IDENTIFICATION OF ALGAL STRAINS BY PCR AMPLIFICATION AND EVALUATION OF THEIR FATTY ACID PROFILES FOR BIODIESEL PRODUCTION

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DEDICATION

This thesis is dedicated to my parents Reynaldo Moreno and Carmen Suyapa Uclés de Moreno who provided their never-ending support, love, inspiration, encouragement to achieve my goals in life, and their efforts to provide me with the best preparation and education possible. Likewise, I extend this dedication to my sisters Karen Suyapa, and Gina Maria, and to my brother Carlos Armando for caring as well and for being such outstanding role models, and to my nieces Karen Daniela, Grace Marie and my nephews Mario Alfredo, Diego Ernesto and Luc Wyatt.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

CLS-Y: Cell Lysis Solution for yeast, algae or fungi containing 1-5 % SDS and 1-5 % DL-Dithiothreitol.

SEWS: Washing solution containing 10-20 % TRIS hydrochloride, less than 1 % TRIS base and 95 % ethanol.

DES: Ultra-pure water DNA elution solution.

TE: 10 mM Tris-HCl and 1 mM EDTA elution buffer (pH 8.0).

QG: pH indicator buffer for optimal DNA binding containing guanidine thiocyanate at 50-100%.

PE: Wash buffer containing ethanol at 70-80 %.

EB: Elution buffer containing 10 mM Tris-Cl (pH 8.5).

P1: Resuspension buffer containing 50 mM Tris-Cl and 10 mM EDTA.

P2: Lysis buffer containing 200 mM NaOH and 1 % SDS.

N3: Purification buffer containing 25-50 % guanidium chloride and 10-25 % acetic acid.

PB: Binding buffer for DNA clean-up containing guanidinium chloride at 25-50 % and propan-2-ol at 25-50 %.

ECO RI buffer: Restriction endonuclease buffer containing 15.8 % Tris buffered with HCl, 3 % NaCl, and 1 % MgCl.

SOC Medium: Medium containing 2 % tryptone, 0.5 % yeast extract, 10 mM sodium chloride, 2.5 mM potassium chloride, 10 mM magnesium chloride, 10 mM magnesium sulfate and 20 mM glucose per liter.

PNNL: Pacific Northwest National Laboratory.

EN 14214: European Standard that describes the requirements and test methods for FAME.

ASTM D6751: American Society for Testing and Materials (ASTM) standards and specifications for biodiesels blended with middle distillate fuels.

ABSTRACT

Four microalgae, obtained from different brackish and fresh water sources within the state of Louisiana's Southeast region, were evaluated for their potential use in the production of biodiesel. The microorganisms were isolated and identified using genomic DNA, and 16S rRNA or 18S rRNA gene amplification followed by sequencing. The resultant sequences were compared with those available on the NCBI website database through the BLAST bioinformatic tool. The results showed high correlation with known nucleotide sequence identities at 99 % with *Synechococcus* sp., 98 % with *Sellaphora pupula*, 99 % with *Chlorella sorokiniana*, 99 % with *Scenedesmus abundans*, and 99 % with *Chlorella vulgaris* (control).

The fatty acid profiles of the identified organisms grown using 5 % CO_2 aeration into the growth media were evaluated and were found to be different to the control group (0.037 % CO_2). In *Synechococcus* sp., total fatty acids (TFA) decreased from 20.63 g kg^{-1} to 17.62 g kg^{-1} dry biomass with the appearance of C18:2 and C18:3, which were absent in the control. TFA from *Sellaphora pupula* decreased from 54.8 g kg^{-1} to 24.4 g kg^{-1} dry biomass and contained the greatest C16:1. The extracted TFA from *Scenedesmus abundans* increased from 14.14 g kg^{-1} to 31.63 g kg^{-1} and displayed the highest content of C18:1. For *Chlorella vulgaris* UTEX 259, TFA content increased from 15.14 g kg^{-1} to 47.83 g kg^{-1}, and 50 % of that total was C18:3. The TFA content from *Chlorella sorokiniana* decreased from 29.82 g kg^{-1} to 23.99 g kg^{-1}; however, it had a lower C18:3 which allowed for a balanced fatty acid profile in terms of cetane number, oxidative stability, viscosity and low temperature conditions. The aforementioned conditions, plus owning the best biomass productivity when using 5 % CO_2, deemed *Chlorella sorokiniana* as the best candidate of the strains evaluated for the production of biodiesel.

Keywords: microalgae• biodiesel• fatty acid methyl esters• carbon dioxide• *Chlorella sorokiniana*

1. LITERATURE REVIEW

1.1. Introduction

The rising need for energy in developing nations is giving place to vehement competition for the world's decreasing energy resources (Pienkos and Darzins, 2009). The increased use of fossil fuels results in larger greenhouse gases (GHG) emissions, and this is usually considered the main reason for global climate change (Wuebbles and Atul, 2001). Fossil fuels are the largest contributor of GHGs to the atmosphere (EIA, 2006). With the increase in anthropogenic GHG emissions due to the extensive use of fossil fuels for transport, new techniques for the development of electricity and thermal energy generation are needed (Brennan and Owende, 2010). The goal of a 5.2 % reduction in GHG emissions worldwide from 1990 values was proposed during The Kyoto Protocol back in 1997 (Wang et al., 2008). One hundred and ninety-three countries are currently part of this protocol, with the United States being the only remaining signatory nation that has not ratified it (Status of ratification of Kyoto Protocol, 2011). Greater use of biofuels, which compete and have partially displaced petroleum based fuels for use in transportation, could help meet that reduction in emissions objective (Macedo et al., 2008).

In terms of CO_2 reduction, first generation biofuels are said to have a limited performance and demand the use of large amounts of land (Schubert, 2006) and have now reached economic levels of production. Examples are food and oil crops (e.g. biodiesel from rapeseed oil, and ethanol from sugarcane, sugar beet, and corn) (FAO, 2008) as well as animal fats (FAO, 2007). Strong controversy surrounds the use of first generation biofuels, usually due to their negative impacts like contributing to the increase in food prices, deforestation and biodiversity losses,

hence, the extent of their ultimate contribution to the reduction of GHG is frequently questioned (Gomez et al., 2011).

Second generation biofuels are produced from lignocellulosic biomass (e.g. agricultural waste products, forest residues, municipal waste) (Aita and Kim, 2010). Technologies for the conversion of lignocellulosic biomass into biofuels have yet to reach the scales for commercialization (FAO, 2008). In order for a biofuel resource to be technically and economically viable, it needs to compete with petroleum fuels' prices, improve air quality (e.g. through CO_2 sequestration) and require low usage of water and land (Khosla, 2009).

Microalgae are projected to be the source for the third generation of biofuels (Vieira Costa and Greque de Morais, 2011). High biomass productivity, accumulation of up to 20-50 % (w/w dry weight) triacylglycerols, the lack of requirement for high quality agricultural land and water renewal when compared to terrestrial crops are among the advantages to be offered by microalgae (Singh and Gu, 2010; Scott et al., 2010). Despite those advantages, several intrinsic and applied research and development obstacles need to be addressed for the commercialization of algal-based fuels at a cost that can compete with that of petroleum-based fuels (Pienkos and Darzins, 2009).

Like plants and some photosynthetic bacteria, algae are photosynthetic organisms, which efficiently utilize energy from the sun to convert water, CO_2 and O_2 into biomass (Sheehan et al., 1998). The most comprehensive research effort regarding the production of fuels from algae has been the "The Aquatic Species Program (ASP)" led by the Department of Energy (DOE) from 1978 to 1996. DOE invested approximately $25 million to study a variety of aquatic species such as macroalgae, cattails and microalgae for use in renewable energy production, and several important advances in the technology arose from this effort (Sheehan et al., 1998).

Microalgae are photosynthetic organisms which can be prokaryotic or eukaryotic and can grow in a wide range of environmental conditions (Mata et al., 2010). Microalgae produce lipids in the form of triacylglycerols or triglycerides (Sheehan et al., 1998). These triglycerides can be reacted with an alcohol in a reaction called transesterification or alcoholysis (Figure 1.1) to obtain fatty acid methyl or ethyl esters for the production of biodiesel (Gouveia and Oliveira, 2009).

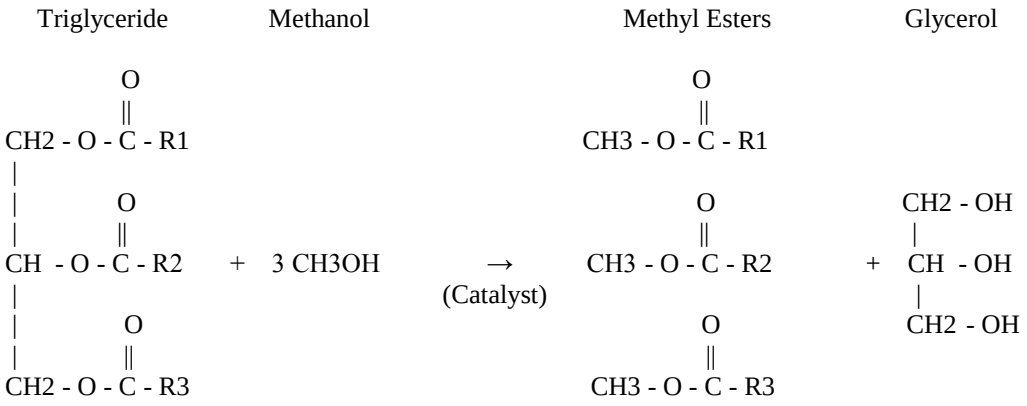

Figure 1.1 Transesterification Reaction.

1.2. Microalgae as a Source for Biodiesel

The use of microalgae as fuel feedstock was first proposed over 50 years ago for the production of methane gas (Meier, 1955). Yields for biodiesel production from microalgae can be 10 to 20 times higher than those obtained from oleaginous seeds and/or vegetable oils (Table 1.1). The oil content in some microalgae can be fairly high and can be induced to produce even higher concentrations of lipids through the implementation of low nitrogen media, varying Fe^{3+} concentration and increased light intensity (Illman et al., 2000; Liu et al., 2007; Rodolfi et al., 2007; Solovchenko et al., 2008; Tornabene et al., 1983). CO_2 removal from power plants through biofixation represents an interesting method for reducing GHG emissions and assisting in the increase of microalgal biomass, lipid and biodiesel yields (Wang et al., 2008).

Several different types of renewable fuels can be obtained from microalgae. Among those, we can include methane produced by anaerobic digestion of the algal biomass (Spolaore et al., 2006); biodiesel derived from microalgal oil (Roessler et al., 1994; Sawayama et al., 1995; Dunahay et al., 1996; Sheehan et al., 1998; Banerjee et al., 2002; Gavrilescu and Chisti, 2005); and biohydrogen which can be produced photobiologically (Ghirardi et al., 2000; Akkerman et al., 2002; Melis, 2002; Fedorov et al., 2005; Kapdan and Kargi, 2006).

Some microalgae have also a convenient fatty acids profile for transesterification and an unsaponifiable fraction which allows for the production of biodiesel with high oxidation stability (Dote et al., 1994; Ginzburg, 1993; Milne et al., 1990; Minowa et al., 1995) and physical and fuel properties (e.g. density, viscosity, acid value, and heating value) which are comparable to those found in fossil diesel (Miao and Wu, 2006; Rana and Spada, 2007).

Table 1.1. Comparison of some sources of biodiesel (Chisti, 2007).

Crop	Oil yield (L ha^{-1})
Corn	172
Soybean	446
Canola	1,190
Jatropha	1,892
Coconut	2,689
Palm	5,950
*Microalgae[a]	136,900
*Microalgae[b]	58,700

* Based on experimentally demonstrated biomass productivity in photobioreactors.
a 70% oil (by wt) in biomass
b 30% oil (by wt) in biomass

Another advantage consists on the potential use of the residual biomass (mainly carbohydrate and protein), collected after the removal of the lipid component for the generation of energy, liquid or gaseous fuels, or higher value by-products (Figure 1.2) (Pienkos and Darzins, 2009). When compared to fossil diesel, biodiesel has advantages in that it is renewable, biodegradable, and produces less SOx and particulate emissions when burned besides not

requiring engine modification for its usage (Sheehan et al., 1998). Microalgae can be an alternative option to produce fuels because of their versatility as biomass source. Microalgae have higher photosynthetic efficiency, higher biomass productivities, and faster growth rates than higher plants, as well as the highest CO_2 fixation and O_2 production rates, positioning microalgae as one of the Earth's most important renewable fuel crops (Campbell, 1997; Chisti, 2007; Chisti, 2008).

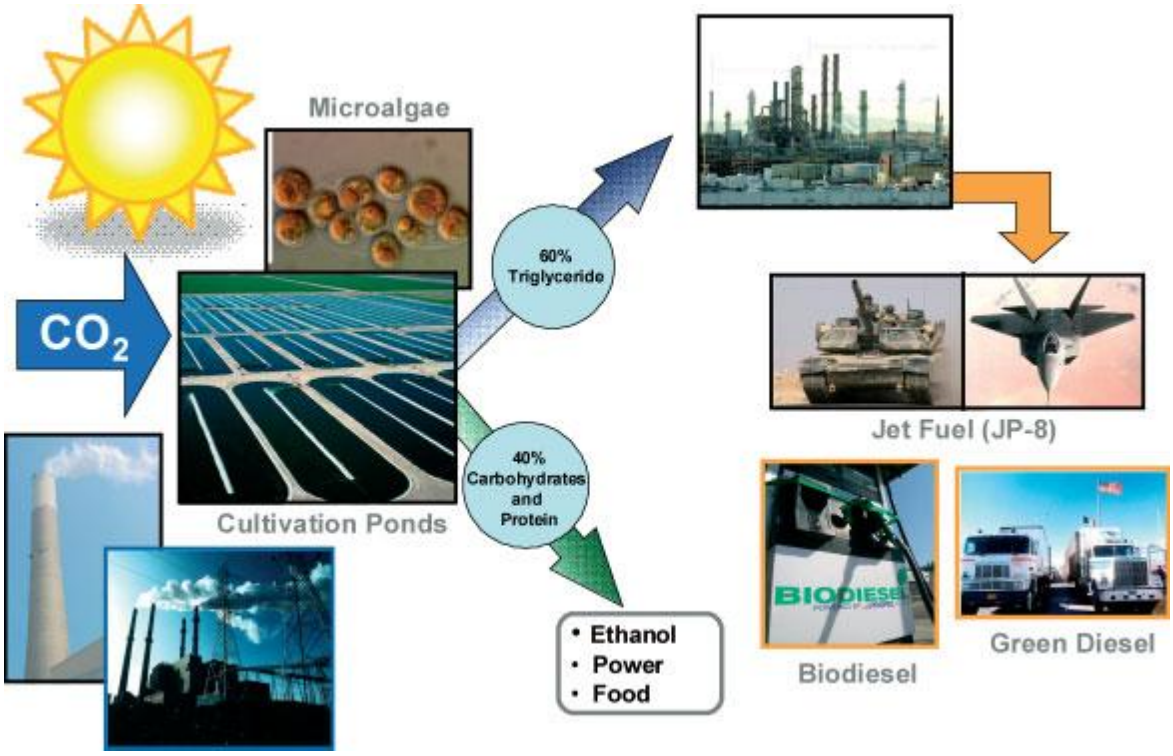

Figure 1.2. Algal Biomass Product Streams (Pienkos and Darzins, 2009).

1.3. Photosynthesis in Microalgae

Photosynthesis is a process where sunlight energy is used to convert CO_2 into glucose and oxygen. In this process CO_2 and light are converted into organic matter by photoautotrophs. Photosynthesis serves as a source of energy for metabolism and growth for basically all forms of life on Earth either in a direct or indirect way (Masojidek et al., 2004). Algae are photosynthetic,

non-vascular plants containing chlorophyll *a* as pigment (Vonshak and Maske, 1982). The chlorophyll *a*, functioning as the principal photochemically active compound, receives light in order for photosynthesis to occur. Hence, the content of this pigment in microalgae influences photosynthetic activity (MacIntyre et al., 2002). The concentration of this light harvesting pigment has an impact on biomass production in microalgae as well as on the accumulation of target products (Su et al., 2007).

Nearly half of the total photosynthesis taking place on Earth is associated with marine phytoplankton (Camacho et al., 2003). Light availability is related to the growth and production performances of a photosynthetic organism. When grown in a photobioreactor, the amount of light absorbed by an algal cell depends on the incident flux on the particular cell, culture density, and cell pigmentation as well as on the photosynthesis-irradiance relationship (the P-I curve). The P-I curve helps predict the culture performance and understand the relationship between the growth observed and the amount of light received (Camacho et al., 2003).

1.4. Microalgae Classification

The main groups of microalgae differ primarily in terms of pigment composition, biochemical constituents, ultrastructure, and life cycle. The groups include diatoms (Class Bacillariophyceae), green (Class Chlorophyceae), golden brown (Class Chrysophyceae), primnesiophytes (Class Prymnesiophyceae), eustagmatophytes (Class Eustagmatophyceae), and blue-green or cyanobacteria (Class Cyanophyceae) (Sheehan et al., 1998).

1.4.1. Diatoms.

Diatoms, with close to 100,000 known species, are among the most common group of algae in existence and tend to dominate the phytoplankton in oceans, but are also commonly found in fresh and brackish water habitats (Sheehan et al., 1998). The golden-brown color is due to the presence of fucoxanthin and β-carotene which mask the green color chlorophyll *a* and chlorophyll *c* (Tomaselli, 2004). Two major groups of diatoms can be

distinguished: the pennates having bilateral symmetry and the centrals with radial symmetry (Tomaselli, 2004). The main storage compounds of diatoms are lipids and a β-1,3-linked carbohydrate known as chrysolaminarin. Substantial amounts of polymerized silicate (Si) can be found in their cell walls (Sheehan et al., 1998).

1.4.2. Green Algae. Members of this group are among the most common microalgae, especially in freshwater (Neenan, 1986). Approximately 8,000 species are estimated to be in existence (Sheehan et al., 1998). This group contains chlorophyll *a* and chlorophyll *b* and several carotenoids which can be synthesized under stress conditions and make the alga change in color (Tomaselli, 2004). Starch is their primary storage component. In certain species, however, N-deficiency has been found to boost lipid accumulation (Sheehan et al., 1998). Higher plants are considered descendants of the green algae, and for that reason, have received more attention than other algal groups with *Chlorella* and *Chlamydomonas* being two commonly studied genera (Sheehan et al., 1998).

1.4.3. Golden-Brown Algae**.** This group is also known as chrysophytes and shares similarities in biochemical and pigment composition with diatoms. Approximately 1,000 species are known, and are primarily present in freshwater habitats (Sheehan et al., 1998), especially in oligotrophic waters low in calcium (Tomaselli, 2004). Major carbon storage forms in this group are usually lipids and chrysolaminarin (Sheehan et al., 1998).

1.4.4. Prymnesiophytes. They are also known as haptophytes. Prymnesiophytes are constituted by approximately 500 species (Sheehan et al., 1998). They are mostly found in the oceans, and form a major part of marine phytoplankton (Tomaselli, 2004). Just like in diatoms and chrysophytes, fucoxanthin imparts a brown color to the cells, with both lipids and chrysolaminarin constituting the major storage products (Sheehan et al., 1998). Scales embedded

in a mucilage can be usually found covering the cells and these can sometimes be calcified (Tomaselli, 2004).

1.4.5. Eustigmatophytes. This group encompasses a significant portion of the"picoplankton" (Sheehan et al., 1998). Eustigmatophytes include unicellular and coccoid organisms that produce a small number of zoospores and are mostly found in soil and fresh waters (Tomaselli, 2004). *Nannochloropsis,* a marine species within this class, is a source of polyunsaturated fatty acids with high amounts of eicosapentaenoic acid (Tomaselli, 2004).

1.4.6. Cyanobacteria. Cyanobacteria are prokaryotic organisms that contain chlorophyll *a* (Tomaselli, 2004). The name of blue–green has been given because of the presence of phycocyanin and phycoerythrin which usually masks the chlorophyll pigmentation (Tomaselli, 2004). They are similar to bacteria in that their cells lack a nucleus, chloroplasts, and also, have a different gene structure (Neenan et al., 1986; Sheehan et al., 1998). An estimated 2,000 species belong to this group. Some cyanobacteria are known to assimilate atmospheric nitrogen (N) thus eliminating the need to provide fixed N to the cells. None of them have been found to produce significant quantities of lipid as storage (Sheehan et al., 1998).

1.5. Growth Requirements of Microalgae

The biochemical composition of microalgae can be affected by such factors as growth rate, environmental conditions, and life cycle (Richmond, 1986). Microalgal growth and chemical composition are mainly controlled by light, temperature, available carbon dioxide, pH, and nutrients (Tzovenis et al., 1997, Zhu et al., 1997). Other factors, such as salinity, can be of vital importance to some species (Chu et al., 1996). As for synergistic or symbiotic effects, association with bacteria can be beneficial to algae for growth. *Azospirillum* sp. is a known plant-growth- promoting bacterium that aids in the growth and yields of many plants and can also promote the growth of several freshwater species belonging to the *Chlorella* genus (Bashan and

Levanony, 1990; Okon and La-bandera Gonzalez, 1994; Bashan and Holguin, 1997; Bashan et al., 2004). Increased nitrogen content, mass, length and yields have been shown in crops grown using strains of *Azospirillum lipoferum* (Govedarica et al., 1993; Govedarica et al., 1994; Favilli et al., 1993). Studies have found an accumulation of lipids with a greater variety of fatty acids when *Azospirillum brasilense* was co-immobilized in alginate beads in conjunction with certain *Chlorella* species (de-Bashan et al., 2002).

1.5.1. Light. The use of monocultures is required in various microalgal applications and controlled cultivation systems. This requirement has favored the development of closed photo bioreactors (Barbosa et al., 2001). Efficient utilization of light is one of the major challenges in microalgal biotechnology, especially when an increase in the biomass yield is desired (Barbosa et al., 2001). Photoacclimation or photoadaptation is a process that controls the effect of light on the biochemical composition of photosynthetic algae. During this process, algal cells undergo changes in cell composition and alterations in their ultrastructural, biophysical and physiological properties can also be observed (Dubinsky et al., 1995). Lee and Lee (2001) showed that cell concentration on a *Chlorella kessleri* inoculum with a density of 10^5 cells ml^{-1} increased to 1.6 x 10^7 cells ml^{-1} after three days of continuous light. However, the total cell concentration in flasks with L/D (light/dark) lighting scheme was increased to 9.1 x 10^6 cells ml^{-1} during the same period, only 57 % of that under continuous illumination. This difference in cell concentration between the two lighting schemes was proportional to the duration of the light exposure. The reduction in cell biomass under L/D lighting scheme seemed to be caused by night biomass loss by respiration. In one study using *Chaetoceros* sp. under a continuous photon flux of 125 µmol photons m^{-2} s^{-1}, provided by either a Cool White (CW) or by Gro-Lux and Gro-Lux wide spectrum agricultural lamps (GRO and GRO/WS), the protein concentration was found to be

higher whereas lipids were lower at the end of the exponential growth compared to the stationary phase (Sanchez and Voltolina, 2006). The concentration of proteins, however, remained consistently higher than other treatments with GRO lamps in both stages, possibly due to the "high emission of blue light". As for lipids, the highest yields were observed in algae grown using CW; whereas, algae grown using GRO/WS yielded the best carbohydrate production during both growth phases.

1.5.2. Temperature. The effect temperature exerts on biochemical reactions and how it affects the biochemical composition of algae, makes temperature one of the most important environmental factors (Hu, 2004). Renaud et al. (2002) evaluated the effect of temperature on fatty acid composition of four tropical Australian microalgal species (the diatom *Chaetoceros* sp. (CS256); two cryptomonads, *Rhodomonas* sp. (NT15) and *Crytomonas* sp. (CRFI01) and an unidentified prymnesiophyte (NT19) and in a commercially available *Isochrysis* sp. used as control. Results showed that the diatom *Chaetoceros* had the highest percentage of lipid (16.8 % dry weight) when cells were cultured at 25°C. However, *Rhodomonas* sp., *Cryptomonas* sp., *NT19,* and *Isochrysis* sp. had significantly higher amounts of lipids at 15.5, 12.7, 21.4, and 21.7 % dry weight, respectively, when grown at temperatures within the range of 27-30°C. The effect of temperature on fatty acid composition of *Chaetoceros* is summarized in Table 1.2.

1.5.3. Carbon Dioxide and Organic Carbon Sources. Algae use carbon dioxide (CO_2) as the source of carbon to synthesize organic compounds such as lipids. Since carbon demand and productivity increase proportionally, intensive mass culture of microalgae requires a vast source of CO_2 (Neenan et al., 1986). Carbon dioxide is present in air at a very small concentration (0.037 % by volume in dry air). On the marine diatom *Chaetoceros wighamii*, addition of CO_2 increased the protein content, lowered the concentration of carbohydrates, but had no significant

Table 1.2. Fatty acid composition of laboratory batch cultures of *Chaetoceros* sp. (CS256) grown at different temperatures (Renaud et al., 2002).

	Temperature (°C)				
	25	27	30	33	35
Saturated fatty acids					
12:0	– [1]	–	–	–	–
14:0	23.6[m,2]	22.3[m]	22.3[m]	23.8[m]	28.3n
16:0	9.2	8.6	8.9	8.7	8.4
17:0	–	–	–	–	–
18:0	0.7	0.7	0.9	1.3	0.9
Sum%	33.5	31.6	32.1	33.8	38.2
Monounsaturated					
16:ln-7	36.5 [m]	36.4 [m]	39.1 [m]	36.9 [m]	33.5[n]
18:1n-9	1.7	1.4	1.4	1.9	1.8
18:1n-7	1.2	1.2	1.3	1.1	1.2
Sum%	39.4	39.3	42.5	40.0	36.5
Polyunsaturated					
16:2n-7	0.9	1.2	1.4	1.4	1.8
16:3n-4	2.6	4.3	3.9	4.2	4.8
16:4n-1	0.5	0.7	0.7	0.7	0.7
18:2n-6	0.4	1.2	1.1	1.3	1.2
18:3n-6	0.9	0.6	0.8	0.8	1.0
18:3n-3	0.5	0.6	0.4	0.5	1.0
18:4n-3	0.6	0.4	0.2	0.5	0.3
18:5n-3	–	–	–	–	–
20:4n-6	4.1 [m]	4.5 [m]	4.0 [n]	3.6 [n]	2.7 [n]
20:5n-3	8.0 [m]	6.7n	6.5 [n]	6.9 [n]	6.6 [n]
22:5n-3	–	–	–	–	–
22:6n-3	1.0	0.6	0.4	0.4	0.3
Sum%	19.5	20.8	19.0	19.8	20.4

Data as mean percentage of total fatty acids; n=3 for flask cultures.

1 (–) Indicates that fatty acid was less than 0.1% of total fatty acids.

2 Different superscripts across a row (m to n) indicate significant difference between means (ANOVA, Tukey's test; $P<0.05$).

effect on the lipid content (Castro and Tavano, 2005). As for microalgae grown on the heterotrophic mode, experiments using diverse sources such as molasses (containing 25 %

glucose, 25 % fructose and 30 % sucrose on average (Becker, 1994a), acetic acid, and hydrocarbons (such as *n*-heptadecane) have been performed and shown to work at different concentrations (Becker, 1994a).

1.5.4. Nutrients. Nutrients are inorganic or organic compounds other than carbon dioxide and water, used for growth whose presence in the cell is necessary for cellular function (Neenan et al., 1986). Some algae require specific organic compounds synthesized by other organisms. However, many algae require only inorganic nutrients, and it is likely that these algae could be used as feedstock for biomass fuel production. Limiting nutrients to algae are nitrogen, phosphorus, silica (for diatoms) and iron (Neenan et al., 1986).

1.5.4.1. Nitrogen. After carbon, nitrogen (N) is the most important nutrient contributing to the production of biomass. The nitrogen content in the microalgal biomass can range from 1 % to more than 10 % and depending on its supply and availability, it can vary between different groups (e.g. low in diatoms) and within particular species (Grobbelaar, 2004). Discoloration of the cells is a frequent response to nitrogen limitation due to a decrease in chlorophyll content and an increase in carotenoids, as well as the accumulation of organic carbon compounds such as polysaccharides and certain oils like polyunsaturated fatty acids (PUFAs) (Becker, 1994b). Nitrogen is mostly supplied as nitrate, but often ammonia and urea are used, both displaying similar growth rates (Kaplan et al., 1986). Many microorganisms tend to prefer ammonia as a nitrogen source, and the assimilation of either nitrate or ammonia is said to be related to the pH of the growth media (Hu, 2004). A drop on pH can be observed when ammonia is used as the only source of N, especially during active growth owing to the release of H+ ions (Grobbelaar, 2004). An increase in pH is observed when nitrate is used as the only N source (Grobbelaar, 2004). Ammonia loss due to volatilization is an important factor to be considered when deciding

whether to supply either one. Urea can be considered another attractive source of nitrogen. About 42 % of the weight of urea is nitrogen; therefore, more material than that of ammonia has to be handled. Lin and Lin (2011) reported that the microalgae fed with a combination of urea and sodium nitrate had the highest ash-free dry biomass content with a yield of 4.15 ± 0.38 g L^{-1} . The urea molecule contains a carbon atom as well as two nitrogen atoms. This carbon atom is released as CO_2 when urea is utilized and presumably, it is available for photosynthetic assimilation (Neenan et al., 1986), therefore, urea has the potential of providing both the nitrogen and 1.5 to 10 % of the carbon requirement (Neenan et al., 1986).

1.5.4.2. Phosphorus. Phosphorus is essential for growth in many cellular processes such as energy transfer and during the biosynthesis of nucleic acids. Orthophosphate is the preferred form in which it is provided to algae and its uptake is said to be energy dependant (Grobbelaar, 2004). In spite of the fact that algal biomass contains less than 1 % phosphorous (P), it usually becomes one of the most important growth limiting factors in algal culture (Neenan, et al., 1986). This happens because P binds easily to other ions (e.g. carbonate and iron) resulting in its precipitation. The insolubility of the resulting phosphate makes this essential nutrient unavailable for algal uptake (Grobbelaar, 2004). The supply of P also influences the composition of the produced biomass. Similar effects to the ones obtained in algae grown under nitrogen starvation, such as the tendency to accumulate large amount of lipids, with a decreased amount of proteins, chlorophyll and nucleic acids content have been reported on phosphate deficient cultures (Becker, 1994a).

1.5.4.3. Other Macro, Micronutrients and Chelates. Sulfur, potassium, sodium, iron, magnesium, calcium and trace elements like boron, copper, manganese, zinc, molybdenum, cobalt, vanadium, and selenium are also important in algal nutrition (Grobbelaar, 2004). Silicon, present in the cell

walls of many algal groups, is an important component in diatoms where it constitutes an essential nutrient for their growth and production (Healy, 1973). Silicon limitation, which is prone to happen, can also lead to the accumulation of secondary metabolites, such as lipids (Grobbelaar, 2004). Experiments conducted with silicon-deficient cells of *Cyclotella cryptic* (a diatom species that accumulates lipids under non-growing conditions) indicated that lipid accumulation occurs as a function of both increased partitioning of newly photo-assimilated carbon into lipids and slow conversion of non-lipid compounds (Roessler, 1987).

1.5.5. Salinity. Protein, lipids and carbohydrates seem slightly affected by a wide range of salinity for most microalgae species. However, in some species, increases in ash and lipid content were observed at higher salinity. Studies by Raghavan et al. (2008) indicated that for *Chaetoceros calcitrans,* in terms of growth and chemical composition, a salinity of 25 mM NaCl was optimum. This study also showed that at a salinity of 35 mM NaCl, carbohydrates were increased while lipids and protein decreased (Raghavan et al., 2008). Vasquez-Duhalt and Arredondo-Vega (1991) tested two strains of *Botryococcus Braunii* (Austin and Gottingen) under different NaCl concentrations. The protein content in the cell biomass of the Austin strain showed a decrease with increasing salinity. The effect was small in the case of the Austin and more pronounced in the Gottingen strain. Carbohydrate content of Austin strain cells was not affected by media NaCl concentrations up to 0.2 M. Higher concentrations of NaCl induced an increase in the carbohydrate content of cells. However, large standard deviations were reported (Vasquez-Duhalt and Arredondo, 1991). The lipid content in both strains was not affected by the salt concentration. This suggests that saline stress does not induce lipid accumulation in these organisms (Vasquez-Duhalt and Arredondo-Vega, 1991). Mishra and Jha (2009) found similar results on *Dunaliella* cultures harvested 20 days after inoculation and grown at different salt

concentrations. Salt induces osmotic pressure in the broth which causes the microorganisms to protect themselves from it by producing exopolysaccharides (EPSs) (Abbasi and Amiri, 2008). EPSs of 944 mg l^{-1} were obtained from *Dunaliella* grown in 5M NaCl relative to the 56 mg l^{-1} observed in media containing 0.5 M NaCl (Mishra and Jha, 2009).

Salinity can have an effect on growth rate along with other factors like temperature. Castro and Tavano (2005) observed that temperature had a significant effect on the growth of *Chaetoceros wighamii* under salinity of 25 mM, but not at 35 mM. At 30°C, the growth rate was lower at 25 mM when no CO_2 had been added. Rao et al. (2006) identified a marginal increase in carbohydrate content at 17 mM and 34 mM salinity cultures with *Botryococcus braunii*. The total fat content of alga grown at different salinity varied from 24-28 % (w/w) compared to only 20 % (w/w) for control without salts. The fatty acid profile indicated the presence of C16:0, C16:1, C18:0, C18:1, C18:2, C22:0, C22:1 and C24:0 fatty acids. Stearic and linoleic acids were higher in proportion in the control culture while palmitoleic and oleic acids were the major fatty acids for cultures grown in 34mM and 85mM salinity (Rao et al., 2006).

1.6. Microalgae Identification

1.6.1. Polymerase Chain Reaction.

The polymerase chain reaction (PCR) is a powerful and sensitive technique which amplifies specific DNA sequences exponentially through a three-step process done in multiple cycles (Saiki et al., 1985). First, the double-stranded DNA template is denatured at a high temperature. Then, sequence-specific primers are annealed to the target sequence followed by the addition of a thermostable DNA polymerase, such as *Taq* DNA polymerase (Chien et al., 1976; Kaledin et al., 1980; Lawyer et al., 1993; Longley et al., 1990; Lyamichev et al., 1993). This enzyme is responsible for extending the annealed primers, and doubling the amount of the original DNA sequence. The new product then becomes an additional template for subsequent cycles of amplification. These three steps are usually repeated in cycles

for 20 to 30 times, resulting in an increase of target DNA concentration of 10^5 to 10^9 times the original amount.

The use of PCR to obtain large amounts of a desired product can have both positive and negative aspects. If amplification is not successful, this can lead to the generation of many undesired products leading even to the exclusion of the target product (Roux, 1995). The opposite would be that no product may be amplified. In regards to optimization, several variables have been recognized to contribute to this effect (Saiki et al., 1988). Main optimization variables include magnesium (Mg^{2+}) concentrations, buffer pH, and cycling conditions. Within cycling conditions, the annealing temperature is of utmost importance. The interdependence between variables adds difficulty to the situation. For instance, increasing the amount of deoxynucleotide triphosphates (dNTPs) lowers the concentration of free Mg^{2+} available to exert an effect on polymerase function because dNTPs directly chelate a proportional number of Mg^{2+} ions (Roux, 1995).

1.6.2. Enhancing Agents. Several additives and enhancing agents such as dimethyl sulfoxide (DMSO), N,N,N,-trimethylglycine (betaine), formamide, glycerol, non-ionic detergents, bovine serum albumin, polyethylene glycol and tetramethyl ammonium chloride can be included in PCR reactions with the aim of increasing yield, specificity and consistency (Frackman et al., 1998).

Application of the Taguchi method (Taguchi, 1986), which focuses only on the main effects and two factor interactions, can eliminate the need of a full multivariate matrix analysis for each of the variables tested, which can become a burdensome and costly task. With this method, the size of the matrix can be trimmed down significantly and several key variables can be altered simultaneously (Cobb and Clarkson, 1994).

1.6.3. Magnesium Concentration. Magnesium chloride serves as an essential co-factor for the DNA polymerase and optimization for every primer/template pair should always be attained (Kolmodin and Felton, 1997). Magnesium ion is closely bound to the phosphate-sugar backbone in nucleotides and nucleic acids; therefore, different amounts can have strong and complex effects on experiments on which nucleic acids take part (Blanchard et al., 1993). Because of the needed role of free magnesium as enzyme co-factor in PCR, its total ion concentration has to exceed the total dNTP concentration. Generally, magnesium ion's concentration varies in series of 1.5 - 4 mM with 0.5-mM step increments (Kolmodin and Felton, 1997).

1.6.4. Amplification of 16S rRNA, 18S rRNA and Other Genes. 16s rRNA is the most commonly used genetic marker for the study of bacterial phylogeny and taxonomy (Janda and Abbott, 2007). Reasons for this include: (1) its presence in almost all bacteria as operons, (2) no change in 16s rRNA gene's function has been observed over time and, (3), the 16s rRNA gene's length (1,500 bp) is suitable for informatics purposes (Janda and Abbott, 2007). Erwin and Thacker (2008) characterized partial 16S rRNA and the entire 16S- 23S internal transcribed spacer (ITS) sequences from *Synechococcus spongiarum* in an attempt to assess the phylogenetic utility of rRNA sequence data in resolving the phylogeny of sponge-associated bacteria. Their results showed that 16S rRNA sequences were highly conserved, exhibiting less than 1 % sequence divergence among symbiont clades; whereas, ITS gene sequences displayed a much higher variability than 16S rRNA sequences.

In addition to molecular techniques based on PCR amplification targeting conserved regions inside the 16S rRNA gene (Komarek , 2006), other genes such as *hetR* (heterocyst differentiation control protein) (Han et al., 2009; Schleifer, 2009) and *nifH*, which encodes for nitrogenase reductase (Zehr and McReynolds, 1989; Janson et al., 1998), have also demonstrated

to have potential for phylogenetic discrimination. However, considering that several disagreements between traditional morphological classification and phylogenetic analysis still remain, the utilization of chemotaxonomic markers, such as lipids and their fatty acids, have been considered as complementary approaches since they provide data for taxonomic position assignment as well as some correlations with morphological properties of cyanobacteria (Galhano et al., 2011). In their study, Galhano et al. (2011) obtained molecular information regarding the 16S rRNA gene as well as the *hetR* and *nifH* genes from filamentous cyanobacterial strains isolated from two Portuguese freshwater ecosystems, a eutrophic shallow lake (genus *Aphanizomenon*) and rice fields (genera *Anabaena* and *Nostoc*). Features such as genotypic, morphological and biochemical attributes (fatty acid methyl ester profiles) were used for strain characterization. Their results showed that the unknown *Aphanizomenon* had 99 % similarity with 16S rDNA sequence with *Aphanizomenon gracile* 219 (isolated from a Danish lake), the unknown *Anabaena* had 99 % with *Anabaena flosaquae* PCC 7905 (type strain) and 99 % similarity with *Anabaena cylindrica* NIES19. The unknown *Nostoc* showed 98 % 16S rDNA sequence similarity with *Nostoc* sp. 8938 and *Nostoc muscorum* I. The *hetR* gene fragments with approximately 450 bp showed 97 % similarity with *Aphanizomenon* sp. TR183 and 98 % similarity with *Anabaena flos-aquae* SAG 30.87; whereas, a 100 % similarity was seen with *Nostoc* PCC 7906. The sequence of the *nifH* gene fragment (319 bp) of the unknown *Aphanizomenon* had high similarity (98 %) with a nitrogenase reductase sequence of an uncultured cyanobacterium clone Gt1463 and a different strain's *nifH* partial sequence showed high similarity (99 %) with *Nostoc muscorum* CC1090A1; whereas, the *Anabaena's* strain's *nifH* partial sequence had similarities of 99 and 100 % with the partial nitrogenase reductase gene

sequences of a freshwater strain of *Anabaena oscillarioides* and *Anabaena cylindrica* UTEX B629, respectively (Galhano et al., 2001).

Considered one of the most important molecular markers, 18s rRNA is a gene used in several applications among which molecular phylogenetic analyses and biodiversity screening can be included (Meyer et al., 2010). Buchheim et al., 1990, 1996, 1997, have conducted phylogenetic studies using 18S rRNA gene which have demonstrated significant diversity within the green algal genus *Chlamydomonas*. Armbrust et al. (2001) used 18S primers to amplify the 18S rRNA genes from three closely related centric diatoms. Degenerate PCR primers were designed and used to amplify a portion of *Sig1* (sexually induced gene 1), a gene strongly upregulated during sexual reproduction in the centric diatom *Thalassiosira weissflogii.* This gene has been hypothesized to encode a protein involved in the gamete recognition in three closely related species of the cosmopolitan genus *Thalassiosira* sp., *oceanica, guillardii,* and *pseudonana*. In their study, identification of *Sig1* facilitated the development of this gene as a molecular marker for diatom sexual events and how these events affect diatom speciation and population dynamics.

Kaczmarska et al. (2005) amplified the small subunit rDNA (SSU rDNA) gene from the domoic acid-producing diatom *Pseudo-nitzschia multiseries* by PCR using the primer pair 27F and 1492R. Isolation and identification of bacteria associated with the *P. multiseries* clones and sub-clones to the closest characterized bacterium was done using the Ribosomal Database Project II. Their results showed that five of the eight bacterial strains belonged to the Alphaproteobacteria; whereas, the remaining bacteria were related to the Gammaproteobacteria and the bacteroides. Additionally, BLAST analysis of some of the above mentioned bacterial strains showed that they shared their highest SSU rRNA gene sequence identities (99.1, 99.0,

93.9 and 99.0 %, respectively) with the bacteria affiliated to *Stappia* (AY258082), *Sulfitobacter* (AY258079 and AJ534214), *Flexibacter* (AY258133) and *Sphingomonas* (AY258097). BLAST (Basic Local Alignment Search Tool) is a bioinformatic tool that helps identify members of gene families by finding regions of local similarity between sequences and by comparing nucleotide or protein sequences to sequence databases. Statistical significances are calculated for those matches (http:// www.ncbi.nlm.nih.gov/blast/). A method described by Rasoul-Amini et al. (2009) used DNA extraction and PCR amplification of the 18S rRNA gene to obtain sequences on 12 microalgae species belonging to the class Chlorophyceae. Bioinformatic tools (BLAST) were also used. The results of PCR products BLASTed with other sequenced microalgae in NCBI showed similarity to the 18S small subunit rRNA of other microalgae at 99-100 %.

1.7. Microalgae Lipid Content

Lipids are extractable to non-polar solvents (e.g. ether, chloroform, alkanes). This definition includes not only the triglycerides (triesters of fatty acids and glycerol), but compound lipids (e.g. phospholipids, glycolipids), steroids, chlorophylls, carotenoids, and hydrocarbons as well (Benemann and Weissman, 1984). The main phospholipids in algae are phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, phosphatidylglycerol, phosphatidic acid, and diphosphatidytil glycerol (Pohl, 1982). The major algal glycolipids are monogalactosyldyglyceride, digalactosyldiglyceride, and sulphoquinovosyldiglyceride (Pohl, 1982). A novel class of algal lipids are chlorosulpholipids which are derivatives of N-docosane-1,14-diol and of N-tetracosane-1,15-diol disulphates found in Chrysophyceae, Xanthophyceae, Chlorophyceae, and Cyanophyceae (Pohl, 1982).

Several reports exist on lipid production by microalgae, but the majority of this data refers only to fatty acids, most of which are C14 to C20 (Benemann and Weissman, 1984). Most of the fatty acids of algae are bound to the above lipids. In some algae, however, minor quantities of free

fatty acids can be found. Larger amounts of even-numbered saturated fatty acids (12:0, 14:0, 16:0,18:0) are primarily produced by algae, with only minor quantities of odd numbered fatty acids (13:0 to 19:0) [(Pohl, 1982)]. Small amounts of branched chain fatty acids have also been reported (Pohl, 1982). Fatty acids with C12 to C22 carbon chains and 1 to 6 double bonds are predominant. These double bonds are usually in the cis- configuration being an exception trans-3-(16:1) which is found in the phosphatidylglycerol of photosynthesizing algae. Different from bacteria and fungi, it seems apparent that algae do not synthesize fatty acids with unusual structures, such as acetylenic, hydroxyl, epoxy, oxo, cyclopropanoic, and cyclopropenoic acids (Pohl, 1982). The average lipid content in algae can range between 1 and 40 % dry weight (Becker, 1994b). However, numbers as high as 85 % dry weight can be achieved under certain conditions (Becker, 1994b). The lipid content of various species of microalgae is presented in Table 1.3.

1.8. Goal of This Study

The goal of this research was to compare different media and growth conditions for the isolation and identification of microalgal strains that are native to both Louisiana's fresh and brackish water bodies for their potential use in the alternative fuel industry. Selected strains were identified through genomic DNA in sequencing of the 16S rRNA and 18SrRNA genes. Lipid and fatty acid content were characterized and quantified post genomic identification.

Table 1.3. Lipid content of some microalgae (Gouveia and Oliveira, 2009). Originally adapted from Becker (1994b); Illman et al. (2000); Liu et al. (2007); Miao and Wu. (2006); Natrah et al. (2008); Spolaore et al. (2006); Tornabene et al. (1983); and Xiong et al. (2008).

Species	Lipids (% dry matter)
Scenedesmus obliquus	11–22/35–55

(Table 1.3, continued)

Scenedesmus dimorphus	6–7/16–40
Chlorella vulgaris	14–40/56
Chlorella emersonii	63
Chlorella protothecoides	23/55
Chlorella sorokiana	22
Chlorella minutissima	57
Dunaliella bioculata	8
Dunaliella salina	14–20
Neochloris oleoabundans	35–65
Spirulina maxima	4–9

2. MATERIALS AND METHODS

2.1. Sampling and Collection of Organisms

Water samples were collected from sources at different locations (gulf, bays, lakes, bayous, ditches) within Louisiana's Southeast region with the aim of isolating and identifying algae as potential feedstock for the production of biodiesel. On April 17, 2010, water samples were taken from Dulotte Canal (Empire, LA), Bay Adams (Empire, LA), Myrtle Grove Marina (Port Sulphur, LA) and on May 18, 2010 from Magnolia ditch and End of Magnolia (Raceland, LA), Cocodrie Marina (Chauvin, LA), and Chacahoula ditch, Lake Palourde, Brownell Memorial's lake, and Adam's landing (Morgan City, LA) (Figure 2.1). Samples were stored at 4°C. Salinities of water samples were analyzed by total conductivity and are depicted in Table 2.1.

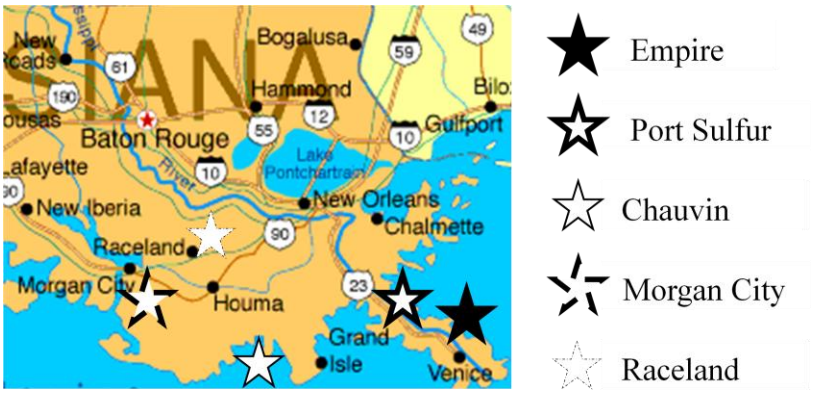

Figure 2.1. Map of Sampling Locations. (Adapted from www.maps.com).

2.2. Growth Conditions

Media for the isolation of algal strains were prepared by mixing filtered (55 mm, Whatman filters, Kent, United Kingdom) and sterilized natural brackish or freshwater (same source from where samples were taken) with either 50X Guillard's (F/2) (APPENDIX 1) marine

water enrichment solution (Sigma-Aldrich, St. Louis, MO) for brackish water or Chu's Media for freshwater samples. The Chu's Media contained 40 mg Ca (NO_3), 0.8 mg $FeCl_3$, 100 mg

Table 2.1. Salinities of sampling locations through total conductivity.

Location	Salinity **(mg/L)
Dulotte Canal	3,727
Bay Adams	4,304
Myrtle Grove	1,064
Magnolia Ditch	58
End of Magnolia	51
Cocodrie Marina	3,317
Chacahoula	53
Lake Palourde	108
Brownell Memorial	65
Adam's Landing	93

** The sum of chromatographic sodium, potassium, magnesium and calcium.

K_2HPO_4, 250 mg $MgSO_4.7H_2O$, 20 mg Na_2CO_2, 25 mg $NaSiO_3$**.** 9 H_2O per liter. Petri plates of each medium were prepared containing 1.5 % w/w agar (Difco Bacterius Limited, Houston, Texas). Supplemental $Na_2\ SiO_3$**.** 9 H_2O w/v at 1 % using a stock solution containing 30 g L^{-1} was added to F/2 enriched media at pH 7 to enhance the isolation of diatoms (Perez, 2006).

2.3. Colony Selection

One ml of each water sample (brackish or freshwater) was inoculated on both plates and into flasks containing 25 ml broth of each medium. Inoculated plates and flasks were placed in a Gyromax^{TM} 777 incubator (Amerex Instruments Inc., Lafayette, CA) under continuous fluorescent lighting at 23 µmol photons m^{-2} s^{-1} measured using an LI-193 spherical quantum sensor along with a LI-1400 data logger (LI-COR, Lincoln, NE) at 30°C ± 2 and slow swirling (flasks only) at 80 RPM for 2 to 4 weeks.

2.4. Colony Homogenization

Colonies were selected based on color differences and transferred to fresh agar plates. In the case of samples grown in liquid media, a 1 ml aliquot was transferred to agar plates and incubated under similar conditions for an additional 2 to 3 weeks. Homogenization and purification of isolated colonies were carried out through the inoculation of colonies on both Blue Green Media (BG-11) (APPENDIX 2) which favors blue greens and diatoms, and Bold's Basal Media (BBM) (APPENDIX 3) which favors green algae. Microscopic observations at 100X magnification with oil immersion (DC3-163 Microscope, National Optical and Scientific Instruments Inc., San Antonio, Texas) and serial dilution were made until a unialgal culture was obtained.

2.5. Microorganism Selection and Harvesting

Out of 17 isolates, four algae were selected for identification. Their selection was based on rapid growth, morphological and class diversity, and culture homogeneity. Based on morphological differences observed through microscopic analysis, they were tentatively identified as a cyanobacteria (N3) isolated from Cocodrie Marina, a diatom (FC2) isolated from a Chacahoula ditch and two green algae isolated from a Chacahoula ditch (FC1) and Dulotte Canal (D2), respectively (Figure 2.2). *Chlorella vulgaris* UTEX 259 (University of Texas Culture Collection of Algae, Austin, TX), provided by Dr. Misook Kim from the Audubon Sugar Institute (St. Gabriel, LA), was used as control during the identification process. N3 and FC2 isolates were grown in 1 L flasks containing 800 ml of BG-11; whereas, FC1, D2, and *Chlorella vulgaris* were grown in BBM. Flasks were placed under continuous fluorescent lighting 20 µmol photons m^{-2} s^{-1} at 30°C with slow shaking at 80 RPM in a shaker incubator Gyromax 777 and harvested after 15 days by centrifugation (Sorvall RC-5 Superspeed Refrigerated Centrifuge, Dupont, England) at either 1,200 x g for N3 or 6,500 x g for FC2, FC1, D2 and *Chlorella*

vulgaris UTEX 259 at 4ºC. Post harvest, 100 mg of wet cell biomass were washed with 100 mM Tris buffer (pH 7.5) and stored at -20°C.

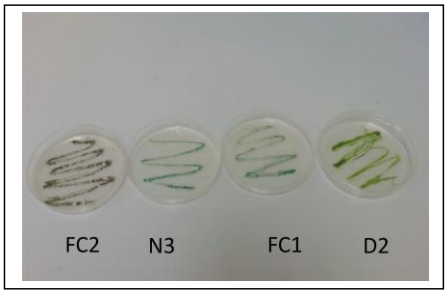

Figure 2.2. Tentatively Identified Microorganisms. FC2: diatom, N3: cyanobacteria, FC1 and D2: green algae.

2.6. Genomic DNA Extraction

Frozen cells were thawed and genomic DNA extraction was performed using a Fast DNA Kit (MP Biomedicals, Solon, OH). Briefly, 1 ml of CLS-Y was added to each tube containing 100 mg of wet cells and then transferred to a new set of tubes each containing a ball and garnet. Cells were processed using a FastPrep®-24 Instrument (MP Biomedicals) for 10 s at a speed setting of 5 m s^{-1}, placed on ice for 1 h and then centrifuged for 15 min at 4°C at 15,700 x g (Hermle Z233 MK-2 High Speed Refrigerated Centrifuge, Hermle Labortechnik, Wehingen, Germany). After centrifuging, 600 µl of supernatant fluid was placed in a fresh tube and to that, 600 µl of binding resin (guanidine thiocyanate at 60-70%, water at 10-20 % and silica at 10-20 %) was added. The mixture was thoroughly mixed and incubated for 5 min at 25ºC. Tubes were centrifuged for 1 min at 15,700 x g (Centrifuge 5415 D- Eppendorf, Hamburg, Germany) and after supernatant removal, 500 µl of SEWS solution was added to each tube, mixed, and centrifuged for 1 min, and then supernatants were discarded. To re-wash, another 500 µl of SEWS was added, mixed, centrifuged, supernatants removed again, and tubes were centrifuged for 10 s to remove any residual supernatants. To elute DNA from the resin, 100 µl of DES

solution was added and incubated for 3 min at 25°C. After incubation, DNA was collected by pipetting, centrifuged for 1 min at 15,700 x g, and supernatants were collected; an additional centrifugation was carried out to make certain no resin was present. Studies were conducted in duplicate.

2.7. DNA Purification

Ten µl of 3M sodium acetate solution (pH 5.2) and 275 µl of 95 % v/v ethanol were added to each sample's DNA, mixed by flipping and placed in dry ice for 15 min. The samples were then centrifuged for 15 min at 15,700 x g at 4°C (Hermle Z233 MK-2 High Speed Refrigerated Centrifuge) to remove the supernatant. The pellets were each washed once with 1 ml of 95% v/v cold ethanol and centrifuged for 5-6 mins. After supernatants were discarded, DNA pellets were placed in an oven (Isotemp Vacuum Oven Model 280A, Fisher Scientific, Pittsburgh, PA) at 37°C for 10 min to dry under a vacuum of -25 inches of Hg. After drying, 80 µl of TE buffer was added and the DNA concentration was determined with a ND-1000 Spectrophotometer (Nanodrop, Wilmington, DE). Two readings were taken per sample (2 µl).

2.8. Standard Polymerase Chain Reaction (PCR) and Optimization

Nine different conditions were tested using a set of primers (Integrated DNA Technologies, Coralville, IA) for either a 16S rRNA gene (cyanobacteria), since this gene is better suited for prokaryotes, or an 18S rRNA gene (diatom and greens) which is a universal gene commonly used for the identification of eukaryotes (Table 2.2).

Three PCR master mixes were prepared using a Taq PCR Kit (New England Biolabs, Ipswich, MA). The components of each PCR master mix are indicated in Table 2.3. Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO) and glycerol (Sigma-Aldrich, St. Louis, MO) were added to the second and third master mixes, respectively. Each mix was divided into three tubes of 80 µl and combined with 20 µl of three $MgCl_2$ concentrations (15 mM, 30 mM and 45

mM) , resulting in a PCR reaction mixture with a final volume of 100 µl. The PCR reactions were performed on a thermocycler (PTC-100, MJ Research, Inc., Waltham, MA) using a program designed for the amplification of either 16S rRNA or 18S rRNA genes (Tables 2.4 and 2.5).

Table 2.2. 16S rRNA and 18S rRNA primers used for PCR reactions.

Primer	Sequence
16S rRNA	
16S bottom[a]	5'- AGAGTTTGATCMTGGCTC-3'
16S top[a]	5'-ACGGGCGGTGTG-3'
16S bottom 2[b]	5'-TTGGGCGTAAAGCGT-3'
18S rRNA	
18S-C[a]	5'-TGATCCTTCYGCAGGTTCAC-3
18S-D[a]	5'-ACCTGGTTGATCCTGCCAG-3'
18S C-2 [b]	5'- ATTGGAGGGCAAGTCTGGT-3'
18S D-2 [b]	5'- ACTAAGAACGGCCATGCAC-3'

a = Used for PCR amplification; b = Used for sequencing

2.9. Gel Electrophoresis

DNA quality and PCR products were determined using 0.8 % agarose cast gels (Genepure LE agarose, ISE Bioexpress, Kaysville, UT) on Tris-acetate-EDTA (TAE) buffer. Ethidium bromide was added to the gel mix to a final concentration of 0.2 µg/ml, and samples were mixed with 6X loading buffer (0.25 % w/v bromophenol blue and 40 % w/v sucrose). Ten µl of 2 log DNA ladder (0.1-10 kb, New England Biolabs) were loaded onto the agarose gel and the electrophoresis was run at 105 watts for 45 min (BRL-Model 250, Life Technologies, Carlsbad, CA). Bands were visualized with a UV lamp at a wavelength of 312 nm (UV Fotoprep I- Fotodyne Kodak, Rochester, NY).

Table 2.3. Reaction volumes and concentrations for PCR master mixes.

Component	**Master Mixes***			**Final Concentration**
	Volume			
	1	**2**	**3**	
	µl	µl	µl	
10X Mg-free buffer	40	40	40	1X
Primer 1 (50 µM)	4	4	4	0.5 µM
Primer 2 (50 µM)	4	4	4	0.5 µM
Template (sample)	X	X	X	0.25 ng/µl
dNTPs (10 mM)	8	8	8	0.2 mM
Taq DNA (5U/µl)	2	2	2	0.025 U/µl
DMSO (neat)	0	20	0	5%
Glycerol (50%)	0	0	80	10%
Nuclease Free Water (to a final volume of 320 µl)	Y	Y	Y	

X= Amount of template required to reach final concentration.
Y= Amount of Nuclease free water up to 320 µl.
*Each master mix was divided into 3 tubes of 80 µl. Next, 20µl of a $MgCl_2$ stock solution (Low (L)=15 mM, Medium (M)=30 mM, and High (H)=45 mM) was added to get the following combinations: 1L,1M 1H, 2L, 2M, 2H, 3L, 3M, and 3H . Final concentrations of $MgCl_2$ were 1.5, 3, and 4.5 mM for L, M, and H, respectively.

Table 2.4. PCR cycle conditions for 16S rRNA.

Initial Denaturation	95°C	30 s
30 cycles	95°C 61°C 68°C	30 s 1 min 1 min
Final Extension	68°C	5 min
Hold	4°C	∞

Table 2.5. PCR cycle conditions for 18S rRNA.

Initial Denaturation	95°C	30 s
30 cycles	95°C 67°C 72°C	30 s 1 min 1 min
Final Extension	72°C	5 min
Hold	4°C	∞

2.10. Gel Extraction and Purification

Electrophoresis was conducted as previously described. DNA bands were visualized using a UV lamp and the gel bands excised using a clean glass cover slip and then placed in a 1.5 ml microfuge tube. DNA was extracted using a QIAquick Gel Extraction Kit (Qiagen,Valencia, CA). Briefly, 1 ml of buffer QG was added to each microfuge tube and mixed by vortexing (VortexGenie 2-VWR, Radnor, PA) for 30 s. The tubes were then incubated on a heating block (ISOTEMP 125D, Fisher Scientific, Pittsburg, PA) at 50°C for 10 min with vortexing for 2-3 s every 2 min. The contents of each tube were transferred to a QIAquick spin column (QIAquick Gel Extraction Kit) containing a silica membrane with a 10 kb cut-off and centrifuged at 15,700 x g for 1 min. The DNA was bound to the membrane in the spin column. Buffer PE (750 µl) was used to wash each column followed by 1 min centrifugation. After the removal of supernatant columns were centrifuged once more to remove residual buffer PE, then they were placed into a new set of tubes and 25 µl of buffer EB was added to elute the DNA. After 1 min, tubes were centrifuged for 1 min and the DNA concentration was measured with a ND-1000 spectrophotometer and stored at 4°C.

2.11. Cloning of PCR Products

2.11.1. LB Agar Plates and Broth Media Preparation. Preceding the cloning reaction and transformation with chemically competent *Escherichia coli (E. Coli)* cells, plates containing Luria- Bertani (LB) (10g Tryptone, 5g Yeast extract, and 10g sodium chloride per liter) [(Fisher Scientific, Pittsburgh, PA)], with 1.5 % agar, and broth were prepared. Kanamycin to a final concentration of 50 µg/ml was added for the selection of transformant colonies.

2.11.2. Colony Screening. Transformant colonies which were grown at 37ºC for 18 h were determined by blue/white screening by evenly distributing 50 µl of 2 % X-gal to each LB agar

plate containing kanamycin. Mostly white cells were observed which was indicative of positive cell transformation.

2.11.3. Cloning Reaction and Transformation with Chemically Competent *E. coli*. The TOPO TA Cloning Kit for sequencing with the plasmid vector PCR® 4-TOPO® and one shot®-TOP 10 chemically competent cells (Invitrogen, Carlsbad, CA) was used. Four µl of gel purified DNA from each sample was mixed with 1 µl of salt solution (containing 1.2 M NaCl and 0.06 M $MgCl_2$) and 1 µl of TOPO vector (6 µl total reaction volume). After gentle mixing by tapping, the mixes were left to stand for 30 min at 25°C. Vials containing the chemically competent *E. coli* kept at -80°C were thawed on ice (~ 0°C) for 5 mins. After the 30 min incubation, 2 µl of the cloning reaction mix was transferred to the vial containing the *E. coli* competent cells, gently mixed by tapping 6-8 times and incubated on ice (~ 0°C) for 30 min. The vials were placed in a water bath (C76 Water Bath Shaker, New Brunswick Scientific, Edison, NJ) at 42°C for 30 s to produce a heat shock, and then immediately placed on ice followed by the addition of 250 µl of SOC medium. Vials were placed in a shaker incubator at 37°C for 1 h at 200 RPM. The contents of each vial were split and spread onto two LB agar plates containing kanamycin and x-gal, adding 80 % of the content to one plate and 20 % to the other one, and incubated overnight at 37°C. After 18 h of incubation, three white colonies were selected from each sample. Each selected colony was grown overnight in 3 ml of LB broth containing 50 ug/ml kanamycin at 37°C at 250 RPM.

2.12. Purification of Plasmid DNA

The QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA) was used for the purification step. *E. coli* cells grown overnight were centrifuged for 3 min at 9,300 x g and the supernatant decanted. The pelleted bacterial cells were resuspended in 250 µl of buffer P1 (containing lyse-

blue reagent which is used as a visual identification system to prevent handling errors), thoroughly mixed followed by the addition of 250 µl of buffer P2. Samples were mixed gently with lysis time not exceeding 5 min. Buffer N3 (350 µl) was added last and samples were centrifuged for 10 min at 15,700 x g. Centrifugates containing the plasmid DNA were then transferred to a column containing a 50 kb cut-off silica membrane and centrifuged for 1 min. Buffer PB (0.5 ml) was used to wash the columns and then centrifuged for 1 min. All flow-through material was discarded. This step was repeated twice to remove all supernatant fluid. The DNA was eluted by transferring the columns to new 1.5 ml tubes and adding 50 µl of EB buffer. Columns were let stand for 1 min followed by centrifugation at 15,700 x g for 1 min. DNA concentration was determined as previously described.

2.13. Restriction Enzyme Digestion

EcoRI restriction enzyme and 10X EcoRI Buffer (New England Biolabs, Ipswich, MA) were used. Sample DNA up to a maximum amount of 800 ng was mixed with nuclease-free water up to a volume of 17.5 µl, followed by the addition of 2 µl of 10X Eco RI buffer and 0.5 µl of restriction enzyme in a 1.5 ml centrifuge tube. The contents were gently mixed by tapping. Sample tubes were incubated in a water bath at 37ºC for 2 h. The desired enzyme cleavage was confirmed by electrophoresis following the conditions previously described.

2.14. Sequencing

Plasmid DNA from each sample was sent out for sequencing to the GeneLab in the School of Veterinary Medicine at Louisiana State University, Baton Rouge, LA. Nucleotide sequences were determined by automated sequence analysis using a Perkin Elmer/ABI Prism 3130 four capillary based DNA sequencer (Perkin Elmer, Foster City, CA) and Applied BioSystems BigDye Terminator version 3.1 (Perkin Elmer, Foster City, CA) and analyzed using ABI Sequencing Analysis 5.3.1 software. The sequences obtained were compared against

sequences in the GenBank nucleotide collection through the Basic Local Alignment Search Tool (BLAST) available on the National Center for Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov/). Sequencing was done twice from independent PCR runs to compensate for any Taq polymerase errors (Cline et al., 1996) that could have occurred during the PCR process.

2.15. Lipid Characterization and Quantification

2.15.1. Algae Inoculum Density. Cell counts for all seed cultures were determined by the use of an Improved Neubauer 0.1 mm Deep Bright-Line hemacytometer (American Optical Corporation, Buffalo, NY). In brief, dilutions of the four strains and control were made (APPENDIX 4), thoroughly mixed, placed on the hemacytometer, and finally observed under a microscope (Leitz SM-LUX Microscope, Wetzlar, Germany). The cells present on either four or five specific squares (APPENDIX 5) were counted and the cell concentration per milliliter determined using the following calculations:

• Total cell count in 4 squares x 2, 500 x dilution factor [For Diatom (FC2) only]

• Total cell count in 5 squares x 50,000 x dilution factor

2.15.2. Growth Conditions. Two batches (A and B) were grown using slightly different conditions to evaluate the fatty acid content of the identified strains.

Batch A: Five liters of each of the identified strains were grown in 3 gallon glass carboys using BG-11 media (Cyanobacteria-N3), 50X F/2 enriched with 1 % $Na_2 SiO_3 . 9 H_2O$ % w/v (Diatom-FC2) and BBM (Green algae FC1, D2 and *Chlorella vulgaris* UTEX 259). The inoculum used was 50 ml L^{-1} on all strains except for the Diatom-FC2, for which 100 ml L^{-1} were used. Air (0.037 % CO_2) was used for mixing at a flow rate of 190 SCCM (standard cubic centimeters per minute) under continuous illumination (59 µmol photons m^{-2} s^{-1}, measured using an LI-193 spherical quantum sensor along with a LI-1400 data logger) at 25ºC for 15 days with the

exception of FC2 (diatom), which was grown for 21 days. Cultures were harvested by centrifugation at 13,300 x g using a Sorvall RC-5 Superspeed refrigerated centrifuge. Harvested biomass was washed with DI water 2-3 times to reduce the salt content. Microalgae paste was subsequently freeze-dried using a Millrock Technology LD53 freeze-dryer (Millrock Technology Inc., Kingston, NY) with a preset program (APPENDIX 6) for 15 h. The total dry weight was determined for each of the strains. Analyses were run in duplicate.

Batch B: Strains were grown using the same conditions as in Batch A, but providing mixing through pumping of 5 % CO_2 enriched air (American Air Liquide, Houston, TX) and the addition of a 0.05 M Sodium Phosphate buffer to adjust the pH in the growth media. Cultures were harvested after 10 days and after 21 days for FC2 (diatom). Analyses were run in duplicate.

2.15.3. Lipid Extraction and Chlorophyll Determination. Lipids were extracted from freeze-dried biomass using a variation of the Folch Method (Folch et al., 1957; Iverson et al., 2001). In brief, 20 parts of 2:1 chloroform/methanol were added to 1 part biomass. Next, 8-10 glass beads were placed in each tube, closed tightly and vortexed for 2 min. This mixture was then filtered using Whatman Grade 42 ashless filters with vacuum and then washed several times with a solution of 2:1 chloroform/methanol. A solution containing 0.88 % NaCl in water was added to obtain a combined filtrate with a final ratio of 8:4:3 chloroform/methanol/water. The final biphasic system was centrifuged, and the lower phase was collected into a preweighed glass tube. Chlorophyll *a* and *b* were determined at 664 and 646 nm, respectively, using a Beckman Coulter DU 800 UV/VIS spectrophotometer (Beckman Coulter Inc., Brea, CA). Absorbance units obtained were divided by molar absorptivity coefficients proposed by Jeffrey and Welschmeyer, (1997) where:

- Chlorophyll a: 87.67 L g^{-1} cm^{-1}

• Chlorophyll b: 51.36 L g^{-1} cm^{-1}

in the equation to calculate pigment concentration:

• Concentration of Pigment (mg/L) : $\left(\frac{\text{Absorbance units (Au)}}{\text{Specific Absorption Coefficient (L } g^{-1} \text{ } cm^{-1})} \right)$

The results for both types of chlorophyll were subtracted from the total lipid. Following this, the extract was evaporated to dryness under N_2 using a water bath at 30°C and a handmade evaporator. The tubes were wiped dry using kimwipes, dried in an oven at 55°C for an hour, let cool to 20°C in a dessicator for 30 min and the weights were recorded. Extractions for each sample were done in duplicate.

2.15.4. Fatty Acids Analysis. Fatty acid methyl esters (FAME) were prepared by transesterification of lipid extracts as described by Xu et al. (2010). Briefly, 40 µl of tricosanoic acid (1000 µg/ml) were added to each of the tubes containing a minimum of 2 mg of lipid. Two ml of MeOH:Benzene at a ratio of 4:1 (ρ = 0.8045 ±0.012 g/cm^3) were added to each tube and vortexed for 30 s. The samples were chilled at -74ºC for 15 min followed by the addition of 200 µl of acetyl chloride. All tubes were flushed with N_2 for five seconds. Samples were transesterified by keeping them in the dark at 20°C for 24 h. Five ml of a saturated solution of $NaHCO_3$ was added to each tube to neutralize the mixture and stop the reaction. Samples were vortexed for 30 s and 40 µl of nervonic acid methyl ester (1000 µg/ml) was added as internal standard. Samples were centrifuged for 10 min at 900 x g. The top layer (150 µl) was collected and 3 µl injected into a GC (5890 Series II gas chromatograph, Hewlett-Packard, Avondale, PA) equipped with a flame ionization detector. FAME analyses were carried out with a 60 m x 0.32 mm X 0.5 µm ZB-Wax + (Phenomenex, Torrance, CA) column. The GC temperature program

was as follows: 150ºC for 3 min, 200°C at increments of 8°C per minute for 15.5 min, 250°C at increments of 8°C per minute for 20.5 min and at 280°C at increments of 10°C per minute for 11 min. Helium was used as the carrier at a flow rate of 1.2 ml/min. Fatty acid identification was done by comparison of retention times with known standards (Supelco F.A.M.E. mix C4-C24, product number 18919-1AMP, Sigma-Aldrich, St. Louis, MO). Percent recovery for tricosanoic acid and nervonic acid methyl ester was calculated using the formula: C_{exp} / C_{Theo} x 100, where C_{exp} = concentration of the standard based on the GC peak area (chromatogram) and C_{Theo} = concentration of the standard added to each sample.

3. RESULTS

3.1.Algal Isolation

After growth for 2-3 weeks, cultures were heterogeneous at this stage. Colony homogenization was achieved by a serial dilution process. Samples were plated onto BG-11 and BBM for 2-4 weeks and observed under a light microscope at 250X. Four out of the 17 initially isolated colonies were selected for this study based on rapid growth, morphological and color diversity, and homogeneity. They were tentatively identified as cyanobacteria (N3), diatom (FC2), and green algae (FC1 and D2) (Figure 3.1).

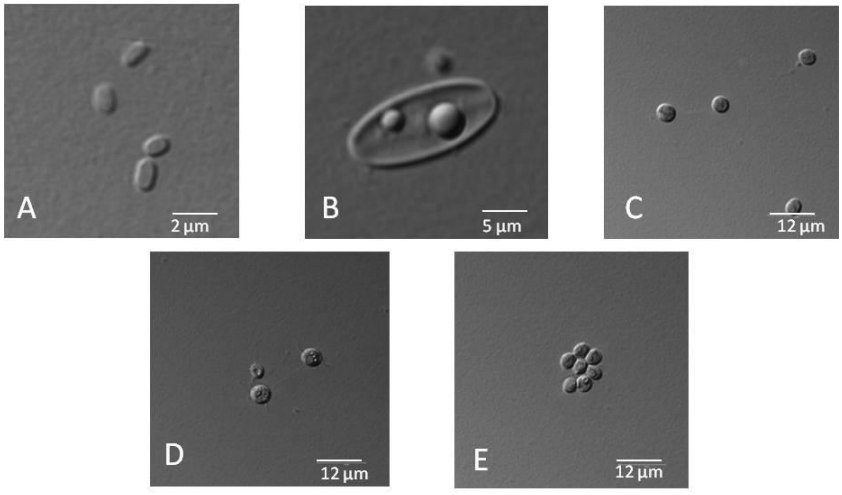

Figure 3.1. Microscopic Images for A) N3: *Synechococcus* sp., B) FC2: *Sellaphora pupula*, C) FC1: *Chlorella sorokiniana*, D) D2: *Scenedesmus abundans*, and E) *Chlorella vulgaris* UTEX 259 (control) as identified by BLAST. Images were taken at 250X using a Leica TCS SP2 spectral confocal microscope.

3.2. DNA Extraction

Cells (N3, FC2, FC1, D2 and *Chlorella vulgaris* UTEX-259) were harvested through centrifugation and their genomic DNA extracted (in duplicate) and quantified. DNA concentration ranged from 13- 440 ng/µl. Samples with DNA bands that showed high molecular weights and brightness were selected for PCR analysis.

3.3. PCR Products

Following PCR reactions, the quality of the PCR products was determined through agarose gel electrophoresis. Bands were observed in all nine conditions with isolate N3; whereas, five conditions showed bands with isolate D2 and only three conditions showed bands with isolates FC2 and FC1 (Figure 3.2). The molecular weights obtained for PCR products were 1.6 kb for N3 using the 16S primers, 1.8 kb for FC2 with the 18S primers, 1.8 kb for FC1 using the 18S primers, and 2.2 kb for D2 with the 18S primers. A PCR product of 1.8 kb was obtained for *Chlorella vulgaris* UTEX 259 using the 18S primers.

3.4. PCR Optimization

The best PCR conditions, based on PCR product quality results, were determined for each organism and were as follow: N3 (1L) no DMSO or glycerol at 1.5 mM $MgCl_2$, FC2 (1H) no DMSO or glycerol at 4.5 mM $MgCl_2$, FC1 (2M) with DMSO at 3 mM $MgCl_2$, D2 (3M) with glycerol at 3 mM $MgCl_2$ and *Chlorella v*ulgaris UTEX-1 (3M) (gel not shown) with glycerol and 3 mM $MgCl_2$ (Figure 3.2).

3.5. Purification of Plasmid DNA

Plasmid DNA concentration for each microorganism was determined after purification using 2 µl of sample DNA and reading it on a nanodrop spectrophotometer. Plasmid DNA concentration for the samples averaged 390 ng/µl for N3, 109 ng/µl for FC2, 273 ng/µl for FC1, 300 ng/µl for D2, and 405 ng/µl for *Chlorella vulgaris* UTEX.

3.6. Restriction Enzyme Digestion

After EcoRI enzymatic digestion all selected transformant colonies had inserts (Figure 3.3). For N3, two bands that were close together could be seen on each of the samples which corresponded to the PCR product insert bands. With regards to the samples amplified using 18S

rRNA gene primers, it was noticeable that the inserts for D2 (Lanes 7- 9) were larger than the rest.

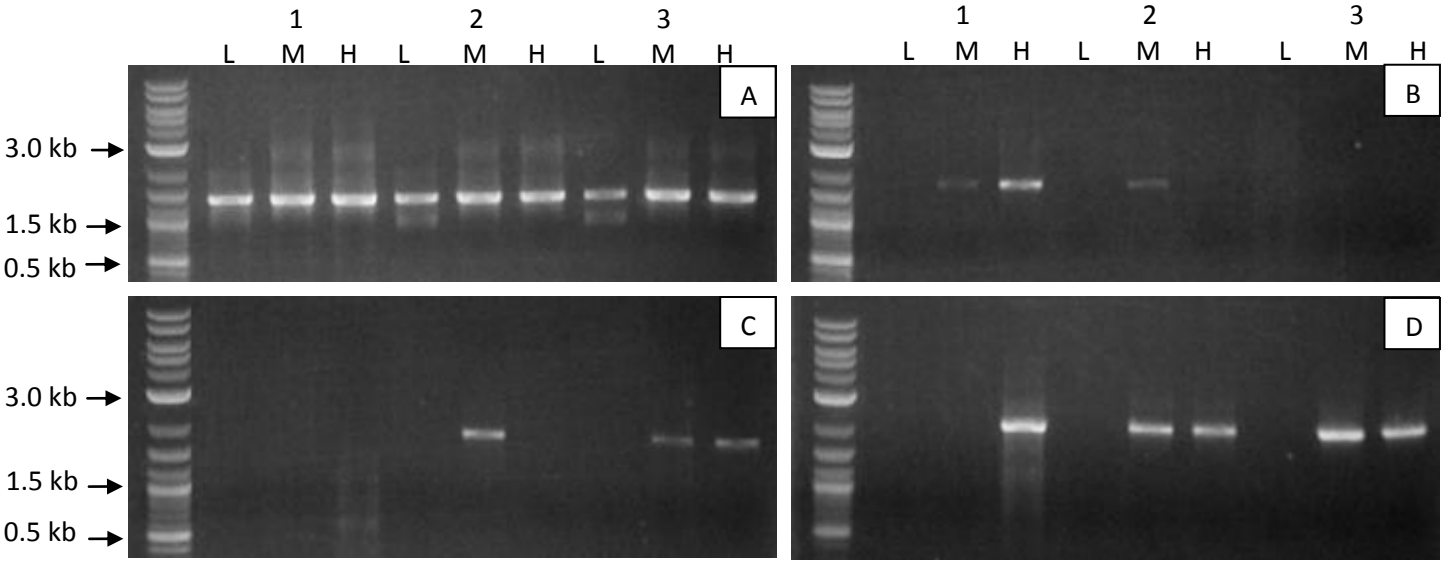

Figure 3.2. PCR Amplification Results Gel. PCR product quality of nine conditions for N3 (A), FC2 (B), FC1 (C), and D2 (D) mixed with $MgCl_2$ at 1.5 mM (low concentration, L), 3 mM (medium concentration, M), and 4.5 mM (high concentration, H).

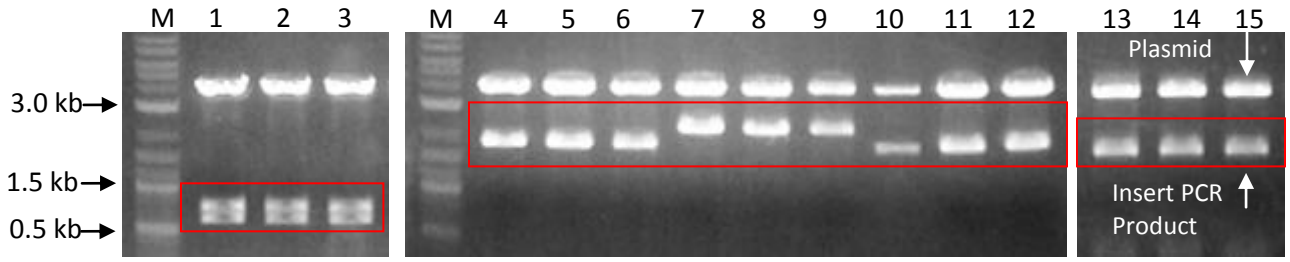

Figure 3.3. Restriction Enzyme Digestion Gel. Lane M, molecular weight marker. Lane 1, N3-1. Lane 2, N3-2. Lane 3, N3-3. Lane 4, FC1-1. Lane 5, FC1-2. Lane 6, FC1-3. Lane 7, D2-1. Lane 8, D2-2. Lane 9, D2-3. Lane 10, *C. vulgaris* UTEX-1. Lane 11, *C. vulgaris* UTEX-2. Lane 12, *C. vulgaris* UTEX-3. Lane 13, FC2-1. Lane 14, FC2-2. Lane 15, FC2 -3.

3.7. Sequencing

Plasmid DNA for each strain was sent out for sequencing and the sequences obtained were compared against sequences on the GenBank at the NCBI website. It was noticed that the

sequences amplified using M13 forward and M13 reverse primers (GeneLab) did not possess the length required to cover the amplified sequence of either 16S rRNA or 18S rRNA genes on the samples. Therefore, new primers were designed to fill that gap (Table 2.1). After the new sequences were obtained from all microorganisms from the LSU GeneLab, they were analyzed by pair-wise comparison and a final sequence was assembled. The obtained sequences were once again compared to the sequences available on the GenBank at the NCBI website.

3.8. BLAST

BLAST on the NCBI website indicated that N3 had a 99 % maximum identification with a *Synechococcus* sp. TAG 16S ribosomal RNA gene partial sequence with the accession number AF448066.1. FC2 results showed a 98 % maximum identification with a *Sellaphora pupula* clone RBG1 18S rRNA gene partial sequence with accession number EF151962.1. FC1 showed a 99 % maximum identification with a *Chlorella sorokiniana*18S rRNA gene, strain Prag A14 with accession number X74001.1. D2 showed a 99 % maximum identification with *Scenedesmus abundans* gene for 18S small subunit rRNA with accession number X73995.1. For the control *Chlorella vulgaris* UTEX 259, there was a maximum identification of 99 % with *Chlorella vulgaris* genes for 18S rRNA, ITS1, 5.8S rRNA, and ITS2 with accession number AB162910.1 (Table 3.1). Both runs for sequences for each organism, which were obtained from independent PCR runs, were found to be consistent showing only minimal nucleotide differences between them.

3.9. Algae Inoculum

Cell counts from inoculums for both batches are depicted in Table 3.2. Some differences were noticed between batches with *Synechococcus* sp. having a higher cell count (1.12×10^8) in Batch A as compared to Batch B (4.82×10^7).

Table 3.1. Codes for isolated microorganisms with tentative identification based on morphology, similarity between amplified sequences and nearest recorded sequence in NCBI using BLAST and accession numbers for NCBI sequences.

Microorganism	Similarity with the nearest recorded sequence	Accession number
N3 (cyanobacteria)	99 % with *Synechococcus* sp. TAG 16S	AF448066.1
FC2 (diatom)	98 % with *Sellaphora pupula* clone RBG1	EF151962.1
FC1 (green algae)	99 % with *Chlorella sorokiniana* strain Prag A14	X74001.1
D2 (green algae)	99 % with *Scenedesmus abundans*	X73995.1
*C. vulgaris**	99 % with *Chlorella vulgaris*	AB162910.1

*= *Chlorella vulgaris* UTEX 259 (control).

Table 3.2. Algae inoculums for Batch A and Batch B in cells ml^{-1}

Strain	Density	
	Batch A	Batch B
Synechococcus sp.	1.12×10^8	4.82×10^7
Sellaphora pupula	2.75×10^4	1.50×10^4
Chlorella sorokiniana	4.80×10^6	6.20×10^6
Scenedesmus abundans	1.06×10^7	1.06×10^7
Chlorella vulgaris UTEX 259	3×10^6	8.1×10^6

3.10. Biomass for Lipid Extraction

The dry weights of each microorganism used for lipid extraction studies are presented in Table 3.3. Compared to control (0.037 % CO_2), most strains resulted in an increase in total dry biomass when grown using 5 % CO_2, with the exception of *Synechococcus* sp., which mass decreased from 435 to 168 mg of dry biomass.

3.11. Total Extracted Lipid and Chlorophyll Content

Extracted lipids on dry weight biomass for each strain are summarized in Table 3.4. A decrease in the total lipid portion was observed on three out of the five evaluated strains when compared to the control batch (Batch A), with the exception of *Scenedesmus abundans* which resulted in an insignificant increment and *C. vulgaris* UTEX 259 with almost a two-fold increment.

Fatty acid profiles for identified strains are summarized in Tables 3.5. The total amount of fatty acids decreased for three of the strains when using CO_2. The most significant difference in the amount of fatty acids was observed with *Sellaphora pupula* when grown using air (54.8 mg g^{-1} dry biomass) as compared to using CO_2 (24.4 mg g^{-1} dry biomass). The amount of fatty acids for S*ynechococcus* sp. and *Chlorella sorokiniana* decreased slightly from 20.63 mg g^{-1} dry biomass and 29.82 mg g^{-1} dry biomass to 17.62 mg g^{-1} dry biomass and 23.99 mg g^{-1} dry biomass, respectively. However, for *Scenedesmus abundans* an increment was seen from 14.14 mg g^{-1} dry biomass to 31.63 mg g^{-1} dry biomass. *Chlorella vulgaris* UTEX 259 presented the most significant increment from 15.14 mg g^{-1} dry biomass to 47.83 mg g^{-1} dry biomass, with more than half of that total belonging to the polyunsaturated fatty acid (PUFA) fraction. Recoveries for internal (methyl nervonate) and external (methyl tricosanoate) standards were 88% and 82% on average, respectively. The total chlorophyll concentration was negligible averaging 0.007 mg g^{-1} of oil.

Table 3.3. Total dry biomass for lipid extraction.

	mg dry biomass	
Strain	Batch A *	Batch B**
Synechococcus sp.	435	168
Sellaphora pupula	62	100
Chlorella sorokiniana	158	847
Scenedesmus abundans	210	682
Chlorella vulgaris UTEX 259	294	575

* = Strains grown for 15 days with mixing using air (0.037 % CO_2). **= Strains grown for 10 days with mixing using 5 % CO enriched air.

Table 3.4. Total lipid extracted from biomass (g/100 g dry biomass).

Strain	Batch A*	Batch B**
Synechococcus sp.	9.17	6.9
Sellaphora pupula	19.52	12.12
Chlorella sorokiniana	11.56	10.95
Scenedesmus abundans	11.61	11.8
Chlorella vulgaris UTEX 259	7.36	13.65

* = Strains grown for 15 days with mixing using air (0.037 % CO_2). **= Strains grown for 10 days with mixing using 5 % CO_2 enriched air.

Table 3.5. Fatty acid profiles for evaluated strains grown in air (0.037 % CO_2) or 5 % CO_2 in mg g^{-1} dry biomass.
Results are the average means of duplicates along with standard deviations.

Fatty Acid	*Synechococcus* sp.		*Sellaphora pupula*		*Chlorella sorokiniana*		*Scenedesmus abundans*		*Chlorella vulgaris* UTEX 259	
	Air (0.037 % CO_2)	5 % CO_2	Air (0.037 % CO_2)	5 % CO_2	Air (0.037 % CO_2)	5 % CO_2	Air (0.037 % CO_2)	5 % CO_2	Air (0.037 % CO_2)	5 % CO_2
Saturated Fatty Acids (SFA's)										
C14:0	5.85 ± 0.03	5.24 ± 0.64	3.73 ± 0.14	1.33 ± 0.05	1.01 ± 0.008	1.22 ± 0.03	0.78 ± 0.04	2.24 ± 0.01	0.56 ± 0.06	2.29 ± 0.05
C16:0	3.57 ± 0.03	3.13 ± 0.16	8.99 ± 0.57	6.02 ± 0.39	7.76 ± 0.96	6.90 ± 2.24	3.15 ± 0.11	6.56 ± 0.84	3.20 ± 0.42	10.35 ± 0.77
C18:0	0.50 ± 0.05	0.85 ± 0.02	2.85 ± 0.13	1.26 ± 0.005	0.93 ± 0.01	1	2.09 ± 0.03	ND	0.79 ± 0.06	ND
Total SFAs	9.92	9.22	15.57	8.61	9.7	9.12	6.02	8.8	4.55	12.64
Monounsaturated Fatty Acids (MUFA's)										
C16:1	10.23 ± 0.004	6.24 ± 0.56	16.07 ± 1.50	9.24 ± 0.78	1.54 ± 0.01	2.03 ± 0.82	1.22 ± 0.03	4.13 ± 0.56	0.41 ± 0.09	5.41 ± 0.53
C18:1	0.48 ± 0.07	0.89 ± 0.03	3.06 ± 0.06	1.40 ± 0.007	1.42 ± 0.14	1.52 ± 0.21	1.14 ± 0.02	3.73 ± 0.47	0.71 ± 0.06	2.02 ± 0.45
Total MUFAs	10.71	7.12	19.13	10.64	2.96	3.55	2.36	7.86	1.12	7.43
Polyunsaturated Fatty Acids (PUFA's)										
C18:2	ND	0.34 ± 0.02	1.43 ± 0.03	0.73 ± 0.22	6.91 ± 0.16	5.87 ± 1.18	2.41 ± 0.07	2.20 ± 0.19	1.68 ± 0.24	3.59 ± 0.56
C18:3	ND	0.94	1.62 ± 0.05	1.87 ± 0.0001	10.25 ± 0.19	6.65 ± 0.21	3.35 ± 0.12	12.77 ± 1.81	7.79 ± 1.31	24.17 ± 4.78
C20:4	ND	ND	17.08 ± 0.06	2.55 ± 0.27	ND	ND	ND	ND	ND	ND
Total PUFAs	0	1.28	20.13	5.15	17.16	12.52	5.76	14.97	9.47	27.76
Fatty Acids*	**20.63**	**17.63**	**54.8**	**24.4**	**29.82**	**23.99**	**14.14**	**31.63**	**15.14**	**47.83**

ND= None detected; *= total fatty acids.

4. DISCUSSION

First described in the late 1980's, PCR is one of the most widely used methods in molecular biology (Saiki et al., 1988). Several decades later, attempts for PCR optimization continued to be made to meet its specific objectives (Dieffenbach and Dveksler, 2003). Templates known for being difficult to sequence include those with high guanine-cytosine (G/C) content, high adenine-thymine (A/T), as well as sequences with marked secondary structure or large regions of homopolymer (Stirling, 2003). In regards to sequences with high G/C content, several approaches have been taken to solve this problem and perhaps the most successful method for improving results is the inclusion of certain organic additives in the reaction mixture, such as DMSO, betaine, polyethylene glycol, glycerol and formamide (Chakrabarti and Schutt, 2001). The effect DMSO exerts in the PCR amplification of some GC-rich sequences is a largely studied one (Pomp and Medrano, 1991; Sun et al., 1993; Sidhu et al., 1996).

In this study, the DNA treatment containing neat DMSO and 3 mM $MgCl_2$ was determined to be the best PCR condition for FC1. Kang et al. (2005) conducted a study to find the best PCR conditions for the uniform amplification of random sequence templates, which can be disrupted by high GC content. They determined that the effect of additives on PCR was more outstanding in the presence of 5 % DMSO, which increased the ratio of full length to short products by 120 % (Kang et al., 2005). For D2 and *Chlorella vulgaris* UTEX 259, the best condition was determined to be the one that had glycerol at 50 % and $MgCl_2$ at 3 mM. Nagai et al, (1998) conducted a study to evaluate the effect of various additives, among them glycerol, on PCR. Their results indicated that the addition of either one in the reaction mixture allowed the specific amplification when an enterohemorrhagic *E. coli* DNA fragment was used as template. The improvement of glycerol on PCR efficiency and specificity can possibly be explained by (1)

glycerol's enhancement of the hydrophobic interactions between protein domains (Back et al., 1979); (2) lowering of the strand-separation temperature (Wang et al., 1993); and (3) a raise on proteins' thermal transition temperature (Back et al., 1979; Gekko and Timasheff, 1981).

Magnesium Chloride ($MgCl_2$) is a necessary co-factor for all Type II enzymes which include restriction endonucleases and polymerases. Mg^{2+} binds to the enzyme inducing a shift which puts the subunits in a proper configuration. It is assumed that a significant reduction in $MgCl_2$ concentration may prevent a sufficient number of enzyme molecules from being in the correct conformation for good amplification to happen (IDT, 2005). However, it is also known that excessive $MgCl_2$ can be equally harmful many times, with a substantial increase in secondary products produced by non-specific priming being the usual response to excess Mg^{2+} (IDT, 2005).

In the present study, three different concentrations of $MgCl_2$ were tested (1.5, 3, 4.5 mM). Three mM of $MgCl_2$ was optimum for the amplification of FC1, D2 and *C. vulgaris*, 1.5 mM for N3 and 4.5 mM for FC2. A study performed using genomic DNA from selected strains of *Vibrio vulnificus* to obtain DNA fingerprint profiles with arbitrarily primed polymerase chain reaction (AP-PCR) demonstrated that when using a $MgCl_2$ concentration of 2.5 mM and PCR cycling parameters in combination with 1 µg of purified genomic DNA, 1.04 mM of R-PSE420 oligonucleotide primer and thermal cycling protocols with stepwise increments in the annealing temperatures, reproducible DNA fingerprints free of primer artifacts were generated (Vickery et al., 1998). The aforementioned $MgCl_2$ concentration is close to the medium (3 mM) concentration used in the present study which was optimum for the amplification of three out of the five organisms subjected to algal identification.

The size of the PCR products obtained in this study were ~1.6 kb for N3, using a set of 16S rRNA primers, and ~1.8 kb for FC2, FC1 and *Chlorella vulgaris* UTEX 259. The PCR

product for D2; however, had a size of 2.2 kb, which were observed on both, the PCR product gel and on the restriction enzyme digestion gel. This was confirmed by the fact that the final sequence assembled for D2 had a total of 2, 200 bp, suggesting that this particular gene on D2 was different compared to the other isolates amplified by the 18S rRNA primers. Therefore, this organism could be considered a different strain within its genus since the query coverage for D2 was only 81 % (results not shown) and it was the lowest among the sample strains (query coverage for the other microorganisms was between 98-100 %).

The NCBI website identified matches for all four sample strains. N3 was identified as *Synechococcus* sp. TAG 16S ribosomal RNA gene, partial sequence, FC2 as *Sellaphora pupula* clone RBG1 18S rRNA gene, partial sequence, FC1 as *Chlorella sorokiniana*18S rRNA gene, strain Prag A14, and D2 as *Scenedesmus abundans* gene for 18S small subunit rRNA.

PNNL Biofuels Scientific Focus Area (BSFA) have conducted research in cyanobacteria in an attempt to develop a better understanding of metabolic subsystems and regulatory networks involved in solar energy conversion to biofuel products (Bryant et al., 2011). A system approach to understanding issues such as metabolic modules, physiological constraints and maximum rates of carbon processing is being undertaken by exploiting the fast-growing cyanobacterium, *Synechococcus* sp. PCC 7002, which has the added benefit of a well-developed genetic system (Bryant et al., 2011). Cyanobacteria are said to be attractive candidates for biofuel producing microbial systems since they possess the favorable characteristics of both prokaryotes and plants. Cyanobacteria are photosynthetic organisms able to absorb solar energy and fix carbon dioxide, whereas other biofuel-producing microbes such as *E. coli, Zymmomonas mobilis, Saccharomyces cerevisiae* are not capable of doing both (Lu, 2010).

Studies on oleaginous diatoms indicate that the average lipid content can be 22.7 % dry cell weight when grown under normal conditions, whereas under stress conditions, a total lipid content of 44.6 % dry cell weight was seen (Hu et al., 2008). *Sellaphora pupula* (Kutzing) Mereschkowsky (formerly *Navicula pupula Kutzing*) is a common, freshwater diatom species and a complex organism containing many pseudo- and semi-cryptic species (Mann, 1984, 1989, 1999; Mann and Droop, 1996; Mann and Kocioleck, 1990; Behnke et al., 2004; Evans et al., 2007, 2008). A member of this genera, namely *Navicula* sp., has been found to possess a fatty acid profile where 16:0, 16:1 and 20:5*n*3 make up more than 75 % of the total lipid content (Dunstan et al., 1994), and other studies have determined the presence of C_{25} triene III, considered probably the most abundant and widely occurring highly branched isoprenoid (HIB) alkene observed in marine diatoms (Belt et al., 2001).

Chlorella species can be encountered in all water habitats, fresh and marine sources (Iwamoto, 2004). It contains essential amino acids, protein, minerals, vitamins, chlorophyll, and bioactive substances (Borowitzka, 1988; Schubert, 1988). Bio-energy generation from *Chlorella* is a relatively new aspect in renewable energy research (Phukan et al., 2011). Illman et al, (2000) studied calorific values of *Chlorella* strains (*Chlorella protothecoides, vulgaris, emersonii, sorokiniana*, and marine strain, *minutissima*) using medium with low nitrogen content and pointed out that *Chlorella* strains may be good prospects for diesel replacements. Chader et al. (2011) analyzed the major fatty acids in *Chlorella sorokiniana* using Gas Chromatography and by growing it on different types of media. It was observed that the strain was mainly composed of a mixture of unsaturated fatty acids, such as oleic (18:1), linoleic (18:2) and linolenic acid (18:3). Saturated fatty acids, palmitic (16:0) and stearic (18:0), were also present to a small extent. In a study by Knothe (2008), palmitic, stearic, oleic, and linoleic acids were recognized as the most

common fatty acids present in the biodiesel. In the three media tested (TAP, BG-11 and media +N), C18:1 and C18:2 were commonly dominant. Chader et al. (2011) reported that the most important fatty acids were C16:0, C18:0, C18:1, C18:2 and C18:3 regardless of the medium utilized. Scragg et al. (2003) used an emulsion consisting of transesterified rape seed oil, a surfactant and slurry of *Chlorella vulgaris* in an unmodified single cylinder diesel engine with good results; whereas, Xu et al. (2006) was able to produce high quality biodiesel using heterotrophically grown *Chlorella protothecoides.*

The genus *Scenedesmus*, belonging to the green algae, have become the equivalent of laboratory rats in many fields in limnology or fresh water science (Wiltshire et al., 2000). If one algal species had to be chosen for biodiesel production, the one with the most suitable fatty acid profile, specifically in terms of linolenic and polyunsaturated fatty acids, would be *Scenedesmus obliquus* (Gouiveia and Oliveira, 2009). Mandal and Mallick, (2009), studied lipid accumulation in *Scenedesmus obliquus* when grown under nitrogen deficiency and observed that lipid was 43 % of dry cell weight compared to 12.7 % (dry cell weight) in the control; whereas, the lipid content increased up to 30 % when phosphorus deficiency and thiosulphate supplementation were used. When cultured for 8 days, the application of response surface methodology in combination with central composite rotary design (CCRD) resulted in a lipid yield of 61.3 % (against 58.3 % obtained experimentally) at 0.04, 0.03, and 1.0 g L^{-1} of nitrate, phosphate, and sodium thiosulphate, respectively .

The four strains (*Synechococcus* sp., *Sellaphora pupula*, *Chlorella sorokiniana*, and *Scenedesmus abundans*) isolated from Louisiana's fresh and brackish water bodies represent potential candidates for the production of biodiesel. In regards to the algal biomass obtained, an increase in final dry biomass was observed with most strains when aeration with 5 % CO_2 was

used for culturing, with the exception of the cyanobacteria *Synechococcus* sp. *Synechococcu*s sp. strain exhibited the lowest pH of all strains showing susceptibility to dissolved levels of CO_2. Wang et al. (2011) observed that significant inhibitory effects on the growth of two cyanobacterial species, *Microcystis aeruginosa* and *Anabaena spiroides*, were seen when three pH conditions (5.5, 6.0 and 6.5) were reached using aeration with CO_2. *Chlorella sorokiniana,* on the contrary, was the strain with the best response to CO_2 aeration resulting in a six-fold increase in dry biomass. A two-fold increase was observed for *Sellaphora pupula* and *Chlorella vulgaris* UTEX 259 and above a three-fold for *Scenedesmus abundans*. Huertas et al. (2000) reported that cultures of *Nannochloropsis gaditana* grown in the absence of dissolved inorganic carbon (DIC) and with aeration of less than 0.0001% (v:v) CO_2 considered to be low DIC conditions, presented a reduction in final cell biomass of approximately 56 % when compared to the biomass obtained in cultures grown under control conditions which equaled 2 mM DIC in the medium and 0.03% (v:v) CO_2. No growth was observed in *Nannochloris maculata* cultured under low DIC conditions. However, when a concentration of 1% (v:v) CO_2 in air (high DIC conditions) was used, only the growth of *N. maculata* was enhanced, concluding that the response to DIC was species dependent. It was also observed that the uptake of nitrate and phosphate by *N. maculata* was linked to the inorganic carbon level and determined that no nutrient absorption had been observed in the low DIC-culture. Growth, however, at the highest inorganic carbon concentration caused an acceleration of the uptake of these nutrients.

A decrease in the amount of total fatty acids extracted from *Synechococcus* sp., *Sellaphora pupula* and *Chlorella sorokiniana* was observed when the growth media was aerated with 5 % CO_2. The amount of total lipids decreased from 20.63, 54.8 and 29.82 g kg^{-1} to 17.62, 24.4 and 23.99 g kg^{-1}, respectively. Similar observations have been reported by Sato (1989), when

supplementing 1 % and 3 % CO_2 to *Chlamydomonas reinhardtii* cells grown photoautotrophically. Total polar lipids decreased from 60.3 nmol/10^7 using 0.04 % CO_2, to 44.2 nmol/10^7 and 45.7 nmol/10^7 when the media was supplemented with 1 and 3 % CO_2, respectively. The opposite was observed with *Scenedesmus abundans* and *Chlorella vulgaris* UTEX 259 where total lipids extracted increased from 14.14 g kg^{-1} to 31.63 g kg^{-1} and from 15.14 g kg^{-1} to 47.83 g kg^{-1}, respectively. Tang et al. (2011) reported similar trends with *Scenedesmus obliquus* and *Chlorella pyrenoidosa* grown in media supplemented with 0.03 %, 5 %, 10 %, 20 %, 30 %, and 50 % CO_2. The total lipid content for *Scenedesmus obliquus* increased from 15.15 wt. % at 0.03 % CO_2 to 16.45 wt. % at 5 % CO_2, whereas for *Chlorella pyrenoidosa* the total lipid content decreased from 20.9 wt. % at 0.03 % CO_2 to 20.65 wt. % at 5 % CO_2. These results demonstrate that even within the green algae classification various responses could be observed.

Changes were exhibited in the distribution of saturated (SFAs), monounsaturated (MUFAs) and polyunsaturated (PUFAs) fatty acids when 5 % CO_2 was used. The trend observed for *Synechococcus* sp., *Sellaphora pupula* and *Chlorella sorokiniana* was a decrease on SFAs, MUFAs and PUFAs with the exception of a slight increase (~0.5 g kg^{-1}) in MUFAs for *Chlorella sorokiniana*. An increase in all three categories of fatty acids was observed for *Scenedesmus abundans* and *Chlorella vulgaris* UTEX 259. Similar observations on strains of *Scenedesmus obliquus* and *Chlorella pyrenoidosa* with an increase in total fatty acids when providing 5 % CO_2 compared to 0.03 % were reported by Tang et al. (2011). Tsuzuki et al. (1990) observed an increase in the composition of saturated fatty acids over that of unsaturated fatty acids for *Chlorella vulgaris* after 1h under CO_2 concentrations ranging from 0.036 % to 2 %.

The main fatty acids in *Synechococcus* sp. grown in media supplemented with air were C16:1, C14:0 and C16:0. These findings agree with those reported by Pratoomyot et al. (2005)

and Patil et al. (2007). For *Sellaphora pupula*, the main fatty acids were C20:4, C16:1 and C16:0. Dunstan et al. (1994) described 16:1*n*7, 16:0 and 20:5*n*3 as the main fatty acids found on *Navicula* sp. The main fatty acids detected in *Chlorella sorokiniana* were C18:3, C16:0, C17:0 and C18:2. Hu et al. (2008) reported C16:0, C18:2 and C18:3 as being the major fatty acids in a *Chlorella sorokiniana* strain. Significant amounts of 16:3 and 16:2*n*7 were also detected, which may possibly correspond to the C17:0 content identified for this strain in the present study since neither 16:3 nor 16:2*n*7 were part of the F.A.M.E. mix used for the characterization. Chader et al. (2011) reported a similar profile on a locally isolated *Chlorella sorokiniana* strain. The strain when grown using TAP media contained C18:3, C18:2, C18:1 and C16:0 as the main fatty acids. The main fatty acids for *Scenedesmus abundans* were C18:3, C16:0, and C18:2. These findings were in agreement with those reported by Pratoomyot et al. (2005). Isik et al. (1999) reported C16:0, C18:0 +1 and C18:3 as the main fatty acid components in *Scenedesmus abundans*. The main fatty acids detected in *Chlorella vulgaris* UTEX 259 were C18:3, C16:0 and C18:2 and are in agreement with the results reported by Isik et al. (1999).

The primary emission in flue gas is CO_2, which may be present at concentrations ranging from 3 % to 25 % (Packer, 2009). Microalgal fixation of CO_2 by photosynthesis is considered the most favorable potential method for CO_2 sequestration from flue gas through the incorporation of CO_2 into a biomass carbon source such as lipids (Lee and Lee, 2003; Doucha et al., 2005; Wang et al., 2008; Brune et al., 2009; Yoo et al., 2010; Ho et al., 2011). The use of biomass generated by capturing CO_2 from industrial processes through microalgae, followed by its utilization for transportation needs, can aid on CO_2 sequestration and it would also help to diminish overall carbon emissions (Chiu et al., 2011). Chiu et al. (2011) reported a *Chlorella* strain that when treated with intermittent flue gas aeration in a double-set photobioreactor system, had an average

efficiency of CO_2 removal from the flue gas that reached 60 %, and NO and SO_2 removal efficiency that was maintained at approximately 70 % and 50 %, respectively. A typical composition of emissions from flue gases from two different power plants is given in Tables 4.1 and 4.2.

Table 4.1. Flue gas composition of Wyodak PC power plant (Robertson, 2007).

Flue gas component	**Concentration (by volume)**
CO_2	11.8 %
N_2	67 %
H_2O	8 %
O_2	12 %
SO_2	180 ppm
NOx	150 ppm
CO	300 ppm

Table 4.2. Typical untreated flue gas composition from a power plant burning low sulfur eastern bituminous coal. (Granite and Pennline, 2002).

Flue gas component	**Concentration (by volume)**
CO_2	15-16 %
O_2	3-4 %
H_2O	5-7 %
SO_2	800 ppm
SO_3	10 ppm
NOx	500 ppm
CO	20 ppm
HCl	100 ppm
Total Hg	1 ppb
N_2	Balance
Hydrocarbons	10 ppm

The fatty acid profiles in this study changed on each of the strains when grown in 5 % CO_2 enriched media. For *Synechococcu*s sp., C16:1, C14:0 and C16:0 remained the major fatty acids with the appearance of C18:2 and C18:3 which were absent in the batch grown in the presence of air only. This can be attributed to the desaturation effect due to higher concentration of CO_2 as described by Tang et al. (2011). According to Knothe (2008), the most common fatty acid esters in biodiesel are C16:0, C18:0, C18:1, C18:2 and C18:3. This is true for biodiesel feedstocks such as soybean, sunflower, rapeseed, palm and peanut oils (Knothe, 2008).

The fatty acid profile of *Sellaphora pupula* changed considerably when grown using 5 % CO_2. In the case of the PUFA C20:4, it was reduced from 17.08 g kg^{-1} to 2.55 g kg^{-1} and a smaller yet not significant decrease was also observed for C16:1 and C16:0. A high content of PUFAs is usually not a desirable feature in a feedstock for biodiesel production since this can result in biodiesel with low oxidative stability, low viscosity and low cetane number, being the latter a dimensionless descriptor of the ignition quality of a diesel fuel (Knothe, 2008). *Sellaphora pupula* demonstrated the greatest lipid percentage yield (19.52 %) when grown with air fed into the media, and, just like in *Synechococcus* sp., additional testing to determine an ideal CO_2 level could result in better biomass yields. For *Chlorella sorokiniana*, C18:3, C16:0 and C16:2 remained the main fatty acids after CO_2 aeration. *Chlorella sorokiniana* had the best growth response yielding the most biomass when treated with 5 % CO_2. For *Scenedesmus abundans*, C18:3 and C16:0 remained the main fatty acids, but the MUFAs C16:1 and C18:1 displaced the PUFA C18:2 when grown using 5 % CO_2. This can be considered a positive shift since *Scenedesmus abundans* displayed the highest content of C18:1. Oleic acid has been suggested in the past as a compound for improving biodiesel fuel properties due to its low melting point (-20°C) (Knothe, 2008). Oleic acid possesses better oxidative stability than C18:2 and C18:3 and it

exceeds the minimum cetane number standards given by the EN 14214 and ASTM D6751 (Knothe, 2008). In addition, out of the four isolated strains, *Scenedesmus abundans* produced the most total fatty acids with 31.63 g kg^{-1}. The main fatty acids detected for *Chlorella vulgaris* UTEX 259 were C18:3, C16:0 and C16:1 in the presence of 5 % CO_2, compared to C18:3, C16:0 and C18:2 without CO_2 aeration. It can be highlighted here that a substantial increment in C18:3 from 7.79 g kg^{-1} to 24.17 g kg^{-1} was observed. However, since this was mostly PUFAs, *Chlorella vulgaris* UTEX 259, under these circumstances, would not have the fatty acid profile most suitable for biodiesel production; but, it did produce the most total fatty acids at 47.83 g kg^{-1}

Oxidative stability, poor low-temperature properties and a slight increase in NOx exhaust emissions remain as the major challenges with biodiesel (Knothe, 2008). The challenge when addressing these problems is that solving one of them usually negatively affects another one and this observed pattern is related to the dependence of fuel properties on the fatty acid profile (Knothe, 2008). Cha et al. (2011) suggested that the use of a binary blending of microalgae system to attain an "ideal mix" of fatty acids could be pursued. In their study, they proposed a mixture of C16:1, C18:1 and C14:0 fatty acids in a ratio of 5:4:1 for biodiesel of very low oxidative potential but that could still offer both, a favorable cold filter plugging point (CFPP) rating and cetane number. Following this suggestion, not only could individual strains isolated in the present study be tested at a larger scale, but could also be combined to obtain a mixture that can offer an even better fatty acid profile and biomass productivity for biodiesel production when paired with the use of CO_2.

5. CONCLUSIONS

Four algae, two of them obtained from different brackish sources and the other two obtained from a fresh water source within the state of Louisiana's Southeast region, were tentatively identified as *Synechococcus* sp., *Sellaphora pupula*, *Chlorella sorokiniana*, and *Scenedesmus abundans*. The use of other taxonomical approaches is recommended to complement these findings.

The fatty acid profiles of the above mentioned organisms were altered when CO_2 aeration was incorporated into the growth media. *Synechococcus* sp.'s profile contained the major fatty acids that traditional biodiesel feedstocks possess. *Sellaphora pupula* had the best lipid percentage yield and the highest content of C16:1, which is considered to be the best fatty acid to improve biodiesel's characteristics after C18:1. *Scenedesmus abundans* displayed the highest content of C18:1. Oleic acid has been suggested in the past as a compound for enrichment in biodiesel fuels for improving biodiesel properties. *Scenedesmus abundans* can be considered as the second best candidate of all the strains analyzed for biodiesel feedstock. However, the lower C18:3 content observed in *Chlorella sorokiniana* allowed for a balanced fatty acid profile in terms of cetane number, oxidative stability, viscosity and low temperature conditions. In addition to the aforementioned properties, *Chlorella sorokiniana* had the greatest productivity when grown using CO_2, making it the best candidate for the production of biodiesel. The opposite can be said for the control *Chlorella vulgaris* UTEX 259 which contained 50 % of total fatty acid as C18:3 making it a less desirable candidate for biodiesel production in terms of oxidative stability.

An interesting approach could be the establishment of a binary production system between *Chlorella sorokiniana*, with a high biomass production, and *Scenedesmus abundans*, with a better fatty acid profile, to produce oil with superior quality and characteristics for the production of

biodiesel. Additionally, further testing should be made to determine if the isolated strains can tolerate higher CO_2 concentrations and evaluate their effect on biomass and fatty acid profile.

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APPENDIX 1. F/2 MEDIA COMPOSITION

Component	Stock Solution	Quantity per liter of medium	Molar Concentration in Final Medium
$NaNO_3$	75 g/L dH_2O	1 mL	8.82×10^{-4} M
NaH_2PO_4 H_2O	5 g/L dH_2O	1 mL	3.62×10^{-5} M
Na_2SiO_3 $9H_2O$	30 g/L dH_2O	1 mL	1.06×10^{-4} M
trace metal solution	(recipe below)	1 mL	---
vitamin solution	(recipe below)	0.5 mL	---

Trace metals solution

Component	Primary Stock Solution	Quantity per liter of medium	Molar Concentration in Final Medium
$FeCl_3$ $6H_2O$	---	3.15 g	1.17×10^{-5} M
Na_2EDTA $2H_2O$	---	4.36 g	1.17×10^{-5} M
$CuSO_4$ $5H_2O$	9.8 g/L dH_2O	1 mL	3.93×10^{-8} M
Na_2MoO_4 $2H_2O$	6.3 g/L dH_2O	1 mL	2.60×10^{-8} M
ZnSO4 $7H_2O$	22.0 g/L dH_2O	1 mL	7.65×10^{-8} M
$CoCl_2$ $6H_2O$	10.0 g/L dH_2O	1 mL	4.20×10^{-8} M
$MnCl_2$ $4H_2O$	180.0 g/L dH_2O	1 mL	9.10×10^{-7} M

F/2 Vitamin Solution

Component	Primary Stock Solution	Quantity per liter of medium	Molar Concentration in Final Medium
thiamine HCl (vit. B_1)	---	200 mg	2.96×10^{-7} M
biotin (vit. H)	0.1 g/L dH_2O	10 mL	2.05×10^{-9} M
cyanocobala-min (vit. B_{12})	1.0 g/L dH_2O	1 mL	3.69×10^{-10} M

APPENDIX 2. BLUE-GREEN 11 MEDIA COMPOSITION

Component	Quantity per liter of medium	Stock Solution Concentration	Final Concentration
$NaNO_3$	10 mL	30 g/200 mL dH2O	17.6 mM
K_2HPO_4	10 mL	0.8 g/200 mL dH2O	0.22 mM
$MgSO_4 \cdot 7H_2O$	10 mL	1.5 g/200 mL dH2O	0.03 mM
$CaCl_2 \cdot 2H_2O$	10 mL	0.72 g/200 mL dH2O	0.2 mM
Citric Acid$\cdot H_2O$	10 mL	0.12 g/200 mL dH2O	0.03 mM
Ammonium Ferric Citrate	10 mL	0.12 g/200 mL dH2O	0.02 mM
$Na_2EDTA \cdot 2H_2O$	10 mL	0.02 g/200 mL dH2O	0.002 mM
Na_2CO_3	10 mL	0.4 g/200 mL dH2O	0.18 mM
BG-11 Trace Metals Solution	1 mL		

BG-11 Trace Metals Solution

Component	Quantity per liter of medium	Stock Solution Concentration	Final Concentration
H_3BO_3	2.86 g	----	46 µM
$MnCl_2 \cdot 4H_2O$	1.81 g	----	9 µM
$ZnSO_4 \cdot 7H_2O$	0.22 g	----	0.77 µM
$Na_2MoO_4 \cdot 2H_2O$	0.39 g	----	1.6 µM
$CuSO_4 \cdot 5H_2O$	0.079 g	----	0.3 µM
$Co(NO_3)_2 \cdot 6H_2O$	49.4 mg	----	0.17 µM

APPENDIX 3.BOLD'S BASAL MEDIA COMPOSITION

Component	400 mL Stock Solution	1 Liter Stock Solution	quantity per liter of medium	Molar Concentration in Final Medium¶
Major Stock Solutions				
$NaNO_3$	10 g L^{-1} dH_2O	25.00 g L^{-1} dH_2O	10 mL	2.94 x 10^{-3}M
$CaCl_2 \cdot 2H_2O$	1 g L^{-1} dH_2O	2.50 g L^{-1} dH_2O	10 mL	1.70 x 10^{-4} M
$MgSO_4 \cdot 7H_2O$	3 g L^{-1} dH_2O	7.50 g L^{-1} dH_2O	10 mL	3.04 x 10^{-4} M
K_2HPO_4	3 g L^{-1} dH_2O	7.50 g L^{-1} dH_2O	10 mL	4.31 x 10^{-4} M
KH_2PO_4	7 g L^{-1} dH_2O	17.50 g L^{-1} dH_2O	10 mL	1.29 x 10^{-3} M
NaCl	1 g L^{-1} dH_2O	2.50 g L^{-1} dH_2O	10 mL	4.28 x 10^{-4} M
Alkaline EDTA Stock Solution			1 ml per liter of medium	
EDTA anhydrous		50 g L^{-1} dH_{2O}		4.28 x 10^{-4} M
KOH		31 g L^{-1} dH_2O		1.38 x 10^{-3} M

Acidified Iron Stock Solution			1 mL per liter of medium	Molar Concentration in Final Medium¶
$FeSO_4 \cdot 7H_2O$		4.98 g L^{-1} dH_2O		4.48 x 10^{-5} M
H_2SO_4		1.0 mL		
Boron Stock Solution			1 mL per liter of medium	
H_3BO_3		11.42 g L^{-1} dH_2O		4.62 x 10^{-4} M
Trace Metal Stock Solution			1 mL per liter of medium	
$ZnSO_4 \cdot 7H_2O$		8.82 g L^{-1} dH_2O		7.67 x 10^{-5} M
$MnCl_2 \cdot 4H_2O$		1.44 g L^{-1} dH_2O		1.82 x 10^{-5} M
MoO_3		0.71 g L^{-1} dH_2O		1.23 x 10^{-5} M
$CuSO_4 \cdot 5H_2O$		1.57 g L^{-1} dH_2O		1.57 x 10^{-5} M
$Co(NO_3)_2 \cdot 6H_20$		0.49 g L^{-1} dH_2O		4.21 x 10^{-6} M

APPENDIX 4. SAMPLES DILUTIONS FOR HEMACYTOMETER

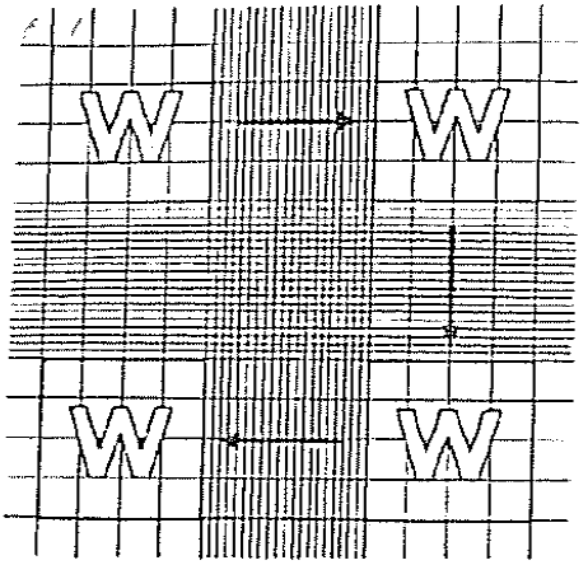
Batch A

Sample	Dilution factor	Method
N3	1:5	B
FC2	1:1	A
FC1	1:3	B
D2	1:3	B
Chlorella v.UTEX 259	1:2	B

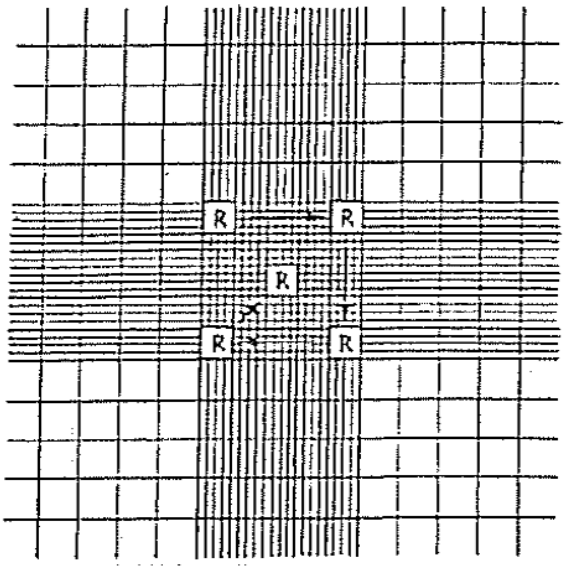
Batch B

Sample	Dilution factor	Method
N3	1:5	B
FC2	1:1	A
FC1	1:5	B
D2	1:3	B
Chlorella v.UTEX 259	1:3	B

APPENDIX 5. COUNTING PROCEDURES FOR HEMACYTOMETER READINGS



Method A (4 squares)



Method B (5 squares)

APPENDIX 6. FREEZE-DRYER PROGRAM

Freeze

Step #	1	2
Tº (ºC)	-40	-40
Minutes	0	240

Final Freeze

Final Freeze(ºC)	-44
Extra Time	0
Prim Vac (mTorr)	200

Primary Dry

Step #	1	2	3	4	5	6
Tº (ºC)	-20	-20	0	0	20	20
Minutes	25	120	0	120	0	360
Vacuum (mTorr)	100	100	100	100	100	100

Tº (ºC)	0
Minutes	0
Vacuum (mTorr)	200
Final Setpoint (º C)	4

VITA

Reynaldo Moreno Uclés, son of Reynaldo Moreno Regalado and Carmen S. Uclés de Moreno, was born in San Pedro Sula, Honduras, in 1978. He graduated from Academia Americana High School in 1995. He attended Universidad de San Pedro Sula from 1997 to 2002, where he received a Bachelor of Science in Agricultural Engineering and Business Administration. In August 2005, he received a scholarship from Louisiana State University, Baton Rouge, to study music, and in 2008 he earned his Bachelor of Music. In August 2009, he began his Master's program in the Department of Food Science at Louisiana State University, Baton Rouge. In May 2012, he will receive his degree of master of science in food science.