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Value-added Lipids Extraction From Wet Microalgae Using Ionic Liquids

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Abstract

Microalgae have gained interest as sources of renewable lipids in the biofuel sector due to their ability to sequester carbon dioxide into triacylglycerol (TAG), a biodiesel feedstock. However, industrial-scale production of microalgae exclusively for fuel production is limited by technical and economic challenges. Some marine microalgae can accumulate large amounts of polyunsaturated fatty acids (PUFAs) such as docosahexaenoic acid (DHA) and other unsaturated fatty acids, which are high-value compounds linked to the prevention of various cardiovascular diseases. This thesis, therefore, examines the extraction of lipids and DHA from two microorganisms (*Chlorella vulgaris*, a model organism for lipid production and *Thraustochytrium sp.*, an industrially relevant DHA producer).

Ionic liquids (ILs) have been shown to assist in cell disruption and lipid extraction from microalgae. Recently, a recently described ionic liquid was therefore synthesized and characterized for lipid extraction. It was used in comparison with commercially available ILs throughout this thesis. Initially, lipid extraction was evaluated by dry samples of the model organism *C. vulgaris*, followed the processing of wet extraction from fresh algae samples. Treating wet biomass is technically more challenging, but a necessity, as complete dewatering and drying economically is not feasible. To further enhance the IL-mediated extraction and cell disruption, the previously evaluated process was combined with electrolysis. Electrolytic pretreatment of microalgae can disrupt the cell wall, aiding in the release of intracellular lipids. ILs have high electric conductivity and hence synergies were expected when combining these two treatments. The obtained extraction efficiency was up to 44.5 ± 2.4% for the combined process compared to 28.8 ± 1.0% with ILs and 1.5 ± 0.7% with electrolysis.

Extraction technologies developed with *C. vulgaris* were subsequently transferred to extract value-added lipids from *Thraustochytrium sp.* (T18) which possesses high polyunsaturated fatty acids (PUFAs) content, with a specifically large percentage of ω-3 fatty acids such as docosahexaenoic acid (DHA). Two ILs were assessed for their ability to facilitate the extraction of PUFAs from dry and fresh *Thraustochytrium sp.* (T18) cultures. The results show that ILs could facilitate the extraction of over 90% (w/w) of the available oils from dried T18 biomass and still maintain an extraction efficiency of around 80% (w/w) when using wet slurry.

Subsequently, value-added oil extraction from T18 using the synthesized IL was also optimized by a central composite design (CCD) and response surface methodology (RSM). The total extraction efficiency was up to around 97% of DHA-rich oil, showing that ionic liquid based methods might be suitable to process marine microalgae with value-added lipid composition.

Keywords: microalgae; ionic liquid; omega-3; DHA; biodiesel, wet extraction; electrolysis; response surface methodology; value-added products

Summary for Lay Audience

Microalgae, as sources for various valuable products including biodiesel, ω-3 fatty acids, astaxanthin, phenolics, etc., have been regarded as renewable and sustainable solutions to mounting energy demands and environmental issues. However, some conventional extraction processes of these products from microalgae are time- and energy-consuming, such as mechanical and biological techniques. Ionic liquids have been widely shown to pretreat and disrupt cells to assist lipid extraction in a short period time around one hour. Thus, ionic liquid based extraction processes were investigated in this work, focusing on the extraction of lipids for biodiesel and DHA production from two microorganisms *Chlorella vulgaris* and *Thraustochytrium sp.*, respectively.

In this work, some ionic liquids were screened for the above two kinds of microalgae. Extracting lipids from wet biomass is more challenging than from dry biomass due to the eliminations of dewatering and drying steps. Initially, dry samples of *Chlorella vulgaris* were evaluated and followed by wet extraction from fresh algae samples. The extraction efficiencies of dry microalgae are around 75%. Also, electrolysis pretreatment, aiming in aiding the release of lipids from cells, was added to pretreat the fresh samples, where the synergic effect of ionic liquid and electrolysis was expected. The extraction efficiency was up to around 45% for the combined pretreatment compared to around 29% with ILs and around 2% with electrolysis.

The developed extraction technologies were subsequently applied to extract value-added lipids from *Thraustochytrium sp.*(T18) with a high content of ω-3 fatty acids such as DHA. The results show that the extraction efficiency was around 80% (w/w) when using wet slurry. A central composite design was used to optimize the extraction efficiency when extracting

from the dry T18, and the extraction efficiency was found around 97%.The results show that ionic liquid based methods are capable of efficiently assisting value-added lipid extraction from marine microalgae.

In summary, Ionic liquid based extraction process has high potential to efficiently gain valuable materials from various microalgae with relatively low energy and time consumption.

Co-Authorship Statement

Dr. Lars Rehmann provided full supervision to this PhD thesis study.

Chapter 2 has been submitted as a review chapter for a book. Entilted "Application of ionic liquids to extract high-value compounds from marine biomass", **Zhang Y** and Rehmann L. "Innovative and Emerging Technologies in the Bio-marine Food Sector" edited by Marco Garcia-Vaquero and Gaurav Rajauria, 2020 Elsevier Inc.

Chapter 3-5 was coauthored with researchers from Queen's University Belfast. Dr. Natalia V. Plechkova synthesized the ionic liquids used. I performed all ionic liquid experiments and drafted the manuscript in all chapters.

Chapter 6 was coauthored with researchers from the University of Waterloo, Queen's University Belfast, and Mara Renewables. I performed all ionic liquid experiments and drafted the manuscript in the chapter. Other authors provided technical advice and revised the manuscript. **Zhang Y**, Ward V, Dennis D, Plechkova NV, Armenta R, Rehmann L. Efficient extraction of a docosahexaenoic acid (DHA)-rich lipid fraction from *Thraustochytrium sp.* using ionic liquids. *Materials*. **2018** Oct;11(10):1986.

Chapter 7 was coauthored with researchers from the University of Waterloo, Queen's University Belfast, and Mara Renewables. I performed all ionic liquid experiments and drafted the manuscript in the chapter. Other authors provided technical advice and revised the manuscript. **Zhang Y**, Ward V, Dennis D, Plechkova NV, Armenta R, Rehmann L. Extraction of a docosahexaenoic acid (DHA) rich oil from *Thraustochytrium sp.* using a novel hydrophobic ionic liquid (in submission).

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Abbreviations:

ILs	Ionic liquids
PUFAs	Poly unsaturated fatty acids
EPA	Eeicosapentaenoic
DHA	Docosahexaenoic
T18	Thraustochytrium sp.
TAG	Triacylglycerol
FAME	Fatty acid methyl ester
PBPs	Phycobiliproteins
HAp	Hydroxyapatite
FTIR	Fourier transform infrared
NMR	Nuclear magnetic resonance
TGA	Thermal gravimetric analysis
DSC	Differential scanning calorimetry
TAP	Tri-acetate-phosphate
HIP	Hexane/isopropanol extraction
FFD	full factorial design
CCD	Central composite design
2-HEAA	*N*-methyl-2-hydroxyethylammonium acetate
2-HEFA	*N*-methyl-2-hydroxyet-hylammonium formate
$[C_2mim][DBP]$	1-ethyl-3-methylimidazolium dibutylphosphate
$[C_2mim][EtSO_4]$	1-ethyl-3-methylimidazolium ethylsulfate
$[P_{4444}][Prop]$	Tetrabutylphosphonium propionate
$[C_{10}mim][BTMPP]$	1-decyl-3-methylimidazolium bis(2,4,4-trimethylpentyl) phosphonate

Symbols:

η	Viscosity	cP
T	temperature	K
$C_{C15:0}$	Concentration of C15:0	µg/mL
C_{total}	Concentration of total lipids	µg/mL
C_{FAME}	Concentration of FAME	µg/mL
m_{FAME}	Mass of FAME	mg
$m_{microalgae}$	Mass of microalgae	g
m_{oil}	Mass of oil	g
$m_{oil\ sample}$	Mass of sampled oil (GC)	mg
m_{slurry}	Mass of biomass slurry	g

1 Introduction

The dependence on fossil resources for products from industrial chemicals to transportations fuels has created a series of problems, such as greenhouse gas emission, resource shortage, and negative impacts on the environment [18]. Renewable energy production is gaining substantial traction and economically competitive technologies exist for most geographical areas leading to the decarbonization of the energy sector. However chemical building blocks still require carbon-based feedstock and biofuels are a suitable intermediate solution for the decarbonization of the transportation sector [19, 20]. Microalgae, have the potential to become an important biomass feedstock of biodiesel and value-added products such as polyunsaturated fatty acids (PUFAs), due to their high energy conversion efficiency together with the fast growth rates and high lipid contents [21, 22]. Additionally, they are unicellular photosynthetic organisms, without the demand for fertile land, which can be cultivated in the freshwater, sea, and even wastewater [23, 24]. The commercial utilization of microalgae for biodiesel production still faces many challenges, owing to the high cost related to the downstream processes, especially drying processes, cell wall disruptions, and extraction technologies [25, 26]. Additionally, the renewability and sustainability of biofuels need to be investigated.

1.1 Research Background

1.1.1 Lipids

Lipids are molecules such as fatty acids and their derivatives, including triacylglycerides (TAGs), diacylglycerol (DAG), monoglycerides, and phospholipids, as well as other sterols. Oils are mainly composed of TAGs, which are esters of glycerol and long-chain carboxylic acid with the chain length from 4 to 22 carbon atoms [27]. Fig. 1.1 shows the basic chemical structures of free fatty acid and a TAG containing palmitic acid (C16:0), linoleic acid (C18:2), and oleic acid (C18:1). Fatty acids are abbreviated by the number of carbons in their chain followed by the number of unsaturated C–C bonds and the carbon number of the double bonds (e.g., linoleic acid (C18:2) represents a C18 chain with two double bonds). The most common classification of fatty acids is based on their degree of saturation: saturated fatty acids without C=C double bonds and unsaturated fatty acids with one or more C=C double bonds. Crude fats and oils may be isolated by various disruption and extraction technologies, but further purification is critical for most fields, such as foods, paints, detergents and lubricants, varnishes, and biodiesel fuels where they, to a certain extent, compete with petroleum derivatives [28, 29].

A general production process for biodiesel is shown in Fig. 1.2. Compared with petroleum diesel, biodiesel can reduce the toxicity of emissions due to a lack of sulphur and high molecular weight compounds. In addition, photosynthesis can offset the CO_2 released by the combustion process of biodiesel, hence can be carbon neutral depending on the cultivation and processing conditions of the feedstock. Due to the high oxygen content of biodiesel, there is less soot in the combustion exhaust, and the fuel itself is highly biodegradable (in case of spills). Moreover, the combustion residue is slightly acidic, extending the service life of engine

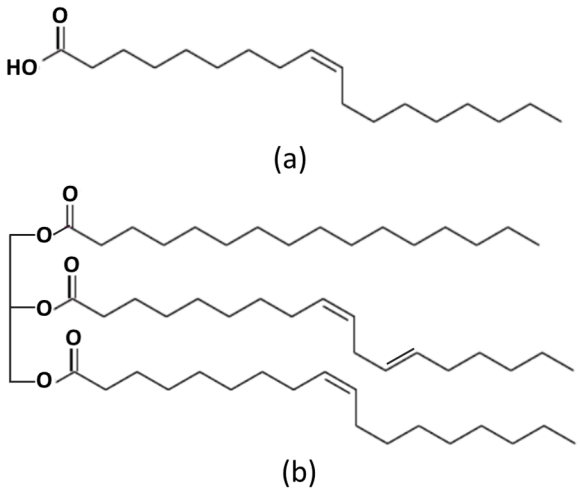

Figure 1.1: Chemical structures. a. oleic acid (C18:1), b. a TAG containing palmitic acid (C16:0), oleic acid (C18:1), and linoleic acid (C18:2).

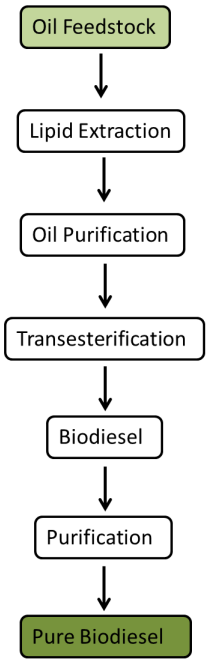

Figure 1.2: A general production process for biodiesel.

oil. There is no need to modify the diesel engine, and it can be directly added and used. Moreover, it is unnecessary for additional fueling equipment, storage and transportation equipment and technical personnel training.

PUFAs, as essential fatty acids, are attracting great attention from the biotech industry

due to lifestyle and dietary requirements [30]. For instance, docosahexaenoic acid (DHA, C22:6, n-3) and eicosapentaenoic acid (EPA; 20:5, n-3) have been proven to have a significant role in the prevention of various diseases [31, 32]. Their chemical structures are shown in Fig. 1.3. Fatty acids are represented by the number of carbons in their chain followed by the number of unsaturated C–C bonds and the carbon number of the first unsaturated bond (e.g., C20:5, n-3 represents a C20 chain with five unsaturated bonds, the first one occurring at carbon 3). Based on the length of the carbon backbone, they are classified into two groups, short-chain polyunsaturated fatty acids (SC-PUFA), with 16 or 20 carbon atoms and long-chain polyunsaturated fatty acids (LC-PUFA) with more than 18 carbon atoms [33].

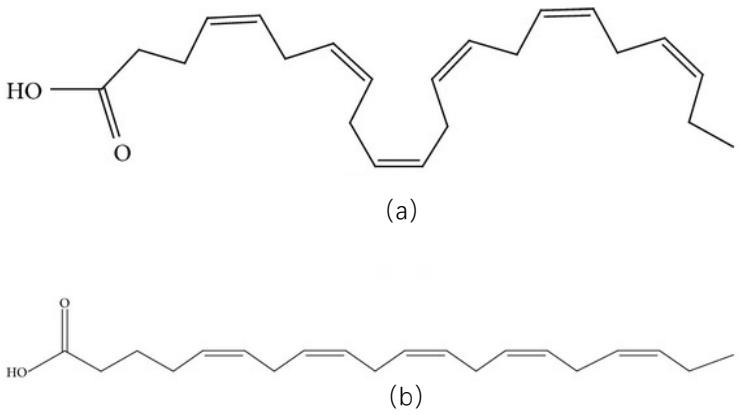

Figure 1.3: Chemical structures. a. docosahexaenoic acid (DHA; C22:6, n-3), b. eicosapentaenoic acid (EPA; C20:5, n-3)

1.1.2 Lipids Feedstocks

Biodiesels are generally produced from oil crops, animal fatty acids, and even waste edible oil. The major concern relating to biodiesel is the struggle of land for food and improvement of the downstream process. Following the EASAC report 2012, the biodiesel feedstocks are typically classified as four generations. The first generation of biodiesels is produced from

the edible feedstocks, for instance, coconut oil, corn oil, olive oil, rapeseed oil, soybean oil, palm oil, mustard oil, etc [8, 34]. Edible feedstock for biodiesel production is fairly and widely accepted at the beginning of biodiesel research due to the ample availability of crops and facile conversion procedures. However, with further investigation, the limitation in the food supply is the major shortcoming [35]. Meanwhile, limited cultivation area, high cost, and adaptability to local climate conditions all impede the development of biodiesel production from edible feedstock [36]. The second generation is obtained from non-edible feedstocks such as jatropha oil, rubber seed oil, Karanja oil, etc. The prime benefit of this generation of biodiesel is no requirement of food plants or agricultural land, but the yields of these plants fall down that can not meet the demand [8].

As for the third generation, the biodiesels are produced from microalgae and waste oils. It is evident that the advantages of microalgae are excellent growth rate, high productivity and so on. The production of biodiesel from microalgal biomass has attracted many researchers to improve the extraction process, reduce the cost, and enhance the yield of biodiesels. Given that fact, this work also focuses on exploring a highly efficient extraction process. Finally, electrofuels and photobiological solar fuels are both regarded as the fourth generation of biodiesels. Conversion from solar energy into biodiesel using some cheap and widely available materials [37]. However, it has high investment at the beginning of the research. To sum up, the advantages and disadvantages of four-generation biodiesels are summarized in Table1.1, which appears in [8] based on information in [9, 10, 11, 12, 13].

Therefore, although over 90% of biodiesels over the world is produced and synthesized from edible oils such as canola oil [38, 39], it is evident that food-grade oil plants or animal fats are both not appropriate feedstocks to produce biodiesel because economic competition could

quickly eliminate this type of low cost-effective product as a result of the high price of the raw materials. The use of plants as the second common feedstocks also has inevitable problems in all countries. On the one hand, the plants must be suitable for the local climate and achieving high and stable yield. On the other hand, the shortage of land resources is inevitable during the planting process to cause the resulting increase in prices of other crops [40, 41]. It is also the waste cooking oils or industrial waste oils that have a lower price than the 1st generation feedstocks for biodiesel production. However, the cost of deriving biodiesels from these wastes is significantly high due to the complexity of downstream, collectin, drying, pretreatment, etc.

Table 1.1: Benefits and limitations of four generations of biodiesel [8, 9, 10, 11, 12, 13]

	First generation	**Second generation**	**Third generation**	**Fourth generation**
Benefits	Easy biodiesel conversion process, easy availability of crops	Not affect on food supply, Feedstocks can be grow on non-arable land, less production cost	Waste food oil can be use for biodiesel production, growth rate of algae is high, not affect on food supply, can be use seawater or waste water for algae growth	More lipid content, more CO_2 absorbing ability, high energy content, rapid growth rate
Limitations	Affect food supply, Low crop yield, Limited area of cultivation, less adaptability of crop to environmental conditions	Less cost-effective conversion technology, low crop yield for some feedstocks	High energy consumption for algae cultivation, low lipid content in open pond system, expensive oil extraction process from algae	High intial investment, research on infancy level

For the production of PUFAs, the compositions typically depend on the different feedstocks, such as some plant seeds, marine fish, and microalgae [28]. PUFAs, such as DHA and EPA are mainly produced from marine fish oil and fish by-products for a long time [42]. Nevertheless, the route for the production of PUFAs is full of rising challenges because of more frequent and severe marine pollution and seasonal variations of fish production [43]. Hence, searching for alternative feedstocks of PUFA-rich oils is necessary for the long-term

development with lower processing costs, less environmental burden, and greater production sustainability.

A promising alternative source of non-edible oil for the production of biodiesel and PUFAs is microalgal lipids. Microalgal lipids present some crucial advantages for cultivation than other feedstocks, such as higher productivity, a faster growth rate, good adaptability in wastewater, and greater independence of climate and agricultural land [27, 44]. Moreover, microalgal lipids possess other merits over marine fish and by-products, such as less impact on ecological balance, greater sustainability of the production, and greater independence of seasons and regions, etc [45, 46, 47].

1.1.3 Microalgal Oil

Microalgae, which is considered as the most promising feedstocks of lipids for the production of biodiesel, mainly benefits from the capacity of synthesis of lipids from CO_2 and sunlight [48, 49]. In the face of many problems in the production of biodiesel from plant materials, the use of microalgae to produce oil has obvious advantages in not competing for land with agriculture, and seawater can be used as a natural medium for mass propagation. Nonetheless, their high cost of downstream processing like dewatering, drying, and extraction steps have limited the widespread commercial applications to value-added products like carotenoids, PUFAs, antioxidants [50, 51].

The utilization process of microalgal oil on different microalgae is shown in Fig. 1.4. After drying microalgae, some methods can be used for oil extraction, such as ultrasound, microwave, mechanical pressing, solvent extraction, supercritical fluid extraction, ionic liquids, etc. Fresh microalgae can also be extracted by several same methods as dry algae shown

in Fig. 1.4, and it can be efficiently treated by enzymatic hydrolysis, pulsed electric field as well. The resultant crude oil is further refined and used as biodiesel or value-added oil. The resulting residue could be utilized as animal food or fermentation to produce acetone, and butanol [52, 53].

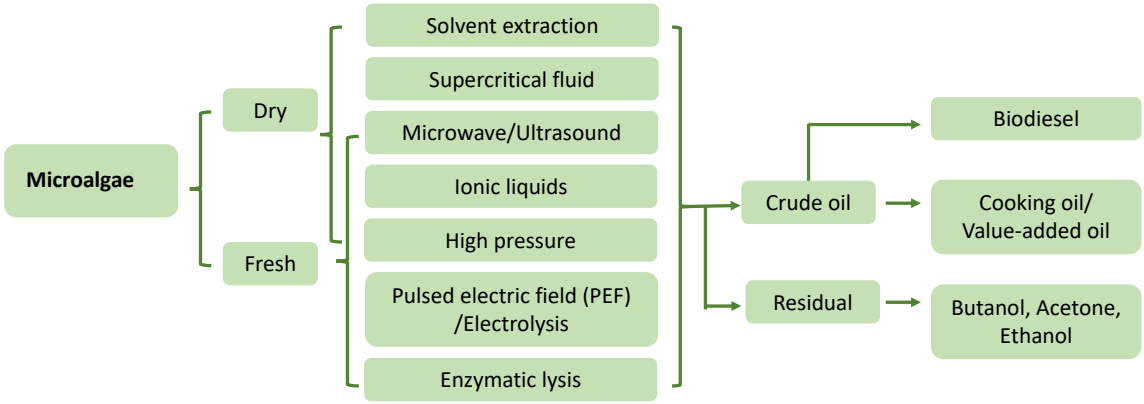

Figure 1.4: Utilization processes of microalagal lipids

1.1.4 Lipid Extraction Process of Microalgal Oil

Microalgae are usually cultivated in aqueous media. Thus the first steps in the lipid extraction process of microalgal oil are harvesting and dewatering steps, which are energy-intensive using liquid-solid separations. Therefore, the development of low-cost dewatering methods has been paid immense attention to, such as centrifugation, sedimentation, flocculation, and filtration [54, 55]. After that, the microalgal cells are supposed to be pretreated to lyse the cells and then release the lipids or other value-added biomolecules. Commonly, the cells are directly dried and milled as a powder for organic solvent extraction [56]. The residual solids of microalgae may contain microalgal lipids, so they are supposed to be recovered by the same solvent. Last but not least, the crude extract requires purification for high yields, which depends on the type of organic solvents [57].

1.1.4.1 Organic solvent extraction

Organic solvent extraction is the most common means for lipids extraction, such as some non-polar organic solvents like chloroform or hexane [58]. Typically, microalgae need to be collected and dewatered for lipid extraction. The extraction process includes several steps [59, 60]: a. Dewatered microalgae contact with solvents through the cell membrane and the cytoplasm. b. The solvents combine with lipids by Van der Waal's forces to become complex. c. The complex goes across the cell membrane and lipids enter into the organic phase, which could be self-divided. d. Extracted lipids are then transesterified to obtain biodiesel. TAGs are regarded as the most readily lipids using one organic solvent like hexane, but the polar lipids are believed to be complexed with proteins or carbohydrates via hydrogen bonding [61, 62]. Two or more solvents can be used simultaneously to improve lipid yield, e.g., methanol/chloroform or hexane/isopropanol. This method is highly effective, but it would produce volatile organic compounds (VOC)-emission and carbohydrates waste, which leads to the environmental problem. Additionally, a pair of common mixtures of organic solvents, to a certain extent, can be used for lipid extraction, such as hexane and isopropanol, and chloroform and methanol [63, 64].

1.1.5 Pretreatment Methods

To improve the extraction efficiency of organic solvent, various pretreated methods have been investigated for cell disruption as shown in 1.5 [65]. The principles of the pretreatment of microalgal biomass are based on the disruption of microalgal cells by using mechanical, chemical, or biological techniques to achieve better bioproduct yield [66]. Once the cell is

disrupted, intracellular products readily release from cells and are extracted by organic solvents, mainly because the rigid cell walls and membranes reduce the biodegradability of the cells [67]. The efficient biomass pretreatment method could enhance the lipid recovery illustrated in Fig. 1.6 [1]. Thus, it is known that cell disruption, which is generally prior to lipid extraction, is an essential step to disrupt the cell walls of microalgal cells to improve the lipid extraction yield because of much more accessible intracellular products in solvent extraction [68, 69].

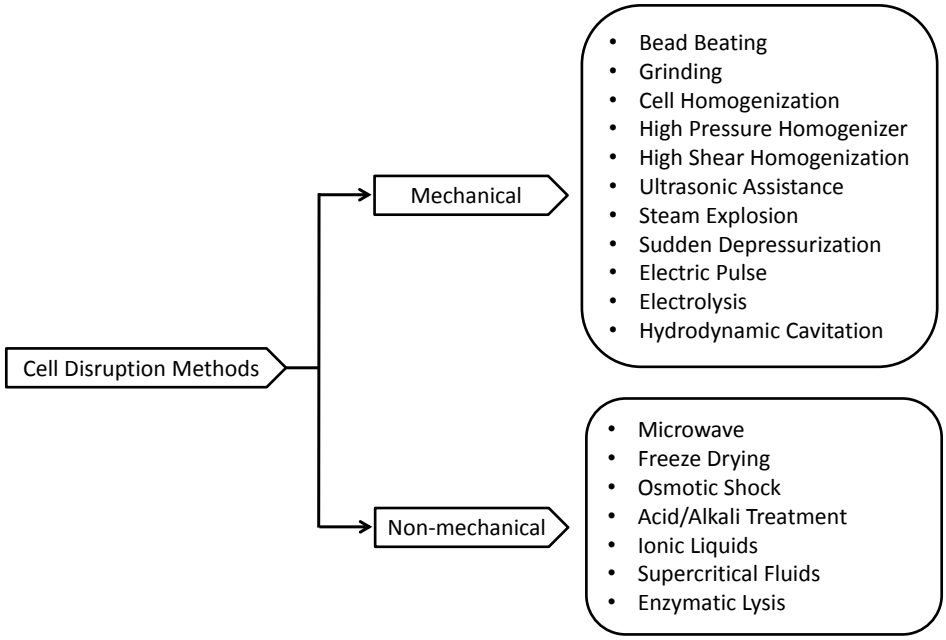

Figure 1.5: Different pretreatment methods for cell disruption process.

Enzymatic lysis is a common pretreatment method for cell disruption. It could destroy and degrade the cell wall by applying biological enzymes so that the oil can be released1 [70]. Firstly, pH is supposed to be adjusted by general acid-base regulation followed by adding enzymes into the cell solution to break the cell wall to prepare extraction processing. It is an eco-friendly but cost consuming approach for cell disruption. The ultrasonic wave has a unique mechanical vibration and cavitation effect on the microalgae for ultrasonic-assisted wall dis-

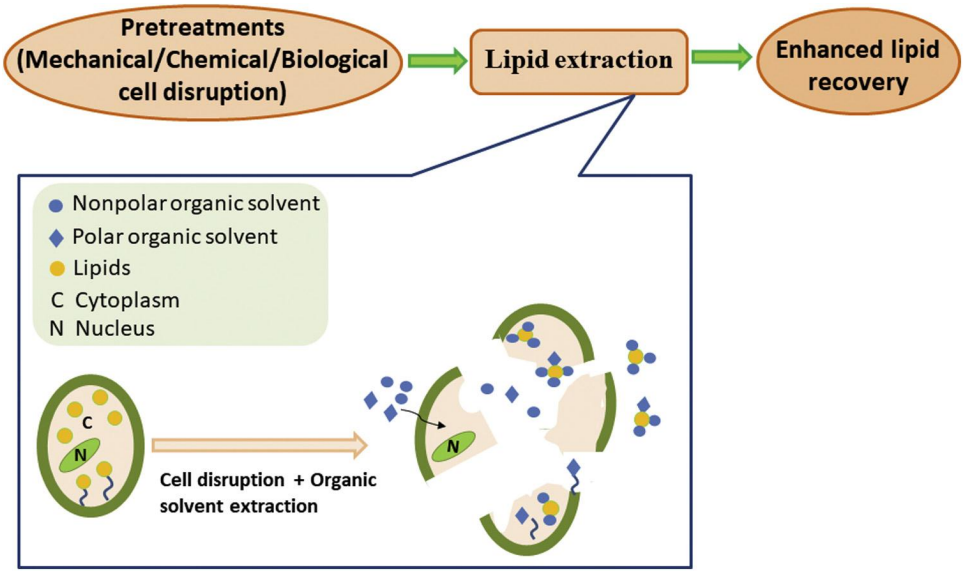

Figure 1.6: Schematic diagram of enhanced lipid extraction through complete cell disruption by pretreatments [1].

ruption. This vibration can generate and spread powerful energy, causing liquid cavitation, which helps to change the cell wall structure. Besides, many secondary effects such as heating, ultrasonic emulsification, dispersion, crushing, chemical, and biological flocculation can also accelerate to ruin the cell wall [71]. The other common method is the microwave. Due to the absorption of microwave energy, the temperature inside the cell increases rapidly, which makes the pressure of the cell exceed the capacity of the cell wall, resulting in cell disruption. The electromagnetic field generated by the microwave accelerates the extracted components diffuse to the extraction solvent interface, so the extraction rate may increase several times [72].

1.1.5.1 Electrical pretreatment

Electrical pretreatment, to some extent, is a relatively new one pretreatment method. The electromagnetic field influences most of the polar molecules in the living cells, which may lead to the non-repair rupture of the cell membrane [73, 74]. These effects are helpful to disrupt the cell wall and enhance lipid extraction [75]. A schematic diagram for enhanced microalgal biomolecules extraction by electrical pretreatment is shown in Fig. 1.7 [2].

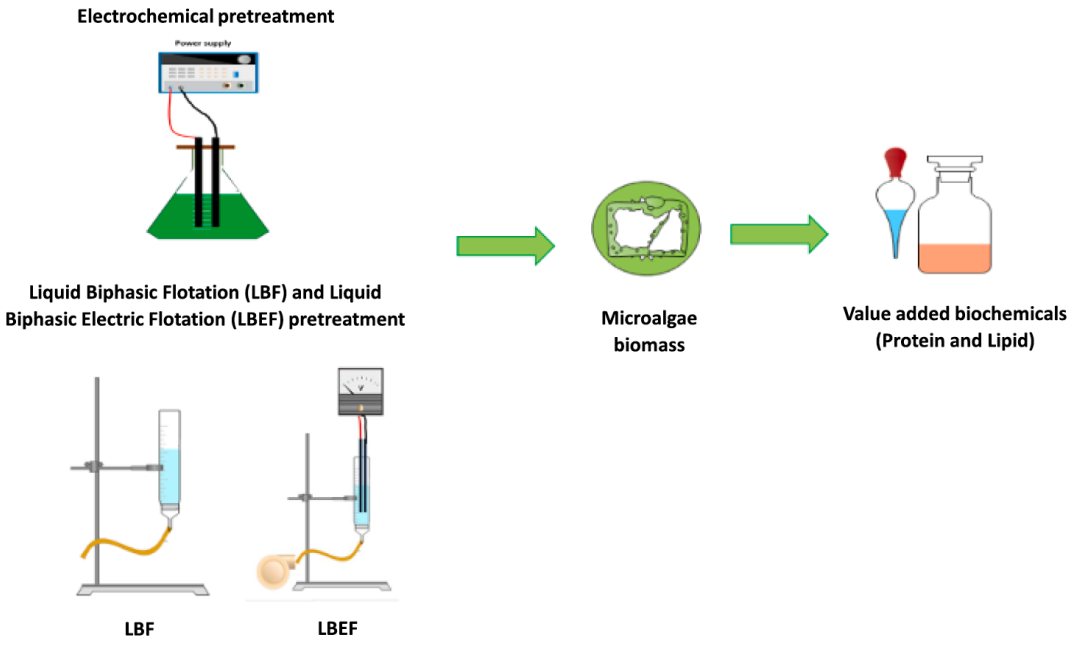

Figure 1.7: Schematic diagram of electrical pretreatment for enhanced microalgal biomolecules extraction [2].

Nowadays, there are four main electrical pretreatment methods for biomass, as shown in Fig. 1.8 [75]. Pulsed electric field (PEF) in microalgae extractions utilizes electrical pulses of high intensity (100 V/cm to 300 kV/cm) to alter the properties of cell walls. This process is also called electroporation for the fact that the cell membrane permeability is enhanced after treatment [76]. The use of high voltage electrical discharges (HVED) is also a cell dis-

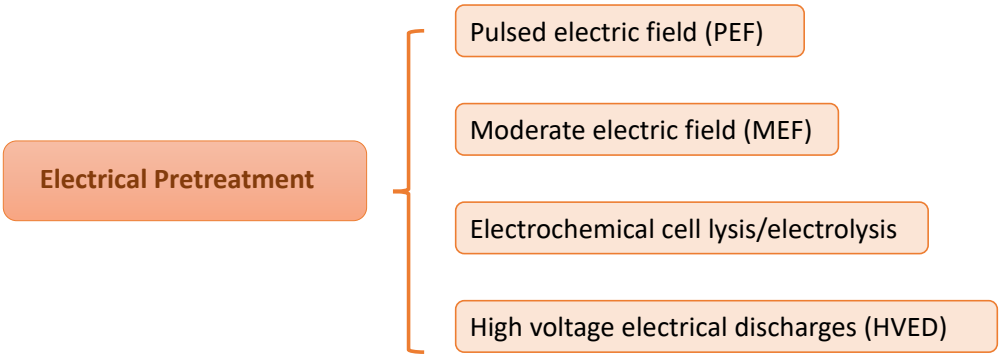

Figure 1.8: Main electrical pretreatment methods.

ruption method, which has been proposed to perform the pretreatment of microalgal cells at reduced diffusion temperature and time and increased extraction rate [77]. Moderate electric field (MEF), or also known as alternating current (AC), is considered as a technique involving a relatively low-intensity electric field varying from 1 to 1 × 103 V/cm [78]. In comparison to PEF, microalgae biomass is also exposed to electrical fields under the pretreatment of electrolysis processes. However, the voltages involved are from 6 to 30 V, and the time of the process is longer. There are still few studies relating to this field. Electrochemical cell lysis for the extraction of microalgal lipid is supposed to be paid more attention [79].

1.1.6 Emerging Lipid Extraction Process

Some emerging technologies for lipid extraction from microalgal cells need to be mentioned here. They include supercritical or subcritical fluid extraction (SFE), direct transesterification, and ionic liquid assisted lipid extraction. The purpose of these emerging Lipid extraction technologies is mainly to eliminate energy-intensive drying or dewatering steps and make the extraction process much more readily.

Supercritical CO_2 fluid extraction separation processes are based on the relationship between the solubility of supercritical fluid and its density, indicating that the effect of pressure

and temperature on the solubility of the supercritical fluid. In the supercritical state, the supercritical fluid is contacted with the material to be separated so that the component with various polarities, the boiling points, and the molecular weights are selected to be extracted in line [80, 81]. This eco-friendly approach has other advantages such as low operating temperature and high extraction rate, but the equipment is relatively expensive and energy-consuming, hindering its industrial application.

Transesterification, known as alcoholysis, is the most common method for directly generating lipids to biodiesel. The biodiesel production by transesterification was first patented in the 1930s by a Belgian researcher, G. Chavanne at the University of Brussels [82]. The transesterification reaction is represented by the general equation as given in Fig 1.9. To some extent, the process is similar to hydrolysis using alcohol, including methanol, ethanol, butanol, propanol, or amyl alcohol. Among them, ethanol, especially methanol, is used most frequently due to its low cost and good physicochemical properties. If methanol is used in this process it is called methanolysis. Methanolysis of triglyceride is displayed in Fig 1.9. The fatty acid ester can be synthesized by two mechanisms through the effect of an acid or base, from fatty acids and/or glycerides and short-chain alcohol [83]. This process has been confirmed to reduce the viscosity of triglycerides and thus enhance the physicochemical properties of renewable fuels. Therefore, biodiesel obtained by transesterification possesses good properties to be directly used as an alternative fuel [84].

In the past ten years, ionic liquids have been regarded as a novel class of solvents with wide application fields [85, 86, 87]. Additionally, ILs have also been considered as a green alternative to solvent extraction for microalgal lipids [61, 88]. The ionic liquid is composed of organic cations and inorganic or organic anions, as a liquid organic salt at approximately

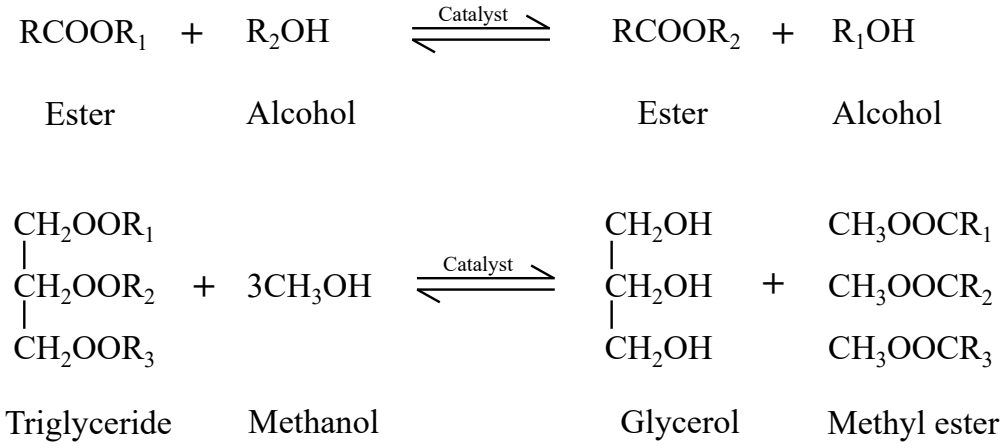

Figure 1.9: The general transesterification reactions.

room temperature. Although ionic liquids are normally liquid at room temperature, higher processing temperature still has a positive for shorter times in most cases. The structure of ionic liquid is complex with a wide range [89]. Hence, they are also regarded as "designable" liquid organic salts because the cationic and cationic could be, to a certain extent, functionalized [90]. Compared with conventional organic solvents, ILs have many advantages such as good thermal stability, low volatility, wide electrochemical window, high conductivity, non-flammable, recyclability, etc [91, 92, 93]. Those physicochemical properties and special structures make ILs promising candidates for various applications [94, 95]. However, the studies referring to wet extraction by ionic liquids are much less from fresh biomass. The compatibility of ionic liquids with wet microalgae needs to be addressed for lipid extraction with high efficiency and low energy consumption.

1.2 Research Objectives and Overview

The overall objective of this research was dedicated to obtaining renewable microalgal lipids for low-energy and high-efficiency bio-oil production. Exploring compatibility with aqueous solutions can reduce the energy consumption of drying microalgae during the production process. In addition, based on the biodiesel production using ionic liquids, this work further explored the utilization of ionic liquids for extraction of value-added oil from marine microalgae instead of fish and by-products as feedstocks. Meanwhile, extraction conditions and process optimization combined simulation and experiments were investigated to develop meaningful, feasible, and effective extraction methods.

1.2.1 Specific Objectives

1. Production of microalgal lipids from dry *Chlorella Vulgaris*

    (a) Characterization of a novel synthesized ionic liquid for further application;

    (b) Exploration of ionic liquids based lipid extraction processes;

    (c) The analysis of the main terms in lipid production from *Chlorella Vulgaris*;

2. Wet extraction of crude oils from fresh *Chlorella Vulgaris*

    (a) Exploration of ionic liquids based wet extraction processes;

    (b) Wet extraction of microalgal lipid with water from *Chlorella Vulgaris*.

    (c) Identification of enhanced extraction of bio-crude oil extraction under the synergy of electrolysis treatment and ionic liquids

(d) Optimizing and simulation of bio-crude oil extraction under the synergy of electrolysis treatment and ionic liquids from fresh microalgae

3. Development of value-added lipids based on ionic liquid process

(a) Exploration of efficient extraction of a DHA-rich lipid fraction from *Thraustochytrium sp.*

(b) Assessment of the compatibility of ionic liquid-based lipid extraction method with water

(c) Evaluation of the reuse/recycling of the ionic liquid

(d) Optimizing and simulation of value-added lipid extraction of a DHA-rich Oil from *Thraustochytrium sp.*

1.3 Thesis Structure

An overview of the thesis is shown in Fig 1.10. This thesis is divided into 8 chapters.

Chapter 1 The introduction overviews the research background and specific objectives of the thesis.

Chapter 2 Literature review covers the literature from a series of topics referring to ionic liquid processing of biomass.

Chapter 3 The characterization of a novel synthesized ionic liquid is described based on various characterization methods. Its potential is also shown in future applications.

Chapter 4 The chapter includes the exploration of ionic liquids based lipid extraction processes from dry *Chlorella Vulgaris*. A full factorial design (FFD) is selected to assess the response pattern with four parameters.

Chapter 5 This chapter develops a wet extraction from fresh culture. It also proposes a combined pretreatment of electrolysis and ionic liquid to assist in extracting lipids from fresh microalgae *Chlorella Vulgaris*.

Chapter 6 This part of the work emphasizes an efficient extraction of a DHA-rich lipid fraction from *Thraustochytrium sp.* Meanwhile, the compatibility of ionic liquid-based lipid extraction method with water and the reuse/recycling of the ionic liquids are both assessed in the chapter.

Chapter 7 It contains optimizing and simulation of value-added lipid extraction of a DHA-rich Oil from *Thraustochytrium sp.* using the synthesized ionic liquid.

Chapter 8 This chapter summarizes the results from this work, and it provides possible avenues for future work.

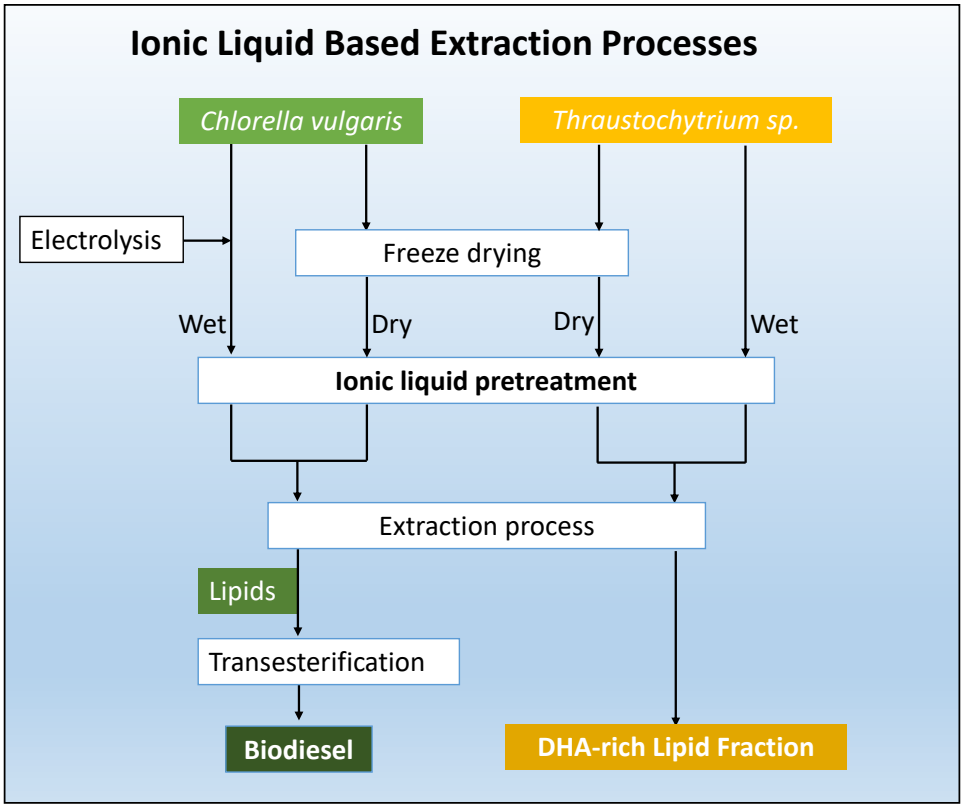

Figure 1.10: An overview of the thesis.

2 Literature Review

2.1 Preface to Chapter 2

The nature of this thesis includes topics from the chemistry of ionic liquids to value-added lipid extraction from microalgae. As such, a literature review was mainly prepared on the use of ionic liquids for the extraction of value-added products from biomass.

This chapter was included in this thesis in order to provide a comprehensive background of the mechanisms of ionic liquids processing of biomass and their aid in the extraction of value-added products from biomass. Furthermore, this chapter summarized the current work in the field of ionic liquids based value-added products from marine microalgae.

Results of future chapters are also cited in this work as already published data with comparison to most recent published works. Finally, environmental and economic outlook on ionic liquid was also presented in the chapter. It comprehensively refers to the pros and cons of ionic liqiuds, as well as the obstacles in the development.

With minor editorial changes to fulfill formatting requirements, this chapter is submitted as a chapter for the handbook of "Innovative and Emerging Technologies in the Bio-marine Food Sector", 2020 Elsevier Inc. (in press) Permission to reproduce figures within this chapter was awarded.

2.2 Overview

Recently, sustainable approaches towards extraction processes of value-added compounds have received much attention [96, 97]. Traditional extraction and separation methods to obtain target compounds from source materials include microwave, high-pressure, mechanic and ultrasound, as well as the use of volatile organic solvents [97]. Generally, the reason for these methods to be successful lies in their simplicity, versatility and effectivity, especially with the convenience of liquid samples. However, there are several disadvantages, such as high time- and energy-consumption, material-waste, and the use of volatile organic solvents. Given these concerns, attempts have been made to develop alternative processes through more sustainable routes.

ILs are molten salts typically composed of large organic cations with inorganic or organic anions, which remain liquid at relatively low temperatures below 100℃, have been shown to increase the extraction efficiency of various products from biomass [6, 98, 99]. The advantageous properties of ILs are low melting points, non-flammability, negligible volatility and outstanding chemical and thermal stability [98, 100]. Moreover, their solvation ability for many materials and compounds makes them suitable as media for extractions of proteins, nucleic acids and other bio-molecules [96, 101]. Thus ILs have evolved as promising solvents to accomplish biomass dissolution followed by the extraction of natural compounds [3, 102, 100]. Aqueous solutions of ILs exhibit good solvation performance, as revealed by their hydrotropic nature and as surface-active ingredients, enhancing extraction efficiency [96, 103]. ILs are typically designable solvents for different applications owing to various possible combinations of ions. As a result, ILs have the potential to replace traditional organic solvents and overcome

the limited options in the extraction field. Furthermore, nonvolatile ILs are able to be recycled and have much fewer emissions to the environment than conventional solvents. An increasing number of room-temperature ILs (over two hundred) are currently commercially available.

Taking into account all-natural sources of high-value bio-molecules, microalgae biomass, as sustainable and renewable resources, are the most employed in recent research. A wide range of high-value bio-compounds can be derived from them, such as polyunsaturated fatty acids (PUFAs) including ω-3 fatty acids: eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) [104, 105], phycobiliproteins [106], phenolics [14], carotenoids [107], fucoxanthin [108], biodiesel [109] and so on. Regarded as high-value compounds, they are linked to various applications from food ingredients to pharmaceuticals, from health-care products to cosmetic products [110, 111]. Therefore, new methodologies for extracting and/or purifying high-value compounds from biomass with less cost and waste have been drawn much attention in recent years.

This review is aimed to discuss and summarize the extraction approaches based on ILs for selected value-added products from microalgae. It reveals the potential applications in practical scale and other possible fields, while also reviewing the limitations of these kinds of extraction technologies. It is demonstrated in this review that ILs are promising solvents for extracting value-added products from microalgal biomass and other resources in a facile and recyclable process (Fig. 2.1).

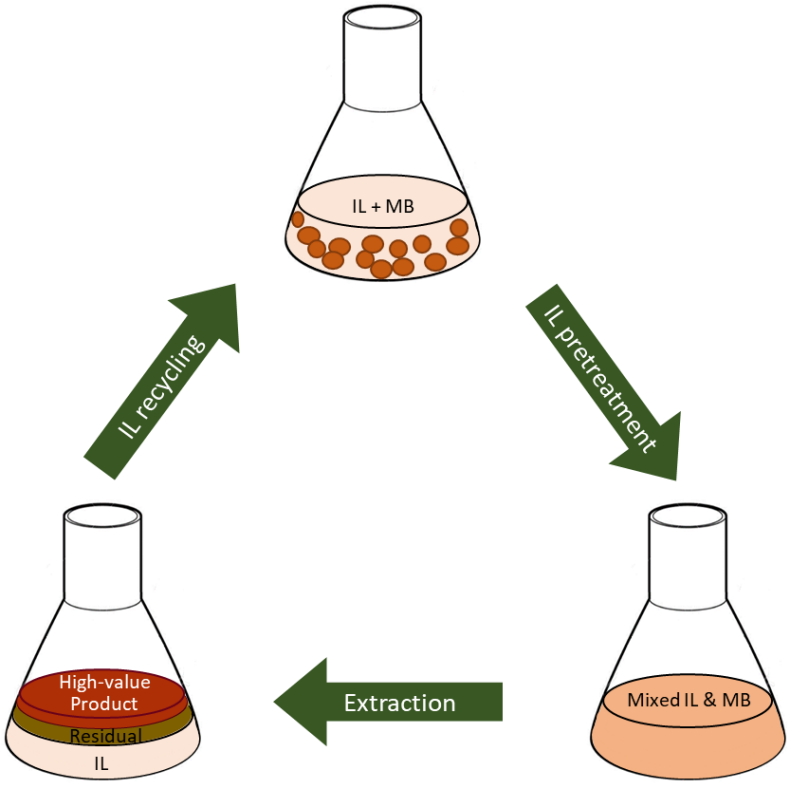

Figure 2.1: Summary of basic IL-based microalgal biomass (MB) fractionation process. The high-value products are recovered following cell disruption with ILs. The residual biomass separates from the ILs, allowing for IL recycling.

2.3 General Structure and Properties of ILs

From a historical viewpoint of IL studies, ethyl ammonium nitrate (EAN) was the first reported room-temperature ionic liquid in 1914 [112]. However, this report attracted little attention in academia and industry [113]. 1-ethyl-3-methylimidazolium tetrafluoroborate ($[C_2mim][BF_4]$) was later reported in 1992 as a stable liquid salt [114]. After that, these ILs were considered to be "green and safe solvents" for different purposes because of negligible volatility. ILs are organic salts composed of an organic cation and an inorganic or organic anion and are generally classified according to the types of cations: 1) imidazolium, 2) ammonium, 3) pyridinium and 4) phosphonium ILs. Passos *et al*. [3] summarized the ILs chemical cation and

anion structures that are shown in Fig. 2.2 [3]. Their name and abbreviations are presented in Table 2.1 [3]. Nowadays, the majority of ILs reported are imidazolium-based with the cation of 1-alkyl-3-methylimidazolium $[C_nC_1im]^+$ because of commercial availability of ILs such as 1-ethyl-3-methylimidazolium ethylsulfate ($[C_2mim][EtSO_4]$), 1-butyl-3-methylimidazolium chloride ($[C_4mim]Cl$), 1-hexyl-3-methylimidazolium methanoate ($[C_6mim][O_2CH]$) and others.

In the last few years, some exceptionally desirable properties of ILs have drawn considerable attention and make ILs different from traditional organic solvents. In general, ILs with very low melting points (< 100℃) are liquids showing salts' properties such as negligible vapor pressure and high ion density. Instead, conventional organic solvents are seldom found with those characteristics. Some ILs have high boiling points over 400℃ [115]. The reason why most ILs are known for shallow vapor pressures is that there are substantial charges in ILs preventing them from evaporating. ILs are often regarded as "green solvents", which contributes from a few emission of volatile organic compounds (VOCs) caused by the low volatility. However, due to the non-renewable synthesis processes of ILs, increasing numbers of studies on their toxicity show that ILs are not inherently "green". Therefore, the assessment of ILs must be explicitly undertaken for any given application.

Some relationships between melting point/viscosity and structures have been reported and revealed. Pinkert found that the anion type has a significant effect on the viscosity and melting temperature of ILs, although the cation does play a minor role [116, 117]. On the other hand, ILs with shorter chains or greater side chains have higher melting temperatures. The presence of double bonds or oxygen in the side chains can decrease viscosity. Moreover, small anions, such as chloride or bromide, can increase melting temperature and viscosity [116]. Further-

more, the presence of co-solvents can decrease viscosity owing to reducing the aggregation of the ions. Again, experimental results show that the amount of co-solvent does have less influence than its nature for most ILs [116, 118].

Table 2.1: Name and acronym of common cation-anions combinations in ILs [3].

Cations			Anions		
	Name	Acronym		Name	Acronym
i	1-Alkyl-3-methylimidazolium	$[C_nC_1im]^+$	i	Bromide	Br^-
ii	1-Alkylimidazolium	$[C_nim]^+$	ii	Chloride	Cl^-
iii	1-Alkylpyridinium	$[C_npy]^+$	iii	Iodide	I^-
iv	1-Alkyl-1-methylpyrrolidinium	$[C_nC_1pyr]+$	iv	Hydroxide	$[OH]^-$
v	1-Allyl-3-methylimidazolium	$[aC_1im]^+$	v	Thiocyanate	$[SCN]^-$
vi	1-Hydroxyethyl-3-methylimidazolium	$[(OH)C_2C_1im]^+$	vi	Acesulfamate	$[Ace]^-$
vii	1-Carboxymethyl-3-methylimidazolium	$[(HOOC)C_1C_1im]^+$	vii	Tetrafluoroborate	$[BF_4]^-$
viii	1-Propylamine-3-methylimidazolium	$[(NH_2)C_3C_1im]^+$	viii	Perchlorate	$[ClO_4]^-$
ix	1-(4-Sulfonylbutyl)-3-methylimidazolium	$[(HSO_3)C_4C_1im]^+$	ix	Dicyanamide	$[N(CN)_2]^-$
x	1-Cyclohexyl-3-methylimidazolium	$[C_6H_{11}C_1im]^+$	x	Nitrate	$[NO_3]^-$
xi	1-Benzyl-3-methylimidazolium	$[C_7H_7C_1im]^+$	xi	Bis(trifluoromethylsulfonyl)imide	$[NTf_2]^-$
xii	*N*,*N*-Dimethyl(cyanoethyl)ammonium	$[N_{11(3N)0}]^+$	xii	Hexafluorophosphate	$[PF_6]^-$
xiii	2-(Dodecyloxy)-*N*,*N*,*N*-trimethyl-2-oxoethanaminium	$[N_{111(C2O(O)C12)}]^+$	xiii	Tosylate	$[Tos]^-$
xiv	*N*,*N*-Dimethylammonium	$[N_{1100}]^+$	xiv	Saccharinate	$[Sac]^-$
xv	Cholinium	$[N_{111(2OH)}]^+$	xv	Dimethylcarbamate	$[N(C_1)_2CO_2]^-$
xvi	*N*,*N*-Dimethylethanolammonium	$[N_{11(2OH)0}]^+$	xvi	Sulphate	$[SO_4]^-$
xvii	*N*,*N*-Dimethyl-*N*-(2-hydroxyethoxyethyl)ammonium	$[N_{11(2(O)2OH)0}]^+$	xvii	Hydrogenosulphate	$[HSO_4]^-$
xviii	*N*,*N*-Dimethyl(2-methoxyethyl)ammonium	$[N_{11(2(O)1)0}]^+$	xviii	Dihydrogenophosphate	$[H_2PO_4]^-$
			xix	Dialkylphosphate	$[(C_n)_2PO_4]^-$
			xx	Alkylsulphate	$[C_nSO_4]^-$
			xxi	Methylsulfonate	$[C_1SO_3]^-$
			xxii	Trifluoromethanesulfonate	$[CF_3SO_3]^-$
			xxiii	Lactate	$[C_2OCO_2]^-$
			xxiv	Isobutyrate	$[C_{i3}CO_2]^-$
			xxv	Alkylcarboxylate	$[C_nCO_2]^-$

Low flammability and good conductivity are distinct advantages of most ILs for replacing organic solvents, especially in an extraction process. The low flammability of ILs is the result of a high ignition point due to the active forces between ions and Van der Waals forces/hydrogen bond. Also, plenty of cations and anions obviously lead to good conductivity. Based on these advance, ILs are widely applied to electrochemical studies for energy conversion, such as supercapacitors [119, 120], solar cells [121] and Li-based batteries [122]. The structure of ILs can also be complemented with different functionality, including some polymeric chains, nanoparticles and monomeric polymerizable groups that serve as not only an electrolyte but also an electrolyte additive [121, 123].

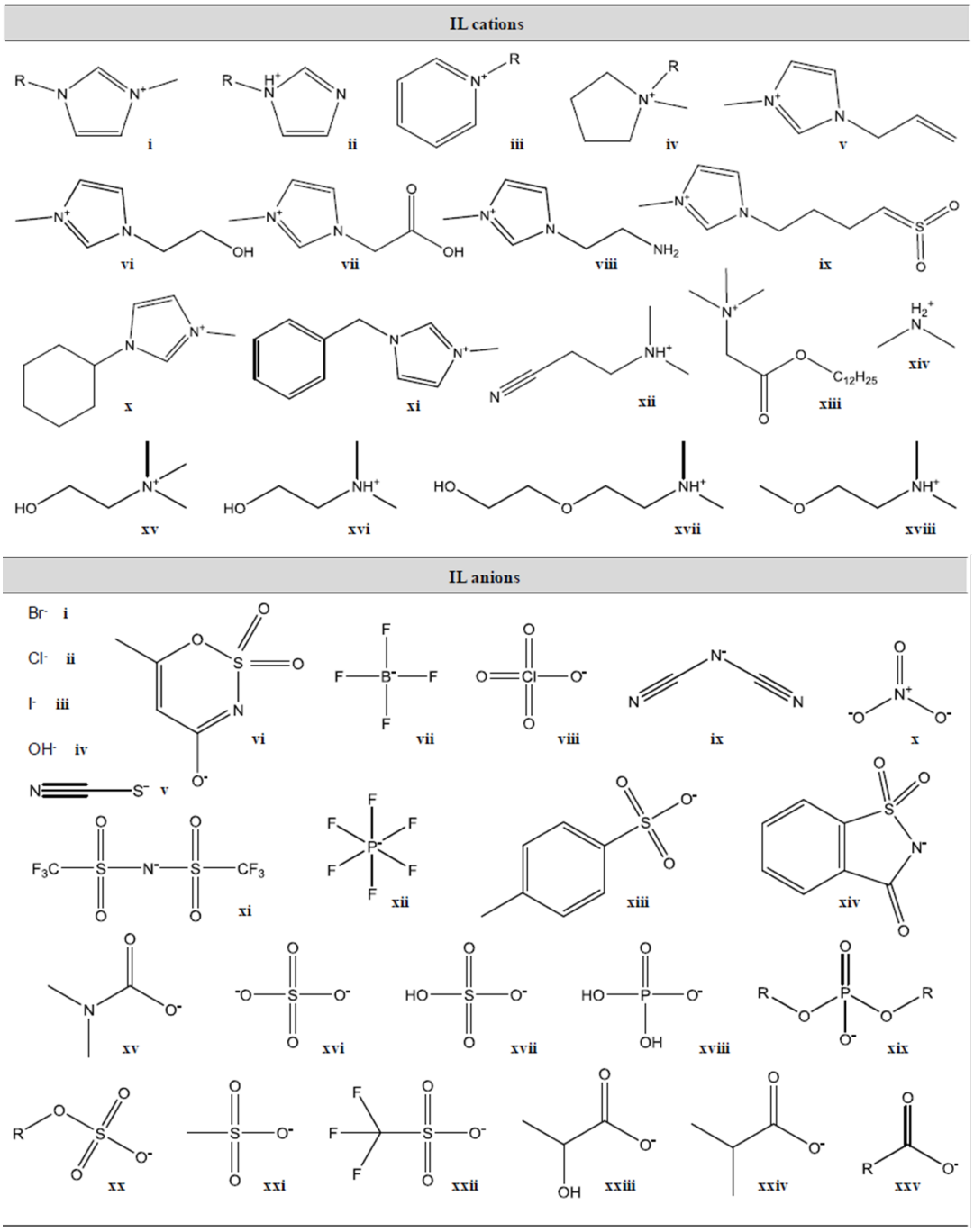

Figure 2.2: Chemical structure of IL cations and anions employed in the extraction of value-added compounds from natural sources. The nomenclature of each ion is presented in Table 2.1 [3].

One of the most characteristic properties of ILs is high thermal stability with T_{onset} (onset decomposition temperatures) of 300-400℃ [116, 124]. Mostly, phosphonium-based ILs possesses better thermal stability than ammonium-based ILs. The anions usually decompose via dealkylation, whereas the cations undergo primarily alkyl migration and elimination reactions. In general, imidazolium salts show more stability than tetraalkylammonium salts [124]. The exceptions are protic ILs with at least one proton which could dissociate. These structures may not bond to the generalization due to their significant reactivity and volatility, also affecting their thermal stability [125].

ILs can also present a range of polarities. However, solvent polarity has not been abundantly quantified even for traditional molecular solvents. Thus it is hard to apply these imperfect matrices to ILs which bring in an additional coulombic aspect to the system. Another critical property of ILs is hydroscopic behavior which partly results from the polarity. Hence, some ILs are easily dissolved in water. However, water impurities can affect the extraction efficiency. Another important reason for the hydroscopic behavior is the hydrophobicity/hydrophilicity of the anion and cation. Generally, hydrophobic ILs are made with cations with long side chains. One sub-category of ILs named switchable solvents is ILs capable of changing polarity or hydrophilicity when a trigger is employed. These are typically made from amidines where carbon dioxide is used as a trigger to convert them to carbonate or bicarbonate salts [126]. These types of solvents might be useful where a given process requires solvents with multiple polarities. Most biomass processing studies employ hydrophilic ILs so far. Moreover, water is always used as an anti-solvent to recover the IL and separate it from the residual biomass. Many water-soluble ILs are also soluble in short-chain alcohols or acetone.

To create a design protocol for ILs with targeted properties, lots of research investigated the

physicochemical properties of ILs. High purity ILs were necessary, and the effects of additives on the properties of ILs were also studied. However, there are limits to designing a pure IL with multiple desirable characteristics by modifying the structures of anions and cations. As an extension, there has been a growing interest in improving the properties of ILs by adding molecular liquids such as water. It is noteworthy that the extraction performance and purity level of the target compounds are the key parameters to be considered when trying to develop new extraction processes.

2.4 Effect of Cellulose Solubility in Ionic Liquids

Cellulose, as the main component in the microalgal cell wall, has been widely proven to be soluble in ionic liquids [127, 128, 129, 130]. Thus ionic liquids could be promising and efficient solvents for pretreatment of the microalgal cells to enhance the lipid extraction efficiency. The dissolution of crystal cellulose models was systematically evaluated in imidazolium-based ILs, indicating that ionic liquids possess high dissolving power such as 1-Allyl-3-methylimidazolium chloride [Amim]Cl and 1-Ethyl-3-methylimidazolium chloride [C_2mim]Cl in Fig. 2.3 [4]. The mechanism may be summarized as a) the hydrophilicity of ILs will be reduced with the increase of alkyl chain length to weaken the affinity between the ILs and cellulose; b) the increase in the size of the ILs will be caused by the increase of substituent chain length increases [127]. With comparison to odd-numbered alkyl chains, cellulose is found with better solubility in chloride-based ILs with even-numbered alkyl chains [131]. However, this kind of effect is found nonexistent in ILs with other anions such as acetate [132]. Therefore, it is significantly evident that ionic liquids with the ability to dissolve cellulose are confirmed as good candidates for cell disruption and lipid extraction processes.

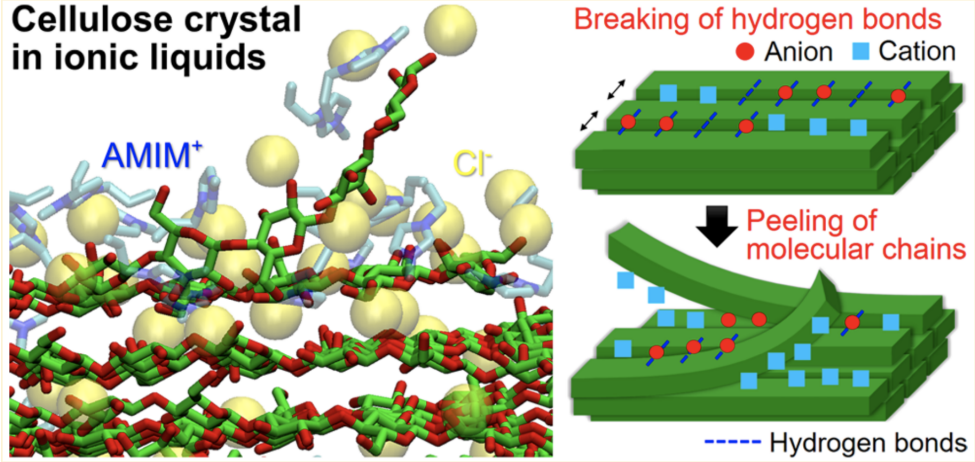

Figure 2.3: Dissolution of crystal cellulose models in powerful solvents ([Amim]Cl and [C_2mim]Cl) [4].

2.5 Value-added Products from Marine Biomass

Ionic liquids (ILs) have been suggested as promising media to separate and extract bioactive compounds from a broad range of natural feedstocks. The unique physicochemical and solubilization properties of ILs have also led to new separation science and materials science developments in recent years. This review highlights recent accomplishments in the extraction processes of diverse high-value compounds from different kinds of marine biomass, such as fish and marine algae via using ILs. High-value products targeted in recent studies through ILs-based processes include lipids, small organic extractable compounds, proteins, etc. Industrial applications of ILs for extraction processes often employ combinations with traditional organic solvents. Achievements, as well as challenges of ILs-based processes are reviewed.

Marine life differs substantially from terrestrial life, on a geno- and phenotype level [133, 134]. The marine environment is relatively extreme such as higher salinity, low light, more radiation, etc., which means marine organisms live in complex habitats and have diverse secondary metabolites and biological activities [135, 134]. Moreover, a large number of com-

pounds from natural marine-based resources have been proved to be functional high-value products, such as polyunsaturated proteins, polyunsaturated fatty acids (PUFAs), antioxidants, sterols, polysaccharides, carotenoids, and pigments [136]. These bio-molecules are capable of being employed in various industries, from pharmaceutics and cosmetics to functional food ingredients and nutritional supplements [137, 138]. Therefore, considering those facts, marine organisms take an essential field of basic and applied research [139]. In recent years, the number of studies on the application of ILs as alternative solvents to extract high-value compounds from biomass gradually increased. Some reports are shown in Table 2.2.

Table 2.2: Summary of research about the extraction of high-value compounds from biomass by ionic liquid.

ILs	Products	Sources	Recovery	Ref.
$[C_2mim][HSO_4]$	Astaxanthin	*Haematococcus pluvialis*	≥ 99% (w/w)	[5]
$[C_2mim][CH_3SO_3]$	Astaxanthin	*Haematococcus pluvialis*	≥ 99% (w/w)	[5]
$[C_2mim][(CF_3SO_2)_2N]$	Astaxanthin	*Haematococcus pluvialis*	≥ 99% (w/w)	[5]
2-HEAA+2-HEAF	Phycocyanin	*S pirulina platensis*	1.65 g/L	[106]
2-HEAA+2-HEAF	Allophycocyanin	*S pirulina platensis*	1.70 g/L	[106]
2-HEAA+2-HEAF	Phycoerythrin	*S pirulina platensis*	0.64 g/L	[106]
$[C_2mim][EtSO_4]$	DHA-rich Lipid	*Thraustochytrium*	83.9%(w/w)	[140]
$[C_4C_1im][BF_4]$	Phenolics	Brown seaweed	125.25mg/g	[14]
$[C_4mim]Ac$	Carrageenan	*Kappaphycus alvarezii*	78.75%(w/w)	[141]
[Ch]Cl	Phycobiliproteins	*Gracilaria sp.*	46.5%(w/w)	[142]
$[C_4mim]Ac$	Hydroxyapatite	Fish scales	32%(w/w)	[143]
$[C_2mim][DBP]$	Astaxanthin	*Haematococcus sp.*	≥ 70%(w/w)	[6]
[Ch]Cl	Astaxanthin	Shrimp waste	76-102% (w/w)	[144]
$[C_4mim]HSO_4$/Cl	Galactose	*G.amansii*	81% (w/w)	[145]
$[C_2mim]OAc$	DHA-rich lipids	*Aurantiochytrium sp.*	145 mg/g	[17]

2.5.1 ω-3 Fatty Acids

Polyunsaturated fatty acids (PUFAs) as high-value products caught much attention due to the effect on the modulation and prevention of various diseases [17, 42, 140, 146]. Some species such as *Aurantiochytrium*, *S chizochytrium*, *Thraustochytrium*, and *Ulkenia* genera are

common research targets on account of their high PUFA content [24]. They are rich in valuable ω-3 polyunsaturated fatty acids, such as the eicosapentaenoic (EPA, C20:5 n-3) and docosahexaenoic (DHA, C22:6 n-3) acids [105, 147]. Some PUFAs have the potential to prevent diseases such as asthma, neural disorders, cardiovascular diseases, arthritis, and dermatosis [17, 42].

Choi *et al*. [17] performed lipid extraction from *Aurantiochytrium sp.* using a molten-salt/ionic-liquid mixture $FeCl_3{\cdot}6H_2O$. As reported in their work, the total lipid of *Aurantiochytrium sp.* was 478.8 mg/g cell, and 30.3 % of total lipid was docosahexaenoic acid (DHA). A high lipid extraction yield (207.9 mg/g cell) was obtained in the presence of $FeCl_3{\cdot}6H_2O$ when compared to that of $[C_2mim]OAc$ (118.1 mg/g cell). However, the extraction yield reached to 478.6 mg/g cell by adopting the mixture of $FeCl_3{\cdot}6H_2O$ and $[C_2mim]OAc$ (5:1, w/w) based on the experimental conditions of 90℃, 30 min. The results also show that a high purity 997.7 mg/g lipid was achieved in the extracted DHA (30.2 % of total fatty acids). Moreover, the $FeCl_3{\cdot}6H_2O/[C_2mim]OAc$ mixture at the 5:1 (w/w) was recycled five times under the same conditions. Updated results from Choi *et al*. [5] compared ten types of 1-ethyl-3-methylimidazolium ($[C_2mim]$)-based ILs and evaluated their capabilities to disrupt *Haematococcuspluvialis* cyst cells for astaxanthin as well as lipid extraction. After comparisons and selections, 3 ILs (i.e.,$[C_2mim][HSO_4]$, $[C_2mim][CH_3SO_3]$, and $[C_2mim][(CF_3SO_2)_2N]$) were deemed better for further experimentation, based on their astaxanthin/lipid extraction ability and the existing costs of chemical synthesis from Sigma Aldrich. The experimental results in Figure 2.4 show that the lipid extraction efficiencies of $[C_2mim][HSO_4]$, $[C_2mim][CH_3SO_3]$, and $[C_2mim][(CF_3SO_2)_2N]$ had increasing trends with time from 5 to 120 min, while obtaining maximal extraction efficiencies of 82.2 ± 1.4, 76.1 ± 0.3, and 70.3 ± 3.0%, respectively. High astaxanthin extraction efficiencies could be achieved to over 99% within 1 h

for the three different ILs. Also, it indicated that lipid and astaxanthin extractions need different times, mainly because of lipid's and astaxanthin's different biochemical sensitivities to ILs. Proper incubation time was a vital factor for the IL-based extraction process for *H.pluvialis*.

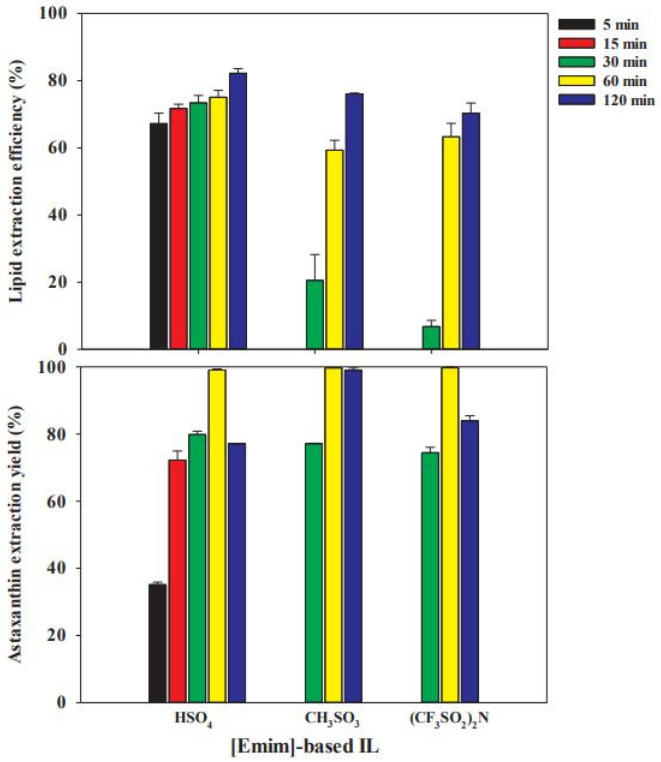

Figure 2.4: Effect of operating time on lipid and astaxanthin extraction efficiencies for three ILs at the same concentration of 6.7% (v/v) in aqueous solution. *H.pluvialis* cyst cells were pretreated by one IL at 30°C and followed by extracting with hexane at room temperature for 2 h [5].

A comparable study employed two ILs to extract DHA-rich lipid from *Thraustochytrium sp.* (T18). The ILs imidazolium 1-ethyl-3-methylimidazolium ethylsulfate $[C_2mim][EtSO_4]$ and phosphonium (tetrabutylphosphonium propanoate $[P_{4444}][Prop]$) were assessed for their capabilities to improve extraction of PUFA-containing lipids by efficient disruption of the cell walls. The extracted lipids after IL pretreatment was further characterized with respect to fatty acid methyl ester (FAME), while some process parameters were considered such as the mass ratio of microalgae to the mixture of ionic liquid and the type of co-solvent for both ILs. The results

indicate that these ILs could facilitate the recovery of lipids (over 90% (w/w) of the available lipids from dried T18 biomass) under the experimental conditions of 60 min and ambient temperature. However, wet T18 slurry (77.2 wt% water) was much more challenging to process. $[P_{4444}]$[Prop] could achieve a lipid yield of over 80% (w/w) from wet T18 biomass so that it was considered a promising candidate for further wet extraction studies. Also, the lipid recovery was not decreased much by ILs recycling (up to five times) [140].

2.5.2 **Phycobiliproteins**

Phycobiliproteins (PBPs) form complexes with photosynthetic pigments, helping to capture light energy in red algae, cryptophytes, cyanobacteria, and glaucophytes [148, 149]. Phycocyanin, allophycocyanin and phycoerythrin are the main PBPs, absorbing light within specific regions of the spectrum [150]. Due to the bright coloration and high solubility in water, PBPs are suitable in various fields, such as foods, energy, cosmetics, and pharmaceutical [142, 151, 152]. Other physiological performances of PBPs also attract much attention, for instance, antioxidant activity [153, 154],anticancer [155, 156, 157], anti-inflammatory [158, 159], and immunomodulatory [160, 161].

Some authors applied ILs to obtain PBPs from different kinds of biomass. Rodrigues *et al*. synthesized the ILs, *N*-methyl-2-hydroxyethylammonium acetate (2-HEAA) protic ionic liquid and *N*-methyl-2-hydroxyet-hylammonium formate (2-HEAF) and also mixed these protic ILs (2-HEAA + 2-HEAF) (1:1, v/v) as solvent to obtain phycobiliproteins from *Spirulina (Arthrospira) platensis* [106]. 2-HEAA + 2-HEAF was applied to extract for 150 min, at 35℃, pH 6.50, at a ratio (solvent:biomass) of 6.59 mL/g which were the optimum conditions. The main products were phycocyanin, allophycocyanin and phycoerythrin with concentrations of

1.65 g/L, 1.70 g/L and 0.64 g/L, respectively. Furthermore, the obtained PBPs presented a good purity with a purification index around 0.50. Therefore, the resultant PBPs are proposed to possess potential applications in the food and cosmetic fields.

There is another work by Martins *et al*. screening aqueous solutions of ILs for the extraction of PBPs from *Gracilaria sp*. [142]. In their work, various families of ILs were evaluated for the extraction of PBPs. Considering the capacity to extract more PBPs with less chlorophylls, aqueous solutions of cholinium chloride ([Ch]Cl) were found with the best extractive activity. The extraction efficiency for PBPs was demonstrated up to 46.5% compared with that of the conventional methodology. The operational conditions were optimized in terms of extraction time (20 minutes), solvent concentration (1 M), pH (5.9), and solid–liquid ratio (0.7) to develop a new approach with higher efficiency to extract PBPs.

2.5.3 **Astaxanthin**

Astaxanthin, a red–orange ketocarotenoid, is ubiquitous in the marine environment. It is widely regarded as a color additive in different fields such as cosmetics and aquaculture [162]. Currently, it is mainly produced from the green microalgae *Haematococcus pluvialis*. There are two stages for the production of astaxanthin from *H. Pluvialis*: (i) A green vegetative phase, where *H. Pluvialis* is cultivated under optimized conditions of light, pH, temperature, and nutrition concentrations where the cells accumulate lutein and β-carotene; (ii) a red-cyst phase, where astaxanthin formation is induced by specific conditions of pH, light, and nutrient starvation, converting almost 80% of the carotenoid fraction [163]. Astaxanthin has antioxidant properties that have potential benefits to human health [96, 164]. Its high market value drives the research for efficient production.

Recently, a novel technology was introduced using ILs to permeabilize Haematococcal cyst cells under mild temperatures and improve astaxanthin extraction [6]. The results show that ≥70% astaxanthin can be extracted from *H.pluvialis* with 1-ethyl-3-methylimidazolium dibutylphosphate ([C_2mim][DBP]) at a concentration of 40% w/w in an aqueous solution at 45 ℃. The ionic liquid treatment was undertaken before the actual extraction. The pretreated cells were processed with ethyl acetate. It was shown that cells only treated with solvents remained intact with astaxanthin inside the cell (Figure 2.5 a and b). However, ILs pretreated cells also kept intact but turned colourless after solvent extraction under similar conditions (Figure 2.5 c and d). In this particular application, ILs weakened the cell wall to facilitate subsequent astaxanthin extract with solvents. Leaving the cells intact is advantageous as not cell-debris removal is necessary.

To develop an emerging green approach, Zhang *et al*. used deep eutectic solvents to extract astaxanthin from shrimp byproducts [144]. A deep eutectic solvent is a different ionic solvent type generated by mixing two ideally low toxicity ionic components. The compounds are chosen to create a eutectic mixture with a melting point substantially lower than the melting points of the individual compounds. Deep eutectic solvent (DES) can be obtained by heating choline chloride ([Ch]Cl) and various hydrogen-bond donors to 80°C with constant stirring until a homogeneous liquid is formed. Such DES were applied to powdered shrimp by-products. When comparing the DES process to a traditional organic solvent (ethanol), the amount of extracted astaxanthin was 146 μg/g and 102 μg/g, respectively. Owing to those excellent properties, DES are regarded as promising and inexpensive solvents to extract a wide range of bioactive compounds from marine organisms.

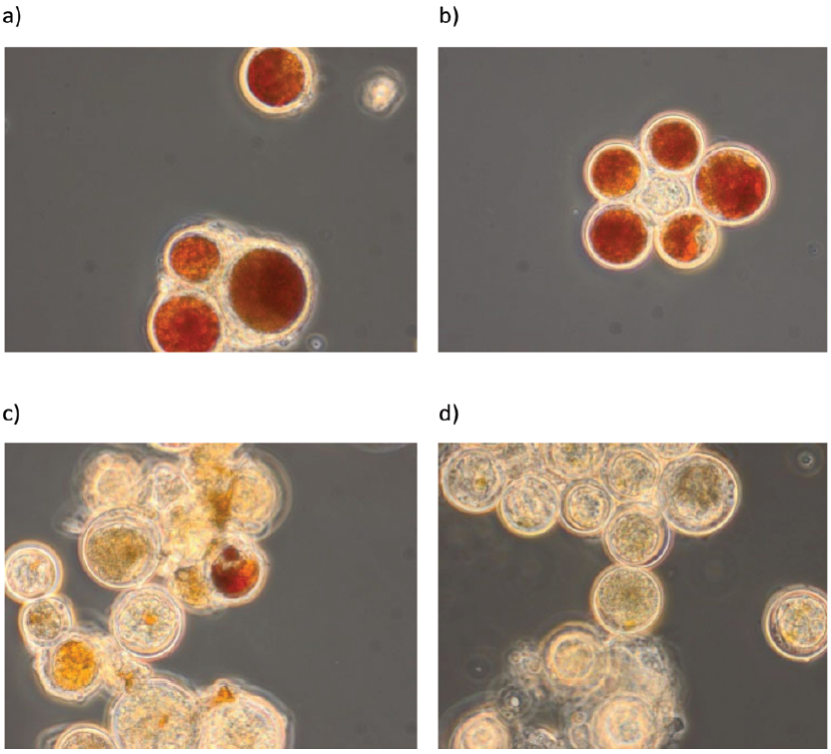

Figure 2.5: Microscopy of H. pluvialis (a) acetone at 45℃; (b) ethyl acetate at 65℃; (c) BMIM-DBP at 45℃; (d) EMIM-DBP at 45℃ [6].

2.5.4 **Phenolics**

Phenolics compounds that can be obtained from marine algae are varying from molecules with simple structures (phenolic acids) to compounds with complex structures such as (phlorotannins) [165]. Typical antioxidant compounds are natural phenolic compounds containing one aromatic ring with two or more hydroxyl groups and functional derivatives. These structural aspects have two different functions. First, phenolics can combine with lipid radicals as a hydrogen atom donor to decrease the rate of autoxidation processes. Second, as an electron donor, it turns too stable after scavenging the free oxidants to react with the substrates [166]. Recently, different techniques have been applied to collect natural phenolic substances. Among them, sub-critical water extraction (SWE) draws much attention as its sustainability has the po-

tential to replace conventional methods. ILs have also been evaluated to extract phenolics from natural resources.

IL-assisted SWE (SWE + IL) has been utilized to obtain various phenolic compounds from a kind of brown seaweed *Saccharina japonica* [14], where the imidazolium-based IL, 1-butyl-3-methylimidazolium tetrafluoroborate ($[C_4C_1im][BF_4]$), was adopted as a catalyst. When determining the extraction efficiency of gallic, gentisic, p-hydroxybenzoic and other compounds, it was found that two SWE techniques determined the highest content of phenolics at 175℃. In contrast, it was hard to detect phenolic compounds by conventional SLE methods. In this work, the phenolic content was termed by the ratio of the weight of phloroglucinol equivalent and dry weight (mg PGE/g DW). As shown in Table 2.3, gallic acid (6.32 ± 0.02 mg PGE/g DW), chlorogenic acid (52.68 ± 0.72 mg PGE/g DW), gentisic acid (10.71 ± 1.03 mg PGE/g DW), protocatechuic acid (34.37 ± 0.50 mg PGE/g DW), p-hydroxybenzoic acid (8.22 ± 0.67 mg/g DW), caffeic acid (11.01 ± 0.75 mg PGE/g DW), and syringic (2.03 ± 0.17) were extracted at temperatures below 175℃. Furthermore, the quality of phenolic bioactive extracts was verified by correlation testing and principal component analysis. Therefore, SWE is a promising method for the extraction of phenolics from natural sources to marine algae.

Table 2.3: Quantification of individual phenolic compounds (mg/g DW) from different extracts using HPLC [14].

Extraction method	Gallic	Chlorogenic	Gentisic	Protocatechuic	*p*-Hydroxybenzoic	Vanillic	Caffeic	Syringic	Total
SWE + IL									
100 °C	0.35 ± 0.02^{m}	$0.72 \pm 0.10^{i,j}$	ND	2.15 ± 0.07^{h}	ND	ND	$0.62 \pm 0.02^{g,h}$	0.02 ± 0.01^{i}	$3.85 \pm 0.08^{l,m}$
125 °C	1.38 ± 0.02^{j}	6.25 ± 0.27^{f}	ND	10.97 ± 0.40^{d}	$0.51 \pm 0.04\ j^{h}$	1.29 ± 0.07^{c}	$0.55 \pm 0.02^{g,h}$	1.33 ± 0.12^{e}	$22.28 \pm 0.58\ j$
150 °C	10.72 ± 0.03^{a}	11.44 ± 0.38^{e}	6.75 ± 0.48^{f}	26.18 ± 0.14^{c}	$1.84 \pm 0.11^{f,g}$	0.85 ± 0.10^{d}	6.89 ± 0.19^{d}	1.73 ± 0.16^{d}	66.42 ± 1.13^{d}
175 °C	6.23 ± 0.02^{c}	52.68 ± 0.72^{b}	10.71 ± 1.03^{d}	34.37 ± 0.50^{a}	8.22 ± 0.67^{e}	ND	$11.01 \pm 0.\ 75^{b}$	2.03 ± 0.17^{c}	125.25 ± 1.53^{b}
200 °C	6.48 ± 0.03^{b}	64.16 ± 0.99^{a}	19.53 ± 0.79^{b}	33.52 ± 0.86^{a}	19.68 ± 0.53^{c}	ND	13.43 ± 0.55^{a}	3.83 ± 0.07^{a}	160.62 ± 3.04^{a}
225 °C	5.97 ± 0.03^{d}	51.13 ± 1.17^{c}	8.46 ± 0.34^{e}	31.43 ± 1.41^{b}	9.96 ± 0.36^{d}	ND	14.00 ± 0.62^{a}	3.23 ± 0.10^{b}	124.18 ± 2.64^{b}
250 °C	5.62 ± 0.07^{e}	14.53 ± 0.54^{d}	6.62 ± 0.22^{f}	11.23 ± 0.67^{d}	8.21 ± 0.40^{e}	ND	7.88 ± 0.50^{c}	1.26 ± 0.09^{e}	55.33 ± 1.04^{e}

Data are expressed as the mean of triplicate ± SD.
Subscript letters within a column indicate significant differences between samples at the level of $p < 0.05$.

2.5.5 Hydroxyapatite

Hydroxyapatite (HAp), with the formula $Ca_5(PO_4)_3(OH)$, is a natural mineral form of calcium apatite. Generally, up to 50% by volume and 70% by weight of human bone is a modified form of HAp, known as bone mineral. Also, carbonated calcium-deficient HAp is the main mineral of teeth [167]. HAp is increasingly adopted to make bone grafting materials as well as dental prosthetics and repair. Muhammad and co-authors carried out a series of experiments were HAp was extracted from carp fish scales(FS) by 1-butyl-3-methylimidazolium acetate([C_4mim]Ac) [143]. The IL [C_4mim]Ac was observed having the ability to dissolve the organic portion of FS powder completely at 100°C for 12 h. Therefore the inorganic portion (HAp) could be easily separated as an insoluble product with a yield of 32 ± 2%. As for the mechanism, ILs have been known to dissolve some biopolymers such as cellulose through breaking the hydrogen bond linkage [143, 168]. When employing pretreatment with ILs, the collagen part, which belonged to FS, could interact with the IL by hydrogen bonding, resulting in its dissolution. At the same time, HAp, as the inorganic part, remains undissolved solid-state so that HAp, as a precipitate, could be obtained and collected easily by centrifugation (11,000 rpm) for 30 min [143, 169, 170].

2.6 Environmental and Economic Outlook on the ILs Extraction

According to all reviewed works relating to the outstanding potential extraction approaches, high-value products can be efficiently obtained by various ILs. There is no doubt that low volatility is a vital advancement in extraction processes in terms of environmental impact. Meanwhile, this property also comes with more complexity during the separation and extrac-

tion of high-value compounds. Some improved strategies have already been proposed and discussed to overcome these issues. However, the high production costs related to ILs are the main impediment to the feasibility of their applications on an industrial scale. In general, the raw materials for their synthesis majorly determine the price of ILs. A rough price estimate of these materials was already carried out, shown in Figure 2.6 [3]. Quaternary imidazolium-based ILs are more expensive than their ammonium- (including cholinium-) based counterpart [3]. Additionally, ILs composed of fluorinated anions should be replaced by halogen- or carboxylate-based ones as a consequence of higher cost. Although multiple ILs production processes have been developed and optimized, the price of ILs can not still compare with that of available organic solvents [3]. However, nonvolatile ILs need to be considered for wide applications with many advantages but not discarded only because of the high price. Furthermore, the reported studies have been shown that ILs are capable of increasing the extraction efficiencies of high-value compounds from natural marine resources, generally, at high solid–liquid ratios, lower temperatures and shorter operation time. Therefore, an IL-based methodology is still a viable alternative for extraction processes.

On the other hand, the costs of the extraction processes will also be variable depending on the various biomass. For those applications based on currently regarded by-products such as residues and wastes, for example, marine raw materials and microalgae, are remarkable sources of biomass, which were reviewed in this work. These resources could be of much attention in the coming years. However, for some processes based on human food or animal feed, they are hard to compete with the established markets owing to the high and inevitable cost.

Considering a sustainable employing cycle, the extraction processes by ILs need to recycle the ILs, which can minimize the processing costs as well as the environmental effects.

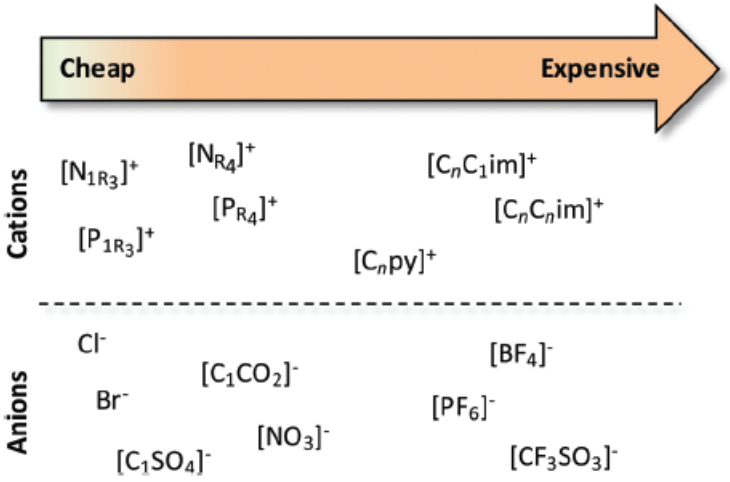

Figure 2.6: Diagram of the relative prices of raw materials used in ILs' synthesis [3].

Therefore, the recovery and reusability of ILs has recently attracted some researchers interest [171, 172, 173, 174, 175, 176]. The authors proved that ILs could be easily and almost totally recovered and recycled with keeping the efficiency on the value-added materials extraction after the isolation of the target value-added products. For example, the recovery and reusability of 1-ethyl-3-methylimidazolium ethylsulfate $[C_2mim][EtSO_4]$ was studied after the lipid extraction from *Chlorella vulgaris* [176]. The results demonstrated that the averaged recovered IL was up to 98.0 ± 5.2 wt %. The IL was collected and reused with the significant selective ability for lipid for at least five times.

However, the eco-toxicity and biodegradability of ILs also attracts many researchers, especially for pharmaceutical or food industries. Several novel fluorinated ILs with excellent surfactant properties have been studied for pharmacological applications as drug delivery systems [177]. The results clearly showed that the short-chain imidazolium-based fluorinated ILs are not readily biodegradable. Moreover, cholinium- and pyridinium-based fluorinated ILs could be more prone to biodegradation, particularly the cation. Therefore, the biodegra-

dation of fluorinated anions is still a concern for their practical applications. The work also demonstrates the necessity to find a greener design with both highly biodegradable anions and cations. Additionally, some other literature also indicate that ILs have low biodegradability [178, 179, 180, 181], but other researchers have proposed solutions to obtain biodegradable ILs, such as the catalytic wet peroxide oxidation (CWPO) [182], ester-functionalized pyridinium ILs [183], choline and acetate as components of ILs [184], and others.

Accordingly, the economic and environmental analysis of ILs has demonstrated that applying ILs as extraction solvents in the industry is likely possible in the coming years. However, it has to be noted that ILs-based extractions are only feasible if the concentration of targeted products is considerably high (> 5 wt %), or they are high-value compounds, such as reviewed in this work. The feasibility of astaxanthin extraction from marine microalgae is dependant only on the high concentration of the target compounds from the biosource. Moreover, the cost and efficiency of ILs-based extraction processes are critical factors in the industrial production of high-value compounds. Nevertheless, other factors such as extra assisted technologies, scale-up concerns, and a specific economic and environmental analysis should all be considering if ILs are applied to a particular extraction process.

In this review, it is clear that ILs can be widely applied to extract high-value products from various marine biosources. Biomass is a ubiquitous, sustainable and renewable resource for producing high-value compounds, such as DHA-rich lipids, phycobiliproteins and astaxanthin, etc. This review briefly listed recent research relating to high-value compounds extraction from marine biosources by ILs and exhibited the extraction processes of some high-value products. Excellent reusability and high extraction performance are the significant bases of extraction processes of high-value products by ILs. Also, the environmental and economic outlook of

ILs-based extraction processes has been comprehensively summarized and analyzed in the review. Overall, ILs have some desirable advantages in the extraction of high-value compounds from biomass, but the commercialization and industrial applications of ILs still need more investigations in the future.

3 Characterization of A Novel Synthesized Ionic Liquid

3.1 Preface to Chapter 3

Previous chapters present the unique properties of ionic liquids and their various application in value-added products.

Over the past ten years, interests and concerns relating to ionic liquids have increased exponentially. Thus they have mainly been utilized in many fields with a wide range, from electrochemistry to separations. Ionic liquids are well known for high thermal stability, good chemical stability, recyclability etc [185]. Thus, they can aid processes with less by-effect and even are able to be recycled and reused in some cases [22, 186].

Chapter 3 presents the synthesis and characterization of a lab-synthesized ionic liquid. The ionic liquid structure was identified by some characterization methods, such as nuclear magnetic resonance(NMR) spectroscopy and Fourier transform infrared (FT-IR). In addition, a series of physicochemical properties, such as viscosity, density, miscibility, and acidic property, were measured and analyzed, respectively.

This chapter supports subsequent chapters to use it as a solvent to pretreat microalgae for further applications.

3.2 Abstract

In this work, a novel ionic liquid, 1-decyl-3-methylimidazolium bis(2,4,4-trimethylpentyl) phosphonate ([C_{10}mim] [BTMPP]), was synthesized and its physicochemical properties were studied. Fourier transform infrared (FT-IR) and nuclear magnetic resonance (NMR) spectroscopy were applied to identify the molecular structure. The thermal properties of [C_{10}mim] [BTMPP] were characterized by thermal gravimetric analysis (TGA) and differential scanning calorimetry (DSC). The polarity was studied by miscibility in a series of organic solvents. The characterization results confirmed the basic structure of the ionic liquid, and it possesses high thermal stability. The physicochemical properties matched the design criteria used for this ionic liquid. Therefore, it was used in subsequent chapters to explore its potential as a solvent for lipid extraction from microalgae.

Keywords: lipid content; microalgae; ionic liquid; characterization

3.3 Introduction

Ionic liquids are composed of anions and cations, so the structures of ionic liquids are diverse[187, 188]. Due to the fact that ionic liquids have high thermal stability, high chemical stability, non-volatility, low clogging point, and non-flammability, they can be used as additives in select fields (such as lubrication, etc.) [189, 190]. Also, ionic liquids have high conductivity. Thus they have a wide range of applications in the electrochemical field [191, 192]. The structure and properties of high-conductivity ionic liquids (such as pH, polarity, etc.) can be adjusted so that it has the dual characteristics of a solvent and catalyst, and can be used in polymerization reactions, chemical reaction media, catalysts, etc [193, 194]. Moreover, it should be emphasized that the polarity and other physicochemical properties of the ionic liquid can be changed by adjusting the anion and cation structure of the ionic liquid, which can dissolve many inorganic and organic substances and form a liquid-liquid two-phase system with the solvent [96, 28]. This laid the foundation for the application of ionic liquids in the field of extraction and separation. The structural tunability of ionic liquids makes it very widely used in extraction and separation, which is better than traditional organic solvents. They have shown their superiority in the extraction of metal ions [195, 196], desulfurization/denitrogenation [197, 198], aromatic/aliphatic hydrocarbon separation [199, 200], the extraction and separation of natural biological oils [22, 201], and other fields. Ionic liquids can be designed and synthesized based on specific compounds to enhance their selective recognition performance for a certain type of compound [202].

In the past ten years, ionic liquids have been known for their ability to solvate highly refractory biopolymers (such as lignin or cellulose) and have become a promising solvent for

extracting lipids from microalgae [203]. There is a high tendency to disrupt the hydrogen bond network between microalgae structure and microfibrils [22, 116]. IL destroys microalgae cell walls by dissolving cellulose and polysaccharides, which are the main components of microalgae cell walls [204, 205]. Krishnan et al. found that the lipid extraction efficiency increased with the amount of IL and the lipid yield obtained up to 37% with FAME profile at the 1.5% IL by hydrophobic IL-based extraction [206]. Choi et al. compared 10 types of 1-ethyl-3-methylimidazolium [C_2mim]-based ionic liquids, and evaluated their abilities to disrupt *Haematococcus pluvialis* cyst cells for astaxanthin/lipid extraction.It was demonstrated that the lipid extraction of [C_2mim][HSO_4], [C_2mim][CH_3SO_3], and [C_2mim][$(CF_3SO_2)_2N$] obtained maximum extraction efficiencies of 82.2 1.4, 76.1 0.3, and 70.3 3.0%, respectively. Gao et al. summarized that the cation has better extraction performance after replacing the longer alkyl group for ILs with the same anion [207]. In this work, we synthesized an ionic liquid [C_{10}mim] [BTMPP] based on the patent [7] and then adopted it in the subsequent works, which has never been applied in lipid extraction processes. The cation($[C_{10}mim]^+$) and anion($[BTMPP]^-$) both had good properties in previous literature [208, 209]. Therefore, we first explored the basic properties of this ionic liquid, followed by lipid extraction performances in the next few chapters.

3.4 Materials and Methods

3.4.1 Materials

As mentioned, the ionic liquid [C_{10}mim] [BTMPP] was synthesized according to the patent [7]. The other related chemicals, H_3PO_4, H_2PO_4, $MgSO_4$, $CaCl_2$, NH_4Cl and glacial acetic were all purchased from Sigma-Aldrich, Inc. and used to prepare the culture solution.

3.4.2 The Synthesis of [C_{10}mim][BTMPP]

Kenneth Richard Seddon et.al invented the synthesis method (Patent:WO2006111712A2) [7]. [C_{10}mim][BTMPP] was synthesized by Dr. Natalia V. Plechkova from Queen's University Belfast. The reactions were shown in Fig. 3.1 [7]. In brief, 1-methylimidazole was added to 1-Bromododecane, and they were allowed to react at 40°C, and then it was mixed with ethyl acetate and cooled to enhance the separation. The ethyl acetate fraction was kept, and the water was removed. The ethyl acetate was removed using a rotary evaporator, and the IL was rewashed with additional ethyl acetate. Diisooctylphosphinic acid was converted to sodium salt in water using NaOH in a molar ratio of 1:1.05. Then the solution was neutralized using HCl to pH 7, which was validated by pH paper. 1-decyl-3-methylimidazolium bromide was mixed in a 1:1.05 by weight ratio with the sodium salt of diisooctylphosphinic acid in water at pH 7. The IL was recovered by extraction with ethyl acetate and separation in a separatory funnel. The ethyl acetate was removed using a rotary evaporator and washed again. The resulting IL was similarly washed with hexane two times and treated using a rotary evaporator.

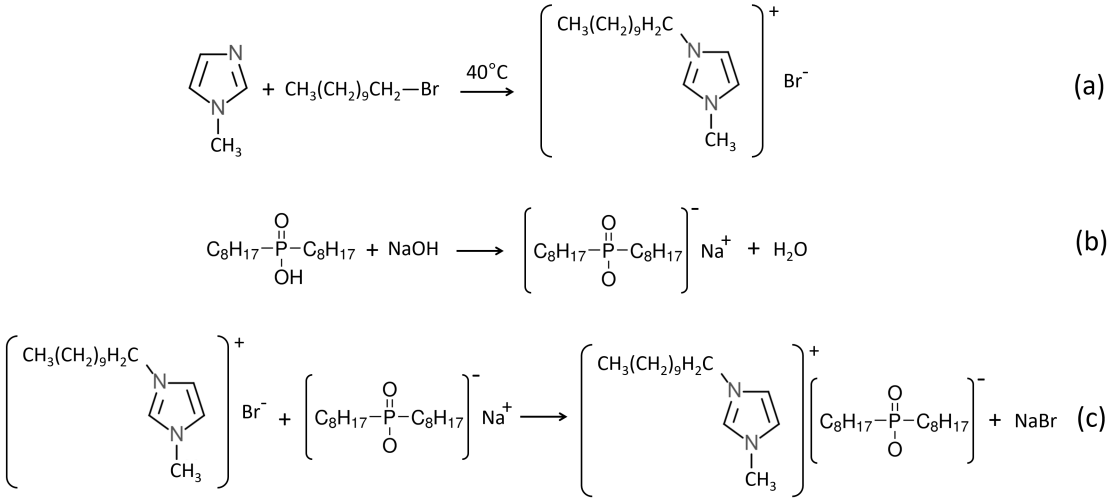

Figure 3.1: The synthesis of [C_{10}mim] [BTMPP] [7].

3.4.3 IL Characterization Methods

3.4.3.1 Thermo-Gravimetric Analyzer (TGA)

Measurements of decomposition temperature were conducted using a Thermo-Gravimetric Analyzer (TGA/SDTA851e, Mettler Toledo, Chicago, United States). The sample was measured in an aluminum crucible with a pierced lid for gas release, and the temperature was set at an increment of 10°C min^{-1} in the temperature range of 10°C to 600°C under nitrogen purge at a flow rate of 40mL min^{-1}.

3.4.3.2 Differential scanning calorimetry (DSC)

Thermal properties of [C_{10}mim][BTMPP], such as melting point, glass transition temperatures and enthalpy, were measured by differential scanning calorimeter (DSC/882e, Mettler Toledo, Chicago, United States). The sample was approximately 10 mg and analyzed in a hermetically sealed aluminum crucible. For each experiment, an empty hermetically sealed pan was referenced as the blank. The ramp temperature was set at 10°C min^{-1} from the range of -70°C to 150°C under nitrogen purge at a flow rate of 40mL min^{-1}.

3.4.3.3 Fourier transform infrared (FTIR)

Fourier transform infrared (FTIR) spectroscopy was used to investigate the functional groups in this synthesized ionic liquid. Each sample was analyzed directly by PerkinElmer Fourier Transform Infrared Spectroscopy (FTIR) with a universal ATR accessory in the range of 500-4000 cm^{-1} (scans = 16) with attenuated total reflectance.

3.4.3.4 Nuclear magnetic resonance (NMR)

NMR spectra were recorded on a Bruker Avance spectrometer DPX 300 at 21°C, using deuterated propanone as solvent (10–20 mM solutions for 1 H and 31P NMR spectroscopy; 30–35 mM solutions for 13C NMR spectroscopy), referred to TMS for 1 H and 13C NMR spectra and 85% H_3PO_4 for 31P NMR spectra.

3.4.3.5 Viscosity, Density, Acidic Property, and Miscibility

The viscosity of this ionic liquid was determined using a Brookfield viscometer dv-e, LVDVE115. All viscosity measurements were performed with spindle number 3 at 100 rpm in atmospheric pressure. The temperature varied from 22 to 70°C using a water base to see the trend of the viscosity of this ionic liquid. The density of [C_{10}mim] [BTMPP] was evaluated by a precision graduated cylinder (10 mL). Three different volumes of [C_{10}mim] [BTMPP] were weighed by an analytical balance and calculated to get average density at room temperature. To analyze the acidic property of [C_{10}mim] [BTMPP], different volumes of the H_2SO_4 solution (1.5 mol/L) were added into 1 mL [C_{10}mim] [BTMPP] to investigate the relationship between the pH of [C_{10}mim] [BTMPP] and the volume of H_2SO_4 added. The miscibility of this ionic liquid was determined as follows: an ionic liquid sample (around 5mL) was added into a beaker followed by 50 mL of solvents (water, ethanol, methanol, hexane, ethyl acetate, 2-propanol, and acetone). This mixed solution was fully stirred and observed for 10 minutes at room temperature in order to preliminary obtain its miscibility in various solvents.

3.5 Results and Discussion

3.5.1 TGA

Ionic liquids are widely used in many fields due to their excellent thermal stability. The synthesized ionic liquid is expected with good purity and stable physicochemical properties to facilitate subsequent liquid-liquid extraction or liquid-solid extraction processes. Here TGA was employed to characterize the decomposition temperature measurement of the self-synthesized [C_{10}mim][BTMPP]. Fig. 3.2 shows a declined trend of decomposition temperature of [C_{10}mim][BTMPP], which is similar to that of [P_{4444}][prop] synthesized in previous work [140, 210]. The weight of [C_{10}mim][BTMPP] lost have a slower decline between 30 to 230°C. This slowdown in the decrease is likely caused by a slightly contained impurity, such as water and ethyl acetate. Even though the purification process has been carried out, extremely high purity is hard to be guaranteed. [C_{10}mim][BTMPP] starts to decompose at around 230°C. However, the IL [C_{10}mim][BTMPP] burn out at around ~320°C. An acceptable reason is that it contains phosphorus and the sublimation temperature of phosphorus oxide is ~320°C. The TGA results indicate that the self-synthesized [C_{10}mim][BTMPP] has high purity and good thermal stability.

3.5.2 DSC

The pure and water-diluted [C_{10}mim][BTMPP] were analyzed, as shown in Fig.3.3. There is a relatively narrow peak around 4°C when the ionic liquid is diluted by water, which is the phase transition (ice melts into water) enthalpy of H_2O. The broad peak around 100°C is another phase transition (liquid water vaporizes into gas) enthalpy of H_2O. However, no

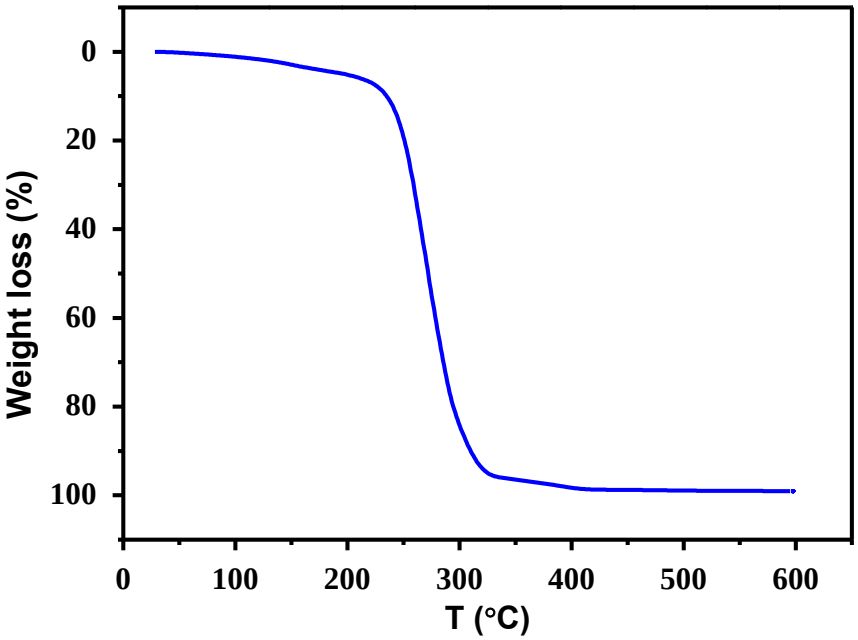

Figure 3.2: The TGA of [C_{10}mim][BTMPP].

significant heat absorption was found when detecting the pure sample, indicating no phase transition in the sample in the tested range of temperature. Thus, it can be concluded that [C_{10}mim][BTMPP] is amorphous and does not have a specific melting point in the measured range of temperatures.

3.5.3 **FT-IR**

IR is used to analyze the molecular structure of the self-synthesized [C_{10}mim][BTMPP]. The infrared absorption spectrum as shown in Fig.3.4 is the characteristic peaks of some functional groups, such as C-N at 1150, 1233, and 1346 cm^{-1}, C=C at 1571 and 1466 cm^{-1}, P=O at 1027 cm^{-1}, C=N at 1643 cm^{-1}, and C-H at 2932, 2926, 2858, and 809 cm^{-1}. These functional groups are greatly consistent with the molecular structure of [C_{10}mim][BTMPP]. IR analysis further confirms that the synthesized material is believed to be [C_{10}mim][BTMPP].

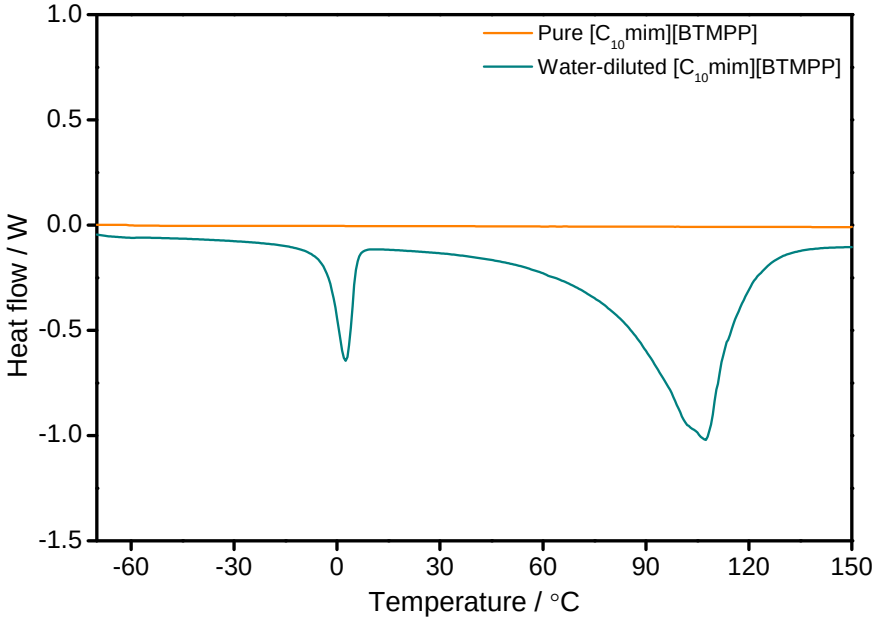

Figure 3.3: Differietial scanning calorimetry trace for [C_{10}mim][BTMPP].

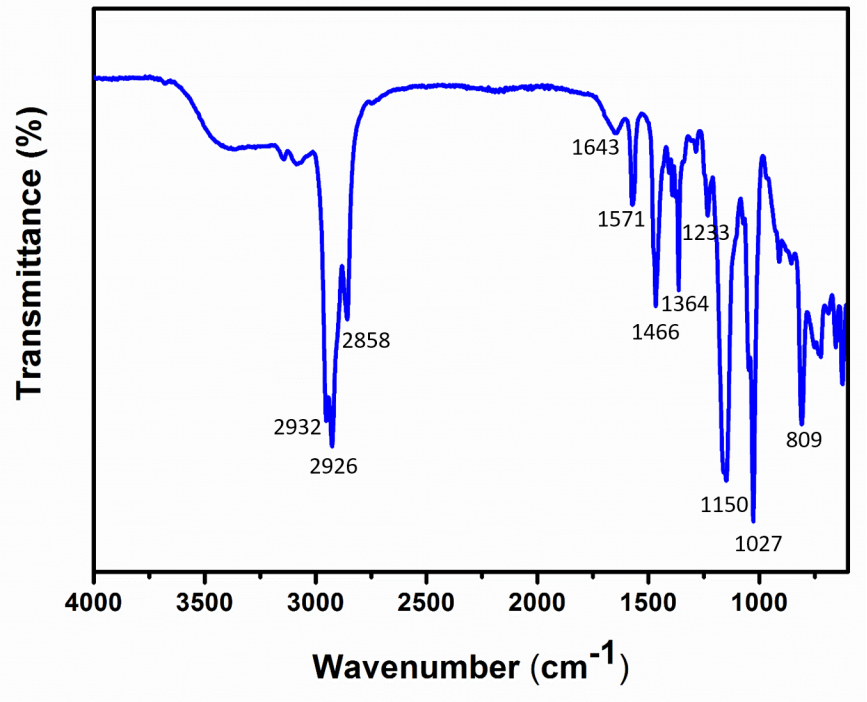

Figure 3.4: The FT-IR of [C_{10}mim][BTMPP].

3.5.4 NMR

NMR is also used to further confirm the structure of the synthesized [C_{10}mim][BTMPP], and ^{13}C NMR and related peaklist are shown in Fig. 3.5. The solvent (MeOD) peak at 49.5 ppm

is found. Two small peaks at high chemical shift (~120 ppm) are observed, and they should result from unsaturated carbons. Here, the two peaks found means that the two unsaturated carbons are asymmetrical. These findings are consistent with the structure of [C_{10}mim][BTMPP] that has precisely one carbon-carbon double bond. Since the element of nitrogen increases the chemical shift of carbon atoms on the ring, a series of peaks are observed near 53, 48, and 35 ppm. Two increased chemical shifts at about 41 ppm have also been seen, mainly caused by the influence of phosphorus (P–CH_2–). The rest of the saturated carbons are considered to be responsible for the remaining peaks between 0-40 ppm.

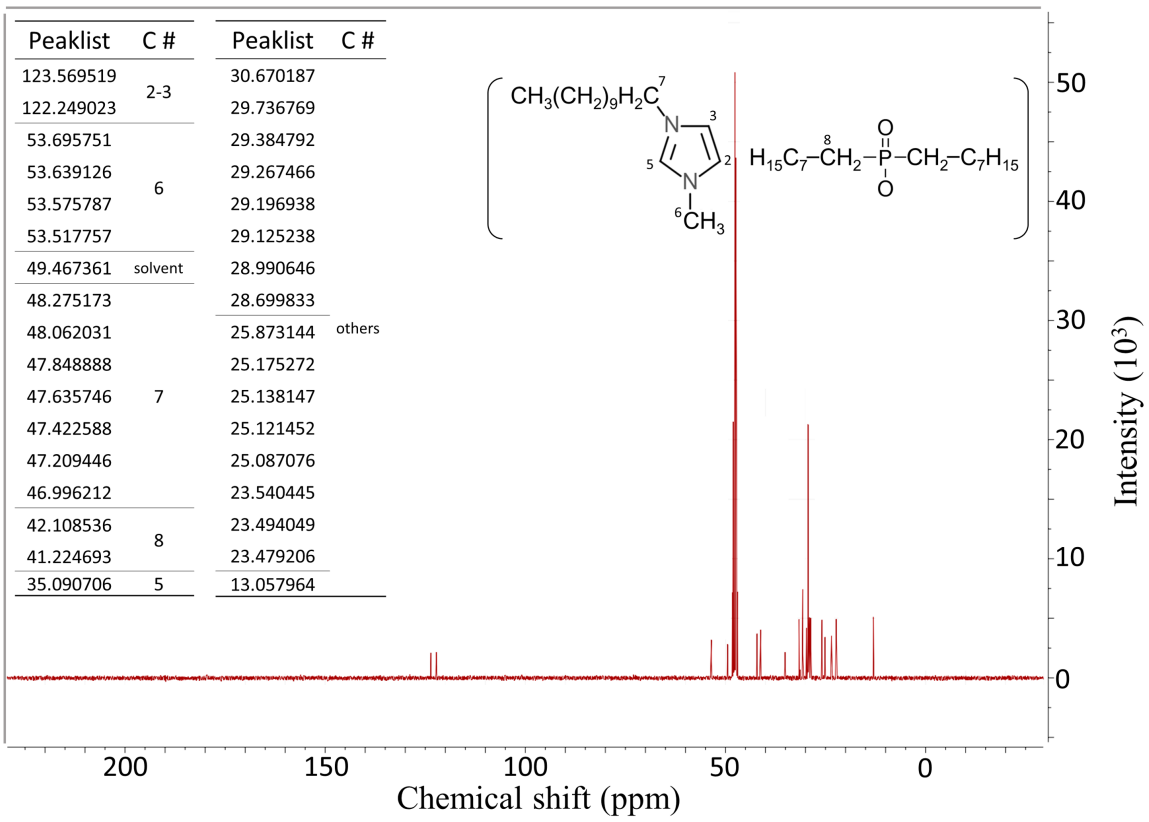

Figure 3.5: The ^{13}C NMR results of [C_{10}mim][BTMPP].

The proton NMR is presented in Fig. 3.6. The peak around 4.8 ppm is caused by the small amount of water in the [C_{10}mim][BTMPP]. These peaks between 0-2 ppm should be the result

of alkyl groups. Due to the induction effect of P and N, the chemical shift of H in P(N)-CH_2 are 3.2, 3.9 and 4.1, respectively. The two small peaks near 7.5ppm result from the conjugation of H at position 2 and 3 (Fig. 3.5). The tiny peak around nine ppm is caused by the H at position 5 due to the conjugation of the ring.

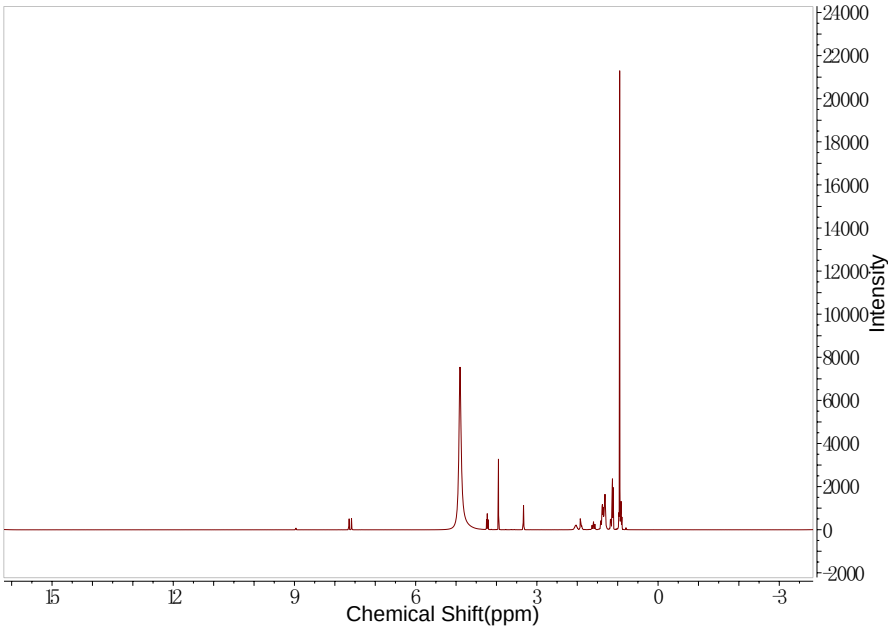

Figure 3.6: The proton NMR results of [C_{10}mim][BTMPP].

The result of ^{13}P NMR is given in Fig. 3.7. The highest peak is around 27 ppm, which is believed to be the result of the four-coordinated P. Meanwhile, some microscopic peaks are found near 50 ppm, which may come from a trace amount of $(C_8H_{17})_2$-P(O)-OH that were formed in the H_3PO_4 solution during the measurement of ^{32}P NMR spectrum.

3.5.5 Density

The density of [C_{10}mim] [BTMPP] was evaluated by a precision graduated cylinder (10 mL). Three different volumes of [C_{10}mim] [BTMPP] were weighed by an analytical balance, as shown in Table 3.1. The density of [C_{10}mim] [BTMPP] is found to be about 0.96 g/cm^3 at 25°C

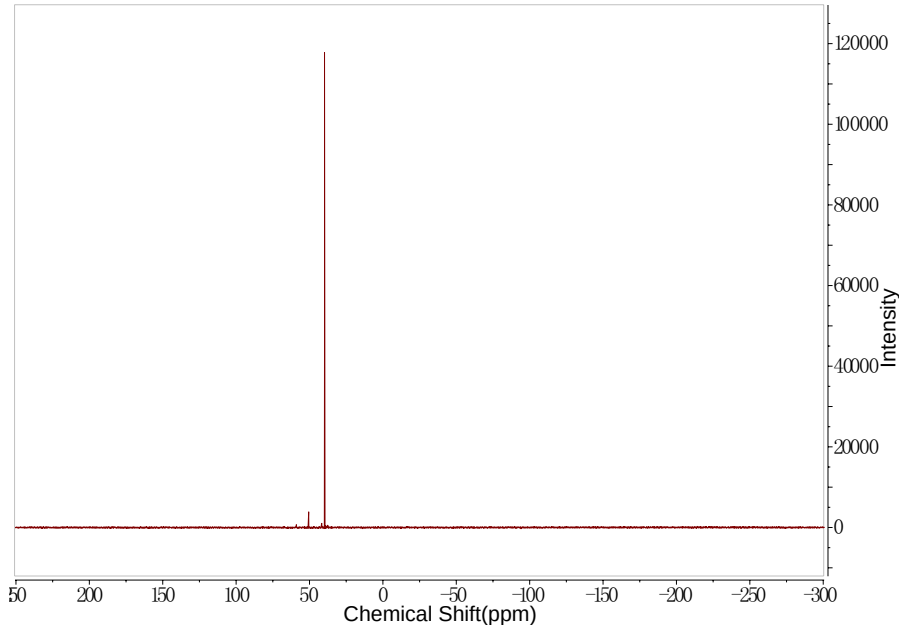

Figure 3.7: The ^{32}P NMR results of [C_{10}mim][BTMPP].

that is a little bit smaller than the density of water (0.997 g/cm^3). This smaller density indicates that [C_{10}mim] [BTMPP] will be the middle layer after the separation during the extraction process.

Table 3.1: The density of [C_{10}mim] [BTMPP].

	#1	#2	#3	Ave.
Volume/mL	5.03	8.12	9.87	
Mass/g	4.8008	7.8982	9.4384	
Density/(g/cm^3)	0.95	0.97	0.96	0.96±0.01

3.5.6 **Viscosity**

At room temperature (22°C), the viscosity of pure [C_{10}mim][BTMPP] is as high as 147 cP, and it cannot be magnetically stirred, hinting that diluent is necessary while extracting without heating. Accordingly, a water-diluted [C_{10}mim][BTMPP] (60% water) was used to extract oil in the present study, and its viscosity at different temperature is plotted in Fig. 3.8.

Theoretically, the viscosity of a fluid can be evaluated by the Vogel equation [211]:

$$Ln\,\eta \;=\; A + \frac{B}{T - C} \tag{3.1}$$

where η is viscosity, cP; T is temperature, K; A, B, and C are the coefficients of a specific material. The viscosity decreases with the increase in temperature, and the declining trend fits the Vogel equation. The fitted Vogel equation is shown in Fig. 3.8, and the values of A, B and C are found to be -2.82714, 1642.54, and 56.0157. The viscosity analysis helps to understand the extraction process, especially when the temperature changes.

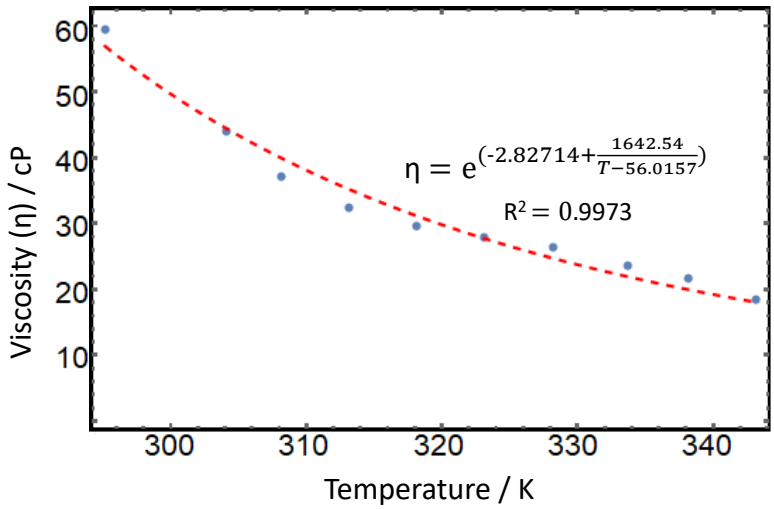

Figure 3.8: The viscosity of [C_{10}mim][BTMPP](60% water) at different temperatures.

3.5.7 **Miscibility**

In the extraction process, the ionic liquid needs to be determined to be immiscible with related solvents. The miscibility of [C_{10}mim] [BTMPP] in several common solvents are shown in Table 3.2. The analysis of miscibility helps to guide the selection of diluent for [C_{10}mim] [BTMPP]. On the one hand, since the viscosity of [C_{10}mim] [BTMPP] is fairly high, the diluent

is necessary to improve the flow property of [C_{10}mim] [BTMPP] in the extraction process. On the other hand, to recover the [C_{10}mim] [BTMPP] during the extraction, the diluent has to be immiscible with [C_{10}mim] [BTMPP]. Hence, water, methanol, hexane and acetone can be used with [C_{10}mim] [BTMPP] according to the miscibility analysis.

Table 3.2: The miscibility of [C_{10}mim] [BTMPP].

Solvent	Miscibility
D.I. Water	Immiscible
Methanol	Immiscible
Ethanol	Miscible
2-propanol	Miscible
Hexane	Immiscible
Ethyl acetate	Miscible
Acetone	Immiscible

3.5.8 **Acidic Property**

According to the preliminary experiments, the synthesized ionic liquid is sensitive to pH to pretreat microalgae. To analyze the acidic property of [C_{10}mim] [BTMPP], different volumes of the H_2SO_4 solution (1.5 mol/L) were added into 1 mL [C_{10}mim] [BTMPP]. Fig 3.9 shows the relationship between the pH of [C_{10}mim] [BTMPP] and the volume of H_2SO_4 added. The pH of pure [C_{10}mim] [BTMPP] is 6.46, and the pH was adjusted by H_2SO_4. The relationship will be helpful for adjusting the pH of [C_{10}mim] [BTMPP] to an expected value.

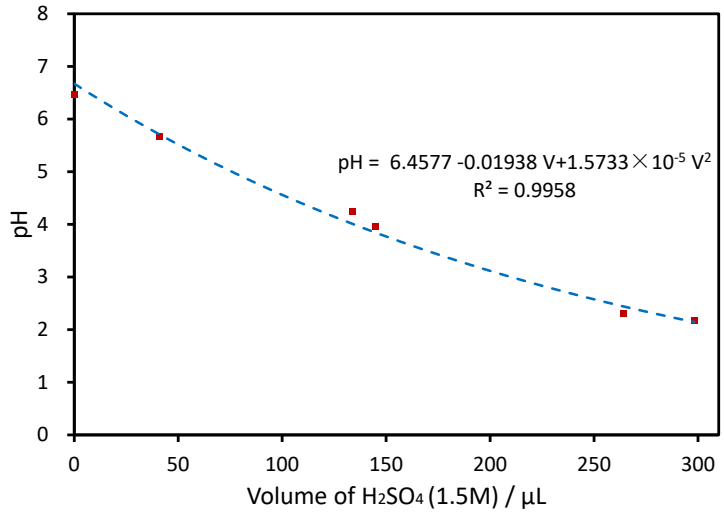

Figure 3.9: The pH values of [C_{10}mim] [BTMPP] when adjusting with H_2SO_4 solution (1.5 mol/L).

3.6 Conclusions

In conclusion, it is confirmed that the self-synthesized ionic liquid [C_{10}mim][BTMPP] has been obtained by the structural characterizations using FT-IR and NMR. In addition, the TGA results indicate that the self-synthesized [C_{10}mim][BTMPP] has a high purity and good thermal stability. Moreover, other fundamental properties, such as viscosity, density and so on, are given in this chapter to clearly understand the physicochemical properties of this ionic liquid. Among the miscibility tests, [C_{10}mim][BTMPP] is immiscible in water, hexane, and methanol. Consequently, these also support for subsequent extraction applications.

4 Explorations of Oil Extraction from Dry *Chlorella vulgaris* Using ILs

4.1 Preface to Chapter 4

The previous chapter describes the structure and properties of the synthesized ionic liquid. Then ionic liquid based lipid extraction was conducted in chapter 4. *Chlorella vulgaris* was chosen as a well-studied model microalgae in order to be able to compare the results found here to the vast body of existing literature on this organism. *C. vulgaris* has a particularly thick cell wall hence rendering successful treatments here also suitable for other organisms. Furthermore the initial characterization of the extraction process was done on dried algae, to avoid complications by the presence of water.

4.2 Abstract

Ionic liquids (ILs) have been proven to be promising in pretreating microalgae and extracting lipid or other bio-active compounds. *Chlorella vulgaris* (*C. vulgaris*) as a typical representative of microalgae, has a faster growth rate and easier cultivation in comparison to most microalgae. Therefore, *C. vulgaris* has been considered as a promising microalgae for lipid production. Additionally, a thicker cell wall makes it relevant model organism to test novel extraction technologies and demonstrate general effects observed from other microalgae. In this work, several ionic liquids were employed to test the potential of the extraction process. A good candidate ionic liquid [C_{10}mim] [BTMPP] was found in the process. Meanwhile, extracted oils by the ionic liquid pretreatment were further characterized, referring to fatty acid methyl ester (FAME) composition.

Keywords: microalgae; ionic liquid; *Chlorella vulgaris* ; lipid yield; dry weight

4.3 Introduction

Microalgae have received increasing attention over the last decades, owing to their high CO_2 sequestering capability and high energy conversion efficiency, together with the fast growth rates and elevated lipid levels [48, 212, 213]. Theoretically, microalgae have the capacity to offer great photosynthetic conversion efficiency without occupying agricultural land for the cultivation [22]. Unlike other microorganisms, many microalgae species have a cellulosic cell wall that is challenging to be mechanically disrupted hence hampering the recovery of lipid or other intracellular products [26]. Therefore, the commercial viability of microalgae-based lipid is limited mainly due to the cost of downstream processing, especially drying processes and cell wall disruption. Lipid extraction using conventional solvents is slow, energy-intensive and creates environmental issues [214, 215, 216]. Simultaneously, conventional solvents may affect the quality of the product by co-dissolving some unwanted compounds, such as chlorophyll [22, 116]. *Chlorella vulgaris* is considered a promising resource for lipid production due to its fast growth and easy cultivation. The cell wall of *C. vulgaris* is particularly challenging to disrupt. It is composed of cellulose, hemicellulose and saccharides that impede the intracellular lipids releasing process [217]. Therefore, ILs can likely be applied to many strains of microalgae once shown efficient for *C. vulgaris*.

Recently, ionic liquids (ILs) have been demonstrated to increase the lipid extraction efficiency from microalgae [38, 218]. ILs are often described as designable solvents, having steady physicochemical performance, such as excellent thermal stability, low melting points, and negligible vapor pressure [219]. Some ILs can even enhance cellulose-dissolving capacity [99, 116]. Shankar et al. synthesized protic ionic liquids and found that protic ionic liquids

treatment(PIL-treatment) could obtain significantly higher lipid yields than the conventional Bligh and Dyer method. Moreover, butyrolactam hexanoate (BTH), among the PILs, was confirmed to exhibit the highest lipid yield, up to 1.86 times the yield obtained with the control method for *Chlorella* [16]. Kim et al. adopted the ionic liquid [C_4mim][$MeSO_4$] to extract microalgal lipids from *Chlorella vulgaris* with the pretreatment of ultrasound. Compared with that of Bligh and Dyer's method and the Soxhlet method (diethyl ether as solvent), the lipid yield is 2 fold and 1.6 fold higher, respectively [220]. Zhou et al. also pointed out that ionic liquid [C_4mim][$MeSO_4$] was an efficient solvent to extract lipids from *Neochloris oleoabundans* at 70°C for 2 hours. The ratios (ILs:methanol) of 1:7 and 1:3 were the optimum conditions in their work to achieve the lipid extraction from the microalgae. Moreover, it was considered the main reason for reducing the extraction rate that the loss of ionic liquid in the reaction was with the increase in the water content of ionic liquid [221]. Other ionic liquids, such as [C_2mim][$EtSO_4$], [C_6mim]Cl, etc., were also widely regarded as candidates for microalgal lipid extraction [17, 22].

In the chapter, several ILs, 1-ethyl-3-methylimidazolium ethylsulfate [C_2mim][$EtSO_4$], 1-hexyl-3-methylimidazolium chloride [C_6mim]Cl, and [C_{10}mim][BTMPP] mentioned in chapter 3, were utilized to pretreat dry microalgae *C. vulgaris*, separately. All these ILs worked under the conditions tested. The preliminary results indicate that further optimization of one promising ionic liquid would improve extraction efficiencies using gravimetric analysis of total extractable oils. In addition, extracted oils using ionic liquid pretreatment would be further characterized referring to FAME composition.

4.4 Materials and Methods

4.4.1 Materials

As mentioned in chapter 3, the ionic liquid [C_{10}mim] [BTMPP] was used [7]. However, it must be diluted by D.I. water because of its high viscosity. Thus the work related to this ionic liquid kept this definition, [C_{10}mim] [BTMPP] (60% water). The other ionic liquids, [C_2mim][$EtSO_4$], and [C_6mim]Cl as well as other chemicals were all purchased from Sigma-Aldrich, Inc.

4.4.2 Strain and Cultivation Conditions

The green algae *Chlorella vulgaris* strain UTEX 2714 was purchased from the Algae Culture Collection at the University of Texas Austin. Aseptic technique was applied to maintain the culture in liquid culture in a 200 cm^3 tri-acetate-phosphate (TAP) medium pH 7.0 in a 500 cm^3 shake flasks. The seed culture was grown under circulating light including 16h on: 8h off (100 µmol m^{-2} s^{-1}) at a speed of 150 rpm at 25°C. The TAP medium consisted of 20 mM tris base, 2.4 mM K[H_2PO_4], 1.58 mM K_2 [HPO_4], 0.83 mM $MgSO_4$, 0.34 mM $CaCl_2$, 7.0 mM [NH_4] Cl, 1 cm^3 L^{-1} glacial acetic acid and 1 cm^3 L^{-1} of Hutner's trace element solution [222]. After 3 d, the exponentially growing seed culture (100 mL) was inoculated into a 900 mL medium at a concentration of 10% (v/v) and cultured in TAP media with reduced [NH_4] Cl (5 mM) and supplement of glucose (20 g L^{-1}) to induce lipid production for 5 days at 25°C and 150 rpm.

4.4.3 Total Lipid Content by Direct Transesterification

The fatty acid methyl ester (FAME) content by weight, based on the freeze-dried cells, were determined by a direct transesterification protocol, a standard FAME laboratory analytical procedure, developed by the National Renewable Energy Laboratories (NREL) briefly as follows [223]:

1. In triplicate, approximately 10 mg ($m_{microalgae}$) of the sample were mixed with 20 µL of the recovery standard pentadecanoic acid methyl ester (C15:0Me at 10 mg mL^{-1}).

2. 200 µL of a mixture of trichloromethane/methanol (2:1 v/v) and 300 µL of 0.6 M HCl were added, and then they were incubated and stirred in a water bath on a magnetic hot plate at 85°C at 1000 rpm for 1h. This step must be carried in a fumehood.

3. After samples cooled to room temperature, 1 mL of hexane was added using a gas-tight syringe and the mixture was stirred at room temperature for 1h at 1000 rpm.

4. Then, those samples were centrifuged and 50 µL of the clear supernatant (hexane phase) was transferred into a GC vial followed by 400 µL hexane.

5. Last, 50 µL of the internal standard undecanoic acid methyl ester (C11:0Me) was subsequently spiked to obtain a final concentration of 100 µg/mL.

6. Run on GC and record total FAME concentration(C_{total}) and concentration of C15:0Me ($C_{C15:0}$)

FAME was separated and analyzed using the following oven ramp: 50°C, 1 min, 10°C min^{-1} to 200°C, 3°C min^{-1}1 220°C, 10 min by an FID equipped Agilent 7890 Series GC with

an Agilent DB-Wax capillary column (30m, 0.25 mm, 0.25 μm). Helium was used as carrier gas. Individual FAMEs were quantified according to the standard analytical mixture and the internal standard (Supelco 37, Sigma Aldrich). Total FAME content by weight was calculated using the following equations, according to the NREL LAP. The weight of cumulative FAME is revised by the recovery standard C15:0Me followed by dividing the total by the dry weight of microalgae cells of this work.

Adjusted factor can be calculated. Above all, the recovery rate (R) can be calculated by:

$$R = \frac{C_{C15:0}}{200\mu g/mL} \tag{4.1}$$

The lipid concentration can be calculated by:

$$C_{FAME}(\mu g/mL) = \frac{(C_{total} - C_{C15:0})}{R} \tag{4.2}$$

The total mass of FAME in the sample can be calculated:

$$m_{FAME}(mg) = C_{FAME}(\mu g/mL)\ \frac{500\mu L(measured\ \ volume)}{450\mu L(transferred\ \ volume)}\ \ \frac{1mL(initial\ \ volume)}{1000\mu g/mg}$$

$$= 1.1111 \times 10^{-3}\ \ C_{FAME}\ (mg) \tag{4.3}$$

The FAME content in the sample:

$$FAME\ \ content = \frac{m_{FAME}(mg)}{m_{microalgae}(mg)}\ \ \times 100\% \tag{4.4}$$

4.4.4 Extracted by Hexane/Isopropanol

Hexane/Isopropanol Extraction (HIP) was based on the procedure from Hara & Radin [224]. This procedure is used to determine the mass of neutral lipids (a.k.a. triglycerides).

1. In triplicate, around 250 mg of samples were added into an air-tight test tube with solvent compatible caps. Then record the weight of each sample, m_{sample}.

2. 5 mL of hexane/isopropanol mixture (HIP) (hexane:isopropanol, 3/2, v/v) and a miniature stir bar was subsequently both added to each tube.

3. The mixture was stirred and mixed for 16 h on a magnetic stir plate in the fume hood.

4. A fine porosity Buchner funnel was applied to filter the mixture using the vacuum and collect the filtrate in a pre-weighted foil pan(m_{pan}) until the filtrate was colorless.

5. The pan was weighed again until the measurements were stable ($m_{pan+oil}$) once the solvent had evaporated.

The crude oil content in the sample:

$$Crude\ oil\ content = \frac{m_{pan+oil}(g) - m_{pan}(g)}{m_{microalgae}(g)} = \frac{m_{oil}(g)}{m_{microalgae}(g)} \times 100\% \tag{4.5}$$

4.4.5 Extraction Method of Dry *Chlorella vulgaris* with the Pretreatment of Ionic Liquids

1. Around 250 mg of dry cells (m_{sample}) were added into a test tube with a miniature stir bar.

2. Some amount of ionic liquids was added into the tubes and stirred on a magnetic stir plate for 1h.

3. 5 mL of hexane was added and the mixture was vortexed for 30s.

4. Then the mixture was kept for 5 min to separate and the supernatant layer (hexane phase) was transferred to a preweighed pan, m_{pan}.

5. Step 4 was then repeated twice and the pan was weighed as above, $m_{pan+oil}$.

This method uses the IL to pretreat the cell and then hexane was adopted to extract the lipids from dry biomass sample. Use Eq. 4.5 to calculate the crude oil content.

4.4.6 Oil Yield and Extraction Efficiency

Crude oil yield calculated in the above method needs to be investigated for the FAME amount to obtain oil yield and even extraction efficiency. Therefore, the extracted oil samples were analyzed by GC via the following procedures:

1. Approximately 10 mg ($m_{oil\ sample}$) of oil sample was added into a GC vial or a small test tube.

2. Then, 1 mL of hexane was spiked by a gas-tight syringe.

3. 50 μL of 2 M KOH (in methanol) was also added into the vial using a gas-tight syringe and the mixture was vortexed.

4. After that, the mixture was centrifuged and 50 μL of the supernatant was transferred to a new GC vial using a gas-tight syringe.

5. 400 µL of hexane was added to dilute and 50 µL of internal standard (C11:0Me) was spiked to each vial before GC analysis.

6. Run on GC to determine FAME amount and composition and record the concentration of the lipids (C_{total}).

According to above procedures, the oil yield of the extraction process can be calculated by the equation:

$$\begin{aligned} Oil\ yield &= \frac{0.5mL \times 1.05mL\ C_{total}(\mu g/mL)}{0.05mL \times 1000(\mu g/mg)\ m_{oil\ sample}(mg)}\ Crude\ oil\ content \times 100\% \\ &= \frac{0.0105\ C_{total}}{m_{oil\ sample}}\ Crude\ oil\ content \times 100\% \end{aligned} \tag{4.6}$$

If the oil yield from HIP method is considered as the total lipid content, the extraction efficiency of ILs can be calculated by:

$$Extraction\ efficiency = \frac{Oil\ yield}{Total\ lipid\ content} \times 100\% \tag{4.7}$$

4.4.7 Experimental Design

To investigate the importance of various experimental parameters, one ionic liquid was conducted by experimental design after the explorations and comparisons by several ionic liquids. A full factorial design (FFD) was selected to assess the response pattern with four parameters. The combination of mass ratio (IL: microalagae), temperature, processing time, and mixing time was evaluated for the selection of significant influence factors. In this work, processing time means the pretreatment time of [C_{10}mim][BTMPP]. The definition of mixing time is the amount of time necessary to attain homogeneity under constant mixing conditions [225]. The

uncoded values for each factor were as follows [low point, high point]: mass ratio of ionic liquid to *C. vulgaris* [0.5, 10], temperature in degree Celsius [20, 80], processing time [0.5, 2], and mixing time [10, 30]. The experimental design was preliminary established using Design Expert 10.0.4 (Stat-Ease, Inc., Minneapolis, MS, USA). Also, all conditions were carried out in triplicates. Thus, there were 16×3 factorial conditions.

4.5 Results and Discussion

4.5.1 Total Lipids

As shown in Table 4.1, the total crude oil content of the *C. vulgaris* was analyzed by the conventional method (HIP). It was found that the crude oil content is around 25.6±3.3% by three parallel extractions and calculated by Eq. 4.5. Moreover, the crude oil content above was further investigated for the FAME amount in order to obtain oil yield. The results are presented in Table 4.2. According to Eq. 4.6, the total lipid content (maximum oil yield) turned out to be about 19.33±2.2%. The difference between the two values should be the chlorophyll and other components extracted by hexane/isopropanol at the same time [226, 227].

Table 4.1: Crude oil content of the *C. vulgaris*.

	m_{oil}/g	$m_{microalage}$/g	Crude oil content/%	Ave. content/%
#1	0.0727	0.2544	28.6%	
#2	0.0545	0.2463	22.1%	25.6±3.3
#3	0.0648	0.2485	26.1%	

Table 4.2: The oil yield of *C. vulgaris*.

	C_{C15}/(μg/mL)	$m_{oil\ sample}$/g	m_{oil}/g	Maximum oil yield/%	Ave. oil yield/%
#1	732.343	10.8	0.0727	20.3	
#2	729.124	10.1	0.0545	16.8	19.3±2.2
#3	778.114	10.2	0.0648	20.9	

4.5.2 Lipid Extraction of *C. vulgaris* via ILs

$[C_2mim][EtSO_4]$ was first employed to extract lipid from the cultured *C. vulgaris*. After the pretreatment of $[C_2mim][EtSO_4]$, the solution separated into three layers as shown in Fig. 4.1. In detail, the three layers are $[C_2mim][EtSO_4]$, the residue of *C. vulgaris*, and the supernatant layer of hexane/oil, respectively. To explore the pretreatment effects of different mass of ionic liquids, the oil yields at various mass ratios are given in Fig. 4.2. While the mass ratio of *C. vulgaris* to $[C_2mim][EtSO_4]$ increased from 1:1 to 10:1, and the oil yield changed in the range of 1.8-4.8 wt%. Compared to the total lipid content, 19.33±2.2% (Fig. 4.2), it is obvious that the maximum of the extraction efficiency, among these mass ratios, is around 24.83% in Fig. 4.2.

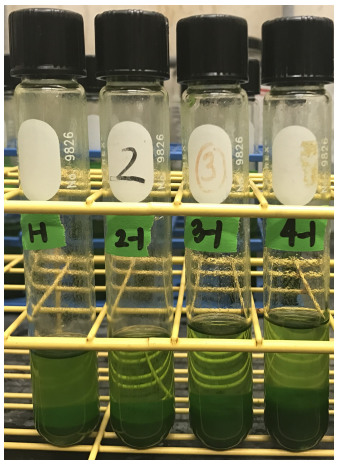

Figure 4.1: The photograph of *C. vulgaris* extracted under different mass ratio ($[C_2mim][EtSO_4]$:*C. vulgaris*). Left to right: 1:1, 2:1, 5:1 and 10:1.

Then $[C_6mim]Cl$ was also tested for helping extract lipids from dry *C. vulgaris* cells, which are shown in Fig. 4.3. The mass ratio of *C. vulgaris* to $[C_6mim]Cl$ changed from 0.25:1 to 5:1. It is notable that less amount of $[C_6mim]Cl$ made it possible to achieve relatively more lipid than that of $[C_2mim][EtSO_4]$. However, the oil yield attained increased from 5.3±0.6% to

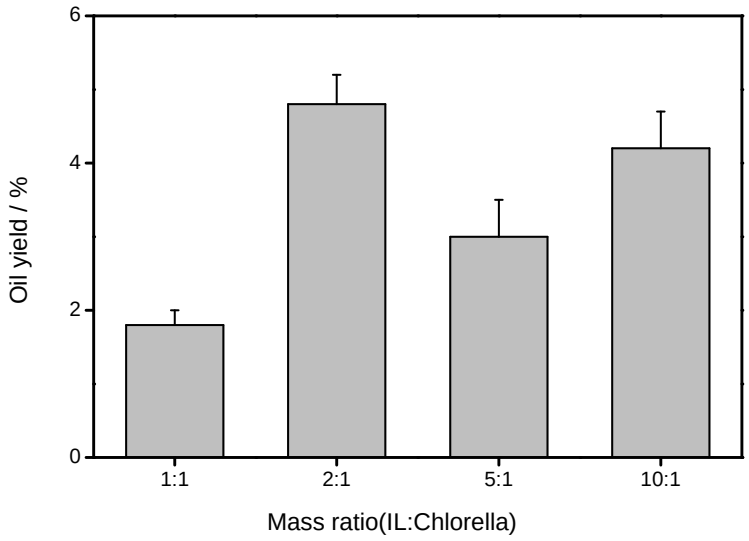

Figure 4.2: Oil yields of *C. vulgaris* under different mass ratio ($[C_2mim][EtSO_4]$:*C. vulgaris*).

8.1±0.5% with the mass ratio between 0.25:1 and 5:1. It is vital to highlight that although there is a 20 times difference in the initial conditions, the final oil yield of grease is not fairly evident, especially when compared with that of $[C_2mim][EtSO_4]$ (10 times difference in the initial condition). This implies that the mass ratio shows less influence on the oil yield when extracting by $[C_6mim]Cl$.

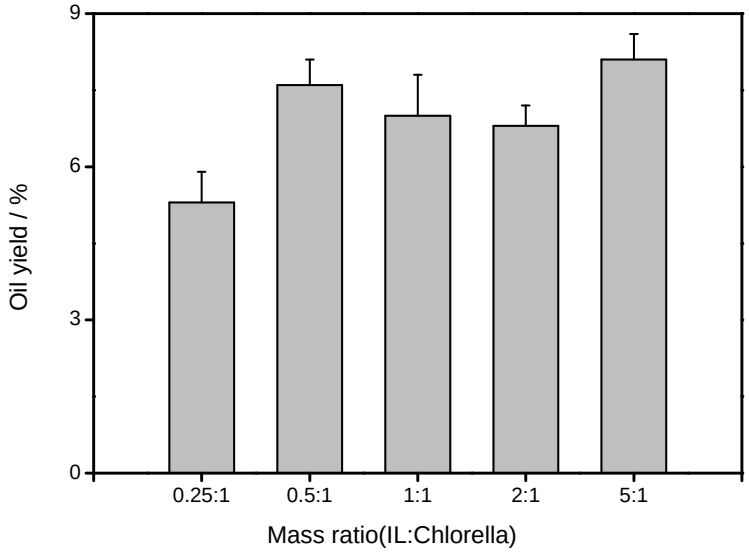

Figure 4.3: Oil yields of *C. vulgaris* under different mass ratio ($C_6mim]Cl$:*C. vulgaris*).

On the basis of the explored extraction results of pretreatment by common ionic liquids, the lab-synthesized ionic liquid [C_{10}mim][BTMPP](60 wt.% water) was subsequently used to extract lipids from *C. vulgaris* for preliminary attempts. It shows that the solutions separated into three layers (from bottom to top): water, [C_{10}mim][BTMPP], and the supernatant of hexane phase in Fig. 4.4. The color of the layer of [C_{10}mim][BTMPP] became much green when the mass ratio increased, this is likely due to more cell disruption and an increase in chlorophyll release from more [C_{10}mim][BTMPP] added. The results of oil yields given in Fig. 4.5 show that there is a trend of small increase when the mass ratio increase from 2:1 to 10:1. The total lipid is about 19.3 wt% (Table 4.2), so the highest extraction efficiency in the three tests is approximately 61% as calculated by Eq. 4.7. The extraction efficiency is greater than those of [C_6mim]Cl (41.90%) and [C_2mim][$EtSO_4$] (24.83%), indicating [C_{10}mim][BTMPP] has a good potential to be a pretreatment agent with highly efficient extraction for further research. This might have resulted from a similar reason that ionic liquids have good desulfurization and extraction performance with longer carbon chains. As the length of carbon chain length increases, the interaction between anions and cations of ionic liquids is weakened, which also has a non-negligible effect on the extraction efficiency [207]. To further evaluate the main factors for the extraction process, lipid extraction from dry *C. vulgaris* by [C_{10}mim][BTMPP] was conducted under a full factorial design.

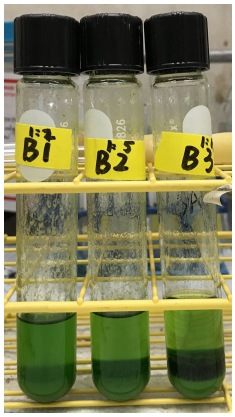

Figure 4.4: The picure of extracting oil from dry *C. vulgaris* by different mass ratios of [C_{10}mim][BTMPP] to *C. vulgaris*. Left to right: 2:1, 5:1, and 10:1.

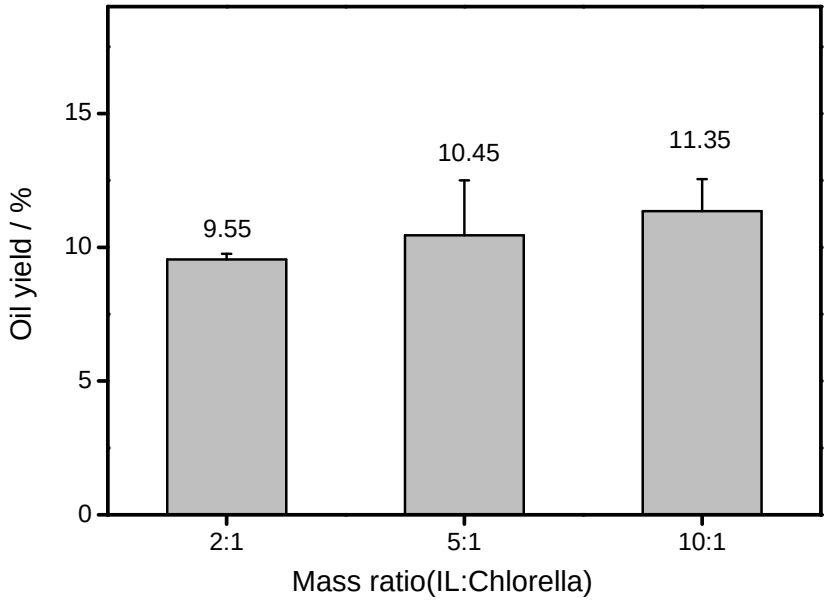

Figure 4.5: Oil yields from dry *C. vulgaris* by [C_{10}mim][BTMPP].

4.5.3 Evaluation of Main Factors of Lipid Extraction

4.5.3.1 Experimental design

In view of the effect of mass ratios, other significant factors also need to be confirmed. A full factorial design was introduced to investigate the factors including temperature, processing time, and mixing time, which might have significant effects on oil yield. There are four factors

with 16 conditions designed and carried out.

Table 4.3: The oil yields (average ± standard deviation) under designed conditions.

Mass ratio	Temperature/℃	Processing time/h	Mixing time/s	Oil yield/wt%
10	20	2	10	8.46±0.77
10	20	0.5	10	11.58±1.53
10	80	2	30	10.47±1.17
0.5	80	0.5	30	8.89±0.93
10	20	2	30	10.52±0.36
10	80	0.5	10	11.72±1.02
10	80	2	10	11.32±1.39
0.5	20	2	30	10.60±1.32
10	80	0.5	30	10.51±0.74
0.5	80	2	10	7.98±0.55
0.5	80	0.5	10	8.52±0.31
0.5	20	0.5	10	8.66±1.09
0.5	20	0.5	30	9.68±1.22
0.5	80	2	30	9.86±1.26
10	20	0.5	30	5.23±0.57
0.5	20	2	10	7.61±1.07

4.5.3.2 The model analysis

The normal probability plot indicates whether the standardized effect follows a normal distribution. If the set of real numbers follows normal distribution, the normal probability graph will be a straight line. The normal probability plot of the actual response shown in Fig. 4.6a implies that most of the points are on or near the straight line, meaning that the standardized effects fit the normal distribution well. In addition, the orange points show a positive impact on the oil yield, but blue ones are negative effects, so mass ratio, temperature, and processing time are positive factors, while the mixing time is a slightly negative one.

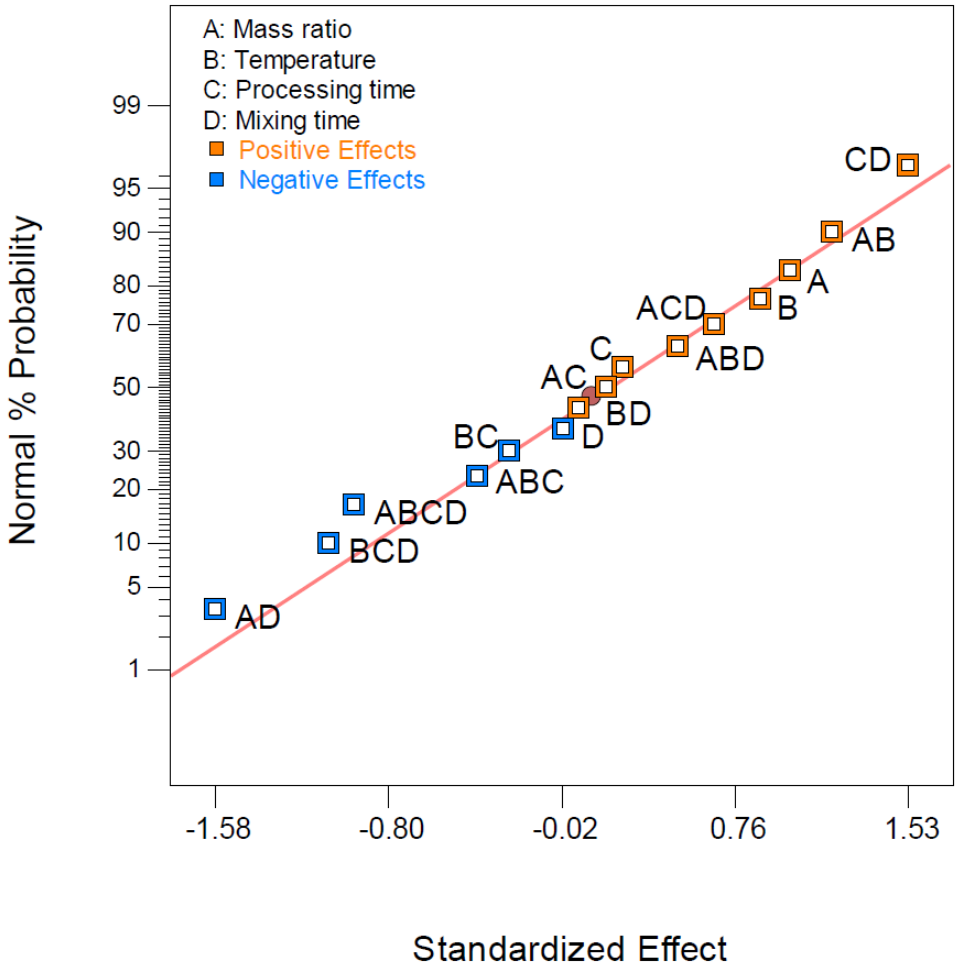

Figure 4.6: Normal probability plot of standardized effect.

4.5.3.3 The analysis of the main terms

Table 4.4 shows the standardized effect and contribution of each model term. From analysis of the effect terms, the contribution of mass ratio (A) and temperature (B) are far more than that of processing time (C) and mixing time (D). The interactive effects, such as AD, C, and AB are observed with great contributions.

Table 4.4: Analysis of the effect terms of the model.

Terms	Standardized effect	Sum of squares	%Contribution
Intercept			
A	1.0032	4.0259	9.0994
B	0.8687	3.0186	6.8228
C	0.2551	0.2604	0.5885
D	-0.0132	0.0007	0.0016
AB	1.1897	5.6616	12.7964
AC	0.1778	0.1264	0.2857
AD	-1.5760	9.9349	22.4550
BC	-0.2567	0.2635	0.5957
BD	0.0574	0.0132	0.0298
CD	1.5338	9.4103	21.2692
ABC	-0.3974	0.6319	1.4281
ABD	0.4997	0.9990	2.2579
ACD	0.6616	1.7506	3.9568
BCD	-1.0641	4.5294	10.2375
ABCD	-0.9509	3.6172	8.1757

A is mass ratio; B is temperature; C is processing time; D is mixing time.

The influence degrees of the four factors (Mass ratio, Temperature, Processing time and Mixing time) on the oil yield are analyzed in Fig.4.7. A higher correlation means a more substantial impact on the oil yield. The correlations of the mass ratio and temperature are 0.302 and 0.261, respectively. The correlation values of processing time and mixing time are much smaller, just 0.077 and -0.004, respectively. These analyses are consistent with previous conclusions.

Hence, the evaluation of the contribution of four factors shows that mass ratio and temperature have a strong effect on the extraction. The processing appears to occur fast, as extending the processing time did not have a strong effect on the extraction, neither did the mixing time. Therefore, the results given by the FFD model indicates that detailed optimization of these two factors, mass ratio, and temperature, are advisable for further experiments.

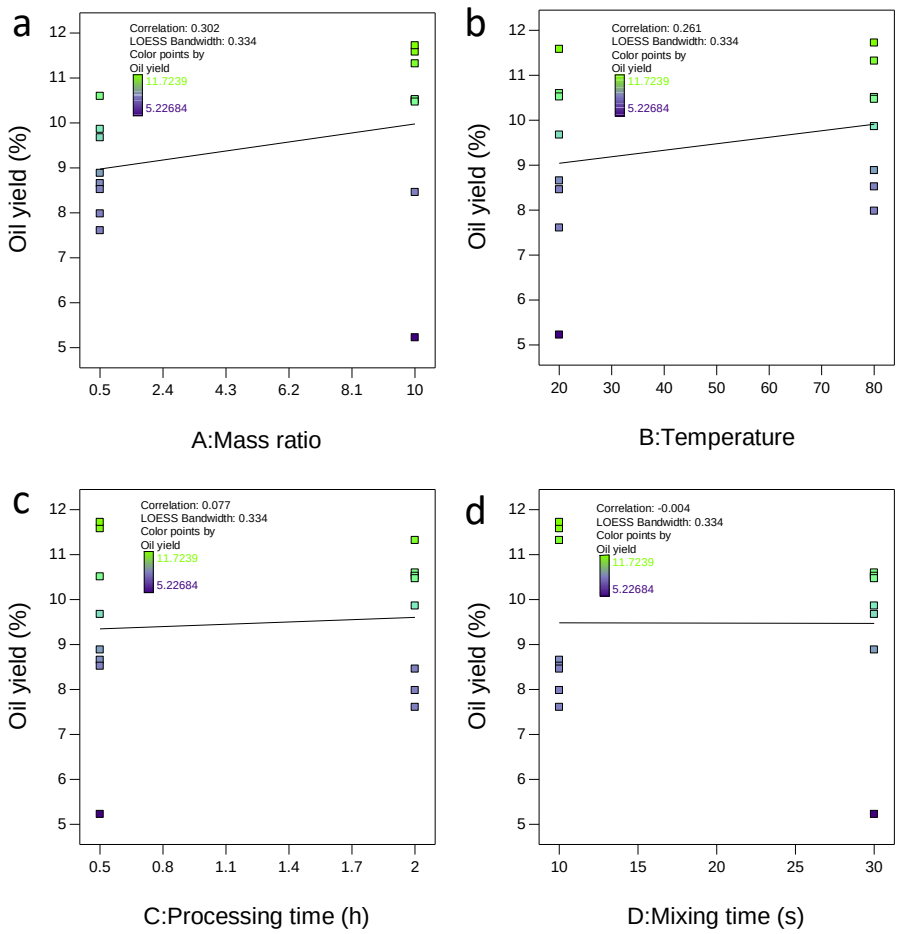

Figure 4.7: Correlation coefficients of the four factors. a) mass ratio; b) temperature; c) processing time; d) mixing time.

4.5.3.4 Oil composition

The composition of the extracted oil is shown in Fig. 4.8. The major composition of the oil from dry *C. vulgaris* are proved to be C18:1n9c, 40.9%. The weight percentages of C18:0, C18:2, and C16:0 contained in the oil are all a little more than 10%. The content of C17:1 and C15:0 are just around 3% in the oil, and the rest lipids are less than 2%. In addition, the results also suggest that [C_{10}mim][BTMPP] extract most of the lipids available without oxidizing the unsaturated ones.

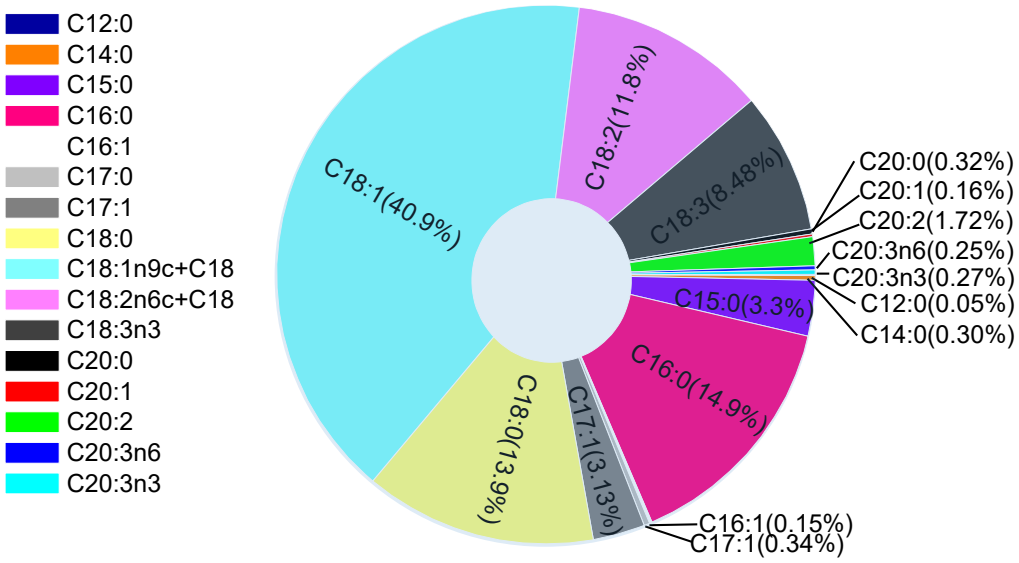

Figure 4.8: The composition of the oil.

4.6 Conclusions and Perspective

In this chapter, three ionic liquids were utilized in order to see the effect of assisting in the extraction of oil from dried *C. vulgaris*. The preliminary analysis above has provided critical information. Among them, the performance of them is showed as follows: $[C_2mim][EtSO_4]$ $<[C_6mim]Cl$ $<[C_{10}mim]$ [BTMPP]. The extraction efficiency increased with the length of cations of these ionic liquids. The extraction efficiency by $[C_{10}mim]$ [BTMPP] reached around 74.6%. The results of preliminary exploration proved that lipid extraction of dried *C. vulgaris* can be improved by ionic liquids to obtain a relatively good oil extraction efficiency. The results of the full factorial design indicated that mass ratio and the temperature had a significant effect on extraction, but processing time and mixing time did not. The FAME composition of extracted oil showed that most of them were extracted, and C18:1n9c accounted for the most. However, the drying process is still a stage of high energy consumption, and it cannot compete with traditional petroleum, so further exploration is needed. The extraction effect under wet

algae conditions is more meaningful for the application of this extraction method.

5 Lipid Extraction from fresh *Chlorella vulgaris* under the Synergistic Pretreatment of Electrolysis and ILs

5.1 Preface to Chapter 5

The previous chapter investigated the extraction of lipid from lyophilized microalgae using ionic liquids. However, a significant limitation in biodiesel production based on microalgal lipids is the cost of the downstream processes, such as dewatering, drying, cell disruption, and extraction. Lipid extraction processes are typically incompatible with water, such as wet biomass. It generally requires a low moisture content of biomass to obtain the greatest yields. Conventional oil crops have less moisture due to the growth in the fields. However, microbial species usually grow in an aqueous medium, making them significantly challenging to be dried. Due to the high cost of drying on specialized equipment and biodiesel production, searching for a highly efficient and low-cost technology is crucial in biodiesel production.

Wet extraction based on fresh biomass gets rid of complicated drying procedures. However, the difficulty in wet extraction hinders its development, mainly because fresh cells with recalcitrant and complete cell walls are much more difficult to disrupt than dry biomass. Ionic liquids have been well known for their ability to dissolve cellulose in the cell wall that can significantly decrease cell wall resistance and aid in lipid extraction. Hence, ILs are supposed to be a promising solvent for microalgal lipid extraction and biodiesel production. The first part of the work in chapter 5 is lipid extraction from fresh *C. vulgaris* biomass. Therefore, several ILs were utilized to explore the wet extraction efficiency based on their excellent extraction capacity in some reports [228]. One of them would be a candidate for further study.

More combined technologies are currently identified with a superposition of their performance and properties in one process [75, 229]. Electrolysis treatment is also an emerging technology with a capacity to increase the permeability of the cell membrane [230]. Therefore, the selected ionic liquid based preliminary experiments was first used with electrolysis treatment as a combined pretreatment for improving cell disruption and lipid extraction in this chapter. This method first confirms the synergy exists with a better extraction performance.

Therefore, the improvements established in this chapter develop a novel pretreatment method for the greater microalgal research community.

5.2 Abstract

The combined pretreatment of electrolysis and an ionic liquid was first exploited on wet *C. vulgaris* biomass to produce biodiesel. Several ionic liquids were used for screening wet extraction of lipids from *C. vulgaris*. Although [C_{10}mim][BTMPP] achieved great extraction efficiency from the last chapter, its extraction capacity is slightly less than satisfactory. Another ionic liquid, tetrabutylphosphonium propionate ([P_{4444}][Prop]), was also utilized to assist lipid extraction from a fresh culture of *C. vulgaris*, in addition to some ionic liquids mentioned before. Recently, electrical pretreatment for enhanced microalgal biomolecules extraction has been given much attention, but it is still less applied in lipid extraction processes. In this work, the variables influencing the oil production such as the mass ratio of IL to fresh algae, voltage, and electrolysis time were optimized using response surface methodology (RSM). The synergetic effect of combining IL and electrolysis at optimized conditions enabled the production of an enhanced extraction efficiency per dry biomass of from ~26% to ~45% with comparison to that without electrolysis.

Keywords: wet extraction; electrolysis; ionic liquid; fresh culture; microalgae.

5.3 Introduction

Increasing needs for energy coupled with negative environmental effects of fossil fuel have driven the development and commercialization of alternative energy strategies such as bio-energy [231, 232]. Microalgae have gained considerable attention in recent years for their ability to convert solar energy and carbon dioxide into triacylglycerides (TAGs) precursors for biodiesel, making the biodiesel production potentially carbon-negative [233, 234]. However, currently, economic barriers hindered the commercial development of algal biodiesel due to the cost-prohibitive harvesting, drying, and extraction steps. Unlike land plants, which are composed of a large portion of differentiated tissue, the unicellular microalgae are cultivated in aqueous media, requiring them to undergo more energy-intensive dewatering or drying steps [22]. Besides, many microalgae species possess a cellulosic cell wall that is resistant to mechanical disruption and impedes diffusion during solvent extraction processes for lipid recovery. There are a lot of disruption approaches as the prep-step of extractions. The main limitation of biodiesel production in industrial applications is the high cost of the microalgae drying, dewatering, and cell wall disruption steps, which contribute 30-50% of the overall process cost of biodiesel from microalgae [36, 235, 236, 237]. In addition, it is also laborious to capture lipids from microalgae cells owing to the complexity and rigidity of the cell wall structure, especially for fresh microalgae [238]. Given that wet biomass impede lipid extraction, ionic liquids have been frequently reported to pretreat dry or even wet microalgae to evidently improve lipid extraction [205, 239].

Ionic liquids have been widely known for their ability to dissolute biopolymers with substantial intra and inter-molecular hydrogen bonding networks, such as cellulose and polysac-

charides, which are the main components of microalgae cell walls [89, 116, 204]. Thus ILs have become promising solvents for lipid extraction from microalgae [238, 240]. There have been some emerging technologies to pretreat cells before lipid extraction processes. Electrical pretreatment, a method of cell disruption, is being studied and regarded as a remarkably efficient method by a few researchers [75]. The basic principle of the electrochemical process, as a pretreatment method for microalgae, is that microalgae cells are usually exposed under the electric field to increase the permeability of the cell membrane, followed by the release of lipids or other value-added biomolecules [241]. Thus, it is much easier to extract them as the surroundings of microalgae cells break down. In general, electrical pretreatment can be mainly divided into 4 categories as shown in Fig. 1.8, which include pulsed electric field (PEF), High voltage electrical discharges (HVED), Moderate electric field (MEF), and electrochemical cell lysis/electrolysis [75]. The electrolysis is conducted with electrodes connected to a direct current supply that provides electric current and voltages. The approach is relatively new and this method to recover proteins and lipids is a promising approach, so it has been gaining much attention recently. Joannes et al. studied lipid extraction from *Ankistrodesmus sp.* using electrolysis treatment. Compared to the extraction without electrolysis treatment, the lipid extraction with electrolysis treatment was improved up to 1.40 times higher. Fourier Transform Infrared Spectroscopy (FTIR) was performed on the samples with and without electrolysis treatment, which showed consistent results [230]. Sankaran et al. conducted the protein recovery with and without the aid of electric supply, and the results also had evident greater recovery efficiency with electrolysis treatment than that without electrolysis [79].

In this work, the effect of electrolysis and ionic liquid combined was first studied on lipid extraction efficiency with and without electrolysis from *C. vulgaris*. Besides, a screening on

ionic liquids was carried out. This combination is also somewhat beneficial for wet microalgae extraction. The ionic liquid tetrabutylphosphonium propionate ([P_{4444}][Prop]) with good conductivity is the first time for treating wet *C. vulgaris* with electrolysis. The synergistic pretreatment of electrolysis and ILs was confirmed and this provides a meaningful basis for subsequent research.

5.4 Materials and Methods

5.4.1 Materials

[C_2mim][$EtSO_4$] was purchased from Sigma-Aldrich. [P_{4444}]Cl donated by Solvay (Niagara Falls, Canada) was used to synthesize [P_{4444}][Prop] by anion exchange with the sodium salt of propanoic acid and the structure was confirmed using standard methods [210]. Thraustochytrium sp. (T18) was obtained from the Canadian Phycological Culture Center (CPCC) strain PTA-6245. The cultivation conditions are described elsewhere [43]. [C_{10}mim] [BTMPP] (60 wt.% water) was used as mentioned in chapter 4 [7]. The work related to this ionic liquid kept this definition. The other ionic liquids, [C_2mim][$EtSO_4$], and [C_6mim]Cl as well as other chemicals were all purchased from Sigma-Aldrich, Inc. Pt electrodes were adopted in the electrolysis system, as shown in Fig. 5.1.

5.4.2 Strain and Cultivation Conditions

The green algae *C. vulgaris* strain UTEX 2714 was purchased from the Algae Culture Collection at the University of Texas Austin. Aseptic technique was applied to maintain the culture in liquid culture in a 200 cm^3 tri-acetate-phosphate (TAP) medium pH 7.0 in a 500 cm^3 shake flasks. The seed culture was grown under circulating light including 16 h on: 8 h off

Figure 5.1: Platinum electrodes.

(100 µmol m^{-2} s^{-1}) at a speed of 150 rpm at 25°C. The TAP medium consisted of 20 mM tris base, 2.4 mM K[H_2PO_4], 1.58 mM K_2 [HPO_4], 0.83 mM $MgSO_4$, 0.34 mM $CaCl_2$, 7.0 mM [NH_4] Cl, 1 cm^3 L^{-1} glacial acetic acid and 1 cm^3 L^{-1} of Hutner's trace element solution [222]. After 3 days, the exponentially growing seed culture (100 mL) was inoculated into a 900 mL medium at a concentration of 10% (v/v) and cultured in TAP media with reduced [NH_4] Cl (5 mM) and supplement of glucose (20 g L^{-1}) to induce lipid production for 5 days at 25°C and 150 rpm.

5.4.3 Dry Weight Percent

The dry weight percent of the microalgae in the slurry was tested by the following method: 1) weight the mass of the slurry (m_{slurry}); 2) dry it up for 24 hours at 45°C and record the mass of dry microalgae, $m_{mciroalage}$. The dry weight percent can be calculated by:

$$Dry\ wt. = \frac{m_{microalage}}{m_{slurry}} \times 100\% \tag{5.1}$$

5.4.4 Total Lipid Content by Direct Transesterification

The fatty acid methyl ester (FAME) content by weight, based on the freeze-dried cells, was determined by a direct transesterification protocol, a standard FAME laboratory analytical procedure developed by the National Renewable Energy Laboratories (NREL) as Chapter 4 showed.

5.4.5 Synergistic Pretreatment of Electrolysis and ILs

The diagram of the electrolysis device is shown in Fig. 5.2.

1. Around 12g of cell slurry (m_{sample}) was added into a beaker with a stir bar.

2. A certain amount of ionic liquids (based on the mass ratio of IL to microalgae) was added and stirred on a magnetic stir plate.

3. DC power supply (NJE CORPORATION RB 36-2-M DC POWER SUPPLY) was on at a certain voltage for a certain period

4. After the pretreatment of electrolysis and ionic liquid, 2g of the mixture was added into a test tube.

5. The last steps were the same as the method mentioned above for fresh *C. vulgaris* only by the pretreatment of ionic liquids. A control experiment was also done at the central point designed without any pretreatment under the same conditions.

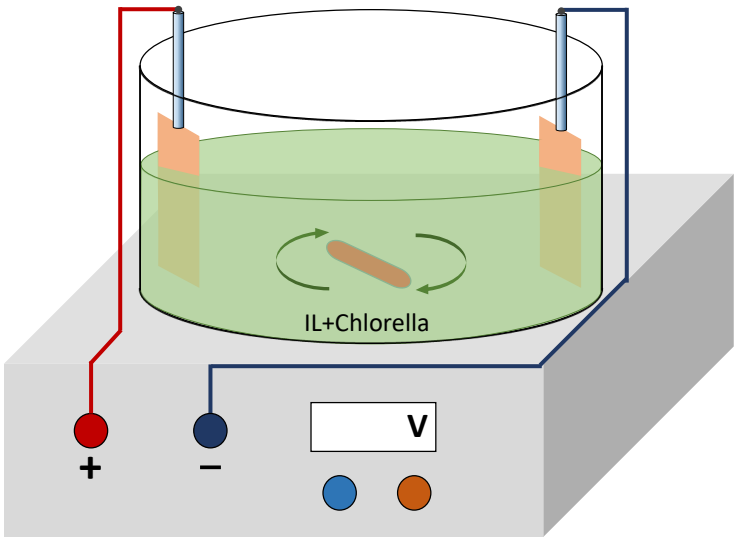

Figure 5.2: Schematic diagram of electrolysis setup.

5.4.6 Extraction Method Fresh Culture by the Pretreatment of Ionic Liquids

The wet extraction method of fresh *C. vulgaris* by the pretreatment of ionic liquids is shown in Fig. 5.3. This method is similar to the method of lipid extraction from dry biomass in Chapter 4. The ILs are utilized to pretreat the cell and then hexane was adopted to extract the lipids from dry biomass sample. Use the Eq. 4.5 to calculate the crude oil content.

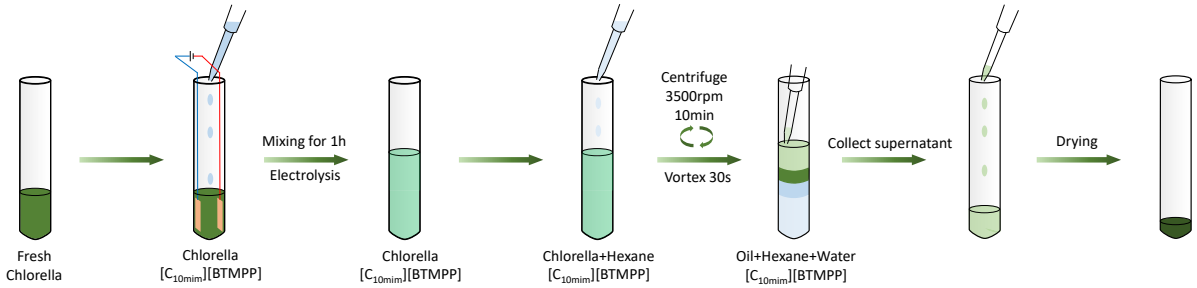

Figure 5.3: The extraction process of fresh *C. vulgaris* via ILs.

1. Around 2g of cell slurry (m_{sample}) were added into a test tube with a miniature stir bar.

2. A certain amount of ionic liquids (based on the mass ratio of IL to microalgae) was added into the tubes and stirred on a magnetic stir plate for 1h.

3. 5 mL of hexane was added and the mixture was vortexed for the 30s.

4. Then the mixture was stood still for 5 min to allow to separate and the supernatant layer (hexane phase) was transferred to a preweighed pan, m_{pan}.

5. Step 4 was then repeated twice and the pan was weighed as above, $m_{pan+oil}$.

5.4.7 **Experimental Design**

A central composite design (CCD) was set up to assess the response pattern with three parameters. The uncoded values for each factor were as follows [low star point, low central point, center point, high central point, high star point]: mass ratio of ionic liquid to T18 [0.05, 0.5, 2.75, 5, 5.45], voltage/V [7.5, 10, 22.5, 35, 37.5], and electrolysis time/min [5, 10, 35,60,65]. The purpose was to evaluate the maximum extraction efficiency using the experimental design by Design Expert 10.0.4 (Stat-Ease, Inc., Minneapolis, MS, USA). Additionally, all conditions were carried out in triplicates including three center points.Hence, there were 51 randomized conditions (8 $\times$ 3 factorial + 6$\times$ 3 augmented + 3$\times$ 3 center points).

5.5 Results and Discussion

5.5.1 Dry Weight Percent and Total Lipid of Fresh *C. vulgaris*

The dry weight percentage of *C. vulgaris* in the slurry measured are listed in Table 5.1. It is found that the fresh slurry contains about 9.21% of *C. vulgaris*. All of the later calculations are based on the dry weight of *C. vulgaris* using Eq. 5.1, such as mass ratio, oil yield, and total lipid.

Table 5.1: The dry weight of *C. vulgaris* in the slurry.

	m_{slurry}/g	$m_{C.\ vulgaris}$/g	Dry wt./%	Ave./%
#1	1.0040	0.0927	9.23	
#2	1.0051	0.0926	9.21	9.21±0.02
#3	1.0078	0.0926	9.19	

Then the maximum oil yield was analyzed by HIP method, and the total lipid content is around 43.77 wt% after three repeated tests, as shown in Table 5.2. This means that the cultured *C. vulgaris* contains 43.77% of extractable lipids. Also, this number is utilized for calculating the extraction efficiency of ionic liquids with Eq. 4.7.

Table 5.2: The total lipid content (wt%) of the cultured *C. vulgaris*.

	m_{slurry}/g	m_{dry}/g	m_{oil}/g	Crude oil con./wt%	Total lipid/wt%	Ave./wt%
#1	1.0001	0.0921	0.0446	48.43	42.79	
#2	1.0292	0.0948	0.0473	49.90	43.20	43.77±1.36
#3	1.0223	0.0942	0.0479	50.87	45.33	

5.5.2 Screening on Pretreatment of Ionic Liquids

To initially study lipid extraction from fresh culture, several ILs were individually applied to extract oil from the cultivated *C. vulgaris*. Firstly, $[C_2mim][EtSO_4]$ was tested at three

different mass ratios of [C_2mim][$EtSO_4$] to *C. vulgaris* (dry weight) as presented in Fig. 5.4. Even though the extraction efficiency has a small increase trend when the mass ratio rises, all of the extraction efficiencies are around 1%. These extremely low values indicate that [C_2mim][$EtSO_4$] is not suitable for extracting fresh *C. vulgaris* alone.

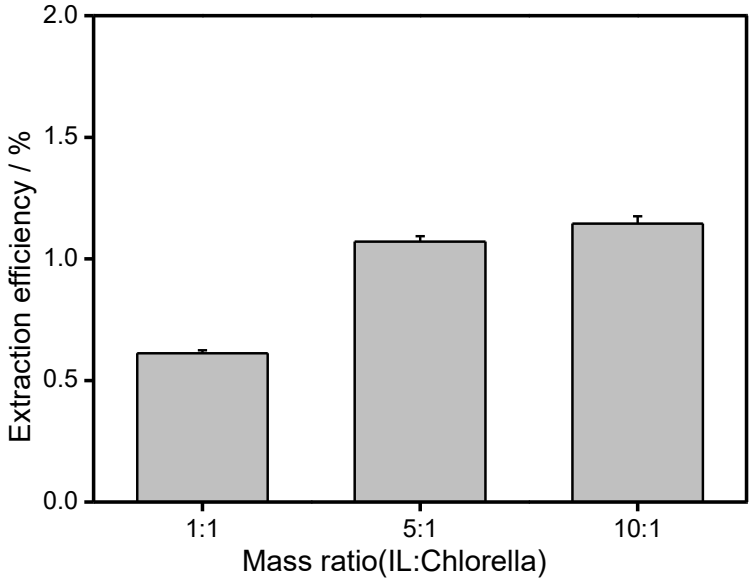

Figure 5.4: Extraction efficiency at different mass ratio of [C_2mim][$EtSO_4$] to *C. vulgaris*.

Hereafter, [C_6mim]Cl was used to help extract oil from the fresh *C. Vulgaris*. Here, six mass ratios ([C_6mim]Cl:*C. vulgaris*) were carried out and the extraction efficiency are plotted in Fig. 5.5. The extraction efficiency are relatively higher than those of [C_2mim][$EtSO_4$], but all of these values are below 5%, suggesting [C_6mim]Cl is not a good candidate for lipid extraction from the fresh *C. vulgaris*.

Then the fresh *C. vulgaris* was pretreated by [C_{10}mim][BTMPP](60% water), and the extraction efficiency are given in Fig. 5.6. There is a clear increase in extraction efficiency when the mass ratio rises from 0:1 to 5:1, and the highest value in the test range is around 24%. It is well known that cell walls pretreated by the more ionic liquid, are much more easily to be dis-

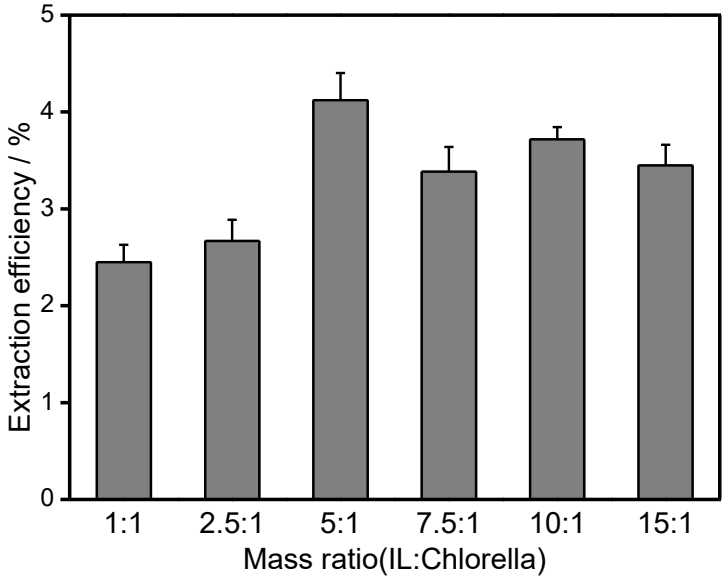

Figure 5.5: Extraction efficiency at different mass ratio of $[C_6mim]Cl$ to *C. vulgaris*.

rupted, which results in higher extraction efficiency. However, it was not obtained after further increasing the mass ratio to 10:1. The probable reason is that the ratio of 5:1 is enough for the cell wall disruption, so much more dosage of $[C_{10}mim][BTMPP]$ has few impacts on the oil extraction. Additionally, the inserted image displays that four separated layers are formed after centrifugation at the mass ratio of 5:1. The layers were water, from bottom to top, residual of *C. vulgaris*/water, $[C_{10}mim][BTMPP]$, and hexane/oils.

At last, to screen more ionic liquids, $[P_{4444}][Prop]$ was employed to extract lipids from the fresh *C. vulgaris* slurry and the results are shown in Fig. 5.7. It shows a similar trend with $[C_{10}mim][BTMPP]$ extraction, and a higher mass ratio leads to a better extraction effect. At first, the extraction efficiency evidently increased from ~15% to ~26% with mass ratio(IL:Chlorella) varying from 1:1 to 5:1. However, if the amount of ionic liquid is doubled from 5:1 to 10:1, the extraction efficiency was not greatly improved, only from ~26.04 ± 0.58% to ~28.79 ± 0.97%. This may result from excessive ionic liquid not participating in the

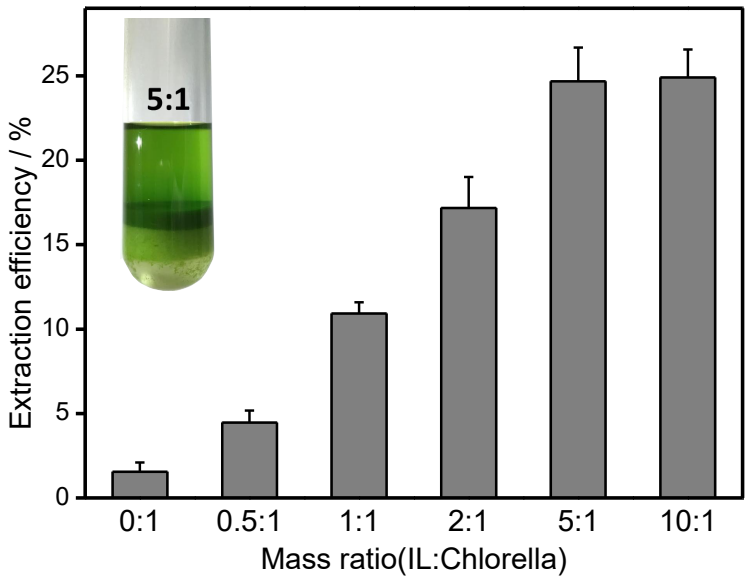

Figure 5.6: Extraction efficiency at different mass ratio of $[C_{10}mim][BTMPP]$ to *C. vulgaris*.

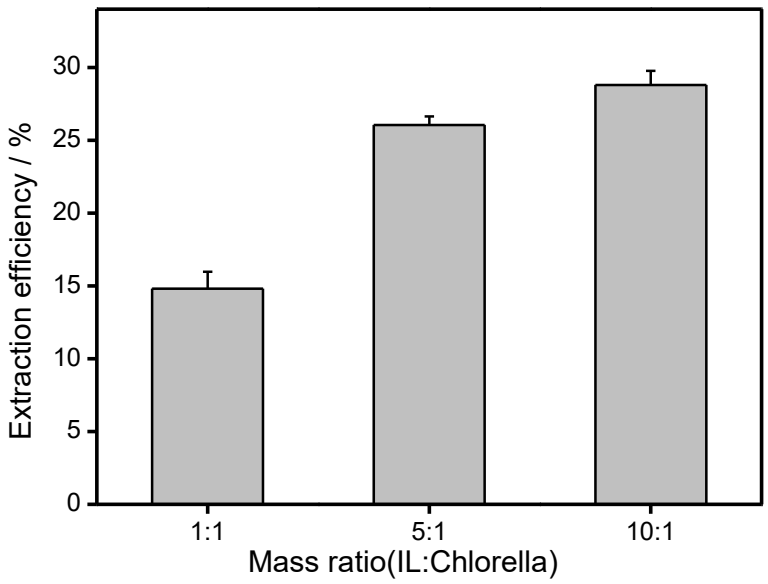

Figure 5.7: Extraction efficiency at different mass ratio of $[P_{4444}][Prop]$ to *C. vulgaris*

pretreatment process, which was similar to that of [C_{10}mim][BTMPP]. Compared to the extract efficiency by [C_{10}mim][BTMPP] (~24%), the extraction process based on [P_{4444}][Prop] shows better extraction efficiency (~29%) at the same mass ratio. Hence, [P_{4444}][Prop] was selected for the further investigation of oil extraction from fresh *C. vulgaris*.

5.5.3 Extraction via [P_{4444}][Prop] and Electrolysis

5.5.3.1 Dry weight and Total Lipid Content

As the previously cultured *C. vulgaris* have been used up after the screening tests, a new batch of *C. vulgaris* were cultivated, and its dry weight percent is about 10.72 ± 0.02% as shown in Table 5.3. The total lipid content of the new batch of *C. vulgaris* is determined to be 37.14 ± 1.54 wt% (Table 5.4), which is a bit lower than that of the former one. This is probably because some cultural conditions are not the same. Performance comparison will be based on extraction efficiency, so the total oil content will not affect the evaluations.

Table 5.3: The mass percent of the *C. vulgaris* in the fresh slurry.

	m_{slurry}/g	$m_{C.\ vulgaris}$/g	Dry wt./%	Ave./%
#1	0.9865	0.1057	10.71	
#2	1.0131	0.1087	10.73	10.72±0.02
#3	1.0043	0.1075	10.70	

Table 5.4: The total lipid content of the *C. vulgaris*.

	m_{slurry}/g	m_{dry}/g	m_{oil}/g	Crude oil con./%	Total lipid/%	Ave./%
#1	2.1021	0.2253	0.0955	42.40	37.52	
#2	2.0122	0.2157	0.0931	43.15	38.45	37.14±1.54
#3	2.0012	0.2145	0.0865	40.32	35.45	

5.5.3.2 Phenomena of Pretreatment of [P_{4444}][Prop] and Electrolysis

Electrolysis was the first time to pretreat microalgae with ionic liquid. Fig. 5.8 shows that there are some tiny bubbles at the surface of the electrode and more small bubbles floating at the liquid surface. These bubbles are mainly produced from the electrolysis of water during the extraction process. The exist of ionic liquid could accelerate the phenomena as well. Meanwhile, based on microalgae cell surfaces that negatively are charged, the accumulation of biomass at the surface of the anode can be observed in Fig.5.8b. This phenomenon was also observed by other researchers when using electrolysis to treat the biomass [79]. The left and right tubes are the mixtures with and without electrolysis during the extraction, respectively in Fig 5.8c. There are three layers in the right tube, but only two layers are found in the left tube (Fig 5.8c). A possible reason may be responsible for this, is that the electrolysis temporally changes the distribution of the anions and cations in the mixture and then forms a suspension of water, [P_{4444}][Prop] and the residual of *C. vulgaris* at the bottom. The supernatants in the two tubes are both extracted oils in hexane (Fig 5.8c).

5.5.3.3 Experimental Design and Model

Considering the electrolysis process during the oil extraction, the mass ratio, electrolytic voltage, and electrolytic time are the three crucial factors. The range of mass ratio (IL:Chlorella) is set from 0.5 to 5, based on the preliminary results of wet extraction assisted by [P_{4444}][Prop] in Fig. 5.6. The electrolytic voltage and time were designed between 10-35V and 5-60 min in view of some relating reports [75]. According to the central composite design, seventeen conditions were designed and the actual responses are displayed in Table 4.3. The extraction

efficiency obtained ranges from 2.48% to 44.53% under these various experimental conditions. The highest extraction efficiency (44.53 ± 2.38%) of the conditions are at a mass ratio of 5.45:1, the voltage of 22.5V, and electrolysis time of 35 min, which is even higher than the efficiency when only using [P_{4444}][Prop] at a very high mass ratio of 10:1 (~28.79 ± 0.97%) in Fig. 5.7. Moreover, the extraction efficiency grows from ~26% to ~38% after applying electrolysis (35V and 60min) at the same mass ratio of 5:1, implying that electrolysis greatly benefits the enhancement of the pretreatment process.

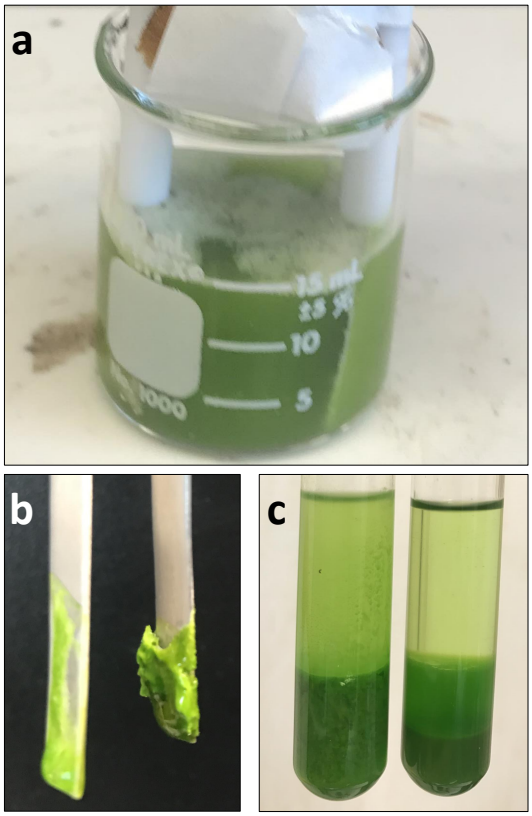

Figure 5.8: The phenomena of the oil extraction via [P_{4444}][Prop] with electrolysis. a) The situation of electrolysis; b) the enrichment of *C. vulgaris* at the surface of anode; c) The difference between the extraction with (left) and without (right) electrolysis.

Table 5.5: Analysis of oil yield (average ± standard deviation) under designed conditions.

Mass ratio (IL:Chlorella)	Voltage/V	Electrolysis time/min	Extraction efficiency /%
0.05	22.5	35	2.48±0.57
0.5	10	60	2.73±0.21
0.5	10	10	2.37±0.14
0.5	35	60	4.75±0.33
0.5	35	10	2.13±0.41
2.75	7.5	35	9.45±1.02
2.75	22.5	5	12.00±1.33
2.75	22.5	65	25.20±1.84
2.75	22.5	35	9.78±0.89
2.75	22.5	35	13.01±0.55
2.75	22.5	35	12.10±1.77
2.75	37.5	35	22.97±2.50
5	10	10	25.16±2.46
5	10	60	31.77±2.53
5	35	60	37.93±4.20
5	35	10	26.75±0.75
5.45	22.5	35	44.53±2.38

5.5.3.4 Analysis of variance of the fitted model

Table 5.6 summarizes the analysis of the variance of the fitted model. The Model F-value of 27.08 implies only a 0.01% chance that an F-value this large could occur due to noise. On the one hand, values of "Prob >F" less than 0.05 indicate model terms are significant. Thus, mass ratio and electrolysis time are considerable model terms in this case. On the other hand, values greater than 0.10 indicate the model terms are not significant, so electrolysis voltage is an insignificant model term in the tested range. The electrolysis voltage is quite important when compared to the one without electrolysis as mentioned before. The "Lack of Fit F-value" of 10.05 implies a 9.38% chance that an F-value this large could occur due to noise. The "Pred R-Squared" of 0.8362 is in reasonable agreement with the "Adj R-Squared" of 0.8670 because the difference is less than 0.2. "Adeq Precision" of 15.488, more extensive than 4.0, indicates

an adequate signal. Above all, this model can be applied to navigate the design space.

Table 5.6: Analysis of variance of fitted model for oil yield.

Sourse	Sum of squares	Freedom Deg.	F value	P value Prob>F	Remark
Model	2566.6	4	27.08	< 0.0001	significant
A	2355.14	1	99.4	< 0.0001	
B	60.95	1	2.57	0.1347	
C	123.05	1	5.19	0.0418	
AC	27.46	1	1.16	0.3029	
Residual	284.33	12			
Lack of Fit	278.78	10	10.05	0.0938	not sig.
R-Squared				0.9003	
Adj R-Squared				0.8670	
Pred R-Squared				0.8362	
Adeq Precision				15.488	

A is mass ratio; B is voltage; C is electrolysis time.

Based on the selected significant variables in Table 5.5, the linear model for the extraction efficiency in terms of actual factors in the tested range is shown as follows:

$$Extraction\ efficiency = -7.0088 + 5.3862 \times Mass\ ratio + 0.04394 \times Electrolysis\ time \tag{5.2}$$

The equation in terms of actual factors can be used to make predictions about the response for given levels of each factor. This equation is not supposed to determine the relative impact of each factor because the coefficients are scaled to accommodate the units of each factor. It indicates that extraction efficiency has a linear relationship with mass ratio and electrolysis time from the equation.

5.5.3.5 The model diagnostics

It shows whether the set of real responses fit normal distribution in Fig. 5.9a. If the points in the graph are straight lines or close to straight lines, the assumption of the normal distribution of samples is acceptable. In this case, all of the points are fairly close to the straight line, suggesting the actual responses basically conform to normal distribution. The plot aims at analyzing how the actual data correlate with those predicted by a model. For a good fit, the points should be close to the fitted line with narrow confidence bands. Fig. 5.9b shows that most of the points are relatively close to the fitted line, illustrating that a good match between actual and predicted values.

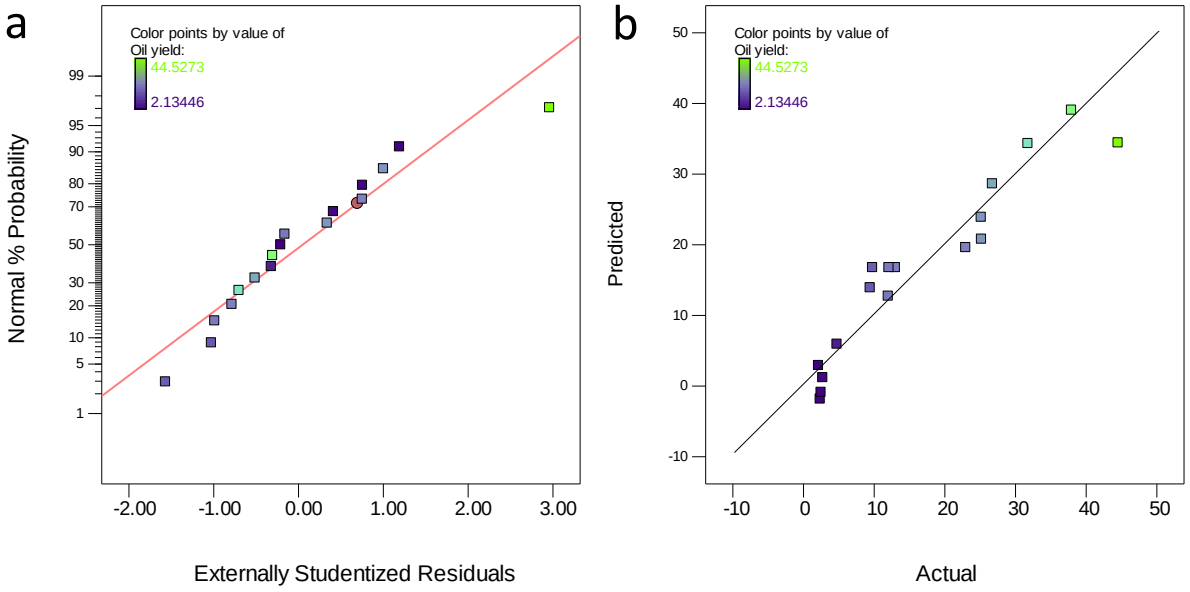

Figure 5.9: a) Normal probability plot of residual; b) Predicted vs. Actual.

The assumption of constant variance was tested in Fig. 5.10. The plot should be a random scatter (constant range of residuals across the graph). Expanding conflict (cross the range) in this plot indicates the need for a transformation. All of the points are randomly scatter inside the field, meaning that there is no need for a transformation for the model. The residuals vs. run

is a plot of the residuals versus the experimental run order, which checks for lurking variables that may have influenced the experiment's response. Fig. 5.10 shows that the points are in a random scatter inside the range, implying that there are no lurking variables.

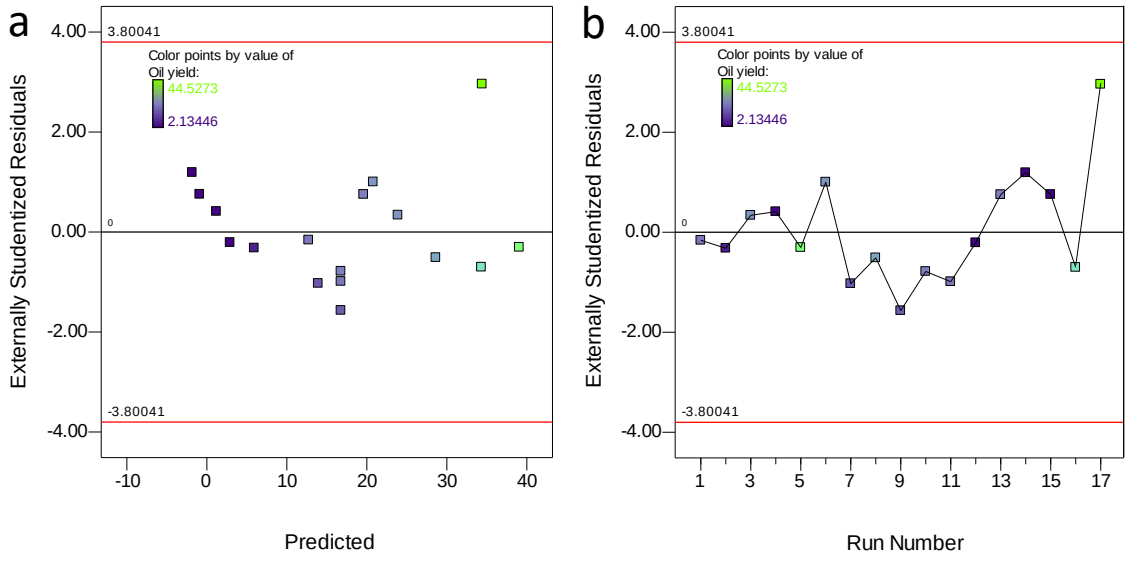

Figure 5.10: a) Residuals vs. Predicted; b) Residuals vs. Run.

5.5.3.6 The plots of factors

Fig 5.11 exhibits the surface plot of the two factors for the extract efficiency in terms of response surface methodology. With the electrolysis time of 35 min, the surface plot appears to be a diagonal plane. It indicates that a higher mass ratio and voltage result in higher extraction efficiency, which is in line with the previous conclusion: mass ratio is a significant term, while electrolytic voltage, to a certain extent, is insignificant (Table 5.6). The surface plot in Fig 5.12 shows that the combined effect of the mass ratio of electrolytic time on the extract efficiency. With the voltage of 22.5V, the extraction efficiency has a slight positive increase trend when the electrolytic time rises.

To have a more precise sense of the influence of each factor on the extraction efficiency,

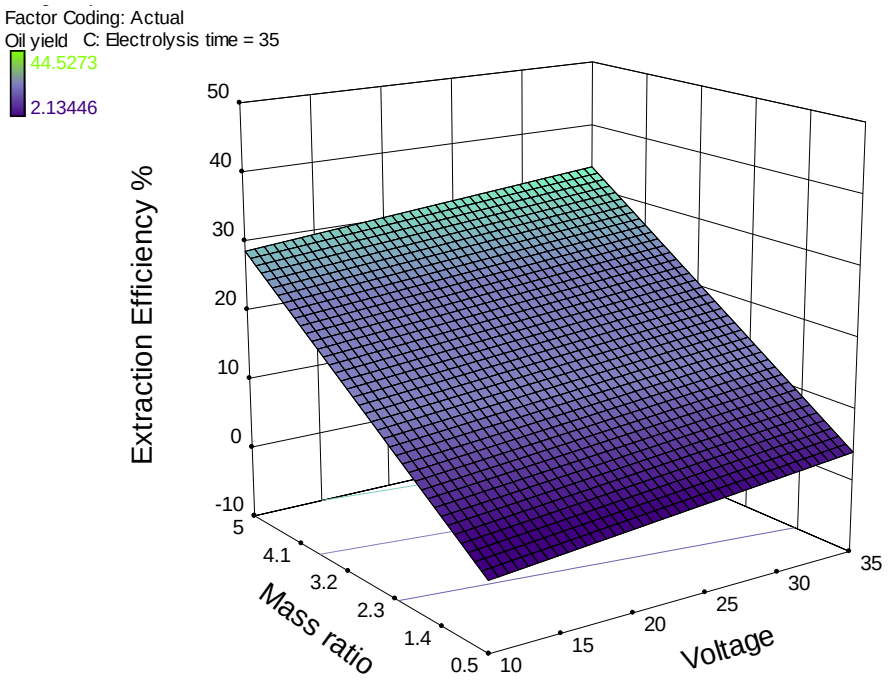

Figure 5.11: Surface plot of combined effect of experimental variables of mass ratio and voltage on extraction efficiency (electrolysis time=35min).

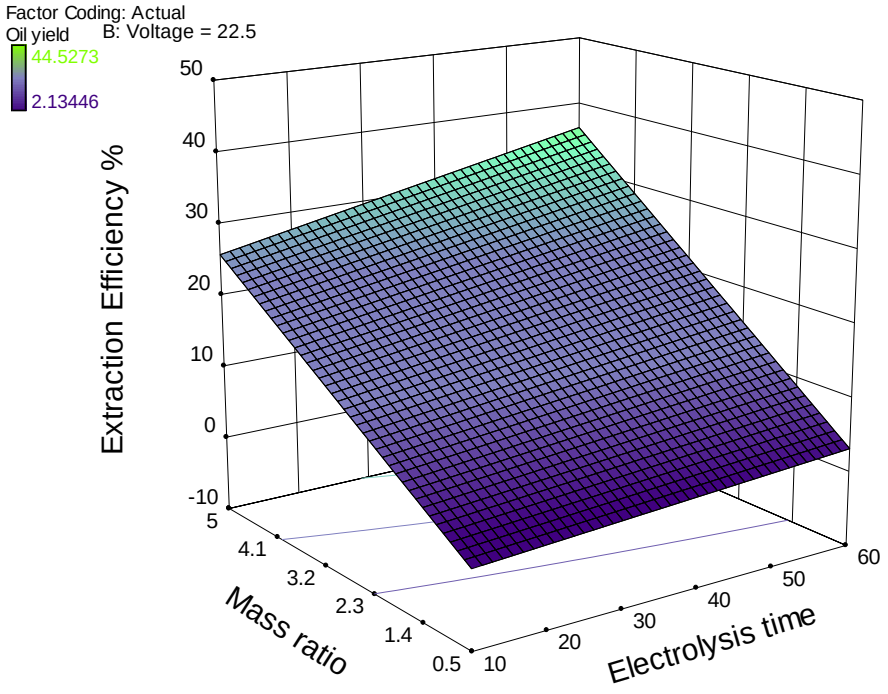

Figure 5.12: Surface plot of combined effect of experimental variables of electrolysis time and voltage on extraction efficiency (mass ratio=2.75).

Fig 5.13 shows individual factor on extraction efficiency. When the mass ratio increase from 0.5 to 5, the extraction efficiency goes up from 2 to 30%. However, the growth of electrolytic voltage and time only caused about a 5% change in extraction efficiency. Even the picked ranges of electrolytic voltage and time chose have exerted less influence than the mass ratio. The extraction efficiency increases from 28% to 45% by intruding the electrolysis process into oil extraction and that of the control sample, without the pretreatment of IL or electrolysis, was only 1.52 ± 0.74%.

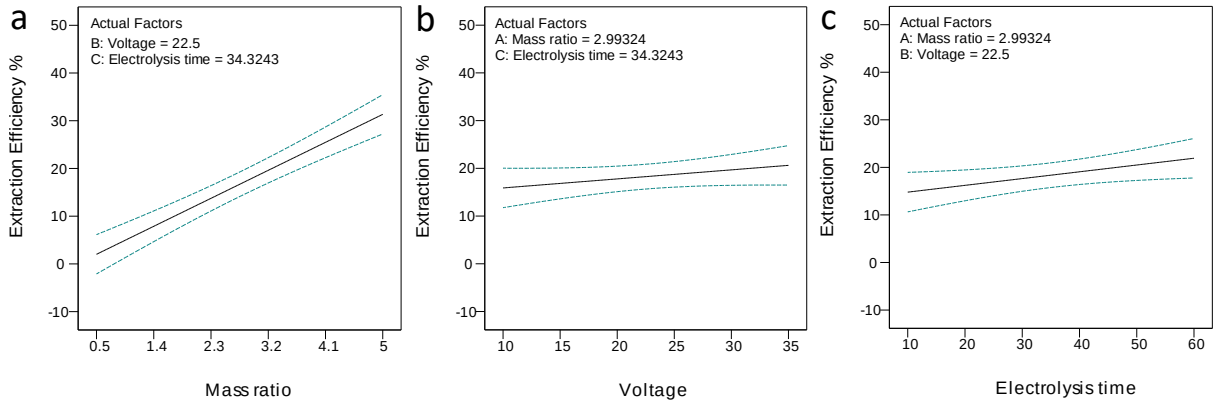

Figure 5.13: Single factor plots. a) mass ratio; b) voltage; c) electrolysis time.

5.5.3.7 Mechanism of Synergy Electrolysis and IL

Given that the above results showed the effect of combined pretreatment by electrolysis and IL, the synergy is significantly evident compared to the control result. According to the model, the mass ratio is the most significant factor, which is consistent with previous work. Electrolysis time is also a significant factor, which has a slight positive effect on lipid extraction efficiency. Although the existence of an electric field can promote the disruption of the cells, thereby improving the extraction efficiency [238], the voltage within the range of this model will not bring significant changes to the results. Additionally, a greater extraction effi-

ciency ~45% at the mass ratio of 5.45:1, was found with comparison to the extraction only by the pretreatment of $[P_{4444}][Prop]$ without electrolysis ~28.79 ± 0.97%, at the higher mass ratio of 10:1. The control experiment without any pretreatment only obtained a low extraction efficiency, less than 2%. Hence, the extraction process with less amount of ionic liquid combined with electrolysis treatment achieves greater extraction efficiency, which confirms the existence of synergy of the two pretreatment methods. Overall, the planned optimization could not be done as non of the quadratic effects were significant. No curvature was observed in the model, and hence it can be only concluded that more IL and longer electrolysis time will lead to more lipid extraction.

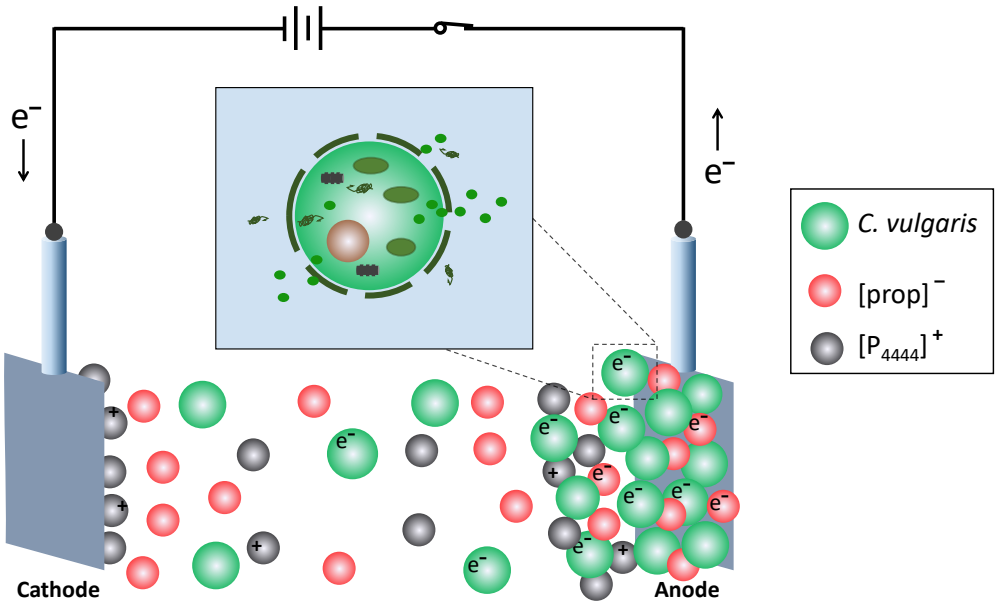

Figure 5.14: The possible effect of electrolysis during the extraction via $[P_{4444}][Prop]$.

There has been some mechanism for extraction improvement of value-added products via electrolysis treatment. Cells are exposed to an electric field during an electrochemical process. It leads to a transmembrane potential difference across the cell membrane, which induces the

breakdown of the membrane followed by increasing permeability. Once the size of cells increases, the content inside will release to the surroundings [75, 238]. The electrolysis treatment also creates gas bubbles due to the electrolysis of water, which leads to an uplift of the flocculated microalgae. Moreover, the charged particles move in an electric field. Microalgae cells are known to be negatively charged, so they move towards the anode and accumulate, which is consistent with the phenomena [79, 238].

On the basis of the results in this work, the application of an electric field will cause the *C. vulgaris* to concentrate on the anode. The anions and cations of the ionic liquid will also move due to the electric field and concentrate on the anode and cathode, which may increase the chance of contact and increase the dissolution of cellulose in the cell wall. The electric field will also increase the cell membrane permeability due to the potential difference in the cell [75]. Moreover, increasing the conductivity of the medium should also lead to a greater cell disruption base on some relating reports [242, 243]. Hence, the combination of the two pretreatment methods and increasing the conductivity of the medium can achieve higher extraction efficiency, thus forming a synergistic effect of ionic liquid and electrolysis treatment.

5.5.3.8 Oil composition

FAME compositions were also characterized by GC after the pretreatment of electrolysis and ionic liquid. It presents that the FAME compositions of the oils extracted by the two methods, the conventional method (HIP) and the novel method (combined pretreatment of $[P_{4444}]$[Prop] and electrolysis) in Fig. 5.15. There is no visible difference seen in the two compositions. The most amount of FAME in fresh *C. vulgaris* is found to be C18:2n6c+C18. Also, C24:1, C18:3n3, C18:2n6c+C18, C18:0, C16:0, whose content is around 10 wt%, are all

critical components. Above all, the results intimated that the new method is full of potential to be a highly efficient extracted process without side effects on FAME composition.

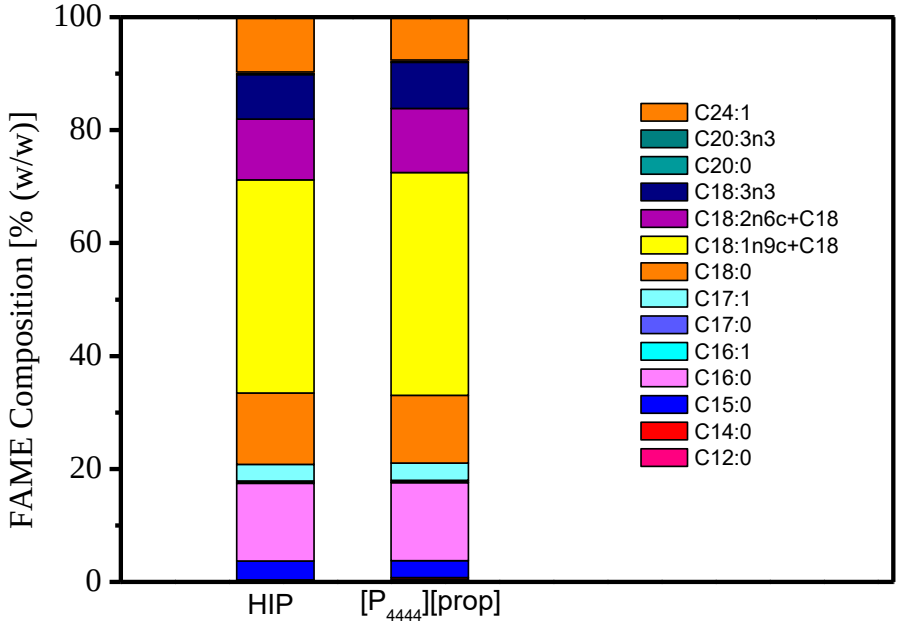

Figure 5.15: The oil composition.

5.6 Conclusions and Perspective

In conclusion, a synergy of ionic liquid with electrolysis was first found and applied to extract lipid from fresh *C. vulgaris*. Wet extraction is initially concerned and the extraction effects assisted by ionic liquids as follows: $[C_2mim][EtSO_4] < [C_6mim]Cl < [C_{10}mim][BTMPP] < [P_{4444}]$ [Prop]. A relatively high extraction efficiency $\sim 45\%$ at the mass ratio of 5.45:1, was found as compared to $\sim 28\%$, that of extraction only by $[P_{4444}]$ [Prop] without electrolysis, at the higher mass ratio of 10:1. Less amount of ionic liquid combined with electrolysis treatment obtains a greater extraction efficiency which confirms the existence of synergy of the two pretreatment methods. The results also verify that mass ratio plays the most significant role in the whole process and electrolysis time has a slightly positive effect for this ionic liquid based ex-

traction process. However, the voltage shows little effect within the designed conditions. The voltage is quite important when compared to the ones without electrolysis. The results indicate better extraction efficiency has been achieved with the existence of an electric field. Therefore, this work develops a novel and facile method to extract lipids from fresh microalgae, which has a significant potential application on the extraction of oils or related microalgal molecules.

6 Extraction of a DHA-rich Lipid Fraction from T18 (*Thraustochytrium sp.*) Using Ionic Liquids

6.1 Preface to Chapter 6

The previous two chapters are involved with *Chlorella vulgaris* for biodiesel production, while chapter 6 presents the enhanced extraction of a value-added lipid fraction, docosahexaenoic acid (DHA, 22:6 n-3) from the marine microalgae *Thraustochytrium sp.* As a kind of ω-3 Polyunsaturated fatty acids, DHA has been considered to prevent cardiovascular diseases, asthma, arthritis, etc [244].

Lyophilized *Thraustochytrium sp.* biomass was first pretreated. DHA-rich lipid was extracted by the pretreatment of two kinds of ionic liquids, respectively. 1-ethyl-3-methylimidazolium ethylsulfate $[C_2mim][EtSO_4]$ and the phosphonium-based IL (tetrabutylphosphonium propanoate $[P_{4444}][Prop]$ were chosen based on previous work. The extraction efficiency of lipid fraction was shown in this chapter. Additionally, the two ionic liquids could be reused with excellent recyclability.

Afterward, fresh biomass was also pretreated using these two ionic liquids in order to investigate and compare the possibility of wet extraction for the value-added lipid. It presents $[P_{4444}][Prop]$ could aid in lipid extraction without any co-solvent, which is a promising candidate for wet extraction. The results are consistent with chapter 5 on wet extraction.

*With minor editorial changes to fulfill formatting requirements, this chapter is substantially as it appears in the Materials 11.10 (**2018**): 1986.*

6.2 Abstract

Polyunsaturated fatty acids (PUFAs) play a significant role in the modulation and prevention of various diseases and hence are attracting increasing attention from the biotech industry. Thraustochytrids are marine heterokonts that exhibit robust growth rates, high PUFAs content, and, more specifically, a large percentage of ω-3 fatty acids like docosahexaenoic acid (DHA). Recently, ionic liquids (ILs) have been shown to improve the efficiency of organic solvent extraction of oils from wet oleaginous yeast and microalgae under mild conditions. Two ILs, the imidazolium-based IL 1-ethyl-3-methylimidazolium ethylsulfate [C_2mim][$EtSO_4$] and the phosphonium-based IL (tetrabutylphosphonium propanoate [P_{4444}][Prop] where assessed for their ability to facilitate extraction of PUFAs from *Thraustochytrium sp.* (T18). The oil extracted after ILs pretreatment was further characterized with respect to fatty acid methyl ester (FAME) composition, while the effects of process parameters such as the ratio of ionic liquid to co-solvent, the mass ratio of algae to the mixture of ionic liquid and co-solvent were also investigated for both ILs. The results indicate that these ILs can disrupt the cells of *Thraustochytrium sp.* when mixed with a co-solvent, methanol, and facilitated the recovery of oils over a large degree of dewatered Thraustochytrium biomass (0-77.2 wt % water) in a short period of time (60 min) at ambient temperature, hence demonstrating a water-compatible, low-energy, PUFAs recovery method.

Keywords: DHA, ω-3, DHA, ionic liquids, microalgae.

6.3 Introduction

The consumption of ω-3 PUFAs such as docosahexaenoic acid (DHA, 22:6 n-3) or eicosapentaenoic acid (EPA, 20:5 n-3) have been linked to the prevention of neural disorders, cardiovascular diseases, arthritis, asthma, and dermatosis [17, 42, 245, 246]. PUFAs are involved in many vital physiological functions and are essential components of brain cell membranes [247, 248, 249]. Their possible use in reducing cholesterol when consumed as food supplements has made their production for nutritional use highly desirable. Currently DHA is mainly produced from marine fish oil, but this route is becoming increasingly challenging due to marine pollution and seasonal variations in fish production [42, 43]. Therefore, alternative sources of oils rich in PUFAs are being developed which aim to reduce the processing costs, increase the sustainability of their production and reduce their environmental burden. One promising source of PUFAs is the heterokonts called thraustochytrids which exhibit robust growth rates and can accumulate high oil contents, particularly DHA which can account for over 30% of the total cellular lipids [250, 251]. Commonly used species belong to the Aurantiochytrium, Schizochytrium, Thraustochytrium, and Ulkenia genera [24]. In addition to their high PUFA contents, thraustochytrids are capable of breaking down complex organic materials and using them as their primary carbon source [252, 253, 254], making them easy to cultivate on a wide range of feedstocks, including waste materials such as wastewater [255].

Like other sources of single-celled oils (SCOs) thraustochytrids are cultivated in aqueous media and current organic solvent extraction processes require energy-intensive and time-consuming drying steps to prepare the biomass for extraction [24]. Harvesting, drying, and extraction steps can account for up to 70% of the processing costs for SCOs [26, 22]. Enhancing

extraction efficiency depends on increasing the interfacial area of the cellular matrix to solvent through cell disruption [22]. Cell disruption can be accomplished through a variety of mechanisms, many of which are time-consuming or energy-intensive. These include mechanical methods (comminution, ultrasonication and high-pressure homogenization) [256, 257], physical methods (thermochemical wall-breaking technology, microwaves and repetitive freeze-thaw cycles) [258, 259] and biochemical methods (the acid-heated method, the alkali-heated method and enzyme hydrolysis) [260, 261, 262]. The diversity of the composition of the cell walls of heterokonts makes it difficult to predict the application of one process to a new species. Thraustochytrids possess a laminated cell wall made of scales which are predominately protein-based (30-43%) with some carbohydrate content (21-36%) [263], while other species like diatoms could have a silica cell wall [264]. As a result, assessing developing technologies on many species of oleaginous biomass is necessary to determine their applicability. Therefore, the commercial viability of thraustochytrid based lipid production will be significantly improved by assessing new cell disruption techniques and developing processes that enhance water compatibility and reduce the overall energy requirements of these costly downstream processing steps. Ionic liquids (ILs) have been shown to increase the lipid extraction efficiency from microalgae by dissolving both dry and wet microalgae under relatively mild conditions (80-40°C) with and without a co-solvent (Table 6.1) [15, 16, 17]. ILs are often described as "green" designer solvents with many desirable physical-chemical properties such as excellent thermal stability, low melting points and negligible vapor pressure [100]. In bioprocessing, ILs are best known for the capability of some ionic liquids to solvate highly recalcitrant biopolymers like cellulose [99, 265, 266].

Until now, imidazolium-based ILs have been the main focus for the cellular disruption of

microalgae and other SCOs, likely owing to their greater commercial availability [236, 267]. In most cases, hexane/isopropanol (HIP; 3:2 v/v) extraction or chloroform/methanol (2:1 v/v) was used for the analytical determination of the theoretical maximum lipid yield [210]. In this work, two ILs were assessed for their ability to improve lipid-extraction from the thraustochytrid sp., T18. Both dry and wet T18 was pretreated with either 1-ethyl-3-methylimidazolium ethyl-sulfate $[C_2mim][EtSO_4]$ or tetrabutylphosphonium propionate $[P_{4444}]$ [Prop] and lipids were briefly extracted using hexane. Extracted oils were further characterized for their lipid composition. Fig. 6.1 presents the methodology employed for oil extraction from dewatered T18 biomass using ILs and the subsequent IL recycling process.

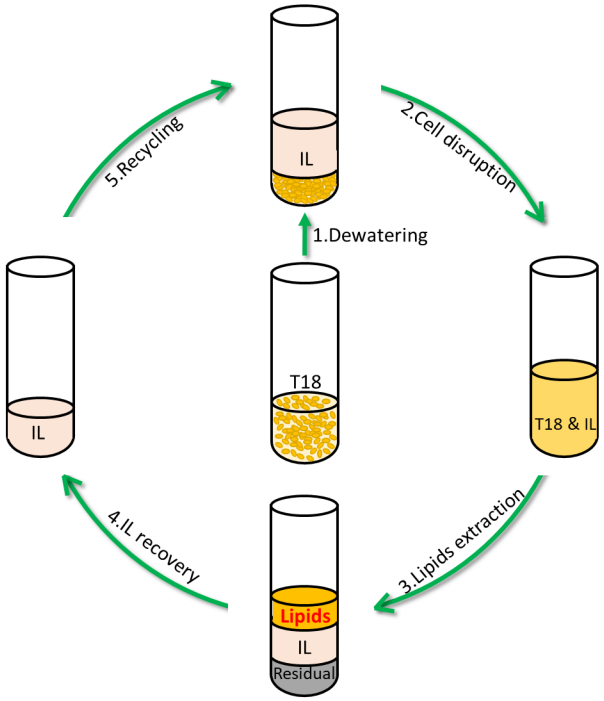

Figure 6.1: Summary of basic IL-based marine biomass (MB) fractionation process. The high-value products are recovered following cell disruption with ILs. The residual biomass separates from the ILs, allowing for IL recycling.

Table 6.1: Ionic liquid-based extraction of lipids and PAF from algae [15, 16, 17].

Algal Species	Operating Conditions	Extraction Solvent/Method	Yield
Chlorella sp.	ILs: cells, 10:1 (w/w). Incubated for 24 h at room temperature with constant low speed stirring.		Oil yield (mg/g algae)
		Bligh and Dyer	38.13
		Butyrolactam formate	48.0 ± 0.4
		Butyrolactam acetate	39.1 ± 5.0
		Butyrolactam hexanoate	46.8 ± 5.8
		Caprolactam formate	36.3 ± 6.8
		Caprolactam acetate	38.3 ± 4.5
		Caprolactam hexanoate	42.9 ± 2.0 PAF 15.4 ± 2.6
		Propylammonium formate	15.4 ± 2.6
		Propylammonium acetate	12.8 ± 4.9
		3-Hydroxypropylammonium formate	8.1 ± 2.1
		3-Hydroxypropylammonium acetate	10.1 ± 1.2
Chlorococcum sp.	ILs: cells, 10:1 (w/w). Incubated for 24 h at room temperature with constant low speed stirring.	Bligh and Dyer	11.55 mg/g
		Butyrolactam formate	36.4 ± 1.4
		Butyrolactam acetate	44.4 ± 1.8
		Butyrolactam hexanoate	51.1 ± 1.9
		Caprolactam formate	45.7 ± 2.5
		Caprolactam acetate	49.1 ± 2.3
		Caprolactam hexanoate	46.7 ± 2.0
		Propylammonium formate	18.9 ± 6.3
		Propylammonium acetate	16.7 ± 3.2
		3-Hydroxypropylammonium formate	13.4 ± 4.7
		3-Hydroxypropylammonium acetate	5.9 ± 2.7
Aurantiochytrium sp.	$FeCl_3$ H_2O:[Emim]OAc, 5:1 (w/w). 90 °C for 60 min (5% *Aurantiochytrium* sp. loading, w/w)		DHA content (mg/g lipid)
		Bligh and Dyer	235.2
		Hexane	259.4
		Hexane:Methanol = 7:3	275.7
		1-ethyl-3-methyl imidazolium acetate	301.3
Chlorella vulgaris	*C. vulgaris* (500 mg) was mixed with a mixture of 2.5 mL IL and 2.5 mL methanol under magnetic stirring at 65 °C for 18 h.		Lipid contents (%)
		Bligh and Dyer	11.1
		[Bmim][CF_3SO_3]	19.0
		[Bmim][$MeSO_4$]	17.4

6.4 Materials and Methods

6.4.1 Materials and Strain

Thraustochytrium sp. (T18) was obtained from the Canadian Phycological Culture Center (CPCC) strain PTA-6245. The cultivation conditions are described elsewhere [43]. All chemical sources are the same as Chapter 5.

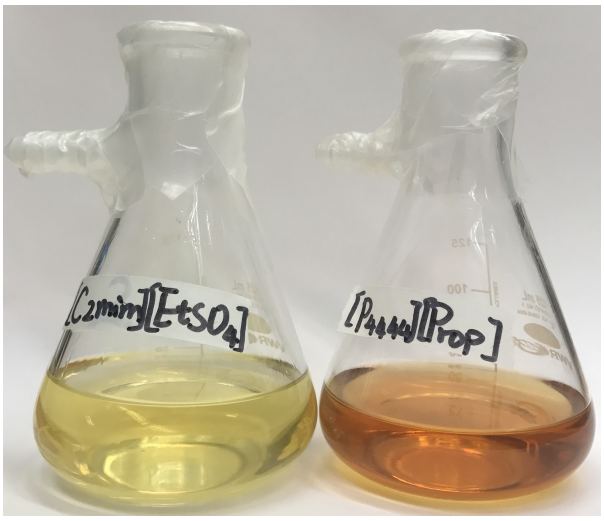

Figure 6.2: $[C_2mim][EtSO_4]$ (left) and $[P_{4444}][Prop]$ (right).

6.4.2 Harvesting and Freeze-Drying

T18 cultures were harvested by centrifugation at 3500 rpm at 4°C in a Sorvall RT centrifuge (Fisher Scientific) for 20 min. Residual salts were removed from the cell pellets by washing three times with deionized water by repeated resuspension and centrifugation. After that, the cell pellets were frozen at -80°C for a minimum of 12 h and then lyophilized using a 4.5 L freeze-drier (Labconco) for 24 h, and stored in a desiccator until further use. For wet extractions, fresh T18 was harvested by centrifugation and resuspended in deionized water as above. After the last centrifugation, the T18 slurry was kept for the next extraction step. Dry weight was determined by overnight drying in an oven at 60°C.

6.4.3 Analytical Determination of Total Lipid Content

The standard total lipid content was determined in triplicate by adding 0.10 g of freeze-dried algae to 5 mL of hexane/isopropanol solution (HIP; 3:2 v/v) [224] and stirring for 12 h. Then the mixture was filtered through a Buchner funnel with a fine porosity fritted disc.

The residual solids were washed with acetone until they were colorless. The appropriate phase was then transferred to a preweighed foil pan in a fume hood to evaporate the solvent. The mass of extracted lipids was measured using an analytical balance until the weight no longer fluctuated. When extracting from wet biomass, the first step was to concentrate the biomass via centrifugation. The wet slurry was washed three times with DI to eliminate the residual media and salt. The resulting slurry was the raw material for extraction via the same operation steps as mentioned above.

6.4.4 IL Pretreatment

IL pretreatments were done in triplicate by mixing 0.10 g of freeze-dried algae with the indicated mass of IL in tubes for 1h with a magnetic stirrer at ambient temperature. Then 5 mL of hexane was added to the IL T18 mixture and mixed by vortexing for 30 s followed by 5 min of stirring before removing the hexane layer to a new container. This step was repeated three times and the mixture was centrifuged at 3500 rpm for 10 min, and the top layer was added to the same container. As a negative control, hexane by itself (without IL pretreatment) using the same process as above was only capable of extracting 6.5 ± 0.7% (w/w) oils.

6.4.5 IL Recycling

$[C_2mim][EtSO_4]$ as well as $[P_{4444}][Prop]$ were tested in triplicate for the extraction performance after recycling using freeze-dried T18 as follows: 0.10 g of T18 was added at a ratio of 1:4, 1:10 mass ratio of dry equivalent T18 to $[C_2mim][EtSO_4]$ and $[P_{4444}][Prop]$ respectively. They were incubated with agitation for 1 h at ambient temperature. Lipids were extracted with hexane as previously described. After three extractions, IL was recovered by adding 5 mL of

MeOH to precipitate dissolved solids followed by filtration using a fine porosity Buchner funnel. The recovered ionic liquid/methanol phase was then transferred to a round-bottom flask and the methanol was removed by evaporation using a rotary vacuum evaporator (BUCHI, Switzerland) at 150 rpm, 200 mbar and 70°C for 20 min or until there was no further solvent removal. This experiment was repeated five times to determine if performance was greatly affected by repeated use. This method was normalized to the extraction yield obtained in the first use with previously unused IL.

6.4.6 Lipid Composition

FAME was prepared by dissolving 100 mg of extracted oil in 10 mL of hexane followed by the addition of 100 µL of 2 M methanolic KOH. Samples were vortexed for 30 s, followed by centrifugation, and 500 µL of the clear supernatant was spiked with the internal standard methyl undecanoate (Sigma) and separated on an FID equipped Agilent 7890 Series GC. The FAME mixture was separated using an Agilent DBWAX capillary column (30 m, 0.25 mm, 0.25 µm) with helium as the carrier gas at a linear velocity of 30 cm s^{-1}. Samples were injected in split mode (50:1). The FID detector was operated at 280°C, and FAMEs were eluted using the following program: 50°C, 1 min, 10°C min^{-1} to 200°C, 3°C min^{-1}, 220°C, 10 min. Individual FAMEs were quantified and identified using analytical standards (Sigma) and the internal standard C11:0. Unidentified FAMEs were estimated using an averaged RF factor.

$$Extraction\ efficiency = \frac{Oil\ yield}{Total\ lipid\ content} \times 100\% \tag{6.1}$$

6.5 Results and Discussion

To confirm that $[C_2mim][EtSO_4]$ and $[P_{4444}][Prop]$ are able to disrupt the cell walls of T18, images of T18 with or without IL pretreatment were captured using an optical microscope, as shown in Fig. 6.3. Freeze-dried T18 which is a light yellow powder due to lack of chloroplast (Fig. 6.3b). Its cellular morphology demonstrates that these cells were intact before the pretreatment of ILs (Fig. 6.3b). After pretreatment with $[C_2mim][EtSO_4]$ (Fig. 6.3a) and $[P_{4444}][Prop]$ (Fig. 6.3b) for one hour, the mixtures were observed for cell disruption. The photos of T18 treated with $[C_2mim][EtSO_4]$ (Fig. 6.3a) show that these cells were broken. Similar results were seen for treatment with $[P_{4444}][Prop]$(Fig. 6.3c).

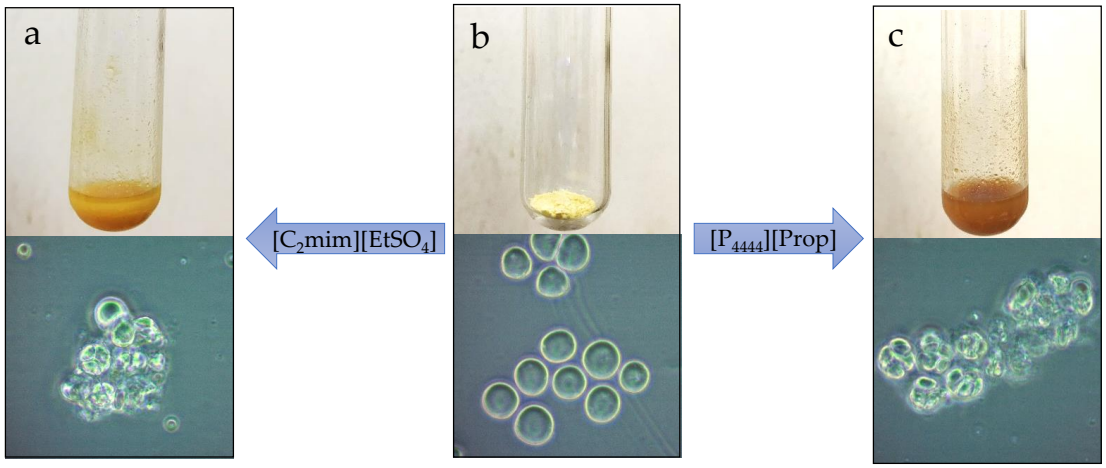

Figure 6.3: Lysis of freeze-dried T18 after the pretreatment of ionic liquid as visualized under a standard bright field microscope.

6.5.1 Total Lipid Contents of Dry and Fresh T18

6.5.1.1 Total Lipid Content of Dry T18

The total lipid content of the dry T18 was tested by HIP method, as shown in Table 6.2.

Table 6.2: The total lipid content of dry T18.

	#1	#2	#3	#4	#5	#6	Ave.
Total lipid/wt%	59.70	64.72	60.25	57.05	56.81	66.02	60.76±3.85

6.5.1.2 Dry Weight Percent and Total Lipid Content of Fresh T18

The dry weight percent of the fresh T18 is shown in Table 6.3.

Table 6.3: The dry weight percent of the fresh T18 in the slurry.

	m_{slurry}/g	m_{T18}/g	Dry wt./%	Ave./%
#1	0.9455	0.2108	22.3	
#2	1.0546	0.2478	23.5	22.8±0.6
#3	1.0224	0.2321	22.7	

The total oil content of the fresh T18 cells is presented in Table 6.4.

Table 6.4: The mass percent of the T18 in the fresh media.

	#1	#2	#3	Ave.
Total lipid/wt%	67.3	72.9	70.2	71.1±2.8

6.5.2 IL Extraction of Oils from Dried T18

First, to determine the theoretical maximum oil content of dried T18 biomass HIP extractions were performed [224]. Dried T18 was found to contain 60.76 ± 3.85wt% oils. Previous results indicated that in some cases a co-solvent was required to increase lipid recoveries from *Chlorella vulgaris* [22]. To see if this was the case for dried T18 biomass, $[C_2mim][EtSO_4]$ was mixed with the co-solvent, methanol (MeOH), at different ratios from 4:1 to 1:7 (Fig. 6.4a), and oils were separating by briefly extracting with hexane for 5 min three times. To compare, untreated T18 was subjected to the same extraction and only 6.5 ± 0.7% (w/w) oil content was extracted in this manner. With IL treatment, the oil yield increased until around 91.0 ±

2.8% of the theoretical maximum when the IL/MeOH ratio was 2:1. However, ratios of MeOH greater than 2:1, had a negative effect on yield, decreasing to as low as 81.3 ± 1.1%. The ratio of dried T18 biomass to the mixture of 2:1 IL/MeOH was further studied to minimize the required amount of solvent per gram of dried biomass (Fig. 6.4b), which was found that a mass ratio 1:4) had the best oil extraction efficiency, 92.6 ± 1.2%. Reducing the solvent used to 1:2 was not enough to fully immerse the T18 biomass in the tube. However, increasing the solvent (1:7) may have caused less direct shear stress when stirring the T18 solvent mixture in the tubes.

$[P_{4444}]$[Prop] was similarly assessed for comparison. First, the effect of the mass ratio of IL to MeOH (Fig. 6.4c) and subsequently the mass ratio of T18 to solvent (Fig. 6.4d) were studied. The ratios of IL to MeOH were varied from 4:1 to 1:7 using a mass loading ratio of 1:4 for T18 to solvent (Fig. 6.4c). Oil yield increased to around 83.9 ± 1.7% when using a 1:1 ratio and then decreased in either direction. The mixture of 1:1 IL/MeOH was further characterized to optimize the ratio of T18 biomass to solvent (Fig. 6.4d). It was found that more $[P_{4444}]$[Prop] (1:10) than $[C_2mim][EtSO_4]$ (1:4) was required to achieve similar oil extraction yields, 91.0 ± 1.1%.

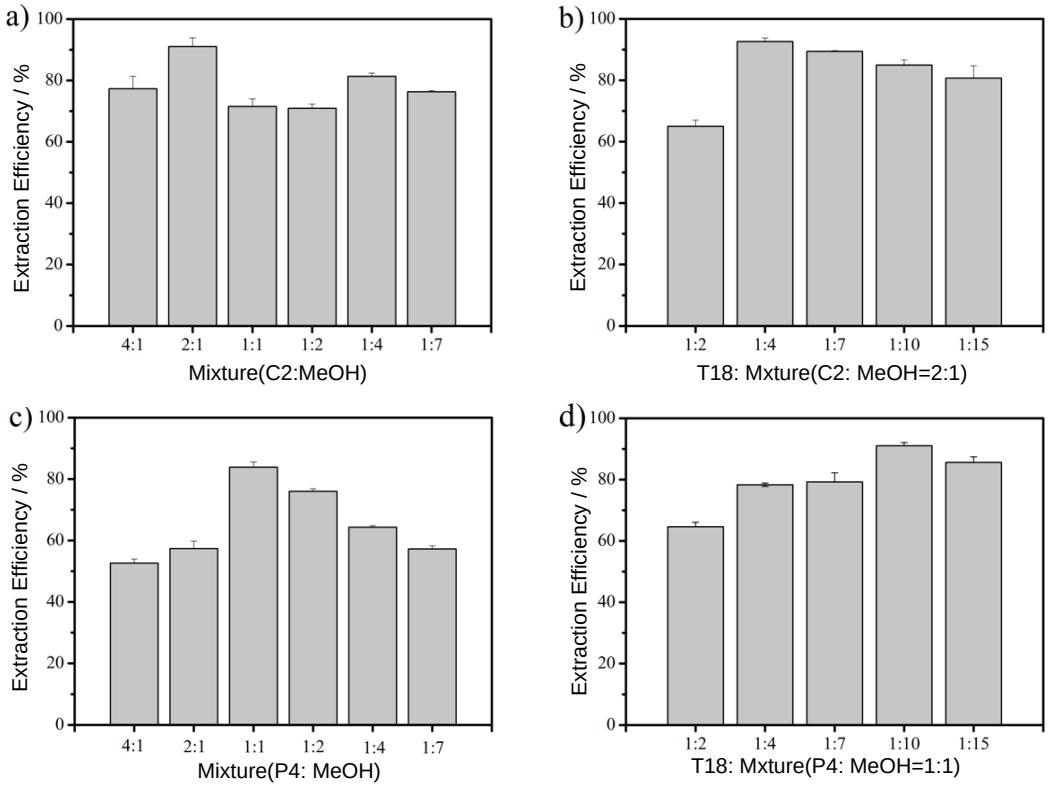

Figure 6.4: Extraction of dried T18 using $[C_2mim][EtSO_4]$(C2): (a)The effect of the mixture ratio (C2:methanol) on the extraction of oils from dewatered T18 at loading ratio of 0.1g:0.4g (T18:mixture). (b) The effect of increasing mass ratios of T18 to the mixture of IL and MeOH (2:1); Extraction of dried T18 using $[P_{4444}][Prop]$(P4): (c)The effect of the mixture ratio (P4:methanol) on the extraction of oils from dewatered T18 at loading ratio of 0.1g:0.4g (T18:mixture). (d) The effect of increasing mass ratios of T18 to the IL/MeOH mixture (1:1).

6.5.3 IL Extraction of Oils from Fresh T18

As in the previous section, the initial oil content of wet biomass was determined via HIP extraction and the batch contained 71.1 ± 2.8% (w/w) of oil. When the $[C_2mim][EtSO_4]$ process was applied to wet T18 the IL extraction worked poorly, extracting only a small fraction of the available lipids (Fig. 6.5a). Therefore, the IL: MeOH ratio was reassessed. It can be seen that the amount of extracted oil were the highest at the ratio of 1:7, but the maximum oil yield was still only 22.6 ± 0.3%. Since the optimal conditions were not consistent with those determined for dry biomass, the next step was to re-evaluate the loading ratio of T18 slurry to

the IL/MeOH mixture (Fig. 6.5b). Here the best ratio was again 1:4 which could recover 31.6 ± 0.7% of the oil. Thus, this ionic liquid was not a suitable candidate for wet extraction. In contrast, the optimal ratio of IL:MeOH did not change with wet T18 biomass processed with [P_{4444}][Prop] (Fig. 6.5c), however, the solid loading ratio of biomass to solvent decreased from 1:10 to 1:4 (Fig. 6.5d).

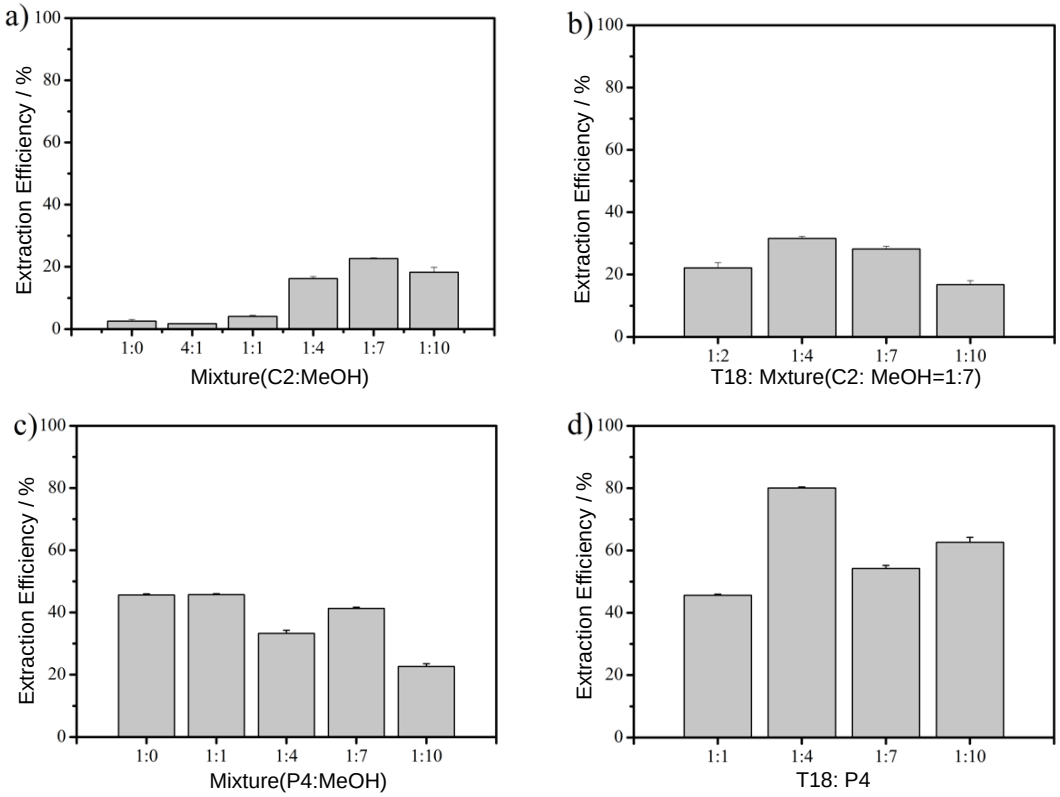

Figure 6.5: Extraction of wet T18 using [C_2mim][$EtSO_4$]: (a) The effect of the mixture ratio (C2:methanol) on the extraction of oils from wet T18 slurry at loading ratio of dry weight of T18:mixture(2g:2g). (b) The effect of increasing mass ratios of T18 to the mixture of IL and MeOH (1:7); Extraction of wet T18 using [P_{4444}][Prop]: (c)The effect of the mixture ratio (C2:methanol) on the extraction of oils from wet T18 slurry at loading ratio of dry weight of T18:mixture(0.5g:2g). (d) The effect of increasing mass ratios of T18 slurry to the IL.

6.5.4 Composition of Extracted Lipids

To analyze the fatty acid composition of extracts, the extracted oils were transesterified to FAME and quantified using a GC-FID (Fig. 6.6). The FAME compositions using [C_2mim][$EtSO_4$]

were no different from those extracted using the HIP method. However, the composition of the lipids recovered using [P_{4444}][Prop] were slightly different. The lipid extracted in this way had a greater proportion of saturated fatty acids such as C18:0. The largest constituent was C22:6 n-3 (DHA), confirming that T18 is a good source of DHA. The results further show that the ILs applied in this study did not selectively extract DHAs, but extract the overall available lipids without oxidizing the unsaturated bonds, hence not compromising the quality of the SCO.

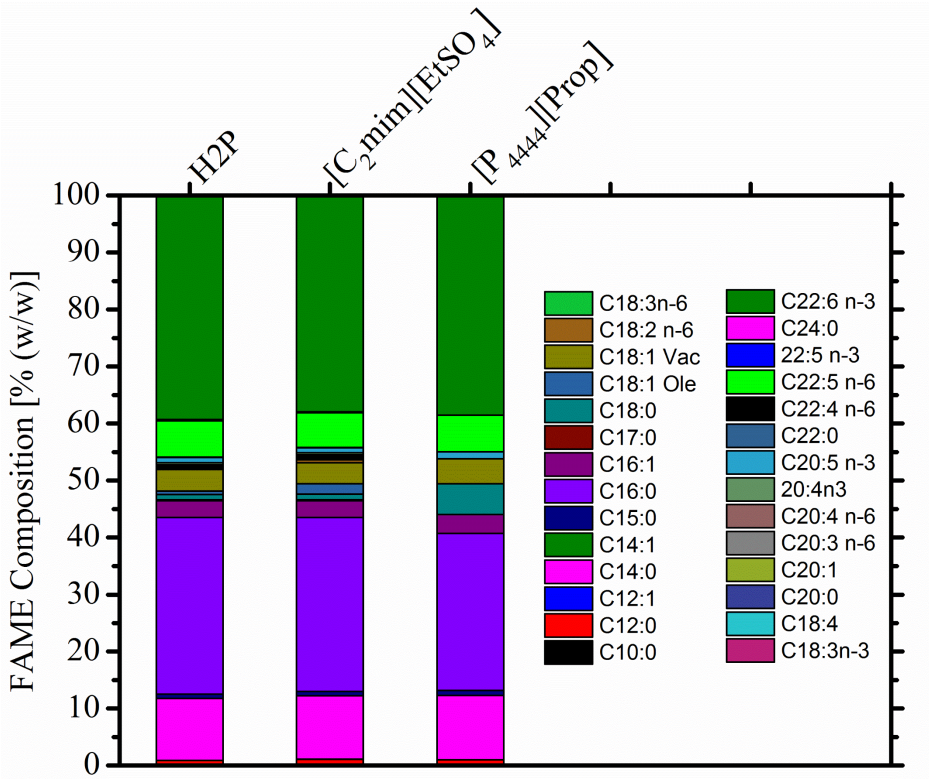

Figure 6.6: Comparison of the relative FAME composition of the oil extracted using HIP, [C_2mim][$EtSO_4$] and [P_{4444}][Prop]. Fatty acids are represented by the number of carbons in their chain followed by the number of unsaturated C–C bonds and the carbon number of the first unsaturated bond (e.g., C20:4n-6 represents a C20 chain with four unsaturated bonds, the first one occurring at carbon 6).

6.5.5 Ionic Liquid Recycling

To reduce the cost of the IL and improve the economic feasibility of a potential process, the reusability of the two ILs was addressed. Ionic liquids were recovered from dried T18

after oil extraction by antisolvent precipitation of the residual biomass using MeOH. After the residual solids were separated by filtration, the MeOH was evaporated using a vacuum evaporator. The pretreatment performance of the recovered IL towards dried T18 oil extraction was observed for a drop in performance. The results show (Fig. 6.7) that the amount of oil recovered by the recycled ILs, $[C_2mim][EtSO_4]$ and $[P_{4444}][Prop]$ over 5 cycles did not change. The average oil recovery by $[C_2mim][EtSO_4]$ and $[P_{4444}][Prop]$ were 98.5 ± 3.7 % and 97.2 ± 2.5 %, respectively. It is likely that impurities carried over between cycles did not negatively affect the oil recovery, which illustrates both ILs possess excellent recyclability.

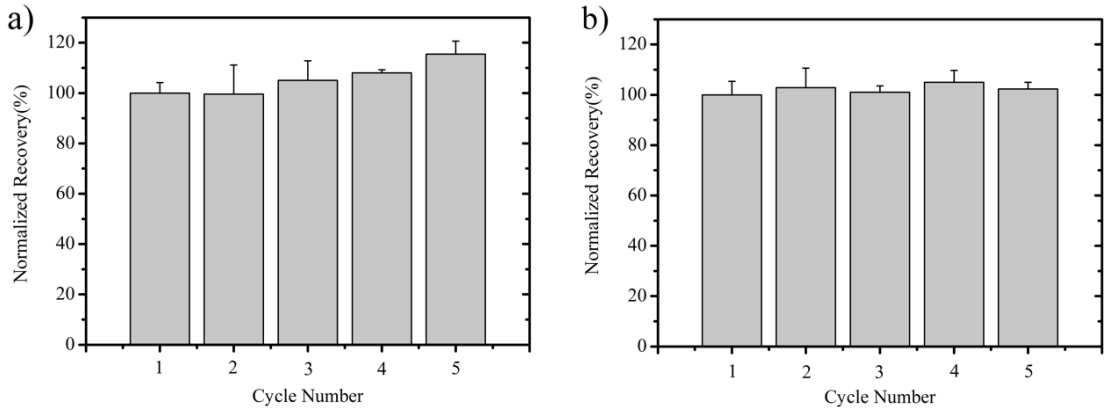

Figure 6.7: Comparison of the relative FAME composition of the oil extracted using HIP, $[C_2mim][EtSO_4]$ and $[P_{4444}][Prop]$.

6.6 Conclusions

The ability of ILs to aid in the extraction of thraustochytrids was assessed in a sustainable and recyclable process for DHA production. Two ILs were characterized under a series of conditions followed by lipid composition analysis. The results demonstrate that while $[C_2mim][EtSO_4]$ and$[P_{4444}][Prop]$ can extract over 90% (w/w) of the available oils from dried T18 biomass, wet T18 slurry was much more difficult to process. However, $[P_{4444}][Prop]$ achieved an oil yield of over 80% (w/w) making it a promising candidate for further studies.

One significant concern when using ILs for possible food processing applications will be the toxicity of these processing agents. While the ILs used in this work are strongly hydrophilic mitigating the concerns to some extent, toxicity of these ILs will need to be evaluated moving forward. The lipid composition by FAME analysis showed that the major component of T18 oil was DHA. Moreover, both ILs were readily recycled with no decrease in performance over 4 cycles. In contrast to conventional extraction methods with require dried biomass, this work demonstrates a simple process that is compatible with wet T18 biomass.

7 Extraction of A DHA-rich Oil from *Thraustochytrium sp.* Using A Novel Hydrophobic Ionic Liquid

7.1 Preface to Chapter 7

The previous chapter showed a water-compatible, low-energy, PUFAs recovery method using two kinds of common ionic liquids from *Thraustochytrium*(T18). In this chapter, to investigate the applicability of the ionic liquid based processing, the synthesized ionic liquid [C_{10}mim][BTMPP] characterized in chapter 3, was first used for DHA-rich oil extraction without co-solvent. The extraction processes were conducted from lyophilized T18 followed by fresh T18.

Based on the previous chapters, a central composite design (CCD) was set up to assess the effect of three parameters, such as mass ratio, pH, and temperature on oil yield. Response surface methodology was used to optimize the reaction conditions in terms of mass ratio, pH, as well as temperature. Additionally, the interaction of the factors was analyzed. The extraction efficiency reached up to 97% at the obtained optimal conditions. Hence, the experimental results are consistent with the model by Design Expert. In addition, FAME composition was also analyzed by GC.

With minor editorial changes to fulfill formatting requirements, this chapter is substantially as it was prepared for ACS Sustainable Chem. Eng. (currently in submission)

7.2 Abstract

Thraustochytrium sp., is a marine heterokont that exhibits a fast growth rate and accumulates a high proportion of polyunsaturated fatty acids (PUFAs) content, particularly docosahexaenoic acid (DHA). The extraction of DHA-rich oil from marine algae and fish is challenging due to the difficulty in disrupting cell walls, the incompatibility of solvents with wet biomass, and the large volumes of organic solvents required by traditional extraction techniques. To address these concerns, ionic liquids (ILs) have been used to improve the oil yield of oils from wet microalgae and oleaginous yeast at milder conditions. In this study, a synthesized hydrophobic ionic liquid, 1-decyl-3-methylimidazolium bis(2,4,4-trimethylpentyl) phosphonate ([C_{10}mim][BTMPP]) was first applied as pretreatment to aid in the extractiong of DHA-rich oil from T18 in aqueous phase. A central composite design and response surface methodology were used to design and study the effect of different extraction variables (temperature, pH, and the mass ratio of IL to dried T18). The optimal extraction conditions were numerically optimized to maximize the oil yield within the experimental range. It was found that up to 69.4 ± 0.5% (w/w) oil yield, without using any co-solvents under the optimized conditions (mass ratio of 4.2, pH of 5.0, and the temperature of 76 °C). The extracted oil contained 44.7 ± 0.2% DHA and the extraction process took 1 h. Hence, this work opens a new approach for a time-saving, efficient and water-compatible DHA-rich oil recovery.

Keywords: DHA, ionic liquids, marine heterokont, value-added products.

7.3 Introduction

Docosahexaenoic acid (DHA, C22:6, n-3) is an unsaturated ω-3 polyunsaturated fatty acid (PUFA), present in the human brain, retina, and skin, and its dietary consumption may have benefits in the treatment of arteriosclerosis, depression, arthritis, adult-onset diabetes mellitus, etc [31, 268, 269]. Owing to these benefits, the global ω-3 PUFA market will grow continuously with a compound annual rate of 7.4% until the year of 2025 [269]. Currently, PUFAs are mainly obtained from marine fish, which competes with food supply and can negatively affect fish supply due to over-harvesting of PUFA rich species, and the oils extracted in this manner exhibit climatic variations and can contain heavy metals and toxic pollutants present in the environment [31, 270, 271]. To circumvent these disadvantages, some plants and algae have been studied as potential sources of PUFA [272], including *Camelina sativa* [273, 274], *Dracocephalum kotschyi*[275, 276] and marine algae like *Thraustochytrium*, *Aurantiochytrium*, *Schizochytrium*, and *Ulkenia* genera [43, 140]. Thraustochytrids have a relatively high PUFA content, high growth rate and high productivity, and do not require freshwater [24]. These marine microorganisms are usually cultivated in aqueous media, and not limited by land/area or seasonality [22]. Thraustochytrids can break down complex organic materials for use as their main carbon source, which makes it possible to cultivate them on various feedstocks, such as waste materials. [24, 251, 255].

Notwithstanding the high level of PUFAs accumulation in marine algae, extraction and separation methods of PUFAs from biomass is still challenging, particularly the steps of cell wall disruption, the high cost of dewatering, and the large volumes of solvents used in traditional methods. A number of advanced extraction techniques such as supercritical fluid extraction

(SFE), microwave-enhanced extraction, and ionic liquid extraction have been used for PUFA extraction from thraustochytrid biomass [17, 140, 269]. SFE was applied to *Aurantiochytrium sp.* biomass using $C0_2$, which achieved the maximum of DHA content (39.3 wt.%) after optimizing pressure, flowrate and temperature [269]. Leone et al. investigated *Nannochloropsis sp.* using SF-CO_2 extraction, which showed that the maximum extraction yield was 9.428 wt.%, with a purity of 19.51% at 550 bar, 75°C and a CO_2 flow rate of 14.48 g/min [277].

Zeb et al. designed a novel microwave-assisted method combined with a three-liquid-phase salting-out extraction technique (MA-TLPSOE) to separate DHA-rich oils from *Schizochytrium limacinum* SR21 biomass, which obtained 100 ± 0.64% DHA separation in the extraction solvent (n-hexane) under optimized conditions of 30% (w/w) n-hexane/ 13% (w/w) ethanol /9% (w/w) sodium carbonate combined with a high microwave power of 650 W for 30 s [278]. Antoine Delbrut et al. recently extracted oil from Tisochrysis lutea biomass using 96% ethanol with a solvent/biomass ratio of 20:1 (v/w). They achieved a 100% yield for both fucoxanthin and DHA within 1 h. Similarly they achieved 95% and 89% oil yield for fucoxanthin and EPA from *Phaeodactylum tricornutum* biomass within 8h, respectively [279]. Choi et al. performed acid-catalyzed hot-water extraction of DHA-rich lipids from *Aurantiochytrium sp.* KRS101, and the optimal DHA yield was 29.3% of total fatty acids using a sulfuric acid concentration of 1.00% at 100°C, 30 min [280].

However, SFE and microwave assisted extraction methods still have a number of challenges affecting their commercialization. While SFE with CO_2 has many advantages, like the non-polar character of CO_2 which is ideal for lipid extraction, its nontoxicity, the extraction rate is low, efficient CO_2 recovery is required, and the technology has a high capital cost [269]. Microwave assisted extraction mainly has problems related to penetration depth when scaling

up reactions [281]. Solvent extraction is the most common method for lipid extraction because it is well understood and established process with high yields and low cost, but elicits serious concerns related to sustainability due to the large volumes of toxic solvents that can damage the environment [58].

Ionic liquids (ILs) are a reusable type of solvent consisting of cations and anions which have been regarded as a promising alternative in the field of biomass extraction owing to their favourable physiochemical characteristics, such as non-flammability, high chemical and thermal stability, and negligible volatility [282, 283]. Moreover, ILs have the ability to accomplish the dissolution of many types of biomolecules, which make them capable of extracting some high-value compounds from natural feedstocks [6, 96, 140, 282]. Furthermore, nonvolatile ILs have been shown to be reusable allowing the recycling of solvents during bioprocess of biomass making them potentially more environmentally friendly than conventional organic solvents [284, 285]. To date, ILs have been combined with many other pretreatment technologies such as microwave and ultrasonication to enhance the efficiency of the IL extraction process [286, 287]. With the increasing number of potential applications of ILs, a rising number of ILs that are now commercially available [140].

Herein, we present a new ionic liquid 1-decyl-3-methylimidazolium bis(2,4,4-trimethylpentyl) phosphonate, [C_{10}mim][BTMPP], was first used for the pretreatment of T18 biomass in the aqueous phase to aid in the extraction of DHA-rich oil from T18. The factors affecting extraction efficiency on total oil yield and DHA recovery were identified and optimized using response surface methodology. The pretreatment of T18 with [C_{10}mim][BTMPP] resulted in cell disruption making the proceeding extraction steps more efficient.

7.4 Experimental

7.4.1 Materials and Methods

Thraustochytrium sp.(T18) is derived from the Canadian Phycological Culture Center (CPCC) strain PTA-6245. [C_{10}mim][BTMPP] (60% water) was synthesized and the structure was confirmed using standard methods as described in chapter 3. All other chemicals used in this work were purchased from Sigma-Aldrich, Inc.

7.4.2 Harvesting and Freeze-Drying

The cultivation conditions of T18 are described in previous work [140]. T18 cultures were harvested by centrifugation at 3500 rpm at 4°C for 20 min by a Sorvall RT centrifuge (Fisher Scientific). Residual salts were washed from the biomass three times by resuspending the cell pellets in deionized water. Then the cell pellets were then frozen at -80 °C for a minimum of 12 h and then lyophilized using a 4.5 L freeze-drier (Labconco) for 24 h and stored in a desiccator until further use.

7.4.3 Direct Transesterification of Total Lipid Content

The total lipid content of the T18 biomass was analyzed using fatty acid methyl esters (FAME) quantification as described by Armenta et al [288]. Briefly, C23:0 in toluene (950 µL of a 1 mg mL^{-1}) was added to 50 mg of dried T18 cells as the internal standard for quantification. The biomass was directly transesterified by adding 6 mL of toluene and 6 mL of 12.5 % (v/v) acetyl chloride in methanol to each sample. Samples were incubated at 80 °C for 120 min followed by the addition of 10 mL of 6% w/v sodium carbonate, then allowed to cool to

ambient temperature. To separate the aqueous and toluene layers, samples were centrifuged at 3500 rpm at 4°C for 20 min. The supernatant now containing FAME was filtered through a silica solid phase extraction (SPE) cartridge before gas chromatography (GC) analysis.

7.4.3.1 IL Pretreatment

As shown in Fig. 7.1, IL pretreatments were conducted in triplicate as follows: a) 0.10 g of freeze-dried T18 was mixed with the indicated amount of DI water followed by the addition of the indicated mass of IL in a tube, followed by mixing for 1h with a magnetic stirrer at 1000rpm at ambient temperature; b) 5 mL of hexane was added to the mixture which was then vortexed for 30 s; c) the mixture was allowed to stand for 5 min before transferring the supernatant layer to a new container; d) this process (steps b/c) was repeated three times; e) the remaining mixture was centrifuged at 4°C and 3500 rpm for 10 min and the top layer was transferred along with the other hexane fractions to a new container to evaporate the solvent. As a negative control, the same process was repeated without the addition of the IL and shown to results in only 0.3±0.1% (w/w) oil yield.

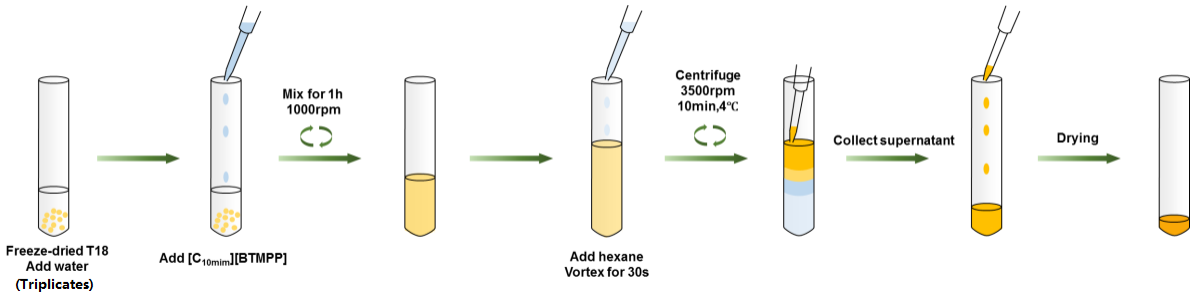

Figure 7.1: Flowchart of the lipid extraction by [C_{10}mim][BTMPP].

7.4.4 Lipid Composition of Extracted Oils

In order to analyze the fatty acid composition of the extracted oils, FAME was prepared as follows: a) 100 mg of extracted oil was dissolved in 10 mL of hexane followed by the addition of 100 μL of 2 M methanolic KOH. b) The samples were then vortexed for 30 s followed by centrifugation at 3500 rpm at room temperature. c) The supernatant was spiked with the internal standard methyl undecanoate (C11:0). d) The FAME mixture was separated via an Agilent DB-WAX capillary column (30 m, 0.25 mm, 0.25 μm) using helium as the carrier gas at a linear velocity of 30 cm s^{-1}. Individual FAMEs were quantified and identified using analytical standards (Supelco-37) and the internal standard. Unidentified FAMEs were estimated using an averaged RF factor. The FID detector operated at 280°C and FAMEs were eluted using the following program: 50°C, 1 min, 10°C min^{-1} to 200°C, 3°C min^{-1}, 220°C, 10 min. Oil yield (%) was defined as the following equation 4.6 in chapter 4. Extraction efficiency (%) was defined as the amount of oil extracted divided by the maximum amount of oil available in the T18 biomass using the equation 4.7 in chapter 4.

7.4.5 Experimental Design

A central composite design (CCD) was selected to assess the effect of three parameters: mass ratio, pH, and temperature on oil yield. The five uncoded values of each factor level [-α, -1, 0, 1, +α] are as follows: mass ratio of ionic liquid to T18 biomass [0.05, 0.5, 2.75, 5, 5.45], pH [2.65, 3, 4.75, 6.5, 6.85], and temperature (°C) [19.5, 25, 52.5, 80, 85.5]. The experimental design was determined using Design Expert 10.0.4 (Stat-Ease, Inc., Minneapolis, MS, USA). In addition, all conditions were carried out in triplicate. As a result, 51 randomized conditions

(8 × 3 factorial + 6× 3 augmented + 3× 3 center points) were run in a randomized order.

7.5 Results and Discussion

7.5.1 IL Pretreatment for Dry T18

The separations of T18 biomass without IL pretreatment and after 60 minutes of pretreatment are compared in Fig. 7.2. Dry T18 (Fig. 7.2a) is mixed with water and IL (Fig. 7.2 b) and immediately mixed with hexane (Fig. 7.2 c, without IL pretreatment) and centrifuged, or stirred for 60 min (Fig. 7.2 d), mixed with hexane and centrifuged to completely separate the phases. Addition of the water was used to dilute the ionic liquid and reduce its viscosity, while simulating wet extraction conditions based on our previous work [140]. To facilitate extraction and aid in phase separation, 5 mL of hexane was added to the tube. The T18 biomass used was determined to contain 71.2 ± 1.4% (w/w) lipid by direct transesterification. It can be seen that the sample without IL pretreatment (Fig. 7.2 c) separates into two layers, however the supernatant is transparent indicating very little lipid and pigment extraction. The oil yield was shown to be negligible at 0.3 ± 0.1% (w/w). In comparison, the pretreated slurry (Fig. 7.2 d) clearly shows four distinct phases, which are, from top to bottom: hexane containing the extracted oil and pigments, T18 residual solids, the hydrophobic IL, and water.

In order to elucidate the effect of the IL pretreatment on the T18 biomass, the treated and untreated biomass were observed using a bright field microscope as shown in Fig.7.3. The untreated T18 biomass can be seen as uniform spheres, however, the morphology of the T18 cells after IL pretreatment is non-uniform indicating that [C_{10}mim][BTMPP] is disrupting the cell structure during the pretreatment. Since lipids are stored intracellularly, cell disruption can potentially enhancing the extraction of lipids from the biomass. Almost all of the cells appear

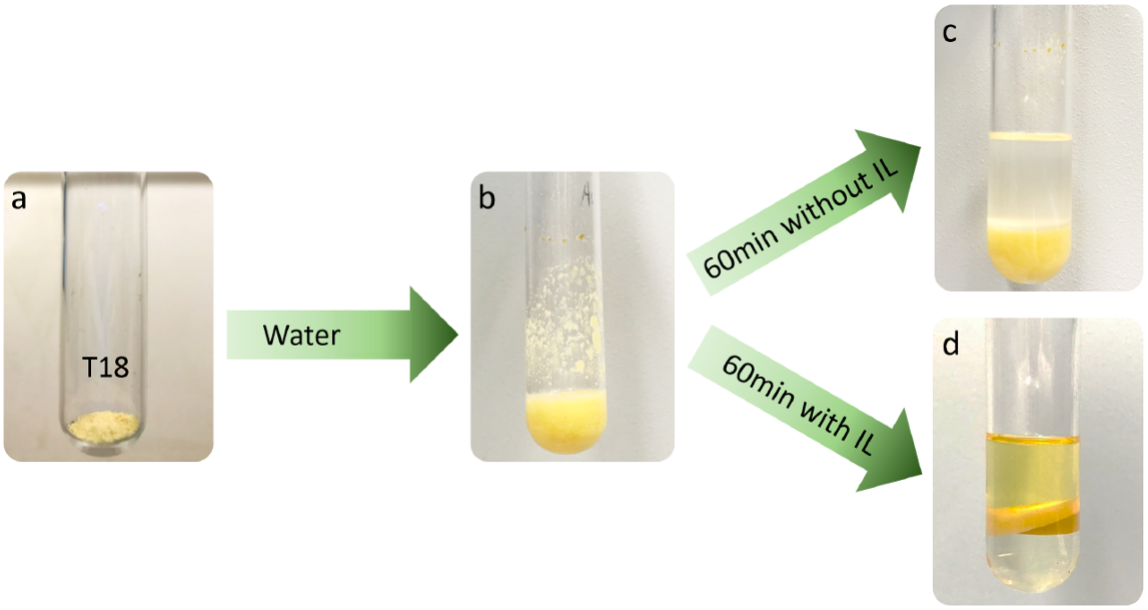

Figure 7.2: The pictures of T18 without/with IL pretreatment.

to be completely broken (Fig.7.3 b) and the broken cells appear to aggregate with each other. These agglomerations made aid in the residual cells solids to separate from the lipids dissolve in hexane, and the water phase during the extracting process as was seen in Fig. 7.2.

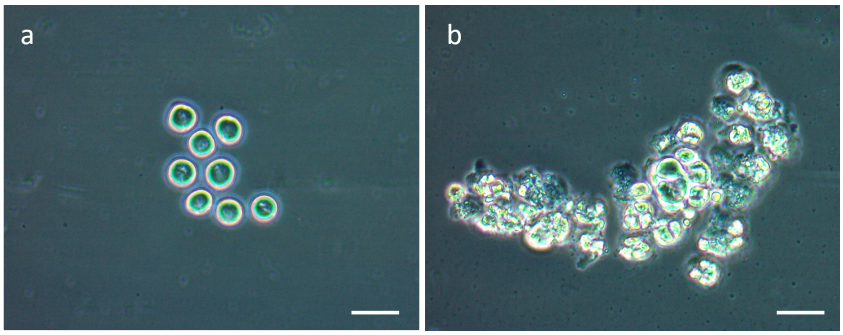

Figure 7.3: The surface morphology of virgin and pretreated T18. The scale bars are both 10μm.

7.5.2 Optimization of Oil Extraction Conditions

To understand the effects that the extraction conditions could have on the extraction efficiency and optimize the oil extraction conditions, response surface methodology was applied to this process. Based on our exploratory experiments, the ranges of the mass ratio of IL:T18,

pH, and temperature were set to 0.5-5, 3-6.5, and 25-80°C, respectively. Experimental conditions were determined using a CCD and the values of the designed conditions as well as the measured responses are presented in Table 7.1

7.5.2.1 Response Surface Model Validation

As seen in Table 7.1, a large range of oil yields were achieved between 5.05 to 60.48% (w/w) under the conditions studied. The dataset was fit with the cubic equation shown in Equation 7.1. Table 7.2 presents the analysis of variance (ANOVA) for the fit model. The model was found to be significant (p ¡0.0001), while the lack of fit was not (p = 0.1064), indicating the model was a good fit for the data. The coefficient of determination (R^2 = 0.9992) was in agreement with the adjusted R^2 which accounts for the number of model terms indicating the model fit is good and the R^2 of prediction (Pred R^2 = 0.9441) indicates that the mode has good predictive ability within the design space.

Based on the selected significant variables, the cubic model for the DHA-rich oil in terms of actual factors is shown as follows:

$$\begin{aligned} Oil\ yield = {} & -0.73 + 0.043 \times Mass\ ratio + 0.37 \times pH + 0.13 \times Mass\ ratio \times pH - 2.28 \\ & \times Mass\ ratio \times Temperature + 5.03 \times pH \times Temperature - 0.06 \times Mass\ ratio^2 \\ & - 0.04 \times pH^2 - 2.70 \times Temperature^2 + 2.41 \times Mass\ ratio \times pH \times Temperature \\ & + 4.43 \times Mass\ ratio^2 \times Temperature - 0.01 \times Mass\ ratio \times pH^2 \end{aligned} \tag{7.1}$$

Table 7.1: Analysis of oil yield (average ± standard deviation) under designed conditions.

Temperature(°C)	Mass ratio (IL:T18)	pH	Oil yield (% dry weight)
19.5	2.75	4.75	56.1±7
25	5	3	11.1±0.5
25	5	6.5	17.9±0.1
25	0.5	3	10.7±0.6
25	0.5	6.5	6.8±0.2
52.5	5.45	4.75	57.1±5.9
52.5	2.75	2.65	20.4±4.3
52.5	2.75	6.85	35.6±4.6
52.5	2.75	4.75	60.5±2.2
52.5	2.75	4.75	60.2±2.0
52.5	2.75	4.75	59.4±3.2
52.5	0.05	4.75	13.8±0.4
80	5	3	22.1±1.7
80	5	6.5	58.3±0.4
80	0.5	3	5.1±0.7
80	0.5	6.5	6.0±0.6
85.5	2.75	4.75	54.0±2.6

Table 7.2: Analysis of variance of fitted model for oil yield.

Sourse	Remark	Sum of squares	Freedom Deg.	F value	P value Prob>F
Model	significant	8424.67	11	594.84	< 0.0001
A	significant	936.06	1	727.02	< 0.0001
B	significant	314.45	1	244.23	< 0.0001
AB	significant	262.81	1	204.12	< 0.0001
AC	significant	414.69	1	322.08	< 0.0001
BC	significant	146.71	1	113.94	0.0001
A^2	significant	1407.44	1	1093.12	< 0.0001
B^2	significant	2396.50	1	1861.30	< 0.0001
C^2	significant	58.29	1	45.27	0.0011
ABC	significant	74.64	1	57397	0.0006
A^2C	significant	252.90	1	196.42	< 0.0001
AB^2	significant	133.49	1	103.68	0.0002
Lack of fit	not-sig.	5.80	3	6.07	0.1446
R-squared					0.9992
Adj R-squared					0.9976
Pred R-squared					0.9441
Adeq Precision					58.028

A is mass ratio; B is pH; C is temperature.

7.5.2.2 The Analysis of the Model

The normal probability plot indicates whether the residuals follow a normal distribution. If the set of real numbers accord with normal distribution, the normal probability graph will be a straight line. The normal probability plot of the actual response shown in Fig. 7.4a implies that most of the points are on or near the straight line, meaning that the experimental results fit the normal distribution well. Fig. 7.4b is the predicted against the actual plot which shows the predicted response values versus the actual response values. The purpose is to detect a value, or group of values, that are not easily predicted by the model. For a good fit, the points should be close to the fitted line with narrow confidence bands. Ideally, the fitted line should correspond to a slope of 1 and an intercept of 0. In this case, most of the points are on the line, indicating the predicted response matches the actual response values well.

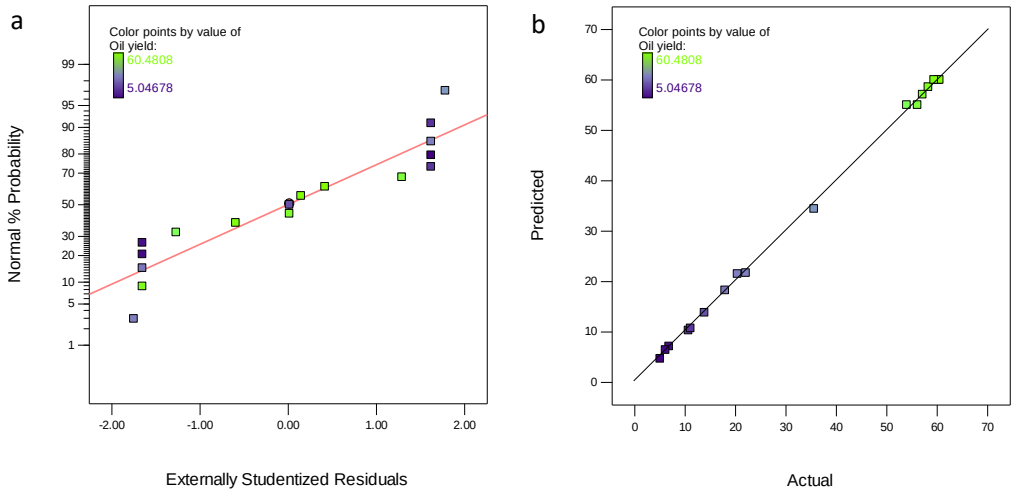

Figure 7.4: a) Normal probability plot of residual; b) Predicted vs. Actual.

Fig. 7.5a displays the plot of residuals vs. predicted. This is a plot of the residuals versus the ascending predicted response values. It tests the assumption of constant variance. The plot is a random scatter, and all of the points are inside the constant range of residuals across

the graph, meaning that there is no need for a transformation. Fig. 7.5b presents the graph of residuals vs. run that is a plot of the residuals versus the experimental run order. It checks for lurking variables that may have impacted the response during the experiment. In addtion, the plot is random scatter and the points are inside the constant range of residuals. The trend indicates that there is no time-related variable lurking in the background.

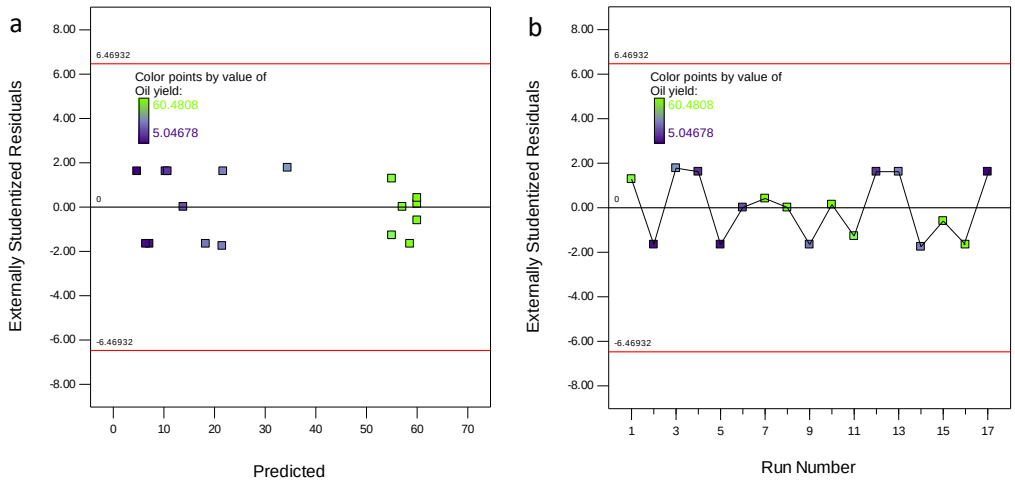

Figure 7.5: a) Residuals vs. Predicted; b) Residuals vs. Run.

Fig. 7.6 checks whether the variance not accounted for by the model is various for different levels of a factor. The two plots exhibit random scatters inside the constant range of residuals that indicates the model is fine.

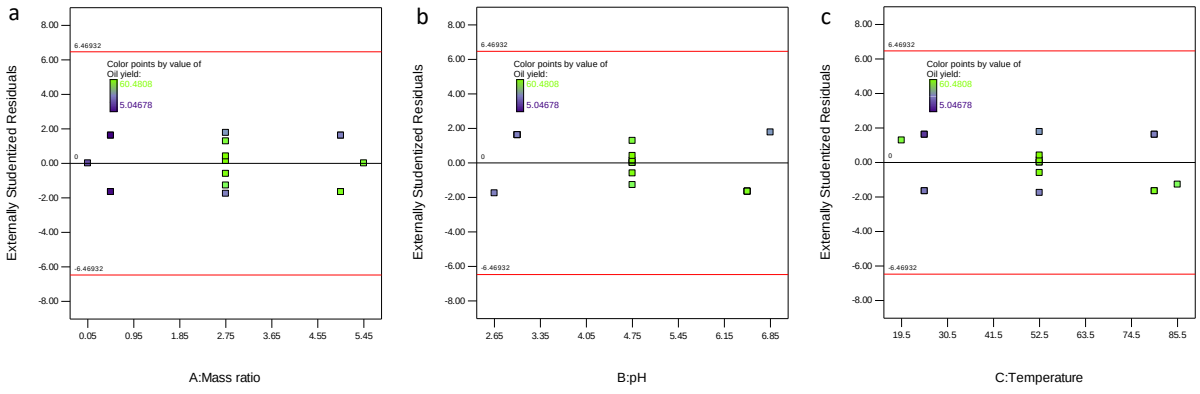

Figure 7.6: Residuals of factors: a) Mass ratio; b) Temperature; c) Processing time.

7.5.2.3 Interaction Effect of Various Factors

Fig. 7.7 illustrates the effect of the interaction between pH and the mass ratio of IL (Fig. 7.7a), pH and temperature (Fig. 7.7b), mass ratio and temperature (Fig. 7.7c) on oil yield. The plots indicate at both low and high pH the yield decreases, however, at higher temperatures the effect of pH can be compensated by the increase in temperature. Similarly, increasing the amount of IL also compensates for non-optimal pH. This suggests that the IL is less effective at disrupting the biomass at pH outside the range of 4.5-6.0. The existence of optimal pH may result from the hydrogen-bond basicity of the involved ionic liquid (Fig. 3.1). It was known that the anions of ionic liquids have a primary impact of hydrogen-bond interactions in the formation of hydrated complexes between proton donor substance and ionic liquids [289]. The long-chain anion ($[BTMPP]^-$) of the involved ionic liquid has an considerable electron-donating effect, which is the main reason for the strong hydrogen-bond basicity [290]. Thus the ionic liquid [C_{10}mim] [BTMPP] working with less or more acids may result in the various amount of hydrated complexes, which has impact on the pretreatment of microalgae cells.

For having a more direct understanding of the impact of each factor on the oil yield, the single factor plots are given in Fig. 7.8. The oil yield increases when the mass ratio increase from 0.5 to 4.1 and then remains around 60% (Fig. 7.8a). Meanwhile, the curve of the pH vs. oil yield is in "inverse-U" shape (Fig. 7.8b), suggesting there is an optimal pH, about 4.8, for the oil extraction. As mentioned in Chapter 3 (Fig. 3.9), the pH of [C_{10}mim][BTMPP] can be adjusted. Also, an appropriate pH value will enhance the cell wall breaking during the oil extraction, which is the probable reason for the existence of optimal pH. Fig. 7.8c presents that the increase of temperature results in a higher oil yield. The high temperature weaken the cell

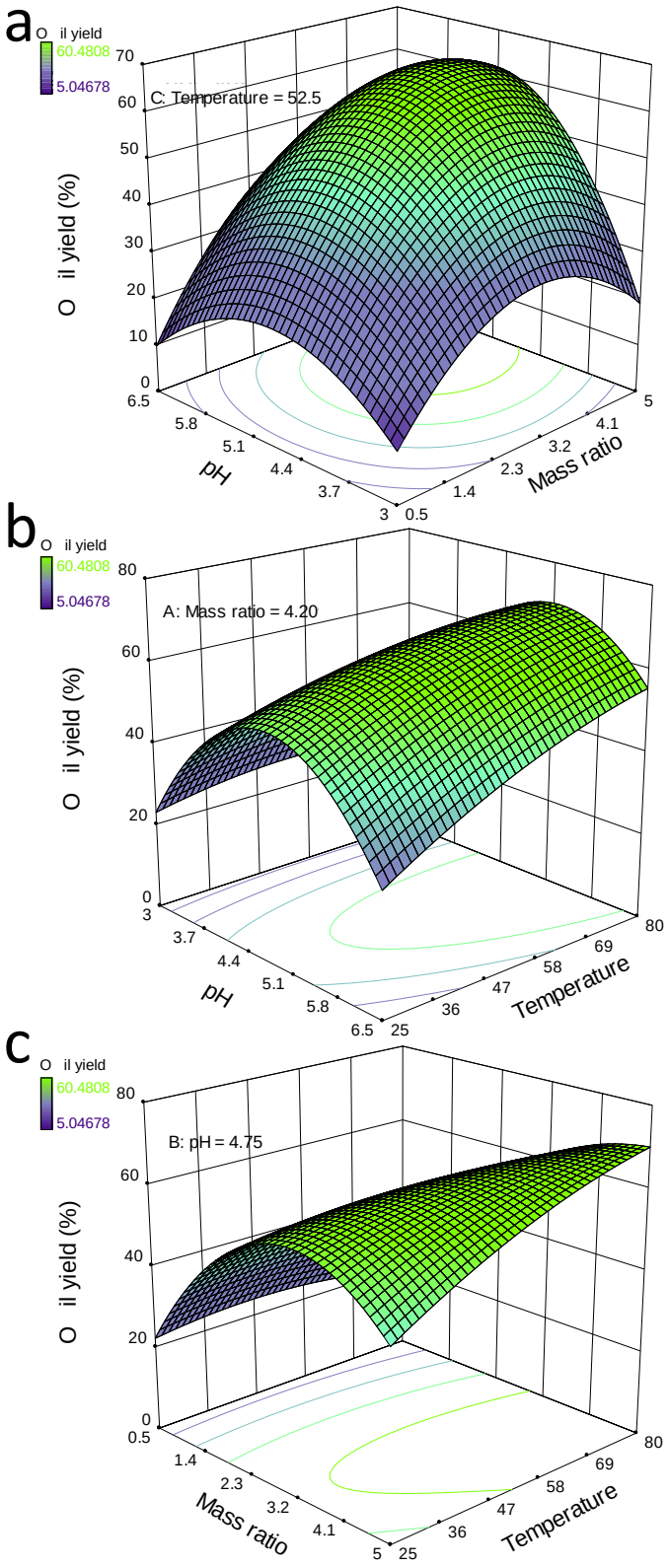

Figure 7.7: Surface plot of combined effect of experimental variables on oil yield(w/w). **a)** pH and mass ratio (temperature = 52.5°C); **b)** pH and temperature (mass ratio = 4.2);**c)** mass ratio and temperature (pH = 4.75).

and reduce the viscosity of the IL which partially helps to improve the oil yield.

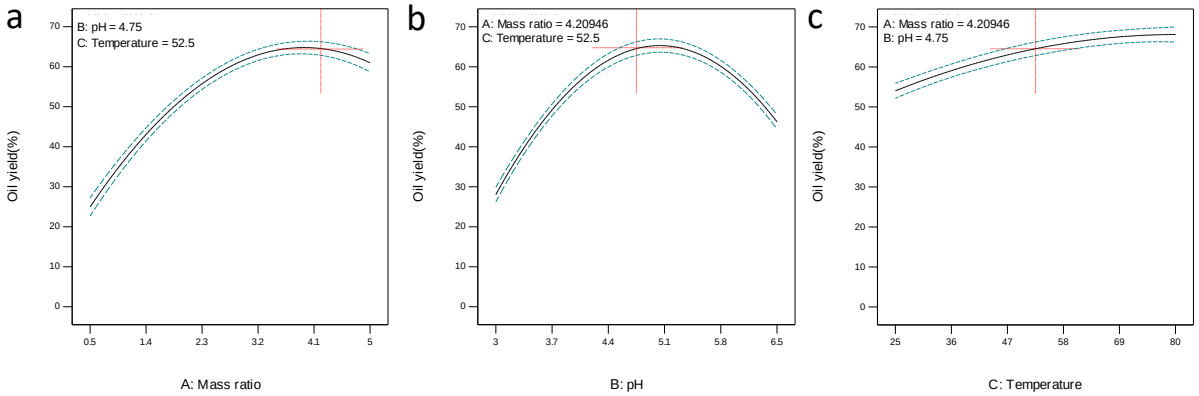

Figure 7.8: Single factor plots of experimental variables on oil yield. **a)** mass ratio (temperature = 52.5°C, pH = 4.75); **b)** pH (mass ratio = 4.2, temperature = 52.5); **c)** temperature (mass ratio = 4.2, pH = 4.75).

7.5.2.4 Response optimization and model validation

Using the fitted model, the optimal combination of process parameters required to achieve the maximum oil yield (69.5 ± 1.1%(w/w)) was obtained using numerical optimization in Design Expert. The optimal conditions were determined to be a mass ratio of 4.2, pH of 5.1, and a temperature of 76°C, as shown in the first row of the Table 7.3. The predicted oil yield at the optimal conditions was validated experimentally resulting in a yield of 69.4 ± 0.5% (w/w) which was in excellent agreement with the yield predicted by our model. This yield is equivalent to an extraction efficiency of 97.4 ± 0.4% based on the lipid content of the T18 biomass. Two more conditions at lower temperatures were chosen to verify the precision of the model as shown in the second and third rows of Table 7.3. Since DHA is sensitive to thermal degradation and using a lower temperature during the pretreatment may reduce the energy cost of the process, a moderate (53°C) temperature and a low (25°C) temperature were selected. The experimental oil yields were found to agree with the predicted values

with both experimental values falling within the 95% confidence intervals of the predictions. The decrease in temperature resulted in a small decrease in overall extraction efficiency (4.5% decrease and 11.6% decrease for 53 vs. 25°C, respectively, at the conditions in Table 7.3). A more in depth study of the process economics is necessary to determine whether the decrease in energy cost would offset the decrease in extraction efficiency. Regardless, the validated RSM model is a useful and accurate model for predicting the oil extraction yield from thraustochytrid biomass under the conditions tested using [C_{10}mim][BTMPP].

Table 7.3: Model validation at optimal and two good conditions, prediction interval, measured values ± standard deviation.

	Temperature (°C)	Mass ratio (IL:T18)	pH	Oil yield (% dry weight)		Confidence
				Predicted	Experimental	
#1	76	4.2	5.1	69.5±1.1	69.4±0.5	> 95%
#2	53	3.8	4.8	64.9±1.1	63.5±2.6	> 95%
#3	25	3.3	4.8	57.9±1.1	60.0±0.5	> 95%

7.5.2.5 Lipid Characterization

To determine the DHA content of the extracted oils, the oils extracted under the #1 and #3 conditions (Table 7.3) were transesterified to FAME and quantified using a GC-FID. The percent composition of the lipids as FAME is shown in Fig. 7.9. The major fatty acids present in T18 extract oil was DHA (C22:6n-3) which encompassed approximately 44% of the extracted oil under both conditions. The other major components were found to be C16:0 (~21%) and C22:2 n-6 (~10%). These similarities imply that the temperature does not affect the composition of the extracted oil, however, the relatively high proportion of DHA found in the extracted oil is promising since PUFAs are prone to oxidation during extraction resulting in a high quality extraction of DHA-rich oil.

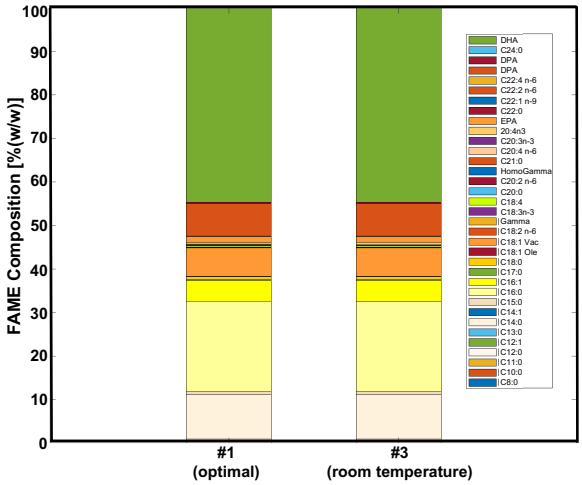

Figure 7.9: FAME composition at optimal condition and a good condition(room temperature of 25.03, Mass ratio (IL:T18) of 3.31, pH 4.79.

7.5.2.6 Effect of pretreatment time on oil yield

In order to simply the preceding experiments, a pretreatment time of 60 min was consistently applied to all runs. To determine if the pretreatment time could be reduced, a series of experiments were carried out at the optimal conditions (temperature of 76°C, mass ratio of 4.17, and a pH of 5.05) while the pretreatment time was varied between 0 and 90 min. The results shown in Fig. 7.10. It was determined that the pretreatment time could be decreased to 40 min as oil yield did not increase after this time.

7.5.3 IL Pretreatment for fresh T18

7.5.3.1 Extraction with MeOH

In this section, $[C_{10}mim][BTMPP]$ with co-sovent MeOH was also tested to extract lipids from fresh T18. The dry weight percent is 22.8±0.6 wt% (Table 6.3) and the total oil content is 78.1±2.8 wt% (Table 6.4). First, the mixture of IL and methanol (total mass is 0.5g) with various ratios (IL: methanol) were added into tubes that had a certain amount of T18 slurry

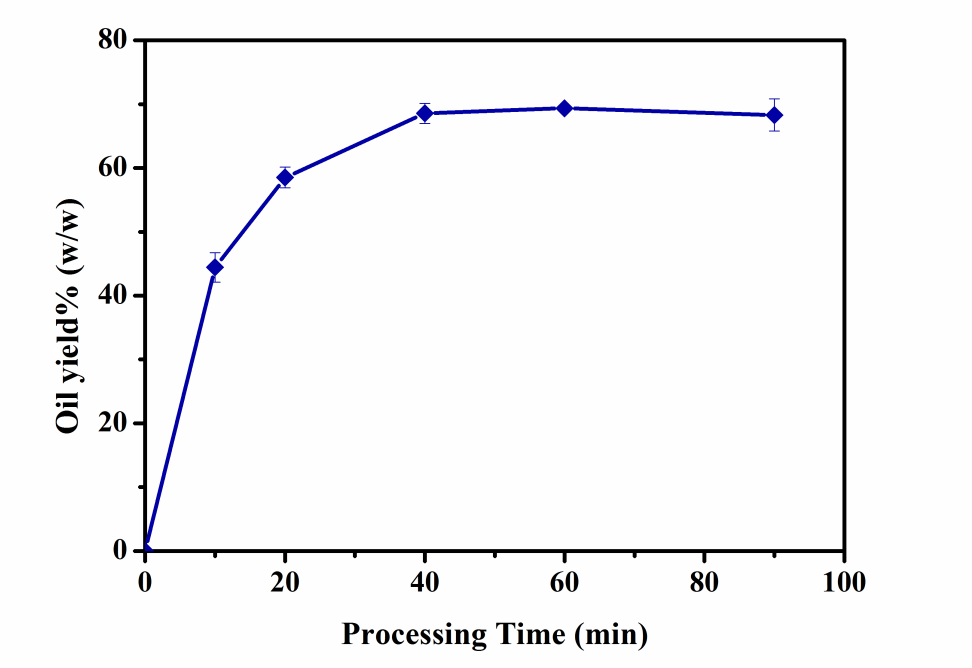

Figure 7.10: The trend of oil yield by processing time.

(2.0g). The results show oil yield is the highest, up to 30.7%, at the ratio of 1:7(IL: Methanol) (Fig.7.11). The effect of loading ratio (T18: mixture) was also studied at the IL to methanol ratio of 1:7. Fig. 7.12b show that the extracted oil yield at a loading ratio of 1:1, was 30.7 wt.%. A higher dosage of the mixture of IL and MeOH led to a lower oil yield, partially due to the insufficient stirring which may comes from excess solution in tubes with the increase of volume of the mixture. There is still potential to use less amount of IL and MeOH.

7.5.3.2 Extraction without MeOH

In order to avoid using volatile solvents, such as MeOH and ethanol, here the water-diluted [C_{10}mim][BTMPP] (60 wt% water) with different pH values were performed to extract oil form fresh T18 at the constant loading ratio of 1:1. Different pH values (from 2.91 to 6.25) were adjusted by the addition of 1.5 M sulfuric acid (H_2SO_4). In this method, fresh T18 culture without centrifugation was adopted, which was different from all mentioned methods. Also,

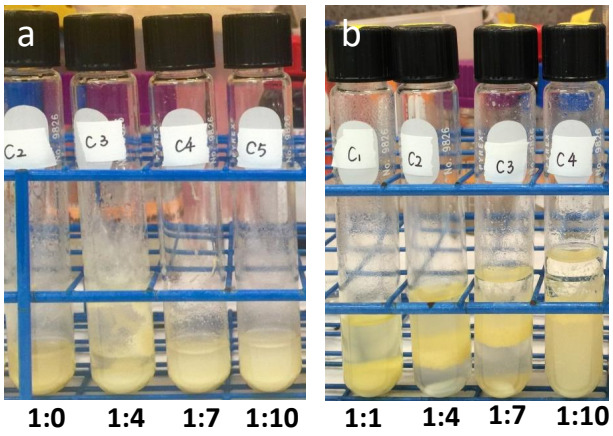

Figure 7.11: a) T18 slurry with different co-solvent of [C_{10}mim][BTMPP] labeled below. b) T18 slurry with different ratio of T18 and IL labeled below (T18: IL) at loading fresh T18 0.5g. Separation was induced with hexane.

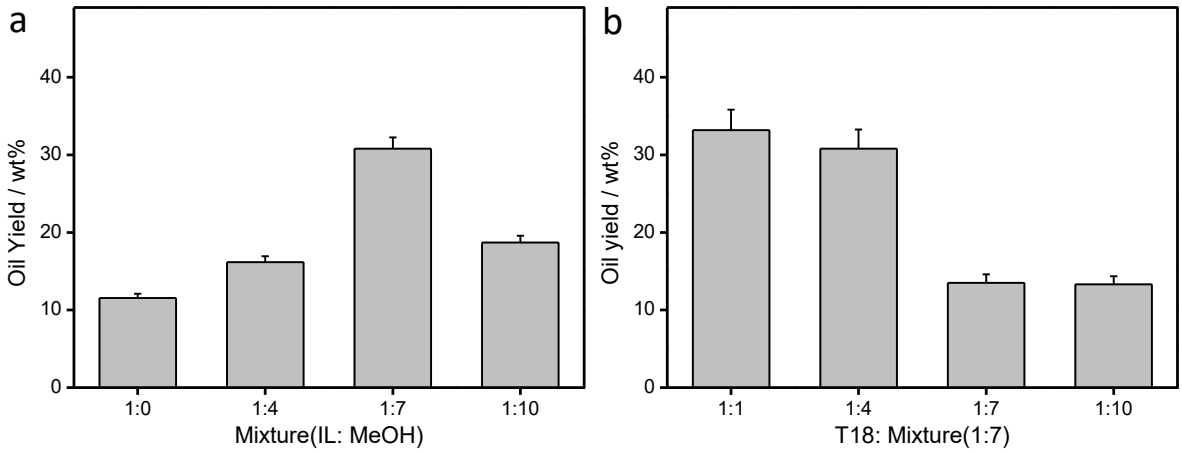

Figure 7.12: The oil yield of extraction with co-solvent. a) the ratio of IL to MeOH; b) the ratio of T18 to the mixture(IL and MeOH).

there was no methanol and no stirring in the process. The T18 fermentation broth and IL were added into tubes and incubated for 2 hours. After the addition of hexane, the solution separated into three layers as shown in Fig 7.13.

The results of Fig. 7.14 indicate that the pH of 4.97 is the best for T18 culture within the certain conditions (30.7% oil yield). Therefore we inferred the pH between 4 and 5 should be the right condition in this process. That result is likely connected to the pH sensitivity of [C_{10}mim][BTMPP]. At the pH of 4.94, the loading ratio was increased to 1:5 and 1:10, and

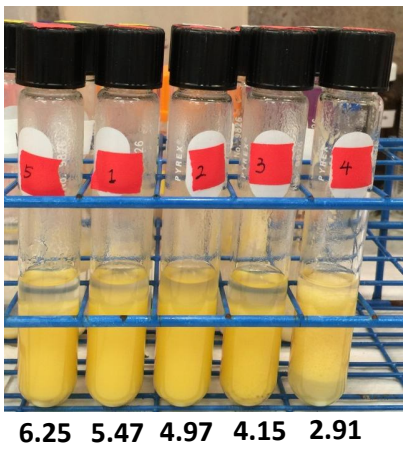

Figure 7.13: T18 slurry with different pH labeled below at loading ratio of 1:1(0.5g fresh T18: 0.5g IL). Separation was induced with hexane.

higher oil yields were found to be about 42 wt.% and 49 wt.%. The results further show that water separation via centrifugation is not a requirement for the technique to work.

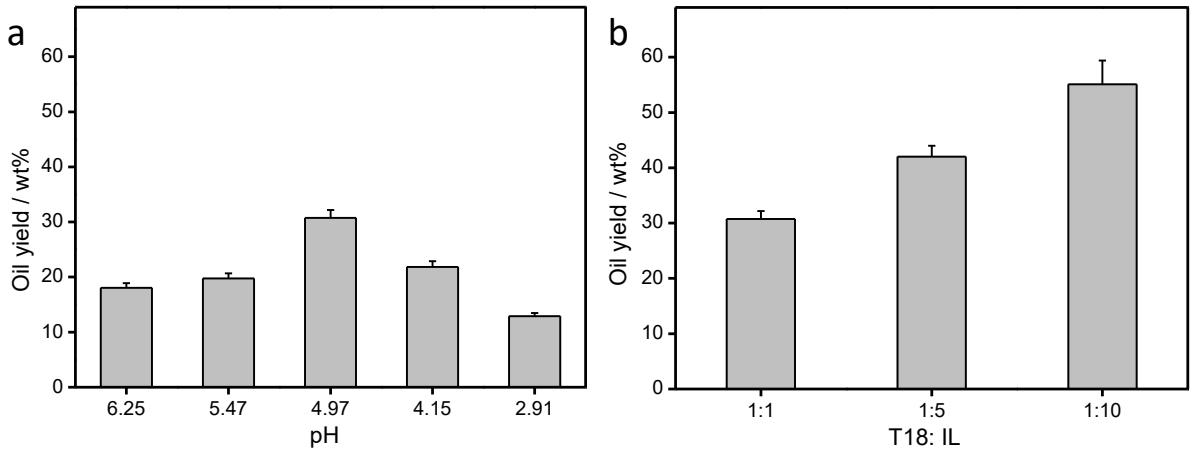

Figure 7.14: Effect of pH and mass ratio on extraction without methanol a) pH effect at the constant mass ratio of 1:1; b) mass ratio effect at the constant pH of 4.94.

7.6 Conclusions

In this work, a novel water-compatible approach for DHA-rich oil extraction without the use of co-solvent from the marine algae *Thraustochytrium sp.* was first achieved using a newly synthesized ionic liquid [C_{10}mim][BTMPP]. Response surface methodology was used to opti-

mize and study the effects of pretreatment conditions on oil yield (temperature, pH, and mass ratio). The optimized extraction conditions were determined by numerical optimization and validated experimentally. It was found that an oil yield of up to 69.4 ± 0.5 wt% under the optimal conditions (mass ratio of 4.2, pH of 5.0, and the temperature of 76 °C), and that the extracted oils were composed of 44.7 ± 0.2% DHA. The extraction efficiency was 97.4 ± 0.4% based on total lipid content of the T18 biomass.

[C_{10}mim][BTMPP] also has reasonable potential for oil extraction from fresh T18. In this part of work, 30.7 wt.% was obtained at 1:7 (IL: Methanol) and 1:1(T18: co-solvent). At last, the oil yield of around 49 wt% ((extraction efficiency is around 69 %)) was obtained from pH 4.97 at the mass ratio of 1:10. Overall, this ionic liquid is also a promising candidate for wet oil extractions from fresh T18, which has consistency with the results in other chapters.

8 Summary and Conclusions

8.1 Summary

The overall objective of this thesis was to develop a biorefinery process for the production of biodiesel and value-added oils from two various kinds of microalgae. This work mainly focuses on ionic liquids that help break the cell wall and assist in the extraction of microalgal oil. It examines the effects of treatment using different ionic liquids on two completely different types of microalgae. A composite treatment method combining electrolysis and the ionic liquid was first proposed, and the mechanism of synergy between electrolysis and the ionic liquid was also speculated. Response surface methodology was employed combining simulation and experiment to optimize the experimental conditions to design, analyze the experiments, and to optimize within the scope of the experiments. The effects of ionic liquids were summarized on the extracted oil yield and the extraction efficiency of different types of oils. The thesis was composed of three specific objectives (i) production of microalgal lipids from dry microalgae, (ii) wet extraction of crude oils from fresh *Chlorella vulgaris*, and (iii) development of value-added lipids based on the ionic liquid process.

First, a ionic liquid 1-decyl-3-methylimidazolium bis(2,4,4-trimethylpentyl) phosphonate ([C_{10}mim] [BTMPP]) was synthesized and characterized in order to be adopted to further application. The structure characterizations both showed great correspondence to the molecular structure of [C_{10}mim][BTMPP]. The thermal analysis indicated the ionic liquid possessed good thermal stability, which is the significant precondition for the extractions under heating. Other physicochemical properties of the ionic liquid provided much more basic information such as

the high viscosity at room temperature, the low solubility in most of solvents such as hexane, methanol, water, etc. Those results are critical guiding significance for further application of the ionic liquid. Then the ionic liquid and the other two ionic liquids were applied to assist in extracting oil from dry *C. vulgaris*. The extraction efficiency was found to increase with the length of cations of these ionic liquids as follows: $[C_2mim][EtSO_4]$ <$[C_6mim]Cl$ <$[C_{10}mim][BTMPP]$. The extraction efficiency by $[C_{10}mim]$ [BTMPP] reached around 74.6%. Meanwhile, the composition of extracted oil is confirmed by GC with the major content is C18:1, 40.9%. The results also suggest that this ionic liquid could help to extract the overall available lipids without oxidizing the unsaturated ones. The preliminary exploration proved that this synthesized ionic liquid presented a good effect on assisting lipid extraction from dry *C. vulgaris*.

The second objective of this work is to achieve the wet extraction of crude oils from fresh microalgae. The lipid in fresh *C. vulgaris* was found much more difficult to be disrupted and extracted. This may result from the fact that there was already a certain degree of rupture in the cell wall because of the freeze-drying procedure. Although $[C_{10}mim][BTMPP]$ achieved great extraction efficiency in lipid extraction from dry *C. vulgaris*, the extraction efficiency was turned out to decrease from around 75% to 24%. Several ionic liquids based lipid extraction were conducted on the fresh culture of *C. vulgaris*. Their capacities were found as follows: $[C_2mim][EtSO_4]$<¡$[C_6mim]Cl$<¡$[C_{10}mim][BTMPP]$<¡$[P_{4444}][Prop]$. Furthermore, the composite treatment method combining electrolysis and ionic liquid was first proposed. The experiments designed by a central composite design indicated that this response surface methodology could not find the optimized conditions. Although voltage and electrolysis time were found to have less effect for this ionic liquid, the synergy of electrolysis and the ionic liq-

uid was still found compared to the control sample and the samples under the pretreatment of only electrolysis or ionic liquid. This work advanced a novel and facile wet extraction method to obtain lipids, which has far-reaching significance for future research on the extraction of related microalgal molecules.

As for the final part of the work, the objective is to develop value-added lipids based on the ionic liquid extraction process. *Thraustochytrium sp.* was adopted mainly because of the high content of ω-3 fatty acids like docosahexaenoic acid (DHA). This work first employed ionic liquids to assist in extracting DHA-rich lipid fraction from dry and fresh *Thraustochytrium sp.* biomass(0-77.2 wt % water). $[C_2mim][EtSO_4]$ and $[P_{4444}][Prop]$ were found with over 90% of the available oils from dried T18 biomass. However, wet T18 slurry was found much more difficult to extract lipids. $[P_{4444}][Prop]$ achieved an extraction efficiency of approximately 80% that makes it a promising candidate for the water-compatible, low-energy, DHA-rich oil recovery method. Moreover, both ILs were readily recycled with no decrease in performance over four cycles. According to the excellent performance of $[C_{10}mim]$ [BTMPP] without co-solvent in previous work, it was also used for DHA-rich oil extraction from *Thraustochytrium sp.*. A central composite design and response surface methodology were adopted to design and study the effect of various extraction variables (pH, temperature, and the mass ratio of IL to dry T18). The model was proven significant and the optimal conditions were the mass ratio of 4.16, pH of 5.0, and the temperature of 76 °C, where the DHA-rich oil yield reached up to 69.36 ± 0.49% (w/w), and DHA constituted 44.65 ± 0.15% of resultant oil. Hence, this part of the thesis indicated that ionic liquid-based extraction technology worked not only on common microalgae for biodiesel production, but also on marine microalgae for value-added product extraction. Overall, this work opens a new approach for a time-saving, efficient and

water-compatible DHA-rich oil recovery.

8.2 Scientific Contributions

Several contributions of this work were brought to the scientific community.

Firstly, the synthesized ionic liquid [C_{10}mim] [BTMPP] was comprehensively characterized, which is not commercially available (chapter 3 and 4). Those characterizations not only supports the thesis but also provides the scientific community with the basic information of this novel ionic liquid. The exploration of several ionic liquids for lipid extraction from *C. vulgaris* showed that the extraction efficiency was found to increase with the length of cations of these ionic liquids in this work. The ionic liquid [C_{10}mim][BTMPP] was found to have a positive effect in lipid extraction from dry *C. vulgaris*.

Secondly, this part of the work made several significant contributions. The electrolysis was first applied with ionic liquid as a pretreatment method to assist lipid extraction from microalgae. With the exception of each one, the results first investigated the existence of the synergy effect of electrolysis and ionic liquid(chapter 5). Wet extraction efficiently worked in lipid extraction from fresh *C. vulgaris* using [P_{4444}][Prop]. The extraction capacity could vary greatly from dry to fresh biomass using the same ionic liquid. Finally, this work first employed the electrolysis with ionic liquid for wet extraction of lipids from fresh *C. vulgaris*.

Thirdly, a water-compatible, low-energy, PUFAs recovery method (chapter 6) was first developed for fresh *Thraustochytrium sp.* DHA-rich lipid was firstly extracted after the pretreatment of the two ionic liquids [C_2mim][$EtSO_4$] and [P_{4444}][Prop]. The extracted oil by the two ionic liquids had a large percentage of DHA, which was shown in FAME composition. The existence of unsaturated fatty acids indicated the ionic liquid-based extraction process had

no negtive impact on the structure of the lipids. Moreover, both ILs have been easily recycled without decrease in performance over four cycles. Finally, this work first adopted ionic liquids to extract DHA-rich lipid from marine microalgae.

Finally, a synthesized ionic liquid was applied to extract value-added oil (PUFAs) from marine microalgae. The preliminary experiments have shown pH is a key factor for the synthesized ionic liquid. Additionally, a central composite design and response surface methodology were adopted to study the effect of various variables of the experiments (temperature, pH, and mass ratio). Experiemtnal data well fitted these models. The predicted and actual extraction efficiency at optimal conditions were close, which means the design and simulation method also had a outstanding experimental design and an efficient analysis capability. This efficient combined analysis method can be applied to many extraction situations. Furthermore, this part of the work opens a new approach combined with response surface methodology for a time-saving efficient and water-compatible DHA-rich oil recovery.

8.3 Conclusions

In conclusion, it is found in the work that the synthesized ionic liquid [C_{10}mim] [BTMPP] has relatively high purity. It is an amorphous transparent liquid at the temperature of -70~150°C. The density of [C_{10}mim][BTMPP] is a little smaller than that of water at the same temperature. The viscosity of pure [C_{10}mim][BTMPP] is as high as 147 cP at room temperature and it has to be diluted before pretreating microalgae.

When extracting crude oil from dry *C. vulgaris*, the performance of pretreatment of ILs: [C_2mim][$EtSO_4$] <[C_6mim]Cl <[C_{10}mim][BTMPP]. The mixing time and processing time are not significant influencing factors on the oil yield compared to the mass ratio and temperature.

Additionally, higher mass ratio and temperature appears to result in a higher oil yield in the tested ranges.

Among the four ILs studied ([C_2mim][$EtSO_4$], [C_6mim]Cl, [C_{10}mim][BTMPP], and [P_{4444}] [Prop]), pretreating by [P_{4444}][Prop] seems to have a higher extraction efficiency. Both electrolysis temperature and time show a positive impact on the oil yield, but not as strong as the mass ratio. The extraction efficiency increases from 28% to 44% after introducing the electrolysis. The compositions of oils extracted via HIP and [P_{4444}][Prop] are fairly similar, indicating [P_{4444}][Prop] will not oxidize the non-saturated lipid fractions.

Both [C_2mim][$EtSO_4$] and [P_{4444}][Prop] have excellent extraction efficiency (~90%) when pretreating dry *Thraustochytrium sp.* with cosolvent MeOH. Additionally, T18 pretreated only by [P_{4444}] [Prop] without co-solvent obtains a relatively high extraction efficiency (~80%). Impurities carried over between cycles did not negatively affect the oil recovery which illustrates both ILs possess excellent recyclability.

The cosolvent was not neccesarry for DHA-rich oil extraction using [C_{10}mim][BTMPP] pretreatment from dry T18, and the extraction efficiency was as high as around 97% under the optimized conditions. When pretreating fresh T18, the extraction efficiency reached about 69% without any cosolvent. A validated RSM model, as a useful and accurate model, was set up to predict the oil extraction yield from microalgal biomass. The results comprehensively reveal that [C_{10}mim][BTMPP] is a significantly promising ionic liquid in both dry and wet extraction processes of the value-added product.

8.4 Future Work

The results presented in this work showed the potential of electrolysis combined with ionic liquid in lipid extraction from fresh microalgae. A synergistic effect was found for electrolysis and ionic liquid [P_{4444}][Prop]. However, the conditions were restricted by the DC power supply from 0V-35V. Hence a wider voltage range would need to be utilized. Other ionic liquids can be adopted to study lipid extraction efficiency. Additionally, other fresh microorganisms, such as other microalgae, yeast, and fungi with value-added biomolecules, could also be pretreated by this novel and facile approach. The mechanism would need to obtain further verification.

The results of the ionic-liquid-based lipid extraction process were conducted at the bench scale in this work. The explorations have material limitations, but in order to confirm the cost-effective advantages in practical production, the whole process would need to be scaled up. Furthermore, other pretreatment technology could be adopted with ionic liquids. The mixtures of ionic liquids with various selectivity could be employed to achieve the production of multiple target products. Other fresh and dry microorganisms could also be applied via this approach. Although ionic liquids could be reused for 4-5 cycles, the design of the ionic liquid-based process should be explored as it could reduce the amount of materials during the process.

Finally, the development of the ionic liquid-based process showed good selectivity on DHA-rich lipid extraction. Although this process appeared to work on the value-added bio-products, the resultants would need to be characterized by much more food safety tests, such as acid value (AV), peroxide value and p-anisidine value, iodine value (IV), etc., which is a prerequisite for the practical application of this technology to the industrial field. Microbial

cells can often produce more than one kind of value-added bioproducts, such as ω-3, carotene, astaxanthin, lutein, etc. Therefore, multiple complex extractions would need to be efficiently achieved by the ionic liquid-based extraction process.

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Appendix A

A.1 **Design expert for Chapter 4**

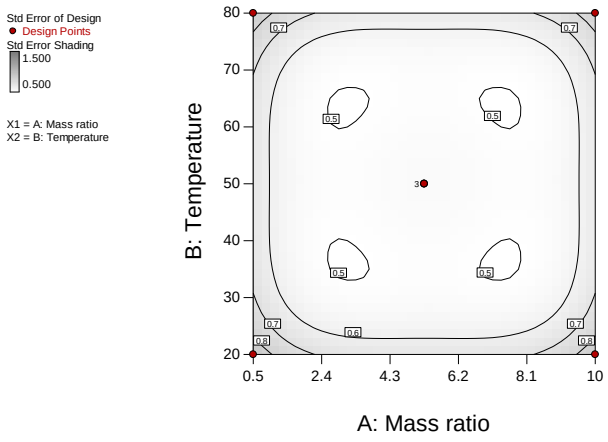


Figure A.1: Standard error of design.

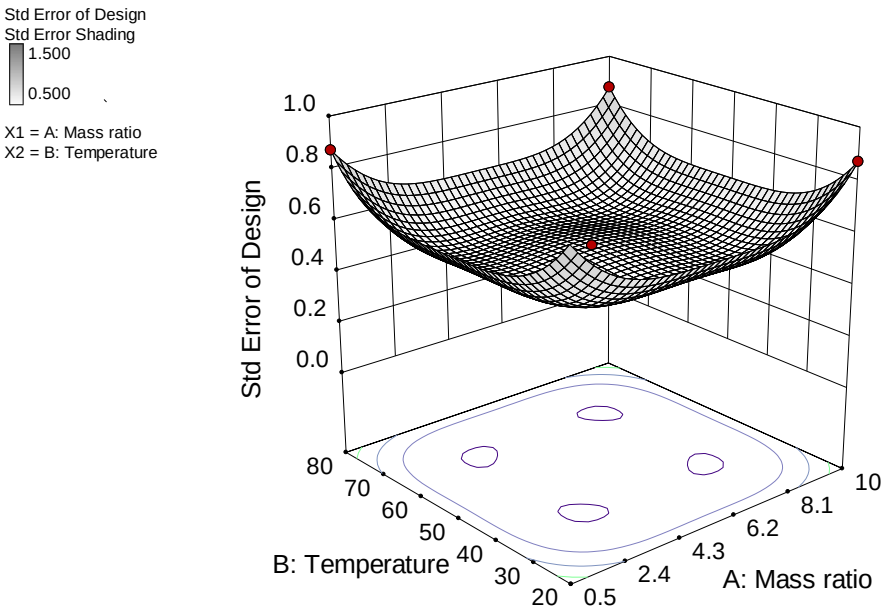


Figure A.2: 3D surface plot of standard error of design.

A.2 Design expert for Chapter 5

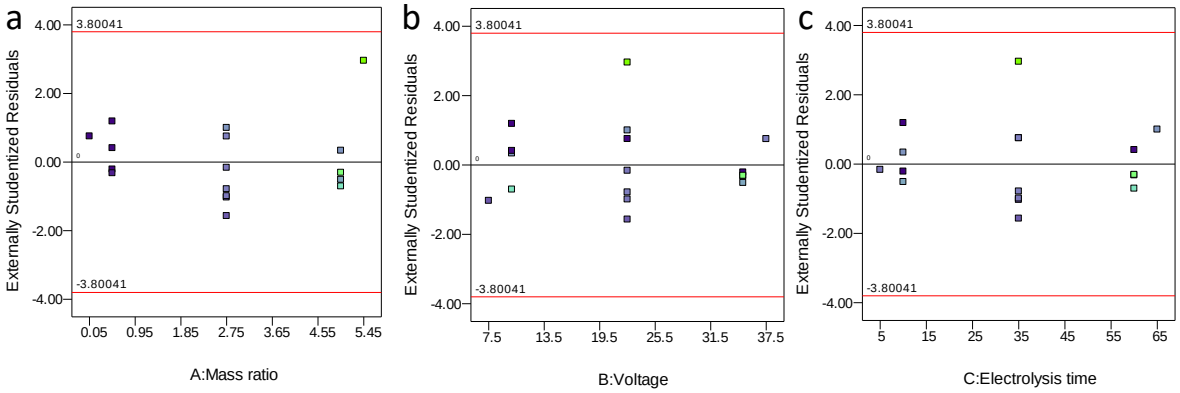


Figure A.3: Residuals of factors: a) Mass ratio; b) Temperature; c) Processing time.

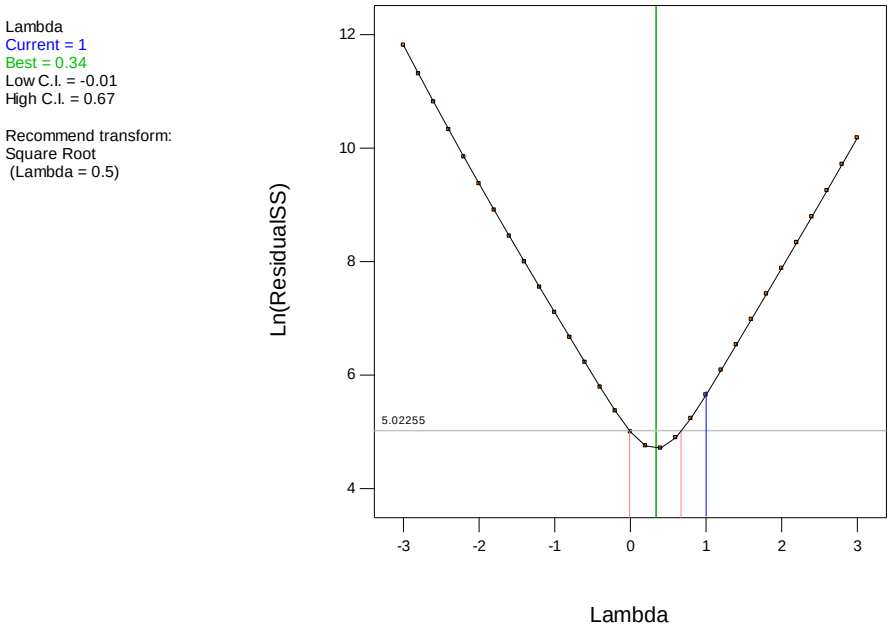


Figure A.4: Box-Cox plot for power transforms.

Table A.1: Some of the optimal solutions for the extracted conditions via $[P_{4444}][Prop]$.

Mass ratio	Voltage/V	Time/min	Oil yield/%	Mass ratio	Voltage	Time	Oil yield/%
5.00	35.00	60.00	39.25	4.71	35.00	60.00	37.29
5.00	34.91	60.00	39.21	5.00	35.00	49.25	37.25
5.00	35.00	59.68	39.19	5.00	35.00	46.27	36.70
5.00	35.00	59.37	39.14	5.00	35.00	45.31	36.52
4.98	35.00	60.00	39.10	4.94	35.00	47.40	36.46
5.00	34.56	60.00	39.06	5.00	28.19	60.00	36.19
5.00	35.00	58.84	39.04	5.00	35.00	41.58	35.82
5.00	34.38	60.00	38.97	5.00	35.00	40.75	35.67
4.95	35.00	60.00	38.94	5.00	26.89	60.00	35.61
5.00	35.00	57.87	38.86	5.00	35.00	39.86	35.50
5.00	34.02	60.00	38.81	5.00	26.19	60.00	35.29
4.93	35.00	60.00	38.75	5.00	35.00	37.64	35.09
5.00	35.00	57.15	38.72	5.00	25.45	60.00	34.96
4.92	35.00	60.00	38.71	5.00	24.94	60.00	34.73
5.00	33.52	60.00	38.59	5.00	35.00	34.16	34.44
5.00	33.31	60.00	38.49	5.00	25.23	52.86	34.09
5.00	35.00	55.66	38.44	5.00	22.39	56.44	33.28
5.00	32.96	60.00	38.34	5.00	22.55	51.11	32.89
5.00	33.62	58.23	38.32	5.00	19.10	60.00	32.10
5.00	34.99	54.48	38.22	5.00	21.65	45.45	32.10
4.84	35.00	60.00	38.19	5.00	27.82	27.91	31.90
5.00	34.18	56.06	38.17	5.00	22.94	34.88	31.58
5.00	32.43	60.00	38.10	5.00	17.04	60.00	31.18
5.00	32.31	60.00	38.05	5.00	25.21	23.42	30.91
5.00	35.00	53.37	38.02	5.00	35.00	15.15	30.90
5.00	35.00	52.84	37.92	5.00	33.69	14.04	30.59
5.00	35.00	52.16	37.79	5.00	15.23	60.00	30.36
5.00	34.49	51.86	37.54	5.00	22.18	14.84	29.72
5.00	35.00	50.40	37.47	5.00	17.86	20.84	29.64
5.00	30.99	60.00	37.45	5.00	19.56	11.60	29.29
5.00	14.16	10.03	28.93	5.00	13.99	14.96	29.01
5.00	13.88	10.00	28.92				

A.3 Design expert for Chapter 7

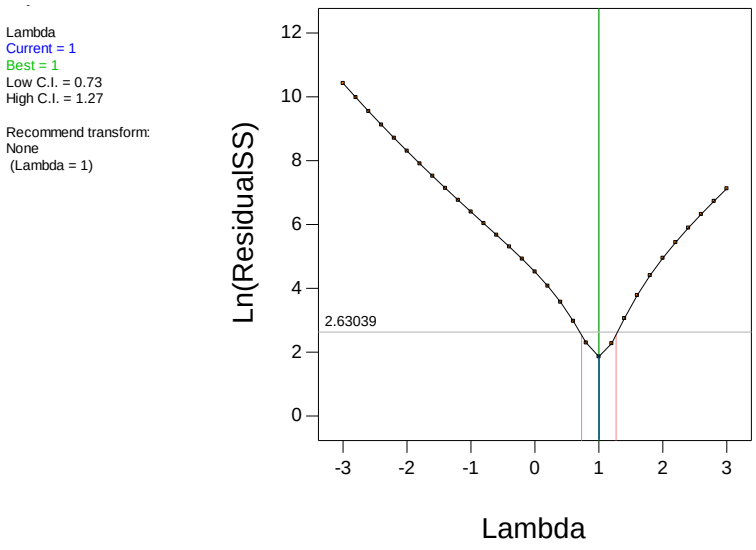
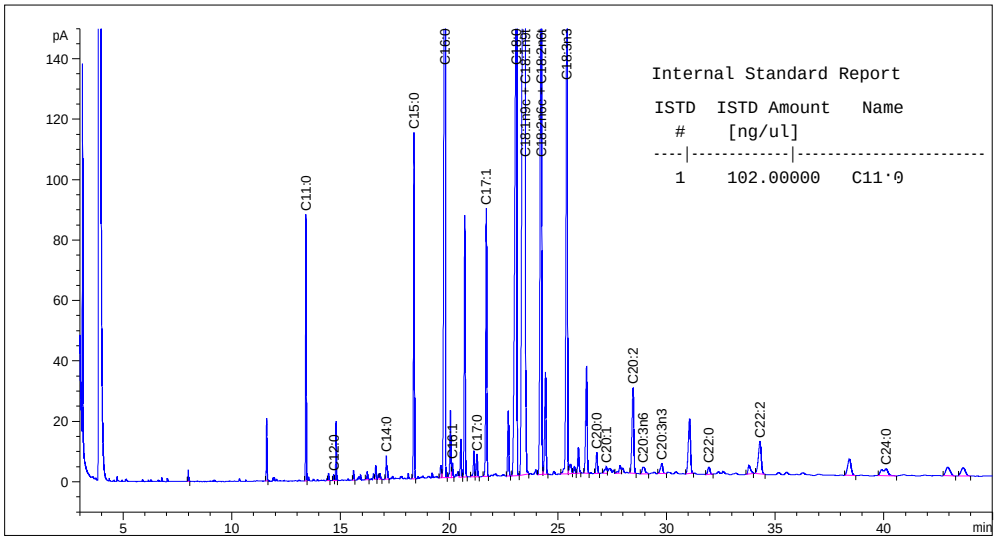


Figure A.5: Box-Cox plot for power transforms.

Table A.2: Part of the optimal solutions for the extracted conditions via [C_{10}mim][BTMPP].

Mass ratio	pH	Temp./°C	Oil yield/%	Mass ratio	pH	Temp./°C	Oil yield/%
4.66	5.02	34.07	54.86	3.39	5.82	49.86	58.29
3.44	5.56	49.15	61.25	3.37	5.32	27.70	56.88
3.60	3.83	57.41	53.51	3.85	4.09	49.23	57.58
2.03	5.22	47.02	52.08	4.85	4.24	51.57	55.98
4.44	4.67	63.82	66.26	4.08	5.49	78.43	68.82
3.03	6.21	66.88	52.46	3.20	3.90	46.05	54.07
3.09	4.77	69.40	61.85	4.84	4.34	36.47	50.89
2.92	3.86	37.90	52.30	3.44	3.88	64.76	53.58
4.15	4.47	57.80	63.47	4.34	4.97	41.94	61.15
3.32	3.79	26.92	50.03	2.65	6.03	71.30	51.38
3.67	5.17	49.96	64.41	4.09	3.85	72.69	53.43
5.00	6.50	80.00	58.60	3.43	4.78	40.84	62.21
2.75	4.75	52.50	60.03	3.23	6.19	59.16	53.52
3.56	5.19	43.85	62.78	4.47	5.76	69.99	66.62
4.89	5.11	67.27	68.91	4.41	5.17	66.97	68.99
3.25	4.68	39.60	61.32	2.60	5.83	71.37	53.39
4.81	4.08	43.86	50.70	4.18	4.93	71.24	68.74
2.19	5.16	38.32	53.67	3.18	5.47	51.05	61.33
4.74	4.94	51.29	62.90	3.01	5.29	78.03	60.72
3.58	5.11	73.93	66.25	4.34	4.91	26.26	53.65

Appendix B



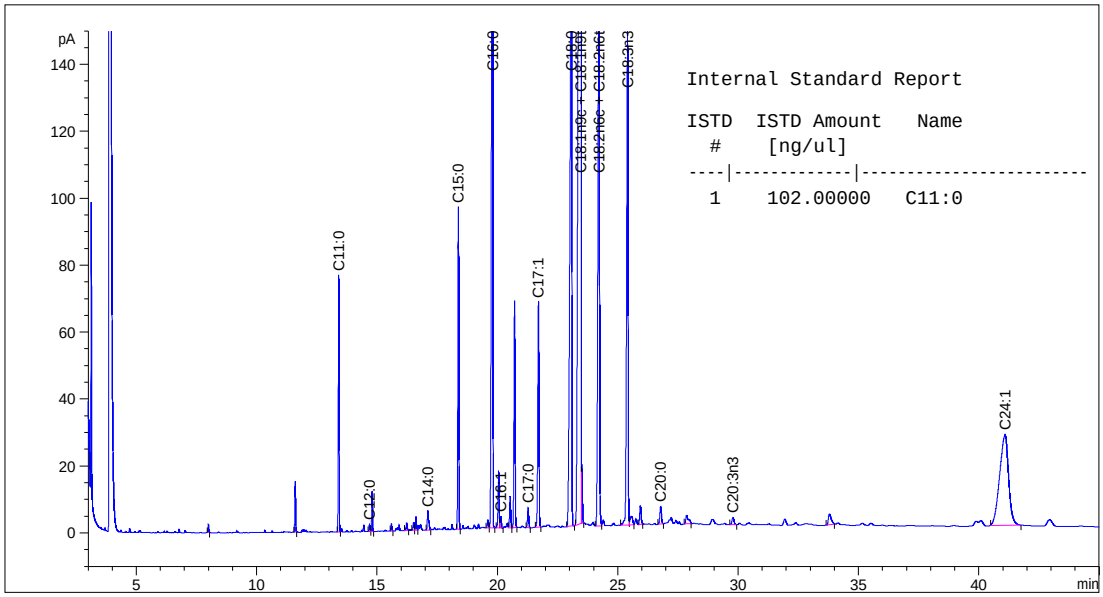
Signal 1: FID1 A, Front Signal

Name	Total Area [pA*s]	Amount [ng/ul]
C4:0	0.00000	0.0000
C6:0	0.00000	0.0000
C8:0	0.00000	0.0000
C10:0	0.00000	0.0000
C11:0	197.30515	102.0000
C12:0	5.02676	2.5497
C13:0	0.00000	0.0000
C14:0	28.43311	14.2308
C14:1	0.00000	0.0000
C15:0	320.73108	159.9710
C15:1	0.00000	0.0000
C16:0	1438.22473	713.8758
C16:1	14.94840	7.4186
C17:0	27.69346	16.0922
C17:1	308.00391	150.3155
C18:0	1347.94409	667.7618
C18:1n9c + C18:	3976.97778	1.961e3
C18:2n6c + C18:	1092.82520	566.9546
C18:3n6 + C19:0	0.00000	0.0000
C18:3n3	805.51355	406.5625
C20:0	30.63896	15.3126
C20:1	15.51028	7.6513
C20:2	162.05409	82.5864
C20:3n6	21.22026	11.9572
C21:0	0.00000	0.0000
C20:3n3	23.24754	12.8768
C20:4	0.00000	0.0000
C20:5	0.00000	0.0000
C22:0	16.38984	8.6952

Signal 1: FID1 A, Front Signal

Name	Total Area [pA*s]	Amount [ng/ul]
C22:1	0.00000	0.0000
C22:2	99.66631	51.2145
C23:0	0.00000	0.0000
C24:0	46.01724	42.1524
C24:1	0.00000	0.0000
C22:6	0.00000	0.0000
Totals :		5001.3356

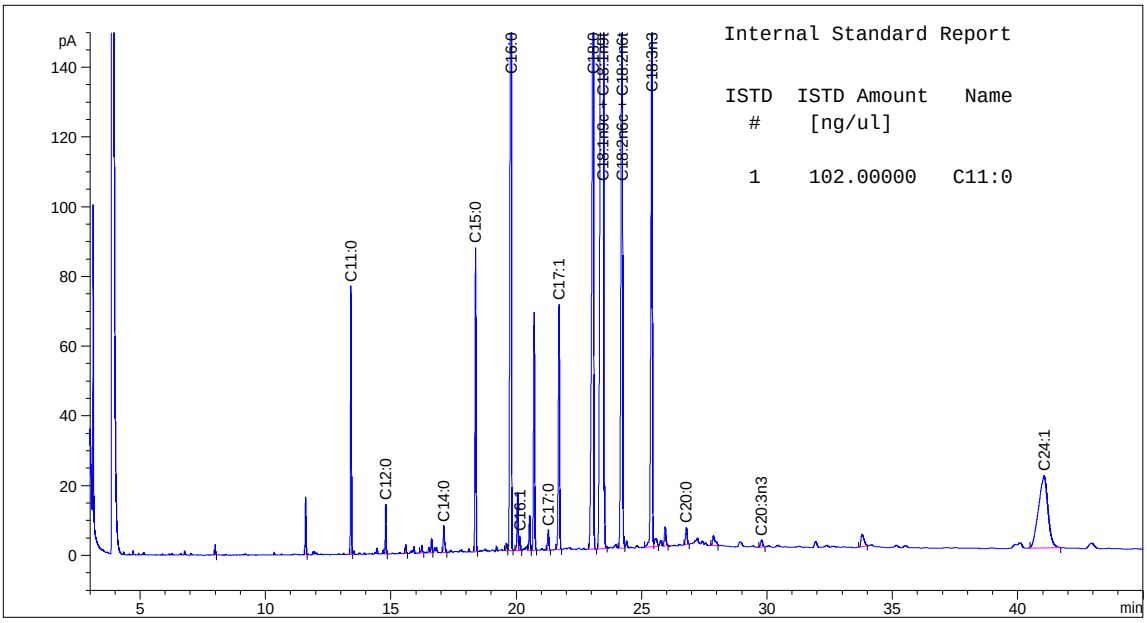
Figure B.1: The GC raw data of Fig. 4.8.



Name	Total Area [pA*s]	Amount [ng/ul]
C4:0	0.00000	0.0000
C6:0	0.00000	0.0000
C8:0	0.00000	0.0000
C10:0	0.00000	0.0000
C11:0	173.00822	102.0000
C12:0	5.89718	3.4113
C13:0	0.00000	0.0000
C14:0	21.60891	12.3342
C14:1	0.00000	0.0000
C15:0	266.52808	151.6055
C15:1	0.00000	0.0000
C16:0	1098.40698	621.7716
C16:1	10.70561	6.0591
C17:0	20.91499	13.8601
C17:1	235.47310	131.0571
C18:0	1013.45520	572.5664
C18:1n9c + C18:	3033.36523	1.706e3
C18:2n6c + C18:	823.52057	487.2408
C18:3n6 + C19:0	0.00000	0.0000
C18:3n3	617.88617	355.6597
C20:0	23.90468	13.6248
C20:1	0.00000	0.0000
C20:2	0.00000	0.0000
C20:3n6	0.00000	0.0000
C21:0	0.00000	0.0000
C20:3n3	12.49831	7.8950
C20:4	0.00000	0.0000
C20:5	0.00000	0.0000
C22:0	0.00000	0.0000
C22:1	0.00000	0.0000
C22:2	0.00000	0.0000

Name	Total Area [pA*s]	Amount [ng/ul]
C23:0	0.00000	0.0000
C24:0	0.00000	0.0000
C24:1	701.11469	438.8969
C22:6	0.00000	0.0000
Totals :		4623.8903

Figure B.2: The GC data of the sample extracted by HIP in Fig. 5.15.



Name	Total Area [pA*s]	Amount [ng/ul]
C4:0	0.00000	0.0000
C6:0	0.00000	0.0000
C8:0	0.00000	0.0000
C10:0	0.00000	0.0000
C11:0	173.36316	102.0000
C12:0	32.08478	18.5217
C13:0	0.00000	0.0000
C14:0	26.42402	15.0517
C14:1	0.00000	0.0000
C15:0	237.17084	134.6304
C15:1	0.00000	0.0000
C16:0	1088.86621	615.1089
C16:1	12.30013	6.9474
C17:0	20.81971	13.7687
C17:1	242.86392	134.8938
C18:0	948.56635	534.8093
C18:1n9c + C18:	3138.89844	1.762e3
C18:2n6c + C18:	853.69440	504.0593
C18:3n6 + C19:0	0.00000	0.0000
C18:3n3	637.22766	366.0418
C20:0	21.35499	12.1466
C20:1	0.00000	0.0000
C20:2	0.00000	0.0000
C20:3n6	0.00000	0.0000
C21:0	0.00000	0.0000
C20:3n3	11.47709	7.2351
C20:4	0.00000	0.0000
C20:5	0.00000	0.0000

Name	Total Area [pA*s]	Amount [ng/ul]
C22:0	0.00000	0.0000
C22:1	0.00000	0.0000
C22:2	0.00000	0.0000
C23:0	0.00000	0.0000
C24:0	0.00000	0.0000
C24:1	540.24084	337.4977
C22:6	0.00000	0.0000
Totals :		4564.3561

Figure B.3: The GC data of the sample extracted by [P_{4444}][Prop] in Fig. 5.15.

Table B.1: GC data (from Mara Inc.) of the oils extracted by HIP, [C_2mim][$EtSO_4$], and [P_{4444}][Prop] in Fig. 6.6.

	Concentration (ng/uL)		
	HIP	[C_2mim][$EtSO_4$]	[P_{4444}][Prop]
C10:0	0.50208	0.75200	0.34656
C11:1	0.00000	0.00000	0.00000
C12:0	8.27822	8.52599	6.36328
C12:1	0.00000	0.41241	0.00000
C14:0	103.84526	94.90521	76.20077
C14:1	0.18444	0.00000	0.00000
C15:0	7.28211	6.47443	6.20708
C16:0	296.57636	260.73769	185.91532
C16:1	28.13824	24.93345	22.47953
C17:0	1.75378	1.55956	0.00000
C18:0	8.84865	8.68675	36.12611
C18:1 Ole	5.83679	15.53661	0.00000
C18:1 Vac	36.17655	31.46993	29.89842
C18:2 n-6	1.80174	4.52781	0.00274
C18:3n-6(Gamma)	0.95896	0.81328	0.00081
C18:3n-3	0.60503	1.67996	0.00058
C18:4	2.04487	1.82478	0.00170
C20:0	0.67289	0.79700	0.00082
C20:1	0.00000	0.29990	0.00000
C20:3 n-6(HomoGamma)	0.59142	0.53759	0.00048
C20:4 n-6	1.44935	1.20787	0.00100
20:4n3	3.58190	3.04357	0.00246
C20:5 n-3(FPA)	8.55414	7.43938	8.37495
C22:0	0.33028	0.43657	0.00039
C22:2 n-6	0.00000	0.00000	0.00000
C22:4 n-6	0.22153	0.22718	0.00017
C22:5 n-6(DPA)	61.36843	52.04482	43.47307
22:5 n-3(DPA)	2.20796	1.63395	0.00125
C24:0	0.11994	0.00000	0.00000
C22:6 n-3(DHA)	375.42343	323.22191	259.95030

Table B.2: GC data (from Mara Inc.) of the oils extracted by [C_{10}mim][BTMPP] at one optaml and a facile coronations in Fig. 7.9.

	Concentration (ng/uL)	
	Optimal	Room temperature
C8:0	17.43877	19.54811
C10:0	319.3634	321.4711
C11:0	73.02146	71.0896
C12:0	7250.62	7253.25
C12:1	10.75477	12.49872
C13:0	535.3265	535.0746
C14:0	88508.7	88327.58
C14:1	643.5008	642.7413
C15:0	5255.816	5263.726
C16:0	181501.4	180915.5
C16:1	42237.92	42119.65
C17:0	1220.048	1217.649
C18:0	5816.986	5833.15
C18:1 Ole	1012.832	1041.699
C18:1 Vac	57256.35	57060.84
C18:2 n-6	441.5973	426.7538
C18:3n-6(Gamma)	1092.44	1091.716
C18:3n-3	110.4781	108.1358
C18:4	2186.301	2176.786
C20:0	280.6628	274.3362
C20:2 n-6	87.38223	91.23605
C20:3 n-6(HomoGamma)	562.3096	559.8851
C21:0	64.2333	66.12116
C20:4 n-6	1990.751	1972.613
C20:3n-3	59.91051	55.43094
20:4n3	3619.995	3607.343
C20:5 n-3(EPA)	12363.96	12316.73
C22:0	91.83607	97.96172
C22:1 n-9	45.9113	32.94884
C22:2 n-6	155.9581	176.5421
C23:0(Int Std)	37766.85	37528.48
C22:4 n-6	275.6225	295.7841
C22:5 n-6(DPA)	65381.42	65257.48
22:5 n-3(DPA)	2376.199	2376.179
C24:0	115.2737	107.0628
C22:6 n-3(DHA)	389455.7	388684.1
C24:1 n-9	0	0

Curriculum Vitae

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Publications:

Zhang Y, Ward V, Dennis D, Plechkova NV, Armenta R, Rehmann L. Efficient extraction of a docosahexaenoic acid (DHA)-rich lipid fraction from *Thraustochytrium sp.* using ionic liquids. Materials. 2018 Oct;11(10):1986.

Zhang Y, Rehmann L. A review on extraction of high-value compounds from marine biomass via ionic liquid-based techniques, Elsevier Book :Innovative and Emerging Technologies in the Bio-marine Food Sector, Chapter 19: Application of ionic liquids to extract high-value compounds from marine biomass. (in press)

Zhang Y, Ward V, Dennis D, Plechkova NV, Armenta R, Rehmann L. Extraction of a docosahexaenoic acid (DHA) rich oil from *Thraustochytrium sp.* using a novel hydrophobic ionic liquid (in submission)