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جامعة الإمارات العربيـة المتحدة
United Arab Emirates University

United Arab Emirates University

College of Engineering

Department of Chemical and Petroleum Engineering

USING SWITCHABLE SOLVENTS FOR SIMULTANEOUS MICROALGAE LIPIDS' EXTRACTION AND BIODIESEL PRODUCTION

Mariam Sultan Saeed AlAmeri

This thesis is submitted in partial fulfillment of the requirements for the degree of Master of Science in Chemical Engineering

Under the Supervision of Professor Sulaiman Al-Zuhair

June 2018

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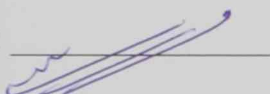
Declaration of Original Work

I, Mariam Sultan Saeed AlAmeri, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled "*Using Switchable Solvents for Simultaneous Microalgae Lipids' Extraction and Biodiesel Production*", hereby, solemnly declare that this thesis is my own original research work that has been done and prepared by me under the supervision of Professor Sulaiman Al-Zuhair, in the College of Engineering at UAEU. This work has not previously been presented or published, or formed the basis for the award of any academic degree, diploma or a similar title at this or any other university. Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my thesis have been properly cited and acknowledged in accordance with appropriate academic conventions. I further declare that there is no potential conflict of interest with respect to the research, data collection, authorship, presentation and/or publication of this thesis.

Student's Signature:  Date: 28/6/2018

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Abstract

Biodiesel produced from microalgae biomass has been pursued as a possible replacement to petroleum diesel. Among the main steps in microalgae biodiesel production are the drying and cell walls disruption, which are energy intensive and/or time consuming, and oil extraction, which is conventionally done using toxic organic solvents that contaminate the left over biomass and require additional solvent recovery. Therefore, these steps are considered the major obstacles facing the commercialization of microalgae biodiesel.

In this work, switchable solvents (SSs), which can reversibly alter their hydrophobicities, have been tested for oil extraction and biodiesel production. Three switchable solvents, namely N,N-dimethylcyclohexylamine (DMCHA), n-ethylbutylamine (EBA), and dipropylamine, were used to extract oil from wet microalgae, while avoiding the drying step. Their effectiveness was compared to that of conventional organic solvent, *n*-hexane, and hydrophobic ionic liquid (IL), 1-Butyl-3-methylimidazolium hexafluorophosphate [Bmim][PF_6]. The optimum extraction protocol was determined for the switchable solvent that showed the highest performance. The switchable solvent was also used for simultaneous extraction-reaction process, in which oils are extracted from wet microalgae and enzymatically converted to biodiesel using the same solvent in the same reaction cell. The successful use of a single solvent for extraction-reaction from wet biomass has never been reported in literature, which has a significant effect on the simplification of biodiesel production from microalgae.

A parametric study was performed using the response surface methodology (RSM) to evaluate the effects of temperature (in the range of 15-55°C) and solvent program, consisted of cell disruption and extraction periods (in the range of 0-3 hrs) on the oil extraction yield. The results were used to develop a statistical model to predict the oil yield under different conditions and to optimize the process. In addition, effects of the solvent program and methanol to oil ratio on the simultaneous extraction-reaction process were also tested with and without the use of immobilized enzyme. At the same extraction conditions, the extracted oil yields from wet biomass were 12.35±3.18%, 6.95±1.34% and 13.30±0.42% using EBA and dipropylamine with 1:1 v/v water and DMCHA, respectively. Using *n*-hexane, and [Bmim][PF_6], resulted in insignificant yields of 0% and 0.70±0.28%, respectively. The SSs were also shown to be effective in simultaneous oil extraction and biodiesel production, and superior to [Bmim][PF_6]. By the addition of Novozyme®435 enzyme, with DMCHA, the fatty acid methyl esters (FAMEs) yield increased by 33% from 19% when no enzyme was used to 25%. .

Keywords: Switchable solvents, Polarity-switching, Cell disruption, Effective extraction, Biodiesel, Simultaneous Extraction-Reaction.

Title and Abstract (in Arabic)

استخدام المذيبات القابلة للتحويل بالتزامن في استخلاص دهون الطحالب وإنتاج وقود الديزل الحيوي

الملخص

إنتاج وقود الديزل الحيوي من الكتلة الحيوية الطحلبية عُقِب كبديل محتمل للديزل النفطي . من الطرق الرئيسية لإنتاج وقود الديزل الحيوي من الطحالب، التجفيف وتعطيل جدران الخلايا اللاتي تستنزفان الطاقة والوقت، واستخلاص الدهن، الذي يتم تقليدياً باستخدام المذيبات العضوية السامة التي تلوث الكتلة الحيوية وتحتاج وحدة إضافية لاسترداد المذيب. لذلك، تشكل هذه الخطوات العقبات الرئيسية التي تواجه تسويق وقود الديزل الحيوي من الطحالب الدقيقة.

في هذا العمل، تم اختبار المذيبات القابلة للتحويل، اللاتي تغير قطبيتها في استخلاص الدهون وإنتاج الديزل الحيوي. ثلاثة مذيبات قابلة للتحويل أي، EBA, DMCHA و dipropylamine استخدمت لاستخلاص الدهن من الطحالب الرطبة مع تجنب خطوة التجفيف. فعالية المذيبات القابلة للتحويل في استخلاص الدهن قورنت مع مذيب عضوي تقليدي *n*-hexane وسائل أيوني نافر للماء $[Bmim][PF_6]$. تم تحديد بروتوكول الاستخلاص الأمثل للمذيب القابل للتحويل الذي أظهر أعلي أداء. المذيب القابل للتحويل استخدم أيضا بالتزامن في استخلاص الدهن وانتاج الديزل الحيوي، حيث الدهون تم استخلاصها من الطحالب الرطبة وحولت إنزيميا لوقود ديزل حيوي باستخدام المذيب نفسه في نفس خلية التفاعل. لم يتم تقرير الاستخدام الناجح لسائل واحد في استخلاص الدهون بالتزامن مع انتاج الديزل الحيوي من طحالب رطبة في الأدبيات حيث ان لها أثر كبير في تبسيط إنتاج الديزل الحيوي من الطحالب الدقيقة.

تم إجراء دراسة بارامترية باستخدام منهجية سطح الاستجابة لتقييم آثار الحرارة (في نطاق ١٥-٥٥ درجة مئوية) وبرنامج المذيب المتكون من فترات تعطيل جدران الخلية واستخلاص الدهن (في نطاق ٠-٣ ساعات) علي عائد استخلاص الدهن. هذه النتائج تم استخدامها لتطوير نموذج إحصائي للتنبؤ عن إنتاجية الدهن تحت ظروف مختلفة ولتحسين عملية الاستخلاص. بالإضافة إلي ذلك، تم أيضاً اختبار تأثيرات برنامج المذيب والنسبة المولية للميثانول إلي الدهن على عملية الاستخراج المتزامن مع التفاعل مع وبدون استخدام الإنزيم المثبت. في نفس ظروف الاستخلاص، كانت عوائد الدهن المستخرجة من الكتلة الحيوية الرطبة ١٢.٣٥±٣.١٨٪، ٦.٩٥±١.٣٤٪ و ١٣.٣٠±٠.٤٢٪ باستخدام EBA و dipropylamine مع ١:١ نسبة حجمية من الماء و DMCHA على التوالي. أدى استخدام *n*-hexane و [Bmim][PF_6] إلى إنتاجية ضئيلة من ٠٪ و ٠.٧+٠.٢٨٪، على التوالي. كما تبين أن المذيبات القابلة للتحويل فعالة في استخلاص الدهن المتزامن مع إنتاج الديزل الحيوي، ومتفوقة على [Bmim][PF_6]. من خلال إضافة الإنزيم Novozyme®435 مع DMCHA، ارتفعت انتاجية وقود الديزل الحيوي بنسبة ٣٣٪ من ١٩٪ عند عدم استخدام أي إنزيم إلى ٢٥٪.

مفاهيم البحث الرئيسية: المذيبات القابلة للتحويل، تبديل القطبية، تحطيم الخلايا، الاستخلاص الفعال، وقود الديزل الحيوي، الاستخلاص التفاعل المتزامن.

Acknowledgements

My deep sense of gratitude to Allah for making the journey smoother than I expected. My sincere thanks and appreciation to my husband and family for their constant prayers, support and encouragement.

With a grateful heart, the completion of this work could not have been possible without the expertise of Prof. Sulaiman Al-Zuhair, my thesis advisor.

Much of my experimental work would not have been completed without the help of Eng. Sami Abdulla and Eng. Anvar. My thanks extended also to the Library Research Desk for providing me the access to many reference materials.

Dedication

" I don't know what your destiny will be, but one thing I know; the only ones among you who will be really happy are those who have sought and have found how to serve."

- Albert Schweitzer

I dedicate my thesis work to my beloved husband, parents and siblings who have supported me throughout the entire process.

Last but not the least, I dedicate my humble effort to my thesis advisor Prof. Sulaiman Al-Zuhair who was always a great source of knowledge and experience.

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List of Abbreviations

[Bmim][PF_6]	1-Butyl-3-methylimidazolium hexafluorophosphate
[Bmim][BF_4]	1-Butyl-3-methylimidazolium tetrafluoroborate
[Bmim][TfO]	1-Butyl-3-methylimidazolium triflate
[Bmim][methide]	1-Butyl-3-methylimidazolium methide
[Bmim][DCA]	1-Butyl-3-methylimidazolium dicyanamide
[Bmim][NO_3]	1-Butyl-3-methylimidazolium nitrate
[Bmim][Tf_2N]	1-Butyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide
BBM	Bold’s Basal Medium
CO_2	Carbon dioxide
CH_3ONa	Sodium methoxide
C14:0	Myristic acid
C16:0	Palmitic acid
C18:0	Stearic acid
C18:1	Oleic acid
C18:2	Linoleic acid
C20:0	Arachidonic acid
$CaCl_2 \cdot 2H_2O$	Calcium chloride
DMCHA	N,N-dimethylcyclohexylamine
DBU	1,8-diazabicyclo-[5.4.0]-undec-7-ene
EBA	N-ethylbutylamine
[$EtNH_3$][NO_3]	Ethylammonium nitrate
[Emim][DEP]	1-Ethyl-3-methylimidazolium diethyl phosphate

FAME	Fatty acid methyl ester
FAAEs	Fatty acid alkyl esters
FFAs	Free fatty acids
FID	Flame ionization detector
GHG	Greenhouse gases
GC	Gas chromatography
[Hmmim][Tf_2N]	2,3-Dimethyl-1-hexylimidazolium bis(trifluoromethylsulfonyl)imide
ILs	Ionic liquids
IEA	International Energy Agency
KOH	Potassium hydroxide
KH_2PO	Potassium di-hydrogen orthophosphate
K_2HPO	Di-potassium hydrogen orthophosphate
M:O	Methanol:oil molar ratio
$MgSO_4 \cdot 7H_2O$	Magnesium sulphate
N_2	Nitrogen
NaOH	Sodium hydroxide
$NaNO_3$	Sodium nitrate
NaCl	Sodium chloride
PBR	Photobioreactor
RSM	Response surface methodology
R/P	Reserves/production
R=C_6H_{17}[Hmim][Tf_2N]	1-Hexyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide
R=C_8H_{17}[Omim][Tf_2N]	1-Octyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide
SSs	Switchable solvents

SC-CO_2	Supercritical carbon dioxide
SCFs	Supercritical fluids
SC-H_2O	Supercritical water
SPS	Switchable polarity solvents
SHS	Switchable hydrophobicity solvents
SW	Switchable water
SSSs	Switchable solvent systems

Chapter 1: Introduction

1.1 Overview

The rapid growing demand for energy has been outstripping the reserves of fossil fuels. According to statistics published by British Petroleum, the reserves/production (R/P) ratio of the world's fossil oil is around 54 years (Dudley, 2012). This have rekindled a strong interest in pursuing alternative and renewable energy sources, especially biodiesel which has been pursued as a possible replacement to petroleum diesel (Lee et al., 2009). Of the available biodiesel feedstocks, microalgae seems to be the only source that can be sustainably developed in the future. The other possible feedstocks are either inefficient for the large-scale production, inconsistent or expensive (Ahmad et al., 2011).

Several process steps are needed to produce lipids from microalgae for biodiesel. These steps include cultivation of microalgae, cell harvesting, dewatering and disruption, and lipid extraction, which are then followed by the transesterification of extracted lipids to produce biodiesel. For efficiently and economically viable conversion of microalgae into biodiesel, efforts are still needed to enhance the extraction and transesterification processes.

Several methods are available for extracting lipids from microalgal biomass, such as Soxhlet extraction using hexane as a solvent and Bligh and Dyer's method using a mixture of chloroform and methanol (Bligh and Dyer, 1959). However, due to the regulatory health and security problems associated with the use of organic solvents and the energy requirements associated with the regeneration operation of the solvent, efficient solvent recovery processes are needed to commercialize this

process. Among the possible alternatives, supercritical carbon dioxide (SC- CO_2) has shown promising results (Kumar et al., 2017). However, the high cost associated with the high pressure needed to bring the solvent to its supercritical conditions makes the process costly (Akoh and Min, 2008).

The extracted oils are converted to biodiesel by reaction with a short chain alcohol in the presence of a catalyst. Efforts have been recently directed towards replacing the alkaline catalysts with more environmentally-benign biocatalysts; such as lipases (Amini et al., 2017a). When reactions are catalyzed by lipases, the production occurs under mild conditions, coupled with easier product separation, and lower energy consumption and waste generation (Marchetti et al., 2007). However, due to the high cost of enzymes, using them in immobilized form is necessary to allow their easy separation and repeated use. The loss of activity caused by the short-chain alcohols used in the reaction and the deposition of the by-product glycerol remain the main obstacles. To avoid the loss in the enzyme activity, organic solvents are conventionally used to dissolve the inhibitors, and enhance the activity and stability of the immobilized lipase. However, as mentioned earlier, these organic solvents are toxic, volatile and require addition separation units for their recovery. In recent years, ILs have also attracted significant attention for their use as green replacements for the organic solvents as a reaction medium (Young et al., 2010). ILs are organic salts that melt below 100°C, which are also referred to as “designer solvents” because of their synthetic flexibility (Freemantle, 1998). The low volatility of ILs further simplifies their separation from the reaction medium for repeated use. Above that, hydrophobic ILs can stabilize enzymes and enhance their activity. Their successful use in lipase-catalysed reactions has been proven (Taher et al., 2017).

Despite the attractive features of ILs as extraction solvents and reaction media, their high cost remains the main barrier for their commercial use (Taher and Al-Zuhair, 2016). In addition, hydrophobic ILs, which are the ones needed for oil extraction and as transesterification media are incapable of disrupting the rigid walls of microalgae and require complete drying for efficient oil extraction. Therefore, the employment of a single IL in a multi-step processes, extraction-reaction-product separation, is not practical due to the need of different solvents of different hydrophobicities in each step. Above that, these separate solvents need to be completely recovered before the next step is carried out.

Recently, SSs have been proposed as an alternative to ILs. SSs are liquids that can be converted from a hydrophobic form to a hydrophilic form by bubbling carbon dioxide (CO_2) and can be converted back to their initial state by bubbling nitrogen (N_2). These solvents have been successfully used in the extraction of oils from soybeans (Phan et al., 2009) and microalgae cells (Du et al., 2013). However, they have not been tested in a simultaneous use as media for reactions and extraction.

1.2 Statement of the Problem

The biodiesel production from microalgae goes through several steps, namely cultivation, cell harvesting, lipid extraction and transesterification of extracted oils. Oils extraction from microalgae cells is generally done by solvent extraction using organic solvents. However, their high toxicity and volatility, render them environment and health hazardous, and therefore other greener solvents have been tested. Prior to any extraction technique, the microalgae rigid cells' walls need to be disrupted to open the structure, and the cells need to be thoroughly dried to allow the

solvent to reach the oils and dissolve them. These steps are energy intensive and/or time consuming.

In this thesis, SSs have been used to produce biodiesel from wet harvested microalgae in simultaneous cell disruption, oil extraction and transesterification in a single step, while eliminating the need for drying step. The SSs which are non-volatile liquids that can reversibly alter their hydrophilicities from one form to the other for easier product separation by phase switch, eliminating the need for distillation or evaporation units for the solvent recovery. The use of one solvent for simultaneous extraction-reaction of oils extracted from wet microalgae has never been reported in the literature before. The results reported in this thesis would have a significant effect on the simplification of biodiesel production from microalgae.

1.3 Relevant Literature

1.3.1 Introduction

Fossil fuels are the dominant energy sources, which meet greater than 80% of the world's energy demand (Olejarnik, 2013). However, fossil fuels are nonrenewable sources of energy and the rapid growing demand for energy outstrips their limited reserves. It has been reported that at the current consumption rates, the remaining supply of petroleum, natural gas and coal will only last for another 45, 60 and 120 years, respectively (Guo et al., 2015). It has been expected that the transportation sector alone will account for 63% of total global liquid fuel consumption from 2010 to 2040 (Sieminski, 2014). The International Energy Agency (IEA) reported that with the current dependence on fossil fuels, the emission of greenhouse gases (GHG) from the transport sector will increase by 92% between

1990 and 2020 and also estimated that 8.6 billion metric tons of CO_2 will be released to the atmosphere from 2020 to 2035 (Mahmudul et al., 2017).

The threats posed by the increasing scarcity of fossil fuels drove the scientists to look over other sustainable energy sources such as nuclear, solar, wind and biomass. The drawbacks associated with the use of nuclear power are its costs, environmental risks of radioactive waste and above all the strong public opposition, which peaked after the Chernobyl accident in 1986 (Li et al., 2009). Although the use of solar energy has grown rapidly in the past few years, the current global nature of solar power output is equivalent to only one coal or gas-fired thermal power plant (Cicia et al., 2012). Wind power is less efficient, has seasonal characteristics and the lands to be occupied by a wind plant are its main drawbacks, hindering its widespread use.

Biofuels on the other hand, have been pursued as a promising sustainable energy sources as a result of their advantages such as renewability, cleanliness and economic efficiency. Biofuels are renewable energy sources produced from biomass (waste or natural plant materials), which can be used as replacement for petroleum fuels. A variety of fuels can be produced from biomass resources including liquid fuels, such as ethanol, methanol, biodiesel, Fischer-Tropsch diesel, and gaseous fuels, such as hydrogen and methane (Demirbas, 2008a). It has been reported that the global biofuel production has increased rapidly from 10,000 thousand tons of oil in 2001 to the equivalent of 58,500 thousand tons of oil in 2010, an increase of approximately by 500% (Dudley, 2012). IEA predicts that using biomass feedstock in all transportation fuel will increase from 2% in 2012 to up to 20% globally by 2040 (Azadi et al., 2017).

The energy security and climate change are the two major driving forces that have promoted researchers to look over the biodiesel development as a replacement to the conventional petroleum diesel. Biodiesel is a mixture of fatty acid alkyl esters that can be used as an environmentally friendly alternative liquid fuel in any diesel engine without modifications (Demirbas, 2008b). In addition to its renewability, biodiesel is better than problem diesel in terms of lower sulfur content, higher flash point, aromatic content and biodegradability. Furthermore, it improves lubricity, while enhancing the performance and life of the engine (Taher and Al-Zuhair, 2016).

1.3.2 Different feedstocks of biodiesel

Biodiesel has been produced from a variety of sources including vegetable oils, cooking oils, animal fats, and cellular biomass sources. The triglyceride feed is a water-insoluble hydrophobic substance made of triglyceride molecules in which esters (three fatty acid groups) are attached to one glycerol molecule (Demirbas, 2008b).

1.3.2.1 Conventional feedstock for biodiesel

Global vegetable oil production increased from 56 million tons in 1990 to 88 million tons in 2000, following a below-normal increase (Demirbas, 2005). It has been estimated that 77% of bioethanol production was from maize and sugarcane, while 81% of biodiesel production was obtained from vegetable oils between 2013 to 2015 (Correa et al., 2017).

Many virgin vegetable oils have been used like rapeseed, soybean, corn, palm and sunflower, cottonseed and peanut oils to produce biodiesel (Demirbas, 2008a). Nut oils such as almond, and other oils such as argan, castor have also been used

(Hassan et al., 2013). Leading the gains in vegetable oils' production is the palm oil, which can be held responsible for an increase in the world's global production of 2.2 million tons from 1997 to 1999 (Demirbas, 2005). Table 1 shows the oil content and biodiesel production yield from selected crops.

Table 1: Various vegetable oils used for production of biodiesel

Vegetable oil used	Oil content (%)	Production yield (kg $ha^{-1}yr^{-1}$)	References
Peanut	36-56	890	(Röbbelen et al., 1990; Song et al., 2009; Sanders et al., 2002)
Soybean	21	375	(Röbbelen et al., 1990; Song et al., 2009; Gunstone, 2006)
Palm	40	4000	(Röbbelen et al., 1990; Williams, 2005)
Sunflower	44-51	655	(Röbbelen et al., 1990; Song et al., 2009; Gupta, 2002)
Rapeseed	35	1000	(Röbbelen et al., 1990; Song et al., 2009; Williams, 2005)

As shown in Table 1, the highest production yield of biodiesel from a vegetable oil source has been reported to palm oil (Fukuda et al., 2001, Al-Zuhair, 2007). In Malaysia and Indonesia, palm oil is used significantly as a biodiesel source. In Europe, rapeseeds are commonly used in biodiesel production. Soybeans are becoming the primary sources for biodiesel production used in US (Demirbas, 2008a).

1.3.2.2 Microalgal feedstock in biodiesel production

Eventhough vegetable oils are considered renewable and potential sources of energy with energy content close to that of a diesel fuel, food versus fuel is still considered a dilemma. The risk of diverting farmland or crops for liquid biofuels'

production on a global scale has made many people believe that it will be competing with human needs and may cause a starvation in developing countries. In addition, by using straight vegetable oils is economically not feasible to produce biodiesel. It has been reported that 60-90% of biodiesel production cost arises from the cost of the vegetable oil feedstock (Lai et al., 2005). Therefore, used cooking oil has been suggested as a cheap alternative feedstock for biodiesel production. Animal fats have also been suggested, but due to their high melting points, they have to be dissolved in an organic solvent, which would require additional solvent recovery unit (Al-Zuhair, 2007). Besides being cheap, the use of waste materials such as used cooking oils and animal fats, is considered a waste management process. The main drawbacks of using waste materials are their inconsistent supply and logistics' complications. In addition, these sources cannot satisfy the global demand for biodiesel.

Recently, microalgae has been identified as the most promising alternative feedstock for biodiesel production. It has been estimated that the yield of oil from algae is over 200 times that of vegetable oils (Kole et al., 2012). Algae feedstock produces large quantities of neutral lipids, needs less water than terrestrial crops and do not require pesticides applications to be maintained. Currently, the production of algae for biodiesel yet to reach a large commercial scale. A recent study "Algae 2020" has identified five key strategies for successful commercialization of algae biofuels (Thurmond, 2009).

1.3.2.2.1 Characteristics of microalgae

Microalgae are microscopic organisms that comprise a vast group of photosynthetic species which have an extraordinary potential of cultivation and grow robustly with the ability to live in diverse environments such as in freshwater,

wastewater, and marine water due to their unicellular or simple multicellular structure. The biochemical composition of a typical algae feedstock contains proteins, carbohydrates, lipids, nucleic acids, and other valuable components such as pigments, antioxidants and vitamins that vary in proportions (Singh and Gu, 2010). Algal oil contains saturated and monounsaturated fatty acids where the contents of each vary depending on culture conditions. The chemical composition of microalgae biomass can be modulated by controlling different variables affecting the metabolic of microalgae cells such as CO_2 and light (Moreno-Garcia et al., 2017).

1.3.3 Process steps for biodiesel from microalgae

1.3.3.1 Cultivation of algal biomass

Microalgae cultivation is done in the presence of light, CO_2, water and inorganic nutrients. There are two widely used cultivation systems, open pond system and closed photobioreactor (PBR) system. The highest productivity in open ponds is obtained in raceway systems to ensure continuous mixing needed to avoid algae settlement and provide maximum gas exchange. Nevertheless, the productivity is considered low in these systems and long times are needed for considerable algal productivity (Singh et al., 2011). However, the capital and operational costs in raceway systems are low and only weekly monitoring is needed to survey the biomass and nutrients.

Microalgae can also be cultivated in PBR closed systems, where water is circulated by pumps. The advantages of PBR are the high productivity, low contamination and efficient CO_2 capture (Burton et al., 2009). However, there are many designs and operational challenges needed to be resolved before

commercializing the production of microalgae using PBR. The high capital and operating cost, fouling on external and internal walls of the reactor and the accumulation of dirt and algae that prevent the light from reaching the algae cells are the major drawbacks (Singh et al., 2011). Thus, eventhough open systems are susceptible to contaminations; they are more commonly used on a large scale (Brennan and Owende, 2010). To overcome the poor biomass productivity in open ponds, a two-step cultivation process which involves the combinations of raceway and PBR has been tested (Singh et al., 2011). The first step is a PBR where a good production of biomass is obtained and CO_2 capture is maximized. After that, the algal suspension is transferred to an open pond with low nitrogen and high CO_2 levels. The nitrogen starvation step is used to enhance the oil accumulation.

1.3.3.2 Harvesting of algal biomass

In a typical culture of microalgae, cells are evenly distributed in a highly diluted medium. Harvesting refers to the step of concentrating this diluted algae suspension into a thick algae paste or slurry with at least 2-7% dry matters basis (Singh et al., 2011). There are several factors to be considered in selection of a suitable harvesting method such as the characteristics of the microalgae (size and properties of algal strain) and the growth medium (Oilgae, 2010).

Different harvesting techniques are currently applied, which include centrifugation, filtration, sedimentation, flocculation and flotation. Flotation harvesting consists of collecting the floating cells, which have a natural tendency to float at the surface of a tank. However, since it is limited to a small number of cells, this technique is not widely used (Brennan and Owende, 2010). Flocculation on the other hand by aggregates cells, which simplifies filtration and speedup settling. The

flocculation takes place using chemical flocculants (organic or inorganic) or through culture autoflocculation. Microalgal biomass flocculation is an electrolytic process where positively charged flocculation agent is used to neutralize the negative charge on the surface of microalgae resulting in sticking them together (Eisenberg et al., 1981). The efficiency of chemical flocculants varies depending on the type of microalgae strains. For example, ferric and aluminum inorganic salts have been used successfully in harvesting *Scenedesmus* and *Chlorella* strains. The main drawback of using chemical flocculants is the contamination of the harvested biomass by the flocculant materials.

Gravity sedimentation that depends on the cell density or filtration that depends on cell sizes can also be used. Centrifugation harvesting is the most reliable one among the other harvesting techniques. It is feasible for most species and results in high biomass recoveries despite its high energy demand (Grima et al., 2003).

1.3.3.3 Drying and cell disruption

After harvesting, a dewatering or a drying step is necessary for further process of lipid extraction. It has been reported that dried algae feed can increase the yield of algae oil (Show et al., 2013). Drying methods may include natural solar drying, or using other advanced methods such as freeze drying, drum drying, spray drying and fluidized bed drying (Guldhe et al., 2014). Despite the low cost and energy consumption of sun drying, it is unreliable and highly weather dependent. In addition, it is much slower than other techniques. Freeze drying is widely used for drying microalgae, which has the concurrent advantage of disrupting the microalgae cell wall (Brennan and Owende, 2010; Chen et al., 2010; Halim et al., 2012). However, freeze drying is a very expensive process.

Microalgae's cells wall disruption is needed for intracellular lipids' extraction. Several approaches have been used to break the cells and extract the oil from them such as autoclaving, bead-beating, ultrasonication, microwaving and osmotic shock. After cell disruption, an organic solvent such as hexane is usually used to dissolve the oil. It has been reported that by using hexane solvent together with pressing, about 95% of the oil in algae can be extracted (Packer, 2009). In microwave, the microwave energy increases the rotation of the molecular dipole resulting in breaking the weak hydrogen bonds. The movement of the molecular structure increases the solvent diffusion into microalgal biomass. In bead-beating, the high-speed spinning with fine beads results in direct mechanical damage of the cell wall. In addition, cells can be ruptured and its cellular components released by sudden reduction in osmatic pressure through osmatic shock. In ultrasonication, ultrasonic waves are used to create bubbles of solvent near the cell and collapsing these bubbles results in cells breakage.

Algal drying and cell disruption are the key steps to increasing the lipid extraction efficiency. However, they are considered as high cost and energy input processes. In some cases, cost of the drying may represent about 75% of the overall cost of algal biodiesel production (Mohn and Soeder, 1978). Efforts for developing effective drying and cell disruption methods are still required to overcome the challenges of algal biodiesel production.

1.3.3.4 Lipid extraction technologies

During lipid extraction, the microalgal cells are exposed to an eluting extraction solvent, which extracts the lipids out of their cellular matrices. Different

solvents have been used for lipid extraction from algal cells such as the conventional organic solvents, supercritical fluids (SCFs), and ILs.

1.3.3.4.1 Conventional organic solvents

The principle underlying the use of organic solvents in lipid extraction is based on the basic chemistry concept of “like dissolves like”. For lipid extraction from algal biomass, Soxhlet extraction using hexane as a solvent, and Bligh and Dyer’s method using a mixture of chloroform and methanol have been conventionally used (Bligh and Dyer, 1959). When the microalgae cells are exposed to an organic solvent, the solvent penetrates the cell membrane into the cytoplasm where Van der Waals forces between the non-polar solvent and the neutral lipids form a complex. Later on, due to concentration gradient, solvent-lipids complex will diffuse out of the cells and thus, the lipid extracted and remain dissolved in the hydrophobic organic solvent. However, the neutral lipids might be found as a complex with polar lipids linked to the proteins present in the cell membrane via hydrogen bonds (Halim et al., 2012). In this case, a polar organic solvent such as methanol or isopropanol is used to facilitate the extraction (Medina et al., 1998). By using the mechanism of polar/non-polar organic solvent mixture, the non-polar organic solvent forms Van der Waals interactions with the neutral lipids in the complex while the polar organic solvent forms hydrogen bonds with the polar lipids. It has been reported that the use of isopropanol as a co-solvent in lipid extraction from *Chlorococcum sp.* improved the total lipid yield where the lipid yield of a pure hexane system was 0.015 g lipid/ g dried microalgal biomass and the final lipid yield of the hexane/isopropanol system (3/2 v/v) was 0.068 g lipid/ g dried microalgal biomass (Halim et al., 2011).

However, due to the high volatility of organic solvents, using them could pose several environmental risks. In addition, since the lipids are dissolved in a non-polar solvent, a downstream separation process would be needed to separate the extracted lipids from the non-polar organic solvent. Therefore, increasing efforts have recently been on replacing these toxic chemicals with more environmental friendly solvents for easier separation.

1.3.3.4.2 Supercritical fluids

SCFs are substances at temperatures and pressures above their critical points, which are the highest values at which the vapor and liquid phases coexist in equilibrium (Taher and Al-Zuhair, 2016). In 1822, the first discovery of SCF was by Baron Charles Cagniard de la Tour in his famous cannon barrel experiments. While listening to the discontinuities in the sound of rolling flint ball in a sealed cannon filled with fluids at various temperatures, he observed the critical temperature where above it the densities of liquid and gas phases become equal and the distinction between them disappeared resulting in a single SCF (Berche et al., 2009). After the sharp increase of interest in SCFs technology in 1980s, the amount of patent applications has been around 100 per year in 1990s concentrating on applications for the food, pharmaceutical or chemical industry (Sihvonen et al., 1999).

Among the different SCFs, SC-CO_2 and supercritical water (SC-H_2O) are the commonly used in lipid extraction since they are cheap, non-toxic and widely available (Taher and Al-Zuhair, 2016). Generally, the effectiveness of using SCFs in extraction depends on the applied pressure and temperature, which affect the solubility of the solvent (Taher and Al-Zuhair, 2016)

SCFs have been effectively used to substitute the highly volatile organic solvents in lipid extraction due to their high selectivity, low extraction time and less toxicity (Kumar et al., 2017). In addition, SCFs do not require a downstream separation process since, for example, CO_2 exists in gaseous state at ambient pressure (Yen et al., 2015). Many experiments on lipid extraction from different species of microalgae were conducted to compare the performance of SC-CO_2 and the conventional organic solvents. Using SC-CO_2, the lipid extraction yields from *C. vulgaris* (Mendes et al., 1995), *Nannochloropsis sp* (Andrich et al., 2005), *S. platensis* (Andrich et al., 2006), *Chlorococum sp* (Halim et al., 2011) and *S.maxima* (Mendes et al., 2003) were found to be comparable to those achieved using *n*-hexane, with a slightly better performance of SC-CO_2 from *Nannochloropsis sp* and *Chlorococum sp.*

However, challenges still encounter the use of SCFs in lipid extraction and the general process of biodiesel production. The main reason being the high pressure needed to bring the solvent to its supercritical condition making the overall process costly. For example, above 72 bars is needed to bring CO_2 to its supercritical conditions (Akoh and Min, 2008).

1.3.3.4.3 Ionic liquids

ILs are organic salts that exist in liquid phase at ambient conditions and melt below 100°C. Chemically, they are composed of positively and negatively charged ions; large organic cations associated with inorganic anions. The structure of ILs is very similar to the table salt, however, while salts do not melt below 800°C, most of ILs remain liquid at room temperature. This is mainly because their ions do not pack well (Renner, 2001). Furthermore, in some cases, the asymmetrical anions are

relatively large and play a role in lowering the melting point too (Yang and Dionysiou, 2004). ILs have synthetic flexibility where the combination cations and anions can easily be altered to design a solvent according to the desired application (Abu-Eishah, 2011). Thus, ILs are referred to as “designer solvents” (Candeias et al., 2009).

ILs have been known for a long time, but their usage as solvents in chemical processes for synthesis and catalysis has become recently significant. Ethylammonium nitrate ($[EtNH_3][NO_3]$) was first discovered in 1914, which exists as a liquid in room temperature with a melting point of 12°C (Sugden and Wilkins, 1929). In 1980, there were only few ILs based patent applications which increased to 100 by the year 2000 and 800 by the end of 2004 (Vancov et al., 2012). The number of applications of ILs have been increased rapidly in the literature as a solvents, reagents and catalysts. Most widely used ILs are 1-Butyl-3-methylimidazolium tetrafluoroborate $[bmim][BF_4]$, 1-Butyl-3-methylimidazolium triflate [bmim][TfO], 1-Butyl-3-methylimidazolium methide [bmim][methide], 1-Butyl-3-methylimidazolium dicyanamide [bmim][DCA], 1-Butyl-3-methylimidazolium hexafluorophosphate $[bmim][PF_6]$, 1-Butyl-3-methylimidazolium nitrate $[bmim][NO_3]$, 1-Butyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide $[bmim][Tf_2N]$, 1-Hexyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide; R= C_6H_{17} $[hmim][Tf_2N]$, 1-Octyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide; R= C_8H_{17} $[omim][Tf_2N]$, and 2,3-Dimethyl-1-hexylimidazolium bis(trifluoromethylsulfonyl)imide $[hmmim][Tf_2N]$ (Aki et al., 2004).

ILs have been reported to extract lipids from oil rich feedstocks, such as oil seeds and microalgae cells (Taher and Al-Zuhair, 2016). They have been proposed in replacing the conventional volatile organic molecules due to their high thermal stability and negligible vapor pressure. Hydrophilic ILs can be used to disrupt the rigid cell walls of the microalgae by dissolving the lignocelluloses. This technique was confirmed by testing the extraction of oil from wet *Chlorella vulgaris* using 1-Ethyl-3-methylimidazolium diethyl phosphate [emim][DEP] at 120°C (Choi et al., 2014). However, the main barrier of using the ILs is their high costs, which are much higher than those of conventional organic solvents. For example, the price of [bmim][PF_6] is €1643 L^{-1}, which is ten times the price of *n*-hexane that costs only €130 L^{-1} (Taher and Al-Zuhair, 2016).

1.3.3.4.4 Switchable solvents

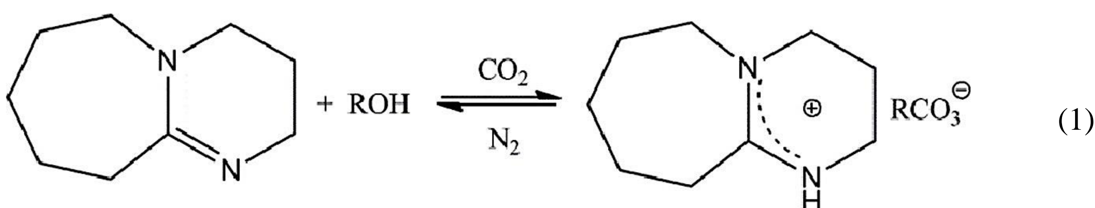
Beside the drawbacks of using organic solvents, SCFs and ILs in lipid extraction and biodiesel production, the common solvent regeneration technologies such as distillation, evaporation and stripping are energy intensive. It has been found that a recovery method based on phase splitting might offer an energy efficient and promising alternative. This could be induced by changing the nature of the solvent. In 2005, Philip Jessop and co-workers of Queen's University, in Kingston, Ontario, devised the first SSs, which show great potential in this field (Jessop et al., 2005).

SSs are liquids that can be converted from a non-ionic form to an ionic form of different physical properties such as conductivity, polarity, solubilizing capability and viscosity by bubbling CO_2. This process can be reversed back by N_2 stripping.

1.3.3.4.4.1 Classifications of switchable solvents

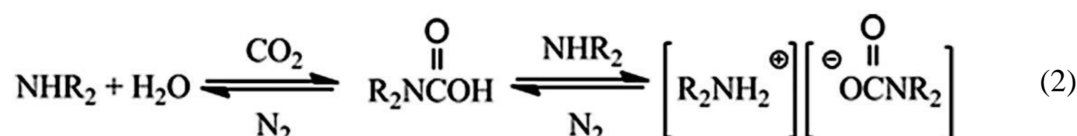
There three principal classes of SSs: switchable polarity solvents (SPS), switchable hydrophobicity solvents (SHS), and switchable water (SW), which related to their ability to change their properties between high ionic strength and low ionic strength (Jessop et al., 2012).

When a SS can change its properties between polar and non-polar, it is called SPS. In physics, the dielectric constant is defined as the quantity that measures the ability of a substance to store electrical energy in an electric field. The dielectric constant is also commonly known as relative permittivity, which is a relative measure of the chemical polarity of the solvent. The higher the dielectric constant, the higher the polarity and vice versa. Different switchable solvent systems (SSSs) have been described as SPS. Generally, these solvents have low polarity until they are exposed to atmosphere of CO_2, which increases their polarity. The first discovered SSS that was classified as SPS consisted of an equimolar mixture of an alcohol and an amidine such as 1,8-diazabicyclo-[5.4.0]-undec-7-ene (DBU). It has been found at operating condition of one atmosphere and room temperature, the exposure of 1:1 mixture of binary liquids, namely, 1-hexanol and DBU to gaseous CO_2 converts the DBU and 1-hexanol into the ions DBUH+ and RCO_3- which has an ionic liquid properties. Equation (1) shows the general polarity switching reaction of the two-components SSS.



(1)

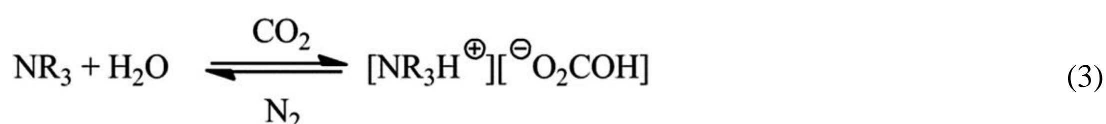
More recently, guanidine/ alcohol mixture, amidine/primary amine mixtures, guanidine/acidic alcohol mixtures, and other single-component SSs have been described acting as SPS (Herrero et al., 2017). The single-component SPS includes secondary amines, primary amines, diamines, hydroxyamines and hydroxyguanidines (Phan et al., 2008; Blasucci et al., 2010; Heldebrant et al., 2010). The secondary amines are cheaper than amidines, have significantly lower polarity and less sensitive than amidine/alcohol SSS to small amount of water (Du et al., 2015). As shown in Equation (2), carbamate salts form when they react with CO_2. According to Du et al. (2015), light secondary amines such as methylamine, diethylamine, and methylpropylamine are less preferable since they are very volatile and highly flammable. However, EBA, N-ethyl-N-propyl amine, dipropylamine and benzylmethylamine have been reported to be more preferable SSs than the light secondary amines which are very volatile and highly flammable (Phan et al., 2008).



(2)

On the other hand, when a SS changes its properties between hydrophilic and hydrophobic, it is defined as SHS. In chemistry, the hydrophilic and hydrophobic are definitions that describe the combination or miscibility and repulsion or immiscibility (biphasic mixture) of the molecule when mixed with water. Generally, very hydrophobic solvents become hydrophilic when they are in contact with CO_2 at atmospheric conditions. Jessop et al. (2012) described several SHS including an amidine such as N,N,N-tributylpentanamidine and tertiary amines. In contrast to amidine, tertiary amines are easy to prepare and often commercially available (Jessop et al., 2011). Tertiary amines give a water-soluble bicarbonate salt by

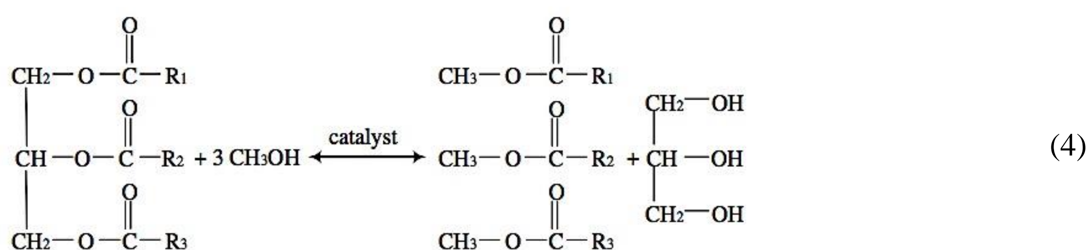
chemical reaction with CO_2 as shown in Equation (3). Furthermore, it has been found that operating at an elevated temperature is recommended to shift the equilibrium to the side of hydrophobic tertiary amine (Du et al., 2013). In addition, these solvents are less reactive towards CO_2 than secondary amines, which means longer reaction time is needed to switch the solvent however, less energy input is needed to reverse the reaction (Du et al., 2015).



(3)

1.3.3.5 Biodiesel production technologies

The first use of vegetable oil (peanut oil) as an engine fuel was in 1900 by Rudolf Diesel, the inventor of compression-ignited diesel engine. Later on, experiments conducted in Belgium, Germany, Italy, France, Japan, China, Argentina, and other countries found that the high viscosity of vegetable oils causes severe operational problems with engine deposits is the main drawback (Guo et al., 2015). To overcome the problem of high viscosity, the oil requires chemical modifications by transesterification, also known as alcoholysis (Meher et al., 2006). Transesterification is a reversible reaction between the triglycerides from a biomass source with a short chain alcohol, such as methanol, in presence of catalyst to produce fatty acid alkyl esters (FAAEs) and glycerol as described in Equation (4).



(4)

The type of the catalyst affects the performance of the transesterification reaction. There are three different approaches used in biodiesel production, namely, acids, bases and biocatalysts technologies.

1.3.3.5.1 Conventional techniques

Biodiesel is normally produced in industry using the conventional alkaline homogenous catalysts, such as potassium hydroxide (KOH), sodium hydroxide (NaOH), and alkoxides such as sodium methoxide (CH_3ONa) to promote the transesterification reaction between the oil and methanol. The advantages of alkali-based process include the mild conditions of temperature about 60°C and atmospheric pressure (Santacesaria et al., 2012), low cost of catalyst and the high attainable yields that can reach 98% within a short reaction time (Atadash et al., 2012). However, even though the alkali-catalyzed processes have been commercialized, there are many drawbacks associated with the use of homogenous catalysts. The process requires the use of highly refined oils, which contain very low amount of free fatty acids (FFAs) and moisture. This is because the FFAs react with the catalysts to produce soap forms emulsions that strongly complicate and prolong the separation time (Ma et al., 1998; Sivasamy et al., 2009). In addition, the moisture favors the formation of more FFAs by hydrolyzing the triglycerides (Taher and Al-Zuhair, 2016). As a consequence of using refined feedstock in the alkali-catalyzed process, most of the cost of biodiesel is estimated to be around 85% by the cost of the feedstock (Haas et al., 2006). Above that, a neutralization unit is needed after the reaction to neutralize the used basic catalyst. Therefore, the catalyst cannot be re-

used and the formed salt contaminates the glycerol increasing the costs of its purification (Santacesaria et al., 2012).

Acid-catalyzed reactions using for example sulfuric acid (H_2SO_4) have been suggested to preliminarily esterify the FFAs prior to the transesterification of the oil by alkali-based catalyst (Tesser et al., 2010). However, the main obstacle is that the pre-treatment is very slow, requires high methanol:oil (M:O) ratio and concentration of catalysts, and requires high energy (Al-Zuhair, 2007; Akoh et al., 2007; Marchetti et al., 2007).

Non-catalytic transesterification by supercritical alcohol has also been proposed to eliminate the use of chemical catalysts. Both esterification and transesterification spontaneously occur at high temperature. It has been reported that by operating with supercritical methanol, the reaction rate increases rapidly and it is possible to be completed within only 10 min at about 350°C (Cao et al., 2005). Moreover, at that temperature the rate of reaction is not affected by the presence of water. However, to make this technology more attractive, efforts are still devoted to reduce the cost associated with the high temperature and pressure (Santacesaria et al., 2012).

1.3.3.5.2 Enzymatic techniques

Currently, there is a favorable trend to use heterogeneous biocatalysts in order to avoid the problems associated with the use of chemical catalyzed reactions. Lipases are enzymes that exist in animals, plants and microorganisms, which are purified from fungal, bacterial, algal and yeast resources (Amini et al., 2017a).

Lipase acts on the ester bonds in triglycerides and can be used in oils transesterification for biodiesel production at mid operating conditions, with energy input and fewer steps compared to the conventional techniques (Marchetti et al., 2007). The first use of lipases in biodiesel production to replace the chemical catalysts was in 1990 where different lipases were tested for biodiesel production from sunflower oils (Mittelbach, 1990). Comparing it to conventional chemical catalysts, they are capable of converting oils from different feedstock without pre-treatment or soap formation with high efficiency (Fukuda et al., 2001). Moreover, since there is no soap formation, the separation and purification processes are easier (Taher and Al-Zuhair, 2016). Comparison of biodiesel production by enzymatic and chemical catalysts is shown in Table 2.

Table 2: Comparison of biodiesel production by enzymatic and chemical catalyzed transesterification reactions (Sebastian et al., 2016)

Catalyst	Alkali	Acid	Enzyme
Energy consumption	High	High	Moderate
Rate of reaction	Short	Long	Long
Reaction temperature	High	High	Mild
FFA influence on catalyst	Yes	No	No

The use of microorganisms in lipase production gained wide industrial importance and they share about 5% of the world enzyme market (Treichel et al., 2010). Currently, lipase, namely Novozyme®435, which is from *Candida Antarctica* is commonly used in enzymatic transesterification with efficiency above 90% (Amini et al., 2017a). According to Taher et al. (2014b), lipase from this resource has shown promising results with oils extracted from different microalgae strains.

Novozyme®435 has been successfully used for biodiesel production from oil extracted from *Nannochloropsis gaditana* (López et al., 2015), waste cooking oil (Hama et al., 2013), soybean oil (Seong et al., 2011), corn oil (Ciftci and Temelli, 2013), and palm oil (Talukder et al., 2011) with yields between 80-95%. Other lipases from *Pseudomonas fluorescens* (Guldhe et al., 2015; Devanesan et al., 2007), *Pseudomonas cepacia* (Noureddini et al., 2005), *Candida rugosa* (Tan et al., 2014; Lee et al., 2011a), *Rizhomucor miehei* (Huang et al., 2012; Huang et al., 2014) and *Thermomyces lanuginose* (Verdugo et al., 2011) have also shown good results.

There are several factors affecting the lipase activity and stability, such as the temperature, M:O ratio, enzyme source and dosage. In presence of lipase, the reaction rate increases with temperature usually below 50°C. More than 90% of lipase efficiency is achieved from 30°C to 50°C (Fjerbaek et al., 2009). At higher temperatures, denaturation of the protein structure of the enzyme occurs resulting in a drop in its activity (Taher and Al-Zuhair, 2016). For example, the FAME yield using Novozyme®435 in transesterification of seed oil increased from 58% to 88% when the reaction temperature increased from 30°C to 40°C (Amini et al., 2017b). However, beyond this optimum temperature, the FAME yield decreased to 76% because of the sharp decrease of the enzyme activity due to denaturation.

For complete conversion of oil to FAME, at least a stoichiometric amount of methanol is required. According to Amini et al. (2017b), the conversion gradually increases by increasing M:O ratio from 3:1 to 12:1. However, it declined from 12:1 to 15:1 due to enzyme deactivation in the presences of excess methanol. Other reports show that the lipase is inhibited when more than 1.5 molar equivalents of methanol are present in the reaction mixture (Taher and Al-Zuhair, 2016). This due

to the fact that at these concentrations, the methanol strips-off the water from lipase surface resulting in a loss of its activity. Generally, most studies reported M:O ratios in the range of 3:1 and 4:1 to be the optimum (Kaieda et al., 2001; Kumari et al., 2009).

Typically, the higher the concentration of the enzyme, the more the active sites available and thus, higher reaction rate and yield. It has been reported that lipase loading of 2-10% w/w on an oil weight basis is necessary for efficient transesterification (Tupufia et al., 2013; Maceiras et al., 2009). However, after specific high enzyme loadings, which typically differ from lipase to other, the addition of more enzyme no longer affects the yield. Studies on Novozyme®435 showed that the effect of enzyme loading becomes less significant at enzyme loadings in the range of 20-35% on an oil weight basis (Taher et al., 2011).

Despite lipases advantages over chemical catalysts, the high cost of enzymes (Stoytcheva et al., 2011), inhibition by the reactant methanol and by-product glycerol remain major obstacles hindering their commercial use. To reduce the overall cost, enzymes have to be used in immobilized form to simplify reusability and enhance stability. It has been reported that immobilized lipase can maintain over 80% of its initial activity after 20 cycles (Hama et al., 2007, Babaki et al., 2016). Above that, when tert-butanol was used in transesterification reaction for biodiesel production form rapeseed oil at 4:1 M:O molar ratio and 35°C, Novozym 435 maintained its activity for over 200 cycles, with 12 hour reaction time in each cycle (Lu et al., 2009).

The other major obstacle facing enzymatic biodiesel production is the inactivation of lipase by the acyl acceptor, which is the polar chain alcohol (Shimada

et al., 2002). As mentioned earlier, when methanol is used, the lipase inhibition occurs when more than 1.5 molar equivalents are present in the reaction mixture, which is mainly due to the outstripping of essential water from the lipase surface resulting in a loss in activity (Taher and Al-Zuhair, 2016). To overcome this problem, three solutions have been suggested, namely methanol stepwise addition, using other acyl acceptor alterations and solvent engineering (Tan et al., 2010). Stepwise addition of methanol was commonly used in earlier studies. For biodiesel production from vegetable oil using immobilized *Candida antarctica* lipase, methanol was added in a 1:1 molar ratio (Shimada et al. 1999). The first addition was at the beginning of the reaction, and the second and third additions were added after more of the methanol was consumed. By doing this, a yield of 98.4% was achieved after 24 hrs of reaction. A similar process was also used with lipases from *Candida* 99-125 (Lu et al., 2007), *Pseudomonas flurescens* (Soumanou and Bornscheuer, 2003) and *Rhizopus orzyae* (Chen et al., 2006). Replacing methanol with another acyl acceptor, such as methyl or ethyl acetate, which has less inhibition effect, was also rested (Tan et al., 2010). In biodiesel production from soybean oil using Novozyme®435 lipase, 92% of FAME yield was achieved using methyl acetate with 12:1 M:O molar ratio and no detected loss in lipase activity was recorded even after being reused for 100 batches (Du et al., 2004). However, the rate of the reaction with methyl acetate was significantly lower than that with methanol.

Improving the methanol solubility in the reaction medium, by adding another solvent, was also used to overcome lipase inhibition by insoluble methanol. The solvents used dissolved both reaction substrates and in addition decreased the viscosity and thus, enhanced substrate diffusion to the enzyme active sites and reduced the enzyme blocking by the deposited by-product, glycerol. Increasing the

hydrophobicity of the used solvent was found to enhance the biodiesel production rate and enzyme stability (Samukawa et al., 2000; Doukyu and Ogino, 2010; Yang et al., 2004). Among the organic solvents, *n*-hexane has been the most widely, due to its cheap price and high hydrophobicity. The FAME yield using *Mucor miehei* lipase increased 5 times when *n*-hexane was added. Compared to solvent-free system at the same conditions. However, organic solvents are highly volatile and toxic and therefore, greener and safer alternative solvents, such as ILs and SC-CO_2, have currently been proposed (Zhang et al., 2011; Lozano et al., 2011). By using IL, [emim][TfO], the biodiesel production yield using Novozyme®435 increased by about eight folds in comparison to solvent-free system. In addition, using IL was found to have a better effect on enzymatic biodiesel production. For example, an increase of 20% in the FAME yield was achieved by using [bmim][PF_6] compared to *n*-hexane. Using SC-CO_2 was also found to give favorable results. For example, 80% FAME yield was achieved when microalgae lipids were transesterified using Novozyme®435 at 50°C, 200 bar, 4:1 M:O molar ratio and 30% enzyme loading (Taher et al., 2014a). However, this process is rather costly, mainly due to the high cost of pumping the CO_2 to bring it to its supercritical state.

Another problem facing the commercialization of immobilized lipase in biodiesel production is the inhabitation by the by-product glycerol. Glycerol forms a hydrophilic environment around the enzyme making it difficult for the hydrophobic substrate to reach the active sites of the enzyme (Taher and Al-Zuhair, 2016). Furthermore, glycerol increases the viscosity of the medium, lowering the mass transfer (Aguieiras et al., 2015). The negative impact of glycerol can be prevented by the addition of an organic co-solvent to the medium. Biodiesel yield from rapeseed oil within 12 hours reaction time increased from 10% to 75% by the addition of tert-

butanol (Li et al. 2006). By recirculating a mixture of organic solvents, yields from sunflower oil using immobilized *Rhizomucor miehei* over 80% were achieved (Dossat et al. 1999). Enzyme washing to remove the glycerol after the reaction was also reported by adding hydrophilic substances such as silica gel (Chen and Wu, 2003; Hama et al., 2011; Lee et al., 2011b; Ko et al., 2012).

1.3.3.6 Switchable solvents in lipids extraction and biodiesel production

The main advantage of the SS is the ease in using it when different properties are needed. For instance, when it is used as a reaction medium and in solvent separation and extraction processes. In case of lipid extraction, the high affinity of SSs towards non-polar compounds has been proven to efficiently extract oil from soybeans and microalgae (Phan et al., 2009; Samorì et al., 2010).

Furthermore, comparing the SSs efficiency with the conventional organic solvents in the lipid extraction from algae strains, DBU/octanol system shows better results than *n*-hexane with hydrocarbons yield of 7.8% and 5.6% from dried and wet *Botryococcus braunii* algae, respectively (Samorì et al., 2010). However, in order to avoid the formation of DBU/alcohol, a further step to remove the water was necessary before treating the SPS with CO_2. Therefore, due to its sensitivity to the presence of water, DBU/ alcohol mixture was not recommended for oil extraction (Zeng et al., 2016).

The lipid extraction using DMCHA was shown to achieve a higher yield than that of the typical extraction using chloroform/methanol mixture. By using DMCHA for lipid extraction from wet algae samples of 80% water content (50 mg/ml, 24 h extraction), it was found that the total lipid content, expressed as per algal dry weight

basis, was $29.2 \pm 0.9\%$, $57.9 \pm 1.3\%$ and $31.9 \pm 1.5\%$ from microalgae strains *Demodesmus communis*, *Nannochloropsis gaditana*, and *Tetraselmis suecica*, respectively (Samorì et al., 2013). Beside that, tertiary DMCHA was shown to have a great capacity to extract oil from dry *Botryococcus braunii* (Boyd et al., 2012).

From an energy consumption point of view, besides drying, cell breaking is another energy intensive step in algae biorefinery processes. Du et al. (2013) investigated the extraction performance of different secondary amines from wet non-broken strain *Desmodesmus sp.*. The extracted oil yield of fresh non-broken algae was 16.81±0.45% and 15.39±0.51% using EBA and dipropylamine as extractants, respectively. These results were similar to those for oil extraction from fresh broken algae (16.74±0.46% and 15.82±0.62% in EBA and dipropylamine respectively). In addition, Du et al., (2016) used the secondary EBA as an extractant from non-broken *Neochloris oleobundans* algae strain. The lipid yield for the non-broken algae reached up to 13 wt.% at 18 h.

These remarkable results point the possibility of using switchable solvents where the energy intensive drying and grinding steps can be omitted. Additionally, from these few works proposed on extracting lipids from algae by switchable solvents, promising results have been obtained when compared with the traditional extraction methods.

1.3.4 Critical discussion

Practical development of biofuels' production from microalgae faces significant challenges. The cost of biofuels production from algal biomass is approximately 50€/L, which is considered not attractive for commercial production

(Ahrens and Sander, 2010). Mainly, the economical production of biodiesel from microalgae is limited by the energy cost of extraction (Boyd et al., 2012). Lipid extraction prior to transesterification remains an obstacle toward the commercialization of the idea of biodiesel production from microalgae. In addition, high-energy intensity associated with drying and cell disruption of algae and solvent recovery afterwards hinders the progress of algae biorefinery (Du et al., 2016).

The goal of microbial biotechnology is to improve the productivity to meet the demands of a rapidly growing large-scale market. The first step is to get the right price in order to compete with petroleum diesel. Recently, some successful improvements have been gained in the area of improving oil extraction efficiency. New procedures that rely on replacing the conventional solvents needed to disrupt the cell and extract the oil from dried and wet samples with SSs have recently been suggested. A hydrophilic solvent is needed for cell disruption, whereas a hydrophobic one is needed for oil extraction, and also as a transesterification medium. Therefore, the employment of the same IL or organic solvent in an extraction-reaction-product separation multi-step processes is not possible due to the need of different solvents of different hydrophobicities in each step. Above that, these separate solvents need to be completely recovered before the next step is carried out. On the other hand, the use of the same SS for (1) cell disruption, (2) oil extraction, (3) as a transesterification medium, and (4) biodiesel separation and solvent recovery seems very promising.

In this thesis, the capacity of different SSs in simultaneous oil extraction and biodiesel production from microalgae strains have been studied. The polarity switching of the SSs has been successfully used in different steps of the biodiesel

production, namely cell disruption, oil extraction, transesterification and product separation. Producing biodiesel from wet biomass using a single solvent in a single cell, minimized the cost. The suggested process eliminates the need for drying, cell disruption and the use of multiple solvents in different stages of biodiesel production.

1.3.5 Novelty statement

A hydrophilic SS, namely DBU, in combination with alcohol, showed an excellent efficacy to extract oils from soybean flakes (Phan et al., 2009) and from *Botryococcus braunii* microalgae (Samorì et al., 2010). However, with the latter biomass, the capacity of the SS to disrupt the cells walls was not assessed, as the cells were lyophilized prior to extraction. In addition, due to DBU's sensitivity towards water (Heldebrant et al., 2005), complete drying of both solvent and algae was inevitable. DBU was also used in combination with methanol (Bao et al., 2015) and ethanol (Xue et al., 2014) in biodiesel production. However, the SS was used in these cases as a base catalyst.

More interest has recently been focused on SSs, which could be used with aqueous samples. DMCHA was used to extract oils from wet biomass of *Botryococcus braunii* (Jessop et al., 2012), *Tetraselmis suecica*, and *Desmodesmus communis* (Samori et al., 2013), and the extraction yields were higher than those obtained through typical extraction procedure with chloroform-methanol mixture.

To the best of the knowledge of the researchers, no previous work considered the use of SSs as a reaction medium in enzymatic biodiesel production process, as suggested in this work. In addition, the use of the same SS for (1) cell disruption, (2) oil extraction from wet biomass, (3) as a transesterification medium, and (4)

biodiesel separation and solvent recovery has never been reported in literature before. The results reported in this work would have a significant effect on the simplification of biodiesel production from microalgae.

1.4 Aims of the Study

The aim of this study is to investigate the feasibility of SSs to extract lipids as a green and potentially energy saving system for the production of biofuels. In order to achieve it, the following objectives have been set:

1. Demonstrate the effectiveness of SSs in cell disruption and lipid extraction enhancement from wet microalgae, as compared to ILs and organic solvents
   Hypothesis: The SSs would be more effective than conventional solvents in extracting lipids from wet biomass.
2. Identify the optimum conditions of SS's extraction.
3. Demonstrate the dual effect of SSs for extraction and reaction in one cell, by altering the hydrophobicity.
   Hypothesis: By altering the hydrophobicity, SSs can be successfully used for simultaneous oil extraction and reaction in one unit.
4. Identify the optimum conditions of simultaneous lipid extraction-reaction in a SS system.

Chapter 2: Methods

2.1 Materials and Methods

2.1.1 Algae strains, chemicals and reagents

N,N-Dimethylcyclohexylamine 99% (DMCHA), n-ethylbutylamine (EBA) ≥ 98.0%, di-n-propylamine 99%, *n*-hexane, and chloroform were purchased from Sigma-Aldrich, USA. Zero air (ultra-pure), helium, nitrogen, and carbon dioxide were supplied by Sharjah Oxygen Company, UAE. Novozyme®435 (activity 11,900 PLU g^{-1}) was a kind gift from Novozymes, Denmark. Analytical grade methanol with a purity of ≥ 99% was obtained from Fisher chemicals, USA. 1-Butyl-3-methylimidazolium hexafluorophosphate, [Bmim][PF_6], with a purity ≥ 99%, was obtained from io-li-tec, Germany. A standard solution of high purity FAMEs consisting of: 4% myristic acid (C14:0), 10% palmitic acid (C16:0), 6% stearic acid (C18:0), 35% oleic acid (C18:1), 36% linoleic acid (C18:2), 2% of arachidonic acid (C20:0), and behenic acid (C22:0) was obtained from Sigma–Aldrich, USA.

Chlorella sp. was grown in an open raceway system of a 150 L volume, with continuous aeration and illumination. A single paddlewheel was used to maintain the suspension of the algal cells, which were grown in Bold's Basal Medium (BBM) (Agrawal and Sarma, 1982) at room temperature. The BBM medium contained 8.82 mM sodium nitrate ($NaNO_3$), 0.17 mM calcium chloride ($CaCl_2{\cdot}2H_2O$), 0.3 mM magnesium sulphate ($MgSO_4 \cdot 7H_2O$), 1.29 mM potassium di-hydrogen orthophosphate (KH_2PO_4), 0.43 mM di-potassium hydrogen orthophosphate (K_2HPO_4), 0.43 mM sodium chloride (NaCl), and 0.1% v/v vitamin B12. The culture was replenished with nutrients once a week to maintain it on a semi-continuous

basis. After establishing a sufficiently green and concentrated culture, the algae were harvested by centrifugation using an IEC-CL Multispeed centrifuge (Model No. 11210913, France) at 6,000 rpm for 5 minutes.

2.2 Oil Extraction

In the screening tests for oil extraction, the capacity of several SSs -- namely DMCHA, EBA, and Dipropylamine -- to extract oils from undisrupted wet algae paste was tested and compared to that of conventional organic solvents, *n*-hexane and hydrophobic ionic liquids, [Bmim][PF_6]. The first SS was used with only the water found in the wet biomass, whereas the latter two were prepared by mixing with an equivalent amount of water. The dry weight content of the harvested wet microalgae paste was determined from the difference in the weight of a sample before and after drying at 60°C for 24 h.

The experimental set-up is shown in Figure 1. The Gasses (N_2 or CO_2) cylinders were connected to a regulator (R_1), from which the flow of the gases that passed thought the system was controlled to allow sufficient bubbling while at the same time avoiding liquid entrainment using the valve (V_1). The sample was kept in a 10-mL glass vial placed in a temperature-controlled water bath (DaihanLabtech, Korea).

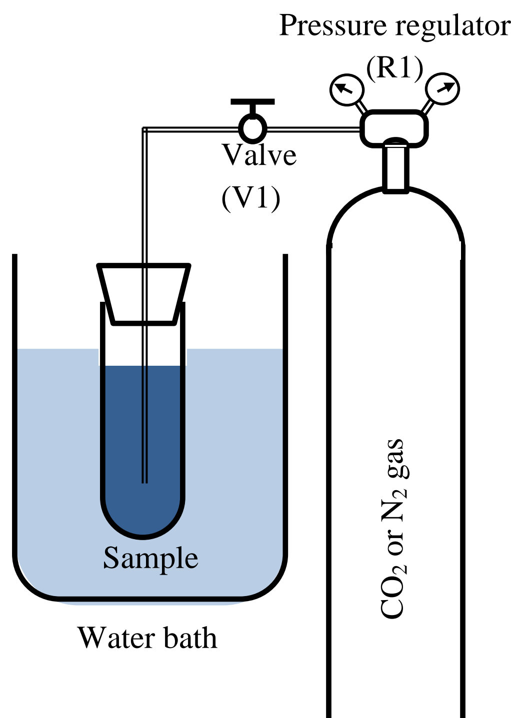


Figure 1: Schematic diagram of SS experimental setup

A sample of 1 g wet microalgae paste, of predetermined dry content, was mixed with 10 ml of the SS, which was kept hydrophilic for 30 minutes to disrupt the cells and liberate the oils. After that, it was switched to a hydrophobic state by bubbling N_2 through the solution and was left for another 30 minutes to dissolve the liberated oils. At the end of the experiment, the solvent was switched back to the hydrophilic form by bubbling CO_2 through the solution to separate the extracted oil. The separated oil was then collected in 10 ml of *n*-hexane, which was added 1 ml at a time and mixed thoroughly, and then centrifuged at 8,000 rpm for 2 minutes to separate the two layers where the upper layer contained *n*-hexane with the dissolved oil and the lower layer contained the SS as shown in Figure 2. The extracted oil was determined gravimetrically after evaporating the solvent in an oven at 50°C. The same procedure was repeated but by replacing the SS with an equal amount of *n*-hexane or [Bmim][PF_6]. The oil extraction effectiveness of the conventional

solvents, *n*-hexane and [Bmim][PF_6], was also determined from completely dried biomass, with the same weight as the dry weight content of the wet biomass. The extracted oil percentage was determined using Equation (5):

$$\text{Extracted oil} = \frac{m_{oil}}{m_{dry\ algae}} \times 100\% \tag{5}$$

Where m_{oil} and $m_{dry\ algae}$ are the weights of the extracted oil and the dry biomass, respectively.

Figure 2: Separation of SS and oil-hexane layers

2.3 Experimental Design and Optimization

Different extraction operation parameters were changed to determine their respective effects and to identify the optimum conditions for oil extraction from wet paste using the best SS in oil extraction, as identified in the previous screening step. The tested parameters were: temperature (in the range of 15 to 55°C) and the exposure times to each form of the SS, referred to in this work as the "solvent program." To evaluate the relationship between the response (i.e., the yield) and the

effects (i.e., the process variables), response surface methodology was applied. The levels of the independent variables were based on preliminary experimental results (not shown in this thesis). Independent process variables and their respective levels are listed in Table 3.

Table 3: Uncoded levels of independent variables

Factor	Symbol	Unit	Levels				
			$-\alpha$	-1	0	1	$+\alpha$
Cell disruption time	x_1	h	0.0	0.5	1.5	2.5	3.0
Oil extraction time	x_2	h	0.0	0.5	1.5	2.5	3.0
Temperature	x_3	°C	15	25	35	45	55

Experiments were carried out in a random manner to eliminate various types of biases order, which was developed using MiniTab 17, as shown in Table 4. A central composite design was developed to generate a polynomial model between the extracted lipid yield and the three variables -- cell disruption and extraction durations, and extraction temperature.

Table 4: Central composite design experiments for the three selected process variables and experimental extracted oil yields

Factor			Extracted oil yield (%)
x_1	x_2	x_3	
0	0	0	45.53
0	0	$-\alpha$	31.57
+1	+1	-1	54.89
+1	-1	+1	52.92
+1	+1	+1	56.72
0	$+\alpha$	0	50.74
$-\alpha$	0	0	4.11
$+\alpha$	0	0	76.31
-1	-1	+1	43.93
0	$-\alpha$	0	17.75
-1	-1	-1	41.29
0	0	$+\alpha$	69.99
-1	+1	+1	45.93
-1	+1	-1	44.57
+1	-1	-1	49.26

In this part, a new batch of *Chlorella sp.* was used, which had a total oil content of $8.56 \pm 1.56\%$, which was determined using the Folch method using a chloroform:methanol solvent mixture of 2:1 (v:v) (Eggers and Schwudke, 2016). Wet *Chlorella sp.* strains were dried in oven at 60°C. A ratio of 2:1 (*v/v*) of chloroform/methanol equivalent to 16 ml and 8 ml, respectively, were added to 1.2 g dried algae. Ultrasonication (Model No. Branson Sonifier 450) was used for 3

minutes. Afterwards, the mixture was agitated in orbital shaker for 20 minutes at the room temperature and centrifuged to recover the liquid phase at 1000 rpm for 3 minutes. Subsequently, the mixture was washed with 0.2 times volume of water (4.8 ml for 24 ml) and mixed in a vortex mixer prior to centrifugation to separate the two phases. Finally, a separatory funnel has been used to separate the upper and lower phases. The lower layer containing chloroform and dissolved lipids was collected and dried in the oven to measure the lipid weight gravimetrically. The extracted oil yield in this part was determined using Equation (6):

$$\text{Extracted oil yield} = \frac{m_{oil}}{m_{oil\,content}} \times 100\% \tag{6}$$

A polynomial, as defined by Equation (7), was used to express the extracted oil yield as a function of the independent variables. MiniTab 17 statistical software (MiniTab, Inc.) was used for the statistical analysis.

$$Y = a_o + \sum_{i=1}^{3} a_i x_i + \sum_{i=1}^{2} \sum_{j=i+1}^{3} a_{ij}\, x_i x_j \tag{7}$$

Where Y is the extracted oil yield, and the constants, a_i and a_{ij} are the linear and interaction coefficients, respectively; and x_i and x_j are the levels of the independent variables. The t-test was performed to judge the significance of the estimated coefficients in the model. To validate the model, additional two independent runs were carried out, and the experimental results were compared to those predicted by the model. Three-dimensional surface response plots were generated by varying two variables within the studied range and holding the third constant.

2.4 Simultaneous Extraction-Reaction

Similar procedures to those described in Section 2.2 were followed here,

except that the wet biomass paste was mixed with the SS and predetermined amounts of methanol and enzyme. The reaction went through three steps: first the SS was kept hydrophilic for cell disruption, then it was converted to hydrophobic for extraction-reaction, and finally converted back to hydrophilic for product separation. The total FAMEs produced were analyzed using Gas Chromatography (GC), (Shimadezo, GC-2010, and Japan), equipped with a flame ionization detector (FID) and a SP-2560 capillary column. Injected samples of 1 μL were filtered through a 0.45 μm pore size filter syringe. Helium was used as a carrier gas at a flow rate of 40 mL/min. The oven temperature was set to 195°C. After an isothermal period of 4 min, the GC oven was heated at a rate of 5°C min^{-1} to 240°C, and held for 12 min. A split ratio of 30 was used with injector and detector temperatures at 240°C and 260°C, respectively. Prior to samples analysis, a FAMEs standard of known composition was injected and used to calibrate the instrument.

Different dilutions of known concentrations of standard were prepared in 5 ml *n*-hexane and were injected into the GC. The retention times and peak areas were used to identify and determine the concentrations of the FAME in the experimental sample, respectively. A table of identified peaks and concentrations of standards were incorporated within the generated method. A calibration curve was created representing the standards concentration levels specified in the compound table. The calibration curve presented as concentration levels versus the peak areas was used to determine the correlation coefficients of the best-fit equation line. The developed method contained a reference to the calibration curve used to estimate the unknown concentrations of any injected sample. The collected FAMEs, produced in the experiments, were diluted in 5 ml of *n*-hexane before injecting into the GC. The

actual amounts produced were then determined by multiplying the measured concentrations by 5. The quantities of the produced FAMEs were reported as percentages of the total oil content, determined using the Folch method as given by Equation (8).

$$\text{FAME yield} = \frac{m_{FAME}}{m_{oil\,content}} \times 100\% \qquad (8)$$

All experiments were carried out in duplicates, and the presented results are the average values (with the standard deviation shown as error bars in the figures, and deviation ranges in the tables).

Chapter 3: Results and Discussion

3.1 Screening Tests of Oil Extraction from Wet Biomass

The capacity of a conventional organic solvent, *n*-hexane, and a hydrophobic IL, [Bmim][PF_6], was tested for extracting oil from dry and wet microalgae cells. Within 30 min of extraction using *n*-hexane, the extracted oil from dried microalgae cells was $9.38 \pm 0.73\%$, as shown in Figure 3. By subjecting the dried cells to [Bmim][PF_6], for the same duration, a lower extraction of $3.8 \pm 1.13\%$ was achieved. However, when wet biomass of the same dried cell content was used, no oil was detected when *n*-hexane was used, and an insignificant oil extraction of $0.70 \pm 0.28\%$ was achieved when [Bmim][PF_6] was used. The main structural element of the microalgae cell wall is the cellulose, which consists of thousands of D-glucose molecules bonded to each other through strong hydrogen bonds (Wang et al., 2016). Hydrophobic solvents, such as *n*-hexane and [Bmim][PF_6] do not have the ability to disrupt the rigid cell walls of microalgae and hence, the solvents cannot reach the oil within the cell to dissolve them. By drying or ultrasonication, the cell walls are disrupted, exposing the oils to the solvents. The dramatic drop in oil extraction, when wet biomass was used, is due to the unbroken polar structure of the cell walls, which prevents the hydrophobic solvents from reaching the cells' oil. On the other hand, hydrophilic ILs have been successfully used to disrupt the rigid cell walls of the wet algae strains by dissolving the lignocelluloses (Choi et al., 2014).

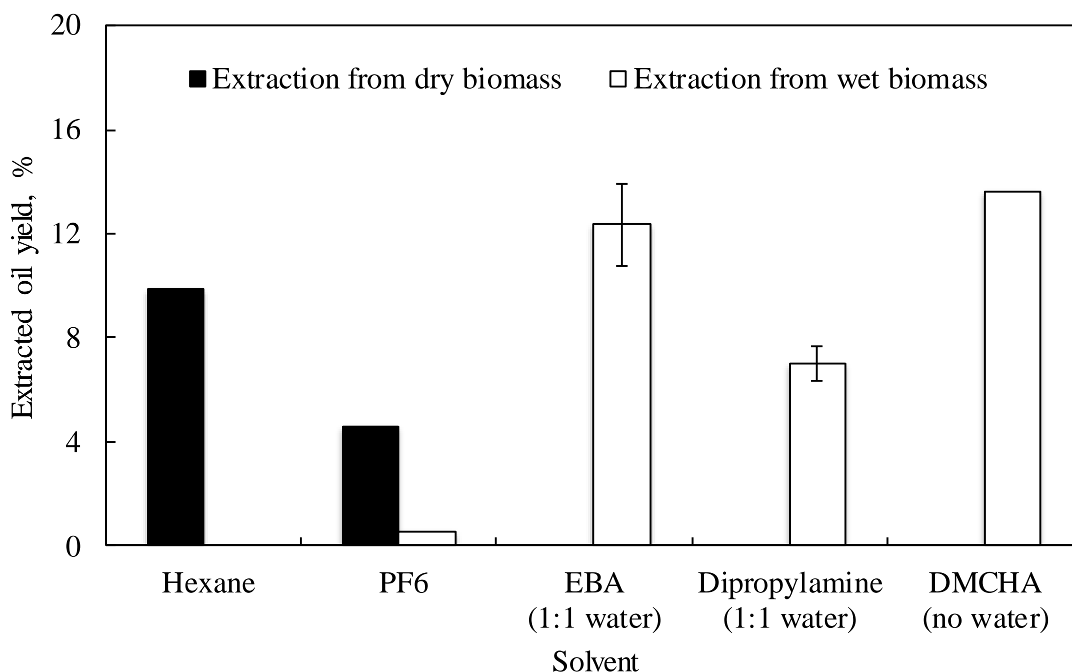


Figure 3: Extracted oil from wet and dry microalgae cells using different solvent

The capacity of three SSs -- namely EBA, dipropylamine, and DMCHA -- to extract oil from wet biomass was tested. By keeping the SSs hydrophilic for 30 minutes, and then changing them to hydrophobic for 30 minutes (which is the same extraction time used with the other conventional solvents), extractions of $12.35 \pm 3.18\%$, $6.95 \pm 1.34\%$, and $13.30 \pm 0.42\%$, were achieved using EBA, dipropylamine and DMCHA, respectively. The hydrophobicity switching characteristic of the switchable solvents allowed them to effectively extract the oil from the wet biomass while achieving yields even higher than those achieved from the dry biomass using the conventional solvents. The reason for the lower extraction yields using the conventional solvents with the dried biomass was because the cells were not disrupted, which is a required step when oil is to be extracted from microalgae (Roux et al., 2017). The higher extraction yield of the tertiary amine, DMCHA, is due to its higher hydrophobicity compared to the secondary amines,

EBA and dipropylamine. Furthermore, the secondary amines react with CO_2 to form carbamate salts as described by Equation [2]. It was also observed, as shown in Figure 2, that with both secondary amines, white carbamate salts of amine accumulated in the lower layer, which was not the case with tertiary amine DMCHA. This implies that the reaction with secondary amines, EBA and dipropylamine, was not completely reversed (Du et al. 2013) unlike in the case of DMCHA at the same extraction conditions. In other words, the formation of the salts indicated that not all the solvent has been converted back to its non-polar phase and thus, less oil extraction was achieved.

It is worth mentioning that the DMCHA was used without the addition of water, and the moisture in the wet harvested biomass was sufficient. However, the other two secondary amines required the addition of water with a 1:1 v/v ratio, as suggested by Du et al. (2015). All experiments in this part of the study were carried out in duplicates, and the presented values were the average of the two runs, with the standard deviation shown as error bars in the figures.

The results presented in this work agreed with those reported for the extraction of oil from wet paste of *Desmodesmus sp.* using different secondary amines (Du et al., 2013). At ambient conditions and within 24 hours, the extracted oil of fresh, non-broken algae was 16.81% and 15.39% in EBA and dipropylamine, respectively. A higher oil extract was reported using DMCHA for lipid extraction from wet algae samples of 80% water content. After 24 hours, extracted oil of 29.2%, 57.9% and 31.9% were reported from microalgae strains *Desmodesmus communis*, *Nannochloropsis gaditana*, and *Tetraselmis suecica*, respectively (Samorì et al., 2013).

3.2 Optimization of Oil Extraction Using DMCHA

Since DMCHA showed the highest oil extraction and FAMEs yield, this SS was selected for all subsequent tests. It was important to study the effect of the two main steps in the oil extraction process using the SS; these are the cell disruption and extraction durations. In addition, the effect of temperature was tested to optimize the extraction process. The ranges of the independent variables are shown in Table 3. In this study, a new batch of *Chlorella sp.* was used, which had a total oil content of $8.56 \pm 1.56\%$, determined using the Folch method using a chloroform:methanol solvent mixture of 2:1 (v:v) (Eggers and Schwudke, 2016). All reported data are yields with respect to the total oil content, as described by Equation (6).

At a temperature of 35°C, the effect of the cell disruption duration on oil extraction yield was examined, while keeping the extraction duration constant at 1.5 hours. As shown in Figure 4, when there was no cell disruption, a very low extraction yield of only 3.25% was achieved. The extraction yield increased significantly to $45.53 \pm 7.90\%$ by increasing the cell disruption time to 1.5 hours. The yield was further increased to 76.31% by increasing the disruption time to 3 hours. This was because the longer the SS was kept hydrophilic, the more time was available to break the cell walls.

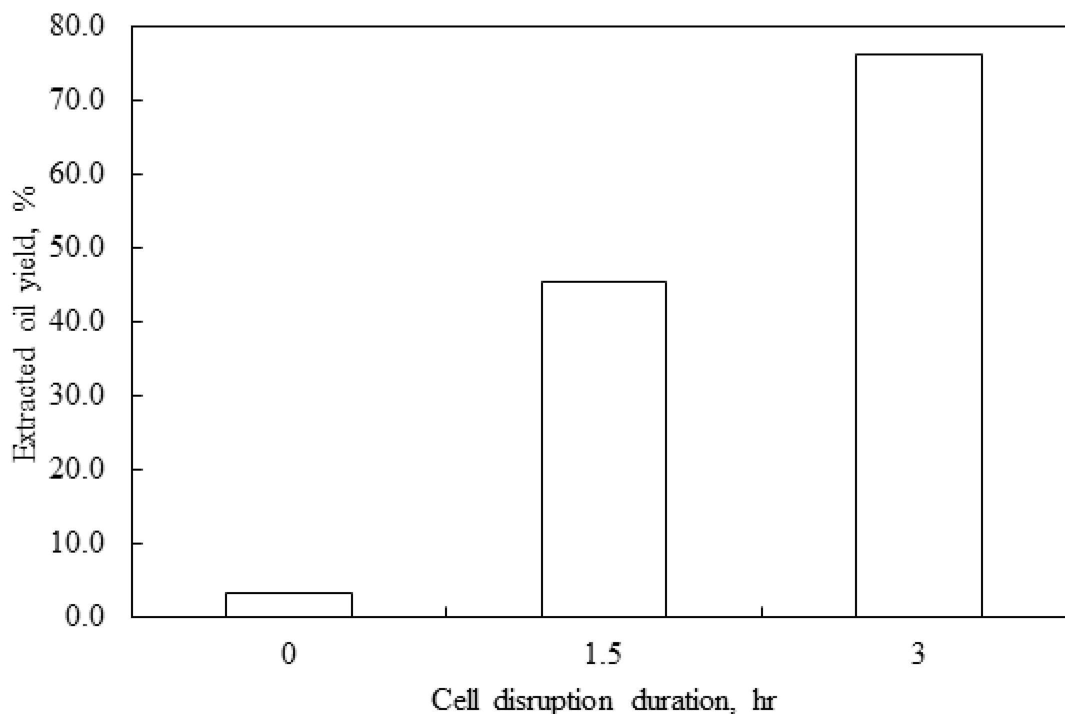


Figure 4: Effect of cell disruption duration on extraction yield at the extraction duration of 1.5 h using DMCHA at 35°C

At a temperature of 35°C, the effect of the extraction duration on oil extraction yield was examined, while keeping the cell disruption duration constant at 1.5 h. As shown in Figure 5, a low extraction yield of only 17.75% was achieved when the extraction step was ignored (extraction duration= 0), which was extracted during the cell disruption and separation steps. Increasing the extraction duration to 1.5 hours resulted in a significant increase in the oil extraction yield to 45.53%. As the duration over which the SS was kept hydrophobic increased, more time was available for it to diffuse through the algal cells and to dissolve the oil. However, doubling the extraction time to 3 hours showed an insignificant increase in the yield, reaching only 50.74%. This proves that the effect on yield of the disruption time was more significant than the extraction time.

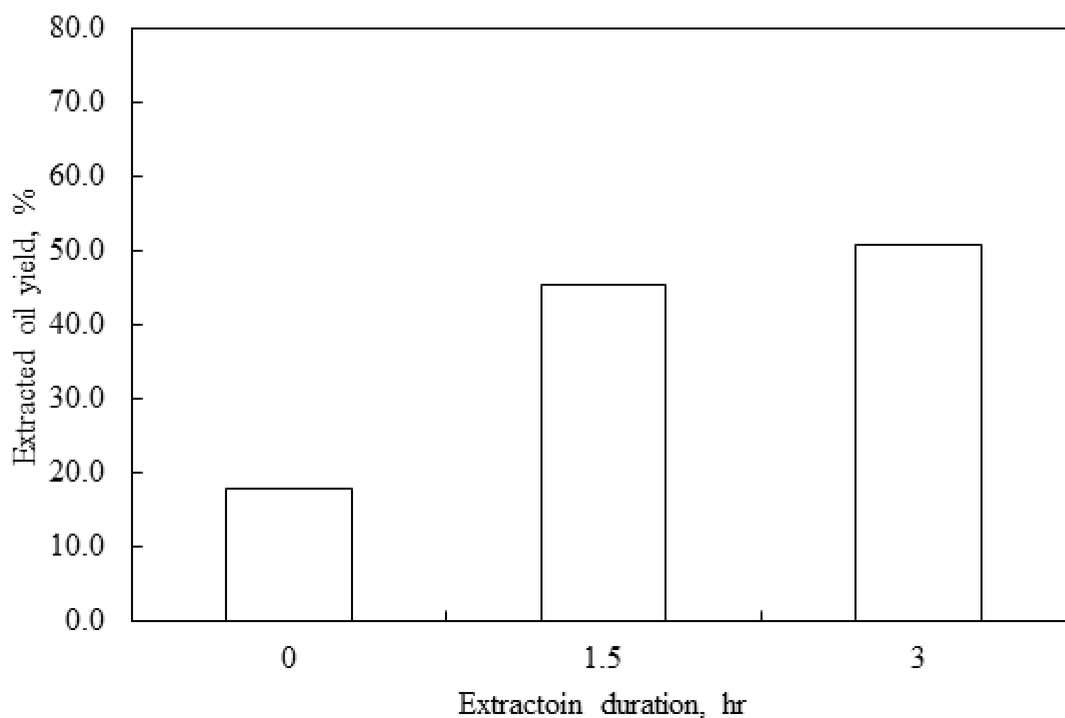


Figure 5: Effect of extraction duration on extraction yield at a cell disruption duration of 1.5 h using DMCHA at 35°C

At a constant cell disruption and extraction duration of 1.5 hour, the effect of temperature on oil extraction yield was tested, and the results are shown in Figure 6. It has been found that the yield increased significantly by increasing the temperature. This is mainly due to the expected enhanced diffusion and mass transfer with temperature.

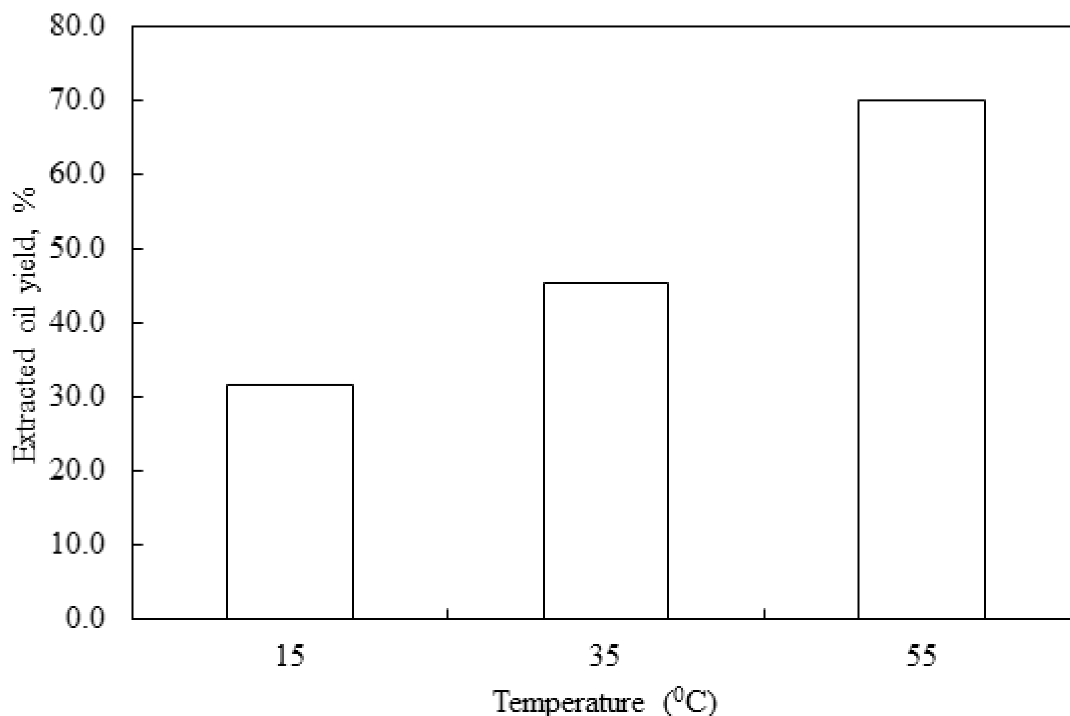


Figure 6: Effect of temperature on extraction yield at cell disruption and extraction durations of 1.5 hour each using DMCHA

The oil extraction yields at all tested conditions are shown in Table 4. MiniTab 17 statistical software was used to fit the experimental results shown in Table 4 to generate a polynomial model between the extracted oil yield, Y, and the three effects: namely cell disruption duration, x_1, extraction duration, x_2, and temperature, x_3. The resultant polynomial with the determined coefficients is shown in Equation (9):

$$Y = 4.1 + 10.3x_1 + 5.7x_2 + 0.51x_3 + 0.52x_1x_2 + 0.018x_1x_3 - 0.039x_2x_3 \quad (9)$$

To validate the model, the results predicted by the model were compared with the experimental results of an additional independent run, which was carried out with a solvent program involving cell disruption for 1.5 h, extraction for 3 h, and phase separation at 35°C for 1 h. These conditions were the optimum for the simultaneous

extraction-reaction system, as shown in Section 3.4. The experiments were carried out in duplicate to further confirm the reproducibility of the data. The experimental extracted oil yield was 51.02±5.25%, which was very close to that predicted by the model, i.e., 53.69% with 4.97% error. An additional run was also tested, using a solvent program involving cell disruption for 0.5 h, extraction/reaction for 3 h, and phase separation at extraction at the same temperature for 1 h. The measured experimental extracted oil yield was 44.33±4.10%, compared to the model predicted value of a 41.20% yield with 7.59% error.

The assumption that the errors are normally and independently distributed must be satisfied before statistically analyzing experimental data. In other words, if these assumptions were valid, the statistical procedures would then be an exact test of the hypothesis been made to test the effect of the factors namely, cell disruption and extraction durations, and extraction temperature on the response variable, namely the extraction yield. Model adequacy has been investigated by examining the residuals, which are defined as the differences between the experimental values and the fitted value as per the model equation. As shown in the normal probability plot in Figure 7, the p-value is 0.147 which is larger than 0.05 generally required to accept the null hypothesis and agree that the residuals are normally distributed. Furthermore, the blue points almost fall on the straight line, which indicates that the differences between observed and the fitted values, presented by the diagonal, is small. The plot of the residuals versus fitted value, shown in Figure 8, reveals no obvious pattern, which suggests a constant variance of the residuals. It also means that the predicted values of the dependent variable (i.e., extraction yeild) by the regression model (Equation 9) was consistant across all the experimental values.

If the residuals were dependent, then a current value would depend on the previous value and thus, there would be an unexplained pattern in the response variable. Figure 9 shows the residuals versus the observations order, which clearly indicates that the residuals were randomly distributed around the zero line. This suggests that there is no correlation between the residuals in case of observations order and thus, the residuals are independent.

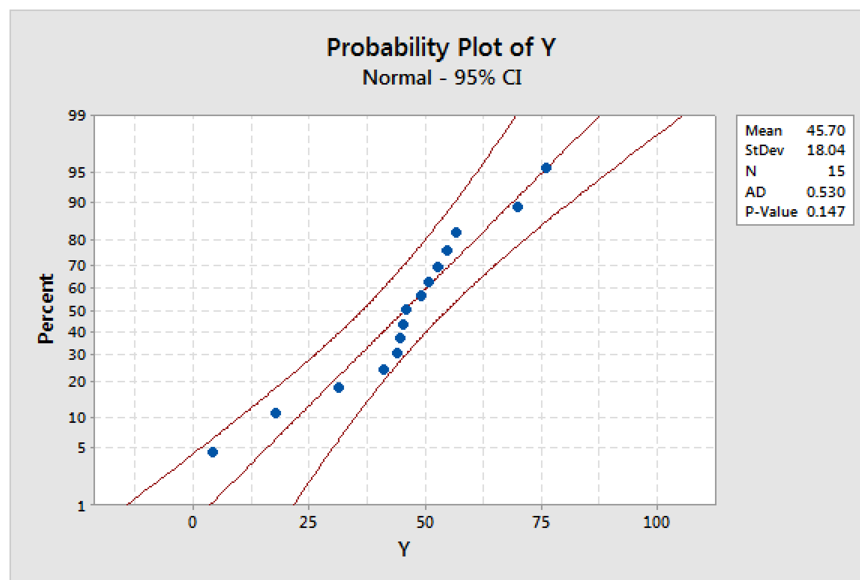


Figure 7: Normal probability plot of residuals

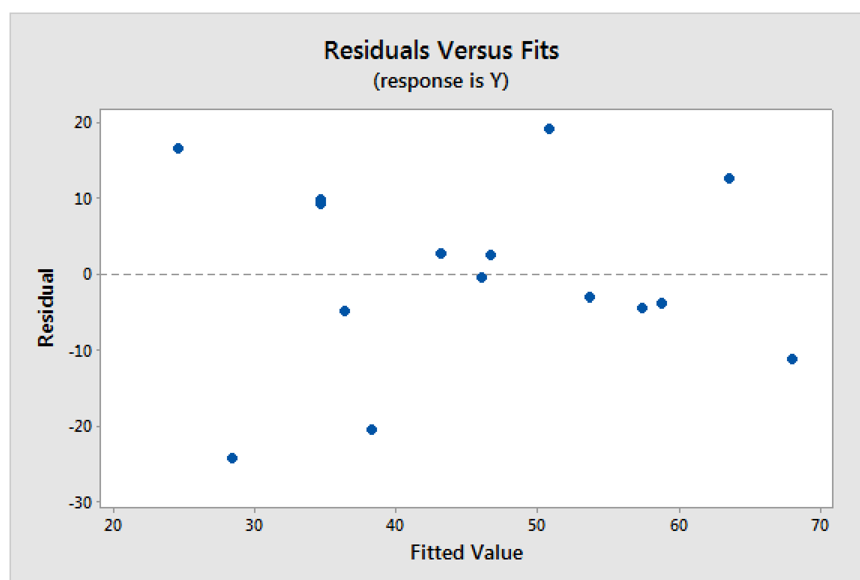


Figure 8: Residual versus fitted value

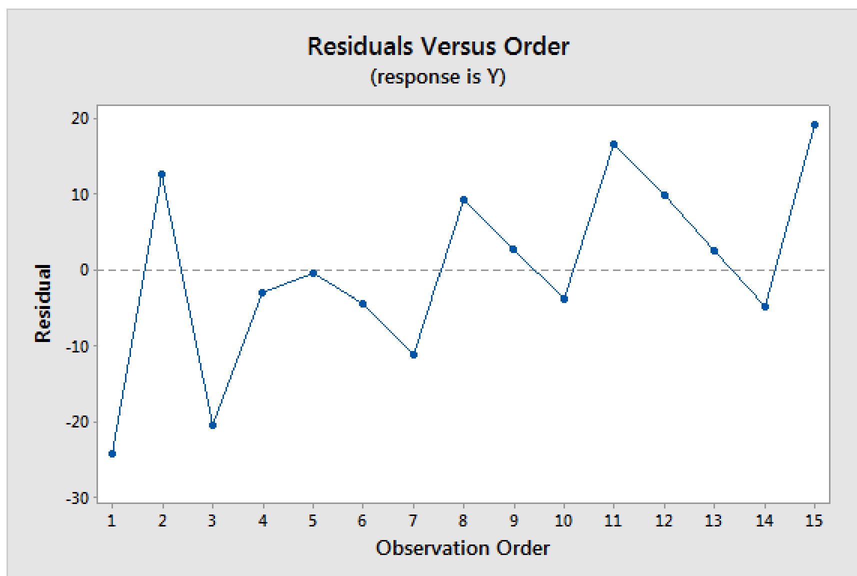


Figure 9: Residual versus observations order

The model equation (Equation 9) was then used to present the combined effects of the cell disruption time, extraction time, and temperature on the extracted oil yield in 3D plots. Figures 10-a and -b show the effects of cell disruption, extraction times, cell disruption time, and temperature, respectively. The results in Figure 10-a show that the extraction yield initially increased with the increase in extraction time, but then the effect starts to subside. On the other hand, the yield increased with the increase in disruption time in almost a linear manner. These results agree with those presented in Figures 4 and 5. The results in Figure 10-b show that the effect of temperature on the extraction yield was also linear, but less significant than that of the cell disruption time.

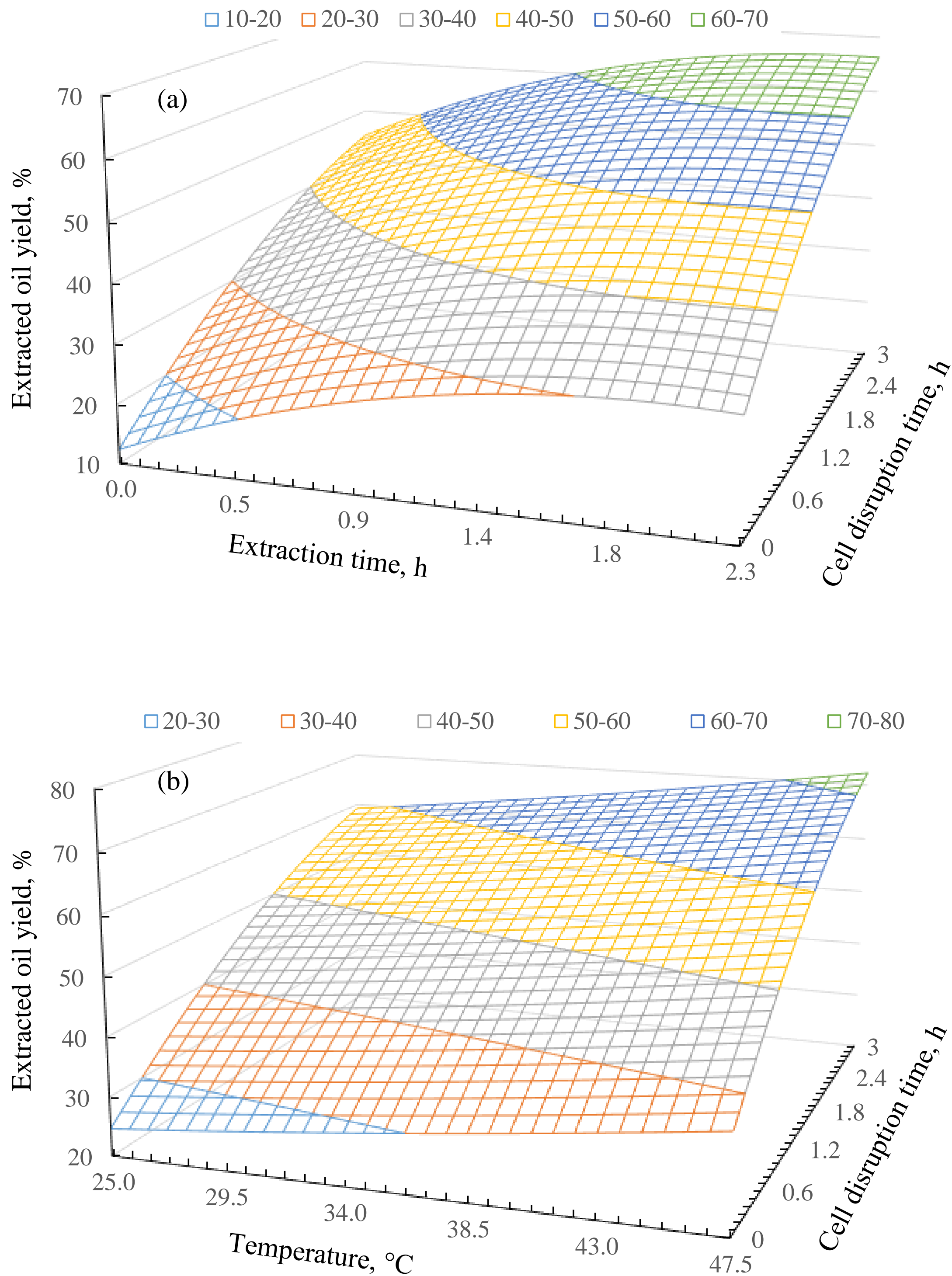


Figure 10: 3-D plot of the extracted oil yield as a function of (a) cell disruption and extraction times at 35°C and (b) cell disruption time and temperature at an extraction time of 1.5 h

3.3 Simultaneous Oil Extraction-Transesterification System

The tertiary amine, DMCHA, which showed the best oil extraction yield, was used to prove the concept of SSs effectiveness for simultaneous oil extraction and transesterification from wet microalgae cells. Initially, no catalyst was used, as tertiary amines, such as DMCHA, were reported to show a catalytic activity (Van et al., 2005; Deshpande et al., 2017; Qian et al., 2012; Xu et al., 2013). A FAMEs yield of 19.04±2.00% was achieved using a M:O molar ratio of 6:1, based on the oil content, at 35°C and using the following solvent switching program: cell disruption for 1.5 h, extraction-reaction for 3 h, and product separation for 1 h. This clearly shows the effectiveness of the SS for simultaneous oil extraction-reaction from wet microalgae cells. This is a very important finding that was never reported in the literature before and promises to simplify biodiesel production from microalgae in a single system using one solvent, without the need for drying. To further enhance the yield, the experiment was repeated under the same conditions, but with the addition of the enzyme, Novozyme®435, at 30% w/w with respect to the dried biomass. A higher FAMEs yield of 25.36±0.82%, equivalent to a 33.18% increase.

The effectiveness of using DMCHA was compared to EBA with 1:1 (v:v) water at 45°C and 6:1 M:O and 30% enzyme, using the following solvent program: cell disruption for 1 h, extraction reaction for 1 h, and product separation for 1 h. As shown in Figure 11, by reducing the disruption period to 1 h, a higher FAMEs production yield of 47.5 ± 3.54% was achieved, compared to the yield of 25.36±0.82% achieved in the previous test using a disruption period of 1.5 h with the same SS (DMCHA) and the same amounts of enzyme and methanol, even when the extraction-reaction time was reduced to 1 hour (compared to 3 hours in the

previous test). Although a longer disruption period is expected to enhance the extraction, it has, however, showed a negative effect on the enzyme activity. This is expected to be due to the negative effect of the hydrophilic solvent on enzyme activity, and it clearly proves the importance of optimizing the solvent program in the extraction-reaction system without relying on the optimum extraction conditions only.

The FAMEs yield using EBA was compared to that found using DMCHA, under the same conditions and solvent program, and the results are shown in Figure 11. A higher FAMEs yield was achieved using DMCHA, and the p-values were found to be equal to 0.0132 and 0.0034 between DMCHA and EBA at M:O of 6:1 and 12:1, respectively, which indicate significant differences. The higher yield obtained using DMCHA was due to its better oil extraction effectiveness, as shown in Figure 3. In addition, EBA required the addition of water, which was not needed with DMCHA. This additional water is expected to have a negative effect on FAMEs production, as it shifts the reaction towards hydrolysis to fatty acids instead (Atadashi et al., 2012). This is also because in the hydrophobic form, the hydrophobicity of the tertiary amine DMCHA that has three hydrocarbon groups is higher than that of the secondary amine EBA that has two hydrocarbon groups. It was reported that rate of the enzymatic biodiesel production increases with the increase of the hydrophobicity of the solvent used (Samukawa et al., 2000, Taher and Al-Zuhair, 2016).

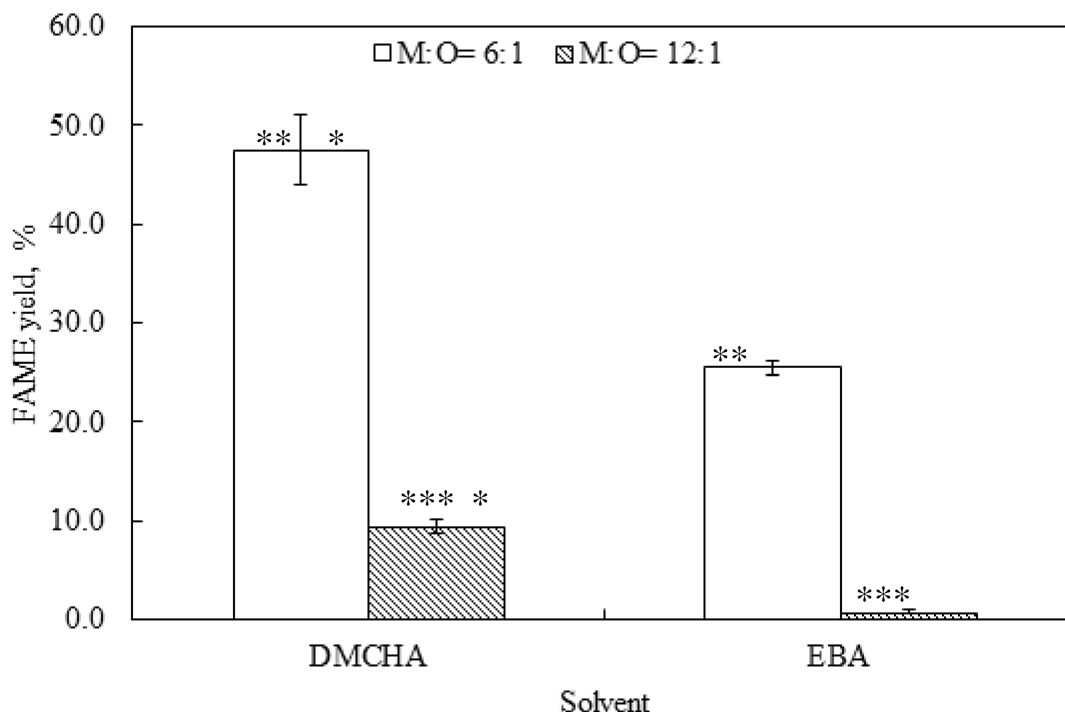


Figure 11: FAME yield at 45°C, M:O molar ratios of 6:1 and 12:1, 30% enzyme loading, and different SSs, using the following solvent program: cell disruption for 1 h, extraction/reaction for 1 h, and phase separation for 1 h
* Comparing DMCHA at two direct M:O ratios (p-value= 0.0045)
** Comparing M:O ratio of 6:1 for different SSs (p-value= 0.0132)
*** Comparing M:O ratio of 12:1 for different SSs (p-value=0.0034)

At a higher M:O ratio of 12:1, a clear negative effect on the FAMEs yield was observed, with the FAMEs yield dropping significantly to 9.5±0.71% and 0.8±0.14% for DMCHA and EBA, respectively. The p-value between M:O of 6:1 and 12:1 using DMCHA was found to be equal to 0.0045, which suggests a significant effect of the M:O ratio. The drop was due to the enzyme inhibition of the alcohol, which is reported in most studies using enzymes for biodiesel production (Taher and Al-Zuhair, 2016). In addition, the high alcohol presence is expected to affect the hydrophobicity switching of the SSs.

To check the effect of water on DMCHA, the experiment was repeated at 45°C, 6:1 M:O, and 30% enzyme loading, using the same solvent program involving

cell disruption for 1 h, extraction-reaction for 1 h, and product separation for 1 h, but using dry biomass with amounts equal to that found in the wet biomass. A much lower FAMEs yield (8.5±0.71%) was achieved, than that (47.5±3.54%) achieved using wet biomass under the same conditions. This is believed to be due to the ineffective hydrophobicity switching of the DMCHA in a completely dried system. It was reported that the chemical reaction of CO_2 and DMCHA with water results in a water-soluble bicarbonate salt that can be switched back to the hydrophobic phase when bubbling N_2 through the system (Du et al., 2015).

To further confirm the superiority of the SSs over conventional solvents, the effectiveness of SSs, DMCHA and EBA, has been compared to the hydrophobic [Bmim][PF_6] using 6:1 M:O and 30% enzyme loading at 35°C, and the following solvent program: cell disruption for 3 h, extraction/reaction for 3 h, and phase separation for 1 h. As shown in Figure 9, the FAME yields were 2.1±1.07%, 8.52±0.56%, and 15.97±0.45% when using [Bmim][PF_6], EBA, and DMCHA as the reaction media, respectively. These results agree with those of the extracted oil yield, with the highest FAMEs obtained when the solvent resulting in the highest oil extraction yield was used.

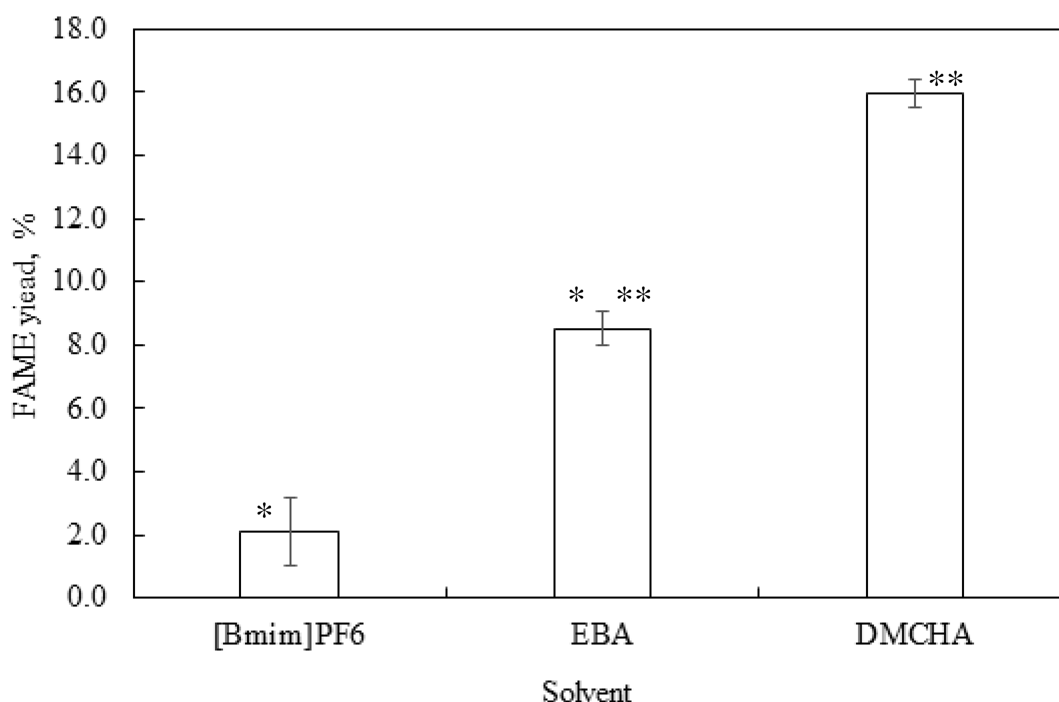


Figure 12: FAME yield produced at with 6:1 M:O ratio and 30% enzyme loading at 35°C, using the following solvent program: cell disruption for 3 h, extraction/reaction for 3 h, and phase separation for 1 h
* Comparing EBA to [Bmim][PF_6] (p-value= 0.0172)
**Comparing DMCHA to [Bmim][PF_6] (p-value= 0.0035)

It was noticed that operating at a lower temperature of 35°C resulted in a lower yield, as shown in Figure 12, compared to the results obtained at 45°C, shown in Figure 11. The effect of the reaction temperature was therefore tested using DMCHA at 6:1 M:O ratio, 30% enzyme loading, using the following solvent program: 3-h cell disruption, 3-h extraction/reaction and 1-h phase separation. At 25°C, the FAMEs yield was $9.51 \pm 1.18\%$, and it increased to $15.97 \pm 0.45\%$ (equivalent to a 6.47% increase) at 35°C. The increase in the yields with increasing temperature was due to the increase in reaction rate constants and mass transfer. The p-value was found to be equal to 0.0186, which indicates that the effect of the temperature on the FAME yield is significant.

As discussed earlier, it was found that the cell disruption duration was a very important factor in both the extraction alone, and the extraction-reaction system. Therefore, it was essential to determine the effect of this important factor on the simultaneous extraction-reaction system. Figure 13 shows the FAMEs yield at cell disruption duration equal to 0.5, 1.5 and 3 hours at fixed extraction time and temperature of 3 h and 35°C, respectively. It was found that increasing the disruption period from 0.5 to 1.5 h resulted in increasing the FAMEs yield from 21.05$\pm$2.24% to 25.36$\pm$0.82%. This is mainly due to the enhanced oil extraction with the increase in cell disruption time, as discussed in section 3.2, yet the p-value was found to be equal to 0.1251 that suggests insignificant effect of increasing the cell disruption time from 0.5 to 1.5 hrs. However, increasing the cell disruption time to 3 h resulted in a drop in the FAMEs yield to 15.97$\pm$0.45%. The drop in the FAME yield was significant, with a p-value of 0.0049. As mentioned earlier, this is mainly due to the negative effect of the hydrophilic solvent on enzyme activity. The optimum solvent program was then determined to be: cell disruption for 1.5 h, extraction for 3 h, and phase separation for 1 h. These conditions are the ones used to validate the statistical model (Equation 9) in Section 3.2.

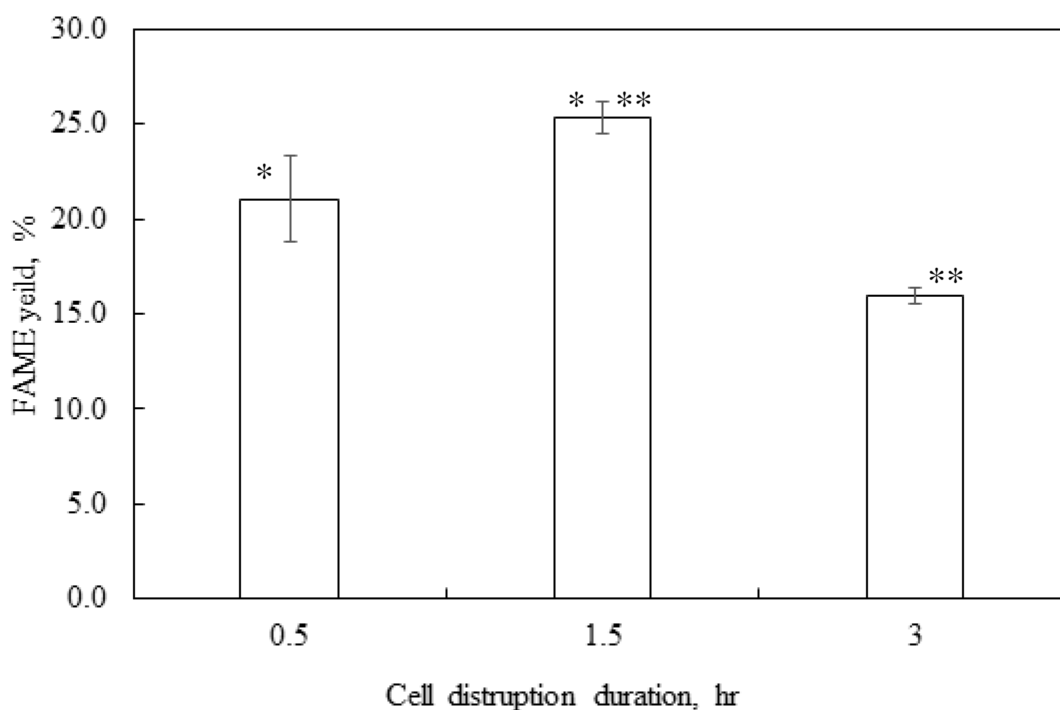


Figure 13: FAMEs yield % using DMCHA at different cell disruption times with 6:1 M:O ratio, 30% enzyme loading, and fixed extraction duration of 3 h at 35°C
* Comparing cell disruption time of 0.5 h and 1.5 h (p-value= 0.1251)
** Comparing cell disruption time of 1.5 h and 3 h (p-value= 0.0049)

Production of biodiesel from wet microalgae biomass was tested using sulfuric acid catalyst and heating in a single step (Im et al., 2014). The highest FAME yield obtained was 91% from wet *N.oceanica* strain, by mixing 0.3 ml sulfuric acid with 2/1 *v/v* mixture of chloroform and methanol and subjecting the sample to 95°C. However, the use of an acidic catalyst is highly corrosive and not recommended when fuels are to be produced. Furthermore, the high reaction temperature would make the process energy intensive.

3.4 Future Work

The use of SSs in the present work has been effectively tested for simultaneous lipid's extraction and biodiesel production. The optimization of the oil

extraction was done by testing the effects of the cell disruption and extraction durations and extraction temperature on the extracted oil yield. Further studies could be carried out to examine the effect of solvent volume on the extraction efficiency. Extended investigations on the reusability of the SS-enzyme system several batches of wet biomass is essential. To lower the cost of the overall process of the biodiesel production the use of continuous system should also be designed.

Chapter 4: Conclusions

In this thesis, SSs were used for cell disruption biomass and oil extraction from wet microalgae biomass simultaneously with reaction of the extracted oils for biodiesel production. At the same extraction conditions, the extracted oil yields from wet biomass were $12.35 \pm 3.18\%$, $6.95 \pm 1.34\%$ and $13.30 \pm 0.42\%$ using EBA and dipropylamine with 1:1 v/v water and DMCHA, respectively. Using conventional organic solvent, *n*-hexane, and hydrophobic IL, [Bmim][PF_6], resulted in insignificant yields of 0% and $0.70 \pm 0.28\%$, respectively. The SSs were shown to be effective in simultaneous oil extraction and biodiesel production, and superior to the hydrophobic IL, [Bmim][PF_6]. By the addition of enzyme, with DMCHA, the FAMEs yield increased by 33% from 19% when no enzyme was used to 25%. The successful use of a single solvent for extraction-reaction from wet biomass has never been reported in literature, which has a significant effect on the simplification of biodiesel production from microalgae.

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