ENHANCING TRIGLYCERIDE PRODUCTION USING CARBON DIOXIDE

by

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Abstract

Biofuels have recently emerged as renewable, biodegradable, and cost-effective alternatives to fossil fuels. Microalgae serve as promising feedstocks to produce biofuel because of their fast growth rates, ability to grow on non-arable land or in wastewater, and high lipid content, which is essential for biofuel production. Unfortunately, current laboratory methods for the extraction of microalgal lipids have high capital/energy costs. Therefore, this thesis examines liquid CO_2 as a potential greener technique for extracting these intracellular lipids.

In the literature review, microalgal cell disruption and extraction techniques are discussed extensively. Current extraction techniques, with or without cell disruption, have high energy and capital costs. Therefore, it is essential to discover greener extraction techniques. Other improvements to reduce cost include using microalgal slurries, combining process steps, and minimizing solvent use. Life cycle assessments are necessary to identify the environmental impact associated with each lipid extraction method.

The lipid extract yields from *Chlorella vulgaris* using liquid CO_2, co-solvents, and/or additional modifiers, were investigated. When *C. vulgaris* in the presence of dodecyltrimethylammonium bromide and water was exposed to liquid CO_2, the highest extract yield was obtained compared to all other co-solvents and/or modifiers tested.

In a study of the transesterification of soybean oil using a switchable-hydrophilicity solvent, 2-(dibutylamino)ethanol, fatty acid methyl ester yields and the recyclability of 2-(dibutylamino)ethanol were reported. After 5 cycles, the yield of fatty acid methyl esters was 80-85 % of the original mass of soybean oil and the recovery of 2-DBAE was 65-90

% of the original volume. This project served as a proof of concept for future applications to microalgae.

The extraction of lipids from *Scenedesmus sp.* using liquid CO_2, methanol, and/or prior cell disruption techniques was investigated. When microwave radiation in the presence of distilled water followed by extraction using liquid CO_2 and methanol, the highest neutral lipid and free fatty acids yields were obtained. Moreover, fluorescence microscopy was able to effectively monitor cell disruption upon microwave radiation and grinding following freezing with liquid N_2.

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Statement of Originality

I hereby certify that all the work described in this thesis is the original work of the author. Any published (or unpublished) ideas and/or techniques from the work of others are fully acknowledged in accordance with the standard referencing practices.

(Kelsey Janet Viner)

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List of Abbreviations

2-DBAE: 2-(Dibutylamino)ethanol

3-DAPS: 3-(Decyldimethylammonio)-propanesulfonate

AG: Acylglycerol

ASE: Accelerated solvent extraction

BPR: Back-pressure regulator

CMC: Critical micelle concentration

CSHD: Cationic surfactant-based harvesting and cell disruption

CSTR: Continuously-stirred tank reactor

CTAB: Cetyltrimethylammonium bromide

CXL: CO_2-expanded liquid

CXM: CO_2-expanded methanol

DAG: Diacylglycerol

DTAB: Dodecyltrimethylammonium bromide

dw: Dry cell weight of microalgae

FA: Fatty acid

FAME: Fatty acid methyl ester

FFA: Free fatty acid

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

GHG: Greenhouse gas

GL: Glycolipid

HPLC: High-pressure liquid chromatography

IL: Ionic liquid

LCA: Life cycle assessment

LC-NH_2: Liquid chromatography aminopropyl-bonded

lCO_2: Liquid CO_2

MAE: Microwave-assisted extraction

MAG: Monoacyglycerol

Mt: Megatonnes

MTAB: Myristyltrimethylammonium bromide

NL: Neutral lipid

PL: Phospholipid

PUFA: Polyunsaturated fatty acid

RES: Renewable energy sources

$scCO_2$: Supercritical CO_2

SCF: Supercritical fluid

scH_2O: Supercritical H_2O

SFE: Supercritical fluid extraction

SHS: Switchable-hydrophilicity solvent

SPE: Solid phase extraction

SPS: Switchable-polarity solvent

TAG: Triacylglycerol

TMBDA: N,N,N’,N’-Tetramethyl-1,4-butanediamine

UAE: Ultrasound-assisted extraction

Chapter 1

INTRODUCTION

1.1 Energy overview

Energy is one of the most important resources for the sustainable development of our world.[1] Fossil fuels make up 80% of the world's energy supply[2] and for this reason, there is a huge dependence on them. The most common types of fossil fuels used today by industrialized and developing countries are oil, coal, and natural gas. Among these, oil is the most consumed for energy conversion, which is the process of changing one form of energy into another, followed by coal, then natural gas.[3] When fossil fuels are burned, greenhouse gases (GHGs), such as CO_2, NO_x, SO_x, and CO, are emitted to the atmosphere creating impacts on our environment (climate change), economy, and health. GHGs are defined as a gas that contributes to the greenhouse effect (an increase in heat) by absorbing infrared radiation. At present, consumption of fossil fuels is dramatically increasing in concert with improvements in the quality of life, industrialization of developing nations, and increases in the world's population. It has been recognized that this excessive fossil fuel consumption not only leads to diminishing fossil fuel reserves, but also has a significant adverse impact on the environment, resulting in increased health risks and the threat of global climate change.[4] According to the US Environmental Protection Agency, approximately 65 % of GHG emissions are generated by fossil fuel combustion.[5] In 2015, coal was responsible for 41 %, oil for 34 %, and natural gas for 19 % of global CO_2 emissions[6] and in 2016, fossil fuel combustion gave rise to 36,400 megatonnes (Mt) of global CO_2 emissions.[6] Also, Canada's total GHG emissions in 2015 were 722 Mt of CO_2

equivalent (eq), or 18 % (111 Mt of CO_2 eq) above the 1990 emissions of 611 Mt of CO_2 eq.[7] The main contributor of CO_2 emissions to the atmosphere is the transportation sector (buses, ships, and vehicles), which has a strong dependence on fossil fuels. However, fossil fuels are depleting, non-renewable, and variable in cost. According to the U.S. Energy Information Administration, there has been a total world consumption of 98.26 million barrels per day of global petroleum and other liquids in 2017.[8] As the world population continues to grow and the consumption of petroleum increases releasing significant amounts of GHGs into the atmosphere, researchers are continually seeking alternative energy sources, especially renewable energy sources, to meet the rising global demand for energy in the long-term.

1.2 Renewable energy sources

Renewable energy sources (RES) will play a crucial role in the world's future. RES are naturally replenished energy sources on a human timescale, which include biomass, hydropower, geothermal, solar, and wind energies. These RESs are considered as clean, contributing less pollution or GHG emissions, and optimal use of these resources minimizes environmental impacts, produces minimum secondary wastes and is sustainable based on current and future economic and social societal needs.[9] Due to our world's growing energy needs, society is slowly moving towards seeking more sustainable production methods, waste minimization, reduced air pollution from vehicles, and reduction of GHG emissions.[10] The total world energy supply consists of 14% RES.[11] Implementation of RES will allow for improved fuel economy, address issues related to

local energy and water supply, enhance the standard of living and level of employment of local populations, ensure sustainable development of remote regions in desert and mountain zones, and meet obligations of different countries with regard to fulfilling their international agreements related to environmental protection.[12] Development and implementation of renewable energy projects in rural areas can create job opportunities, thus minimizing migration towards urban areas.[13] Decentralizing renewable energy harvesting is one of the options to consider in order to meet rural and small scale energy needs in an affordable and environmentally sustainable way.[14,15]

1.3 Biofuel

Biofuels are solid, liquid or gaseous fuels predominantly produced from biomass or from agricultural, commercial, and/or industrial wastes.[1] The production of renewable biofuels generally involves carbon fixation, as occurs in plants or microalgae through the process of photosynthesis. Biofuels can be broadly classified as primary and secondary.[16] Primary biofuels, such as wood chips, are used in their unprocessed form to supply cooking fuel, heating, or electricity production needs in small and large-scale applications. Secondary biofuels are modified primary biofuels that have been processed and produced in the form of solids (charcoal), liquids (ethanol, biodiesel, and bio-oil), or gases (hydrogen and methane). They can be used for a range of applications including transportation and industrial processes. Using renewable sources to produce biofuels along with agricultural residuals and organic waste as raw materials can minimize the conflict between food and

fuel. Biofuels produced from lignocellulosic materials generate low GHG emissions, which reduce environmental impacts.[17]

The most promising alternative to fossil fuel for diesel engines is biodiesel. Advantages of using biodiesel in place of fossil fuels include its less toxic emissions, biodegradability, and its renewability.[11] It also burns cleaner than regular diesel, in that it emits less particulate matter and sulfur compounds.[18] Biodiesel is made up of fatty acid alkyl esters and can be produced by chemically combining an oil or fat with an alcohol such as methanol or ethanol.[19] Methanol has been the most commonly used alcohol in the commercial production of biodiesel. A number of studies on biodiesel have shown that the fuel made from vegetable oil can be used effectively in diesel engines.[20–23] In fact, the energy density of biodiesel is quite close to regular diesel.[24]

1.3.1 Feedstocks for biofuel

Secondary biofuels can be divided into four catergories based on the raw material and technology used for their production.

1.3.1.1 First-generation biofuels

First-generation biofuels are derived from edible feedstocks, such as canola, wheat and sugar.[25] The most well-known first-generation biofuel is ethanol, which is made by fermenting sugar extracted from crop plants and starch.[26] Another well-known first-generation biofuel is biodiesel produced from the vegetable oils of oleaginous plants by a

process known as transesterification. Transesterification can use alkaline, acid or enzymatic catalysts along with methanol to produce fatty acid methyl esters (FAMEs; primary components of biodiesel) and glycerol as a by-product.[27] Presently, first-generation fuels are produced commercially in a number of countries; however, their production has raised an ethical dilemma since the feedstock leading to their production conflicts with our food supply.[28] Moreover, the finite availability of arable land is a major limitation to producing these biofuels. Limited available arable land is driving the search for efficient methods to convert non-edible biomass into biofuel.

1.3.1.2 Second-generation biofuels

Second-generation biofuels are generally derived from agricultural lignocellulosic biomass, which are typically non-edible residues of food crop production or non-edible whole plant biomass.[29] The main advantage of the production of second-generation biofuels from non-edible feedstocks is that it addresses the food versus fuel dilemma associated with first-generation biofuels.[30] Feedstocks involved in the process can be grown specifically for energy purposes, enabling higher production per unit land area, and therefore, a greater amount of material can be used to produce biofuel. As a result, these non-edible feedstocks have the potential to be lower in cost, as well as offer significant energy and environmental benefits. A major limitation of second-generation biofuels is the high capital costs associated with the equipment needed for their production.[31] Moreover, like first-generation biofuels, land-use competition with agriculture is a major limitation to producing these second-generation biofuels. To develop techno-economically feasible

production, further research, development, and applications are required with respect to feedstock production and conversion technologies.[31]

1.3.1.3 Third-generation biofuels

Third-generation biofuels, specifically derived from microorganisms, such as algae, have emerged as a viable alternative energy resource that is devoid of the major drawbacks associated with first and second-generation biofuels.[32] Algae are recognized as one of the oldest life-forms and are present in all existing earth ecosystems, representing a large variety of species living under a wide range of environmental conditions.[33] Algae are photosynthetic organisms that produce lipids, proteins, and carbohydrates as stored energy sources. However, their production of lipids, proteins, and carbohydrates may be limited by available sunlight due to diurnal cycles and seasonal variations, thereby limiting the viability of commercial production to areas with high solar radiation. Microalgae can produce lipids, proteins, and carbohydrates in large amounts over short periods of time.[34] These constituents can be processed into both biofuels and valuable co-products.

1.3.1.4 Fourth-generation biofuels

Fourth-generation biofuels, like first-, second-, and third-generation biofuels, are derived from biomass materials that have absorbed CO_2 while growing.[35] These materials are then converted into fuel using the same processes as second-generation biofuels. These types of biofuels, photobiological solar fuels and electrofuels,[36] differ from first-, second-,

and third-generation biofuels because during all stages of production, CO_2 is captured using various processes, primarily oxy-fuel combustion.[37] Upon capture, the CO_2 can then be geosequestered by storing it in oil and gas fields or saline aquifers. The carbon capture processes make fourth-generation biofuel production carbon negative, which is highly desirable as it captures more carbon than it produces.[38] Overall, fourth-generation biofuels are aimed at producing sustainable energy by way of capturing and storing CO_2, reducing CO_2 emissions by replacing fossil fuels.[35]

1.3.2 Microalgae

Microalgae are microscopic algae existing as unicellular organisms. They partake in a process known as photosynthesis (Eq. 1), by which carbon dioxide gets converted to glucose as a stored energy source.

$$6CO_2 + 6H_2O + \text{sunlight} \rightarrow C_6H_{12}O_6 + 6O_2 \quad (1)$$

Microalgae are responsible for fixing more than 40% of fixed CO_2 globally.[39] Moreover, microalgae grow in a higher density than traditional feedstock, have a relatively fast growth, and have the ability to accumulate a higher quantity of cellular lipids and carbohydrates than conventional feedstocks.[40,41] The lipid and carbohydrate productivity of microalgae is strongly linked to microalgal strain (e.g. marine or freshwater species) and climatic conditions (e.g. light intensity, temperature).[42] Some microalgae have been reported to produce up to 50% of dry cell weight (dw) of triacylglycerols, as lipid storage,

under photo-oxidative stress or other adverse environmental conditions.[43] In addition, microalgae can be produced year-round and have an oil productivity (12,000 l/ha) that greatly exceeds that of oilseed crops, such as rapeseed (1190 l/ha).[33] They can be grown on marginal or infertile land with adequate moisture, CO_2, and sunlight, which minimizes competition with land for agricultural food sources, and in wastewater, whereby they participate in municipal wastewater remediation.[44] This remediation involves the removal of nitrogen and phosphorus from the wastewater, diminishing the potential for eutrophication.[44] Microalgae can also be used to produce valuable co-products, such as proteins and residual biomass after lipid extraction, which may be used as fertilizer or animal feed,[45] or bioethanol or biomethane through fermentation processes.[46] For these reasons, microalgae are currently considered to be one of the most promising feedstocks for renewable energy.[47]

1.3.3 Lipid composition of microalgae

Microalgae produce different kinds of cellular lipids, including fatty acids (FAs), phospholipids (PLs), glycolipids (GLs), and acylglycerols (AGs). Depending on the type of species, growth conditions, and ambient environment, microalgae can produce different quantities of these lipids. FAs are carboxylic acids that contain various numbers of carbons and double bonds, and make up most of the lipids found inside the cells. When FAs are bound to a glycerol backbone, they form AGs, and when FAs are bound to a hydrogen atom, they are known as free fatty acids (FFAs).[48] The AGs, which are ester molecules, can be further categorized depending on the number of FAs bound to the glycerol

backbone: monoacylglycerol (MAG), diacylglycerol (DAG), and triacylglycerol (TAG). Lipids can generally be classified into two categories based on the polarity of the molecular head group: 1) neutral lipids (NLs) which comprise AGs and FFAs and 2) polar lipids which can be further sub-categorized into PLs and GLs. Figure 1.1 displays structures of FFAs (stearic and oleic acid), a PL, and a TAG. In microalgal cells, NLs are hydrophobic molecules lacking charged groups primarily used for energy storage, while PLs are used to form the bilayer cellular membranes. Depending on the microalgal species, there may be additional NLs that do not contain FAs, such as pigments (carotenes and chlorophyll), sterols, and ketones. These types of NLs are not useful in the production of biodiesel.

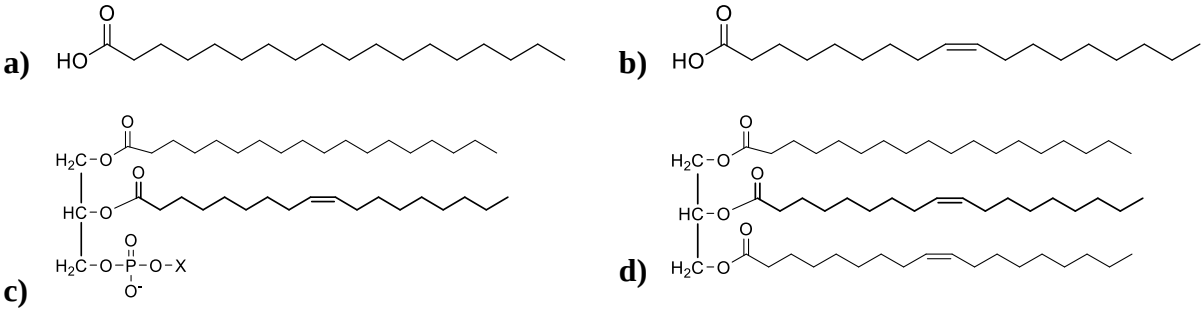


Figure 1.1. Structures of some examples of the types of microalgal lipids: a) Stearic acid (C18:0), b) Oleic acid (C18:1), c) Phospholipid (polar lipid), and d) TAG (NL).

1.3.4 Selection of microalgae species and cultivation

In any algal process, species selection is a key factor influencing the choice of growth location, reactor design, culture conditions, harvesting method and product range.[33,49,50] Species are selected based on biomass and lipid productivities under specific cultivation conditions. Lipid productivity has been suggested as the most appropriate kinetic parameter in the comparison of species for biodiesel production.[49] Table 1.1 lists

the lipid content and productivities of various microalgal species under different cultivation conditions.[51] Lipid content is defined as the total lipids found inside of microalgal cells and lipid productivity is the product of lipid content and biomass productivity. As can be seen, lipid content and productivity varies between species. The most common algae: *Chlorella*, *Crypthecodinium*, *Cylindrotheca*, *Dunaliella*, *Isochrysis*, *Nannochloris*, *Nannochloropsis*, *Neochloris*, *Nitzschia*, *Phaeodactylum*, *Porphyridium*, *Schizochytrium*, and *Tetraselmis*, generally have lipid contents between 20-50%. Moreover, higher lipid contents can be achieved by these species, but only when placed under induced cultivation stresses and at the expense of biomass productivity. For biodiesel production, the most desirable microalgal species are those with higher lipid contents, such as *Botryococcus braunii*, *Chlorella sp.*, and *Dunaliella sp.*

Table 1.1. Lipid content and productivities of marine and freshwater microalgae species.[51]

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	1.2-40.0	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 tertiolecta	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	-
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 salina	10.0-25.0	-	-	13.5
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	-

Cultivation of microalgae can be performed in either indoor or outdoor systems with different growth media.[52] Indoor cultivation systems normally use photobioreactors, while outdoor systems employ either raceway pond or photobioreactor configurations. In outdoor systems, microalgae are grown in the open environment where cultivation parameters (temperature and light intensity) are dependent on day-to-day climatic conditions. The microalgae grown in such systems often suffer from inconsistent growth rates and are more susceptible to local species invasion, which is why these are not grown as monocultures. On the other hand, the microalgae grown in indoor systems are placed in a greenhouse-type structure where cultivation conditions can be more actively controlled. Despite providing better protection against local species invasion, indoor systems generally require higher operating costs.[52] Throughout their cultivation, microalgal cultures need to be replenished with growth medium consisting of essential nutrients, such as nitrogen, phosphorous, and iron.[52] Depending on the growth medium used, the biomass productivity and lipid productivity can vary with the species of microalgae.

During the cultivation of microalgae, lipid productivity can be enhanced by depriving microalgal cells of nutrients. Nitrogen depletion has been reported to enhance

the amount of lipids and triglycerides produced in the cells. Also, deprivation of nutrients can cause the ratio of FFAs to NLs to differ.

The overall scheme of microalgae to biodiesel production is shown in Figure 1.2.

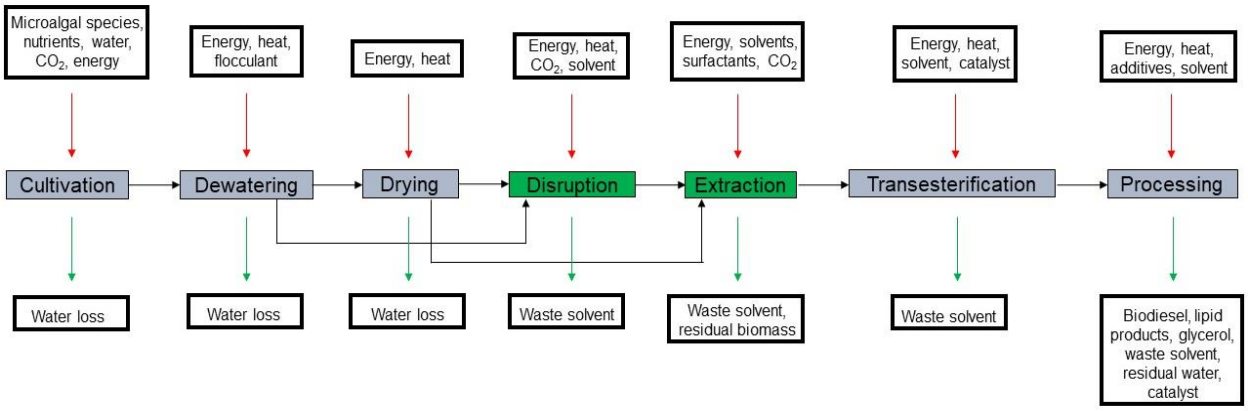


Figure 1.2. Overview of the production of biodiesel from microalgae. Inputs into the biofuel production pathway are shown above the pathway, while waste streams and products are shown below.

1.3.5 Harvesting, dewatering, and drying

Recovering microalgal biomass, typically requiring one or more solid-liquid separation procedures, is one of the most challenging steps in the production process,[53] and accounts for 20-30 % of the total costs of production.[54] Harvesting the biomass requires large amounts of energy and there are currently no standard harvesting techniques due to variable sizes, densities, and species of microalgae. Low biomass concentration (1-5 g/L) due to the limit of light penetration and the small cell sizes (2-20 μm) are major challenges in the recovery process.[55] Therefore, the choice of harvesting technology is very important to make it economically viable.[55] In most cases, microalgae harvesting exists as a two-phase process involving bulk harvesting and thickening. Bulk harvesting isolates the

biomass from the dilute aqueous suspension (0.1 to 2 g of dried microalgal biomass/L culture, depending on the cultivation method),[47] concentrating it down to approximately 2-7 % of solid matter.[33] The main harvesting methods for this phase include flocculation, flotation, or gravity sedimentation. To reduce the cost of downstream processing, this aqueous suspension needs to be concentrated.[53,56] Thickening, also known as dewatering, concentrates the dilute suspension into a slurry through centrifugation, filtration, or ultrasonic aggregation. This phase is generally more energy intensive compared to bulk harvesting. When dewatered beyond 200 g dried microalgal biomass/L of culture, the concentrated microalgal biomass, or concentrate, is transformed to a suspension that is often referred to as a paste or pellet.[47] Among these harvesting and dewatering processes, flocculation appears to be most advantageous due to its low energy requirement.[57,58] During flocculation, microalgal cells adhere to one another and to a flocculant to form heavy aggregates which then settle to become concentrate. Cationic, anionic, and non-ionic polyelectrolytes (or polymer) are typically used to flocculate microalgal cells. Overall, developing an economically viable and an energy-efficient dewatering technology is currently an active field of research.

After dewatering, drying the biomass slurry is another important step to remove residual water held within the biomass. This is typically performed to ensure effective mass transfer of the lipids during the subsequent extraction process. Even though removing water from microalgal biomass improves lipid extraction efficiency, drying the biomass is energy intensive. According to one life cycle assessment, the drying step accounts for 89 % of the required energy input for biodiesel production from microalgae.[59] Some studies have reported that the total energy expenses for processing microalgae to biodiesel is more than

doubled when drying the microalgae takes place.[60] Therefore, extraction methods using microalgal slurries (2-20 wt% solids) have been the focus of a number of research efforts in recent years.[61–64]

1.3.6 Cell disruption: microalgae cell lysis

Microalgal cellular membranes are rigid and often require cell disruption to allow the release of intracellular lipids. Cell disruption can be applied prior to or simultaneously with the lipid extraction step by mechanical, chemical, or biological methods.

Mechanical disruption can break through most biomass membranes by physical force. These include bead mill, press, high-pressure homogenization, ultrasonication, lyophilization, and microwave. The first three are the most widely used methods for laboratory-scale microalgal cell disruption.[65] Bead milling involves physically grinding the microalgal cells against the solid surfaces of glass beads in a violent agitation. High-pressure homogenization involves pumping microalgal concentrate through a narrow hole of a valve under high pressure. It then releases the concentrate into a low-pressure chamber. This pressure drop induces shear stress on the microalgal concentrate, allowing for an effective cell disruption of the cellular membranes and walls. Ultrasonication disrupts microalgal cells via transmission of sonic waves through the microalgal concentrate. These waves create a series of microbubble cavitations on the cell surface and eventually disintegrate the cell membrane/wall.[65] Lyophilization involves the dehydration of frozen samples of microalgal cells at a low pressure of 1 kPa and a temperature of -50 °C, in which ice crystals sublime.[66] This process weakens the microalgal cell walls whereby intracellular

microalgal lipids are released. Microwave is a process that provides electromagnetic radiation within a specific frequency range to microalgal cells.[67] When the cells receive this radiation, they experience a dramatic increase in temperature and intracellular microalgal lipids are released. Among the mechanical cell disruption methods, bead milling appears to be the most suitable for large-scale application due to its low operating cost.[65]

Chemical disruption techniques often involve surfactants, acids, bases, solvents, and osmotic shock. Most chemical disruption techniques will destroy the cellular membrane/wall through intermolecular forces. Chemical disruption is often conducted simultaneously with lipid extraction most commonly using polar and/or non-polar solvents.

Biological disruption methods involve the use of enzymes, which degrade membrane polysaccharides and/or proteins. The use of enzymes offers great selectivity and the mildest reaction conditions; however, they are of such high cost that their implementation to date has been limited. Mechanical and chemical disruption methods disrupt microalgae in a non-selective manner, whereby mechanical cell disruption damages all species present in solution and chemical cell disruption could partake in side-reactions with NLs (MAG, DAG, TAG) and FFAs from microalgal cells. Biological methods can selectively target which bonds to break for lipids to be retrieved without side-reactions or destroying everything completely.

1.3.7 Microalgal lipid extraction

During microalgal lipid extraction, the pre-treated microalgal biomass, either in the form of a concentrate, disrupted concentrate, or dried powder, is exposed to an eluting

extraction solvent which extracts the lipids out of the cellular matrices. Concentrate and disrupted concentrate forms of biomass contain some residual water while dried powder does not. In a typical lipid extraction process, polar solvents interact with the polar lipids and non-polar solvents interact with the NLs.[47]

A major challenge in research presently is the efficient extraction of desirable lipids from microalgal cells primarily due to the rigidity of their cell walls. These walls are made up of a polysaccharide and glycoprotein matrix, providing the cells with a formidable defense against the environment.[68] For the extraction from dried microalgae, efficient extraction of desirable lipids does not pose a problem because the drying process breaks down the cell walls. However, microalgal slurries still have intact cell walls and therefore the extraction of desirable lipids is not as efficient. This has made the extraction of industrially useful quantities of lipids from microalgae difficult.

This difficulty in isolating the desirable lipids has led to the development of energy intense extraction techniques, resulting in higher economic and environmental costs. Some of the main extraction techniques used to recover microalgal lipids are conventional organic solvent, Soxhlet, and supercritical carbon dioxide ($scCO_2$) extractions.[69] In recent years, new solvent extraction approaches have been proposed to minimize the environmental and economic costs of the process by choosing solvents that are inexpensive, non-toxic and easily removable. Conventional methods use flammable and/or chlorinated solvents, such as n-hexane or a mixture of chloroform/methanol, resulting in increased toxicity and carcinogenicity.[70] Moreover, the use of these solvent systems causes a decreased selectivity for the extraction of NLs and FFAs. Soxhlet extraction typically uses a 2:1 v/v mixture of chloroform/methanol solvent system, a temperature of 80 °C, and

is conducted for 24 hours.[71] This technique has been reported to extract the most lipids, but is not economically viable because of the environmental damage caused by the use of chlorinated solvents. It exhibits poor selectivity for desirable NLs and FFAs, and requires large amounts of energy for heating. Supercritical CO_2 extraction is a promising greener extraction technology usually conducted at temperatures of 50-80 °C, high pressures of 200-300 bar, for 80 minutes.[72] It has many advantages compared to Soxhlet extraction and conventional organic solvent extraction, including higher selectivities, shorter extraction time, and absence of halogenated organic solvents. Moreover, the energy cost is not at a disadvantage since the energy cost for distillation of organic solvents is comparable to the energy costs of recompressing CO_2. However, this technique does require high pressures to obtain yields comparable to conventional organic solvent extractions and so the capital cost of the equipment to operate these pressures is of concern. As a result, the economical production of biodiesel from microalgal lipids is limited by the energy and capital costs associated with the extraction techniques described.

1.3.8 Production of biodiesel from microalgal lipids

As mentioned previously, biodiesel consists of fatty acid alkyl esters and is made from natural oils, such as vegetable, or animal fats. Biodiesel can be used in diesel engines in its pure form or can be mixed with petroleum-based diesel due to their similar physical and chemical properties. Some advantages it has over conventional diesel is that it has a higher flash point (150 °C),[73] it has better lubricant properties and is oxygenated which enhances engine life,[74,75], cleaner emissions,[11] and it offers safer transportation, handling,

and storage.[73] A major setback with the wide-scale application of biodiesel is its high production cost compared to conventional diesel. Since biodiesel is less volatile and more viscous than conventional diesel, it requires fine tuning to adjust its viscosity and volatility to match that of diesel. Some techniques that have evolved to produce biodiesel effectively include pyrolysis, microemulsification, and transesterification from crude vegetable oil.[76] Pyrolysis is a thermochemical process that heats (400-500 °C) biomass in the absence of oxygen to create bio-oil that resembles crude oil. This bio-oil is then converted to transportation fuels by hydrotreating, whereby hydrogen reacts with the bio-oil to remove sulfur and oxygen followed by hydrocracking, whereby the treated bio-oil reacts with hydrogen to create smaller-chained hydrocarbons to meet the specifications of diesel fuels.[77] Microemulsification involves reducing the viscosity of vegetable oils and enhancing the miscibility of polar and oil phases when producing biodiesel.[78] Of these three methods, transesterification is the most common way to transform crude bio-oil to biodiesel.[74]

1.3.9 Transesterification/esterification

In general, a transesterification reaction occurs between an ester and an alcohol to produce a different ester and alcohol (Figure 1.3). Upon isolation of microalgal lipids, transesterification is the reaction between AG and an alcohol producing fatty acid alkyl esters and glycerol (Figure 1.3.1). Esterification is the reaction between an FFA and an alcohol producing a fatty acid alkyl ester and glycerol (Figure 1.3.2). When methanol is used in the transesterification/esterification processes, it is called methanolysis. After

extraction of the lipids from microalgae, methanolysis is performed to specifically produce FAMEs, the primary class of chemicals found in biodiesel.

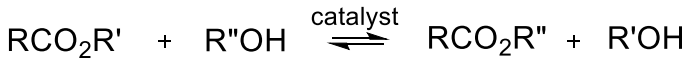


Figure 1.3. General transesterification reaction.

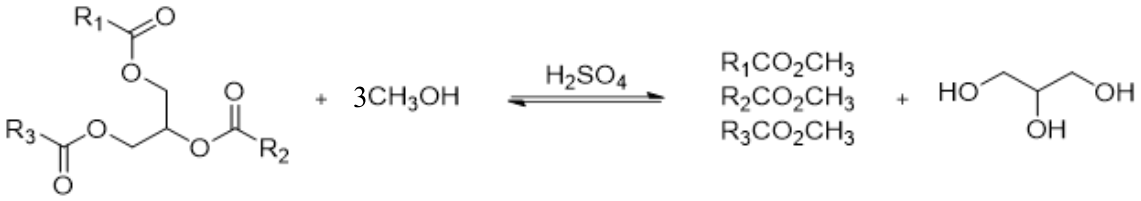


Figure 1.3.1. General transesterification reaction of a TAG using an acid catalyst.

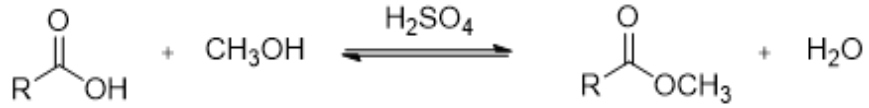


Figure 1.3.2. General esterification reaction of an FFA using an acid catalyst.

The catalysts used in transesterification/esterification processes are heterogeneous, homogeneous or enzymatic.[79] Typically, an alkali or base-catalyzed transesterification/esterification is faster than acid-catalyzed reactions; however, in the presence of FFAs, the base will participate in an acid-base side reaction giving rise to the production of soap alongside the FAMEs. Therefore, the acid-catalyzed transesterification/esterification minimizes this side reaction and is able to effectively achieve high yields of FAMEs. Some of the drawbacks associated with acid-catalyzed reactions is the use of higher reaction temperatures, longer reaction times required to produce FAMEs, and weaker catalytic activity compared to base-catalyzed reactions.[80,81]

For these reasons, industry has limited its use of acid catalysts and continues to use alkali catalysts for these processes.[82–85] The use of enzymes as catalysts for these reactions eliminates most of the setbacks associated with acid- or base-catalyzed techniques; however, enzymes are quite costly and in the presence of FFAs and short chain alcohols, they have a tendency to become denatured.[86,87] A greener catalyst-free method that has recently been reported involves the use of supercritical methanol at high temperatures and pressures that can convert NLs and FFAs, in the presence/absence of water, to biodiesel rapidly.[88] Moreover, soap is not produced and the catalyst removal step is eliminated. However, the use of high pressure and temperature conditions yield energy production costs similar to the catalytic production routes.[89] Overall, greener methods (lower energy and capital costs) for these processes need to be examined to effectively convert NLs and FFAs to biodiesel.

1.4 Thesis outline and objectives

The purpose of this research is to enhance lipid extraction of microalgal slurries using greener technologies compared to conventional organic solvent techniques. This is accomplished by using liquid CO_2 (lCO_2) for the extraction of lipids from microalgae. Chapter 2 discusses the literature surrounding cell disruption and microalgal lipid extraction. Chapter 3 examines the extraction of lipids from *Chlorella vulgaris* slurries using lCO_2 and additional modifiers. Chapter 4 discusses the transesterification of soybean oil using a switchable-hydrophilicity solvent (SHS) as a proof of concept for future applications to microalgal lipids. Chapter 5 discusses various cell disruption techniques

applied prior to extraction using lCO_2 and methanol on *Scenedesmus sp.* slurries. Extracting lipids from microalgal slurries directly would reduce the energy expense associated with drying the cells, and using lCO_2 would eliminate the use of hazardous halogenated organic solvents.

The extractions were performed under moderate temperature (≤ 25 °C) and pressure (150 bar) to reduce the future larger scale capital and processing costs. The use of these methods for the extraction of lipids from microalgal slurries could present an advantage compared to conventional organic solvent extraction because they require little to no flammable, chlorinated or volatile organic solvents.

The work contained in Chapters 2, 4, and 5 of this thesis will be submitted to the following refereed journals:

Harris, J., **Viner, K.**, Champagne, P. & Jessop, P.G. (2017) Advances in microalgae lipid extraction for biofuel production: a review. *BioFPR* (in preparation)

Viner, K., Roy, H., Lee, R., Champagne, P. & Jessop, P.G. (2017) Transesterification of soybean oil using switchable hydrophilicity solvent, 2-(dibutylamino)ethanol. *Green Chemistry* (in preparation)

Viner, K., Champagne, P. & Jessop, P.G. (2017) Comparison of cell disruption techniques prior to lipid extraction of wet *Scenedesmus* sp. for biodiesel production using liquid CO2. *Green Chemistry* (in preparation)

Chapter 2

REVIEW OF MICROALGAL CELL DISRUPTION AND LIPID EXTRACTION FOR THE PRODUCTION OF BIODIESEL

2.1 Introduction

Petroleum-derived fuels have been the focus of increasing concerns from both an economical and environmental perspective. Their prices vary substantially, their supply is dwindling in the long term, and their use could have a massive environmental impact in the form of greenhouse gas emissions. There is increasing agreement between public, political, and scientific communities that renewable, safe, and affordable alternatives to petroleum fuels should be sought out.

Biomass-derived fuels (biofuels) have been identified as potential alternatives to petroleum-based fuels that are greener and more sustainable.[90] One potential feedstock for next generation biofuels is microalgae, as it is an excellent source of lipids that can be readily converted to biofuel through various techniques.[91] The overall scheme of microalgae biofuel production is illustrated in Figure 2.1. The processing steps are generally undertaken separately and sequentially, although some methods have been developed to combine two or more process steps. This review primarily focuses on methods for the extraction of lipids from microalgae; however, cell disruption will also be briefly discussed.

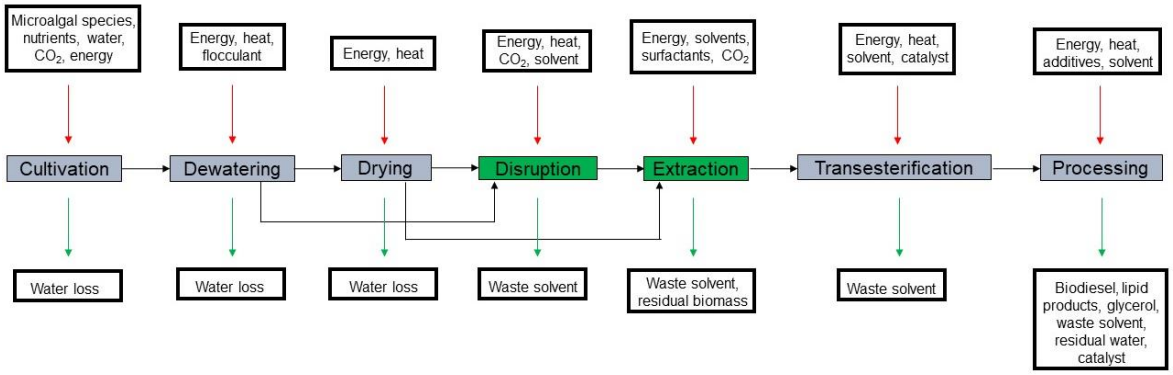


Figure 2.1. Overview of the production of biodiesel from microalgae. Inputs into the biofuel production pathway are shown above the pathway, while waste streams and products are shown below.

There are, however, important considerations to bear in mind related to microalgal strain cultivation before discussing lipid extraction approaches. Unlike a chemical feedstock that is unaffected by its origin, the extraction of microalgal lipids is influenced by biological variables, such as species and cultivation conditions. The choice of microalgal species and growth conditions will also have a large impact on the final lipid content,[49,54,92,93] as well as the resistance of the microalgae to cell disruption.[94] Studies focused on microalgal lipid extraction often do not described growth conditions in detail, making it difficult to assess or reproduce the effects of microalgae cultivation on lipid content or ease of extraction. It is, therefore, difficult to meaningfully compare lipid yields achieved in different studies that utilized different cultivation conditions, as the effect of changes in biomass composition will alter the results.

The effective extraction of lipids from microalgal biomass is the most important process in the production of biodiesel. Extraction methods should be measured with respect to a number of requirements. For example, it is not sufficient for a method to be environmentally friendly but not economically viable, as the marketplace will not adopt it.

A review by Chisti provided a detailed overview of the major challenges facing microalgal biofuels.[95] Of the challenges identified, there were three major concerns pertaining to extraction: environmental impact, energy ratio, and economic costs.

1) Environmental impact: Any method for microalgal biofuel production should have an improved environmental impact compared to fossil fuels. Greenhouse gas emissions, toxicity of reagents and waste products, and environmental costs upstream of the extraction step (e.g. synthesis of extracting solvents) constitute most of the environmental impact of the extraction process.
2) Energy ratio: The energy ratio is a measure of the energy productivity of a given process, and compares the amount of energy in the fuel to the energy consumed in its production.

$$\text{Energy ratio} = \frac{\text{Energy produced by the fuel}}{\text{Energy consumed to make the fuel}}$$

One of the considerations for a fuel to be economically and environmentally sustainable is that it will need to produce more energy than it consumes. Energy ratio estimates for microalgal biofuel production processes to date have been reported to be energy-inefficient.[96] This must be improved to make microalgal biofuels an economically competitive option. Lipid extraction has been identified as one of the most energy-intensive steps in the production of microalgal biofuel.[96] Drying the microalgae is also another very energy-intensive step. Therefore, extraction methods that collect microalgal lipids with low energy inputs and can be easily scaled to an industrial level would be most attractive.

3) Economic costs: Microalgal biofuel production approaches will ultimately need to be both environmentally friendly and cost-effective. Economic assessments of microalgal biofuel to date have typically indicated that they will have difficulty competing with traditional fuels.[59] It is anticipated that process optimization will lead to lower costs, but approaches that require the use of specialized equipment or expensive solvents will not likely be commercialized.

2.2 Lipid extraction

Cell disruption can effectively liberate microalgal lipids from biomass for the production of microalgal biofuel, but an extraction step is required to separate the valuable neutral lipids and fatty acids from the cellular matrix and water. However, methods relying on conventional solvent systems for lipid extraction have struggled to meet economic and environmental requirements. Most studies have investigated extraction methods that rely on alternative solvents such as supercritical CO_2 ($scCO_2$),[97,98] liquid CO_2 (lCO_2),[99] gas-expanded liquids,[99] ionic liquids (ILs),[100] and switchable solvents.[70] Below is a summary of the rationale behind each approach, as well as recent advances in the field and challenges the technology faces.

2.2.1 Conventional organic solvent and co-solvent extractions

Early methods of microalgal lipid extraction relied on conventional organic solvents. An ideal solvent for this process would be specific towards neutral lipids (NLs)

and have a high volatility in order to minimize energy distillation costs when separating the lipids from the solvent following extraction.[101] The extraction of lipids from the microalgal biomass can be performed using polar organic solvents such as methanol, acetone, and ethanol, as well as low-polarity organic solvents such as hexane, benzene, toluene, diethyl ether, ethyl acetate, and chloroform. Low-polarity organic solvents alone are unable to effectively extract NLs as they cannot access those that form strong hydrogen bonds with the polar lipids in the cell wall.[102] To access these NLs, a polar organic solvent must be paired with a low-polarity organic co-solvent; the polar organic solvent is intended to disrupt the neutral-polar lipid complexes and the low-polarity organic solvent aims to solubilize the intracellular NLs. Therefore, solvent systems containing a mixture of low-polarity and polar organic solvents generally maximize the extraction efficiency of NLs.[102]

In 1956, Folch used a 2:1 v:v mixture of chloroform:methanol to extract lipids from animal tissues; this came to be known as the Folch method.[103] In 1959, the Bligh and Dyer method was developed using 2:1:0.8 by volume of chloroform:methanol:water for total lipid extraction and purification.[104] When wet tissue has been homogenized using a mixture of chloroform and methanol, a monophasic system is observed; however, additional water produces a biphasic system, in which the chloroform layer should contain the lipids and the methanol-water layer should contain the non-lipids. These two methods are considered the standards for conventional organic solvent extraction of microalgal lipids. More recent work by Hidalgo et al. found that a 1:3 v:v mixture of chloroform:methanol was able extract 98.9 % of esterifiable lipids from *Botrycoccus braunii*, while a 3:1 mixture of methanol:diethyl ether could extract 96.9 % of esterifiable lipids without the use of halogenated solvent.[105] Unfortunately, diethyl ether, due to its extreme flammability,

would be just as unacceptable industrially as chloroform. Some researchers have also found that the addition of formic acid can enhance the overall lipid yield. Dingyaw et al. examined the extraction of lipids from dry *Chlorella protothecoidesis*.[106] The highest lipid yield of 42 % dw was achieved with a FAME yield of 89 % dw with a mixed solvent of 70 % dichloromethane, 20 % formic acid and 10 % methanol.

There are a number of major obstacles associated with methods relying on conventional organic solvent extraction. The costs associated with large-scale extraction of lipids from microalgae by conventional solvent systems are unlikely to make these processes economically viable. Solvent losses to evaporation represent a liability from both environmental and economic perspectives. Although conventional organic solvent and co-solvent extraction approaches are efficient for the extraction of lipids from dried microalgal biomass, they suffer from important limitations including the use of halogenated organic solvents and inefficiencies encountered during extraction of lipids from microalgal slurries. Halogenated organic solvents are unlikely to be used industrially due to the associated environmental and health issues, such as ozone depletion, groundwater contamination, and carcinogenicity. Moreover, most organic solvent and co-solvent extraction methods require large volumes of chlorinated, flammable, and/or volatile solvents to achieve high lipid yields, which could have significant environmental impacts, including contamination of the aqueous waste stream and the impacts associated with solvent manufacture. Residual water in microalgal slurries hinders mass transfer of the lipids from the microalgal cell, which decreases the efficiency of lipid extraction; energy-intensive drying is usually performed to avoid this problem.[107] Therefore, it is essential to develop greener extraction processes to improve the overall techno-economic viability of algal biofuel production.

2.2.2 Supercritical CO_2 extraction

Supercritical fluid extractions (SFEs) evolved as an alternative to traditional organic solvent-based extraction processes because of the health and safety concerns and environmental impact generated by organic solvents.[72] "A supercritical fluid (SCF) is a compound, mixture or element above its critical temperature and pressure, but below the pressure required to condense it to a solid." [108] SCFs have a number of attractive features as extraction solvents, including the adjustable solvent power of the fluid. CO_2 in particular has a very low polarity, allowing for the preferential extraction of NLs.[109] SCFs also allow for rapid mass transfer, resulting in higher total lipid yields and shorter extraction times.[109] The crude lipids obtained are also free from extraction solvents as any residual SCF evaporates following extraction. SFE can also be relatively energy efficient if the fluid is not completely decompressed when it is separated from the oil.[110]

The most commonly used fluid in SFE studies reported to date is $scCO_2$. Supercritical CO_2 presents advantages such as low toxicity, minimal oxidation or thermal degradation of extracts, rapid penetration through cellular matrices, high diffusivity, and facile product/solvent separation compared to conventional solvent extraction.[111] Moreover, CO_2 does not contaminate the aqueous phase, and does not require distillation to produce a solvent-free extract.[112–115] Supercritical CO_2 is non-polar and therefore only interacts with a portion of the NLs; specifically, the unbound neutral lipids that do not form complexes with the polar lipids present in the cell wall. However, with the addition of a polar co-solvent, affinity towards the NLs that form complexes with polar lipids can be

enhanced, leading to greater biodiesel production.[116] Some common co-solvents are methanol, ethanol, and toluene.[98]

Other possible SCFs are methanol, ethane, propane, and CHF_3, and are generally either flammable or halogenated and therefore lack the safety and environmental benefits of $scCO_2$. Propane and methanol require much higher temperatures. Supercritical H_2O (scH_2O) shares the environmental advantages of $scCO_2$, but its critical temperature is too high to be used for extractions.[117]

There are a number of recent studies that have been published focusing on the enhancement of $scCO_2$ extraction techniques. Taher et al. investigated the $scCO_2$ extraction of lipids from dried *Scenedesmus sp.* for biodiesel production.[118] The best results were obtained at 50 MPa, 53 °C, and a continuous $scCO_2$ flow rate of 1.9 g/min, recovering a lipid extraction yield of 7.4 % dw. This study also compared $scCO_2$ extraction to Soxhlet extraction, chloroform/methanol, n-hexane extraction as well as n-hexane/iso-propanol extraction, and the optimized $scCO_2$ method exhibited the highest yields of both total lipids and triacylglycerols.[118] McKennedy et al. investigated the effects of $scCO_2$ extraction conditions and co-solvent selection on the distribution of the FAMEs recovered.[119] They showed that $scCO_2$ with co-solvents such as methanol or hexane could effectively extract the lipids beneficial in biofuel production without the need for extreme temperatures. Viguera et al. described the effects of operational conditions and water contents on the $scCO_2$ extraction of lipids from *C. protothecoides*.[120] At 30 MPa CO_2, 70 °C, and a flow rate of 72 kg CO_2/h•kg biomass a maximum lipid yield of 21 % dw was achieved, which is among the highest reported for microalgae.

Though $scCO_2$ avoids the use of environmentally damaging organic solvents, it still requires high capital cost to build and operate equipment for high pressure conditions. An ideal extraction method would not require the use of high pressures, and would also avoid the need for a distillation step. Gas-expanded liquid extraction offers a possible solution to these challenges.

2.2.3 Gas-expanded liquid extraction

A gas-expanded liquid is generated by dissolving a compressible gas, such as CO_2 or light olefin into the traditional liquid phase at mild pressures (tens of bars).[121] When CO_2 is used as the expansion gas, the resulting liquid phase is termed a CO_2-expanded liquid (CXL). CXLs have been used in a variety of applications including extractions, separations, and reactions.[122,123] Life cycle assessment (LCA) has determined that the environmental impact of CXL extraction of microalgal oil was one-tenth of that associated with other extraction methods, including chloroform/methanol, dichloromethane/methanol, isopropranol/hexane, $scCO_2$, and CO_2-expanded ethanol, particularly as it relates to inhalation toxicity and climate change impacts.[124] Moreover, another LCA conducted by Collotta et al. compared a conventional co-solvent-based extraction system (chloroform:methanol), non-expanded methanol in a flow-through reactor and CO_2-expanded methanol (CXM) in a flow-through reactor.[125] CXM demonstrated the lowest environmental impact in all categories tested.

The use of CXLs for the extraction of lipids from natural products, such as microalgae, is a relatively new field of study. The primary benefits of CXLs compared to

$scCO_2$ are the greater tunability of the physicochemical properties of the liquid solvents, such as polarity and solubilizing power, at much lower pressure requirements. This was shown in trials conducted by Paudel et al. examining the use of CXM for lipid extraction from freeze-dried *B. braunii*.[99] It was found that CXM at 35 °C and 7.2 MPa extracted lipids with a yield of 24 % dw, when the w/w ratio of methanol to dry mass of microalgae was 7:1. Moreover, CXLs offer environmental benefits compared to traditional organic solvents, including substantial volume replacement of organic solvents with environmentally benign CO_2, and process safety advantages such as reduced flammability associated with the presence of CO_2 in the vapor phase.[121] Further work will be required to assess the limits of this technology.

2.2.4 Liquid CO_2 extraction

The high capital costs of $scCO_2$ are primarily due to the requirement of high pressure equipment[112] and represent a major limitation of this technology. Liquid CO_2 extraction has emerged as a possible substitute, requiring lower temperatures and pressures than $scCO_2$ extraction while avoiding the toxic or flammable organic solvents employed in conventional solvent extraction. For example, in order to obtain a density of 0.8 g/mL, high enough for acceptable solubilizing power, $scCO_2$ at 50 °C would require a pressure of 21 MPa while lCO_2 at 25 °C would only require 9 MPa.[126] lCO_2 has a low polarity compared to most organic solvents, it can exhibit higher selectivity towards the neutral lipids that are most desirable for biodiesel production, and minimize the simultaneous extraction of polar lipids, which are less desirable in biodiesel production.[97,127] Unfortunately, there are

limited studies on the topic of lCO_2 extraction. Paudel et al. found that lCO_2 extraction conducted on freeze-dried *B. braunii* at 6.8 MPa and 25 °C lead to a lipid yield of 19 % dw.[99] Chen et al. demonstrated that high yields could be achieved with lCO_2 in a continuous lipid extraction from a slurry of pre-dried commercial microalgae rich in docosahexaenoic acid (DHA; an omega-3 fatty acid) under pressures of up to 20 MPa.[128] Although these initial results are promising, more experimental results will be needed to assess the efficacy of lCO_2 technology.

2.2.5 Ionic liquids as extraction solvents

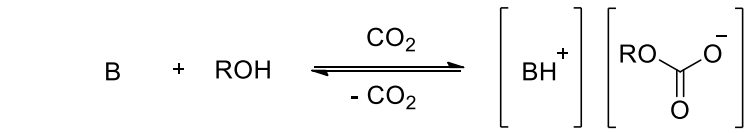
Ionic liquids (ILs) are organic salts that melt below 100 °C and are typically composed of large asymmetric organic cations coupled with smaller anions.[129–131] Their thermal stability, synthetic flexibility, non-volatility, non-flammability, and recyclability[131,132] have positioned them as alternatives to traditional organic solvents in microalgal lipid extraction. Orr and Rehmann recently provided a comprehensive summary of research completed in the field.[100] Although ILs appear to improve greatly reported yields, there are concerns that their use as a solvent for industrial scale algal biofuel applications may be unrealistic due to their environmental and economic costs.[133] Many ILs are not particularly hazardous to humans, but their production requires many synthetic steps, uses toxic and/or volatile reagents and solvents, and is far more environmentally damaging than the manufacturing of conventional solvents.[133,134] Some ILs with much simpler and more efficient syntheses have been reported, offering hope that more economically viable and environmentally sound ILs may become available for algal lipid

extraction. For example, George et al. examined the design of low-cost ILs for lignocellulosic biomass pre-treatment.[135] They showed that as a result of the large reduction in synthetic steps (from 30 steps to 7), the synthesis of protic ILs, especially [$HNEt_3$][HSO_4], have the potential to exhibit a lower environmental footprint due to reductions in waste by-products, solvent losses, energy usage and CO_2 generation. Although the potential for ILs in lipid extraction is alluring, it is likely that more progress will need to be made in the synthesis of ILs before they are able to meet environmental and economic standards.

2.2.6 Switchable solvents for extraction

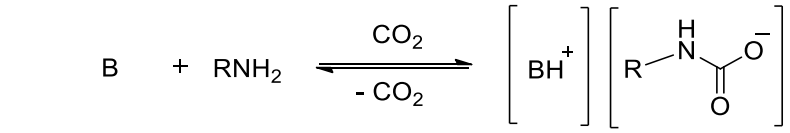
Switchable solvents, also known as "reversible" or "smart" solvents, can reversibly change their properties upon application or removal of a "trigger".[136,137] Switchable-polarity solvents (SPSs), invented by Jessop et al., switch from a low polarity to a high polarity IL when exposed to CO_2 (the trigger).[138] Many solutes are selectively soluble in either the low polarity or high polarity solvent.[136] The polarity of the solvent can be reversed by removing the CO_2, which can be achieved by heating or sparging the solution with a non-acidic gas such as air, N_2 or Ar gas. There are three classes of SPSs, and these can either be single component or two component species. Two-component SPSs consist of either a base with an alcohol (Scheme 2.1a)[139] or base with an amine (Scheme 2.1b),[140] while single component systems utilize a primary or secondary amine (Scheme 2.1c).[136]

a)



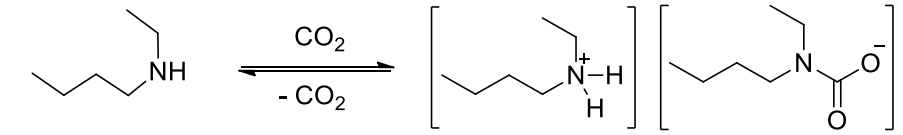
Low polarity form High polarity form

b)



Low polarity form High polarity form

c)



Low polarity form High polarity form

Scheme 2.1. Three classes of SPSs: (a) two-component SPS, consisting of an alcohol and a base such as an amidine or guanidine, (b) two-component SPS, consisting of a primary amine and a base such as an amidine or guanidine, and (c) a single-component SPS, N-ethylbutylamine.[141]

In the case of microalgal lipid extraction, the low polarity form of the SPS is used to extract the lipids. Subsequently, the addition of CO_2 causes the SPS to change to its high polarity form, which then allows the SPS to naturally separate from the lipids. Although any of the three classes of SPS can be used in lipid extractions, it should be noted that amidine/alcohol SPSs are poorly suited for extraction from wet biomass. Samori et al. found that SPSs of the amidine/alcohol class could extract lipids to a higher extent than

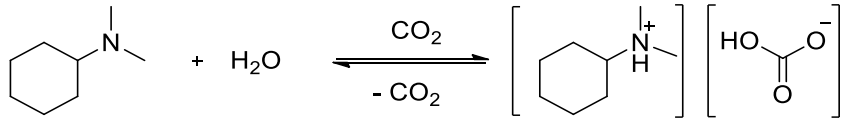
hexane from both microalgal slurries and dried samples of *B. braunii*.[140] They reported that DBU/octanol exhibited the highest lipid yields from freeze-dried microalgal samples (16 % dw). However, the yield decreased by half when microalgal slurries were used. This limitation is particular to amidine/alcohol SPSs. Amidine/amine or secondary amine type SPSs do not exhibit the same decreases in yield when extractions are performed on microalgal slurries.[141]

Secondary and primary amines can act as single-component SPSs if they form liquid carbamate salts when exposed to CO_2 at atmospheric pressure (Scheme 2.1c).[141] Unlike the amidine/alcohol SPS, secondary amine SPSs function in the presence of water. Du et al. used single-component SPSs to extract lipids directly from microalgal slurries of *Desmodesmus sp.*[141] They obtained a lipid yield of 17 % dw using N-ethylbutylamine (a secondary amine). A LCA was also conducted to compare methods for extraction of lipids from microalgal slurries using traditional organic solvents, scCO_2, and CO_2 switchable solvents.[142] The results indicated that a significant positive energy balance for lipid extraction could only be achieved using a switchable solvent extraction method, making this a promising approach for extracting lipids from microalgae for energy applications.

Another class of switchable solvents are switchable-hydrophilicity solvents (SHSs), unusual solvents discovered by the Jessop group.[143,144] Unlike an SPS, an SHS is a switchable solvent designed for use with water, therefore its potential application extends to the extraction of lipids from microalgal slurries. An SHS is defined as a solvent that can be reversibly converted between a hydrophobic form that forms a biphasic mixture with water, and a hydrophilic form that is miscible with water. The switch to the hydrophilic form is achieved by the introduction of CO_2 at 1 bar while the reverse is achieved by the

removal of CO_2.[143,144] In the case of microalgal lipid extraction, an SHS in its hydrophobic form will extract lipids from microalgae, after which it will be switched to its hydrophilic form upon the addition of carbonated water. The solvent will then partition out of the lipids and into the water. After separating out the lipids, the CO_2 is flushed away with N_2 or air causing the solvent to become hydrophobic and separate from the water. SHSs normally consist of an amine or amidine that, when exposed to 1 bar of CO_2 in the presence of water, becomes a water-soluble bicarbonate salt (Scheme 2.2). Moreover, since SHSs can be removed from the products by a carbonated water wash, rather than distillation, there is no need for SHSs to be volatile. For that reason, most of them are non-flammable.[70] This approach should allow for lipid extraction without drying of biomass or energy intensive distillation of solvents. Boyd et al. used N,N-dimethylcyclohexylamine, an SHS, to extract lipids from freeze-dried *B. braunii*.[70] The crude lipid fraction contained high concentrations of long chain MAG, DAG, and TAG without phospholipids. N,N-Dimethylcyclohexylamine extracted up to 22 % crude lipid on a microalgae dry mass basis.

Although results using switchable solvents are promising, further research focusing on SPS and SHS extraction optimization and scale up is necessary to determine their viability in commercial applications.



Hydrophobic form Hydrophilic form

Scheme 2.2. N,N-Dimethylcyclohexylamine is an example of an SHS.[70]

2.3 Simultaneous cell disruption and extraction

Several methods have been proposed that combine the cell disruption and lipid extraction steps of the microalgal biofuel production process. There are several economic and environmental advantages to combining process steps (process intensification), including reduced capital costs and energy savings. Some of the most common methods for simultaneous disruption and extraction are summarized below.

2.3.1 Microwave-assisted extraction

Microwave-assisted extraction (MAE) has been extensively studied for the purpose of extracting medicinal compounds from plants.[145–147] Although the technology is well established in other fields, research applying microwave technology to lipid extraction for biofuel production from microalgae began only in 2011.[67] The principle behind the method involves a rapid oscillation of molecules within an electric field, causing friction, and therefore heat. Rapid heating causes cell membranes to rupture, which allows desirable cell materials to be more readily extracted.[148] With the assistance of an extraction solvent, the microwave cell disruption and extraction steps generally takes place simultaneously.

There have been a number of studies reported since 2011 that utilize MAE for simultaneous cell disruption and lipid extraction. A 2013 study by Iqbal and Theegala examined the use of biodiesel as an extraction solvent during MAE.[149] It was found that extracting *Nannochloropsis sp.* with 40 % biodiesel in ethanol at 120 °C could exceed the lipid yields obtained through conventional Soxhlet extraction. Pan and co-workers utilized

the IL [BMIM][HSO_4] as an extractant while performing MAE on *C. sorokiniana, N. salina* and *Galdieria sulphuraria*.[150] The IL assisted in the dissolution of the cell membrane in conjunction with the microwave heating. The authors reported almost 20-fold, 4-fold and 12-fold increases in yields from *C. sorokiniana*, *N. salina*, and *G. sulphuraria*, respectively, when using microwave heating compared to oil bath heating (experiments preformed at 120 °C). In an effort to assess the energy balance of MAE, Ali and Watson performed extractions on *N. oculta* slurries (23 wt% solids) using ethanol and hexane as extraction solvents over a range of times and energy levels.[151] They demonstrated that using 5 min of 1021 W microwave irradiation would contribute <1 % to the energy input, while increasing lipid extraction 3-fold over a control.

2.3.2 Ultrasound-assisted extraction

In the fields of biochemistry and molecular biology, sonication has long been used as a method of cell disruption. This process works by generating high frequency sound waves that cause bubbles to form and collapse rapidly within the sample. This phenomenon, known as cavitation, imparts mechanical stress on the cells through shear force and shock waves, which causes the breakdown of the cell membrane and allows lipids to escape. The use of ultrasound-assisted extraction (UAE) as a means of microalgal cell disruption has risen in popularity in the research community since 2010.[152–155] In UAE, the sonication is typically simultaneous with the extraction step, so that lipids are released into an extraction solvent.

Researchers in the field of microalgal UAE have worked to develop sonication techniques capable of achieving higher lipid yields and being used at larger scales. Yamamoto et al. found that higher frequency sonication resulted in more effective cell disruption and that the frequency requirements were tied to cell membrane characteristics.[154] Other researchers, rather than focusing on the use of high frequency sonication, have developed UAE methods for use over prolonged periods at lower frequencies for the purpose of developing low-energy UAE strategies.[155] D'Oca et al. studied ultrasonication in combination with a variety of solvents such as chloroform:methanol (2:1 v/v mixture), methanol, chloroform, ethanol, and hexane for extraction from dry *C. pyrenoidosa* biomass.[152] They reported that using chloroform:methanol (2:1 v/v mixture) with ultrasonication showed the highest lipid extraction of 19 % dw. Adam et al. developed a "solvent free" ultrasonic extraction method, a strategy focused on eliminating the environmental impact and economic burden of organic solvents.[153] Subjecting a 5 % microalgal suspension in aqueous media to 1000 W of power for 30 min yielded a maximum oil recovery of 4.2 % dw. Wang and Yuan investigated the use of nozzle spraying with ultrasonication, and noted that low concentration microalgal suspensions (0.05-0.4 wt% solids) were well disrupted using this technique.[156] Although currently limited to low density samples, nozzle spraying with ultrasonication may offer a scalable cell disruption method.

A major concern for both MAE and UAE relates to scalability and energy costs. MAE and UAE may be too expensive to be cost-competitive on a commercial scale.[157,158] Energy usage is also a major concern as large-scale microwave or sonication systems may require large amounts of electricity in their operation. Additional scale-up research and

development is required to determine the viability of such technologies in industrial settings.

2.3.3 Surfactant-assisted extraction

Surfactants are compounds that lower the surface or interfacial tension between a liquid and a second phase. They are often used to disrupt cell membranes for the purpose of accessing DNA from microalgal biomass.[159] As a result, surfactants have been examined as an alternative approach to disrupt microalgal cell membranes for greater accessibility to the intracellular lipids.

Lai et al. examined a novel method for improving lipid recovery from *Scenedesmus sp.* slurries using surfactants such as myristyltrimethylammonium bromide (MTAB) and 3-(decyldimethylammonio)-propanesulfonate (3-DAPS), which are cationic and zwitterionic surfactants, respectively.[160] Treatment with 50 mM surfactant overnight improved lipid yield as much as 16-fold over untreated (without surfactant) biomass. Corre et al. measured the effect of dodecylbenzene sulfonate and Triton X-100 (anionic and neutral surfactants) on two species of dried microalgae, *C. vulgaris* and *C. emersonii*.[161] Since *C. emersonii* has a trilaminar sheath in addition to a polysaccharidic cell wall, it was nearly unaffected at high concentrations (1 g/L), whereas trilaminar sheath-devoid *C. vulgaris* exhibited a high sensitivity, especially when actively growing cells were tested. A novel approach called the cationic surfactant-based harvesting and cell disruption (CSHD) method was studied by Huang & Kim to determine its effectiveness in simultaneous microalgal biomass harvesting and cell disruption.[162] CSHD exhibited a powerful ability

to disrupt the cells; the lipid recovery was increased 133 % compared to not using CSHD and it allowed the extraction of up to 100 % of the total lipids from *C. vulgaris* slurries (20 wt% solids). Ulloa et al. examined the role of non-ionic surfactants (0.1-5 % concentration) as cell lysis agents on freeze-dried *Tetraselmis suecica*.[163] Among the Tween and Triton X series, Triton X-114 was shown to possess the best lytic effect.

A major advantage of surfactant treatment is that it allows for the extraction of lipids from microalgal slurries at room temperature, which removes the energy cost associated with the heating and drying of the biomass during the extraction process.[162] In addition, it does not need to be paired with halogenated organic solvents for efficient extraction. A disadvantage of surfactant treatment is that the concentration of surfactant must be above the critical micelle concentration, with trace amounts being insufficient. Moreover, the surfactant contaminates the water phase and needs to be recovered, and the recovery process of the surfactant is often difficult.[164] Advances in surfactant-assisted extraction of microalgae will likely rely on new research in surfactant selection and recovery.

2.4 Conclusions

The field of microalgal biofuel has expanded significantly in recent years due to increasing pressure to find viable fossil fuel alternatives. However, there is considerable progress to be made, especially in the area of microalgal lipid extraction, before microalgae can effectively compete with fossil fuels. To improve upon microalgal lipid extraction processes, the following must be considered:

1) Using microalgal slurries is essential due to the economic and environmental expenses associated with drying of microalgal slurries. Future work will be directed at overcoming challenges such as low lipid extraction yields and the loss of solvents, surfactants, or catalysts to the aqueous phase.
2) Combining process steps is necessary. By combining steps, such as cell disruption and extraction, capital costs and energy use will be reduced. Studies combining processing or process integration should remain a focus of the research community moving forward.
3) LCAs are important to highlight the true environmental costs associated with each lipid extraction method. Unfortunately, limited data is available to perform meaningful comparisons between the various lipid extraction methods since each study uses different microalgal strains, each containing a variable amount of water. Moreover, these microalgal strains are cultivated and harvested under different conditions, which gives rise to the production of different quantities and ratios of lipids and fatty acids. Future work should be concerned with identifying the complete environmental impact of a given process and to make adjustments as necessary to achieve a greener technology.

Chapter 3

EXTRACTION OF LIPIDS FROM *CHLORELLA VULGARIS* SLURRIES USING LIQUID CO_2, CO-SOLVENTS, AND/OR ADDITIONAL MODIFIERS

3.1 Introduction

Fossil fuels are currently the main source of energy employed in all forms of transportation; however, their use is detrimental to the environment because upon combustion, they emit greenhouse gases (GHGs), particularly large quantities of CO_2.[165] Not only are fossil fuels detrimental to the environment, but they are non-renewable resources and are slowly depleting over time.[165] For these reasons, along with their fluctuating costs, there is an economic motive for replacing them with a fuel that is both renewable and cost-effective. Since commercial vehicles, such as tractor trailers, are typically powered by diesel engines as opposed to gasoline ones, replacing diesel fuel could potentially make the greatest impact on fossil fuel usage in this class of vehicles.[166]

Biodiesel represents an attractive alternative to traditional petroleum-derived fuels. Not only does the combustion of biodiesel result in fewer harmful emissions of CO, SO_x, and particulate matter than petroleum-derived fuel,[167] but it also is a renewable resource, biodegradable, and can be made from a variety of feedstocks.[11] Importantly, the combustion of biodiesel releases similar amounts of CO_2 to comparable fossil fuels; however, the CO_2 is consumed in the growth of the biomass, which reduces the net CO_2 emissions. Currently, there are four generations of biofuels.[35] First-generation biofuel is

derived from food crops, such as corn and wheat;[25] however, the underlying competition with food supplies and the limited availability of arable land renders first-generation feedstocks unsustainable. Second-generation biofuel feedstocks include wood and waste products,[29] although this still indirectly competes for land with food or agriculture for other purposes. The issue of arable land use was finally addressed with the use of third-generation biofuel feedstocks, such as microalgae.[32] Microalgae require no land use and can be applied in municipal wastewater remediation by removing nitrogen and phosphorus from this wastewater.[168] Moreover, they have high productivities, high intracellular lipid contents, and rapid growth rates.[40] Therefore, microalgae avoid competing with food sources for land, as well as diminish environmental pollution. Fourth-generation biofuels involve not only producing fuel from biomass materials, but also the capture and storage of CO_2 taken up from these materials using oxy-fuel combustion.[35]

One of the major challenges hindering the commercialization of biodiesel from microalgal slurries is the extraction of the neutral lipids (NLs) that are necessary for biodiesel production. Microalgal cell walls are made up of a polysaccharide and glycoprotein matrix that protects the cells against the environment and harmful species.[68] Hence, this makes it difficult to access the desirable lipids and has conventionally required the use of energy intensive extraction techniques. Currently, conventional organic solvent extraction, Soxhlet extraction, and supercritical CO_2 ($scCO_2$) are used as the main extraction techniques in research, but each technique presents significant challenges.[69] Conventional organic solvent extraction often uses halogenated organic solvents, such as chloroform and dichloromethane, resulting in increased toxicity and carcinogenicity.[70] Eliminating the need for these halogenated organic solvents could potentially increase the

benefits for this extraction method. Soxhlet extraction also suffers from the use of halogenated organic solvents and, furthermore, very long reaction times and high temperatures are required which is disadvantageous.[99] Compared to conventional organic solvent extraction and Soxhlet extraction, the use of $scCO_2$ has been examined as a greener extraction approach.[72] CO_2 is essentially non-toxic, inexpensive, and easily removable, which eliminates the cost of downstream solvent separation processes.[169] Moreover, this technique does not use any halogenated organic solvents and can achieve effective extractions in shorter periods of time.[47,111] However, as the process requires high pressures (200-300 bar), the capital costs to operate equipment that consistently runs at these pressures is not economically viable.[72] Overall, the extraction techniques that exist currently are limited by their potential environmental impacts, as well as energy and capital costs. Therefore, other approaches to microalgal lipid extraction for biodiesel production are actively being researched.

Liquid CO_2 (lCO_2) has emerged as an innovative extraction technique that offers many of the same benefits as $scCO_2$, but can operate at lower temperatures and pressures, reducing capital costs and safety concerns.[99] Since this method operates under milder conditions compared to $scCO_2$, complete access to intracellular microalgal lipids continues to present an important challenge. For this reason, lCO_2 with additional co-solvent, and/or variety of acid, base, and/or surfactant modifiers has been proposed to increase lipid yield.

In recent years, the use of $scCO_2$ extraction, acid/base disruption, and surfactant-assisted extraction have been reported to enhance microalgal lipid extraction. Choi et al. examined the lipid extraction from *Nannochloropsis sp.* using $scCO_2$ with or without methanol as the co-solvent.[170] Both extractions were performed at 50 °C, 400 bar, and 4.0

mL/min of $scCO_2$ and, in the co-solvent extraction, methanol was added at 0.4 mL/min.[170] The crude lipid yields obtained for $scCO_2$ were 6.9±0.6 % dw, while $scCO_2$ and methanol achieved yields of 12.5±0.6 % dw, demonstrating a beneficial effect of the added co-solvent. Laurens et al. investigated an acid-catalyzed pre-treatment method on *Scenedesmus sp.* using 0.3 mL of hydrochloric acid and 5 % v/v methanol in water for 1 h at 85 °C and reported a yield of up to 97 % of total fatty acids from microalgal biomass slurries.[171] Lastly, Lai et al. examined surfactant-assisted disruption on *Scenedesmus sp.* to improve lipid recovery from microalgal biomass slurries.[160] Myristyltrimethylammonium bromide (MTAB) and 3-(decyldimethylammonio)-propanesulfonate (3-DAPS) (50 mM) surfactant treatments were applied overnight, and were found to improve FAME recovery as much as 16-fold over untreated (without surfactant) biomass.[160]

In this study, lipid extractions from *Chlorella vulgaris* slurries were conducted using lCO_2 and a co-solvent: methanol, ethanol, isopropanol, butanol, pentanol or acetone. Further extractions were performed using lCO_2, with or without the co-solvent that was deemed most efficient from the first set of experiments, and an additional modifier: sodium hydroxide, sulfuric acid, cetyltrimethylammonium bromide (CTAB), or dodecyltrimethylammonium bromide (DTAB). Finally, the resulting FAME profiles of *C. vulgaris* lipid extracts, when subjected to certain additional modifiers, were produced to quantify the number of carbon chain lengths that would be considered desirable for biodiesel production.

3.2 Materials and methods

All materials were used as received from the suppliers. Carbon dioxide (99.99%) was obtained from Praxair. Sodium hydroxide pellets (≥97.0%), sulfuric acid (99.999%), CTAB (≥99%), and DTAB (~99%) were obtained from Sigma-Aldrich. HPLC grade methanol (>99.9%), ethanol (99.5%), isopropanol (99.9%), and butanol (≥99.4%) were obtained from Fisher Scientific. Acetone (≥99.5%) was obtained from Sigma-Aldrich. The microalgae species used for the study was *C. vulgaris*. Dr. Shijian Ge grew this species of microalgae locally in the Department of Civil Engineering at Queen's University and generously supplied the microalgal slurry for lipid extraction. The microalgal species was grown in a batch mode in Bold's Basal Medium under continuous illumination for 14 days and then harvested through centrifugation at 10,000 x g for 10 min. The microalgal slurry contained approximately 14 wt% solids. Prior to extraction, the frozen algal slurry was allowed to thaw for 30 min in a hot water bath.

3.2.1 Extraction using lCO_2

A lCO_2 tank was connected to a JASCO PU-980 Intelligent High-Pressure Liquid Chromatography (HPLC) pump. This pump was then connected to a 160 mL continuously stirred tank reactor (CSTR; Parr T316SS stainless steel vessel). A sample of 1.5 g of algal slurry, a magnetic stir bar, and 20 mL of co-solvent, with or without additional modifiers, was placed inside the CSTR. The CSTR was then placed on a stir plate and connected to a back-pressure regulator (BPR; JASCO 880-81). The BPR was used to maintain a constant

pressure throughout all extractions and was set to 150 bar. To collect the microalgal extract, a 125 mL sample collection flask (Erlenmeyer flask) containing acetone, as the collection solvent for the microalgal extract, was placed under the BPR. To initiate the extraction, the lCO_2 tank was turned on and the pump was set to a flow rate of 9 mL/min until the pressure in the system reached 150 bar. Then, the flow rate was set to 2 mL/min for an extraction period of 3 h. After 3 h, the lCO_2 tank was turned off, the remaining lCO_2 in the system was vented by slowly decreasing the pressure on the BPR, and the flask containing the extract was collected. The acetone was evaporated using a rotary evaporator, and the extract was dried overnight in a vacuum oven at 55 °C. The extract yield was determined gravimetrically.

A process flow diagram is shown in Figure 3.1. All extractions were conducted in duplicate at room temperature (20 °C) for approximately 3 h.

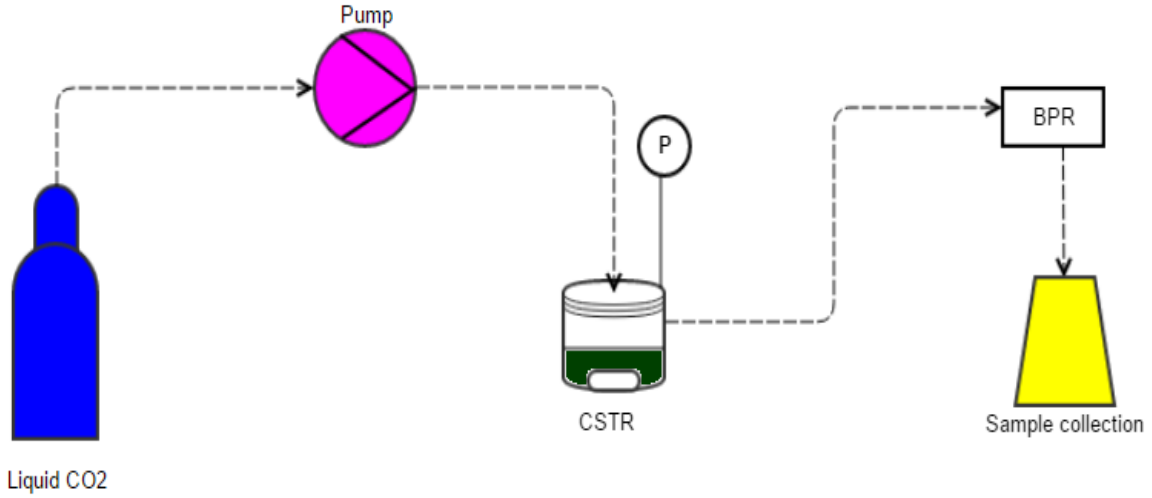


Figure 3.1. Process flow diagram for the extraction of microalgal lipids using lCO_2.

One set of experiments examined the effects of different co-solvents on extract yield. The co-solvents used included: methanol, ethanol, propanol, butanol, or acetone. Further experiments tested the effects of additional modifiers on extract yields obtained

using methanol. Additional modifiers were: 10 µL of NaOH, 10 µL of H_2SO_4, 10% (w/w) of CTAB in water, and 10% (w/w) of DTAB in water.

3.2.2 FAME preparation and analysis

Acid-catalysed methanolysis was carried out to prepare fatty acid methyl esters (FAMEs) from the microalgal extracts. The methanolysis procedure was modified from Lam & Lee.[172] In this method, 50 mg of microalgal extract was placed in a 50 mL round bottom flask along with 11 mg of concentrated sulfuric acid, 1.5 mL of methanol, 1 mL of tetrahydrofuran, and a magnetic stir bar. The round bottom flask was then connected to a condenser. The system was placed on a hot plate at 90 °C for 3 h with continuous stirring. After 3 h, the mixture was neutralized with sodium bicarbonate and FAMEs were extracted with hexanes using a separatory funnel. The extracted FAMEs were analyzed by a Perkin Elmer Clarus 680 gas chromatograph equipped with a flame ionization detector and Thermo Scientific TG-Polar column. The oven temperature was initiated at 50 °C, for 5 min, raised to 260 °C at a rate of 7 °C/min and was held at 260 °C for 5 min. The injector and detector temperatures were 260 °C. Helium was used as the carrier gas. The FAME peaks in the samples were identified by comparing their retention times with those of a standard (Supelco TM 37 component FAME mix, Sigma-Aldrich).

3.3 Results and Discussion

3.3.1 Extractions using lCO_2 and co-solvent

The results of the extractions with lCO_2 and co-solvents are shown in Figure 3.2. The extracts were obtained with the addition of 20 mL of co-solvent: methanol, ethanol, isopropanol, butanol, pentanol, acetone, or none to the microalgal slurry in a CSTR followed by pressurization of the vessel/system using lCO_2. As can be seen, all co-solvents tested (methanol, ethanol, isopropanol, butanol, and acetone) achieved similar extract yields. Since the co-solvents tested exhibited similar efficiencies, methanol was employed as the co-solvent of choice for further experiments because it has the highest polarity (it has been demonstrated that the combination of polar and non-polar solvents typically yield the highest lipid extracts)[47] and the lowest boiling point, so it could be easily evaporated.

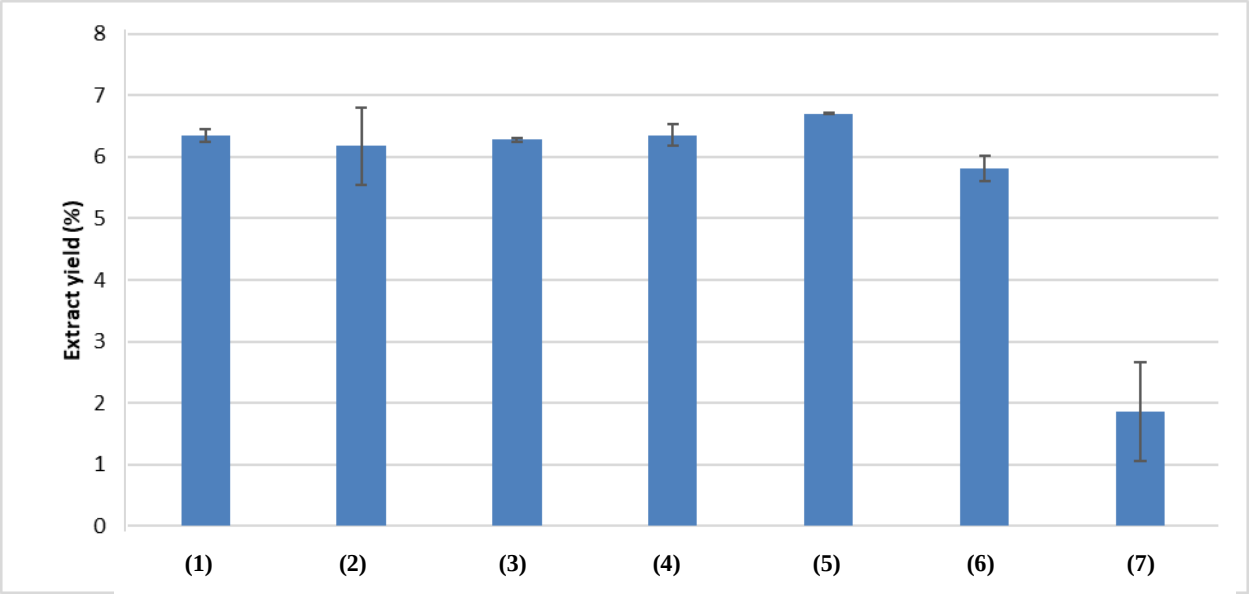


Figure 3.2. Lipid extractions from *C. vulgaris* slurries using lCO_2 and co-solvents. (1) methanol, (2) ethanol, (3) isopropanol, (4) butanol, (5) pentanol, (6) acetone, and (7) no co-solvent. Extract yield (%) is measured on a dry mass basis (dw; g dry extract/g dry algae). All extractions were performed in duplicate (n=2).

3.3.2 Extractions using lCO_2, methanol, and NaOH or H_2SO_4

Results of the lipid extractions from *C. vulgaris* slurries using lCO_2 and other modifiers are shown in Figure 3.3. As can be seen when 10 µL of NaOH was added to the microalgal slurry in the reactor followed by pressurization of the vessel/system using lCO_2 (Figure 3.3(1)), an increase in extract yield was observed (4.7±0.8 % dw) compared to lCO_2 without additional modifiers (1.9±0.8 % dw). The extraction yield using only methanol was 4.3±0.4 % dw. The addition of 20 mL of methanol and 10 µL of NaOH to the microalgal slurry in the reactor followed by pressurization of the vessel/system using lCO_2 further increased the extract yield to 7.4±1.1 % dw, which was the highest yield obtained. Conversely, the use of 10 µL of H_2SO_4 appeared to have a detrimental effect on

extract yield (0.26±0.03 % dw) compared to lCO_2 without additional modifiers (1.9±0.8 % dw).

The addition of 20 mL of methanol and 10 µL of H_2SO_4 to the microalgal slurry in the reactor followed by pressurization of the vessel/system using lCO_2 increased the extract yield (5.3±0.7 % dw) compared to H_2SO_4 (0.26±0.03 % dw) alone, consistent with the trend observed in experiments using NaOH.

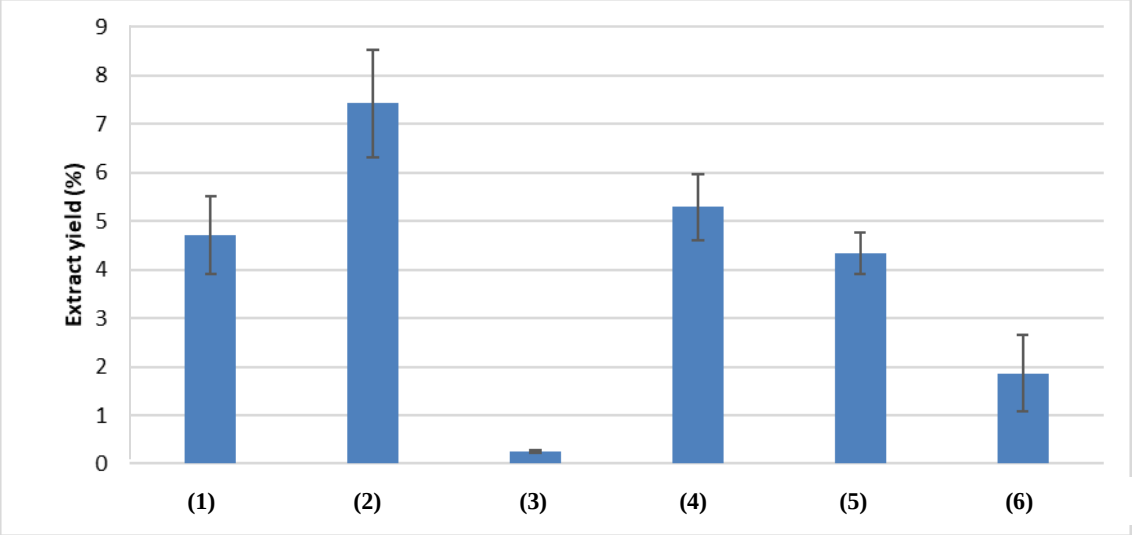


Figure 3.3. Lipid extractions from *C. vulgaris* slurries using lCO_2 and other modifiers. (1) NaOH, (2) NaOH and methanol, (3) H_2SO_4, (4) H_2SO_4 and methanol, (5) methanol, and (6) no modifiers. Extract yield (%) is measured on a dry mass basis (dw; g dry extract/g dry algae input). All extractions were performed in duplicate (n=2).

3.3.3 Extractions using lCO_2 and surfactant

From Figure 3.4, it can be seen that when 10 % (w/w) of DTAB in water was added to the microalgal slurry in the reactor followed by pressurization of the vessel/system using lCO_2, a yield of 13.2±0.5 % dw was achieved, which was more than a two-fold increase

compared to the yield obtained with 10 % (w/w) of CTAB in water (4.9±1.0 % dw). Moreover, the yield of extract obtained with DTAB was more than 4 times that obtained with lCO_2 alone as the solvent (1.9±0.8 % dw). The higher extract yields achieved using DTAB compared to CTAB could likely be attributed to the difference in chain length, critical micelle concentration (CMC), and aggregation number. DTAB is a 12 carbon chain length surfactant with a CMC of 15 mM,[173] while CTAB is a 16 carbon chain length surfactant with a CMC of 0.9 mM.[174] Since CTAB has a longer chain length and lower CMC compared to DTAB, it tends to self-aggregate and form micelles more readily at lower concentrations. CTAB also has a greater aggregation number (the number of surfactant molecules per micelle) of 170 compared to 70 for DTAB.[175] Having a large number of surfactant molecules participating in a micelle implies that there are fewer unbound surfactant molecules present and these surfactant monomers are needed to partition into the bilayer and solubilize the cell wall.[176] Unfortunately, further surfactant experiments could not be performed due to a limited supply of microalgal slurry. It would be suggested that future work examine various ionic surfactants, both cationic and anionic, taking into account their CMCs and aggregation numbers, to determine their effect on lipid extraction.

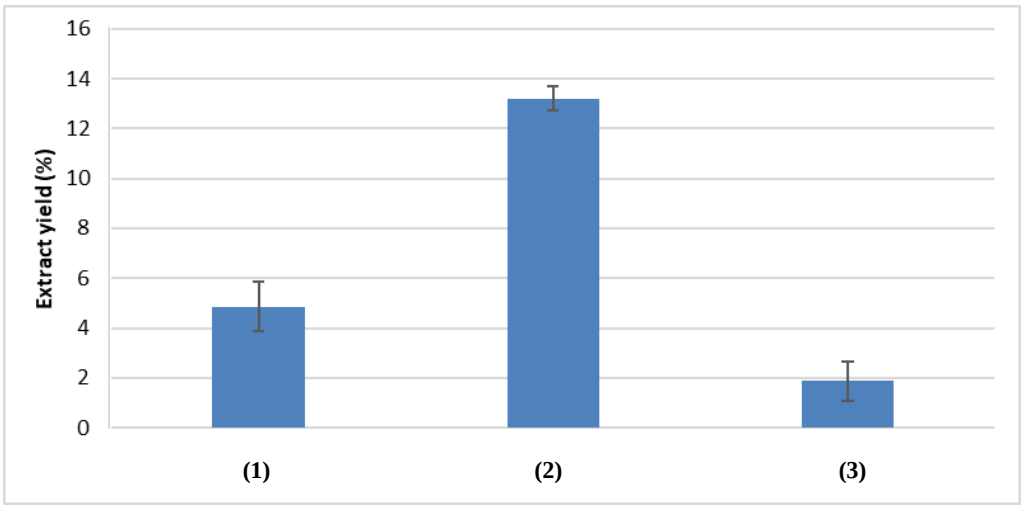


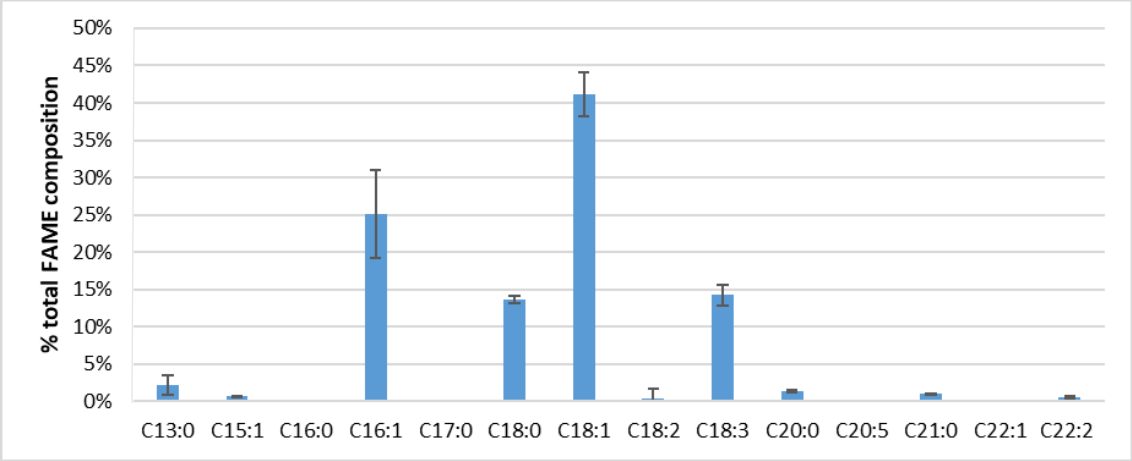
Figure 3.4. Lipid extractions from *C. vulgaris* slurries using lCO_2 and surfactants. (1) CTAB, (2) DTAB, and (3) no surfactant. Extract yield (%) is measured on a dry mass basis (dw; g dry extract/g dry algae input). All extractions were performed in duplicate (n=2).

3.3.4 FAME analysis of extractions using lCO_2, methanol, and NaOH or H_2SO_4

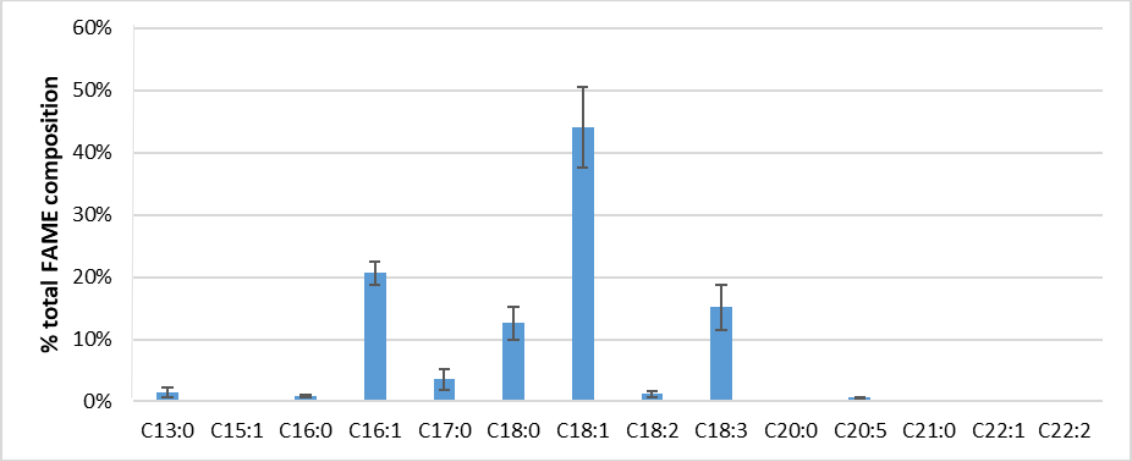
FAME profiles of *C. vulgaris* extracts using lCO_2, methanol, and NaOH or H_2SO_4 are shown in Figure 3.5. According to a study by Knothe,[177] the most desirable FAMEs for high-quality biodiesel production would include the 16 and 18 carbon chain lengths. These chain lengths lead to cetane numbers (47 minimum), viscosities (1.9-6.0 mm^2/s at 40 °C), and oxidative stabilities (3 minimum), which are within the ideal range as stated by ASTM D6751 (standard specifications for biodiesel). The FAME profiles for the extractions with NaOH (Figure 3.5a) and NaOH and methanol as modifiers and co-solvent (Figure 3.5b) were relatively similar, which was expected since the same modifier was employed in both extractions. From Figure 3.5c, it can be seen that, with the addition of H_2SO_4, the only biodiesel-desirable FAME produced was C18:1 with a total FAME composition of 29.6±3.3 %. The addition of H_2SO_4 and methanol (Figure 3.5d) yielded a similar FAME

profile, however, C16:0 and C16:1 were also present, which would render it more desirable for biodiesel production. Figure 3.5e (methanol alone) exhibited a similar FAME profile to H_2SO_4 and methanol (Figure 3.5d) with the exception of the presence of C18:0. Lastly, it can be noted that when no additional modifiers were employed (Figure 3.6f), a FAME composition consisting of mainly C13:0 with the presence of some C18:0, C18:1, and C18:3. The relative proportions of these FAMEs varied depending on the additional modifier with or without the presence of a co-solvent. Based on the FAME profiles, *C. vulgaris* would be considered a desirable feedstock for the production of biodiesel.

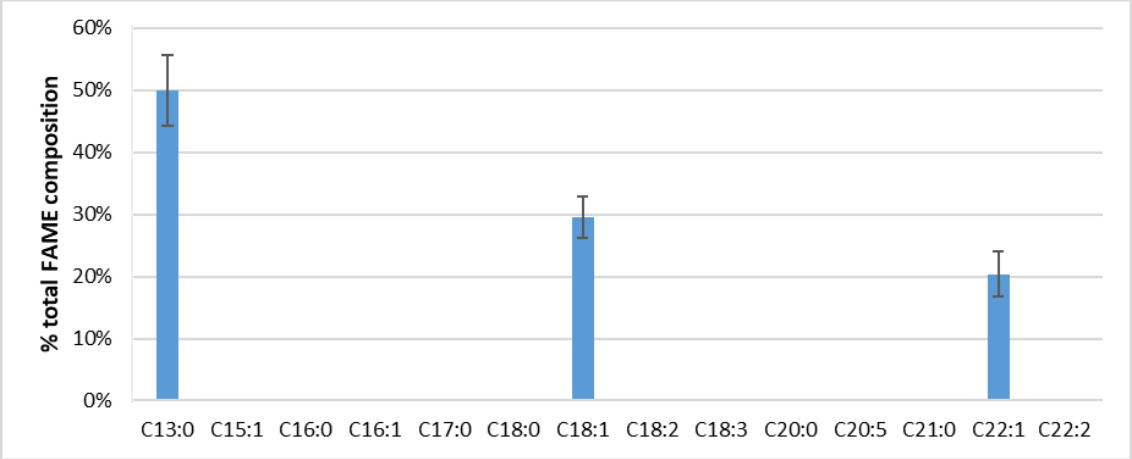
a)



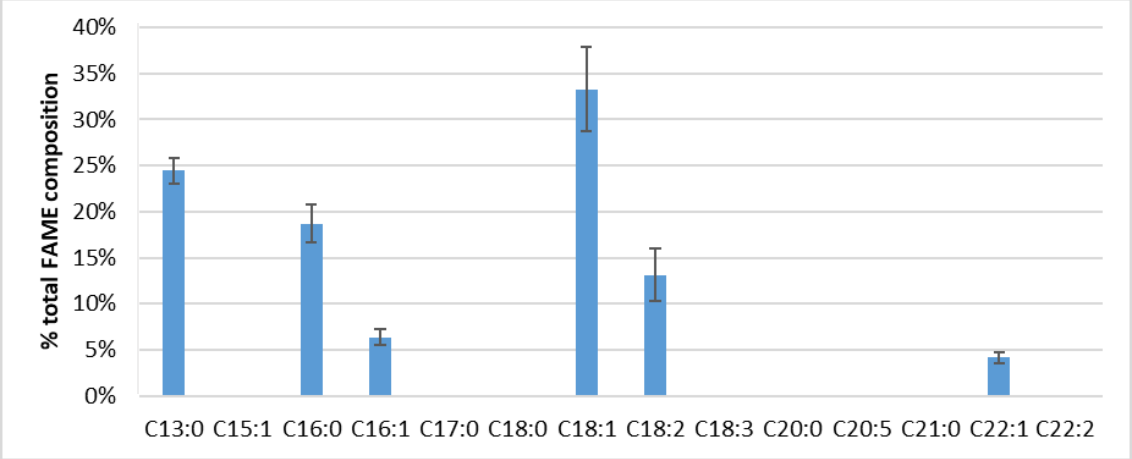
b)


% total FAME composition
60%
50%
40%
30%
20%
10%
0%
C13:0 C15:1 C16:0 C16:1 C17:0 C18:0 C18:1 C18:2 C18:3 C20:0 C20:5 C21:0 C22:1 C22:2

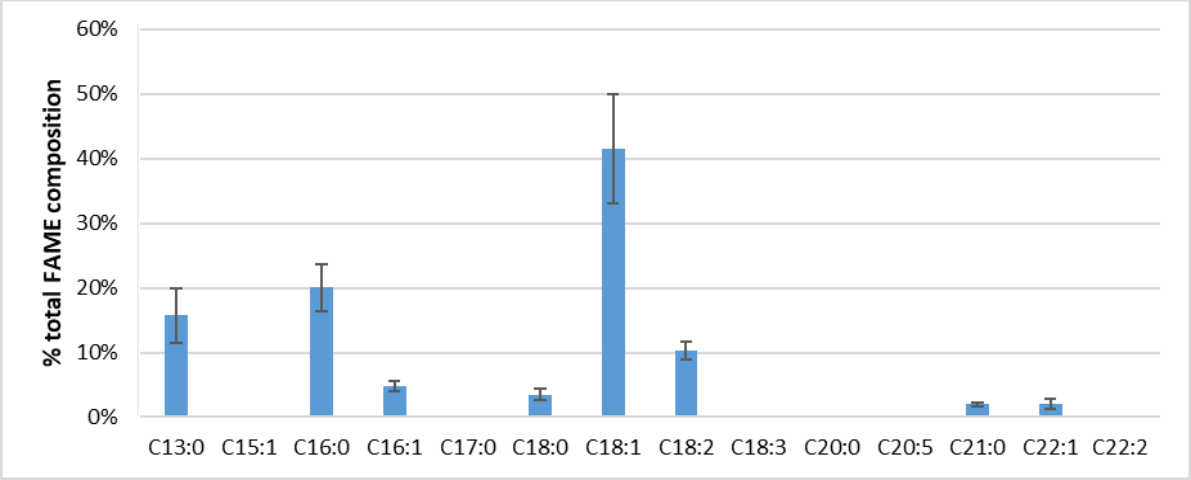
c)


% total FAME composition
60%
50%
40%
30%
20%
10%
0%
C13:0 C15:1 C16:0 C16:1 C17:0 C18:0 C18:1 C18:2 C18:3 C20:0 C20:5 C21:0 C22:1 C22:2

d)


% total FAME composition
40%
35%
30%
25%
20%
15%
10%
5%
0%
C13:0 C15:1 C16:0 C16:1 C17:0 C18:0 C18:1 C18:2 C18:3 C20:0 C20:5 C21:0 C22:1 C22:2

e)



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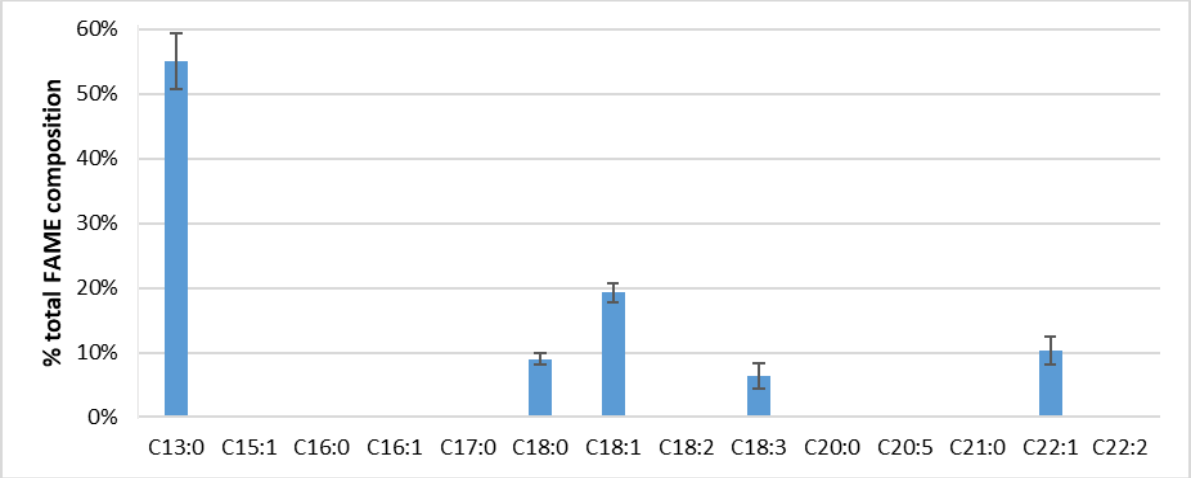

Figure 3.5. FAME profiles of extracts of *C. vulgaris* upon extraction using lCO_2 and other modifiers. a) NaOH, b) NaOH and methanol, c) H_2SO_4, d) H_2SO_4 and methanol, e) methanol, and f) no modifiers.

3.4 Conclusions

Lipid extractions from *C. vulgaris* slurries were conducted using lCO_2, co-solvents, and/or additional modifiers in the form of an acid, base, or surfactant. All co-solvents tested were able to facilitate NL, FFA, and other materials from *C. vulgaris* during the extraction.

Based on the results of the extractions performed using co-solvents/modifiers, the combination of NaOH and methanol showed the most promising extract yield; however, further experiments should be conducted to determine whether these results were statistically significant. From the modifiers tested in this study, DTAB produced the highest extract yields (13.2±0.5 % dw). However, this study represented a proof of concept and it would be suggested that other surfactants also be investigated. The FAME composition was found to vary for each of the extraction approaches, indicating that different modifiers may target some FAMEs over others. Moreover, *C. vulgaris* could be employed as a valuable feedstock for biodiesel production based on the presence of C16 and C18 carbon chain lengths noted in the FAME profiles.

Chapter 4

TRANSESTERIFICATION OF SOYBEAN OIL USING A SWITCHABLE-HYDROPHILICITY SOLVENT, 2-(DIBUTYLAMINO)ETHANOL

4.1 Introduction

Negative environmental effects, such as the emission of greenhouse gases, caused by non-renewable fossil fuels, have prompted the search for renewable alternatives.[178] Biodiesel has emerged as a renewable, biodegradable, and environmentally friendly replacement for some of these fossil-derived fuels.[76] Biodiesel is made up of long chain fatty acid methyl esters (FAMEs), which are typically produced from animal fats or vegetable oils. Soybean oil has gained much attention in recent years as a potential renewable feedstock for biodiesel production.[179]

Currently, most biodiesel is produced by transesterification/esterification of triacylglycerols (TAGs) and free FAMEs using homogeneous basic catalysts, such as sodium or potassium hydroxides, carbonates or alkoxides and an alcohol.[180,181] Martins et al. examined the transesterification of soybean oil to biodiesel using hydrotalcite, a basic catalyst, and they reported a promising FAME conversion of 94.8 %, obtained using a methanol to oil ratio of 20:1, 5.0 % (w/w) hydrotalcite catalyst, and a reaction time of 10 h at 64 °C.[182] Li et al. studied the use of potassium hydroxide in the transesterification of soybean oil and noted that a yield of 96 % of FAMEs was possible using a methanol to oil ratio of 4.5:1, 1.0 % (w/w) KOH, and a reaction time of 2 h at 45 °C.[183] Another study by

Liu et al. investigated the use of calcium oxide as a basic catalyst for the conversion of soybean oil to biodiesel.[184] With a methanol to oil ratio of 12:1, 8 % (w/w) CaO catalyst, and a reaction time of 3 h at 65 °C, FAME yields exceeding 95 % were achieved.[184] However, the use of some homogeneous basic catalysts for transesterification is expensive and energy intensive because several washing steps to remove the catalyst from the products are required, resulting in a substantial consumption of water and a significant generation of waste.[185] Moreover, the presence of an alkali metal can produce large amounts of soap by-product, posing significant downstream processing challenges, including poor product separation and decreased yield.[186,187] Methanol is the alcohol of choice for the production of biodiesel because of its low cost and industrial availability.[188] There is a pressing need to develop novel processes for solvent removal from a hydrophobic product without the use of distillation. One recently proposed approach involves the use of switchable solvents.[143]

Switchable solvents are liquids that can be reversibly converted from one form to another, where the two forms differ in their physical properties.[189] A switchable-hydrophilicity solvent (SHS) is a switchable solvent that typically has very little miscibility with water, but can become completely miscible with water when an atmosphere of CO_2 is bubbled through the solution.[143,144] An SHS generally consists of a liquid amine that, when an atmosphere of CO_2 is bubbled through the liquid in the presence of water, becomes a water-soluble bicarbonate salt. A solution of CO_2 dissolved in water is also referred to as carbonated water. An example of an SHS is 2-(dibutylamino)ethanol (2-DBAE), a tertiary amine (Figure 4.1). CO_2 is preferred as the trigger for the switching process because it is essentially non-toxic, benign, inexpensive and easily removed.[169]

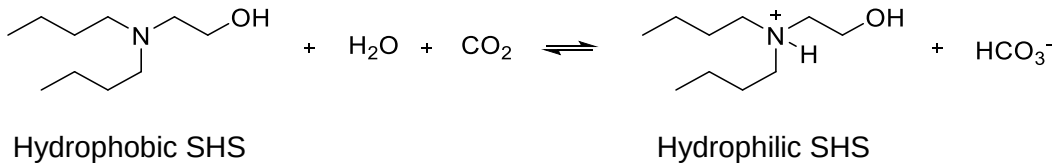

Figure 4.1. Protonation of 2-DBAE in the presence of CO_2 and water.

Potential applications for SHSs include the rapid removal of the solvent from products, such as soybean oil,[143] algae oil,[70,190] bitumen,[191] and polystyrene powder[144] without distillation. They could also extend to the transesterification of triglycerides from soybean oil due to their hydrophobic nature. Because SHSs can be removed from products by bubbling with CO_2 in the presence of water, rather than distillation, there is no need for SHSs to be volatile. This approach allows for transesterification using an SHS without energy intensive distillation of solvents.

The SHS used in this research was selected based on the design and evaluation of various SHSs done by Vanderveen et al.[192] This study reported 13 new secondary and tertiary amine SHSs, whereby most of them were commercially available and were more easily prepared compared to earlier generations of SHSs, which were expensive to manufacture and had health and safety concerns associated with them.[143,144] 2-DBAE was one of the SHSs formulated that exhibited switchable behaviour and minimal health and safety concerns with a log K_{ow} of 2.20 and a pK_{aH} of 9.67.[192]

In the current study, soybean oil was converted to FAMEs in the presence of a hydrophobic solvent, 2-DBAE, and a transesterifying agent, methanol. Methanol was used to methylate the TAGs in soybean oil. The FAMEs produced were separated from 2-DBAE and water by bubbling CO_2 into the system in the presence of water. The SHS was recovered from water by subsequent bubbling with Ar (releasing CO_2). The recyclability

of 2-DBAE was analyzed volumetrically. The residual 2-DBAE was assumed to be with the FAMEs. The yield of FAMEs was calculated gravimetrically. The carbon chain lengths of the FAMEs were analyzed using a gas chromatograph equipped with a flame ionization detector (GC-FID). Figure 4.2 displays a flow diagram of the proposed process.

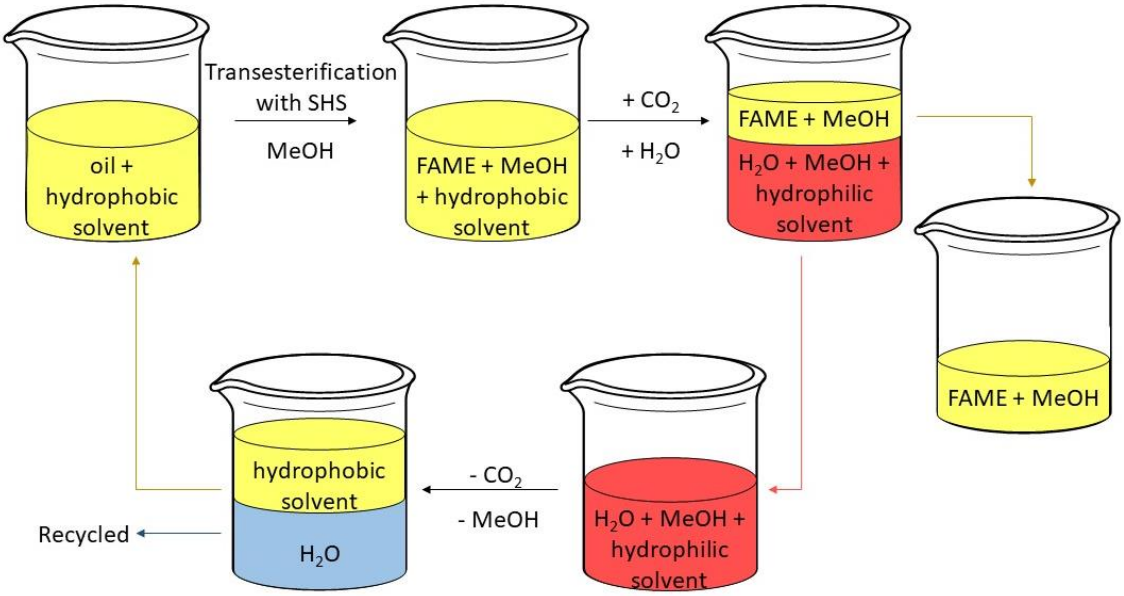

Figure 4.2. Method for the transesterification of soybean oil using an SHS, 2-DBAE. This schematic illustrates one complete cycle.

4.2 Materials and Methods

All chemicals were used as received. 2-DBAE (99%) was obtained from Sigma-Aldrich. Carbon dioxide (4.0 grade, 99.99%) and argon gas (>99.998%) were obtained from Praxair. Methanol HPLC grade (>99.9%) was obtained from Sigma-Aldrich. Distilled water was used in all reactions. Soybean oil was of PC brand and purchased from Loblaws. GC-FID was used to analyze the FAMEs produced and ^{1}H NMR was used to quantify any residual 2-DBAE remaining with the FAMEs.

4.2.1 Transesterification of soybean oil to FAMEs

Soybean oil (25 g), methanol (25 mL), and 2-DBAE (50 mL) were added to a round bottom flask with a magnetic stir bar. This round bottom flask containing the mixture was then connected to a condenser and placed on a stirring hot plate. The reaction took place at 90 °C for 24 h under reflux. Approximately 25 mL of distilled water was added to this mixture and was then bubbled with CO_2 at 25 °C under 1 bar for 2 h, causing the 2-DBAE to transition from being hydrophobic to hydrophilic. FAMEs were isolated using a separatory funnel and were washed again with 25 mL of fresh distilled water and further carbon dioxide bubbling for 2 h. Excess methanol was removed from the FAMEs using rotary evaporation at 55 °C and the FAMEs were isolated and recovered for analysis. These experiments were repeated over a period of 5 cycles and performed in duplicate. The yield of FAMEs was calculated in two steps:

(1) $Mass\ of\ FAMEs\ (g) = Volume\ of\ FAMEs\ (mL) \times Density\ of\ FAMEs\ (\frac{g}{mL})$

whereby the volume of FAMEs was recorded after each cycle and the density of methyl linolenate (C18:3; the primary FAME in Figure 4.4) is defined as 0.895 g/mL at 25 °C.[193]

(2) $Yield\ of\ FAMEs\ (\%) = \frac{Mass\ of\ FAMEs\ (g)}{Mass\ of\ soybean\ oil\ (g)} \times 100$

4.2.2 Recyclability of 2-DBAE

Excess methanol was removed from the water, 2-DBAE, and methanol layer from the experiment section in 4.2.1 using rotary evaporation at 55 °C. Then, the solution of water and 2-DBAE was bubbled with Ar at 70 °C under 1 bar for 2 h to remove CO_2 from

the mixture, causing the 2-DBAE to become hydrophobic again, allowing the 2-DBAE to separate from water. 2-DBAE was isolated from the water using a Pasteur pipette, analyzed based on the original volume used, and placed in vials for further experiments. Water was also stored for subsequent steps. The water used for the first and second CO_2 bubbling steps was recycled and topped back up to 25 mL if needed. These experiments were repeated over a period of 5 cycles and performed in duplicate.

4.2.3 Quantification/Analysis of FAME

The FAMEs were analyzed by a Perkin Elmer Clarus 680 GC-FID and Thermo Scientific TG-Polar column. The oven temperature was initiated at 50 °C, for 5 min, raised to 260 °C at a rate of 7 °C/min and was held at 260 °C for 5 min. The injector and detector temperatures were 260 °C. Helium was used as the carrier gas. The FAME peaks in the samples were identified by comparing their retention times with those of the standards (Supelco TM 37 component FAME mixture, Sigma-Aldrich Co.)

4.3 Results and Discussion

4.3.1 Transesterification of soybean oil to FAMEs using 2-DBAE

The transesterification of soybean oil to FAMEs using 2-DBAE was investigated and this technique generated consistently high yields of FAMEs. The yield of FAMEs over a period of 5 cycles is shown in Figure 4.3, with a cycle being defined as the entire process

as depicted in Figure 4.2. The FAME yield ranged from 80-85 % of the original mass of oil after each cycle for 5 cycles.

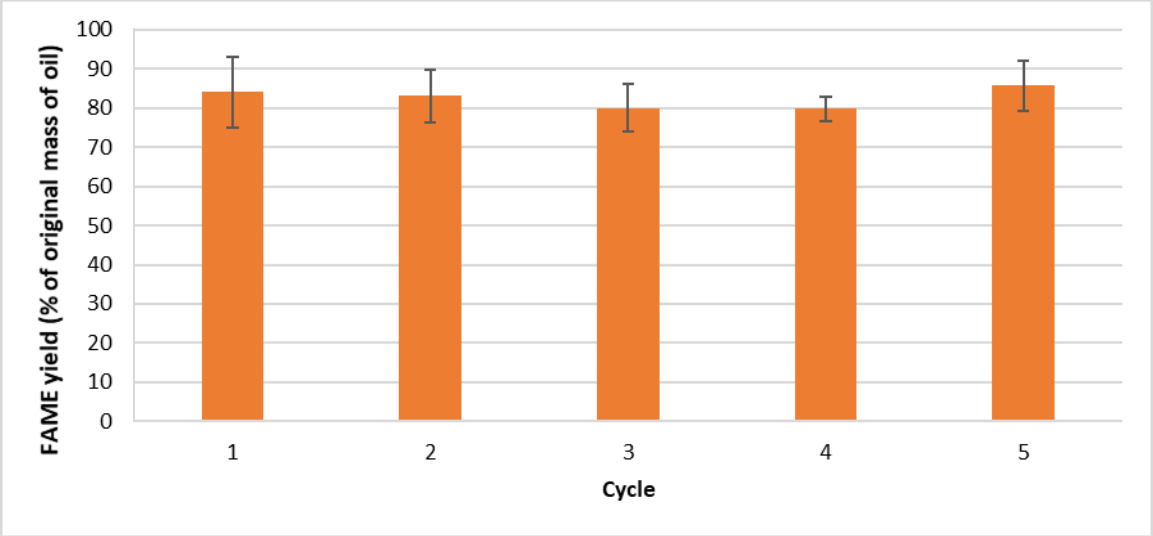

Figure 4.3. FAME yield (% of the original mass of oil) after each cycle for 5 cycles. A cycle consisted of the transesterification of soybean oil to FAMEs at 90 °C and isolation of FAMEs from 2-DBAE by bubbling with 1 bar of CO_2 in the presence of water at 25 °C.

4.3.2 FAME composition

The composition of FAMEs was measured at the end of the 5 cycles and the average standard deviation is displayed in Figure 4.4. The FAME composition ranged from C4-C24 and was calculated based on a ratio of each type of fatty acid over the total fatty acids found in the individual sample. Different biodiesel properties are highly dependent on the type of FAMEs recovered. Polyunsaturated fatty acids (PUFAs) were predominant with a total percentage of 90.9±0.8 % and the most abundant FAMEs were of C18, C20, and C24 chain lengths. Normally, high levels of some PUFAs are not favourable for biodiesel production because they contain double bonds that do not biodegrade quickly.[194] However,

some PUFAs, such as C18:2 and C18:3, representing 6.8±0.04 % and 83.6±2.6 % of total FAMEs, respectively, are omega-6 fatty acids. C18:2 and C18:3 are particularly useful for biodiesel production because they can biodegrade more quickly than other PUFAs, but not quickly enough that biodiesel would begin to degrade a week after putting it in an engine. Knothe[177] reported that the most desirable fatty acids for biodiesel quality were of C16 and C18, which made up the majority of soybean oil composition according to Figure 4.4. The remainder of the FAME composition consists of saturated fatty acids, with a total percentage of 7.5 %. Since soybean oil mainly consists of C18, it would represent an ideal feedstock for the production of biodiesel.[177]

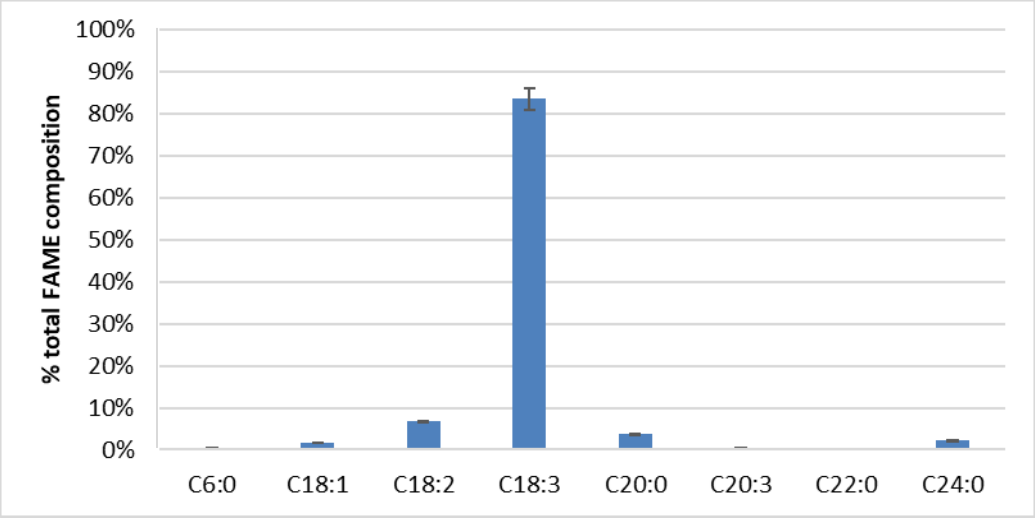

Figure 4.4. FAME profile of soybean oil upon transesterification using 2-DBAE.

4.3.3 Recyclability of 2-DBAE from the hydrophilic layer

The recyclability of 2-DBAE was assessed over a period of 5 cycles (Figure 4.5). After 5 cycles, the percentage of solvent recovered was roughly 65-90 % of the original volume of SHS used. The decrease in recovered 2-DBAE after each cycle suggests that

some 2-DBAE did not protonate upon initial CO_2 bubbling. Instead, the remaining 2-DBAE can be found with the FAMEs as well as in the water phase upon deprotonating the hydrophilic solvent using Ar. In Figure 4.5, the amount of 2-DBAE residing with the FAMEs after a carbonated water wash was 10-35 % of the original volume. Since the water is recycled for subsequent cycles, the 2-DBAE that remains with the water will also be recycled. Overall, 2-DBAE displayed good recyclability when bubbled with argon at 70 °C for 2 h. However, further optimization should be attempted to minimize the 2-DBAE with FAMEs before this technology could be implemented on an industrial scale.

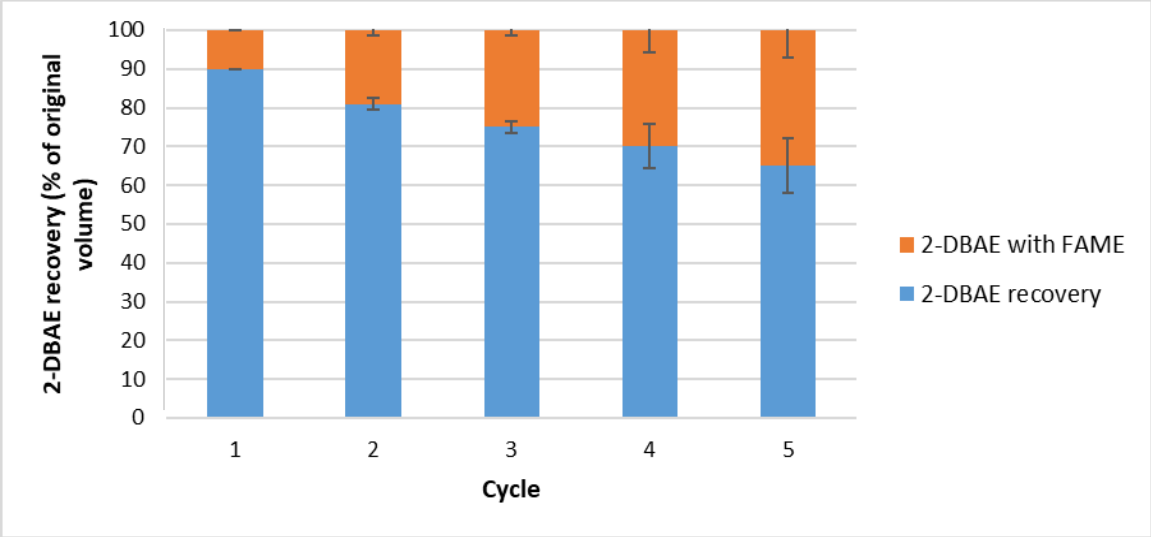

Figure 4.5. Cumulative 2-DBAE recovery (% of the original volume) over a period of 5 cycles. An increasing amount of 2-DBAE remained with the FAMEs upon exposure to CO_2 in the presence of water.

4.4 Conclusions

2-DBAE was shown to be an effective solvent for the transesterification of soybean oil to FAMEs. FAMEs were recovered from the FAME layer efficiently with yields of 80-85 % of the original mass of oil, respectively. Additionally, 10-35 % of the original volume

of 2-DBAE that resided with the FAMEs was recovered. The cumulative recyclability of 2-DBAE was 65-90 % of the original volume. FAMEs produced from soybean oil mainly consisted of C18:3, which is very useful for biodiesel production. SHSs can be removed from the FAMEs using carbonated water, eliminating the need for distillation. The use of an SHS does not require flammable, highly volatile, or chlorinated solvents, which is highly preferable compared to the use of conventional solvents. Moreover, it does not produce soap that is generated using basic catalysts. The amount of SHS remaining in the FAME layer would need to be reduced to improve its recyclability and before this approach could be employed at a commercial scale. Two ways to improve this method is to choose a more hydrophilic SHS ($\log K_{ow} < 2.20$) and a more basic SHS ($pKa_H > 9.67$). As a result, the SHS would be protonated more readily upon exposure to CO_2 in the presence of water and therefore, separate from the FAMEs more effectively. Moreover, diamine SHSs could be used as they have been shown to separate better from oil than monoamine SHSs.[195]

Chapter 5

CELL DISRUPTION PRIOR TO LIPID EXTRACTION FROM *SCENEDESMUS SP.* SLURRIES USING LIQUID CO_2 AND METHANOL

5.1 Introduction

Society has become increasingly dependent on fossil fuels for production of transportation energy and electricity generation. However, fossil fuels are non-renewable, depleting, and their combustion leads to greenhouse gas (GHG) emissions, especially CO_2, that contribute significantly towards global warming.[165] Biodiesel has recently emerged as a greener and more sustainable alternative of its fossil fuel-derived counterpart, where advantages include its lower toxic emissions of CO, SO_x, and particulate matter, its biodegradability, and its renewability.[11] Importantly, the combustion of biodiesel releases similar amounts of CO_2 to comparable fossil fuels; however, the CO_2 is consumed in the growth of the biomass, which reduces the net CO_2 emissions.

There are several feedstocks that could be employed to produce biofuel. First-generation biofuels are derived from edible crops as a feedstock, while second-generation biofuels are derived from non-edible crops.[196] However, the escalating demand for edible feedstocks as food sources, in addition to the finite availability of arable land for the cultivation of edible and non-edible feedstocks, makes first and second-generation biofuel production unsustainable in the long term. In the last decade, third-generation biofuels,

such as those derived from algae, have emerged as a possible alternative to the rising global demands for transport fuels.[32]

Microalgae represent an ideal feedstock for the production of renewable biodiesel because of their rapid growth rates, high productivities, and high intracellular lipid contents.[40] Moreover, they can be grown on marginal or infertile lands, thereby avoiding competition with the production of agricultural food crops, and can be grown in wastewater, providing nutrient (nitrogen and phosphorus) removal, thereby diminishing environmental pollution.[168] Overall, biodiesel produced from microalgae has been widely considered to be one of the most sustainable alternatives to fossil fuel-derived diesel. It is a viable means for energy security and environmental and economic sustainability.[44]

One of the major challenges in achieving techno-economic viability remains the efficient extraction of the desirable lipids from the microalgal cells due to the rigidity of their cell walls. These walls are made up of a polysaccharide and glycoprotein matrix, providing the cells with a formidable defense against the environment.[68] For the extraction from dried microalgae, efficient extraction of desirable lipids does not pose a problem because the drying process breaks down the cell walls. However, microalgal slurries still have intact cell walls and therefore the extraction of desirable lipids is not as efficient. This makes extracting industrially useful quantities of lipids from microalgae difficult, requiring the use of energy intensive extraction or drying techniques, leading to higher economic and environmental costs. Some of the main extraction techniques employed in the recovery of microalgal lipids are conventional organic solvent extraction, Soxhlet extraction, and supercritical CO_2 ($scCO_2$).[69] The environmental and economic costs of conventional organic solvent extraction can be minimized by choosing a solvent or co-solvent that is

inexpensive, non-toxic and easily removable. Current methods often use flammable and/or chlorinated solvents, such as n-hexane or a chloroform/methanol mixture, which increase risks associated with fire, acute toxicity, neurotoxicity, and carcinogenicity.[70] Moreover, the use of these solvents is associated with a decreased selectivity in the extraction of lipids and free fatty acids. Soxhlet extraction typically uses a 2:1 v/v mixture of chloroform and methanol, a temperature of 80 °C, and is conducted for 24 hours.[70] This technique extracts the most lipids, but is not economically viable because of the high-energy consumption and the environmental concerns associated with the use of chlorinated solvents. In addition, it exhibits poor selectivity for the desirable nonpolar lipids and free fatty acids. Supercritical CO_2 extraction, which is usually conducted at temperatures ranging from 50-80 °C and pressures of 200-300 bar for 80 minutes, is considered to be a promising and greener extraction approach.[72] It offers advantages compared to Soxhlet extraction and conventional organic solvent extraction, including higher selectivity for neutral lipids (NLs) and shorter extraction times, while avoiding the need for halogenated organic solvents.[47,111] However, this technique does require high pressures to obtain yields comparable to liquid conventional organic solvent extractions. As such, the economical production of biodiesel from microalgal lipids is strongly limited by the energy and capital costs associated with the lipid extraction approaches currently available.

Liquid CO_2 (lCO_2) has emerged as an innovative extraction technique that offers many of the same advantages as $scCO_2$, but at a lower pressure (150 bar) and temperature (25 °C), thereby reducing the energy costs.[99] Since lCO_2 has a similar polarity to $scCO_2$, it could exhibit comparable selectivity towards NLs, and which would be higher than for most organic solvents.

Obtaining complete access to intracellular microalgal lipids using lCO_2 extraction continues to present a significant challenge. For this reason, cell disruption prior to extraction has been considered as a means to enhance microalgal lipid extraction. Mechanical cell disruption methods include bead milling, high-pressure homogenization, ultrasonication, autoclaving, freeze-drying, and microwave radiation. Non-mechanical methods involve lysing the microalgal cells with acids, alkalis, enzymes, or osmotic shock.[47] Ultrasonication, involving the disruption of cell walls by cavitation, along with a 2:1 v/v mixture of chloroform:methanol was shown to extract 19 % dw from *C. pyrenoidosa*.[152] Freeze-drying, involving the removal of water from frozen microalgae, could disrupt *Botryococcus braunii* cells effectively and, with an extraction using lCO_2, could achieve a lipid yield of 19 % dw.[99] Microwave radiation, involving rapid oscillation of water molecules causing friction and heating, using ethanol and hexane as extraction solvents was conducted on *Nannochloropsis oculta*.[151] It was shown that, under optimal conditions, the addition of microwave treatment to a biofuel production process contributed <1 % of energy expenses, while it increased lipid extraction 3-fold compared to a control. Osmotic shock, involving rapid changes in solute concentration, along with both polar and non-polar organic solvents was noted to increase lipid recovery approximately 2-fold when performed on *Chlamydomonas reinhardtii* slurries.[197] Moreover, it was shown that cooling microalgal suspensions below their growth temperatures affected the composition of fatty acids in the cell membrane and led to cell disruption.[198–200]

In this study, mechanical and chemical cell disruption techniques, such as ultrasonication, microwave radiation, grinding with liquid nitrogen, switchable osmotic shock, cooling, and freeze-drying, were investigated prior to the extraction of lipids from

Scenedesmus sp. slurries using lCO_2. The switchable osmotic shock method was unconventional and was a test of a switchable osmotic shock agent, N,N,N',N'-tetramethyl-1,4-butanediamine (TMBDA), rather than a test of the more conventional osmotic shock method using NaCl.[201] A switchable osmotic shock agent can reversibly change from an organic solvent to a salt in the presence of carbonated water. This salt is used to create a super-concentrated brine solution that draws water out of the microalgal cells. Using a conventional salt, there are substantial downstream costs associated with separation of the salt from water, whereas a switchable salt can revert back to its organic solvent form in the presence of Ar or air without the need for separation. This study will provide a comparative analysis into which technique can provide the most cell disruption, and hence, release the most available lipids overall for downstream biodiesel production. Also, FAME profiles of *Scenedesmus sp.* lipid extracts, when subjected to each of the cell disruption techniques, were characterized to quantify the production of biodiesel-desirable chain lengths.

5.2 Materials and methods

All chemicals were used as received from the suppliers. Carbon dioxide (99.99%) was obtained from Praxair. Methanol of HPLC grade (>99.9%) was obtained from Fisher Scientific. A wet sample of microalgae species *Scenedesmus sp.* was obtained from the National Research Council (NRC), Halifax, Canada and was kept frozen at -81 °C to allow for the extended storage of the microalgal biomass without degradation and to minimize cell disruption prior to extraction by flash-freezing.

5.2.1 Cell disruption methods

For each cell disruption technique, except for freeze-drying, 10 g of microalgal slurry aliquots (20 wt% solids) were used and all experiments were conducted under the conditions specified in Table 5.1. For freeze-drying, 2 g of dry mass was used. Experiments were conducted in duplicate and a control, without cell disruption, was also performed in duplicate. Prior to cell disruption, the frozen algal slurry was allowed to thaw for 30 min in a hot water bath.

5.2.1.1 Ultrasonication

Algal slurry (20 wt%) aliquots were mixed with 20 mL of methanol in a 100 mL beaker and sonicated using a sonicator probe (Fischer Scientific Sonic Dismembrator Model 500) at amplitudes of 30 % or 60 % for 15 min.

5.2.1.2 Microwave radiation

Algal slurry (20 wt%) aliquots were mixed with 10 mL of methanol or distilled water in a 50 mL round bottom flask and subjected to microwave radiation (CEM Corporation Discover LabMate) at 300 W for 15 s followed by 15 min of cooling under ambient laboratory conditions (20 °C, 1 atm). This procedure was repeated 3 times.

5.2.1.3 Grinding following freezing with liquid N_2

Algal slurry (20 wt%) aliquots were placed in a mortar and subjected to liquid N_2 until frozen. A pestle was then used to grind the frozen biomass for 2 min.

5.2.1.4 Switchable osmotic shock

Algal slurry (20 wt%) aliquots were subjected to 20 mL of 60 % v/v mixture of TMBDA in distilled water in a 100 mL beaker. This mixture was then subjected to a carbonated water wash at ambient laboratory conditions (20 °C, 1 atm) using a gas dispersion tube for 2 h, and then placed on a stir plate overnight. TMBDA is a switchable salt, which means that in the presence of carbonated water, it will transform from an organic solvent to a salt. This high concentration of solute outside the algal cells has been reported to cause the rupture of the cell walls.[197]

5.2.1.5 Cooling

Algal slurry (20 wt%) aliquots contained in 20 mL plastic vials were placed in a refrigerator (4 °C) to remain cool and in the dark overnight.

5.2.1.6 Freeze-drying

Algal slurry (20 wt% solids) aliquots contained in 20 mL plastic vials were frozen in liquid N_2 and freeze-dried overnight (12-15 h) using a ThermoSavant ModulyoD freeze-dryer. The freeze-dried powder was stored under ambient laboratory conditions (20 °C, 1 atm).

5.2.2 Extraction using lCO_2

A lCO_2 tank was connected to a JASCO PU-980 Intelligent HPLC pump and the flow rate was maintained at 2 mL/min. This pump was then connected to a 160 mL CSTR (Parr T316SS stainless steel vessel), which contained the cell-disrupted algal slurry, a magnetic stir bar, and 20 mL of methanol. A BPR was attached to the CSTR to maintain a constant pressure at 150 bar throughout the extraction. In a typical extraction using lCO_2, the biomass was contacted with methanol in the CSTR for 3 h and from this point, the microalgal extract was collected by allowing it to pass through the BPR and into a sample collection flask. A process schematic is shown in Figure 5.1. All extractions were conducted in duplicate at room temperature (20 °C) for approximately 3 h.

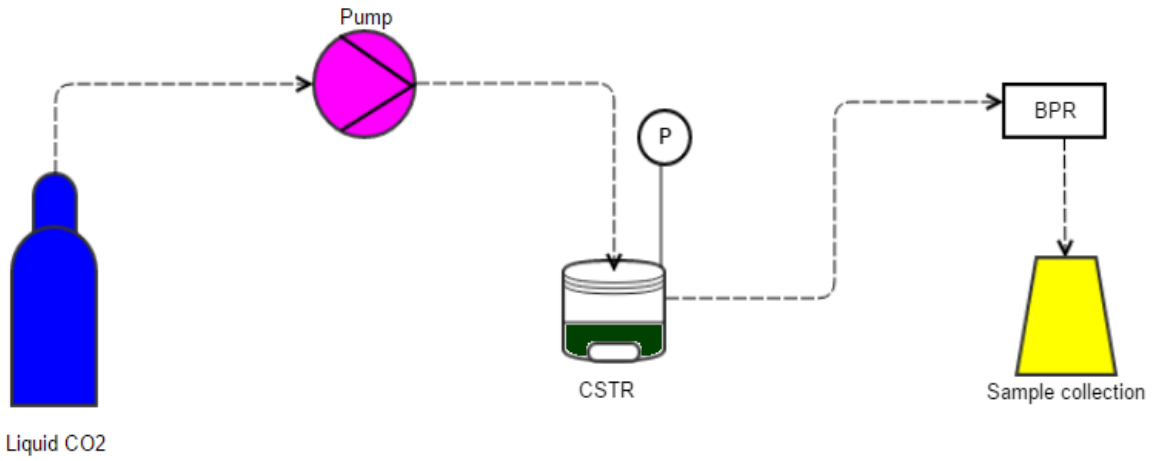

Figure 5.1. Process flow diagram for the extraction of microalgal lipids using lCO_2.

5.2.3 Solid phase extraction (SPE)

Microalgal extract obtained from the extractions using lCO_2 was fractionated using SupelcleanTM LC-NH_2 (500 mg) cartridges. A cartridge was placed on a vacuum manifold and 6 mL of hexane was allowed to seep through. An aliquot (25 mg) of extract was diluted in 1 mL of hexane and placed on the preconditioned cartridge without vacuum. The cartridge was then eluted with three different solvent systems and 3 individual sample fractions were collected. Fraction A collected NLs using 5 mL of 17:3 (v/v) hexanes:ethyl acetate. Fraction B collected the chlorophyll, pigments, and other extracted constituents considered not to be useful in the production of biodiesel, using 4 mL of 23:1 (v/v) chloroform:methanol. Fraction C collected FFAs using 3 mL of 46:1 (v/v) diethyl ether:acetic acid. The fractions were placed on a rotary evaporator to remove residual solvent and allowed to air-dry for 3 h. The weight of each of the three fractions was measured gravimetrically, using a modified procedure from Bodennec et al.[202] Moreover,

Fraction D consisted of the remaining extract in the cartridge and was added to Fraction B. SPEs were performed on the extracts obtained from Soxhlet extraction.

5.2.4 Soxhlet extraction

Based on a modified procedure reported by Paudel et al.,[99] duplicate Soxhlet extractions were performed on frozen microalgae and were employed as a reference (indicating the total available NL and FFA) to assess the efficiency of the lCO_2 extraction with and without cell disruption. Moreover, duplicate Soxhlet extractions were performed on the fresh microalgae to determine whether there is comparable difference between the lipid extractions or their resulting FAME profiles obtained from the fresh and frozen microalgal slurries. In this procedure, 250 mg of oven-dried algal biomass (95 wt% solids) was placed in a glass thimble and extracted using 150 mL of a 2:1 v/v mixture of chloroform:methanol mixture in a Soxhlet extractor placed in an oil bath at 80 °C. After a 24 h extraction period, the lipid extracts were placed on a rotary evaporator to remove any residual solvent and allowed to air-dry for 3 h. The weights of the total lipid extracts were measured gravimetrically on a dry mass basis.

5.2.5 FAME preparation and analysis

Acid-catalysed methanolysis was carried out to prepare FAME from the microalgal extracts. The methanolysis procedure was modified from Lam & Lee.[172] Briefly, 50 mg of microalgal extract was placed in a 50 mL round bottom flask along with 11 mg of

concentrated sulfuric acid, 1.5 mL of methanol, 1 mL of tetrahydrofuran, and a magnetic stir bar. The round bottom flask was then connected to a condenser. The system was placed on a hot plate at 90 °C for 3 h under continuous stirring. After 3 h, the mixture was neutralized with sodium bicarbonate and FAMEs were extracted with hexanes using a separatory funnel. The extracted FAMEs were analyzed by a Perkin Elmer Clarus 680 gas chromatograph equipped with a flame ionization detector and Thermo Scientific TG-Polar column. The oven temperature was initiated at 50 °C, for 5 min, raised to 260 °C at a rate of 7 °C/min and held at 260 °C for 5 min. The injector and detector temperatures were set to 260 °C. Helium was used as the carrier gas. The FAME peaks in the samples were identified by comparing their retention times with those of a standard (Supelco TM 37 component FAME mix, Sigma-Aldrich).

5.2.6 Fluorescence microscopy using Nile Red

To effectively monitor cell disruption, Nile Red has been used as a dye of choice due to its ability to fluorometrically determine the neutral lipid content in microalgal cells.[203] Nile Red was diluted in acetone (10 µg/mL) and 100 µL was added to samples of *Scenedesmus sp.*, placed on microscope slides and their respective cover slips, before and after cell disruption. These samples were then observed under a Leica fluorescence microscope (40x magnification) containing a rhodamine filter with an excitation and emission of 520 nm and 650 nm, respectively. Photographs were taken using AxioVision 4.7 software.

5.3 Results and Discussion

5.3.1 Soxhlet extractions – fresh vs. frozen microalgae

To determine the effect of freezing the microalgae at -81 °C on cell disturbance and lipid extraction and composition, Soxhlet extractions and SPEs were conducted on both fresh and frozen *Scenedesmus sp.* (20 wt% solids) and results are shown in Figure 5.2.

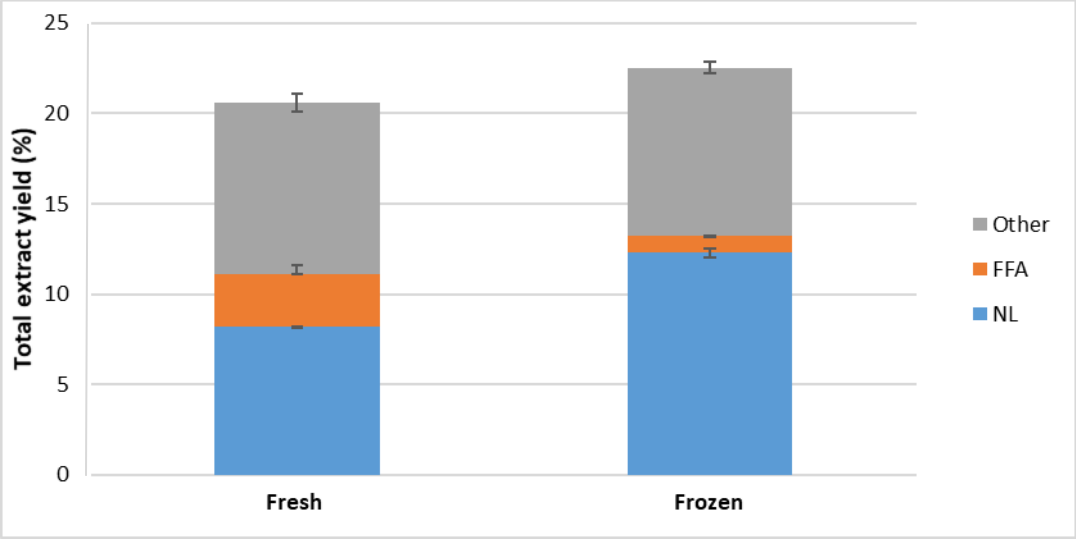

Figure 5.2. Percent of total extract yield (dry mass) from *Scenedesmus* sp. (fresh and frozen). Soxhlet extractions were conducted at 80°C for 24 h using a 2:1 (v/v) chloroform:methanol and performed in duplicate (n=2). SPE determined the percentage of NL, FFA, and other constituents (Other) recovered in the Soxhlet extract. Error bars are from SPE performed in duplicate (n=2).

The total extract yields obtained for fresh and frozen *Scenedesmus sp.*, respectively, were 21±1.0 % dw and 22.5±0.5 % dw. Although the total extract yields did not appear to be substantially different, the percentage of NLs extracted was comparatively different. The fresh microalgal slurry extract contained 8.2±0.2 % dw of NLs, while the frozen

microalgae extract contained 12.3±0.1 % dw. This demonstrated that freezing *Scenedesmus sp.* could positively affect the total amount of NLs extracted.

5.3.2 Cell disruption followed by extraction using lCO_2

Each of the experiments performed used the same lipid extraction method with lCO_2 and 20 mL of methanol. These experiments only differed in the type of mechanical/chemical cell disruption applied prior to lipid extraction to effectively liberate valuable NL and FFA from frozen *Scenedesmus sp.* From Table 5.1, microwave radiation in the presence of distilled water followed by lipid extraction was found to be most effective as it extracted NL and FFA fractions of 9.6±1.5 % dw compared to a total available NL and FFA yield of 13.2±0.3 % dw obtained from the Soxhlet extraction. Therefore, it was determined when microwave radiation in the presence of distilled water followed by lipid extraction was applied, it could extract approximately 73 % of the total extractable NL and FFA. This result was consistent with a study by Lee et al. where it was noted that microwave radiation followed by a lipid extraction involving a 1:1 v/v mixture of chloroform and methanol produced the highest lipid extract yield of 11 % dw for *Scenedesmus sp.*[201] In Table 1, when comparing microwave radiation in the presence of water (9.6±1.5 % dw) to microwave radiation in the presence of methanol (4.4±0.7 % dw), the yield of NL and FFA decreased by roughly half. This was likely because water is a more polar solvent than methanol and microwave radiation has been reported to act on polar solvents more effectively.[204] The lowest NL and FFA yield of 2.8±0.1 % dw was obtained by grinding following freezing with liquid N_2 as the cell disruption method.

Freeze-drying, ultrasonication (30 and 60 %), cooling, microwave in the presence of methanol, and switchable osmotic shock achieved NL and FFA yields of approximately 4.3-5.1 % dw, which was in a similar range as that extracted without prior cell disruption (5.1±0.7 % dw). Further experiments need to be performed in order to understand why these cell disruption methods attained similar NL and FFA yields, while microwave radiation achieved a higher yield.

Table 5.1. Extraction yields obtained after various cell disruption methods.

Cell disruption method	Cell disruption solvent (20 mL)	Extraction method*	Total yield (wt% of dry algae)	Yield of algae lipid components (wt% of dry algae)		
				NL	FFA	Other
Oven-dried	None	Soxhlet	22.5 ± 0.5	12.3 ± 0.3	0.9 ± 0.0	9.3 ± 0.3
None	None	lCO_2/MeOH	6.0 ± 1	1.5 ± 0.4	3.6 ± 0.6	1.0 ± 0.3
Freeze-drying	None	lCO_2/MeOH	6.4 ± 0.3	1.4 ± 0.4	3.3 ± 0.1	1.6 ± 0.4
Ultrasonication (30%)	Methanol	lCO_2/MeOH	5.6 ± 0.2	1.0 ± 0.0	4.1 ± 0.1	0.6 ± 0.1
Ultrasonication (60%)	Methanol	lCO_2/MeOH	4.6 ± 0.4	0.8 ± 0.1	3.5 ± 0.2	0.3 ± 0.1
Cooling	None	lCO_2/MeOH	5.7 ± 0.1	1.2 ± 0.4	3.3 ± 0.2	1.3 ± 0.7
Microwave	Water	lCO_2/MeOH	12.1 ± 1.3	5.5 ± 1.2	4.1 ± 0.9	2.5 ± 0.7
	Methanol	lCO_2/MeOH	5.6 ± 1.7	1.5 ± 0.3	2.9 ± 0.6	1.2 ± 0.3
Grinding following freezing with liquid N_2	None	lCO_2/MeOH	3.1 ± 1.4	0.5 ± 0.1	2.3 ± 0.1	0.3 ± 0.1
Switchable osmotic shock	60 % TMBDA	lCO_2/MeOH	5.8 ± 1.2	1.4 ± 0.3	1.8 ± 0.1	2.6 ± 0.4

*All extractions were performed with 10 g of *Scenedesmus sp.* slurry (20 wt% solids). After cell disruption, the extraction process was run for 3 h at 25 °C using lCO_2 (150 bar) and 20 mL methanol. Each value represents the mean ± S.D. (n=2).

5.3.3 FAME analysis

The FAME profiles of *Scenedesmus sp.* lipid extracts using no disruption, disruption, and Soxhlet extraction alone are shown in Figure 5.3. According to Knothe[177], the most desirable FAMEs for high quality biodiesel production include the C16 and C18 carbon chain lengths as they lead to cetane numbers (47 minimum), viscosities (1.9-6.0 mm^2/s at 40 °C), and oxidative stabilities (3 minimum), which are within the ideal range as stated by ASTM D6751 (standard specifications for biodiesel). For all FAME profiles,

the sum of C16:0, C16:1, C18:0, C18:1, C18:2, and C18:3 accounted for 75-83 % of the total composition, with the exception of those extracted after ultrasonication (69 %). The relative proportions of these FAME varied depending on the disruption technique used. For instance, both FAME profiles for cooling and no cell disruption contained ~82 % of C16 and C18 carbon chain lengths, but cooling exhibited higher amounts of C16:0, C18:0, C18:2, and C18:3, while no cell disruption displayed higher amounts of C16:1 and C18:1. The difference in FAME constituents could be due to variations in freezing times during storage of the microalgal samples. The FAME profiles confirmed that *Scenedesmus sp.* is a desirable feedstock containing high quantities of FAME that would be beneficial in biodiesel production.

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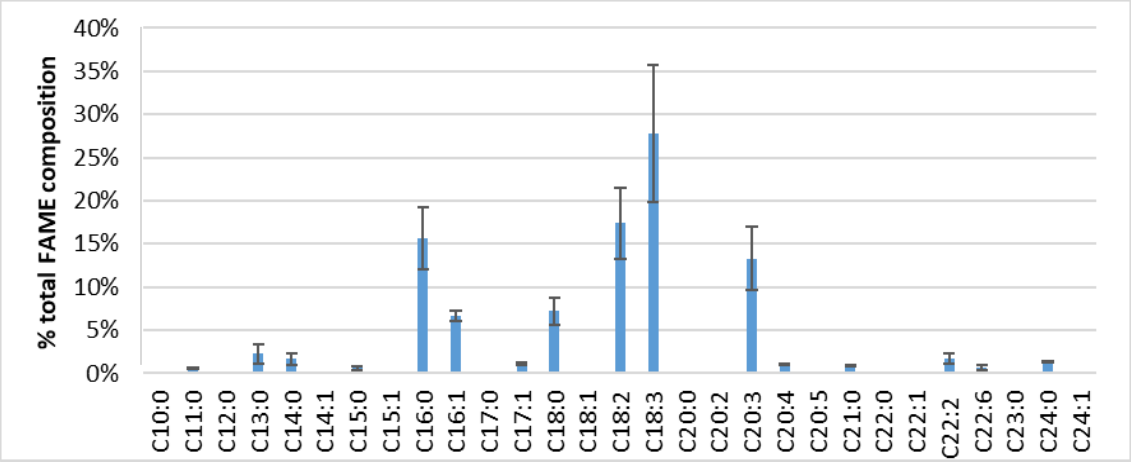

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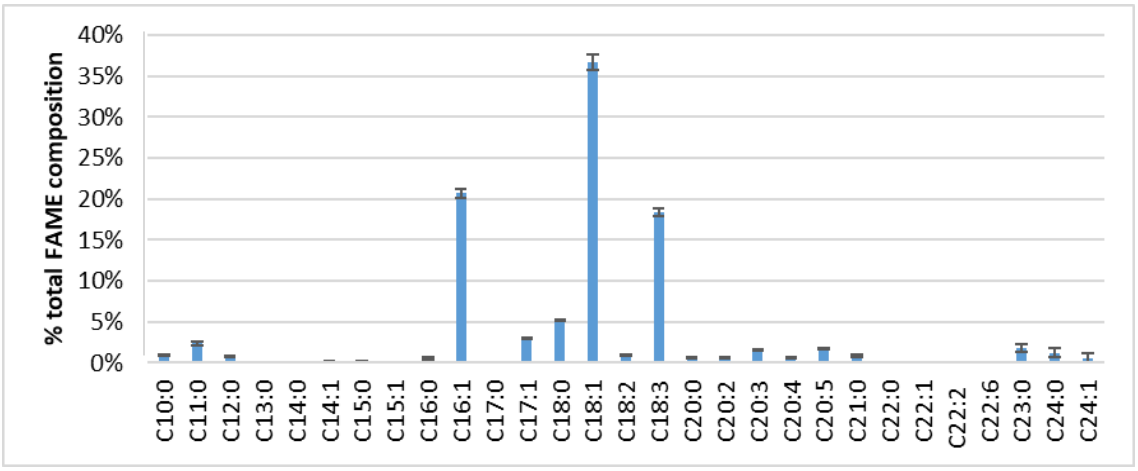
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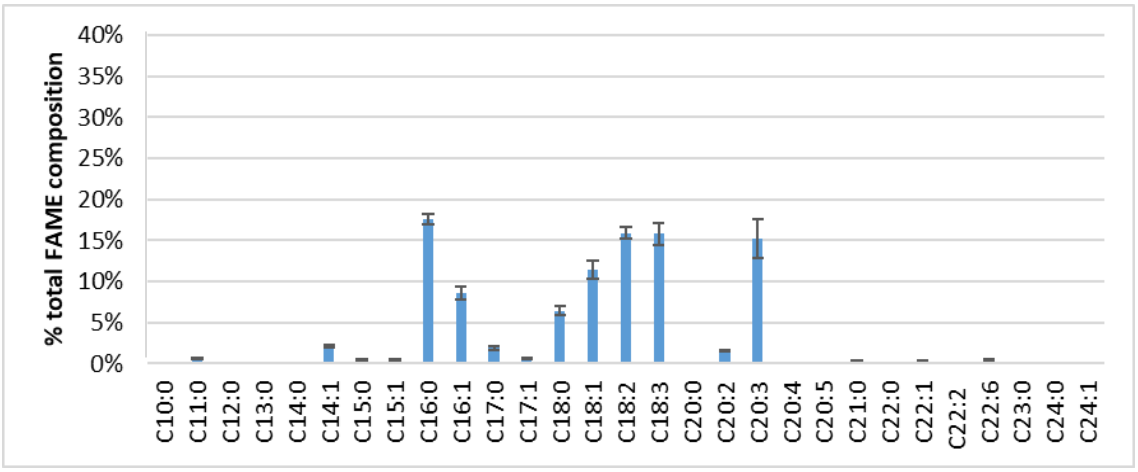
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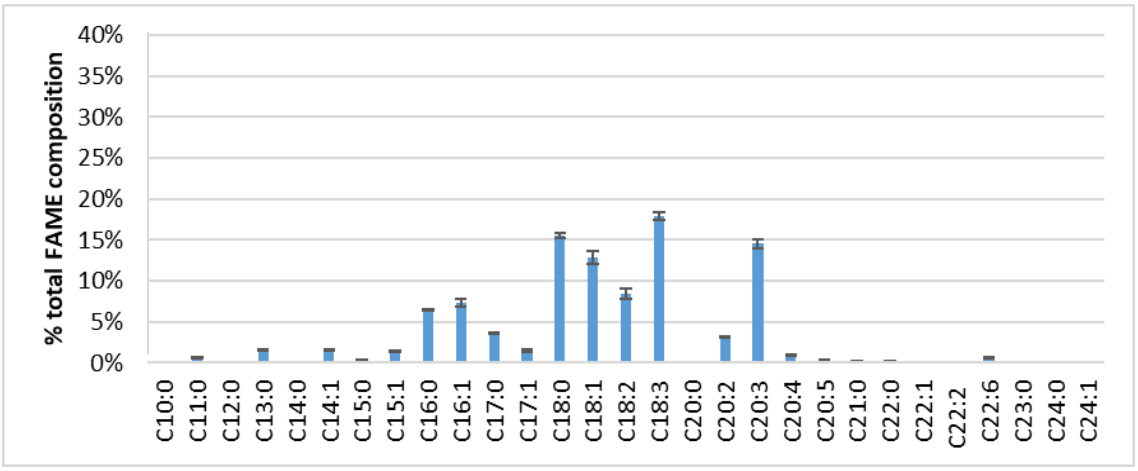
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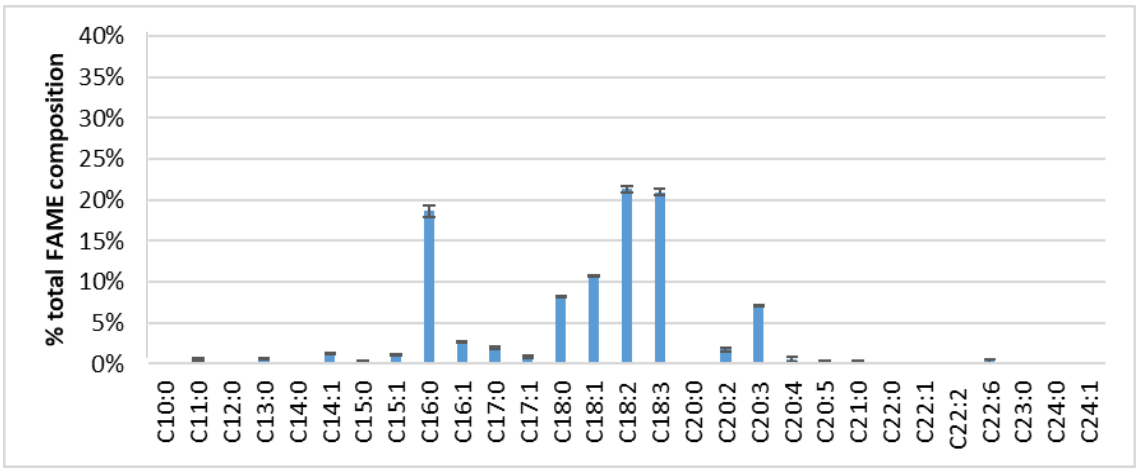
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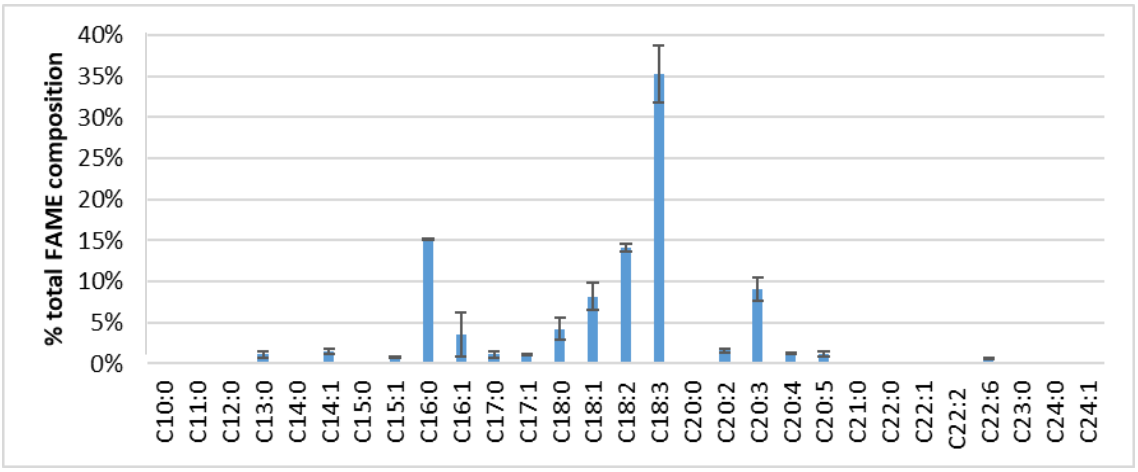
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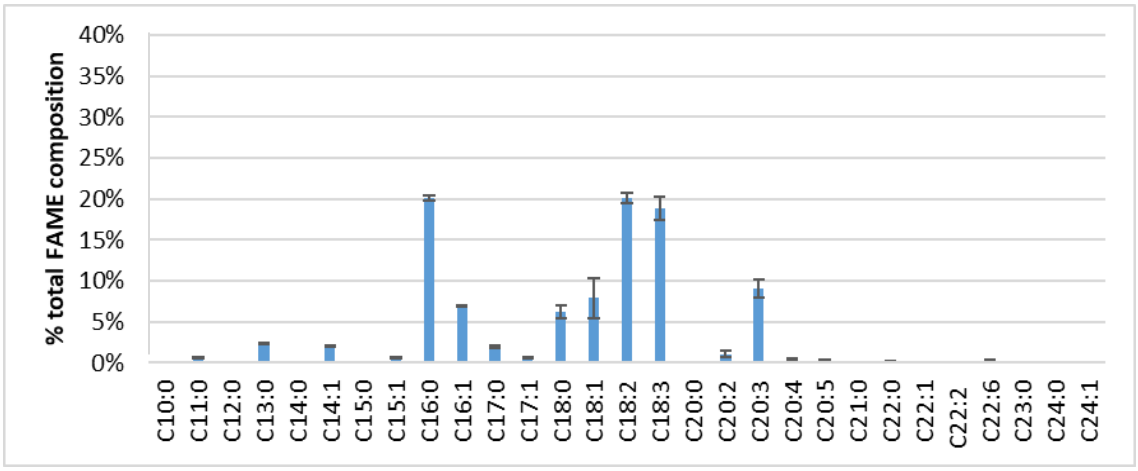
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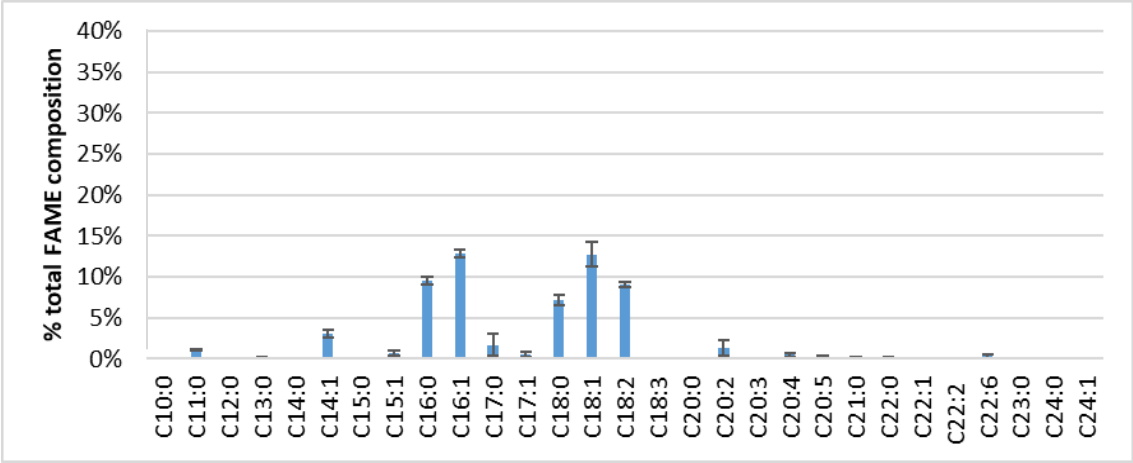


Figure 5.3. FAME profiles of *Scenedesmus sp.* upon mechanical/chemical cell disruption followed by extraction using ICO_2 and 20 mL methanol. Mechanical/chemical cell disruption techniques used are a) Soxhlet extraction with chloroform and methanol, b) none c) freeze-drying, d) ultrasonication, e) cooling, f) microwave radiation in the presence of water, g) grinding following freezing with liquid N_2, and h) switchable osmotic shock.

5.3.4 Fluorescence microscopy using Nile Red

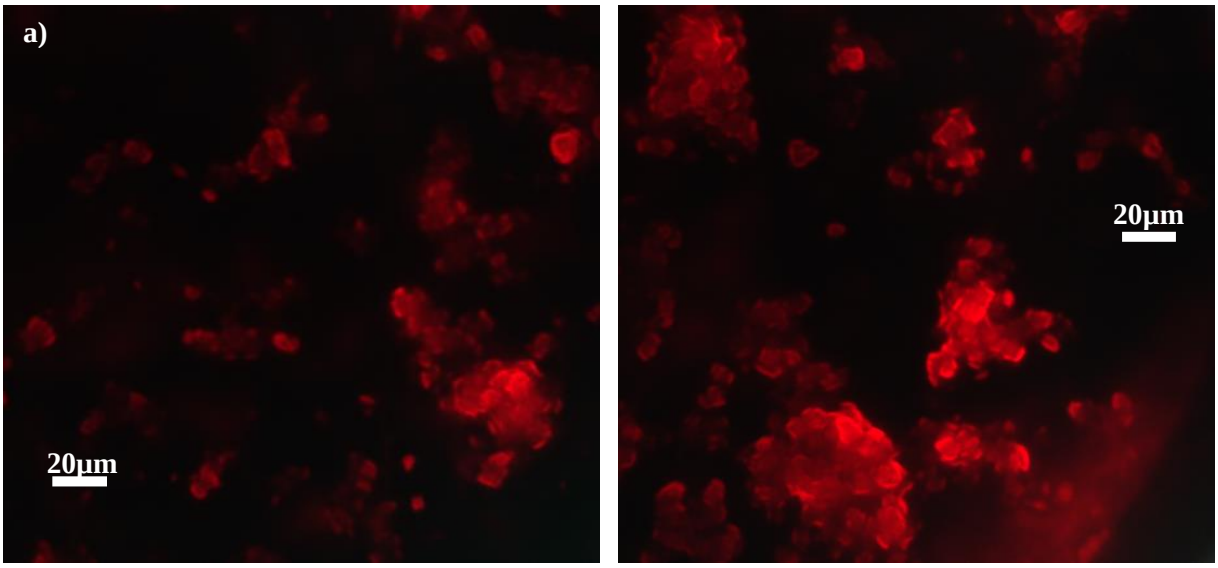
Fluorescence microscopy was used to visually assess whether cell disruption had taken place. Nile Red dye, when in the presence of a lipid-rich environment, can be intensely fluorescent. When it encounters polar membrane lipids, it fluoresces a deep red and upon contact with NLs in intracellular storages, it exhibits a strong yellow-gold emission.[205] This implies that yellow fluorescence should only be visible when microalgal cell walls were disrupted prior to the dye application.

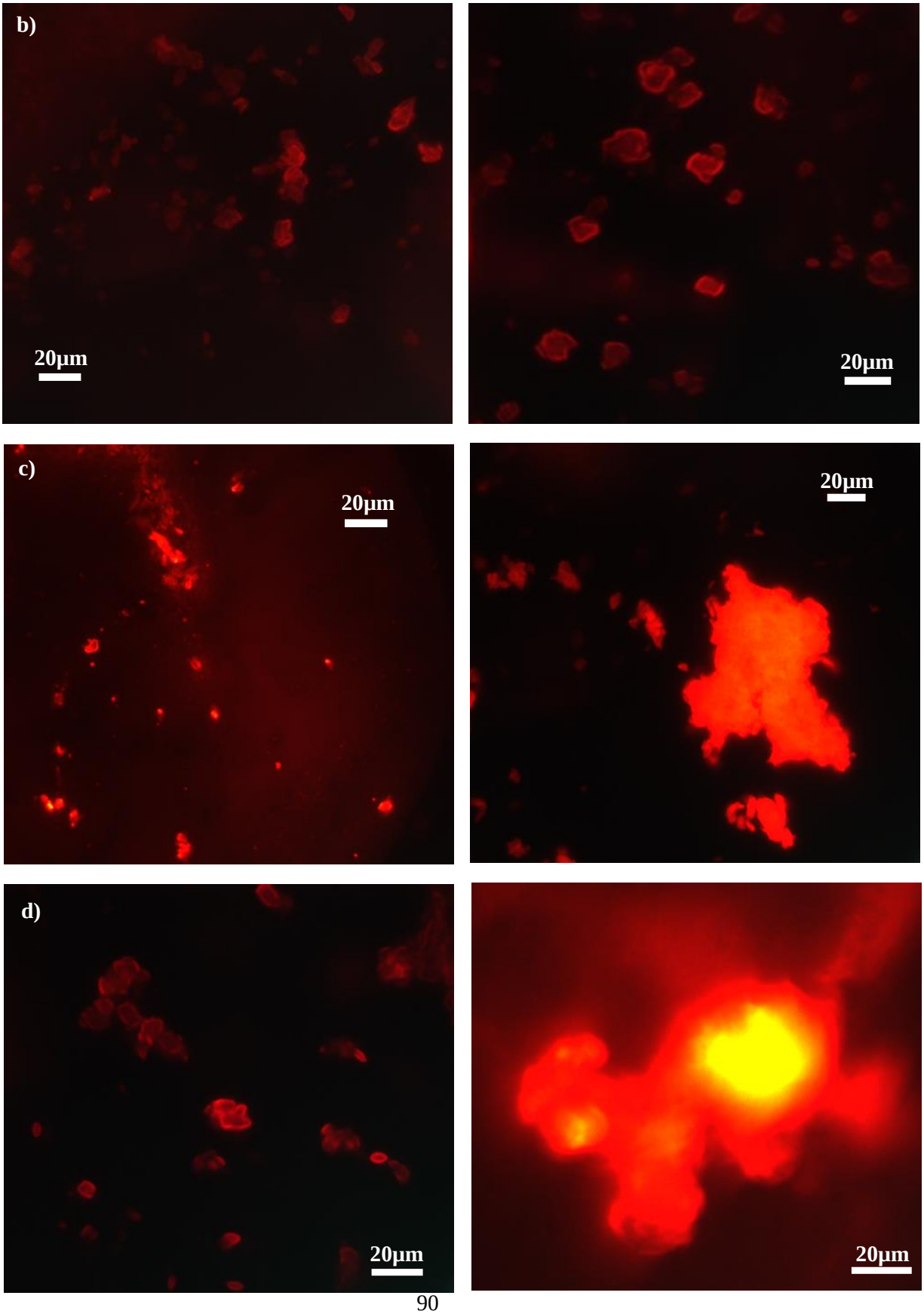
As can been seen from the images taken prior to all cell disruption applications, red autofluorescence due to the presence of chlorophyll, deep red fluorescence upon contact with the polar membrane lipids, and no yellow fluorescence were observed in each of the

samples. After microwave radiation and grinding following freezing with liquid N_2, yellow fluorescence was explicitly detected (Figures 5.4c, d, e). Microwave radiation in the presence of water appeared to impose the greatest amount of cell disruption (Figure 5.4d), which was also consistent with the higher extract yields measured (Table 5.1). Grinding following freezing with liquid N_2 appeared to exhibit the second greatest amount of cell disruption (Figure 5.4e); however, after lipid extraction, it displayed the lowest yields of NLs and FFAs (Table 5.1). After freeze-drying, cooling, and switchable osmotic shock (Figures 5.4a, b, f), yellow fluorescence was not observed, which would suggest that the cell walls were not disrupted enough for the Nile Red dye to interact with the intracellular NLs. On the other hand, freeze-drying, cooling, and osmotic shock, followed by lipid extraction, achieved higher NL and FFA yields than grinding followed by freezing with liquid N_2, which displayed an observable cell disruption. Based on these results, no observable trends between the amount of cell disruption and the amount of NLs and FFAs extracted could be identified.

Microwave treatment is potentially more economical compared to conventional thermal treatments because a shorter treatment time is required, it is more energy efficient, and it has lower-operating costs.[151,206] For these reasons, this technique could possibly be used industrially; however, some disadvantages are the capital costs associated with the equipment and the temperature is difficult to control.[206] Ali and Watson demonstrated that using 5 minutes of 1021 W microwave radiation would contribute <1 % of the total energy process costs, while increasing lipid extraction 3-fold in comparison to a control.[151] They also found that the energy consumption to dry the microalgal slurry (23 wt%) accounted for roughly 80% of the total energy.[151] In the case of results presented in the current study,

microwave radiation was applied in 3 intervals of 15 seconds each using 300 W of power, which would therefore contribute even less than 1 % to the total energy expenses and could lead to a 2-fold lipid extraction yield compared to the control. In a study by Lee et al., a variety of cell disruption methods were assessed, including microwave radiation, bead beating, autoclave, sonication, and osmotic shock followed by a lipid extraction (1:1 v/v mixture of chloroform and methanol) on three wet microalgal slurries of *Scenedesmus sp.*, *Chlorella vulgaris*, and *Botryococcus sp.*[201] Among the cell disruption methods investigated, they reported that microwave radiation prior to solvent lipid extraction achieved the highest lipid yield and appeared to be the most efficient for all three microalgal species.

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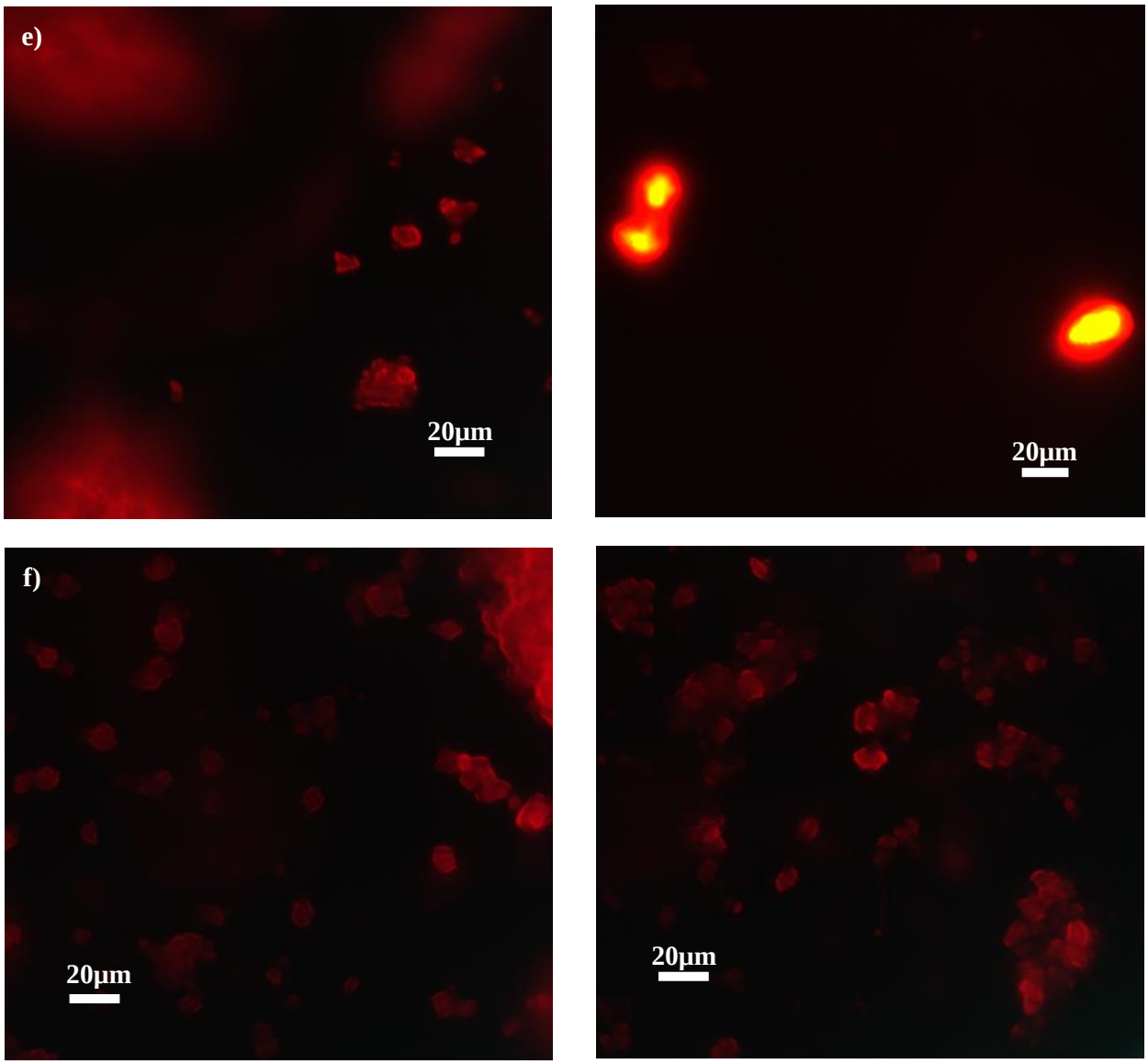

Figure 5.4. Fluorescence microscopy images of *Scenedesmus sp.* before (left) and after (right) cell disruption: a) freeze-drying, b) ultrasonication (30%), c) cooling, d) microwave in the presence of water, e) grinding following freezing with liquid N_2, and f) switchable osmotic shock using Nile Red. Red autofluorescence indicates presence of chlorophyll and yellow fluorescence indicates NL.

5.4 Conclusions

Microwave radiation in the presence of distilled water exhibited the highest potential for releasing NL and FFA from *Scenedesmus sp.* prior to extraction using lCO_2 and methanol. All other cell disruption techniques yielded extracts with roughly the same or less than the amount of NL and FFA achievable without prior cell disruption. The FAME profiles of the *Scenedesmus sp.* lipid extracts were highly variable due to variations in freezing times during storage of the microalgal samples. Freezing alone was as effective as most of the cell disruption techniques investigated; however, microwave displayed the greatest extract yield. Fluorescence microscopy was employed to effectively monitor cell disruption upon freeze-drying, ultrasonication, microwave radiation, and grinding following freezing with liquid N_2 techniques. Upon closer examination of the FAME composition profiles, it was noted that C16 and C18 made up 69-83 % of the total FAMEs identified, indicating that *Scenedesmus sp.* could be employed as a valuable feedstock for biodiesel production.

Chapter 6

CONCLUSIONS AND FUTURE DIRECTIONS

6.1 Conclusions

Microalgae have high lipid productivities, rapid growth rates, the ability to capture CO_2 for photosynthesis, and the ability to grow in wastewater. One of the major disadvantages of the use of microalgae for biodiesel production is the need for energy-intensive extraction techniques. Microalgae-derived biodiesel is a promising alternative to conventional diesel; however, energy and capital costs of the process of creating biodiesel need to be effectively minimized before it can be utilized as a suitable replacement. With this goal in mind, this thesis first examined the potential of liquid CO_2 (lCO_2) as a greener microalgal lipid extraction technique compared to conventional halogenated organic solvent methods. Compared to the greener extraction gold standard, supercritical CO_2 ($scCO_2$), lCO_2 operates at a lower temperature (room temperature) and lower pressure. However, lCO_2 alone could not access much of the lipids available due to the rigidity of the cell wall. Additional polar co-solvents were used to further disrupt biodiesel-desirable lipids covalently linked to polar lipids. Additional acid/base/surfactant modifiers were tested to facilitate lipid extraction via disruption of the rigid cell wall. Moreover, microalgae slurries were employed for all extractions to minimize the energy costs associated with drying.

The process step that accounts for the highest energy and capital costs is microalgal lipid extraction, and this is primarily due to the techniques applied to extract the microalgal

lipids through rigid microalgal cell walls. In Chapter 2, some improvements for microalgal lipid extraction are discussed:

1) Using microalgal biomass slurries is an important approach to minimize the economic and environmental expenses associated with drying.
2) Using one solvent consistently throughout and combining process steps (process intensification) to reduce energy and capital costs.
3) Quantifying the environmental and economic costs associated with each microalgal lipid extraction method using life cycle assessments.
4) Avoiding the use of halogenated organic solvents, and unnecessary high temperature and pressure conditions.

In Chapter 3, lipid extraction from microalgal slurries was performed on *Chlorella vulgaris* using lCO_2, co-solvents, and additional modifiers. The major discovery was the double and quadruple extract yields achieved using surfactants compared to the control. Additionally, all co-solvents tested were able to obtain similar amounts of extracts from *C. vulgaris* during extraction. For this reason, surfactants displayed great promise as chemical disrupters of the rigid microalgal cell walls. Interestingly, the fatty acid methyl ester (FAME) composition changed based on which modifiers were added to the extraction. The properties of each modifier were different (e.g. acids can be deprotonated, bases can be protonated, and surfactants can interact through hydrophobic interactions), and, therefore, likely targeted the TAGs and FAs differently.

In Chapter 4, use of a switchable-hydrophilicity solvent (SHS) for the transesterification of soybean oil was shown to be beneficial. The FAMEs derived from soybean oil mainly consisted of C18:3, which is very useful for biodiesel production. SHSs

could be removed from FAMEs using carbonated water, eliminating the need for distillation. The use of an SHS does not require flammable, highly volatile, or chlorinated solvents. These features suggest that SHSs are highly preferable compared to the use of conventional solvents, but an LCA would be required to confirm this. Moreover, it does not produce soap that is generated when basic catalysts are employed. However, the amount of SHS remaining in the FAME layer would need to be lowered before this technology could be applied on an industrial scale.

In Chapter 5, chemical and mechanical cell disruption techniques followed by extraction using lCO_2 and methanol were performed on *Scenedesmus sp.* slurries. Of all cell disruption techniques tested, microwave radiation in the presence of distilled water exhibited the highest potential for releasing neutral lipids (NLs) and free fatty acids (FFAs) from *Scenedesmus sp.* when applied prior to the extraction. All other cell disruption techniques achieved NL and FFA yields in roughly the same or lower quantities than those achieved without prior cell disruption. Fluorescence microscopy could effectively monitor cell disruption upon microwave radiation and grinding following freezing with liquid N_2 techniques. Upon closer examination of the FAME composition profiles, it was noted that C16 and C18 made up most of the total FAMEs identified, indicating that *Scenedesmus sp.* could be employed as a valuable feedstock for biodiesel production.

Overall, extractions using lCO_2 showed great promise for the future of microalgae to biodiesel; however, life cycle assessments need to be conducted to quantify if this extraction is techno-economically viable for industrial purposes.

This thesis has contributed knowledge in the development of greener technologies for the replacement of fossil fuels, major contributors of global warming. Several methods

were investigated to improve the conversion of microalgae to biodiesel, which may eventually lead to large-scale applications, most importantly for transportation.

6.2 Future work

In extractions performed using microalgal slurries (14-20 wt% solids), the mass transfer of lipids was hindered. As mentioned earlier, drying wet microalgae has a significant economic cost associated with it and therefore, future extractions should focus on varying the wt% of algae (increase or decrease water content) to measure its effect on the yield of extract.

According to the properties of lCO_2, an increase in the pressure increases the density of lCO_2 and in turn, increases solvent power. However, the NL and FFA yields obtained from lCO_2 extractions are moderate. For this reason, longer extraction times than 3 h should be undertaken to monitor the mass transfer of the lipids to the lCO_2 phase. The environmental costs associated with this process should be investigated using life cycle assessments and should be compared to current extraction techniques, such as conventional organic solvent extraction, Soxhlet extraction, and $scCO_2$ extraction. It would be beneficial to also perform these extractions with dry microalgae and assess the associated cost with compressing CO_2 at 150 bar.

All lCO_2 extractions used a flow rate of 2 mL/min in this study, but perhaps a higher flow rate would improve the extraction yields by allowing higher contact time leading to a better solvent/lipid interaction. However, an increased flow rate would consume more CO_2, which may have an adverse effect on the overall economic and environmental costs.

In Chapter 3, since the extraction using lCO_2 and dodecyltrimethylammonium bromide (DTAB) exhibited the most promising yield, future work should examine whether other surfactants (cationic, anionic, zwitterionic, and non-ionic) could enhance these yields further, and if so, at what concentrations these could best be employed (above or below the critical micelle concentration). Moreover, additional modifiers, such as acids, bases, and solvents should be examined with DTAB to determine their effect on the extract yield. The effect of these surfactants on the recycling of the water and the overall environmental impact should also be considered.

In Chapter 4, it would be beneficial to examine a more hydrophilic SHS ($\log K_{ow} < 2.20$) and a more basic SHS ($pKa_H > 9.67$). Some SHSs that exhibit both of these properties are N,N-dimethylcyclohexylamine, N,N-dimethylbutylamine, and 1-ethylpiperidine. With these SHSs, optimizing the ratio of SHS:methanol:water used for the transesterification of soybean oil should be examined. Moreover, diamine SHSs have recently been discovered as potential solvents that can separate more effectively than monoamine SHSs from oil, and therefore, FAMEs. Once the SHSs that can effectively separate from FAMEs have been identified, these SHSs can be applied to a wide variety of oils, such as soybean, canola, and vegetable oils. If these SHSs can effectively transform each of these oils into FAMEs, then they can potentially be used to convert microalgae to FAMEs.

In Chapter 5, each of the cell disruption techniques need to be examined in greater detail to understand why they all obtained comparable NL and FFA yields, except for that obtained with microwave radiation in the presence of water. For microwave radiation disruption, experiments should be performed to optimize the reaction conditions (volume of solvent, choice of solvent, temperature, power). As well, microwave radiation should be

tested on fresh microalgal slurries and these should be compared to frozen-then-thawed microalgal slurries. This would indicate whether freezing the microalgae plays a role in cell disruption directly. For the osmotic shock method, experiments would need to be conducted comparing conventional salts, such as NaCl and Na_3PO_4, to switchable salts to determine whether switchable salts are substantially better as cell disruption agents. For the ultrasonication method using a sonicator probe, the vibration frequency and amplitude need to be optimized for disrupting microalgal cells. Imaging of all cell disruption techniques could be performed using confocal fluorescence microscopy in future studies because of this technique's greater magnification and minimal blurring artefacts. Moreover, performing each of the extractions using lCO_2 and methanol a second time, with or without prior cell disruption, should be assessed to evaluate if more NLs and FFAs are obtained. Lastly, examine why the variation in freezing times could cause such a change in each of the FAME profiles.

REFERENCES

(1) Huang, D.; Zhou, H.; Lin, L. Biodiesel: An Alternative to Conventional Fuel. *Energy Procedia* **2011**, *16* (PART C), 1874–1885.

(2) Energy Information Administration, Primary Energy Production by Source. *Mon. Energy Rev.* **2015**.

(3) Shafiee, S.; Topal, E. When Will Fossil Fuel Reserves Be Diminished? *Energy Policy* **2009**, *37* (1), 181–189.

(4) Farhad, S.; Saffar-Avval, M.; Younessi-Sinaki, M. Efficient Design of Feedwater Heaters Network in Steam Power Plants Using Pinch Technology and Energy Analysis. *Int. J. Energy Res.* **2008**, *32*, 1–11.

(5) Boden, T. A.; Andres, R. J.; Marland, G. Global, Regional, and National Fossil-Fuel CO2 Emissions. **2017**.

(6) Le Quéré, C.; Andrew, R. M.; Canadell, J. G.; Sitch, S.; Ivar Korsbakken, J.; Peters, G. P.; et al. Global Carbon Budget 2016. *Earth Syst. Sci. Data* **2016**, *8* (2), 605–649.

(7) Kavuri, S. Greenhouse Gas Emissions. **2013**, No. 2012.

(8) Short-Term Energy Outlook - U.S. Energy Information Administration (EIA) https://www.eia.gov/outlooks/steo/report/global_oil.cfm (accessed Sep 19, 2017).

(9) Panwar, N. L.; Kaushik, S. C.; Kothari, S. Role of Renewable Energy Sources in Environmental Protection: A Review. *Renew. Sustain. Energy Rev.* **2011**, *15* (3), 1513–1524.

(10) Sims, R. E. H. Bioenergy to Mitigate for Climate Change and Meet the Needs of Society, the Economy and the Environment. *Mitig. Adapt. Strateg. Glob. Chang.* **2003**, *8* (4), 349–370.

(11) Demirbas, A. Political, Economic and Environmental Impacts of Biofuels: A Review. *Appl. Energy* **2009**, *86* (SUPPL. 1), S108–S117.

(12) Zakhidov, R. a. Central Asian Countries Energy System and Role of Renewable Energy Sources. *Appl. Sol. Energy* **2008**, *44* (3), 218–223.

(13) Bergmann, A.; Colombo, S.; Hanley, N. Rural versus Urban Preferences for Renewable Energy Developments. *Ecol. Econ.* **2008**, *65* (3), 616–625.

(14) Reddy, A. K. N.; Subramanian, D. K. The Design of Rural Energy Centres. *Proc. Indian Acad. Sci.* **1979**, *2* (3), 395–416.

(15) Ravindranath, N. H.; Hall, D. O. (David O. *Biomass, Energy, and Environment : A Developing Country Perspective from India*; Oxford University Press, 1995.

(16) Nigam, P. S.; Singh, A. Production of Liquid Biofuels from Renewable Resources. *Prog. Energy Combust. Sci.* **2011**, *37* (1), 52–68.

(17) Hoekman, S. K. Biofuels in the U.S. - Challenges and Opportunities. *Renew. Energy* **2009**, *34* (1), 14–22.

(18) Biofuels - Biofuel Information - Guide to Biofuels http://biofuel.org.uk/ (accessed Aug 29, 2017).

(19) Chen, M.; Liu, T.; Chen, X.; Chen, L.; Zhang, W.; Wang, J.; Gao, L.; Chen, Y.; Peng, X. Subcritical Co-Solvents Extraction of Lipid from Wet Microalgae Pastes of Nannochloropsis Sp. *Eur. J. Lipid Sci. Technol.* **2012**, *114* (2), 205–212.

(20) Usta, N.; Ozturk, E.; Can, A; Conkur, E. S.; Nas, S.; et al. Combustion of Biodiesel Fuel Produced from Hazelnut Soapstock/waste Sunflower Oil Mixture in a Diesel Engine. *Energy Convers. Manag.* **2005**, *46* (5), 741–755.

(21) Predojević, Z. J. The Production of Biodiesel from Waste Frying Oils: A Comparison of Different Purification Steps. *Fuel* **2008**, *87* (17–18), 3522–3528.

(22) Apostolakou, A. A.; Kookos, I. K.; Marazioti, C.; Angelopoulos, K. C. Techno-Economic Analysis of a Biodiesel Production Process from Vegetable Oils. *Fuel Process. Technol.* **2009**, *90* (7–8), 1023–1031.

(23) Hayyan, M.; Mjalli, F. S.; Hashim, M. A.; AlNashef, I. M. A Novel Technique for Separating Glycerine from Palm Oil-Based Biodiesel Using Ionic Liquids. *Fuel Process. Technol.* **2010**, *91* (1), 116–120.

(24) Benjumea, P.; Agudelo, J.; Agudelo, A. Basic Properties of Palm Oil Biodiesel-Diesel Blends. *Fuel* **2008**, *87* (10–11), 2069–2075.

(25) Biofuels - First Generation Biofuels http://biofuel.org.uk/first-generation-biofuel.html (accessed Sep 1, 2017).

(26) Balat, M.; Balat, H. Recent Trends in Global Production and Utilization of Bio-Ethanol Fuel. *Appl. Energy* **2009**, *86* (11), 2273–2282.

(27) Taher, H.; Al-Zuhair, S.; Al-Marzouqi, A. H.; Haik, Y.; Farid, M. M. A Review of Enzymatic Transesterification of Microalgal Oil-Based Biodiesel Using Supercritical Technology. *Enzyme Res.* **2011**, *2011*, 1–25.

(28) Patil, V.; Tran, K. Q.; Giselr??d, H. R. Towards Sustainable Production of Biofuels from Microalgae. *Int. J. Mol. Sci.* **2008**, *9* (7), 1188–1195.

(29) Jose, S.; Bhaskar, T. *Biomass and Biofuels : Advanced Biorefineries for Sustainable Production and Distribution*. Date accessed: August 29th, 2017.

(30) Naik, S. N.; Goud, V. V.; Rout, P. K.; Dalai, A. K. Production of First and Second Generation Biofuels: A Comprehensive Review. *Renew. Sustain. Energy Rev.* **2010**, *14* (2), 578–597.

(31) Balan, V. Current Challenges in Commercially Producing Biofuels from Lignocellulosic Biomass. *ISRN Biotechnol.* **2014**, *2014* (i), 1–31.

(32) Ullah, K.; Ahmad, M.; Sofia; Sharma, V. K.; Lu, P.; Harvey, A.; Zafar, M.; Sultana, S.; Anyanwu, C. N. Algal Biomass as a Global Source of Transport Fuels: Overview and Development Perspectives. *Prog. Nat. Sci. Mater. Int.* **2014**, *24* (4), 329–339.

(33) Brennan, L.; Owende, P. Biofuels from Microalgae-A Review of Technologies for

Production, Processing, and Extractions of Biofuels and Co-Products. *Renew. Sustain. Energy Rev.* **2010**, *14* (2), 557–577.

(34) Cuellar-Bermudez, S. P.; Garcia-Perez, J. S.; Rittmann, B. E.; Parra-Saldivar, R. Photosynthetic Bioenergy Utilizing CO<inf>2</inf>: An Approach on Flue Gases Utilization for Third Generation Biofuels. *J. Clean. Prod.* **2015**, *98*, 53–65.

(35) Generations of Biofuels - Energy from waste and wood http://energyfromwasteandwood.weebly.com/generations-of-biofuels.html (accessed Sep 5, 2017).

(36) Aro, E. M. From First Generation Biofuels to Advanced Solar Biofuels. *Ambio* **2016**, *45* (1), 24–31.

(37) Reed, M. National Energy Technology Laboratory Increasing Security and Reducing Carbon Emissions of the U . S . Transportation Sector : A Transformational Role for Coal with Biomass Increasing Security and Reducing Carbon Emissions of the U . S . Transportation Se. *Transportation (Amst).* **2007**.

(38) Carbon Capture and Storage: the “Fourth Generation” Biofuels- Crop Biotech Update (10/12/2007) | ISAAA.org/KC http://www.isaaa.org/kc/cropbiotechupdate/article/default.asp?ID=1008 (accessed Aug 29, 2017).

(39) Hannon, M.; Gimpel, J.; Tran, M.; Rasala, B.; Mayfield, S. Biofuels from Algae: Challenges and Potential. *Biofuels* **2010**, *1* (5), 763–784.

(40) Sharma, K. K.; Schuhmann, H.; Schenk, P. M. High Lipid Induction in Microalgae for Biodiesel Production. *Energies* **2012**, *5* (5), 1532–1553.

(41) Schenk, P. M.; Thomas-Hall, S. R.; Stephens, E.; Marx, U. C.; Mussgnug, J. H.; Posten, C.; Kruse, O.; Hankamer, B. Second Generation Biofuels: High-Efficiency Microalgae for Biodiesel Production. *BioEnergy Res.* **2008**, *1* (1), 20–43.

(42) Ge, S.; Champagne, P.; Plaxton, W. C.; Leite, G. B.; Marazzi, F. Microalgal Cultivation with Waste Streams and Metabolic Constraints to Triacylglycerides Accumulation for Biofuel Production. *Biofuels, Bioprod. Biorefining* **2017**, *11* (2), 325–343.

(43) Abou-Shanab, R. A. I.; Hwang, J. H.; Cho, Y.; Min, B.; Jeon, B. H. Characterization of Microalgal Species Isolated from Fresh Water Bodies as a Potential Source for Biodiesel Production. *Appl. Energy* **2011**, *88* (10), 3300–3306.

(44) Saifullah, A.; Karim, A.; Ahmad-Yazid, A. Microalgae: An Alternative Source of Renewable Energy. *Am. J. Eng. Res.* **2014**, *3* (3), 330–338.

(45) Spolaore, P.; Joannis-Cassan, C.; Duran, E.; Isambert, A. Commercial Applications of Microalgae. *J. Biosci. Bioeng.* **2006**, *101* (2), 87–96.

(46) Hirano, A.; Ueda, R.; Hirayama, S.; Ogushi, Y. CO2 Fixation and Ethanol Production with Microalgal Photosynthesis and Intracellular Anaerobic Fermentation. *Energy* **1997**, *22* (2–3), 137–142.

(47) Halim, R.; Danquah, M. K.; Webley, P. A. Extraction of Oil from Microalgae for Biodiesel Production: A Review. *Biotechnol. Adv.* **2012**, *30* (3), 709–732.

(48) Nascimento, I. A.; Marques, S. S. I.; Cabanelas, I. T. D.; Pereira, S. A.; Druzian, J. I.; de Souza, C. O.; Vich, D. V.; de Carvalho, G. C.; Nascimento, M. A. Screening Microalgae Strains for Biodiesel Production: Lipid Productivity and Estimation of Fuel Quality Based on Fatty Acids Profiles as Selective Criteria. *Bioenergy Res.* **2013**, *6* (1), 1–13.

(49) Griffiths, M. J.; Harrison, S. T. L. Lipid Productivity as a Key Characteristic for Choosing Algal Species for Biodiesel Production. *J. Appl. Phycol.* **2009**, *21* (5), 493–507.

(50) Griffiths, M. J.; Dicks, R. G.; Richardson, C.; Harrison, S. T. L. Advantages and Challenges of Microalgae as a Source of Oil for Biodiesel. *Biodiesel - Feed. Process. Technol.* **2011**, 178–200.

(51) Mata, T. M.; Martins, A. A.; Caetano, N. S. Microalgae for Biodiesel Production and

Other Applications: A Review. *Renew. Sustain. Energy Rev.* **2010**, *14* (1), 217–232.

(52) Chisti, Y. Biodiesel from Microalgae. *Biotechnol. Adv.* **2007**, *25* (3), 294–306.

(53) Molina Grima, E.; Belarbi, E.-H.; Acién Fernández, F. G.; Robles Medina, A.; Chisti, Y. Recovery of Microalgal Biomass and Metabolites: Process Options and Economics. *Biotechnol. Adv.* **2003**, *20* (7–8), 491–515.

(54) Barros, A. I.; Gon??alves, A. L.; Sim??es, M.; Pires, J. C. M. Harvesting Techniques Applied to Microalgae: A Review. *Renew. Sustain. Energy Rev.* **2015**, *41*, 1489–1500.

(55) Li, Y.; Horsman, M.; Wu, N.; Lan, C. Q.; Dubois-Calero, N. Optimization of Biomass Production of Spirulina Maxima. *J. Algal Biomass Util.* **2010**, *1* (2), 20–32.

(56) Danquah, M. K.; Ang, L.; Uduman, N.; Moheimani, N.; Forde, G. M. Dewatering of Microalgal Culture for Biodiesel Production: Exploring Polymer Flocculation and Tangential Flow Filtration. *J. Chem. Technol. Biotechnol.* **2009**, *84* (7), 1078–1083.

(57) Uduman, N.; Qi, Y.; Danquah, M. K.; Hoadley, A. F. A. Marine Microalgae Flocculation and Focused Beam Reflectance Measurement. *Chem. Eng. J.* **2010**, *162* (3), 935–940.

(58) Wijffels, R. H.; Barbosa, M. J.; Eppink, M. H. M. Microalgae for the Production of Bulk Chemicals and Biofuels. *Biofuels, Bioprod. Biorefining* **2010**, *4* (3), 287–295.

(59) Quinn, J. C.; Davis, R. The Potentials and Challenges of Algae Based Biofuels: A Review of the Techno-Economic, Life Cycle, and Resource Assessment Modeling. *Bioresour. Technol.* **2015**, *184*, 444–452.

(60) Lardon, L.; Helias, A.; Sialve, B.; Steyer, J.-P.; Bernard, O. Policy Analysis Life-Cycle Assessment of Biodiesel Production from Microalgae. *Envir. Sci. and Technol.* **2009**, 6475–6481.

(61) Kanda, H.; Li, P.; Ikehara, T.; Yasumoto-Hirose, M. Lipids Extracted from Several Species of Natural Blue-Green Microalgae by Dimethyl Ether: Extraction Yield and Properties. *Fuel* **2012**, *95*, 88–92.

(62) Liu, C. Z.; Zheng, S.; Xu, L.; Wang, F.; Guo, C. Algal Oil Extraction from Wet Biomass of Botryococcus Braunii by 1,2-Dimethoxyethane. *Appl. Energy* **2013**, *102*, 971–974.

(63) Boonnoun, P.; Kurita, Y. Wet Extraction of Lipids and Astaxanthin from Haematococcus Pluvialis by Liquefied Dimethyl Ether. *J. Nutr. Food Sci.* **2016**, *4* (5).

(64) Saga, K.; Hasegawa, F.; Miyagi, S.; Atobe, S.; Okada, S.; Imou, K.; Osaka, N.; Yamagishi, T. Comparative Evaluation of Wet and Dry Processes for Recovering Hydrocarbon from Botryococcus Braunii. *Appl. Energy* **2015**, *141*, 90–95.

(65) Chisti, Y.; Moo-young, M. Disruption of Microbial Cells for Intracellular Products. *Enzyme Microb. Technol.* **1986**, 194-204.

(66) Unterlander, N.; Champagne, P.; Plaxton, W. C. Lyophilization Pretreatment Facilitates Extraction of Soluble Proteins and Active Enzymes from the Oil-Accumulating Microalga Chlorella Vulgaris. *Algal Res.* **2017**, *25* (June), 439–444.

(67) Balasubramanian, S.; Allen, J. D.; Kanitkar, A.; Boldor, D. Oil Extraction from Scenedesmus Obliquus Using a Continuous Microwave System - Design, Optimization, and Quality Characterization. *Bioresour. Technol.* **2011**, *102* (3), 3396–3403.

(68) Gerken, H. G.; Donohoe, B.; Knoshaug, E. P. Enzymatic Cell Wall Degradation of Chlorella Vulgaris and Other Microalgae for Biofuels Production. *Planta* **2013**, *237* (1), 239–253.

(69) Harris, J.; Viner, K.; Jessop, P. G.; Champagne, P. Advances in Microalgae Lipid Extraction for Biofuel Production: A Review. **2016**, in preparation.

(70) Boyd, A. R.; Champagne, P.; McGinn, P. J.; MacDougall, K. M.; Melanson, J. E.; Jessop, P. G. Switchable Hydrophilicity Solvents for Lipid Extraction from Microalgae for Biofuel Production. *Bioresour. Technol.* **2012**, *118*, 628–632.

(71) Paudel, A. Liquid CO2 and CO2-Expanded Methanol for Lipid Extraction from Microalgae, Queen's University Thesis, 2015.

(72) Santana, A.; Jesus, S.; Larrayoz, M. A.; Filho, R. M. Supercritical Carbon Dioxide Extraction of Algal Lipids for the Biodiesel Production. *Procedia Eng.* **2012**, *42* (August), 1755–1761.

(73) Al-Zuhair, S. Production of Biodiesel: Possibilities and Challenges. *Biofuels, Bioprod. Biorefining* **2007**, *1* (1), 57–66.

(74) Robles-Medina, A.; González-Moreno, P. A.; Esteban-Cerdán, L.; Molina-Grima, E. Biocatalysis: Towards Ever Greener Biodiesel Production. *Biotechnol. Adv.* **2009**, *27* (4), 398–408.

(75) Vasudevan, P. T.; Briggs, M. Biodiesel Production—current State of the Art and Challenges. *J. Ind. Microbiol. Biotechnol.* **2008**, *35* (5), 421.

(76) Ma, F.; Hanna, M. A. Biodiesel Production: A review1. *Bioresour. Technol.* **1999**, *70* (1), 1–15.

(77) Hsu, D. D. Life Cycle Assessment of Gasoline and Diesel Produced via Fast Pyrolysis and Hydroprocessing. *Biomass and Bioenergy* **2012**, *45*, 41–47.

(78) Attaphong, C.; Sabatini, D. A. Phase Behaviors of Vegetable Oil-Based Microemulsion Fuels: The Effects of Temperatures, Surfactants, Oils, and Water in Ethanol. *Energy and Fuels* **2013**, *27* (11), 6773–6780.

(79) Helwani, Z.; Othman, M. R.; Aziz, N.; Fernando, W. J. N.; Kim, J. Technologies for Production of Biodiesel Focusing on Green Catalytic Techniques: A Review. *Fuel Process. Technol.* **2009**, *90* (12), 1502–1514.

(80) Freedman, B.; Butterfield, R. O.; Pryde, E. H. Transesterification Kinetics of Soybean Oil. *Journal of the American Oil Chemists' Society.* **1986**, *63* (10).

(81) Schuchardt, U.; Sercheli, R.; Matheus, R. Transesterification of Vegetable Oils : A Review General Aspects of Transesterification Transesterification of Vegetable Oils Acid-Catalyzed Processes Base-Catalyzed Processes. *J. Braz. Chem. Soc.*, **1998**, *9* (1), 199–210.

(82) Schwab, A. W.; Bagby, M. O.; Freedman, B. Preparation and Properties of Diesel Fuels from Vegetable Oils. *Fuel* **1987**, *66* (10), 1372–1378.

(83) Ejikeme, P. M.; Anyaogu, I. D.; Ejikeme, C. L.; Nwafor, N. P.; Egbuonu, C. A. C.; Ukogu, K.; Ibemesi, J. A.; Chemistry, I.; Polytechnic, F. Catalysis in Biodiesel Production by Transesterification Processes-An Insight. *E-Journal Chem.* **2010**, *7* (4), 1120–1132.

(84) Aksoy, H. A.; Becerik, I.; Karaosmanoğlu, F.; Yatmaz, H. C.; Civelekoğlu, H. Utilization Prospects of Turkish Raisin Seed Oil as an Alternative Engine Fuel. *Fuel* **1990**, *69* (5), 600–603.

(85) Gryglewicz, S. Alkaline-Earth Metal Compounds as Alcoholysis Catalysts for Ester Oils Synthesis. *Appl. Catal. A Gen.* **2000**, *192* (1), 23–28.

(86) Lisboa, P.; Rodrigues, A. R.; Martín, J. L.; Simões, P.; Barreiros, S.; Paiva, A. Economic Analysis of a Plant for Biodiesel Production from Waste Cooking Oil via Enzymatic Transesterification Using Supercritical Carbon Dioxide. *J. Supercrit. Fluids* **2014**, *85*, 31–40.

(87) Silva, C. Da; Oliveira, J. V. Biodiesel Production through Non-Catalytic Supercritical Transesterification: Current State and Perspectives. *Brazilian J. Chem. Eng.* **2014**, *31* (2), 271–285.

(88) Bunyakiat, K.; Makmee, S.; Sawangkeaw, R.; Ngamprasertsith, S. Continuous Production of Biodiesel via Transesterification from Vegetable Oils in Supercritical Methanol. *Energy and Fuels* **2006**, *20* (2), 812–817.

(89) Kusdiana, D.; Saka, S. Biodiesel Fuel for Diesel Fuel Substitute Prepared by a Catalyst-Free Supercritical Methanol. *Fuel* **2001**, *80* (2), 225–231.

(90) Durrett, T. P.; Benning, C.; Ohlrogge, J. Plant Triacylglycerols as Feedstocks for the Production of Biofuels. *Plant J.* **2008**, *54* (4), 593–607.

(91) Ferrell, J.; Sarisky-Reed, V. National Algal Biofuels Technology Roadmap. *U.S. Dep.*

Energy **2010**, No. May, 140.

(92) Milledge, J. J.; Heaven, S. A Review of the Harvesting of Micro-Algae for Biofuel Production. *Rev. Environ. Sci. Biotechnol.* **2013**, *12* (2), 165–178.

(93) Japar, A. S.; Takriff, M. S.; Yasin, N. H. M. Harvesting Microalgal Biomass and Lipid Extraction for Potential Biofuel Production: A Review. *J. Environ. Chem. Eng.* **2017**, *5* (1), 555–563.

(94) Yap, B. H. J.; Crawford, S. A.; Dagastine, R. R.; Scales, P. J.; Martin, G. J. O. Nitrogen Deprivation of Microalgae: Effect on Cell Size, Cell Wall Thickness, Cell Strength, and Resistance to Mechanical Disruption. *J. Ind. Microbiol. Biotechnol.* **2016**, *43* (12), 1671–1680.

(95) Chisti, Y. Constraints to Commercialization of Algal Fuels. *J. Biotechnol.* **2013**, *167* (3), 201–214.

(96) Khoo, H. H.; Sharratt, P. N.; Das, P.; Balasubramanian, R. K.; Naraharisetti, P. K.; Shaik, S. Life Cycle Energy and CO2 Analysis of Microalgae-to-Biodiesel: Preliminary Results and Comparisons. *Bioresour. Technol.* **2011**, *102* (10), 5800–5807.

(97) Crampon, C.; Mouahid, A.; Toudji, S. A. A.; Lepine, O.; Badens, E. Influence of Pretreatment on Supercritical CO2 Extraction from Nannochloropsis Oculata. *J. Supercrit. Fluids* **2013**, *79*, 337–344.

(98) Soh, L.; Zimmerman, J. Biodiesel Production: The Potential of Algal Lipids Extracted with Supercritical Carbon Dioxide. *Green Chem.* **2011**, *13* (6), 1422–1429.

(99) Paudel, A.; Jessop, M. J.; Stubbins, S. H.; Champagne, P.; Jessop, P. G. Extraction of Lipids from Microalgae Using CO2-Expanded Methanol and Liquid CO2. *Bioresour. Technol.* **2015**, *184*, 286–290.

(100) Orr, V. C. A.; Rehmann, L. Ionic Liquids for the Fractionation of Microalgae Biomass. *Curr. Opin. Green Sustain. Chem.* **2016**, *2*, 22–27.

(101) Molina-Grima, E.; Gonzalez, J. I.; Gimenez, A. Solvent Extraction for Microalgae Lipids. *Biotechnol. Advances*. **2013**, *20*, 491-515.

(102) Tang, Y.; Zhang, Y.; Rosenberg, J. N.; Sharif, N.; Betenbaugh, M. J.; Wang, F. Efficient Lipid Extraction and Quantification of Fatty Acids from Algal Biomass Using Accelerated Solvent Extraction (ASE). *RSC Adv.* **2016**, *6* (35), 29127–29134.

(103) Folch. A Simple. **1987**, *55* (5), 999–1033.

(104) Bligh, E. G.; Dyer, W. J. A Rapid Method of Total Lipid Extraction and Purification. *Can. J. Biochem. Physiol.* **1959**, *37* (8), 911–917.

(105) Hidalgo, P.; Ciudad, G.; Navia, R. Evaluation of Different Solvent Mixtures in Esterifiable Lipids Extraction from Microalgae Botryococcus Braunii for Biodiesel Production. *Bioresour. Technol.* **2016**, *201*, 360–364.

(106) Kuan, D.; Du, W.; Dai, L.; Ma, G.; Liu, D. Effect of Solvent on the Extraction of Microalgae Lipid for Biodiesel Production. *Chem. Res. Chinese Univ.* **2016**, *32* (4), 625–629.

(107) Chen, C.-L.; Chang, J.-S.; Lee, D.-J. Dewatering and Drying Methods for Microalgae. *Dry. Technol.* **2015**, *33* (4), 443–454.

(108) Jessop, P. G.; Leitner, W. Supercritical Fluids as Media for Chemical Reactions. In *Chemical Synthesis Using Supercritical Fluids*; Wiley-VCH Verlag GmbH: Weinheim, Germany, 2007; pp 1–36.

(109) Mendes, R. L.; Nobre, B. P.; Cardoso, M. T.; Pereira, A. P.; Palavra, A. F. Supercritical Carbon Dioxide Extraction of Compounds with Pharmaceutical Importance from Microalgae. *Inorganica Chim. Acta* **2003**, *356*, 328–334.

(110) Martin, L.; Skinner, C.; Marriott, R. J. Supercritical Extraction of Oil Seed Rape: Energetic Evaluation of Process Scale. *J. Supercrit. Fluids* **2014**, *105*, 55–59.

(111) Li, Y.; Ghasemi Naghdi, F.; Garg, S.; Adarme-Vega, T.; Thurecht, K. J.; Ghafor, W.;

Tannock, S.; Schenk, P. M. A Comparative Study: The Impact of Different Lipid Extraction Methods on Current Microalgal Lipid Research. *Microb. Cell Fact.* **2014**, *13* (1), 14.

(112) Liau, B. C.; Shen, C. T.; Liang, F. P.; Hong, S. E.; Hsu, S. L.; Jong, T. T.; Chang, C. M. J. Supercritical Fluids Extraction and Anti-Solvent Purification of Carotenoids from Microalgae and Associated Bioactivity. *J. Supercrit. Fluids* **2010**, *55* (1), 169–175.

(113) Sahena, F.; Zaidul, I. S. M.; Jinap, S.; Karim, A. A.; Abbas, K. A.; Norulaini, N. A. N.; Omar, A. K. M. Application of Supercritical CO2 in Lipid Extraction - A Review. *J. Food Eng.* **2009**, *95* (2), 240–253.

(114) Macías-Sánchez, M. D.; Serrano, C. M.; Rodríguez, M. R.; Martínez de la Ossa, E. Kinetics of the Supercritical Fluid Extraction of Carotenoids from Microalgae with CO2 and Ethanol as Cosolvent. *Chem. Eng. J.* **2009**, *150* (1), 104–113.

(115) Mercer, P.; Armenta, R. E. Developments in Oil Extraction from Microalgae. *Eur. J. Lipid Sci. Technol.* **2011**, *113* (5), 539–547.

(116) Halim, R.; Gladman, B.; Danquah, M. K.; Webley, P. A. Oil Extraction from Microalgae for Biodiesel Production. *Bioresour. Technol.* **2011**, *102* (1), 178–185.

(117) Marshall, W. L.; Jones, E. V. Liquid-Vapor Critical Temperatures of Several Aqueous-Organic and Organic-Organic Solution Systems. *J. Inorg. Nucl. Chem.* **1974**, *36* (10), 2319–2323.

(118) Taher, H.; Al-Zuhair, S.; Al-Marzouqi, A. H.; Haik, Y.; Farid, M.; Tariq, S. Supercritical Carbon Dioxide Extraction of Microalgae Lipid: Process Optimization and Laboratory Scale-Up. *J. Supercrit. Fluids* **2014**, *86*, 57–66.

(119) McKennedy, J.; ??nen??, S.; Pala, M.; Maguire, J. Supercritical Carbon Dioxide Treatment of the Microalgae Nannochloropsis Oculata for the Production of Fatty Acid Methyl Esters. *J. Supercrit. Fluids* **2016**, *116*, 264–270.

(120) Viguera, M.; Marti, A.; Masca, F.; Prieto, C.; Calvo, L. The Process Parameters and Solid Conditions That Affect the Supercritical CO2 Extraction of the Lipids Produced by Microalgae. *J. Supercrit. Fluids* **2016**, *113*, 16–22.

(121) Subramaniam, B. Gas-Expanded Liquids for Sustainable Catalysis and Novel Materials: Recent Advances. *Coord. Chem. Rev.* **2010**, *254* (15–16), 1843–1853.

(122) *Gas-Expanded Liquids and Near-Critical Media*; Hutchenson, K. W., Scurto, A. M., Subramaniam, B., Eds.; ACS Symposium Series; American Chemical Society: Washington, DC, 2009; Vol. 1006.

(123) Lin, H.-W.; Yen, C. H.; Hsu, H.; Tan, C.-S. CO2 Promoted Hydrogenolysis of Benzylic Compounds in Methanol and Water. *RSC Adv.* **2013**, *3* (38), 17222.

(124) Wang, T. H.; Hsu, C. L.; Huang, C. H.; Hsieh, Y. K.; Tan, C. S.; Wang, C. F. Environmental Impact of CO2-Expanded Fluid Extraction Technique in Microalgae Oil Acquisition. *J. Clean. Prod.* **2016**, *137*, 813–820.

(125) Collotta, M.; Busi, L.; Champagne, P.; Romagnoli, F.; Tomasoni, G.; Mabee, W.; Alberti, M. Comparative LCA of Three Alternative Technologies for Lipid Extraction in Biodiesel from Microalgae Production. *Energy Procedia* **2017**, *113*, 244–250.

(126) NIST Chemistry WebBook http://webbook.nist.gov/chemistry/ (accessed Sep 4, 2017).

(127) Moyler, D. A. Extraction of Essential Oils with Carbon Dioxide. *Flavour Fragr. J.* **1993**, *8* (5), 235–247.

(128) Chen, K. T.; Cheng, C. H.; Wu, Y. H.; Lu, W. C.; Lin, Y. H.; Lee, H. T. Continuous Lipid Extraction of Microalgae Using High-Pressure Carbon Dioxide. *Bioresour. Technol.* **2013**, *146* (13), 23–26.

(129) Baumann, M. D.; Daugulis, A. J.; Jessop, P. G. Phosphonium Ionic Liquids for Degradation of Phenol in a Two-Phase Partitioning Bioreactor. *Appl. Microbiol. Biotechnol.* **2005**, *67* (1), 131–137.

(130) Wahidin, S.; Idris, A.; Shaleh, S. R. M. Ionic Liquid as a Promising Biobased Green Solvent in Combination with Microwave Irradiation for Direct Biodiesel Production. *Bioresour. Technol.* **2016**, *206*, 150–154.

(131) Choi, S. A.; Lee, J. S.; Oh, Y. K.; Jeong, M. J.; Kim, S. W.; Park, J. Y. Lipid Extraction from Chlorella Vulgaris by Molten-Salt/ionic-Liquid Mixtures. *Algal Res.* **2014**, *3* (1), 44–48.

(132) Huddleston, J. G.; Visser, A. E.; Reichert, W. M.; Willauer, H. D.; Broker, G. A.; Rogers, R. D. Characterization and Comparison of Hydrophilic and Hydrophobic Room Temperature Ionic Liquids Incorporating the Imidazolium Cation. *Green Chem.* **2001**, *3* (4), 156–164.

(133) Zhang, Y.; Bakshi, B. R.; Demessie, E. S. Life Cycle Assessment of an Ionic Liquid versus Molecular Solvents and Their Applications. *Environ. Sci. Technol.* **2008**, *42* (5), 1724–1730.

(134) Jessop, P. G. Searching for Green Solvents. *Green Chem.* **2011**, *13* (6), 1391.

(135) George, A.; Brandt, A.; Tran, K.; Zahari, S. M. S. N. S.; Klein-Marcuschamer, D.; Sun, N.; Sathitsuksanoh, N.; Shi, J.; Stavila, V.; Parthasarathi, R.; et al. Design of Low-Cost Ionic Liquids for Lignocellulosic Biomass Pretreatment. *Green Chem.* **2015**, *17* (3), 1728–1734.

(136) Jessop, P. P. G.; Heldebrant, D. J. D.; Li, X.; Eckert, C. A. C.; Liotta, C. C. L. Green Chemistry: Reversible Nonpolar-to-Polar Solvent. *Nature* **2005**, *436* (August), 1102.

(137) Jessop, P. G. *Switchable Solvents as Media for Synthesis and Separations: An Update from the Co-Creator of GreenCentre Canada*; 2015; Vol. 48.

(138) Phan, L.; Chiu, D.; Heldebrant, D. J.; Huttenhower, H.; John, E.; Li, X.; Pollet, P.; Wang, R.; Eckert, C. A.; Liotta, C. L.; et al. Switchable Solvents Consisting of Amidine/alcohol or Guanidine/alcohol Mixtures. *Ind. Eng. Chem. Res.* **2008**, *47* (3), 539–545.

(139) Yamada, T.; Lukac, P. J.; George, M.; Weiss, R. G. Reversible, Room-Temperature Ionic Liquids. Amidinium Carbamates Derived from Amidines and Aliphatic Primary Amines with Carbon Dioxide. *Chem. Mater.* **2007**, *19* (5), 967–969.

(140) Samorì, C.; Torri, C.; Samorì, G.; Fabbri, D.; Galletti, P.; Guerrini, F.; Pistocchi, R.; Tagliavini, E. Extraction of Hydrocarbons from Microalga Botryococcus Braunii with Switchable Solvents. *Bioresour. Technol.* **2010**, *101* (9), 3274–3279.

(141) Du, Y.; Schuur, B.; Samor??, C.; Tagliavini, E.; Brilman, D. W. F. Secondary Amines as Switchable Solvents for Lipid Extraction from Non-Broken Microalgae. *Bioresour. Technol.* **2013**, *149*, 253–260.

(142) Du, Y.; Schuur, B.; Kersten, S. R. A.; Brilman, D. W. F. Opportunities for Switchable Solvents for Lipid Extraction from Wet Algal Biomass: An Energy Evaluation. *Algal Res.* **2015**, *11*, 271–283.

(143) Jessop, P. G.; Phan, L.; Carrier, A.; Robinson, S.; Dürr, C. J.; Harjani, J. R. A Solvent Having Switchable Hydrophilicity. *Green Chem.* **2010**, *12* (5), 809–814.

(144) Jessop, P. G.; Kozycz, L.; Rahami, Z. G.; Schoenmakers, D.; Boyd, A. R.; Wechsler, D.; Holland, A. M. Tertiary Amine Solvents Having Switchable Hydrophilicity. *Green Chem.* **2011**, *13* (3), 619–623.

(145) Pan, X.; Niu, G.; Liu, H. Comparison of Microwave-Assisted Extraction and Conventional Extraction Techniques for the Extraction of Tanshinones from Salvia Miltiorrhiza Bunge. *Biochem. Eng. J.* **2002**, *12* (1), 71–77.

(146) Pan, X.; Niu, G.; Liu, H. Microwave-Assisted Extraction of Tea Polyphenols and Tea Caffeine from Green Tea Leaves. *Chem. Eng. Process.* **2003**, *42* (2), 129–133.

(147) Mandal, V.; Mohan, Y.; Hemalatha, S. Microwave Assisted Extraction - An Innovative and Promising Extraction Tool for Medicinal Plant Research. *Pharmacogn. Rev.* **2007**, *1* (1), 7–18.

(148) Choi, I.; Cho, S. J.; Chun, J. K.; Moon, T. W. Extraction Yield of Soluble Protein and Microstructure of Soybean Affected by Microwave Heating. *J. Food Process. Preserv.* **2006**, *30* (4), 407–419.

(149) Iqbal, J.; Theegala, C. Microwave Assisted Lipid Extraction from Microalgae Using Biodiesel as Co-Solvent. *Algal Res.* **2013**, *2* (1), 34–42.

(150) Pan, J.; Muppaneni, T.; Sun, Y.; Reddy, H. K.; Fu, J.; Lu, X.; Deng, S. Microwave-Assisted Extraction of Lipids from Microalgae Using an Ionic Liquid Solvent [BMIM][HSO4]. *Fuel* **2016**, *178*, 49–55.

(151) Ali, M.; Watson, I. A. Microwave Treatment of Wetalgal Paste for Enhanced Solvent Extraction of Lipids for Biodiesel Production. *Renew. Energy* **2015**, *76*, 470–477.

(152) D'oca, M. G. M.; Viêgas, C. V.; Lemões, J. S.; Miyasaki, E. K.; Morón-Villarreyes, J. A.; Primel, E. G.; Abreu, P. C. Production of FAMEs from Several Microalgal Lipidic Extracts and Direct Transesterification of the Chlorella Pyrenoidosa. *Biomass and Bioenergy* **2011**, *35* (4), 1533–1538.

(153) Adam, F.; Abert-Vian, M.; Peltier, G.; Chemat, F. "Solvent-Free" ultrasound-Assisted Extraction of Lipids from Fresh Microalgae Cells: A Green, Clean and Scalable Process. *Bioresour. Technol.* **2012**, *114*, 457–465.

(154) Yamamoto, K.; King, P. M.; Wu, X.; Mason, T. J.; Joyce, E. M. Effect of Ultrasonic Frequency and Power on the Disruption of Algal Cells. *Ultrason. Sonochem.* **2015**, *24*, 165–171.

(155) Ferreira, A. F.; Dias, A. P. S.; Silva, C. M.; Costa, M. Effect of Low Frequency Ultrasound on Microalgae Solvent Extraction: Analysis of Products, Energy Consumption and Emissions. *Algal Res.* **2016**, *14*, 9–16.

(156) Wang, M.; Yuan, W. Microalgal Cell Disruption via Ultrasonic Nozzle Spraying. *Appl. Biochem. Biotechnol.* **2015**, *175* (2), 1111–1122.

(157) Shirsath, S. R.; Sonawane, S. H.; Gogate, P. R. Intensification of Extraction of Natural Products Using Ultrasonic Irradiations-A Review of Current Status. *Chem. Eng. Process. Process Intensif.* **2012**, *53* (March 2015), 10–23.

(158) Ciriminna, R.; Carnaroglio, D.; Delisi, R.; Arvati, S.; Tamburino, A.; Pagliaro, M. Industrial Feasibility of Natural Products Extraction with Microwave Technology. *ChemistrySelect* **2016**, *1* (3), 549–555.

(159) Fawley, M.; Fawley, K. A Simple and Rapid Technique for the Isolation of Dna From Microalgae1. *J. Phycol.* **2004**, *225* (40), 223–225.

(160) Lai, Y. S.; Francesco, F. De; Aguinaga, A.; Parameswaran, P.; Rittmann, B. E. Improving Lipid Recovery from Scenedesmus Wet Biomass by Surfactant-Assisted Disruption. *Green Chem.* **2016**, *18* (5), 1319–1326.

(161) Corre, G.; Templier, J.; Largeau, C. Influence Of Cell Wall Composition On The Resistance Of Two Chlorella Species (*Chlorophyta*) To Detergents. *J. Phycol.* **1996**, *32* (April), 584–590.

(162) Huang, W. C.; Kim, J. D. Cationic Surfactant-Based Method for Simultaneous Harvesting and Cell Disruption of a Microalgal Biomass. *Bioresour. Technol.* **2013**, *149*, 579–581.

(163) Ulloa, G.; Coutens, C.; Sánchez, M.; Sineiro, J.; Fábregas, J.; Deive, F. J.; Rodríguez, A.; Núñez, M. J. On the Double Role of Surfactants as Microalga Cell Lysis Agents and Antioxidants Extractants. *Green Chem.* **2012**, *14* (4), 1044.

(164) Tharapiwattananon, N.; Tharapiwattananon, N.; Scamehorn, J.; Scamehorn, J.; Osuwan, S.; Osuwan, S.; Harwell, J.; Harwell, J.; Haller, K.; Haller, K. Surfactant Recovery from Water Using Foam Fractionation. *Sep. Sci. Technol.* **1996**, *31* (9), 1233–1258.

(165) Höök, M.; Tang, X. Depletion of Fossil Fuels and Anthropogenic Climate Change - A Review. *Energy Policy* **2013**, *52*, 797–809.

(166) Demirbas, A. Importance of Biodiesel as Transportation Fuel. *Energy Policy* **2007**, *35* (9),

4661–4670.

(167) Ahmed, A. S.; Khan, S.; Hamdan, S.; Rahman, R.; Kalam, A.; Masjuki, H. H. Biodiesel Production from Macro Algae as a Green Fuel for Diesel Engine. *J. Energy Environ.* **2010**, No. 2010, 1–5.

(168) Sriram, S.; Seenivasan, R. Microalgae Cultivation in Wastewater for Nutrient Removal. *J. Algal Biomass Util.* **2012**, *3* (2), 9–13.

(169) Hallett, J. P.; Kitchens, C. L.; Hernandez, R.; Liotta, C. L.; Eckert, C. A. Probing the Cybotactic Region in Gas-Expanded Liquids (GXLs). *Acc. Chem. Res.* **2006**, *39* (8), 531–538.

(170) Choi, K.; Ryu, J.; Park, D.; Oh, S.; Kwak, H. Lipid Extraction from Nannochloropsis sp. Microalgae for Biodiesel Production Using Supercritical Carbon Dioxide. *Korean Chem. Eng. Res.* **2015**, *53* (2), 205–210.

(171) Laurens, L. M. L.; Nagle, N.; Davis, R.; Sweeney, N.; Van Wychen, S.; Lowell, A.; Pienkos, P. T. Acid-Catalyzed Algal Biomass Pretreatment for Integrated Lipid and Carbohydrate-Based Biofuels Production. *Green Chem.* **2015**, *17* (2), 1145–1158.

(172) Lam, M. K.; Lee, K. T. Catalytic Transesterification of High Viscosity Crude Microalgae Lipid to Biodiesel: Effect of Co-Solvent. *Fuel Process. Technol.* **2013**, *110*, 242–248.

(173) In, M.; Aguerre-Chariol, O.; Zana, R. Closed-Looped Micelles in Surfactant Tetramer Solutions. *J.Phys.Chem.B* **1999**, *103* (37), 7747–7750.

(174) Li, W.; Zhang, M.; Zhang, J.; Han, Y. Self-Assembly of Cetyl Trimethylammonium Bromide in Ethanol-Water Mixtures. *Front. Chem. China* **2006**, *1* (4), 438–442.

(175) Linke, D. *Chapter 34 Detergents. An Overview*, 1st ed.; Elsevier Inc., 2009; Vol. 463.

(176) Anatrace. Detergents and Their Uses in Membrane Protein Science. **2007**, 1–17.

(177) Knothe, G. " Designer " Biodiesel : Optimizing Fatty Ester Composition to Improve Fuel Properties. *Energy and Fuels.* **2008**, *22*, 1358–1364.

(178) Hill, J.; Nelson, E.; Tilman, D.; Polasky, S.; Tiffany, D. Environmental, Economic, and Energetic Costs and Benefits of Biodiesel and Ethanol Biofuels. *Proc. Natl. Acad. Sci.* **2006**, *103* (30), 11206–11210.

(179) Qiu, F.; Li, Y.; Yang, D.; Li, X.; Sun, P. Biodiesel Production from Mixed Soybean Oil and Rapeseed Oil. *Appl. Energy* **2011**, *88* (6), 2050–2055.

(180) Silva, C. C. C. M.; Ribeiro, N. F. P.; Souza, M. M. V. M.; Aranda, D. A. G. Biodiesel Production from Soybean Oil and Methanol Using Hydrotalcites as Catalyst. *Fuel Process. Technol.* **2010**, *91* (2), 205–210.

(181) De Lima Da Silva, N.; Batistella, C. B.; Filho, R. M.; Maciel, M. R. W. Investigation of Biofuels Properties. *Chem. Eng. Trans.* **2011**, *25*, 851–856.

(182) Martins, M. I.; Pires, R. F.; Alves, M. J.; Hori, C. E.; Reis, M. H. M.; Cardoso, V. L. Transesterification of Soybean Oil for Biodiesel Production Using Hydrotalcite as Basic Catalyst. *Chem. Eng. Trans.* **2013**, *32*, 817–822.

(183) Li, Y.; Qiu, F.; Yang, D.; Sun, P.; Li, X. Transesterification of Soybean Oil and Analysis of Bioproduct. *Food Bioprod. Process.* **2012**, *90* (2), 135–140.

(184) Liu, X.; He, H.; Wang, Y.; Zhu, S.; Piao, X. Transesterification of Soybean Oil to Biodiesel Using CaO as a Solid Base Catalyst. *Fuel* **2008**, *87* (2), 216–221.

(185) de Lima, A. L.; Ronconi, C. M.; Mota, C. J. A. Heterogeneous Basic Catalysts for Biodiesel Production. *Catal. Sci. Technol.* **2016**, *6* (9), 2877–2891.

(186) Veljković, V. B.; Lakićević, S. H.; Stamenković, O. S.; Todorović, Z. B.; Lazić, M. L. Biodiesel Production from Tobacco (Nicotiana Tabacum L.) Seed Oil with a High Content of Free Fatty Acids. *Fuel* **2006**, *85* (17–18), 2671–2675.

(187) Sharma, Y. C.; Singh, B. Development of Biodiesel: Current Scenario. *Renew. Sustain. Energy Rev.* **2009**, *13* (6–7), 1646–1651.

(188) Leung, D. Y. C.; Wu, X.; Leung, M. K. H. A Review on Biodiesel Production Using

Catalyzed Transesterification. *Appl. Energy* **2010**, *87* (4), 1083–1095.

(189) Phan, L.; Andreatta, J. R.; Horvey, L. K.; Edie, C. F.; Luco, A. L.; Mirchandani, A.; Darensbourg, D. J.; Jessop, P. G. Switchable-Polarity Solvents Prepared with a Single Liquid Component. *J. Org. Chem.* **2008**, *73* (1), 127–132.

(190) Samorì, C.; López Barreiro, D.; Vet, R.; Pezzolesi, L.; Brilman, D. W. F.; Galletti, P.; Tagliavini, E. Effective Lipid Extraction from Algae Cultures Using Switchable Solvents. *Green Chem.* **2013**, *15* (2), 353.

(191) Holland, a; Wechsler, D.; Patel, a; Molloy, B. M.; Boyd, a R.; Jessop, P. G. Separation of Bitumen from Oil Sands Using a Switchable Hydrophilicity Solvent. *Can. J. Chem. Can. Chim.* **2012**, *90* (10), 805–810.

(192) Vanderveen, J. R.; Patiny, L.; Chalifoux, C. B.; Jessop, M. J.; Jessop, P. G. A Virtual Screening Approach to Identifying the Greenest Compound for a Task: Application to Switchable-Hydrophilicity Solvents. *Green Chem.* **2015**, 5182–5188.

(193) Chemical Book http://www.chemicalbook.com/ChemicalProductProperty_EN_CB5422197.htm (accessed Oct 2, 2017).

(194) Dijkstra, A. J. Revisiting the Formation of Trans Isomers during Partial Hydrogenation of Triacylglycerol Oils. *Eur. J. Lipid Sci. Technol.* **2006**, *108* (3), 249–264.

(195) Vanderveen, J. Personal Communication. 2017.

(196) Mubarak, M.; Shaija, A.; Suchithra, T. V. A Review on the Extraction of Lipid from Microalgae for Biodiesel Production. *Algal Res.* **2015**, *7*, 117–123.

(197) Yoo, G.; Park, W. K.; Kim, C. W.; Choi, Y. E.; Yang, J. W. Direct Lipid Extraction from Wet Chlamydomonas Reinhardtii Biomass Using Osmotic Shock. *Bioresour. Technol.* **2012**, *123*, 717–722.

(198) Vigh, L.; Gombos, Z.; Joo, F. Selective Modification of Cytoplasmic Membrane Fluidity

by Catalytic-Hydrogenation Provides Evidence on Its Primary Role in Chilling Susceptibility of the Blue-Green-Alga, Anacystis- Nidulans. *FEBS Lett.* **1985**, *191* (2), 200–204.

(199) Vigh, L.; Joo, F. Modulation of Membrane Fluidity by Catalytic-Hydrogenation Affects the Chilling Susceptibility of the Blue-Green-Alga, Anacystis-Nidulans. *FEBS Lett.* **1983**, *162* (2), 423–427.

(200) VIGH, L.; JOÓ, F.; CSÉPLÓ, Á. Modulation of Membrane Fluidity in Living Protoplasts of Nicotiana Plumbaginifolia by Catalytic Hydrogenation. *Eur. J. Biochem.* **1985**, *146* (2), 241–244.

(201) Lee, J. Y.; Yoo, C.; Jun, S. Y.; Ahn, C. Y.; Oh, H. M. Comparison of Several Methods for Effective Lipid Extraction from Microalgae. *Bioresour. Technol.* **2010**, *101* (1 SUPPL.), S75–S77.

(202) Bodennec, J.; Koul, O.; Aguado, I.; Brichon, G.; Zwingelstein, G.; Portoukalian, J. A Procedure for Fractionation of Sphingolipid Classes by Solid-Phase Extraction on Aminopropyl Cartridges. *J. Lipid Res.* **2000**, *41* (9), 1524–1531.

(203) Cooksey, K. E.; Guckert, J. B.; Williams, S. A.; Callis, P. R. Fluorometric Determination of the Neutral Lipid Content of Microalgal Cells Using Nile Red. *J. Microbiol. Methods* **1987**, *6* (6), 333–345.

(204) Rodríguez, A. M.; Prieto, P.; De La Hoz, A.; Díaz-Ortiz, Á.; Martín, D. R.; García, J. I. Influence of Polarity and Activation Energy in Microwave-Assisted Organic Synthesis (MAOS). *ChemistryOpen* **2015**, *4* (3), 308–317.

(205) Wang, Z. T.; Ullrich, N.; Joo, S.; Waffenschmidt, S.; Goodenough, U. Algal Lipid Bodies: Stress Induction, Purification, and Biochemical Characterization in Wild-Type and Starchless Chlamydomonas Reinhardtit. *Eukaryot. Cell* **2009**, *8* (12), 1856–1868.

(206) Ranjith Kumar, R.; Hanumantha Rao, P.; Arumugam, M. Lipid Extraction Methods from

Microalgae: A Comprehensive Review. *Front. Energy Res.* **2015**, *2* (January), 1–9.