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Development of cost-effective and benign lipid extraction system for microalgae

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DEVELOPMENT OF COST-EFFECTIVE AND BENIGN LIPID EXTRACTION SYSTEM FOR MICROALGAE

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This dissertation is dedicated to my parents who's prayers and support throughout my life helped me reach this stage

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LIST OF ACRONYMS

AA – Arachidonic acid

ASE – Accelerated solvent extraction

B100 – 100% biodiesel

BD20 – 20% biodiesel in ethanol

BD40 – 40% biodiesel in ethanol

CFLES – Continuous flow lipids extraction system

DHA – Docosahexaenoic

EPA – Eicosapentaenoic

FAMEs – Fatty acid methyl esters

FMASE – Focused Microwave-assisted Soxhlet extraction

HHV – High heating value

HTL – Hydrothermal liquefaction

KB value – Kauri-butanol value

MAE – Microwave-assisted extractions

MSTFA – *N*-methyl-N-trimethylsilyltrifluoracetamide used as silyating agent

PAHs – Polyaromatic hydrocarbons

PBR – Photobioreactor

PLE – Pressurized liquid extraction

PSE – Pressurized solvent extraction

PUFAs – Long-chain polyunsaturated fatty acids

SC-CO_2 – Supercritical CO_2 extraction

SEM – Scanning electron microscope

SFE – Supercritical fluids extraction

TAGs – Triacylglycerides

UAE – Ultrasound-assisted extraction

ABSTRACT

A laboratory-made continuous flow lipid extraction system (CFLES) was devised to extract lipids from microalgae *Nannochloropsis sp.*, a potential feedstock for biodiesel fuel, with a focus to assess the workable temperatures and pressures for possible scale-up applications. Using conventional solvents, the CFLES recovered 100% of the lipids recovered with conventional Soxhlet extraction (USEPA method 3540) at moderate 50 psi pressure and 100°C temperature; conditions significantly lower than those normally used in pressurized liquid extractions requiring specialized equipment. Approximately 87% of the extracted oil was successfully transesterified into biodiesel fuel. For exploring the solvent potential of biodiesel, CFLES was also tested with 40% methyl-soyate (BD40) as co-solvent with ethanol. Both the solvents are less toxic to health and environment compared to conventional solvents. The system extracted 67% of lipids at 50 psi pressure and 100°C temperature. The system also extracted 64% and 65% lipids at pressure/temperature combinations of 50psi/120°C and 500psi/120°C respectively. Energy efficiency of CFLES was 48.9%. Compared to a lab-scale Soxhlet extraction system (150 mL), the solvents consumption in CFLES was reduced by 80% and 67% for conventional and biodiesel co-solvents, respectively, while extraction time was notably reduced from 8h to 0.25h and 0.67h, respectively. The estimated savings in extraction cost and energy at scaled-up CFLES systems are expected 57% and 60%, respectively, as compared to solvent extraction coupled with mechanical extractor. Based on previous studies, the total cost of microalgae oil production was estimated in the range of \$13.73 to \$44.60gal^{-1}. The solvent potential of biodiesel was further investigated with the use of methyl-soyate in a closed-vessel microwave-assisted lipid extraction (MAE). Approximately 66%, 78%, and 116% lipids were extracted with BD40 at 80°C, 100°C, and 120°C temperatures, respectively, compared to that extracted with Soxhlet. Maximum efficiency for BD20 (20% methyl-soyate) was 34%. MAE

using chloroform/ethanol mixture extracted 32%, 93%, and 108% of lipids at 80°C, 100°C and 120°C, respectively, compared to Soxhlet. Efficiency increased with increased biodiesel proportion in the co-solvent system and increased temperature. To our knowledge, this is the first study to report CFLES and the use of biodiesel as a co-solvent for extraction of biochemicals.

CHAPTER 1

INTRODUCTION

1.1 Overview

One of the primary hurdles preventing biodiesels from getting established as a viable renewable fuel is the availability of feedstock; the vegetable oil. It is estimated that feedstock accounts for 45 – 58% of total production cost for second generation biofuels (Hamelinck and Faaij, 2006; Al-Zuhair, 2007). Therefore, cheaper feedstock which does not interfere with the food market is the key to the future of renewable biodiesel fuel. Numerous non-crop feedstock have been explored as possible substitutes for vegetable oil e.g. Chinese tallow tree (Boldor et al., 2010) and Jatropha (Kaushik et al., 2007). Microalgae is reported as the most promising substitute for the current vegetable oil obtained from non-food crop along with its nutraceutical value (Roessler et al., 1994; Sawayama et al., 1995; Sheehan et al., 1998; Benerjee et al., 2002; Miao and Wu 2006; Chisti, 2007). Microalgae has the potential to yield 15 – 300 times more oil for biodiesel production than traditional crops (Chisti, 2007). Under optimum conditions, a potential yield of 5,000 – 15,000 gallons of microalgal oil per acre per year has been estimated (Sheehan et al., 1998; Ferrentino et al., 2006). Triglycerides, the building blocks of energy, obtained from microalgae have successfully been transesterified into biodiesel (Bartholomew, 1981; Pryde, 1983; Shay, 1993; Chisti, 2007). However, there are issues to resolve before reaping the full benefits of microalgae as an alternative diesel fuel. Cost-effective esxtraction of oil is one of the primary concerns. It is an important and costly step, which often involves the use of toxic solvents. The use of solvent extraction requires extra energy input to recover the solvents, and it has the potential to contaminate the algal solids; thereby restricting their end use applicability.

1.2 Biomass and Biofuels

Biomass is biological material derived from living, or recently living organisms; often termed plant- based material with renewable energy potential. Biomass energy is primarily the sun energy trapped into the mass of biological material. Therefore, energy derived from biomass is usually termed as bio-energy. Biomass include woody biomass (woody shrubs and trees), and agriculture biomass (corn, sugar cane, soybean etc.). Different types of woody biomass and agricultural biomass can be utilized for heat, power, electricity, fuel, and other bioproducts. Agriculture biomass can be either food crop (e.g. soybean) or non-food crop (e.g. microalgae). Fuel derived from biomass is called biofuel. This does not encompass fossil fuel which also has its origin from biomass but has been out of the carbon cycle for a very long time (Demirbas, 2009). Biofuels can be in the form of liquid, solid or gas. The most preferred one is the liquid form because of its potential to replace fossil fuels in the transportation industry. Liquid biofuels are categorized into (1) bioethanol; (2) vegetable oils and biodiesel; and (3) biocrude and synthetic oils (Demirbas, 2007). Bioethanol is the most used non-fossil alternative engine fuel in the world (Demirbas, 2009). Biodiesel from conventional food crop oil and animal fats account for only approximately 0.3% of the current demand for transport fuels (Schenk et al., 2008). Large scale thermochemical or hydrothermal liquefaction to produce biocrude or bio-oil by converting biomass in water at high temperatures and pressures; is limited by economic and process viability factors.

The first generation biofuels are derived from edible biomass, primarily corn and soybeans which compete with the food market e.g. bioethanol from corn and sugarcane or biodiesel from soybean or palm oil. The second generation biofuel are derived from cellulosic biomass e.g. sawdust, corn stalks, wheat straw, and fast growing grasses. Second generation biofuels do not compete with the food market but has high production cost. The third generation

biofuels are derived from algae biomass and cyanobacteria. The current work is based on the extraction part of the algal lipids as potential source for third generation biofuels.

1.3 Potential of Microalgae for Fuel and Non-fuel Products

1.3.1 Fuel Products

1.3.1.1 Biodiesel

Several microalgae strains are reported significantly rich in oil which can be converted into biodiesel using existing technologies (Benerji et. al. 2002; Chisti, 2007). Compared to the best oil-producing crops, microalgal biodiesel has been reported for it potential to completely displace petroleum-derived transport fuels without adversely impacting supplies of food and other agricultural products (Chisti, 2007). Currently, biodiesel production from microalgae is at least ten times more expensive than the regular diesel (Canakci et. al. 2008).

1.3.1.2 Bioethanol

Alcoholic fermentation of microalgae such as *C. vulgaris* is reported as a good source of ethanol due to the high starch content (37% dry wt.) with up to 65% ethanol conversion efficiency (Hirano et. al. 1997). Ethanol can be used as a supplement or substitute for petrol in cars. The solid residues from the process can be used for cattle-feed or gasification.

1.3.1.4 Biohydrogen

Microalgae convert water molecules into hydrogen ions (H^+) and oxygen during photosynthesis. The hydrogen ions are then subsequently converted by hydrogenase enzymes into H_2 under anaerobic conditions (Cantrell et. al. 2008). Melis and Happe (2001) reported that using the two-stage photosynthesis process and H_2 production a theoretical maximum yield of hydrogen by green algae could be achieved as 198 kg H_2 ha^{-1} per day.

1.3.1.5 Gasification

Partially oxidized biomass gasified at 850 – 1000^{o}C generates gas with highest theoretical yield of 0.64 g methanol equivalents from 1 g of biomass (Hirano et. al. 1998). Minowa and Sawayama (1999) gasified the *C. vulgaris* microalgae in a novel system with nitrogen cycling to obtain methane-rich fuel.

1.3.1.6 Crude Bio-Oil

Thermochemical or hydrothermal liquefaction is used to convert wet microalgal biomass into crude bio-oil fuel at 395^{o}C temperature and 1500 psi pressure in the presence of a catalysts (Minowa et. al. 1995; Brown et. al. 2010).The liquefied bio-oil contain C17 – C18 *n*-alkanes and polyaromatic hydrocarbons (PAHs). The gas fraction contains mostly CH_4, CO_2 and traces of H_2 and CO. Miao and Wu (2010) reported a fast pyrolysis of microalgae *Chlorella prothothecoides* to yield bio-oil up to 57.9% dry wt. Results indicated that bio-oils from microalgae are of a higher quality than those extracted from lignocellulosic materials (Miao et. al. 2004; Demirbas, 2006). Bio-oil however, is not suitable as substitute for transport fuel.

1.3.1.7 Anaerobic Digestion

Anaerobic digestion process of microalgae biomass is reported to yield methane gas at a rate of 1.17 ml l^{-1} (Yen and Brune, 2007). Microalgae biomass has lower C/N ratio which was adjusted by co-digesting with addition of paper wastes at 50/50 v/v ratio.

1.3.2 Non-Fuel Products

1.3.2.1 Nutraceuticals

Antioxidant compounds e.g. dimethylsulfoniopropionate, mycosporines or mycosporine-like amino acids, β-carotene, astaxanthin and other carotenoids are isolated from microalgal to protect against oxidative stress (Barrow and Shahidi, 2008). Microalgae is capable of accumulating high levels of carotenoids e.g. lutein (also present in leafy green vegetables, corn,

and egg yolk), which is beneficial for prevention and treatment of degenerative diseases (Mata et. al. 2010; Del Campo et al., 2007). Other chemicals of nutritional significance derived from microalgae include glycerol, β-carotenes, vitamins A and C (Mata et. al. 2010; Barrow and Shahidi, 2008).

1.3.2.2 Pharmaceuticals

Long-chain polyunsaturated fatty acids (PUFAs), especially of ω3 and ω6 series such as eicosapentaenoic (EPA), docosahexaenoic (DHA), and arachidonic acid (AA) are considered pharmacologically important for dietetics and therapeutics (Pulz and Gross, 2004). They have been used for prophylactic and therapeutic treatment of chronic inflammations (e.g. rheumatism, skin diseases, and inflammation of the mucosa of the gastrointestinal tract) along with positive effect on cardio-circulatory diseases, coronary heart diseases, atherosclerosis, hypertension, cholesterol, and cancer treatment (Barrow and Shahidi 2008; Mata et. al. 2010). Astaxanthin produced from *Haematococcus pluvialis* (1.5–3% of dry weight) has potential clinical applications due to its higher antioxidant activity (Miki, 1991).

1.3.3 Other Valuable Products from Microalgae

The biomass can be used as aquaculture and animal feed if toxic solvent used for extraction is not a concern. The biomass can be fed to aquatic animals e.g. larvae and juveniles of many commercially important fish and crustaceans. Algae cake is used as high protein animal feed in industries like poultry, cattle farming, and aquaculture (Kretschmer et al., 1995). Microalgae also contain neurotoxic substances e.g. saxitoxin, neosaxitoxin and gonyautoxin in different derivatives are produced by dinoflagellate *Alexandrium lusitanicum* causing interruption conduction in the neurons responsible for shellfish paralytic poisoning. Cytotoxic activity is important in anticancer drugs (Sirenko et al., 1999).

1.4 Algal Lipids

All algae are primarily made up of lipids, proteins, carbohydrates, and nucleic acids in different proportions. Lipids are a source of energy which makes membrane components, metabolites, and storage products. Their accumulation usually occurs during times of environmental stress, or nutrient deficient conditions. Lipids are hydrophobic molecules soluble in organic solvents. They are energy rich organic compounds made of carbon, hydrogen and oxygen. Lipids may be neutral or polar; a property important for selection of an appropriate extraction solvent. Neutral lipids include triglycerides, pigments, and trace amounts of hydrocarbons. Polar lipids include phospholipids, phosphatidylcholine, sterols, as well as prenyl derivatives such as tocopherols, carotenoids, terpenes, and quinines (Mata et al, 2010; Barrow and Shahidi, 2008).

1.5 Microalgae Production

Research and development interest of microalgae production has been grown significantly in the current millennium because of its high productivity, serving as non-food feedstock source for biofuels, and environmental benefits (Chisti, 2007; Pienkos and Darzins, 2009). Despite these benefits, the technology is still in its infancy stage. There are many R&D as well as economic challenges to meet before the algae biofuel are produced on commercial scale. Currently, no microalgae biofuels are produced commercially in the USA. Approximately 5000–10,000 tons worldwide of algal biomass is produced commercially for production of high-value, low-volume food supplements and nutraceuticals (Pulz and Gross, 2004; Spolaore et al., 2006; Pienkos and Darzins, 2009).

1.5.1 Cultivation

Microalgae can be produced either in open ponds or closed photobioreactor (PBR). A hybrid system involves both the systems where a continuous culture is maintained in a

photobioreactor feeding the pond (Christenson and Sims, 2011). Other methods include immobilized cultures (Hoffmann, 1998) and algal biofilms (Middelbrook et al., 1974; Wuertz et al., 2003; Christenson and Sims, 2011).

1.5.1.1 Open Ponds or Raceways

Open ponds or raceways consist of independent closed-loop recirculation channels where the flow is usually facilitated by paddle wheels (Sheehan et al., 1998). The concentration of algal biomass per liter is typically 0.5 – 1.0 g L^{-1} (Chisti, 2007). The shallow water depth is normally in a range of 0.2 – 0.5 m, and the area is 200 ha for extensive ponds or 0.5 to 1 ha for raceway ponds (Pienkos and Darzins, 2009; Brennan and Owende, 2010). Open systems are more economical due to their simplicity and low cost (Sheehan et al, 1998), however, challenges such as culture contamination with unwanted strains, evaporation, harvesting, and supply of CO_2 are required to be dealt with for optimal productivity. Typically, building and operation of raceways are relatively inexpensive. However, open systems have low productivity, culture contamination, poor mixing, and inefficient in use of CO_2 (Chisti, 2007; Mata et al., 2010; Christenson, 2011).

1.5.1.2 Closed Systems

Closed photobioreactor technology is designed to overcome major problems encountered with the open pond production systems and include tubular, flat plate, and column photobioreactors (Brennan and Owende, 2010). Tubular PBR are used for large scale systems. Vertical, horizontal, and helical designs are common (Carvalho et al., 2006; Chisti, 2007). In closed systems the concentration of algal biomass per liter is approximately 5 – 10 g L^{-1} (Chisti, 2007). Challenges include oxygen removal (Carvalho et al., 2006), relatively high construction and operating costs (Huntley and Redalje 2007; Rodolfi et al., 2009; Greenwell et al., 2011)

1.5.2 Harvesting

Harvesting is the most energy intensive part of the algal biofuels production. Different harvesting methods have been adopted which are chemical, mechanical, electrical, and biological based (Christenson and Sims, 2011). Selection of an appropriate harvesting technique is dependent upon microalgae strain, density, size, and the final product of interest (Brennan and Owende, 2010; Chen et al., 2011). The overall goal of the different approaches used has been to reduce the energy consumption and increasing the efficiency.

Chemicals based methods included chemical flocculation, chemical coagulation, and combined flocculation. Microalgal cells in the suspension are concentrated by a factor of 100 – 800 times which brings the total solids contents to 2 – 7% (Brennan and Owende, 2010). Mechanical harvesting approaches include gravity sedimentation, centrifugation, filtration, and flotation. Gravity settling is a low cost harvesting approach however, it has been reported the least efficient and time consuming in most of the cases concentrating the cells to 0.5 – 3% solids (Uduman et al., 2010; Greenwell et al., 2010; Christenson and Sims, 2011). Centrifugation is reported the most efficient and reliable harvesting approach concentrating the cells by a factor of 250 – 2500 to 5 – 22% solids with more than 90% recovery. The method is energy intensive with high cost (Shelef et al., 1984; Christenson and Sims, 2011). Filtration by conventional approaches is good for relatively large size microalgae (>70μm) while for small size microalgal cells (<30 μm), membrane microfiltration is preferred (Mohn, 1980; Petrusevski et al., 1995; Brennan and Owende, 2010). Cells are concentrated to 27% solids (Christenson and Sims, 2011). Flotation methods employ dispersed micro-air bubbles to trap and float algae cells as opposed to flocculation (Brennan and Owende, 2010). Dissolved air flotation (DAF) is used in wastewater treatment sludge removal (Friedman et al., 1977). DAF produces 10 – 100 μm bubbles in water stream presaturated with air at excessive pressures (Uduman et al., 2010). Dispersed air flotation

produces 700 – 1500 μm bubbles by using a high speed mechanical agitator with an air injection system (Rubio et al., 2002; Chen et al., 2011).

The electrical based approaches include electrocoagulation or electroflocculation mechanisms. The negatively charged algal cells are concentrated by movement in an electric field towards the anode in order to neutralize the charge forming aggregates (Kumar et al., 1981; Christenson and Sims, 2011). No chemical is required but the high power requirements affect the economics of the system for large-scale applications (Uduman et al., 2010). The efficiency of this method in algal removal is 80 – 95% (Poelman et al., 1997).

Biological based harvesting approaches involve autoflocculation or bioflocculation (Sukenik and Shelef, 1984). Autoflocculation occurs at high pH (8.5 – 9) causing supersaturation of calcium and phosphate ions. The calcium phosphate precipitates are positively charged which are attracted towards the negatively charged algae cells (Sukenik and Shelef, 1984). Algae removal efficiency was noted above 90% (Christenson and Sims, 2011). Other biological based harvesting approaches reported in literature include biofilms (Shipin et al., 1999), and microbial flocculation of algae (Lee et al., 2008).

1.5.3 Lipids Extraction

Extraction is another energy intensive step which determines the sustainability of microalgal biofuels production. Various methods have been used to extract lipid oil from algae. The sensitivity of the method selected depends upon the final product desired. Most of these methods do not offer long-term solutions because they are dominantly used either at lab scale or small pilot scale for nutraceuticals or uses other than biofuels. Most of the efficient methods are chemical based employing organic solvents. Algae oil has to be extracted by using suitable solvents coupled with some kind of cell disruption technique. Extraction methods are generally categorized as mechanical, chemical, or a combination of both. Mechanical extraction methods

include homogenization, milling, expression/expeller press, ultrasonic-assisted extraction, microwave assisted extraction, bead milling, and osmotic shock. Oil presses including mechanical expeller/repelling press, are the simplest methods and most popular of the techniques employed on commercial scale for seed plants. Chemical extraction methods include solvent extraction, supercritical fluid extraction, enzymatic extractions, thermal liquefaction, and pressurized liquid extraction. All of these methods have their individual benefits and drawbacks. Table 1.1 shows some of the commonly used extraction methods and their efficiency. Different methods work differently on different strains of microalgae due to the algal cell shape, size, and wall structure. For instance, supercritical CO_2 extraction is reported for 25% of oil recovery from *Nannochloropsis sp*., (Andrich et al., 2005), 40% from *Arthrospira (spirulina) maxima* (Mendes et al., 2006), 77.9% from *Spirulina (arthrospira) platensis* (Andrich et al., 2006) and 8.6% from *Crypthecodinium cohnii* (Couto et al., 2010).

1.5.3.1 Challenges in Microalgal Oil Extraction

Properties of microalgal cell wall play an important role in selection of oil extraction method and the extraction solvent because of its high resistivity (Hejazi and Wijffels, 2004a). Algae oil extraction is also significantly affected by the small algal cell sizes, and presence of water. It is, therefore, a challenge to determine the most efficient and cost effective extraction method which can reduce the energy requirements for lipid extraction. Exploring environmentally acceptable and health friendly solvent alternatives to the presently used toxic solvents is also a challenge.

Extraction efficiency depends on different factors including contact between the cellular material to be extracted and the solvent. Potential of extractability also depends on the hydration and permeability of the microalgal cell wall. Solvent plays an important role in cell lyses to increase the extraction yield from cells with strong walls. Several extraction procedures have

been reported, where extraction yields depend upon the microalgal strain, and the extraction technique employed (Table 1.1).

Table 1.1. Some commonly used extraction methods; their efficiency, advantages, and limitations

Extraction Method	Efficiency	Advantages	Limitations	Reference
French press	21.2%	Easy to use, no solvent required	Slow and least efficient, require more than one extraction and dry biomass	Shen et al 2009
Supercretical-CO_2	77.9%	No organic solvent residue in extracts	Expensive, difficult to scale-up	Pawliszyn, 1993; Macıas-Sanchez et al., 2005; Andrich et al., 2006
Solvent extraction/Bligh and Dyer/Folch	47% - 80%	Solvents relatively inexpensive; results are reproducible	Use significant vol. of toxic solvent, solvent recycling cost	Fajardo et al., 2007; Burja et al., 2007; Widjaja, 2009
Microwave-assisted extractions (MAE) and Ultrasound-assisted (UAE)	21% - 84%	Reduced extraction time; greater penetration of solvent into cellular materials; improved recovery	High power consumption; toxic solvents, difficult to scale up	Balasubramanian et al., 2011; Burja et al., 2007 Shen et al 2009
Thermochemical liquefaction	35% - 65%	Can assimilate wet biomass	Require high pressure and high temperature, difficult to scale up. Limited use of end product	Sawayama, et al., 1995; Brown et al., 2010; Valdez et al., 2011
Pressurized Liquid Extraction	40%	Reduced extraction time and solvent used	High temperature and pressure; difficult to scale up, require specialized instrument	Rodriguez-Meizoso et al., 2008
Direct saponification	34% - 46%	Good for lab scale fatty acid profile	Loss of useful cellular materials	Burja et al., 2007; Lewis et al., 2000
Solvent/saponification	60%	Good for lab scale applications	Loss of useful cellular materials	Guil-Guerrero et al., 2000

The current study hence focused on the development of an algal lipids extraction system which uses relatively less toxic and economical co-solvents coupled with high process temperatures and pressures, for effective lipid extraction.

1.6 Objectives

The current study was designed to meet the following objectives:

(1) To develop and optimize a continuous flow lipid extraction system (CFLES) which employs temperature and pressure as cell disruption tools for extraction of microalgal lipids

(2) Use of biodiesel as environmentally safe and health friendly solvent for extraction of algal lipids in microwave-assisted extraction (MAE) and continuous flow lipids extraction system

(3) To analyze the process economics in terms of extraction cost and energy usage for the devised extraction and co-solvent systems.

CHAPTER 2

LITERATURE REVIEW

2.1 Overview

Microalgae are reported to contain important compounds significant for industries in the areas of energy, fuels, nutraceuticals, pharmaceuticals, food, and chemicals. Predominantly, microalgae has been used for non-fuel products since as early as 2500 years ago in China (Tseng, 2004; Edwards, 2008). Health food products have been the dominant market for microalgae production e.g. polyunsaturated fatty acids (PUFAs) (Pulz and Gross, 2004). Lately, increased level of CO_2 in the atmosphere causing global warming, and limitation of non-renewable fossil fuel reserves being two critical issues have drawn the attention of researchers and scientists (Chisti, 1980-81; Sawayama et al., 1995; Gavrilescu and Chisti, 2005). Microalgae is regarded as one of the best solutions to curb with these issues in the short and long run (Chisti, 2007).

Various methods have been reported for the extraction of oil from microalgal biomass (Mercer and Armenta, 2011). The extraction yields depend upon the nature of the microalgal cell structures and the extraction techniques employed (Lewis et al., 2000). Some of the most commonly used methods and their efficiencies are given in Table 1.1. A brief description of the most common methods reported in the literature is given below.

2.2 Solvent Extraction

Solvent extraction employs the use of organic solvents. Hexane is dominantly used in food industry for extraction of oil from food grains. In case of microalgal lipid extraction, the solvent extractions determine the physical and chemical properties of the extracted biomass in terms of the extent the cell wall is damaged (Cooney et al., 2009). Therefore extraction systems that are effective over a broad range of species and cell wall structures are encouraged.

2.2.1 Conventional Soxhlet Extraction

Conventional Soxhlet extraction (EPA method 3540) is a classical solvent extraction technique to extract nutraceutical or pharmaceutical products from plants and has been widely used for microalgal extractions (Cheung et al., 1998; Garcia-Ayuso and Luque de Castro, 2001; Krichnavaruk et al., 2008; Balasubramanian et al., 2011). In conventional Soxhlet apparatus, the sample is placed in an extraction thimble fitted in the thimble-holder. A fresh solvent distillated at its boiling point, condensates back on top of the sample in the thimble. Once full, solvent from the thimble aspirates back into the distillation flask. This cycle of fresh solvent continues until extraction is complete. In Soxhlet method, the analytes are extracted at the boiling temperature of the solvent for extended hours, 8 h or more. Soxhlet is a standard method and the main reference for evaluating the performance of other extraction techniques. The method is well known for using large amounts of solvent, and the possible thermal decomposition of the target analytes (Wang and Weller, 2006). Modifications to this method reported to accelerate the extraction process includes focused microwave assisted Soxhlet extraction and ultrasound assisted Soxhlet extraction (Garcia-Ayuso and Luque de Castro, 2001; Luque-Garcia and Luque de Castro, 2004).

2.2.2 Folch Extraction

Folch et al., (1957) was the first reported method to develop a chloroform/methanol/water phase system for extraction of lipids from biological material. The method is still considered a classic and most reliable for quantitative extraction of lipids (Iverson et al., 2001). The method uses a ratio of 1 part of sample to 20 parts of co-solvent system (2:1, chloroform/methanol), followed by several washings of the crude extract with water. The washing process entails 1% loss of lipids (Folch et al., 1957). The method can also be applied to tissues containing 80% water (Iverson et al., 2001). This method uses large volumes of solvent (Bligh and Dyer, 1959).

Efficiency of Folch method and the Bligh & Dyer methods are reported comparable (Iverson et al., 2001).

2.2.3 Bligh and Dyer Method

Bligh & Dyer (1959) is the most cited reference method in the literature for extraction of lipids from biological materials (Burja et al., 2007). This method is a modified version of the Folch method. The advantage of Bligh & Dyer method is the use of reduced volume of solvent/sample ratio (i.e. 1 part sample to 3 parts of co-solvent system which is 1:2 chloroform/methanol followed by 1 or 2 parts chloroform (Iverson et al., 2001). This is a laboratory scale quantitative extraction technique using polar and non-polar solvents in a monophasic ternary system of chloroform:methanol:water (1:2:0.25, v/v/v). The method involves blending of sample and solvent mixture for 2 to 3 minutes. After the extraction, the system is converted into a biphasic solution with addition of chloroform and water yielding a chloroform layer at the bottom containing lipids and a methanolic layer on top containing nonlipids. Several modifications to this method have been reported including the use of various cell wall disruption techniques, such as ultrasonication (Dunstan et al., 1992, Burja et al., 2007), pressurized/accelerated hot solvents at high temperature (Macnaughton et al., 1997, Lewis et al., 2000), bead-beating and shaking (Lee et al., 2010), and the use of lyophilized samples (Dunstan et al., 1992). Sonication is carried out in an ultrasonic bath or by inserting an ultarasonicating probe into a mixture of biomass (usually 100 mg freeze dried) and solvent. Phase separation of the disrupted cells is achieved in a separatory funnel by adding chloroform and water (Dunstan et al., 1992; Burja et al., 2007).

Burja et al., (2007) reported miniaturized Bligh & Dyer method with recovery 47.5% greater than the classic Bligh & Dyer. They used 0.25 g of biomass placed in a tinted screw cap test tube containing 12.5 mL of chloroform, 25 mL of methanol, and 10 mL of a 50 mM K_2HPO_4

buffer solution (pH 7.4) followed by agitation for 1 h. After the extraction, the sample was transferred to a stoppered graduated cylinder. Adding 12.5 mL of chloroform and 12.5 mL of buffer created a biphasic solution for further phase separation. Burja et al., (2007) reported 73% efficiency of using ultrasonic bath compared to that of the miniaturized Bligh & Dyer method and 84% efficiency while using ultrasonic probe. Similarly, Lewis et al., (2000) reported 34.2% of fatty acids compared to 40.3% of that of the direct transesterification. Lee et al., (2010) reported the least efficiency of 8.8% using sonication method. Ultrasonication uses significant amounts of solvent and usually the sample is extracted more than once to complete the extraction process.

2.3 Supercritical Fluids Extraction (SFE)

Supercritical fluids extraction (SFE) or supercritical CO_2 (SC-CO_2) extraction use extremely high pressure and high temperature (Mendes et al., 1995; Valderrama et al., 2003; Krichnavaruk et al., 2008). The solubility of the compounds in carbon dioxide increases with pressure up to 30.0MPa (4351 psi). It is an efficient method for extraction of natural substances from foods (Mendes et al., 2003; Sun and Temelli, 2006) such as decaffeination of coffee, the synthesis of polymers, as well as purification and formation of nanoparticles (Lim et al., 2002; Kopcak and Mohamed, 2005). Supercritical CO_2 extraction has been employed for lipid extraction from microalgae. Several authors have reported extraction of lipids or bioactive compounds from microalgae using supercritical carbon dioxide extractions (Valderrama et al., 2003; Aresta et al., 2005; Krichnavaruk et al., 2008; Kitada et al., 2009). Polak et al. (1989) used SC-CO_2 to extract lipids from algae. The authors reported extraction of 25% eicosapentaenoic acid (EPA) from freeze dried microalgae *Skeletonema costatum*, and *Ochromonas danica*. The method does not employ toxic solvents, but the high power consumption to maintain high pressure and temperature have economic implications for scale-up microalgae production.

2.4 Thermal Liquefaction

Thermochemical liquefaction or hydrothermal liquefaction yield biocrude or bio-oil from microalgae biomass under high temperature up to 395°C, and high pressure (up to 1450 psi), and a holding time up to 60 min or more (Minowa et al., 1995; and Sawayama et al., 1995; Aresta et al., 2005; Brown et al., 2010). The physical and chemical properties of bio-oil are strongly dependent on the feedstock and production conditions employed (Shuping et al., 2010). Thermal liquefaction is performed in autoclave (Shuping et al., 2010; Huang et al., 2011), or stainless-steel tubular reactor (Brown et al., 2010). Bio-oil yields typically range from 35 – 65 wt %, and the heating value range from 35 – 50 MJ kg^{-1}. Brown et al. (2010) reported the heating value for bio-oil obtained by hydrothermal liquefaction of *Nannochloropsis sp*. as 39 MJ kg^{-1}. The bio-oil contained phenol and its alkylated derivatives, heterocyclic N-containing compounds, long-chain fatty acids, alkanes and alkenes. Similarly, Valdez et al. (2011) reported hydrothermal liquefaction of *Nannochloropsis sp*. They obtained 39% dry weight percent of bio-oil. The bio-oil obtained cannot be directly used as liquid fuels for transportation, but could be used for heating purposes unless refined (Vardon et al., 2011). The method is difficult for scaled up production because attaining and maintaining high temperature and pressure in thermochemcial liquefaction affect the economic feasibility of extraction system.

2.5 Saponification or *in-situ* Transesterification

Lewis et al. (2000) reported lipid extraction combined with *in-situ* (or direct) transesterification at 90°C for 15 – 120 min, followed by recovery of the fraction of fatty acid methyl esters (FAMEs) using a biphasic solvent system of hexane: chloroform (4:1). Direct transesterification is however, reported to underestimate the true fatty acid content of the biomass. Cartens et al. (1996) reported direct saponification of biomass using two solvents that contained KOH for lipid saponification: ethanol (96%) and hexane: ethanol (96%) (1:2.5, v/v).

The authors reported that the direct saponification works well for extracting lipids from fish tissue; however, it does not necessarily work for lipid extraction from microbial biomass. The method is regarded as good tool for fatty acid profiling at laboratory scale extractions.

2.6 Pressurized Liquid Extraction (PLE)

Pressurized liquid extraction (PLE), or accelerated solvent extraction (ASE), or pressurized solvent extraction (PSE) uses solvents at high temperature above their boiling point, and high pressure to maintain the solvent in the liquid state during extraction. Accelerated solvent extraction was first reported by Richter et al. (1996) for extraction of chemicals from environmental samples. The extraction technique is well known for higher selectivity, shorter extraction times, and small amount of toxic organic solvents used (Herrero et al., 2004; Jaime et al., 2005; Rodriguez-Meizoso et al., 2008). The sample placed in an extraction cell (11 mL) is extracted statically i.e. unlike Soxhlet extraction where a fresh solvent is recycled all the time; a fixed small volume of solvent (usually 10 to 11 mL) is used for the entire extraction. Herrero et al. (2004) employed this technique for extraction of bioactive compounds from *Spirulina platensis* microalgae using ASE-200® system from Dionex (Sunnyvale, CA, USA) at extraction temperature up to 170°C) and 1500 psi pressure. These authors reported improved recovery at higher temperature, higher extraction time, and higher dielectric constant of the solvent. The best yields were obtained with ethanol at the higher extraction temperature and time. Ethanol was found to be the better solvent than hexane. Denery et al. (2004) optimized this method for extraction of bioactive compounds, carotenoids and kavalactones from *Haematococcus pluvialis* and *Dunaliella salina* respectively. They found the optimum temperature as 60°C, pressure as 2000 psi, and 0.75 g sample size.

Similarly, Rodriguez-Meizoso et al. (2008) reported extraction with pressurized fluid extraction of bioactive compounds from *Phormidium species* at temperature up to 200°C, and

1500 psi pressure. The authors reported improved recoveries with increase in temperature as was found elsewhere (Herrero et al., 2004). These authors have, however, reported the degradation of chlorophylls into pheophytins at 150°C and 200°C temperatures. Similar degradation of total pigments was noted by Denery et al. (2004) at temperature up to 100°C.

To our knowledge, the pressurized liquid extraction (PLE), accelerated solvent extraction (ASE), or pressurized solvent extraction (PSE) has been used primarily for extraction of bioactive compounds including β-carotene, antioxidants, and chlorophyll as well as natural products from plant materials (Denery et al., 2004). The method has not been reported for extraction of lipids with focus of scaled-up production of biofuels from microalgae. One reason is that the method, reported so forth, requires a specialized instrument e.g. ASE-200® (Dionex, Sunnyvale, CA, USA). The second reason is that attaining and maintaining high temperature and pressure are expected to affect the economic feasibility at an industrial scale. The third reason is that the yield of heat-sensitive compounds is expected to be reduced at higher temperature (Kaufmann et al., 2001). Furthermore, the ASE® is an efficient technique for solid and semi-solid samples requiring drying of samples to an extent which can affect the economics of biofuel production from microalgae. However, large scale extraction using this technique has not been investigated.

2.6.1 Advantages of Elevated Pressure and Temperature

Pressurized liquid extraction (PLE) is carried out at an elevated pressure and temperature, allowing liquid extraction above the boiling point of the solvent. The analytes solubility is therefore enhanced and the desorption kinetics are accelerated. The extraction is generally completed within a few minutes. Liquids under pressure act as solvents; therefore, higher extraction efficiency is expected at higher pressure which may be accelerated further with

increasing temperature. PSE is reported for reduced solvent consumption, as well as comparable solute recoveries.

Temperature increases the solvent potential of a solvent (Richter et al., 1996) by accelerating diffusion rates (Denery et al., 2004). The thermal energy helps overcome the cohesive (solute-solute i.e. lipids-lipids) interactions and adhesive (solute-matrix, i.e. lipids-cell matrix) interactions (Richter et al., 1996; Cooney et al., 2009). Increase in thermal energy increases molecular motion of the molecules and thereby decreasing their molecular interactions of hydrogen bonds, van der Waals forces, and dipole interactions (Cooney et al., 2009). Similarly, pressure facilitates increased transport of solvent to hard-to-reach corners, pores, surfaces and matrices (Richter et al., 1996; Cooney et al., 2009). Pressure also increases the penetration power of the solvents through the cell wall to contact the lipids inside microalgal cells. Elevated pressure is reported to reduce the dielectric constant of immiscible solvents to values that better match the polarity of the lipids (Richter et al., 1996; Herrero et al., 2006; Cooney et al., 2009). Pressurized solvents at elevated temperature hence improve the efficiency of traditional extraction systems resulting in shorter extraction time and lower solvent consumption (Cooney et al., 2009). The mass transfer rates are thereby increased.

2.7 Microwave Assisted Extraction

Microwave is a non-contact heat source which heats the whole sample volume simultaneously as compared to conductive heating. The weak hydrogen bond is disrupted by promoting the rotation of molecular dipoles, an effect opposed by the viscosity of the medium and strongly dependent upon the solvent and matrix (Cravotto et al., 2008). Luque de Castro and Garcia-Ayuso (1998) designed a focused microwave-assisted Soxhlet extractor (FMASE), for food industry using two sources of energy – microwave plus electrical heating applied at the bottom of the extraction flask accelerating the performance of Soxhlet extraction. Focused

microwave-assisted Soxhlet extractor has been reported for high yield to extract different compounds and lipids from environmental solid samples or food products (Freyburger, et al., 1988; Loque-Garcia and Luque de Castro, 2004; Priego-Capote and Luque de Castro, 2005; Virot et al., 2007). The system is reported for maximum recovery of oils and fats at sample moisture contents sample between 20 and 90%. Recently, the microwave energy has also been extended to fast preparation of biodiesel via transesterification process from vegetable oil (Leadbeater and Stencel, 2006; Barnard et al., 2007) where a commercially available microwave was employed in batch or continuous mode. Furthermore, a pilot scale continuous microwave system has also been reported for extraction of oil from Chinese tallow tree, soybean and rice bran (Boldor et al., 2010; Terigar et al., 2011).

Lee et al. (2010) compared the performance of various lipid extraction methods including microwave from three species of microalgae *Botryococcus sp*., *Chlorella vulgaris*, and *Scenedesmus sp*. The authors reported higher lipid content extracted from the three species when using microwave oven method rather than autoclaving, bead-beating, sonication or osmotic shock with 10% NaCl solution (Lee et al., 2010). Balasubramanian et al. (2011) reported successful extraction of lipids from microalgae up to 77% at 95°C employing continuous microwave system using hexane as solvent. The study shows the feasibility of microwave assisted lipid extraction from microalgae. The authors reported 76 – 77% recovery of oil in 20 – 30 min hold time at 95°C as compared to 43 – 47% for water bath heating. Because of the polar nature, a recovery for ethanol was 36% higher than hexane. The question of environment friendly solvent is still under investigation since the organic solvents commonly used in microalgal extraction such as hexane, are well known for their toxic effects on human health and ecosystem. The volatile nature of these solvents at lower temperature further reduces their contact with

analytes to be extracted and excessive quantities have to be used to ensure contact between the solvent and solute for efficient extraction.

2.8 Other Alternative Methods

More recently, a single-step oil extraction has been introduced by a company with the name OriginOil (www.originoil.com). Little details about the process are available; however the process is reported to involve a combination of ultrasound and electromagnetic pulses to disrupt algal cell walls. Carbon dioxide is then injected into the resulted slurry of algae biomass to lower the pH facilitating separation as the biomass sinks to the bottom and the oil floats to the top (http://www.technologyreview.com/energy/22572; Mercer and Armenta, 2011). The top portion is passed through gravity clarifier to separate the lipids, biomass, and water. The technology is claimed to take a matter of minutes for the whole process.

Another extraction process called "cell milking" involve the extraction of oil from microalgal biomass without damaging the cell walls using non-toxic biocompatible solvents e.g. decane and dodecane (Hejazi et al., 2004b; Mojaat et al., 2008). The solvents suggested have higher hydrophobicity where the effect of the solvent on the membrane is decreased with consequent decrease in extraction efficiency. The triglycerides are supposedly extracted without the loss of cell viability. The cells are supposedly returned to the growth medium for further growth and lipid production. The method needs further investigation to determine the long-term effects of milking on the cell viability and lipid production (Cooney et al., 2009).

CHAPTER 3

OPTIMIZING A CONTINUOUS FLOW LIPID EXTRACTION SYSTEM (CFLES) USED FOR EXTRACTING MICROALGAL LIPIDS

3.1 Introduction

The use of high pressure and temperature with forced flow of solvent is recently adopted method for extraction of chemicals from solid and semi-solid matrices for environmental analysis (Schantz, 2006; Richter et al., 1996; Herrero, 2004). Pressurized liquid extraction (PLE), or pressurized fluid extraction (PFE) and also know by the trade name "Accelerated Solvent Extraction" (ASE) (Dionex, Sunnyvale, CA, USA); was introduced in 1996 (Richter et al., 1996). The advantages of PLE over Soxhlet extraction include less solvent consumption, and less time required for extraction. Pressurized accelerated hot solvent extraction improves the speed and extraction efficiency of lipid. Higher temperature increases the extraction kinetics while high pressure keeps solvents below their boiling point, thereby enabling safe rapid extraction (Macnaughtona et al., 1997; Richter et al., 1996). However, the limitations of PLE are its requirement for specialized instrumentation to achieve relatively high pressures and temperatures. Maintaining excessive temperature and pressure is a cost deriving factor in terms of maintenance and operation. Therefore an optimum temperature and pressure is expected to help lower this cost. Moreover, selection of a suitable extraction solvent is probably the most important step in optimizing PLE for micro-algal extractions as the solvents generally used involve environmental and health implications.

The main disadvantage of an extraction process is the high cost associated with its infrastructure and operation. This study involves a laboratory made continuous flow lipid extraction system (CFLES) to improve the process economics of microalgae oil extraction

simpler and less expensive for ultimate production of biodiesel, nutraceuticals, pharmaceuticals, and recovery of other possible value added bioproducts contained in microalgae. Traditionally, pressurized liquid extraction (PLE) uses extreme temperatures up to 200°C or higher and extreme pressures up to 3000 psi or higher. The current study examined moderate temperatures (80 – 120°C) and moderate pressures (ambient to 500 psi) in the custom-developed CFLES. These are normally workable temperature and pressure parameters for scale-up or industrial applications. Pressurized liquid extraction systems use a specialized sample cartridge filled with an extraction fluid, which statically extract the oils under elevated temperature and pressure. The current CFLES system on the other hand used a continuous flow of solvent through the extraction cell containing biomass.

Microalgal strain has a significant impact on the efficiency of lipid extraction in terms of its cell wall strength, structure, and chemical composition (Cooney et al., 2009). *Nannochloropsis sp.*, a marine microalgae, was selected because of its tougher cell wall and its ability to tolerate a wide range of temperatures. Insoluble and non-hydrolysable biopolymers called algaenans are reported to form a chemically resistant part of the outer cell wall in *Nannochloropsis sp*. (Gelin et al., 1999; Tyson, 1995; Sukenik, 1999, Rodolfi, 2003). Due to high growth rates and lipid contents, *Nannochloropsis sp.* is recognized as a potent renewable resource for production of biofuel (Rodolfi et al., 2009; Brown et al., 2010). Furthermore, to our knowledge, a pressurized liquid extraction system with continuous solvent flow has not been reported for lipid extraction from microalgae. The effect of temperature and pressure on the fatty acids profile of *Nannochloropsis sp.* is reported in more details than previously reported (Sukenik and Carmeli, 1990; Rebolloso-Fuentes et al., 2001; Rodolfi, 2009; Brown et al., 2010). The extraction yield was compared with baseline lipid concentrations in the biomass obtained with conventional Soxhlet extraction using chloroform and ethanol.

3.2 Material and Methods

3.2.1 Microalgae Strain and Culture Condition

Nannochloropsis sp. along with modified Guillards f/2 formula, Micro Algae Grow™, and Crystal Sea® marine mix at 33 g l^{-1} salinity were acquired from Aquatic Ecosystems Inc. (Apopka, FL, USA, cat# LAC1Q, F2A6 and CM2 respectively). The growth chambers were four 10 gal aquarium tanks filled with 3 gal of growth media each. The illumination source for 16/8 hours light/dark was a 400W high pressure sodium light bulb (Figure 3.1). Air was continuously bubbled through the media. Ambient temperature was recorded in the range of 22 to 28°C. Approximately 40 g of wet paste was produced in two weeks (equivalent to 64 mg l^{-1} d^{-1} of wet paste). Water was separated through centrifugation in Thermo IEC, K centrifuge (Needham Heights, MA) at 3500 rpm for 5 min, bringing down the moisture contents to 80%. The paste was then dried at 38°C overnight to contain 30 wt% solids. The biomass was homogenously mixed before extraction.

3.2.2 Conventional Soxhlet Extraction

Soxhlet extraction was performed according to the method mentioned by Luque de Castro and Garcia-Ayuso (1998). Briefly, 3.3 g of algal paste (equivalent to 1 g dry wt.) was placed in a cellulose extraction thimble (Whatman # 2800–338). A co-solvent system based on well-known Bligh and Dyer (1959) method consisted of 50 mL of chloroform, 100 mL of ethanol, and 40 mL of DI water, was used. The extraction was performed for 8h to achieve a complete extraction. The extracts were transferred to a stoppered graduated cylinder and 40 mL of DI water was added. The cylinder was inverted 30 times and allowed to settle for 1h to recover the bottom layer containing lipids and chlorophyll dissolved in chloroform. The chloroform layer was transferred to a 250 mL flask to evaporate the excess chloroform using rotary evaporator (Rotavapor R-210, Buchi Inc.). The final extraction volume was adjusted to 10 mL.

Figure 3.1. Microalgae growth chambers and illumination

3.2.3 Continuous Flow Lipid Extraction System (CFLES)

As given in Figure 3.2, the laboratory made CFLES consisted of an HPLC solvent injection pump (Model 510, Millipore, Milford, MA) capable of delivering liquid in the range of 0.1 to 10 mL min^{-1} at pressure up to 3000 psi. The pump was connected to a stainless steel check-valve, a ball valve (V1), and a 10 feet long, ¼ inch ID copper tubing, coiled inside a temperature controlled oven (Isotemp vacuum oven, Model 285A, Fisher Scientific, USA). The long copper tube facilitates pre-heating the solvent before entering the sample extraction cell, and a pressure gauge. The sample extraction cell was a 3 in long and 3/8 in ID stainless steel tubing (Figure 3.3). The other end of the sample extraction cell opened into ¼ in copper tubing exiting the oven and attached to a stainless steel ball-valve (V3). After V3, the tubing ended in a 250 ml clear glass bottle to hold the liquid extracts exiting CFLES. Flow control through V3 controlled the system pressure. The entire system was fully leak proof at maximum operating pressures and temperatures. The entire system was flushed with clean ethanol before the test runs and in between the sample runs to overcome any carry over.

Approximately 3.3 g of microalgal wet paste (70% moisture) was fed into the sample extraction cell. Both ends of the sampling cell were plugged with a mass of steel wool to filter and contain the microalgal cells inside the extraction cell (Figure 3.3). The extraction cell was connected tightly with the copper tubing using stainless steel nuts, front and back ferules. Valve V2 was open all the time, except during replacement of the extraction cell. Pressure inside the copper tubing and extraction cell was regulated using V3, for a given pump flow.

3.2.4 Solvents Used for CFLES

A co-solvent extraction system consisted of alcohol (ethanol) as a polar solvent and chloroform, a relatively non-polar organic solvent in 1:2 proportions. Fresh solvent flushed through the microalgal cells in CFLES improved the extraction rates. The flow rate of co-solvent was adjusted to 2 mL min^{-1}. All the extractions were performed in triplicate. Starting with ambient temperature and pressure, the extractions were performed at 80°C, 100°C and 120°C temperatures, and 50 psi and 500 psi pressures (Table 3.1). The sample extraction was terminated with a clear solvent draining into the extracts collection bottle.

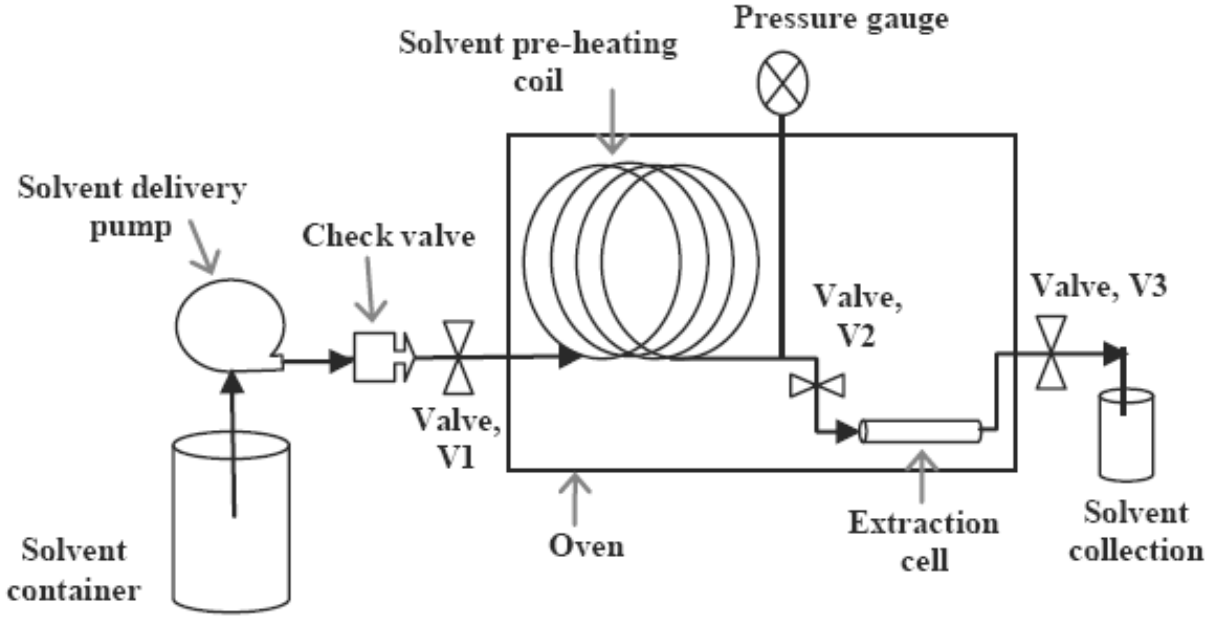


Figure 3.2. Schematic of continuous flow lipid extraction system (CFLES)

Figure 3.3. Sample extraction cell used in continuous flow lipid extraction system (CFLES), along with a steel wool plug/filter.

3.2.5 Post-extraction Process

The extracts were transferred to a graduated cylinder. DI water, approximately equal to the amount of ethanol, was added forming a biphasic system. The top layer, containing water and ethanol, was removed. The bottom layer contained lipids and chlorophyll contents dissolved in chloroform. Final extraction volume was set to 10 mL.

Table 3.1. Temperature and pressure parameters used in continuous flow lipid extraction system (CFLES)

Test name	**Temperature,** ^{o}C	**Pressure,** psi	**Test name**	**Temperature,** ^{o}C	**Pressure,** psi
AmbT,P	Ambient	Ambient	100T,AmbP	100	Ambient
AmbT,50psi	Ambient	50	100T,50psi	100	50
AmbT,500psi	Ambient	500	100T,500psi	100	500
80T,AmbP	80	Ambient	120T,AmbP	120	Ambient
80T,50psi	80	50	120T, 50psi	120	50
80T,500psi	80	500	120T,500psi	120	500

3.2.6 Glycerides Analysis

Mono-, di-, and triglycerides were analyzed using 1 mL of the extracts according to the method mentioned in Balasubramanian et al., (2011). Briefly, 1 mL of the extracts was silyated with 20 µL of N-methyl-N-trimethylsilyltrifluoracetamide (MSTFA) (ThermoScientific catalog# TS-48913) in a 5 mL vial (ASTM D6584). The solution was mixed thoroughly and reacted for 10 min at 70°C in an oven. One µL of the final diluted aliquot was injected into a gas chromatograph (SRI, 8610C, Torrance, CA, USA) equipped with flame ionization detector. A siltek-treated stainless steel capillary column (14 m, 0.53 mm id, 0.16 µm df) with two meter Integra-Gap® built-in retention gap (Restek, MXT-Biodiesel TG w/inl Gap, catalog# 70289) was used for mono-, di-, and tri-glycerides. The initial column oven temperature was 50°C held for 2 min; raised to 380°C at 15°C min^{-1}. Helium was used as a carrier gas at 4mL min^{-1}. Peaksimple software (SRI instruments) was used to quantify peak areas and converted to concentrations using appropriate response factors.

3.2.7 Fatty Acid Methyl Esters (FAMEs) Analysis Using GC/MS

FAMEs were determined using GC/MS by transesterifying 1 mL of the extracts with 15 µL of 2N methoxide solution (11.2g KOH in 100 mL methanol). The sample was centrifuged at 3000 rpm for 2 min to precipitate the free glycerol and chlorophyll. Hexadecanoic acid, 2-hydroxy-, methyl ester (CAS No. 16742-51-1, Indofine Chemicals, NJ, USA, Cat.# 24-1602) was used as an internal standard.

Fatty acid methyl esters were carried out with a Varian 450-GC gas chromatograph (Walnut, CA, USA) with 1179 injector, equipped with Varian 250-MS ion trap mass spectrometer, and Varian CP-8400 autosampler. FAMEs were separated with a Varian FactorFour WAXms column (30 m, 0.25 mm, and 0.25 µm df; Varian catalog# CP9205). The MS electron multiplier voltage was set to 1400V, ionization time of 25,000 µs in electron impact

(EI) mode, with transfer line, ion trap and manifold temperatures set to 250°C, 200°C, and 50°C. The MS was set to scan 50–1000 m/z with an ionizing voltage of 70eV. One µL sample was injected with a split ratio of 20 and injector temperature set to 240°C. The initial temperature of the column oven was set to 100°C, held for 2 min, then raised to 255°C at 12°C min^{-1} held for 7min; Helium was used as a carrier gas at 1 mL min^{-1}. Data acquisition and analysis was performed using Varian MS Workstation version 6.5. The instrument was calibrated using a 37-component standards mix (Supelco No. 18919, Ca, USA) containing C4 – C24 FAMEs (2 to 4% relative concentrations).

3.2.8 Chlorophyll *a* Analysis.

Chlorophyll *a* determination was done according to US EPA method 446.0. The method employs Jeffrey and Humphrey's Trichromatic Equations (1975). The UV-VIS Spectrophotometer (Helios Aquamate, ThermoSpectronic, UK) was calibrated using a chlorophyll standard (MP Biomedicals, OH, USA; Catalog# 210221). Absorbance was measured at 750, 664, 647 and 630 nm. Chlorophyll *a* (mg L^{-1} of extracts) was calculated according to the following equation:

$$Chl_a = 11.85\ (Abs_{664} - Abs_{750}) - 1.54\ (Abs_{647} - Abs_{750}) - 0.08\ (Abs_{630} - Abs_{750}) \quad \ldots\ldots \text{Eq. 3.1}$$

where, Chl_a is the concentration (mg L^{-1}) of chlorophyll *a* in the extracts, converted to mg g^{-1} dw.

3.2.9 Gravimetric Analysis

Total lipids were determined gravimetrically according to Bligh-Dyer modified method (Burja et al., 2007) using the following equation:

$$\text{Total lipids (mg of oil per g of sample)} = [(W_{dry} - W_{dish}) \times V_{ext}] / [V_{dry} \times W_S] \quad \ldots\ldots \text{Eq. 3.2}$$

where, W_{dry} is the weight (mg) of aluminum dish and residues dried at 60°C, W_{dish} is the weight (mg) of empty aluminum dish, V_{ext} is the volume (mL) of final extracts, V_{dry} is the

volume (mL) of extracts transferred to the aluminum dish, W_S is the weight (g) of the sample extracted.

3.2.10 Statistical Analysis.

Analyses of variance (ANOVA) of different treatments and Fisher's protected least significant difference (PLSD) test for pair wise comparison was performed using STATISTICA version 9 software (StatSoft Inc., Tulsa, OK, USA).

3.3 Results and Discussion

3.3.1 Conventional Soxhlet Extraction

Prolonged 8 h lipid extraction from *Nannochloropsis sp.* with conventional Soxhlet apparatus yielded 498.9 mg g^{-1} of oil (49.9% as total bound glycerides), 443 mg g^{-1} (44.3%) of fatty acid methyl esters (FAMEs), and 4.19 mg g^{-1} of chlorophyll *a*. The Soxhlet extraction consumed approximately 150 mL of solvent, which is one of the drawbacks associated with conventional Soxhlet extractions (Luque de Castro and Garcia Ayuso, 1998; Wang and Weller, 2006). A complete extraction was achieved in 8 hr, which was indicated by clarity of solvent filtered through the sample and discoloration of the algal biomass (Rao et al., 2007). Though conventional Soxhlet extraction is widely reported for its more efficient extraction, its longer time required for complete extraction, large volume of solvents wasted, and the high energy requirement for continuous distillation restrict its use at industrial scale (Halim et al., 2011).

3.3.2 Efficiency of the Continuous Flow Lipid Extraction System (CFLES)

Oil contents (as total bound glycerides) extracted from *Nannochloropsis sp.* was 627.5 mg g^{-1} dw (62.8% dry wt.) using CFLES, which was approximately 13% higher ($p<0.05$) than that of the conventional Soxhlet extraction (49.9%) (Figure 3.4). Although these results agree with those previously reported for *Nannochloropsis sp.* to contain 31– 68% oil on dry weight (dry wt.) basis (Rodolfi et al., 2009; Chisti, 2007; Gouveia and Oliveira, 2009; Rebolloso-

Fuentes et al., 2001), only 87% of the CFLES extracted oil (i.e. 546 mg g^{-1} or 54.6% dry wt.) was saponifiable which could be converted into FAMEs. High concentrations may also incorporate analytical errors associated with standards recovery during glycerides analysis using GC-FID instrument (112.3±5.6%). The final results for all analysis including those of the Soxhlet extraction were not corrected for standard's recovery. Many microalgae strains naturally have high lipid content (20–50% dry weight) (Brennan and Owende, 2010; Hu et al., 2008), Soxhlet extraction showed 491.2 mg g^{-1} of triglycerides. CFLES extracted 100% of the triglycerides (494.3 mg g^{-1}) along with additional diglycerides (116.2 mg g^{-1}), which was not extracted with conventional Soxhlet extraction (Table 3.2). This shows significantly higher lipid contents extracted with CFLES, though the excess lipids failed successful esterification into FAMEs with the methylation techniques. This is likely due to the fact that *Nannochloropsis sp.* has a variety of polar and non-saponifiable lipids, including complex phospholipids, glycolipids, and phosphatidylglycerol (Halim et al., 2011; Schnieder and Roessler, 1994). Phospholipids are suggested as a source of catalyst destruction during transesterification (Schnieder and Roessler, 1994) and the phosphorus compounds in the oil do not easily carry over into the methyl esters (Gerpen and Dvorak, 2002).

Non-polar lipids (e.g. sterol esters, glycerides, hydrocarbons and carotenoids) are bound weakly by Van der Waals forces and are relatively easy to extract if in contact with suitable solvents (Enssani, 1990). However, the impermeability of the microalgal cell wall is a physical barrier for solvents to reach the lipids (Bligh and Dyer, 1959). Therefore, combination of chemical and moderate physical processes (100°C/50psi, and the solvents) used in this study was sufficient enough to break the barrier between the solvents and the lipids, unlike pressurized solvent extraction processes, which require extreme pressure and temperature to achieve similar extraction efficiencies. Morrison and Conventry (1989) reported that fatty acids were more

extractable at 100°C as compared to ambient temperature, particularly saturated acids e.g. C16:0 palmitic and C18:0 stearic acid, while using hot propanol–water (3:1, v/v), water saturated butanol, methanol and methanol–water (85:15, v/v).

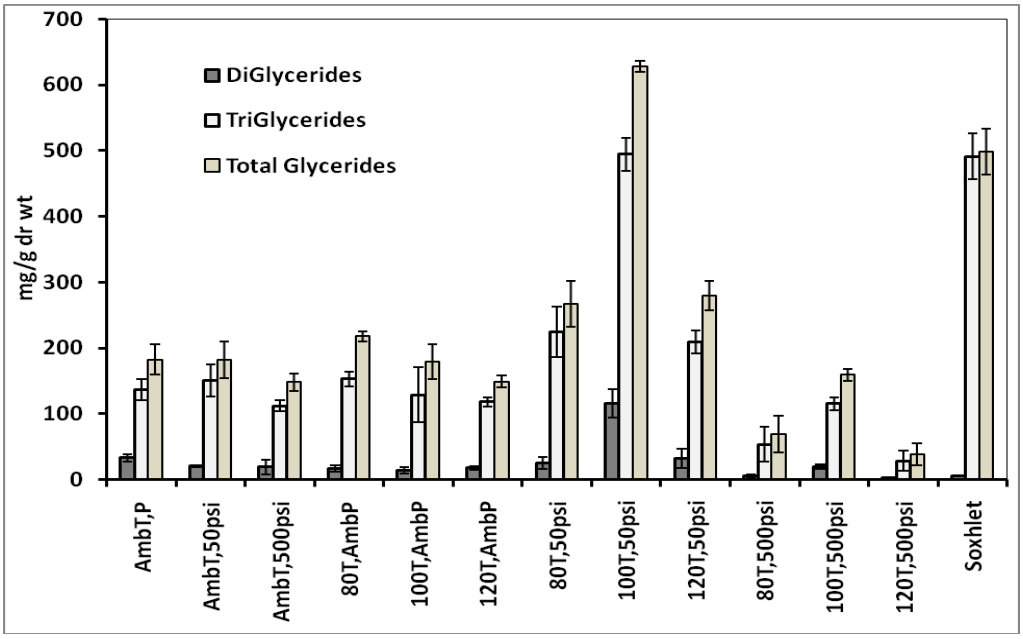


Figure 3.4. Total bound glycerides (mono-, di-, and triglycerides) concentrations (mg g^{-1} dry weight) extracted from *Nannochloropsis sp.* under different temperature and pressure combinations in continuous flow lipid extraction system (CFLES) and Soxhlet extraction (n=3).

The maximum total fatty acid methyl esters (FAME) produced from the total bound glycerides extracted using CFLES was approximately 87% of FAME produced from total glycerides using Soxhlet extraction (Figure 3.5). This maximum yield was achieved at 100°C temperature, 50 psi pressure, and 15 min time compared to Soxhlet's 8 h extraction. The total FAMEs extracted under the aforementioned conditions was 385 mg g^{-1} (38.5% dry wt.) compared to 443 mg g^{-1} (44.3% dry wt.) of the Soxhlet extraction (i.e. 87% efficiency).

Fatty acids, being the main focus for biofuel and nutraceutical value in microalgae, indicated a significant response to an optimum temperature and pressure, with the best CFLES extraction performance comparable to that of the 8 h Soxhlet extraction. The CFLES used only 30 mL of the solvent compared to 150 mL of the Soxhlet (80% less solvent used). Surprisingly,

increasing pressure above 50 psi or temperature above 100°C had no useful effect on the extraction process, a beneficial energy saving consideration.

Extractions at too low or too high pressures had no significant effect unless augmented with temperature (Figure 3.4 and 3.5). Only up to 21% of oil, 17% of which was esterifiable to FAMEs, was extracted at lowest or highest pressures. Once combined with temperature, the extraction of oil, and consequent FAMEs, at lowest pressure (ambient) promoted to 38% and 30%, respectively. An optimum pressure of 50 psi further maximized the extraction of oil and FAMEs yield significantly ($p<0.05$) to 97% and 87%, respectively. Pressure higher than 50 psi did not show any beneficial effect. Temperature around 80°C was not enough to attain maximum yield, while 120°C had deleterious effects on physical and chemical properties of the biomass, including the mass transfer and reduced extraction of lower molecular weight fatty acids (Table 3.3). Temperature above 100°C seems to deteriorate the lipids. For instance, C12:0 and C14:0 fatty acids reduced by 50% at 120°C temperature and 500 psi pressure, compared to that of 100°C and 500 psi. The effect of solvent temperature was complex. As temperature increased, the biomass clumped into a hard cake inside the extraction cell, reducing the solvent diffusion and mass transfer (Figure 3.6). The temperature increase also reduced the solvent's density considerably, thereby reducing the solvent-lipids contact, which in turn offset the lipids volatility, resulting in a net lower lipid mass transfer rates (Halim et al., 2011).

A co-solvent system has a limited carrying capacity (or the lipids have a limited solubility in co-solvent) (Cooney et al., 2009). At a certain point the carrying capacity of the co-solvent was exhausted unless a fresh solvent was either added or re-circulated like that in Soxhlet extraction. In similar fashion, a fresh solvent was flushed continuously through the biomass in the CFLES extraction cell so that the carrying capacity of the solvent was never exhausted, resulting in high yield. Conversely, lower yields reported for PLE (Jaime et al., 2005; Rodriguez-Meizoso et al.,

2008) were attributed to the exhaustion of carrying capacity of the solvent system. In such systems, the extraction cell was filled with biomass and solvent up to the desired pressure. Extraction was performed statically for a certain time. The same solvent along with solute was then removed for further processing and analysis.

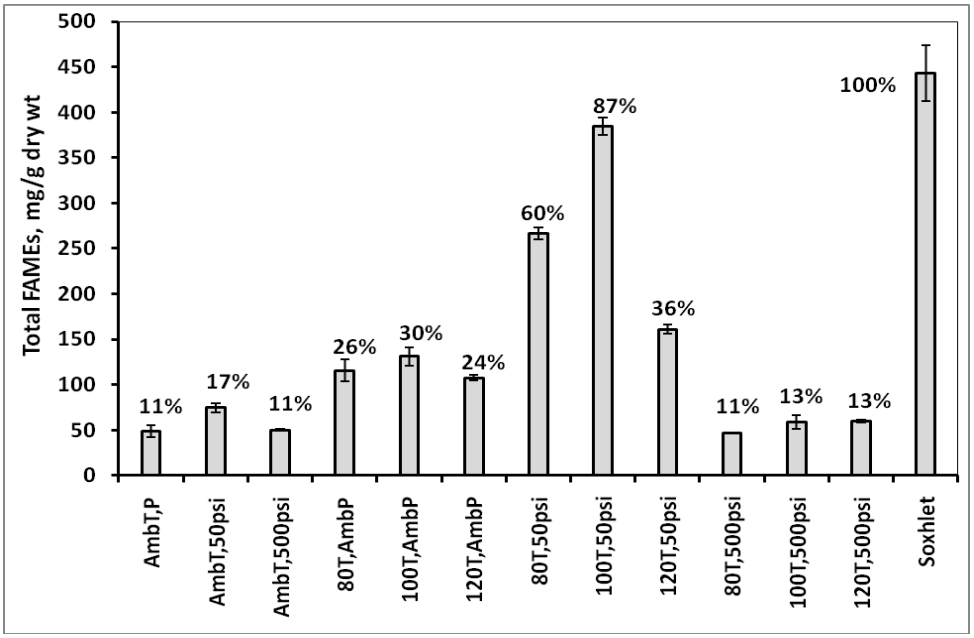


Figure 3.5. Total fatty acid methyl esters (FAMEs) concentrations (mg g^{-1} dry weight) extracted from *Nannochloropsis sp.* under different temperature and pressure combinations in continuous flow lipid extraction system (CFLES) and Soxhlet extraction (n=3).

Fatty acids profile of CFLES extracted lipids showed C18:1 as the major component (~38%) followed by C16:0 (~23%), C12:0 (~12%), and C14:0 (~8.5%) (Appendix A1). The C12:0 has not been reported in *Nannochloropsis sp.* before. Total saturated fatty acids were slightly higher (~54%) than the unsaturated ones (~46%). No significant concentrations of polyunsaturated fatty acids (PUFA), eicosapentaenoic acid (EPA) [C20:5(n-3)], or docosahexaenoic acid (DHA) [C22:6(n-3)] were found in the algal culture, though the strain has been reported for significant production of EPA (Brown et al., 2010; Sukenik, 1999). Fatty acid profile of microalgal species, however, was reported to be a function of its culturing conditions (Rao et al., 2007; Andrich et al., 2005). The Soxhlet extracted lipids were dominated by C16:1

(~21%), C18:ω3 fatty acid (~16%), C12:0 (~15%), C18:1 (12%), and C14:0 (~8%). Fatty acid profile, in agreement with previously reported results (Chisti, 2007; Umdu et al., 2009) indicated the potential of *Nannochloropsis sp.* for biodiesel production.

Figure 3.6. Microalgal biomass extracted at 100°C temperature and 50 psi pressure (R), and 120°C/500psi (L) in the continuous flow lipid extraction system (CFLES).

The CFLES showed a similar significant yield of 229.3 mg g^{-1} dry wt. (22.9%) of oil at 100°C and 50 psi pressure compared to 207.3 mg g^{-1} dry wt. (20.7%) with Soxhlet extraction ($p<0.05$), i.e. 10% higher yield than the Soxhlet extraction when tested with *Botryococcus Braunii* (data not shown). This was a green microalgal species that was known to produce large quantities of triterpenes as opposed to traditional fatty acid triglycerides. FAMEs concentration was similarly higher by 10% (223 mg g^{-1} vs. 201 mg g^{-1}; $p<0.05$). This yield was significantly higher than any of the other temperature and pressure combinations ($p<0.05$).

No specific trend was observed in the ratios of total mono-, di-, or tri-unsaturated fatty acids to the total unsaturated fatty acids. However, the highest fraction of mono-unsaturated fatty acids (94.8%) was observed in the 100°C/50 psi experiment compared to 59.2% in the Soxhlet extracts. This was indicative of the effect of high temperature and pressure on the fatty acid profile of oil. This may be a special concern for certain analytes, including some chemicals of nutraceutical or pharmaceutical significance. Overall, the potential of biodiesel production from microalgae was not jeopardized at this temperature and pressure.

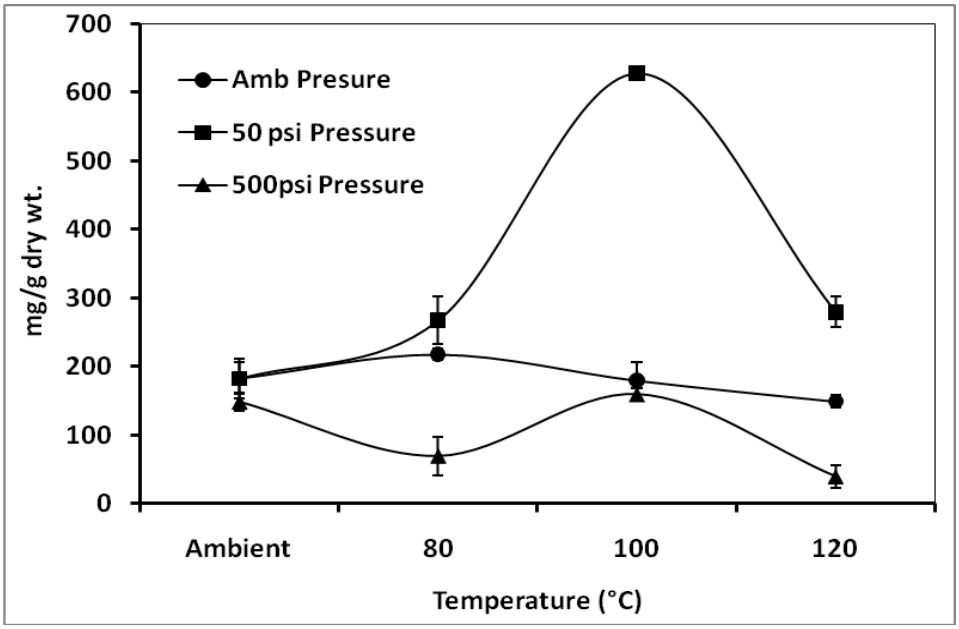
Table 3.2. Total free and bound glycerides (% of dry wt.) extracted from *Nannochloropsis sp.* under different temperature and pressure conditions in CFLES and Soxhlet extraction (n=3)

Test name	Free glycerin	Mono-glycerides	Di-glycerides	Tri-glycerides	Total bound glycerides
AmbT, P	0.29±0.01	1.21±0.2	3.32±0.5	13.72±1.6	18.26±2.2
AmbT, 50psi	1.32±0.5	1.09±0.5	2.09±0.1	15.02±2.4	18.22±2.8
AmbT, 500psi	0.25±0.01	1.64±0.6	1.95±1.1	11.22±0.8	14.82±1.3
80T, AmbP	0.38±0.1	4.76±0.2	1.71±0.4	15.28±1	21.76±0.8
100T, AmbP	2.68±0.3	3.64±1.2	1.39±0.5	12.9±4.1	17.95±2.6
120T, AmbP	0.13±0.01	1.27±0	1.78±0.2	11.83±0.7	14.89±0.8
80T, 50psi	0.34±0.01	1.72±0.2	2.57±0.8	22.43±3.8	26.74±3.4
100T, 50psi	0.97±0.1	1.69±0.2	11.62±2.1	49.43±2.5	62.75±0.8
120T, 50psi	0.3±0.01	3.74±0.3	3.23±1.4	20.95±1.7	27.93±2.2
80T, 500psi	0.06±0.01	0.9±0	0.62±0.2	5.38±2.5	6.91±2.7
100T, 500psi	0.08±0.01	2.42±0.2	1.95±0.3	11.54±0.9	15.92±0.9
120T, 500psi	–	0.68±0.1	0.3±0.1	2.87±1.4	3.88±1.6
Soxhlet	–	0.19±0	0.57±0.1	49.12±3.4	49.89±3.4

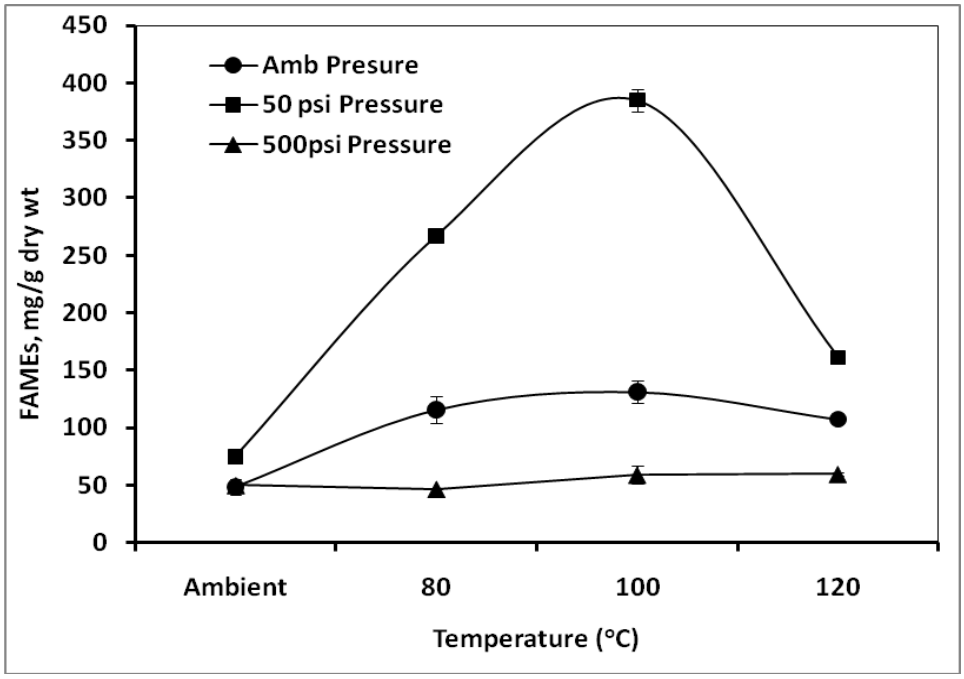
Amb = Ambient; T = Temperature, ^{o}C; P = pressure, psi; "–" = Non detect

3.3.3 Effect of Temperature and Pressure

As shown in Figure 3.7, the extraction performance increased from ambient to 100°C temperature and decreased with further increase. Similarly, the extraction performance increased from ambient to 50 psi pressure and decreased with further increase. Similar trends were observed for FAMEs, total bound glycerol, and gravimetric lipids contents.



A



B

Figure 3.7. Effect of temperature and pressure on: (A) lipids as total glycerides extracted from *Nannochloropsis sp.* in continuous flow lipid extraction system (CFLES), and (B) lipids transesterified into fatty acid methyl esters (FAMEs).

Extraction at 120°C resulted in a hardened biomass which stayed green at the end of the extraction process (Figure 3.6). A part of the biomass was also seen burnt. Similar results were observed at 150°C temperature and 1000 psi pressure (data not shown). On the other hand,

microalgal cells turned white at the end of extraction at 100°C, indicating an efficient or complete extraction (Eroglua and Melis, 2010), as was observed in Soxhlet extraction. Extracted biomass at the end of 80°C was light green indicating incomplete extraction.

This study has demonstrated that residual water (~70%) in the biomass did not affect the extraction performance. Water was reported to aid extraction through its swelling of the cellular matrix and its natural role as a polar co-solvent (Pourmortazavi and Hajimirsadeghi, 2007; Schwartzberg, 1997). This was important since (1) drying biomass before extraction has economic implications for microalgae as biodiesel feedstock (2) industrial-scale implementation of the extraction system is more economical with wet paste compared to dried biomass.

The solvents in the copper tubing and sample cell created a closed environment. Once vaporized at high temperatures and pressures, the solvent forced its way into the microalgal cells, thereby, increasing the heat and mass transfer along with disruption of cell wall. Increase in temperature also increased the diffusivity of the solvent and enhances the interaction between the solvent and the solute in the complex cellular matrix (Krichnavaruk et al., 2008). Diffusion rates have been reported to increase roughly 2–10 folds upon increasing the temperature from 25 to 150°C (Richter et al., 1996; Perry et al., 1984). Soxhlet extraction on the other hand was performed at the boiling point of the solvent (Richter et al., 1996).

3.3.4 Effect of Higher Temperature on Fatty Acids Profile

Changes in fatty acid composition at high temperature have been reported previously (Tyagi and Vasishtha, 1996). They reported a significant decrease in unsaturated fatty acids with temperature during frying oil. Tri-unsaturated fatty acids (trienes) deteriorated faster than the di-unsaturated (dienes), which in turn deteriorated faster than mono-unsaturated (monoenes). The concentration of saturated fatty acids was reported increasing with temperature simultaneously. We found similar results while heating soybean oil at 100°C for one hour in a Rancimat

oxidation stability test (data not shown), wherein linolenic acid methyl ester (C18:3ω-3) reduced by 98%, C18:2 reduced by 63%, and C18:1 reduced by 36%. The γ-linolenic acid methyl esters (C18:3ω-6) reduced by 27%. Almost similar results were observed here in the CFLES (Figure 3.8). High concentration was seen for C18:1 compared to C18:2 and C18:3 contrary to that of Soxhlet extraction. Generally metals like nickel are used as a catalyst in hydrogenation of oil (Fernandez and Tonetto, 2007). Copper also works as a catalyst for hydrogenation of oil, which has a much higher preference for linolenic acid (C18:3) and is further accelerated with increasing temperature (Coenen, 1976). Since copper tubing and high temperatures were used in this study, hydrogenation of the double bonds may have occurred during the extraction process.

3.3.5 Gravimetric Lipids and Chlorophyll *a*

Gravimetric lipids concentration indicated similar trends as observed for bound glycerol and FAMEs (Figure 3.9). Lipids concentration recovered at 100°C/50psi (662±14 mg g^{-1}) were not significantly different ($p>0.05$) than that in the Soxhlet extracts (682±22 mg g^{-1}). None of the other temperature and pressure combinations had yields comparable to these two extractions ($p<0.05$).

Significantly higher concentrations of chlorophyll *a* were extracted at 120°C temperature and ambient pressure than the 100°C and 50 psi or the Soxhlet ($p<0.05$) in *Nannochloropsis sp.* (Figure 3.10). Results showed that high Chlorophyll *a* extracted were not associated with high yields of oil or FAMEs, or even the gravimetric lipid contents. Gravimetric lipids at 120°C temperature and ambient pressure were 157±6 mg g^{-1}, which was significantly less than that of the 100°C/50 psi or Soxhlet extractions ($p<0.05$). The chlorophyll *a* contents hence do not indicate any relationship with the lipid contents extracted at a specific temperature or pressure. Degradation of chlorophyll may occur at a high temperature. For instance, pheophytins as

degradation products of chlorophylls have been reported at 200°C (Rodriguez-Meizoso et al., 2008).

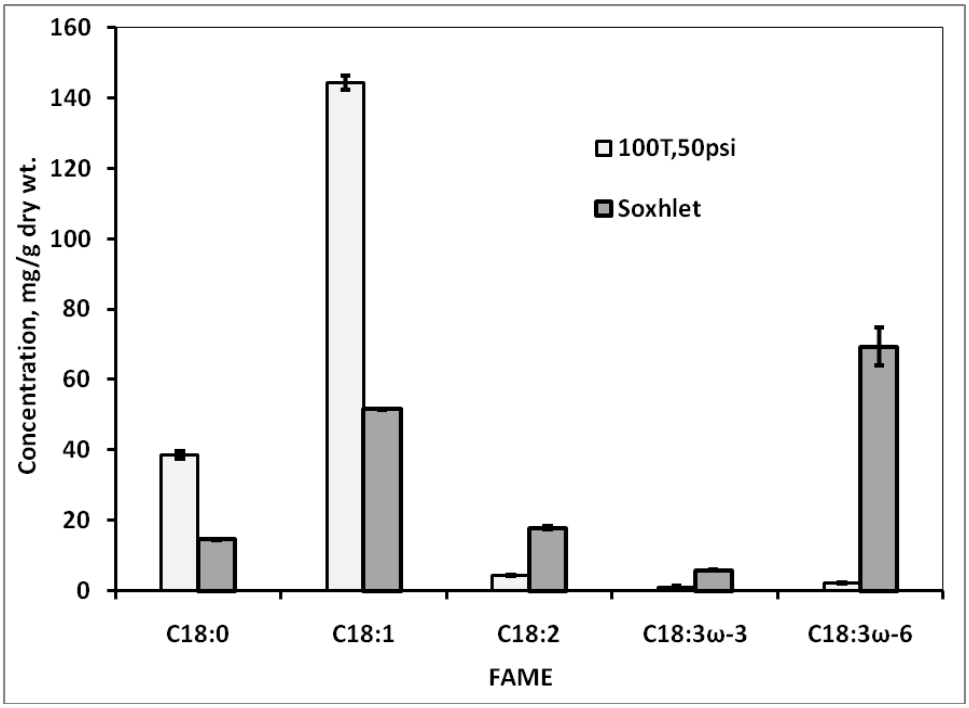


Figure 3.8. Concentrations of saturated and unsaturated fatty acid components of C18 extracted from *Nannochloropsis sp.* at 100°C temperature and 50 psi pressure in continuous flow lipid extraction system (CFLES) compared to that of the Soxhlet extraction.

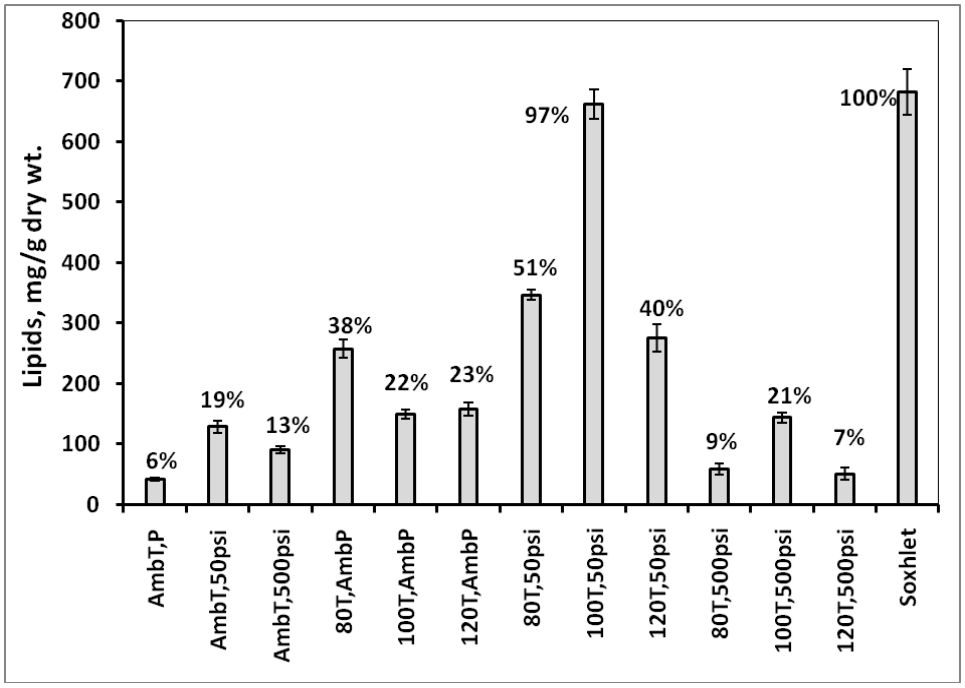


Figure 3.9. Gravimetric lipid concentrations extracted from *Nannochloropsis sp.* using continuous flow lipid extraction system (CFLES) and Soxhlet extraction**.**

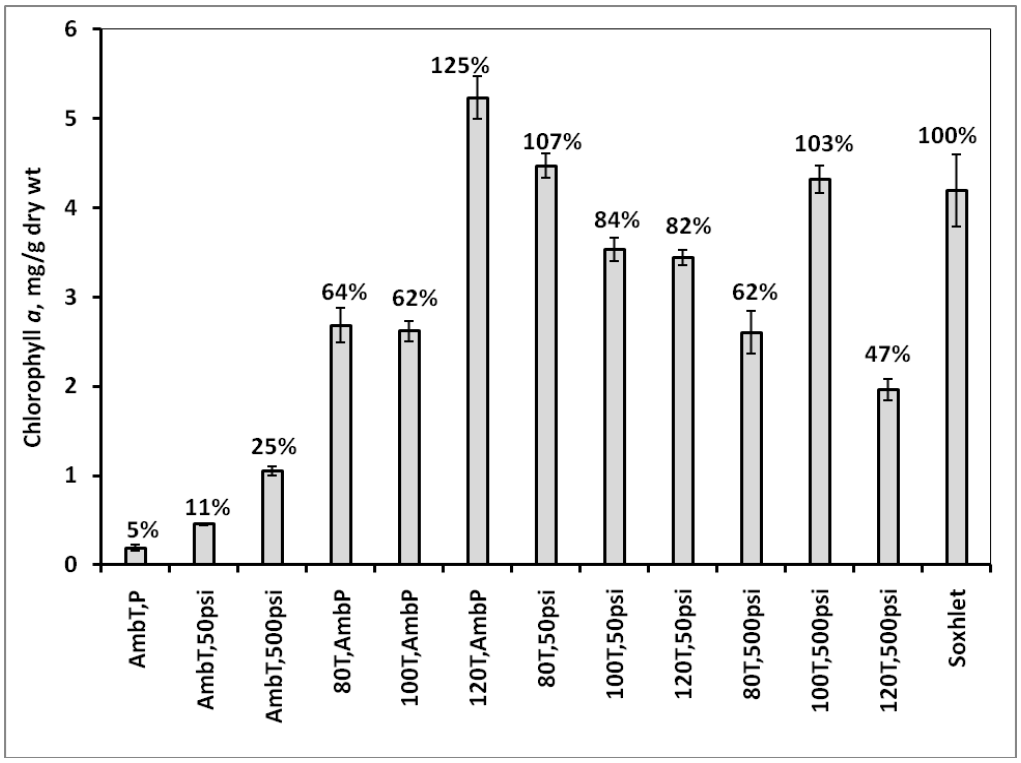


Figure 3.10. Chlorophyll *a* contents extracted from *Nannochloropsis sp*. using continuous flow lipid extraction system (CFLES) and Soxhlet extraction.

The microalgal lipid extracts had a dark green color due to chlorophyll presence. In order to make it a viable feedstock for biodiesel, further refining to remove these unusable constituents would be necessary. The transesterification process in the current study precipitated significant concentrations of these constituents. Various studies have been conducted to effectively remove chlorophyll from oil (Bahmaei et al., 2005).

3.3.6 Energy Efficiency and Energy Savings

The cost of extraction (both energy and solvent) is anticipated to be significantly lower for CFLES. Although the true cost savings can only be assessed at an industrial plant level, preliminary calculations on the tested lab-scale systems indicated an energy consumption of 0.3 kWh for the CFLES (0.25 hr, 1200W of solvent pumping and heating, continuous power consumption) and 8.64kWh for Soxhlet extraction (8 h of 1200W heating and condensing, assuming a heater on time of 90%), which corresponds to approximately 96.5% energy savings.

Dulong formula (Eq. 3.3) was used to estimate the heating value of the dry microalgae and extracted oil.

$$\text{Heating value (MJ kg}^{-1}) = \frac{33.5\times wt\%C}{100} + \frac{142.3\times wt\%H}{100} - \frac{15.4\times wt\%O}{100} \quad \text{…….. Eq. 3.3}$$

where C, H, and O are the weight percentages of carbon, hydrogen, and oxygen, respectively. The elemental composition and the heating value for microalgae feedstock and bio-oils are given in Table 3.3. The heating value for bio-oils (~33.6 MJ kg^{-1}) was significantly lower than that of petroleum crude oil (43MJ kg^{-1}) and biodiesel (~41MJ kg^{-1}) but significantly higher than that of the dry microalgal feedstock (~21.06 MJ kg-1). Energy efficiency calculated as:

{(heating value of product – energy input to process)/heating value of starting material} Eq. 3.4

was found to be 48.9% for the CFLES. Based on the heating values per 100 g of dry feedstock, energy recovered from algae was approximately 100% using CFLES compared to 79% of the Soxhlet extraction (Figure 3.11). The calculations were based on 62.8% and 49.9% dry wt. oil contents extracted with CFLES and Soxhlet respectively.

Table 3.3. Elemental composition of bio-oil and *Nannochloropsis sp.* feedstock

	C %	H %	N %	O % *	High heating value MJ kg^{-1}
Dry feed	48.74±0.37	7.34±0.07	6.9±0.08	37.03±0.4	21.06±0.1
Bio-oil	67.95±0.46	9.85±0.05	1.23±0.3	20.97±0.4	33.61±0.3

*Oxygen contents were determination by subtracting the sum of C, H, and N from 100

The solvent usage for the CFLES was also 80% less when compared to Soxhlet extraction without solvent recycling. Significant time and labor savings are also anticipated. Despite these promising figures, it has to be kept in mind that these savings may or may not reflect the true economics of continuous flow, industrial-scale lipid extraction systems.

The optimum temperature and pressure of 100^{o}C and 50 psi respectively used in the CFLES readily extracted the microalgal oil than is possible at extreme (low or high) temperatures and pressures. The extraction efficiency is higher than most of the extraction methods reported previously for microalgae including supercritical CO_2 extraction, thermochemical and hydrothermal liquefaction, pressurized liquid extraction, and microwave assisted extraction. The operating temperature and pressure are workable for scaled up continuous CFLES if environment and health friendly solvents are used.

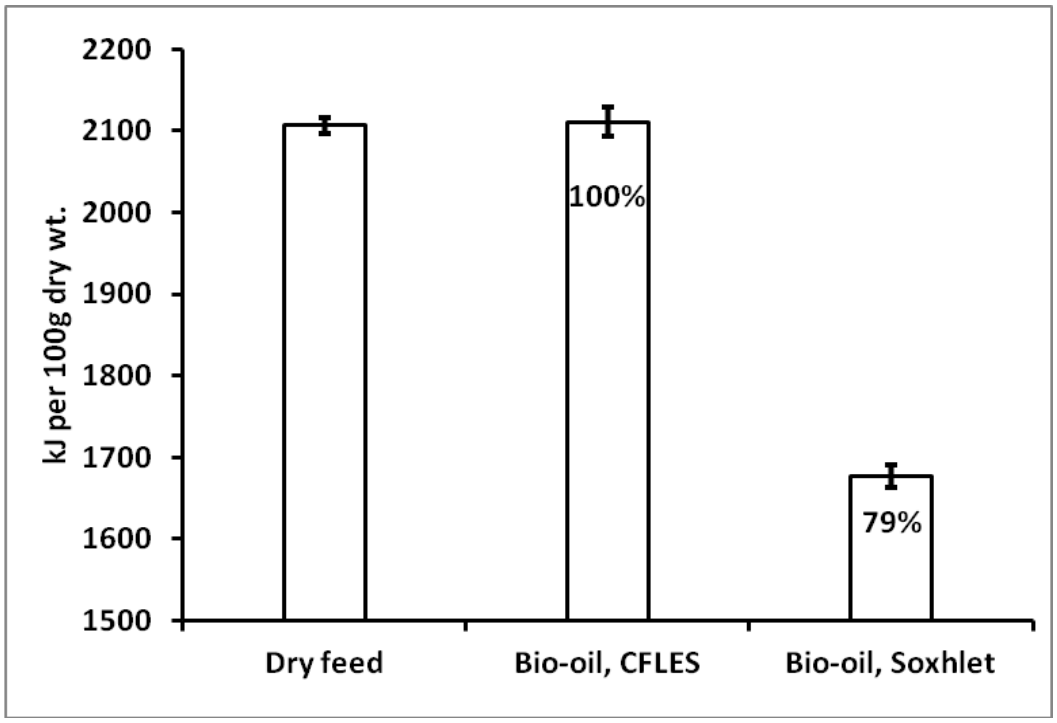


Figure 3.11. High heating values (HHVs) for dry microalgae feedstock and bio-oil extracted with continuous flow lipid extraction system (CFLES) and Soxhlet. Percent values in the bars shows energy recovered compared to that of the dry feed.

CHAPTER 4

MICROWAVE ASSISTED LIPID EXTRACTION FROM MICROALGAE USING BIODIESEL AS CO-SOLVENT

4.1 Introduction

Extraction of oil from microalgal cells is an important and costly procedure, which often involves the use of toxic solvents. The use of solvent extraction requires extra energy input to recover the solvents, and it has the potential to contaminate the algae solids, thereby restricting options for their end use. The contact between the cellular material to be extracted and the solvent can be determinant to the amount of extracted products.

Several procedures have been developed to extract lipids from microalgal biomass e.g. solvent extraction using Soxhlet (Bligh and Dyer, 1959; Lee et al., 2010), Supercritical CO_2 Extraction (Mendes et al., 1995; Valderrama et al., 2003), thermochemical liquefaction (Aresta et al., 2005; Minowa et al., 1995; and Sawayama et al., 1995), pressurized liquid extraction (Herrero et al., 2004; Jaime et al., 2005; Rodríguez-Meizoso et al., 2008). Two of the latest methods, ultrasound assisted extraction (UAE) and microwave assisted extraction (MAE), are reported for efficient extraction, shorter extraction time, increased yield and quality of extracts (Cravotto et al., 2008, Balasubramanian et al., 2011).

Microwave heating (MW) is a non-contact heat source which heats the whole sample volume simultaneously as compared to conductive heating. After being reported for extraction of chemicals from environmental matrices (Freyburger, et al., 1988; Loque de Castro et al., (1998); Priego-Capote and Luque de Castro, 2005. Virot et al., 2007), microwave assisted extraction has been used for efficient extraction of lipids from microalgae using conventional solvents (Balasubramanian et al., 2011). In conventional solvent extraction, mass transfer occurs from the

inside to the outside while heat transfer occurs from the outside to the inside. In case of microwave assisted solvent extraction, mass and heat transport occurs from the inside of the extracted material to the bulk solvent (Virot et al., 2008). Extraction efficiency is increased with increase in moisture contents in the biomass (Virot et al., 2007).

This study investigated the use of an environment friendly solvent which has the least potential of toxicity to human health and ecosystem. One of such solvents is biodiesel (methyl soyate; monoalkyl esters of fatty acids). Biodiesel is rapidly biodegradable and non-toxic solvent. Solvency power of biodiesel has been proved successfully in recent studies (Hu et al., 2004; Spear et al., 2007; Salehpour et al., 2009; Knothe and Steidley, 2011). Biodiesel has been reported as promising industrial solvent, cleaning and degreasing agent, resin cleaning and removal as well as cleaning up of oil spills (Hu et al., 2004; Wildes, 2002; Miller and Mudge, 1997; Von Wedel, 2001). Studies indicate the good solvency potential of biodiesel (Wildes, 2002, Hu et al., 2004; Srinivas, et al., 2009; Salehpour et al., 2009) due to its partial polar behavior (Asap and Augustin, 1986; March, 1992). The use of biodiesel as co-solvent reduces one important step of separating the extracted lipids for subsequent transesterification reaction whereas ethanol is used as one of the reactants. Use of biodiesel as co-solvent for transesterification reaction is reported to increase the reaction rate for solid acid catalyst system (Lam and Lee, 2010). The solvent extraction system selected coupled with microwave assisted extraction, therefore, can drastically reduce the cost, time and labor.

Very limited literature is available on the solvent potential of biodiesel. To our knowledge, biodiesel as solvent for any extraction purpose has not been reported. Therefore this is the first of its kind study to report the solvent potential of biodiesel in the extraction chemistry. Biodiesel as co-solvent is expected to improve the process economics of microalgal oil extraction

since the main disadvantage of an extraction process is high cost associated with its infrastructure and operation.

4.2 Material and Methods

4.2.1 Microalgae Strain and Culture Condition

Microalgae *Nannochloropsis sp.* (Aquatic Ecosystems Inc., Apopka, FL, USA; catalog# LAC1Q) was cultured in 10 gal aquarium tanks illuminated for 16/8 hours light/dark using a 400W high pressure sodium light bulb at 22 – 28°C temperature. Culture media was a modified Guillards f/2 formula Micro Algae Grow™, and Crystal Sea® marine mix at 33 g l^{-1} salinity (Aquatic Ecosystem Inc., Apopka, FL, USA; product# F2A6, and CM2 respectively). Biomass was prepared as mentioned in Section 3.2.1.

4.2.2 Biodiesel Production

Methyl soyate was produced from commercially available soybean oil. A base-catalyzed transesterification reaction was carried out at 60°C using KOH as a catalyst at a concentration of 1.8 wt % of vegetable oil and methanol (6 mol), 100% excess, to obtain high conversion of oil into esters (Hu et al., 2004). Transesterification was repeated twice to insure complete conversion of the oil. Methyl esters were separated and washed with distilled water to remove the catalyst and unreacted methanol until the pH of wash water was around 7. The esters were dried at 60°C for 2 h. The final product was tested to meet ASTM D6751 specifications for total and free glycerides.

4.2.3 Conventional Soxhlet Extraction

Conventional Soxhlet extraction was performed according to the method mentioned by Luque de Castro and Garcia-Ayuso (1998) and also mentioned in Section 3.2.2. Briefly, approximately 3.3 g of algal paste which is equivalent to 1 g dry wt., was extracted vigorously in

the Soxhlet apparatus for 8 h. The extracts were treated according to Section 3.2.2. The final extraction volume was adjusted to 10 milliliter.

4.2.4 Kauri-butanol (KB) Values

The kauri-butanol test is primarily used for evaluating solvent power of hydrocarbon solvents. This test provides a scaleless index called KB value. The higher the KB value, the more aggressive or active the solvent is to dissolve or clean certain materials. KB values for oxygenated compounds including biodiesel are recently reported by Konthe and Steidley (2011). The reported the KB values for methyl soyate and methyl oleate in the range of 55 – 60. The KB values for 100% biodiesel and its blends with ethanol were determined according to ASTM method D1133-09.

4.2.5 Microwave Assisted Extraction

Microwave Accelerated Reaction System (MARSXpress, CEM Corporation, Matthews, NC) provided with 40-vessels turntable was used (Figure 4.1). The fluorocarbon vessels were supplied with single ported Teflon caps equipped with pressure relief valve to regulate excessive pressure. The system used 1.2 kW of microwave energy at a frequency of 2.45 GHz. The instrument was pre-calibrated according to manufacturer specifications. Method parameters included 5 min ramp to a desired temperature, then hold for 15 min, followed by 30 min cool-down step. Approximately 3.3 g of wet paste was loaded in to the vessels. Triplicate samples were extracted at 80°C, 100°C, and 120°C using BD40 and BD20.

4.2.6 Solvent System

Methyl soyate was used as co-solvent with ethanol in 20% and 40% proportions (BD20 and BD40 respectively). Twenty milliliter of the solvent and 50 µl of tricosanoic acid methyl ester (C23:0) surrogate standard (Sigma-Aldrich, cat#T9900) were added to the sample; thoroughly mixed before irradiation. MAE was also performed using conventional solvents

chloroform and ethanol (1:2 v/v). Final results were corrected for recovery of tricosanoic acid methyl ester and the baseline methyl soyate, as well as total bound glycerides concentrations.

Figure 4.1. Microwave Accelerated Reaction System used for extraction of microalgal lipids using biodiesel as co-solvent (source: MARSXpress, CEM Corporation, Matthews, NC)

4.2.7 Post-extraction Process

The extracts were transferred to a 50 mL centrifuge tubes. Five milliliter of DI water and 5 mL of hexane was added to obtain a biphasic system. Extracts were mixed followed by centrifugation at 3500 rpm for 5 min. The bottom layer, containing water and ethanol, was removed while the top layer contained extracted lipids, biodiesel, and chlorophyll contents dissolved in hexane. Final extraction volume was set to 10 mL using hexane for subsequent analysis.

4.2.8 Glycerides Analysis

Total bound glycerol (mono-, di-, and triglycerides) were analyzed using 1 mL of the extracts according to the method mentioned in Section 3.2.6.

4.2.9 Fatty Acid Methyl Esters (FAMEs) Analysis Using GC/MS

One mL sample aliquot of the 10 mL extracts was used for fatty acids profile. The sample was diluted to 2 mL with hexane in a screw cap tube (Lee et al., 2010). The sample was transesterified with 5 µL of 2N methoxide solution (11.2g KOH in 100 mL methanol) .The sample was homogenized thoroughly in a vertex for 30 s and allowed to react in 10min. The sample was treated two more times with 5 µL of methoxide solution to ensure complete transesterification of the lipids. The sample was centrifuged at 3000 rpm for 2 min to precipitate the free glycerol and chlorophyll. Exactly 20 µL of the supernatant layer was diluted to 1 mL in hexane for fatty acid profile using GC/MS. Hexadecanoic acid, 2-hydroxy-, methyl ester (CAS No. 16742-51-1, Indofine Chemicals, NJ, USA catalog# 24-1602) was used as internal standard.

Fatty acid methyl esters were carried out with a Varian 450-GC gas chromatograph (Walnut, CA, USA) with 1179 injector, equipped with Varian 250-MS ion trap mass spectrometer, and Varian CP-8400 autosampler. Complete GC/MS instrument information, instrument conditions, and temperature program are given in Section 3.2.7.

4.2.10 Chlorophyll *a* Analysis

Chlorophyll *a* determination was done according to Section 3.2.8 using US EPA method 446.0, using Jeffrey and Humphrey's Trichromatic Equations (1975).

4.2.11 Scanning Electron Microscope (SEM) Imagery

SEM images of the biomass were taken using Scanning Electron Microscope (Joel, JSM-6610LV; Tokyo, Japan) available at the Socolofsky Microscopy Center, Department of Biological Sciences, Louisiana State University, Baton Rouge, LA. The method used is mentioned in Balasubramanian et al. (2011). Briefly, 5 mL of the cell suspension (before or after extraction) was fixed for 1 h with 5 ml of 4% glutaraldehyde and 2% formaldehyde solution in 0.2 M cacodylate buffer (pH 7.2). One mL of the mixture was diluted with 9 mL of 2%

glutaraldehyde, 1% formaldehyde in 0.1 M cacodylate buffer solution. The solution was 5 lm pore polycarbonate filter and fixed for an additional 1 h. The filter membrane was rinsed with 0.1 M cacodylate buffer followed by DI water and then dehydrated in ethanol. The membrane was dried with liquid CO_2 in a Denton DCP-1 critical point dryer, mounted on aluminum SEM stubs, coated with gold:palladium (60:40) in an Edwards S150 (Crawley, England) sputter coater and imaged with JSM-6610 (JEOL Ltd., Japan) high vacuum mode SEM (Balasubramanian et al., 2011).

4.3 Results and Discussion

Lipid extraction is usually performed with solvents like *n*-hexane, a non-polar solvent not absorbing microwave. Methyl esters used as a co-solvent with ethanol readily absorb microwave energy to develop hot solvent in contact with extracting materials which are expected to improve the extraction process. The presence of the ester linkage imparts partial polarity to the FAME molecule which helps to offset the high volatility during microwave irradiation. Temperature of 100% biodiesel irradiated in the closed MAE vessel for 5 min hardly reached to 100°C which means partial polarity imparts partial absorption of microwave heat energy. However, as a co-solvent with ethanol the temperature reaches to the desired temperature within a matter of seconds (Terigar et al., 2010). Used as such, the good advantage of the non-volatile nature of biodiesel is its capacity to hold conductive heat while in constant contact with biomass. Biodiesel readily dissolves the neutral lipids extracted. In traditional extraction, on the other hand, a hot solvent is passed through solids/biomass several times where the extraction efficiency is set by the diffusion rates which increase with temperature (Cravotto et al., 2008) though the temperature does not significantly exceed the solvent's boiling point. For instance, temperature of traditional solvents in the extraction chamber of Soxhlet apparatus was reported 63 – 65°C for hexane (boiling point 69°C) and 73 – 75°C for ethanol (boiling point 78°C) (Balasubramanian et

al., 2011). On the other hand, solvents in the closed vessels supplied with the MARSXpress microwave are expected to boil at temperature higher than normal because the vessels are equipped with pressure release valves to maintain pressure up to 200 psi. The high temperature and pressure in microwave assisted extractions could further enhance the extraction efficiency of the MAE. Ethanol for example will boil at 100°C with 32 psi pressure, and 120°C under 60 psi pressure. This property helps maintain considerable concentrations of solvents especially ethanol available to biomass for subsequent heat and mass transfer.

4.3.1 Conventional Soxhlet Extraction

Approximately 49% dry wt. of oil (total bound glycerols) was recovered from *Nannochloropsis sp.* using 8 h Soxhlet extraction which agrees with previously reported results (Chisti, 2007) (Table 4.1). Approximately 48% of the lipids (i.e. 98% of the extracted lipids) was converted into biodiesel (FAMEs) dominated by saturated fatty acids (60%). Dominant fatty acid chains included those of C16s and C18s; a distribution typical of most green algae (Volkman et al., 1989). Significant concentrations of C12:0 (dodecanoic acid) were also observed in the extracts.

4.3.2 Microwave Assisted Extraction Using Chloroform and Ethanol Co-solvents

Using chloroform and ethanol as a co-solvent system in microwave assisted extraction (MAE) yielded approximately 53% dry wt. of lipids at 120°C comparable to that of the Soxhlet extraction ($p>0.05$). The co-solvent extracted approximately 45% dry wt. of lipids (93% that of the Soxhlet) at 100°C; significantly lower than that of the MAE at 120°C ($p<0.05$). The efficiency is comparable to that previously reported (Balasubramanian et al., 2011) recovering 77% of the total oil from microalgae *Scenedesmus obliquus* using continuous microwave system at 95°C using hexane. The difference, however, is that the current study used a closed vessel system compared to continuous microwave system. Current study indicated a positive role of

temperature in MAE from 80°C to 120°C when compared to Soxhlet extraction (Figure 4.2). MAE extraction efficiency dropped to 32% at 80°C; a temperature close to ethanol's boiling point of 78°C.

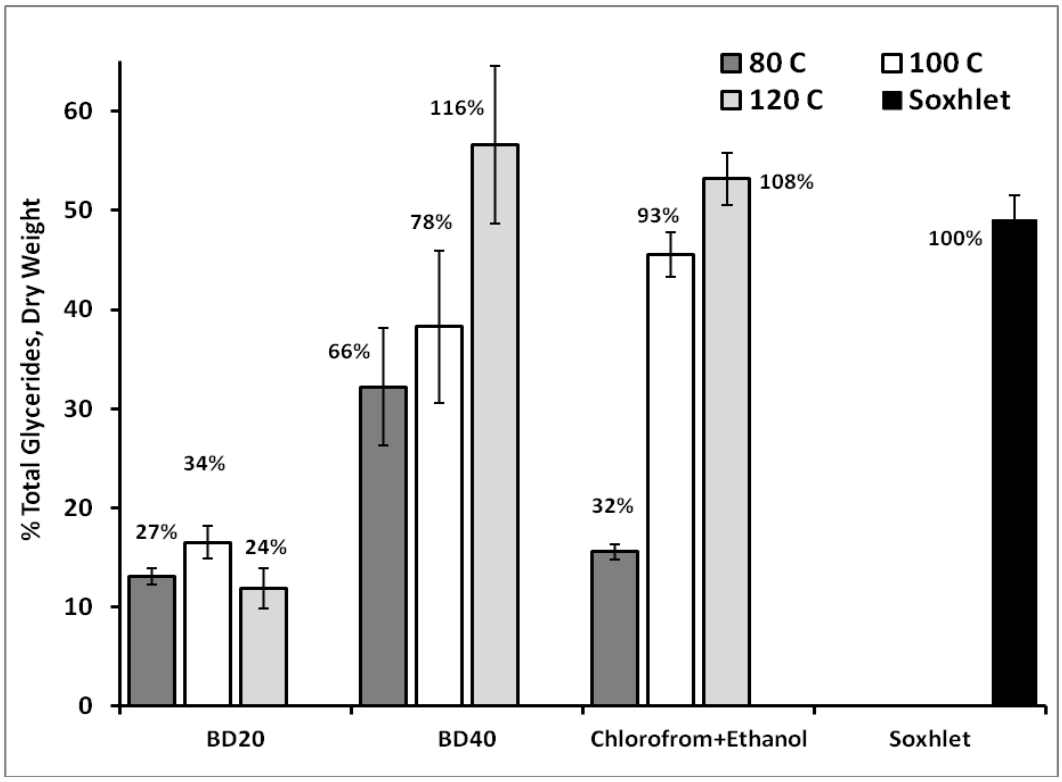


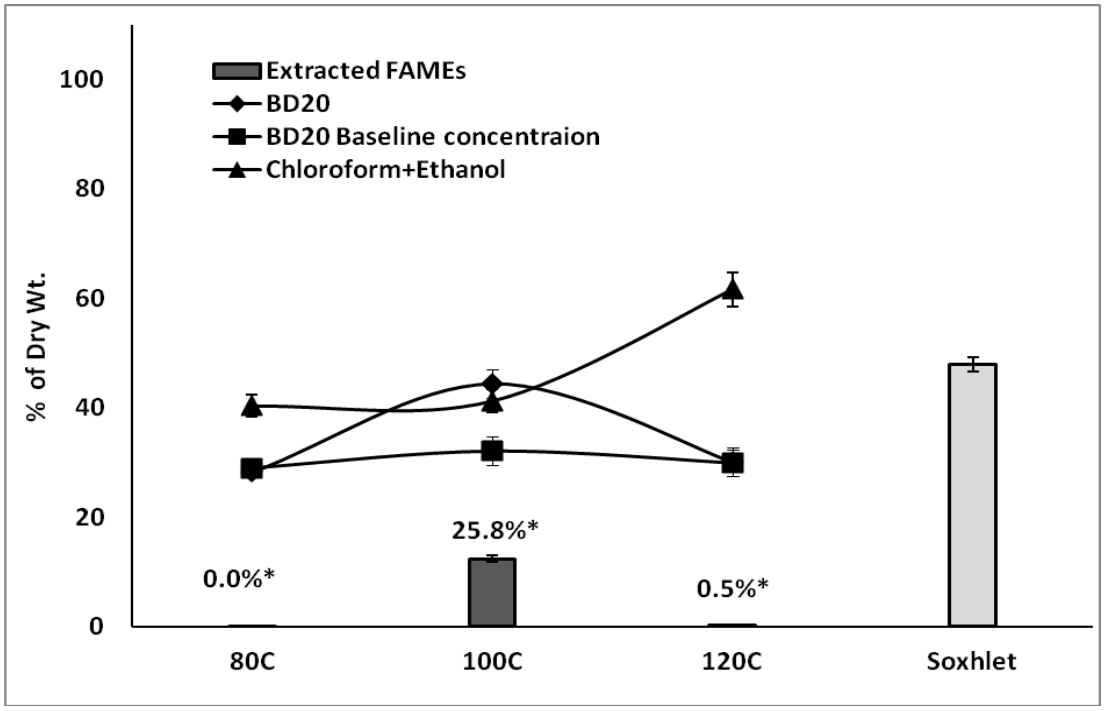
Figure 4.2. Percent of bound glycerides (sum of mono-, di-, and triglycerieds) extracted with BD20 and BD40 (20% and 40% of biodiesel respectively in ethanol) and chloroform with ethanol using microwave and conventional Soxhlet extraction (baseline concentrations have been substracted). Percent values indicate efficiency compared to that of Soxhlet extraction.

FAMEs concentrations indicated similarly higher efficiency of chloroform and ethanol at higher temperatures in the MAE (Figure 4.3). High levels of free fatty acids and free glycerols were noticed at higher temperature. Fatty acids profile of chloroform and ethanol extracts was found similar to that of the Soxhlet extracts with the exception that the concentrations of more volatile fatty acid chains (C12 to C14) were found decreased with increase in temperature as would be expected. Extraction of longer chain fatty acids on the other hand increased with increased temperature.

High concentrations of FAMEs were extracted at 100°C using BD20 (25.8%) compared to the 80°C and 120°C (Figure 4.3). BD40 has similar higher efficiency (64.9%) at 100°C. However, significant concentrations of free fatty acids were also noted in 100°C and 120°C using BD40 as compared to 80°C. Microalgae are reported for significant concentration of free fatty acids which further increase with the extraction procedure employed (Dunston et al., 1994; Pernet and Tremblay, 2003).

Table 4.1. Lipid oil extracted with BD20, BD40 (20% and 40% of biodiesel respectively in ethanol) and chloroform with ethanol using microwave and conventional Soxhlet extraction (% of dry wt.).

	Microwave assisted extraction using biodiesel and ethanol				Soxhlet extraction
Analytes	Temperature	BD20	BD40	Chloroform +Ethanol	
Triglycerides	80°C	5.4±0.6	11.9±2.1	5.5±0.2	25.54±1.2
	100°C	6.8±0.4	15±1.1	20.8±1	
	120C	5.7±0.3	20.6±2.6	30.4±1.5	
Diglycerides	80°C	0.7±0.4	0.7±0.9	3.7±0.1	14.9±0.7
	100°C	0±0	0.2±0	3.1±0.1	
	120°C	0.9±0.9	4.2±0.7	2.5±0.1	
Monoglycerides	80°C	6.8±1	19.4±3.3	6.2±0.3	8.6±0.4
	100°C	9.7±1.9	22.9±6.6	21.5±1	
	120°C	5.2±1.4	31.6±4.7	20.1±1	
Total Glycerides	80°C	13.1±0.8	32.2±5.9	15.5±0.7	49±2.4
	100°C	16.5±1.6	38.2±7.7	45.5±2.2	
	120°C	11.8±2	56.6±7.9	53.1±2.6	



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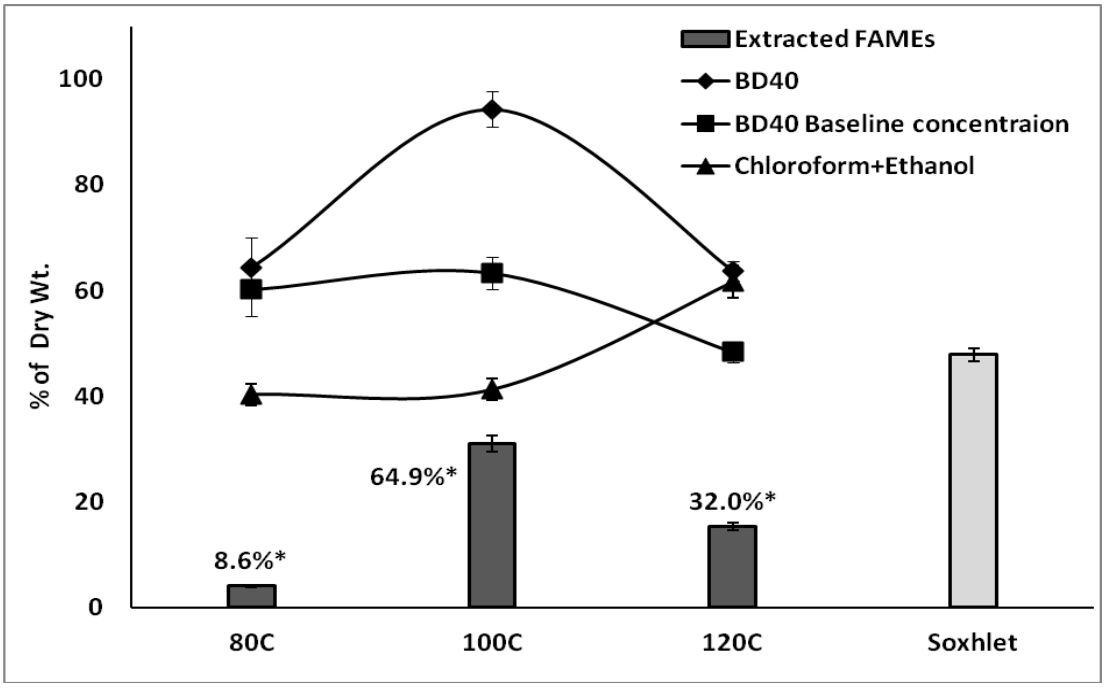


B

Figure 4.3. Total fatty acid methyle esters (FAMEs) extracted with: (A) BD20 and (B) BD40; (20% and 40% of biodiesel respectively in ethanol) and chloroform with ethanol using microwave and conventional Soxhlet extraction (*Percent of that extracted with Soxhlet).

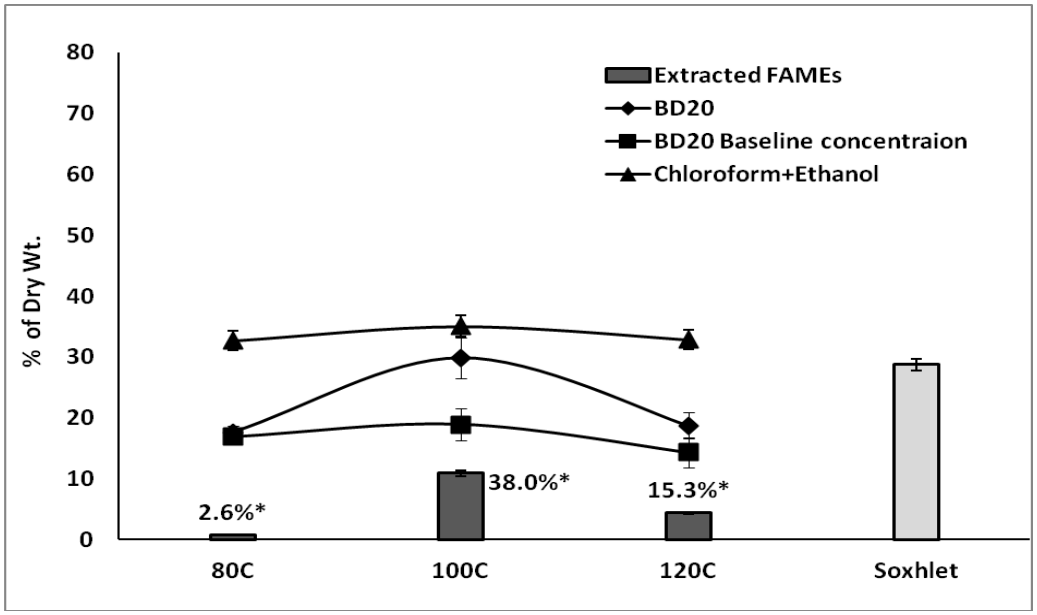
4.3.3 Microwave Assisted Extraction Using Biodiesel and Ethanol Co-solvent System

Biodiesel and ethanol co-solvent, containing 40% methyl soyate (BD40), indicated comparable results with those of chloroform plus ethanol as well as Soxhlet extraction. BD40 extracted 48.7% to 64.6% dry wt. of lipids at 120°C compared to 49% dry wt. of the Soxhlet extraction (99 to 116% efficiency compared to Soxhlet). Results indicated approximately 78% efficiency at 100°C which was not significantly different from 66% of the 80°C extracts ($P>0.05$). Temperature showed positive effect on extraction efficiency while using both BD40, and chloroform with ethanol. BD20 containing 20% methyl soyate, on the other hand showed comparatively lower efficiency of 27%, 34%, and 24% lipids at 80°C, 100°C, and 120°C temperatures respectively. One of the possible reasons for lower efficiency is that BD20 contained 80% ethanol which more likely dissolved polar lipids than the non-polar lipids.

Saturated fatty acids dominated the fatty acids profile compared to unsaturated ones in most of the higher temperature extracts. At high temperature the unsaturated fatty acid are expected to saturate (Tyagi and Vasishtha, 1996). Higher levels of 89.7% saturated fatty acids were extracted at 100°C using BD40 compared to 38% using BD20 (Figure 4.4). Similarly, BD40 extracted 29.7% of the unsaturated fatty acids compared to 8.1% of the BD20 (Figure 4.5). At 120°C temperature the extraction efficiency was better than the 80°C but less than the 100°C because of the reduced concentrations of volatile fatty acids which may have been degraded at higher temperature.

Unlike conventional solvent extraction, heat and mass transfer occurs in the same direction; from the inside of the extracted material to the bulk solvent (Virot et al., 2008). This helps in efficient disruption of cells especially at higher temperature at indicated by the SEM images in Figure 4.6. The technique is reported for its benefits in terms of efficient heating, faster

energy transfer, reduced equipment size, faster start-up, increased production, and elimination of process steps (Pare and Belanger, 1997; Virot et al., 2007; Virot et al., 2008).



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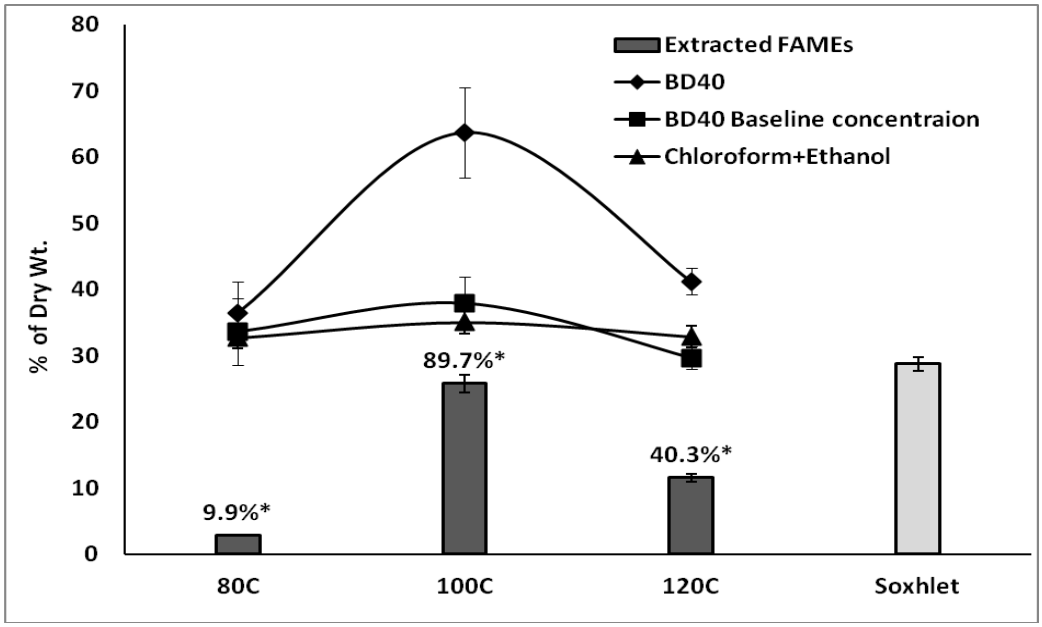
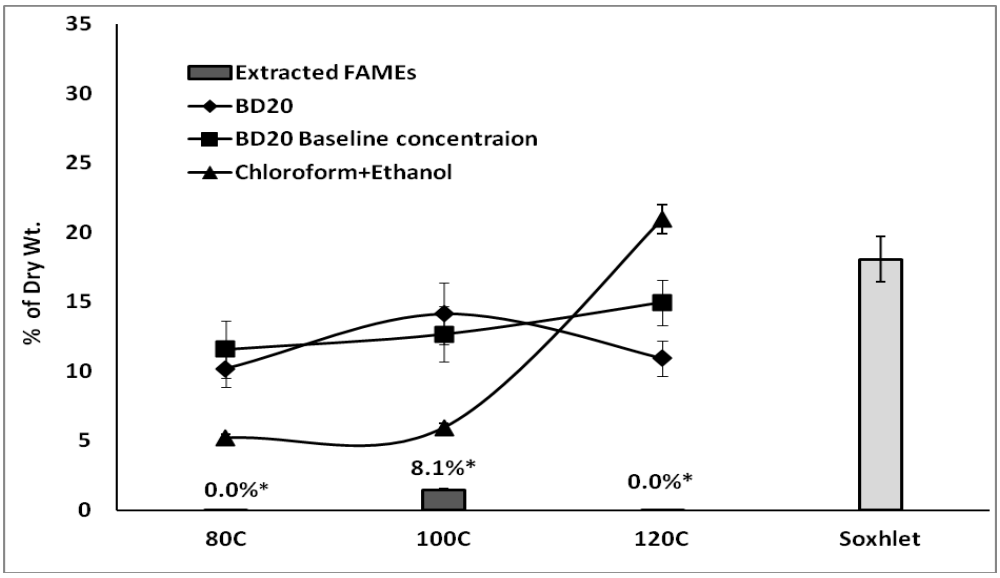


B

Figure 4.4. Total saturated fatty acid methyle esters (FAMEs) extracted with: (A) BD20 and (B) BD40; (20% and 40% of biodiesel respectively in ethanol) and chloroform plus ethanol using microwave and conventional Soxhlet extraction (*Percent of that extracted with Soxhlet).



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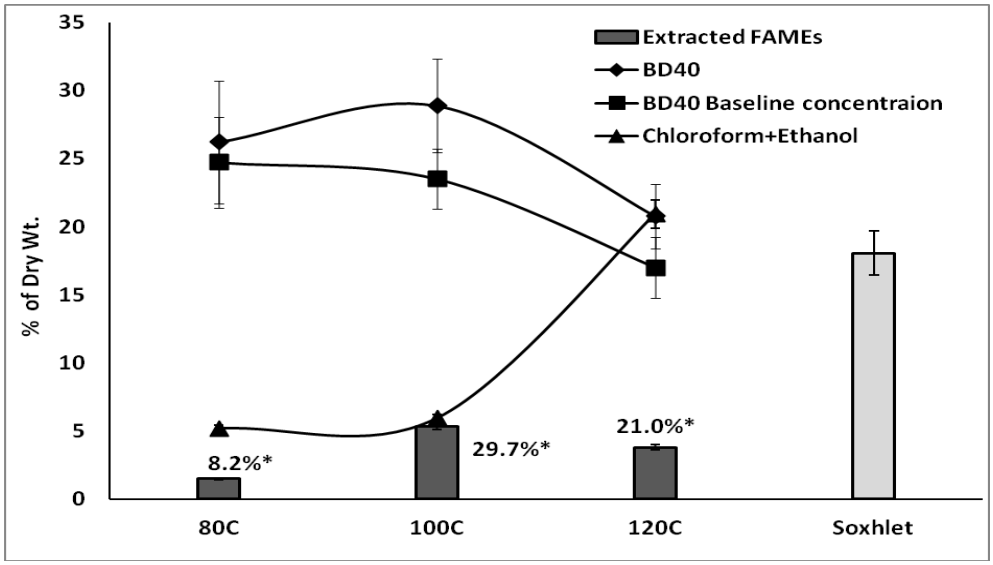


B

Figure 4.5. Total unsaturated fatty acid methyle esters (FAMEs) extracted with: (A) BD20 and (B) BD40; (20% and 40% of biodiesel respectively in ethanol) and chloroform with ethanol using microwave and conventional Soxhlet extraction (*Percent of that extracted with Soxhlet).

Dielectric constant or relative static permittivity of a solvent is a relative measure of its polarity. Water being a very polar molecule, has a dielectric constant of 80.1 at 20°C while *n*-hexane, being a very non-polar molecule, has a dielectric constant of 1.89 at 20°C (Lide, 2005). Regular petro-diesel, and biodiesel has dielectric constant of 2.2, and 3.35 respectively (Gonzalez Prieto et al., 2008; Sorichetti and Romano, 2005). Dielectric constatnt for chloroform

is 4.8. The dielecriv constatnt for biodiesel is close to coneventional solvent which indicate the potential of biodiesel as an alternative solvent to *n*-hexane or chloroform in extraction of lipids. However, because of the low volatility of methyl soyate (<50mg/mL of VOCs, and high boiling point, >400^{o}F) (Wildes, 2002; Hu et al., 2004), solvent penetration power of methyl soyate is low compared to *n*-hexane and chloroform. Therefore, it is suggested to use biodisel as co-solvent with another polar solvent like ethanol which helps in penetration power of methyl soyate along with capability to hold heat energy while in constant contact with microalgal cells.

4.3.4 Kauri-butanol (KB) Value

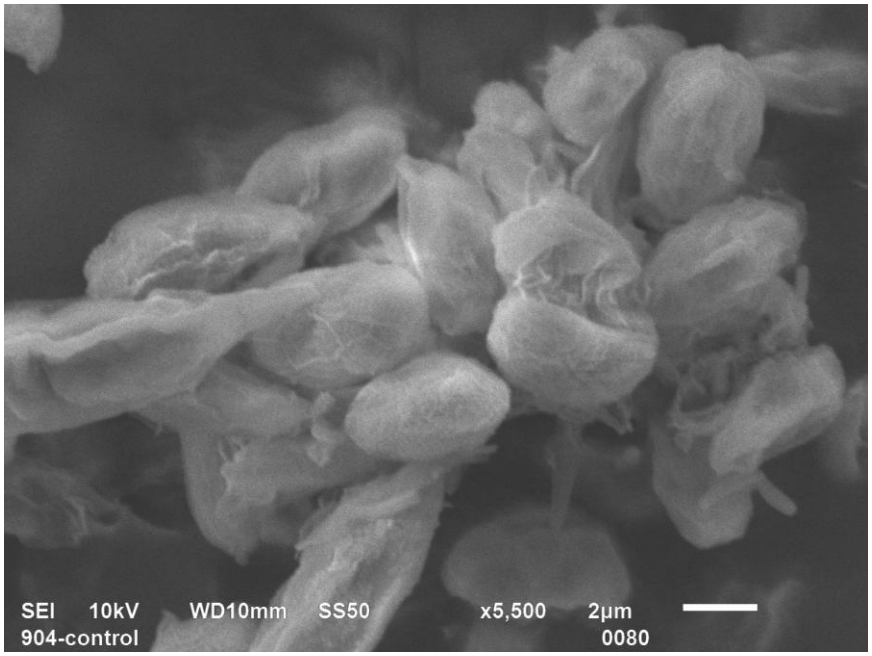
The KB value for pure methyl soyate (B100) was found as 57.5 which is in agreement with 58 reported by Hu et al. (2004) and 56 – 58 reported by Srinivas et al. (2009). Knothe and Steidley (2011) reported the KB values for methyl soyate and methyl oleate in the range of 55 – 60 while 19.1 for efined soybean oil. Methyl soyate indicate better solvency compared to 26.5 for *n*-hexane (Stauffer, 2008). Hu et al. (2004) reported the KB values of refined sunflower oil, corn oil, canola oil, and soybean oil biodiesels as 83.7, 83.4, 82.3, and 82.7 respectively. KB value of vegetable oil methyl esters is however reported proportional to the fatty acid profile (Knothe and Steidley, 2011). The number of double bonds of the unsaturated fatty acid of biodiesel esters were reported for very little effect on the solvent power while saturated fatty acid esters had weaker solvent power than unsaturated fatty acid esters. Similarly, methyl esters were reported for stronger solvency power than the ethyl esters (Hu et al., 2004). The KB value for pure ethanol (200 proof) was found as 102.7. This value is however in contrast to 84.2 reported by Knothe and Steidley (2011). It was found that the KB value of methyl soyate increased with increasing the ethanol proportion (Figure 4.7). The predicted KB values indicate maximum solvency strength for 40% biodiesel in ethanol. The presence of oxygen imparts higher KB values (knothe and Steindley, 2011). Although KB value usually determine the relative solvent power of

hydrocarbon solvents, however, other compounds including methyl esters have also been investigated for KB values (Knother and Steidley, 2011). Methyl esters are moderate hydrogen bonding liquids and hardly self-associate due to their lack of hydrogen-bond-donating ability (March, 1992). Once combined with strong hydrogen bonding liquid like alcohol, the cohesive solvent power is strengthened.

4.3.5 Chlorophyll *a*

Chlorophyll *a* concentrations in the extracts shows BD40 as more efficient solvent than the BD20 at 80°C as compared to 100°C and 120°C (Figure 4.8). Chlorophyll *a* concentrations in the extracts are adversely effected by high temperature. Exposure to heat may cause degradation of thermally labile compounds. The same drawback affects Soxhlet extraction (Cravotto et al., 2008). Balasubramanian et al. (2010) reported extraction of eicosapentaenoic acid (EPA) [C20:5(n-3)] and docosahexaenoic acid (DHA) [C22:6(n-3)] from green algae *Scenedesmus obliquus* using microwave where any of these fatty acids was not extracted with conventional Soxhlet extraction. That shows insignificant effect of microwave on nutraceuticals present in microalgae.

Biodiesel, as methyl soyate, along with ethanol as a co-solvent system was found to yield comparable results to those of chloroform plus ethanol or conventional 8 h Soxhlet extraction. This study confirms that toxic solvents like hexane and methanol can successfully be substituted with less toxic, environment friendly, biodegradable solvents to extract oil from microalgae. Employing such a solvent system is comparatively economical; avoiding one step of separating solvents and lipid oil since both serve as reactants in transesterification reaction. More research work is, however, suggested here to further investigate the effect of biodiesel, high temperature and microwaves on fatty acids of nutraceutical significance e.g. EPA and DHA, as well as their further refining.



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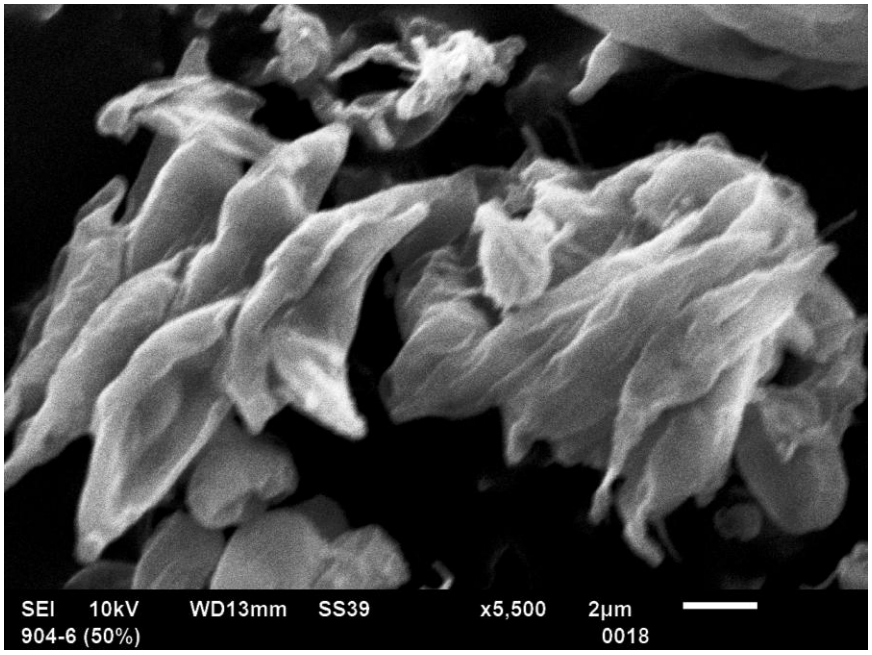
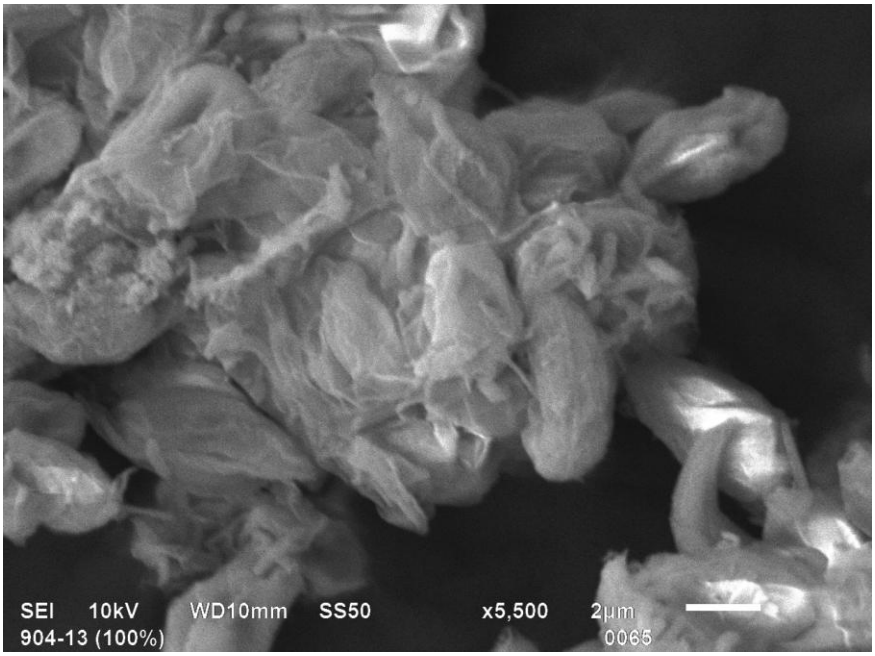


B

Figure 4.6. Scanning electron microscope (SEM) images of *Nannochloropsis sp.* showing microwave energy efficiently disrupted the microalgal cell structures. Compared to the undisrupted cells before extraction (A); BD40 with 40% biodiesel in ethanol being the most efficient co-solvent in microwave assisted extraction at 100°C as shown by the disrupted cells structures (B). Cells disrupted with microwave-assisted extraction (MAE) using chloroform and ethanol in 1:2 (v/v) proportions was also significant (C).

Figure 4.6. Continued.



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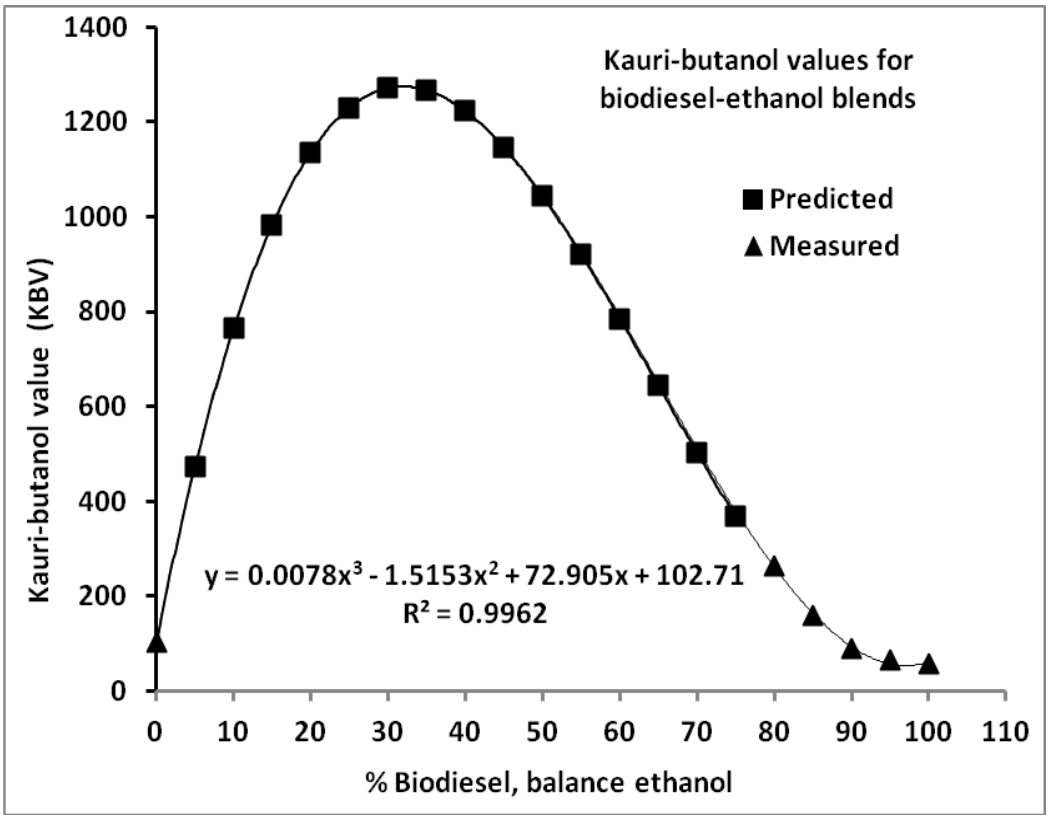


Figure 4.7. Kauri-butanol (KB) solvency power of biodiesel used as co-colvent with ethanol (values for biodiesel less than 80% were predicted based on the higher percentage blends due to large amounts of solvent required)

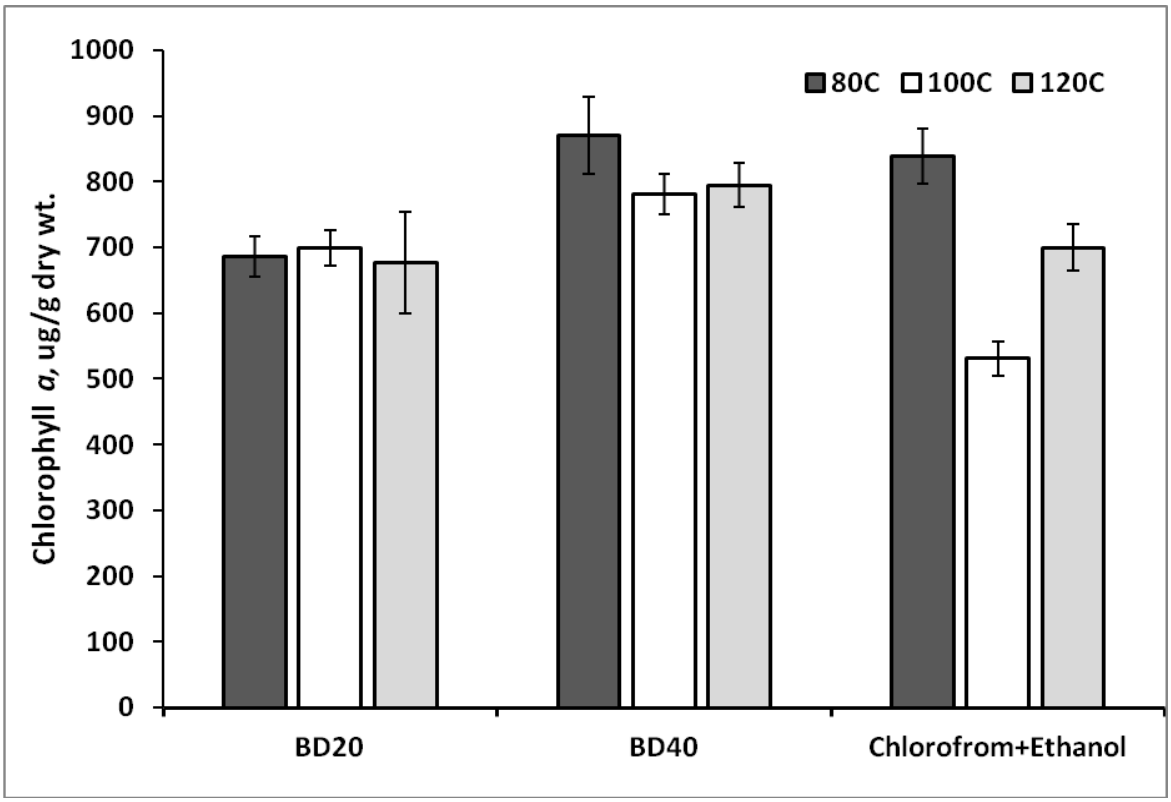


Figure 4.8. Chlorophyll *a* contents extracted with BD20 and BD40 (20% and 40% of biodiesel respectively as co-solvent with ethanol) and chloroform with ethanol (1:2 v/v) using microwave-assisted extraction.

CHAPTER 5

CONTINUOUS FLOW LIPID EXTRACTION SYSTEM FOR MICROALGAE USING BIODIESEL AS CO-SOLVENT

5.1 Introduction

High cost associated with cultivation, harvesting and extraction is the determining factor in mass production of microalgae as a viable source of renewable energy and other value added bioproducts. Harvesting costs contribute 20% – 30% of the total cost of algal biomass production while harvesting and extraction processes together contribute 40 – 60% of the total cost (Grima et al., 2003; Gudin and Thepenier, 1986). Harvesting and lipid extraction hence require focusing on all valuable algae materials and co-products for both fuel and non fuel products. Efficient extraction of lipids from microalgae is a determining factor in sustainability of algae-based biofuel.

Literature reveals that most of the efficient lipid extraction techniques involve solvents coupled with mechanical or physical disruption techniques e.g. accelerated solvent extraction (ASE), (Herrero et al., 2004; Jaime et al., 2005; Rodríguez-Meizoso et al., 2008), microwave assisted extraction (Balasubramanian et al., 2011), other solvent extraction based on Bligh and Dyer method (1959) and its modifications to include various cell wall disruption techniques such as sonication (Dunstan et al., 1992, Burja et al., 2007) or pressurized/accelerated hot solvents at high temperature (Macnaughtona et al., 1997, Lewis et al., 2000), and shaking (Lee et al., 2010). Solvents facilitate recovery of the physical extraction techniques while physical disruption techniques are required to facilitate the penetration of the solvent into the cells for physical contact between solvent and lipids. On the other hand, non-solvent techniques such as mechanical press and milling, requires dry biomass making it more energy intensive and least efficient (Shen et al., 2009). Therefore, because of its indispensable role in extraction, selection

of suitable solvent plays an important role in the economics of microalgal lipids extraction. A green extraction system involving an environmentally safe, least toxic or non-toxic, and least expensive co-solvent system is hence explored in the current study.

The CFLES system devised in the previous chapter (Section 3.2.3) has been demonstrated for highest efficiency (100% of glycerides recovered with Soxhlet extraction EPA method 3540) while using traditional solvents (chloroform and ethanol; 1:2 v/v). The system employed temperature and pressure as the physical disruption techniques coupled with continuous flow of co-solvent system through the biomass in an inline sample cell. The system is different than the traditional accelerated solvent extraction, ASE (or pressurized liquid extraction, PLE) in that CFLES undergo continuous flow of solvent while ASE statically extracts the sample. In CFLES, the solvency capacity of the solvent is not exhausted due to continuous flow of fresh solvent through the biomass (mimicking the Soxhlet extraction in this regard; but faster). ASE on the other hand has specific amount of solvent in contact with the biomass throughout the extraction process limited by the solvency capacity of the solvent. ASE (or PLE) uses high temperature and pressure while CFLES use moderate workable temperature and pressure. Maximum efficiency was noted at moderate 100°C temperature and 50 psi pressure. The system has the potential for scaled up lipid extraction from microalgae. Current study employed CFLES using biodiesel (methyl soyate) instead of chloroform or hexane blended with ethanol. The goal of this study was to further reduce the environmental and economic cost of solvents used in microalgal lipid extraction. To our knowledge, this would be the first study to demonstrate biodiesel as solvent in extraction of biochemical products from biomass.

Nannochloropsis sp. was selected for this study based on previous reports to contain 31 to 68% of oil (Chisty 2007, Sheehan et al., 1998; Hu and Gao, 2006). Gouveia and Oliveira (2009) and Rebolloso-Fuentes et al. (2001) reported *Nannochloropsis sp*. to contain high lipids (about

46%), and is considered to be a promising green microalgae for fuel products (Gouveia and Oliveira, 2009; Rebolloso-Fuentes et al., 2001). Rodolfi et al. (2009) reported *Nannochloropsis sp.* to attain 60% lipid content after nitrogen starvation.

5.2 Material and Methods

5.2.1 Microalgae Strain, Culture Condition and Sample Preparation

A culture of *Nannochloropsis sp.* was acquired from Aquatic Ecosystems Inc., Apopka, FL, USA (catalog# LAC1Q) and cultured as mentioned in Section 3.2.1. Samples were prepared as mentioned in Section 3.2.1. The biomass used for extraction contained 70% moisture.

5.2.2 Conventional Soxhlet Extraction

Approximately 3.3 g of algal paste (equivalent to 1 g dry wt.) was extracted using Soxhlet extraction apparatus as mentioned in Section 3.2.2. Chloroform and ethanol (1:2 v/v) was used as solvents. The final extraction volume was adjusted to 10 mL. Constituents in Soxhlet extracts were used as baseline for CFLES extracts.

5.2.3 Biodiesel Production

Methyl soyate was prepared from commercially available soybean oil as mentioned in Section 4.2.2 using base-catalyzed transesterification process repeated three times to ensure complete transesterification of all the oil (Hu et al., 2004; Sorichetti and Romano, 2005) . Total bound glycerols were determined using gas chromatograph with FID detector (SRI, 8610C, Torrance, CA, USA) to insure that all the oil is completely transesterified. Biodiesel was thoroughly tested to insure it meet ASTM D6751 biodiesel specifications. The total bound glycerides, if any, were considered as baseline concentrations in biodiesel co-solvent.

5.2.4 Continuous Flow Lipid Extraction System (CFLES)

Detailed description and operation parameters of the laboratory made CFLES are given in Section 3.2.3, Figure 3.2. The system was flushed with clean ethanol before the test runs and in

between the sample runs to overcome any carry over. Approximately 3.3 g of microalgal paste (equivalent to 1 g dry wt.) was fed into the sample extraction cells.

5.2.5 Solvents Used

Biodiesel as methyl soyate was used as co-solvent with ethanol in 40% proportions mentioned hereafter as BD40 (biodiesel:ethanol; 40:60 v/v). BD40 blend was selected based on results found in previous study mentioned in Section 4.3.3 where BD40 has better performance than BD20. The flow rate of co-solvent system was adjusted to 2 mL per min. Triplicate extractions were performed under the parameters give in Table 5.1. Sample extraction was terminated with a clear solvent draining into the solvent collection bottle of the CFLES system.

5.2.6 Post-extraction Process

The extracts were transferred to a graduated cylinder. DI water approximately equal to the amount of ethanol was added forming a biphasic system. Extracts were mixed by swirling ten times and then let settled for 30 min. The bottom layer containing water and ethanol was pipette out while the top layer contained lipids and chlorophyll contents dissolved in biodiesel. The extract was then centrifuged into a centrifuge tube at 3600 rpm to ensure complete separation of biodiesel from ethanol and water where the later was pipetted out with a pasteur pipette. The final extraction volumes varied between 25 to 80 mL since some of the extraction consumed more solvent than the others.

5.2.7 Glycerides Analysis

One milliliter of the extracts was silyated with 20 µL of N-methyl-N-trimethylsilyltrifluoracetamide (MSTFA) (ThermoScientific catalog# TS-48913) in a 5 mL vial (ASTM D6584). The solution was mixed thoroughly and reacted for 10 min at 70°C in an oven. After appropriate dilution, one µL of the reacted sample was manually injected into a gas chromatograph (SRI, 8610C, Torrance, CA, USA) equipped with flame ionization detector. More

details about the conditions of the instrument and temperature program are given in Section 3.2.6.

5.2.8 GC/MS FAMEs Analysis

One milliliter sample aliquot of the final extracts was used for fatty acids profile. Further details of the transesterification method, instrument conditions, and temperature program are given in Section 3.2.7. Hexadecanoic acid, 2-hydroxy-, methyl ester (CAS No. 16742-51-1, Indofine Chemicals, NJ, USA catalog# 24-1602) was used as internal standard. The instrument was calibrated using a 37-component standards mix (Supelco No. 18919, Ca, USA) containing C4–C24 FAMEs (2 to 4% relative concentrations).

5.2.9 Total Nitrogen and Carbon

Total nitrogen was quantified by CHN analysis. Dry samples were combusted in a CHN elemental analyzer (Elementar, Vario EL III). Helium was used as a carrier gas. Acetanilide (C = 71.09%; N = 10.36%; H = 6.71%) was used to calibrate the instrument. Nitrogen-to-protein conversion factor (N-Prot factor) is used to estimate the protein contents in microalgae biomass by measuring the total nitrogen contents (Bradford 1976, Lowry et al., 1951; Lourenço et al., 2002 and 2004; Gonzalez Lopez et al., 2010). Protein contents in the biomass before and after extraction were therefore estimated to determine the extraction efficiency of the system. The latest conversion factor N x 4.44 suggested by Gonzalez Lopez et al. (2010) to estimate the protein content in microalgal biomass, was used in the current study.

5.2.10 Chlorophyll *a*

Chlorophyll *a* was measured as mentioned in Section 3.2.8.

5.2.11 Scanning Electron Microscope (SEM) Imagery

SEM images of the extracted and feedstock was conducted according to the method mentioned in Balasubramanian et al. (2011) and Section 4.2.11.

5.2.12 Statistical Analysis

Analyses of variance (ANOVA) of different treatments and Fisher's protected least significant difference (PLSD) test for pair wise comparison was performed using *STATISTICA* version 9 software (StatSoft Inc., Tulsa, OK, USA).

Table 5.1. Temperature and pressure parameters used in continuous flow lipid extraction system (CFLES) using biodiesel as co-solvent with ethanol.

Test	**Temperature,** ^{o}C	**Pressure,** psi
AmbT,P	Ambient	Ambient
80T,AmbP	80	Ambient
80T,50psi	80	50
80T,500psi	80	500
100T,AmbP	100	Ambient
100T,50psi	100	50
100T,500psi	100	500
120T,AmbP	120	Ambient
120T,50psi	120	50
120T,500psi	120	500

5.3 Results and Discussion

5.3.1 Conventional Soxhlet Extraction

Soxhlet extraction yield 414 mg g^{-1} of bound glycerides (41% dry wt.) as given in Figure 5.1. These concentrations agree with most of the previous findings for *Nannochloropsis sp.* under normal environmental conditions (Chisti, 2007). This was considered as baseline concentration for comparing the performance of other extractions in the current study. The 8 h extraction consumed approximately 150 mL of solvent. Complete extraction was indicated by white

coloration of the biomass and clear solvent draining into the flask. Mono-glycerides dominated the glycerides profile (25% dry wt.) followed by di-glycerides and tri-glycerides (16% dry wt.) (Table 5.2). Soxhlet extraction is well known for its drawback (Wang and Weller, 2006, Garcia-Ayuso and Luque de Castro, 2001) affecting the composition of chemicals. Tri-glycerides are decomposed into di-, and mono-glycerides and free fatty acids due to the high boiling temperature of the solvent over a longer extraction time. Extracts kept boiling for 8 h are expected to breakdown heat sensitive compounds. Approximately 89% of the extracted oil was transesterified into fatty acid methyl esters (FAMEs) which is 37% of the dry weight of *Nannochloropsis sp.* biomass. Yu et al. (2007) found approximately 79% of the lipids as non polar triglycerides while 9% as polar. Polar lipids in marine organisms are reported to occur as glyco- and phospholipids which interfere with transesterification (Volkman et al., 1998). The biomass contained approximately 1% and 0.2% dry wt. of eicosapentaenoic acid (EPA) [20:5(*n*-3)] and docosahexaenoic acid (DHA) [22:6(*n*-3)] respectively. Both constitute approximately 3% of the total fatty acids profile. *Nannochloropsis sp.* is a marine eustigmatophyte which is reported to contain significant concentrations of EPA but little DHA (Volkman et al., 1998). Current study also reveals similar composition. The C16:1 made approximately 30% of the total fatty acids, while C16:0 and C18:0 were found 10% and 12% of the total fatty acids respectively as compared to 14% and 16% of those found in soybean oil. The high concentrations of C16:1 in *Nannochloropsis sp.* agrees with 27.4% reported by Sukenik (1999) and Hu et al. (2008). These authors, however, reported 34.9% of EPA in *Nannochloropsis sp.* compared to 3% found in the current study. Ratio of saturated to unsaturated fatty acids was 35:65 contrary to that of the soybean oil which was 58:42 containing most of the saturated fatty acids. Mono-unsaturated fatty acids (MUFA) constituted 44%, polyunsaturated fatty acids (PUFA) were 21% which included diunsaturated fatty acids as 6% and triunsaturated or higher as 15% (Appendix A2).

Table 5.2. Total free and bound glycerides (% of dry wt.) extracted from *Nannochloropsis sp.* under different temperature and pressure conditions in CFLES using biodiesel co-solvent and Soxhlet extraction using conventional solvents (n=3)

Component	Mono-glycerides	Di-glycerides	Tri-glycerides	Total glycerides
Amb T,P	5±0.6	83.8±8.2	4.9±0.6	93.6±9
80 T,AmbP	1.3±0.2	110.7±12.2	15±2	127±8.9
80T,50psi	11.2±1.2	175.4±17.4	18.5±2.6	205.1±6.1
80T,500psi	129±12.1	42.7±1.9	15.7±3.1	187.5±17.2
100T,AmbP	77.3±6.1	49.7±2.1	18±2.7	145.1±8.7
100T,50psi	80.9±9.7	156±9.6	40±3.9	276.9±22.4
100T,500psi	5.9±0.2	188±12	47.2±7.1	241.1±1.5
120T,AmbP	51.9±5.5	96±6.1	9.5±1.2	157.4±10.8
120T,50psi	125.2±9.4	99.9±4.8	41.6±4.7	266.6±19.2
120T,500psi	55.3±8.8	135.3±1.7	78.1±7.8	268.7±31.4
Soxhlet	251.2±0.9	48±0.2	115.3±10.8	414.4±10.5

Total nitrogen content in the biomass was 6.7±0.03 % dry wt., total C was 44.2±0.1, and total protein contents were calculated as 30%. Total protein contents agree with 30.1% reported by Zamora et al. (2004). However, Brown et al. (2010) found 52% dry wt. of protein in *Nannochloropsis sp*. Protein composition vary greatly with nitrogen contents in the medium which is inversely proportional to the lipid contents in biomass (Richardson et al., 1969; Piorreck et al., 1984; Chisti, 2007; Converti et al., 2009). The high heating value (HHV) as calculated with Dulong's formula was 18.69 MJ kg^{-1}.which agree with 19 MJ kg^{-1} reported by Brown et al. (2010) for *Nannochloropsis sp.* (Table 5.3).

5.3.2 Continuous Flow Lipid Extraction System (CFLES)

CFLES has successfully extracted 100% of lipids while using conventional solvents chloroform and ethanol (Section 3.3.2). The efficiency of the system, however, dropped to 67% while switching to biodiesel as co-solvent with ethanol (BD40). The efficiency is still comparable to most of the extraction techniques reported using conventional solvents. Table 5.4 shows several methods with efficiencies reported greater than 40%. Using biodiesel as co-solvent for extraction in CFLES has advantage over most of the methods in that it is a renewable product obtained from the biomass extracted. Biodiesel can help avoid an additional step of separating solvent and solute after extraction. It is non-toxic and easily biodegradable.

High lipid content of 28% dry wt. (as total bound glycerides) was extracted at 100^{o}C temperature and 50 psi pressure (100^{o}C/50 psi) (Figure 5.1). This is equivalent to 67% of the total available bound glycerides in the biomass. Approximately 27% dry wt. of lipids was extracted under each 120^{o}C/50 psi and 120^{o}C/500 psi parameters which were equivalent to 64% and 65% of the total bound glycerides respectively. Extractions efficiencies for two aforementioned parameters were, however, not significantly different than that of the 100^{o}C/50psi extractions. Total bound glycerides concentrations in the extracts were significantly different than those of the baseline (Figure 5.2). Variability among three extractions performed under similar temperature and pressure conditions increased with increase in temperature and pressure beyond 100^{o}C and 50 psi although recoveries were similar (Figure 5.2) since high temperature is expected to decompose triglycerides into di- or mono-glycerides and free fatty acids (Table 5.2). Lowest recoveries were seen for extractions performed under ambient temperature and pressure followed by those obtained at 80^{o}C. Extractions involving ambient pressure had significantly low recoveries than those of the high pressure or temperature. This explains the positive effect of pressure on efficiency of the CFLES. Under ambient temperature

or pressure, the oil recoveries ranged 23% to 38%. Increasing the pressure to 50 psi, recoveries increased to the range of 50% to 67%. Further increase in pressure, however did not increase the recoveries significantly (Figure 5.3). Similar trend was observed while using conventional solvents as mentioned in Section 3.3.3, Figure 3.7.

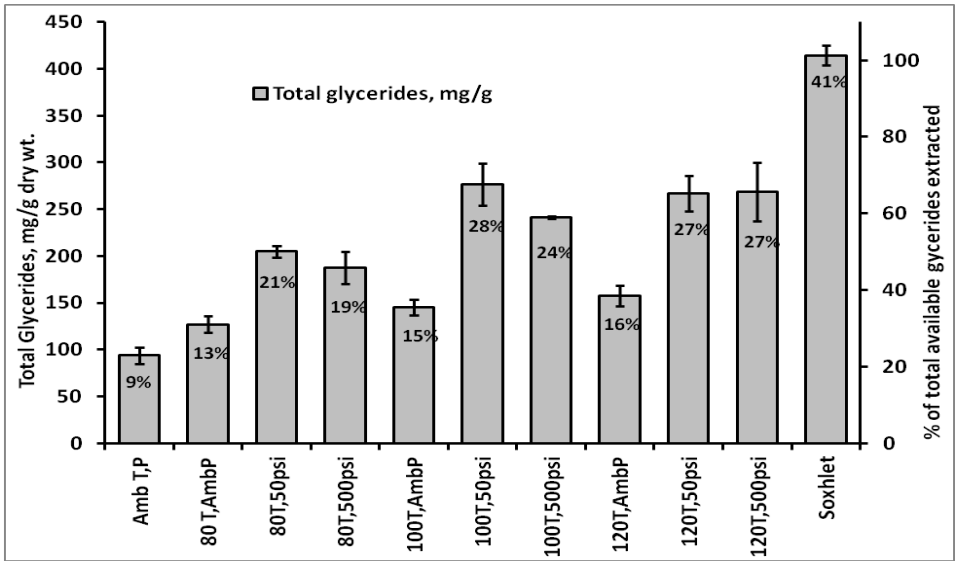


Figure 5.1. Total bound glycerides (sum of mon-, di-, and tri-glycerides) extracted at different temperature and pressure using CFLES and biodiesel as co-solvent. Percent values show percent of total glycerides on dry weight basis. Results are corrected for baseline glycerides concentrations in biodiesel.

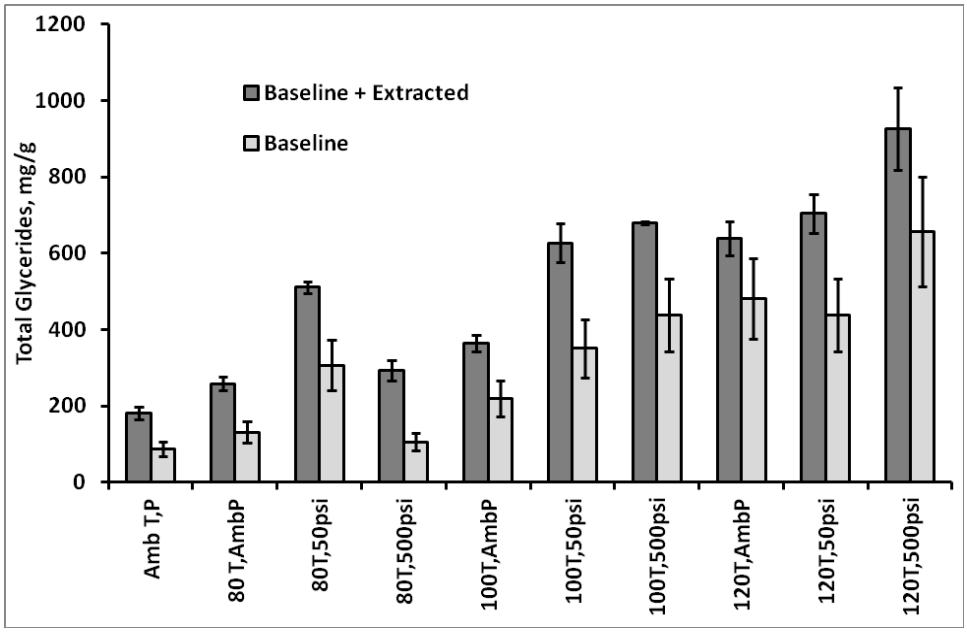


Figure 5.2. Total glycerides (sum of mon-, di-, and tri-glycerides) concentrations in the extracts (baseline + extracted) and biodiesel co-solvent (baseline) at different temperature and pressure using continuous flow lipid extraction system (CFLES).\

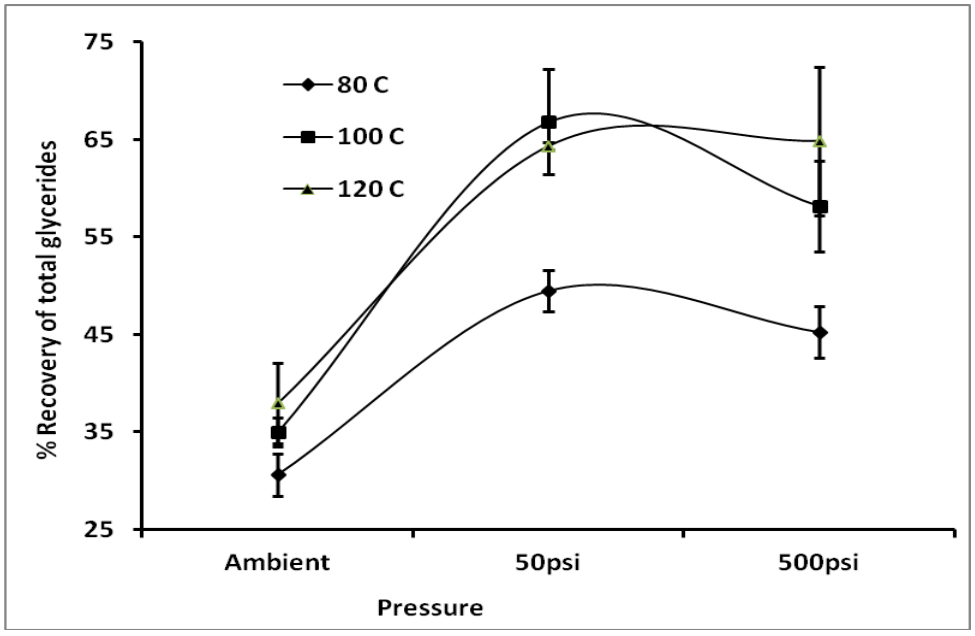


Figure 5.3. Effect of temperature and pressure on recovery of total glycerides while using biodiesel as co-solvent in CFLES

Table 5.3. Chemical composition (dry wt. basis) of *Nannochloropsis sp*.

Proteins	29.82 %
Lipids	414.4 mg g^{-1}
Elemental analysis:	
C	44.21 %
H	7.25 %
O*	41.82 %
N	6.72 %
High heating value	18.69 MJ kg^{-1}

* Calculated as 100 – (C+H+N)

Keeping in view the biodiesel potential of the extracts, maximum concentration of FAMEs, 28 % dry wt. (or 75% of the total FAMEs), was extracted at 100°C temperature and 50 psi pressure as compared to the baseline concentration of approximately 37% dry wt. found in

Nannochloropsis sp. using Soxhlet extraction (Figure 5.4). Recoveries under the aforementioned parameters were not significantly different than 27% dry wt. recovered at 100°C and 500psi. Low recoveries in the range of 22% to 24% dry wt. were seen at 80°C and also at 120°C. As expected, only 4% dry wt. or 12% of the total FAMEs were extracted under ambient conditions of temperature and pressure. Lowest recoveries in the range of 33% to 43% of the total FAMES (or 12% to 16% dry wt. respectively) were seen at ambient pressures regardless of high temperature.

The nutraceutical significance of CFLES is explained by 100% recovery of EPA at 100°C or lower. Less than 75% recovery was noted at high temperature of 120°C. Recovery of DHA was around 62.5% or less in most of the extractions performed under ambient conditions or low temperature of 80°C (Appendix A2). High temperature up to 100°C did not affect the DHA recovery. Balasubramanian et al. (2011) while using temperature of 95°C in a microwave assisted extraction successfully recovered both DHA and EPA which was not recovered while using conventional Soxhlet extraction. Concentrations of high carbon chain fatty acids, (C22:1, C22:2, C23:0, and C24:0) were significantly low or non-detected in biodiesel and Soxhlet extractions while their concentrations in CFLES extractions, especially 100°C/50 psi, were detected significantly. *Nannochloropsis sp.* has been reported for significant concentrations of high carbon chain fatty acids however extraction technique has a role in their detectable concentrations (Brown et al., 2010).

The conventional solvent used in CFLES was 30 mL to reach a complete extraction. Consumption of biodiesel co-solvent, on the other hand varied depending upon the clear color of the extracts draining into the extracts collection bottle. The range was 25 to 80 mL. The time of the extraction similarly varied between 15 to 40 min. Extractions performed at ambient temperature used the most solvents as compared to those of the high temperature. The 100°C/50 psi extraction used 25 mL while most of the extractions used 40 to 60 mL (averaged to 50 mL for

calculations purposes) of the solvents. Compared to 150 mL of the Soxhlet extractions, CFLES used approximately 67% less solvent while using biodiesel as co-solvent.

Table 5.4. Efficiency (more than 40%) of some common extraction methods and Continuous Flow Lipid Extraction System (CFLES).

Extraction Method	**% Efficiency**	**Reference**
Supercretical-CO_2	78	Andrich et al., 2006
Solvent	47.5% - 80%	Fajardo et al., 2007; Burja et al., 2007
Bligh and dyer (dry)	53	Widjaja, 2009
Microwave assisted extractions (MAE)	77	Balasubramanian et al., 2011
Extraction-transesterification	53	Lewis et al., 2000
Direct transesterification	51	Lewis et al., 2000
Thermochemical liquefaction	64	Sawayama, et al., 1995
Pressurized Liquid Extraction	40	Rodriguez-Meizoso et al., 2008
Direct saponification	46	Burja et al., 2007
Solvent/saponification	60	Guil-Guerrero et al., 2000
CFLES using traditional solvents	100	Current study
MAE using biodiesel as co-solvent	78	Current study
CFLES using biodiesel co-solvent	67	Current study

Similar to conventional solvents used in CFLES, yield with biodiesel co-solvent at 80°C temperatures was also noted significantly low in terms of total glycerides and FAMES ($P<0.05$). Maximum yield was noted for extractions performed at 100^{o}C. The biomass did not clump into a hard mass at 120^{o}C extractions as was noted with conventional solvents. However, the yield decreased at this temperature. One of the possible reasons is that biodiesel has a boiling point

more than 150°C. Therefore, at 120°C the biodiesel is expected to be available to the biomass for diffusion and mass transfer. Being a high boiling point liquid, the density of biodiesel in CFLES is not reduced and stay in constant contact with the biomass thereby increasing the heat and mass transfer along with disruption of cell. Increase in temperature is expected to increase its diffusivity and enhance the interaction between the solvent and the solute in the complex cellular matrix (Krichnavaruk et al., 2008) which increase roughly 2–10 folds upon increasing the temperature from 25 to 150°C (Perry, et al., 1984; Richter et al., 1996). However, the density of ethanol may be decreased at high temperature which lowers the extraction capability of the co-solvent system.

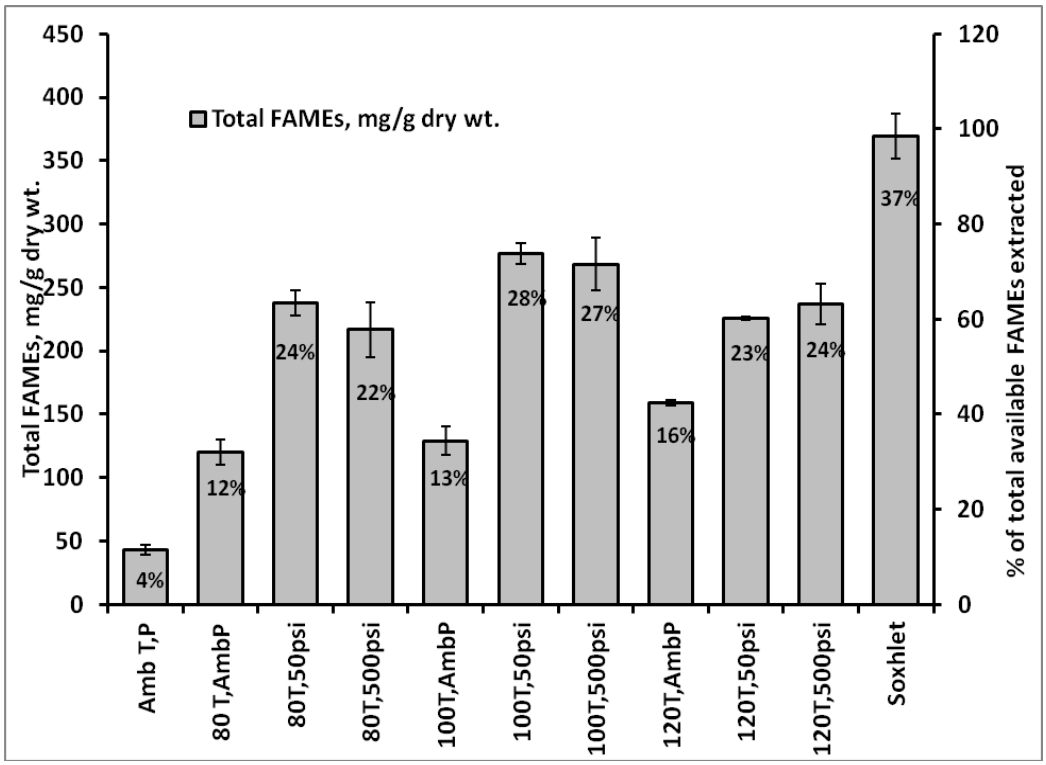


Figure 5.4. Total fatty acid methyl esters (FAMEs) extracted at different temperatures and pressures using CFLES and biodiesel as co-solvent. Percent values show percent of total FAMEs based on dry weight. Results are corrected for baseline FAMEs concentrations.

Results indicated higher concentrations of chlorophyll *a* extracted at 80°C and 100°C temperature (Figure 5.5). The concentration dropped at 120°C temperature. Significantly high

concentrations were observed at 100°C temperature and ambient pressure (P<0.05). Effect of high temperature has been found to decrease chlorophyll content significantly while chlorophyll *a* been less thermostable (Loey et al., 1998. Pheophytins are reported as the degradation products of chlorophylls (Ferrentino et al., 2006)

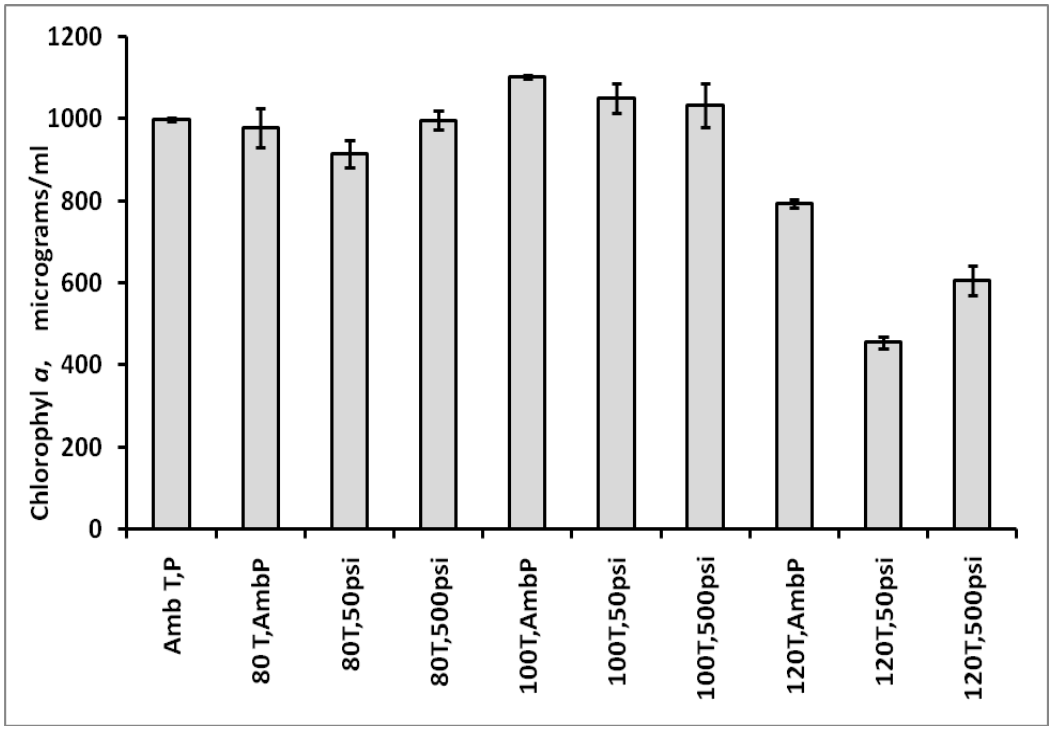


Figure 5.5. Chlorophyl *a* concentrations in the extracts of CFLES at different tmperature and pressure comibinations using biodiesel as co-solvent

5.3.3 Total N and Protein Analysis

Algal proteins associated with cell membrane are hard to extract. Efficient extraction of algal proteins hence is one of the major problems in protein analysis (Fleurence, et al., 1995). Therefore, elemental analysis including total N was determined in the biomass before and after the extraction. Difference in calculated protein contents was used as an indicator of extraction performance of CFLES system. Dry *Nannochloropsis sp.* biomass contained up to 44% Carbon (C), 6.72% Nitrogen (N) and 7% Hydrogen. The protein content was calculated as 29.82±0.1%. The protein contents agree with 28.8% reported by Rebolloso-Fuentes et al. (2001) and 36% reported by Fabregas et al. (2004) for *Nannochloropsis sp*. The CFLES efficiency in protein

extraction conforms to the lipids and FAMEs data with high yield in the range of 48 to 57% of the total protein extracted at 100^{o}C temperatures (Figure 5.6). The efficiency increased with increase in temperature from 80^{o}C to 100^{o}C. Similar trend was also indicated by glycerides and FAMEs data. However, the proteins extraction decreased significantly beyond 100^{o}C whereas the lipids or FAMEs concentration dropped slightly (Figure 5.3 and 5.4). Increasing pressure has positive effect up to 100^{o}C, and 500 psi. With further increase, the efficiency dropped. Extractions at 80^{o}C temperatures were comparable to those of ambient temperature and pressure. No significant extraction of proteins occurred at 120^{o}C and 500 psi pressure. This indicates a reduced contact between the solvent and the cellular material.

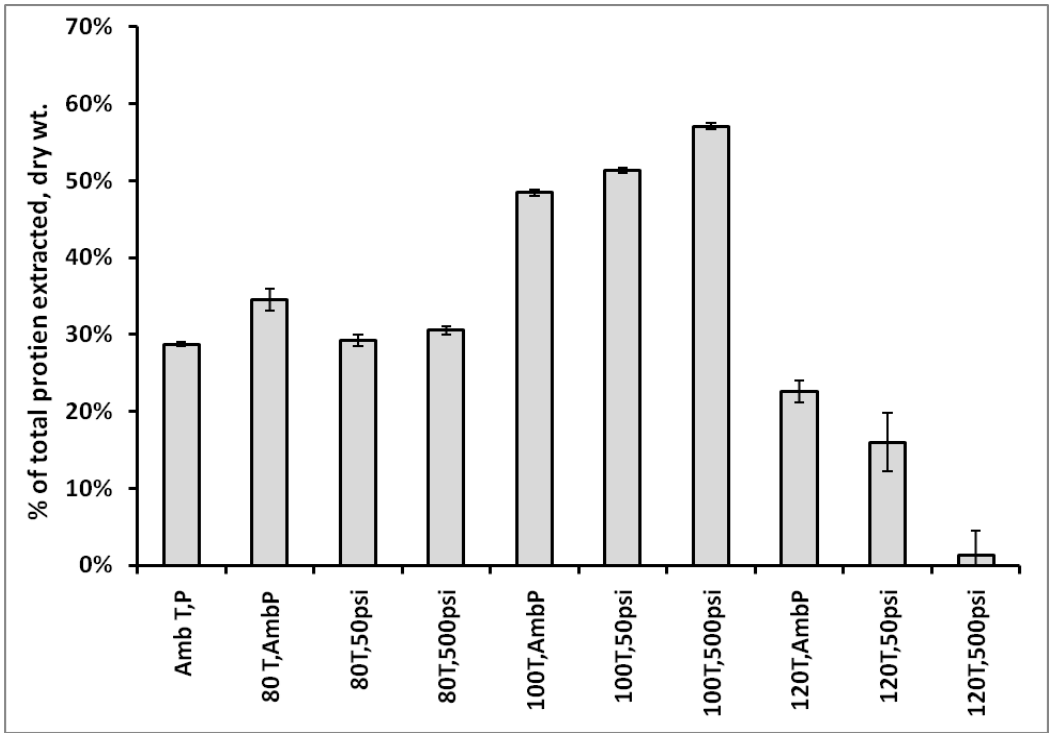
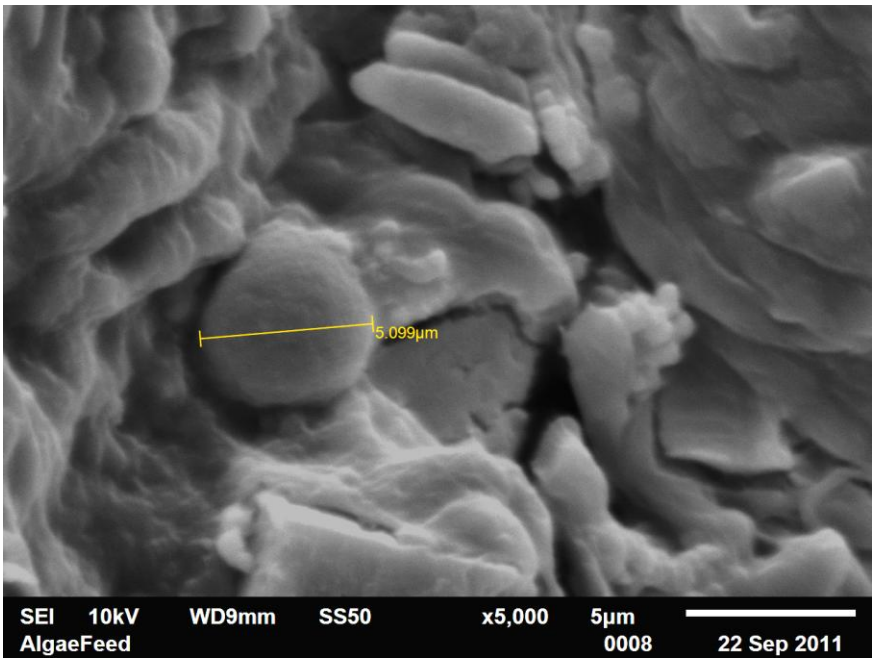


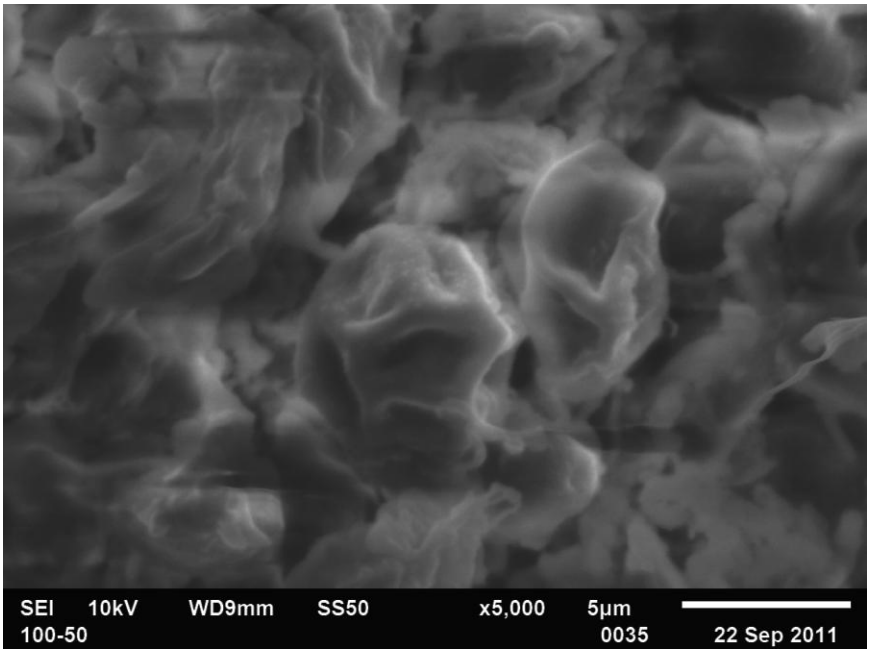
Figure 5.6. Percent of total protein extracted as an indicator of extraction performance of the CFLES system at different temperatures and pressures

5.3.4 Scanning Electron Microscope (SEM) Images

SEM images shown in Figure 4.7 shows the *Nannochloropsis sp.* cells before and after extraction. Disruption of cells at 100^{o}C and 50 psi pressure is more conspicuous compared to those extracted at 120^{o}C and 50 psi pressure.



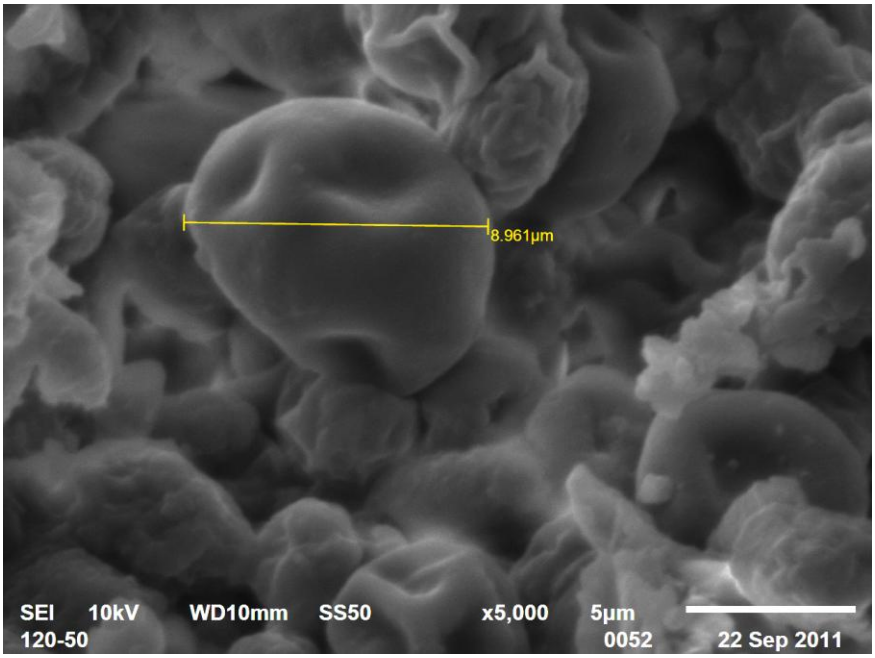
A



B

Figure 5.7. Scanning Electron Microscope (SEM) images of *Nannochloropsis sp.* cells: (A) cells before extraction, (B) cell extracted at 100^{o}C and 50 psi pressure using biodiesel as co-solvent, and (C) cells extracted at 120^{o}C and 50 psi using biodiesel as co-solvent.

Figure 5.7. Continued.



C

5.3.5 Energy Efficiency and Energy Savings

Energy consumption for the CFLES is calculated as to 0.8kWh (0.67h, 1200W of solvent pumping and heating) compared to 8.64kWh for the Soxhlet extraction (8hr of 1200W heating and condensing, assuming a heater on time of 90%), which corresponds to approximately 90.7% energy savings.

The average solvent consumption for CFLES was 50 mL of BD40 compared to 150 ml used in conventional Soxhlet extraction which corresponds to 67% savings in solvent consumption. Assuming biodiesel is obtained from the oil extracted and the cost is negligible, the estimated solvent saving increases to 80% along with additional environmental and health benefits. With 50% of ethanol recycled, the solvent savings are calculated as 90%.

Biodiesel as co-solvent for extraction of biochemicals is reported here for the first time. The study demonstrates that continuous flow lipid extraction system (CFLES) has efficiency comparable to most of the extraction techniques available in the literature. Biodiesel being the ultimate renewable product of microalgae has an advantage of eliminating one step of separating

the solvent from the solute. The co-solvent, ethanol, can be recycled to further reduce the extraction cost. The study shows that bioproducts of nutraceutical significance, EPA and DHA, were successfully extracted with biodiesel as co-solvent; 100% and 75% respectively. The study also confirms that moderate pressures and temperatures of 50 psi and 100°C used in the CFLES were the most favored conditions based on less variability and performance; although extractions at 120°C had comparable results with the 100°C/50 psi. Results suggest that at temperature and pressure above 100°C and 50 psi respectively, the solvents flow in the CFLES has to be increased to ensure sufficient solvent-solute interaction.

Residual water, approximately 70%, in the biomass did not affect the extraction performance. Therefore, drying biomass before extraction is not required, hence lowering the economic cost of harvesting and extraction. The operating temperature and pressure (100°C, 50 psi) are workable conditions for scaled up continuous CFLES. Biodiesel and ethanol has less environmental and health cost compared to conventional solvents like chloroform, hexane and methanol.

This study shows that biodiesel is a good solvent. However, because of the high flash point, its penetration power into the biomass matrix is very low which in turn lowers the diffusion rates and mass transfer of the solute. Therefore, addition of co-solvent like ethanol is required to synergistically enhance the diffusion and mass transfer rates.

CHAPTER 6

ECONOMIC ANALYSIS

6.1 Introduction

Research interest has grown up in third generation biofuel, the algal biofuel, during recent years. Algae biofuel has the potential to produce transportation fuel as an alternative fuel on a sustainable basis. Microalgae production does not compete with other food crop or agriculture land. Algae have a potential to produce up to 40,000 L/ha (4,222 gal/acre) of biodiesel (Weyer et al., 2010) as compared to oil yield of 455 L/ha (48 gal/acre) from soybeans or 5,685 L/ha (600 gal/acre) from oil palms grown in tropical regions (Murthy, 2011). Sustainable production of aglal biofuel however, still requires extensive research in the areas of its production, recovery, and processing (Murthy, 2010).

6.1.1 Production

The main focus at production level is to cultivate a strain with high lipid contents and keep its integrity in the medium. Algal strains with high lipid contents are reported more sensitive to contamination compared to strains with low lipid contents (Sheehan et al., 1998; Schenk et al., 2008). Similarly, strains with high lipid contents are slow growers as compared to fast growing species (Sheehan et al., 1998; Pienkos and Darzins, 2009). Environmental conditions also have significant impact on production. Production system either involves cultivation in open ponds or closed more controlled environment of photobioreactors (PBR). PBR systems have obvious advantages in terms of contamination and environmental controls as compared to pond systems. The disadvantages include comparatively higher capital investment as well as operation & maintenance cost.

6.1.2 Recovery of Biomass

Recovery is the most energy intensive aspect of algal biofuel production. Recovery of minute microalgal cells suspended in more than 99.9% water (typically between 0.02% to 0.07%) requires economical harvesting to a concentration between 5% – 25% depending on the extraction process employed (Bremann and Oswald, 1996). Several mechanisms exist to harvest microalgae. Flocculation (Elmaleh, 1991), dissolved air floatation, centrifugation, and drum filtration (Sim et al, 1988) are some of the well known techniques. Harvesting is reported to cost 20 – 30% of the production cost (Gudin and Thepenier, 1984; Molina Grima et al., 2003; Pienkos, Darzins, 2009). Lardon et al., (2009) reported 25.6% of energy consumed by algae culture and harvesting, and 74.4% for wet oil extraction. Biomass is generally concentrated by 50- to 200-fold (Murthy 2010). Reducing its cost through simple, reliable and low-cost processes is expected to help with economics of algal biofuel production. For instance, dehydration involving thermal drying is very costly option compared to mechanical dewatering (Mohn 1980, Murthy 2010). Further research is suggested to investigate more economical harvesting techniques.

6.1.3 Recovery of Oil

Extraction of lipids from biomass is the next energy intensive process in biofuel production from algae and is dependent upon the desired end product. It should be cost effective without jeopardizing environmental and food values of the products and byproducts. Harvesting and extraction are coherent processes in a sense that the end product of harvesting depends on the extraction approach used. If the extraction is performed with solvents and mechanical pressing, the harvested biomass has to be dried significantly (up to 10%). This drying process is the most energy intensive step requiring heating. Sun drying is not reliable because of the requirement of large drying surfaces and the risk of loss of lipids or other useful ingredients which may

decompose or spoil within few hours in hot climate (Brennan and Owende, 2010). Extraction techniques requiring algae paste, e.g. CFLES or microwave-assisted, where the biomass can contain up to 80% moisture, the end product of the harvesting process may contain approximately 20% solids. Similarly, extraction of many proteins requires biomass that has not been dried significantly (Bermejo Roman et al., 2002). In case of CFLES, the 80% water contents are expected to help the extraction process since water is reported to work as solvent under high temperature and pressure (Briones et al., 1990). Temperature around 100^{o}C and 50 psi in CFLES has shown maximum extraction (for comparison, a standard kitchen pressure cooker or an autoclave operates at 15 psi pressure). The moisture is phase separated at the end of the extraction process instead of thermal drying. This saves significant amount of energy. For instance, the solvent extraction with mechanical press requires the biomass to be dried to 10% moisture. Dehydration to this level costs approximately 60% of the entire extraction cost. Therefore, processes based on dry biomass are likely to be more uneconomical due to the energy inputs involved, and so methods that work with algal slurries or wet paste are preferred (Pienkos and Darzins, 2009).

6.1.4 Processing

Processing of the extracted products involve the comparatively least number of complexities as compared to production and recovery steps. Challenges at this level include removal of impurities, refining of the final product for specific use, and creating market niche for products where the market does not exist previously. The extraction process also plays a significant role in the end use of the final products, especially the food products which could be impacted with the use of toxic solvents.

Economic analysis of algal biofuel production is a challenge because the commercial sector is still in the research and development phase (Kovacevic and Wesseler, 2010). Numerous

uncertainties exist with the yield and evolving technologies which makes it hard to obtain reliable cost estimates (Carriquiry et al., 2011). The goal of this economic analysis was to evaluate the economics of different extraction techniques performed in the current study. Though results of microalgal oil extraction at lab scale are difficult to extrapolate to an industrial scale, the analysis can still help identify cost-reduction tools in future biofuel production initiatives.

6.2 Process Economics of Lipid Extraction Based on a Case-Scenario

Studies so far published in the literature related to performance of microalgal oil extraction are predominantly referenced to that of the Soxhlet extraction. Soxhlet extraction is very efficient method used to set a bench-mark for comparison with other methods in laboratory situation. Being a longer duration but very intensive method, Soxhlet extraction provides a good baseline concentration by extracting all the lipids contained in the biomass, to which the performance of other methods is assessed. This method is however, not workable for scaled-up operation.

Current research work mentioned in previous chapters compares the CFLES and microwave-assisted extraction with Soxhlet extraction in terms of performance and efficiencies. Savings in terms of energy, solvents and labor were evident as compared to Soxhlet in the laboratory situation. This comparison is however, not directly applicable for process economics at scaled up operation. Therefore, a case-scenario based on pilot production scale was developed to better understand the process economics of microalgae oil extraction. The case study covers only the extraction part while assuming the biomass was already harvested to a paste containing 80% moisture. Further dehydration, if required, was based on the extraction approach proposed.

6.2.1 Variables Studied for the Case-Scenario

A large number of variables are involved with the economic analysis of microalgal fuel production at large scale. The current economic analysis covers the operational expenditures

involved with the oil extraction part only. Variables involved with cultivation, harvesting, or final processing of the extracted oil is not within the scope of the current work. The analysis is based on the following few but important variables involved in the extraction process:

- Moisture content in the biomass
- Cost of thermal dehydration
- Solvent type and volume used
- Heating cost for solvent recycling
- Cost of solvent lost (~2.5% by volume)
- Cost of electricity
- Glycerides (TAGs) recovered based on efficiency of the method used
- Cost per gallon

The cost of solvent lost is included in this analysis. However its purchase price was not considered assuming it was an initial investment.

6.2.3 Economy of Scale

It was assumed that 3,420 gallons of algae paste (*Nannochloropsis sp*.) containing 80% moisture was recovered from a 3.35M gallon pond. The dry weight of the biomass was approximately 2,588 kg containing 20% lipid content of which 80% were glyceride precursors to biodiesel (TAG). Assumptions were based on results obtained in the current laboratory studies mentioned in previous chapters. Complete extraction of the biomass, 100% efficiency, was expected to yield approximately 100.7 gallons of TAGs (Table 6.1).

The process economics involved the amount of heat energy required for dehydration, recovery of solvents, and approximate electrical energy required to operate the extraction system. The cost of energy consumed was calculated using $0.10 /kWh. The heat energy consumed for dehydration and solvent recovery was calculated using Equation 6.1.

$$E = m C_\rho \Delta T + m H_v \quad \text{Eq. 6.1}$$

where,

E – energy consumed to evaporate a liquid, kJ

m – mass of liquid evaporated, kg

C_ρ – specific heat of liquid, kJ/kg. ^oC

ΔT – change in temperature (from room temperature to boiling point of the liquid), ^oC

m – mass of liquid evaporated, kg

H_v – Latent heat of vaporization of the liquid, kJ/kg

For cost estimation, the energy calculated was converted into kWh. The analysis included 2.5% of the solvent lost during extraction and recovery cycles (Huang, and Chang, 2010).

Table 6.1. Assumptions made for model extraction case scenario

Production capacity, Gal	$3.35x10^6$
Harvested biomass with 80% moisture, gal	3,353
Harvested dry biomass, kg	2,588.22
Lipid contents at 20% dry wt., kg	517.65
TAG contents, 80% of lipids, kg	414.12
TAG contents, based on 0.92 g/mL density, L	380.99
TAG contents dry wt., Gal	100.68
Electricity Price	$\$0.10\ kWh^{-1}$

TAGs: Triacylglycerides

6.2.4 Technological Assumptions

Solvent extraction method using hexane is the most economical method reported for extraction of oil from crop like soybean (Naksuk et al., 2009). The solvent extraction is coupled with mechanical press or extractors. This technology, however, has not been utilized for

extraction of oil from microalgae at large scale (Greenwell et al., 2010). The method was selected based on its potential for microalgae oil extraction. The estimated costs using this method were compared with those of the estimated cost for CFLES and microwave assisted extractions (MAE). It was assumed that both CFLES and MAE process one gallon of post-harvest biomass at one time in a continuous or batch mode whichever is applicable. Cost-per-gallon estimated with the following techniques and combinations of solvents were compared in the current economic analysis:

1. Solvent extraction with mechanical extractor (Sol-Mech.)
2. CFLES with conventional solvents (CFLES-CS-100^{o}C)
3. CFLES with biodiesel and ethanol (CFLES-BD-100^{o}C)
4. Microwave-assisted extraction with conventional solvents; 2:1 of solvent to feed (MAE-CS-100^{o}C)
5. Microwave-assisted with biodiesel and ethanol; 2:1, solvent to feed, (MAE-BD-100^{o}C)
6. Microwave-assisted with 40% biodiesel in ethanol; 0.8:1.2:1 of biodiesel: ethanol: feed (MAE-BD-120^{o}C)

6.3 Estimated Extraction Costs

6.3.1 Solvent Extraction with Mechanical Extractor (Sol-Mech.)

This is one of the most common methods for extraction of vegetable oil, nutraceuticals, botanicals, specialty chemicals, and pharmaceuticals using continuous solid/liquid extraction. In this method the solvent extraction is coupled with a mechanical extractor. The extraction solvent predominantly used is hexane because: (1) its low boiling point (68.7°C /155.7°F), (2) high solubility of oils and fats in hexane, and (3) comparatively lower price. Example of mechanical extractor is an immersion extractor (model IV) manufactured by Crown Iron Works Company, Minneapolis, MN (Figure 6.1). This is used for extraction of chemicals from granular or coarse

products that sink in the solvent bath such as soybean. Although differences between microscopic algal cells and the seeds of oil-bearing plants require different processes for oil recovery (Pienkos and Darzins, 2009), the extraction of oil from dry algae is more or less similar to soybean extraction, both with solid contents around 90% (Lardon et al 2009). The extractor uses slow motion conveyors which pull solid materials through a solvent bath (3:1 ratio of solvent to feed, v/w) in a continuous counter-current direction providing good contact between solvent and soybean flakes. Complete immersion of solids ensures good contact with the solvent. Soybean flakes in the extractor are washed counter currently with various hexane/oil mixtures and, finally, with pure hexane producing a solvent/oil mixture (micella). The solvent from the micella and solvent-laden, defatted flakes is then evaporated in the next step (i.e. desolventization). The initial oil content of the soybeans is approximately 18 percent to 20 percent by weight. After extraction, the defatted soy flakes contain approximately 0.5 percent to 2.0 percent oil by weight (U.S. EPA, 1995).

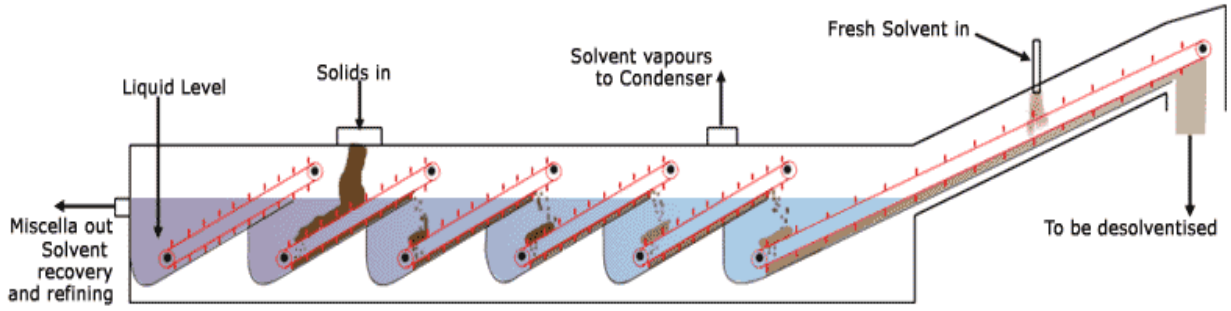


Figure 6.1. Continuous Solid/Liquid Immersion Extractor (Model IV) by Crown Iron Works Company, Minneapolis, MN (http://www.crowniron.com/technologies/spx_model4.cfm)

The extractor has not been reported for algal oil extraction. Rupture of cell algal wall through mechanical friction is only possible when dry. The disadvantage of solvent extraction

with mechanical extractor for microalgal oil is that the biomass has to be dried to 10% moisture (Erickson, 1995), which consumes significant amount of thermal energy i.e. approximately 0.627 kWh/kg water (Mohn 1980; Molina Grima, 2003; Murthy, 2011). In general, the combined total thermal and electric energy required for hexane extraction from soybean with mechanical extruders is reported as 23,151 Btu per gallon of oil which is equivalent to 1.65kWh per kg (Pardhan et al., 2009).

Economic analysis of the case-scenario using this approach indicated that the extraction cost of one gallon of algal oil estimated was $23.64 (Table 6.2). Approximately 34% of the cost is associated with thermal dehydration of the biomass to 10% moisture; calculated as 0.714kWh/kg (estimated from room temperature of 25°C) (Figure 6.2). The solvent recycling consumed the least energy (approximately 5%) of the total extraction. The total cost of evaporative heating is approximately 39%. Electricity cost for equipment operation accounts for approximately 34% of the total extraction cost. The total energy consumption contributes approximately 73% of the total cost. In terms of energy consumption, Xu et al. (2011) reported that nearly 70% of the energy input is required as heat for extraction from microalgae. Similarly, according to Lardon et al. (2009) the energy required for the dewatering process account for 84.9% of the total energy consumption. Loss of solvents up to 2.5% contributes approximately 27% to the total extraction cost based on the current market price of hexane ($370 for 55 gal drum).

The ratio of oil recovered to the cost per gallon (oil:cost ratio) was used to set a performance based criterion for the extraction techniques compared (Figure 6.3). Higher ratio means the most efficient techniques and the least cost per unit of the product. Based on this ratio, the solvent extraction was ranked fifth among the techniques (Table 6.2).

Table 6.2. Cost estimates for different extraction systems analyzed

Extraction method:	Sol-Mech.	CFLES-CS-100°C		CFLES-BD-100°C		MAE-CS-100°C		MAE-BD-100°C		MAE-BD-120°C	
Moisture in biomass	10%	70%		70%		80%		80%		80%	
Dehydration cost	$646.83	$92.40		$92.40		$00		$00		$00	
Total vol., solvent gal	3078	3077		3077		6838		6838		6837	
Solvent(s)	Hexane	Hexane	Ethanol	Biodiesel	Ethanol	Hexane	Ethanol	Biodiesel	Ethanol	Biodiesel	Ethanol
Vol., solvent (gal)	3078	1231	1846	1231	1846	855	5983	855	5983	2735	4102
Solvent recovery cost	$99.02	$39.61	$151.85	$00	$151.85	$27.51	$492.09	$00	$492.09	$00	$337.43
Cost, solvent loss, ~2.5%	$517.66	$206.99	$324.03	$00	$324.03	$143.80	$1050.10	$00	$1050.10	$00	$720.07
Electricity cost	$640.59	$213.75		$213.75		$277.53		$277.53		$277.53	
Total cost	$1,904.10	$1,028.63		$782.03		$1,991.03		$1,819.72		$1,335.87	
Efficiency	80.00%	100.00%		67.00%		93.00%		93.00%		100.00%	
TAGs recovered, gal	80.5	100.7		67.5		93.6		93.6		100.7	
Cost per gallon	$23.64	$10.22		$11.59		$21.26		$19.43		$13.27	
Oil:Cost ratio	2.80	9.85		5.82		4.40		4.82		7.59	
Ranking	6	1		3		5		4		2	

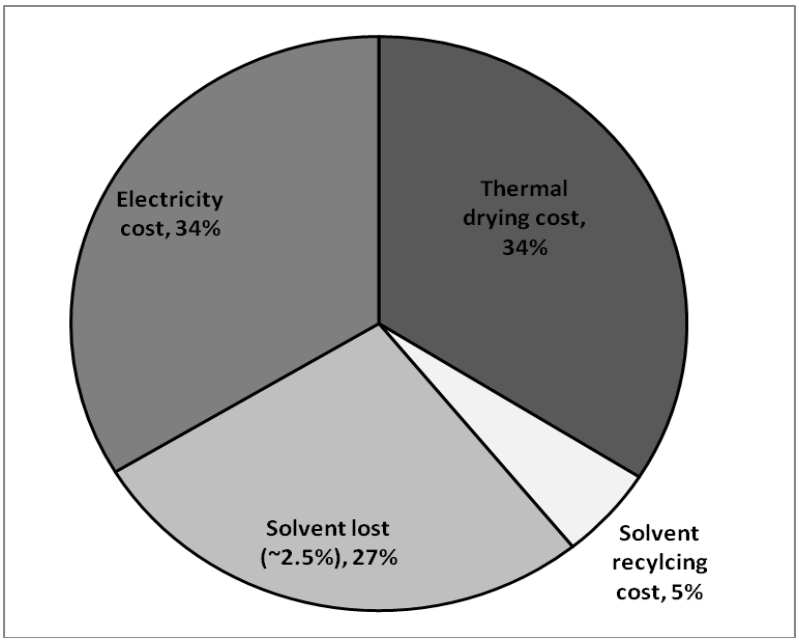


Figure 6.2. Estimated cost distribution of solvent and mechanical extractor (Sol-Mech) for use with microalgae

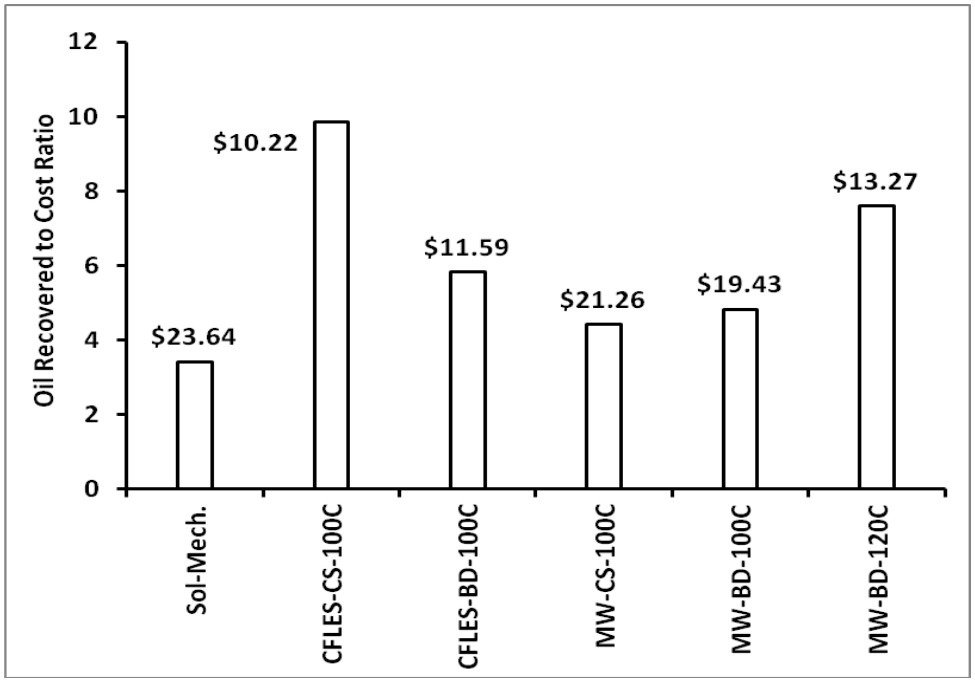


Figure 6.3. Oil recovery to cost per gallon ratio (oil:cost). Higher ratio value represents the most efficient method in terms of performance and cost. Values on the bars show estimated extraction cost-per-gallon for each method. Abbreviations used: Sol-Mech. – Solvent and mechanical extraction; CFLES-CS-100^{o}C – Continuous flow lipid extraction system with conventional solvents, hexane and ethanol, at 100^{o}C temperature; CFLES-BD-100^{o}C – CFLES using 40% biodiesel as co-solvent with ethanol at 100^{o}C; MAE-CS-100^{o}C – Microwave-assisted extraction using conventional solvents at 100^{o}C; MAE-BD-100^{o}C – Microwave assisted extraction using biodiesel as co-solvent with ethanol at 100^{o}C; MAE-BD-120^{o}C – Microwave-assisted extraction using 40% biodiesel with ethanol at 120^{o}C.

The extraction efficiency for Sol-Mech. method was assumed 80% based on a lab study using microalgae extracted with solvents and mechanical disruption with cell homogenizer (Mendes-Pinto et al., 2001; Fajardo et al., 2007; Murthy, 2011). To our knowledge, the actual mechanical extractor with hexane solvent has not been reported for microalgae oil extraction. Extraction efficiency for microalgae is expected lower than soybean feedstock because microalgae are well known for their tougher cell wall, which is resistant to solvent diffusion.

6.3.2 CFLES with Conventional Solvents (CFLES-CS-100^{o}C)

The continuous flow lipid extraction system (CFLES) mentioned in previous chapters has significant efficiency (100%) in the lab using conventional solvents. Estimates were based on a co-solvent system consisting of hexane and ethanol in 40% and 60% proportions, respectively. The co-solvent and biomass proportion considered was 1:1, v/w. Projecting its processing capacity to one gallon indicated $10.22 per gallon as compared to $23.64 for the solvent extraction (Table 6.2). The CFLES with conventional solvents is the most efficient and least expensive techniques ranked top on the oil recovery to cost ratio (Figure 6.3). The advantage of CFLES is that the biomass does not need to be dry. The system works well with 70% moisture. Drying the post harvest algae paste from 80% to 70% moisture contributes approximately 12% of the extraction cost compared to 60% of the Sol-Mech. The highest estimated cost, 41%, is associated with the 2.5% solvent loss (Figure 6.4). This means reducing the solvent loss will further minimize the extraction cost. According to Lardon et al. (2009), the heating and electricity energy consumption for wet oil extraction, similar to CFLES-CS, was estimated 54.1% and 20.3% respectively as compared to 25.6% required for algae culture and harvesting. The CFLES study mentioned in Chapter 3 indicated that the moisture content in the biomass has a beneficial effect on the extraction explained by: (1) the water at elevated temperature and pressure work as solvent (Briones et al., 1990), (2) moisture keeps the algal cells loose and

suspended as compared to those compacted in the form of a dry algae cake, thus easily penetrable by the solvents, enhancing mass transfer, and (3) moisture helps create a biphasic solvent system in post-extraction process separating the oil rich extracts, biomass, and water+ethanol solution. Drawback of using wet biomass is that comparatively large amount of solvent is required than that of the dry biomass used in solvent and mechanical extraction. This also enhances the solvent recycling cost (in this case approximately 19% of the extraction cost). In CFLES-CS-100^{o}C, the electrical energy input is approximately 13% lower than the Sol-Mech. method. Significant savings compared to Sol-Mech. include approximately 57% in extraction cost, and approximately 60% in energy savings (Table 6.3).

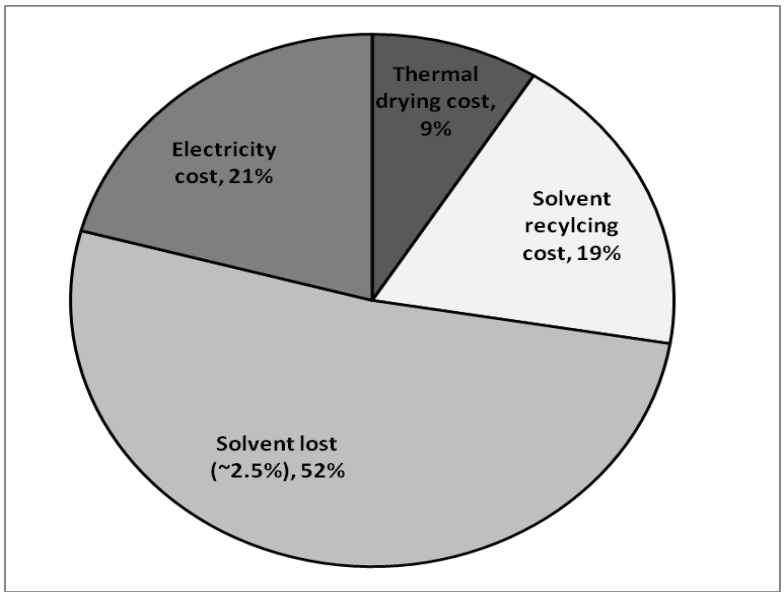


Figure 6.4. Cost distribution of continuous flow lipid extraction system using conventional solvents at 100^{o}C (CFLES-CS-100^{o}C)

6.3.3 CFLES with Biodiesel and Ethanol Co-solvent (CFLES-BD-100^{o}C)

The CFLES-BD using biodiesel and ethanol instead of hexane and methanol solvents certainly has intrinsic value in terms of environmental and health safety. Estimates were based on a co-solvent system consisting of biodiesel and ethanol in 40% and 60% proportions respectively. The co-solvent and biomass proportion considered was 1:1, v/w. The cost per gallon ($11.59) is

higher than that of CFLES-CS but lower than the Sol-Mech. The oil to cost ratio ranks this technique as the third preference (Figure 6.3). The efficiency of CFLES-BD was found approximately 33% lower than the CFLES-CS at 100^{o}C (Chapter 5). However there are some downstream benefits of using this technique including (1) separation of biodiesel from the extracted oil is not necessary, (2) no cost of evaporative recycling or loss of biodiesel as happen in case of conventional solvents, (3) biodiesel may ease the transesterification reaction, if biodiesel is the ultimate goal.

Being the first study to investigate the solvent potential of biodiesel, this technique requires input from further research to enhance its efficiency and reduce its cost. For instance, increasing biodiesel proportion from 40% to 60% in the co-solvent system may help reduce the solvent cost if the efficiency is also increased proportionally. A major component of the extraction cost (41%) under this technique is that of the solvent lost (ethanol in this case). A significant portion of the extraction cost (approximately 61%) is due to the solvent recycling or solvent lost in the process (Figure 6.5). Significant savings for this technique compared to Sol-Mech. are approximately 51% reduction in extraction cost, and approximately 64% in energy savings (Table 6.3).

6.3.4 Microwave-assisted Extraction with Conventional Solvents; 2:1 of Co-solvent to Feed (MAE-CS-100^{o}C)

The continuous MAE has been reported at a small pilot scale for extraction of oil from soybean (Terigar et al., 2011). The extraction efficiency was reported as more than 93.0% using ethanol as solvent in a 3:1 proportion of solvent to feed containing less than 10% moisture. The authors reported that the extraction time and the flow rate do not have a significant influence on extraction yield. The system processed 1 L/min of the solution with retention time of 21 min.

For the current economic analysis the MAE system was assumed to process one gallon of biomass either in continuous or batch mode with 15 min retention time. This assumption was

also applied to closed vessels microwave system used in the current study (Chapter 4). Furthermore, the cost of hexane (1:8, hexane:ethanol, v/v) was included in this analysis to create a biphasic system for recovery of oil after the extraction.

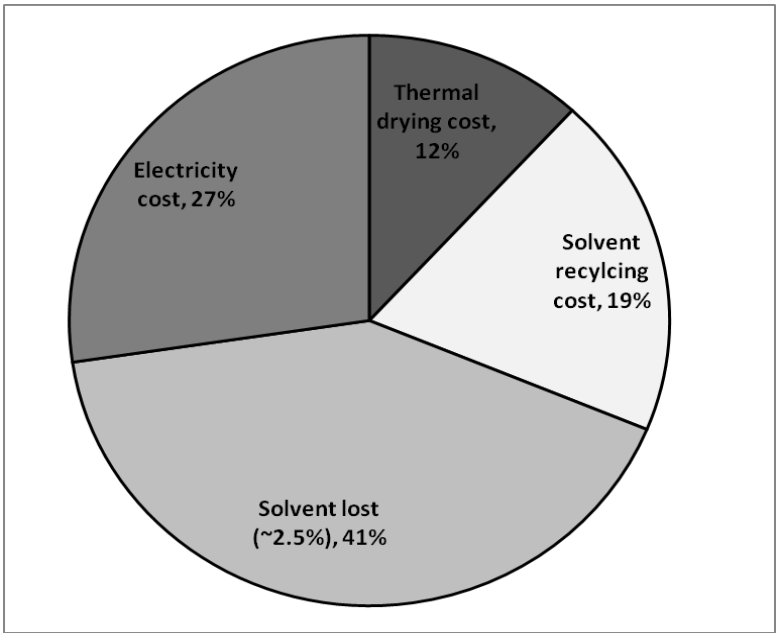


Figure 6.5. Cost distribution of CFLES using biodiesel as co-solvent

Cost estimates for MAE-CS-100^{o}C technique was $21.26 per gallon as compared to $23.64 for the Sol-Mech. This technique ranked sixth on the oil recovery:cost ratio. The technique required significant amounts of solvent, controlling a significant proportion of the cost (approximately 86%). The advantage of MAE is that the post-harvest algal paste can be used without further drying. This avoids the cost of thermal drying. Compared to Sol-Mech., the electricity cost (equipment operation) for MAE was 57% less, but, 23% higher than the CFLES. Because of the large amount of solvent used, approximately 60% and 26% of the cost is associated with solvent loss and solvent recovery respectively (Figure 6.6). Efficiency of microwave energy is not accounted for in these calculations, however, extraction efficiency is assumed to compensate for this deficiency. Generally microwave energy is reported 60 – 65% efficient (Barnard et al., 2007; Moseley and Wooman, 2009) which is expected to further

increase the extraction cost. The cost per gallon was approximately 10% lower than that of the Sol-Mech. along with significant energy savings (approximately 43%) due to lack of thermal drying.

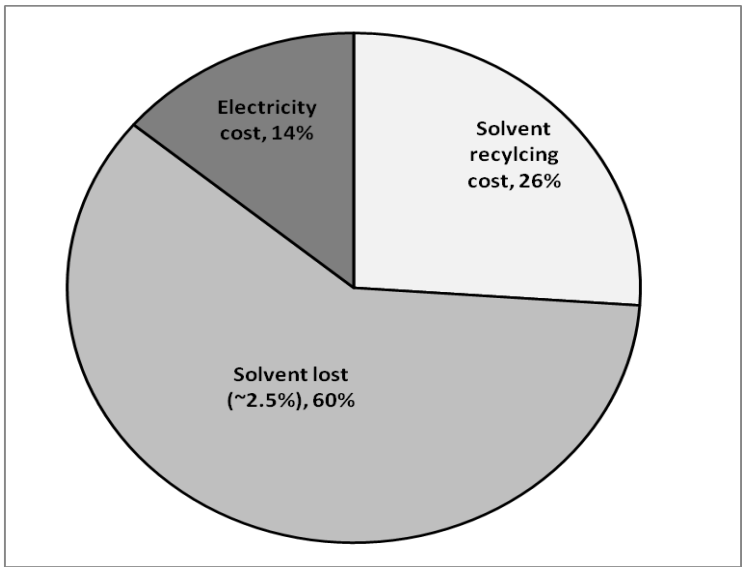


Figure 6.6.. Microwave-assisted extraction with conventional solvents; 2:1 of solvent to feed (MW-CS-100^{o}C)

6.3.5 Microwave-assisted With Biodiesel and Ethanol; 2:1 of Co-solvent to Feed, (MAE - BD-100^{o}C)

This technique is similar to MW-CS-100^{o}C except the 12.5% of hexane is replaced with biodiesel. Biodiesel acts like hexane to creat a biphasic system in the presence of water. This reduced the cost of extraction by approximately 9% (from $21.26 to $19.43 per gal). An important assumtion in this case is that the efficiency of extraction is not affected significantly while replacing the 12.5% hexane fraction with an equal volume of biodiesel. The main purpose of hexane or biodieel is to create a biphasic solution for easy separation of the extracts. Results of the study mentioned earlier (Chapter 4) however indicate that the use of 40% biodisel instead of conventional solvents in MAE at 100^{o}C reduced the extraction efficiency from 93% to approximately 78%. The oil:cost ratio ranked this method as fourth. The cost distrubution was close to that of the MAE-CS-100^{o}C (Figure 6.7). Increasing the proportion of biodiesel in the

co-solvent system may help reduce the cost if the efficiency is increased proprtionally. The value of environmental and health safety can not be ignored when compared to conventional solvents. The cost per gallon was approximately 10% lower than that of the Sol-Mech. Significant energy saving compared to Sol-Mech. (approximately 45%) was observed while avoiding thermal drying or recovery of biodiesel. Electricity energy consumption was approximately 56% lower than Sol-Mech.

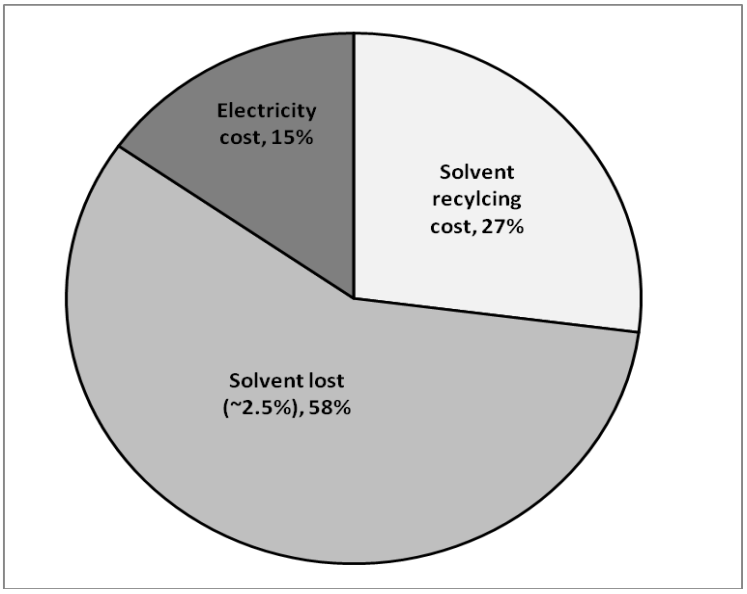


Figure 6.7. Microwave-assisted with biodiesel and ethanol; 3:1, solvent to feed, (MW-BD-100^{o}C)

6.3.6 Microwave-assisted with 40% Biodiesel in Ethanol; 0.8:1.2:1 of Biodiesel: Ethanol: Feed (MAE-BD-120^{o}C)

This technique is based on the findings of 40% biodiesel as co-solvent with ethanol used in closed-vessel microwave assisted extraction mentioned earlier (Chapter 4). The lipid extraction efficiency of microwave assisted extraction was found more than 100% at 120^{o}C temperature in 15 min. Compared to MAE-BD-100^{o}C, the proportion of biodiesel solvent was increased from 12.5% to 40%. Since solvent recovery and loss are the dominant driving forces to affect the cost of extraction, the total cost of extraction decreased significantly with decrease in solvent volume. The cost per gallon was estimated as $13.27. Cost of solvent lost, solvent

recycling, and electricity contributed approximately 54%, 25%, and 21% respectively to the overall extraction cost (Figure 6.8). Among the MAE techniques analyzed this was found to be the most favored technique based on the oil:cost ratio rank of second. The drawbacks of this method include high temperature which may cause degradation of thermosensitive chemicals, although in the current study mentioned earlier it was shown that essential fatty acids such as DHA and EPA were not affected significantly by the high temperature. High temperature extraction has one important advantage in downstream process having reduced heating requirement for co-solvent recovery; ethanol in this case. Significant savings for this technique compared to Sol-Mech. are approximately 43% reduction in extraction cost, and approximately 56% in energy savings (Table 6.3) since thermal drying is not required and the reduced volume of solvent reduced the recovery cost and energy. Electricity cost was estimated lower than the Sol-Mech. by approximately 57%.

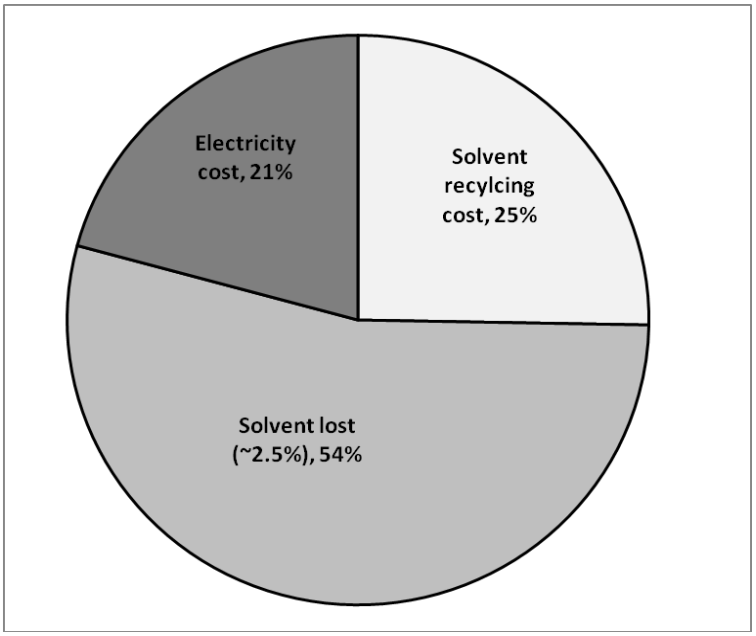


Figure 6.8. Microwave-assisted with 40% biodiesel in ethanol; 0.8:1.2:1 of biodiesel: ethanol: feed (MAE-BD-120^{o}C)

Table 6.3. Breakdown of energy consumption (kWh) estimated for extraction systems analyzed

		Sol-Mech.	CFLES-CS-100°C	CFLES-BD-100°C	MAE-CS-100°C	MAE-BD-100°C	MAE-BD-120C
Thermal drying		6468.30	924.04	924.04	-	-	-
Solvent recycling	Hexane	990.22	396.09	-	275.06	-	-
	Ethanol	-	1518.45	1518.45	4920.91	4920.91	3374.34
Electricity		6405.93	2565.00	2565.00	2775.33	2775.33	2775.33
Total Energy consumed		13864.46	5403.58	5007.49	7971.30	7696.24	6149.67
Savings compared to Sol-Mech.		-	61.0%	63.9%	42.5%	44.5%	55.6%

6.4 Discussion

The main economic challenge for algae biofuel production is to produce low cost harvesting and lipid extraction methods so the price per gallon is competitive to that of petro diesel. Estimates reported so far, as well as the current analysis, indicate that the cost of algal biofuel is several folds higher than petro diesel. The Algae 2020 study has reported the estimated costs to produce algae biofuels between $9 and $25 per gallon in ponds, and $15–$40 in photobioreactors (PBRs) (Thurmond, 2009; Sing and Gu, 2011). Lowering the production costs will require reducing the number of costly steps in production, harvesting, extraction, and drying systems (Sing and Gu, 2011).

Current analysis indicated that the extraction cost is predominantly controlled by the cost associated with the use of solvents as compared to electricity. Losing a fraction of the solvents affects the overall extraction cost estimates significantly. This is more accurate for the CFLES or MAE extractions than the Sol-Mech. extraction. In the later case, the dominant cost is associated with the thermal drying of biomass. Recovery of solvents is more economical as compared to thermal dehydration. Energy consumption for the later is 0.714kWh kg^{-1} as compared to 0.129kWh kg^{-1} for hexane and 0.275kWh kg^{-1} for ethanol (calculated at $25^{o}C$ initial temperature). Thermal drying hence attracts the least priority as compared to solvent recovery. Cost estimates are greatly affected by solvent lost. Minimizing solvent loss significantly reduce the costs. Based on these findings, biodiesel as co-solvent has the potential to avoid the cost of thermal dehydration, solvent recovery, as well as solvent loss. Further research to enhance its extraction efficiency, toxicological effects, and recovery of useful bioproducts other than TAGs or fatty acids is recommended. Total extraction cost for the techniques assessed in the current economic analysis range between $10.22 and $23.64 per gallon. CFLES cost estimates are the lowest ($10.22) followed by MAE with biodiesel. By replacing conventional solvent with

biodiesel, the CFLES cost decreased by approximately 24%, and MAE cost decreased by 9%. The CFLES cost was however offset by 33% drop in its efficiency which actually increased the cost estimates by 13%. Raising MAE temperature from 100^{o}C to 120^{o}C increased the efficiency to 100% thereby decreasing the cost per gallon significantly by approximately 32%.

Economic analysis of algal biofuel production has been reported previously by different authors. Sun and co-workers (2011) reported that the estimated cost of production varies between \$1 to \$42 gal^{-1} based on previous studies conducted by different authors. Most of the cultivation systems analyzed were open ponds but also included were hybrid and PBR systems. The estimated average cost per gallon for biofuel production was reported as \$19.3 gal^{-1} with a standard deviation of \$28.8 gal^{-1} (Sun et al., 2011). Similarly, Lardon et al. (2009) reported the energy consumption for dry and wet extraction for biodiesel production from microalgae. The wet extraction consumed 74.4% energy while the dry extraction consumed 93% (84.9% for drying and 8.1% for oil extraction) of energy. The balance is consumed by algae culture and harvesting. Assuming the cost of wet extraction estimated in the current analysis (Table 6.2) covers 74.4% of the biodiesel production cost (reported by Lardon et al., 2009), the cost of one gallon of algal oil is now estimated for CFLES-CS-100^{o}C as \$13.73 gal^{-1}; CFLES-BD-100^{o}C as \$15.58 gal^{-1}; MW-CS-100^{o}C as \$28.58 gal^{-1}; MW-BD-100^{o}C as \$26.12 gal^{-1}; and MW-BD-120^{o}C as \$17.83 gal^{-1}. Similarly, assuming the cost estimated for dry extraction using Sol-Mech. (\$23.64 gal^{-1}) covers 93% of the cost, the new estimated cost using Sol-Mech. is \$25.42 gal^{-1}. The average estimated cost based on Lardon et al. (2009) is \$21.21 gal^{-1}.with a standard deviation of \$6.25 gal^{-1} which is not significantly different ($p>0.05$) than those estimated by Sun et al. (2009). The analysis reported are dominantly based on energy consumption which does not include labor or any returns associated with other bioproducts derived from algae. Another

estimate based on different studies compiled by Carriquiry et al. (2011) indicated the median cost as \$16.27 gal^{-1} which is higher than that of the CFLES in the current analysis.

Another significant cost analysis was performed by Molina Grima and co-workers (2003). They estimated the biomass production and harvesting cost as 43%, extraction-esterification as 53%, and processing to the final product as 4%. The estimates were based on wet extraction process using wet biomass more or less similar to CFLES or MAE. Applying Molina Grima et al. (2003) cost distributions to those obtained in the current analysis, assuming the current cost per gallon in Table 6.2 covers the above mentioned 53% as extraction-esterification cost, the estimated production cost for one gallon of algal biodiesel is: Sol-Mech., \$44.60; CFLES-CS-100^{o}C, \$19.28 gal^{-1}; CFLES-BD-100^{o}C, \$21.28 gal^{-1}; MW-CS-100^{o}C, \$40.12 gal^{-1}; MW-BD-100^{o}C, \$36.67 gal^{-1}; and MW-BD-120^{o}C, \$25.03 gal^{-1}.The average cost is \$31.26 gal^{-1} with a standard deviation of \$10.55 gal^{-1} which is approximately 32% higher than those based on Sun et al. (2009). The cost estimated is however, not significantly different than those reported by Sun et al. (2009) ($p>0.05$).

The current economic analysis shows high cost estimates compared to some of the analysis reported previously (Benemann and Oswald, 1996; Huntley et al., 2006). The differences are attributed to the variation in the assumed yields (Carriquiry et al., 2011). Benemann and Oswald (1996) estimated the operation cost for open ponds in the \$51 – \$90 per barrel range (\$1.21 – 2.14 gal^{-1}, accounted for inflation to 2011 by Carriquiry et al., (2011)), for two different yield levels and CO_2 supply methods. This estimate was based on 400 hectares of open ponds with productivity assumptions of 30 – 60 g m^{-2} day^{-1} and 50 dry weight % lipid contents. Such high yields are theoretically possible but have not been demonstrated (Schenk et al., 2008). Carriquiry et al. (2011) reported that almost all of the recent estimates are much higher than the numbers presented by Benemann and Oswald, which serves as starting point for many

researchers. Another estimate provided by Huntley et al. (2006) shows algae oil production costs as $84 US/bbl ($2/gal) based on assumptions of Benemann and Oswald (1996) but utilized a hybrid system consisting of open ponds inoculated with a desired strain cultivated in a bioreactor with an aerial productivity of 70.4 g m^{-2} day^{-1} and 35 dry weight % lipid contents (Huntley et al., 2006; Schenk et al., 2008).

In summary, the current economic analysis indicated that continuous flow lipid extraction system (CFLES) with conventional solvents reduced the estimated extraction cost significantly (approximately 57% along with energy savings up to 61%) as compared to solvent extraction coupled with mechanical extractor (Sol-Mech.). Use of biodiesel further increased the energy savings of CFLES to 64%. CFLES efficiency was dropped by 33% with the use of biodiesel, which requires further investigation to enhance its efficiency. Microwave-assisted extraction (MAE) with conventional solvents has demonstrated cost estimates comparable to those of the Sol-Mech. However, extraction at 120^{o}C and the use of 40% biodiesel with ethanol reduced the cost significantly (44%), as compared to Sol-Mech., along with 57% energy savings.

The average extraction cost for all the techniques assessed in the current analysis was estimated as $16.57 with a standard deviation of $5.59. Based on the estimated extraction cost and previous studies, the total cost of microalgae oil production is estimated in the range of $13.73 to $ 28.58 gal^{-1} based on Lardon et al. (2009) or $19.28 to $44.60 gal^{-1} based on Molina Grima et al. (2003). Lowest extraction cost estimates were observed with CFLES using conventional solvents. The post-extraction microalgae residue is expected to help the process economics as a value added product for animal food and energy.

CHAPTER 7

SUMMARY AND CONCLUSION

Besides nutraceutical and pharmaceutical products obtained from microalgae, it is the most important source of renewable biofuels in the near future. Many species of algae are rich in oil with potential for biodiesel. Some species are potentially reported to contain oil exceeding 80% of dry weight of algae biomass (Demirbas, 2011). Economic viability of the process in terms of minimizing the operational and maintenance costs, along with maximization of oil-rich microalgae production has been reported the key factors in commercialization of microalgae-based fuels (Sing and Gu, 2010). For instance harvesting costs contribute 20 – 30% to the total cost of algal biomass (Grima et al., 2003). Similarly, harvesting and extraction processes together may contribute 40 – 60% of the total cost (Grima et al., 2003; Gudin and Thepenier, 1986). As of now, low-cost microalgal oil appears to be a long term goal. Challenges exist at different levels of the production process namely cultivation, harvesting, extraction, and conversion. Significant research work has been conducted to meet the challenges confronted during cultivation e.g. identification of oil rich strains, environmental conditions and infestation of foreign unwanted strains. Research and development work is still needed for economical and energy efficient harvesting. Harvesting microalgal biomass from more than 99% of water is the most energy intensive step in production process. Harvesting method and the water content in the harvested biomass has a crucial role in determining the right lipids extraction approach. Significant research work is still needed to explore cost-effective techniques. An extraction approach capable of assimilating significantly wet biomass can minimize the cost of production e.g. CFLES and hydrothermal liquefaction. The later is, however, not cost-effective because of the high temperature and pressure requirements. Extraction techniques requiring dry biomass (such as

supercritical CO_2 extraction and mechanical extraction) is similarly not cost-effective because drying microalgae is very energy intensive. The next step in production process is conversion which is dependent upon the extraction approach selected. Bio-oil obtained with thermochemical or hydrothermal liquefaction cannot be used as transportation fuel unless further refined or hydro-treated. Extractions leading to production of triglycerides (TAGs) and other valuable products is the best case scenario. TAGs can easily be converted into biodiesel which do not require any modification in the existing vehicles engine.

This project was an attempt to help improve the process economics of microalgal lipids extraction by devising a laboratory made continuous flow lipid extraction system (CFLES) and testing biodiesel as a nonhazardous as well as economical co-solvent. The CFLES was used to extract lipids from *Nannochloropsis sp.* biomass. The system was tested at ambient, 80°C , 100°C and 120°C temperatures and ambient, 50 psi, and 500 psi pressures to reach an optimum conditions of temperature and pressure in terms of efficiency.

Initially the CFLES was tested with conventional solvents (chloroform and ethanol). The study confirmed that the moderate temperature and pressure of 100°C and 50 psi used in the CFLES readily extracted the microalgal oil than what was extracted at extreme (low or high) temperatures and pressures. The extraction efficiency is higher than most of the extraction methods reported previously for microalgae including supercritical CO_2 extraction (36%) (Valderma et al., 2003; Krichnavaruk et al., 2008), thermochemical and hydrothermal liquefaction (37 – 64%) (Sawayama et al., 1995; Minowa et al., 1995; Brown et al., 2010), pressurized liquid extraction (20 – 40%) (Jaime et al., 2005; Rodriguez-Meizoso et al., 2008), and microwave assisted extraction (28 – 77%) (Lee et al., 2010; Balasubramanian et al., 2011). The operating temperature and pressure are workable for scaled up continuous CFLES if environment and health friendly solvents are used.

The CFLES was then tested with 40% biodiesel (methyl soyate) as co-solvent in ethanol (BD40). Biodiesel and ethanol has less environmental and health concerns when compared to conventional solvents like chloroform, hexane and methanol. This appears to be the first study to report biodiesel as co-solvent for extraction of biochemicals. Efficiency comparable to most of the available extraction techniques was achieved using biodiesel as a co-solvent. The use of biodiesel as a co-solvent has the potential to avoid one step of separating solvent from the extracts if the desired end product is biodiesel. Nutraceutical products (EPA and DHA, 100% and 75% recoveries respectively) were successfully extracted. Most favored conditions were found 50 psi and 100°C, though comparable results were obtained at higher temperature and pressure. Further investigation is suggested to see the effect of higher than 40% proportion of biodiesel on the extraction performance. Approximately 70% moisture in the biomass had no effect on the extraction performance. The operating temperature and pressure (100^{o}C, 50 psi) are workable for scaled up continuous CFLES. Most of the techniques for microalgae lipid extraction have been tested at laboratory bench scale. Therefore, there is a need for extraction methods tested beyond the laboratory scale i.e. pilot or larger scale production. The study showed that biodiesel is a good solvent. The energy saving with CFLES and biodiesel co-solvent was approximately 91%, while the solvent savings were approximately 90%, assuming 50% of the ethanol co-solvent is recycled. Because of the high flash point, its penetration power into the biomass matrix is expectedly low lowers the diffusion rates and mass transfer of the solute. Addition of a co-solvent like ethanol is hence suggested.

To further investigate the solvent potential of biodiesel for microalgal lipids extraction, microwave assisted extraction (MAE) was employed. The co-solvent system had comparable results to those of chloroform plus ethanol or conventional 8 h Soxhlet extraction. This study also confirmed that toxic solvents like hexane and methanol can successfully be substituted with

less toxic, environment friendly, biodegradable solvents to extract lipids from microalgae. Employing such a co-solvent system is comparatively economical; avoiding one step of solvents separation since ethanol serve as reactant in transesterification reaction. Approximately 66%, 78%, and 116% efficiency was noted with BD40 (40% biodiesel) at 80°C, 100°C, and 120°C respectively as compared to 8 h Soxhlet extraction. The BD20 (20% biodiesel) on the other hand extracted 27%, 34%, and 24% of oil at 80°C, 100°C, and 120°C temperatures respectively. More research work is, however, suggested to further investigate the effect of biodiesel, high temperature and microwaves on fatty acids of nutraceutical significance e.g. EPA and DHA, as well as their further refining.

The current research work indicates that continuous flow lipid extraction system (CFLES) has optimum efficiency at 100°C temperature and 50 psi pressure while using conventional solvents or biodiesel. The efficiency dropped from 100% to 67% after switching from conventional solvents to biodiesel. Approximately 78% efficiency of biodiesel co-solvent in microwave assisted extraction (MAE) was also found comparable to most of the extraction techniques.

A case-scenario postulated to determine the process economics of the extraction part of microalgae biofuel production indicated that the cost to extract one gallon of oil using CFLES was $10.22, compared to $23.64 for solvent extraction coupled with mechanical extractor commonly used in soybean industry. The CFLES with conventional solvents hence save approximately 57% of extraction cost, and approximately 61% of energy consumption as compared to solvent extraction coupled with mechanical extractor. Solvent extraction with mechanical extractor requires dry biomass which was considered the most energy consuming step. In CFLES on the other hand, solvent recovery and loss were the dominant factors affecting the cost of scaled up extractions. Replacing conventional solvents (i.e. hexane, methanol,

chloroform etc) with biodiesel and ethanol reduced the cost associated with solvent recovery and loss. These savings were however, offset with reduced efficiency of 67% compared to 100% of using conventional solvents. It is worth mentioning that efficiency of biodiesel and ethanol co-solvent was found comparable to most of the other methods reported (Table 5.4mentioned earlier) besides environmental and health benefits. Compared to solvent extraction coupled with mechanical extractor, use of biodiesel co-solvent estimates approximately 51% less in extraction cost and 64% less in energy consumption.

Microwave-assisted extraction has been successfully performed in the lab studies for microalgal extraction. Large or pilot scale microwave assisted extraction has not been reported except for grain crops like soybean. A major proportion of the cost estimates were associated with the use of solvents (86%). The use of microwave extraction has the advantage that moisture contents in the biomass can help reduce the cost of drying. One major disadvantage of MAE, compared to CFLES, was that the electricity cost for using microwave energy was estimated 23% higher (but 57% lower than solvent plus mechanical extraction). Cost per gallon using conventional solvents containing 12.5% hexane was estimated as $21.26. Switching hexane with biodiesel solvent reduced the cost per gallon estimates by approximately 9% to $19.43. Based on the current study, the biodiesel proportion was further bumped up to 40% in ethanol (2:1, v/v, co-solvent:biomass) with 100% efficiency at 120^{o}C temperature. The estimated cost per gallon reduced significantly to $13.27, which is approximately 38% lower than the microwave-assisted extraction using conventional solvents.

As mentioned earlier, the previous reports estimated the average cost per gallon for biofuel production as $19.30 gal^{-1} with a standard deviation of $28.80 gal^{-1} (Sun et al., 2009). Subjecting the cost of extraction estimated in the current analysis to that reported by Lardon et al (2009), the average cost of algal biofuel production is $21.21 gal^{-1}, with a standard deviation of

$6.25gal^{-1}, which is not significantly different ($p>0.05$) than those estimated by Sun et al. (2009). Similarly, by applying the cost distribution reported by Molina Grima et al. (2003) to those obtained in the current analysis, the average cost is estimated as $31.26 gal^{-1}, with a standard deviation of $10.55 gal^{-1}, which is approximately 32% higher than those based on Sun et al. (2009), although not significantly different than that reported by Sun et al. (2009) ($p>0.05$).

The current study indicated that continuous flow lipid extraction system (CFLES) reduced the estimated extraction cost significantly and was further assisted by the use of biodiesel co-solvent. Biodiesel co-solvent used in CFLES and MAE has shown extraction efficiencies comparable to most of the extraction methods reported in the literature. The study suggests further investigation of biodiesel as solvent in the extraction chemistry.

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APPENDIX A1. FATTY ACID PROFILE; CFLES EXTRACTS USING CONVENTIONAL SOLVENTS

Fatty acid profile (% of total fatty acid methyl esters) extracted from *Nannochloropsis sp.* under different temperature and pressure combinations in CFLES using conventional solvents chloroform and ethanol (1:2 v/v) and Soxhlet extraction (n=3).

Fatty acid	AmbT,P	AmbT,50psi	AmbT,500psi	80T,AmbP	100T,AmbP	120T,AmbP
C10:0	0±0	0±0	0±0	0±0	0±0	0±0
C11:0	0±0	0±0	0±0	0±0	0±0	0±0
C12;0	20.37±0.9	19.57±1.1	23.02±0.1	7.17±0.4	22.1±1.2	9.54±0.2
C13;0	0±0	0±0	0±0	0±0	0±0	0±0
C14;0	15.97±0.6	14.33±1	16.36±0.2	5.1±0.4	15.33±0.8	5.67±0.1
C14:1	1.76±1.7	3.25±0	0±0	0.22±0.2	0±0	0±0
C15:0	0±0	0±0	0.06±0	0.09±0	0.04±0	0.09±0
C15:1	0±0	0±0	0±0	0±0	0±0	0.42±0.4
C16:0	20.17±1.6	15.65±1.9	23.78±0.8	26.95±1.4	28.83±1.4	22.29±0.5
C16:1	20.36±1.7	23.9±2.4	10.41±0.4	15.9±0.6	4.04±0.3	2.26±0
C17:0	0±0	0±0	0±0	0±0	0.04±0	0±0
C17:1	1.85±1	0.63±0.4	0.77±0.7	0.61±0.3	0.43±0.2	0.82±0.1
C18:0	7.37±1.9	4.64±0	6.5±0.4	5.64±0.4	9.02±0.3	4.9±0.1
C18:1	4.33±0.5	10.99±6.3	8.16±0.1	20.69±3.8	13.13±3.4	34.58±1.1

C18:2	4.39±2.3	3.69±2	3.85±1.5	4.03±0.9	2.54±0.7	7.3±0.3
C18:3	1.61±0.1	1.17±0.3	2.01±0.6	6.74±0.5	1.12±0.3	2.67±0.6
C18:3	0.91±0.1	0.85±0.1	3.89±0.5	5.85±1.6	1.99±0.1	7.56±0.1
C20:0	0±0	0±0	0±0	0.06±0	0±0	0±0
C20:1	0.46±0.1	0.99±0.4	0.93±0.1	0.42±0.2	1.03±0.1	0.92±0.1
C20:2	0±0	0±0	0±0	0±0	0±0	0±0
C20:3	0±0	0±0	0±0	0±0	0±0	0±0
C20:4	0±0	0±0	0±0	0±0	0±0	0±0
C20:3	0±0	0±0	0±0	0±0	0±0	0±0
C20:5	0±0	0±0	0±0	0±0	0±0	0±0
C22:0	0±0	0±0	0±0	0.18±0.1	0.13±0	0.14±0
C22:1	0±0	0±0	0±0	0±0	0±0	0±0
C22:2	0.34±0.1	0.22±0	0.18±0.1	0.23±0	0.15±0	0.43±0.1
C23:0	0±0	0±0	0±0	0±0	0±0	0±0
C24:0	0.04±0	0.02±0	0±0	0.03±0	0.01±0	0.06±0
C24:1	0±0	0±0	0±0	0±0	0±0	0±0
C22:6	0±0	0±0	0±0	0±0	0±0	0.26±0.1

Total Sat	63.95±1.9	54.24±3.6	69.75±1.4	45.25±2.9	75.49±3.7	42.72±1
Tot Unsat	36.04±1.9	45.75±3.6	30.24±1.4	54.74±2.9	24.5±3.7	57.27±1
1	28.77±2.8	39.79±4.2	20.29±0.2	37.87±3.6	18.63±3.1	39.01±0.7
2	4.73±2.3	3.92±2.1	4.04±1.6	4.26±0.9	2.7±0.7	7.74±0.3
3	2.52±0.2	2.03±0.4	5.91±0.7	12.59±2	3.11±0.4	10.24±0.5
4 or >4	-	-	-	-	-	0.26±0.1

Amb = Ambient; T = Temperature, ^{o}C; P = pressure, psi; "–" = Non detect; Sat = Saturated fatty acids; Unsat = unsaturated fatty acid; 1, 2, 3, 4 = mono-, di-, tri-, and polyunsaturated FAMEs

APPENDIX A1. (continued) ……..

Fatty acid	80T,50psi	100T,50psi	120T,50psi	80T,500psi	100T,500psi	120T,500psi	Soxhlet
C10:0	0±0	0.08±0	0±0	0±0	0±0	0±0	0.83±0
C11:0	0±0	0±0	0±0	0±0	0±0	0±0	0±0
C12;0	20.68±0.6	11.93±0.6	12.91±0.2	18.8±0.7	20.04±3	9.56±0.4	14.83±0.4
C13;0	0±0	0±0	0±0	0±0	0±0	0±0	0±0
C14;0	12.8±0.3	8.47±0.2	9.91±0.2	10.63±0.4	12.24±1.8	5.77±0.2	8.05±0.8
C14:1	0±0	1.07±0.5	2.24±0.3	0±0	0±0	0±0	10.18±3.5
C15:0	0.04±0	0.08±0	0.08±0	0.04±0	0±0	0.04±0	0.17±0
C15:1	0±0	1.1±0.1	0±0	0±0	0±0	0±0	0±0
C16:0	26.16±0.6	22.46±0.7	28.46±0.3	27.3±0.6	30.94±0.7	14.96±0.7	21.27±0.2
C16:1	11.54±0.6	3.47±0	12.27±0.2	10.88±0.3	6.65±0.7	19.79±1.4	6.62±2.5
C17:0	0±0	0.31±0	0.01±0	0±0	0±0	0±0	0.06±0
C17:1	0.42±0.2	0.51±0.1	0.24±0.2	0.82±0.8	2.14±1.1	0.15±0.1	1.87±0
C18:0	6.4±0.1	10.04±0.3	5.79±0.2	6.35±0.5	5.32±0.8	2.92±0.1	3.28±0
C18:1	8.61±0.3	37.48±0.6	10.45±0.2	10.54±0.2	8.2±1.7	37.03±0.9	11.63±0
C18:2	2.65±0.1	1.16±0	4.88±0.6	4.39±1.4	5.05±1.9	3.32±1.2	4.03±0
C18:3	2.68±0.8	0.24±0	5.04±0.8	0.61±0.3	1.39±0.8	1.22±0.4	1.33±0
C18:3	6.99±0	0.56±0	6.84±0.8	7.68±0.3	5.64±2.1	2.8±0.2	15.62±0.6
C20:0	0±0	0±0	0±0	0±0	0.03±0	0.09±0	0±0
C20:1	0.52±0	0.12±0	0.2±0.1	1.38±0.5	1.26±0.2	1.75±0.2	0±0
C20:2	0±0	0±0	0±0	0±0	0±0	0±0	0±0

C20:3	0±0	0±0	0±0	0±0	0±0	0±0	0±0
C20:4	0±0	0±0	0±0	0±0	0±0	0±0	0±0
C20:3	0±0	0±0	0±0	0±0	0±0	0±0	0±0
C20:5	0±0	0±0	0±0	0±0	0±0	0±0	0±0
C22:0	0±0	0.56±0	0.08±0	0±0	0.09±0	0.03±0	0.2±0
C22:1	0±0	0±0	0±0	0±0	0±0	0±0	0±0
C22:2	0.41±0.2	0.11±0	0.42±0.2	0.51±0	0.8±0.4	0.4±0.2	0.16±0
C23:0	0±0	0±0	0±0	0±0	0±0	0±0	0±0
C24:0	0.03±0	0.15±0	0.1±0	0±0	0.13±0	0.09±0	0.13±0
C24:1	0±0	0±0	0±0	0±0	0±0	0±0	0±0
C22:6	0±0	0±0	0±0	0±0	0±0	0±0	0±0
Total Sat	66.13±1.2	53.8±0.5	57.35±0.6	63.15±1.3	68.83±4.3	33.5±1.1	48.79±1.6
Tot Unsat	33.86±1.2	46.19±0.5	42.64±0.6	36.84±1.3	31.16±4.3	66.49±1.1	51.2±5.3
1	21.1±0.4	43.78±0.4	25.42±0.1	23.64±0.6	18.27±1.8	58.73±0.7	30.32±6
2	3.07±0.1	1.28±0	5.31±0.8	4.91±1.5	5.85±2.4	3.73±0.9	4.2±0.1
3	9.68±0.8	0.8±0	11.89±0.2	8.29±0.6	7.03±2.9	4.02±0.6	16.96±0.7
4 or >4	-	-	-	-	-	-	-

Amb = Ambient; T = Temperature, ^{o}C; P = pressure, psi; "–" = Non detect; Sat = Saturated fatty acids; Unsat = unsaturated fatty acid; 1, 2, 3, 4 = mono-, di-, tri-, and polyunsaturated FAMEs

APPENDIX A2. FATTY ACID PROFILE; CFLES EXTRACTS USING BIODIESEL CO-SOLVENT (BD40)

Fatty acid profile (% of total fatty acid methyl esters; FAMEs) of soya methyl esters, algal oil, and the change in their profile during extraction of lipids from *Nannochloropsis sp.* with biodiesel co-solvent under different temperature and pressure combinations in CFLES (n=3).

Compound Name	Soya methyl esters	Algal methyl esters	Amb T,P	80 T, AmbP	80T, 50psi	80T, 500psi
Decanoic acid methyl ester (C10:0)	0±0	0±0	0±0	0±0	0±0	0±0
Undecanoic acid methyl ester (C11:0)	0±0	0±0	0±0	0±0	0±0	0±0
Dodecanoic acid, ME (C12:0) M.Laurate	0.1±0	0.6±0.1	0±0	0±0	0±0	0±0
Tridecanoic acid, ME (C13:0)	0±0	0±0	0±0	0±0	0±0	0±0
Methyl Tetradecanoate (C14:0)	1.4±0.1	3.8±0.3	1.1±0.1	1.3±0	1.3±0.1	1.1±0
Myristoleic Acid ME (C14:1)	0±0	0.5±0.1	2.9±0.1	2.6±0.2	2±0.1	1.8±0
Pentadecanoic acid, ME (C15:0)	0.3±0	1.8±0.2	0.3±0	0.3±0	0.3±0	0.3±0
Cis-10-Pentadecenoic Acid ME (C15:1)	0±0	0±0	0±0	0±0	0.3±0	0.2±0.1
Methyl Palmitate (C16:0)	14.3±2.9	10±2.7	14.5±2.1	15.4±2.8	13.9±2.5	14.4±0.3
Methyl Palmitoleate-Cis-9 (C16:1)	4.3±0.2	28.9±1.7	3.3±0.1	3.7±0.1	3.9±0.2	3.3±0.2
Heptadecanoic acid, ME (C17:0)	2.9±0.1	1±0.1	2.4±0.1	2.7±0.1	2.6±0.1	2.4±0
Heptadecenoic Acid-Cis10, ME (C17:1)	1.5±0.1	3.4±0.2	1.2±0	1.3±0	1.4±0	1.2±0
Methyl stearate (C18:0)	16.4±2.2	11±0.8	17.9±3	17.2±2.7	19±1.7	24.6±6.9

Cis-9-Oleic ME (C18:1)	10.9±0.4	7.9±1.8	11.1±0.6	11.7±1.7	10.7±0.5	9.9±0.5
Methyl Linoleate (C18:2)	8.6±0.3	5.7±0.5	6.1±1.1	7.7±0.5	7.1±1.3	7±0.3
Methyl Linolenate (C18:3)	0±0	2.5±2.5	3.6±0.1	1.3±0.1	1.2±0.1	1.6±0.1
Gamma-Linilenic Acid ME (C18:3)	7.7±1	9.4±2.6	6.7±1.8	7.6±2.1	8.6±3.3	4.9±0.1
Methyl Arachidate (C20:0)	9.9±1.3	2.6±0.2	9±0.4	8.4±1.6	6.3±1.6	9.8±1
Cis-11-Eicosenoic acid, ME (C20:1)	5.6±0.1	2.9±0.1	4.5±0.3	4.5±0.1	4.9±0.2	4.5±0.2
Cis-11,14-Eicosadienoic acid ME (C20:2)	1.3±0.1	0.4±0.2	1±0	1.1±0	1.1±0.1	1±0.1
Cis-8,11,14-Eicosatrienoic ac ME (C20:3)	0.8±0	0±0	0.7±0.1	0.3±0.3	0.8±0.1	0.2±0.2
Cis-5,8,11,14-Eicosatetraenoic..(C20:4)	0±0	0.5±0.1	0.1±0.1	0±0	0±0	0±0
Cis-11,14,17-Eicosatrienoic Acid (C20:3)	0±0	0±0	0.4±0.4	0.6±0.6	0±0	1.1±0.6
Cis-5,8,11,14,17-Eicosapentaenoic (20:5)	0.8±0.1	2.6±0.1	2.1±1.5	0.7±0	2.3±1.4	2.6±2
Methyle Behenate (Docosanoate) (C22:0)	11.7±0.3	3.9±2	9.8±0.3	10.5±0.3	10.7±0.2	6.4±3.2
Cis--13-Docosenoic acid, me (C22:1)	0±0	0±0	0.2±0.2	0±0	0±0	0.5±0.3
Cis-13,16-Docosadienoic acis me (C22:2)	0.1±0	0.1±0	0±0	0±0	0.1±0	0±0
Tricosanoic acid, methyl ester (C23:0)	1.2±0.1	0.1±0	1±0	1±0.1	1.2±0	1±0
Tetracosanoic acid, methyl ester (C24:0)	0±0	0±0	0±0	0±0	0±0	0±0
15-Tetracosenoic acid, methyl ester, (Z)	0±0	0±0	0±0	0±0	0±0	0±0

4,7,10,13,16,19-Docosahexaenoic..(C22:6)	0±0	0.4±0.1	0±0	0.1±0.1	0.2±0.1	0.2±0.1
Saturated FAMEs	58.3±7	34.9±6.3	56±6	56.8±7.6	55.4±6.2	60±11.4
Unsaturated FAMEs	41.7±2.3	65.1±10.1	44±6.4	43.2±5.8	44.6±7.3	40±4.6
∑ mono-unsaturated FAMEs	22.3±0.7	43.5±4	23.2±1.4	23.9±2.2	23.2±1.1	21.3±1.2
∑ di- unsaturated FAMEs	10.1±0.4	6.1±0.7	7.1±1.1	8.8±0.6	8.3±1.3	8.1±0.4
∑ Tri- and higher unsaturated FAMEs	9.4±1.2	15.5±5.3	13.7±4	10.5±3.1	13.2±4.9	10.6±3

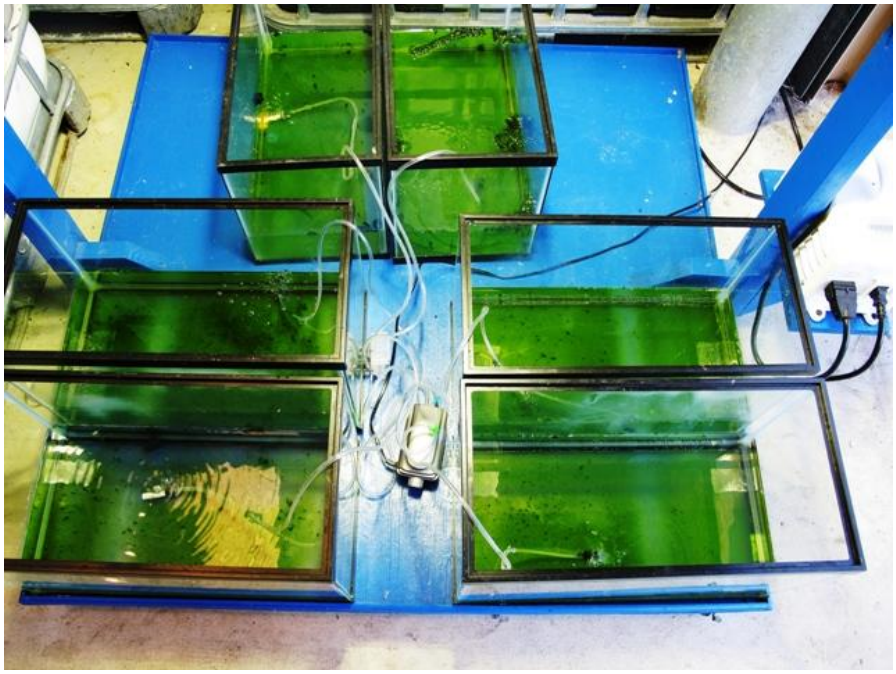
APPENDIX A2, continued.

Compound Name	**100T, AmbP**	**100T, 50psi**	**100T, 500psi**	**120T, AmbP**	**120T, 50psi**	**120T, 500psi**
Decanoic acid methyl ester (C10:0)	0±0	0±0	0±0	0±0	0±0	0±0
Undecanoic acid methyl ester (C11:0)	0±0	0±0	0±0	0±0	0±0	0±0
Dodecanoic acid, ME (C12:0) M.Laurate	0.1±0	0.1±0	0±0	0±0	0.1±0	0±0
Tridecanoic acid, ME (C13:0)	0±0	0±0	0±0	0±0	0±0	0±0
Methyl Tetradecanoate (C14:0)	1.2±0	1.4±0	1.1±0	1.2±0	1.4±0.1	1.2±0.1
Myristoleic Acid ME (C14:1)	1.9±0.1	4±0.7	2.4±0.1	2.7±0.1	3.1±0.5	2±0.4
Pentadecanoic acid, ME (C15:0)	0.3±0	0.3±0	0.3±0	0.3±0	0.3±0	0.3±0
Cis-10-Pentadecenoic Acid ME (C15:1)	0.2±0.2	0.6±0.1	0.3±0	0.3±0.1	0.6±0.1	0.3±0
Methyl Palmitate (C16:0)	15±1.2	10.4±1.4	10±0.8	12.8±2.2	13.4±1.6	14.5±0.3
Methyl Palmitoleate-Cis-9 (C16:1)	3.8±0.1	4.2±0	3.5±0.1	3.5±0.1	4.2±0.4	3.5±0.1
Heptadecanoic acid, ME (C17:0)	2.6±0	2.8±0.1	2.5±0	2.6±0	2.9±0.2	2.5±0.1
Heptadecenoic Acid-Cis10, ME (C17:1)	1.3±0	1.4±0	1.3±0	1.3±0	1.6±0.1	1.2±0
Methyl stearate (C18:0)	17.7±1.9	21.8±4	17.1±2.5	19.3±3	18.1±2.1	23.5±7.2
Cis-9-Oleic ME (C18:1)	8.7±0.6	6.9±1.8	10.9±0.5	10.5±0.5	9.5±0.6	9.5±0.3

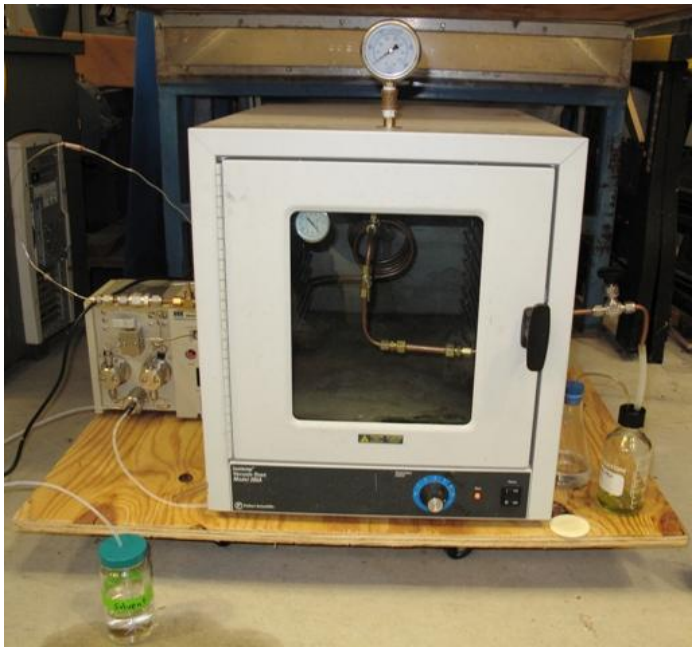
Methyl Linoleate (C18:2)	7.2±0.3	6.5±0.2	7.8±0.4	5.2±1.1	7.9±0.4	7.9±0.3
Methyl Linolenate (C18:3)	1.4±0	1.4±0.1	1.6±0.1	1.4±0.1	1.4±0.1	1.5±0.1
Gamma-Linilenic Acid ME (C18:3)	7.8±2.6	5.5±0.5	7.8±2.7	5.1±0.1	6.7±1.3	4.8±0.2
Methyl Arachidate (C20:0)	11.3±1.1	11.2±0.1	11.2±0.9	11.3±0.2	11.4±1.1	8.5±0.7
Cis-11-Eicosenoic acid, ME (C20:1)	5.1±0	4.8±0.3	4.4±0.2	4.8±0.1	5.5±0.3	4.5±0.2
Cis-11,14-Eicosadienoic acid ME (C20:2)	1.1±0	1±0.1	1±0	1±0	1.1±0.1	1±0
Cis-8,11,14-Eicosatrienoic ac ME (C20:3)	0.8±0	0.9±0.1	0.9±0	0.6±0.3	0.4±0.4	0.5±0.3
Cis-5,8,11,14-Eicosatetraenoic..(C20:4)	0±0	0±0	0±0	0±0	0±0	0±0
Cis-11,14,17-Eicosatrienoic Acid (C20:3)	0±0	0.7±0.7	0.5±0.5	0.7±0.7	0±0	0.6±0.6
Cis-5,8,11,14,17-Eicosapentaenoic (20:5)	0.7±0.1	2.1±0.8	2.9±2.2	3.4±2.6	1.6±0.8	0.8±0.2
Methyle Behenate (Docosanoate) (C22:0)	10.8±0.3	10.8±0.3	10.9±0.5	10.7±0.1	7.5±3.7	9.8±0.2
Cis--13-Docosenoic acid, me (C22:1)	0±0	0.4±0.3	0.3±0.3	0.2±0.2	0±0	0±0
Cis-13,16-Docosadienoic acis me (C22:2)	0±0	0.1±0	0.2±0	0.1±0	0±0	0±0
Tricosanoic acid, methyl ester (C23:0)	0.9±0	0.6±0.2	0.9±0.1	0.8±0	0.9±0.1	1.1±0.1
Tetracosanoic acid, methyl ester (C24:0)	0±0	0±0	0±0	0±0	0±0	0.1±0.1
15-Tetracosenoic acid, methyl ester, (Z)	0±0	0±0	0±0	0±0	0±0	0±0
4,7,10,13,16,19-Docosahexaenoic..(C22:6)	0.2±0.1	0.2±0.1	0.1±0.1	0.2±0.1	0.2±0	0.1±0.1

Saturated FAMEs	59.9±4.5	59.4±6.1	54.1±4.9	59.1±5.6	56.1±9	61.6±8.8
Unsaturated FAMEs	40.1±4.1	40.6±5.6	45.9±7.2	40.9±6	43.9±5	38.4±2.9
∑ mono-unsaturated FAMEs	20.9±1	22.4±3.2	23.2±1.2	23.3±1.1	24.5±2	21.1±1
∑ di- unsaturated FAMEs	8.4±0.3	7.5±0.2	9±0.5	6.3±1.1	9.1±0.5	9±0.3
∑ Tri- and higher unsaturated FAMEs	10.9±2.8	10.7±2.1	13.8±5.5	11.3±3.8	10.3±2.5	8.3±1.5

APPENDIX A3. MICROALGAE GROWTH CHAMBERS



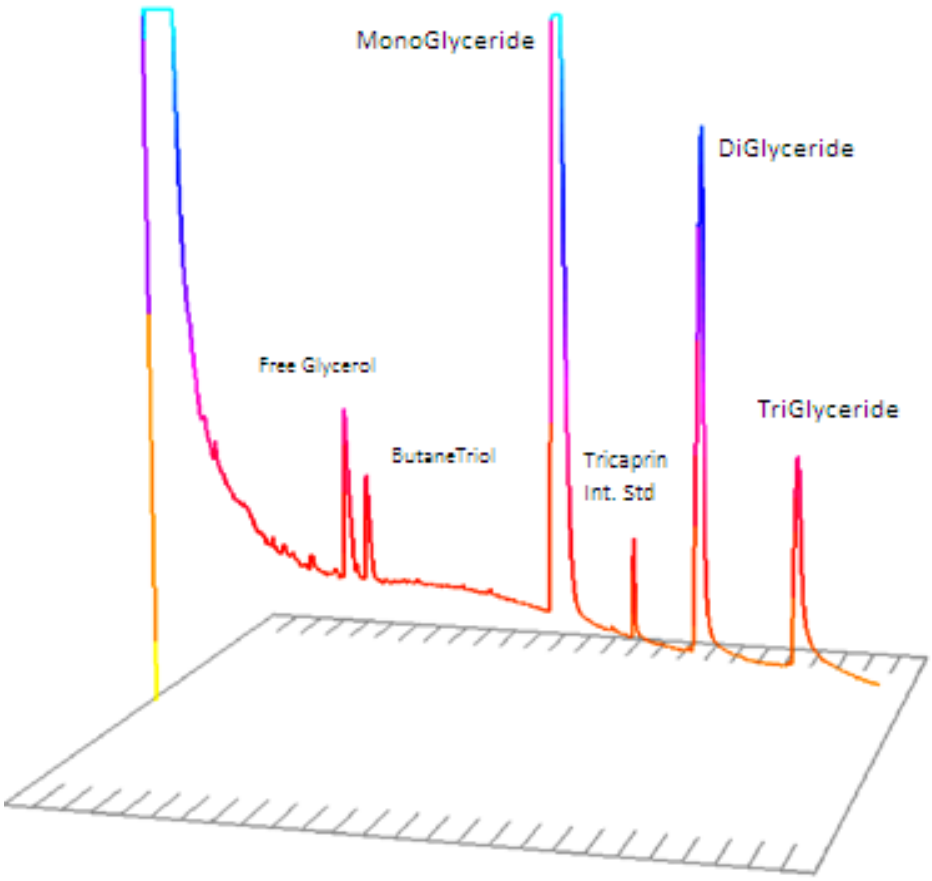
APPENDIX A4. CONTINUOUS FLOW LIPID EXTRACTION SYSTEM (CFLES) SETUP



CFLES setup

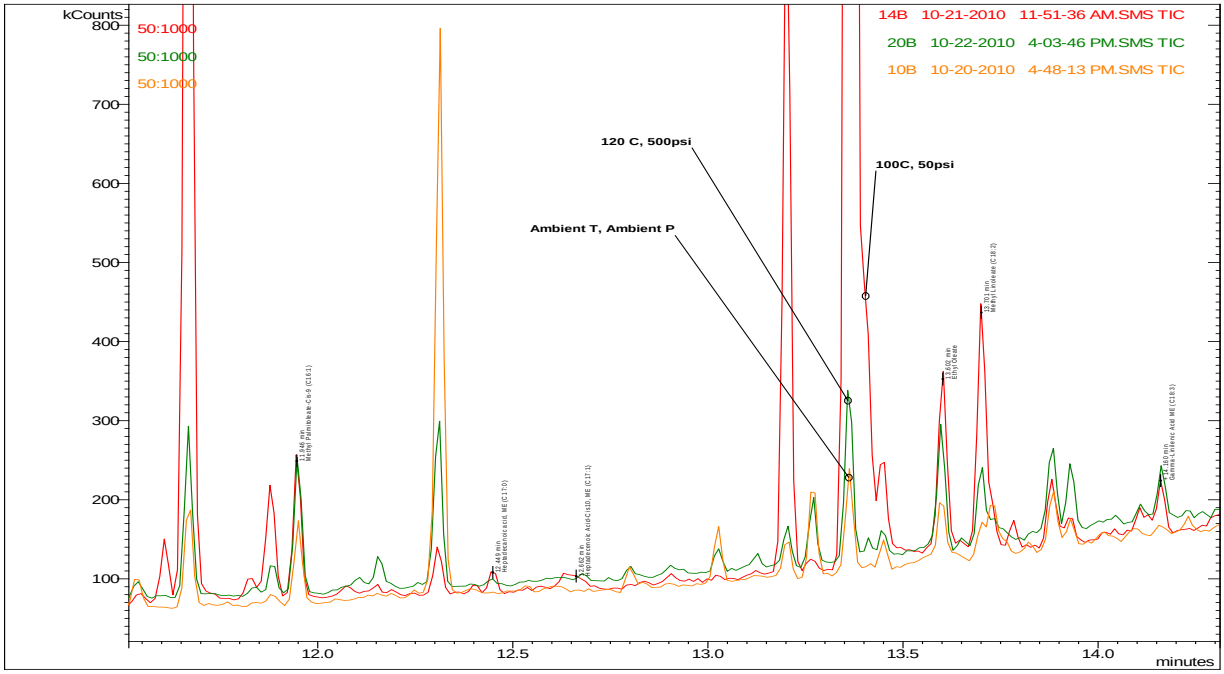
CFLES Oven, sample extraction cell, and copper tubing column

APPENDIX A5. GC CHROMATOGRAM OF TOTAL AND BOUND GLYCERIDES



GC/FID Chromatogram showing peaks of Triglyceride, Diglycerides, Monoglycerides, and free Glycerol along with peaks of Tricaprin and Butanetriol internal standards in a 25 ppm reference standard.

APPENDIX A6. GC/MS CHROMATOGRAM.



GC/MS chromatogram showing difference in fatty acid methyl esters (FAMEs) concentrations extracted from samples with CFLES using conventional solvents. Top showing - 100°C/50psi; Middle - 120°C/500psi; Bottom - ambient pressure and ambient temperature

VITA

The author was born in Pakistan in a small town of Swat Valley. He graduated from Peshawar University with a Bachelor of Science in biological sciences in 1992. In 1996, he graduated from Department of Environmental Planning & Management, University of Peshawar with a Master of Science in environmental planning and management. In 1997, he started working for a state Environmental Protection Agency (EPA-GoNWFP, Pakistan) as monitoring officer for urban-industrial environment protection project. In 2001 he went back to school at Louisiana State University, Department of Environmental Sciences and graduated with Master of Science in environmental sciences. He started working for LSU in 2003 as research associate. He switched to LSU Agriculture Center as extension associate in 2004. Currently he is working as Laboratory Manager at W.A. Callegari Environmental Center, LSU AgCenter.

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