LIQUID CO_2 AND CO_2-EXPANDED METHANOL FOR LIPID EXTRACTION FROM MICROALGAE

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Abstract

Interest in developing biofuels, which are biodegradable, clean burning and renewable energy sources alternative to petroleum-based fuels, has increased in recent years. Microalgae are considered to be the most promising alternative energy resources and have potential to replace non-renewable fossil fuels. However, the economical extraction of lipids from microalgae for the production of biofuel is limited in part by the energy costs related to solvent use, as well as the potentially harmful effects of traditional solvents. This study investigated the use of CO_2-expanded methanol and liquid CO_2 as greener alternatives to traditional solvents to extract lipids from dried or wet biomass of microalgae. Unlike extractions using conventional solvents, these new methods require little to no volatile, flammable, or chlorinated organic solvents.

CO_2-expanded methanol (cxMeOH) consists of pressurized gaseous CO_2 dissolved in methanol. When compressed CO_2 dissolves in methanol, the solvent expands in volume and decreases in polarity, reducing the amount of organic solvent needed and using much lower pressures than those required for supercritical CO_2. The decreased polarity of methanol in cxMeOH increased the selectivity of the solvent towards the extraction of biodiesel-desirable neutral lipids (NLs) and free fatty acids (FFAs). The selectivity was up to 82% for those desired lipids when cxMeOH was used, compared to about 50% when a conventional solvent was used. Moreover, the use of cxMeOH reduces the volume of organic solvent needed for the extraction by up to five times.

The use of liquid CO_2 (lCO_2) presents many advantages of sustainability, safety and selectivity over the use of conventional solvents because this technique requires no flammable,

highly volatile, or chlorinated solvents. The lCO_2 with its low polarity has high affinity for non-polar lipids. The lCO_2 was used to extract lipids from dried as well as the wet biomass of microalgae. Using lCO_2, it is possible to extract up to 10% neutral lipids relative to the mass of dry algae. Also, the lCO_2 extracts were up to 96% biodiesel desirable lipids with high content of oleic acid compared to only about 50% desirable lipids in conventional solvent extracts.

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List of Abbreviations

AG: Acylglycerides

BPR: Back-pressure regulator

CCS: Carbon dioxide capture and storage

CCU: Captured carbon dioxide utilization

CO_2: Carbon dioxide

CXL: CO_2-expanded liquids

cxMeOH: CO_2-expanded methanol

dw: Dry weight of microalgae

EIA: Energy information administration

FA: Fatty acids

FAME: Fatty acid methyl ester

FFA: Free fatty acids

FID: Flame ionization detector

GC-FID: Gas chromatograph equipped with a flame ionization detector

GHG: Greenhouse gas

GXL: Gas expanded liquids

LC-NH_2: Liquid chromatography aminopropyl-bonded

lCO_2: Liquid carbon dioxide

MeOH: Methanol

MTBE: Methyl-tert-butyl ether

NEB: Net energy balance

NL: Neutral lipid

OPEC: Organization of the petroleum exporting countries

PUFA: Polyunsaturated fatty acid

scCO_2: Supercritical carbon dioxide

SPE: Solid phase extraction

TMA: Trimethylammonium bicarbonate

UTEX 572: The university of Texas at Austin strain number 572

Chapter 1

INTRODUCTION

1.1 Energy overview

Energy plays a crucial role in sustaining life on the Earth. Energy is one of the vital inputs to the socioeconomic development of any country. All societies require energy to meet basic human needs such as lighting, cooking, space comfort, mobility, and communication (Moomaw et al., 2011). The sources of global energy can be classified into two groups: non-renewable and renewable. The non-renewable energy sources include fossil fuels such as coal, petroleum and natural gases, which are no longer available once used. The renewable energy sources are biomass, hydro, geothermal, solar, and wind energy. According to U.S. Energy Information Administration (EIA), about 80% of the world's current energy supply comes from non-renewable sources.

The continuously growing human population and, consequently their high energy consumption, and industrialization and rapid modernization in developing countries lead to increasing energy demands all over the world (Satyanarayana et al., 2011). Current primary sources of global energy (fossil fuels) are unstable and/or unsustainable because of the environmental, economic, and geopolitical concerns that will effect in the future. According to the U.S. Energy Information Administration (EIA), members of the Organization of the Petroleum Exporting Countries (OPEC) are responsible for 40 percent of the world's oil production and hold the majority of the world's oil reserves (EIA, February 2015). Fortunately,

renewable energy resources are more evenly distributed than fossil fuel resources. Although the petroleum reserves are found in abundance in nature, the frightening rate at which they are being consumed has resulted in substantial depletion of their stocks (Singh & Singh, 2012). Therefore, sustainable renewable energy sources will play an important role in the globe's future energy supply.

One of the major concerns with using fossil fuels is the consequent carbon dioxide (CO_2) emission as a result of fossil fuel combustion. Burning of such fuels releases CO_2 and some other gases that can lead to disastrous effects in the environment and impacts human health (EPA, 2014). Carbon dioxide is one of the primary greenhouse gases directly associated with the global warming observed in recent decades. The rise in the temperature of the earth has resulted in melting of polar ice caps, flooding of low lying areas, and rising sea levels. Reducing the use of fossil fuels would considerably reduce the amount of CO_2 produced, as well as reduce the levels of the pollutants (Kralova & Sjöblom, 2010). In addition, rather than treating CO_2 as a waste product, it could be regarded as a chemical feedstock for the synthesis of other chemicals that do not rely on a petrochemical source (Styring et al., 2011). For example, cultivation of oil-rich microalgae using flue gases as an inorganic carbon supply. Therefore, to address the threat of global warming effectively, the energy systems must be transferred to sustainable, renewable and cleaner sources, while reducing current fossil fuel dependency.

Additionally, fluctuating prices of petroleum fuels have also underlined the need for non-petroleum alternatives. Because the members of the OPEC control the majority of the world's crude petroleum oil supply, they have a great influence on fuel prices around the world. The OPEC controls the prices by either increasing or decreasing the amount of oil available. While

prices of fossil fuels are expected to remain relatively low over the next few years, if the trend of increasing global consumption continues, resources will become exhausted. The amount of time until this happens will depend on efforts from individual countries to make the change to more renewable resources (Riddell et al., 2014).

1.2 Renewable energy sources

Renewable energy sources provide energy in a sustainable manner while mitigating global climate change. Greenhouse gas (GHG) mitigation is one of the vital motivating forces behind a growing demand for renewable energy (Moomaw et al., 2011). Currently, about 20 % of global energy consumption is being provided by renewable energy sources and the number is expected to continue to grow continuously (REN21, 2014). The major sources of alternative energy are biomass, solar, wind or geothermal energy, and hydropower. These new and renewable fuels have the potential to solve many of the current social problems and concerns, from global climate change, and the depletion of fossil fuels to sustainability issues (MacLean & Lave, 2003). For instance, biomass-based liquid transportation biodiesel contains 11 % oxygen by weight and no sulfur (Kralova & Sjöblom, 2010). The higher oxygen content of the biodiesel promotes a more complete ignition. As a result, the renewable energy sources produce lower levels of pollutants as compared to fossil energy sources. Thus, the biomass-based fuels or bio-fuels may represent promising alternatives to fossil fuels and have attracted increasing attention as evidenced by the growing research and development efforts found in the literature (Durrett et al., 2008; Solomon, 2010).

1.3 Biofuel

Biofuels are solid, liquid or gaseous fuels derived from biomass or bio-waste through a process of biological carbon fixation, usually by plants. They are divided into primary and secondary biofuels. The primary biofuels such as fuel wood are used for heating, cooking or electricity production in their unprocessed form while the liquid or gaseous secondary biofuels such as bioalcohols (biomethanol, bioethanol or biobutanol), biodiesel, or biogas (biomethane) that are produced by processing biomass can be used in transport and industrial sectors (Nigam & Singh, 2011). Table 1.1 compares the secondary biofuels with their fossil fuels counterparts.

Table 1.1 Comparison of biofuels with their fossil fuel counterparts (Biofuels, 2014. Retrieved from http://biofuel.org.uk in November, 2014).

Biofuel	Fossil Fuel	Differences
Ethanol	Gasoline/Ethane	Ethanol has about half the energy per mass of gasoline, which means it takes twice as much ethanol to get the same energy. Ethanol burns cleaner than gasoline, however, producing less carbon monoxide. Nevertheless, ethanol produces more ozone than gasoline and contributes substantially to smog. Engines must be modified to run on ethanol.
Biodiesel	Diesel	Has only slightly less energy than regular diesel. It is more corrosive to engine parts than standard diesel, which means engines have to be designed to take biodiesel. It burns cleaner than diesel, producing less particulate and fewer sulfur compounds.
Methanol	Methane	Methanol has about 1/3 to 1/2 as much energy as methane. Methanol is a liquid and easy to transport whereas methane is a gas that must be compressed for transportation.
Biobutanol	Gasoline/Butane	Biobutanol has slightly less energy than gasoline, but can run in any car that uses gasoline without the need for modification of engine components.

Bioethanol and biodiesel are the most common biofuels, which can replace gasoline and diesel. Bioethanol is commonly made by fermentation of biomass rich in carbohydrates and is mostly used as a blending agent with gasoline to increase octane number and reduce carbon monoxide emissions. Biodiesel is made of fatty acid alkyl esters and is produced by the transesterification of vegetable oils, animal fats or recycled cooking grease with short-chain alcohols (Chen et al., 2012). It can be used as an additive, a 5% (B5) or 20% (B20) blend, to reduce vehicle emissions or in its pure form (B100) as a renewable alternative fuel for diesel engines (Ataya et al., 2007; NREL). The demand for biodiesel has increased in recent years because it is a non-toxic, biodegradable, clean burning, and renewable fuel (Ranganathan et al., 2008).

1.3.1 Feedstocks for biofuel

The secondary biofuels can be classified into three generations based on the type of feedstock, processing technology or their level of development. The first generation biofuel feedstocks are from agricultural products such as oilseeds, corn, wheat, and sugarcane. These feedstocks have a high carbohydrate or lipid content, which can be transformed into fuels such as alcohol, biodiesel and biogas. Edible feedstocks that are currently used for biofuel include sugarcane (for bioethanol) in Brazil, corn (for bioethanol) in the USA, and rapeseed (for biodiesel) in European countries (Srirangan et al., 2012). The first generation feedstocks are good alternatives for biofuel production because of their high carbohydrate content, abundance and facile cultivation and processing. Unfortunately, because many traditional feedstocks require nutrient rich soils and they may compete with food resources, and because demand for biofuels is rapidly increasing, the feasibility of expanding and using first generation feedstocks is limited.

Also, the extensive use of land with excessive water, fertilizer and pesticide applications can lead to significant environmental concerns (Schenk et al., 2008).

Second generation biofuel feedstocks are comprised of lignocellulosic materials, the wooded segment of plants that do not compete with food production. Agricultural residues such as corn stalks and wheat straw, forest harvesting residue or wood processing waste offer large and readily available second-generation resources. Unlike the conventional energy crops, the lignocellulosic resources can be cultivated on a wider range of lands and environments with comparable yields. Furthermore, an efficient processing of crude lignocellulosic residues for biofuel production could reduce the current disposal problems associated with these materials. Nevertheless, the conversion of lignocellulosic biomasses into biofuels requires complex and sophisticated processes because of their intractable nature associated with complex polysaccharides such as lignin cellulose and hemicellulose (Srirangan et al., 2012). Moreover, excess elimination of residue can deteriorate soils leading to soil erosion, and exhaust the nutrients and organic matter from the soil. Harvesting forest residue for biomass raises concerns of soil nutrient management in terms of long-standing soil productivity (BR&Di, 2012).

1.3.2 Net energy balance of biofuels

Net energy balance (NEB) of the biofuels needs to be considered while examining their benefits. The energy balance is the ratio of the output (amount of energy available after transformation process) and the input (total energy used in the process) energy. The NEB of bioethanol and biodiesel depends on several factors such as geographical location of production, the kind of agro-system and the kind of feedstock (Basset et al., 2010). The first generation biofuels, where inputs must be invested to cultivate, fertilize and harvest the feedstocks, have

high production cost, and low net energy balance (Randelli, 2009). Hill et al. (2006) reported that corn grain ethanol yields 25% more energy than the energy invested in its production, whereas soybean biodiesel provides 93% more energy than is required in its production. This advantage of biodiesel over ethanol comes from lower agricultural inputs and more efficient conversion of the feedstocks to fuel. The use of lignocellulosic feedstocks (agricultural and wood residues) may reduce the overall biofuel production cost compared to specially cultivated energy crops. However, the factors such as soil conservation, livestock feed, seasonal variation and the energy costs associated with the collection and transportation of the feedstocks to the processing site, lower the energy and the carbon balance (Antizar-Ladislao & Turrion-Gomez, 2010). Therefore, third generation biofuels derived from microscopic organisms such as microalgae are considered to be viable alternative energy resources that have potential to overcome the major limitations associated with first and second generation biofuels (Chisti, 2007).

1.3.3 Microalgae

Microalgae are a diverse group of unicellular organisms and are the most promising feedstock for an alternative renewable energy source (Chisti, 2007). They can grow in a wide range of aquatic environments, from freshwater through brackish water. Also, they can be found in some other extreme environments such as snow or desert including hot springs. They effectively use atmospheric CO_2 and are responsible for more than 40% of global carbon fixation (Hannon et al., 2010). They use sunlight, CO_2, and water with dissolved nutrients to produce lipids, proteins, carbohydrates and other valuable organic compounds as pigments (Batista et al., 2013). During photosynthesis, microalgae absorb CO_2 to produce sugars and oxygen. Sugars are then converted to lipids, proteins and carbohydrates.

$6\ CO_2 + 6\ H_2O + \text{sunlight (energy)} \rightarrow C_6H_{12}O_6\ \text{(sugar)} + 6\ O_2$,

Microalgae exhibit some unique properties that make them well suited for use in commercial-scale biodiesel production. Recent interest in using microalgae as a nonedible biodiesel feedstock has grown considerably, mainly on the promise of high oil yields (5,000 to 100,000 L/ha/y), the opportunity to capture waste CO_2, and the ability to cultivate algae on abandoned or unproductive land using brackish, salt, or wastewaters instead of freshwater (Levine et al., 2010). Moreover, microalgae are reported to produce 15–300 times more oil for biodiesel production than traditional crops on an area basis (Schenk et al., 2008). Many algal species show rapid growth and high productivity, with some capable of being manipulated to accumulate substantial quantities of lipids, often greater than 60% of their biomass (Sheehan et al., 1998). In particular, some species double in mass in as few as 6 h while many exhibit two doublings per day (Huesemann et al., 2009). Compared to traditional crop plants that are usually harvested once or twice a year, microalgae have a very short harvesting cycle (1-10 days depending on the process), which allows continuous harvests with substantially increased yields. In fact, microalgae are able to produce 15–300 times more lipids for biodiesel production than conventional crop plants produce on an area basis (Schenk et al., 2008). Other benefits of microalgae include their nominal land requirements, use of wastewater as nutrient feed and lack of reliance on chemicals such as herbicides and pesticides (Rawat et al., 2011). However, because the microalgae are unicellular and are in suspension it is very difficult to harvest them. Also, lipid extraction, refining and residual biomass exploitation processes are complex and still are in developing phase (Hannon et al., 2010).

1.3.4 Lipid composition of microalgae

Microalgae produce several types of lipids (Figure 1.1) such as glycerides (mono-, di- and tri-), phospholipids, and glycolipids in different ratios depending on the species and their growth conditions. These lipids contain fatty acids (FA), which may consist of different numbers of carbon atoms and double bonds along the hydrocarbon chain. Most of the fatty acids are confined to the glycerol backbone molecules forming acylglycerides (AG) while others are bonded to a hydrogen atom and exist as free fatty acids (FFA) (Nascimento et al., 2012). Based on the polarity of the molecular head group, the lipids can be can be categorized into two groups: neutral lipids (AG, FAA and hydrocarbons), and polar lipids, which can be further sub-classified into phospholipids and glycolipids. In microalgal cells, the neutral lipids are used as energy storage, while the polar lipids stack to form bilayer cell membranes. In addition, microalgae may have some other types of neutral lipids that do not contain fatty acids. These lipids include ketones, sterols, and pigments (carotenes and chlorophyll) (Halim et al., 2012). These lipids cannot be converted into biodiesel.

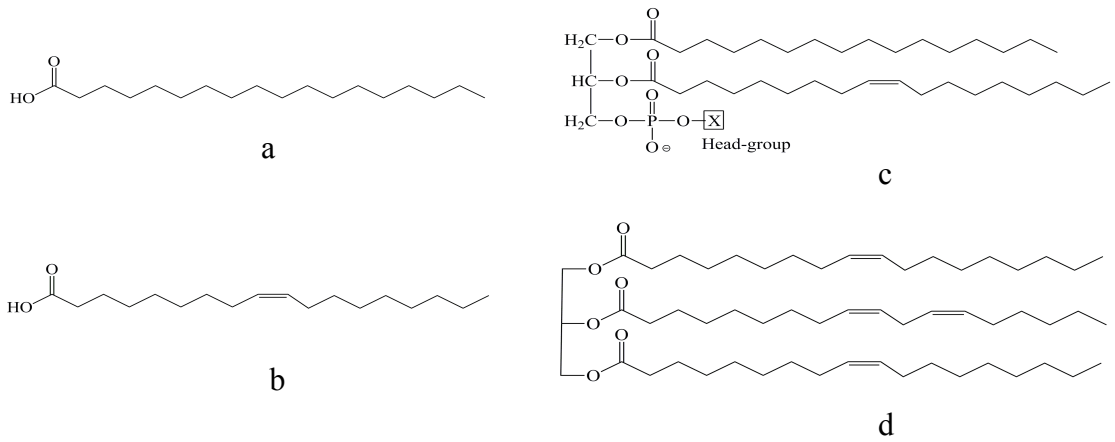

Figure 1.1. Types of microalgal lipids. (a) Stearic acid (C18:0), (b) Oleic acid (C18:1), (c) Negatively charged phospholipid molecule (polar lipid) (d) Triacylglycerol (neutral lipid).

1.3.5 Selection of microalgae species and cultivation

About 35,000 species of microalgae have been reported so far, and it is estimated that the actual number of species is significantly higher (Borowitzka, 2013). Any microalgal species selected for commercial cultivation must exhibit fast growth, high lipid content, good adaptability, and specific lipid composition. Many microalgal species have the ability to accumulate substantial amount of lipids (Slocombe et al., 2013). The average lipid content of microalgae ranges from 1 to 70% however under certain conditions some species can reach 90% of dry weight (Mata et al., 2010). For example, *Botryococcus braunii* can produce significant levels of lipids, which can reach as high as 75% by weight of dry biomass (Table 1.2). However, the total lipid yield depends on both areal productivity and lipid content, which in turn depends on the growing conditions and the growth phase (Xiao et al., 2013). Moreover, the major factors that can affect the economic feasibility of algal oil for biodiesel production are biomass productivity, lipid content, and overall lipid productivity. The biomass concentration and lipid content affect the downstream processing cost, whereas the overall lipid productivity determines the costs of the cultivation process. Therefore, an ideal cultivation process should have a high lipid productivity with maximum lipid content. However, cells produce high lipid content under stress, typically nutrient deprivation, which is associated with low biomass productivity and therefore low overall lipid productivity (Li et al., 2008).

The results presented in Table 1.2 show significant differences in lipid content between the various microalgal species. Most common algal species such as *Chlorella*, *Cylindrotheca*, *Crypthecodinium*, *Dunaliella*, *Isochrysis*, *Nannochloropsis*, *Nannochloris*, *Neochloris*, *Nitzschia*, *Porphyridium*, *Phaeodactylum*, *Schizochytrium*, and *Tetraselmis* have lipid content between 20

and 50 wt% relative to the dry weight of microalgae (dw). Also, as can also be seen in Table 1.2, higher lipid contents in microalgae can be achieved but are typically associated with low productivities. Among the microalgal species listed, some species such as *Chlorella sp., Botryococcus braunii, Dunaliella salina*, and *Nannochloropsis sp.,* appear to be good options as microalgal feedstocks for biodiesel production.

Table 1.2. Lipid content and productivities of different microalgal species under different cultivation conditions (Mata et al., 2010).

Microalgae species	**Lipid content (% dw)**	**Lipid productivity (mg/L/day)**	**Volumetric productivity of biomass (g/L/day)**	**Areal productivity of biomass g/m^2/day)**
Ankistrodesmus sp.	24.0–31.0	-	-	11.5–17.4
Botryococcus braunii	25.0–75.0	-	0.02	3.0
Chaetoceros muelleri	33.6	21.8	0.07	-
Chaetoceros calcitrans	14.6-16.4/39.8	17.6	0.04	-
Chlorella emersonii	25.0–63.0	10.3–50.0	0.036–0.041	0.91–0.97
Chlorella protothecoides	14.6–57.8	12-14	2.00–7.70	-
Chlorella sorokiniana	19.0–22.0	44.7	0.23–1.47	-
Chlorella vulgaris	5.0–58.0	11.2–40.0	0.02–0.20	0.57–0.95
Chlorella sp.	10.0–48.0	42.1	0.02–2.5	1.61–16.47
Chlorella pyrenoidosa	2.0	-	2.90–3.64	72.5/130
Chlorella	18.0–57.0	18.7	-	3.50–13.90
Chlorococcum sp.	19.3	53.7	0.28	-
Crypthecodinium cohnii	20.0–51.1	-	10	-
Dunaliella salina	6.0–25.0	116.0	0.22–0.34	20–38
Dunaliella primolecta	23.1	-	0.09	14
Dunaliella primolecta	16.7–71.0	-	0.12	-
Dunaliella sp.	17.5–67.0	33.5	-	-
Ellipsoidion sp.	27.4	47.3	0.17	-
Euglena gracilis	14.0–20.0	-	7.70	-

Microalgae species	Lipid content (% dw)	Lipid productivity (mg/L/day)	Volumetric productivity of biomass (g/L/day)	Areal productivity of biomass g/m^2/day)
Haematococcus pluvialis	25.0	-	0.05–0.06	10.2–36.4
Isochrysis galbana	7.0–40.0	-	0.32–1.60	-
Isochrysis sp.	7.1–33	37.8	0.08–0.17	-
Monodus subterraneus	16.0	30.4	0.19	-
Monallanthus salina	20.0–22.0	-	0.08	12
Nannochloris sp.	20.0–56.0	60.9–76.5	0.17–0.51	-
Nannochloropsis oculata.	22.7–29.7	84.0–142.0	0.37–0.48	-
Nannochloropsis sp.	12.0–53.0	37.6–90.0	0.17–1.43	1.9–5.3
Neochloris oleoabundans	29.0–65.0	90.0-134.0	-	-
Nitzschia sp.	16.0–47.0	-	-	8.8–21.6
Oocystis pusilla	10.5	-	-	40.6–45.8
Pavlova salina	30.9	49.4	0.16	-
Pavlova lutheri	35.5	40.2	0.14	-
Phaeodactylum tricornutum	18.0–57.0	44.8	0.003–1.9	2.4–21
Porphyridium cruentum	9.0–18.8/60.7	34.8	0.36–1.50	25
Scenedesmus obliquus	11.0–55.0	-	0.004–0.74	-
Scenedesmus quadricauda	1.9–18.4	35.1	0.19	-
Scenedesmus sp.	19.6–21.1	40.8–53.9	0.03–0.26	2.43–13.52
Skeletonema sp.	13.3–31.8	27.3	0.09	-
Skeletonema costatum	13.5–51.3	17.4	0.08	-
Spirulina platensis	4.0–16.6	-	0.06–4.3	1.5–14.5
Spirulina maxima	4.0–9.0	-	0.21–0.25	25
Thalassiosira pseudonana	20.6	17.4	0.08	-
Tetraselmis suecica	8.5–23.0	27.0–36.4	0.12–0.32	19
Tetraselmis sp.	12.6–14.7	43.4	0.30	-

Microalgae cultivation can be carried out either in open ponds or in closed photobioreactors. Open pond systems are cheaper to operate and can be scaled up more easily to several hectares. However, microalgae grown in an open environment may suffer from inconsistent growth rates depending on day-to-day weather conditions and biological invasion by other species and organisms. On the other hand, the cultivation conditions can be controlled in a closed system for better protection of the microalgae, but such a system has higher operating costs (Benemann, 2008). Throughout the cultivation period, the microalgal culture needs to be supplied with CO_2 and essential micro- and macro-nutrients such as nitrogen, phosphorus, and iron (Chisti, 2007).

As previously noted, lipid accumulation within the microalgal cells varies with growth conditions. Nitrogen starvation is an effective environmental stress used to increase both lipid and triglyceride content in microalgal cells. The general principle is that when there is insufficient nitrogen for the protein synthesis required for cell division and growth, the excess carbon from photosynthesis is channeled into storage molecules such as triglyceride or starch (Zhang et al., 2013). It has been reported that nitrogen deficiency applied to all the microalgal species studied so far induced neutral lipid (triacylglycerides) production (Sharma et al., 2012). For instance, the lipid content in *chlorella vulgaris* could be increased by up to three times under nitrogen starvation conditions (Converti et al., 2009) and a linear relationship between the nitrogen source concentration and the lipid content has been observed (Hsieh & Wu, 2009). It has also been reported that changing from normal nutrient supply to nitrogen depleted media can result in a change the lipid composition from free fatty acid to triglyceride (Takagi et al., 2000).

1.3.6 Harvesting and dewatering

Growth of microalgae in dilute aqueous suspension, at about 0.1-2 g dry solid biomass/L culture, presents significant challenges in achieving a sustainable energy balance in microalgal biofuel production (Halim et al., 2012). In addition, the nature and small size of microalgal cells, the similar density of the cells to the culture medium, the biomass growth rate that requires frequent harvesting, and high capital equipment costs are the other potential difficulties associated with harvesting (Milledge & Heaven, 2013). Selection of an efficient harvesting method is of great importance for scaled biodiesel production as harvesting alone can account for 20%-30% of the total production costs (Pragya et al., 2013; Rawat et al., 2011).

A number of solid-liquid separation techniques such as flocculation/coagulation, ultrasonic aggregation, gravity and centrifugal sedimentation, and filtration are used to dewater the microalgal growth culture. In fact, the appropriate harvesting method often depends upon the characteristics of the microalgal species (Rawat et al., 2011). The biomass recovery techniques can be single stage (individual technique) or multistage (combination of techniques) depending on the microalgal species and desired product concentration. Figure 1.2 represents the general stages of microalgal biomass recovery. Almost all of the current recovery techniques are microalgae species specific and not readily scalable. A single best method or a series of processes suitable in all situations and for all microalgal species has yet to be developed (Pahl et al., 2013).

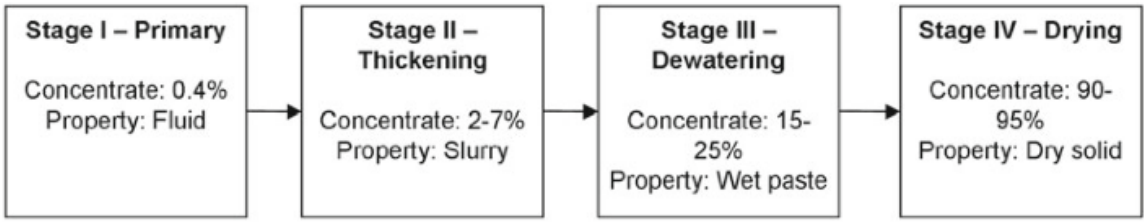

Figure 1.2. Different stages of microalgae biomass recovering showing typical biomass concentration achieved (Pahl et al., 2013).

1.3.7 Lipid extraction

Lipids are extracted out of the cellular matrices by exposing the microalgal biomass to an eluting extraction solvent (Figure 1.3). The choice of extraction solvent is crucial in determining the energy consumption of the drying, extraction, and solvent recovery steps. An ideal solvent for lipid extraction should be inexpensive, non-flammable, non-toxic, easily removable, lipid specific to minimize the co-extraction of non-lipid constituents, and be more selective towards the desirable neutral (mono-, di-, and tri-acylglycerols) lipids and free fatty acid fractions (Halim et al., 2012).

The efficiency of the extraction of microalgal lipids will depend on the polarity of the eluting solvent. A proposed mechanism for organic solvent extraction technique is shown in Figure 1.3. A non-polar organic solvent (e.g. hexane or chloroform) penetrates the microalgal cell membrane and interacts with neutral lipids forming a solvent-lipid complex. A concentration gradient drives the solvent-lipid complex across the cell membrane and finally out of the cell. However, some neutral lipids, which form a complex with other polar lipids, are strongly linked via hydrogen bonds to proteins in the cell membrane. A surface (Van der Waals) interaction formed between the neutral lipids and non-polar solvent is unable to disrupt the membrane-based

lipid–protein interactions. In contrast, a polar solvent such as an alcohol (methanol or isopropanol) helps in the disruption of the lipid-protein interaction and the dissolution of the lipids (Halim et al., 2012; Medina et al., 1998). The mechanism involves penetration of the non-polar and polar organic solvent mixture through the cell membrane into the cytoplasm and the solvent interactions with the lipid complex. During such interaction, the polar solvent forms hydrogen bonds with polar lipids in the complex, while the non-polar solvent interact with neutral lipids in the complex. Once the solvent-lipids complex is formed, it diffuses across the cell membrane and finally out of the cell. Thus, a polar/non polar mixture solvent system facilitates the extraction of membrane associated neutral lipids. However, the polar solvents may also extract other cellular constituents such as amino acids, sugars, pigments and hydrophobic proteins undesired for biodiesel production.

Several extraction techniques have been investigated worldwide to extract lipids from microalgae for commercialization. Conventional extraction methods use organic solvents or solvent mixture such as hexane, chloroform/methanol, petroleum ether and isopropanol, which must subsequently be separated from the bio-oil using distillation. In recent years, supercritical carbon dioxide ($scCO_2$) has been implemented for microalgal lipid extraction. Details about these methods will be described in the following chapters.

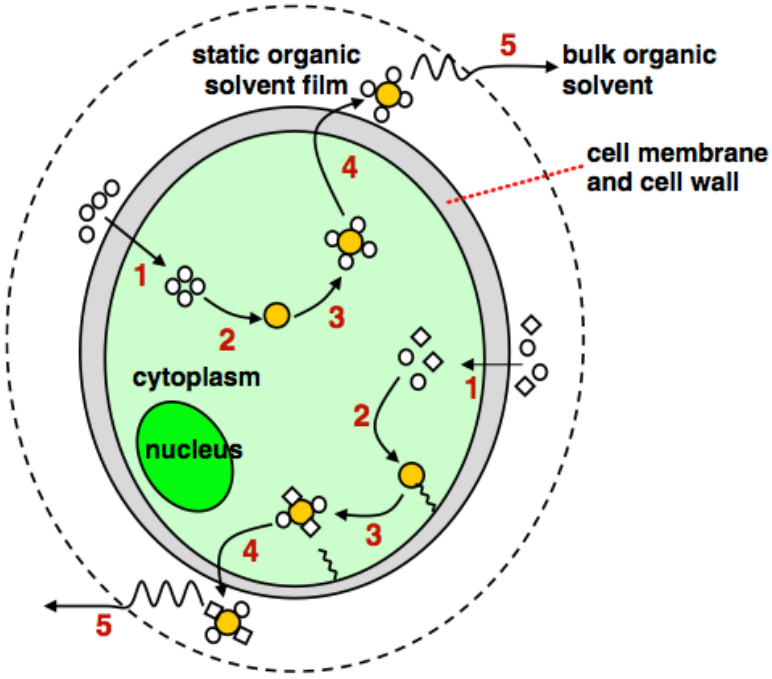

Figure 1.3 schematic diagram of the proposed organic solvent lipid extraction mechanisms. Pathway shown at the top of the cell: mechanism for non-polar organic solvent. Pathway shown at the bottom of the cell: mechanism for non-polar/polar organic solvent mixture. (Yellow dot): lipid molecule, (O): non-polar organic solvent, and (◊): polar organic solvent. Both mechanisms comprise 5 steps. Step 1: penetration of organic solvent through the cell membrane. Step 2: interaction of organic solvent with the lipids. Step 3: formation of organic solvent–lipids complex. Step 4: diffusion of organic solvent–lipids complex across the cell membrane. Step 5: diffusion of organic solvent–lipids complex across the static organic solvent film into the bulk organic solvent (Halim et al., 2012).

1.3.8 Pre-treatment: microalgae cell lysis

The extraction of lipids from microalgae is one of the most important steps in microalgae-based biodiesel production. As mentioned earlier, in a typical lipid extraction method, microalgal biomass is contacted with an organic eluting solvent, which diffuses through

the cell wall/membrane into the cytoplasm and interacts with the lipids. However, the cell wall/membrane poses a barrier to permeation by the eluting solvents. Therefore, a pre-treatment aimed to disrupt the microalgal cell wall/membrane prior to extraction is necessary (Steriti et al., 2014).

The microalgal cell wall is a thick and rigid layer composed of complex carbohydrates and glycoproteins with high mechanical strength and chemical resistance (Steriti et al., 2014). The cell disruption methods can be classified into three categories: mechanical, chemical, and biological methods. Mechanical methods can be applied to any biomass regardless of the species and break cells through physical force. These methods include the use of bead beating, grinding using mortar and pestle, ultrasonication, electroporation, and high-pressure homogenization. Chemical methods comprise the use of chemical treatments and osmotic shock. The chemical means such as treatment of biomass with acids, alkalis, and surfactants degrade chemical linkages of the cell wall by interacting with certain components of the cell wall or membrane whereas the external osmotic pressure induces the cell rupture. Biological methods typically involve the use of enzymes, which degrade polysaccharides and/or proteins. The enzymatic methods offer the mildest reaction conditions and the highest selectivity, but their industrial implementation is limited due to the high cost associated with the technique. An enzyme can selectively degrade a specific chemical linkage, whereas mechanical methods destroy almost every particle existing in the solution, and chemical methods sometimes induce side-reactions of the target products (Kim et al., 2013).

1.3.9 Production of biodiesel from microalgal lipid

Biodiesel consists of the mono alkyl esters of long chain fatty acids derived from vegetable oils or animal fats, for use in diesel engines. The physical and chemical properties as well as the energy content of biodiesel are similar to the conventional petroleum-based diesel fuel. Therefore, the biodiesel can be used either as pure biodiesel or blended with petroleum-based diesel with little or no modification to existing diesel engines. Moreover, since biodiesel is oxygenated and has better lubricant properties than petroleum-based diesel, it enhances engine yield and extends engine life (Robles-Medina et al., 2009; Vasudevan & Briggs, 2008). Also, biodiesel has a relatively high flash point (150 °C) and offers safer transportation, handling and storage than conventional diesel (Al-Zuhair, 2007). However, the high production cost compared to that of petroleum-derived diesel fuel limits the wider application of biodiesel. A major contribution to the final production cost results from the processing of highly viscous and less volatile crude bio-oil to acquire the properties that are similar to the petroleum-based diesel. Pyrolysis, microemulsion and transesterification are some techniques than can be used for the production of biodiesel from crude vegetable oil (Ma & Hanna, 1999). Of these, transesterification is the most common way to transform crude bio-oil to biodiesel (Robles-Medina et al., 2009).

1.3.10 Transesterification

Transesterification is the reaction of vegetable oil or animal fat with an alcohol to form alkyl esters and glycerol. The reaction is facilitated with a base or an acid catalyst (Figure 1.4). Polar, short-chain methanol is mostly used because of its low cost. When methanol is used in the transesterification process it is called methanolysis. The methanolysis reaction of triglycerides

produces diglycerides and monoglycerides as intermediates (Figure 1.5).

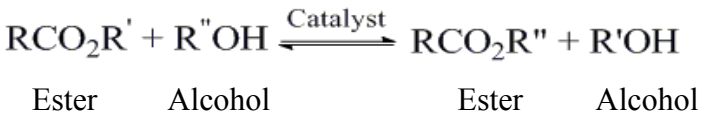

Figure 1.4 General transesterification reaction.

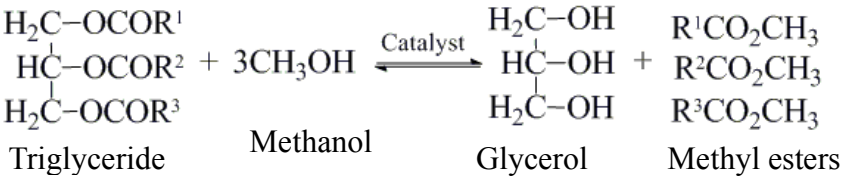

Figure 1. 5 General transesterification reaction of triglyceride.

The catalysts used for the transesterification process can be classified as homogeneous, heterogeneous or enzyme (Helwani et al., 2009). Although the alkali catalyzed transesterification is faster than the acid catalyzed reaction, the use of alkali catalyst is limited by the drawbacks such as a requirement for low free fatty acid content and anhydrous conditions, saponification and emulsion formation. The acid catalyzed transesterification/esterification avoids soap formation, carries out the esterification and transesterification simultaneously, and so gives very high yields of biodiesel. However, the process is associated with equipment corrosion, waste from neutralization, higher reaction temperature, longer reaction time and weak catalytic activity compared to the alkali-catalyzed method (Schuchardt et al., 1998; Soriano Jr et al., 2009). Therefore, the acid-catalyzed esterification/transesterification has not gained as much attention as the alkali-catalyzed transeterification. The use of an enzyme-catalyzed technique overcomes most of the drawbacks of acid or base catalyzed methods. However, enzymes are expensive and there would be some problems associated with their usage in the presence of FFAs and short

chain alcohols (methanol and ethanol), which denature the enzyme due to deleterious alcohol effects (Lisboa et al., 2014; Silva & Oliveira, 2014). Application of supercritical alcohols (methanol and ethanol) for biodiesel production may be a potential solution. However, the adoption of high temperatures and pressures for the reaction to reach reasonable conversion levels leads to high capital cost (Silva & Oliveira, 2014). Therefore, an efficient method for the production of biodiesel from bio-oil still needs to be developed.

1.4 Thesis objectives and outline

The main objective of this study is to explore greener and more efficient methods of microalgal lipid extraction compared to the conventional organic solvent techniques. This will be accomplished by using liquid CO_2 or gas expanded liquids, specifically, CO_2-expanded methanol, for the extraction of lipids from microalgae. Chapter 2 reports the organic solvent extraction of lipids from dried mass of microalgae. This extraction was performed to determine the total extractable lipid content of the algal mass. Use of CO_2-expanded methanol (cxMeOH) for extraction of lipids from microalgae has been explored in Chapter 3. To avoid the use of any hazardous organic solvents, liquid CO_2 (under mild conditions) was used to extract lipids from dried (water content ~3%) as well as from the wet biomass (water content ~80%). The results obtained from lCO_2 extraction of dry and wet microalgae biomass are reported in Chapters 4 and 5 respectively.

The work contained in Chapters 3 and 4 of this thesis is within the following journal paper (Impact factor: 5.039):

Paudel, A., Jessop, M.J., Stubbins, S.H., Champagne, P., Jessop, P.G. 2015. Extraction of lipids from microalgae using CO_2-expanded methanol and liquid CO_2. *Bioresource Technology*. http://dx.doi.org/10.1016/j.biortech.2014.11.111.

The extractions were carried out under moderate temperature (≤35 °C) and pressure (up to 17 MPa) to reduce the eventual larger scale capital and processing costs. The use of these methods for the extraction of lipids from microalgae could present an advantage to the use of conventional organic solvent techniques because they require little to no flammable, chlorinated or volatile organic solvents.

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Chapter 2

EXTRACTION OF LIPIDS FROM MICROALGAE USING CHLOROFORM/METHANOL SOLVENT MIXTURE

2.1 Introduction

In this technique, the extraction of algal oil is performed with the use of organic solvents. The basic principle of solvent extraction is 'like dissolves like.' That is, non-polar compounds are more soluble in non-polar solvents than in polar solvents, whereas the polar compounds are more soluble in polar solvents. The lipids in the microalgal cells have different kinds of interactions with their molecules or with the cell membrane, which need to be disrupted for effective extraction. Non-polar organic solvents interrupt the interactions between the long hydrophobic fatty acid chains of neutral lipids, while the polar organic solvents disrupt hydrogen bonding between polar lipids. Therefore, the choice of extraction solvent depends on the microalgal species and its lipid composition, the nature of the lipids to be extracted and the cell pre-treatment (Halim et al., 2012; Pragya et al., 2013).

Among several microalgae species studied to date, the green, colonial, and unicellular *Botryococcus braunii* has been reported to have a very high lipid content (up to 75% on a dry weight basis) and is widespread in the fresh and brackish waters of all continents (Banerjee et al., 2002). This algae is characterized by a prominent ability to synthesize and accumulate a variety of lipids such as hydrocarbons and fatty acid-based lipids (Zhang et al., 2011). Depending on the type of hydrocarbons found in their cells, *B. braunii* could be classified into one of three

chemical races: A, B and L. The race A produces odd numbered C_{21} to C_{33} n-alkadienes and trienes. The race B produces triterpenoid hydrocarbons called botryococcenes (C_nH_{2n-10}, n= 30 - 37) and methyl branched squalene, while the race L produces lycopadiene, a C_{40} tetraterpene (Li et al., 2013). In terms of thermal values, these hydrocarbons are more attractive as a biofuel for jet engines than other fatty acid-derived microalgal lipids (Furuhashi et al., 2013). However, *B. braunii* has a slower growth rate compared to other microalgal species. The slow growth of this algal species is due to the production of high-energy containing hydrocarbons (Zhang et al., 2011).

Lipids from microalgae have been extracted using different eluting solvents as described in the literature. Soxhlet extraction using hexane and the Bligh and Dyer (1959) technique using a mixture of chloroform and methanol are the two most commonly reported microalgal lipid extraction methods (Kim et al., 2012; Zhang et al., 2014). The lipid extraction yields depend on the polarity of the organic solvent or mixture of solvents used for the extraction and the extraction conditions. In general, a mixture of non-polar/low polarity organic solvent and polar organic solvent extracts higher amount of lipids (Ryckebosch & Muylaert, 2012). Therefore, several studies have been conducted using solvent mixtures such as chloroform-methanol (2:1, v/v) (Folch et al., 1957), hexane-isopropanol (3:2, v/v), dichloroethane-methanol (1:1, v/v), dichloroethane-ethanol (1:1, v/v), acetone-dichloromethane (1:1, v/v) (Lee et al., 1998), and hexane-ethanol (Grima et al., 1994) to ensure the complete extraction of lipids. Other solvent extraction methods include methyl-*tert*-butyl ether (MTBE) (Matyash et al., 2008), and ethanol (Fajardo et al., 2007). However, concerns about high toxicity towards human health and the surrounding environment, and sustainability issues related to using organic solvents has driven a demand of methods that use lower amounts of these solvents (Li et al., 2014).

The present work presents the extraction of lipids from freeze-dried microalgae in a Soxhlet extractor using a chloroform/methanol solvent mixture. The objective of this extraction is to determine the total extractable lipid contents of the microalgal species used in this research. The results of this extraction are used as a benchmark and compared with the results of the other extraction methods explored in subsequent chapters. The methods are compared on the basis of total extracted lipid mass, biodiesel-desirable lipids (neutral lipids and free fatty acid), and the concentration of key fatty acid residues in the extract after methylation.

2.2 Methods and materials

All chemicals were used as received from the suppliers. The species of *B. braunii* race A UTEX 572, cultivated as described by MacDougall et al. (2011) was obtained from the National Research Council (NRC), Halifax, Canada. Cells were freeze-dried to a final moisture content of 3% (w/w).

2.2.1 Lipid extraction

About 250 mg of dry algae was placed into a Soxhlet extractor glass thimble and extracted using 2:1 (v/v) chloroform/methanol ($CHCl_3$/MeOH) mixture (25 mL) by the extractor in an oil bath at 80 °C (Figure 2.1). After 24 h of extraction, the solvents were removed by rotary evaporation under reduced pressure. The extract was further dried in a vacuum oven at 55 °C for 3 h and the resultant lipid was weighed to determine the gravimetric lipid extract yield. An extraction at lower temperature (35 °C) and for a shorter period of time (2 h) was also performed with $CHCl_3$/MeOH in an Erlenmeyer flask rather than a Soxhlet extractor to compare the extraction yields under similar conditions as was used in the CO_2-expaned methanol method

(Chapter 3). All extractions were completed in triplicate except if otherwise indicated.

Figure 2.1 Soxhlet extractor for the extraction of lipids using organic solvent.

2.2.2 Lipid fractionation by solid phase extraction (SPE)

Algal lipid extract obtained from the Soxhlet extraction was fractionated and collected as described by Bodennec et al. (2000) using Supelclean^{TM} LC-NH_2 (500 mg) cartridges. The cartridge was placed on a Vacuum Manifold and preconditioned with 6 mL of hexane. Extract (25 mg) diluted in a minimum volume of hexane was placed on the preconditioned cartridge and allowed to seep in without vacuum. The cartridge was then eluted with three different solvents (flow rate: ~1 ml/min) to collect three fractions of the sample. The solvents used to collect those fractions were – fraction A, 5 mL of 15:85 (v/v) ethyl acetate:hexane; fraction B, 4 mL of 23:1 (v/v) chloroform:methanol and fraction C, 3 mL of 92:2 (v/v) ethyl ether:acetic acid. The fractions were retrieved and the solvent was removed under reduced pressure on a rotary

evaporator followed by a vacuum oven at 55 °C for 3 h. The fractions were measured gravimetrically.

2.2.3 Fatty acid methyl ester (FAME) preparation and analysis

Acid catalysed transesterification was carried out to prepare FAME from the algal extracts. The transesterification/esterification (methanolysis) reaction was accomplished using the method described by (Lam & Lee, 2013) with slight modifications. In this method, 11 mg of concentrated H_2SO_4 was mixed with 1.5 mL of methanol and 1 mL of THF. The mixture was mixed with 50 mg algal extract in a reaction flask. Next, the reaction mixture was refluxed at 90 °C with continuous stirring for 3 h. The mixture was then neutralized with solid $NaHCO_3$ and extracted with hexane. The FAMEs obtained were analyzed by a Perkin Elmer Clarus 680 gas chromatograph (GC) equipped with a flame ionization detector (FID) and HP-INNOWax column (30 m × 320 μm id × 0.25 μm film of polyethylene glycol). The oven temperature program was initiated at 100 °C, for 5 min, raised to 250 °C at a rate of 10 °C/min and was held at 250 °C for 20 min. The injector and detector temperatures were 250 °C. Helium was used as the carrier gas. The standard FAMES: C14:0, C16:0, C16:3, C18:0, C18:1, C18:2, C18:3 and C20:0 were used to determine the retention times and to construct calibration curves for quantification (R^2= 0.99). The FAME peaks in the samples were identified by comparing their retention times with those of the standards.

2.3 Results and discussion

The chloroform/methanol Soxhlet extraction of *B. braunii* biomass resulted in a total lipid yield of 50 wt% relative to the mass of dry algae (Table 2.1). An extraction, in a flask,

using the same solvent ($CHCl_3$/MeOH) system at lower temperature and for a shorter time (35 °C for 2 h) gave a total lipid yield of only 33 wt% of dry algae suggesting the need for more elevated temperatures and reaction times for maximum yields.

Table 2.1 Results of lipid extraction using $CHCl_3$/MeOH solvent mixture.

Method	Vol. of organic solvent (mL)	Temperature (°C)	Time (h)	Total Yield (Wt % of dry algae)	Yield of algae lipid components (wt % of dry algae)		
					NL	FFA	Other
$CHCl_3$/MeOH	25[a]	80	24	50±1	14±0.3	9.0±0.9	27±2
	25[b]	35	2	33	6.5	15.6	10.9

[a]Extraction was performed in a Soxhlet extractor. Each value represents the mean ± S.D. (n=3). [b]Extraction was conducted in a flask and only one experiment was performed. Abbreviations: Neutral lipids (NL), free fatty acids (FFA), other undesirable materials extracted (Other).

In order to define the selectivity of this extraction method for neutral lipids (NLs) and free fatty acids (FFAs), the NL and FFA contents of the extracts were determined by solid phase extraction. As reported by Bodennec et al. (2000), extracting the lipid extract with the ethyl acetate - hexane mixture collected the less polar lipids such as tri-acylglycerols and 1, 2- and 1, 3- diacylglycerides (Fraction A), which are designated here as NL. A solvent mixture of chloroform - methanol eluted other lipids such as ceramides (Fraction B), which are categorized in Table 2.1 as "Other" because these are not considered to be useful in biodiesel production. Elution of fraction C, the FFA fraction, consisting of normal and α- hydroxy free fatty acids, was achieved by using an acetic acid - diethyl ether solvent mixture as reported by Kaluzny et al. (1985). Further elution of the extract, as described by Bodennec et al., (2000) would not result in

the yield of lipids desirable in biodiesel production and hence was not performed. The remaining mass of the extract plus the mass of fraction B are listed together as 'Other'. The fractionation of the lipids by solid phase extraction showed that, of the total lipid extracted, 14 wt% was neutral lipids and 9 wt% free fatty acid, while the remaining 27 wt% was undesired lipids such as phospholipids and ceramides (Table 2.1).

The selectivity of the $CHCl_3$/MeOH solvent mixture for biodiesel-desirable lipids was further determined by GC-FID analysis of the biodiesel obtained from the algal extract. A previous study of the lipid composition of this batch of algal species showed that the principle fatty acid residues were C16:0, C16:3, C18:1, C18:2 C18:3 and some long chain fatty acids 28:1 and 28:2 (Boyd et al., 2012). Among these, C16:0, C16:3, C18:1, C18:2 and C18:3 are commonly major components of biodiesel made from microalgae (Halim et al., 2012). Therefore, the selectivity of the solvent system for these major acid components was measured by esterifying/transesterifying the extract with methanol and then analyzing the resulting FAME mixture. As shown in Figure 2.2, the $CHCl_3$/MeOH extracts contained significant quantities of C16:0 C18:0 and C18:1 fatty acid residues, while the C18:2 was somewhat less abundant in the extract.

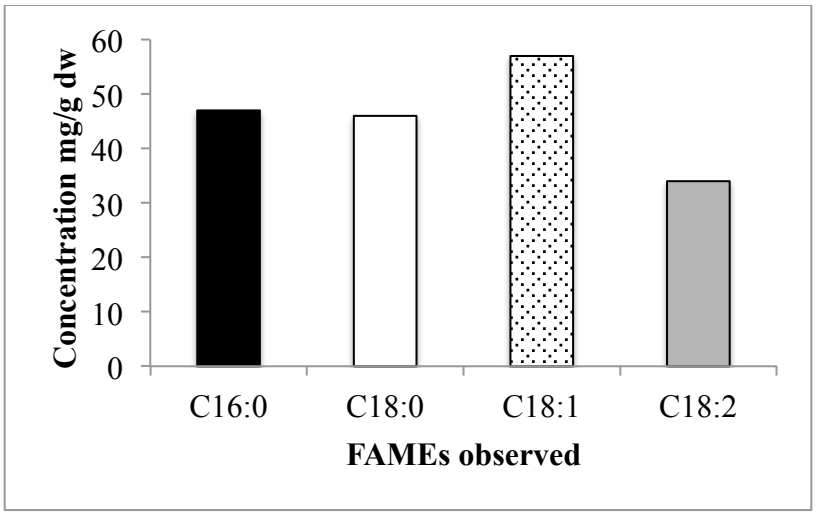

Figure 2.2 Concentrations of key fatty acid residues in the $CHCl_3$/MeOH Soxhlet extract after methylation.

The lipid and the FAME fractionation results showed that the $CHCl_3$/MeOH solvent mixture had a moderate affinity for the neutral lipids, which accounted for an average 23% of the mass of the extract. This value is much higher than reported in a previous work conducted by Boyd et al. (2012). This discrepancy may be explained by batch-to-batch variation and the harvesting times of the algae. Once the culture reaches a stationary phase, the triglycerides are broken down into free fatty acids. Therefore, the later the *B. braunii* culture is harvested, the higher the chances of obtaining lower triglycerides concentrations.

2.4 Conclusion

The extracted total lipid yield from microalgae depends on the solvent used for the extraction as well as on the extraction conditions. The chloroform/methanol solvent system, containing a mixture of non-polar and polar solvent, at 80 °C extracted 50 wt% (relative to the

dry algae) lipids from dried microalgae biomass. The solvent mixture extracted greater amounts of lipids at higher temperature and longer reaction time than at lower temperature and shorter reaction time. The fractionation of the lipids demonstrated by solid phase extraction showed that the $CHCl_3$/MeOH solvent mixture had moderate affinity for the neutral lipids. According to the results obtained with this extraction method, about 23 wt% (relative to the dry biomass) of biodiesel-desirable lipids could be expected from this batch of *B. braunii*. This will be treated as the benchmark by which the less conventional extraction solvents: CO_2-expanded methanol and liquid CO_2, will be compared in this research.

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Chapter 3

EXTRACTION OF LIPIDS FROM FREEZE-DRIED MICROALGAE USING CO_2-EXPANDED METHANOL

3.1 Introduction

Gas expanded liquids (GXLs) are liquids expanded in volume by the application of modest pressures with a compressible gas, CO_2 being one of the most common (Subramaniam et al., 2014). The GXLs consist of a mixture of conventional solvents and compressed gases. As such, GXL solvents have the combined beneficial properties of a compressed gas and organic solvent, resulting in solvent properties that can be adjusted through variations in the pressure (Jessop & Subramaniam, 2007). In particular, the presence of dissolved gas results in a change in polarity of the organic solvent, tunable solubility, and transport properties like viscosity (Zevnik & Levec, 2007). When CO_2 is used as the compressed gas, the resulting GXL is termed a CO_2-expanded liquid (Subramaniam & Akien, 2012). The CO_2-expanded liquids (CXLs) can be created at comparatively mild pressures with a significant replacement of the organic solvent with CO_2. As shown in Figure 3.1, gaseous CO_2 has a substantial solubility in many benign organic solvents at adequate pressures (<8 MPa). For all of these solvents, a 2- to 3-fold volumetric expansion is observed at moderate temperatures at relatively mild pressures reaching as high as six fold expansion for ethyl acetate at around 5.5 MPa (Jessop & Subramaniam, 2007).

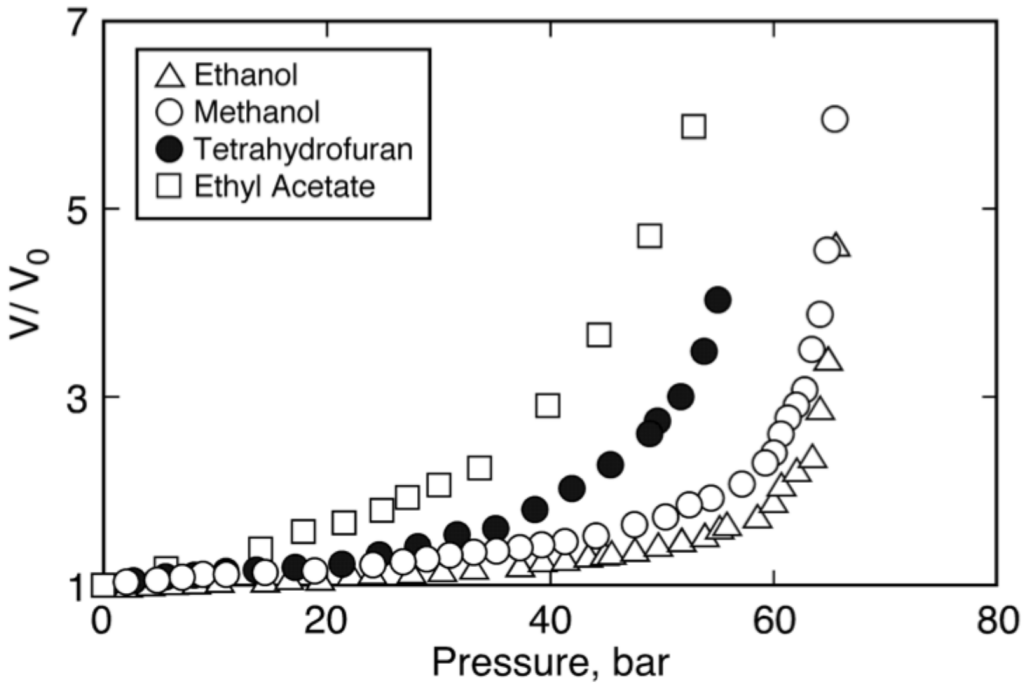

Figure 3.1 Isothermal volumetric expansion of benign solvents by CO_2 at 40 °C (Jessop & Subramaniam, 2007).

CXLs are the most commonly used class of GXLs, representing a continuum of liquid media ranging from the neat organic solvent to supercritical carbon dioxide (*sc*CO_2). The CXLs provide an opportunity to change the physicochemical properties of the liquid solvents, most notably the polarity, dielectric constant, and solubility, at much lower pressures than those required to use a supercritical fluid. Furthermore, CXLs offer many other benefits such as environmental advantages including substantial replacement of organic solvents with environmentally benign CO_2 and process advantages incorporating reduced flammability due to CO_2 presence in the vapor phase and milder process pressures compared to *sc*CO_2 (Subramaniam, 2010). Also, CO_2 is an ideal anti-solvent, because it is easily removed and recyclable (Hallett et al., 2006).

In recent years, CXLs have been used in a variety of applications including extractions, separations, reactions and some other purposes (Scurto et al., 2009). The milder pressures (≤8 MPa) required for working with CXL as compared to *sc*CO_2 make the applications of CXLs more attractive. CO_2-expanded ethanol has been used to extract moderately polar food product (γ-linolenic acid) from Spirulina microalgae (Golmakani et al., 2012). Moreover, CXLs have been used to synthesize methyl esters from extracted triacylglycerols or free fatty acids (Ginosar et al., 2008). Although many of the CO_2-organic solvent systems have been assessed for solvent system, the CO_2-methanol system has been studied most (Bezanehtak et al., 2001; Gui et al., 2011; Román-Ramírez et al., 2010). The CO_2-expanded methanol (cxMeOH) system consists of pressurized gaseous CO_2 as a co-solvent that is dissolved in methanol. As shown in Figure 3.1, compressed CO_2 at 40 °C is soluble in methanol, causing it to volumetrically expand about six fold at around 7 MPa. CO_2-expanded methanol has been used for the production of fatty acid methyl esters, (FAMEs) using H_2SO_4 as a catalyst (Wyatt & Haas, 2009). However, the potential use of CXLs for the extraction of lipids from natural products such as microalgae has not been explored yet.

Conventional methods of lipid extraction, such as Soxhlet extraction, are time consuming and require large amounts of solvents. Therefore, new extraction techniques that offer reduced extraction time, abridged organic solvent intake, and increased environmental protection are in high demand. An efficient extraction of microalgal lipids depends on the polarity of the eluting solvent. In particular, a solvent system containing a mixture of non-polar and polar solvents could extract higher yields of lipids than a single solvent extraction (Ryckebosch & Muylaert, 2012). Thus, a mixture of CO_2 and methanol, like the more traditional chloroform/methanol mixture, should retain the oil-solvating ability of the low polarity component (CO_2 or

chloroform) and the ability of methanol to enhance cell wall degradation. In cxMeOH, the CO_2 not only reduces the volume of the organic solvent required for the extraction process but also decreases the solvent polarity, which could increase the selectivity of the solvent for neutral lipids and free fatty acids.

In the present study, the use of cxMeOH is explored for the extraction of neutral lipids (NL) and free fatty acids (FFA) from microalgae. The extractions were carried out under moderate temperature (35 °C) and pressure (7.2 MPa) to reduce the eventual larger scale capital and processing costs. The use of this method for the extraction of lipids from microalgae could present an advantage to the use of conventional solvents because this requires little to no flammable, highly volatile, or chlorinated solvents.

3.2 Methods and materials

All chemicals were used as received from the suppliers. Carbon dioxide (4.0 grade 99.99%) was obtained from Praxair. HPLC grade methanol was used for the expansion and extraction process. A microalgae species of *Botryococcus braunii* race A UTEX 572, cultivated as described by (MacDougall et al., 2011) was obtained from the National Research Council (NRC), Halifax, Canada. Cells were lyophilized to a final moisture content of 3% (w/w).

The algae used in the extractions were freeze-dried (hereinafter called ‘dry algae’). All extractions were conducted in triplicate except as indicated, and the data reported include mean values and standard deviations.

3.2.1 Lipid extraction using cxMeOH

For the extraction of lipids from dry algae, appropriate amounts of methanol were added to a 160 mL Parr high-pressure vessel fitted with a dip tube inside. The methanol in the vessel was then heated in a water bath at 35 °C. After the temperature had equilibrated, CO_2 was pumped by an ISCO model 500D pump through the system for 15 min at 7.2 MPa. The compressed CO_2 is soluble in methanol causing it to volumetrically expand. The incoming tube carrying CO_2 was vertically positioned above the methanol, while the entrance of the outgoing tube (the dip tube) carrying out the cxMeOH was placed at the bottom of the vessel. Dry algae (300 mg) were placed in a 3 mL stainless steel tube vessel. The vessel was packed, on edges, with glass wool to filter the extract from any solids, as well as filter agent, diatomaceous earth, to provide filtration and to serve as an anchoring co-matrix to increase the contact surface area between the microalgal cells and the eluting solvent (Halim et al., 2011). The cxMeOH from the vessel was passed through the tube vessel, which was also heated at 35 °C, at a constant flow rate of 0.15 mL/min. The pressure in the system was maintained at 7.2 MPa by a back-pressure regulator (BPR). The extract exiting the BPR was captured in isopropanol in an ice-cooled Erlenmeyer flask (Figure 3.2). After 2 h of extraction, the remaining CO_2 was vented into the flask. The vessel and the tubing were not further washed with any other solvent. The extract was transferred into a pre-weighed vial and the solvent was removed by evaporation. The extract was further dried in a vacuum oven at 55 °C under reduced pressure for 3 h and weighed to determine the gravimetric lipid yield.

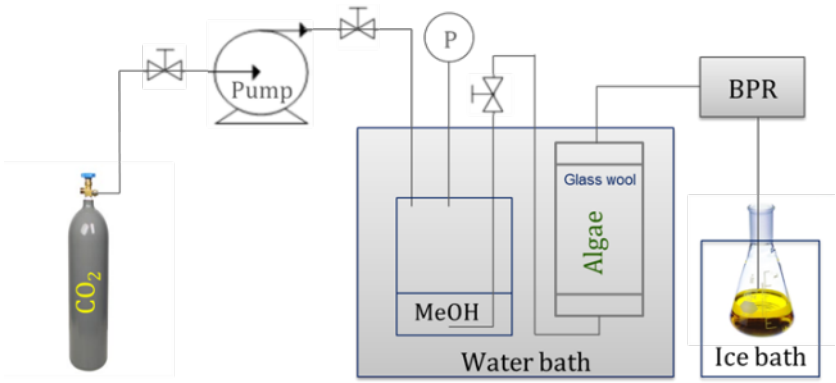

Figure 3.2 Apparatus for extractions using cxMeOH, where P is a pressure gauge and BPR is a back-pressure regulator. Expansion of MeOH and lipids extraction was performed at 35 °C and 7.2 MPa. The extract was collected in ice-cold isopropanol.

3.2.2 Lipid extraction using neat MeOH

The extraction was also repeated with neat methanol to compare the results with the CO_2-expanded MeOH method. An ISCO model 500D pump was charged with 28 mL of methanol at 7.2 MPa (Figure 3.3). While methanol does not require the use of such elevated pressure, this pressure was used in order to ensure an equal comparison to the results with cxMeOH. The pressurized methanol was then used to extract lipids from 300 mg of dry algae in the 3 mL tube vessel at 35 °C with a flow rate of 0.08 mL/min for 2 h. The lower flow rate than that utilized for cxMeOH method (0.08 mL/min vs 0.15 mL/min) was used in order to minimize the volume of methanol used. The volume of methanol used was then 9.6 mL (0.08 mL/min x 120 min). Additionally, ~3.2 ml of the methanol present in the tube vessel and the downstream tubing was also considered a part of the volume of methanol used for the extraction. The total volume of methanol used was then 12.8 (9.6 + 3.2) mL, which was approximately five times greater than

the volume of methanol used in the cxMeOH extraction. The pressure in the system was maintained at 7.2 MPa by BPR. The extract exiting the BPR was captured, separated from the methanol and dried using the same procedure described for the cxMeOH extraction.

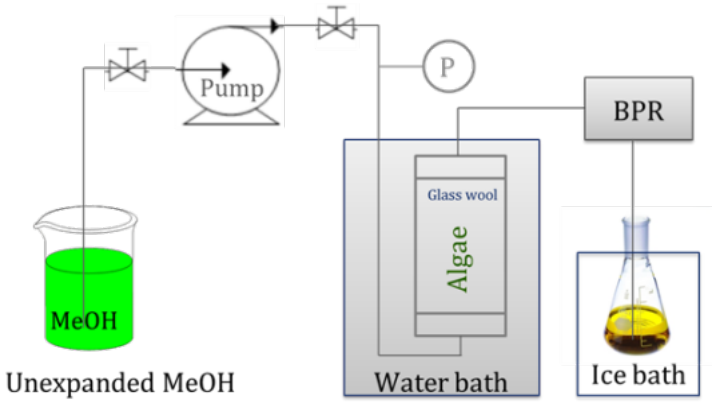

Figure 3.3 Apparatus for extractions using neat MeOH, where P is a pressure gauge and BPR is a back-pressure regulator. Extraction was performed at 35 °C and 7.2 MPa, and the extract was collected in ice-cold isopropanol.

3.2.3 Lipid fractionation

The lipid extract was fractionated and collected as described by Bodennec et al. (2000) using solid phase extraction (SPE) Supelclean^{TM} LC-NH_2 (500 mg) cartridges. In this method, the cartridge was placed on a Vacuum Manifold (Figure 3.4) and preconditioned with 6 mL of hexane. Extract (25 mg) diluted in a minimum volume of hexane was placed on the preconditioned cartridge and allowed to seep in without vacuum. The cartridge was then washed with three rinses of solvents (flow rate: ~1 ml/min) to collect three fractions of the sample. The solvents used to collect those fractions were – fraction A, 5 mL of 15:85 (v/v) ethyl acetate-hexane; fraction B, 4 mL of 23:1 (v/v) chloroform-methanol and fraction C, 3 mL of 92:2 (v/v)

ethyl ether-acetic acid. The fractions were retrieved and the solvent was removed under reduced pressure on a rotary evaporator followed by a vacuum oven at 55 °C for 3 h. The fractions were measured gravimetrically.

Figure 3.4 Vacuum manifold for solid phase extraction of microalgal lipid extract.

3.2.4 Fatty acid methyl ester (FAME) preparation and analysis

Acid catalysed methanolysis was carried out to prepare FAME from the algal extracts. The methanolysis (transesterification/esterification) reaction was accomplished using the method described by Lam & Lee (2013) with slight modifications. In this method, 11 mg of concentrated H_2SO_4 was mixed with 1.5 mL of methanol and 1 mL of THF. The mixture was mixed with 50 mg algal extract in a reaction flask. The reaction mixture was refluxed at 90 °C with continuous stirring for 3 h. The mixture was then neutralized with $NaHCO_3$ and extracted with hexane. The FAMEs obtained were analyzed by a Perkin Elmer Clarus 680 gas chromatograph (GC) equipped with flame ionization detector (FID) and HP-INNOWax column (30 m × 320 µm id ×

0.25 μm film of polyethylene glycol). The oven temperature program was initiated at 100 °C, for 5 min, raised to 250 °C at a rate of 10 °C/min and was held at 250 °C for 20 min. The injector and detector temperatures were 250 °C. Helium was used as the carrier gas. The standard FAMES: C14:0, C16:0, C16:3, C18:0, C18:1, C18:2, C18:3 and C20:0 were used to determine the retention times and to construct calibration curves for quantification (R^2= 0.99). The FAME peaks in the samples were identified by comparing their retention times with those of the standards.

3.3 Results and discussion

To optimize the volume of methanol required, extraction using cxMeOH was performed under a series of mass ratios of dry algae to normal unexpanded methanol (Table 3.1). Of the different mass ratios (dry algae:methanol) tested, a ratio of 1:7 was found to be the optimum ratio required for maximum yields. The results of the extraction showed that the ratio of 1:7 extracted the same 24 wt% yield of bio-oil as the mass ratios of 1:20 and 1:10. The lower ratio of 1:5 resulted in 16 wt% yields.

The lipid yields of pure methanol extraction were comparable to cxMeOH extraction yields. However, the technique required about 5 times more methanol than in the cxMeOH method. The mass of extracted lipids obtained using the more traditional chloroform/methanol solvent mixture Soxhlet extraction at 80 °C for 24 h method was double (50 wt% of dry algae) that obtained with cxMeOH, presumably due to the use of a much higher temperature, greater volume of organic solvent, and longer extraction time. An extraction using the chloroform/methanol ($CHCl_3$/MeOH) mixture at lower temperature and for a shorter extraction time (35 °C for 2 h) gave a total lipid yield of only 33 wt% of dry algae suggesting the need for

more elevated temperatures and reaction times for maximum yields. Thus, when compared at equal temperatures and extraction time, the yield obtained with the $CHCl_3$/MeOH mixture was only moderately higher than that obtained with cxMeOH.

Table 3.1 The conditions and yields of lipid extraction using cxMeOH.

Method	Vol. of Solvent (mL)	Algae: Solvent (w/w)	T (°C)	P (MPa)	Total Yield (wt% of dry algae)	Yield of algae lipid components (wt% of dry algae)		
						NL	FFA	Other
cxMeOH	7.6	1:20	35	7.2	25±0.5	6.9±0.8	12.8±1	4.3±1
	3.8[a]	1:10	35	7.2	24±0.4	-	-	-
	2.7	1:7	35	7.2	24±0.7	6.l±0.4	12.5±0.7	5.2±0.6
	2.7[b]	1:7	50	7.2	24	5.6	13.1	5.1
	1.9[a]	1:5	35	7.2	16±2	-	-	-
MeOH	12.8	1:33	35	7.2	23±1	4.2±0.7	11.5±0.6	7.3±1
$CHCl_3$/MeOH	25.0[c]	1:104	80	0.0	50±1	14±0.3	9.0±0.9	27±2
	25.0[d]	1:104	35	0.0	33	6.5	15.6	10.9

All extractions were performed with 300 mg of dry algae. The extraction processes were run for 2 h. Each value represents the mean ± S.D. (n = 3). [a]For these extractions, n=2 and SPE fractionation was not done. [b]Only one experiment done. [c]The process was carried out for 24 h. [d]The experiment was performed for 2 h by just soaking dried algae in the solvent. Abbreviations: temperature (T), pressure (P), neutral lipids (NL), free fatty acids (FFA), other undesirable materials extracted (Other).

In order to compare the selectivities of each extraction method for NLs and FFAs, the NL and FFA contents of the extracts were determined by solid phase extraction. In SPE, the sequential elution of the extract (a lipid mixture) on aminopropyl cartridges by solvents with different solvent polarities allows the separation of the extract into neutral lipids, free fatty acids, phospholipids and ceramides. The extract was successively eluted by three different solvent

mixtures: ethyl acetate-hexane, chloroform-methanol and ethyl ether-acetic acid. As reported by Bodennec et al. (2000), extracting the lipid extract with the ethyl acetate - hexane mixture collects the non-polar lipids such as tri-acylglycerols and 1, 2- and 1, 3- diacylglycerides (fraction A), which are designated here as NL. A solvent mixture of chloroform - methanol eluted other neutral lipids such as ceramides (fraction B), which are categorized in Table 3.1 as "Other" because these are not considered to be useful in biodiesel production. Elution of fraction C, the FFA fraction, consisting of normal and α-hydroxy free fatty acids, was achieved by using an acetic acid - diethyl ether solvent mixture as reported by Kaluzny et al. (1985). Further elution of the extract, as described by Bodennec et al. (2000) would not result in the yield of lipids desirable in biodiesel production and hence was not performed; the remaining mass of the extract plus the mass of fraction B are listed as 'Other'.

Of the tested solvent systems, cxMeOH, pure MeOH, and $CHCl_3$/MeOH, the cxMeOH extracted the largest amount of FFAs while the pure MeOH extract had comparable amounts of FFAs to cxMeOH extract (Table 3.1). It is not necessarily desirable to simultaneously extract both NL and FFA as it is difficult to transesterify both of these chemical classes in the same step if an alkali catalyst is used. However, algae oil has a high FFA content regardless of the solvent used. A number of studies have been presented to identify suitable methods for biodiesel production from lipids with high FFA content. Bio-catalyzed transesterification (Al-Zuhair et al., 2009; Chesterfield et al., 2012; Fjerbaek et al., 2009; Yan et al., 2012) and two-step catalyzed esterification and transesterification (Awolu & Layokun, 2013; Charoenchaitrakool & Thienmethangkoon, 2011; Sarantopoulos et al., 2014) have been investigated to produce biodiesel from feedstock with high FFA content. Therefore, depending on the subsequent

processing steps, the high FFA content extracted along with NLs could increase overall biodiesel yields without negatively affecting biodiesel production.

Total NL extracted by cxMeOH was comparable to that extracted by $CHCl_3$/MeOH at the same temperature (35 °C) but inferior to that extracted by $CHCl_3$/MeOH at the higher temperature (80 °C). Raising the temperature of the cxMeOH extraction to 50 °C did not increase the yield of NL. The cxMeOH extracted a minimal amount of other lipids compared to both neat methanol and the $CHCl_3$/MeOH method. While the $CHCl_3$/MeOH method extracted a greater amount of NLs, it is unusable in practice because of the long extraction time, high temperature, use of a chlorinated solvent, and its propensity to extract a large amount of other lipids that could interfere with the transesterification process.

Selectivity of the extraction methods for biodiesel-desirable lipids was further determined by GC-FID analysis of biodiesel obtained from the algal extract (Figure 3.5). Previous study on the lipid composition of this batch of algal species showed that C16:0, C:16:3, C18:1, C18:2 C18:3 and some long chain fatty acids 28:1 and 28:2 could be found in the composition of the extract (Boyd et al., 2012). Among these, C16:0, C16:3, C18:1, C18:2 and C18:3 are the primary components in microalgal biodiesel production (Halim et al., 2012). The biodiesel derived from unsaturated fatty acid has advantageous properties. The saturated fatty acid chains form semi crystalline structures at low temperature while the unsaturated chains are less likely to crystallize and so freeze at lower temperature. Therefore, we tried to observe the selectivity of our methods for these major acid components. As shown in Figure 3.5, the cxMeOH extracted comparable amounts of C16:0 and C18:0-2 as $CHCl_3$/MeOH, despite the latter method having extracted a double amount of lipids. It has been reported that $scCO_2$ has designated selectivity towards

certain acids (Halim et al., 2012); the CO_2 dissolved in MeOH might have increased the selectivity of cxMeOH towards specific acid chains.

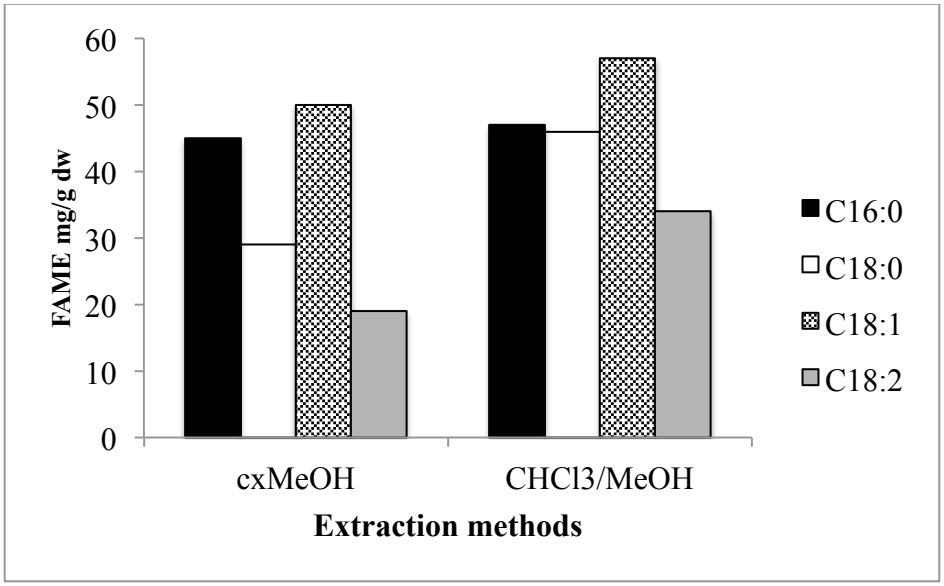

Figure 3.5 Concentrations of key fatty acid residues in the cxMeOH extract, relative to mass of dry algae, compared with $CHCl_3$/MeOH extract after methylation. The FAME composition includes methyl esters of palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1) and Linoleic acid (C18:2).

The energy and environmental costs of an extraction process are related in part to the amount of organic solvent used, because of costs of the manufacturing, purification, removal and disposal of the solvent; thus obtaining the highest lipid content possible per unit volume of organic solvent is desired. We therefore compared these solvents in terms of the yield of FFA and NL per ml of organic solvent. The cxMeOH was superior (21 mg of NLs and FFAs per mL of organic solvent) to neat methanol (3 mg/mL) and $CHCl_3$/MeOH (3 mg/mL), as shown in Table 3.2. Because methanol is subsequently used for biodiesel production process, the

remaining methanol after extraction with neat or expanded methanol could be used for the subsequent esterification or transesterification (methanolysis) process. While CO_2 is not free of cost or environmental impact, it is significantly easier to remove and purify than organic solvent.

Table 3.2 Mass of lipid extracted per gram of dry algae and per mL of organic solvent.

Method	Vol of org. solvent/g dw (mL)	T (°C)	P (MPa)	Total yields (mg/g dw)	mg lipid (in 1 g dw) /mL of organic solvent		
					NL	FFA	Other
cxMeOH[a]	9	35	7.2	243	7.0±0.5	14.1±1	5.8±0.9
MeOH[b]	54	35	7.2	234	0.83±0.2	2.1±0.2	1.4±0.3
$CHCl_3$/MeOH[c]	75	80	0.0	503	1.9±0.1	1.1±0.1	3.7±0.2

The results shown were based on extractions from 0.3 g of dry algae and equivalent to 1 g of the dry mass. [a]Extraction with cxMeOH; ~9 mL (7 g) of MeOH becomes 54 mL upon expansion, ~6-fold expansion under the listed conditions (Jessop & Subramaniam, 2007) [b]Extraction with neat MeOH, an equivalent amount (54 mL of MeOH) to cxMeOH method was considered to be used. [c]Extraction with chloroform/methanol mixture, 75 mL of the mixture of solvents [2:1 (v/v)] was used. Abbreviations: dry weight of algae (dw), neutral lipids (NL), free fatty acids (FFA), other materials extracted (Other).

In terms of the selectivity of the extraction method for NLs and FFAs, cxMeOH exhibited 78-82% selectivity for those desired components compared to normal methanol (68%) and $CHCl_3$/MeOH mixture (46%). The selectivity data represent the percentage of desired lipids in the total lipid extracts. Because other materials such as pigments are undesirable in fuel products (Hossain & Salleh, 2008) and phospholipids reduce the efficiency of the catalyst used in the methanolysis process (Fjerbaek et al., 2009), an extraction system which minimizes the extraction of such undesirable constituents is preferable.

The lipid yields were also comparable to that obtained from *B. braunii* using other solvents (Boyd et al., 2012; Lee et al., 1998; Yamaguchi et al., 1987). The quantity of lipids extracted by cxMeOH were comparable to that extracted by switchable hydrophilicity solvent (22 wt% of dry algae total yield and NL content of 5.4 wt% relative to the total mass of dry algae) (Boyd et al., 2012). Also, the total lipid yield of the cxMeOH method (24 wt% dw) was analogous to bead beating cell disruption followed by chloroform/methanol extraction (28 wt% of dry algae) (Lee et al., 1998), as well as the lipid composition of *B. braunii* suggested by Yamaguchi et al (Yamaguchi et al., 1987) (30% total lipid content, with higher FFA content).

3.4 Conclusion

The use of CO_2-expanded methanol for the extraction of lipids from microalgae is preferable to the traditional elevated temperature chloroform/methanol method because it operates at lower temperature, is more selective for free fatty acids and neutral lipids, does not require any chlorinated solvent and uses less organic solvent. The cxMeOH is also advantageous over neat methanol because the volume of methanol needed for the extraction is reduced by up to five times. Moreover, the CO_2-expanded methanol extracts a larger amount of free fatty acids and neutral lipids per gram of organic solvent than the more polar non-expanded methanol and the conventional chloroform/ methanol method. Thus, the mixture of CO_2 and methanol, like the more traditional chloroform/methanol mixture, retains the oil-solvating ability of the low polarity component (CO_2 or chloroform) and the ability of methanol to enhance cell wall degradation. The maximum yield of lipids was obtained with a 1:7 mass ratio of dry algae to normal unexpanded methanol.

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Chapter 4

EXTRACTION OF LIPIDS FROM FREEZE-DRIED MICROALGAE USING LIQUID CARBON DIOXIDE

4.1 Introduction

Carbon dioxide is a colorless, odorless, non-toxic, and incombustible gas, which occurs naturally in the atmosphere. It is the primary greenhouse gas (GHG) emitted through the combustion of any carbon containing substance. In nature, it is released through the natural processes such as respiration, fermentation, ocean release, and volcanic activity. Human activities such as burning of fossil fuels, deforestation, and other processes substantially increase its concentration in the atmosphere, which disturb its natural balance. Currently, there are more CO_2 emissions being released to the atmosphere than are being absorbed.

There are a number of methods, including increased energy conversion efficiency, low carbon or carbon free energy sources, and carbon sequestration, that can be used to manage the level of CO_2 emitted into the atmosphere (Keffer & Kleinheinz, 2002). Of these, carbon sequestration reduces net emissions either through increasing natural sinks such as forestation, or by capturing the emitted CO_2 and storing it or converting it to benign materials through biological or chemical processes (NELT, 2000). Carbon dioxide capture and storage (CCS) has been widely recognized as a potential technology for mitigating global climate change (Rubin et al., 2013). However, less attention has been given to reusing the captured CO_2. The captured CO_2 utilization (CCU) could play an important role in mitigating climate change. The CCU includes

separation of CO_2 from industrial and energy related sources, its transportation and use in the fabrication or synthesis of new products or as a solvent or working fluid for various industrial processes (Hendriks et al., 2013). Moreover, by utilizing CO_2 as a precursor for the synthesis of chemical feedstocks, as supplemented inorganic carbon source for algae growth or in its pure form rather than treating it as waste, it is possible to retain the carbon within the cycle (Styring et al., 2011). Figure 4.1 shows some potential methods for utilization of captured CO_2.

Pure CO_2 is presently used in carbonation of drinks, and as a solvent in dry fabric cleaning and decaffeination. Liquid and supercritical CO_2 can be used to solubilize many organic molecules and can be recovered easily at the end of the process leaving behind the pure organics without solvent residue. However, the food additive or solvent CO_2 is only stored transiently and is eventually released back to the atmosphere, hence these would be considered recycling rather than mitigation approaches (Styring et al., 2011).

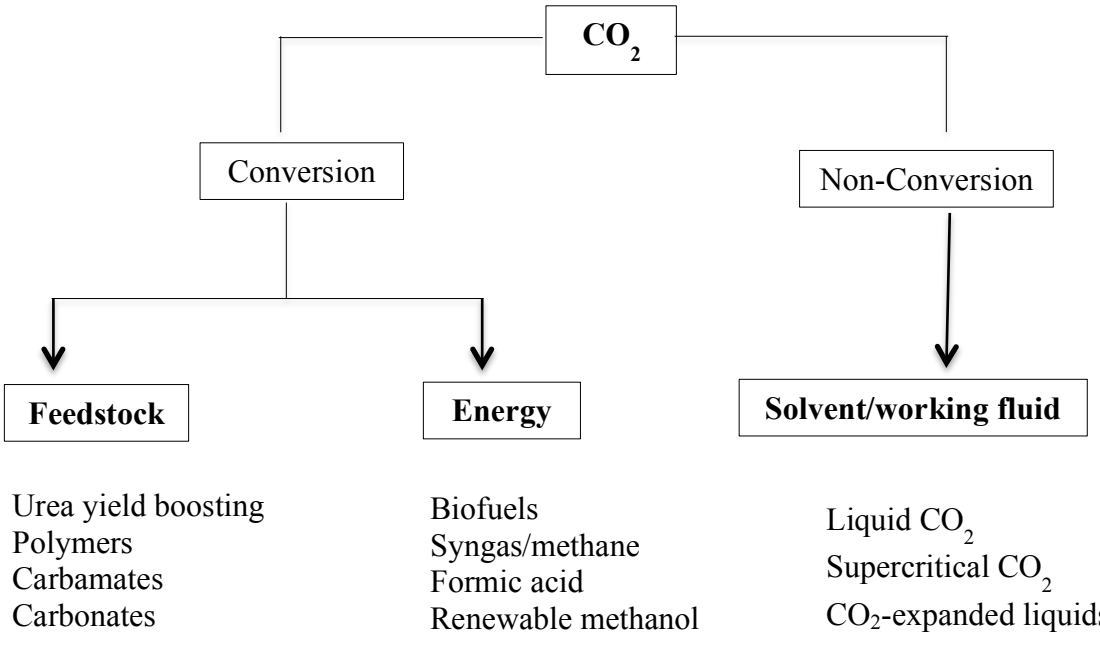

Figure 4.1 CCU application summary.

4.1.1 CO_2 phase diagram

The triple point (at which solid, liquid and gaseous states are in equilibrium) of CO_2 is at -56.6 °C and 0.51 MPa. As can be seen from Figure 4.2, above the triple point and below the critical point (31 °C and 7.38 MPa), carbon dioxide exists as a colorless liquid under mild conditions (~6.5 MPa and 25 °C). Above the critical point it exists as a supercritical fluid phase, the density of which is related to temperature and pressure conditions (Hyatt, 1984).

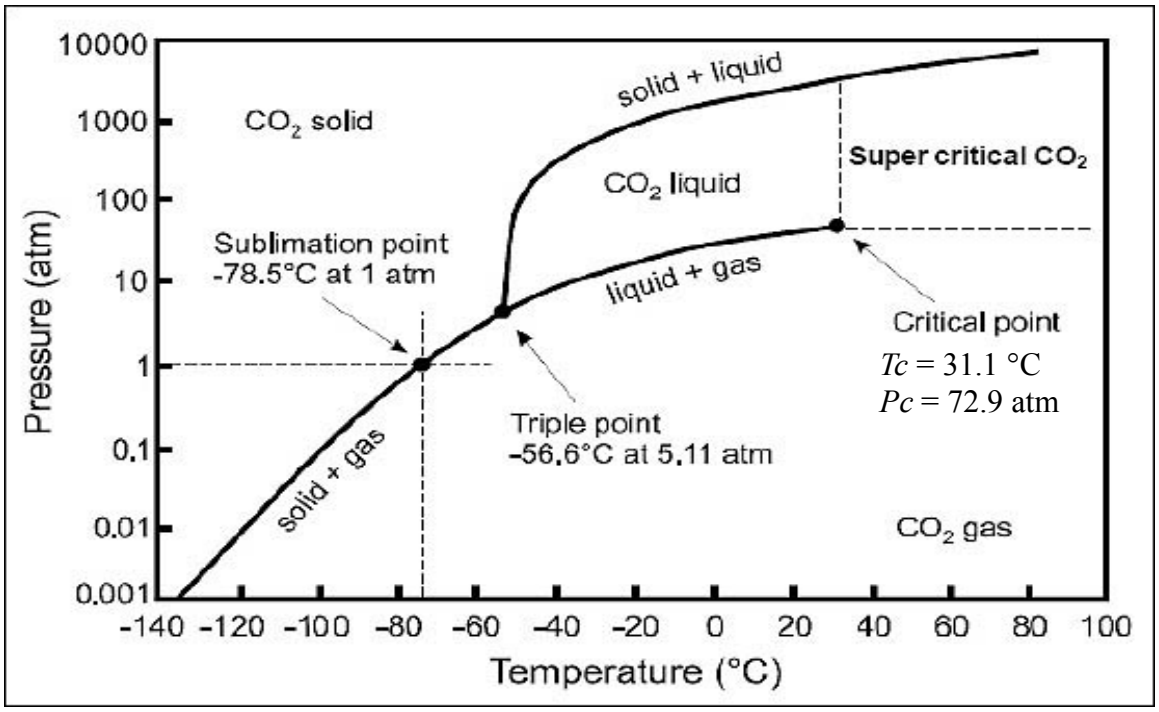

Figure 4.2 Phase diagram of CO_2 (source: http://toplowridersites.com/co2-pressure-temperature-phase-diagram/ accessed January, 2015).

4.1.2 Properties of carbon dioxide

The density of CO_2 is greatly affected by pressure and temperature, especially in the vicinity of the critical point. The density increases with increasing pressure, while it decreases

with rising temperature. As shown in Figure 4.3, a relatively higher pressure is required to achieve specific densities at higher temperature.

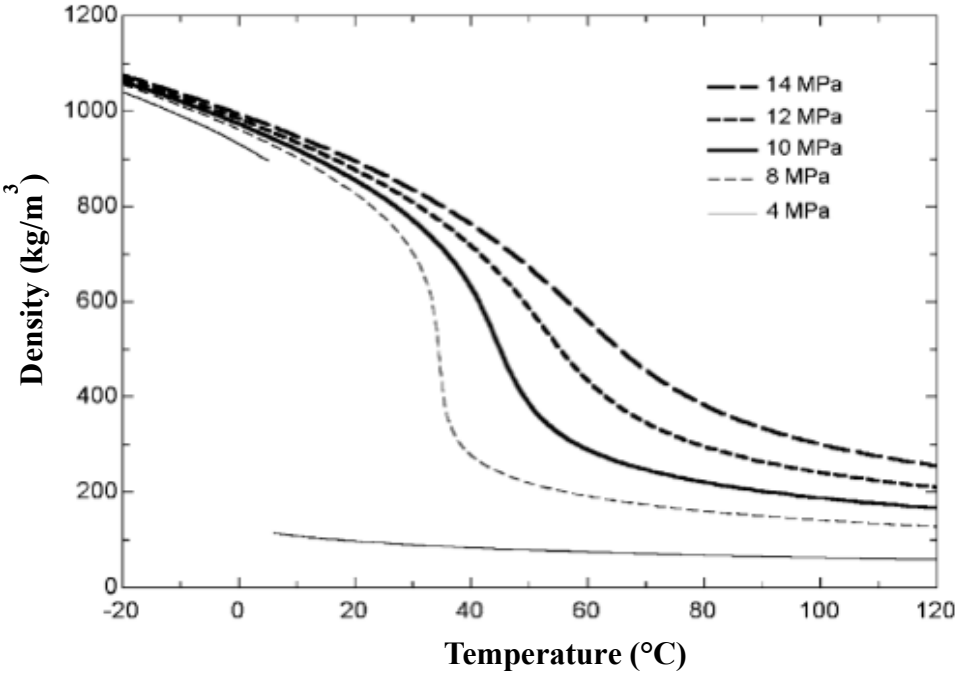

Figure 4.3 Density of CO_2 as function of temperature and pressure (Kim et al., 2004).

Table 4.1 compares the physical properties of gaseous, supercritical and liquid CO_2. As shown in the Table, supercritical CO_2 has a greater liquid like density and consequential greater solvation power than gaseous CO_2. The solvation power of CO_2 depends on its density. The pressure increases the density and hence the solvent power of the CO_2, which is more evident at pressures near the critical point where the CO_2 is more compressible (Orellana et al., 2013). The increased density and subsequently enhanced solvent power of carbon dioxide results in the higher solubility of compounds in the CO_2 (Jia, 2003). Moreover, the mass transfer is faster in supercritical CO_2 compared to liquid CO_2.

Table 4.1 Comparison of physical properties for gaseous, supercritical fluid and liquid CO_2 (Zhari, 2007; Leitner & Jessop, 2010).

Carbon dioxide (Phase)	Density (g/mL)	Dynamic Viscosity (g/cm.sec)	Diffusion Coefficient (cm^2/sec)
Gas	0.0006-0.002	0.0001-0.003	0.1-0.4
Supercritical fluid	0.1 – 1.0	0.0001-0.0003	0.0007
Liquid	0.6-1.6	0.002-0.03	0.000002-0.00002

4.1.3 Use of liquid or supercritical CO_2 as organic solvent

The use of subcritical (liquid) or supercritical CO_2 as a solvent has been exploited and its wider applications are still under investigation. Carbon dioxide is chemically inert under many conditions, and liquefiable at reasonable pressures. Hence, it has been used for a wide variety of applications such as separations, reactions and materials processing. It is miscible with a variety of organic solvents, and is readily recovered after processing because of its high volatility. It is a small linear molecule and thus diffuses more quickly than bulkier conventional liquid solvents (Sahena et al., 2009). Furthermore, CO_2 presents a reasonable compression cost while its critical temperature allows extraction of thermally sensitive compounds without degradation (Santana et al., 2012). Also, the CO_2 solvent facilitates safe extractions due to its low reactivity.

4.1.4 Liquid CO_2 extraction of lipids from microalgae

A solvent used for lipid extraction should be inexpensive, non-toxic, easily removable, lipid-specific to minimize the co-extraction of non-lipid constituents, and selective towards

desirable neutral (mono-, di-, and tri-acylglycerols) lipid and free fatty acid fractions (Halim et al., 2012). The conventional chloroform/methanol extraction method uses flammable and chlorinated organic solvent(s), which results in high extraction yields but also produces low quality lipids due to the co-extraction of undesirable polar lipids (Paudel et al., 2014). The energy requirements for solvent separation by distillation further reduce the overall efficiency of the process. Therefore, as CO_2 is a non-flammable, non-toxic, inexpensive and readily available solvent, it has been studied extensively as an alternative to the conventional solvent extraction methods. For instance, liquid carbon dioxide (lCO_2) and supercritical carbon dioxide ($scCO_2$) extraction techniques offer the advantages of separation with no residual solvents in the lipids, and CO_2 recycling.

Supercritical carbon dioxide ($scCO_2$) has been investigated as a means of extracting lipids from microalgae (Andrich et al., 2005; Bjornsson et al., 2012; Santana et al., 2012; Soha & Zimmerman, 2011). The use of $scCO_2$ has many advantages, including tunable solvent properties and rapid mass transfer (Halim et al., 2012). However, the technique requires high temperatures (100 °C) and pressures (41 MPa) to obtain yields comparable to conventional solvent extractions (Soha & Zimmerman, 2011). Liquid carbon dioxide (lCO_2) tenders many of the same benefits as $scCO_2$, but at a lower pressure and temperature. In addition, because of its low polarity compared to most organic solvents, lCO_2 could exhibit higher selectivity towards the neutral lipids, while exhibiting a limited affinity to non-neutral lipids (Moyler and Heath, 1987), but there is a possibility that the overall lipid yield might be lessened by this lower polarity.

In the present study, the use of lCO_2 for the extraction of lipids from microalgae has been explored. The study appraises the lCO_2 extraction efficiency of algal lipids compared with

conventional solvent methods by determining the selectivity of the solvent for biodiesel-desirable lipids and the major FAME composition of the methylated extract. The extraction was carried out under moderate temperature (25 °C) and pressure (6.8 MPa) to reduce capital and processing costs of the eventual larger scale production. Use of this method for the extraction of lipids from microalgae could present many advantages including sustainability, safety and selectivity when compared to the use of conventional solvents because this technique requires no flammable, chlorinated or volatile organic solvents.

4.2 Methods and materials

All chemicals were used as received from the suppliers. Carbon dioxide (4.0 grade 99.99%) was obtained from Praxair. A microalgae species of *Botryococcus braunii* race A UTEX 572, cultivated as described by (MacDougall et al., 2011) was obtained from the National Research Council (NRC), Halifax, Canada. Cells were freeze-dried to a final moisture content of 3% (w/w).

The algae used for all extraction experiments were lyophilized (hereinafter called 'dry algae')**.** All extractions were conducted in triplicate except if indicated otherwise, and the results reported include mean values and standard deviations.

4.2.1 Lipid extraction using liquid CO_2

Dry algae (300 mg) were placed in the 3 mL tube vessel, which was then connected to the high pressure system and heated in a water bath at 25 °C. Liquid CO_2, pressurized to 6.8 MPa using an ISCO model 500D pump, was then conveyed through the tube vessel for lipid extraction as shown in Figure 4.4. The pump was kept cold at -5 °C (using a coolant: 75% ethyleneglycol in

distilled water) to prevent it from being heated. The purpose of tube coil, as shown in Figure 4.4, was to make CO_2 (coming out from the cold pump) spend more time in the water bath before it got to the vessel, so that it would warm up to the temperature of the water bath before being delivered to the vessel. The liquid CO_2 extraction was conducted at different flow rates, ranging from 0.1 mL/min to 0.38 mL/min, as shown in Table 4.2. Extractions at higher pressure (12 MPa or 17 MPa) were also performed to observe the effects of pressure on lipid yields. The required pressure in the system was maintained by the back-pressure regulator (BPR). After 2 h of extraction, the remaining CO_2 was vented into the flask. The tube vessel and the tubing were not further washed with solvent. The extract exiting the BPR was captured, separated and dried using the same procedure described in Chapter 3.

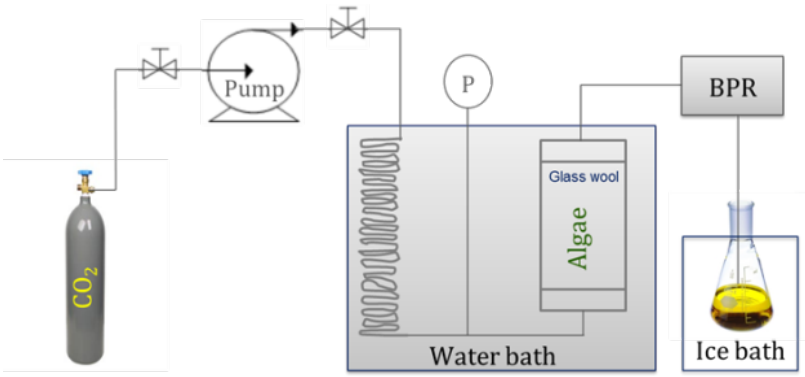

Figure 4.4 Apparatus for lipid extraction using lCO_2, where P is a pressure gauge and BPR is a back-pressure regulator. Extraction was performed at 25 °C and 6.8, 12 or 17 MPa and the extract was collected in ice-cold isopropanol.

4.2.2 Fractionation and methanolysis of extract

The algal lipid extract obtained from lCO_2 extraction was fractionated and collected using solid phase extraction (SPE). In addition, the extract was methanolysed in an acid catalyzed transesterification reaction and the resultant free fatty acid (FAME) sample was then analyzed by gas chromatography coupled to flame ionization detector (GC-FID) technique. All the experimental details of the fractionation, methanolysis and analysis are provided in Chapter 3.

4.3 Results and discussion

Liquid CO_2 extraction of lipids from *B. braunii* was performed over a range of flow rates (0.1 to 0.38 mL/min for 2 h), and hence, different volumes (12.6 to 45.5 mL) of lCO_2 (Table 4.2). Extraction of lipids from 0.3 g of dried algae with 45.5 mL of lCO_2 was found to be the most effective, obtaining a maximum yield (19 wt% of dry algae at 6.8 MPa). The lower yields of total extract with 25 mL (12 wt% of dry algae) and 12.5 mL (11 wt% of dry algae) of lCO_2 suggest that the lCO_2 may have been saturated with the extract within the vessel. A single extraction run was also carried out with liquid CO_2 at higher pressure (12 MPa), but these conditions did not improve the total and NL yields significantly. However, extraction at even higher pressure (17 MPa) increased the total lipid yields to 23 wt% of dry algae. This would suggest that an increase in the pressure of lCO_2 also increases the yields. The increase in extraction yields with increasing pressure may be due to the higher density of the fluid, increased cell destruction or the lower pH of the biomass-water-CO_2 system at higher pressure. Meyssami et al. (1992) reported that the fastest pH decrease in the water-CO_2 system should be expected at temperature between 32 °C to 42 °C and pressures up to about 5.5 MPa. However, at higher pressure, there is no significant decrease in the pH of the system with further pressure increase.

Hence, beyond this pressure, fluid density or increased cell damage would be the most likely contributors to improved lipid yields at 17 MPa. These results are in accord with Chen et al. (2013) and Soha and Zimmerman (2011), as they reported higher yields of lipids with increase in $scCO_2$ pressure at 30 °C or 100 °C.

Table 4.2 The conditions and yields of liquid CO_2 extraction method compared to $CHCl_3$/ MeOH method.

Method	Vol. of Solvent (mL)	Vol. of organic solvent (mL)	T (°C)	P (MPa)	Total Yield (wt% of dry algae)	Yield of algae lipid components (wt% of dry algae)		
						NL	FFA	Other
lCO_2	45.5	0.0	25	6.8	19±2	10.0±4	4.5±1	4.5±2
	45.5[b]	0.0	25	12.0	20	10.6	4.5	4.3
	45.5	0.0	25	17.0	23	10.7	7.2	5.1
	25.0	0.0	25	6.8	12±1	6.6±1	4.3±2	1.1±0.6
	12.6	0.0	25	6.8	11±1	6.5±4	3.5±1	1.0±0.4
$CHCl_3$/MeOH	25.0[c]	25	80	0.0	50±1	14±0.3	9.0±0.9	27±2
	25.0[d]	25	35	0.0	33	6.5	15.6	10.9

All extractions were performed with 0.3 g of dry algae. The extraction processes were run for 2 h except the chloroform/methanol method. Each value represents the mean ± S.D. (n = 3). [b]Only one experiment done. [c]The process was carried out for 24 h. [d]The experiment was performed for 2 h by just soaking dried algae in the solvent. Abbreviations: Temperature (T), Pressure (P), Neutral lipids (NL), free fatty acids (FFA), other undesirable materials extracted (Other).

As anticipated, the SPE fractionation of the extract showed that the non-polar lCO_2 had the highest selectivity for the NLs (53-59%). In terms of selectivity for NLs and FFAs combined, the lCO_2 at 12.5 to 25 mL volumes was superior (91-92%) compared to the other methods tested in this study (Table 4.2). The yield of NL was almost as high with lCO_2 at 25 °C as it was with the $CHCl_3$/MeOH extraction at 80 °C, but the yields of FFAs and especially other constituents were much lower with lCO_2. Such extraction of high NLs with low FFA or other constituents

would be advantageous in the subsequent transesterification process.

The yields from the lCO_2 extraction were comparable to or slightly lower than those previously reported using supercritical CO_2 extractions (Bjornsson et al., 2012; Santana et al., 2012; Soha & Zimmerman, 2011) (Table 4.3). Nevertheless, $scCO_2$ extraction yields from *B. braunii* require higher pressures and temperatures, 25 MPa at 50 °C (Santana et al., 2012) or 35 MPa at 70 °C (Bjornsson et al., 2012) (Table 4.3). Soha and Zimmerman (2011) reported that the lipid extraction efficiency of $scCO_2$ at 42 MPa and 100 °C could be as high as that obtained using the $CHCl_3$/MeOH method. The desirable lipid yields in the lCO_2 extracts obtained in this study were approximately 80% of the NL and FFA yields obtained from the $scCO_2$ yields noted by Soha and Zimmerman (2011); however the lCO_2 technique was operated under milder conditions. Similarly, the quantity of NLs and FFAs extracted by lCO_2 were also comparable to the biodiesel desired hydrocarbons extracted by switchable polarity solvent (Samorì et al., 2010). However, the comparisons between different batches are inaccurate due to variations in lipid content.

Table 4.3 Comparison of reaction conditions and results of liquid CO_2 and supercritical CO_2 extraction methods.

Method	Temperature (°C)	Pressure (MPa)	Total yields (mg/g dry algae)
lCO_2	25	6.8	192
lCO_2	25	17	229
$scCO_2$[a]	50	25	145
$scCO_2$[b]	70	35	110

The $scCO_2$ extraction results obtained from the literature [[a]Santana et al. (2012) and [b]Bjornsson et al. (2012)] were for a different batch of *B. braunii* and extracted by different techniques.

The higher selectivity of lCO_2 towards biodiesel desirable lipids was confirmed by the

FAME profile of the lipids extracted, Figure 4.5. As mentioned earlier, C16:0, C16:3, C18:1, C18:2 C18:3 and some long chain fatty acids 28:1 and 28:2 were the principal fatty acid residues of this algal species. In fact, the presence of oleic (C18:1), palmitic (C16:0), stearic (C18:0) and linoleic (C18:2) acids make the algal extract ideal for quality biodiesel production (Ali & Watson, 2015). Therefore, the selectivity of the solvent system for these key fatty acid components was examined.

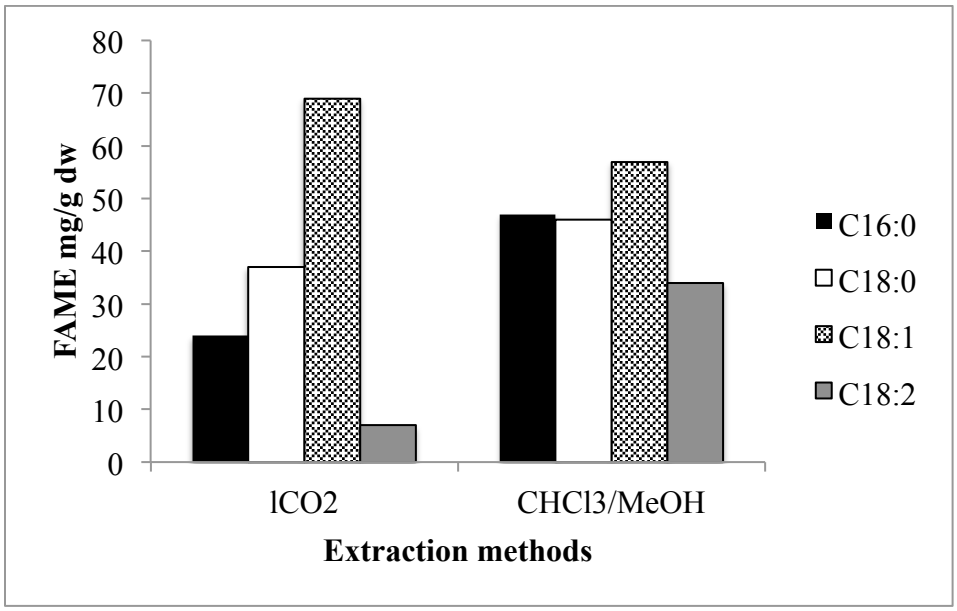

Figure 4.5 Concentrations of key fatty acid residues in the lCO_2 extract compared with $CHCl_3$/MeOH (Soxhlet method performed at 80 °C for 24 h) extract after methylation. The FAME composition includes methyl esters of palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1) and linoleic acid (C18:2).

Fuel properties such as cetane number, cold flow, oxidative stability, viscosity, and lubricity of biodiesel are influenced by the properties of the individual fatty esters in the biodiesel. In general, cetane number, heat of combustion, melting point, and viscosity of fatty

compounds increase with increasing chain length and decrease with increasing unsaturation (Knothe, 2005). Therefore, lipid extracts with a high content of stearic acid (C18:0) and oleic acid (C18:1) are of interest because the alkyl esters of such lipids mimic the properties of high quality biodiesel (Meng et al., 2009). As shown in Figure 4.5, the lCO_2 extracted with a higher selectivity for C18:1 and lower selectivity for C18:2 than the Soxhlet $CHCl_3$/MeOH extraction at high temperature and longer extraction time (80 °C for 24 h). Maheshwari et al. (1992) reported that oleic acid (C18:1) is more soluble in $scCO_2$ than linoleic acid (C18:2) at 40 °C and 13.8 MPa, the lowest pressure and temperature they tested. Halim et al. (2012) reported that $scCO_2$ has a higher affinity for oleic acid. Although liquid rather than supercritical CO_2 is being employed, the selectivity for C18:1 over C18:2 found in this study was still in accord with those findings.

4.4 Conclusion

The use of liquid CO_2 is a promising method of extracting neutral lipids from microalgae. The liquid CO_2 as a means of extracting lipids from microalgae offers a greener solution over traditional solvent systems due to its inert nature and extraction efficiencies. The low polarity lCO_2 exhibits higher selectivity for neutral lipids. The fractionation of the algal extract demonstrated that there is a greater affinity of liquid CO_2 towards the neutral lipids than that of a mixture of chloroform/methanol. Solid phase extraction showed that the lCO_2 extracts were up to 96% neutral lipids and free fatty acids, both used in biodiesel production. In addition, the lCO_2 extracts contained lower amount of other undesired materials than any other methods. The FAME profile of methylated lCO_2 extract also showed a higher selectivity of lCO_2 towards oleic

acid. Moreover, the lCO_2 method operates under milder conditions and requires no organic solvent while offering an easy product separation.

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Chapter 5

EXTRACTION OF LIPIDS FROM WET MICROALGAE USING LIQUID CO_2

5.1 Introduction

The extraction of lipids from microalgae is one of the most limiting processes for the commercialization of biodiesel. Harvested microalgae contains 97-99% water (Chen et al., 2009). Conventional methods of lipid extraction require dried biomass because the residual water in wet microalgae hinders mass transfer of the lipids from the algal cell, which decreases the efficiency of lipid extraction (Yang et al., 2014). Natural drying such as sun and wind is the most common method as it directly utilizes the solar and wind energy (Chen et al., 2009). However, it is a time consuming process and drying rates remain the main challenge of this process. Other faster dewatering processes are energy intensive, which may alter the lipid structure and the protein rich residual biomass (Taher et al., 2014). A lifecycle assessment of biodiesel production from microalgae has shown that the dewatering step accounts for 89% of the required energy input (Sander & Murthy, 2010). Moreover, it has been estimated that the total energy required to produce 1 kg of biodiesel from dried biomass is 4000 times more than that from wet biomass (Taher et al., 2014). Therefore, it is important to develop an extraction process that minimizes the need for complete dewatering of the microalgae.

The microalgae *C. vulgaris* is an attractive algal species for biofuels production because of its fast growth and ease of cultivation. However, the lipid content in this strain under general growth conditions is 14–30% by weight of dry biomass, which cannot meet the commercial requirement for biodiesel production (Liu et al., 2008). Therefore, increasing the lipid content, for example, by altering the growth conditions or by genetic modifications, in this species is a potential research area. To address this issue, there is ongoing research currently being conducted on this species of microalgae in the department of civil engineering, Queen's university.

One of the main reasons for the high energy consumption is the location of microalgae lipids within the cell wall. The cell wall is a thick and rigid layer, which is composed of complex carbohydrates, glycoproteins, and some minerals with high mechanical strength and chemical resistance (Kim et al., 2013; Lee et al., 2012). Therefore, cell disruption is important to increase the lipid extraction efficiency (Lee et al., 2010). A number of different physical, chemical or enzymatic methods have been proposed as viable methods for disrupting algal cells with the aim of lipid extraction (Wang et al., 2014). However, most of these disruption methods are difficult to scale up and involve high energy consumption since they are based on the continuous supply of thermal, electrical or mechanical energy inputs until the cell wall is broken (Steriti et al., 2014).

The conventional methods of extracting lipids from microalgae use flammable or toxic organic solvents such as benzene, cyclohexane, hexane, acetone and chloroform, which cause

adverse health and environmental effects. Moreover, the immiscibility of water in wet biomass with any low-polarity eluting solvent that dissolves neutral lipids is the biggest challenge associated with lipid extraction from wet biomass (Kim et al., 2013). Therefore, conventional solvent extraction methods often suffer from low efficiency yields (Yoo et al., 2014). Conventional methods may also deteriorate the polyunsaturated fatty acids (PUFAs) due to high processing temperatures (Cheng et al., 2011).

In recent years, alternative solvent methods for lipid extraction such as supercritical fluid, especially supercritical carbon dioxide ($scCO_2$), have been developed for microalgae lipid extraction (Yen et al., 2014). The supercritical carbon dioxide ($scCO_2$) technique offers several advantages such as very low toxicity, no oxidation or thermal degradation of extracts, high diffusivity and easy product separation compared to conventional solvent extraction (Li et al., 2014). However, the technique requires high temperatures and pressures to obtain yields comparable to conventional solvent extractions (Soha & Zimmerman, 2011).

Liquid CO_2, a solvent that also readily separates by depressurization, leaves no trace of organic residues in the product and aids in CO_2 gas stream recycling (Orellana et al., 2013), as $scCO_2$ does, but under milder conditions. It is neither highly toxic nor flammable, hence it is recognized as a safe solvent. Furthermore, lCO_2 extracts lipids containing labile PUFAs because the extraction method is conducted at a low temperature, below 31.1 °C (Cheng et al., 2011). Due to its low polarity compared to most organic solvents, lCO_2 should show higher selectivity towards the neutral lipids, while showing limited affinity to non-neutral phospholipids (Crampon

et al., 2013; Moyler & Heath, 1987). As described in chapter 4, extraction of lipids using lCO_2 from microalgae could increase the neutral lipid yields.

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The purpose of this study is to develop an efficient and selective method of lipid extraction from wet algae using liquid carbon dioxide (lCO_2) as a solvent. The extraction of lipids directly from wet microalgae eliminates the need for complete cell drying and, as such, the use of a high-power energy source. The extraction was carried out under moderate temperature 25 °C and pressure up to 17 MPa to reduce the eventual larger scale capital and processing costs. Use of this method for the extraction of lipids from microalgae could present many advantages of sustainability, safety and selectivity to the use of conventional solvents because this technique requires no flammable, chlorinated or highly volatile organic solvents. Therefore, a positive net energy from microalgae biodiesel could be obtained.

5.2 Methods and materials

All materials were used as received from the suppliers. Carbon dioxide (4.0 grade 99.99%) was obtained from Praxair. The microalgae species used for the study was *Chlorella vulgaris*. Ms. Ana Elizondo Ramos grew this species of microalgae locally in the department civil engineering at Queen's university and kindly supplied the biomass (dried and wet) for lipid extraction. The algae were grown in a batch mode on Bold's Basal Medium under continuous illumination for 14 days. The algae were then harvested through centrifugation at 10,000 x g for 10 min. The wet biomass pellet contained about 80% water (w/w). The dry algae used for Soxhlet extraction were dried in a freeze-drier for 24 hours to a final moisture content of 3% (w/w).

5.2.1 Cell lysis methods

A wet microalgae pellet (ca.5 g) was disrupted using one of four different methods: 1) *Ultrasonic vibration* - using ultrasonic bath (FS30 3QT) or ultrasonic probe (Sonic Dismembrator model 100) both manufactured by Fisher Scientific. Initially, the ultrasonic bath was used for the biomass pretreatment, where, the biomass was treated in the bath for 30 min. Later on, the ultrasonic bath was replaced by an ultrasonic probe. The ultrasonication probe delivered ultrasound at a frequency of 20 kHz and the audio power of the equipment ranged from 1 W to 10 W. The probe was immersed into the biomass and ultrasonication was performed at power level 9 W for 25 min. 2) *High-pressure homogenizer* - the biomass suspension (~5 g of wet pellet diluted in 20 mL of distilled water) was pumped through the valve of an Avestin high-

pressure homogenizer (model EmulsiFlex –C5) at a pressure of 103 MPa. 3) *Freezing and grinding* - the wet biomass pellet was frozen in liquid nitrogen and the frozen cells ground using a mortar and pestle. 4) *Osmotic shock* - the wet algal pellet was soaked in a 35% trimethyl ammonium bicarbonate solution (TMA) for 24 h.

5.2.2 Lipid extraction

The cell-disrupted biomass was extracted using liquid CO_2, chloroform/methanol solvent mixture or pure chloroform. All extractions were conducted in duplicate except if indicated otherwise. The methodologies are described in the following sections.

5.2.2.1 Extraction using liquid CO_2

The extraction of lipids was performed in two different sized high pressure vessel: a 3 mL tube vessel or a 160 mL batch type vessel as shown in Figures 5.1 and 5.2 respectively. An aliquot of the lysed cell biomass containing 2.5 g of wet pallet was transferred into an appropriate vessel, which was then connected to the high-pressure system and heated in a water bath at 25 °C. Inside the 3 mL tube vessel, a thin layer of glass wool was packed into the lower (inlet) end, above which ~0.15 g of diatomaceous earth, the 2.5 g of the pretreated wet algae pallet, another ~0.15 g of diatomaceous earth, and another thin layer of glass wool was placed. The vessel was then closed with upper outlet stopper. The vessel was not provided with any stirrer (Figure 5.1). In contrast, inside the 160 mL vessel, the biomass was suspended in the lCO_2, which was continuously stirred throughout the extraction time (Figure 5.2). All of the

extractions were conducted at 25 °C to maintain CO_2 in its liquid phase (critical temperature 31.1 °C). Liquid CO_2, pressurized at a predetermined pressure (6.8, 12 or 17 MPa) by an ISCO model 500D pump, was then conveyed through the vessel for lipid extraction. The liquid CO_2 extraction was conducted at a flow rate of 1 mL/min of CO_2. A back-pressure regulator (BPR) maintained the required pressure in the system. The process was stopped after 2 or 4 h of extraction and the remaining CO_2 in the tubing system was vented into the extract collection flask. The extract coming out of the BPR was captured, separated and dried using the same procedure described in previous chapters: 3 and 4.

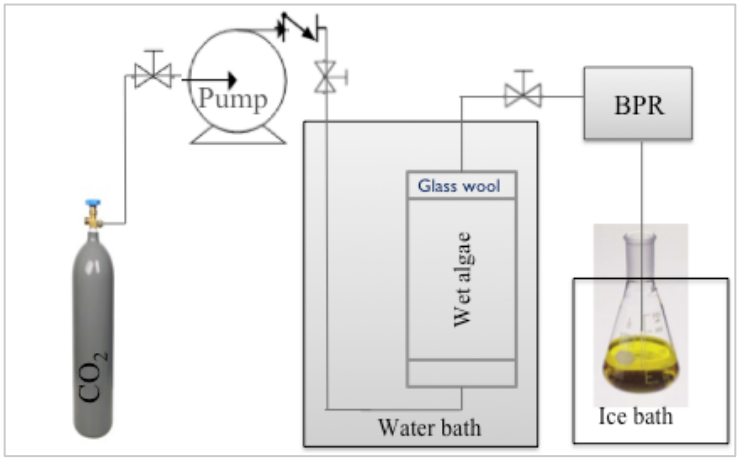

Figure 5.1 Apparatus for lipid extraction in 3 mL tube vessel using lCO_2, where P is a pressure gauge and BPR is a back-pressure regulator. Extraction was performed at 25 °C and 6.8 or 12 MPa. The extract was collected in ice-cold isopropanol.

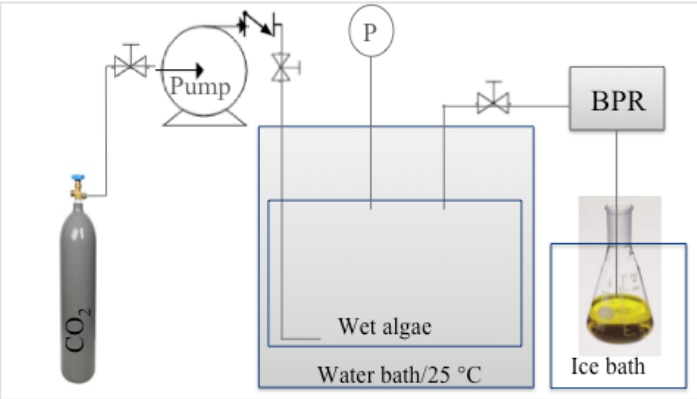

Figure 5.2 Apparatus for lipid extraction in 160 mL batch type vessel using lCO_2, where P is a pressure gauge and BPR is a back-pressure regulator. Extraction was performed at 25 °C and 12 or 17 MPa. The extract was collected in ice-cold isopropanol.

5.2.2.2 Extraction using organic solvent

In order to compare lCO_2 method of extraction with the conventional $CHCl_3$/MeOH solvent method, a Soxhlet extraction was also performed. In this method, 250 mg of freeze dried algae (3% water content) was inserted into a glass thimble and extracted using 2:1 (v/v) chloroform/methanol mixture (25 mL) by Soxhlet extractor in an oil bath at 80 °C. After a 24 h extraction period, the solvents were removed by rotary evaporator followed by vacuum oven at 55 °C for 3 h under reduced pressure, and then weighed to determine the gravimetric lipid yield. A single organic solvent extraction was also performed on wet algae pellet. In this method, 3 g of the wet pellet was taken in a flask and soaked in chloroform at 25 °C and atmospheric pressure for 2 h. In another single extraction, the wet pellet was treated with methanol followed by ultrasonication probe. The biomass was then transferred into the 160 mL high pressure vessel

and extracted with liquid CO_2 at 25 °C and 12 MPa for 4 h, (Figure 5.2). The extract was collected and dried by the same procedure as described above in the previous section 5.2.2.1.

5.3 Results and discussions

5.3.1 Lipid extraction using organic solvent

In order to determine the total extractable lipid content, Soxhlet extraction of lipids from freeze-dried biomass of *C. vulgaris* was performed prior to the extraction from wet biomass (Table 5.1). In this case, the polar methanol releases lipids from the protein-lipid complex within the cell, and the nonpolar chloroform subsequently dissolves the released and other free non-polar lipids. Therefore, the method was considered to extract the total extractable lipid content of the biomass. An average lipid yield of 19 wt% relative to the dry biomass was obtained from two Soxhlet extractions conducted at 80 °C for 24 h. One of the reasons for the high lipid yields obtained from dried biomass by Soxhlet extraction is lyophilization, which disrupts algal cells and makes cell walls more porous (Taher et al., 2014). A single extraction of wet microalgae pellet using pure chloroform was also performed (Table 5.1). As shown in the table, the lipid extraction yield of wet extraction using chloroform was only 1.7 wt% dw compared to 19 wt% achieved from freeze-dried biomass Soxhlet extraction. Such a low extraction yield is likely due to a water film forming over the lipid that would prevent the solvent (chloroform) from contacting it.

5.3.2 Lipid extraction using liquid CO_2

Extraction of lipids from microalgae is a mass transfer process that depends on the nature of the solute (lipid) and the selectivity of the eluting solvent. The cell disruption facilitates the contact between cellular lipids and the eluting solvent with breaking of the microalgae cell wall. It can be assumed that the wet extraction with CO_2 at high temperature may allow the compressed CO_2 to penetrate through the water film and reach the lipids. The liquid CO_2 extraction efficiency is influenced by different factors mainly: pressure, temperature, and extraction time. Therefore, several experiments were conducted using different cell disruption methods followed by lipid extraction using lCO_2 (Table 5.1).

As can be seen from Table 5.1, the initial extraction yields (2 wt% relative to dry weight of microalgae or less) using lCO_2 (Trial 4-7) were comparable to that of the chloroform extraction (Trial 2). These extractions were carried out in a 3 mL tube vessel at two different pressures (6.8 MPa or 12 MPa), while keeping a constant reaction time of 2 h. Several factors such as effectiveness of the cell disruption methods, extraction temperature, pressure and time or the reactor vessel itself could be factors influencing the low yields obtained (Chen et al.; Halim et al., 2013).

Table 5.1 Result of lipid extraction from wet microalgae pellet using lCO_2 under different conditions.

Solvent	Cell disruption	Trial	Time (h)	T (°C)	P (MPa)	Yields (wt% dw)
$CHCl_3$/MeOH	Lyophilization	1	24	80	0	19
$CHCl_3$	Sonication probe	2	2	25	0	1.7
lCO_2	None	3	2	25	6.8	0.26
lCO_2	Sonication bath	4a	2	25	6.8	0.84
		4b	2	25	12	0.93
lCO_2	Sonication probe	5a	2	25	6.8	1.2
		5b	2	25	12	1.9
lCO_2	High-pressure homogenizer	6	2	25	6.8	1.3
lCO_2	Mortar and Pestle	7a	2	25	6.8	1.1
		7b	2	25	12	2.0
$lCO_2 +$ MeOH	Sonication probe	8	4	25	12	18
lCO_2	Osmotic shock	9	4	25	17	5
lCO_2	Sonication probe	10	4	25	17	4.6

Trials 3-7 were performed in a 3 mL flow through tube vessel while the Trials 8-9 were conducted in a 160 mL batch reactor at higher pressure and longer extraction time. Letters a and b after numbers indicate two different extractions, from that biomass, performed under different conditions. A constant lCO_2 flow rate of 1 mL/min was used for all the extractions. Yield expressed is relative to the dry wet (dw) of microalgae.

With respect to the cell disruption, three different lysis methods: sonication (bath or probe), high-pressure homogenizer and grinding were employed. Lipid extraction from wet biomass without pretreatment gave only about 0.3 wt% dw extract yield. Pretreatment of wet algae using the ultrasonication bath followed by lCO_2 extraction also had a poor extract yield,

0.9 wt% dw. Later, the sonication bath was replaced by a sonication probe, which applies the ultrasonic vibration directly to the biomass slurry, considering that it should be more effective than the bath. The use of the sonication probe yielded better results (up to 2 wt% dw) compared to ultrasonic bath but the results were still unsatisfactory. In trial 6, the high-pressure homogenizer required biomass suspension (~5 g of wet pellet diluted in 20 mL of distilled water) to pump the slurry through the valve. There was a concern associated with the use of the homogenizer as it was not possible to use the diluted biomass directly for the extraction in the 3 mL tube vessel. Subsequently, the excess water was removed using centrifugation to reform the wet pellet. However, the lipids released due to breakdown of the cells might also have been removed with decantation aimed at removing water. Therefore, this method was not used in further experiments. Cell disruption by manual grinding using mortal and pestle, in Trial 7, also gave the similar extract yields (up to 2 wt% dw) as the sonication probe.

In order to improve the extraction yields, an extraction was performed using methanol as a co-solvent (Trial 8, Table 5.1). In this method, the wet biomass was treated with methanol, using a mass ratio of 1:7 in terms of dried algae/methanol, prior to the extraction using lCO_2. The treatment of the wet pellet with methanol enhanced the cell wall degradation where methanol penetrated the outer cell wall (Zhang et al., 2014). Moreover, the methanol increased the solvent polarity, thereby increasing extraction efficiency for more polar compounds (Soha & Zimmerman, 2011). Although the extraction with methanol as a co-solvent resulted in high extract yield (18 wt% dw), it involved the use of an organic solvent, which mixed with water and needs to be recovered from the water after the process. However, there is the possibility of direct

methanolysis of the lipids using this method. In that case, economic and environmental costs associated with the use of methanol could be offset by benefits of the simultaneous extraction and methanolysis process.

Furthermore, so as to improve the extract yield without the use of an organic co-solvent, other influencing factors including extraction time and pressure were also tested (Trial 9, Table 5.1). In this method, the cells were disrupted by osmotic shock while the extraction time and pressure were increased to 4 h and 17 MPa, respectively. In changing these parameters, the extraction yield was increased to 5 wt% dw, which is ~26 wt% of the total extractable lipids (19 wt% dw, Soxhlet extraction) of the microalgal biomass. The lCO_2 extraction from freeze-dried microalgae resulted into 38 wt% of the total extractable lipids, as described in chapter 4. Based on the results obtained in Chapter 4 as well as from the Soxhlet extraction described above, a total extract yield of ~ 7-10 wt% dw was expected in this wet extraction. The following parameters were considered to improve the extraction yields.

5.3.2.1 Effect of vessel type and size

The initial extractions, Trial 3-7 (Table 5.1), were conducted in a 3 mL tube vessel, where the biomass was tightly packed without any stirrer. Therefore, it is possible that the lCO_2 was not in optimal contact with all of the biomass. Based on this hypothesis, the later extractions, Trial 8-9 (Table 5.1), were performed in a bigger (160 mL) batch type vessel. In such a vessel, the biomass was suspended in the lCO_2, which was continuously stirred throughout the extraction

time, hence providing a better opportunity for cells to come into contact with lCO_2, which may be the cause of the higher extract yields.

5.3.2.2 Effect of cell disruption method

A number of different methods of cell disruption have been studied and reported (Munir et al., 2013). However, there is no agreement on the most efficient method of cell lysis (Natarajan et al., 2014). One study reported that ultrasonication was the most efficient method (Prabakaran & Ravindran, 2011), while another study noted that grinding with liquid nitrogen resulted in high lipid yields (Zheng et al., 2011). A study by Lee et al. (2010) reported similar extraction results for sonication and osmotic shock on *C. vulgaris*. In this study, the lipid extraction yields obtained from an osmotic shock treated biomass or an ultrasonicated biomass (Trial 9 and 10, Table 5.1) were comparable (4.6 wt% vs. 5 wt% dw). Consequently, the ultrasonication probe and the osmotic shock should have comparable algal cell lysis efficiencies. No extraction was performed from a manually ground biomass under the conditions used in Trials 9 and 10. Moreover, a mechanical method such as ultrasonication or manual grinding is usually preferred as this method is less likely to contaminate the biomass and the lipid product. Therefore, an ultrasonication or manual grinding method would be preferred over osmotic shock.

5.3.2.3 Effect of pressure

In the case of supercritical CO_2 extraction, the extract yields increase with increasing pressure at a constant temperature, as the lipid solubility (in $scCO_2$) increases with increased

pressure (Döker et al., 2010). A similar effect was found in the lCO_2 extraction presented in this study as well. As seen in the Table 5.1, extraction yields at 12 MPa were slightly higher than those at 6.8 MPa. The further increase in pressure from 12 MPa to 17 MPa also increased the extract yields from ~2 wt% to 5 wt% dw. The increase in extraction yields with increasing pressure may be due to the higher density of the fluid and/or increased cell destruction. Although the extractions at 6.8 MPa, 12 MPa, and 17 MPa were performed for two different extraction times (2 h or 4 h), the increase in extract could be because of the increase in pressure. The findings are in accord with the results reported previously (Chen et al. 2013; Soha and Zimmerman 2011), which showed higher yields of lipids with increase in liquid or supercritical CO_2 pressure at 30 °C or 100 °C. However, further investigation at the same extraction time may require to confirm the positive effect of increased pressure on lipid extraction yields.

5.3.2.4 Effect of extraction time

The lipid extraction yield may also be affected by the extraction time. It has been reported that the lipid extraction time depends on the extraction pressure (King et al., 1989). In $scCO_2$ extractions, the extraction of lipids occurs more rapidly at higher pressures than at lower pressures (Cheung et al., 1998). Most of the studies using $scCO_2$ extractions techniques have been performed for 1 to 8 h of extraction time under pressure and temperature conditions of up to 70 MPa and 60 °C respectively (Halim et al., 2012). Because the extraction using lCO_2, in this study, was conducted at milder conditions compared to $scCO_2$ extractions, the lCO_2 may require a longer residence time. As mentioned earlier, increased cell damage is one of the contributors to

improved extract yields at high pressure. Therefore, some of the allocated time at the beginning of the extraction process may be utilized to break the cell wall before the lCO_2 penetrates through the wall. Also, a longer extraction time than 2 or 4 h used in this study may need for the diffusion of lipids from cell matrix to the lCO_2 phase. The extraction times longer than 2 or 4 h were not tested due to the time limitations and the biomass availability.

5.4 Conclusion

The use of lCO_2 for the extraction of lipids from wet microalgae is preferable over the traditional organic solvent method because the CO_2 is readily available, inexpensive, has very low toxicity, non-flammable, chemically inert under many conditions, environmentally acceptable, liquefiable at reasonable pressures, and offers an easy product separation after the process. Extractions using lCO_2 under different pressures (6.8-17 MPa) and a constant temperature of 25 °C were used to extract lipids from wet biomass of *Chlorella vulgaris* that contained about 80% water content. The technique was shown to be a promising method of lipid extraction, which could minimize the energy intensive and time-consuming dewatering step. About 26 wt% of the extractable lipids was extracted directly from the wet biomass by lCO_2. Further study needs be conducted to optimize pressure and extraction time for maximum lipid extraction yields.

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Chapter 6

CONCLUSIONS AND FUTURE DIRECTIONS

6.1 Conclusions

The principal purpose of this study was to explore greener and more efficient methods of microalgal lipid extraction compared to the conventional organic solvent techniques. Microalgae, which can be grown on marginal land in a wide range of environments, give high oil production, capture waste CO_2, and have a rapid growth rate, which make them good candidates as potential feedstocks for renewable energy resources. However, there are a number of impediments such as the requirement of dewatering algal biomass prior to lipid extraction and the use of organic solvents for extraction that prevent the commercialization of algal biofuels. In this study, liquid CO_2 (lCO_2) and CO_2-expanded methanol (cxMeOH) were used for the extraction of lipids from microalgae. The aim of using these two solvents was to avoid or minimize the amount of organic solvent(s) needed for lipid extraction and to increase the selectivity of the solvent towards the biodiesel-desirable lipids, while minimizing the co-extraction of other unwelcomed constituents.

The use of cxMeOH, which consists of pressurized gaseous CO_2 dissolved in methanol, offers advantages over the use of conventional solvents because this technique requires a smaller volume of volatile solvent than required by methods that use pure methanol or methanol/chloroform mixtures, and the cxMeOH requires no chlorinated solvent. The cxMeOH (under pressure and temperature conditions of 7.2 MPa and 35 °C respectively) was used to

extract lipids from dried microalgal biomass. The study showed that by using cxMeOH, it was possible to reduce the volume of organic solvent needed for the extraction by up to five times. Also, the chlorinated solvent (chloroform) could be replaced by a much safer solvent (CO_2). The maximum yield of lipids was obtained with a 1:7 mass ratio of dry algae to normal unexpanded methanol. Moreover, the decreased polarity of methanol by dissolved CO_2 increased the selectivity of the solvent towards biodiesel-desirable neutral lipids (NLs) and free fatty acids (FFAs) exhibiting up to 82% selectivity for those desired lipids. However, such selectivity of pure methanol and $CHCl_3$/MeOH mixture under similar conditions as cxMeOH method were only up to 73% and 67% respectively.

In other methods of extraction, lCO_2 was used to extract lipids from dried, as well as wet algal biomass. CO_2 is readily available, inexpensive, nontoxic, nonflammable, chemically inert under many conditions, environmentally acceptable, and liquefiable at reasonable pressures. Therefore, the use of liquid CO_2 in lipid extraction presents many advantages in terms of sustainability, safety and selectivity when compared to the use of conventional solvents because this technique requires no flammable, highly volatile, or chlorinated solvents. Firstly, the lCO_2 was used for lipid extraction from dried microalgal biomass. The low polarity lCO_2 exhibited higher selectivity for neutral lipids compared to all other methods tested. However, the total extraction yield of lCO_2 method was lower than that of the other methods studied. The lower total extraction yields of lCO_2 could be offset by the fact that no organic solvent was required in this method. Solid phase extraction showed that the lCO_2 extracts were up to 96% neutral lipids and free fatty acids, both used in biodiesel production. Also, the FAME profiling of the lCO_2

extract showed that lCO_2 exhibited higher selectivity towards oleic acid compared to cxMeOH and the $CHCl_3$/MeOH mixture. Secondly, the lCO_2 was used to extract lipids from wet biomass containing about 80% water content. Extraction of lipids from wet microalgae could reduce the energy requirement for dewatering step. Both dry and wet biomass extraction were conducted under pressures of 6.9 MPa to 17 MPa and at the temperature of 25 °C. The lCO_2 extracted up to 38 wt% of the extractable lipids from dried microalgal biomass while it extracted up to 26 wt% of that from the wet biomass.

Table 6.1 compares the cxMeOH and lCO_2 methods with the conventional organic solvent extraction techniques.

Table 6.1 Comparison of cxMeOH and lCO_2 methods with organic solvent extraction methods.

	lCO_2 (6.8 MPa, 25 °C)	cxMeOH (7.2 MPa, 35 °C)	MeOH (7.2 MPa, 35 °C)	$CHCl_3$/MeOH (35 °C)
Total lipid yield	19	24	23	33
Avoid/reduce organic solvent	Yes	Yes	No	No
Selectivity for desired lipids (%)	94	82	68	67

As the table shows, the lCO_2 method is superior to other methods in terms of selectivity for desired lipids. Also, the lCO_2 technique totally avoids the use of organic solvent. The cxMeOH showed higher selectivity for desired lipids compared to pure methanol or $CHCl_3$/MeOH solvent mixture. Moreover, the cxMeOH technique greatly reduced the amount of

organic solvent required.

6.2 Future directions

The extraction of lipids directly from the wet microalgae biomass (water content of ~80%) extracted about 26 wt% of the extractable lipids. However, the total extraction yields could be increased. The major obstacle of lipid extraction from wet microalgae includes high residual water content in the microalgal biomass, which protects the cells from rupturing and impeding the contact of the eluting solvent with lipids. The cell disruption could be improved by increasing extraction pressure and extraction temperature in addition to cell pretreatment whereas an increased residence time could increase the lipid extraction. As discussed earlier in this manuscript, pressure and temperature determine the solvent power of lCO_2. Increase in extraction pressure increases the density of lCO_2 and accordingly its solvent power. The increased lCO_2 density could lead to an increased diffusion rate of the solute (lipid) in the CO_2 film. Therefore, a longer extraction time than 2 or 4 h used in this study may be needed for the diffusion of lipids from cell matrix to the lCO_2 phase. Although increased lipid extraction yields could be possible by increasing pressure, it requires high installation and maintenance costs of the pressure vessels associated with the algal biodiesel production. Consequently, the cost of drying the algal biomass and the costs of CO_2 compression should be evaluated to optimize the tradeoff between these two processes, which could be a topic of further investigation. Even though an increased temperature enhances cell wall degradation, the temperature should be below 31 °C, in this study, to maintain CO_2 in its liquid phase. In addition to these factors, the

lCO_2 flow rate could have affected the extraction yields. A constant flow rate of 1 mL/min was used in this study. A higher flow rate could increase the contact between the solvent and the lipids. However, the higher solvent velocity, with a constant extraction time, may decrease its penetration through the matrix to reach the lipid molecules. Therefore, lCO_2 flow rate also needs to be optimized. After optimization of the method for maximum possible lipid extraction yields, the extract needs to be analysed for biodiesel-desirable lipid content, which could be performed by methanolysis of the extract followed by GC-MS/GC-FID analysis of the resulted fatty acid methyl ester (FAME).

Appendix A

TRANSESTERIFICATION/ESTERIFICATION OF LIPIDS IN CO_2-EXPANDED METHANOL

CO_2-expanded methanol (cxMeOH) was also used for the production of fatty acid methyl ester (FAME) by the methanolysis of lipids. Three different lipids: canola oil, oleic acid and tributyrin were used for the study. In cxMeOH, the methyl carbonic acid formed by MeOH and CO_2 could act as an acid catalyst for esterification or transesterification (methanolysis) of the lipids. The CO_2 in cxMeOH could also decrease the polarity of methanol. The decreased polarity would increase the solubility of lipid in methanol and empower the methanolysis reaction. Also, the methanolysis of lipids in cxMeOH offers an easy product separation, which could greatly reduce the biodiesel production costs.

In this method, the reaction mixture had a 1:50 molar ratio of lipid to methanol in all the experiments. A high-pressure vessel (160 mL) with a glass insert was charged with lipid, methanol and a magnetic stirrer. The vessel was then connected to the high-pressure system and heated in an oil bath at 70 °C. After the temperature had equilibrated, CO_2 was pumped by an ISCO model 500D pump into the system for 15 min at a constant pressure of 7.0 MPa. The system was then kept under these temperature and pressure conditions for 24 h with continuous stirring. The reaction was, subsequently, quenched by cooling it in an ice bath and the pressure

was released through a valve. The reaction mixture was then extracted with hexane for FAME, dried in rotary evaporator, weighed and analyzed by proton nuclear magnetic resonance (^{1}H NMR) spectrometer (Bruker 300 MHz). The NMR samples were prepared in 5 mm NMR tubes by dissolving a known amount of the FAME sample in dimethyl sulfoxide-d_6. A quantified amount of chloroform was also added to the sample as an internal standard. ^{1}H NMR spectra were recorded using the following parameters: pulse width, 13.4 μs; pulse delay, 1 s; spectral width, 4136.2 Hz; data points, 16,384; and number of scans, 16.

The (FAME) yield was determined by using ^{1}H NMR spectroscopy based on the fact that the amplitude of a ^{1}H-NMR signal is proportional to the number of hydrogen nuclei contained in the molecule. The spectrum of FAME obtained from methyl esters of canola oil and oleic acid are presented in Figure A-1. Most of the peaks for glycerides and methyl esters were between 0 and 6 ppm. Therefore, chloroform having a chemical shift value of 7.2 ppm was chosen as an internal standard. The relevant signal chosen for integration was that of methoxy protons in the methyl esters at 3.68 ppm (singlet). The yield of methyl esters was determined by using the relative ratio of the intensity of the methoxy protons in FAME to a known amount of the internal standard ($CHCl_3$).

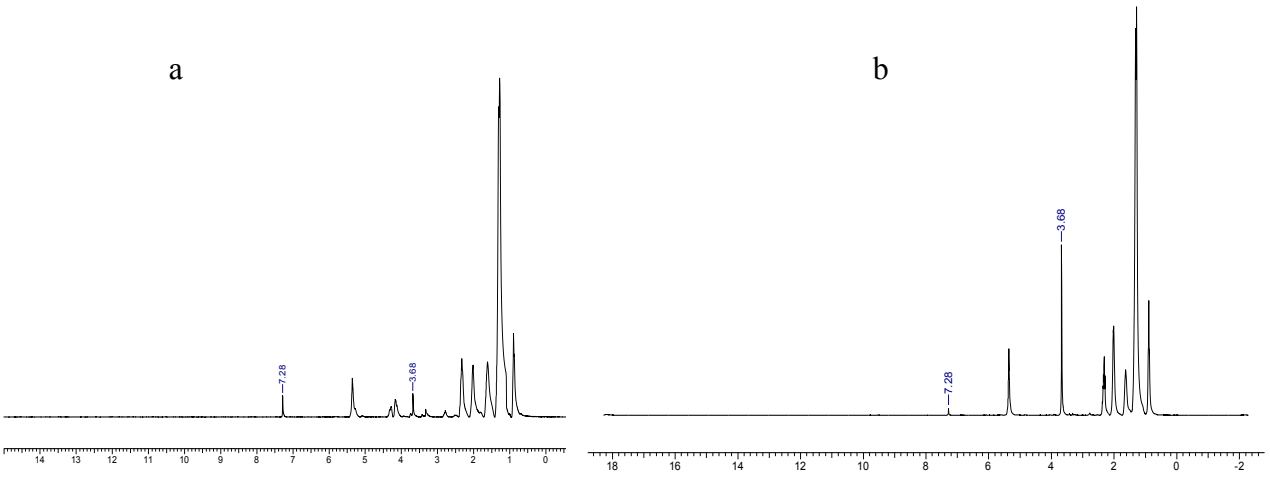

Figure A-1 ^{1}H NMR spectrum of FAME produced from a) canola oil b) oleic acid.

The conversion of three different lipids: canola oil, oleic acid and tributyrin to FAME have been reported in Table A-1. The canola oil conversion yields were calculated by assuming average molecular weights of 880.69 g/mol for the oil and of 293.55 g/mol for FAME. As shown in Table A-1, up to 6 % of the lipids was converted into to the FAME under the tested conditions.

Table A-1 Results of catalyst free methanolysis of lipids in cxMeOH.

Lipids source	*Temperature (°C)*	*Pressure (MPa)*	*Time (h)*	*Lipid conversion (Yield %)*
Canola oil	70	7.0	24	4
Oleic Acid	70	7.0	24	6
Tributyrin	70	7.0	24	4

As evident in Figure A-1 and Table A-1, it was possible to methanolyse lipids into fatty acid methyl esters using cxMeOH without any catalyst, but only with very low conversion. However, it may be possible to obtain higher conversion of lipids by optimizing the reaction conditions for temperature, pressure and the residence time. Therefore, more investigation is required to optimize the method for reaction temperature and residence time in order to improve the lipid conversion yields.

Appendix B

SAMPLE RAW DATA OF GC-FID ANALYSIS OF FATTY ACID METHYL ESTERS

Fatty acid methyl esters (FAME) were produced by an acid catalyzed methylation of the lipid extract and analysed by using a GC-FID technique. The figures below are examples of chromatogram and calibration curve obtained.

1. Chromatogram

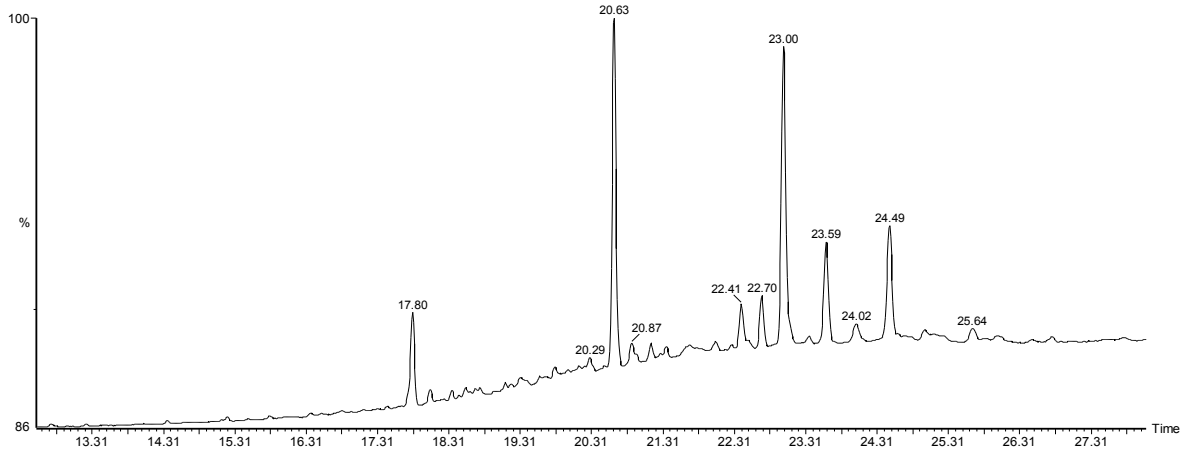

Figure B-1 Analysis of FAMEs in microalgal biodiesel. Methyl palmitate (C16:0)- 20.63 min; methyl stearate (C18:0)- 22.70 min; methyl oleate (C18:1)- 23.00 min; methyl linoleate (C18:2)- 23.59 min.

2. Calibration curve

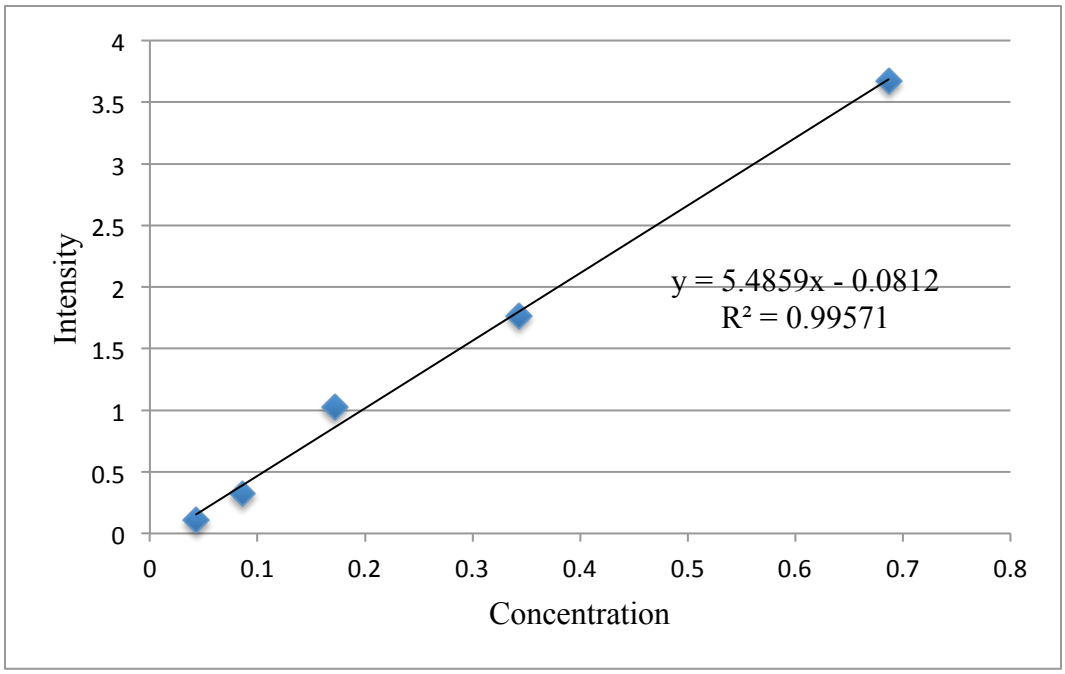

Figure B-2 C18:1 Methyl oleate