in hosts such as E.coli and yeast using glucose as carbon and energy source. (Evans, 2008)
- e. Fischer Tropsch (FT fuels) is produced using the Fischer- tropsch gas to liquid technology. FT fuels are not economically feasible because of the high energy investment for the FT process (Oliver, 2009).
- f. Wood diesel is a new form of biodiesel where oil is extracted and added directly to the diesel engines. The charcoal byproduct is added to the soil as a fertilizer. Since the carbon is put back into the soil, the biodiesel is carbon negative rather than carbon neutral. Carbon negative decreases the carbon dioxide in the air, reducing the green house effect.

Second generation fuels are not produced commercially, but there is a lot of research development going on in nations like the United States and also some emerging economies like India, China and Brazil in order to produce fuel in a more sustainable manner. More research and development of second generation fuels can prove to be an eco-friendly alternative to conventional fuel.

1.2.1.3 Third generation fuels:

Microalgae is one of the most promising alternative and renewable feedstock sources for producing biodiesel. These are the third generation fuels. The algae-for-fuel concept has gained renewed interest recently with the wide fluctuation in energy prices (Hu et al., 2007). Algae have

several advantages over conventional crops. Because of their simple cellular structure, algae have higher rates of biomass and oil production than other conventional crops (Becker, 1994). The per unit area of oil is estimated to be around 5000 to 20,000 US gallons per acre per year (Maryking, 2007). Microalgae have much faster growth rates compared to terrestrial crops. The other advantages of using algae are that they can be grown in marginal areas, thereby ensuring there is no competition with food crops (Tsukahara & Sawayama, 2005). Algae also use less water than the traditional oilseed crops. Algae do not need fresh water and can even tolerate saline or brackish water. Algae can efficiently sequester carbon-dioxide (CO₂), thereby reducing the emission of greenhouse gas (Wang et al., 2009). A variety of high value biofuels are produced from commercial scale cultivation of microalgal biomass. Some of the high value biofuels include methane, bio-hydrogen, bio-ethanol and bio-diesel. Methane is produced by anaerobic digestion of biomass (Spolaore et al., 2006). Bio-hydrogen and Bio-ethanol are produced by photobiologically active algae (Kapdan & Kargi, 2006) and biodiesel is derived from microalgal oil (Banerjee et al., 2002).

A favorable amount of different kind of lipids, hydrocarbons and other oils are produced in different algal species (Metzger & Largeau, 2005). A great deal of attention is given to neutral lipids (triacylglycerols) due to their lower degree of unsaturation and potential diesel fuel substitute (McGinnis et al., 1997). Some species of algae can accumulate about 50%-60% of their dry weight of neutral lipid in the form of Triacylglycerides. TAGs are the best substrates for biodiesel production (Hu et al., 2008). The fatty acids attached to the triacylglyceride structure within the algal cells can be short and long chain hydrocarbons. Fatty acids are either saturated or unsaturated. In most of the algae examined, saturated and mono-unsaturated fatty acids are predominately found (Borowitzka, 1988). The major synthesized fatty acid chain length ranges from C16 to C18, which are identical to conventional oleaginous crops (Ohlrogge and Browse,

1995). Due to the long chain fatty acid, some species of algae are capable of producing jet fuels. The U.S Department of Defense is sponsoring research in creating JP-8 jet fuel from microalgae.

Table 1: Production averages for common oil crops

Source: Adapted from Chisty, 2007

| Plant | Lb | Gallons of |
|--------------------|-------|------------|
| Algae ¹ | 6757- | 700 |
| Coconut | 2070 | 285 |
| Jatropha | 1460 | 201 |
| Rapeseed | 915 | 126 |
| Peanut | 815 | 112 |
| Sunflower | 720 | 99 |
| Soybean | 450 | 62 |

Based on the above mentioned advantages, third generation of fuels, i.e., algae fuels were chosen as the subject of research. This research investigated the native algal strains isolated from Everglades for its potential to produce biodiesel.

1.3 Algae – Definition:

Algae are sunlight- driven cells that convert carbon-dioxide to potential biofuels, foods and feed, and high value bioactives (Spolaore et al., 2006, Banerjee et al., 2005). Algae most commonly occur in water (fresh, marine or brackish) in which they may be suspended (planktonic) or live at the bottom (benthic). Few algae live at the water- atmosphere interface and are defined as neustonic. Some algae grow on moist rocks, wood, trees and on the surface of moist soils. The algae are eukaryotic organisms. The algae cell is surrounded by a thin, rigid cell wall. Algal cell wall contains polysaccharides, such as cellulose (glucan) or a variety of glycoproteins or both (Albert, 2000).

¹ Oil content in terms of dry weight

1.3.1 Classification and Characteristics of Algae:

There are two classifications of algae – Microalgae and Macroalgae. Microalgae are very small, plant like organisms which range from 1 – 50 μm (Chang, 2007) and can be seen only using a microscope. Microalgae have a high biomass yield and have high lipids in them. High amount of lipids within their cell structure makes them an interesting feedstock for biodiesel production. On the other hand, macroalgae can reach upto 60 cm in length (Mchugh, 2003). Macroalgae have a medium biomass yield, and contain only a small amount of lipids.

Selection of fast growing, productive strains are of primary importance to the success of any algal mass culture and for biodiesel production. Biologists have classified microalgae in a variety of classes, which are primarily differentiated by their pigmentation, life cycle and basic cellular structure. The four most important classes are (Borowitzka 1997):

- i. Diatoms (Bacillariophyceae) are mainly dominant phytoplankton of ocean, but are also found in fresh and brackish water, and store carbon in the form of natural oil or as a polymer of carbohydrate.
- ii. Green algae (Chlorophyceae) are commonly found in fresh water and swimming pools. Green algae store their energy in the form of starch, but oils can also be formed under certain growth conditions.
- iii. Blue green algae (Cynophyceae) are much closer to bacteria and play an important role in nitrogen fixation from atmosphere.
- iv. Golden algae (Chrysophyceae) appear yellow, orange or brown in color and they produce natural oil and carbohydrates as storage compounds.

The strains used in this research belong to the Chlorophyta class (Green algae). The green algae are globally distributed in fresh water, marine water and terrestrial ecosystems. They are often dominant in the fresh water environment. According to the fossil record, they are an ancient group of organisms found on this planet that are extremely diverse. Green algae contain

chlorophylls a and b, and store food as starch (Hoek et al., 1995) in their plastids and also secondary pigments like carotenes, lutein and zeaxanthin. The division contains both unicellular and multicellular species. Chlorophyta group of species also form symbiotic relationships with protozoa, sponges and cnidarians. Green algae has maximum growth rate at 30°C. Green algae represent the major taxonomic group from which oil producing candidates have been identified. It is not a result of fact that green algae naturally contains a considerable amount of lipid, but rather they are ubiquitous in diverse natural habitats, thereby easily isolated and grows faster than species from other groups under laboratory conditions. Chlorophyceae contains specific fatty acids that have carbon chains of between 16 to 18 units in length (C16:0 to C18:0). These carbon chain lengths have been shown to be ideal precursors for the production of biodiesel (Hu et al., 2008). Rodolfi et al (2008) screened species for high lipid productivity and found the marine species *Nannochloropsis* and *Tetraselmis* to be promising. Miao and Wu (2004) reported a heterotrophic growth of *Chlorella protothecoides* capable of yielding as high as 55% lipid content and converting the lipid to biodiesel. Allard & Templier (2000) extracted lipid from a variety of freshwater and marine microalgae and reported that lipid content varied from 1 to 26%. The table below shows the amount of lipid accumulation in various microalgae.

Table 1: Oil content of various microalgae.

| Microalga | Oil content (% dry wt) |
|----------------------------------|-------------------------------|
| <i>Botryococcus braunii</i> | 25–75 |
| <i>Chlorella</i> sp. | 28–32 |
| <i>Cryptocodinium cohnii</i> | 20 |
| <i>Cylindrotheca</i> sp. | 16–37 |
| <i>Dunaliella primolecta</i> | 23 |
| <i>Isochrysis</i> sp. | 25–33 |
| <i>Monallanthus salina</i> | > 20 |
| <i>Nannochloris</i> sp. | 20–35 |
| <i>Nannochloropsis</i> sp. | 31–68 |
| <i>Neochloris oleoabundans</i> | 35–54 |
| <i>Nitzschia</i> sp. | 45–47 |
| <i>Phaeodactylum tricornutum</i> | 20–30 |
| <i>Schizochytrium</i> sp. | 50–77 |
| <i>Tetraselmis sueica</i> | 15–23 |

Botryococcus braunii, green colonial microalgae, is unusually a rich source of hydrocarbons (Sawayama et al., 1995). It is found worldwide in freshwater and brackish lakes, reservoirs and ponds. This alga gained a great interest in scientific and commercial world because of its ability to synthesize and accumulate huge amount of various lipids. These lipids are speculated as solutions for the increasing oil-price, as these lipids can be converted into biodiesel, Jet-fuel, gasoline and other important chemicals. *B. braunii* converts simple inorganic compounds and sunlight to potential hydrocarbon fuels and feedstocks for the chemical industry. Based on the hydrocarbons produced, *B. braunii* is classified into A, B and L races (Metzger & Largeau, 2005). Race- A produces C21 to C33 odd numbered n-alkadienes, mono-, tri-, tetra-, and pentanes (Banerjee et al. 2002; Metzger et al. 2005). The L race yields a single C40 isoprenoid hydrocarbon, lycopa - 14(E), 18(E)-diene (Metzger et al. 1991). The B race produces two types of triterpenes called botryococcenes of C30 - C37 of general formula C_nH_{2n-10} as major hydrocarbons and small amounts of methyl branched squalene. Certain strains of the B race also biosynthesizes cyclobotryococcenes (Achitouv et al. 2004). Also a feature common to all three races is the presence of a highly aliphatic, non-hydrolysable and insoluble biomacromolecule (algaenan) found in their outer cell walls (Audino et al. 2002).

Despite the high lipid content of this strain, it is reported that their growth rate is very low (Sheehan et al., 1998). *Botryococcus braunii* has extremely long chain lengths greater than C30 (Banerjee et al., 2002), and hence unsuitable for biodiesel production. Due to its very high lipid content, this strain was chosen as the reference strain in this research.

1.4. Microalgae for Fuel and Aquatic Species Program:

The major reason for developing alternative energy resources by the United States could be attributed to the 1973 oil crisis. There are several impacts because of the dependence of United States on Middle East for oil. Oil dependence endangers U.S economic and national security. In 1970, during the first oil crisis, U.S started developing biofuel technologies. Corn for ethanol and

soybean for biodiesel were some technologies studied. Historically, algae were seen as a promising source of protein and were actively cultured primarily for food. The first attempt in the U.S.A was made at the Stanford Research Institute to translate the biological requirements for algae growth into engineering specifications for a large scale plant (1948-1950). During 1951, Arthur made a little progress to study the growth, physiology and biochemistry of algae by using *Chlorella* species as the model (Burlingame, 1951). The concept of using microalgae for producing methane gas from the carbohydrate fraction of cells was first proposed by Meier in 1955. The idea of using algae for transportation was further developed in the 1950's (Oswald & Golueke, 1960) and since the 1970's many countries have investigated microalgae for cultivation for producing renewable fuels (Benemann et al., 1982).

The U.S Department of Energy (DOE) initiated the Aquatic Species Program (ASP). This is one of the most comprehensive efforts till date to study algae for fuel production. More than US\$25 million was invested in this program were made by this program. More important advances were made by this program in algal strain isolation and characterization, physiology and biochemistry, genetic engineering, process development and algal culture piloting. The program pursued research in three major areas. They are as follows:

1.4.1 Screening and collection of variety algal species:

Initially researchers collected and screened a variety of algal species to assess their potential for high production of lipids by investigating their physiology and biochemistry. Further, molecular biology and genetic engineering techniques were used to enhance the oil yield. Researchers in the ASP collected over 3000 strains from various sites in the western and south eastern USA and Hawaii that represented diversity in the environments and water types. The collection was narrowed to 300 most promising strains. Most of the strains were green algae (Chlorophyceae) and diatoms (Bacillariophyceae). In 1996, remaining cultures were transferred

to the University of Hawaii (<http://prcmb.hawaii.edu/research/HICC/index.html>). Studies on algal physiology under the ASP, centered on the ability of many species to induce the lipid biosynthesis under the condition of nutrient stress (Dempster & Sommerfield, 1998). The study on the diatom *Cyclotella cryptica* indicated that silicon deficiency increased the activity of enzyme Acetyl Co-A carboxylase (ACCase), which catalyses the conversion of acetyl co-A to malonyl Co-A, the substrate responsible for fatty acid synthesis. The ACCase enzyme was extensively characterized and it was also learned that the storage carbohydrate chrysolaminarian competed with the lipid pathway for fixed carbon (Roessler, 1988). In the latter years, the ASP also focused on the genetic engineering of green algae and diatoms for enhanced lipid production. The first accomplishment was made with the transformation of diatom *Cyclotella cryptica* and *Navicula saprophila* to produce a microalgal strain with potential for biodiesel production. The second major success with *Cyclotella cryptica* was that their genes were isolated and characterized which encoded ACCase and UGPase enzymes (Jarvis & Roessler, 1999).

1.4.2 Development of mass cultivation systems:

The second research area involved the development of algal mass production systems. To grow algae in large scale requires proper infrastructure and operational facilities. The photosynthetic algae require light and carbon dioxide for their growth. This culture mode is referred as photoautotrophic (Harel & Place, 2003). Some algae are also capable of growing in dark and using organic carbons such as acetate or glucose as energy and carbon sources and are classified as heterotrophic. In order to maintain cost effectiveness, photoautotrophic mode is preferred over heterotrophic mode. Photoautotrophic microalgae need sufficient amount of light, carbon dioxide, water and inorganic salts. The growth medium provides inorganic elements like nitrogen, phosphorous, iron and sometimes silicon (Grobbelaar, 2004). Algal cells are continuously mixed to ensure settling does not occur (Grima et al., 1999). Currently, open ponds

and enclosed photobioreactors are the two commonly used photoautotrophic modes of algal mass production systems.

Open ponds:

Open ponds are simplest and oldest systems used for cultivating algae. The pond is designed in a raceway configuration. A paddlewheel circulates and mixes the algal cells and nutrients. The system is often operated in a continuous mode. By continuous mode it means that feed is continuously added in front of the paddle wheel and the algal broth is harvested behind the paddle wheel. Several sources of waste water – such as dairy / swine, lagoon effluent can be used for algal culture depending on the nutrients required by the algal species.

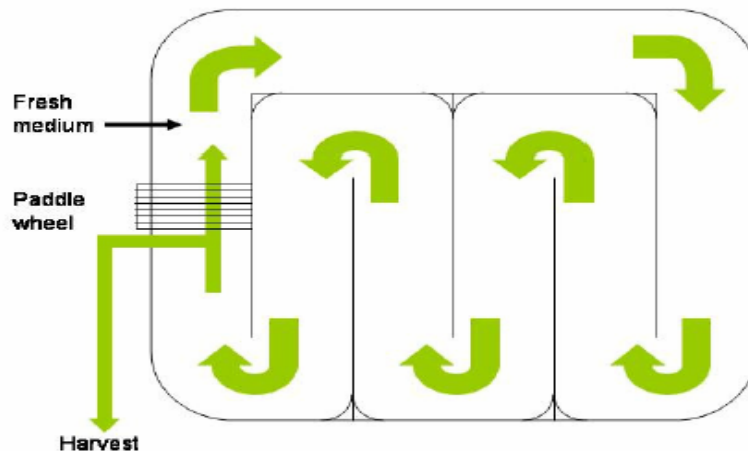


Figure 3: Schematic diagram of an Open pond²

Enclosed Photobioreactors:

To overcome the problems of contamination and evaporation problems, photobioreactors were developed (Grima et al. 1999). These systems are made out of transparent materials that allow light penetration. Harvesting biomass, in the case of photobioreactors is less expensive than open ponds, because the algal biomass is about 30 times as concentrated as the biomass in the open ponds (Chisti, 2007). There are different types of photobioreactors. The most widely used

² Chisty, 2007

photobioreactor is the tubular design. To increase sun penetration, the tubes are devised less than 10 centimeters in diameter. In some bioreactors, the tubes are coiled spirals to form a helical tubular photobioreactor.

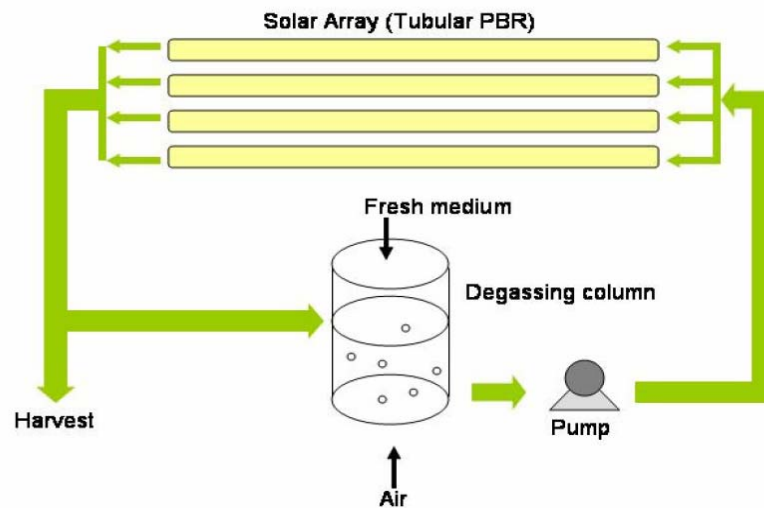


Figure 4: Schematic diagram of an enclosed photobioreactor³

The Department of Energy (DOE) suggested open ponds as the major system for algal fuel production because they were relatively less expensive. The cost of photo bioreactors was still prohibitive for producing biofuels because of capital and maintenance cost.

1.4.3 Analysis of systems for resource availability:

The third research area was to analyze the resource availability, including; land, water and CO₂ resources. DOE concluded that there was significant amount of land, water and CO₂ to support the algal biofuel technology in the South Western part of USA. Algae had the potential to produce many billion gallons of fuel. Cost analysis emphasized the necessity of low cost culture system such as open ponds. The fuel cost was influenced by the biological productivity of the strain used. Estimated fuel costs were determined to range between \$1.40 to \$4.40 per gallon in

³ Chisty, 2007

1995 (Sheehan et al., 1998). The program was closed in 1995 because of budget reduction. However, the ASP demonstrated the feasibility of using algae as a source of oil and resulted in important advances in the technology. The need to develop highly productive strains was apparent to make this technology economically feasible.

A part of the study under the ASP involved collecting and screening of saline chromophytic algae (e.g., diatoms, chrysophytes and pyrrnesiophytes) from the Florida Keys (Rhyter et al., 1987). Growth rates for more than one hundred strains isolated from the Keys were determined. The growth of these strains were evaluated by growing them in test-tubes containing various media including enriched sea water, SERI Type I/25, Type I/40, Type II/25 and Type II/40. Most of the species grew better in Type II medium when compared to Type I medium. *Hymenomonas* HB 152(HYMEN3) was identified as the strain with highest growth rate in Type II/25 medium. Seven strains had growth rates that exceeded 2 doublings d⁻¹ in atleast one media type. The strains included in this group were *Dunaliella* HB37 (DUNAL2), (CHLOR7) and HB 87, *Pyramimonas* HB133 (PYRAM2) and HYMEN3. *Tetralselemis* sp. and an olive green unicell HB 54 had the highest lipid contents over a wide range of conditions. In summary, the ASP research resulted in identification of strains from notable genera based on the growth rates and triacylglyceride production. The strains were *Chlorella*, *Phaeodactylum* and *Amphora*. In summary, the research finally concluded that there was no one strain that was found to be “perfect” for TAG production.

1.5 The fatty acid biochemistry in microalgae and determination of neutral lipid:

The favorable fatty acid profile of algae makes it suitable to produce biodiesel. Triacylglycerides, phospholipids and cholesterol are the various classes of lipids produced. The major lipid precursor for producing biodiesel is the TAGs. TAG synthesis can be divided into three different steps: (i) formation of acetyl co-A (ii) acyl chain elongation (iii) triacylglycerol

formation (Hu et al., 2008; Huang et al., 2009). TAG synthesis has been proposed to occur via the direct glycerol pathway (Ratledge, 1988). Neutral lipid, mainly in the form of TAG is stored in cytosolic lipid bodies of the microalgae (Hu et al., 2008). The total content of lipids varies from 1-85% of the dry weight (Borowitzka, 1988) with values higher than 40% being typically reached under nutrient limitation. The lipid composition and lipid content is affected by factors such as temperature, irradiance and nutrient availability.

The algal lipids are analyzed using several methods. The common methods are fluorometric analysis using a lipid stain such as Nile red or BODIPY (Chen et al., 2009; Cooksey et al., 1987; Elsey et al., 2007) and nuclear magnetic resonance method such as TD-NMR (Time Domain – Nuclear Magnetic resonance) (Gao et al., 2008).

Nile red, a phenoxazine dye is a simple and sensitive method, to screen neutral lipids (Cooksey et al., 1987). Nile red is frequently employed to estimate the lipid content in mammalian cells (Genicot et al., 2005), bacteria (Izard & Limberger, 2003), yeasts (Evans et al., 1985) and microalgae (Elsey et al., 2007). Of the algal strains analyzed by the Nile red method, it was identified that most belong to Bacillariophyceae (McGinnis et al., 1997), Xanthophyceae (Eltgroth et al., 2005), Chrysophyceae (de la Jara et al., 2003) and Chlorophyceae (Lee et al., 1998). Compared to other lipid stains, Nile red is photostable and intensely fluorescent (Fowler & Greenspan, 1985). A study conducted by Green span et al. (1985) showed that the Nile red was superior to the other tested dyes as it stained specifically for neutral lipids in the strains analyzed. Other tested dyes such as Rhodamine and Nitrobenzoxodiazol derivatives stained the cells uniformly without clearly distinguishing for neutral lipids. It was found that antibiotic filipin is used to stain cholesterol (Kruth, 1983). However, it does not efficiently stain TAGs. Greenspan et al. (1985) describes Nile red as the stain that does remarkably well, and that it can be applied to cells in an aqueous solvent and it dissolves well in the lipid.

However, the Nile red method failed to detect lipid in certain algae. This was due to the presence of thick cell wall and this cell wall inhibits the dye from penetrating and binding with the intracellular neutral lipid (Chen et al., 2009). Neutral lipids are the building blocks of biomembranes; they tend to remain in intimate contact with an aqueous phase of electrolytes (Suzuki et al., 1973). Moreover, there are polysaccharides present in the cell wall that makes it much more difficult for the dye to penetrate the cells. In order to increase the penetration of the dye into the cells, there are several solvents used. Some of them include ethanol, glycerin and Dimethyl sulfoxide (DMSO) (Genina et al., 2001). Solvents such as ethylene glycol and acetone were suggested by Castell and Mann (1994) to improve the dye from entering the cell effectively. Elfbaum and Laden (1968) conducted an in vitro skin penetration study employing guinea pig skin as the membrane. They concluded that the passage of picrate ion through this membrane in the presence of DMSO was a passive diffusion process which adhered to Fick's first law of diffusion. Chen et al., (2009) worked using DMSO to enhance the dye penetration in the cells and found that addition of 25% DMSO was effective in dye penetration in the cells. In this study 200 μ l DMSO was added to 800 μ l of the cell culture in order to increase the penetration of dye into the cells. Dimethyl Sulfoxide (DMSO) is an organosulfur compound. This colorless liquid is a polar aprotic solvent that dissolves polar and non-polar solvents. It has a distinctive property of penetrating the skin very readily.

The fluorescent character of Nile red is influenced by polarity, hydrophobic and saturation of the lipids and surroundings (Fowler et al., 1987). By selecting proper excitation and emission wavelengths, greatest sensitivity of the method could be achieved. The wavelengths chosen for determining lipids in zooplankton were 530/580 nm (Alonzo & Mayzaud, 1999). However, the wavelengths used by Lee et al., (1998) to screen *Botryococcus braunii* using the Nile red method (490/585 nm) were different. The wavelengths chosen by Chen et al., (2009) to determine the neutral lipid content in *C.vulgaris* were in the range of 530 nm and 575 nm. The

same excitation and emission wavelengths (530/575) were chosen in our study. Another issue with Nile red method to be considered was that the green algal taxa (Chlorophyceae) contains chlorophyll (1-4% of dry weight) which can increase the fluorescence background (Alonzo & Mayzaud, 1999) which can overestimate or underestimate the neutral lipid content. In a study done by Lee et al. (1998) to determine the lipids in green alga *Botryococcus braunii*, the relative fluorescence intensity of Nile red was obtained after subtracting both, the autofluorescence of algal cells and the fluorescence intensity of Nile red alone in the medium.

BODIPY 505/515 is another fluorescent method to stain lipid bodies of morphologically diverse algae like *O. maius* and *Chlamydomonas* species (Cooper et al., 2009). There are some disadvantages of using this probe such as; they display a short emission wavelength or fluorescence gets influenced by changes in solvent (Sunhara et al., 2007).

Time Domain Nuclear magnetic resonance (TD-NMR) is another method used to estimate the amount of lipids present in the cells. The lipid content in *Chlorella protothecoides* determined using TD-NMR and Soxhlet extractor showed that TD-NMR was as effective as the Soxhlet extractor. The two methods produce similar results (correlation coefficient for TD-NMR and Soxhlet extractor = 0.9773) (Todt et al., 2001). There are several disadvantages of the TD-NMR method. Background interference is a problem and relaxation time is influenced by additional parameters such as temperature, salt concentration and other local environmental conditions. Presence of water in the sample gives signals from fat and water. So, sample preparation is a laborious process. Although this seems to be a very new and trendy method, it requires additional hardware and experiment set up routines which is not a cost effective process.

1.5.1 Extraction of neutral lipid:

The methods used in the extraction of lipids are broadly classified into two types – Mechanical and Chemical methods. Mechanical methods can further be classified into Expeller

press and Ultrasonic assisted extraction (hielscher.com). The use of the expeller press requires dried algae and the oil is obtained with an oil press. It can extract about 70-75% of the contained oil. But the disadvantage of expeller press is that it is not efficient enough to remove the last trace of oil from the raw material. Ultrasound is primarily associated with cell disruption or disintegration (Allinger, 1975) and is based on ultrasonic waves. Sonication can have a constructive and destructive effect to cells depending on the sonication parameters employed. In order to avoid an unhindered release of all intracellular products including cell debris and nucleic acids, proper control of cell disruption is required.

Chemical methods are further classified into Solvent extraction method, Supercritical extraction method and Soxhlet extraction method. The conventional method used for lipid extraction involves solvent extraction for Gravimetric analysis (Bligh & Dyer, 1959). However, these conventional methods are time consuming and labor intensive that makes screening of large number of algal strain very difficult. The methodology used for the extraction of lipids should ensure complete extraction and should avoid decomposition of the lipid constituents. The use of organic solvents can increase the level of extraction by 99%, however, there is an increased cost in processing to achieve this (Metzger & Largeau, 2005). There are several solvents being used for extracting the lipids. Some of them are chloroform/methanol (Bligh & Dyer, 1959), hexane/isopropanol (Hara & Radin, 1978), dichloromethane/methanol, acetone/dichloromethane and dichloroethane/ethanol. A comparison was made between the wet and dry biomass extracted from the fungus *Mortierella alpine* (Zhu et al., 2003). Wet biomass was extracted using the Bligh and Dyer method (1959) and dried biomass was extracted using chloroform: methanol (2:1 v/v ratio). Dry method was much more effective than the wet extraction technique. The extraction of lipids using the solvent chloroform and methanol were found to be the most superior method in comparison to other methods like supercritical or sub critical fluid extraction (Mendes et al., 2003). A study conducted by Lee and group in determining the lipid from the green alga

Botryococcus braunii showed that most effective solvent system for extracting the lipids is chloroform/methanol system. In the case of supercritical method of extraction, high heat and pressure is used to burst the cell walls of the algae. The disadvantage of using this technique involves using of a high pressure equipment which is both expensive and an energy intensive process. Another method of chemical solvent extraction is Soxhlet extraction. In this method, algal oils are extracted through repeated washing, or percolation, with an organic solvent such as hexane or petroleum ether, under reflux in special glassware. The value of this technique is that the solvent is reused for each cycle.

1.6 Other applications of Algae

1.6.1 Algae and waste water treatment:

Algae play a vital role in bioremediation application (Kalin et al., 2005). Both micro and macro algae have characteristics which makes them suitable for waste water treatment. Microalgae has particularly been used for a long time in treatment ponds and lagoons for removing the excess nutrients such as nitrogen and phosphorous from animal manure effluent, specifically swine and dairy (Pizzaro et al., 2006). Algae have also been investigated to remove heavy metals from industrial effluents. Industrial remediation is different from waste water treatment in different ways. Industrial remediation refers to remediation of large scale waste water which contains several contaminants including heavy metal contaminants (Yalcin et al., 2008), chemicals released from diverse refinery process (Joseph & Joseph, 2001) and also pollutants from acid mine drainage (Rose et al., 1998). Algae when used for removing heavy metals are often not expected to survive the process (Brinza et al., 2007). In some cases algae tissue has been reused up to 10 cycles for adsorption or desorption of heavy metals (Brinza et al., 2007). Research has also examined the ability of algae to remove dye like Methylene Blue from industrial waste water. The green algae, *Ulva lactuca* has a biosorbent capacity of 40% of its own biomass under ideal conditions (El Sikaily et al., 2006).

1.6.2 Food products:

Seaweed is most often used as food for both humans and livestock (Hong et al., 2007). Seaweeds are rich source of vitamins like A, B1, B2, B6, C and Niacin. They are also rich in macro and micronutrients like iron, potassium, calcium, magnesium and iodine (Poulickova et al., 2008). Algae are also rich in omega-3 fatty acids and used as diet supplements and a component of livestock feed. They also contain amino acids such as docosohexanoic acid (DHA) (Pyle et al., 2008). These characteristics make algae an attractive supplement for food.

1.6.3 Carbon Sequestration:

The largest fossil energy reserve in the world is coal. Consumption of coal would grow in the coming decades because of population growth and demand which would ultimately lead to increased greenhouse gas levels in the atmosphere. If an algae plant is built close to the coal power plant, microalgae will be capable in absorbing the CO₂ and releasing oxygen. CO₂ released from energy plants can be used as a carbon source for growth and carbon emitted from power plants can be efficiently sequestered reducing the green house gas emissions (Danielo, 2005).

1.7 Disadvantages of using algae for biodiesel production:

Despite the various advantages of using algae fuel, there are some caveats in this technology, which needs to be addressed. There are some caveats in using algae for fuel as well. The major limitation is algal productivity followed by labor and harvest costs (Borowitzka, 1992). Laboratory yields are rarely reached on large scale culture. Several factors like contamination with unwanted species, evaporation of large volumes of water, lack of control over light and temperature in open ponds can reduce the yield (Pulz, 2001). Open ponds, although less expensive to build and operate, does not allow efficient use of carbon dioxide, and therefore

produces limited biomass (Chisti, 2007). According to Grima et al., (1999), biomass recovery from a dilute culture such as an open pond is very expensive.

Therefore, much focus was given to photobioreactors as a means of preventing culture contamination as well as decreasing the vulnerability to fluctuation in temperature changes. A significant downside of using photobioreactors is the capital cost of the current designs. Another disadvantage of using this system is that it requires artificial illumination, in addition to sunlight which adds to the production costs. Other problems like variations in temperature and light can cause suboptimal growth of microalgae (Wen & Chen, 2003). Another common problem with photobioreactors is the buildup of O₂ at levels that can be harmful to algae. To scale up laboratory process to production level is a very expensive process (Grima et al., 2003). Because of their complexity and differences in design and construction, the initial capital cost is very high (Eriksen, 2008). According to Benemann (1996), growing algae is cheap. However, use of enclosed photobioreactors for the commercial production of algal fuel will not be cost effective process. Moreover, light limitation cannot be entirely overcome because light penetration is inversely proportional to the cell concentration. The cells may attach to the tubes walls which may also prevent light penetration. Therefore, substantial focus must be played on designing much lower cost photo bioreactors and also overcome light limitation. Also, the reproduction rate is lowered by storage of energy as oil rather than as carbohydrates. So, the strains with higher oil tend to grow more slowly than low oil strains which may not be favorable for producing fuel on a large scale basis (Vasudevan, 2008). Extremophiles, although capable of tolerating extreme conditions, such as high salinity or alkalinity yielded poor results, in terms of net productivity of the system.

Another important characteristic for biodiesel production is the suitability of lipids for biodiesel in terms of the type and amount produced by an algal species, e.g., chain length, degree of saturation and total lipid proportion made by triglycerides. These factors influence the quality

of biodiesel produced. The proportion of various lipid classes varies significantly with the fluctuations in the environmental conditions (Rodolfi et al., 2002), making it difficult to compare algal species across experimental conditions (Grima et al., 1994). Another current limitation in the use of algal biomass is the removal of lipids from the cells, before it can undergo the direct transesterification process. Lipid extraction from algal tissue is very difficult (Ahlgren 1991). The Bligh and Dyer method was developed in order to extract the lipids. But still more research is needed in order to ensure complete and efficient extraction of lipids. Hence, addressing the above mentioned issues with cutting edge technology can achieve commercial success with algae based biofuel.

1.8 Significance of Research:

In this research, an integrated method has been developed to screen the various native green algal strains to find its potential for biodiesel production using the Nile red method. Previous studies have investigated the potential of various saline algal strains of South Florida. So this work has been an attempt to explore the potential of various native fresh water green algae in serving as feedstocks for biodiesel production. The strains which showed the highest amount of lipid were grown on large scale and lipids were extracted using solvent extraction technique and quantified using the Gravimetric technique. The purpose of this experiment was to determine if the amount of lipids obtained from both Gravimetric and Nile red fluorescence method were the same. The high lipid producing strains were subjected to a growth curve analysis to see the time period where there was maximum accumulation of lipid. The high lipid producing strains were also subjected to various environmental stress conditions like varying concentrations of nitrogen and phosphorous to investigate the effect on lipid production. In all the experiments *Botryococcus braunii* were used as the reference strain due to its significantly high lipid content.

Objective:

I. To screen the various native green algal strains from Florida Everglades to identify those with potential to produce biodiesel using Nile red method.

II. To select a method for fast and reliable screening

Hypothesis: The lipid content determined using Gravimetric technique and Nile red method are the same.

III. To assess the effect of environmental conditions on accumulation of neutral lipid

Hypothesis: Nitrogen and phosphorous depletion increases lipid accumulation

2. MATERIALS AND METHODS:

2.1. Organisms:

Twenty Seven algal strains were obtained from FIU culture collection of Dr. M. Gantar (Tab 3). The reference strain *Botryococcus braunii* was purchased from University of Texas, Austin. All these strains were grown in BG 11 medium.

Table 2: List of strains used in this research

| Genus | Strains |
|----------------------|---------------------------------|
| <i>Chlamydomonas</i> | EV-29 |
| <i>Chlorella</i> | 2-4,71-4 |
| <i>Selanstrium</i> | 2-7,34-4 |
| <i>Scenedesmus</i> | 3-11,66-1,79-1,80-15,81-5,103-4 |
| <i>Chlorococcum</i> | 5-1,45-3,55-2,55-5 |
| <i>Coelastrum</i> | 46-4,108-5 |
| <i>Cocoid green</i> | 56-4,56-5,81-7,103-6,64-12 |
| <i>Stigeoclonium</i> | 64-8 |
| <i>Dactylococcus</i> | 64-10 |
| <i>Pediastrum</i> | 81-6,108-4 |
| <i>Kirchneriella</i> | 104-7 |

2.2. Medium:

Algal cultures were maintained in 125 ml flasks in BG 11 medium (Rippka et al., 1979) and grown under cool white light ($30 \mu\text{E m}^{-2} \text{s}^{-1}$) at 27°C with aeration with sterile air.

2.3 Screening of Lipid:

Prior to measuring the lipid content, the algal cell concentration of each strain was adjusted to 0.2 at OD_{600} using a spectrophotometer based on the method prescribed by Chen et al. (2009). After the homogenization of cells by vortexing, 800 μL of the cell mixture was taken in an eppendorf tube. To increase the penetration of dye into cells, 200 μL of 20% of Dimethyl Sulfoxide was added. The mixture was agitated on a vortex. To 700 μL of this mixture, 7 μL Nile red solution, in acetone (6.5 mg of Nile red in 26 ml acetone) was added and kept aside for 10 min. This mixture was vortexed and then pipetted into a 96- well plate (Chen et al., 2009).

Excitation and emission wavelengths of 530 nm and 575 nm were selected according to the pre-scan of excitation and emission characteristics of neutral lipid standards (Alonzo & Mayzaud, 1999). The relative fluorescence intensity of Nile red was obtained after subtraction of both the autofluorescence of algal cells and the fluorescence intensity of Nile red alone in the medium (Lee et al., 1998). These values were then normalized by dividing them by the chlorophyll absorbance measured at a wavelength of 663 nm in order to eliminate the background fluorescence. The amount of fluorescence obtained was further translated into lipid content present in 100µl of cells by generating a standard curve using lipid standard, Triolein (Fischer Scientific, USA). One ml of the lipid standard which weighed 0.9194g was dissolved in 9ml of chloroform and serially diluted until 10^{-4} dilution was reached. The concentration of the lipid was brought to 90 µg and further diluted in chloroform in order to obtain a concentration in the range of 5 to 50 µg ml⁻¹ with the total working standard volume of 3ml.

To 3ml of solution, 300 µl of chloroform and 150 µl of methanol was added. Also, 7 µl of Nile red was added and boiled for 1- 1.5 min and allowed to cool down to room temperature (Briaud et al., 2001; Raheja et al., 1973) and then the fluorescence was read using the plate reader at 530nm and 575 nm respectively. The blank in this case was chloroform, methanol and Nile red. The calibration curve was constructed with the fluorescence intensity against the lipid concentration. The fluorescence values were translated to amount of lipid present in 100µl of culture.

In order to estimate the amount of biomass present in 100 µl of culture, about 80 -100 ml of the culture with concentration 0.2 optimized at OD₆₀₀ was taken. The trial experiments with 10ml, 20 ml and 30 ml of the culture were pipetted on to a pre-weighed GFC 25mm circle filter paper. The filter paper was placed on to a Petri dish and was dried in an oven at 60° Celsius. After the filter dried, the dry weight of biomass was estimated. The lipid present in terms of percentage dry weight was calculated from the dry weight of biomass.

2.4. Quantification of Lipid by Gravimetric Technique:

Strains that showed a high lipid content by Nile red method were selected for quantification of lipid content and further study. Solvent extraction and Gravimetric techniques were used. The algal strains were grown in 3-liter cultures under cool white light ($30 \mu\text{E m}^{-2} \text{s}^{-1}$) at 27°C with aeration with sterile air and biomass was harvested after 3-4 weeks and freeze dried. One gram of freeze dried algal biomass was placed in a glass test tube. The neutral lipids were extracted using the chloroform: methanol: water (2:1:1, v/v). For the direct transesterification of lipids, about 1 ml of sulfuric acid was added in order to disrupt the cells. After the addition of sulfuric acid, 5 ml of chloroform and 2.5 ml of methanol were added. Then the samples were sonicated for about 15 min. Then about 2.5 ml of distilled water was added and the samples were left over night for phase separation. The aqueous phase was discarded and the organic phase which had the lipid in it was transferred to a pre-weighted glass beaker. The samples were air dried and the weight of the glass beaker was taken. The presented data are the means of three replicate extractions.

2.5 Growth and Lipid Accumulation dependent on Culture Age:

The growth and neutral lipid were assessed over a period of 45 days to determine the time to maximum lipid accumulation and to investigate the correlation between biomass content and lipid accumulation. The growth was assessed by monitoring the chlorophyll content every 5 days. The cell concentration was adjusted to 0.2 at OD_{600} using a spectrophotometer. After adjusting the cell concentration, the sample was centrifuged where the medium was discarded and the pellet was kept in a freezer at -20°C . At the end of the experiment the pellet was re-suspended in 1ml of methanol and centrifuged for 5 minutes. The supernatant was separated and the absorbances were read at 653 nm and 666nm for chlorophyll a determination. The amount of these pigments was calculated by the formulas prescribed by Lichtenthaler and Wellburn (1985). The formula used in the calculation of chlorophyll a with methanol as a solvent is given by:

$$Ca = 15.65 A_{666} - 7.340 A_{653}.$$

The lipid content was monitored every five days simultaneously by using the Nile red method discussed in section 3.3. The new culture was prepared by inoculating the cells from old culture which served as the control for all the experiments. The cells were re-suspended into the new culture on the same day. The lipid content was tested in the old culture as well the new culture on the same day to see how the lipid content got affected when it was transferred to a new medium.

2.6. Assessment of the effect of environmental factors:

The effect of cell lipid content during the growth of culture was assessed in those strains showing highest lipid content under varying nitrogen and phosphorous conditions.

2.6.1. Effect of Nitrogen depletion on Lipid content:

To investigate the effect of nitrogen depletion on lipid accumulation of algae, the old culture of the strains which showed high lipid content were first centrifuged and supernatant was discarded. It was then washed in the nitrogen free media in order to remove the nitrogen present in the old culture and then inoculated into the new medium containing 0%, 50% and 100% of standard nitrogen content in the BG11 medium to see the differences in the lipid accumulation between these concentrations. 500 ml of medium containing 0% N, 50% N and 100% N were prepared. The stock solution had a composition of NaNO_3 - 4.4ml/500ml, K_2HPO_4 - 0.5 ml in 500ml, Na_2CO_3 – 0.5ml in 500ml, MgSO_4 – 0.5 ml in 500ml, CaCl_2 – 0.5 ml in 500ml, Fe chelate – 0.5 ml/500ml and microelements – 0.5 ml in 500ml. The nitrogen free media was prepared by omitting the addition of sodium nitrate. The medium containing 50% and 100% nitrogen were prepared by adding 2.2 ml in 500 ml and 4.4 ml in 500 ml of sodium nitrate respectively. The lipid concentration was again measured using the Nile red method discussed in section 3.3.

2.6.2. Effect of Phosphorous depletion on Lipid content:

To investigate the effect of phosphorous depletion on lipid accumulation in algae, the old culture of the strains were first centrifuged and the supernatant was discarded. It was then washed in phosphorous free medium. Phosphorous free medium was prepared by omitting the addition of potassium hydrogen phosphate from the original BG 11 formulation. Washing with phosphorous free medium ensured removal of phosphorous present in the media. Then the culture was re-inoculated into the new media containing 0%, 50% and 100% phosphorus of the standard BG 11 medium. 50% and 100% phosphorous containing medium were prepared by adding 0.25ml in 500ml and 0.5ml in 500ml of potassium hydrogen phosphate solution respectively. The lipid content in the biomass of cultures grown with different phosphorous concentrations was determined using the Nile red method mentioned in section 3.3.

2.7 Statistical analysis:

All statistical analysis was conducted using SPSS 15.0 software. In this study it was hypothesized that the lipid content determined by both Gravimetric technique and Nile red method were the same. In order to test our hypothesis, Independent sample T test was used by comparing the means. Also it was hypothesized that Nitrogen and phosphorus depletion increases the lipid accumulation in our potential strains. The significant differences in the means were reported using one way repeated measure ANOVA. Comparisons that show p-value < 0.05 were considered significant.

3. RESULTS:

This chapter discusses the results obtained in this research.

3.1. Screening the isolates using NILE RED METHOD:

The fluorescence was read from the plate reader after staining the cells with Nile red. The lipid content in the cells was estimated during the logarithmic phase (Day 13) and during the later stages of the culture (Day 45). The fluorescence intensity was recorded at excitation and emission wavelengths of 530 nm and 575 nm (Table 4).

Table 4: Fluorescence intensity observed on 13th and 45th day in all 27 strains. Data are expressed as the means and standard error of the mean of three replicates.

| Strain | Fluorescence on 13th day | Fluorescence on 45th day |
|--|---------------------------------|---------------------------------|
| <i>Pediastrum</i> 81-6 | 138.86 ± 36.21 | 913.39 ± 18.34 |
| <i>Pediastrum</i> 108-4 | 316.69 ± 22.19 | 835.89 ± 69.03 |
| <i>Coelastrum</i> 108-5 | 209.46 ± 15.46 | 1172.63 ± 6.849 |
| <i>Chlorella</i> 2-4 | 120.57 ± 8.41 | 195.03 ± 10.22 |
| <i>Chlamydomonas</i> EV-29 | 158.17 ± 26.20 | 365.13 ± 14.70 |
| <i>Cocoid green</i> 56-5 | 52.44 ± 12.56 | 743.66 ± 17.56 |
| <i>Selanstrium</i> 34-4 | 138.89 ± 13.46 | 756.74 ± 51.44 |
| <i>Chlorococcum</i> , 55-2 | 36.23 ± 2.93 | 417.58 ± 29.57 |
| <i>Chlorococcum</i> , 55-5 | 85.54 ± 12.79 | 1060.23 ± 118.45 |
| <i>Dactylococcus</i> , 64-10 | 14.89 ± 7.45 | 1790.58 ± 87.01 |
| <i>Coelastrum</i> , 46-4 | 113.47 ± 5.71 | 3704.96 ± 12.83 |
| <i>Chlorococcum</i> , 45-3 | 101.78 ± 14.14 | 905.79 ± 76.83 |
| <i>Scenedesmus</i> , 103-4 | 56.64 ± 13.99 | 636.21 ± 26.61 |
| <i>Cocoid Green</i> , 64-12 | 120.64 ± 7.98 | 3094.99 ± 9.62 |
| <i>Scenedesmus</i> , 66-1 | 195.17 ± 16.83 | 377.31 ± 12.45 |
| <i>Chlorococcum</i> , 5-1 | 255.49 ± 20.31 | 1033.46 ± 25.90 |
| <i>Pediastrum</i> , 80-15 | 56.55 ± 11.97 | 324.36 ± 16.44 |
| <i>Chlorella</i> , 71-4 | 8.32 ± 4.19 | 405.74 ± 23.91 |
| <i>Scenedesmus</i> , 81-5 | 154.39 ± 20.90 | 247.02 ± 23.50 |
| <i>Prochloro</i> , 104-1a | 33.16 ± 16.21 | 296.23 ± 23.17 |
| <i>Cocoid Green</i> , 81-7 | 49.99 ± 8.21 | 530.31 ± 32.37 |
| <i>Scenedesmus</i> , 79-1 | 58.29 ± 3.90 | 1037.76 ± 19.74 |
| <i>Stigeoclonium</i> , 64-8 | 114.29 ± 28.34 | 1431.08 ± 18.88 |
| 111-4 | 80.57 ± 10.42 | 527.55 ± 51.37 |
| <i>Cocoid Green</i> , 56-4 | 98.43 ± 7.01 | 292.32 ± 8.47 |
| <i>Cocoid green</i> , 103-6 | 238.26 ± 1.76 | 523.76 ± 12.88 |
| <i>Kirchorniella</i> , 104-7 | 65.37 ± 11.94 | 454.39 ± 68.82 |
| <i>Botryococcus braunii</i> (Control) | 2316.08 ± 8.67 | 1114.06 ± 13.87 |

The five strains which were considered to be potential candidates for biodiesel production were chosen based on the capability to accumulate the maximum amount of lipid during the later stages of culture (i.e., on the 45th day). The strains were *Coelastrum* 46-4; *Cocoid green* 64-12;

Dactylococcus 64-10; *Stigeoclonium* 64-8 and *Coelastrum* 108-5 with maximum fluorescence of 3704.96 ± 12.83 , 3094.99 ± 9.62 , 1790.58 ± 87.0 , 1431.08 ± 18.88 and 1172.63 ± 6.849 respectively. However, these values indicate only the amount of fluorescence produced by each strain. It is important to estimate the amount of lipid present in the culture.

In order to estimate the amount of lipid present in 100 μ l of the culture, a standard curve was generated with the lipid standard Triolein. The correlation co-efficient (R^2) was found to be 0.9329 which shows that the curve is a good fit and there is a positive correlation between the fluorescence intensity and the amount of lipid.

$$Y = 47.206e^{0.1148x} \quad (1)$$

where, Y represents the fluorescence intensity and the unknown (x) is the lipid concentration present in the algal cells. The unknown was calculated by plugging in the values of fluorescence intensity and the two constants. The lipid concentration in 100 μ l was determined for all the strains (Table 5).

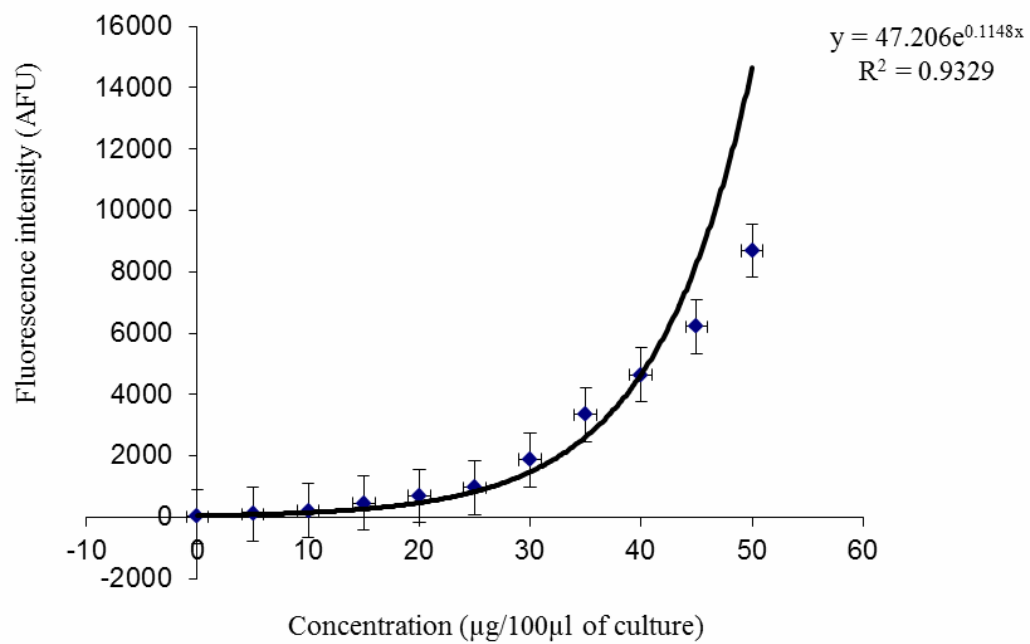


Figure 5: Triolein based calibration curve used to determine the amount of lipid present in 100 μl of cell culture.

Table 5: Concentration of lipid present in the cells on the 13th and 45th day expressed in µg.

| Strain | Lipid content 13 day(µg/100 µl culture) | Lipid content 45 day (µg/100 µl culture) |
|------------------------------|--|--|
| <i>Pediastrum</i> , 81-6 | 9.39 ± 1.55 | 25.81 ± 0.17 |
| <i>Pediastrum</i> , 108-4 | 16.58 ± 0.36 | 25.03 ± 0.50 |
| <i>Coelastrum</i> , 108-5 | 12.98 ± 0.65 | 27.98 ± 0.28 |
| <i>Chlorella</i> , 2-4 | 8.17 ± 0.52 | 12.36 ± 0.32 |
| <i>Chlamydomonas</i> , EV-29 | 10.53 ± 1.59 | 17.82 ± 0.638 |
| <i>Cocoid green</i> , 56-5 | 0.92 ± 0.14 | 24.02 ± 0.21 |
| <i>Selanstrum</i> , 34-4 | 9.40 ± 0.86 | 24.17 ± 0.61 |
| <i>Chlorococcum</i> , 55-2 | 3.96 ± 0.69 | 18.99 ± 0.63 |
| <i>Chlorococcum</i> , 55-5 | 5.17 ± 1.23 | 27.11 ± 1.01 |
| <i>Dactylococcus</i> , 64-10 | 3.25 ± 1.18 | 29.86 ± 0.43 |
| <i>Coelastrum</i> , 46-4 | 7.68 ± 0.45 | 38.00 ± 0.62 |
| <i>Chlorococcum</i> , 45-3 | 6.69 ± 1.27 | 25.73 ± 0.71 |
| <i>Scenedesmus</i> , 103-4 | 1.59 ± 0.87 | 22.65 ± 0.36 |
| <i>Cocoid Green</i> , 64-12 | 8.17 ± 0.60 | 36.44 ± 1.34 |
| <i>Scenedesmus</i> , 66-1 | 12.36 ± 0.79 | 18.11 ± 0.28 |
| <i>Chlorococcum</i> ,5-1 | 14.71 ± 0.71 | 26.88 ± 0.22 |
| <i>Pediastrum</i> , 80-15 | 1.57 ± 1.10 | 16.78 ± 0.45 |
| <i>Chlorella</i> , 71-4 | 2.11 ± 0.60 | 18.79 ± 0.52 |
| <i>Scenedesmus</i> , 81-5 | 10.32 ± 1.28 | 14.42 ± 0.83 |
| <i>Prochloro</i> , 104-1a | 1.02 ± 3.92 | 16.01 ± 0.71 |
| <i>Cocoid Green</i> , 81-7 | 0.50 ± 0.11 | 21.07 ± 0.52 |
| <i>Scenedesmus</i> , 79-1 | 1.84 ± 0.61 | 26.92 ± 0.16 |
| <i>Stigeoclonium</i> , 64-8 | 7.70 ± 2.15 | 29.72 ± 0.58 |
| 111-4 | 4.656 ± 1.21 | 21.03 ± 0.87 |
| <i>Cocoid Green</i> , 56-4 | 6.40 ± 0.60 | 15.88 ± 0.26 |
| <i>Cocoid green</i> , 103-6 | 14.10 ± 0.06 | 20.96 ± 1.97 |
| <i>Kircherniella</i> , 104-7 | 2.84 ± 1.61 | 27.54 ± 1.29 |
| <i>Botryococcus braunii</i> | 33.91 ± 0.40 | 27.54 ± 0.64 |

The screening for lipids during the early exponential phase showed no substantial amounts of lipid accumulation except the reference strain *Botryococcus braunii*. As the age of the culture increased, there was considerable amount of lipid increase in all the native strains with *Coelastrum* 46-4; *Cocoid green* 64-12; *Dactylococcus* 64-10; *Stigeoclonium* 64-8 and *Coelastrum*, 108-5 accumulating the highest amount of lipids (Table.5). The lipid content obtained on the 13th and 45th day was further translated in terms of percentage dry weight (Figure 6). There was a considerable difference in accumulation of lipid observed between 13th and 45th

day. The highest amount of lipid was observed in *Coelastrum* 46-4 accumulating about 74% followed by *Coelastrum* 108-5 at the end of the 45th day accumulating about 73.62%.

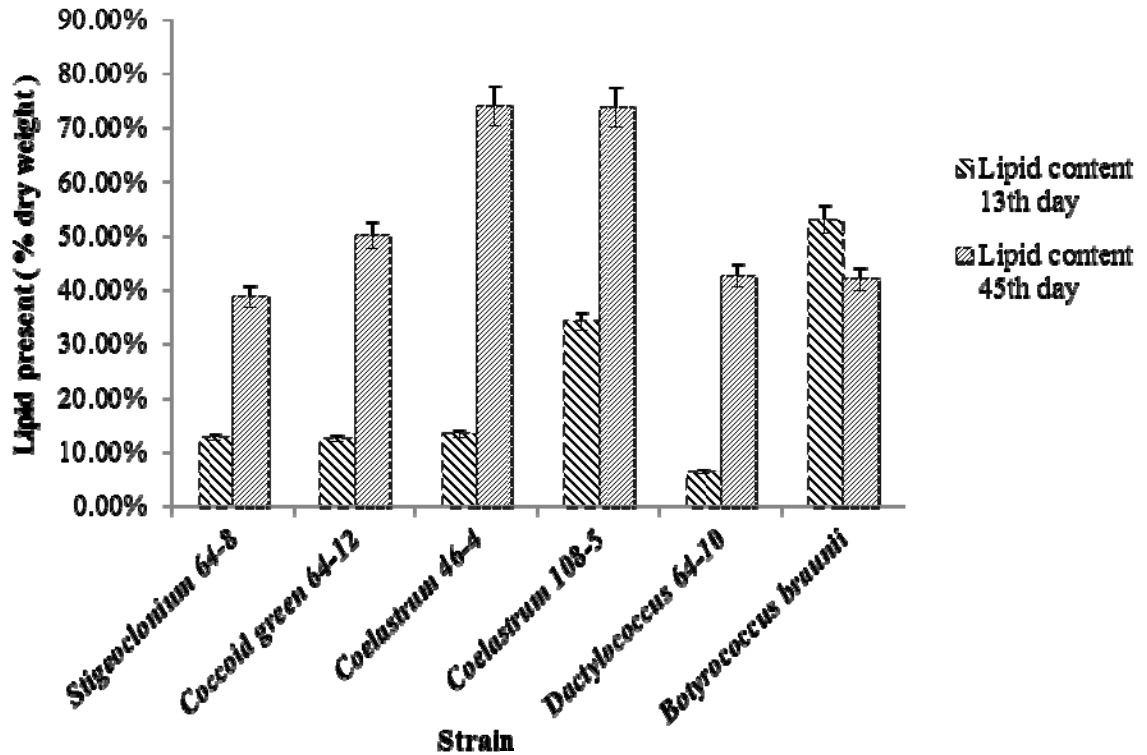


Figure 6: Comparison of lipid content in terms of percent dry weight in six strains on 13th and 45th day by Nile red method. Error bars represent standard error (n=3)

3.2 Lipid quantification by Gravimetric Technique:

The biomass of five selected strains was harvested at the end of the 45th day and the neutral lipid was extracted using the chloroform-methanol-water extraction technique. The presented data are the means obtained in three separate extractions. The highest content of lipids was found in *Coelastrum* 108-5 that had about 70% followed by 64-8 *Stirgeoclonium* with 39%.

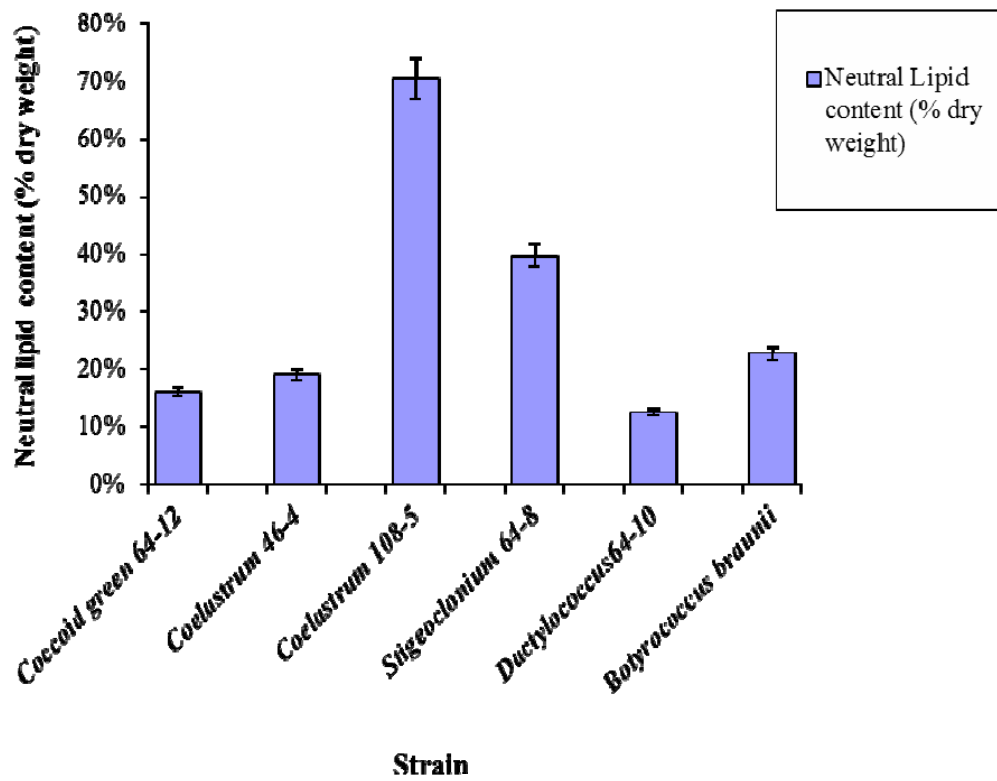


Figure 7: Neutral lipid extraction using Gravimetric method. Data are expressed as the means and standard error obtained from triplicate determinations

3.3. Comparison between lipid yields obtained using Nile red and Gravimetric technique:

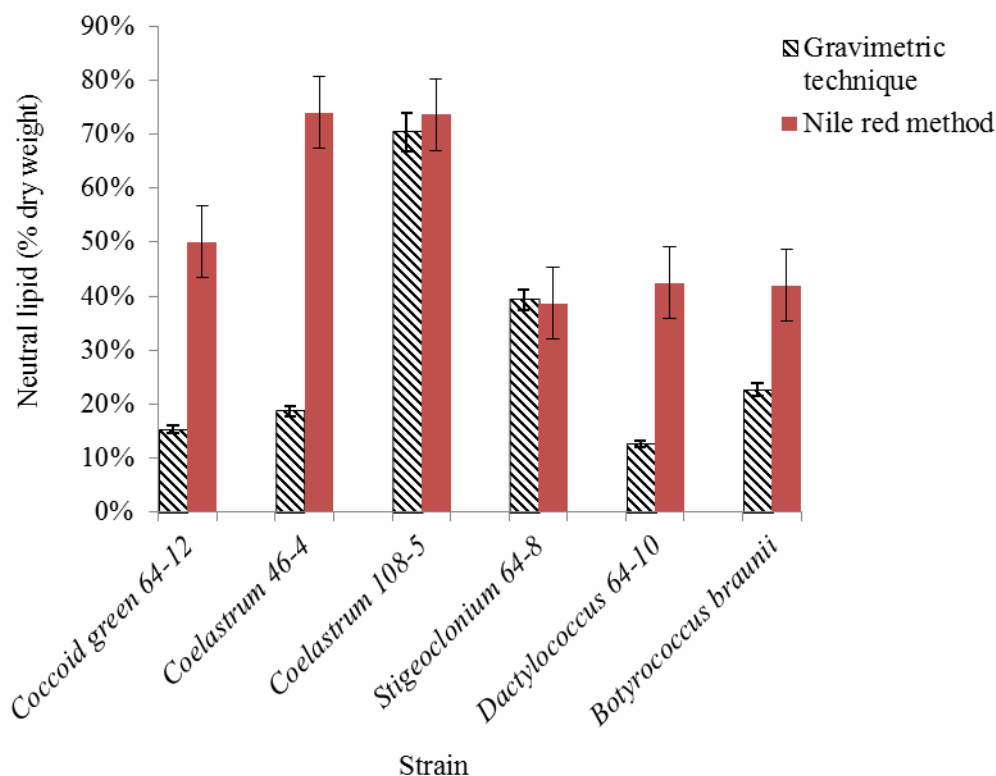


Figure 8: Comparison of neutral lipid yield using Nile red and Gravimetric technique. Data are expressed as the means and standard error obtained from triplicate determinations.

In order to test our hypothesis if the lipid yield obtained from both Gravimetric method and Nile red method were same, Independent sample T test was used. The lipid yield obtained from Nile red and Gravimetric method on the 45th day is shown in figure 8. All the strains except *Coelastrum 108-5* and *Stigeoclonium 64-8* showed a p value less than 0.05 which illustrates that the lipid yields were significantly different between both Gravimetric and Nile red method. However, the p-value of *Coelastrum 108-5* and *Stigeoclonium 64-8* were 0.25 and 0.06

respectively. This indicates that the lipid yielded was not significantly different and same amount of lipid was obtained from both the methods. This was evident from the means obtained from both the methods. For *Coelastrum* 108-5, the Gravimetric technique yielded about 70% of neutral lipid while the Nile red method yielded about 74%. In the case of *Stigeoclonium* 64-8, the amount of neutral lipid obtained using Gravimetric technique was 39% while for Nile red method it was about 35.63%. From figure 8 we can see that for other strains there was a huge difference in the means observed between both the methods.

3.4 Growth and Lipid Accumulation dependent on Culture Age

The objective of this experiment was to observe the growth and lipid accumulation trends in the five potential strains over a period of 45 days. The duration at which maximum amount of lipid and biomass were produced in the culture was studied by monitoring the biomass and lipid accumulation every five days over a period of 45 days. Biomass increased as the age of the culture increased indicating that the cells were still growing. However, the maximum lipid accumulation varied at different time interval depending upon the strain.

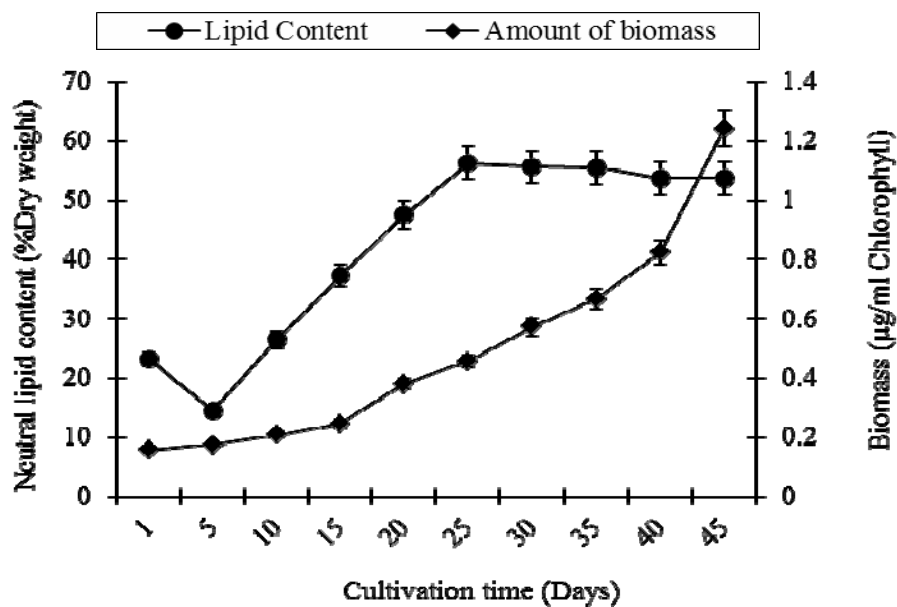


Figure 9: Biomass and neutral lipid content in *Coccoid Green* 64-12 over a period of 45 days. Data are expressed as the means of three replicates.

The biomass of *Coccoid green* 64-12 increased throughout the 45 day growth interval while the maximum lipid accumulation was reached at approximately 25 day and remained constant for the duration (Figure 9). In the initial days of the culture this strain has very less amount of lipid. The biomass keeps increasing which indicates that the cells are still growing.

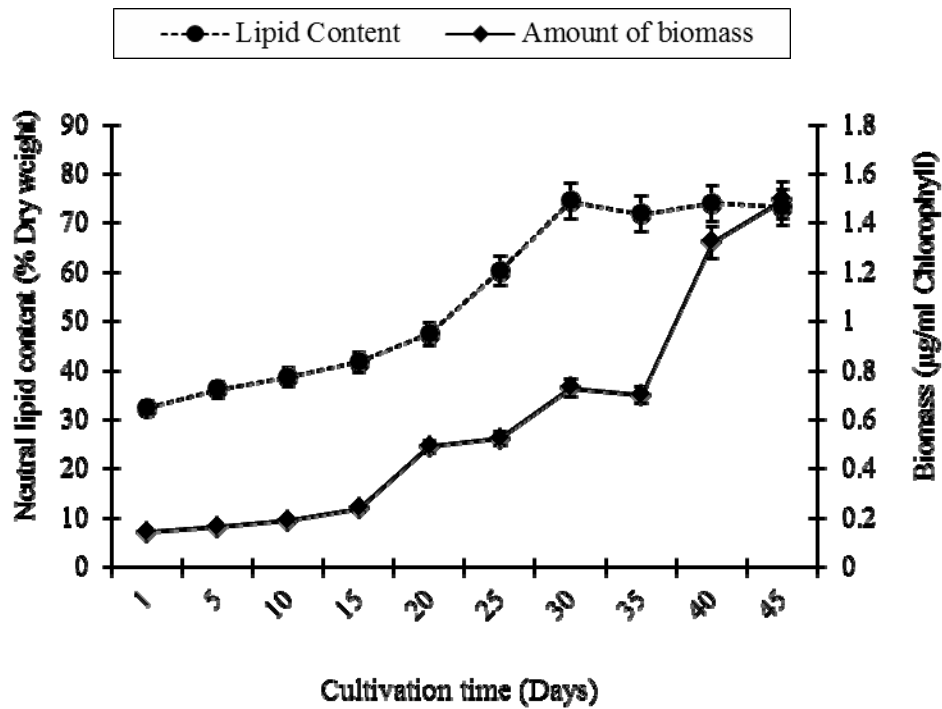


Figure 10: Biomass and Neutral lipid accumulation in *Coelastrum* 108-5 over a period of 45 days. Data are expressed as the mean of three replicates.

The growth and lipid accumulation pattern of *Coelastrum* 108-5 was similar to *Cocoid green* 64-12. The biomass increased throughout the 45 day growth interval with maximum accumulation of 1.49 µg /ml chlorophyll on the 45th day. The lipid accumulation increased slowly accumulating the maximum amount of lipid of about 75% on day 30 and then decreased to 71% and then increased again to 74% (Figure 10). Since the culture was monitored only on a 45 day period, no inference can be made about the lipid accumulation trends after this time period.

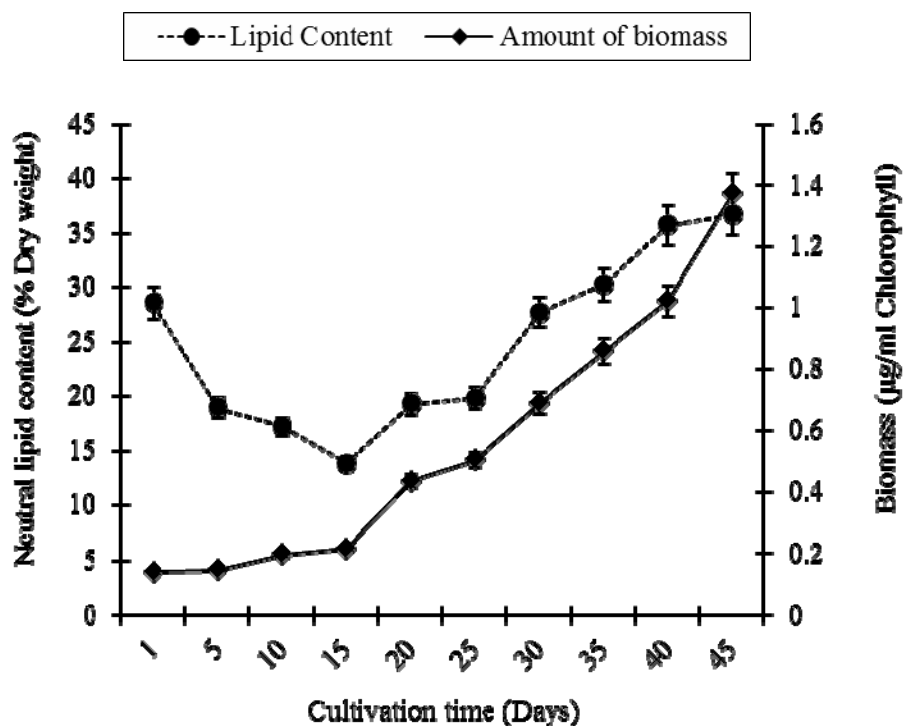


Figure 11: Biomass and Neutral lipid accumulation in *Stigeoclonium* 64-8 over a period of 45 days. Data are expressed as the mean of three replicates.

The biomass, in the case of *Stigeoclonium* 64-8, kept increasing throughout the time period indicating that the cells were still growing. The biomass increased from 0.13 µg/ ml chlorophyll to 1.3 µg/ml chlorophyll from day 1 to day 45. The lipid content also increased and the maximum accumulation was on day 45 with 36% of neutral lipid. So the cells here still did not reach the stationary phase and were probably in the late logarithmic phase as per the details provided by figure 11.

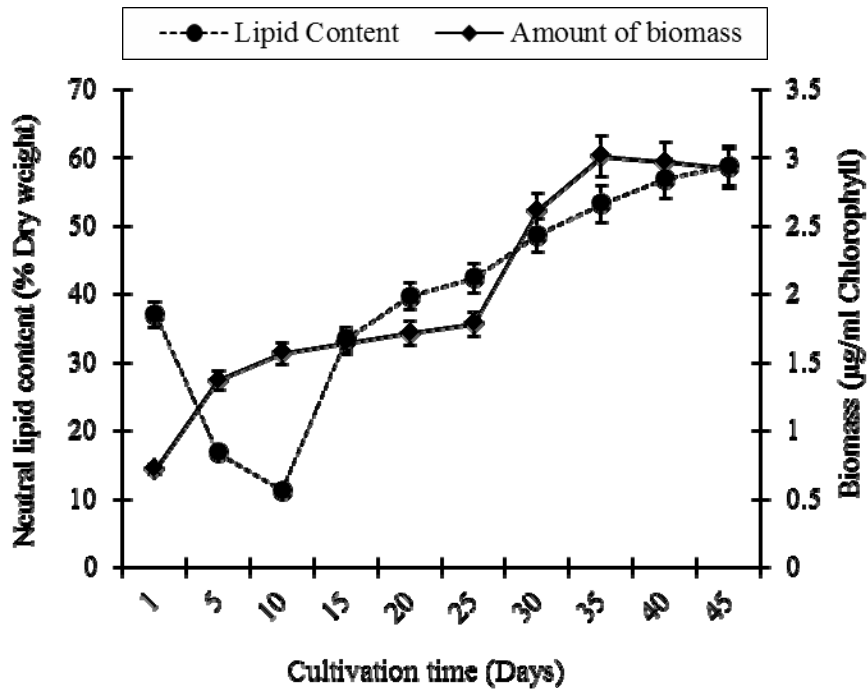


Figure 12: Biomass and Neutral Lipid accumulation on *Coelastrum* 46-4 over a period of 45 days. Data are expressed as the mean differences of the three replicates.

Three distinct growth phases (Fogg, 1965) are shown in figure 12. *Coelastrum* 46-4 underwent a logarithmic growth form day 1 to day 5 with an increase in biomass from 0.71 µg/ml chlorophyll to 1.36 µg/ml chlorophyll. Then it entered the linear phase with an extended period of slow growth until day 35. The onset of stationary phase occurred from day 35 and lasted till day 45. The lipid content on the other hand seems to be high on day 1. This could be because the inoculum from cultures which were in the late stationary phase was used for growing the cells in the new medium (Hongjin & Guangee, 2009). The lipid content increases by from logarithmic phase to stationary phase from 37% to 58%.

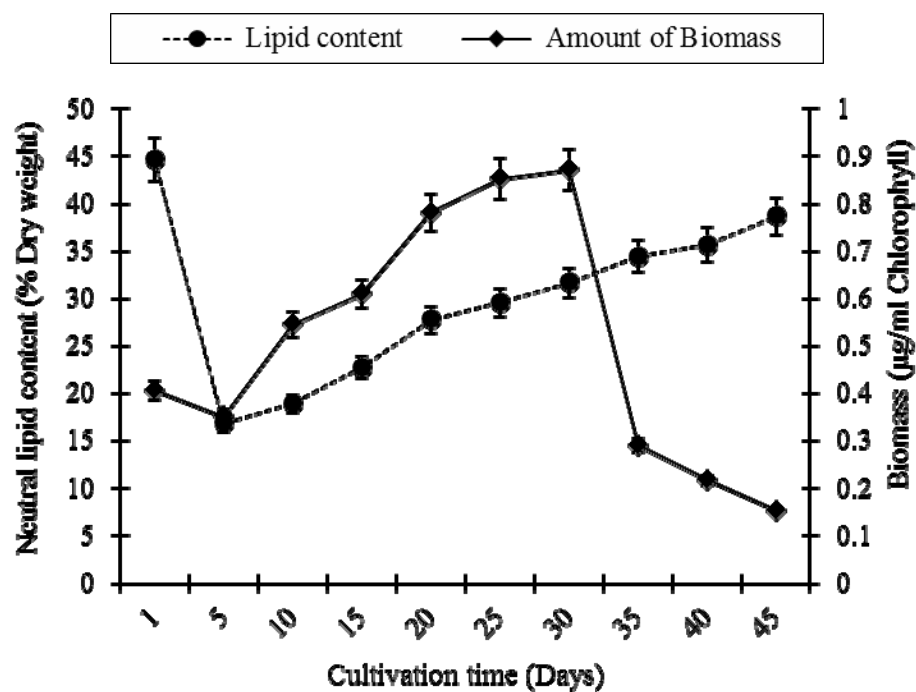


Figure 13: Biomass and Neutral lipid accumulation in *Dactylococcus* 64-10 over a period of 45 days. Data are expressed as mean values of three replicates.

In the case of *Dactylococcus* 64-10 the amount of biomass tends to decrease from day 1 (0.406 µg/ml chlorophyll) to day 3 (0.35 µg/ml chlorophyll) and then tends to increase continuously till day 30 (0.87 µg/ml chlorophyll). After day 30 the cells started degenerating and reaching phase of decline. The lipid content on day 1 was high because the inoculum from cultures which were in late stationary phase was used for growing the cells in the new medium (Hongjin & Guangee, 2009). It then decreased on day 5 and started increasing continuously till day 45.

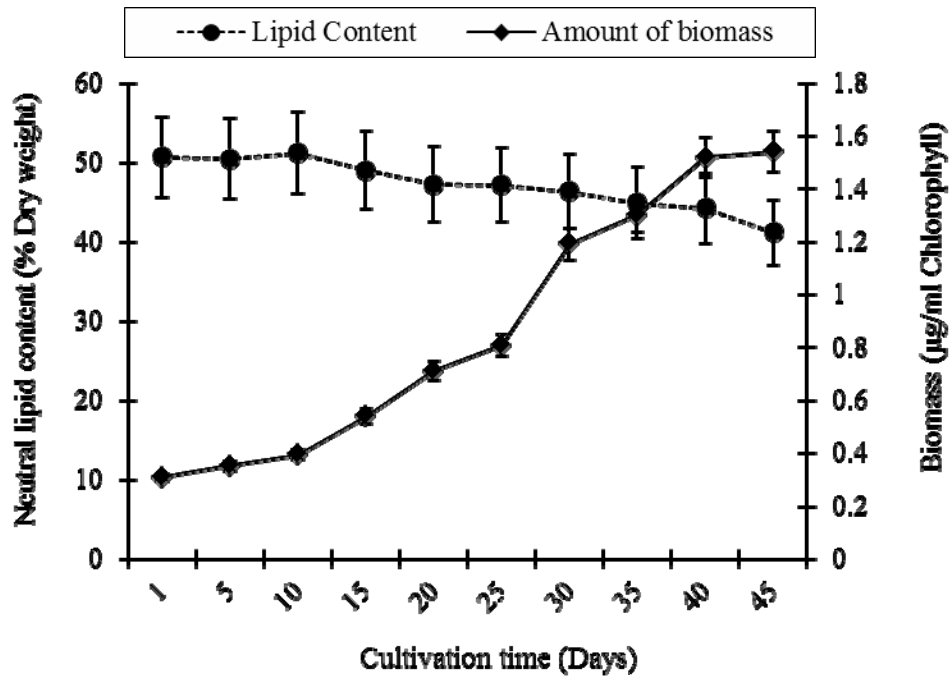


Figure 14: Biomass and neutral lipid accumulation in the control strain *Botryococcus braunii* over a period of 45 days. Data are expressed as the values of three replicates.

The biomass in the control *Botryococcus braunii*, was consistently increasing from day 1 to day 45 indicating that the cells were growing. The cells entered the linear phase from day 5 which was followed by steady growth until day 45. The lipid content on the other hand was higher on day 1 and decreased slightly as the age of the culture increases. The lipid content on day 1 was around 50% and decreased to 41% on day 45.

On the whole, it could be concluded that strains *Cocoid green* 64-12, *Coelastrum* 108-5, *Stigeoclonium* 64-8 and *Botyrococcus braunii* showed a consistent increase in biomass over a period of 45 days indicating that the cells were still in its linear phase of growth .A linear regression test was used to find out if there was a linear relationship between algal biomass and lipid accumulation. *Cocoid green* 64-12, *Coelastrum* 108-5, *Stigeoclonium* 64-8 and *Coelastrum*

46-4 showed a correlation co-efficient (R^2) of 0.834, 0.703, 0.632 and 0.612 respectively indicating that increase in algal biomass increased the lipid accumulation. In *Cocoid green* 64-12 and *Coelastrum* 108-5, the lipid reached its maximum amount on day 25 and day 30 and then remained stationary throughout its period. *Stigeoclonium* 64-8 showed a constant increase in lipid content while *Botyrococcus braunii* showed decrease in the lipid content over the period of study. However, strains like *Coelastrum* 46-4 reached its stationary phase within the 45 day period increasing the lipid content. The biomass in *Dactylococcus* 64-10 started declining within a 45 day period. But still the amounts of lipids were increasing for the period of time. This kind of trend observed in *Dactylococcus* 64-10 is unclear and no conclusion can be made.

3.5 Assessment of effect of environmental factors on Lipid accumulation:

3.5.1 Effect of Nitrogen depletion on Lipid accumulation:

The effects of nitrogen depletion on lipid accumulation were studied. *Cocoid green* 64-12 showed a significant ($P < 0.05$) increase in lipid content over a period of 10 days in the nitrogen deplete concentration. Control represents the lipid concentration in the old culture which was used as an inoculum. However, when the concentration of Nitrogen was 50% of the standard BG11 medium, the lipid content did not seem to increase. Similarly, nitrogen replete cultures also did not increase the lipid content effectively.

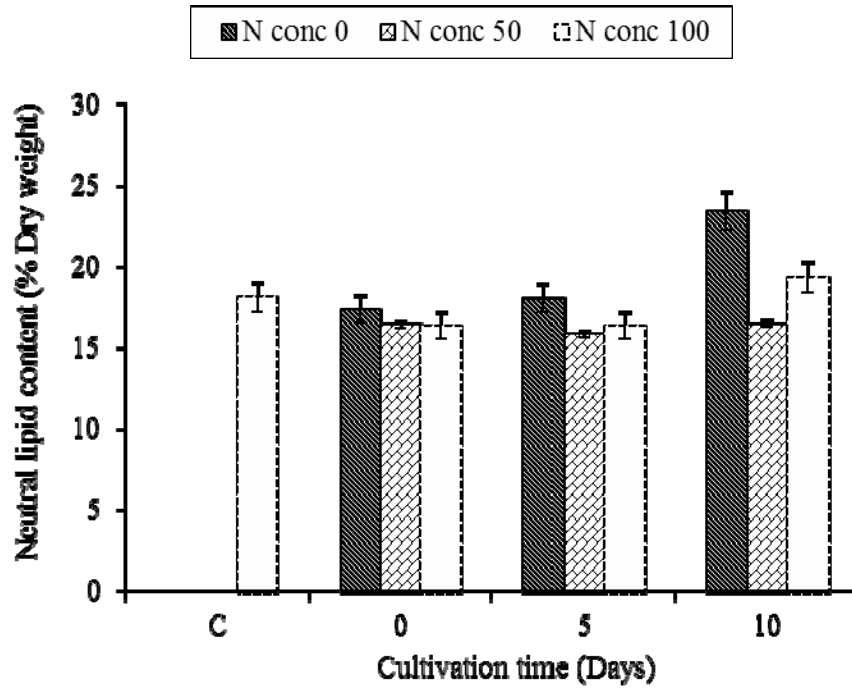


Figure 15: Comparison of neutral lipid accumulation under nitrogen deplete and replete conditions in *Cocoid green* 64-12. Data are expressed as the mean values and standard deviation of three replicates.

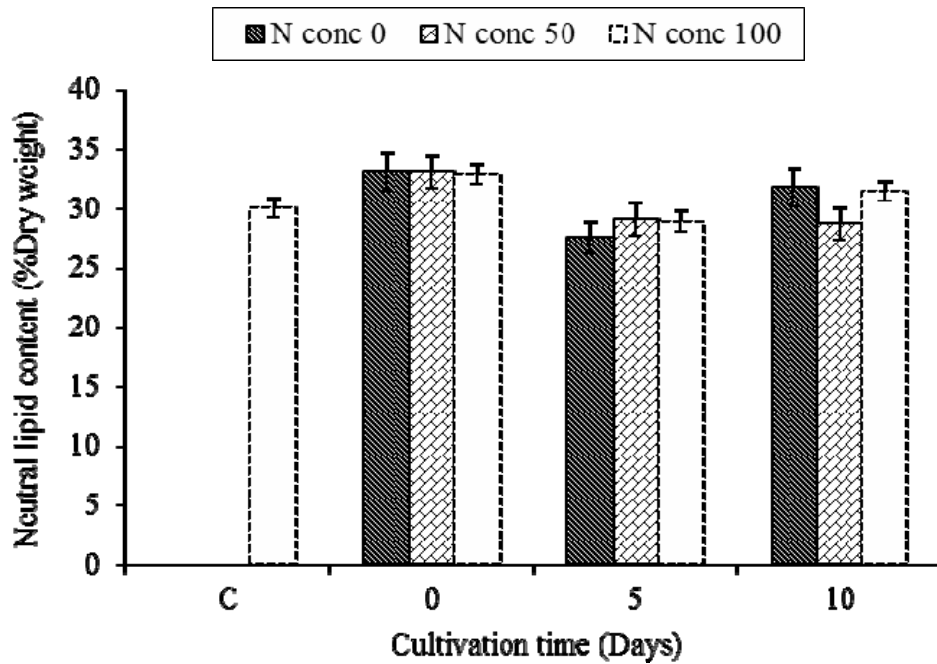


Figure 16: Comparison of neutral lipid accumulation under nitrogen deplete and replete conditions in *Coelastrum* 108-5. Data are expressed as the mean values and standard deviation of three replicates.

Coelastrum 108-5 did not show a significant ($P > 0.05$) increase in lipid accumulation on nitrogen limitation. On the day of inoculation, the lipid content was high in the nitrogen replete conditions (33% dry weight), and then it decreased (29% dry weight) and finally increased (31% dry weight). Nitrogen depletion did not have much influence on this strain.

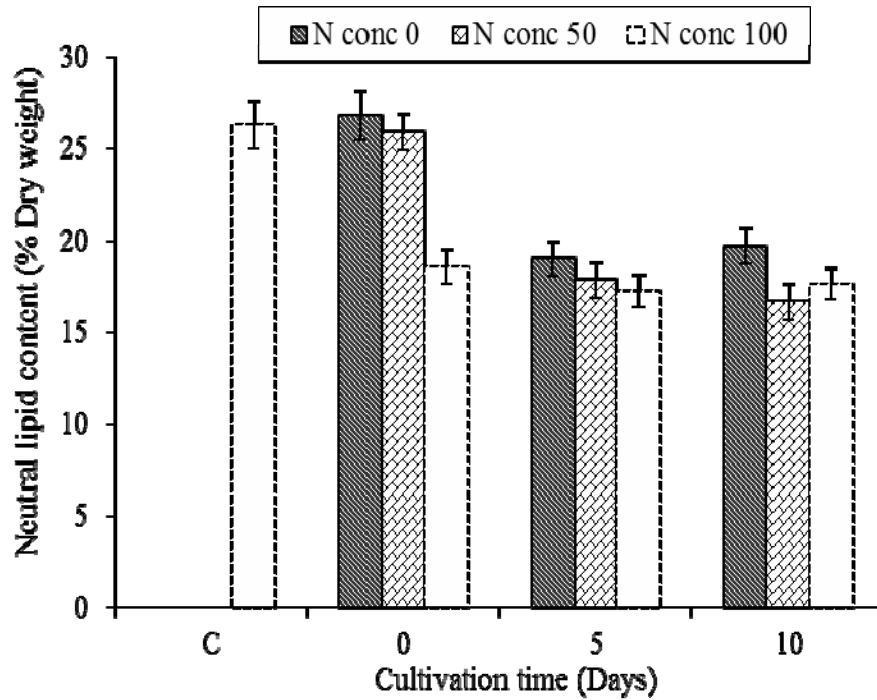


Figure 17: Comparison of neutral lipid accumulation under nitrogen deplete and replete conditions in *Dactylococcus* 64-10. Data are expressed as the mean values and standard deviation of three replicates.

Dactylococcus 64-10 was also not influenced by nitrogen depletion. The initial lipid content was high in all three different concentrations of nitrogen. In the nitrogen deplete conditions, the lipid concentration was around 26% on day 0 and then it decreased to 20% on day 10 indicating that nitrogen depletion did not influence lipid accumulation in this strain. Under nitrogen replete conditions, the lipid content was 19% on day 0 and decreased to 17% on day 10.

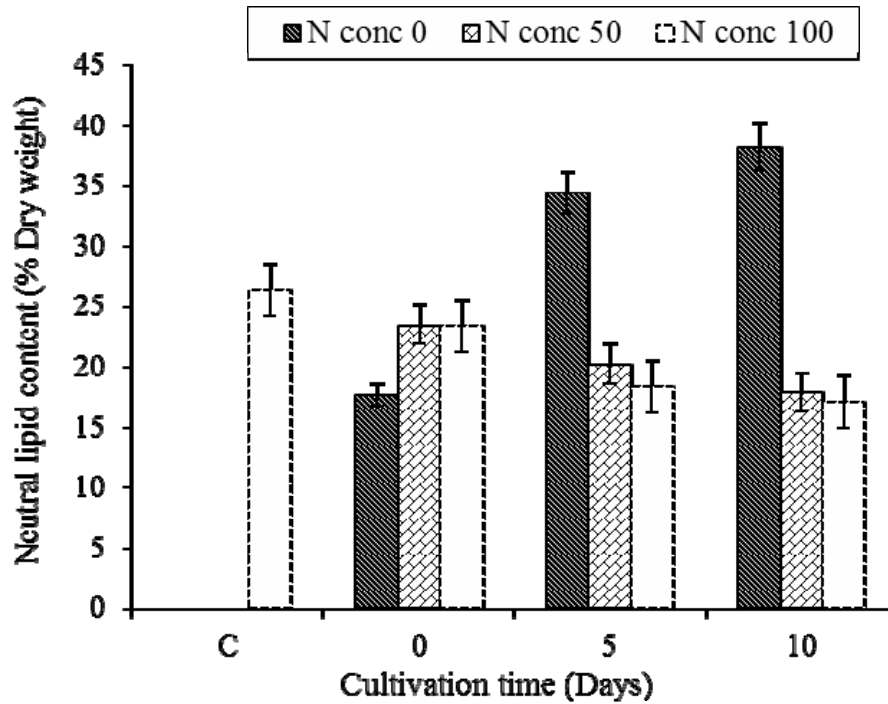


Figure 18: Comparison of neutral lipid accumulation under nitrogen deplete and replete conditions in *Stigeoclonium* 64-8. Data are expressed as the mean values and standard deviation of three replicates.

In the case of *Stigeoclonium* 64-8 (Figure 18), there was a significant ($P < 0.05$) increase in the lipid concentration when it was deprived of nitrogen which is evident from the increase in means from 18% dry weight on day 0 to 38% dry weight on day 10. Under nitrogen replete conditions there is no substantial amount of lipid accumulation observed over the period of 10 days. But the nitrogen depletion seems to be effective in triggering the lipid accumulation.

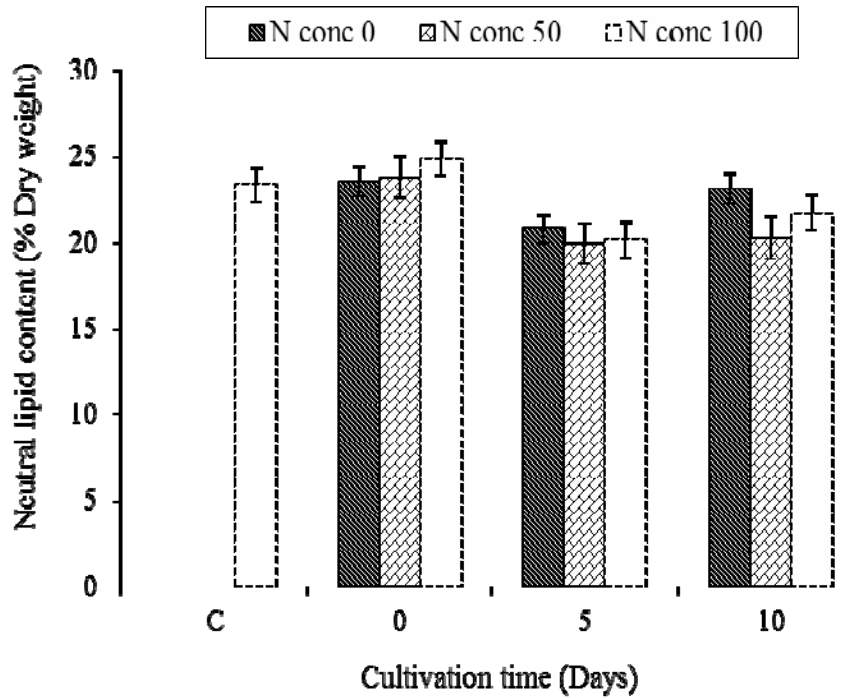


Figure 19: Comparison of neutral lipid accumulation under nitrogen deplete and replete conditions in *Coelastrum* 46-4. Data are expressed as the mean values and standard deviation of three replicates.

Nitrogen depletion does not seem to significantly influence the lipid accumulation in *Coelastrum* 46-4 (Figure 19). When there was no nitrogen in the media, the lipid content initially decreased from 24% on day 0 to 20% on day 5 and then increased further to 22% on day 10. On day 0 the lipid concentration in all three levels of nitrogen is high when compared to day 5 and day 10. When the concentration of nitrogen is 50%, the lipid accumulation is about 24% on day 0 and decreases to 18% on day 10. Similar kind of results was observed in nitrogen replete media where the amount of lipids was around 24% on day 0 and then decreased to 22% on day 10.

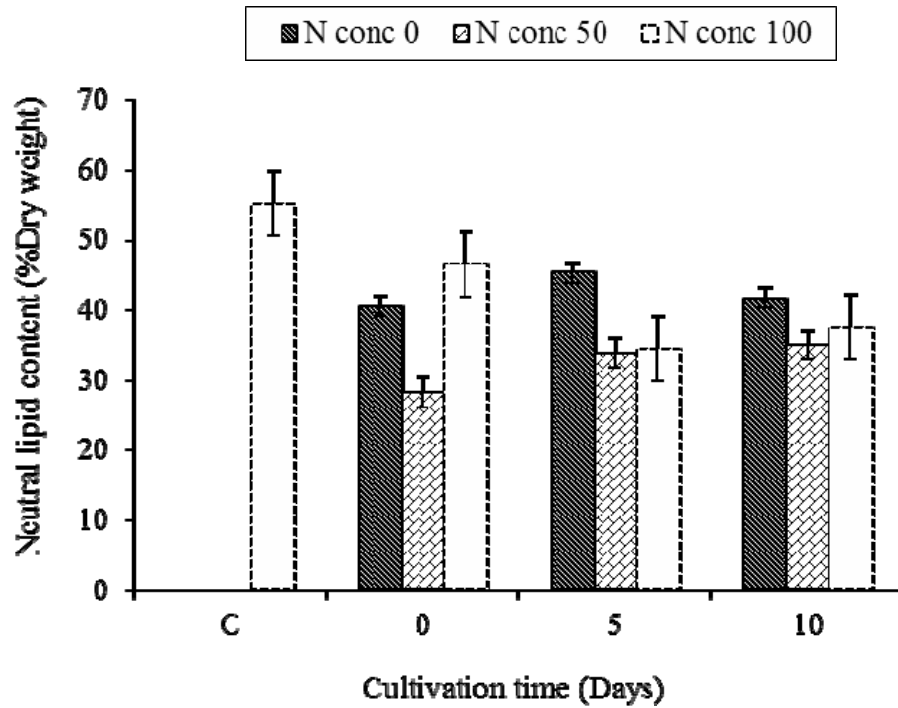


Figure 20: Comparison of neutral lipid accumulation under nitrogen deplete and replete conditions in *Botryococcus braunii*. Data are expressed as the mean values and standard deviation of three replicates.

The reference strain does not seem to respond to nitrogen deprivation (Figure 20). The mean values of the lipid concentrations under nitrogen depleted conditions show a decrease from 45.44% dry weight to 41.75% dry weight. However, when compared to the native strains, it has the highest amount of lipid content under nitrogen replete and deplete conditions. It is evident that no other strain accumulates about 40-50% of lipid within the first few hours of inoculation. Although the reference strain is not greatly influenced by nitrogen depletion, it has a natural tendency to accumulate a large amount of lipids for the first few days. It could be concluded when comparing the native strains with the reference strain only *Stirgeoclonium* 64-8 under Nitrogen depleted conditions tends to accumulate about 38.21% dry weight of lipid within a 10 day period.

3.5.2 Effect of Phosphorous depletion on Lipid accumulation:

The six strains were grown for 10 days under varying phosphorous conditions to determine the effects of phosphorus on lipid accumulation. A variation in phosphorus over time was determined using repeated measures ANOVA. The accumulation of lipid over the period of time in *Cocoid green* 64-12 under varying concentrations of the phosphorous is illustrated in Figure 20. The lipid accumulated over the period of time under 0% phosphorous concentration was found to be statistically significant (P value < 0.05). It was shown that the lipid content increased from 19.83% dry weight to 39.176% dry weight over a period of 10 days under 0% phosphorous concentration. However, in the other two concentrations (50% and 100%) it could be inferred that there was no increase in lipid observed. The control indicates the lipid content in the old culture which was inoculated in the new media. The lipid content estimated in the old culture was of much significance because it reveals the information about any variation in lipid content when the culture in the old media gets re-inoculated in the new media. Figure 20 shows that there was not much difference in the lipid content observed after the re-inoculation into the new media from the old culture.

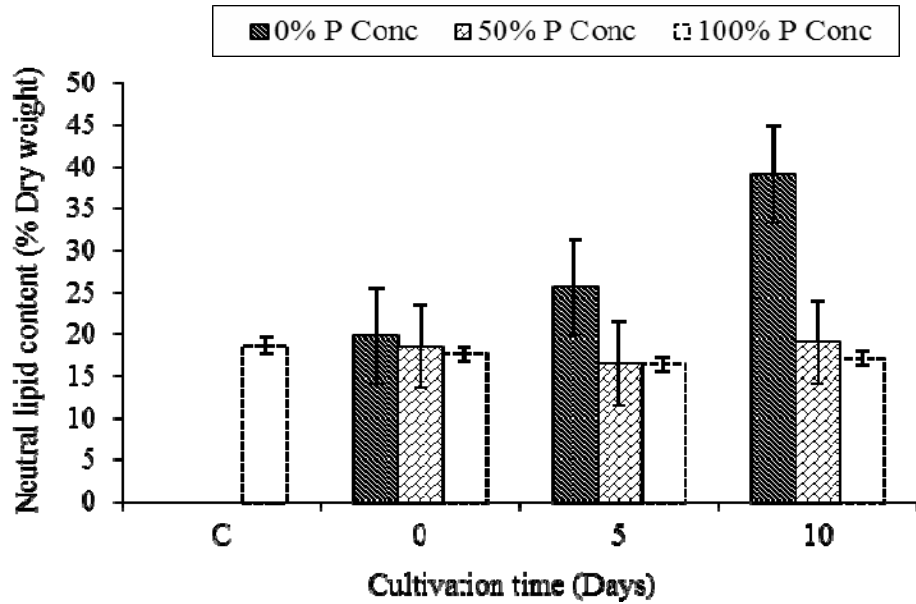


Figure 21: Comparison of neutral lipid accumulation under phosphorous deplete and replete conditions in *Coccoid green 64-12*. Data are expressed as the mean values and standard deviation of three replicates.

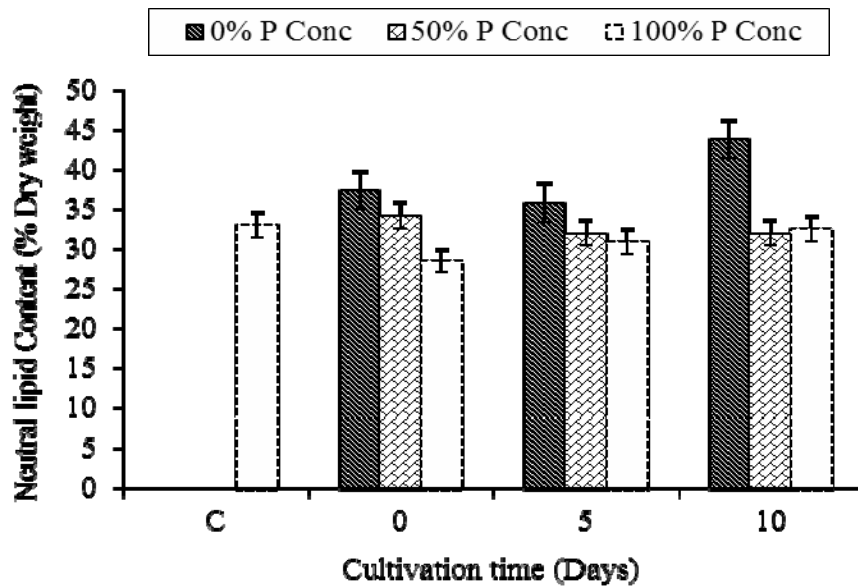


Figure 22: Comparison of neutral lipid accumulation under phosphorous deplete and replete conditions in *Coelastrum 108-5*. Data are expressed as the mean values and standard deviation of three replicates.

In the case of *Coelastrum* 108-5 there was no steady increase in the lipid content observed when it was starved out of nutrients. There was a decrease in the lipid content observed under phosphorous deplete condition from day 0 to day 5 and again increased from day 5 to day 10 (P value < 0.05). However, under 50% phosphorous concentration the lipid concentration was high on Day 0 (34% dry weight) and decreased on day 5 and slightly increased to 32% dry weight. In the phosphorous replete medium, there was a slight decrease on Day 5 (31% dry weight) from Day 0 (28 % dry weight) and then had a slight increase to 32% dry weight.

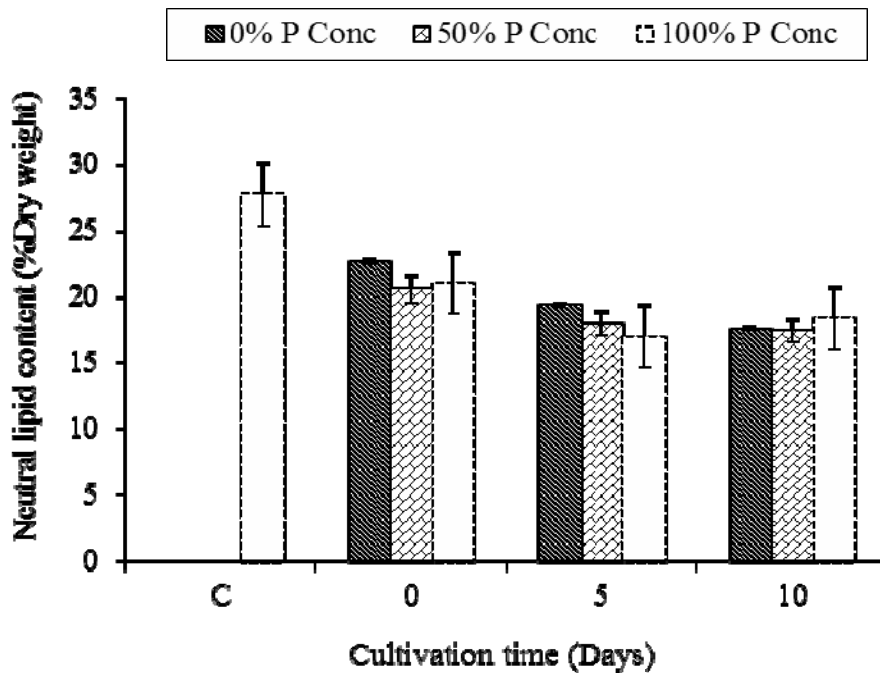


Figure 23: Comparison of neutral lipid accumulation under phosphorous deplete and replete conditions in *Dactylococcus* 64-10. Data are expressed as the mean values and standard deviation of three replicates.

Dactylococcus 64-10 did not respond to phosphorous depletion. There was no increase in lipid over the period of time under phosphorus depleted conditions (figure 23). The control had a

high value of lipid content (28% dry weight) and as it was re-inoculated into the medium; it showed a decrease in the lipid content (21% dry weight). Deprivation of phosphorous did not increase the lipid content which is significant ($P > 0.05$). This shows that the each strain has different behavioral patterns and responds under different conditions.

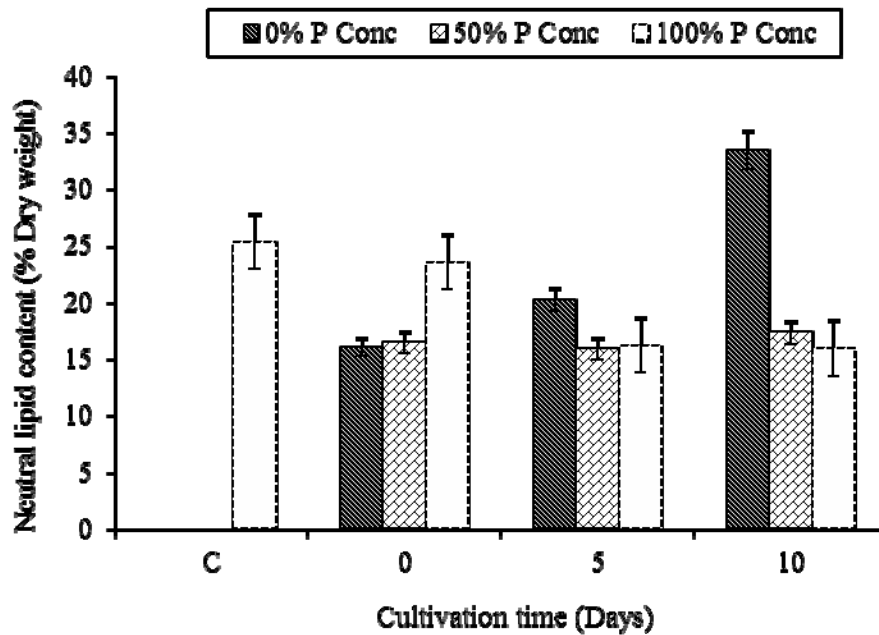


Figure 24: Comparison of neutral lipid accumulation under phosphorous deplete and replete conditions in *Stigeoclonium* 64-8. Data are expressed as the mean values and standard deviation of three replicates.

Stigeoclonium 64-8 showed a high response in increasing the lipid content on phosphorous deprivation. As the phosphorous deprived culture progressed along a time period of 10 days there was a high increase in lipid content from (P value < 0.05). However, in the culture containing 50% phosphorous there was not significant increase or decrease over the time period observed (P value > 0.05).

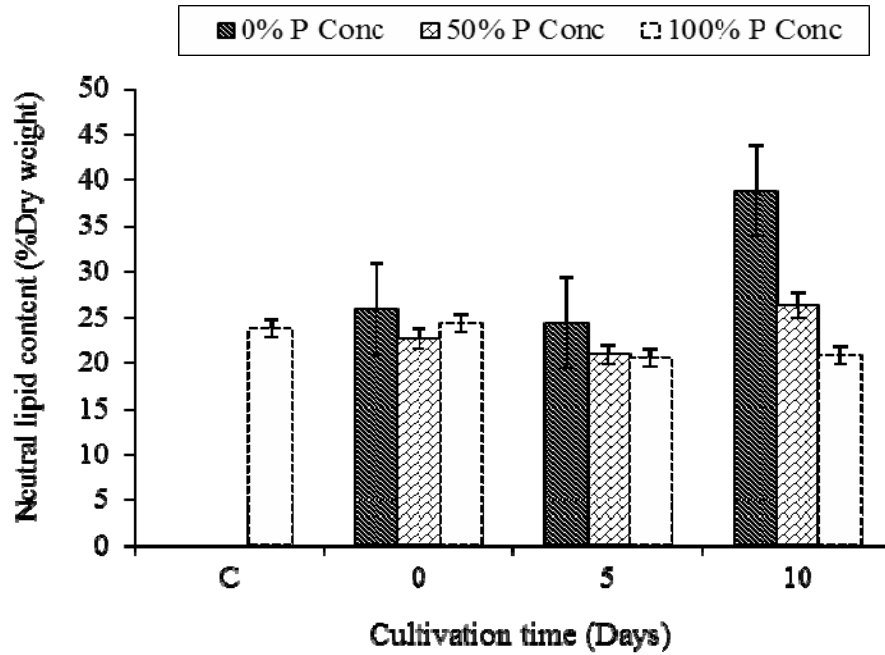


Figure 25: Comparison of neutral lipid accumulation under phosphorous deplete and replete conditions in 46-4 *Coelastrum*. Data are expressed as the mean values and standard deviation of three replicates.

In the case of *Coelastrum* 46-4, there was a significant amount of increase in lipid content under phosphorous depleted conditions. Whereas in 50% phosphorous concentration there was no significant increase in the lipid content observed. The phosphorous replete culture did not show any increase in lipid content over a 10 day period.

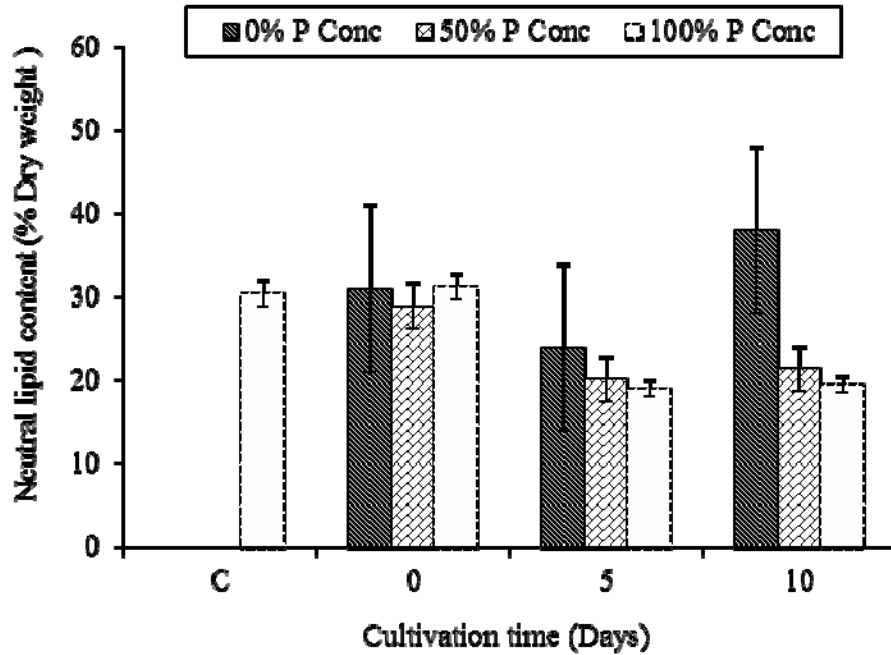


Figure 26: Comparison of neutral lipid accumulation under phosphorous deplete and replete conditions in *Botyrococcus braunii*. Data are expressed as the mean values and standard deviation of three replicates.

The reference strain, *Botyrococcus* did seem to show some response to the depletion of phosphorous. Initially on day 0, the concentration of lipid was almost the same as that of control in all three different concentrations of phosphorous. On day 5, the lipid concentration decreased in all the three different concentrations of phosphorous and then increased on day 10 under phosphorous deplete conditions. Whereas for the other two concentrations of phosphorous there was no significant increase in the lipid observed.

Overall, it could be concluded that both nitrogen and phosphorous depletion can trigger the accumulation of lipid in some strains. In this study, there was an increase in lipid over a period of 10 days under phosphorous deplete conditions observed in *Stirgeoclonium* 64-8 and *Cocoid green* 64-12 more evidently (P value < 0.05). However, other strains did not show any

significant accumulation in lipids. As already discussed, the pattern of lipid accumulation depends on the characteristic of the strain.

4. DISCUSSION:

Earlier studies done by Aquatic Species Program (1996) emphasized collecting chromophytic algae (e.g., diatoms, chrysophytes and pyrmnesiophytes) and analyzing their potential for lipid accumulation. My research attempted to analyze the potential of 27 native Chlorophyta algal strains for producing biodiesel. The strains were tested for their potential to produce biodiesel by estimating the neutral lipid content present in their cells. Relatively simple and less time-consuming methods were needed to screen for the presence of lipids.

The strains were screened on the 13th and 45th day using the Nile red method. A study done by Kalacheva et al. (2002) assessed the effect of temperature on the lipid composition in the green algae *Botyrococcus* and reported that the storage lipids, triacylglyceride were present only in trace amounts during the initial stages of cultivation. In the same study it was reported that neutral lipid accumulates up to 2% after 13 days of cultivation. Therefore, we chose to measure the amount of neutral lipids in our strains on the 13th day to see if there were some strains which were able to accumulate substantially high amount of lipid during the initial stages. TAGs are the important substrate for biodiesel production (Illman et al., 2000). They primarily serve as a storage form of carbon and energy. Since TAGs are the substrate of interest, it is important to measure the amount of TAGs to determine the potential strains.

When the age of the culture increases or cells undergo stressful conditions, the majority of the lipids were neutral lipids (TAGs) (Hu et al., 2008). The reason is, because of the shift in lipid metabolism from membrane lipid synthesis to the storage of neutral lipid. The overall increase in TAGs was contributed by De Novo biosynthesis and conversion of membrane polar lipids into TAGs (Hu et al., 2008). This increase in turn accounts for about 80% of TAG in the total lipid content of the cell. Since, our substrate of interest accumulates more during later stages of culture age; we decided to screen the strains for neutral lipid during the later stages of culture. A study done by Mansour et al. (2003) in marine dinoflagellate *Gymnodium* sp., identified that

the microalgal biomass increased from 8.6 mg L⁻¹ on day 6 (late logarithmic phase) to 127 mg L⁻¹ on day 45 (stationary phase). An increase in lipid yield was also observed from 1.3 mg L⁻¹ during the logarithmic phase to 30.1 mg L⁻¹ during the stationary phase. Based on the previous results showing that amount of neutral lipid accumulation is higher during the stationary phase, we decided to screen our lipids on day 45 assuming that the culture would have reached the stationary phase. However, a growth curve was not generated on a 45 day period for all 27 strains, hence no conclusion can be made that the culture during the 45th day was in the stationary phase. The culture age increased and more accumulation of TAG was observed in all the strains on the 45th day (Table 5). Table 5 gives information that all the strains showed an increase in the neutral lipid content on the 45th day from the 13th day except the reference strain *Botryococcus braunii*. Some papers have published about the accumulation of unusual hydrocarbons and ether lipids in *Botryococcus braunii* (Metzger et al., 2002). These hydrocarbons are classified as: (1) n-alkadienes and trienes, (2) triterpenoid botryococenes and methylated squalenes, (3) a tetraterpenoid, lycopadiene (Metzger & Largeau, 2005). So the presence of these hydrocarbons can make *Botryococcus* the most significant strain in accumulating considerably high amounts of lipids. The strains that accumulated the maximum amount of lipid on the 45th day were chosen because they had highest percentage of TAG, which was the substrate of interest. The strains *Coelastrum* 46-4, *Cocoid Green* 64-12, *Dactylococcus* 64-10, *Coelastrum* 108-5 and *Stirgeoclonium* 64-8 were chosen for further study as they accumulated the maximum amount of neutral lipids on the 45th day. The lipid content in terms of dry weight increased from 13% to 74%, 12% to 50 %, 6% to 42%, 34% to 73% and 13% to 38% in *Coelastrum* 46-4, *Cocoid Green* 64-12, *Dactylococcus* 64-10, *Coelastrum* 108-5 and *Stirgeoclonium* 64-8 respectively. Our results were in accordance with the study done by Chen et al. 2009, in which lipid content increased from 30% on Day 1 to the maximal concentration of 56% by Day 12 in *C.vulgaris*. However, not all the strains responded to culture age in accumulating higher levels of lipid.

Strains like *Botryococcus braunii* did not show a substantial increase in lipid as the age of the culture increased. Similar to this result Alonso et al. (2002) reported that culture age had no influence in accumulating higher levels of TAG in the diatom *P. tricornutum*. So, it could be concluded that not all the strains responds to the increasing culture age in accumulating higher levels of lipid.

The neutral lipids in all the strains were assessed using the Nile red method. In this research, the concentration of lipid present in 100 μ l of cell culture was determined unlike other studies which reported the relative fluorescence intensity. The Aquatic Species Program conducted by NREL concentrated on screening saline microalgae from South Florida using the Nile red fluorescence method. A visual assessment of the lipid contents of the most rapidly growing strains was conducted using the Nile red method. *Tetra 5* and UNKNO4 were the strains that exhibited the maximum accumulation of lipids. However, it was only a visual evaluation and no quantitative measurements of the amount of lipids present in the algae were made. Similarly, in a study done by Huang et al. (2009) the relative fluorescence intensity of *Chlorella vulgaris* was found to be 120.787 AFU using the Nile red fluorescence method which was read from the 96 well plate reader. In our method, we initially read the fluorescence for all the 27 strains from the 96 well plate reader on the 13th and 45th day respectively. The amount of fluorescence was further translated into the amount of lipid present in 100 μ l of culture by constructing a calibration curve using lipid standard Triolein. This gives an absolute measurement of lipid present in the cell.

The strains which exhibited higher amounts of lipids in the Nile red method were further estimated in term of percentage dry weight in order to compare the lipid yield with the Gravimetric technique. The five potential strains grown in 3l flasks were harvested on 45th day and amount of neutral lipids were determined gravimetrically. It was observed that *Coelastrum* 108-5 and *Stigeoclonium* 64-8 accumulated the same amount of lipid in both the methods and

hence could be said that both the methods were effective. Similar kind of results were observed by Chen et al. (2009), which indicated that there was no significant difference between Nile red and Gravimetric method. However, in the other strains like *Coelastrum* 46-4, *Cocoid green* 64-12, *Dactylococcus* 64-10 and *Botryococcus braunii* the lipid yield was different between both the methods. The Gravimetric technique can have noticeable errors because of the several complicated steps involved like lipid extraction, separation and concentration which might also lead to loss of some neutral lipid as observed by other researchers (Elsey et al., 2007). Also, the change in some environmental parameters could have influenced the fatty acid composition in the cell that could have altered the lipid yields in these strains. Nile red method could be more reliable because there are no complicated steps involved and there could be potentially no loss of lipids. Also, estimation of neutral lipid can be done using a small amount of sample. The reliability of Nile red method was assessed by the results obtained for *Botryococcus braunii*. In the case of *Botryococcus braunii*, the amount of lipid accumulated on day 13 was around 52.98%. In accordance to these results, a study done by Kalacheva et al. (2002) indicated that the amount of the fatty acid C16:0 that gets accumulated on day 13 in *Botryococcus* at 25°C was about 54.94 ± 1.94 in terms of % dry weight. The major composition of neutral lipid is C16:0 (Henderson, 1982). Hence, we could say that Nile red method was reliable in estimating the amount of lipids as the neutral lipids yields obtained for *Botryococcus braunii* in both the studies on day 13 were almost the same. Nile red method is very fast and easy method when compared to Gravimetric technique. A small amount of sample is sufficient to analyze the lipid content. This technique was not only faster than traditional Gravimetric analysis, but could detect even a 0.1% difference in lipid content (Huang et al., 2009). Especially, while screening many strains, Nile red method could be an easy qualitative test to analyze the lipids.

The lipid and fatty acid content and composition are affected by culture aging or senescence. The aging algal cells or cells that were maintained under stress conditions showed an

increase in neutral lipid, mainly TAGs. Therefore, growth and lipid accumulation trend on a 45 day period was studied in all the potential strains. There was a steady increase in the biomass observed in *Coccoloba green* 64-12, *Coelastrum* 108-5 and *Stigeoclonium* 64-8, *Coelastrum* 46-4 and *Botryococcus braunii* except *Dactylococcus* 64-10 from day 1 to day 45. However, the accumulation of lipids over a period of 45 days yielded different results for different strains. For instance, in strains like *Coccoloba green* 64-12 and *Coelastrum* 108-5, the maximum lipid got accumulated on day 25 and day 30, and remained constant for the duration. On the other hand, *Stigeoclonium* 64-8 showed a constant increase in lipid content while *Botryococcus braunii* showed decrease in the lipid content over the period of study. *Dactylococcus* 64-10 showed a consistent increase in lipid while there was a decline in the biomass. In strains like *Coccoloba green* 64-12, *Stigeoclonium* 64-8, *Coelastrum* 46-4, *Dactylococcus* 64-10 and *Botryococcus braunii* the neutral lipid content on day 1 i.e., initial phase of the culture was high. This was because the late exponential cultures were used as inoculum to grow these strains in the fresh media (Hongjin & Guangee, 2009). In most of the strains except *Botryococcus*, the lipid content in the initial stages of culture is lower. The reason for this observation could be because when the cells in the initial phase prepare to grow, the lipids were decomposed to supply energy for cell division and growth (Hongjin & Guangee, 2009). However, it was noticed that the lipid content increased as the age of the culture increased. As the cells starts growing, the carbon source gets absorbed and gets assimilated in the form of storage lipids i.e., TAGs (Hongjin & Guangee, 2009). The carbon source in the medium plays an important role in the lipid accumulation (Wen et al., 2003). According to a study done by Jang et al. (2010), the lipid content and biomass concentration in *Chlorella minutissima* UTEX 2341 under heterotrophic fermentation conditions concluded that accumulation of lipids increased quickly when the concentration of glycerin declined sharply; glycerin being the carbon source. Another study done by Hongjin & Guangee, 2009 studied the effect of different carbon sources on growth and lipid accumulation. It was concluded that the

different growth rates occurred due to the response of different metabolism pathways to the added carbon. In our study, we did not add any additional carbon source. The source of carbon in the standard BG-11 medium was Na_2CO_3 . However, no literature has been identified on how Na_2CO_3 stimulates the growth and lipid accumulation in our strains and how it can be utilized in several pathways including the synthesis of fatty acids. This could be an area of further research to study the effect of Na_2CO_3 in stimulating lipid accumulation in algal strains.

Another possible reason for lipid content increase as the culture age increased could be as follows. Another study on assessing the effect of nitrogen deficiency on biochemical composition of *Botryococcus* by Zhila et al. (2004) reports that, nitrogen deficiency influenced the lipid class. It was observed that TAGs increased as the polar lipids decreased. Piorreck and co-workers reported that, during early stages of growth, green algae produced relatively large amounts of polar lipids and polyunsaturated C_{16} and C_{18} fatty acids. On approaching the stationary phase of growth, however, the dominant lipids produced by these algae were neutral, and consisted primarily of saturated 18:1 and 16:0 fatty acids.

In *Cocoid green* 64-12, on a period of 45 days, the lipid content increased about 2 fold while the biomass had a six fold increase. This six fold increase in biomass indicates that, the cells were still in its linear growth phase. Lipid accumulation increased because, sufficient amount of lipids were decomposed to produce energy for growth during the initial phase (day 1-day10) and in the later stages (day 15-25) the excess amount of carbon was absorbed and assimilated to form storage lipids (Hongjin & Guangee, 2009). However, after day 25 the lipid accumulation remained stationary for that period. But, the biomass kept increasing indicating that the culture was still in its linear phase. Neutral lipids would increase if the culture became nutrient limited (Smith et al., 1995). However, if the nutrient became limiting, the biomass would decrease. This is because nutrient limitation decreases the overall cellular activity leading to reduced cell proliferation (Raetz, 2009). In our experiment, the *Cocoid green* 64-12 cells were

still growing but the lipids increased by day 25 and remained stationary throughout. Though, the factors responsible for stationary accumulation of lipids after day 25 remains unclear, possible explanation could be the alteration in the physiological state of the cell. More morphological studies can help in better understanding of the growth and physiology of the cell. Similar kind of result was observed in *Coelastrum* 108-5.

In the case of *Coelatsrum* 46-4, the four phases of growth was observed within a 45 day period. This strain entered the stationary phase on day 30. The lipid increased from 37% on day 1 to 58% on day 45. Whereas, in the case of *Stigeoclonium* 64-8, the lipid content decreased sharply from day 1 to day 15 and then started accumulating lipids. The growth was slow in the initial stages of the culture until day 15 and started increasing after steadily after day 15. So, it can be inferred that as soon as the carbon reserves were utilized for growth, the excess amount of carbon were assimilated as neutral lipids, while the lipids were decomposed until day 15 to produce energy for growth and cell division (Hongjin & Guangee, 2009). While in the case of *Coelastrum* 46-4, the culture reached stationary phase on day 30 and the lipids were increasing (Figure 12). As discussed earlier, we know that when the culture reaches the stationary phase, the dominant lipids produced by algae are mainly in the form of neutral lipids which primarily consists of C16:0 and C18:0 (Piorreck et al., 1984).

Dactylococcus 64-10 showed a different trend in accumulation of biomass over the period of 45 days. The biomass started decreasing after day 30. The lipid content on the other hand kept increasing continuously (Figure 13). The growth almost started declining after day 30; however, the lipid accumulation was still increasing. According to the data presented by Shifrin & Chisholm (1981), the trend of decreasing growth rate with an increase in lipid content was reported. The cells accumulate lipids when the growth and division of cells is put to halt (Greenwell et al., 2009). This apparent inverse relationship could have important implications on the economics of algal biofuels (Greenwell et al., 2009). According to Widjaja et al. (2009),

higher lipid content and higher biomass obtained resulted in higher lipid productivity. The biomass productivity, cell lipid content and overall lipid productivity determines the economic feasibility of algae oil for biodiesel production (Tran et al., 2009). In the case of *Dactylococcus* 64-10 although the lipid content is high during the stationary phase, the biomass is very less, making the overall lipid productivity less.

Botryococcus braunii, showed a decrease in the lipid content as the age of the culture increased. This strain accumulated maximum amount of lipids in the initial phase and decreased as the age of the culture increased. *Botryococcus* produces hydrocarbons 75% of dry weight (Banerjee et al., 2002). According to a study done by Kalcheva et al. (2009) in assessing the effect of temperature on green alga *Botryococcus*, it was identified that, the decrease in lipids was accompanied by the accumulation of polysaccharides. It was also identified by Casadevall et al. (1985) that some *Botryococcus* accumulates more polysaccharides in the stationary phase and synthesis lipids in the exponential and linear growth phases. These results obtained by other researchers were in accordance with our results. A higher amount of lipid accumulation was observed during the initial phase of the culture. However, as the age of the culture increased, the lipids started decreasing. This could be potentially due to the increase in polysaccharides content and decrease in neutral lipids. However, it was observed that the growth rate of this species was comparatively slower than other strains in our study. In the initial stages of the culture, the growth was in lag phase. According to a review report written by Wolf (1983), it was shown reported that the unusual morphology of this alga could possibly be the factor for imposing constrains on growth. It was suggested that sluggish growth may be because of the hindrance of cellular gas exchange to the cells by colonial matrix and alga's ability to metabolize metabolically expensive lipids (Belcher, 1968).

Much research focused on the lipid increase in microalgae caused under environmental stress conditions. In algae, lipids and carbohydrates are both used as energy reserves and their

synthesis is regulated qualitatively and quantitatively by a number of environmental factors. It is generally accepted that depletion of nitrogen from the medium induces lipid accumulation (Evans et al., 1984). For instance, a strain of algae put into nitrogen deficient environment can cause the algae to go from 22% to 58% oil content per dry mass (Sheehan et al. 1998). Once this high oil content algae is obtained, the oil from the algae is extracted using solvents and then turned into biodiesel through a process known as transesterification (Vasudevan & Briggs 2008). In the reports published by the U.S Department of Energy National algal biofuels workshop, it was mentioned that the nutritional stress causes phospholipids and glycolipids to be converted to TAG's. According to Sukenik & Wahnnon, 1991, under nutrient sufficient conditions, proteins are synthesized and during nutrient deprived conditions the cell division is suppressed and a greater amount of carbon is available for lipid storage. Sheehan et al. (1998) reported that the reason for the increase in lipid content was that under nutrient starvation, the rate of production of all cell components is lower, but oil production remains higher, leading to accumulation of oil in cells. Biochemical studies have also suggested that a biotin- containing enzyme acetyl-CoA carboxylase (ACCase), may also be involved in the control of lipid accumulation. Therefore, it may be possible to enhance lipid production rates by increasing the activity of this enzyme via genetic engineering. One particularly important nutrient which influences lipid accumulation is nitrogen (Roessler, 1990). Turcotte & Kosaric (1988) studied the biosynthesis of lipids on *Rhodospiridium toruloides* under nitrogen limiting conditions which enhanced the accumulation of lipids. Nitrogen starved *Chlorella pyrenoidosa* was able to accumulate up to 85% of lipid in its culture while the accumulation of lipids in normal culture was only 5% (Spoehr & Milner, 1949). Illman et al. (2000) studied five strains of the green alga *Chlorella* for growth in Wantanabe and low-nitrogen media in a 2-L stirred bioreactor. Though the growth of *Chlorella* varied in low-nitrogen media, an increase in lipid content was seen in all five strains when compared to the growth in Wantanabe medium. The greatest increases were seen in *C. vulgaris*, in which the lipid

content increased from 18% to 40% dry weight, and *C. emersonii* where the lipid content increased from 29% to 63% dry weight in Wantanabe medium versus low-nitrogen medium (Illman et al. 2000). However, large variability exists in response to nitrogen deficiency. Diatoms, although have a long log- phase do not seem to respond to nitrogen starvation by increasing their lipid content (Benemann & Oswald, 1996). The Chlorophyceae (Green algae) group shows different responses to nitrogen starvation from several fold increases from log phase values (e.g., *Chlorella pyrenoidosa*) to no change (*Dunaliella* species) in the lipid content (Borowitzka, 1988). Environmental stresses like nitrogen depletion lead to inhibition of cell division, but does not immediately slow down the oil production. In our case, the increased lipid content clearly illustrated that nitrogen deprivation stimulated the lipid storage in *Coccolid green* 64-12, *Coelastrum* 46-4 and also *Stigeoclonium* 64-8. The amounts of TAGs are usually the lowest during the exponential phase (Volkman, 1989) and increases in stationary phase as the nutrients in the medium gets depleted (Brown et al., 1996). Under nitrogen stressed cultures, there was an increase in TAGs from 5% of the total cell volume to 70% of the total cell volume in *Chaetoceros gracilis* (Lombardi & Wangersky, 1995). This increase was not observed in *Isochrysis galbana* and *Dunaliella tertiolecta*, utilized in the same study (Lombardi & Wangersky, 1995). In another study conducted by Rodolfi et al. (2008) four strains (two marine and two freshwater) were selected because of their robustness, high productivity and relatively high lipid content and were grown under nitrogen deprivation in 0.6-L bubbled tubes. Only the two marine microalgae accumulated lipid under such conditions; they are eustigmatophyte & *Nannochloropsis* sp. F&M-M24, which attained 60% lipid content after nitrogen starvation. Similarly in our study, *Coelastrum* 46-4, *Stigeoclonium* 64-8 and *Coccolid green* 64-12 responded to nitrogen starvation while other strains showed no response. Thus nitrogen starvation may be a good method for increasing the lipid synthesis and storage only in some strains of green algae. It was suggested (Suen et al., 1987) that since nitrogen and phosphorous participates in the elaboration of

phospholipids that under nutrient limiting conditions lipid synthesis is oriented towards the storage of saturated triglycerides and neutral lipids. Hence, in this study phosphorous limitation was also analyzed in its role in increasing the lipid accumulation.

Phosphorous stress has also been found to decrease the chlorophyll *a* content and increase the lipid accumulation in *Phaeodactylum tricornutum* (Roy, 1988). Many investigators have worked on phosphorous deprivation (Kuhl, 1974). Growing *S. obliquus* under phosphate depleted conditions seems to increase the lipid content to 29.5% dry cell weight (Mandall & Malick, 1984). This approach seems to stimulate the breakdown of the phospholipids in the cell membrane into neutral lipids to obtain phosphate, resulting in an accumulation of neutral lipids in the cell (Beer et al., 2009). In a study done by Khozin-Goldberg & Cohen (2006) in *Monodus subterraneus*, the absence of phosphate increased the proportion of TAG from 6.5% to 39.3% of the total lipids. Similarly, in our study we had three strains responding to phosphorous depletion. *Cocoid green* 64-12 which showed an increase in the lipid content 30.69% to 60.63% of lipid. Similarly *Stigeoclonium* 64-8 also showed an increase in lipid content from 27.20% to 56.71% dry weight which was substantially high. Another strain *Coelastrum* 46-4 showed an increase in lipid content from 25.89% to 38.846% dry weight. In order to increase the oil yield of algae cultures, the cell lipid content should be increased without any loss in lipid productivity. This appears to be a very tough task. Usually under nutrient shortage, the fraction of lipids will increase but cellular growth declines with the decrease in lipid productivity (Huesemann & Benemann, in press). However, in this research we have not measured the lipid productivity. Hence, a balance between lipid production and biomass has to be maintained for higher lipid productivity, which makes this technology feasible from an economic standpoint.

5. Conclusion and Recommendations:

This section summarizes the research findings and discusses how well the results obtained address the research questions formulated to achieve the goals of the study.

5.1 Conclusion:

There is an increasing concern about the long term availability of non-renewable energy sources due to the large consumption of fossil fuels. Also, it is important to take necessary actions in order to mitigate the reduction in CO₂ concentration in the atmosphere which contributes to global warming. One of the best options in the long term is harnessing photosynthetic power of microalgae in which sun's energy is converted into biomass and then converted in to biodiesel. Biodiesel is a clean burning fuel that is renewable and biodegradable. Current research has focused on screening algal strains as potential source of biofuels. The commercial success of microalgae based biofuel production depends on identification of high lipid producing microalgae. More amount of oil can be extracted when the lipid content is high. A strain having a high lipid content, and ability to respond to accumulating lipids under nutrient deficiency and to adapt to unavoidable changes in the environment like temperature, pH and light intensity would be ideal for growing on a large scale for fuel production.

It seems likely that there are billions of strains that colonize every niche of this planet, of which there may be atleast few strains suitable for biofuel production. Florida, being the ideal place with optimum sunlight would be helpful for growing algae for biofuel production. On the other hand, numerous reports suggested that Chlorophyceae is the class of algae which had large pool of organisms for lipid production (Hu et al., 2008). This research investigated the potential of the native green algal strains isolated from the Everglades for producing biodiesel. The Nile red method provided a rapid and easily manipulated method for in-vivo quantification of neutral lipids in these native algae. Out of the 27 strains screened, there were five strains which showed substantially a high amount of lipid in them. The strains were *Coelastrum* 46-4, *Dactylococcus*

64-10, *Stigeoclonium* 64-8, *Cocoid green* 64-12 and *Coelastrum* 108-5. *Coelastrum* 108-5 yielded more than 70% of the neutral lipid when extracted gravimetrically as well as when estimated using the Nile red method. *Stigeoclonium* 64-8 and *Coelastrum* 108-5 yielded the same amount of lipid in both Nile red method and Gravimetric technique. Nile red method was reliable to use as it required very less sample and an easy method for screening large number of samples. Almost all our strains tend to accumulate lipids over a month's period which is definitely not feasible from an industrial stand point. Higher lipid accumulation was observed by exposing the strains to 10 day nitrogen and phosphorous depletion in *Stigeoclonium* 64-8, *Coelastrum* 46-4 and *Cocoid green* 64-12. In short, it could be summarized that *Coelastrum* 108-5, *Cocoid green* 64-12 and *Stigeoclonium* 64-8 were promising in some aspects and could be used for further studies. Our conclusion is in accordance with the results published by the Aquatic Species Program which states that there is no one particular strain which is found to be efficient for biodiesel production.

There is a continued research on microalgae for biodiesel production. The algae-fuel technology is a very complex science that is yet to be fully grasped and that is continually evolving and opportunities for improvement never fail to present them. Commercial-scale use of microalgae for biodiesel would require huge investments in production facilities. A considerable investment by R &D in technological development and technical expertise is still needed to close the gap between micro-algae derived fuels and fossil oil. It is also necessary to sustain the needs of energy in future and earn carbon credit by adopting the green-clean technology (Verma et al., 2009).

5.2 Recommendations:

To reap the benefits of algal fuel technology, focused research fundamental biological questions related to regulation of lipid metabolism is required. The isolation and characterization of algae from different aquatic environments should be a continuing effort. The several thousand algal strains examined for lipid production represent only a small portion of the over 40,000

identified species available in nature (Hu et al., 2008). More research on additional organisms will provide novel insights into the unique mechanisms that algae possess for more efficient lipid production. A modified or new approach is required for the lipid extraction from algal biomass which is another major task. Genetic manipulation of the strains through metabolic engineering represents another promising strategy for producing algal oils. A complete understanding of the control mechanisms underlying the relationship between cell cycle and TAG production will enable genetic manipulation of selected algal strain that exhibit rapid growth and TAG accumulation simultaneously to ensure maximum sustainable biodiesel production.

From this research it could be recommended, that the strains *Pediastrum* 108-4, *Chlorococcum* 5-1 and *Cocoid green* 103-6 can be used for further studies as these strains has the capacity to accumulate considerably a fair amount of lipid within a 13 day period (Table 5). The lipid extracted should further be subjected to GC-MS analysis for the determination of FAME, which determines the biodiesel yield.

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