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Thermoresponsive Switchable Solvents for Enhanced, Simultaneous Microalgae Oil Extraction Reaction for Biodiesel Production

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THERMORESPONSIVE SWITCHABLE SOLVENTS FOR ENHANCED, SIMULTANEOUS MICROALGAE OIL EXTRACTION REACTION FOR BIODIESEL PRODUCTION

Mukhtar Ahmed Ismail

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

Under the Supervision of Professor Sulaiman Al-Zuhair

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Declaration of Original Work

I, Mukhtar Ahmed Ismail, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled *"Thermoresponsive Switchable Solvents for Enhanced, Simultaneous Microalgae Oil Extraction-Reaction for 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 Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my thesis have been properly cited and acknowledged in accordance with appropriate academic conventions.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.

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Abstract

In this study, a thermo-responsive switchable solvent (TSS), with a tunable hydrophobicity by simply changing the temperature (between 25 to 45°C) was used for simultaneous lipids extraction from wet microalgae and biodiesel production. By manipulating the hydrophobicity of the solvent, the cell wall disruption, lipid extraction and transesterification, and product separation steps were all carried out in a single pot, while eliminating the need for the energy intensive and time-consuming drying step. To overcome the problems currently encountered by using conventional alkaline catalysts in the transesterification of lipids, immobilized enzyme has been used. The proposed TSS consisted of an ionic liquid (N,N diethyl-N-methylammonium methane sulfonate), a polymer poly(propylene) glycol (PPG) and water. The effectiveness of the proposed process was compared to that using conventional organic solvent, *n*-hexane, and other CO_2 triggered amine based switchable solvents, namely 1,8-diazabicyclo-[5.4.0]-undec-7-ene (DBU)-1-hexanol and DBU-Mono-ethanolamine (MEA). At the same conditions and solvent switching program, using immobilized lipase as a biocatalyst, the biodiesel yields were 45.5 ± 0.38 %, 37.8 ± 1.03 % and 5.9 ± 1.50 %, using TSS, DBU-hexanol, and DBU-MEA respectively. Using n-hexane resulted in insignificant yield of 3.1 ± 0.43 %. Furthermore, a reusability of the TSS-immobilized lipase system was investigated, and it was shown that the reusability biodiesel yield dropped from 50 ± 1.46 % in the first cycle to 20.4 ± 0.60 % in the fourth.

A parametric study was performed, using response surface methodology (RSM) to evaluate the effects of cell disruption and extraction/reaction durations in the range of 0-3 h, and methanol amount used in the range of 0.02 – 0.2 mL on the biodiesel production yield from 1 g of wet biomass. The results were used to develop a statistical model to predict the biodiesel yield under different conditions and to optimize the process. The optimum conditions were estimated to 0.5 hr, 3 hr and 0.15 mL for the cell disruption time, extraction-reaction time and methanol amount respectively, at which the yield was predicted to be 78.65 %. The experiment was repeated at the optimum conditions, and the actual yield was found to be 75.11 ± 1.03 %.

The successful use of TSS for simultaneous extraction-reaction and product separation from wet biomass has a significant effect on the simplification of microalgae to biodiesel process. By simply changing the temperature, the hydrophobicity of TSS can be manipulated, rendering the overall process easier, as compared to the CO_2 triggered Switchable Solvents. A process similar to the one presented in this work has never been reported before in literature.

Keywords: Thermoresponsive Switchable Solvents, Microalgae, Biodiesel, Simultaneous Extraction-Reaction.

Title and Abstract (in Arabic)

المذيبات المتحولة حراريا لاستخلاص دهون الطحالب بالتزامن مع انتاج وقود الديزل الحيوي

الملخص

تم استخدام وقود الديزل الحيوي، المنتج من الدهون الطحلبية، كبديل واعد لوقود الديزل الاحفوري. من بين الخطوات الرئيسية في إنتاج الديزل الحيوي الطحالب المجففة، فإن تجفيف الخلايا وتعطيل جدرانها هما الأكثر استهلاكًا للطاقة و/أو استهلاكًا للوقت. تتطلب خطوة استخراج الدهون الطحلبية، والتي تتم تقليديًا باستخدام المذيبات العضوية السامة التي تلوث الكتلة الحيوية المتبقية، خطوة إضافية لاسترداد المذيب مما يجعلها غير مناسبة للتطبيقات الغذائية أو الصيدلانية. لذلك، تعتبر هذه الخطوات العقبات الرئيسية التي تواجه عملية تسويق الديزل الحيوي الطحالب.

في هذه الدراسة، تم استخدام مذيب قابل للتحويل حراريا (TSS)، مع درجة نفور من الماء قابلة للضبط من خلال تغيير درجة الحرارة ببساطة (ما بين 25 إلى 45 درجة مئوية) لاستخراج الدهون د من الطحالب الرطبة بالتزامن مع إنتاج الديزل الحيوي. من خلال التلاعب بدرجة النفور من الماء لدى المذيب، تم تعطيل جدار الخلية واستخراج الدهون الطحلبية وانتاج الديزل الحيوي مع فصل المنتج في وعاء واحد، مع التخلص من الحاجة إلى خطوة التجفيف المستهلكة للطاقة والكثير من الوقت. للتغلب على المشاكل الحالية التي تصادف المحفزات القلوية التقليدية في تحويل الدهون الى وقود الديزل حيوي، تم استخدام إنزيم مثبت. يتكون TSS المقترح من سائل أيوني (N، N diethyl-N-methylammonium sulfonate)، بوليمر (propylene) glycol (PPG) وماء. تمت مقارنة فعالية العملية المقترحة مع استخدام المذيبات العضوية التقليدية، n-hexane، وغيرها من المذيبات القابلة للتحويل عن طريق تعرضها لثاني أكسيد الكربون، وهي DBU-hexanol و (MEA) -DBU. في نفس الظروف وبرنامج تحويل المذيبات، باستخدام الانزيم المقيد كحافز حيوي، كانت عائدات الديزل الحيوي ٪٤٥.٤±٠.٣٨، ٪٣٧.٨±١.٠٥ و ٪٥.٩±١.٥٠، باستخدام TSS، DBU-hexanol وDBU-MEA على التوالي. أدى استخدام n-hexane في انتاج ضئيل ٪٣.١±٠.٤٣. علاوة على ذلك، تم التحقق من امكانية إعادة

استخدام الانزيم المقيد، واتضح أن انتاج الديزل الحيوي انخفض من ٥٠±١.٤٦٪ في الدورة الأولى إلى ٢٠.٤±٠.٦٠٪ في الرابعة.

تم إجراء دراسة حدية باستخدام منهجية سطح الاستجابة (RSM) لتقييم آثار مدة تعطيل جدران الخلايا ومدة الاستخراج والتفاعل في حدود ٣-٠ ساعات، وكمية الميثانول المستخدمة في حدود ٠.٢-٠.٠٢ مل على إنتاج الديزل الحيوي العائد من 1 غرام من الكتلة الحيوية الرطبة. تم استخدام النتائج لتطوير نموذج إحصائي للتنبؤ بعائد الديزل الحيوي في ظل ظروف مختلفة ولتحسين العملية. تم تقدير الظروف المثلى إلى ٠.٥ ساعة و٣ ساعات و٠.١٥ مل لمدة تعطيل جدران الخلايا ومدة استخراج الدهون والتفاعل وكمية الميثانول على التوالي، حيث كان من المتوقع أن يكون العائد ٧٨.٦٥٪. تم تكرار التجربة في الظروف المثلى، ووجد أن العائد الفعلي هو ٧٥.١١±١.٠٣٪.إن الاستخدام الناجح لـ TSS للاستخراج المتزامن مع التفاعل لإنتاج وقود الديزل الحيوي وفصل المنتج عن الكتلة الحيوية الرطبة له تأثير كبير على تبسيط عملية انتاج الوقود الديزل الحيوي من الطحالب الدقيقة. ببساطة عن طريق تغيير درجة الحرارة، يمكن معالجة النفور المائي لـ TSS، مما يجعل العملية الكلية أكثر سهولة، بالمقارنة مع المذيبات القابلة للتحويل الناتجة عن ثاني أكسيد الكربون. لم يتم الإبلاغ عن أي عملية مماثلة لتلك المقدمة في هذا العمل من قبل.

مفاهيم البحث الرئيسية: المذيبات القابلة للتحويل الحراري، الطحالب الدقيقة، الديزل الحيوي، الاستخراج بالتزامن مع التفاعل.

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Last but not least I would like to express my utmost appreciation to my colleges who offered a great deal of support and guidance through my experimental work.

Dedication

To my beloved parents and family, and specially to the memory of my late sister

Mona

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List of Abbreviations

ABS	Aqueous biphasic system
BBM	Bold's Basal Medium
BMA	Benzyl alcohol amine
[Bmim][PF$_4$]	1-Butyl-3-methylimidazolium hexafluorophosphate
C14:0	Myristic acid
C16:0	Palmitic acid
C18:0	Stearic acid
C18:1n9c	Oleic acid
C18:1n9t	Elaidic acid
C18:2n6c	Linoleic acid
C18:2n6t	Linolelaidic acid
C18:3	Linolenic acid
C20:0	Arachidonic acid
C22:0	Behenic acid
CaCl$_2$·2H$_2$O	Calcium chloride
CaO	Calcium oxide
[CF$_3$SO$_3$]	Tri-fluoromethane sulfonate
CO$_2$	Carbon dioxide
DAGs	Diacylglycerols
DBU	1,8-diazabicyclo-[5.4.0]-undec-7-ene
DMCHA	N,N-dimethyl cyclohexyl amine
DPA	Di-propyl amine

EBA	N-ethyl butyl amine
[Emim][CH$_3$SO$_4$]	1-Ethyl-3-methylimidazolium methyl sulfate
[Emim][DEP]	1-Ethyl-3-methylimidazolium diethyl phosphate
FAAE	Fatty Acid Alkyl Esters
FAME	Fatty acid methyl ester
FFA	Free fatty acid
FID	Flame ionization detector
GC	Gas chromatography
HC	Hydrodynamic Cavitation
HPH	High-Pressure Homogenization
H$_2$SO$_4$	Sulfuric acid
ILs	Ionic liquids
IPCC	International Panel on Climate Change
K$_2$HPO	Di-potassium hydrogen orthophosphate
KH$_2$PO	Potassium di-hydrogen orthophosphate
LCST	Lower critical solution temperature
MAGs	Monoacylglycerols
MEA	Mono-ethanolamine
MgSO$_4$·7H$_2$O	Magnesium sulphate
N$_2$	Nitrogen
NaCl	Sodium chloride
NaNO$_3$	Sodium nitrate
[N$_{1220}$][C$_1$SO$_3$]	*n,n*-Diethyl-*n*-methylammonium methane sulfonate
PPG	Poly(propylene) glycol

SC-CO$_2$	Supercritical CO$_2$
SHS	Switchable hydrophobicity solvent
SS	Switchable solvents
TAGs	Triacylglycerols
TG	Triglyceride
TSS	Thermo-responsive switchable solvent
UCST	Upper critical solution temperature

Chapter 1: Introduction

One of the most critical technical issues facing humanity in the twenty-first century is to provide the world's population with adequate energy to fulfill the lifestyle's needs. The power usage of the present global population of nearly 7.06 billion (July 2015 estimated) people is 20.96 trillion kWh (2015 estimated), and these numbers are estimated to boost to 9 billion and 30 TWh by 2050 [1]. Fossil fuels, which currently account for 65.3 % of global energy supply, will probably not match this increase in demand. These estimated reserves vary from 150 to 400 years for coal, 40 to 80 years for oil and 60 - 160 years for natural gas. According to the international Energy Agency, the production of conventional (easily recoverable) oil already peaked in 2006 [1].

A far more serious concern associated with the use of fossil fuels is the impact on the environment. The main concern in this regard is the emission of greenhouse gasses, in particular CO_2. Since the beginning of the industrial revolution, the CO_2 level in the atmosphere has risen from 280 to 394 ppm and it is currently rising by about 2 ppm/year. According to the International Panel on Climate Change (IPCC), a CO_2 level above 450 ppm carries a high risk of causing global warming by more than 2°C. Such a rise is likely to have a severe adverse impact on ecosystems and human society, with effects that will be felt throughout the century. If the temperature change can be limited to less than 2°C, there is a good chance that society can adapt. Several studies agree that the current decade, between 2010 and 2020, is a critical one. Unless CO_2 emissions are sharply reduced within the next 10 years, exceeding the 450 ppm level seems unavoidable [2]. To reduce the dependence on fossil fuels and curb the exhaust of CO_2, a large-scale transition toward new, sustainable sources of energy. While the

majority of scientists and political figures nowadays agree that such a transition is inevitable, there is much uncertainty regarding the route to follow and the speed at which this should be done. More often, the viability of a particular route is determined by economic factors, instead of technological impediments.

1.1 Sustainable energy sources

Solar energy is the known renewable or sustainable energy since it is available as long as the sun continues to shine. The other major renewable energies are wind, bioenergy, geothermal, hydro, tides, and waves. Wind energy is derived from the irregular heating of the surface of the Earth as a consequence of more heat input at the equator with the accompanying transfer of water and thermal energy by evaporation and precipitation. The third major aspect of solar energy is the conversion of solar energy into biomass by photosynthesis. Animal products such as oil from fat and biogas from manure are derived from solar energy. Another renewable energy is geothermal energy due to heat from the Earth from decay of radioactive particles and residual heat from gravitation during formation of the Earth. Volcanoes are fiery examples of geothermal energy reaching the surface from the interior, which is hotter than the surface. Tidal energy is primarily due to the gravitational interaction of the Earth and the moon. Overall 14 % of the world's energy comes from bioenergy, primarily wood and charcoal but also crop residue and even animal dung for cooking and some heating. This contributes to deforestation and the loss of topsoil in developing countries. Unlike other renewable energy sources, biomass can be converted directly into liquid fuels, called "biofuels," to help meet transportation fuel needs. Biofuels offer an alternative fuel for all types of internal combustion engines running on gasoline, diesel or kerosene, which are used vehicles, ships and airplanes. REmap shows that biofuels, including both conventional

and advanced forms of ethanol and biodiesel, could account for 10 % of transport sector energy use by 2030, more than triple the share in 2016 [2].

Liquid biofuels will be a key pillar of our future transportation infrastructure if shipping and aviation are to be made more sustainable. These modes of transport make up 20 % of total energy demand from transportation and are the fastest growing segments of the transport sector. Therefore, solutions for advanced biofuels will need to be developed.

1.2 Biodiesel

Biodiesel is a renewable and sustainable replacement to petroleum diesel. It is produced from a diverse mix of feedstock including recycled cooking oil, soybean oil, and animal fats. Meeting strict technical fuel quality and engine performance specifications, it can be used in existing diesel engines without modification. The main benefit of biodiesel is that it can be described as 'carbon neutral'. This means that the fuel produces no net output of carbon in the form of carbon dioxide (CO_2). This is because when the oil crops grow, they absorb the same amount of CO_2 as is released when the fuel is combusted.

There are three possible feedstocks for biofuels. First-generation, which is also known as conventional biofuels, are made from vegetable oil. First generation biofuels are produced through well-understood technologies and processes. However, the main disadvantage in first generation biofuels is the competition with food and the high cost of the feedstock. Second Generation biofuels have been developed to overcome the limitations of first-generation. Second Generation biofuels are also aimed at being more cost competitive in relation to existing fossil fuels [2]. However, some biomasses for second-generation biofuels still compete with fresh water and land use since some

of the biomass grows in the same climate as food crops. The use of the waste cooking oil and animal fat from slaughterers is a very good approach because it adds the advantage of waste minimization in addition to the fact that they are cheap. However, they are inconsistent, and the supply is very small. The third generation of biofuels is based on microalgae. The algae are cultured to act as a low-cost, high-energy and entirely renewable feedstock. It is predicted that algae will have the potential to produce more energy per acre than conventional crops. Algae can also be grown using land and water unsuitable for food production, therefore reducing the strain on already depleted water sources. A further benefit of algae-based biofuels is that the fuel can be manufactured into a wide range of fuels such as diesel, petrol and jet fuel.

1.3 Conventional biodiesel production techniques

Biodiesel feedstock consist of triglyceride (TG) and free fatty acid (FFA), which are converted to Fatty Acid Alkyl Esters (FAAEs). The high viscosity of the feedstock prevents them from direct using in diesel engines and causing major issues including high carbon deposition, injection nozzle failure and gum formation [3]. To overcome these obstacles, the feedstock is chemically reduced to its derivative, which have similar properties to petroleum diesels. The most conventional tetchiness of biodiesel production are Pyrolysis, micro-emulsification and transesterification. Pyrolysis involves chemically reducing triglycerides to FAAEs via extreme heat. Micro-emulsification depends on the solvents to physically reduce the viscosity of the feedstock [4]. Transesterification is the reaction of a fat or oil triglycerides (TGs) with an alcohol in presence of a catalyst to form FAAEs and glycerol as a byproduct. Transesterification showed to be the simplest and most efficient route for biodiesel

production against less environmentally friendly, costly and low yield of pyrolysis and micro-emulsification.

A popular process for producing biodiesel by transesterification with methanol to produce fatty acid methyl esters (FAMEs) in presence of a catalyst where each molecule of triglyceride is reacted with three molecules of methanol to produce 3 molecules of Methyl Ester and one molecule of glycerol as shown in Figure 1. Since the reaction is reversible, excess methanol is usually used to shift the equilibrium to the product side.
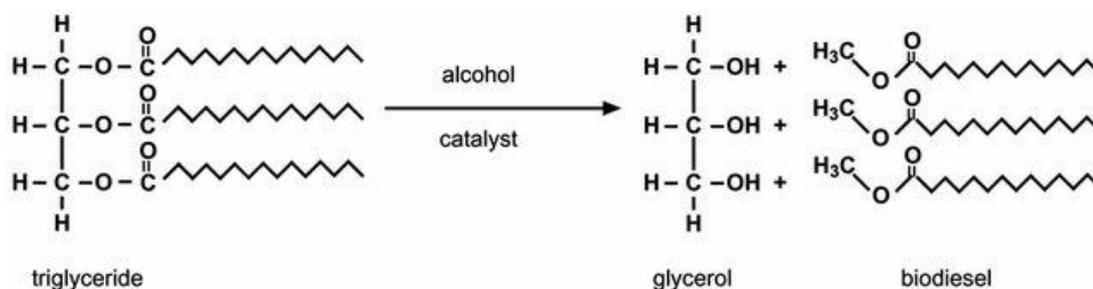
Figure 1: Transesterification of triglycerides with alcohol

The transesterification process is either catalyzed by chemical catalysts, either alkaline or acid, or by enzymes. Alkaline catalysts are more commonly used because of their availability. Alkaline catalysts consist of homogeneous and heterogeneous types. Homogeneous alkaline-catalyzed transesterification is considered economical since the process can be carried out at low temperature and pressure with high yield. However, the use of homogenous catalyst limited to refined fat/oil with less than 0.5 wt % FFA. If an oil or fat containing high FFA is used, the alkaline catalyst reacts with the FFA to form soap, which is highly undesirable excessive soap in the products can drastically reduce the fatty acid methyl ester (FAME) yield and inhibit the subsequent purification process of biodiesel, including glycerol separation and water washing [5].

Numerous researches have been conducted on heterogeneous catalysts to overcome the problems caused by homogeneous catalyst. Most of the heterogeneous catalysts developed for production of biodiesel are either alkaline oxide or alkaline earth metal oxide supported over large surface area [6]. In addition, solid alkaline catalysts, for instance, calcium oxide (CaO) provide many advantages such as higher activity, long catalyst lifetimes, and could run in moderate reaction condition. Although heterogeneous alkaline catalysts are preferable for easier separation process, they still face similar challenges of homogenous alkaline catalyst.

Enzymatic transesterification, especially using lipase has drawn researcher's attention in the last ten years due to the downstream processing problem posed by alkaline catalyst. In contrast, lipase allows the synthesis of specific alkyl esters, easy recovery of the glycerol, and the transesterification of triglycerides with high free fatty acid content under mild conditions [7]. However, one of the common drawbacks with the use of enzyme-based processes is the high cost of the enzyme and the relatively slower reaction rate because of the attachment of reactant (alcohol) and/or byproduct (glycerol) to the enzyme active site which causes the inhibition of the enzyme [8]. In addition to that, enzymes are usually obtained in aqueous form, which makes them hard to recycle where it needs multi separation steps which is time and energy consuming [9].

The transesterification process can be done in either solvent free or with addition of the solvent. In the enzymatic biodiesel production, the addition of solvent has a positive effect in the transesterification process as the solvent helps in the solubility of hydrophilic alcohols and hydrophobic of triglyceride. Solvent is used in enzymatic

assisted transesterification because of its ability to increase the reaction rate and decrease the inhibitory effect of alcohol on the enzyme [8].

Immobilization is a robust tool to enhance enzyme stability. In the last few years, production of biodiesel via immobilized lipase has drawn huge attention. An immobilized enzyme is defined as the enzyme physically confined to a certain defined region while retaining its most catalytic activity. Immobilized lipase has many advantages over the free lipase, especially for large-scale industrial applications, which include easy product separation, reusability of the enzyme which lowers the cost, simple glycerol recovery, improved lipase stability, and the adaptability for continuous operation [7].

Numerous immobilization techniques have been used in recent decades. Lipase immobilization method can be categorized in five different approaches: adsorption, covalent bonding, cross-linking, entrapment, and encapsulation, as shown in Figure 2. Among all available, methods, adsorption is the most favorable, as it is simple and cost-effective [9]. The major two setbacks are the excess methanol deactivates the enzyme and the viscos glycerol that is deposited on the pores of the immobilized. To minimize the effect of methanol inhibition, organic solvents, such as n-hexane, have been proposed. The addition of a solvent reduces the medium viscosity and enhances the mass transfer [10]. Hydrophobic organic solvents are favored compared to other organic solvents because they permit aggregation of water molecules around the enzyme which explains the improved activity [11]. Study has shown that the pre-treatment of enzyme using organic solvent might increase the yield of FAME by 50 % more compared to atmospheric condition as compared to solvent [12]. However, the

addition of the solvent faces many obstacles, one of which is the separation process of the solution from the medium as well as the hazardous nature of the solvent.
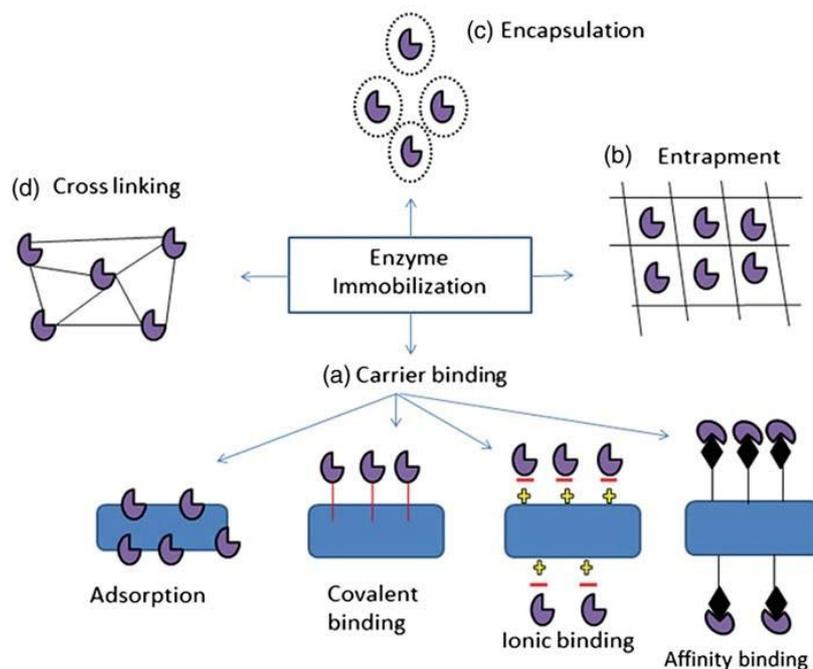
Figure 2: Different techniques for enzyme immobilization [7]

1.4 Biodiesel production from microalgae

Microalgae have emerged as a potential feedstock for biodiesel production. They are prokaryotic or eukaryotic photosynthetic microorganisms that can grow rapidly and survive harsh conditions due to their unicellular structure. They grow by photosynthesis, converting solar radiation into chemical energy, completing an entire growth cycle every few days [13]. Cyanobacteria (Cyanophyceae) are examples of prokaryotic microalgae, whereas green algae (Chlorophyta) and diatoms (Bacillariophyta) are examples of eukaryotic ones.

Numerous species of microalgae with high lipid content have been used for biodiesel production due to the convenient cultivation technique with less freshwater and land needs. Recently, scientific development have been carried out on microalgae biodiesel

production process to bring the process closer to the threshold of becoming economically feasible via improved performance of the production steps [14]. The main steps of producing biodiesel from microalgae includes cultivation, harvesting, drying, lipids extraction and transesterification and produced purification. Effective harvesting of cultured biomass from the growing medium and the extraction of lipids from the collected biomass are among of the major challenges facing the commercial microalgae to biodiesel process. Centrifugation, flocculation and sedimentation are the conventional technique used for harvesting the cultivated biomass. Before lipid recovery from the cultivated microalgae cells, a drying stage is needed. Sun drying is most frequently used because it does not require an external energy. However, this process is time-consuming with a very low drying rate. Using energy-intensive drying processes, such as spray drying, although is faster, but they are generally expensive and could lead to deformation in lipid structure and protein-rich residual biomass [15]. for example, it was reported that the drying stage is responsible for 89 % of the required power input and 70 % of the total production cost [16]. It was also reported that 25 % reduction in energy can be attained by using wet extraction method, due to the elimination of the drying step [17]. In another study, a more drastic effect has been reported, in which the energy needed to produce 1 kg of biodiesel from dewatered biomass was projected to be 4000 times higher than that produced from wet biomass [18]. The drying step is therefore considered a major obstacle for taking algae-based biodiesel to the industrial scale [19].

Hence, it is essential to develop a cost-effective and energy-efficient process that eliminates the need for the drying step, and allows the extraction of oils from wet biomass. Such a process can solve major technical and economic obstacles facing the conventional microalgae to biodiesel production techniques.

1.5 Microalgae lipids extraction

Each species of microalgae has its own lipid content. In addition, the composition and fatty acid profile of lipids obtained from one type of microalgae is influenced by the cultivation conditions, such as temperature, medium composition, illumination intensity, ratio of light/dark cycle and aeration rate [20]. Microalgae lipids are classified based on the polarity of the molecular functional group as: (1) polar lipids, which can be sub-classified into glycolipids and phospholipids and (2) neutral lipids (non-polar) which are made of free fatty acids and acylglycerols. Acylglycerol comprises of fatty acids that are bound to a glycerol backbone via ester-bonds. Depending on the amount of fatty acids chains, they can be categorized as monoacylglycerols (MAGs), diacylglycerols (DAGs) and triacylglycerols (TAGs). Neutral lipids, also known as storage lipids, are formed by microalgae for energy storage. Neutral lipids are linked by relatively fragile non-covalent bonds such as Van der Waals or hydrophobic association via their hydrocarbon bonds to the hydrophobic areas of microalgae proteins and to other lipids [21], which makes them relatively easy to extract due to this week bonding. On the other hand, polar lipids are component of cell membrane molecular structure. These lipids are more difficult to extract because they can form hydrogen and covalent links with neighboring molecules. They contain non-polar lipids without fatty acids such as sterols and ketones that cannot be transformed to biodiesels [20].

The proximate analysis of three fresh-water microalgae strains, namely *Chlorella vulgaria, Chlamydomonas reinhardtii*, and *Scenedesmus sp.* and two saltwater strains, namely *Nannochloropsis sp. and Schizochytrium limacinum* were examined [22]. The cells were freeze-dried before the lipids were extracted using chloroform-methanol (2-1) solvent system combined with ultrasonication to disrupt the cell walls. As shown in

Table 1, *Schizochytrium limacinum* showed the highest lipid content of 57 % and *Scenedesmus sp.* showed the lowest of only 11 %. C. *vulgaria* and *Nannochloropsis sp.* had lipid content in the range of 18−25 %, which is comparable to conventional oil crops, such as soybeans [8]. While having the highest lipid content, *Schizochytrium limacinum* showed the lowest protein content of 12.4 %. The other strains showed a higher protein content in the range of 24-34 %. *Scenedesmus sp.* had an extremely high ash concentration, reaching up to 30 %, whereas the other strains had ash contents in the range of 5-11 %.

Table 1: Microalgae biomass composition on a dry weight basis

Strain	*Chlamyd omonas*	*Chlorella vulgaria*	*Nannochloropsis sp.*	*Scenedesmus sp.*	*Schizochytrium limacinum*
lipid %	24.1	17.9	25	10.5	56.7
Protein %	34.2	28.2	32.2	24.6	12.4
Ash %	6.1	10.5	5.5	29.5	5.6
Carbohydrate %	35.5	43.4	37.3	35.4	25.3
Ref	[22]	[22]	[22]	[22]	[22]

The lipids composition of the strains was also examined, and the results are shown in Table 2. *Nannochloropsis* showed the lowest amount of neutral lipids of (15 %), but the highest amount of polar lipids (25 %). *Schizochytrium limacinum* was mainly composed of TAG (78 %) with less than 1 % polar lipids. All green microalgae had appreciable amounts of chlorophylls (6 – 17 %) and USP (13 – 19 %). *Chlamydomonas reinhardtii* and *Chlorella vulgaria* had similar lipid compositions in terms of neutral lipids (51 – 57 %), USP (13 %), and chlorophylls (15 – 17 %). Freshwater species were found to contain 27 – 31 % FFA, which was attributed to lipid degradation during storage and processing rather than the algae responding to the change of or detrimental growth condition [23]. The lipid composition of microalgae does not only change from one strain to another, but even for the same species, the compositions of fatty acids

and complex lipids in algae heavily fluctuate depending on growth conditions such as light, temperature, nitrogen level, salt stress and the growth stages at which they are harvested [24].

Table 2: Microalgae lipid composition (wt%)

Strain	*Chlamydomonas reinhardtii*	*Chlorella vulgaria*	*Nanno- chloropsis*	*Scened- esmus*	*Schizochytrium limacinum*
Neutral lipids	51.3	57.2	14.5	13.5	78.2
TAG	24.5	26.6	8.6	4.1	77.5
FFA	26.8	30.6	5.9	27.4	0.7
Polar lipids	9.7	0.7	24.6	0.7	0.9
USP	13.1	13.2	14.6	18.7	1.9
Chlorophyllides	16.8	14.6	5.8	14.3	-
others	9.1	14.3	40.5	34.8	19.1
Ref	[22]	[22]	[22]	[22]	[22]

1.5.1 Microalgal cell wall composition and structure

The resilient cell wall structure of microalgae is a major obstacle that limits the industrial production of algal biodiesel. Just like other plants, microalgae cell wall is generally trilaminar; an organized microfibrillar structure embedded in a continuous matrix [25]. However, microalgae cell wall has a higher protein content when compared to other plants, majority of these proteins consist of glycoprotein. The cell wall structure and composition vary from one species to another and can be used as an identifying indicator for its taxonomy. Most microalgae species contain algaenan in their outer cell wall structure, which is a nonhydrolyzable hydrocarbonaceous, which is resilient biopolymer [26]. Algaenan consists of unsaturated ω-hydroxy fatty acids, which are connected to each other by different types of chemical bonds like glycosidic, ester and ether bonds [27]. Algeanan presence in the cell wall structure enhances the durability of the microalgae against degradation by different cell wall disruption techniques. Algaenan is found in species belonging to the *Trebouxiophyceae* and

Chlorophyceae of the *Chlorophyta* [28], such as *Chlorella sp.*, *Tetraedron sp.*, *Scenedesmus sp.* and *Ryocococcus* [29]. As shown in Table 3, algaenan is found in the cell wall of *Chlorella species*, e.g., *C. minutissima, C. zofingiensis* and *C. homosphaera*, Chloroidium, e.g., *C. ellipsoideum*) [30] and *Scenedesmus* [18]. Nevertheless, trilaminar structure is not found in all algenan producing species and the existence of algaenan is not merely indicated by a trilaminar structure [29]. As shown in Table 3, even species that belong to the same taxonomic class may have different cell wall structure and composition. For example, absence of algeanan was reported in *C. saccharophilum* extracellular matrix [29], whereas it is present in *C. ellipsoideum* which belongs to the same taxonomic class [31].

Besides algaenan, cellulose also present in the cell walls of microalgae, reaching in some species up to 70 % per cell wall weight, such as in *C. zofingiensis* [32]. Simple sugars, such as glucose and xylose are also present in some cell walls. A large concentration of these sugars adds stiffening of the cell walls [33], which supports cells structure [34]. For example, the cell walls of *T. suecica* and *T. striata*, was found to contain several sugars, such as arabinose, galactose, mannose, rhamnose, rhamnose, and xylose [35]. These complex sugars creates a rigid wall to intercellular content extraction [36].

The broad variety in the structure and composition of the cell wall among the various species of microalgae highlights the urge to classify the cell wall in order to understand the impact of different cell disruption technique on the microalgae. This is essential in optimizing the extraction of microalgae's intracellular value-added products, which would enhance the process economically.

Table 3: Overview of the diversity of microalgal cell wall structure and biochemical composition based on taxonomic class

Microalgae species	Taxonomic class	Cell size	Cell wall structure	Cell wall composition	Ref.
Chlorella zofingiensis	Trebouxiophyceae	2-4 µm	Glucosamine-rigid wall	Cellulose, glucose, xylose	[37]
Chlorella vulgaris	Trebouxiophyceae	3-4 µm	Lacks trilaminar structure	Extracellular polysaccharides Rhamnose, galactose, xylose	[32]
Scenedesmus	Chlorophyceae	10-12 µm	Non-hydrolyzable algeanan structure	Crystalline glycoprotein, algeann	[29]
Chlorella minutissima	Trebouxiophyceae	2-4 µm	Algeanan trilaminar structure	Algeanan	[30]
Chloroidium ellipsoideum	Trebouxiophyceae	7-5 µm	Outer non-trilaminar layer, an inner microfribrillar layer	Glucose-mannose rhamnose, galactose	[38]
Chloroidium saccharophilum	Trebouxiophyceae	6–16 µm	Algaenan trilaminar structure	Algaenan	[31]
Tetraslemis suecica	Chlorodendrophyceae	10–25 µm	Scales	Extracellular polysaccharides	[35]
Tetraslemis. striata	Chlorodendrophyceae	10–25 µm	Scales	Extracellular polysaccharides	[35]

1.5.2 Cell wall disruption techniques

As mentioned earlier, microalgae cells show high resistance to mechanical and chemical stresses due to their tough cell walls. To be able to extract the lipids, and other valuable cell components, the cell wall needs to be disrupted and several attempts have been made to find out viable methods to achieve that. These methods are classified as mechanical, chemical and biological methods. Although, cell disruption has been performed on both dry and wet biomass, recently attention has been focused

more on utilizing wet biomass to eliminate the energy costs incurred due to the drying step.

1.5.2.1 Mechanical disruption

As the name suggests, in this type of disruption energy inputs in the form of electrical pulses, waves, heat, and shear forces are applied. These processes require large amounts of energy, but result in high yields in processes that can easily be controlled and scaled to the requirement. Due to the intensive energy inputs however, the advantages and disadvantages of large-scale microalgae production should always be considered. Key parameters that affect the process include type and concentration of the microalgae cells and the intensity of energy input. Due to extremely harsh conditions encountered, some of these methods are not suitable to extract sensitive compounds, such as proteins. High pressure, shear stress, and temperature can harm the intracellular compounds, limiting the use of those methods to lipids extraction only. The energy consumption can be reduced using a hybrid process, in which the mechanical method is combined with a non-mechanical method to increase the disruption efficiency.

1.5.2.1.1 Bead milling

Due to its high efficiency in single-pass operations, low labor requirements, and easy scale-up setups, bead milling is considered of great potential for industrial applications. In this process, a tangential force is applied to the cell-wall causing disruption. The movement of solid beads at really high-speed causes sudden compression that disrupts the cells [47]. This process is The fragility of the cell-wall of *Nanochloropsis sp.* was examined by flowing a culture suspension through a high-pressure disrupter based on bead milling to measure the fraction of disrupted cells after

the flow [39]. As shown in Table 4, the results were very encouraging and showed a 98 % cell disruption at a pressure of 1,750 bar. Therefore, the process can be considered a good option for lipid extraction in a wet environment. The high-energy requirement and the amount of heat generated during the process however, are major hurdles facing its application.

Table 4: Different approaches for cell disruption

Strain	Cell-disruption method	Cell-disruption condition	Extraction method	Efficiency	Ref.
Nannochloropsis oculata	Bead milling	bead milling under high pressure (1,750 bar)	chloroform, methanol	98 %	[39]
Chlorella saccharophila	High-pressure homogenization	homogenization at 200 to 1,000 bar	t-butanol, ammonium sulfate	89.9 %	[40]
Nannochloropsis salina	Hydrodynamic cavitation	hydrocavitation (1.27 kW), autoclave 5 kW	hexane	97 %	[41]
Chlorella sp.	Ultrasonication	20 kHz, 0.8 KWh (5 min, 18 to 60°C)	ethanol, dimethyl sulfoxide	75 %	[42]
Scenedesmus sp.	Microwave	80-95°C for 30 minutes	chloroform/methanol	76-77 %	[43]
Nannochloropsis oceanica	Steam explosion	steam at set pressure (1.0 to 2.1 MPa), 0.1 s pressure release for 5 min	hexane:isopropanol	76.5 %	[44]
Chlorella vulgaris	Acid	1 % H_2SO_4 (120°C, 60 min)	hexane/methanol	93.5 %	[45]
Chlamydomonas reinhardtii	Osmotic shock	NaCl or sorbitol (60 g/L)	chloroform/methanol	91 %	[17]
Nannochloropsis oceanica	Enzymatic lysis	cellulose, lipase, protease	hexane	83 %	[46]

1.5.2.1.2 High-pressure homogenization

High-Pressure Homogenization (HPH) are also scalable and can be applied to highly concentrated (20 - 25 % w/w) algal pastes [48]. Microalgae having recalcitrant cell

walls are the best candidates for the cell disruption through HPH. During the HPH process the cell suspension is allowed to flow through a small opening, where turbulence, shear stress, and cavitation stimulate cell lysis. Optimal HPH cell disruption is based on the loading pressure and other properties of cell-suspension, such as viscosity, cell concentration and cell size [41]. The loading pressure helps in increasing the force of impact, which results in an efficient cell disruption and helps in the release of intracellular components. At a pressure of 800 bar and 10 cycles, found to be the optimal, 89.9 % of lipid in *Chlorella saccharophila* was recovered [40].

1.5.2.1.3 Hydrodynamic cavitation

Hydrodynamic Cavitation (HC) occurs by creating cavities inside a homogenous liquid medium by the generation of microbubbles. Once the pressure drops below the vapor pressure, at the vena contracta, these microbubbles are formed, which then collapse once the pressure returns to values above the vapor pressure. The microbubbles collapse generates shock waves that increase the pressure and temperature causing cells disruption [41]. HC treatment was applied to *Nanochloropsis salina* to disrupt the cell-wall for enhanced lipids extraction [41]. At a specific energy input of 500 - 10,000 kJ/kg, a high lipid-recovery recovery, of 97 % was achieved, which was higher than that achieved using ultrasonication (5.4 - 26.9 %). However, to achieve this high yield, the energy required for HC (1.27 kW) was almost double that needed for ultrasonication (0.75 kW). In addition, HC process requires a sufficient cooling system to counter the high energy consumption and heat generation. Above that, for an industrial scale application, a facility is needed to ensure the application of concentrated algal biomass and to sustain high fluid velocity,

necessary not only for the cavitation effect but also to prevent the blockage of vena contracta.

1.5.2.1.4 Ultrasonication

The creation of jet streams in the surrounding medium during the propagation of shockwave causes cell disruption by shear forces [47]. *Chlamydomonas reinhardtii* was disrupted by Ultrasonication to enhance the lipids extraction by hexane. The cells were disrupted within 10 or 30 s using a bench-scale sonication at amplitudes of 16 to 160 µm [49]. To achieve a maximum cell disruption, an energy input of 80 J/mL was necessary. Ultrasonication was also found to be effective for lipids extraction from *Chlorella sp.* [42], achieving 75 % cell-disruption efficiency utilizing 0.8 kWh energy per liter under 20 kHz. and 1 kW sonic processor conditions. Due to reduction of energy within the medium because of the increase in viscosity with higher cell concentrations, a relatively high amount of energy was required. The resulting heat generation from such an intensive energy process requires strict temperature control.

1.5.2.1.5 Microwave treatment

Microwave (MW) treatment is a non-contact, high efficiency method that consumes less energy and takes less processing time. By this treatment, the pectin and cellulose structures in the cell walls are damaged. *Chlorella sp.* cells were subjected to microwave. treatment for 20 min, the wall's pore diameter increased from 0 0.005 to 0.18 µm [43]. With MW treatment at 1.2 kW and 2,450 MHz 77 % of the total recoverable lipids were extracted within 30 min from *Scenedesmus obliquus* in water suspension using chloroform:methanol (1:1, w/w) as solvent. Despite its numerous benefits, similar to other mechanical methods, MW treatment is energy intensive and results in increasing the temperature [43].

1.5.2.1.6 Steam explosion

In steam explosion, the cells are subjected to steam at high temperatures and pressure for few minutes, before being suddenly depressurized to room temperature, resulting in cell-wall disruption. Under pressures ranging from 1 to 2.1 MPa, steam explosion was used to disrupt wet *Nannochloropsis oceanica* cells, resulting increased surface pore area [44]. When hexane/isopropanol (1:1 v/v) solvent was used at 60°C for lipids extraction, 76.5 % recovery was achieved. Having said that, the required high temperature and pressure make this process economically unfeasible.

1.5.2.1.7 Freeze drying

Microalgae cell wall disruption by freeze-drying is achieved by intracellular water expansion. This is a common technique used to recover protein cells. By freeze drying, the extraction of lipid from microalgal biomass resulted in yields in the range of 30 and 45 %, which is lower than other mechanical methods [20]. However, by freeze-drying, the rapid rise temperature, which negatively impacts the quality of high value extracted products, can be avoided. Nevertheless, in addition to the lower lipids yield using freeze drying, the process is energy intensive.

1.5.2.2 Chemical disruption

Numerous chemicals, such as salts, acids, solvents and detergents have been investigated for microalgal cell-wall disruption. These agents effectively disrupt microalgal cell-wall structure.

1.5.2.2.1 Acid disruption

Acid hydrolysis of sugar polymers in cell walls is the basis of the hydrothermal acid treatment, which has been successfully applied to break the cellulosic structure of cell

walls. Sulfuric acid (H_2SO_4) has been the most commonly used acid, because of its high efficiency and low cost [41]. Extraction under 1 % sulfuric acid was tested to extract lipids from wet *Chlorella vulgaris* at 120°C. Within 60 min of treatment, lipid-extraction yield of 33.7 % of the dry biomass was achieved [45]. Despite its effectiveness, using corrosive acids require proper reactor and process design, material selection, safety consideration and wastewater treatment.

1.5.2.2.2 Osmotic shock

The osmotic shock cell wall disruption is achieved by the addition of salt, such as sodium chloride. The technique at a NaCl concentration of 2 % (w/v) was used to enhance lipids extraction from wet *Chlamydomonas reinhardtii* [17]. The lipid - recovery yield from was increased as a result of the osmotic shock by a factor of two compared to that achieved in NaCl-less control. Compared to other disruption methods, the salt addition can be relatively scalable and simple process. Nevertheless, the recovery/clean-up of the salts is expensive. In addition, different microalgal species have different metabolic mechanisms of acclimation/adaptation to osmotic stresses the osmotic salt effect.

1.5.2.3 Biological disruption

Microalgae's rigid cell walls can be disrupted by biological treatments as well, including algicidal treatment or lysis enzymes. The main benefits of biological cell disruption techniques are their biological characteristics such as mild operation temperature and low energy consumption.

1.5.2.3 Enzymatic lysis

To enhance the disruption of the rigid cell walls of microalgae, lysis enzymes like lipase, protease and cellulase have been used [20]. A combinational enzymatic/thermal

lysis process were developed for wet *Nannochloropsis oceanica* biomass to facilitate aqueous lipid extraction High product-recovery efficiencies of 88.3 % and 62.4 % of the total available lipids and proteins, were achieved respectively under three enzymes cocktail (lipase, protease and cellulase) [46]. However, there are still many challenges facing the enzymatic lysis process, which hinder its large-scale implementations. These challenges include the high cost of enzymes, slow reaction time and low enzyme stability.

1.5.3 Lipid extraction from microalgae

1.5.3.1 Physical extraction

The most popular technique used in oils extraction from oilseeds is mechanical squeezing or oil expellers. The biomass mechanically pressed resulting in the extraction of the lipids, causing the biomass to heat up in the process due to friction, which further aids the lipid extraction. Although, oil expellers are simple and suitable for continuous operation, the recovery efficiency of commercially feasible expellers is generally around 75 %. However, to achieve the 75 % efficiency, the biomass has to be subjected to a costly and energy intensive process of drying of up to 95 % dry weight [50]. Screw expeller press was successfully used to recover 68.5 % of the lipids content in filamentous algae without using solvent extraction [51]. However, large amounts of solvent would be required to recover the residual 31.5 % lipid in the formed cake.

1.5.3.2 Solvent extraction method

As mentioned earlier, for effective extraction of lipids from microalgae, the rigid walls of the harvested cells need to be disrupted to open the structure and allow the solvent reaching the lipids. The suitable solvents to dissolve the lipids are 1-butanol, *n*-hexane,

dimethyl ether and DBU (1,8-diazabicyclo-[5.4.0]-undec-7-ene) [52]. Among these hydrophobic solvents, the most commonly used in extracting lipids from microalgae is *n*-hexane [53]. However, the extraction efficiency of this organic solvent is still relatively low, since it is not effective in releasing non-polar lipids from the complex formed with the polar lipids within the cytoplasm of the cells [20]. Due to this restriction, only a part of the polar lipids is obtained. On the other hand, polar lipids in biological membranes are in close contact with the solvent, requiring the existence of membrane wetting mediums, such as a polar solvents to achieve an effective extraction. These constraints resulted to the emergence of co-solvent (hydrophilic/hydrophobic) systems for lipid extraction.

Possible solvents mixtures that can be used for lipids extractions are n-hexane with ethanol, isopropanol or 2-propanol, and chloroform with methanol. Solvents mixtures, namely acetone / chloromethane (1:1), hexane / isopropanol (3:2) and chloroform / methanol (2:1) were tested for extraction of lipids from *Botrycoccus braunii* microalgae with the aid of bead milling, and chloroform/methanol (2:1) solvent achieved the highest lipid yield of 28.6 % within 2 hours [41]. Different solvent extraction methods of lipids from *Pavlova sp.* microalgae with and without pretreatment methods have been investigated [53]. It was found that the highest extraction yield of 44.7 % was achieved using ethyl acetate/methanol solvent within 3 h with ultrasonication as a pretreatment technique. Using single solvent, namely n-hexane, in Soxhlet extraction system for 15 h with bead beating for cell disruption, the extraction yield did not significantly increase.

Two solvent system, namely chloroform-methanol (2-1) and hexane-methanol (3-2) were tested for lipid extraction from *Nannochloropsis sp.* with sonication for cells

disruption. It was found that the efficiency of lipid extraction could be significantly improved by properly disrupting the cell walls, which make it easier for the solvent system to extract the lipids. As shown in Table 5, the only significant difference between the two tested solvents mixture was with using sonication as a pretreatment method, where *n*-hexane-ethanol achieved 23 % and chloroform-methanol achieved 35 %.

Although, the extraction was less efficient than other methods, this technique was much faster and was completed within 5 min. As shown in Table 5, the significance of using co-solvent system can be seen by comparing the oil extraction yield using hexane with methanol as a solvent, which was double that achieved using hexane alone in Soxhlet extraction. However, the use of organic solvents is not recommended, as they have high toxicity and volatility, making them hazardous to use [20]. In addition, they require additional solvent separation unit for their recovery and reuse. Therefore, the focus of research has recently been on finding greener solvents, which can affectively be used for lipids extraction.

Table 5: Different solvent systems for lipid extraction from microalgae

Strain	Cell disruption method	Solvent used	Extraction parameters			Ref.
			Time	Temp.	yield %, of dry weight	
Pavlova sp.	Ultrasonication	Ethyl acetate/methanol	3 h	25°C	44.7	[53]
Pavlova sp.	Ultrasonication	Soxhlet n-hexane	15 h	25°C	13.5	[53]
Pavlova sp.	Bead-beating	Soxhlet n-hexane	15 h	25°C	15.3	[53]

Nannochloropsis sp.	Ultrasonication	Chloroform-methanol (2-1)	12 h	25°C	35	[54]
Nannochloropsis sp.	Not-specified	Soxhlet chloroform-methanol (2-1)	6 h	25°C	34.3	[54]
Nannochloropsis sp.	Ultrasonication	Hexane-methanol (3-2)	12 h	25°C	23	[54]
Nannochloropsis sp.	Not-specified	Soxhlet hexane-methanol (3-2)	6 h	25°C	31.6	[54]
Scenedesmus sp.	Freeze-dried	Soxhlet n-hexane	8 h	25°C	21.1	[55]
Scenedesmus sp.	Lysosome	n-hexane	12 h	25°C	16.6	[55]
Pavlova sp.	Bead-beating	SC-CO_2	6 h	60°C, 300 bar	17.9	[53]
Chlorococcum sp.	Not-specified	SC-CO_2	1.3 h	30°C, 303 bar	7.1	[50]
Scenedesmus sp.	Lysosome	SC-CO_2	30-60 min	50°C 500 bar	12.5	[55]
Chlorella vulgaris	None	[Emim][DEP]	2 h	120°C	25	[56]
Chlorella sp.	None	[Emim][CH_3SO_4]	18 h	65°C	22.5	[57]
Botryococcus braunii	Freeze-dried	DMCHA	18 h	60-80°C	22	[58]
Nannochloropsis gaditana	None	DMCHA	24 h		29.2	[59]
Tetraselmis suecica			24 h		57.9	
Desmodesmus communis			24 h		31.9	
Chlorella sp.	None	DMCHA	3h	35°C	47.5	[60]

1.5.3.3 Supercritical CO_2 extraction

The use of chemical solvents, such as n-hexane, has several drawbacks, which include the leftover biomass contamination with the solvent, long extraction time and the need of additional separation units. These drawbacks can be overcome by using Supercritical CO_2 (SC-CO_2) extraction. SCCO_2 extraction is a much faster and more efficient process compared to solvent extraction. In addition, it results in a greater selectivity towards triglycerides and the separation process of the solvent ycan be easily achieved by simple reduction of the pressure [55]. Numerous studies have shown promising results with SC-CO_2 lipid extraction from microalgae. For example, 7.1 wt% of dry the *Chlorococcum sp.* without any pretreatment technique was

achieved within less than 80 min using SC-CO_2 as a solvent, at 30°C and 303 bar. However, 5.5 h were needed to achieve a similar yield using Soxhlet extraction and hexane as a solvent [50]. The effect of the addition of ethanol to SC-CO_2 to enhance the lipid extraction from A*rthrospira maxima* was tested. It was shown that the addition of the polar component enhanced the extraction yield from 32 % without the co-solvent, reaching 40 % with it at 345 bar and 60°C [61] .

As shown in Table 5, using Soxhlet extraction with *n*-hexane, total lipid extraction yield form *Scenedesmus sp.* was 21.1 % per dry weight of the biomass achieved within 6 hours and freeze-drying as cell disruption method. Using *n*-hexane in static system, the extraction yield dropped to 16.6 % per dry weight within 12 hours. With SC-CO_2 and enzymatic cell disruption using lysosome, the extraction yield dropped further to 12.5 % per dry weight within 1 h. [55]. Although, the lower yield achieved using SC-CO_2, this extraction method was still superior in terms of the extraction time and environmental impact. Having said that, the high costs associated with the high pressure of the SC-CO_2, renders the overall process costly [62].

1.5.3.4 Ionic liquids

Recent studies have focused in ionic liquids (ILs) as a greener solvent for lipid extraction from microalgae, since they have a negligible vapor pressure and are less toxic than organic solvents [63]. Furthermore, ILs can be designed to have a higher selectivity towards desired lipids, which cannot be achieved using organic solvents. They can hence be targeted to selectively extract triglycerides, while minimizing the co-extraction of undesired compounds, such as pigments and phospholipids, which do not contribute to the biodiesel production.

Similar to organic solvents, full drying of cells that is expensive and energy intensive process, is crucial for efficient extraction of lipids from microalgae using hydrophobic ILs. To eliminate the costly process of drying, while maintain an efficient lipid extraction from wet biomass, hydrophilic ionic liquids , which contain hydrophilic anions such as [HSO$_4$], [CF$_3$SO$_3$] or [Ac$^-$] have been suggested to disrupt the cells. However, these hydrophilic ILs do not dissolve the lipids, and are solely used for the disruption of the microalgae tough cell walls [63]. Hydrophobic solvents, including hydrophobic ILs would still be needed to extract the oils, after the cell disruption. ILs extraction technique was investigated for lipid extraction from wet *Chlorella vulgaris* using [Emim][DEP] at 120°C, achieving lipid yield of 25 % per dry biomass within only two hours [56]. This yield was 40 % higher than the that achieved using a mixture of n-hexane and methanol (7:3 v/v) for 12 hours.

The effect of adding different polar solvent with IL was investigated to enhance the lipids extraction from microalgae [57]. The polar solvent [Emim][CH$_3$SO$_4$] mixed with different co-solvents were examined as an extraction solvent system of lipids from *Chlorella sp.* with water content of up to 70 % at 65°C. The highest achieved lipid extraction yield was 75 % using [Emim][CH$_3$SO$_4$] with methanol at a 1:1.2 *(w/w)* ratio within 18 hours. The main obstacle facing the commercializing of ILs in lipids extraction is the high cost of the ILs compared to the conventional solvents. For example, the cost of [Bmim][PF$_6$] is ten times higher than *n*-hexane. Hence, in order to make ILs economically favorable, it should be recycled and reused for at least 10 cycles.

1.6 Switchable solvents

Switchable solvents (SSs) are solvents capable of reversing their properties, such as polarity, conductivity, viscosity or solubilizing capability from one form to another [64]. SSs have several advantages over conventional solvents as a reaction medium and in separations and extractions, especially when there are multiple steps involved in the process. In these kind of systems, the solvent used in one step has to be fully removed prior to the next step that require a solvent with other properties than those of the first one. This makes the overall process energy intensive, economically unfavorable and may result in environmental waste production.

The first reported SS was that composed of an alcohol, 1-hexanol and an amidine, 1,8diazabicyclo-[5.4.0]-undec-7-ene (DBU), which were equimolarly mixed [64]. The hydrophobic solvent became hydrophilic by passing CO_2 at ambient pressure and temperature, and the equimolar mixture of DBU-1hexanol transformed to the ions $DBUH^+$ and RCO_3^- as shown in Equation (1).

$$\text{(1)}$$
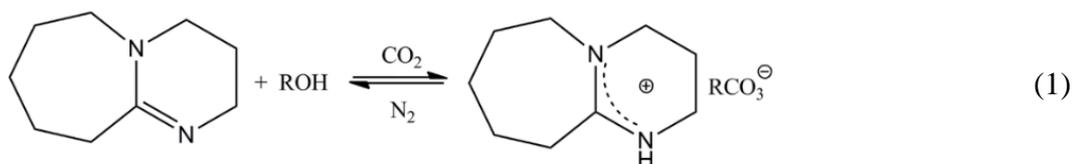
The changes in the physical properties, such as viscosity, miscibility polarity and conductivity, made the solvent technically an ionic liquid. Interestingly, by exposing the formed IL to an inert gas such as N_2 gas, the CO_2 stripped off, and the solvent returned back to the original mixture to its initial state, as shown in Figure 3 [64].
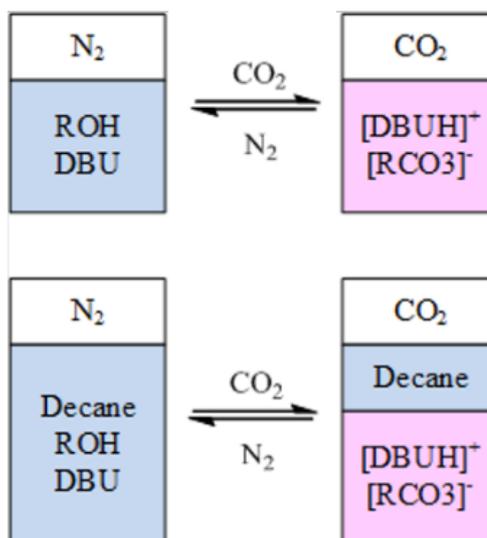
Figure 3: Switchable mechanism of alcohol/amidine (guanidine) mixtures [20] showing the miscibility of decane with the hexanol/DBU mixture under nitrogen, and phase separation of decane once the solvent mixture becomes polar in the presence of CO_2

The exposure of the switchable solvent to CO_2 creates a significant increase in the viscosity of the generated IL. The final viscosity depends on the alcohol used [58]. Therefore, the selection of the alcohol is crucial to maintain an adequate viscosity for optimum extraction efficiency. For instance, when an equimolar mixture of DBU and ethanol, methanol or water is exposed to CO_2 a solid DBU alkyl carbonate salts is formed at room temperature. However, when DBU is combined with a longer alkyne chain, the exposure to CO_2 produces a viscous hydrophilic liquid at room temperature.

1.6.1 Amidines

Switchable hydrophicity solvents (SHS) are a unique class of SS comprised of a single component, such as N,N,N'-tributylpentanamidine. Similar to other SSs, SHSs change their polarity when exposed to CO_2 switching to hydrophilic and switch back to hydrophobic by N_2, making them a suitable choice for lipid extraction and separation [65].

1.6.2 Secondary amines

Similar to amidines, some secondary amines can also operate as switchable solvents with CO_2 as a stimulus. Secondary amines are generally cheaper than amidines and have a lower polarity. Furthermore, their sensitivity to water molecules are significantly lower than DBU/alcohol system [66]. In order to classify a solvent system as a switchable solvent, the carbamate and amine states must be in liquid phase and show a substantial polarity shift. Majority of liquid amines such as primary alkyl amines, allyl amine, benzyl amine, pyrrolidine, and piperidine however transform into solid carbamates [66], whereas some secondary amines form liquid salts at room temperature, and those are the ones of interest. Within the secondary amines, methyl-propyl amine, ethyl methyl amine, and di-ethylamine are less favored because they are highly unstable and extremely flammable. Benzyl alcohol amine (BMA), N-ethyl butyl amine (EBA), N-ethyl propyl amine (EPA) and di-propyl amine (DPA) are more favorable, and their switching is described by Equation (2) [66].

$$NHR_2 + H_2O \underset{N_2}{\overset{CO_2}{\rightleftharpoons}} R_2NCOH \underset{N_2}{\overset{NHR_2}{\rightleftharpoons}} \left[R_2NH_2^{\oplus}\right]\left[^{\ominus}OCNR_2\right] \tag{2}$$
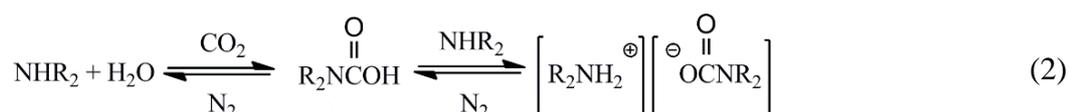
Tertiary amines have been also suggested as another type of switchable solvents, since they are easy to prepare and commercially available, unlike the amidine systems. These tertiary amines are hydrophobic solvents with low miscibility in water under nitrogen atmosphere but are hydrophilic at the existence of CO_2. The miscibility changes are triggered by a chemical reaction between CO_2 and water and the SHS, providing the protonated SHS a water-soluble bicarbonate salt. The reaction is inverted when the CO_2 is removed by introducing nitrogen or air to the mixture. Tertiary amines are generally less sensitive than secondary amines to CO_2, which means they require

longer reaction periods but at the same time, a far less energy to reverse the reaction is required.

The tertiary amine N,N-dimethyl cyclohexyl amine (DMCHA) was investigated for lipid extraction from freeze-dried *Botryococcus braunii* microalgae and 22 % yield based the dry biomass was achieved at 60 to 80°C. When the experiment was repeated at room temperature, the yield dropped to 19 % [58]. DMCHA was also used to extract lipid from wet microalgae with water content reaching up to 80 % using three strains, namley *Desmodesmus communis*, *Tetraselmis suecica* and *Nannochloropsis gaditana* without any pretreatment. At an extraction period of 24 h, the yields were 29.2 %, 57.9 % and 31.9 % from *D. communis*, *N. gaditana* and *T. suecica*, respectively [59].

In the hydrophilic form SS which are usually hydrophilic ionic liquids tend to compromise the integrity of the cell wall structure by the H-bonds of polysaccharides [58], which lead to either complete rapture of the cell wall causing the intercellular matter to spill out or significant reduction in the cell wall thickness, where in this case the cell matter can diffuse through the cell wall [66]. Although the hydrophobic form of the SS is not viable for cell wall disruption, its vital for the extraction of the lipid after the cell has been already disrupted [68], which is done by switching the SS to the hydrophobic form. Finally, the SS is switched back to the hydrophilic form to separate the product from the SS and the cell debris.

To further assess the degree of hydrophobicity at each state contact angle measurement could be conducted. Contact angle is defined geometrically as the angle formed by a liquid at the three-phase boundary where a liquid, gas and solid intersect. The well-known Young equation describes the balance at the three-phase contact of solid-liquid and gas.

From Figure: 4, the low contact angle values indicate that the liquid spreads on the surface while high contact angle values show poor spreading. If the contact angle is less than 90° it is said that the liquid wets the surface, zero contact angle representing complete wetting. If contact angle is greater than 90°, the surface is said to be non-wetting with that liquid. Contact angles can be divided into static and dynamic angles. Static contact angles are measured when droplet is standing on the surface and the three-phase boundary is not moving. Static contact angles are utilized in quality control and in research and product development. Contact angle measurements are used in fields ranging from printing to oil recovery and coatings to implants. When the three-phase boundary is moving, dynamic contact angles can be measured, and are referred as advancing and receding angles.
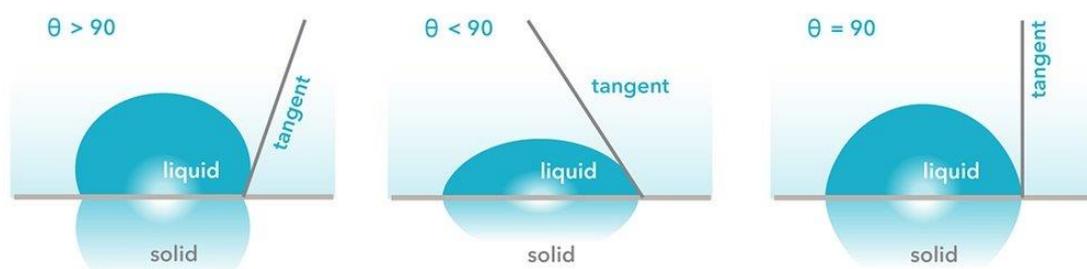
Figure: 4 Contact Angle

1.7 Hypothesis

As mentioned earlier, for effective extraction of oils from microalgae cells, the rigid walls of the harvested cells need to be disrupted to open the structure and allow the solvent reaching the oils. In this regard, hydrophilic solvents have shown better effectiveness in cell disruption compared to hydrophobic ones [69], whereas hydrophobic solvents are the ones needed for oil extraction and as an enzymatic

transesterification medium [68]. At the same time, the separation of the produced biodiesel would be easier from a hydrophilic solvent, in which its solubility is low. Therefore, the employment of the same solvent (of single hydrophobicity) in multi-step processes, i.e. extraction-reaction-product separation, is not possible, as different solvents of different hydrophilicities are needed in each step. Above that, these separate solvents need to be completely removed before the next step can be carried out.

SSs in their polar state are suitable for cell disruption, whereas their high affinity in their non-polar state towards non-polar lipids makes them perfect choice for extraction and as a medium for transesterification. Beside the simplification of the process, by allowing effective extraction from undisrupted wet paste, using switchable solvents can also simplifies the product separation step, which is an energy-consuming process when a conventional hydrophobic solvent is used. In addition to the high amount of energy required for separation, using conventional solvents, which are toxic and volatile has a negative environmental impact. Using SSs, the product separation can be easily achieved by switching the solvent back to polar [70]. Three SSs, namely DMCHA, EBA and Dipropylamin were recently tested for the extraction of oils from wet paste of *Chlorella sp.* [60]. With the tertiary amine, DMCHA, no additional water was needed, and what was present in the wet algal paste was sufficient. However, in the latter two, water in 1:1 ratio was used required. The oil extraction yields were 13.6, 12.3 and 7.0 % for the three solvents, respectively. The performance of the SSs were compared to solvents of single hydrophobicity, namely n-hexane and a hydrophobic IL, namely [Bmim][PF$_6$]. The single hydrophobicity solvents were unable to extract oil and yields of zero and 0.7 were achieved using the two solvents, respectively. The two SSs that resulted in the highest oil yield were used for simultaneous wet

microalgae cell disruption and oil extraction as well as transesterification and biodiesel separation. This was a very promising process that would significantly simplify biodiesel production from microalgae. The reaction was catalyzed by immobilized lipase and 47.5 % conversion was achieved at 35°C, 6:1 methanol:oil molar ratio and 30 % enzyme loading, using DMCHA with a solvent program of 1-h cell disruption, 1-h extraction/reaction, and 1-h phase separation steps... having a similar oil extraction yield, the use of EBA resulted in a significant drop in the yield, achieving only 24 %. This was due to the high amount of water needed with the EBA, which has a negative effect on the reaction.

Despite obtaining successful results using CO_2 triggered SSs, dealing with gases complicated the system, and necessitate the use of reflux condenser to avoid evaporation of methanol [60]. In addition, the high quantities of water, needed with the binary amine EBA, in the reaction medium inhibits the reaction significantly due to the hydrolysis of TGAs forming FFAs [71]. Similar to CO_2-based SSs, Some solvent/IL mixtures display an upper critical solution temperature (UCST) [72] or a lower critical solution temperature (LCST)[73], at which their hydrophobicity switches. An example of such a system is polypropylene glycol (PPG)-IL, which forms aqueous biphasic system (ABS), consisting of the hydrophobic phase PPG and a hydrophilic phase IL. An example of those Thermoresponsive Switchable Solvents (TSS) is polypropylene glycol (PPG)-IL, which forms at low temperatures aqueous biphasic system consisting of a hydrophilic IL phase and a hydrophobic PPG phase, owing to the methylene groups along the backbone of the polymer [74]. At low temperatures hydrogen bonding between PPG and water molecules are greater than the entropy forming monophasic solution. Whereas, at higher temperatures the entropy increases breaking those hydrogen bonds and initiating the phase separation [75].

Moreover, this behavior is not only temperature dependent, but its concentration dependent as well, which means that the cloud point could be lowered by lowering the PPG concentration [74].

Recently, PPG-IL systems have been used for the separation and purification biomolecules, such as proteins and organelles from cells, because of the biocompatibility of PPG and limited solubility of proteins in organic solvents [69]. Six ILs were mixed in different concentrations with PPG for protein separation [74]. It was found that mixing N,N diethyl-N-methylammonium methane sulfonate with PPG and water in ratios of (6 %, 30 % and 64 %) respectively, resulted in a monophasic ternary mixture at 25°C and by increasing the temperature to 45°C phase separation°C curs, this was followed by a test for protein separation from aqueous solution which yielded 99 % protein separation.

The high dependence of the mixture on temperature with small changes in temperature being sufficient to trigger the phase transition suggests that this solvent can be used as a thermo-responsive switchable in the same way the CO_2-based SSs were used. However, with the thermos-responsive SS, the process is expected to be much easier, wherein the switching can be achieved by simpler heating or cooling, as compared to the gas bubbling needed with the CO_2-based SSs.
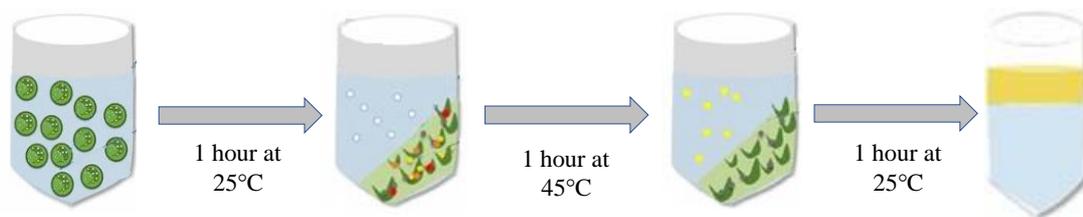
Figure 5: Graphical abstract of the hypothesis of thermoresponsive switchable solvents for simultaneous microalgae oil extraction reaction from wet undisrupted microalgae

Therefore, in this work, a thermos-responsive SS was tested for simultaneous cell disruption, oil extraction-reaction and product separation from wet paste of *Chlorella sp.* without any pretreatment as illustrated in Figure 5. As far as the investigators know, a process similar to the one presented in this work has never been presented before, and the successful results would definitely significantly simplify and can reshape the biodiesel production from microalgae industry.

Chapter 2: Methods

2.1 Chemicals and reagents

1,8-Diazabicyclo[5.4.0]undec-7-ene 98 % (DBU), Mono-ethanolamine (MEA) $\geq$ 98.0 %, 1-hexanol anhydrous 99 %, polypropylene glycol 400 (PPG), *n*-hexane, and chloroform were purchased from Sigma-Aldrich, USA. *n,n*-Diethyl-*n*-methylammonium methane sulfonate, [N_{1220}][C_1SO_3] with a purity of $\geq$ 98 %, was obtained from IO-LI-TEC, Germany. Analytical grade methanol with a purity of $\geq$ 99 % was obtained from Fisher chemicals, USA. Hydrogen, zero air (ultra-pure), helium, carbon dioxide, and nitrogen were supplied by Sharjah Oxygen Company, UAE. Novozyme®435 (activity 11,900 PLU/g) was a kind gift from Novozymes, Denmark. A standard solution of high purity FAMEs mix consisting of 4 % myristic acid (C14:0), 10 % palmitic acid (C16:0), 6 % stearic acid (C18:0), 25 % oleic acid (C18:1n9c), 10 % Elaidic acid (C18:1n9t), 34 % linoleic acid (C18:2n6c), 2 % linolelaidic acid (C18:2n6t), 5 % linolenic acid (C18:3), 2 % arachidonic acid (C20:0), and 2 % of behenic acid (C22:0) was purchased from Sigma-Aldrich, USA.

2.2 Synthesis of the switchable solvent (SS)

DBU based SSs, namely [DBU][Hexanol] and [DBU][MEA], were prepared as reported previously [63, 75]. Briefly, DBU and 1-hexanol were mixed in equimolar ratio and stirred vigorously for 5 min, resulting in a hydrophobic solution. The solution was triggered by CO_2 to turn into hydrophilic ionic liquid and returned to its original state by stripping the CO_2 by the addition of N_2 at 80°C. The SSs used in our previous work [60], EBA mixed with an equal amount of water, despite being effective in oil extraction, had a negative effect on the biodiesel production due to its high water

content. Therefore, in the SSs used in this work, the water was replaced with a hydrophobic alcohol or amine .

TSS was prepared as described earlier [74]. Briefly, a homogeneous mixture, composed of 6 wt % $[N_{1220}][C_1SO_3]$, 30 wt % PPG, and 64 wt % distilled water was prepared. The composition that switched hydrophobicity at 45°C was earlier reported to be suitable for enzyme reaction [77] and was selected for this study. The hydrophobicity of the prepared SSs and TSS were evaluated from the surface contact angle. Briefly, a 0.5 µl drop was placed on a hydrophobic surface made of a glass laminated with wax paper (Falcon wax paper, UAE) and the contact angle was determined using contact-angle instrument (Kyowa, drop master series, Japan). The contact angle of a droplet of water was used as a reference.

2.3 Algae strains and culture conditions

Freshwater microalgae, *Chlorella sp..*, was cultivated in 100-liter indoor open pond made of fiberglass (150 cm length, 80 cm width, 30 cm depth) with a horizontal paddlewheel rotating at 1400 rpm/min to mix the culture and run by a single phase electric motor (ML80B4, China). A white fluorescent tube light of 202 µmol/m^2 s intensity, fixed 35 cm above the culture surface was programmed to provide 12/12 photoperiod using the 24 h timer. The culture was grown at room temperature in Bold's Basal Medium (BBM) composed of 0.17 mM calcium chloride ($CaCl_2·2H_2O$), 0.43 mM di-potassium hydrogen orthophosphate (K_2HPO_4), 0.3 mM magnesium sulphate ($MgSO_4·7H_2O$), 1.29 mM potassium di-hydrogen orthophosphate (KH_2PO_4), 8.82 mM sodium nitrate ($NaNO_3$), 0.43 mM sodium chloride (NaCl), and vitamin B12 (0.1 % v/v). After 2 weeks, the algal biomass was harvested by centrifugation at 6000 rpm for 5 minutes using IEC-CL Multispeed centrifuge (Model No. 11210913, France). Dry

weight of the biomass was analyzed by weighing 1 g of wet paste and measuring the weight difference before and after overnight drying at 70°C.

2.4 Quantifying lipid content

Lipid content was determined using the Bligh and Dyer method [78]. Briefly, the wet harvested microalgae cells were lyophilized overnight (2 h freeze/12 h drying under vacuum) using a freeze dryer (Telstar LyoQuest, Spain) operated at -54°C and 0.02 mbar. Microalgae oil was extracted from 1 g of lyophilized cells, homogenized with 15 ml of chloroform-methanol mixture (1:2). The mixture was vigorously mixed using continuous ultrasonication (Branson Sonifier 450, USA) in five cycles of 5 min each to ensure complete cell disruption. Subsequently, the mixture was kept on orbital shaker (Stuart Lab scale Orbital Shaker/SSL1) at room temperature and rotated at 120 rpm for 20 min. Next, 15 ml of chloroform-distilled water mixture (1:2) was added and mixed thoroughly. The mixture was then centrifuged (IEC CL31 multispeed centrifuge, Thermo Scientific, USA) at 1000 rpm for 3 min to separate the biomass. The supernatant was centrifuged again at 2000 rpm for 5 min to separate the two layers, and was placed in a separation funnel. The lower chloroform layer containing the extracted oil was collected in a pre-weighed dry beaker and dried in the oven (ULE 400, Memmert Universal) at 60°C chloroform evaporated. The amount of extracted lipid was determined from the difference between the final weight of the dried sample in beaker and the weight of the empty dry beaker.

2.5 Simultaneous extraction-reaction

A screening test was performed to assess the effectiveness of the TSS for simultaneous oil extraction-reaction and product separation from wet, undisrupted microalgae paste using immobilized enzyme as catalyst. The effectiveness was compared with *n*-

hexane, and CO_2 triggered (DBU)-1-hexanol and DBU- MEA. The experimental setup of the TSS tests was much simpler than that of the CO_2 triggered amine based SS. Briefly, a 15 ml capped vial was placed on a hotplate magnetic stirrer (DAIHAN hotplate stirrer, Korea). One gram sample of wet, undisrupted microalgae paste of predetermined dry content, was mixed with immobilized lipase (30 % per biomass dry weight), 10 ml TSS and pre-specified amount of methanol. The TSS was maintained hydrophilic for 1.5 h at room temperature and reaction contents were continuously stirred to disrupt the cells and liberate the oils. The TSS was switched to hydrophobic state by increasing the temperature to 45°C and stirred for another 1.5 h to dissolve the liberated oils and simultaneously convert them to biodiesel. Finally, the TSS was switched back to the hydrophilic state by reducing the temperature back to 25°C to separate the biodiesel. To extract the separated product, 10 ml *n*-hexane was added to the system and then sent for analysis. Similar procedure was followed for the experiment with *n*-hexane, except that the TSS was replaced with *n*-hexane.

The procedure for evaluating the CO_2-triggered amine-based SSs was similar to the one followed for the TSS. Briefly, a sample of wet, undisrupted microalgae paste (1 g) was mixed with 10 ml of SS. This was followed by steps of cell disruption and extraction-reaction lasting 1.5 h each at the room temperature, followed by 1 h of FAMEs separation. The extent of the microalgae cell wall disruption was confirmed by imaging cells before and after pretreatment with the TSS using optical microscope equipped with DFC 310 FX camera (Leica microsystem, Germany). To turn the solvent hydrophobic, the temperature was increased to 80°C. Prior to starting the reaction, the system was cooled down to 40°C, and methanol and the enzyme were added. The system was covered throughout the experiment to minimize the loss of methanol.

2.6 Reusability test

Simultaneous extraction-reaction of microalgae lipids was performed to test the reusability of the TSS-immobilized enzyme system for four cycles. In this test, the enzyme leaching was avoided by not exposing the enzyme to the hydrophilic solvent at any stage of the reaction. Briefly, 1 g of wet biomass was mixed with 10 ml of the hydrophilic TSS for 1.5 h to allow cells disruption and oil liberation. The solvent was then switched to hydrophobic state to extract the lipids, and centrifuged to discard the unwanted cell debris. Subsequently, the enzymes (30 % loading) and methanol (1.0 ml) were added to initiate the transesterification process and the reaction was carried out for 1.5 h. Before switching the solvent hydrophilic, the enzyme was separated by centrifugation. Subsequently, the solvent was switched to hydrophilic state and FAMEs were extracted by adding 10 ml n-hexane. The used enzyme was kept in the refrigerator at 4°C before reusing in another cycle with 1 g of fresh undisrupted biomass. The steps were repeated for four cycles.

2.7 Fatty acids methyl esters analysis

Gas Chromatograph GC-2010 (Shimadzu, Japan) equipped with a flame ionization detector (FID) and a SP-2380 capillary column (30 m, 0.25 mm, 0.2 µm film thickness) was used to analyze the extracted FAMEs. Helium was used as the carrier gas at a flow rate of 68.9 ml/min. A total of 1 µl sample filtered through a 0.45 µm syringe filter was injected. The temperature of the oven was set at 185°C and raised to 220°C after an isothermal time period of 16 min. The temperatures of the injector and detector were set at 220°C, and a divided coefficient of 50 was used. The instrument was calibrated using a standard FAME mix (C14-C22, SIGMA-CRM18917) prepared by dissolving 100 mg of standard FAME mix in 10 mL n-hexane. The amount of the

FAMEs produced was presented as a percentage of the total oil in the biomass, as explained in Section 2.4, and represented in Equation (3).

$$\text{FAME yield} = \frac{m_{\text{FAME}}}{m_{\text{oil content}}} \times 100 \ \% \tag{3}$$

2.8 Experimental design and optimization

Three key parameters were changed in order to identify their respective effects on the simultaneous oil extraction-reaction from wet, undisrupted microalgae using TSS. The tested factors were the TSS solvent program (i.e., cell disruption and extraction-reaction durations) and the amount of methanol. The levels of independent variables based on the results of the screening experiments are listed in Table 6.

Table 6: Levels of the independent variables

Factor	Symbol	Unit	Levels				
			$-\alpha$	-1	0	1	$+\alpha$
Cell disruption duration	x_1	h	0.0	0.5	1.5	2.5	3.0
Extraction-reaction duration	x_2	h	0.0	0.5	1.5	2.5	3.0
Methanol amount	x_3	ml	0.02	0.05	0.1	0.15	0.2

MIniTab 2019 was used to develop a central composite design to create a polynomial model between the produced yield (response) and the three parameters (cell disruption. extraction-reaction periods, and the amount of methanol) as shown in Table 7. Experiments were performed randomly to avoid bias.

The response surface methodology (RSM) was applied to determine a polynomial, as shown in Equation (4), to express the yield of produced FAMEs as a function of the independent variables. MiniTab 19 statistical software (MiniTab, Inc.) was used for the statistical analysis.

$$Y = a_o + \sum_{i=1}^{3} a_i x_i + \sum_{i=1}^{2} \sum_{j=i+1}^{3} a_{ij} \ x_i x_j \tag{4}$$

Table 7: Central composite design experiments for the three selected process variables and FAMEs yields

Factor			Response
x_1	x_2	x_3	FAMEs yield %
0	0	0	43.26 ± 1.15
0	0	0	42.61 ± 1.72
+1	-1	-1	36.71 ± 2.75
+1	-1	+1	49.61 ± 1.75
-1	+1	+1	76.23 ± 2.42
-1	-1	-1	18.83 ± 0.24
+1	+1	-1	50.28 ± 1.00
0	0	0	47.11 ± 0.98
0	0	0	44.94 ± 0.01
-1	-1	+1	38.03 ± 0.10
-1	+1	-1	46.34 ± 0.06
+1	+1	+1	72.64 ± 0.55
-□	0	0	33.26 ± 1.10
0	0	0	45.23 ± 0.37
0	0	-□	29.06 ± 2.36
0	-□	0	24.48 ± 0.29
0	0	0	42.63 ± 0.51
0	+□	0	55.93 ± 0.54
0	0	+□	50.53 ± 2.87
+□	0	0	48.46 ± 0.23

where, Y is the extracted FAMEs 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. Three-dimensional surface response plots were generated by varying the two variables within the studied range while holding the third variable constant.

Chapter 3: Results and Discussion

3.1 Using CO_2-triggered switchable solvents for biodiesel production from wet undisrupted microalgae cells

It has been previously reported the successful use of SS in the simultaneous extraction-reaction of oil from wet, undisrupted microalgae paste using 1:1 EBA-water system [60]. Further, EBA-water system was also successfully used to extract oil from *Neochloris oleoabundans* [79]. However, the system was ineffective for biodiesel production and the main reason for the low FAME yield was the excessive use of water that promoted the hydrolysis of the extracted oils rather than transesterification [80].

Therefore, in this study, we investigated the effect of replacing water with a long chain alcohol, thereby rendering the solvent entirely hydrophobic [64]. Figure 6 A shows the DBU-Hexanol mixed with water. The idea of adding the water to show that the SS layer, found on top, is totally separated from the lower water layer. By introducing CO_2 at room temperature, the SS switched its hydrophilicity, to become hydrophilic, and formed a miscible, one phase, solution with water, as shown in Figure 6 B. By bubbling N_2 at 80°C, the CO_2 was liberated and the SS was switched back to its hydrophobic, forming the two layers are again, as shown in Figure 6 C.
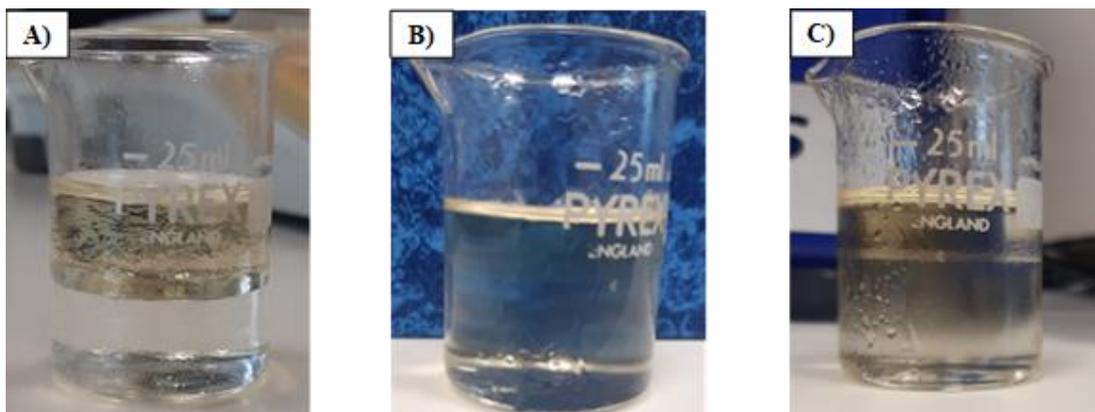
Figure 6: Hydrophobicity change of DBU-hexanol-water system. (A) hydrophobic form of the SS separated from the lower water layer. (B) Hydrophilic form of the SS after addition of CO_2 forming one miscible phase with water. (C) Hydrophobic form of the SS after stripping the CO_2 by N_2 at 80°C, forming again the two layers

When the reaction was carried out without the enzyme to assess the capacity of DBU-Hexanol SS, we did not record FAMEs generation (Figure 7). Since DBU-hexanol SS is known to disrupt the cell wall and release the lipids, the absence of FAMEs could not be attributed to functional inefficiency of DBU-hexanol [81]. When the reaction was repeated in presence of NaOH as a catalyst, a higher yield (10.05±0.32 %) of FAMEs was achieved, indicating that DBU-hexanol system lacked the catalytic activity in absence of NaOH.

Since alkaline catalyst have many drawbacks, for e.g., soap formation [62, 80], the experiment was repeated using Novozyme 435, an immobilized enzyme, under similar experimental conditions. To avoid exposing the enzyme to high temperature, it was added with the methanol once the solvent was cooled to 40°C. This increased the FAMEs yield to 37.77± 0.32 %, which was 1.5 folds higher than that achieved using EBA-water SS under the same conditions and enzyme loading [60]. As explained earlier, this was mainly due to the absence of excessive water used in the DBU-hexanol system. In addition, by using a completely hydrophobic solvent, the solvent was

completely utilized to extract the oil and acted as the reaction medium, unlike EBA-water system, where only half of the volume was hydrophobic.
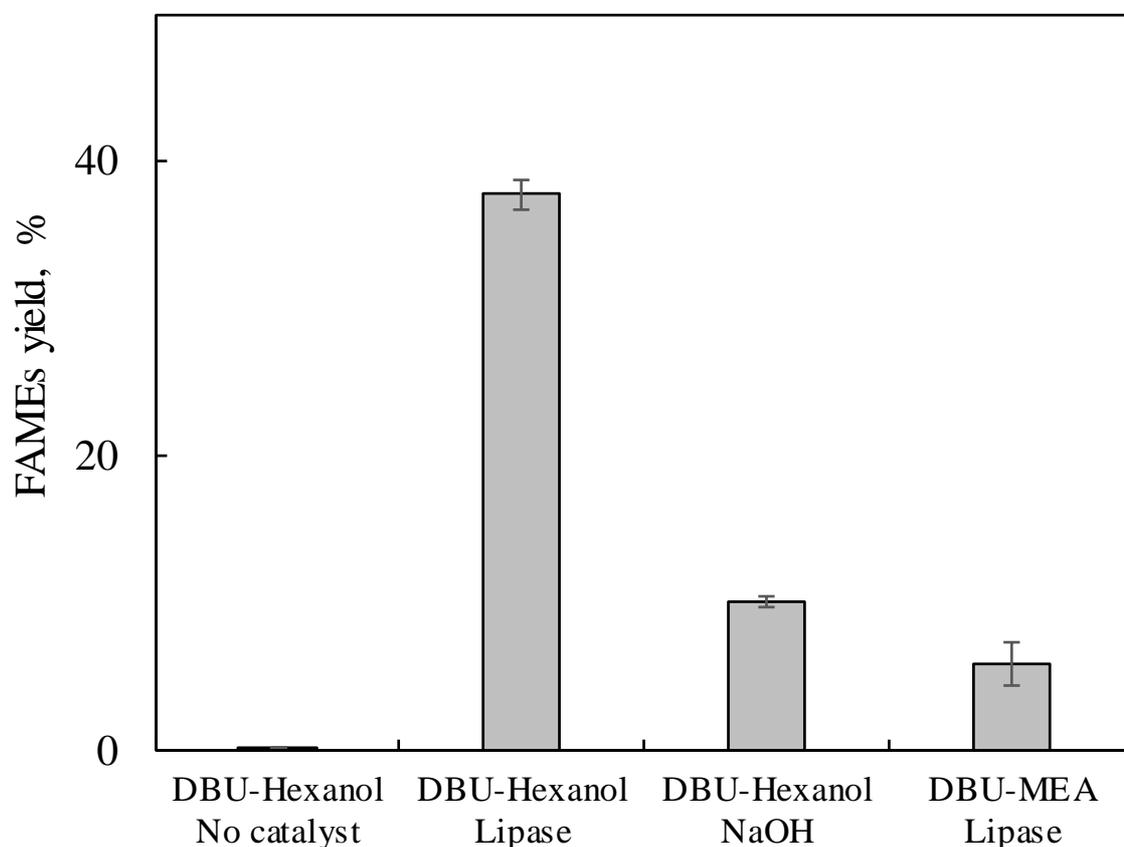

Figure 7: FAME yield at 1.0 ml MeOH, 30 % enzyme loading, and 10 ml SSs, with the solvent program: cell disruption: 1.5 h, extraction/reaction: 1.5 h, and phase separation: 1 h

A previous study reported use of DBU-MEA SS in the delignification of lignocellulosic materials [76]. We tested DBU-MEA SS under the same conditions and solvent program, with Novozyme 435 as a catalyst. As shown in Figure 7, a much lower FAMEs yield (5.86 $\pm$ 1.50 %) was achieved when DBU-MEA was used. This could be explained by the higher viscosity of the DBU-MEA mixture [83], which might have negatively affected the diffusion of the solvent into the biomass matrix and the diffusion of the extracted oil into the pores of the immobilized enzyme. In biodiesel

production catalyzed by immobilized lipase, the hydrophobicity of the solvent is more significant than its viscosity [68]. This was confirmed from the higher biodiesel yield achieved using an [bmim][PF$_6$] as a solvent, as compared to using [bmim][NTf$_2$] with a lower viscosity and hydrophobicity. Next, to understand the better performance of DBU-hexanol than DBU-MEA, the hydrophobicity of both SSs was compared using the contact angle on a hydrophobic surface. As shown in Figure 8, the contact angle of the DBU-hexanol SS was 45.85º, which was 62.5º lower than that of DBU-MEA. The lower contact angle of DBU-hexanol SS suggested a higher hydrophobicity.

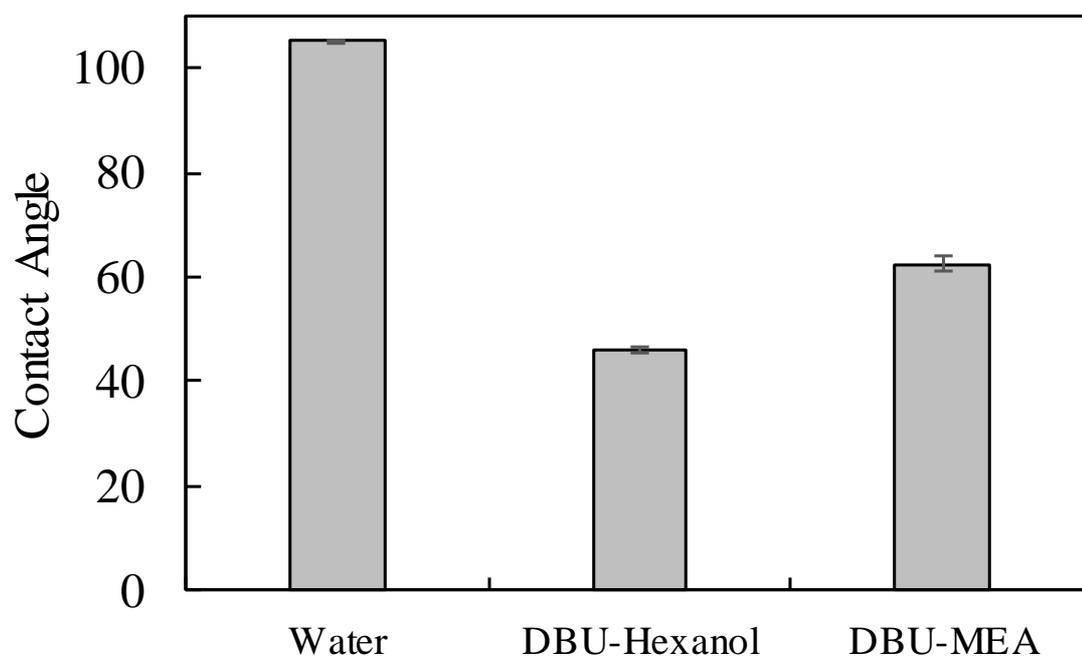Figure 8: Contact angle measurements on a hydrophobic surface, using water as a reference, DBU-Hexanol SS and DBU-MEA SS in their hydrophobic form

3.2 Using TSS solvent for biodiesel production from wet undisrupted microalgae cells

Although improved results were achieved using DBU-hexanol SS system, the need to bubble the system with gases and to alternate between different gases complicated the

process and made scaling up a real challenge. In addition, losing some amount of the methanol with the vented gasses was inevitable. The need for a reflex condenser to completely eliminate the methanol evaporation further added to the complications. Therefore, we tested a thermo-responsive switchable solvent (TSS), composed of 6 wt % $[N_{1220}][C_1SO_3]$, 30 wt % PPG, and 64 wt % distilled water for its ability to produce biodiesel. Firstly, the hydrophobicity changes of the TSS were evaluated by measuring the contact angle at different temperatures. As shown in Figure 9, at 25°C, the TSS-water system formed a hydrophilic miscible solution, which was confirmed by the high contact angle of 75.1°. As temperature was increased to 35°C, the TSS-water solution formed a cloudy biphasic system and the contact angle dropped to 56.3°. Finally, at 45°C, clear biphasic layers were formed, and the contact angle dropped to 48.5°, which was close to that of DBU-hexanol SS in its hydrophobic state.
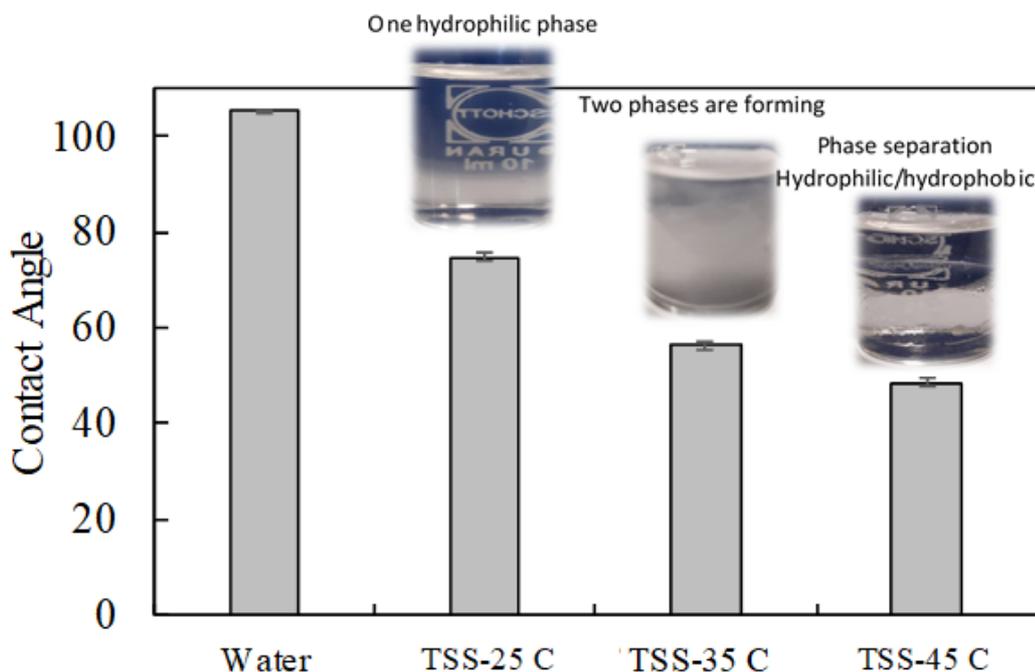

Figure 9: Changes in hydrophobicity of TSS at different temperatures as seen by the reducing contact angle measured on a hydrophobic surface

After confirming the hydrophobicity switch of the TSS with increasing temperature, the solvent was used for simultaneous lipid extraction-reaction with wet and undisrupted microalgae. Although the solvent program was same, the temperature was different at each stage (hydrophilic cell disruption: 1.5 h at 25°C; hydrophobic extraction-reaction: 1.5 h at 45°C; hydrophilic FAMEs separation: 1 h at 25°C). A blank experiment without catalyst was carried out to assess the catalytic activity of the TSS. As shown in Figure 10, although TSS catalyzed reaction led a higher yield of FAMEs (2.45 ± 0.95 %), than the CO_2-triggered SS, it was still insignificant, indicating that the TSS too did not possess catalytic capacity. Using Novozyme 435 and methanol at a load used previously with the CO_2-triggered SS (Figure 10). It was observed that the FAMEs output of mere 15.15 ± 0.36 %, as compared to 37.77 % achieved with the CO_2 -triggered SS. However, the production of FAMEs significantly increased (45.2 ± 0.37 %) upon adding 0.1 ml of methanol. This indicated that just 0.1 ml of methanol was enough to overcome the inhibitory effects of methanol escape in the tightly capped system with the TSS, which has been reported in most studies using enzymatically catalyzed biodiesel production [9, 80].
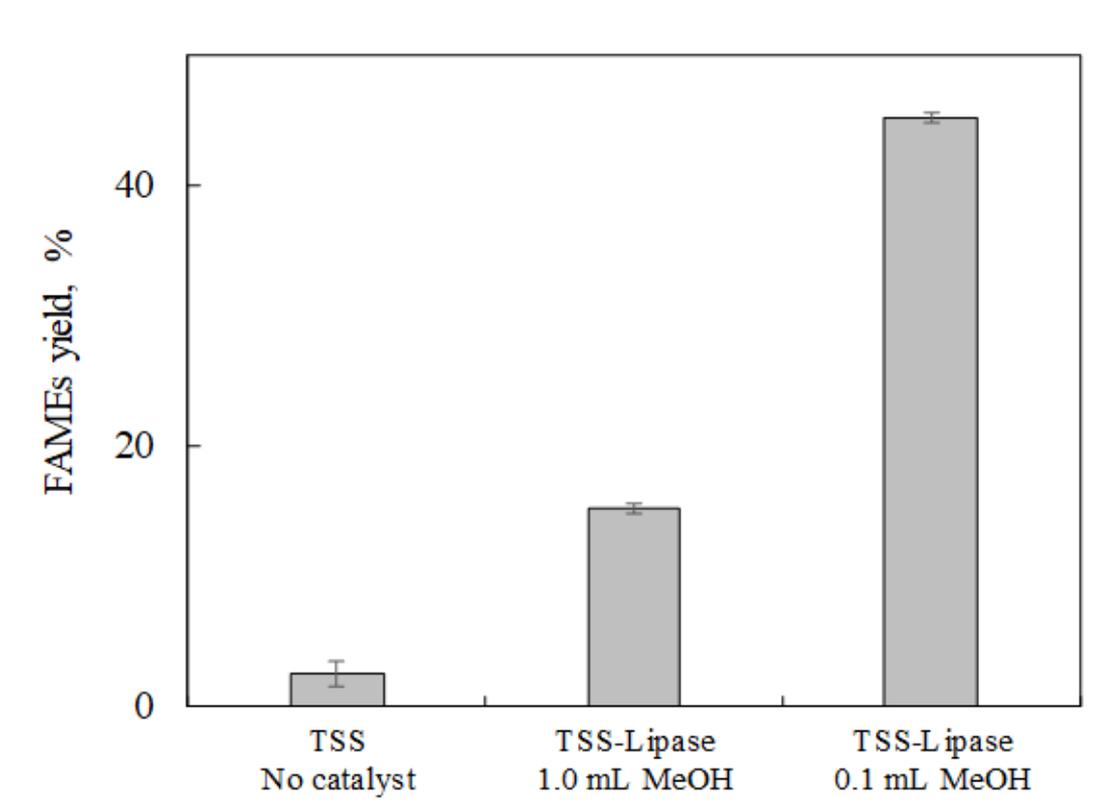
Figure 10: FAMEs yield at 30 % enzyme loading and TSS (cell disruption: 1.5 h, extraction/reaction: 1.5 h, and phase separation: 1 h) at different amounts of methanol

We verified the ability of the TSS to disrupt the rigid cell wall of the *Chlorella sp.* by imaging cells before and after exposure to the TSS. As shown in Figure 11, the thickness of the cell wall decreased after the cells were treated with the TSS. This could be attributed to the protic ionic liquids (PILs) constituent of the TSS which dissociates cellulose in the cell walls, decrease its thickness, thereby facilitating the diffusion of the lipids out of the cells.
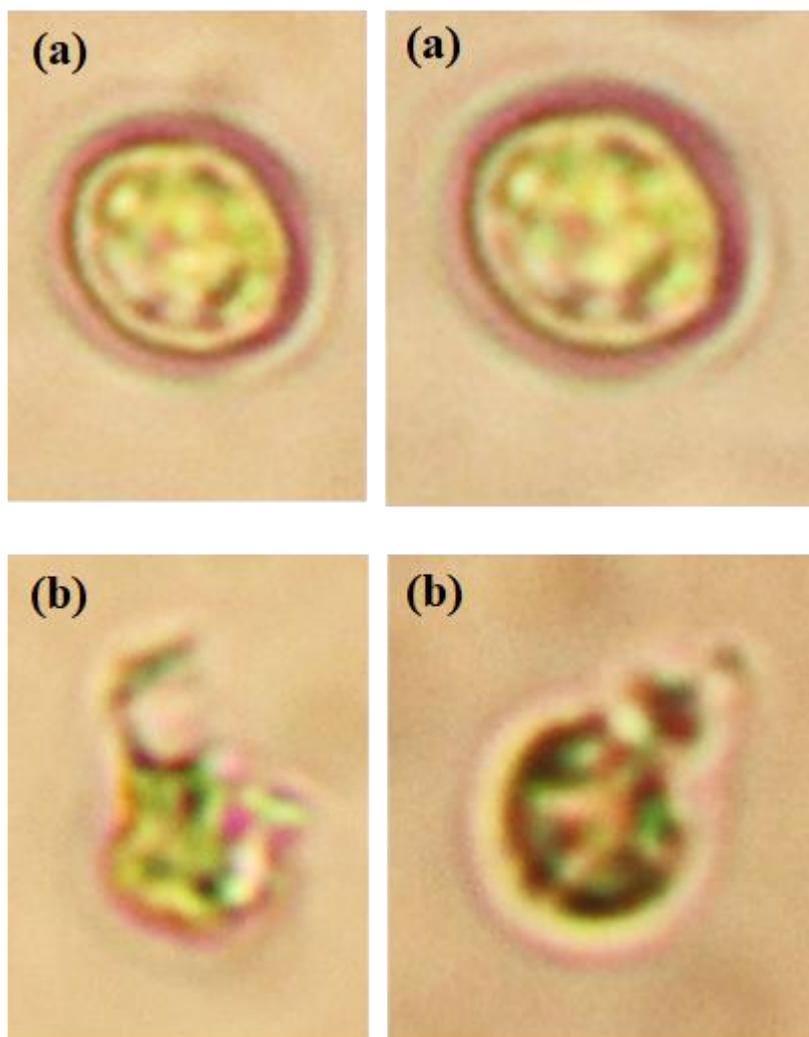
Figure 11: Microscopic images of (a) fresh undisrupted *Chlorella sp.* cell before and (b) after exposure to the TSS

Although both, TSS and CO_2-triggered SS offer the advantage of the simultaneous extraction-reaction of oil from wet, undisrupted microalgae, our results clearly showed that the TSS system has the additional advantage of ease of operation and it does not require reflux condensers. The results presented in this work promise a significant simplification of the biodiesel production rom microalgae.

3.3 Optimization of simultaneous oil extraction-transesterification system using TSS

We analyzed the effects of durations of cell disruption and extraction-reaction, and the amount of methanol used as a reactant, on the simultaneous lipid extraction and transesterification. The ranges of these independent parameters are given in Table 6. The lipid content in the tested conditions was determined to be 8.56 $\pm$ 1.56 %, using a chloroform: methanol (2:1) solvent mixture [84]. All subsequent FAMEs yields with respect to the total lipid content were determined as per Equation (3).

3.3.1 Effect of TSS solvent program

The effect of cell disruption was examined by altering the duration of cell disruption [74], while the extraction-reaction duration (1.5 h) and the methanol amount (0.1 ml) were kept constants. As shown in Figure 12, the yield of FAMEs increased with increasing the duration of cell disruption (from 33.25$\pm$ 1.09 % at 0 h to 48.46 $\pm$ 0.23 % at 3 h). The increase in FAMEs yield was due to the longer exposure to the PILs present in the TSS, which eventually enhanced the lipid extraction. Similar results were reported in an earlier study that used CO_2-triggered SSs for simultaneous cell disruption and extraction-reaction using the same microalgae strain [12]. Next, the duration of extraction-reaction was altered, while the cell disruption duration (1.5 h) and the methanol amount (0.1 ml) were kept constant. As shown in Figure 13, the FAMEs yield increased with increasing the duration of the extraction-reaction (from 24.47 $\pm$ 0.29 % at 0 h to 55.93$\pm$0.53 % at 3 h). Although these results are consistent with those reported in a previous study with CO_2-triggered SSs [12], we observed that the TSS was more effective for cell disruption compared to the CO_2-triggered SS, and hence achieved better cell disruption in lesser time. Interestingly, increasing the cell

disruption duration from 0 to 1.5 h increased the FAMEs yield by 30 %, whereas further increasing the duration to 3 h increased the yield by just 12 %, indicating that most of the cell disruption happened within the first 1.5 h.
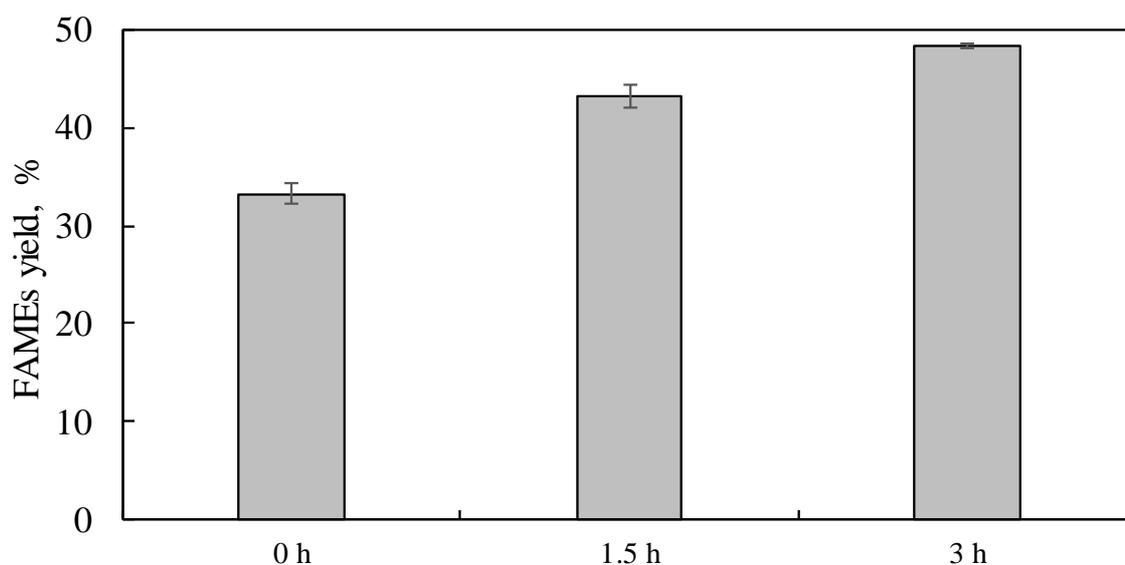

Figure 12: Effect of cell disruption duration on FAMEs yield at constant extraction-reaction duration(1.5 h), methanol amount (0.1 ml), and enzyme loading (30 %)
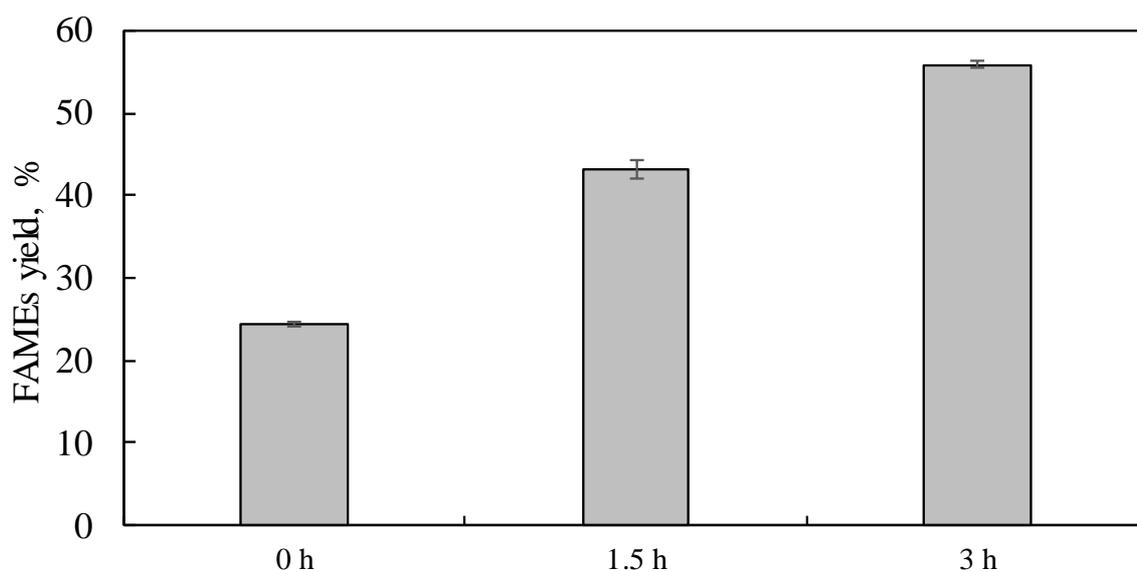

Figure 13: Effect of extraction-reaction duration on FAMEs yield at constant cell disruption duration (1.5 h), methanol amount (0.1 ml), and enzyme loading (30 %)

3.3.2 Effect of methanol amount

To elucidate the effect of methanol, we varied the amount of the methanol used in the reaction in the range of 0.02 to 0.2 ml, while cell disruption and extraction-reaction durations were kept constant at 1.5 h each. As shown in Figure 14, the increase in FAMEs yield was directly proportional to the increase in the methanol amount used (from 25.06 $\pm$ 2.36 % at 0.02 ml to 50.53 $\pm$ 2.87 % at 0.2 ml). Interestingly, while increasing methanol amount from 0.02 to 0.1 ml increased the yield by 73 %, a further increase to 0.2 ml increased the yield by just 17 %. In fact, at higher amounts, methanol actually inhibited the reaction (Figure 10).
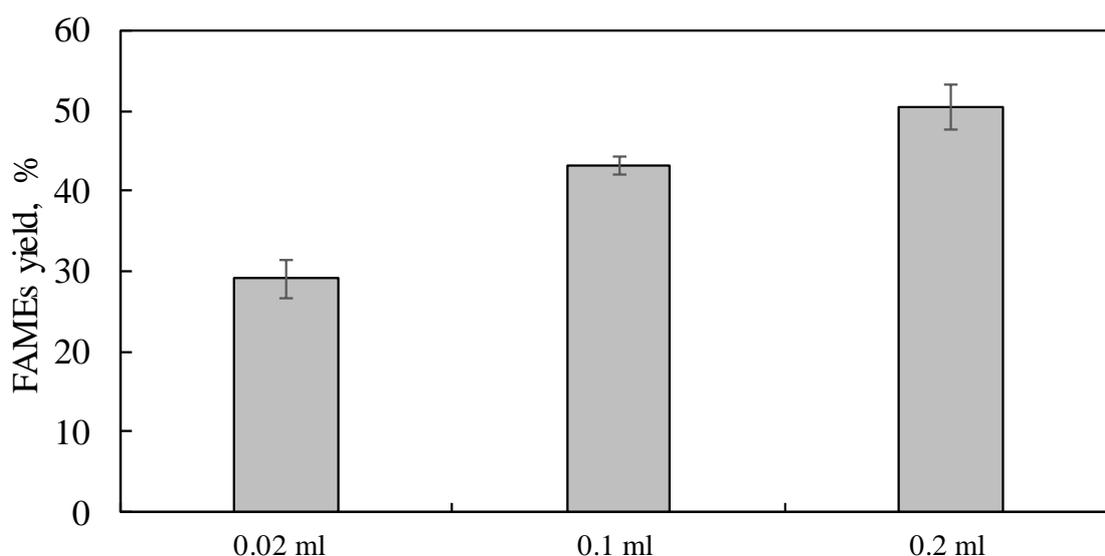
Figure 14: Effect of methanol amount on FAMEs yield at constant cell disruption and extraction reaction durations of 1.5 h each and 30 % enzyme loading

3.3.3 Statistical analysis of combined effects

The regression analysis was performed on the experimental data using MiniTab 19 software and is shown in Table 7. Further, a second order regression interactive model was developed relating the FAMEs yield (Y) and the three independent parameters,

namely cell disruption duration x_1, extraction-reaction duration x_2, and methanol quantity in the system x_3. The significance of the parameters was evaluated based on the P-value, whereas the lack-of-fit value of the model was determined from the analysis of the variance (Table 8). Our analysis showed that all the studied parameters were significant $(P < 0.05)$. However, the coefficients of the quadratic and the interaction terms were insignificant $(P > 0.05)$, which reflected on the linear trend of the FAMEs yield with increasing the independent parameters (Figure 12, Figure 13 and Figure 14). The developed model is shown in Equation (5)

$$Y = -0.6 + 9.11x_1 + 9.62x_2 + 190x_3 + 1.13x_1x_1 + 0.92x_2x_2 - 203x_3x_3 - 3.42x_1x_2 - 30.3x_1x_3 - 46.0x_2x_3 \quad (5)$$

Table 8: Response Surface Regression: FAMEs yield versus cell disruption duration, extraction-reaction duration and methanol quantity (a): Coded Coefficients (b) Analysis of Variance

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	45.46	2.19	20.79	0.000	
x_1	6.07	2.36	2.58	0.028	1.06
x_2	18.49	2.36	7.85	0.000	1.06
x_3	15.19	2.57	5.92	0.000	1.02
$x_1 * x_1$	2.55	3.79	0.67	0.516	1.01
$x_2 * x_2$	2.08	3.79	0.55	0.595	1.01
$x_3 * x_3$	-1.65	4.02	-0.41	0.691	1.03
$x_1 * x_2$	-7.70	4.29	-1.80	0.103	1.00
$x_1 * x_3$	-4.09	5.14	-0.79	0.445	1.06
$x_2 * x_3$	6.21	5.14	1.21	0.255	1.06

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	9	3226.51	358.50	12.35	0.000
Linear	3	2996.29	998.76	34.42	0.000
x_1	1	192.53	192.53	6.64	0.028
x_2	1	1787.48	1787.48	61.60	0.000
x_3	1	1016.27	1016.27	35.02	0.000
Square	3	29.54	9.85	0.34	0.797
$x_1 * x_1$	1	13.17	13.17	0.45	0.516
$x_2 * x_2$	1	8.76	8.76	0.30	0.595
$x_3 * x_3$	1	4.87	4.87	0.17	0.691
2-Way Interaction	3	154.37	51.46	1.77	0.216
$x_1 * x_2$	1	93.66	93.66	3.23	0.103
$x_1 * x_3$	1	18.33	18.33	0.63	0.445
$x_2 * x_3$	1	42.37	42.37	1.46	0.255
Error	10	290.18	29.02		

Table 9: Central composite design experiments for the three selected process variables and FAMEs yields of both predicted and actual response

Factor			Actual Response	Predicted Response
x_1	x_2	x_3	FAMEs yield %	FAMEs yield %
0	0	0	43.26 ± 1.15	46.48
0	0	0	42.61 ± 1.72	46.48
+1	-1	-1	36.71 ± 2.75	36.46
+1	-1	+1	49.61 ± 1.75	55.46
-1	+1	+1	76.23 ± 2.42	56.5
-1	-1	-1	18.83 ± 0.24	18.26
+1	+1	-1	50.28 ± 1.00	55.7
0	0	0	47.11 ± 0.98	46.48
0	0	0	44.94 ± 0.01	46.48
-1	-1	+1	38.03 ± 0.10	37.26
-1	+1	-1	46.34 ± 0.06	37.5
+1	+1	+1	72.64 ± 0.55	74.7
-□	0	0	33.26 ± 1.10	32.83
0	0	0	45.23 ± 0.37	46.48
0	0	-□	29.06 ± 2.36	31.28
0	-□	0	24.48 ± 0.29	32.05
0	0	0	42.63 ± 0.51	46.48
0	+□	0	55.93 ± 0.54	60.91
0	0	+□	50.53 ± 2.87	65.48
+□	0	0	48.46 ± 0.23	60.13

An optimization process was carried out using response optimizer in Minitab. The software-calculated optimum conditions were found to be 0.5 h cell-disruption duration at room temperature, 3 h extraction-reaction at 45°C, and 0.15 ml methanol in the reaction system. At these conditions, the FAMEs yield predicted by the model was 78.65 %. We checked the model by carrying out an additional independent experiment at the calculated conditions, at which the actual FAMEs yield was found to be 75.11 ± 1.03 %, which was close to the value predicted by the model, with 4.0 % error, and a detailed comparison between the predicted FAMEs yield and the actual are shown in Table 9, and to further investigate the predicted model two mor independent runs were conducted at extreme durations of 24 hours and as shown in Figure 15.
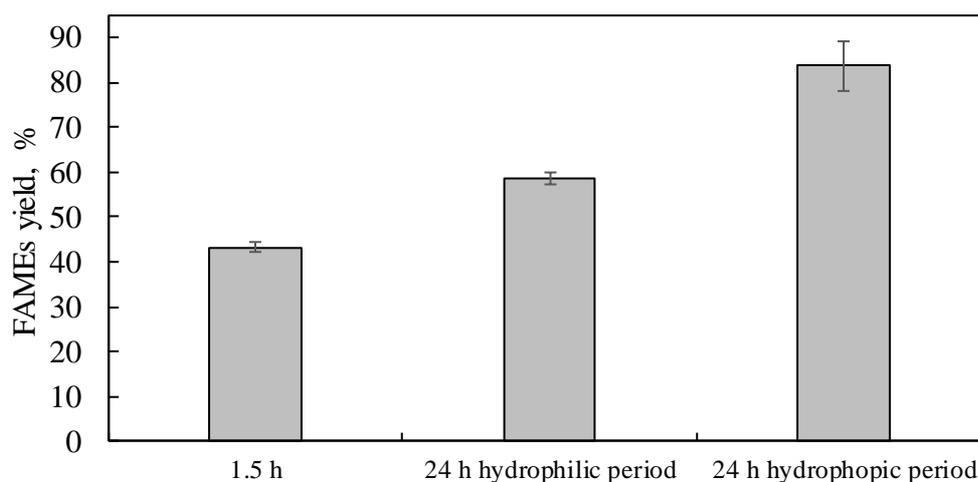
Figure 15: FAMEs yield at extreme of 24 hours for both hydrophilic and hydrophobic durations

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 16, the p-value 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 is small. The plot of the residuals versus fitted value, shown in Figure 16, 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 3) was consistent 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 16 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.
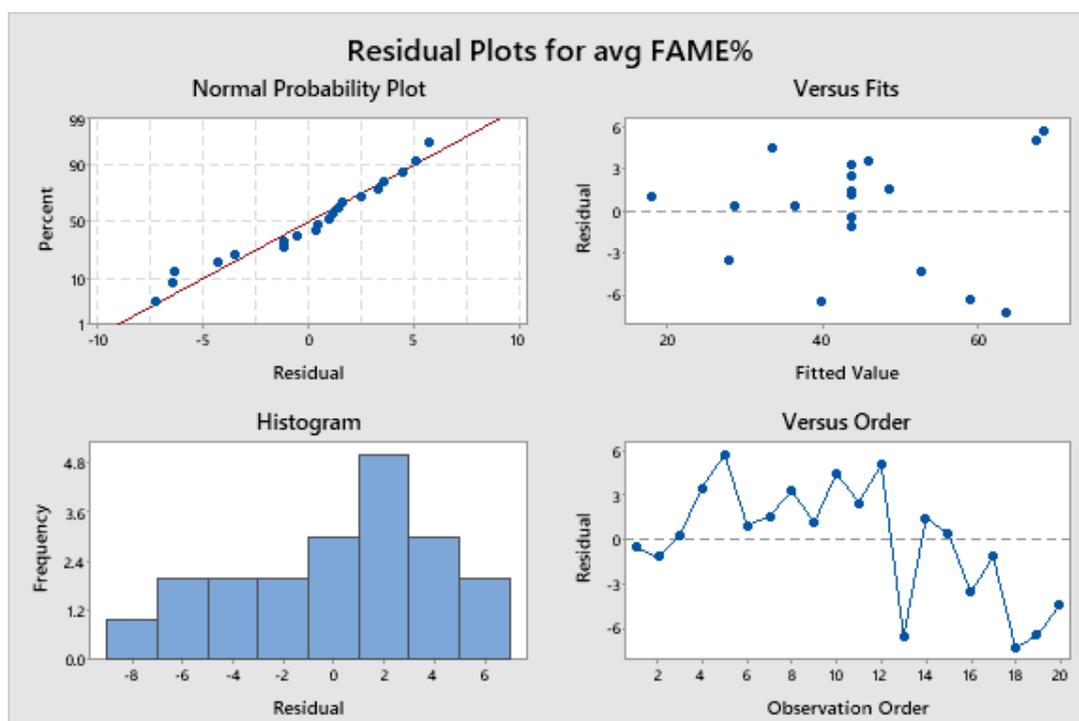

Figure 16: Residual versus Percent, Fitted value, Frequency and Observation order

The combined effects of cell disruption duration, extraction-reaction duration and methanol amounts in the system on FAMEs yield are shown as 3D plots (Figure 17 A, B and C). Our analysis showed that the increase in FAMEs yield was directly proportional to the increase in all the three parameters, with extraction-reaction duration being the most significant parameter. Further, the increase in yield followed a linear pattern with all the parameters, suggesting that the second order terms were less significant than the linear terms.
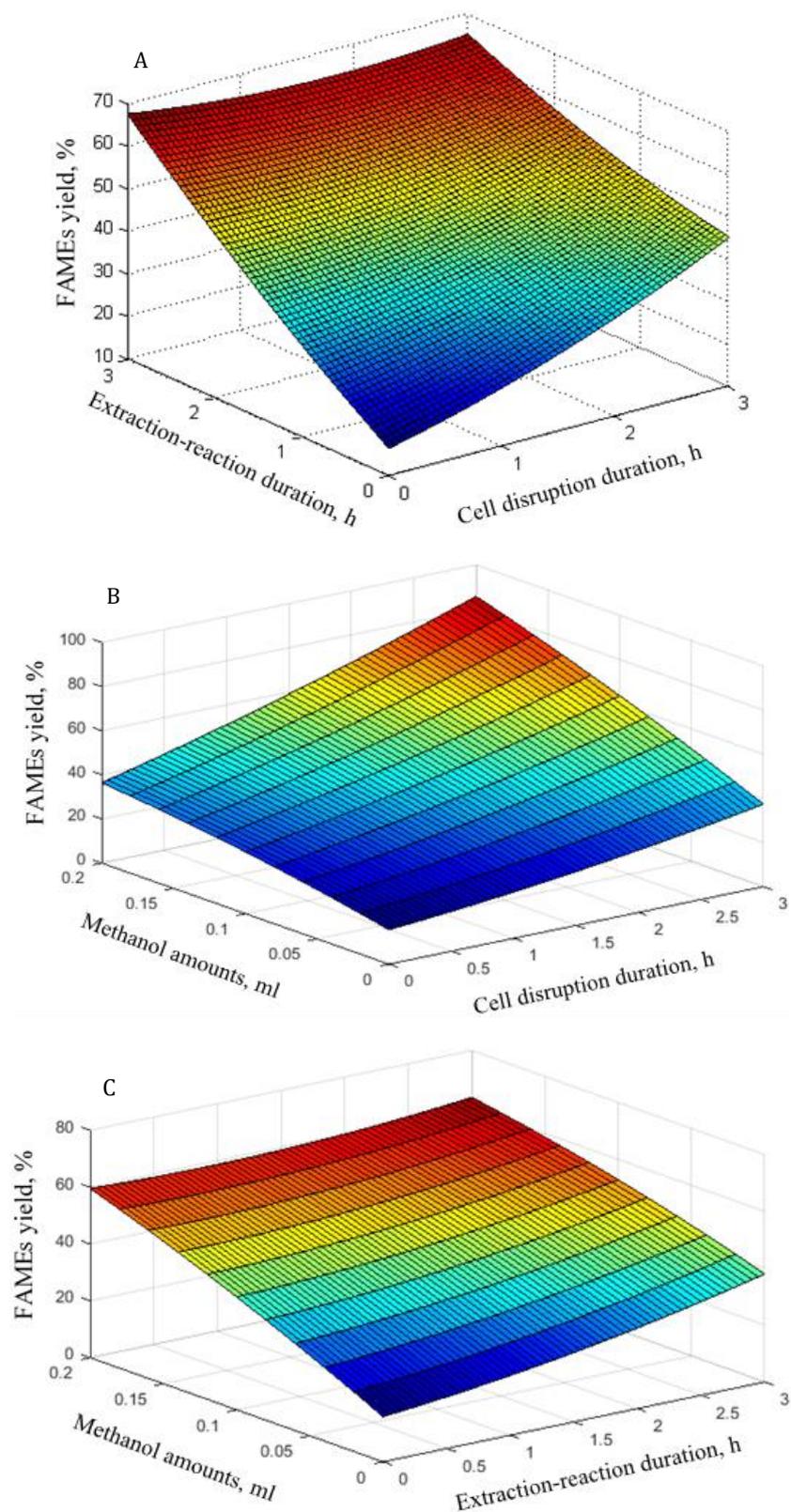
Figure 17: 3-D plot of the FAMEs yield at 30 % enzyme loading as a function of (A) cell disruption and extraction durations at 0.1 ml methanol, (B) cell disruption time and methanol amount at extraction-reaction duration of 1.5 h and (C) extraction-reaction duration and methanol amount at cell disruption duration of 1.5 h

3.4 Reusability of TSS-enzyme system

The reusability of the TSS-enzyme system was examined for 4 consecutive cycles using fresh biomass in each cycle. The test was done without washing enzyme between the cycles. As shown in Figure 18, the stability and reusability were preserved in the second cycle, with a negligible drop in the FAMEs yield. The drop however became prominent in the following cycles, and reached 60 % in the fourth cycle as compared to the first cycle. The drop was expected to be mainly due to the negative effect of enzyme exposure to the TSS in its hydrophilic state [7], and to the deposition of the byproduct glycerol. However, our results indicated that the stability and reusability of the TSS-enzyme system can be achieved, although further work is required to standardize a protocol to enhance the enzyme reusability. One way to do so could be by washing the reaction system with tert-butanol to remove the deposited glycerol. This method has been shown to be successful in enhancing the reusability of IL-enzyme system [85] and may be useful in TSS-enzyme system as well.
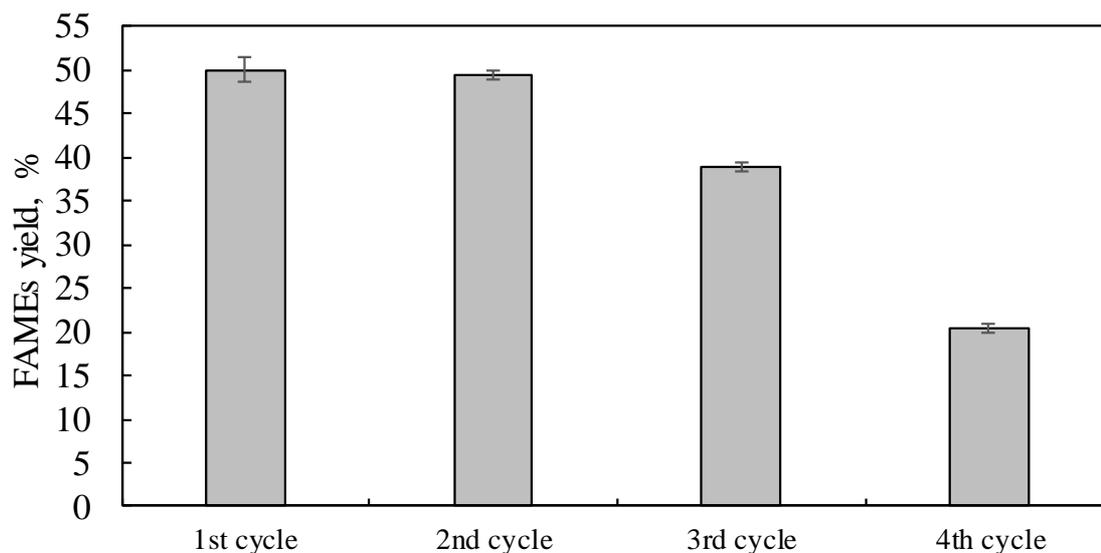
Figure 18: Effect of reusing the TSS-enzyme (without washing) on the FAMEs yield at 30 % enzyme loading and 0.1 ml methanol with cell disruption (1.5 h), extraction/reaction(1.5 h), and phase separation (1 h).

3.5 Future work

The successful results of the TSS opens a new horizon for easing and simplifying the process of biodiesel production from microalgae. However, there are still significant work must be done to enhance the TSS capabilities. An alternative for the water constituent must be investigated due to the many drawbacks of water on the enzymatically produced biodiesel. Furthermore, the effect of the TSS volume on the FAMEs yield could be investigated. Further studies could be carried out to examine and enhance the reusability of the TSS enzyme system by either modifying the TSS or the enzyme.

Chapter 4: Conclusion

This study showed replacing water in CO_2-triggered SS with an alcohol made the solvent suitable for the simultaneous cell disruption, oil extraction-reaction, and product separation in biodiesel production from wet microalgae. With DBU-hexanol SS, a biodiesel production yield of 37.77 ± 0.32 % was achieved, which was 1.5 folds higher than that achieved using EBA-water SS under the same conditions. Further, the TSS enhanced the yield of FAMEs significantly. The reusability of the TSS-enzyme system was tested and our results showed that enzyme retained its activity for two cycles, and that the reusability could be further enhanced by future endeavors. In summary, the results of this work hold potential to significantly simplify the production of biodiesel from the microalgae with enhanced efficiency.

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