In situ Transesterification of Microalgal Oil to Produce Algal Biodiesel

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by

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Abstract

Microalgae are a sustainable energy resource with great potential for CO_2 fixation. With the current demand for renewable fuels, especially the demand from the transportation sector, there is a need to develop a range of sustainable biofuels resources as the combined mix, which will be a significant step towards the replacement of fossil fuels. Combined with development of technologies to optimize the microalgae production, oil extraction and biomass processing has the capacity to make significant contributions towards this goal. A novel process that was developed in this study can convert the algal oils in the microalgae directly without first extracting lipids called *in situ* transesterification. Methanol under sub-or supercritical condition is used as the solvent to extract the lipids out of algae; meanwhile, methanol as a reactant esterifies the free fatty acids and transesterifies the triglycerides in the microalgae into biodiesel in situ. These two steps are performed simultaneously in a single step, which simplifies the processing and is therefore more economical. A high lipid content microalgal strain, *Schizochytrium limacinum*, was selected as the model to perform the *in situ* transesterification. Temperature (170, 210, 250, and 290˚C), reaction time (30, 60, 90 and 120 min), and lipid-to-methanol molar ratio (sRatio; 1:50, 1:75, and 1:100) were investigated for their effects on the conversion efficiency. Temperature appeared as a most influential factor.

Microalgal lipids are composited of multiple fatty acid types including triglycerides, free fatty acids, and phospholipids. Presence of phospholipids and free fatty acids (FFA) in such oils can cause processing difficulties, such as saponification and decrease in catalytic efficiency, in the transesterification of such oils for biodiesel production, thus lead to adverse process efficiency. This phenomenon was also observed in our previous study on converting microalgal lipids to fatty acid methyl esters (FAME) via *in situ* transesterification. This study explored the transesterification of phospholipids and the effects of processing conditions and presence of FFA in biodiesel production from plant oils in sub- and/or super-critical methanol.

Additional, FFA and water are the two common interferences in conventional catalyzed transesterification process. Consequently, high quality or refined oil feedstocks need to

be utilized to avoid side reactions phenomenon that would reduce the product yield. *In situ* transesterification was previously studied to directly convert microalgal lipids into FAME and developed successful achievement. Therefore, the process effectiveness of in situ transesterification on FFA content and water content need to further investigate individually.

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Chapter 1. Introduction

Since the world is aware of the negative effects of burning petroleum fuel and the foreground of petroleum fuel shortage, developing a sustainable energy seems just the right solution to it.[1] In 2009, the International Energy Agency (IEA) investigated the current energy consumption status in US and claimed that United States would strive to achieve 6% of total transportation fuels that will be biofuel by 2030.[2] The United States has worked on investigating renewable energy since 1970's and already became the number one biodiesel and bioethanol producer in the world. So far, people mostly use vegetable oil as the feedstock for biodiesel production and corn as the feedstock of bioethanol production. However, extensively taking agriculture products into biodiesel production is threatened to the world food supplying. Therefore, discovering a different resource, which is not competing with world food supplying, is the vital revolution for biofuel production, and producing biofuel from either freshwater microalgae or marine microalgae could be one of the solutions. Microalgae exhibit several important attributes for futuristic research on renewable energy. With the advantages of simple and inexpensive nutrient regime to culture, faster growth rate as compared to terrestrial energy crops, high biomass productivity, attractive biochemical profile and good energy content, microalgae are the strong candidate for a bioenergy resource.[3–5] The production of biodiesel from microalgae compared to irrigate crops is more beneficial because of the availability of the water and high lipid productivity of microalgae per unit area with lower quality land, in contrast to the productivity of terrestrial energy crops in large areas with high quality farming land.[6,7] Besides, microalgae growth requires primarily carbon dioxide and sunlight with small quantities of other readily available nutrients from power plant fuel gases and agricultural waste streams.[7]

The commercial usage on microalgae is more than 30 years old and the common used microalgae species are *Chlorella, Spirulina, Dunaliella salina* and *Haematococcus pluvialis*. The first commercial application of microalgae is in health and pharmaceutical industry.[8] Microalgae have been proved to contain large amount of Omega 3 oil and chlorophyll, which are frequently occurred in food supplements and pharmacy

ingredients.[9] Thus, from a nutrient point of view, microalgae have remarkable value for human health. From an energy point of view, microalgae can be used directly to generate heat, steam and electricity. Alternatively, algal biomass may be converted to gaseous and liquid biofuels.[10] Through microbial processes, algal biomass can be anaerobic digested to biogas and biohydrogen. Meanwhile, some specific microalgae strains are able to produce high yield of lipids, which are favored for converting to biodiesel. Many different classes of lipids can be produced from microalgal cells, which are similar to vegetable oils, but mainly hydrocarbons. The US Department of Energy's Office of Fuels Development funded the Aquatic Species Program (ASP) program to develop renewable transportation fuels from algae from 1978 to 1996.[11] In this program, approximately 300 species of algae have been proved of producing lipids and most of them are green algae and diatoms.[12] Moreover, they concluded that an open pond system of microalgae cultivation could develop 50 g/m^3 mass per day. Therefore, microalgae have been widely treated and studied for producing biofuels commercially.[8,13] Although microalgae have already been acknowledged as a viable feedstock for making biofuel, currently there is still no commercially microalgae-based biofuel manufacture in production.[10,14,15] According to market analysis and economic modeling of the whole production process, the main obstacle of microalgal biodiesel is the significantly higher labor and harvesting costs than other biofuel processes.[16] To face these obstacles on a large scale of production, increasing productivity efficiency is the key for developing a viable microalgae-base biofuel production. For the purpose of biodiesel production, high oil content and rapid growth rate microalgae are the favorite subjects to determine high process yield and reduce the cost of extraction and purification.[17–20] Compared to the quality of oil from various types of resources which can be used for biofuels production, microalgae have superiorities: 1) the fatty acid constitution is similar to vegetable oils; 2) by using high lipid content microalgae species and optimum culture condition, the oil yield can research 85% of the dry biomass weight; 3) microalgae lipid can be the feedstock for several different types of renewable fuels such as biodiesel, methane, hydrogen, ethanol, among others.[21–23] In contrast, microalgal lipids have disadvantages too: 1) certain types of microalgae lipids

are difficult to convert to biodiesel; 2) the cost of cultivation is higher than common crop oils.[24] Therefore, finding an economic procedure of biofuels processing is highly desired.

Microalgae process, even after being harvested, requires extensive research and development before the technology can be efficiently and economically applied for biofuels production. Conventionally, biodiesel can be produced from algal biomass or lipids by extraction-transesterification methods.[2526] Algal biodiesel is produced from wet algal biomass in a series of steps including preparation of dry algae powder, extraction of algal oils with chemical solvents, and conversion of the algal oil to biodiesel with a catalytic transesterification.[10] However, drying the biomass and extraction of algal oils by conventional methods is both energy- and cost-intensive. An alternative approach to the conventional extraction and transesterification method has been investigated, known as *in situ* transesterification. Although, catalysts play a great role in reducing transesterification time, their presence promotes complications of final product purification. This results in an increased process cost. Therefore, to avert the drawbacks of catalyzed transesterification, *in situ* transesterification is applied. Besides, *in situ* transesterification gives both much shorter reaction time and high conversion rate (oil to ester), and eliminates the catalysts cost. *In situ* transesterification has been introduced to produce biodiesel for several years. However, this technology is still not a favorite option when coming to employ on large manufacture. One of the significant reasons of *in situ* transesterification has not been widely used in industry is that there is no universal optimum operation condition for biomass. For example, microalgae have been approved to be a promising alternative feedstock for making biodiesel. Unfortunately, due to the complicated composition and tough physical structure, microalgal biodiesel has not been applied on the market. Therefore, it is necessary to study on microalgal biodiesel production via *in situ* transesterification, which could achieve that on one day the world would have a real clean sustainable energy.

A novel process that was developed in this study can convert the algal oils in the microalgae directly into biodiesel without first extracting the lipids. Methanol under sub-or supercritical condition is used as the solvent to extract the lipids out of algae;

meanwhile, methanol as a reactant esterifies the free fatty acids and transesterifies the triglycerides in the microalgae into biodiesel. These two steps are performed simultaneously in a single step, which simplifies the processing. In order to thoroughly understand the effect of this new developed process on microalgae biomass, three individual studies were taken care of. The subjects of these three studies are (1) biodiesel production from green microalgae *schizochytrium limacinu*m *via in situ* transesterification, (2) phospholipids transesterification in sub-/super-critical methanol with the presence of free fatty acids, and (3) effects of free fatty acids and water content in transesterification with sub/ super-critical methanol. In the first study, biodiesel production from green microalgae *schizochytrium limacinu*m *via in situ* transesterification, two small objectives were included: (1) investigate the possible contributing factors of lipid esterification and transesterification for algal biodiesel production, (2) systematically evaluate the process efficiency as affected by the process parameters and the treated and untreated microalgae samples.

Several conclusions were made from above listed studies. Each conclusion supported and proved the idea of *in situ* transesterification of microalgae for biodiesel production is feasible. Secondly, this process method worked on phospholipids transesterification and form fatty acid methyl esters. In addition, presence of free fatty acids enhances transesterification efficiency in supercritical methanol. Furthermore, presence of water affects the process in a negatively way.

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Chapter 2. Literature Review

2.1 Microalgae Characterization

2.1.1 Lipid-rich microalgae vs crops as biodiesel feedstocks

Algae are recognized as one kind of the oldest aqueous organism, which were first residents on the earth. They are primitive plants and categorized into two groups, microalgae and macroalgae. Microalgae are tiny, unicellular algae that normally grow in suspension within a body of saline water. In contract, macroalgae are the large, multi-cellular algae often grow in a pound.[1,2] Both microalgae and macroalgae are able to convert CO_2 to organic matters. With over 40,000 species already identified and with many more yet to be identified, algae are classified in multiple major groupings as follows: cyanobacteria (*Cyanophyceae*), green algae (*Chlorophyceae*), diatoms (*Bacillariophyceae*), yellow-green algae (*Xanthophyceae*), golden algae (*Chrysophyceae*), red algae (*Rhodophyceae*), brown algae (*Phaeophyceae*), dinoflagellates (*Dinophyceae*) and 'pico-plankton' (*Prasinophyceae* and *Eustigmatophyceae*).[3] Lipid-rich microalgae are one sub-category of microalgae which can photosynthesize light and carbon dioxide (CO_2) into lipids and preserve approximately 20 wt%-50 wt% of their dry biomass weight.[3,4] Most of them are found in green algae, red algae, diatoms, brown algae and cyanobacteria.[3] Scientists have researched on various species of microalgae, and found lipid contents and productivities are varies from species to species.[5–7] Among the most commonly researched species, *Chlorella protothecoides* and *Neochloris oleoabundans* are found containing the highest lipid contents (wt% dry biomass) and *Chlorella sp.*, and *Chlorella protothecoides* providing the highest lipid productivity (mg/L/day) (table 2-1).

Microalgal cultivation requires less freshwater than terrestrial plants. Microalgae are able to survive in tough environment and utilize non-arable land to relief stress for the production of food crops whilst reducing other environmental effects.[8,9] US Department of Energy delivered a thorough review on biodiesel production from algae in 1998. They concluded that microalgae growth can achieve worldwide fuel demand by using fewer than 6 million hectares landscape which less than 0.4% of arable land for oily crops. The rapid growth rate of microalgae and high lipids content make microalgae

much more competitive than terrestrial crops. Microalgae possess high tolerance on cultivation. Easy to cultivation, alive in vary conditions, consume nutrients from wastewater and easy to obtain nutrients. Microalgal cultivation consumes less water and required smaller spaces than land crops, and thus the cost of algae production is less than planting.[3,6] Some microalgae produce valuable by-products in the form of high value products and in the form of proteins, pigments, biopolymers, and carbohydrates for commercial or pharmaceutical purpose.[6,9] The lipid content of microalgae is dependent on not only species but also other factors, such as cultivation methods (e.g., reactor types of open pound, close photobioreactors, and auto-photo-bioreactors), nutrients in medium, salinity, light intensity and sources, pH, temperature, and dissolved oxygen levels. There are four major types of algae cultivation conditions: photoautotrophic, heterortrophic, mixotrophic, and photoheterotrophic.[3,5,10,11] Different microalgae can grow under one or more cultivation conditions with varied lipid content and biomass productivity. Each of the cultivation possesses its own advantages and disadvantages.

Among these four growth types, heterotrophic has been indicated to be capable of producing much better lipid yield than those under other cultivation conditions. However, heterotrophic culture is easily contaminated, especially in open pond systems, in addition to its high cost requirement of organic carbon as energy source. Phototrophic cultivation can uptake carbon dioxide in fuel gas and is commonly used in lab-scale cultivation. However, the lipid productivity of phototrophic cultivation is typically lower than that of heterotrophic cultivation due to its low cell growth rate and low biomass productivity. Compared to the high operation cost in heterotrophic cultivation, phototrophic cultivation is relatively inexpensive and then widely used.[4,5,10] Recently, literature reported that mixotrophic and photoheterotrophic cultivations would be also used for lipid production. However, these operations are restricted by their high contamination risk and special light requirement, thus involve more operating and processing costs.[12,13]

Table 2-1. Lipid contents and productivities of various microalgae species and multiple cultivation methods.

Species	Cultivation condition	Lipid content (wt% d.b.)	Lipid productivity (mg/L/d)	Biomass productivity (g/L/d)	Reference
Chlorophyta/ Green algae					
Botryococcus braunii UTEX 572	Phototrophic[a]	25.0-75.0	5.5	0.03	Yoo, Lee, Veriansyah, Kim, Kim and Lee[14]
Chlorella emersonii CCAP 211/11N	Phototrophic[b]	25.0-34.0	10.3-12.2	0.04	Scragg and Bonnett[15]
	Phototrophic[a]	29.0-63.0	8.1-49.9	0.03-0.05	Illman, Scragg and Shales[16]
Chlorella sp. F&M-M48	Phototrophic[a]	18.7	42.1	0.23	Rodolfi, Zittelli, Bassi, Padovani, Biondi, Bonini and Tredici[3]
	Phototrophic[a]	32.0-34.0	121.3-178.8	0.37-0.53	Chiu, Kao, Chen, Kuan, Ong and Lin[17]
	Phototrophic[a]	11.0-23.0	0.2-5.4	0.002-0.02	Illman, Scragg and Shales [16]
Chlorella protothecoides CCAP 211/8D	Heterotrophic[f]	43.0-46.0	1881.3-1840.0	4.0-4.4	Cheng, Zhou, Gao, Lan, Gao and Wu[18]
	Heterotrophic[c]	50.3-57.8	1209.6-3701.1	2.2-7.4	Xiong, Li, Xiang and Wu[19]
	Heterotrophic[c,g]	46.1	932.0	2.0	Xu, Miao and Wu[3]
	Heterotrophic[c]	43.0-48.7	732.7-932.0	1.7-2.0	Li, Xu and Wu[3]
Chlorella vulgaris KCTC AG	Phototrophic[a]	6.6	6.9	0.1	Yoo, Jun, Lee, Ahn and Oh[14]
	Phototrophic[a]	33.0-38.0	4.0	0.01	Liang, Sarkany and Cui[3]
	Heterotrophic[c,d]	23.0-36.0	27.0-35.0	0.08-0.15	Liang, Sarkany and Cui[3]
	Mixotrophic[c,e]	21.0-34.0	22.0-54.0	0.09-0.25	
	Phototrophic[b]	5.1	7.4	0.18	Gouveia, Marques, da Silva and Reis[20]
Dunaliella salina	-	6.0-25.0	116.0	0.22-0.34	Mata, Martins and Caetano[6]
Neochloris oleoabundans UTEX 1185	Phototrophic[a,b]	15.9-56.0	10.7-38.8	0.03-0.15	Gouveia, Marques, da Silva and Reis[20]
	Phototrophic[b]	29.0	26.1	0.09	

	Phototrophic[a]	7.0-40.3	38.0-133.0	0.31-0.63	Li, Horsman, Wang, Wu and Lan[21]
Nannochloropsis oculata NCTU-3	Phototrophic[a] -	22.7-29.7	84.0-142.0	0.37-0.48	Chiu, Kao, Tsai, Ong, Chen and Lin[17]
Phaeophyta/ Brown algae					
Pavlova lutheri CS 182	Phototrophic[a]	35.5	50.2	0.14	Rodolfi, Zittelli, Bassi, Padovani, Biondi, Bonini and Tredici[3]
Pavlova salina CS 49	Phototrophic[a]	30.9	49.4	0.16	
Isochrysis sp. F&M-M37	Phototrophic[a]	27.4	37.8	0.14	
Isochrysis sp. *(T-ISO)* CS 177	Phototrophic[a]	22.4	37.7	0.17	
Rhodophyta/ Red algae					
Porphyridium cruentum	Phototrophic[a]	9.5	34.8	0.37	Rodolfi, Zittelli, Bassi, Padovani, Biondi, Bonini and Tredici[3]
Bacillariophyceae/Diatoms					
Phaeodactylum tricornutum	Phototrophic[a]	18.7	44.8	0.24	Rodolfi, Zittelli, Bassi, Padovani, Biondi, Bonini and Tredici[3]
Thalassiosira pseudonana	Phototrophic[a]	20.6	17.4	0.08	

[a] CO_2, [b] Air, [c] Glucose, [d] Acetate, [e] Glycerol, [f] Jerusalem artichoke hydrolysate (JAH), [g] Corn powder hydrolysate (CPH).

Table 2-2. Lipid content, and biomass yield of various biodiesel feedstocks.

Plant	Lipid content (wt%)	Biomass yield (kg/ha/yr)	Reference
Rapeseed	35.0	600-1000	Issariyakul and Dalai[22]
Soybean	21.0	300-450	
Sunflower seed	44.0-51.0	280-700	
Palm	40.0	2500-4000	
Coconut	63.0	600-1500	
Microalgae	Lipid content (wt%)	Biomass yield (kg/L/yr)	Reference
Chlorella protothecoides CCAP 211/8D	50.3-57.8	0.8-2.7	Xiong, Li, Xiang and Wu[19]
Chlorella sp. F&M-M48	32.0-34.0	0.14-0.19	Chiu, Kao, Chen, Kuan, Ong and Lin[17]

Microalgal oil and spent biomass offered good potential sources as biodiesel feedstock. Microalgae lipids contain twice the energy stored per carbon atoms than carbohydrates which leads to a twofold energy increase in fuel energy.[8] More than 95% of biodiesel sources are first generation agricultural edible crop oils. Second generation biofuels such as jatropha oil, waste cooking oil and animal fats as recourses provide not threat to food security, but its poor cold flow properties and saturated fatty acids give rise to production difficulties and possible bio-hazard due to their nature of solidify at room temperature.[23] Microalgal biofuel is recognized as third generation biofuel that resolves the conflict between energy demand and food security. Microalgae possess priorities of fast growth rate, multiple harvests during year around and higher solar energy yields thereby giving superior lipid productivity (table 2-2). Microalgae are commonly known to double their weight with respect to biomass within 24 h. Some species have doubling times as short as 3.5 h. This high productivity decides the potential of a modern high theoretical yield production of 47,000 – 308,000 L^{-1} ha^{-1} $annual^{-1}$ and a possible outcome of 5950 l of biodiesel per hectare per year.[8] Furthermore, microalgae are able to survive in a crude non-arable land which significantly reducing costs and environmental impact. Theoretically, microalgae require 2% of the land required to produce the same amount of biodiesel from lipids bearing crops.[8] Growth of microalgae can effectively remove phosphates and nitrates from wastewater, thus making it an ideal substrate for the cultivation of microalgae while cutting down the nutrient cost. In an assessment worked on the properties of microalgal biodiesel are similar to petroleum diesel.[4] These include density, viscosity, flash point, cold flow, and heating value. None of the other potential biofuel resources has the capability of replacing petroleum diesel as microalgae do. This is mainly contributed by their positive impact on environment.

Since microalgae biomass contains carbohydrates, proteins, and fats, various applications are able to implement in order to generate biofuels. Algal biodiesel has several advantages over petroleum diesel. It is derived from biomass and therefore is renewable, biodegradable, and non-toxic, and contains reduced levels of particulates carbon monoxide, soot, hydrocarbons and SO_x.[4] Furthermore, algal biodiesel emits 78%

less CO_2 than petroleum diesel, which is the major positive feature of algal biodiesel. Thermochemical conversion and biochemical conversion are two primary divisions of algal-fuel production. An experiment studied on partially gasification *Spirulina* from temperature range of 850 to 1000°C and generated a highest methanol yield of 0.64 g methanol from 1 g biomass at 1000°C.[24] Liquefaction, pyrolysis, and transesterification are processes that can be employed to convert algal biomass material into liquid fuel. Liquefaction is a low temperature (300- 350°C), high pressure (5-20 MPa) process aided by a catalyst in the presence of hydrogen to yield bio-oil.[4] In 1994, Dote et al. successfully convert *Botryococcus braunii* to bio-oil through liquefaction process at 300°C and 3 MPa.[25] They achieved an oil yield of 64 wt% d.b. with HHV of 45.9 MJ/kg and positive energy balance of 2.94:1. This technology provides a much higher oil yield than pyrolysis due to the principle of pyrolysis process. Pyrolysis reaction generates bio-oil, syngas and charcoal at medium to high temperatures (350-700°C) in the absence of air.[4] In the work of microalgal pyrolysis, a bio-oil yield of 55.3 wt% d.b. was achieved by pyrolysis *Chlorella prothothecoides* at 502°C and 0.101 MPa.[26] It also provided products of 36.3 wt% d.b. syngas and 8.4 wt% d.b. charcoal. The portion of a single product could be adjusted by varying the retention time. In the process of flash pyrolysis, a high biomass-to-liquid conversion ratio (95.5%) can be reached by shorten hot vapor retention time (about 1 s).[26] Transesterification process is purely focused on biodiesel that conventionally requests lipids extraction before head. *In situ* transesterification is a novel technique that could exclude the step of lipid extraction and form biodiesel in one-step. *In situ* transesterification process undergoes extensive investigation and projects a promising further on becoming an ultimate substitute of petroleum diesel.

2.1.2 Lipid characterization

2.1.2.1 Fatty acid profiles of microalgae

Using algae as raw material of biodiesel production can mitigate the current environment situation and able to provide feedstock for several different types of biofuels, such as biodiesel, methane, hydrogen, ethanol, and among others.[3,11,27]

Microalgae are prokaryotic or eukaryotic unicellular sunlight-driven microorganisms that can grow rapidly and live in harsh condition.[3,6,10] Besides lipid-bearing microalgae are one sub-category of microalgae that contain highly amount of lipid. Lipid-rich microalgae contain roughly 20% - 50% of the dry mass weight of lipid under several specific cultural conditions, such as in a high C/N medium.[3,28] Compared to conventional raw material and oleaginous crops, microalgae are more efficient for biodiesel production. Lipid content of microalgae is different from various species but similar to other potential biodiesel resources. Scientists have used various species of microalgae to extract oil out, and get comparison on their oil content. Two parameters are generally considered for the evaluation of lipid accumulation for biofuel production: one is the lipid content (% lipids pre dry weight of biomass), the other the lipid productivity (amount of lipid produced per liter of working volume per day). They conclude *Botryyococcus braunii*., and *Chlorella vulgaris* have the highest lipid content (% dry weight biomass) and *Cholorella sorokiniana, Nannochloropsis oculata* give the highest lipid productivity (mg/L/day). Apart from species, other influences are also contributing on microalgal lipids capacity, such as cultivation methods (e.g. open pound, close photobioreactors, and autophotobioreactors, etc.), nutrients in medium, salinity, light intensity and resource, pH and temperature, and oxygen dissolving level.[5,10,28]

The property of fatty acids directly affects fuel property. Thus, understanding of fatty acids composition is essential for designing a proper production process. Several papers provide information on fatty acids composition of algal lipids with/ without considering the effect of cultivation condition difference. Although growth condition could modify lipids composition in some extend, genetic inheritance still is the primary discipline. Within a close look of microalgal lipids fatty acid profile of numbers of different strains of microalgae, a universal conclusion was made that microalgal lipids possess the similar composition with vegetable oil.[29] Many studies have reported the lipid content and productivities of different microalgae species, and have a common fatty acids carbon range from C12 to C22, which is crossover of a vegetable oil range of C14 to C20 and a polyunsaturated fatty acids range of C20 to C22.[5–7,9,28] The unique character of microalgal lipids, which possess massive amount of polyunsaturated fatty

acids, draws a quite attention as a valuable health nutrient. For example, 50% of total fatty acids in *S. obliquus* and *C. zofingiensis* is oleic acid (C18:1) while polyunsaturated one constitute 25-32 wt%.[30] This fact supports the statement of that microalgae are well-known feedstock substitute for biodiesel possess some unique properties other than vegetable biodiesel. In a summary reported microalgal lipids composition, myristic acid (C14:0), palmitic acid (C16:0) and stearic acid (C18:0) present in most of the microalgae lipids as dominant component.[3,29,31] But, in some exceptional cases, these prominent fatty acids may not appeared or present as a less dominate constituent.[3] In certain microalgae strains, some odd number fatty acids may appear in lipid compositions[32], such as strain *Chaetoceros, Dunaliella* and *Chlorella.* They are commonly found in a traceable odd number fatty acids of pentadecylic acid (C15:0) and iso-margaric acid (C17:0). Microalgae *Chlorella protothecoides* were determined to contain C17:0 of 15.8 wt% d.b.[3] Furthermore, *Chlorella sp.* contains both C15:0 (0.8 wt%) and C17:0 (0.3 wt%), which possess odd number fatty acids with much less quantity than *Chlorella protothecoides*. Comparing with other classes of fatty acids, saturated fatty acids take the largest portion of microalgal lipids. Besides the content of saturated fatty acids in many species of microalgae is comparable or greater than that of many vegetable oils.[33] Numerous studies have been implemented in order to understand the microalgal biodiesel production from various aspects. They all selected the specie, which has relative high lipids content, or a well-known strain. Most of the selected microalgae strains are green microalgae cultured in either freshwater or marine. Green microalgae are commonly used for algal biodiesel studies due to the high lipids content and high productivity. The green microalgae showed higher contents of unsaturated fatty acids than saturated fatty acids. The major fatty acids in green algae group were C14:0, C16:0, C18:0, C16:1ω9, C18:2ω2, C18:3ω3 and C18:4ω3 that collectively contributed to 58.7-88.9% of total fatty acids pool.[34] Interestingly, no *trans* fatty acids were encountered in any green algae.[35] Some experiments also used diatom and cyanobaterium since they also belong to microalgae. Besides, red microalgae and brown microalgae are also studied but not favored in biodiesel studies due to the fact that carbohydrate is the dominate compound and high ash content.[29] Red algae showed

large variations in Monounsaturated fatty acids and ranged from 3.5% to 26.6% of total fatty acids.[34] Myristic acid, palmitic acid, stearic acid, palmitoleic acid, oleic acid, elaidic acid, dihomogammalinolenic acid (C20:3,n-6), arachidonic acid (C20:4) and eicosapentaenoic acid (C20:5) were among the predominant fatty acids encountered and collectively accounted for 77.6–99.2% of total fatty acids.[34] In the 24 different brown algal species in Kumari et al.[34] study, the major fatty acids encountered were myristic, palmitic, stearic, palmitoleic, oleic, linoleic, and stearidonic acids that collectively contributed to 80.1-92.2% of total fatty acids. In contrast to green algae and red algae, both polyenoic C18 and C20 fatty acids were predominant in these species and varied from 23.6 to 58.0% of total fatty acids. This trend was a typical character of brown algae group that distinguishes them from rest of the green and red algae groups. Table 2-3 summarized fatty acid profiles of some common used microalgae in algal biodiesel studies.

Fatty acids profile of a potential biodiesel feedstock directly affects fuel property. Polyunsaturated fatty acids take the second place of being the primary component.[31,34] Microalgal lipids have been recognized as a suitable oil source of biodiesel regards to its similar fatty acids profile to vegetable oil. However, microalgal lipids still have many different from conventional biodiesel feedstock. The composition of microalgal lipids can be divided into three catalogs, saturated fatty acids, polyunsaturated fatty acids, and impurities (free fatty acids, phospholipids and pigments).[36] The influence on biodiesel property are revealed from several aspects, including cetane number (CN), viscosity, exhaust emissions, oxidative stability, cold flow, and lubricity. Generally, for most properties, a significant difference in fuel-related properties exists between saturated and unsaturated fatty compounds. A trade-off also exists between composition and fuel properties. Saturated fatty esters are favorable for combustion related properties, such as cetane number, exhaust emission, and oxidative stability.

Table 2-3. Fatty acid profiles of common studies microalgae.

Group	Species	Fatty acid profile			Reference
		Saturated	Monounsaturated	Polyunsaturated	
Green algae	*Botryococcus* sp.	16:0/20.56%	16:1ω/7 8.20%	18:2ω6/ 6.36%	Sahu, et al. [37]
		18:0/ 4.56%	C18:1ω9/44.74%	18:3ω3/ 15.55%	
	Chlorococcum sp.	16:0/19%	18:1/63%	18:2/4%	Halim, et al. [38]
		18:0/3%	16:1/4%		
	Chlorella sp.	14:0/2.0%	14:1ω7/0.1%	16:2ω6/3.6%	Zhukova and Aizdaicher [33]
		15:0/0.8%	16:1ω7/0.8%	16:3ω3/12.0%	
		anteiso-15:0/1.6%	16:1ω13tr/2.6%	20:5ω3/1.3%	
		16:0/19.6%	18:1ω9/5.7%	16:2ω4/0.4%	
		17:0/0.3%	18:1ω7/1.6%	18:3ω3/22.3%	
		18:0/3.3%	20:1ω9/0.1%	18:2ω6/11.8%	
				18:3ω6/0.3%	
				20:2ω6/0.2%	
				20:5ω3/1.3%	
	Chlorella sorokiniana	14:0/0.27%	16:1/2.29%	16:2/4.87%	Dong, et al. [39]
		16:0/25.32%	18:1/22.56%	18:2/39.32%	
		17:0/0.21%		18:3/4.62%	
		18:0/1.22%			
		20:0/0.77			
	Chlorella protothecoides	14:0/1.31%	18:1ω9/60.84%	18:2ω6/17.28%	Xu, Miao and Wu [40]
		16:0/12.94%	19:1ω9/0.36%		
		17:0/0.89%	20:1ω9/0.42%		
		18:0/2.76%			
		20:0/0.35%			
	Chlorella vulgaris	16:0/15–18%	14:1/0.5%	14:2/0.1%	Matucha, Zilka and Svihel [41]
		18:0/3–5%	16:1/0.5–1%	16:2/3–5%	
		20:0/0.1–0.2%	18:1/20–25%	18:2/13–18%	
			20:1/0.2%	20:2/0.2%	
				14:3/0.1%	
				16:3/10–12%	
				18:3/13–18%	
	Dunaliella salina	14:0/0.5%	14:1ω7/0.1%	16:2ω6/1.5%	Zhukova and Aizdaicher [33]
		anteiso-15:0/0.9%	16:1ω7/0.8%	16:3ω6/1.7%	
		15:0/0.2%	16:1ω13/1.7%	16:4ω3/18.2%	
		16:0/17.8%	18:1ω9/2.8%	18:2ω6/6.1%	
		18:0/1.5%	18:1ω7/0.6%	16:3ω4/0.3%	

				18:4ω3/0.7% 16:3ω3/2.1% 18:3ω6/2.5% 18:3ω3/36.9%	
	Neochloris oleoabundans	14:0/0.4% 16:0/19.4% 18:0/1.0%	16:1/1.9% 18:1/20.3%	16:2/1.7% 18:3/17.5% 18:4/2.1% 18:2/13.0%	da Silva, Santos and Reis [42]
	Nannochloropsis oculata	14:0/3.9% iso-15:0/1.2% 15:0/0.5% 16:0/20.5% iso-17:0/0.7% 17:0/0.4% 18:0/1.8%	14:1/0.2% 16:1ω7/25.2% 18:1ω9/3.6% 18:1ω7/0.5%	16:2ω6/0.6% 16:2ω4/0.2% 18:2ω6/2.2% 18:3ω6/0.7% 20:4ω6/5.3% 20:5ω3/29.7%	Zhukova and Aizdaicher [33]
Brown algae	*Isochrysis* sp., 0.5 M NaCl; N deficient	14:0 + 14:1/18.6% 16:0/1.4%	16:1/2.9% 18:1/17.2% 20:1/1.1%	18:2/5.0% 18:3/6.3% 18:4/14.0% 22:4/2.7% 22:6/14.9%	Knothe[31]
Red algae	*Phaeodactylum tricornutum*	14:0/ 2.25% 16:0/ 3.65% 18:0/0.11%	16:1ω7/4.48% 18:1ω9/2.46% 18:1ω7/ 0.24%	16:3ω4/0.21% 20:5ω3/2.15%	Ceron Garcia, et al. [43]
	Porphyridium cruentum	14:0/1.3% 16:0/29.7% 18:0/0.8%	16:1/1.4% 18:1ω9/0.8% 18:1ω7/1.9%	18:2ω6/8.0% 20:2ω6/0.7% 20:3ω6/0.7% 20:4ω6/25.0% 20:5ω3/20.9%	Alonso, et al.[44]
Diatoms	*Thalassiosira pseudonana*	14:0/15.5% 15:0/1.0% 16:0/9.7%	16:1/29.5%	16:2/5.5% 18:2/1.0% 16:3(+18:1)/6.3% 20:4/14.2% 20:5/15.0%	Orcutt and Patterson[45]

While unsaturated compounds are favorable for clod flow properties as well as viscosity.[46] Long chain hydrocarbons are ideal for diesel fuel, which bring a high CN and

meaning a shorter ignition delay time and a more complete combustion. Microalgal lipids mainly contain saturated fatty acids. Saturated long chain esters, such as those of C16:0 and C18:0 have high CNs, in the ranges of the corresponding alkanes. However, with the introduction of double bonds in esters, the CN decreases significantly. Fortunately, Knothe[47] summarized that in a mixture of the presence of compounds with high CN, such as methyl plamitate, can compensate for those with low CN. Viscosity is also a factor strongly influenced by compound structure. The longer chain length and higher saturation of a fatty acid leads a poorer viscosity of the corresponded ester. Therefore, the balance of long chain fatty acids and unsaturation level would decide the viscosity of a microalgal biodiesel.

Exhaust emissions are not dealt with in fuel standards; rather, they are regulated by legislation to limit the maximum values that, for a specific type of engine, should not be exceed regardless of the fuel used. Four kinds of exhaust emissions are generally addressed in regulations, namely particulate matter (PM), nitrogen oxides (NO_x), hydrocarbons (HC), and carbon monoxide (CO). Biodiesel generally reduces exhaust emission of PM while other emissions are more based on the level of combustion. As previous discussion, microalgal biodiesel has long chain fatty acids that provides high CN and leads to more completed combustion. Therefore, a reasonable expectation of that microalgal biodiesel has lower exhaust emission could be made. The property of high content of saturated fatty acids and polyunsaturated fatty acids in microalgal oils lead poor cold flow and poor oxidative stability of algal-diesel fuel.[47] Cold flow and oxidative stability are two interacting factors controlled by long-chain saturated fatty acids and level of unsaturated fatty acids. Long-chain fatty acids cause poor clod flow property of corresponding biodiesel but promise a decent oxidative stability property. In contrast, a good portion of microalgal lipids is polyunsaturated and provides compensation to the poor cold flow property. However, it also means that a further hydrogenation step may be needed to reduce the double to lower their susceptibility to oxidation.[48] An investigation focused on the influence of several fatty acids on algal biodiesel properties (table 2-4). In the table 2-4, saturated methyl esters have been qualified oxidative stability but poor cold flow property. Meanwhile all C18 unsaturated

fatty acid methyl esters show oxidative stability values that do not meet biodiesel standards. Besides polyunsaturated fatty acids with even more double bonds, such as C22:5 and C22:6, have extremely unstable oxidative stability and the higher unsaturated level the lower oxidative stability.

In addition, the content of total lipids includes a considerably amount of pigments, phospholipids and free fatty acids (FFAs). The colorful appearance of algae is derived from pigments, and three major classes of photosynthetic pigments that appear in algae are chlorophylls, carotenoids and phycobilins. Algal pigments are steroid type lipids that cannot react in transesterification into methyl esters. Therefore, for algal biodiesel production, this portion of lipids is undesired. Phospholipids mainly exist in algal cell membranes. They are abundant component of microalgal lipids. Phospholipids have issues can poison the catalyst and cause reaction failure.[49] Besides, when algal cells are actively growing, polar membrane lipids (phospholipids and glycolipids) generally predominate[50], but as the cell enters stationary growth phase, many species accumulate triglycerides in order to preserve energy or meet cell metabolism requirement. In order to convert phospholipids via transesterification reaction, catalysts were added to enhance the reaction efficiency. In the paper of Halim et al.[38], they compared supercritical carbon dioxide ($ScCO_2$) and hexane extraction method for biodiesel production from microalgae *Chlorococcum* sp. lipids. They used both acid and alkaline catalyst to transmethylate extracted fatty acids and stated the usage of alkaline catalysts is to further transmethylate acylglycerols and phospholipids. However, the phospholipids were unsuccessfully esterified with the applied methylation techniques. To deal with an oil source that contains large amounts of phospholipids, a pretreatment using acid catalyst, to reduce phospholipids content was commonly employed. Therefore, phospholipids may be able to be convert into alkyl esters under acid catalytic transesterification. So far, there is no evidence to support this proposition. Another special feature of microalgal lipids is the high content of FFAs. High content of FFAs could poison catalyst and stop the transesterification reaction happens.[51] A similar treatment to eliminated phospholipids was usually employed. A more detailed

review of overcoming the barrier in microalgal biodiesel production is discussed in the following section.

Table 2-4. Fuel properties of some common neat fatty acid methyl esters.[46]

Compound	Cetane number	Viscosity, 40°C (mm^2/s)	Oxidative stability (h)	Melting point (°C)
Saturated				
Methyl caprate (C10:0)	51.6	1.72	>24	-13.5
Methyl laurate (C12:0)	66.7	2.43	>24	4.3
Methyl myristate (C14:0)	nd	3.30	>24	18.1
Methyl palmitate (C16:0)	85.9	4.38	>24	28.5
Methyl stearate (C18:0)	101	5.85	>24	37.7
Methyl arachidate (C20:0)	nd[1]	-		46.4
Methyl behenate (C22:0)	nd[1]	-		53.2
Unsaturated				
Methyl palmitoleate (C16:1)	56.6, 51.0	3.67	2.11	-34.1
Methyl oleate (C18:1)	56.6, 59.3	4.51	2.79	-20.2
Methyl linoleate (C18:2)	37.4	3.65	0.94	-43.1
Methyl linolenate (C18:3)	22.2	3.14	0.00	-52
Methyl erucate (C22:1)	74.2	7.33	nd[1]	-3.1

[1] Not detected.

2.1.2.2 Challenge of using microalgal oil as biodiesel resource

Lipids composition depends on the species and culture conditions, as are mainly composed by two fractions: 1) saturated and polysaturated fatty acids and acylglycerols which are suitable for biodiesel production (non-polar lipids) and 2) waxes, sterols, free fatty acids, phospholipids, hydrocarbons, carotenes and chlorophylls or unsaponifiable

matter which are not suitable foe biodiesel production (polar lipids).[52,53] The latter fraction significantly intervenes due to its foaming properties and specifically FFA esterification is a reversible reaction.[51,52] The unsaponifiable matter is not affected during biodiesel preparation and causes engine malfunctions but the poor cloud point makes it difficult to perverse.[52] Therefore, a two-step process of extraction-transesterification does not perform the best outcome for a purpose of efficient biodiesel production. Solvent extraction is the most common method to extract microalgal lipids while the physical method has a much low extraction yield.[54] However, the extractive matter contains undesired polar lipid that requires pretreatment before catalytic transesterification. Krohn et al.[51] reported that FFA in microalgal lipid via solvent extraction can reach as high as 84% (oil weight). High FFAs content is an unique character of microalgal lipid, and is different from other plant oil, and is the reason why algal cellular lipids can be degraded to volatile organic acids[55] or FFAs[56] by enzymes during long term storage. A review was written by Singh et al.[51], summarized the mechanism and challenge in commercialization of algal biofuel. He pointed out that algal lipids (triglycerides, diacylglycerols, phospholipids, etc.) could be degraded to FFAs by lipases, peroxidases and phospholipases or produce by microorganisms present in the algal paste. There are many pretreatment methods have been proposed and established to be effective on reducing FFAs in oil resource, including steam distillation, exaction by alcohol and acid catalyst. Whereas, acid catalyst are commonly used when the FFAs content in lipids is higher than 1 wt% since alkaline catalysts would form soap with exceeded FFAs.[51] The use of acid catalysts can promote both transesterification and esterification reaction of microalgae lipids. Johnson and Wen[57] reported biodiesel production yields higher than 50 wt% using the microalgae *Schizochytrium limacinum* with a shorter reaction time (40min) due to the addition of chloroform in the reaction. Miao and Wu[51] claimed to have achieved a biodiesel yield higher than 70 wt% in 5 h with sulfuric acid using the microalgae *Chlorella protothecoides* with a lipids content of 55 wt%.

Another challenge of converting microalgal lipid to biodiesel is its large amount of phospholipids. Phospholipids form the backbone structure of microalgal membrane.

However, its chemical nature makes it extremely difficult to react with alcohol. The higher the phospholipid content in the oil, the lower is the methyl ester yield. Hence, the quality of algal biodiesel is most influenced by feedstock, which possess large portion of lipids as phospholipids. In the early 20 century, biologists discovered phospholipids content has a range from 10% to 53% (oil weight). A research was conducted by Du et al.[58], where treated crude soybean oil with Novozym 435, a lipase catalyst, to breach the barrier of phospholipid transesterification. A FAME yield of 92 wt% was achieved when methyl acetate was used as the acyl acceptor with a molar ratio of methyl acetate to oil of 12:1 and methyl acetate showed no negative effect on enzymatic activity.[79] Another similar work was done by Du et al.[59] to achieve a 94 % of FAME yield by immersing lipase in crude oil (phospholipid content of 0.36 %, water content of 430 p.p.m., and acid value of 2) at 40°C for 120 h, and the same FAME yield was obtained for refined oils. This research proved that immersing pretreatment of lipase in oils could improve both the reaction rate and methyl ester yield significantly. Watanabe, Shimada, Sugihara and Tominaga [81] also used a lipase-catalyze transesterification method (*Candida antarctica*) to convert crude soybean oil with 0.7% FFAs, 0.08 % phospholipids and 0.12 % water. They used a three-step methanolysis method which add methanol for three times at 30°C and finally achieve 93.8% of oil conversion rate. The experiment showed the lipase could be reused for 25 cycles without any loss of the activity. Other than lipase catalyst transesterification is able to convert phospholipids, and acid catalysts also work on oil sample with high phospholipid content by removing phospholipids from microalgal lipids. The process of removing phospholipids is called degumming. A method described by Chen et al.[51] use a two-step catalytic conversion (degumming and acid catalytic transesterification) to produce microalgal biodiesel with high FFAs content. They experimented on three microalgal oils (*Scenedesmus* sp., *Nannochloropsis* sp., and Dinoflagellate oil). All three oils were degummed by stirring with 1% phosphoric acid and 10% water at 85˚C for 1h to remove most of phospholipids and non-lipid impurities then followed by acid catalytic transesterification. While *Scenedesmus* sp. oil with a highest FFAs content of 14.7% oil weight and phospholipids content of 37.3% oil weight gave a highest FAME yield of

56.2%. Moreover, the phosphorus concentrations in crude biodiesel from *Scenedesmus* sp. was as high as 295.6 ppm while the ASTM limits it to 10 ppm.[51] This study proved that phospholipids could be converted to FAME under appropriate conditions; however, the incomplete reaction of phospholipids can result in loss of the product by as much as 45% due to the precipitation and saponification.[58]

Last but not the least, water is another major factor interfering the transesterification reaction. During transesterification reaction, presence of water reduces the conversion of triglycerides to biodiesel fuel. Therefore, it is essential to minimize the water content in the feedstocks prior to transesterification process. In some works, the negative effects of water on transesterification reaction also present as triglycerides hydrolysis that increase the FFAs content and cause saponification.[58] Canakci and Van Gerpen[60] mentioned that if water higher than 1 wt%, the transesterification reaction will reduce the formation of FAMEs. Microalgae, aquatic organisms, will contain water when directly harvested from culture. Removal of water from the algal cells will be required during the period of pretreatment. Many authors who studied on microalgal biofuel production noted that high cost of oleaginous materials preparation the main problem hindering commercial production of biodiesel.[61] Moreover, water in biodiesel feedstocks can strongly affect catalytic transesterification. In the paper of Liu et al.[62], an investigation into the impact of water on liquid phase sulfuric acid catalyzed esterification of acetic acid with methanol at 60˚C. It was found that the catalytic activity of sulfuric acid was strongly inhibited by water. The catalyst lost up to 90% of activity while the amount of water present increased and the water deactivate the catalyst by being a preferential solvent.[62] Water also has influence on alkali-catalyst transesterification by inhibiting its activity. Singh et al. reported that for an alkali-catalyzed transesterification, the glycerides and alcohol must be anhydrous.[63] The reaction requires high-quality feedstock with a water content less than 0.06 wt%. It was also reported by Chew et al.[64] that alkaline metal alkoxides could give a biodiesel yield higher than 98% when the feedstock has a moisture concentration lower than 0.5%. Thus alkali-catalyzed transesterification are greatly affected by the presence of water that makes the reaction partially change to saponification, leading to soaps formation.

Hass[65] noted that water inhibits transesterification reaction since it competes with the alcohol, thereby the major reaction shifted from ester transformation to hydrolysis into FFAs. The FFAs formation favors saponification that inhibited the transesterification reaction. Therefore, high water content feedstocks require a prior refining in the industrial process. The refined feedstock requires a limited quality with less FFAs content (≤ 3 wt %) and water content (≤ 0.06 wt %) for alkali-catalyzed transesterification.

2.1.3 Transesterification on microalgal lipids

2.1.3.1 Feasibility of using lipid-bearing microalgae as biodiesel feedstock.

Biodiesel feedstocks differ from petroleum diesel in which they are highly viscos and thus not suitable for direct use in modern diesel engines and thus require conversion to meet regulatory standards. The most commonly seen methods for producing biodiesel from biomass base materials are i) direct combustion, ii) blending its oil component with petroleum diesel, iii) obtain crude bio-oil via pyrolysis or liquefaction, and iv) transesterification.[8] There are many reports that methanol to as a resource to transesterification the commodity oil, such as soybean, rapeseed or palm oil with other feedstocks such as animal fats, waste cooking oils or microalgal oil also playing a role. Along with the demand of bioenergy, changes from various aspects, first, second and third generation biofuels were introduced to the world. Algal biodiesel has generated considerable discussion in the literature with numerous review or perspective articles often containing supporting statements regarding the feasibility of algae-derived biodiesel being published.[23,38,63,66] They have emphasized the need to explore the possibilities of producing biodiesel from microalgae, as it will not compete with the land and cereal crops. Microalgae are potential to be used as a raw material for biodiesel production, as it meets the requirements of high lipid production rate which is mostly neutral lipids with lower degree of unsaturation.[6] Moreover, microalgae can potentially produce one to two orders of magnitude higher biodiesel yield than oil palm and cotton.[7,67] So far, numerous experiments of microalgal biodiesel production have been conducted and received positive results. Most of the experiments used a

conventional method (two-step transesterification) on microalgal biodiesel. Conventional transesterification is the one matured method of making biodiesel from oil-rich crops. The oil-rich crops/ seeds were first drawn oil by physical or chemical methods, then following by a catalytic transesterification. An additional refinery of the extracted lipids may need to meet the transesterification reaction condition. Soh et al.[68] used supercritical carbon dioxide ($ScCO_2$) extracted lipids from microalgae *Scenedesmus dimorphus* and implemented an acid transesterification of BF_3 at 105°C for 75 min afterwards. They claimed to get a maximum 80% of FAMEs yield from this method. In spite of the obvious advantages of microalgae as potential biodiesel feedstocks, numbers of unresolved issues still remain. In the review written by Rawat et al.[8], a concern was raised that production of microalgal biodiesel can be an energy intensive process. However, most of them still are based on the two steps catalytic process (extraction-catalytic transesterification), which requires large amount of catalysts and limited in lab-scale production. The major issue of this procedure is the high levels of FFAs and phospholipids in the extracted microalgal lipids. These fatty acids are unwanted in the biodiesel process as they strongly influence the processing and the quality of the product. The biomass with high levels of FFAs, an acid catalytic esterification was recommended along with a lipids extraction before alkaline catalytic transesterification. Lam et al.[69]summarized the advantages and disadvantages of different types of catalysts used in transesterification of waste cooking oil (high FFAs content). This is the major property to decide which kind of transesterification is suitable for microalgae oil-biodiesel production. Since both cooking oil and microalgae oil contain high level of FFAs, the acidic catalyzed transesterification is more suitable than basic catalyzed transesterification to avoid undesired soap formation.[69] High levels of FFAs and phospholipids raise the possibility of catalysts poison and incomplete conversion. A combination of acidic and alkaline transesterification catalysts is more effective than each individually when using the wet microalgae sample.[70] Nevertheless, the conclusion made by Lam et al. claims that homogeneous base catalyzed transesterification is not suitable to convert high FFAs oil. Besides, homogeneous acid catalyzed transesterification requires longer reaction time and could potentially causes

corrosion on equipment. In contrast, heterogeneous catalyzed transesterification, either basic or acidic catalyst, gives better tolerance on high FFAs oil samples. Apart from that, enzymatic transesterification is another possible way to produce biodiesel from high FFAs oil due to its ability of minimized the saponification caused by FFAs. Javidialesaadi et al.[71] designed an experiment to project above propositions with respect of biodiesel production from high free fatty acid-content oils. This study obtained a close 100% FFA conversion at a reaction temperature 50°C for 1 h with a methanol-to-oil ratio of 80% v/v and an acid catalyst (98% sulfuric acid) to methanol ratio of 3% v/v [64]. Another study was conducted in order to produce biodiesel from a high FFAs content algae oil via two-step catalytic conversion.[51]

The best way to determine the feasibility of one potential biodiesel feedstock is to exam the similarity to known fatty acid profile. There is considerable interest in Microalgae as fast growth, increasing production yield, and abundant lipids capacity. There are valuable information regarded to process effect of the fatty acid composition in algal lipids. In a research on comparing and contrasting vegetable oil and algal lipids for purpose of biodiesel production, a few points of algal fatty acid composition were listed. In the fatty acid profiles of algal lipids often tend to both extremes, namely high content of saturated fatty acids and high content of polyunsaturated fatty acids.[47] Although high content of saturated fatty acids and polyunsaturated fatty acids cause poor cold flow property and oxidative stability, they are always can be modified through growth condition control and genetic manipulation. It is obvious from the critical appraisal of the viability of algae projects from a true market perspective that algae-based biofuels deserve a strong attention of further commercialization.

2.1.3.2 Catalytic transesterification

In transesterification reaction, triglycerides are stable chemical that could not react with alcohol at standard condition. Therefore, a catalyst has to be used in order to initiate and catalyze the reaction. To clearly understanding this process, people sub-categorize to catalyst transesterification and non-catalyst transesterification, and sub-categorize catalyst transesterification into three types: homogeneous alkali/ acid catalyzed transesterification, heterogeneous alkali/ acid catalyzed transesterification

and enzymatic transesterification. Transesterification involves the cleaving of an ester bond by an alcohol and can be catalyzed by either a base or an acid. Both homogeneous and heterogeneous catalysts also can catalyze transesterification reaction. The most commonly used alkali catalysts are NaOH, CH_3ONa, and KOH. The reaction mechanism for alkali-catalyzed transesterification was formulated as three steps (Figure 2-1). The first step is an attack on the carbonyl carbon atom of the triglyceride molecule by the anion of alcohol (methoxide ion) to form a tetrahedral intermediate reacts with an alcohol (methanol) to regenerate the anion of alcohol (methoxide ion). In the last step, rearrangement of tetrahedral intermediate results in the formation of a fatty acid ester and a diglyceride. The alkali-catalyzed transesterification of vegetable oils proceeds faster than the acid catalyzed reaction. Biodiesel production with microalgae *Chlorella vulgaris* via alkaline *in situ* transesterification. A maximum biodiesel recovery of 78 wt% was achieved using a catalyst to lipid ratio of 0.15:1 and a methanol to lipid molar ratio of 600:1 at 60°C with a reaction time of 75 min.[72] In contrast to a acid-catalyzed *in situ* transesterification achieved up to 97 wt% of production yield using acid catalyst (H_2SO_4) to lipid ratio of 0.35:1 and methanol to lipid molar ratio of 800:1 at 60°C for 20 h reaction time.[72] The mechanism of base catalyzed transesterification on vegetable oils was discussed by Demirbas[73]. Usually a single catalyst is used and the choice is determined by the characteristics of the different catalysts. Acid catalystic transesterification is an alternative process to use on the feedstock contains higher FFA content due to the acid catalyst has higher tolerant of FFAs than alkali catalyst.

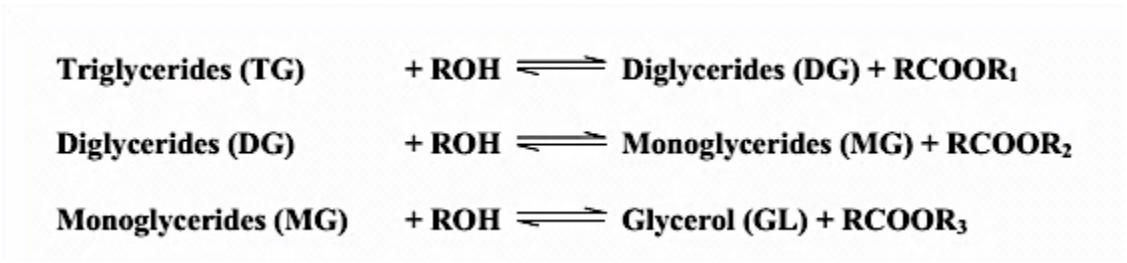

Figure 2-1. Kinetic of transesterification reaction

The most common method for the synthesis of biodiesel involves the use of catalyst. There are many studies conducted of using either homogeneous or heterogeneous

catalysts with oil and all achieved significant success.[74] Currently, the common homogeneous base catalyst for biodiesel production is sodium hydroxide (NaOH) or potassium hydroxide (KOH).[75–77] These catalysts are commonly used due to the reasons of requiring low reaction temperature and time; widely available and economical.[78] However, the use of this catalyst is limited only for refined vegetable oil with less than 0.5 wt% of FFA.[79] Therefore, base catalyst is not suitable to react with lipids such as microalgal lipids, which contain high FFAs. If an oil or fat contains high FFA, alkali catalyst will typically react with FFA to form soap, which is highly undesired. Moreover, excessive soap in the products can drastically reduce the FAME yield and inhibit the subsequent purification process of biodiesel.[80] Apart from that, high water content in the feedstock also affects the FAME yield. When water is present, particularly at high temperatures, it can hydrolyze triglycerides to diglycerides and form FFA.[69]

Acid-catalyzed transesterification was discovered and applied on some extreme situations. To date, the most investigated catalysts for acid-catalyzed transesterification are sulfuric acid (H_2SO_4) and hydrochloric acid (HCl). Acid-catalyzed transesterification hold an important advantage compared with alkali-catalyzed process due to acid catalyst is insensitive to the presence of FFAs and can perform both catalyzed esterification and transesterification reactions simultaneously.[81] It was reported that acid catalysis was more efficient when the amount of FFA in the oil exceeds 1 wt%.[82] In addition, economic analysis has proven that acid-catalyzed transesterification procedure could be reduced to a one-step process which cost much less than base catalysis process with the respect of extra purification treatment.[58]

However, acid-catalyzed system is not always a good choice for commercial applications due to longer reaction time, higher reaction temperature, high molar ratio of alcohol to oil, and serious environmental and corrosion related problem.[79,81] Because both base-catalyze and acid-catalyze systems have serious problem, a heterogeneous catalysis transesterification system was discovered to resolve the conflict. Similar to homogeneous catalysis, heterogeneous catalysis also sub-categories into two types: base catalyst and acid catalyst. Heterogeneous base catalyst is famous due to the fact of higher reaction rate than acid-catalyzed transesterification, works for mild reaction

condition and less energy intensive, easy to separate and high possibility to reuse and regenerate the catalyst.[69] Unfortunately, heterogeneous base-catalyst also has its drawbacks, such as poisoning the catalyst when exposed to ambient air, sensitive to FFA content, decrease FAMEs yield when soap formed, leaching of catalyst active site may result to product contamination.[58,83] Heterogeneous acid catalytic transesterification was investigated to overcome the disadvantages of base catalytic possesses. Heterogeneous acid catalysts are insensitive to FFA and water content, both esterification and transesterification reactions can occur simultaneously. Besides, it is easy to separate after reaction but may raise production cost due to the complicate catalyst synthesis procedures: high reaction temperature, high alcohol demand, longer reaction time, and leaching of catalyst active sites. Furthermore, the catalyst activation process may result in product contamination.[84,85] Although heterogeneous catalysts mainly have the same properties with homogeneous catalysts, they still have different applications regarding to renewable energy production. The development of heterogeneous catalyst system holds an important factor to be incorporated into a continuous flow reactor.[86] Recently, a new class of sulfonated carbon-based heterogeneous catalyst was demonstrated as promising catalysts or the esterification reaction of FFAs for the production of biodiesel.[87] Dong et al.[39] conducted a two-step acid transesterification with green microalgae *Chlorella sorokiniana* (UTEX 1602). In this study an acid heterogeneous catalyst, Amberlyst- 15, was added to pre-esteriication on FFAs then followed by a base-catalyzed transesterification. The two-step *in situ* transesterification resulted in a total FAME recovery up to 95%.[87]

Enzymatic transesterification is another catalyst transesterification. Enzymatic transesterification, especially those using lipase, has drawn researcher's attention in last ten years due to the downstream processing problem posed by chemical transesterification. Enzyme catalysis proceeds has the advantages of none by-products generated, easily recovery of product, mild reaction condition, insensitive to high FFA oil and reusable catalyst.[80] The advantages prove that enzyme catalyzed biodiesel production has high potential to be an eco-friendly process and a promising alternative to the chemical process. However, it still has its fair share of constraints especially

when implemented in industrial scale such as high cost of enzyme, slow reaction rate and enzyme deactivation.[88] A pioneering work by Shimada et al.[89] showed that over 6% conversion in a lipase-catalyzed solvent-free reaction system can be achieved by employing a stepwise process using less than 1:1 methanol to oil molar ratio. They further showed that adding more than 1.5 M equivalents of methanol leads to an irreversible lipase inactivation. The lipase, *Candida Antarctica,* can be used for more than 50 cycles that significantly reduce capital costs.[89] In the work of Hama and Kondo[90], they summarized recent works of enzymatic transesterification of various oil sources. Oleaginous microalgae strain *Chlorella* was studied by using three lipases, *Candia* sp., *Penicilium expansum*, and *Burkholeria* sp., via methanolysis reaction and determined FAME yield of 98%, 91%, and 97% respectively.[90] At this point, lipase *Candida* sp. has superiority on lipid methanolysis. A 98% FAMEs yield over 12 h period was achieved while other two lipases needed 48 h reaction. Enzymatic transesterification also could be combined with other applications to provide higher biodiesel production yield. A study used a mixture of *Candida rugosa* and *Rhizopus oryzae* lipases with a supercritical carbon dioxide process to produce biodiesel from soybean oil. An optimal condition was determined at 130 bar pressure, 45°C, 250 rpm agitation speed, 10% water content, and 20% immobilized *Candida rugosa* and *Rhizopus oryzae* (1:1) and oil to methanol molar ratio of 1:4. When batch process was performed under optimal conditions, the biodiesel conversion yield was 99% at 3 h while biodiesel conversion yield still reached 85% after the lipases were used 20 times.[36]

2.1.3.3 Non-catalytic transesterification and supercritical alcohol transesterification

Catalytic transesterification have been shown to have several limitations, which include sensitivity to high water and FFA content, complicated separation and purification of biodiesel, enormous amount of reaction time and exorbitant cost of catalysts, which make the process uneconomical. Direct transesterification (DT), also known as *in situ* transesterification, was first successfully performed in 1963 by Abel and coworkers. Since then, it has been verified by numerous researchers in a variety of tissues, as a simple and rapid method of quantifying fatty acids by combining extraction and

transesterification into one-step. Diverse methods have been used for DT, but most involve the addition of an organic solvent, methanol, catalyst and heat to a small amount of dried sample. The method was first demonstrated with sunflower seeds and gave a 20% of biodiesel yield increased than conventional method.[91,92] DT has previously, although infrequently, been applied to the biodiesel production from various oil resources. However, the process with no-catalyst involved with cheap oil feedstock (high water content and high FFAs content) would not obtain a positive reaction. The transesterification of triglycerides via supercritical fluid (SCF) has great strength comparing to catalysis transesterification on producing biodiesel. In this non-catalytic process, only reactants are added in the reaction mixture and heated to supercritical alcohol condition to produce biodiesel, which makes the process relatively simple and cost-effective. The primary obstacle of triglycerides transesterification is that they are immiscible fluids. During supercritical fluid treatment, the solubility parameter of alcohol is reduced substantially to a value near to triglycerides which form a homogeneous mixture and leads to higher reaction rate in contrast to catalytic reaction.[93] Under the SCF conditions, various properties of the fluid are placed between those of a gas and those of a liquid. Although the density of a supercritical fluid is similar to a liquid and its viscosity is similar to a gas, its diffusivity is intermediate between the two states. Thus the supercritical state of a fluid has been defined as a state in which liquid and gas indistinguishable from each other, or as a state in which the fluid is compressible even though processing a density similar of liquid and, therefore, similar solvating power. Because of its different physicochemical properties, SCF has been used in several industrial applications, such as supercritical extraction. Due to their low viscosity and relatively high diffusivity, supercritical fluids have better through solid materials and can therefore hive faster extraction yields. One of the main characteristics of a supercritical fluid is the possibility of modifying the density of the fluid y changing its pressure and/ or its temperature. Since density is directly related to solubility by altering the pressure, the solvent strength of the fluid can be modified. Therefore, its extraction efficiency in terms of increasing yields and lower extraction times is much higher than general solvent extraction.[94] Although SCF reaction seems to

be a promising technology which could resolve existing problems of catalytic reactions, there has been a lot debate on the efficiency of SCF reaction in term of energy utilization and safety issue due to the high pressure and temperature employed in this technology (table 2-5). Hence, there are challenges and issues that need to be addressed before SCF technology can play a major role in biodiesel production. Tan et al.[95] combined non-catalyst transesterification with supercritical methanol (SCM) and investigated the impact of several parameters including reaction time, temperature and the molar ratio of alcohol to oil. At temperature of 350˚C, reaction time of 20 min with alcohol to oil ratio of 40, a methyl esters yield of 80% was achieved.[95]

Table 2-5. Critical temperatures and critical pressures of various alcohols and carbon dioxide.

Alcohol	Critical temperature (K)	Critical pressure (MPa)
Methanol	512	8.1
Ethanol	516	6.4
1-Propanol	537	5.1
1-Butanol	560	4.9
CO_2	304	7.4

Supercritical transesterification is able to overcome these obstacles. This novel technology utilizes supercritical alcohol conditions to allow the usually immiscible oil and alcohol to form a single phase of solution due to its unique diffusivity property. The supercritical state of alcohol is believed to solve the two phase nature of oil/alcohol mixture to form a single phase due to a decrease in dielectric constant of alcohol in supercritical state.[93,95–97] Therefore, it would solve the problems of limited contact area between these two reactants and causes the reaction to occur at a slow rate. In consequence, catalysts were commonly applied in SCF transesterification procedure to increase the reaction rate. Some researchers have reported solely using supercritical

methanol to achieve lipid transesterification and provided optimistic results.[98,99] In the study conducted by Warabi et al., a catalyst-free transesterification was used on producing rapeseed oil biodiesel via supercritical alcohols. The results showed that transesterification of triglycerides was slower in reaction rates than alkyl esterification of FFAs for any of the alcohols employed.[99] Kusdiana et al. stated in one of papers that triglycerides transesterification reaction rate got dramatically increased in the SCM state in respect of subcritical methanol (SubCM) state.[74]

In addition, SCF process has a high tolerance towards impurities of FFA and water in oils, and no side reaction of saponification was reported.[100] In the situation of SCF transesterification, both triglycerides and FFAs simultaneously react with alcohol, which leads to higher biodiesel yield.[100,101] Another positive effect of using supercritical conditions is that the alcohol is not only a reactant but also an acid catalyst.[102] The non-catalytic supercritical process has been proved of superior environmental advantages due to the fact of no waste generated from catalyst treatment and separation from the final product. Furthermore, this non-catalytic method requires no pretreatment of the feedstock because impurities in the feed do not affect the reaction very strongly. Besides, it is easy to separate the final product and glycerol due to that the products are not miscible and there is no catalyst in the mixture to remove. In addition, in the SCF transesterification, there is no soap formed, which it may occur in the alkali-catalyzed process due to the FFAs reacting with catalyst.[58,98]

Even though supercritical transesterification has many advantages, there are still some disadvantages: (1) high alcohol to oil ratio used in experiments (>40:1). When operating it for commercial purpose, it would create difficulties in separating the biodiesel from the excess methanol for its recovery and reuse. (2) An additional drawback of employing supercritical conditions is the higher pressure and temperature, which require more energy and/or a process well engineered for energy recovery, and perhaps higher capital costs for the reactor.[98,103] A numbers of advantages of *in situ* transesterification includes much shorter reaction time, high conversion rate (oil to ester) and eliminate the catalysts cost. Tan et al. compared SCM transesterification with conventional catalytic methods.[93] They observed 1hr reaction time for conventional

catalyst on palm oil to biodiesel, but only 20 min reaction time for SCM. They pointed out that the process cost of SCM transesterification was reduced and the products were more environmental friendly. Besides, SCM process gives shorter reaction time than conventional transesterification and requires lower methanol: oil ratio than conventional transesterification. For reactions in SCM and supercritical ethanol (SCE), no catalyst is required and nearly complete conversion can be achieved in a very short time (2 -4 min).[104] Saka and Kusdiana reported 95% oil conversion in first 4 min of reaction with optimum process parameters of alcohol: oil molar ratio of 42: 1, pressure of 430 bar, and reaction temperature of 350˚C.[74] A similar result was given by Madras et al.[104] who experimented on synthesis biodiesel at a temperature range of 200 - 400˚C with a constant molar ratio of alcohol to oil of 40 at a fixed pressure of 200 bar. A highest lipid conversion yield of 100% was achieved for both methanol and ethanol at temperature of 40˚C for 40 min reaction time[104] Compared to Lam et al. gave a highest methanol: oil rate is 245: 1 for homogeneous acid catalyst, which achieved 99% conversion in 4 h at 70 ˚C reaction temperature.[69] SCM without a catalyst has also been used for triglyceride transesterification showing advantages in both system simplicity as well as reaction kinetics, but the higher operating temperature of at least 350˚C makes this option extremely energy intensive.[105] Despite the high temperature requirements, SCM may be the most energy efficient means to extract and convert lipid into biodiesel due to the decreased solvent and energy requirements for the single step process as well as the use of a wet feedstock.[106][107] Therefore, SCF transesterification is more suitable for a complex matter, which may not produce biodiesel via a conventional process, such as cooking oil, lipid rich microalgae. Biodiesel synthesis without catalyst can be accomplished under supercritical reaction conditions. High production yields can be obtained while using short reaction times. This alternative non-catalytic process has fewer unit operation steps than the current commercial process, and it may be better suited for handling inexpensive feedstocks such waste oils and greases. Supercritical transesterification process requires higher temperature, pressures, and alcohol to oil ratio, however, which will tend to drive up some aspects of the processing cost. Besides, supercritical transesterification for biodiesel production

only suitable to apply on pure oil as raw material. Thus, oil extraction has to employ before transesterification, and the quality of oil would directly affect the biodiesel quality and may lead to further downstream purification and separation.

In situ transesterification does not only limit in supercritical fluid system. It generally defines as the direct transesterification of oil-bearing seed without lipids extraction.[107] However, without the presence of catalysts, the reaction rate is too slow for it to produce considerable yield of biodiesel. Hence numerous researches were dedicated to investigate the factors may enhance the performance. To optimum this process, numbers of parameters are studied and a discussion is given on how they affect the process:

Raw materials

Traditional oil resources, such as rapeseed and soybean oil have a fatty acid profile that varies substantially and require different process parameters. Fatty acid profiles are known to influence biodiesel properties such as cetane number and cold filter plugging point. Therefore, a thorough characterization on raw material is essential. Generally speaking, feedstocks have similar property to vegetable oils, such as soybean and rapeseed oil, were recommended. Besides, low water content, low FFAs content, low phospholipids content, and less impurities are favor for biodiesel feedstocks. As previously reviewed, microalgae among other oil resources of biodiesel feedstocks have many superiors with respect to lipid property. However, *in situ* transesterification processes prefer to use more whole biomass rather than oil. Hence, an extra complex factor of physical barrier of cell wall/membrane needs to list in consideration.

Catalysts

In situ transesterification is well known by the ability of achieving conversion without catalysts aid. However, short chain alcohols are poor solvent for lipid extraction. Especially, methanol at room temperature and atmosphere pressure is only able to extracted 4.5 wt% of oil from 20 of soybean, compared with 45 wt% when using n–hexane.[36] Thus acid or alkali catalysts in *in situ* transesterification aim to help on breaking oilseed' cell walls, thereby allowing methanol to access the oil in cotyledon

cells.[107] Acanning electron microscopy (SEM) and light microscopy revealed NaOH dissolve cell walls and leased lipids to environment.[107] There have been intensive reviews of catalyst effects on transesterification in the previous section. Apart from alkali and acid catalysts, some studies suggest that metal reactor induces a catalytic effect on supercritical transesterification reaction. Dasari et al.[108] studied the effect of a metal catalyst on transesterification of soybean oil. Transesterification in a 316 stainless steel (316SS) reactor was compared with reaction in a glass capillary tube, which the experiments were not carried at supercritical condition. The results showed that the conversion from the 316SS reactor was 8% higher than that in the glass tube under the same reaction condition (180˚C, alcohol to oil ratio of 6:1 for 4 h). Moreover, when fine mesh 316SS and nickel shaving were placed into the glass tube reactor, reaction rates were accelerated 30 and 400 fold, respectively.

Effect of temperature and pressure

Temperature and pressure are important parameters for *in situ* transesterification. Madars et al. reported a reaction temperature from 200 to 400˚C to achieve 78% to 96% of conversion rate at 200 bar with a reaction time of 40 min. A similar result was report by Demirbas.[73] In this research, conversion rates happened at 177 ˚C to over 50% and at 250 ˚C to over 95% with a methanol to oil ratio of 41:1. Bunyakiat et al.[109] also found out the conversion of coconut oil almost doubled from 50% to 95% when the temperature was raised from 270 to 350˚C. The conversion yield nearly tripled in the case of palm kernel oil (from 38% to 96%) over the same temperature range with molar ratio of methanol to oil of 2 and reaction time of 7 min. The yield was increased around 20% when the temperature rose from subcritical to supercritical conditions of methanol with cottonseed oil at a methanol to oil ratio of 41:1.[110] Tan et al.[100] combined non-catalyst transesterification with SCM and investigated the influence of several parameters including reaction time, temperature and the molar ratio of alcohol to oil. At a temperature of 350˚C, reaction time of 20 min with alcohol to oil ratio of 40, a methyl esters yield of 80% was achieved.[95] Thus, transesterification reaction can achieves high conversion yield at high temperature at a very short time. Saka and Kusdiana reported 95% oil conversion in first 4 min of reaction with optimum process parameters of

alcohol: oil molar ratio of 42: 1, pressure of 430 bar, and reaction temperature of 350˚C.[74] He et al.[111] reported a conversion yield of 72% at 280˚C with 50 min reaction and 82% at 300˚C with 25-30 min for soybean oil transesterification. There have been reports that long chain methyl esters easily to degrade under a sever reaction condition. The yield of saturated FAME increase with reaction time while the unsaturated FAME participates more readily in side reactions that reduce its yield at longer times. The loss becomes severe for unsaturated FAME with multiple double bonds. The authors showed that these losses could be reduced by gradually heating the reactor from 100 to 320˚C, which improved the yield to 96%. Similar phenomena was found by Silva et al.[112] that the FAME yield declined at 375˚C after a certain reaction time, and the yield reduction was more severed at low methanol to oil ratio. Besides, Xin et al.[113] also found that natural antioxidant was not stable at a temperature higher than 300˚C. In order to obtain the highest yield of biodiesel, reaction temperature should be carried out at a temperature of 300˚C or lower.[114]

According to a work given by He et al.[115], pressure plays a significant role in FAME yield. Their results at a constant temperature of 280˚C, molar ratio of methanol to oil of 42 and reaction time of 30 min showed that at pressure less than 155 bar, this variable had a considerable impact on the reaction yield. The FAMEs yield was 56.1% at 8.7 MPa compared to 81.7% at 15.5 MPa. Increasing the pressure further to 25 MPa led to only a 9% increase in FAME yield. Above 25 MPa, the influence of pressure on yield was negligible. When the pressure increased from 25 to 36 MPa, there was only 1% increase in the ester yield.[115] In the same year, they reported a similar result for the same reaction time, and the yield of FAMEs increased from 43% to 77% when pressure rose from 10 MPa to 40 MPa.[111] However, in this time, they claimed the pressure did not have significantly impact on improvement the FAMEs yield. In contrast, the work conducted by Silva et al. gave a negative effect of increase pressure from 7 MPa to 20 MPa on FAMEs conversion rate. At 350˚C and methanol to oil molar ratio of 40:1, a raise of pressure from 7 MPa to 20 MPa leads to the reaction rate constant decreased from 0.17 to 0.10 min^{-1}.[112] The authors thought that reaction rate tended to be faster due to favorable diffusivity and viscosity at lower pressure. A conclusion could be made from

Lee and Saka[114] that high volumetric concentration of alcohol is favorable for transesterification reaction due to the density of an alcohol decrease with temperature increase (above its boiling point) at same pressure while increase its density with pressure incline. Secondly, high pressure increases the solubility of triglycerides, and thus molecular interaction between alcohol and triglycerides become closer at high pressure.

Effect of water and FFAs in feedstock

It is inevitable to have a microalgal oil contains high level of FFAs and large amount of water. These impurities pose no problem when present in *in situ* transesterification. The reaction tends to increase slightly as the FFA content increased in oil and the water does not slow down the reaction as in catalyzed transesterification. In the research of Tan et al.[100], the effect of levels of water content (4% - 20%) in feedstocks were investigated in terms of biodiesel yield. The results showed that SCM transesterification which has a constant 80% of FAMEs yield, did not influenced by water content in feedstocks. In contrast, FAMEs yield from heterogeneous acid catalytic transesterification was greatly decreased by water content increase. When water content reached 15 wt% of the feedstock, only 13 wt% of FAMEs yield obtained. Kusdiana and Saka reported a successful conversion on a feedstock contains 50% of water content via SCM transesterification reaction.[106] Water at temperatures above 250 ˚C can be solubilized into most nonpolar organic compounds, including hydrocarbons. Water in the feedstock can be hydrolyzed the oil and form FFAs.[116] In addition, this result does not affect transesterification process due to that the FFAs will occur at a rate faster than transesterification. Therefore, all FFAs will be converted to their corresponding esters, and the water will be separated at the end of the SCM process.

Apart from water, FFAs content in oil is also one of the major nuisances in biodiesel production due to the side reaction between FFAs and base catalysts to produce saponified products. However, the study of Tan et al.[100] provided results of that FFAs content does not affect negatively the yield of biodiesel in SCM and heterogeneous acid catalytic transesterification. Instead, both types of reaction show a steady increase in yield with the increment of FFAs content in reaction mixture. The reason of this

phenomenon is believed to be esterification of the FFAs will occur at a rate faster than transesterification. Therefore, all FFAs will be converted to their corresponding esters. Finally, water contributes to an easier separation at the end of the supercritical process

Effect of alcohol to oil molar ratio

Stoichiometrically, 3 mol of alcohol are required to react with 1 mol of triglyceride. In practice, an excess amount of alcohol is usually used to make sure of complete conversion. Higher alcohol to oil ratio also helps to reduce the critical temperature of the mixture, which allows homogeneous reaction conditions at milder temperatures for supercritical processing. Therefore, as more alcohol is used, higher conversions can be obtained, but eventually a point is reached where more alcohol does not help accelerate the reaction. An optimal ratio (~40:1) at temperature of 350˚C and 450 bar to yield over 95% conversion.[105,115] A successful study used methyl acetate instead of alcohol to produce biodiesel from rapeseed oil and an alcohol to oil molar ratio of 42:1 employed and achieved a 97% FAMEs yield at 350˚C for 45 min in 20MPa.[117] However, in a study of producing fatty acid ethyl esters (FAEEs) from soybean oil via a continuous catalyst-free transesterification, a higher FAEEs yield (59.9%) was obtained at ethanol to oil ratio of 1:20 while only 29.6% of FAEEs yield at ethanol to oil of 1:40.[36] The reason of this controversy was due to fact of production model difference. Wang et al.[118] reported a FAMEs yield of 60% in the alcoholysis of soybean oil for oil to methanol molar ratio of 1:20, 15 MPa and 350˚C, while for oil to methanol molar ratio of 1:40 the authors reported 80% yield of soybean oil. Overall, for non-catalytic SCF transesterification, alcohol to oil molar ratio of 42:1 is needed to achieve a close 100% FAMEs yield and triglycerides conversion yield.

Effect of co-solvent

Utilization of co-solvent in SCM transesterification is an important factor, which could improve the performance. Based on previous study on transesterification, the primary barrier is the immiscible of oil and alcohol. Moreover, lipids extraction from whole biomass is hindered by the physical restrain effect of biomass cell wall. Thus, a co-

solvent was usually introduced in non-catalytic transesterification process. N-hexane, heptane, and supercritical CO_2, were commonly used as co-solvent to increase the mutual solubility between methanol and triglycerides and to reduce the critical points of the mixture. This is possible by the excellent solubility between triglycerides and heptane, which subsequently forms a homogeneous phase. The homogeneous phase can react readily with methanol even under milder conditions compared to those without the presence of co-solvent. Hence, supercritical reaction can proceed at lower temperature and pressure, due to the critical conditions of the homogeneous phase are lower than using pure triglycerides alone. In the work of Trentin et al.[36], the effect of co-solvent addition on the alcoholysis reaction was evaluated at 325˚C, adopting the oil to ethanol molar ratio of 1:20, and pressure of 20 MPa with a co-solvent of CO_2 to substrate ratio from 0.05:1 to 0.2:1 resulted in a raise of FAEE yield from 60% to 80 wt%. Han et al.[119] investigated the production of biodiesel from soybean oil at 300˚C, 20 MPa and using oil to ethanol molar ratio of 1:33. A 75 wt% of FAEEs yield was obtained without CO_2 assistant. Meanwhile a FAEEs yield of 95 wt% was obtained with the addition of co-solvent to substrate mass ratio of 0.2:1.

Mixing intensity

Georgogianni et al. compared the difference between the use of a mechanical stirrer (600 rpm) and low frequency unltrasound (24 kHz) as a means of agitation.[120,121] Both agitation methods gave high conversion of methyl ester after 20 min of reaction. However, when ethanol was used, the application of ultrasound produces higher conversion more rapidly. At 40 min, 98% conversion was achieved with ultrasound, whereas mechanical stirring results in lower yield (88%). They asserted that the reason behind this phenomenon was that ultrasound produces less soap because no stirring action was required. In the study conducted by Ehimen et al.[58] working on microalga biodiesel production investigated the effect of stirring on *in situ* transesterification. A 90% of conversion rate of FAME was achieved after 1 h with continuous stirring a mixture of 15 g microalgae biomass with 60 ml methanol containing 0.04 mol of acid catalyst at a reaction temperature of 60˚C. It was therefore envisaged that, without further stirring, the FAMEs conversion rate reaches 89% while an intermittently

stirring has a similar result with continuous stirring 1 h. The above studies were with vegetable oil, and thus stirring may not present a significant role of conversion. When using whole biomass as biodiesel feedstock, stirring is necessary for mixing the biomass with alcohol and promote the miscibility of lipids and alcohol.

2.1.3.4 *In situ* transesterification for microalgal biodiesel production

In the past few decades, numerous studies had focused on producing a quality biodiesel from microalgae via various approaches. Most studies have used two-step procedure involving solvent extraction to remove lipids from the microalgae biomass first, followed by catalytic or supercritical alcohol transesterification.[58,122] Solvent extractions from microalgae are typically based on the methods using chloroform and methanol published in the 1950s by Folch et al. and Bligh and Dyer. It is well recognized that solvent extraction often extracts lipids incompletely, particularly FFAs, and can extract significant quantities of non-nutritive, non-saponifiable materials such as pigments.[103] Therefore, the process of converting microalgal oil to biodiesel requires oil refinery and catalysts assistance. Although catalysts play a great role in reducing transesterification time, their presence promotes complications of final product purification. This results in increased process cost and a hazard to the environment. Therefore, to avert the drawbacks of catalysts transesterification, *in situ* transesterification is applied. *In situ* transesterification mostly refers to a biodiesel production from oil-rich seed with or without catalyst aid and accomplished lots successful achievements. The most superior advantage of *in situ* transesterification is the ability of producing more biodiesel than stepwise method. So far, the research of biodiesel production from microalgae requires either co-solvent (i.e. chloroform, hexane, water) or catalyst assistance.[123–125] A research investigated a biodiesel production with microalgae *Chlorella vulgaris* via alkaline *in situ* transesterification using NaOH as catalyst. A maximum biodiesel recovery of 78 wt% was achieved using a catalyst to lipid ratio of 0.15:1 and a methanol to lipid molar ratio of 600:1 at 60°C with a reaction time of 75 min.[72] Another study used *Chlorella sp.* as feedstock with base-catalyzed transesterification and achieved a highest conversion rate of 84% at 25°C for 10 h, with a concentration of base catalyst (KOH) of 2% of the lipid amount.[125] Compare

to a traditional two-step process with a highest conversion rate of 63.5%, *in situ* transesterification present a 20.5% more efficient conversion rate. Furthermore, the conventional process uses 14% more solvent than the *in situ* transesterification process. In contrast to a acid-catalyzed *in situ* transesterification achieved up to 97 wt% of production yield using acid catalyst (H_2SO_4) to lipid ratio of 0.35:1 and methanol to lipid molar ratio of 800:1 at 60°C for 20 h reaction time.[72] In the research conducted by El-Shimi et al., an acid-catalyzed *in situ* transesterification with non-edible *Spirulina-Platensis* microalgae lipids gave a beat result of 85% FAME yield with a 100% (w/w oil) H_2SO_4 concentration, 80 ml methanol volumes, 8 h reaction time and 65˚C reaction temperature with continuous stirring at 650 rpm. [124] Wahlen et al. implemented catalytic *in situ* transesterification on five microalgae species, *Chaetoceros gracilis* (diatom)*, Tetraselmis suecica, Chlorlla sorokiniana* (green algae)*, Synechocystis* sp*.* PCC 6803 and *Synechococcus elongates* (cyanobactrium).[126] They believed the key parameter, which is essential to the sucessfuel conversion of algal lipids to biodiesel by *in situ* transesterification, was alcohol type, methanol to biomass ratio, temperature and catalyst concentration. To determine the total FAME content of each sample, lyophilized microalgae samples were taken 100 mg reacted with 2 mL of methanol containing 1.8% (v/v) H_2SO_4 for 20min at 80˚C. They achieved the product yields (percent FAME of extractable lipid) of 82% (*Chaetoceros gracilis*), 78% (*Tetraselmis suecica*), 77% (*Chlorlla sorokiniana*), 39% (*Synechocystis* sp*.* PCC 6803), and 40% (*Synechococcus elongates*), respectively. They also experimented on producing FAME from *Chaetoceros gracilis* with a water content of 50% (w/w) using the same method and obtained a FAME yield of 84%. More recently, Tran et al.[127,128] demonstrated an innovative approach using wet microalgae with up to 90 wt% water content as the feedstock to produce biodiesel with a co-solvent aided, an excessive amount of methanol, and immobilized lipase via an *in situ* transesterification process. They achieved a high biodiesel conversion of 97.3% when using wet *Chlorella vugaris* microalgae (lipid content, 63 wt% d.b.) with a 71% water content. A combination of acid and base catalyst was found most effective on converting microalgal lipids with high FFAs content via *in situ* transesterification. A two-step *in situ* process was investigated to

obtain a high FAME yield (94.9 %) from microalgae (*Chlorella sorkiniana* UTEX 1602) biomass that had high FFAs content.[39] This was accomplished with a pre-esterification process using heterogeneous lipase catalyst to reduce FFA content prior to the base-catalyzed (KOH) transesterification. They also compared the FAME recovery of one-step process with acid, base, or enzyme catalysts and two-step process with acid catalyst or enzyme catalyst. The results were shown in the following table 2-6.

Apart from the issue of undesired lipid component, physical cell wall structure also obstructs alcohol reacting with microalgal lipids in *in situ* process. Therefore, external assistance was listed in consideration to aid *in situ* biodiesel production process from microalgae biomass. Cheng et al.[129] used microwave irradiation instead of conventional heating to achieve higher biodiesel rate and yield from wet microalgae (*Chlorella pyrenoidoas)* (water content, 80 wt%) via an *in situ* process. A maximum biodiesel yield (percentage of FAME in dry biomass) of 11% was obtained from a reaction condition of 60°C and 30 min with 1:1:0.05 (v/v/v) of methanol, chloroform, sulfuric acid. They proved that microwave irradiation gave a 78% disruption but transesterification rarely occurred with sulfuric acid and chloroform assistance. However, with sulfuric acid and extraction agents (i.e. methanol), 2 min was sufficient for cell disruption under microwave irradiation at 60°C. The FAMEs yield under microwave irradiation was 94 wt%.[130] *In situ* transesterification process also combined with supercritical fluid to overcome the immiscibility and physical obstacle between microalgal lipids and alcohol. Levine et al.[131] developed a two-step process involving hydrothermal carbonization and supercritical *in situ* transesterification. Microalgae *Chlorella protothecoides* (UTEX #255) biomass were first carbonized and followed by a supercritical ethanol transesterification to achieved a FAEE yield of 79% with a 5:1 ethanol: FA molar ratio in 150 min and a 89% FAEE yield with a 20:1 ethanol: FA molar ratio in 180 min at 275˚C.[131] A substantial thermal decomposition of unsaturated FAEE was observed when reaction temperature above 275˚C. There are about 25 - 50% of lipids were lost in the thermal carbonization, and remaining portion of lipids could completely be converted in an *in situ* transesterification stage. Another similar experiment of a two-step technique was developed by Levine et al.[123] for algal biodiesel production. Wet

microalgae lipids, *Chlorella vulgaris* (water content, 80 wt%), were first hydrolyzed in subcritical water then followed by a catalyst-free supercritical transesterification process. They achieved a highest crude biodiesel yield of 100% with a reaction temperature of 325˚C, 6.6:1 of ethanol to dry hydrolysis solids mass ratio, 10% of water content in 120 min of reaction time.[123] Compares to a 94% crude biodiesel yield for 120 min at 275˚C with an ethanol to hydrolysis solids mass of 7.5 and 9.1% of water content. Both of them were having a similar FAEE yield in the crude biodiesel (66% and 62% respectively) while the highest FAEE yield (45 wt%) happened at 275˚C in 120 min with 2.3 of ethanol to hydrolysis solid mass ratio and 20.4% of water content. But the crude biodiesel yield (68 %) at this condition is much lower than the others.[123] Patil et al. proposed a method combined *in situ* transesterification, supercritical fluid and microwave processes to produce ethyl ester from wet microalgae (*Nannochloropsis salina*) biomass.[96] Based on the experimental analysis for wet algae biodiesel, the optimal conditions for maximum yield of 31 % were reported as: wet algae/ethanol (wt/vol.) ratio of around 1:9, reaction time of about 25 min, respectively, at controlled power dissipation levels. The temperature noted at these optimum reaction conditions was around 260˚C. They also observed a greatly decrease of polyunsaturated ethyl esters when the temperature beyond 230˚C. So far, all existed studies regarding to microalgal biodiesel production through *in situ* transesterification method involved catalysts aid. Thus our research focuses on developing a new procedure of *in situ* transesterification for microalgal biodiesel production without catalysts.

Table 2-6. FAME recovery from microalgae *C. Sorokiniana* using one-step and two-step *in situ* process [53].

Process	Catalyst	FAME recovery (%)
One-step	KOH	26.78 ± 4.30^{a} 41.74 ± 2.28^{b}
One-step	H_2SO_4	60.89 ± 2.37^{c}
Two-step	H_2SO_4 + KOH	65.23 ± 4.11^{d} 82.60 ± 2.19^{e}
One-step	Amberlyst-15	48.87 ± 1.67^{f}
Two-step	Amberlyst-15 + KOH	94.87 ± 0.86^{g}

[a] KOH = 0.3 wt%, methanol to biomass ratio of 4 mL/g, 90°C, 70 min.

[b] KOH = 0.6 wt%, methanol to biomass ratio of 4 mL/g, 90°C, 70 min.

[c] H_2SO_4= 6.9 wt%, equivalent molar of H^+ as 30 wt% Amberlyst-15, methanol to biomass ratio of 4 mL/g, 90°C, 70 min.

[d] Pre-esterification: H2SO4= 6.9 wt%, methanol to biomass ratio of 4 mL/g, 90°C, 60 min; base-catalyzed transesterification: 0.3 wt% (besides the 7.88 wt.% of KOH for neutralization), methanol to biomass ratio of 4 mL/g, 90°C, 10 min.

[e] Pre-esterification: H_2SO_4= 6.9 wt%, methanol to biomass ratio of 4 mL/g, 90°C, 60 min; base-catalyzed transesterification: KOH = 1.8 wt% (besides the 7.88 wt.% of KOH for neutralization), methanol to biomass ratio of 10 mL/g, 90°C, 10 min.

[f] Amberlyst-15 = 30 wt%, methanol to biomass ratio of 4 mL/g, 90°C, 70 min.

[g] Pre-esterification: Amberlyst-15 = 30 wt%, methanol to biomass ratio of 2 mL/g, 90°C, 60 min; base catalyzed transesterification: KOH = 0.3 wt%, methanol to biomass ratio of 4 mL/g), 90°C, 10 min.

2.1.3.5 *In situ* transesterification for biodiesel production from other oil seeds

In original definition of *in situ* transesterification method, it was first applied on oil seed. Whereas, the actual process procedure was alternated due to the difference of physical and chemical properties of oil seeds. A summary of biodiesel production from various feedstocks via *in situ* transesterification is listed below (table 2-7).

Apart from the common oil seed feedstock, rice barn was also used in some studies. The method included an in-situ acid-catalyzed esterification followed by an in-situ base-catalyzed transesterification. Free fatty acids (FFAs) level was reduced to less than 1% in the first step under the following conditions: 10 g rice bran, methanol to rice bran ratio 15 mL/g, H_2SO_4 to rice bran mass ratio 0.18, 6˚0 C reaction temperatures, 600 rpm stirring rate, 15 min reaction time.[132] In the second-step, 8 mL of 5 N NaOH solution was added to allow to react for 60 and 30 min for rice barn A and rice B. FAMEs yields of 96.8% and 97.4% were obtained for rice bran A and rice bran B, respectively, after this two-step in-situ reaction.

Table 2-7. A summary of process conditions and conversion of *in situ* biodiesel production from various raw materials.

Raw material	Solvent	Catalyst (mol/L)	Molar ratio solvent :oil	Reaction time (h)	Temp. (˚C)	Conversion (oil basis) (%)	Ref.
*Jatropha curas**	Methanol	H_2SO_4 (0.2)	300:1	24	60	99.8	Shuit, et al.[133]
Jatropha curas	Methanol/ethanol mix	NaOH (0.02)	512:1	1	60	87	Harvey, et al.[107]
Jatropha curas	Methanol	NaOH (0.04)	100:1	1	60	70	Oloniyo[134]
Jatropha curas	Ethanol	CH_3ONa (0.04)	-	2	30	99.98	Surya et al. [135]
Jatropha curas	Methanol	KOH (0.075)	6:1	5	50	87	Amalia et al.[136]
*Jatropha curas***	Ethanol	H_2SO_4/KOH (0.07/0.05)	-	0.2	60	97.29	Jaliliannosrati, et al.[137]
DDGS	Methanol	NaOH (0.4)	655:1	1.2	35	91.1	Haas et al. [138]

DDGS: Distiller's dried grains with solubles

*: Hexane co-solvent

**: Microwave assistance

2.1.3.6 Perspective of microalgal liquefaction process

In the studies of microalgal biodiesel through *in situ* transesterification method, it is frequently confused with microalgal liquefaction. The thermochemical liquefaction of biomass is a process that requires heating the biomass at high temperature ranging from 200°C to 500°C with pressures greater than 2 MPa in the presence of a catalyst. This process resulted in the production of bio oil yields ranging from 9% to 72% and gas mixture yields ranging from 6 to 20%.[67] This process intents to convert all biomass into oil product for a complete conversion. However, the product of this method could not be easily separated. This process also produced ash yields ranging from0.2-0.5%.[67] The product of the liquefaction process is also comparable with crude fuel, where the energy content of the bio oil range from 30 to 39 MJ/kg and the gaseous product also contain energy content of more than 21 MJ/kg.[139] One advantage of liquefaction compared to other thermochemical process is its high tolerance of feedstock water content, which can be up to 65% of the biomass weight.[140] Therefore, wet microalgae

biomass could be directly used in liquefaction without further drying process. The liquefaction products of microalgae are mainly affected by the biomass composition and the liquefaction conditions of temperature, pressure, residence time and catalyst. The bio oil yield can reach 2-25% higher than the lipid content of microalgae.[141] In the research done by Li et al.[142], conversion efficiencies of hydrothermal liquefaction of both low-lipid high-protein microalgae (*Nannochloopsis* sp.) and high-lipid low-protein microalgae (*Clorella* sp.) were studied. An orthogonal design was applied to investigate the effects of reaction temperature (220-300˚C), retention time (30-90 min), and total solid content (15-25% wt.) of the feedstock. The highest bio-crude yield for *Nannochloropsis* sp. was 55% at 260˚C, 60 min, and for *Chlorella* sp. was 82.9% at 220 ˚C, 90 min.[142] Other studies of microalgal liquefaction of *Dunaliella tertiolecta*, which is mainly composed of crude protein (63.6%) and fat (20.5%), produced approximate 37% of bio oil yield.[140] The *Microcystis virids* strain, which is consisted of 46% carbon, 7.3% hydrogen, and 9.5% nitrogen, was reported to produce yields of up to 33% and 40% of bio oil and energy.[143] All the results above have bio oil yield up to 54% without catalysts lower than the other alternative thermochemical conversion process such as *in situ* transesterification from high lipid content microalgae strain. Besides, crude oil from liquefaction could not be directly used that requires a series of separation and purification to make this product usable. In addition, bio oil from liquefaction contains high sulfur content due to the fact that microalgal proteins also react in the process. Therefore, a process such as transesterification is more suitable to produce microalgal biodiesel. This method will make the microalgal biodiesel production simpler and less energy cost on downstream purification.

2.2 Summary

Microalgae are a sustainable energy resource with great potential for CO_2 fixation. In the past several years, scientists developed numerous productions from it. In addition, they are not only fuels. The usages of microalgae can be concluded from food supplements to new types of energy resource. This review aims to highlight the potential of algal biodiesel production, and the highest oil yield among various stains can be obtained by modulate the cultivation condition and the process variances on

biodiesel production. Producing microalgae biofuels requires large-scale cultivation and harvesting systems, with the challenge of reducing the cost per unit areas. Fortunately, multiple applications of microalgal biomass can pay off the effects cost by cultivation. Microalgae biomass was able to turn into various energy source including biohydrogen, bioethanol, biodiesel, and bioethanol. Lipids are the most readily extractible biofuel feedstock from algae, but potential storage is hindered by the presence of polyunsaturated fatty acids causing oxidation reactions and high moisture content of algal feedstock. However, the microalgae biofuel production still stays in lab-scale research mainly due to the economic issue. It is therefore clear that a considerable investment in technological development and technical expertise is still needed before algal biofuels is economically viable and can become a reality. On the other hand, after economic study, a conclusion that using supercritical direction transesterification method to manufactory microalgae biodiesel costs less than conventional production methods was made. Apart from the limitation of feedstock preparation, a process optimization could improve the productivity of transesterification, which saves process cost from the processing part. Overall, with the current demand for renewable fuels, especially the demand from the transportation sector, there is a need to develop a range of sustainable biofuels resources as the combined mix, which will be a significant step towards the replacement of fossil fuels. Combined with development of technologies to optimize the microalgae production, oil extraction and biomass processing has the capacity to make significant contributions towards this goal. In the following chapters, a thoroughly investigation of *in situ* transesterification of microalgal biodiesel production will employ. The *in situ* transesterification method developed in this study is a new invention due to the natural of a single step, catalyst free process with targeting to produce higher FAMEs concentration rather than a maximum conversion rate.

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Chapter 3. Biodiesel Production from Green Microalgae *Schizochytrium limacinum via in situ* Transesterification

3.1 Abstract

Microalgae are considered as one of the most promising feedstocks for biofuel production because of their environmental and social benefits. However, challenges exist in converting microalgal lipids into algal biofuels due to the unique characteristics of microalgae and the technologies for processing them. This study aims at exploring an alternative technology that combines lipid extraction from whole microalgae and lipid esterification/ transesterification in a single step or in situ transesterification. A high lipid content microalgal strain, Schizochytrium limacinum, was selected as the model to perform the in situ transesterification with sub-/super-critical methanol (SubCM)/ SCM in a batch reactor without catalyst application. Temperature (170, 210, 250, and 290˚C), reaction time (30, 60, 90 and 120 min), and lipid-to-methanol molar ratio (sRatio; 1:50, 1:75, and 1:100) were investigated for their effects on the conversion efficiency. Temperature appeared as a most influential factor. In the range of testing, higher temperature leads a faster transesterification reaction. Additionally, operating temperature over 250˚C caused degradations to the lipids and/or algal biomass thus led to the decline of the ester yield. The combination of reaction time and temperature had a significant impact on the in situ transesterification reaction. The sRatio had a statistically significant impact on the product yield and selectivity, and both these two responds factors reached the maximum levels after sRatio reached 1:75. It was observed that the highest product selectivity (37.5 wt%) happened at sRatio 1:75, 211.6˚C, 120 min, with a product yield of 58.4 mol%. This study shows that the in situ transesterification of microalgae bears some advantages over the traditional two-step processes and has the potential to be applied to large-scale processing microalgae for algal biodiesel production.

3.2 Introduction

Microalgae are considered as one of the most promising feedstocks for biofuel production because of their environmental and social benefits. However, challenges exist in converting microalgal lipids into algal biofuels due to the unique characteristics of microalgae and the technologies for processing them. In the past decades, extensive

research has been conducted on the selection of microalgal strains, cultivation of various microalgal strains for high lipid production, and bioreactor technologies for microalgae production. Studies had also focused on producing biodiesel from microalgae via various approaches. Most of these studies, however, have used the traditional two-step procedure, i.e., solvent extraction to remove the algal lipids from the microalgae biomass first, and lipid transesterification of the extracted lipids in a separate unite.[1–4] Two-step processes have many limitations including the increased processing cost due to the additional purification process. Besides, strong acid or alkaline catalysts are commonly used in two-step transesterification that catalyst disposal will harm the environment in some extant.[5–7] Therefore, to avert the drawbacks of two-step catalytic transesterification process, *in situ* transesterification was studied. Traditionally, *in situ* transesterification in literature refers to a biodiesel production from oil seeds, with or without a catalyst, without conducting the oil extraction beforehand. In an *in situ* transesterification, oil extraction and oil transesterification are performed simultaneously in a single step, which simplifies the processing. The most superior advantage of *in situ* transesterification is the ability to simplify the biodiesel production process. So far, biodiesel production from microalgae requires either co-solvent (i.e., chloroform, hexane, water) and/or catalyst assistance.[3,8–13] *In situ* transesterification of microalgae for biodiesel production is researched by some researchers.[9,14–16] For example, a research investigated the *in situ* transesterification of microalgae *Chlorella vulgaris* for biodiesel production using NaOH as the catalyst and achieved a maximum biodiesel yield of 78 wt% using a catalyst to lipid ratio of 0.15:1 and a methanol to lipid molar ratio of 600:1 at 60°C for 75 min.[4] In contrast, an acid-catalyzed *in situ* transesterification achieved up to 97 wt% of yield with a catalyst (H_2SO_4) to lipid ratio of 0.35:1 and methanol to lipid molar ratio of 800:1 at 60°C for 20 h.[4] Wahlen et al.[3] studied the catalytic *in situ* transesterification of five microalgal species, i.e., *Chaetoceros gracilis* (diatom)*, Tetraselmis suecica, Chlorlla sorokiniana* (green algae)*, Synechocystis* sp*.* PCC 6803 and *Synechococcus elongates* (cyanobactrium). They believed the key parameters, which are essential to the successful conversion of algal lipids to biodiesel by *in situ* transesterification, were alcohol type, alcohol to biomass ratio, temperature and catalyst concentration. They

achieved the product yields (percent FAME of extractable lipid) of 82% (*Chaetoceros gracilis*), 78% (*Tetraselmis suecica*), 77% (*Chlorlla sorokiniana*), 39% (*Synechocystis* sp. PCC 6803), and 40% (*Synechococcus elongates*), respectively, with the lyophilized microalgae of 100 mg reacting with 2 mL of methanol containing 1.8% (v/v) H_2SO_4 for 20 min at 80˚C. They also experimented on producing FAME from *Chaetoceros gracilis* with a water content of 50% (w/w) using the same procedure and obtained a FAME yield of 84%. More recently, Tran et al. demonstrated an innovative approach of using wet microalgae with up to 90 wt% water as the feedstock to produce biodiesel with a co-solvent aided. In their studies an excessive amount of methanol and immobilized lipase were employed and achieved a high biodiesel conversion of 97.3% when using a wet microalga *Chlorella vugaris* (lipid content 63 wt%) in the presence of 71% water content.[17,18] A combination of acid and base catalysts was found most effective on converting microalgal lipids with a high content of free fatty acids (FFA) via *in situ* transesterification. In another research, a two-step *in situ* process was investigated to obtain a high FAME yield (95%) from a microalgae (*Chlorella sorkiniana* UTEX 1602) biomass that had high FFA content of 47% over the total lipids.[9] This was accomplished with a pre-esterification process using heterogeneous lipase catalyst to reduce the FFA content prior to the base-catalyzed (KOH) transesterification. To the author' knowledge, there are currently research reports on using whole microalgae biomass as the feedstock to produce biodiesel through a catalysts-free thermochemical process.

Many researchers have also focused on converting the whole microalgae biomass (i.e., the lipid and the cellulosic components in microalgae) into a liquid fuel through liquefaction processes[19–22], but such a liquid fuel has no direct applications in the real life due to the complex composition of the products before cost effective downstream separation/ purification are developed. Therefore, to take advantages of the microalgal lipids, the high quality energy components, a study of targeting at producing FAME without converting the lignocellulosic and other matters is desired.

Keeping in this objective in mind, we conducted a study on a process of algal biodiesel production directly from microalgae with the oil extraction and esterification/ transesterification in a single step, i.e., *in situ* transesterification without external catalyst application. Methanol under sub-or super-critical condition was used as the

solvent to extract the lipids out of algae and the reactant to esterify the free fatty acids and transesterify the triglycerides in the microalgae into biodiesel simultaneously. The effects of process parameters on the process efficiency were explored. A statistical analysis of selected parameters was conducted and used to predict the optimum conditions, which can predict the highest product yield and productivity. Furthermore, a Fourier transform infrared (FTIR) spectroscopic analysis was also performed on the chemical composition of the products from various operation conditions and the degree of biomass liquefaction. Upon the successful investigation of the *in situ* transesterification process for microalgal biodiesel production, a preferable operation condition could be obtained for use in scaled-up productions in the future.

3.3 Methodology

3.3.1 Microalgae and chemical reagents

A lipid-rich green microalga *Schizochytrium limacinum* was purchased from ENN Energy Service Co., Ltd (Langfang, China) as a dry powder biomass with 1.55% moisture on wet biomass basis. This *S. limacinum* was used in all *in situ* transesterification experiments in this study. The microalgae biomass was manually ground in mortar to get relative uniform particle size (10-20 μm) beforehand.
All reagents used in this research are HPLC grade chemicals. The n-hexane used for Soxhlet extraction was purchased from Fisher Scientific (Pittsburgh, Pa.). Chloroform and methanol used in Modified Bligh & Dyer extraction were bought from EMD Millipore (Darmstadt, Germany). Methanol used in *in situ* transesterification was purchased from Macron Fine Chemicals (Center Valley, Pa.).

3.3.2 Biomass characterization

3.3.2.1 Proximate analysis

A proximate analysis is the determination of moisture, volatile matter, fixed carbon, and ash. Based on the purpose of this study, volatile matter and fixed carbon contents were considered as one analyte, which was determined by subtracting the moisture content and ash content from 100% biomass basis.

A Karl Fischer Titrator (Mettler-Toledo DL38) made by Mettler-Toledo (Columbus, Ohio) was used for determine moisture content in microalgae by reacting with iodine and methanol reagents which were purchased from EMD Millipore (Darmstadt, Germany). Besides, a HB43-S Halogen moisture analyzer from Mettler-Toledo (Columbus, Ohio) was used for confirming the moisture content in microalgae. In order to determine the moisture content in the feedstock, approximately 5 g microalgae was weighed and then heated it up to 155°C in the sample chamber to give off moisture. Then the analyzer based on the weight loss calculated moisture content automatically.

Ash content was determined by following the ASTM Standard D1102-84 (ASTM, 2007) in a furnace made by Thermo Scientific (Asheville, N.C.).

3.3.2.2 Ultimate analysis

In this study, a professional laboratories performed the ultimate analysis on the microalga *S. limacinum*. Contents of carbon (C), nitrogen (N) and sulfur (S) were tested by the Holm Research Centre, Analytical Sciences Laboratory of the University of Idaho. In determining C, S, and N, a complete combustion process converts the elemental carbon, sulfur, and nitrogen into CO_2, SO_2, N_2, and NO_x. These gases then pass through infrared ray cells to convert the information to the carbon and sulfur contents and a thermal conductivity cell to the nitrogen content. The reporting limit (RL) is approximately 0.01-0.02% for all three elements for a sample size of approximately 0.25 g.

Microalgae samples were sent out for oxygen content analysis by Eurofins Scientific Inc. (Des Moines, Iowa). A GLI method (a procedure developed by Galbraith Laboratories, Inc.) was used through a Thermo Finnigan FlashEATM 1112 Elemental Analyzer (Galbraith Laboratories, Inc., Knoxville, Tenn.). Samples of 1.0-4.0 mg were weighed into silver weighing capsules and crimped before being put into combustion furnace for slow pyrolysis. The samples were pyrolyzed in helium environment then nitrogen gas, hydrogen gas, and carbon monoxide gas formed when the samples came in contact with the nickel plated carbon catalyst at 1060°C. The gases were then separated in a chromatographic column. Carbon monoxide was then analyzed automatically in a self-integrating, steady state thermal conductivity analyzer Flash EA 112. The oxygen content was obtained as weight percentages of the original samples.

3.3.2.3 Solvent extraction for lipids composition analysis

As a comparison, the Modified Bligh & Dyer extraction was used to obtain the lipids from the microalga. In Modified Bligh & Dyer extraction, 20 mL of methanol (CH_3OH) and a 10 mL of chloroform ($CHCl_3$) were mixed with 5 g of microalgal sample, and vortexed for 5 min at low speed. Then, 18 mL distilled water was added to the mixture and vortexed for another 2 min. Additional 10 mL of chloroform was added and vertexed for another 5 min to ensure a complete lipid extraction. A centrifuge (755VES Fisher H, Philipsburg, Pa.) was used to separate the solid from liquid and the aqueous layer was separated from biomass and biphasic miscella. Biphasic miscella was separated from biomass through filter paper (#42 Ashless 110 mm dia., Whatman, Maidstone, Kent, England). Lastly, methanol and chloroform were evaporated out of the miscella in the vacuumed evaporator, Büchi Rotavapor R-14 (Flawil, Switzerland) at 60˚C 60 kPa for 60 min.

3.3.2.4 Micaoalgal fatty acid profile and fatty acid methyl esters quantifications via gas chromatography-mass spectrometry (GC-MS)

Fatty acid profile of the lipids extracted by aforementioned procedures was analyzed by gas chromatography (GC) as described by Hammond.[23] A sample of 100 µL algal lipid was mixed with 5 mL of hexane in a 15 mL test tube, and then a 3 mL subsample of the resulting lipid solution was transferred to the 5 mL GC vial to prepare fatty acid methyl esters by treating with 200 µL of a 0.5M solution of sodium methoxide in methanol. Approximately 1 mL of de-ionized water was added to each GC vial to wash the unreacted sodium methoxide out and the upper layer was transferred to an auto-sampler for analysis.

A sample of 1 µL was injected into a Hewlett Packard Model 5890 Series II gas chromatograph equipped with a flame ionization detector, a split injection port set to achieve a 100:1 split ratio with a J&W 30 m x 0.25 mm I.D. DB-23 column (0.25 µm film thicknesses). Injection port temperature and the detector temperature were maintained at 250˚C and 300˚C, respectively. An initial oven temperature of 215˚C was held for 3 min, and then increased at 3˚C/min until reaching 230˚C. Helium carrier gas was used at 1 mL/min, and nitrogen makeup gas was supplied to the detector at 30

mL/min. The area percentages of the fatty acid methyl esters were quantified with a Hewlett-Packard 3396 Series II integrator. Then mass percentages were converted from area percentages by Excel calculations.

The FAME was content in the *in situ* transesterified product was analyzed by a GC-MS (PolarisQ instrument, ThermoFinnigan, West Palm Beach, Fla.) in the electron impact mode. A sample of the transesterified product was transferred into a 2 mL GC glass vial to which a solution of anthracene (0.3-0.5 mg/mL) in dichloromethane (1 mL) was added as an internal standard. Gas separation was achieved by a ZB-1 (30 m x 0.25 mm dia. Phenomenex) capillary column with a helium carrier gas at 0.8 mL/min. The temperature was programed at 5°C/min from 40°C to 250°C while the injector and MS-transfer line were kept at 255°C. An Xcalibur software package (ThermoFinnigan, West Palm Beach, Fla.) was employed in data processing. Comparing the retention times of the MS components with the standards of lignocellulos degradation products, fatty acids, and carbohydrates to perform compound identification.

3.3.3 Experimental design

A respond surface method was used to determine the optimal operation condition for maximizing the yield of FAME. The experimental design was based on a 2^3 full factorial design and was constructed using Design-Expert v. 8.0 (Stat-Ease, Inc., Minneapolis, Minn.). The process of *in situ* transesterification was investigated as a function of three factors, i.e., operating temperature, reaction time, and lipid-to-methanol molar ratio (sRatio). The significance of the regression coefficients was determined at a p-value of 0.05. Microsoft Excel was used for data processing.

3.3.4 Experiments and Procedures

All experiments were carried out in a batch reactor (Pressure Reactor 4560, 300-mL; Parr Instrument Co., Moline, Ill.) controlled by a PC-based 4857 Reactor Controller on temperature and agitation speed. A computer connected to the controller displays the operating temperature, pressure, and agitation speed (set constant at 500 rpm). The reactor system was hosted in a metal-framed chamber and vented out through a duct to ensure safe operation of the system. In the preliminary experiments, the reaction system was operated under supercritical methanol (250°C, 10.3 MPa) and subcritical

methanol (210°C, 7.6 MPa) conditions. Two levels of reaction time, i.e., 30 and 60 min, were employed. The reactor was immediately immersed in to a cold-water bath in order to stop the reaction after the set reaction period. Six sRatio of 1:50, 1:75, 1:100, 1:250, 1:300, and 1:350 were investigated which are approximately equivalent to mass ratios of 1:1, 1:2, 1:3, 1:5, 1:6 and 1:7, respectively. At the beginning of each experiment, carbon dioxide (CO_2) was used to purge the system. After the reaction, the oily products were collected and analyzed by a gas chromatography-mass spectrometry (GC-MS) for the FAME content.

After a proper range of operation parameters was concluded from preliminary experiments, a systematic investigation on the effects of selected operation factors was conducted. Experiments were performed based on a factorial experimental design, mentioned above, that included three numeric factors and one categorical factor. The selected numeric factors are three levels of sRatio (i.e., 1:50, 1:75 and 1:100), four levels of operating temperature (i.e., 170, 210, 250, and 290°C), four levels of reaction time (i.e., 30, 60, 90, and 120 min) with a constant 1.4 MPa (200 psi) gauge CO_2 input to clean-up the oxygen residue. According to this design, 48 sets of experiments were conducted and each experiment was implemented in triplicates for a total of 144 experiments.

3.3.5 Respond Factors

Two respond factors were used to reflect the effects of various operation condition on the *in situ* transesterification, i.e., product yield (mol%) and product selectivity (wt%), based on the following definitions:

$$\text{Product yield (mol\%)} = \left[\frac{mol\ of\ total\ FAME\ in\ product}{3\times mol\ of\ triglycerides\ in\ feedstock + mol\ of\ free\ fatty\ acids\ in\ feedstock}\right] \times 100\% \quad \text{(Eq. 1)}$$

$$\text{Product selectivity (wt\%)} = \left[\frac{mg\ of\ total\ FAME\ in\ prouct}{mg\ of\ product}\right] \times 100\% \quad \text{(Eq. 2)}$$

3.3.6 Spectroscopies on Microalgal Biomass

Spectroscopic analysis of Fourier transform infrared (FTIR) was performed on an Avatar 370 spectrophotometer (Thermo Nicolet) with an attenuated total reflection

(ATR) probe (ZnSe crystal). The spectra were ATR and baseline corrected using Omnic v7.0 software.

Scanning electron microscopy (SEM) images were taken for analyzing microalgae cell structure. An imaging SEM 200F (FEI 200F SEM) at Franceschi Microscopy and Imaging Center, Washington State University was employed to take the images of microalgae biomass samples from various operation conditions. This apparatus has high resolution, low vacuum, and environmental friendly as its signature. Images were taken at three different resolutions and presented for comparison with a unified resolution of 25000×.

3.4 Results and discussion

3.4.1 Microalgae characterization

Characterization was performed on the *S. limacinum* biomass, including proximate analysis, ultimate analysis and fatty acid (FA) composition, before the experiments. Fatty acid profile of the lipids was analyzed by gas chromatography-mass chromatography (GC-MS) as described by Hammond.[23] It was determined that the microalgal biomass contains 1.6 wt % of moisture, 78.5 wt% of volatile matters, 8.9 wt% of fixed carbon and 11.0 wt% of ash content on dry basis. Elementally, the *S. lmacinum* biomass consists of 61.4 wt% carbon, 8.9 wt% of hydrogen, 19.5 wt% of oxygen, 4.0 wt% of nitrogen, and 0.6 wt% of sulfur on dry basis. The gross heating value was calculated as 29.7 MJ/kg, based on the formula proposed by Channiwala and Parikh.[24] Moreover, the FA profile of the *S. limacinum* lipids, as determined via GC-MS after solvent extraction (i.e., hexane, methanol, and chloroform/ methanol separately), was 1.5 wt% of myristic acid (C14:0), 22.3 wt% of palmitic acid (C16:0), 0.7 wt% of stearic acid (C18:0), 3.0 wt% of oleic acid (C18:1), 7.2 wt% of linoleic acid (C18:2), 1.0 wt% of linolenic acid (C18:3), 3.6 wt% of clupandonic acid (C22:5), and 15.1 wt% of docosahexaenoic acid (C22:6) on dry basis. Furthermore, the analysis, according to AOCS official method Ca 5a-40[25], indicated that the extracted microalgal lipids contained 16.6 wt% of FFA.

3.4.2 Biodiesel properties due to algal lipid composition

Fatty acid profiles of microalgal lipids are different among different species. In general, microalgal lipids contain fatty acids that possess two unique signatures of relatively

longer chain length and high unsaturation. Microalgae have a common FA chain length from C12 to C22, which is crossover with typical vegetable oil FA ranges of C14 to C20, and a polyunsaturated FA range of C20 to C22.[26–28] Lipid composition of *S. limacinum* presents most of the fatty acids (i.e., C14, C16 and C18) in typical vegetable oils but with a higher portion of polyunsaturated fatty acids. C16:0 is the most abundant saturated fatty acid in *S. limacinum* lipids, and C22:6 is the highest among the polyunsaturated fatty acids. FA profile can directly affect derived fuel properties. The longer chain length and higher saturation of a fatty acid leads to a higher viscosity of the corresponding FA methyl ester. Therefore, the combination of the long chain and the level of unsaturation in fatty acids decide the viscosity of microalgal biodiesel. Cold flow and oxidative stability are two interacting factors controlled by the chain length and the level of unsaturation of fatty acids. Long-chain saturate fatty acids cause poor clod flow property of corresponding biodiesel but promise a decent oxidative stability property. Polyunsaturated fatty acids are more reactive than saturated fatty acids due to the carbon-carbon double bonds that are easily to open up and react with alcohols. In contrast, a good portion of microalgal lipids is polyunsaturated which provides a compensation to the poor cold flow property caused by the longer chain fatty acids. However, it also means that its susceptibility to oxidation is a concern.[29] Besides, microalgal lipids contain even more polyunsaturated fatty acids such as C22:5 and C22:6, than those found in typical vegetable oils thus the algal biodiesel made from such lipids tends to be oxidatively unstable.

3.4.3 Preliminary experiments of in situ transesterification

Microalgal lipids are mainly composed of triglycerides and some free fatty acids and phospholipids. In this study, the microalgal lipids are extracted by the high temperature methanol and then sequentially converted to FAME (or biodiesel) by transesterification of the microalgal lipids with methanol but without adding any catalyst. This process is referred as the *in situ* transesterification in this study. The ideal operation of such an *in situ* transesterification is to carefully control the operating conditions so that only the microalgal lipids are extracted and converted to FAME and the microalgal lingo-cellulosic biomass structure is kept intact as much as possible. If the operating

conditions are controlled in such a way that the whole microalga, including the microalgal lipids and cellulosic biomass, is converted into liquefied products, the process is then referred as thermal liquefaction as reported extensively in literature, which is beyond the scope of exploration in this study.

To identify the most influential process parameters, preliminary experiments were carried out first. The preliminary experiments were started with two temperatures (210˚C and 250˚C) in the vicinity of the supercritical point of methanol (240˚C and 8.1 MPa), 5 levels of sRatio (1:50, 1:75, 1:100, 1:250 and 1:300), and two duration of reaction time (30 and 60 min). The stirring was set constant at 500 rpm and was not considered as a process parameter.

The primary barrier in transesterification of vegetable oils, animal fats and microalgal lipids is the immiscible nature of the oils, fats or lipids and alcohol. Moreover, lipid extraction from whole microalgal biomass is hindered by the physical restraint of cell wall structure.

The processing temperature and reaction time determine the reaction environment. Higher temperature and longer reaction time would speed up the reaction rate and increase the conversion of the lipids. However, too high a temperature or too long a reaction time would also convert the microalgal biomass to untargeted products, which is undesired in this study. To pre-screen the effective range of the operating temperature, 210˚C and 250˚C were chosen for that they are close to the critical temperature of methanol at 240˚C and presumably lead to favorable fluid properties of methanol as a solvent as well as a reactant without the aid of a catalyst.

Preliminary experimental results showed that the product yield depends on the combined effect of operating temperature and the reaction time at high sRatio. A spike of product yield occurred at 210˚C with a reaction time of 60 min. Within a constant reaction time of 60 min, higher temperature than 210˚C gave negative result in FAME yield. At the lower temperature (210˚C), longer reaction temperature was needed; at higher temperature (290˚C), extended reaction time may not be beneficial. The possible cause of this phenomenon is that some decomposition reactions may occur at the condition of higher temperature and extended reaction time. Saka and Isayama[30] indicated that unsaturated fatty acids are dramatically breakdown at temperature over

300˚C. A general observation was that lower temperature and longer reaction time led to a significantly higher lipid conversion yield (Figure 3-1). This finding was used as a guideline in the following stage of investigation.

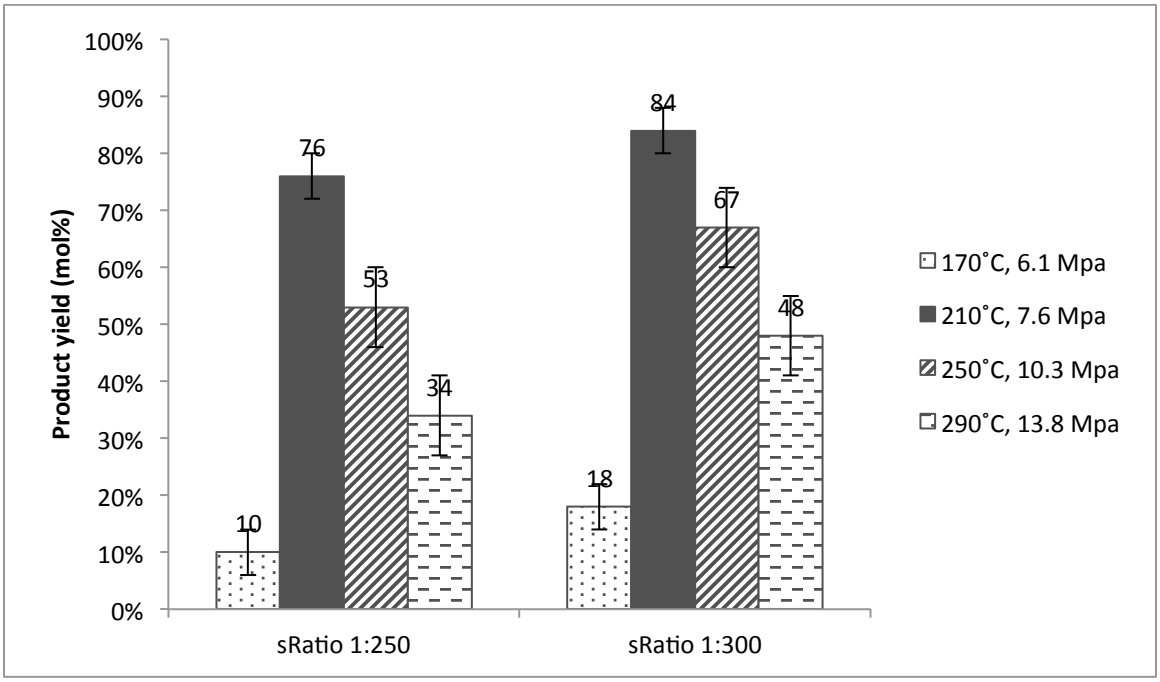


Figure 3-1. Product yields of microalgal FAME at two levels of sRatio and 60 min reaction time.

There was a noticeable difference in the figure 1 while sRatio rose from 1:250 to 1:300 (equivalent to algae to methanol mass ratio of 1:5 and 1:6, respectively), thus the higher sRatio (1:350) was selected to further test the effect of sRatio levels. Besides, reaction time has been pointed as a core parameter of transesterification.[31,32] The results (Figure 3-2) showed no significant impact of sRatio beyond 1:300 regarding to the product yield. Although, for this microalgal strain of *S. limacinum*, sRatio 1:300 is satisfactory in driving the *in situ* esterification/ transesterification to completion, this reaction condition, however, requires enormous amount of methanol, which significantly increase the cost of operation. Therefore, this high reactant ratio was not selected for further study.

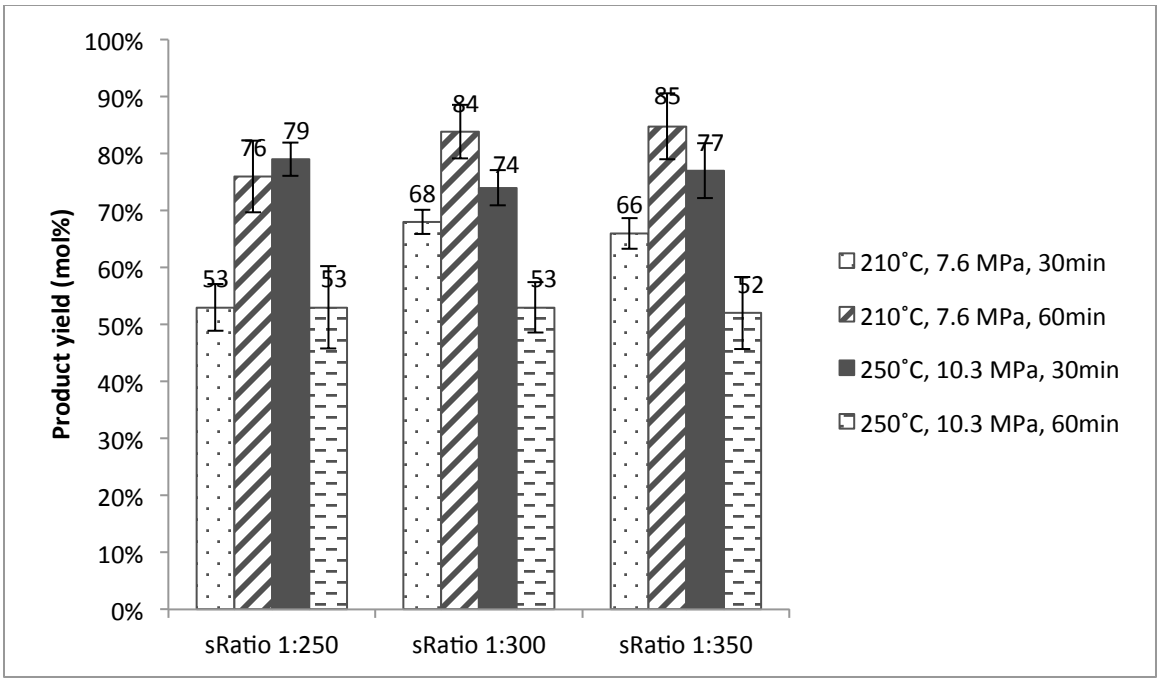


Figure 3-2. Product yields of microalgal FAME at three levels of sRatio.

Carbon dioxide (CO_2) is an excellent solvent for extraction in supercritical fluid status which has been used for oleaginous matters in many fields.[33–35] It has been proved to have a similar behavior to the conventional co-solvent, such as hexane or ethyl acetate.[37] Moreover, its properties allow it to efficiently and selectivity extract non-polar compounds from complex matrices. Especially, long chain hydrocarbon compounds are known to be soluble in supercritical CO_2 ($ScCO_2$).[38] Furthermore, glycerol is almost insoluble in $ScCO_2$ and will precipitate out of methanol upon formation.[33,36–38] Triglycerides are moderately soluble in methanol while FAME product is highly soluble. Therefore, the property of FAME tends to dissolve in $ScCO_2$ will drive the reaction equilibrium forward to FAME formation.[37] The supercritical conditions of CO_2 are 304K (31°C) and 7.4 MPa. It is often used with methanol or ethanol as the co-solvent in supercritical processing.[33,39] In this study, carbon dioxide was applied in all further experiments for enhanced lipid extraction and transesterification reaction. Stoichiometrically, one mole of microalgal lipids as expressed by triglycerides requires three moles of alcohol to react. However, *in situ* transesterification of microalgae requests a much higher oil to alcohol ratio mainly because methanol serves as a solvent first then a reactant. Oil to alcohol molar ratio range of 1:100 to 1:800 with acid/ base

catalysts was commonly selected in microalgae *in situ* transesterification studies.[4,16,40] Meanwhile, other researchers studied *in situ* transesterification with physical method aid, such as microwave. Chee et al. studied base catalyzed transesterification of microalgae lipids with microwave heating assistant.[41] They operated this process with *Nannochloropsis* sp. and *Tetraselmis* sp. biomass and oil to methanol molar ratio of 1:12 at temperature of 50˚C and 16 h reaction time. The maximum yield of microalgal biodiesel was indicated as 83.33% and 77.14%, respectively.[41] Because of all the evidences from previous studies that *in situ* transesterification of microalgae can be done at oil to alcohol molar ratio close to the stoichiometric level. Therefore, some lower levels of sRatio, i.e., 1:50, 1:75, 1:100 and 1:250, were selected to investigate the effects of sRatio.

Experimental results indicated that the sRatio lower than 1:250 does not show clear effect on the product yield (Figure 3-3). When sRatio was increased to 1:250, the product yield reaches the highest at 210˚C. In contrast, product yields showed a decreasing trend after sRatio of 1:75 at 250˚C. This phenomenon suggests that temperature is a more critical parameter than sRatio. From lipid extraction and physical operation point of view, sRatio of 1:50 to 1:100 are preferable because low methanol application than 1:50 would not make a good microalgae-methanol suspension and limit the mass transfer of microalgal lipids into the liquid phase for reaction. Additionally, the interaction of temperature and sRatio affected the results of product yields due to a turning point at sRatio 1:75 to 1:100. Between sRatio of 1:100 and 1:250, reaction temperature seems to be a more important factor since the product yield at sRatio 1:250 has dropped at 250˚C. Furthermore, literature suggests that a sRatio larger than 1:42 is suitable for microalgae biodiesel transesterification.[42] Therefore, in the second stage of this study, sRatio of 1:50-1:100 were chosen for further investigation.

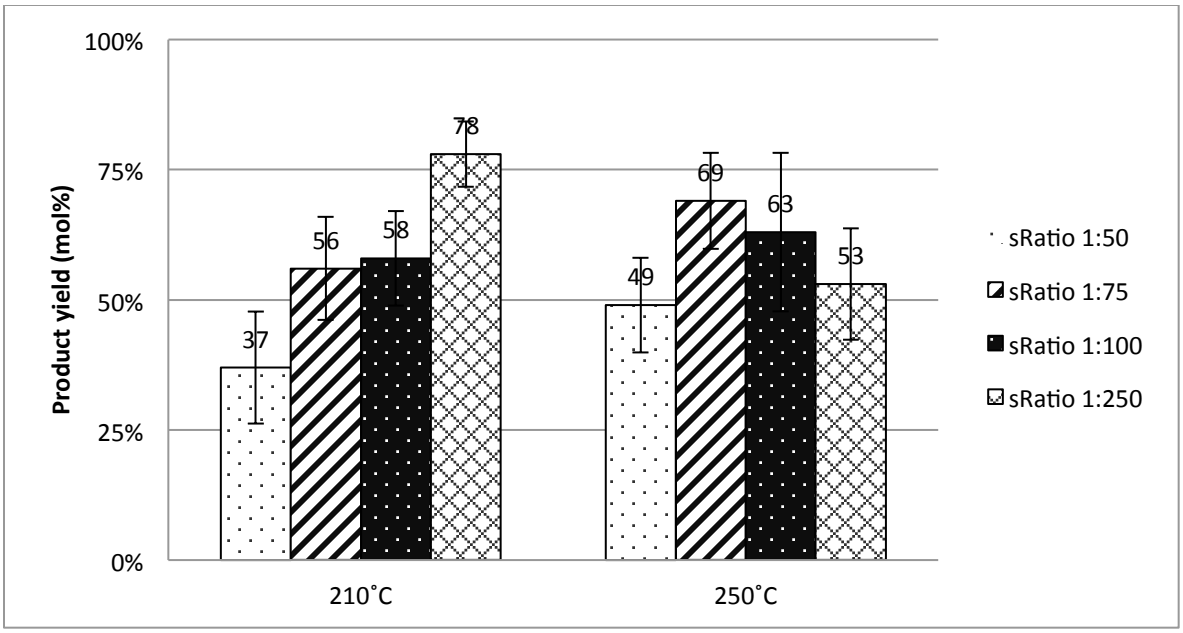
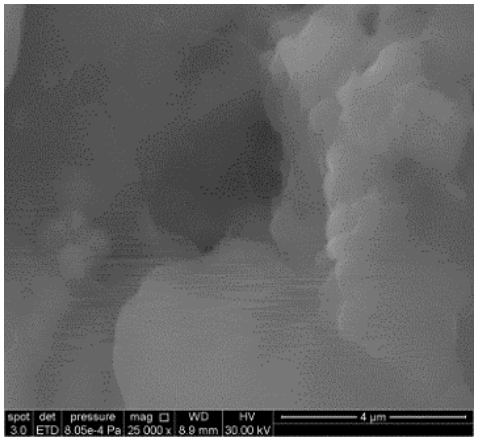
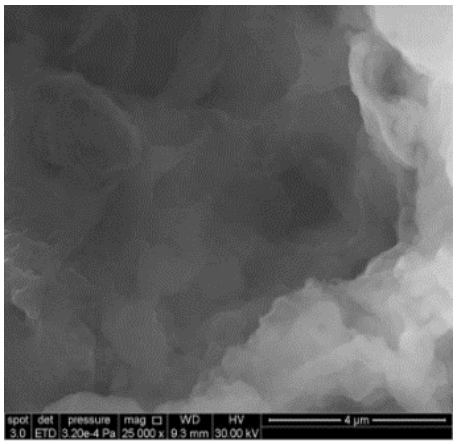


Figure 3-3. sRatio effect on product yields with an initial 1.4 MPa gauge of CO_2 for 60 min.

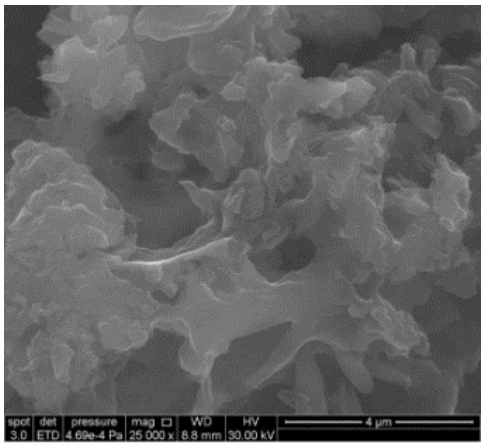
Another possible barrier in the *in situ* transesterification of microalgal lipids is the physical structure of microalgal cells. The lipids need to overcome the resistance from the algal membrane to migrate to the methanol phase for reaction. Besides, the physical structure of cell wall possessed in spatial structure deliver an inevitable possibility of lignocellulosic methylation reaction occurring during *in situ* transesterification. Methanol at elevated temperature possesses strong solvent properties and may break the cell membrane.[29,39,43] Therefore, the sub- and/or super-critical methanol has a strong solvent effect on the microalgal biomass structure in the *in situ* transesterification process. To review the physical changes of microalgae cell wall, images of microalgal biomass undergone different thermal conditions were taken with scanning electron microscope (SEM) (Figure 3-4).



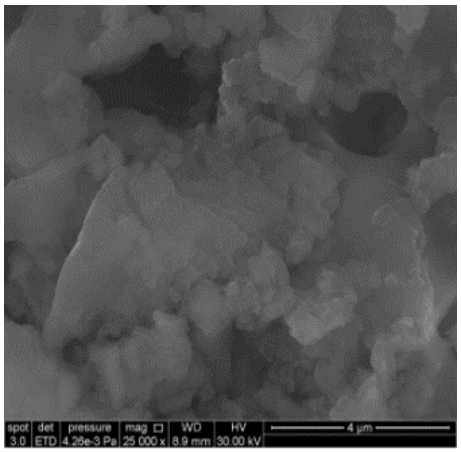
(a) Original biomass



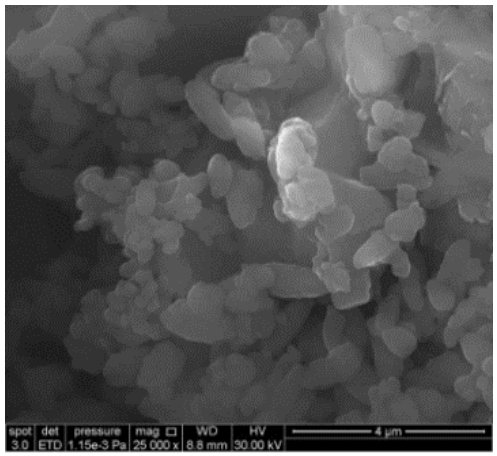
(b) 170˚C



(c) 210˚C



(d) 250˚C



(e) 290˚C

Figure 3-4. SEM images with magnification of 25,000× of microalgal biomass before and after the *in situ* transesterification at various temperatures.

Before processing, the microalgal cell structure was intact with visible pores on the surface. As the processing temperature increased from 170°C, the cell structure gradually collapsed, the pore sizes enlarged and the spherical structure broke down. The particle size gradually decreased as the reaction temperature increased. When temperature reached 210°C the spherical structure began to deform and a greater degree of deformation was obvious. At 290˚C, the cell structure has completely been destroyed, and microalgal biomass no longer held its spherical structure and disintegrated into smaller pieces. This observation suggests that methanol at elevated temperatures is not only able to penetrate the cell wall and reach lipids, but also break the cell structure, which leads to the stage of biomass liquefaction. Under extreme thermal conditions, therefore, methanol properties are enhanced to such a level that the biomass cell structure can physically collapse, the lignocellulosic matters can thermally decompose, and likely also react with methanol in a complicated manner. Therefore, the next study is logically to investigate the levels and combined effects of process parameters systematically in order to maximize the transesterification of microalgal lipids with minimized lignocellulosic biomass decomposition.

3.4.4 Systematic investigation of process parameters

Preliminary experiments revealed that *in situ* transesterification of the microalga is achievable in methanol its vicinity of critical point (240˚C and 8.1 MPa). However, the complexity of the microalgal properties, namely the physical and chemical properties of microalgal lipids versus the microalgal cellulosic biomass, implies that the process operating parameters need to be systematically investigated in order to achieve satisfactory product yield and selectivity which are the valuable outcomes in evaluating the efficiency of the process from the angle of engineering and technological development.

The objective of this systematic investigation was to find the suitable operating conditions at which the highest product yield and/or selectivity can be achieved. Experiments were performed based on a 4×4×3 factorial experimental design that includes four levels of operating temperatures (i.e., 170, 210, 250, and 290°C), four

levels of reaction time (i.e., 30, 60, 90 and 120 min), and three levels of sRatio (i.e., 1:50, 1:75 and 1:100). All experiments were conducted in triplicate to ensure the repeatability and reliability. Tables 3-1 summarizes the experimental results.

Close examination of the experimental results leads to some preliminary conclusions. First, the product yield ranged widely from as low as 17% to as high as 65%, highly depending on the operating conditions. It was observed that the operating temperature affects the product yield significantly. Generally, the product yield increases as the temperature increases in the range of 170-250°C. Once the operating temperature reaches 290°C, the some product yields were even lower than those at temperatures below 290°C. This might be due to the faster rate of decomposition of the formed product FAME, which exceeds the rate of FAME formation from esterification and transesterification.

Table 3-1. Product yield (mol%) and product selectivity (wt%) of FAME via *in situ* transesterification with 1.4 MPa (200 psig) initial CO_2.

Product yield (mol%)	Time (min)	sRatio 1:50				sRatio 1:75				sRatio 1:100			
		170°C	210°C	250°C	290°C	170°C	210°C	250°C	290°C	170°C	210°C	250°C	290°C
	30	16.5±5.7	24.7±12.7	48.6±10.4	27.6±13.1	10.6±3.1	27.3±11.4	62.7±11.1	59.2±11.4	9.3±11.1	28.6±7.6	60.6±13.1	53.9±9.1
	60	16.7±3.2	39.6±13.8	39.4±12.1	26.8±12.2	20.7±3.4	41.9±9.9	60.5±9.2	38.5±9.7	25.9±14.7	48.3±9.1	58.7±15.2	46.6±8.7
	90	26.4±4.7	44.4±12.3	36.4±11.1	26.7±13.1	34.9±2.1	46.2±12.4	52.9±11.4	29.0±10.1	28.8±9.4	52.5±7.7	52.5±11.0	26.6±8.4
	120	36.1±4.8	47.6±13.4	27.4±10.3	17.2±14.3	38.2±1.6	64.5±11.8	47.2±10.0	31.7±11.1	36.7±10.1	60.2±7.6	40.9±12.3	20.7±9.7
Product selectivity (wt%)	Time (min)	sRatio 1:50				sRatio 1:75				sRatio 1:100			
		170°C	210°C	250°C	290°C	170°C	210°C	250°C	290°C	170°C	210°C	250°C	290°C
	30	10.3±7.1	18.2±13.7	23.6±12.4	22.4±10.0	15.1±9.4	21.7±11.3	25.4±3.7	40.6±4.7	18.6±4.1	26.6±10.1	46.8±10.7	28.5±10.1
	60	12.7±8.6	25.9±20.4	22.2±15.1	18.1±5.6	20.4±10.7	30.6±10.9	35.2±4.6	24.1±4.3	23.8±9.3	28.3±12.2	20.6±13.5	19.4±9.6
	90	21.0±11.2	26.9±14.3	20.5±11.6	13.4±6.3	28.7±9.4	29.9±14.2	36.7±3.9	20.5±4.4	37.2±13.1	29.6±10.7	18.4±11.2	22.9±11.5
	120	22.3±11.4	27.1±10.1	15.8±1.7	10.6±5.1	29.3±8.2	33.1±13.7	24.9±4.8	24.0±4.7	34.2±10.2	34.7±9.3	20.8±13.1	20.6±7.6

Meanwhile, reaction time with operating temperature showed a correlated effect on the product yield, with a few exceptions. At lower temperatures (170°C and 210°C), the product yield showed an increasing trend as the reaction time is in the range of 30-120 min. Once the operating temperature was higher than 210°C, especially at 290°C, the product yield changed inversely as the reaction time increased. Similar to the reason discussed above, this decrease in product yield might be due to the decomposition of FAME at high temperatures and extended reaction time. It seems that there is an optimum range of operating conditions at 210˚C, 120 min and 250˚C, 30 min, at which both the product yield and the product selectivity reached their peak values, and then both rapidly declined beyond temperature of 250˚C. Hence, the optimal reaction condition point ought to exist between these two points regardless of the effect of sRatio level.

The sRatio statistically exhibits a significant effect on the product yield. Under the same operating temperature and reaction time, the product yields are approximately the same in sRatio range of 1:75 to 1:100. The highest product yield and selectivity happened at sRatio 1:75. This indicates that the quantity of methanol at sRatio 1:75 and 1:100 are adequate for the purpose of lipid extraction and transesterification, which are higher than that of 1:42 as reported in the literature.[42,44] However, the results of product yield and selectivity are also affected by the interaction of sRatio and other parameters. When sRatio beyond 1:75, the effect of methanol quantity reached a plateau and did not benefit the transesterification reaction significantly. Therefore, another conclusion is that methanol amount does not have an effect on FAME selectivity beyond sRatio of 1:75.

Since the maximum product yield achieved at 210˚C, 120 min with sRatio 1:75 with a product yield of 64.5 mo% and a product selectivity of 33.1 wt%, GC-MS spectra were reanalyzed to indicate the other chemical products generated in the process. Table 3-2 summarizes the chemical compounds appeared at 210˚C for 120 min.

With an operation temperature of 210˚C for 120 min in *in situ* transesterification not only has successfully converted microalgal lipids into FAME but also presents biomass liquefaction reaction. Furthermore, a higher degree of biomass liquefaction reaction

leads to more complex product composition would expected in process at a higher operation temperature. Therefore, the reason of product yield loss in *in situ* transesterification may conclude due to lignocelluloses degradation. A FTIR analysis was applied to further prove this hypothesis.

Table 3-2. Chemical compounds generated in *in situ* transesterification of microalgae at 210˚C for 120 min.

#	Retention Time (min)	Name	Formula
1	24.31	Methyl 10-methyl-undecanoate	C13H26O2
2	24.48	2-Bromotetradecanoic acid	C14H27BrO2
3	28.27	E-11,13-Tetradecadienal	C14H24O
4	28.31	Methyl 10-methyl-undecanoate	C13H26O2
5	31.16	10,12,14-Nonacosatriynoic acid	C29H46O2
6	33.80	1-(+)-Ascorbic acid 2,6,-dihexadecanoate	C38H68O8
7	36.45	Methyl 9-cis,11-trans-octadecadienoate	C19H34O2
8	36.61	2,3,-Dihydroxypropyl elaidate	C21H40O4
9	38.76	i-Propyl 5,8,11,14,17-eicosapentaenoate	C23H36O2
10	38.85	n-Propyl 5,8,11,14,17-eicosapentaenoate	C23H36O2
11	38.95	i-Propyl 5,8,11,14,17-eicosapentatenoate	C23H36O2
12	39.15	i-Propyl 7,10,13,16,19-docosapentaenoate	C25H40O2
13	39.20	Butyl 6,9,12,15-octadecatetraenoate	C22H36O2
14	39.46	9,12,15-Octadecatrienoic acid,2,3-dihydroxypropyl ester, (ZZZ)	C21H26O4
15	42.33	3-Oxatricyclo[20,8,0,0(7,16)]triaconta-1(22),7(16),9,13,23,29-hexaene	C29H42O
16	42.35	Butyl 4,7,10,13,16,19-docosahexaenoate	C26H40O2
17	43.37	Aldosterone-21-acetate	C23H30O6

3.4.5 Response surface methodology for producing microalgal biodiesel

A Response surface methodology (RSM) model was used to statistically analyze and determining the optimum operation condition. Table 3-3 shows the ANOVA results which indicates that sRatio (C) in the 1st order, temperature (A) in the 2nd order and an interaction (AB) of temperature and time (B) were shown to be the most significant variables. Secondly, variable A is another variable that presents a significant effect on the results. The model also included temperature in the 2nd order and time based on their significance ($\alpha = 0.05$). The R-square of the model was 0.85, which is acceptable to

provide a decent prediction on FAME yield under optimal operation conditions. Thus the modeling results could be concluded by the equations 3 and 4:

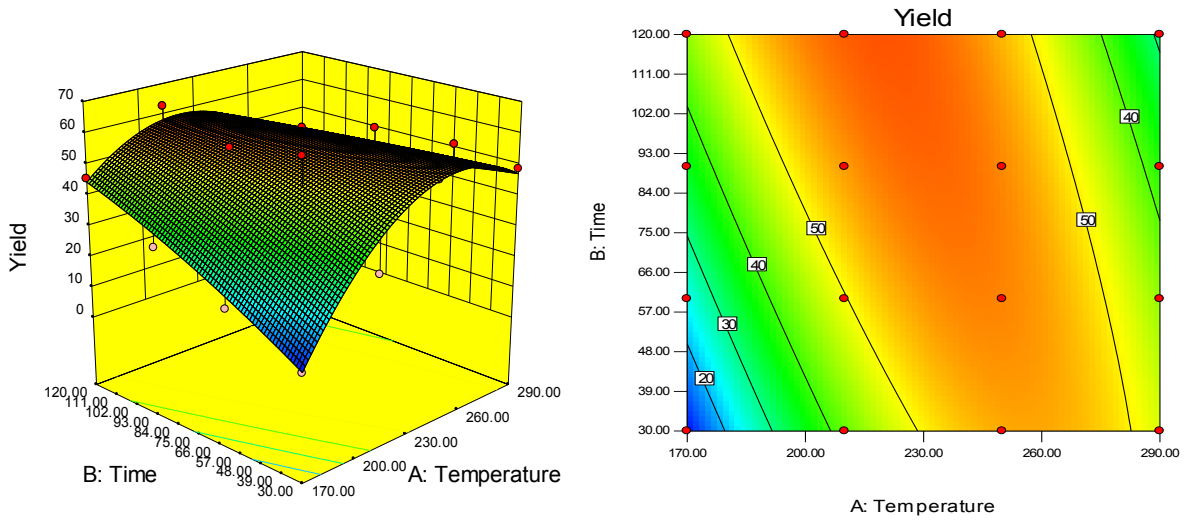
$$\text{Yield} = 51.42 + 4.44 \times A + 1.31 \times B - 8.60 \times C + 5.41 \times C^2 - 13.10 \times AB - 1.31 \times AC + 0.71 \times AC^2 - 0.76 \times BC + 2.48 \times BC^2 - 21.40 \times A^2 - 2.56 \times B^2 \quad \text{(Eq. 3)}$$

$$\text{Selectivity} = 28.92 - 1.83 \times A + 1.21 \times B - 5.24 \times C + 2.99 \times C^2 - 6.74 \times AB + 1.02 \times AC + 0.14 \times AC^2 - 0.95 \times BC + 2.71 \times BC^2 - 7.28 \times A^2 - 0.34 \times B^2 \quad \text{(Eq.4)}$$

With product yield and product selectivity as the related responds factors, RSM provides a direct visual analysis on the overall results. Since the optimum operation condition was given at the sRatio of 1:75, sRatio at 1:75 was fixed in the 3D plot (Figure 3-5A). It is seen that the highest product yield lay at the point of 210˚C and 120 min. The 2D contour plot (Figure 3-5B) indicates the optimization result based on the quadratic model at sRatio of 1:75. The maximum predicted product yield is 64.5 mol% and product selectivity is 33.1 wt% at 210˚C, 120min, and sRatio 1:75.

Table 3-3. ANOVA table of product yield analysis including all four parameters.

Source	Sum of Squares	df	Mean of Square	F Value	p-value Prob > F
Model	9444.55	11	858.60	1862	<0.0001
A - Temperature	525.70	1	525.70	10.68	0.0018
B -Time	46.11	1	46.11	1.48	0.3234
C - sRatio	1815.92	2	907.96	11.10	<0.0001
AB	2542.18	1	2542.18	65.40	<0.0001
AC	23.02	2	11.51	2.41	0.7800
BC	86.10	2	43.05	1.11	0.4016
A^2	4343.41	1	4343.41	97.97	<0.0001
B^2	62.11	1	62.11	0.080	0.2529
Residual	1656.27	36	46.01		
Corrected Total	11100.82	47			
$R^2 = 0.85$, adjusted $R^2 = 0.81$					



(A) (B)

Figure 3-5. (A): 3D plot of RSM for *in situ* transesterification product yield based on a 4×4×3 factorial design (fixed sRatio at 1:75); (B): Contour plot of RSM of *in situ* transesterification product yield at fixed sRatio of 1:75.

3.4.6 Spectroscopic analysis

FTIR spectroscopy was used to further analyze chemical function group changes of the *in situ* transesterification products (Figure 3-6). The main characteristics are attributed to the presence of esters and hydrocarbon content and protein. A strong hydrogen bond O-H stretching band appeared at 3348 cm^{-1} due to the composition of proteins and celluloses matters in the feed which also occurred in the FTIR spectra of microalgae hydrothermal liquefaction biocrude oil.[20] Besides, the absorption bands from 1600 to 1400 cm^{-1} derived from C=C vibration of the aromatic ring were assigned to lignin.[45] The distinct peak at 1435 cm^{-1} is detected in the spectra of product mixture from a reaction of 210˚C 120min. Similar to hydrothermal liquefaction biocrude oil from *Spirulina* algae, greater intensity and higher resolution of bands of C-H stretching at 3000-2840 cm^{-1} appeared in all spectra which suggested a more highly aliphatic in character.[46] Absorption ester bands of C=O stretching at 1741 cm^{-1} from fatty acid, hydroxyl fatty acid, and diacids in lipids were also detected in all product mixtures.[19,20,22,46] Moreover, the characteristics of the fatty acids, esters and carbohydrate derivatives, C-O stretch at 1360 cm^{-1} and C-O alcohol stretch at 1196 cm^{-1} and 1168 cm^{-1} are also presented in all spectra. Along with ester banding presented as

signature feature of microalgal biodiesel, a prominent N-H bending peaks (1680-1600 cm^{-1}) associated with amide and amine compounds shown in all spectra.

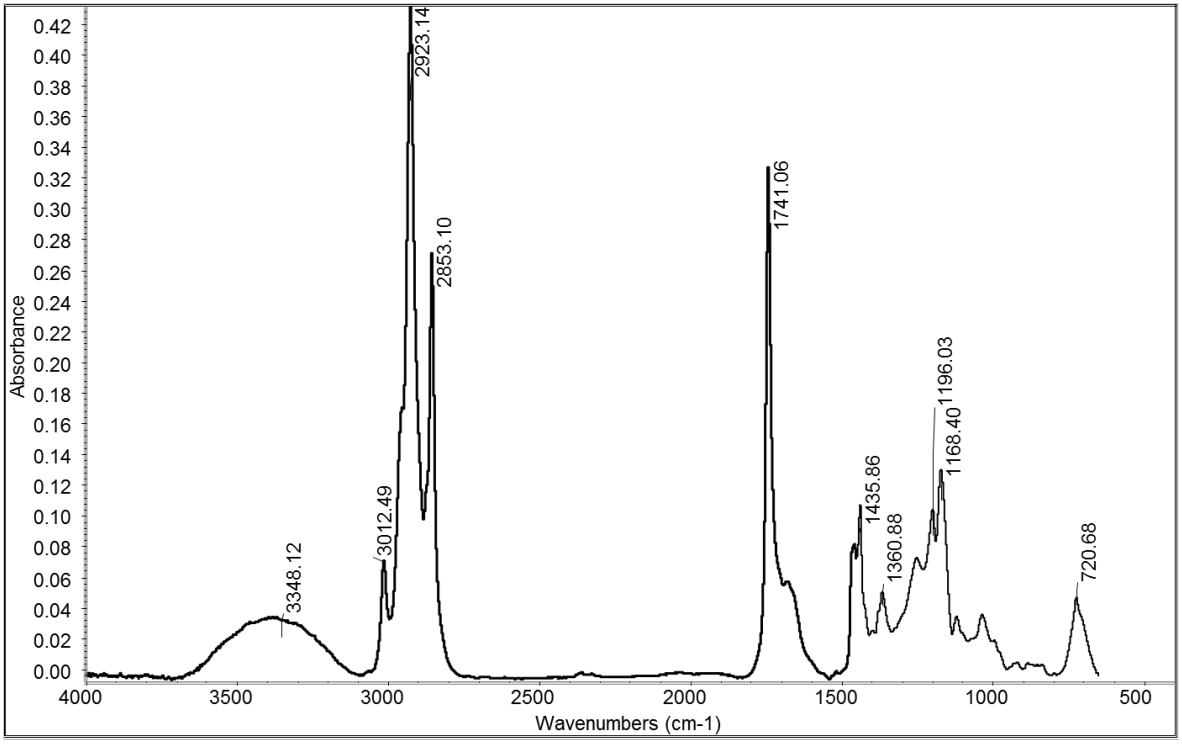


Figure 3-6. FTIR spectra of the product mixture from *in situ* transesterification at 210˚C and 120 min.

However, some distinct differences could also be observed when comparing the spectra of *in situ* transesterification oil products from different operation conditions. Figure 3-7 compares the spectra differences of biocrude oils from operation conditions of 210˚C and 290˚C at 120 min. The spectra clearly indicate the esters yield loss at a higher reaction temperature. The strong ester band (C=O stretching) at 1739 cm^{-1} is significantly reduced. In contrast, O-H stretching at 3373 cm^{-1}, C-O stretching at 1290 cm^{-1}, C-O alcohol vibration at 1196 cm^{-1} and 1170 $cm^{-1,}$ and N-H bending at 1661 cm^{-1} are amply increased. This phenomenon reveals the fact that more cellulose, lignin and protein were reacted at 290˚C than at 210˚C and less esters formed which could be explained by the possibility of degradation reactions occurred after transesterification. The esters were further reacted with the components of the microalgal biomass and formed other compounds (i.e., amide, amine, hydroxyl group and aromatic compounds). This statement was also claimed in a work by Demirbas.[47]

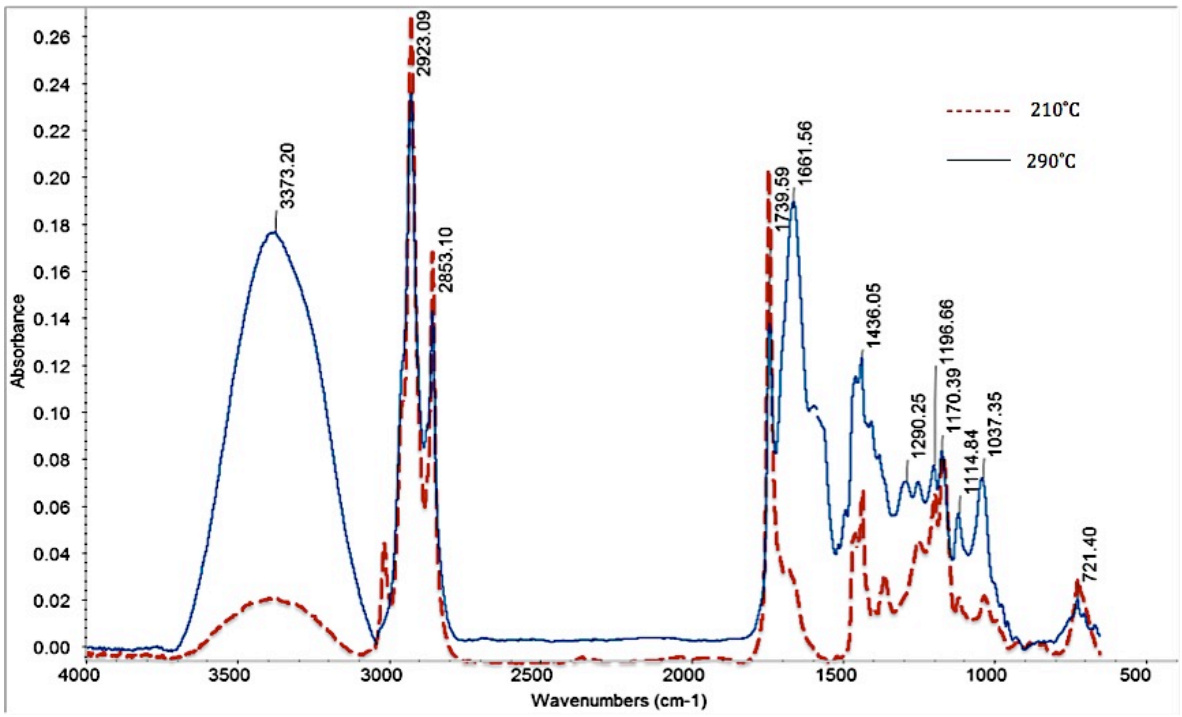


Figure 3-7. FTIR spectra of in situ transesterification product mixture at 210˚C and 290˚C for 120 min.

The effect of reaction time was examined by comparing the FTIR spectra of product mixture from the same operation temperature but different reaction time (Figures 3-8 and Figure 3-9). When the *in situ* transesterification was operated at 210˚C for 30 min and 120 min, the compositions of product mixture did not significantly changed except the O-H stretching and C=O stretching had a slightly increase. It proves the fact that at 210˚C, biomass liquefaction was not promoted with the elongated time period. Figure 8 presents the comparison of FTIR spectra of product mixture from reaction conditions of 30 min and 120 min at 250˚C. A dramatic change of the product composition was detected for different period of time at 250˚C. At this temperature, biomass liquefaction became the dominate reaction with a reaction time of 120 min. Therefore, an interaction of operation temperature and reaction time affected the process greatly, which is consistent with the results of statistical analysis.

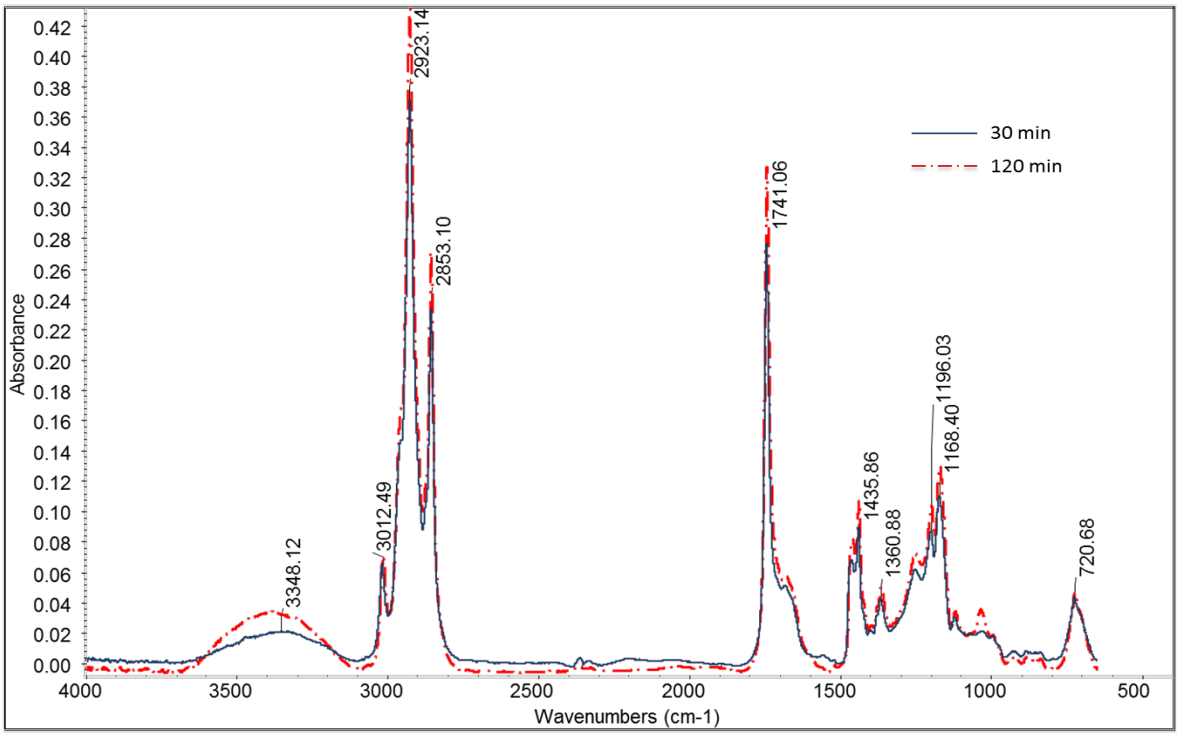


Figure 3-8. FTIR spectra of the product mixture from *in situ* transesterification 30 min and 120 min at 210˚C.

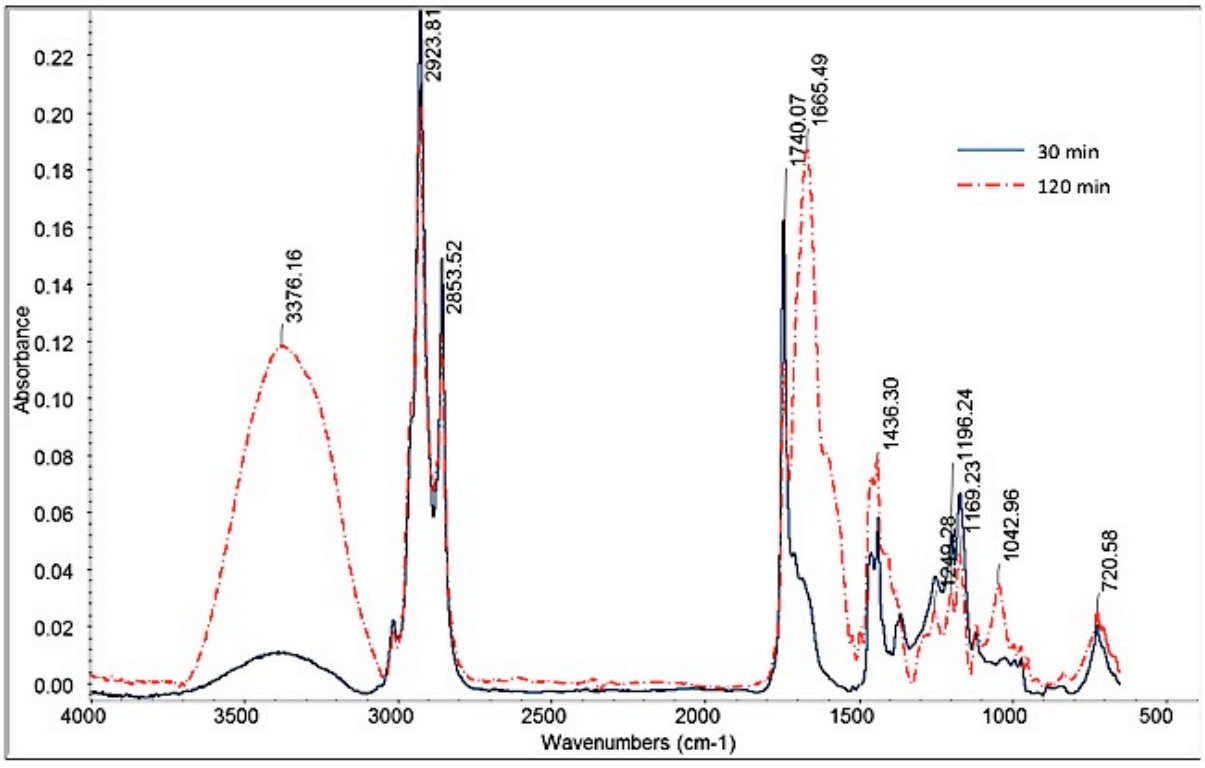


Figure 3-9. FTIR spectra of the product mixture from *in situ* transesterification for 30 min and 120 min at 250˚C.

3.5 Conclusion

The experimental results showed that the direct conversion of algal lipids of microalga *Schizochytrium limacinum* can be achieved in one step. The sub-/ supercritical *in situ* transesterification without catalyst application has significant results with respecting to product yield and selectivity. The actual effects on product yield are collectively contributed by the operating temperature, which determines the fluid status of methanol, and sRatio. The effect of reaction time was determined not significant but affected the system by its interaction with operating temperature. Another important response factor is the selectivity of the targeted microalgal methyl esters or FAME. Experimental data on product selectivities lead to similar conclusions as those observed on product yield. Generally, the product selectivity increases as the operating temperature increases in the range of 170-250˚C. A clear trend was noticed that when the operating temperature was at 290˚C, the selectivities were generally lower than those at lower temperatures. The FTIR spectroscopy analysis proved the deduction of the statistical analysis and also gave an idea that the formed FAME may decompose and reacte with other components at higher temperature (i.e., 250˚C and 290˚C) and longer reaction time (i.e., 90 and 120 min). Overall, an optimum operation condition for *in situ* transesterification was determined at 210˚C, 120 min with sRatio 1:75 and the highest product yield was 64.5 mo% and product selectivity of 33.1 wt%. The advantage of such a proposed one-step, *in situ* transesterification without addition of catalysts is to simplify the microalgae processing process and overcome the technological challenges in traditional processes thus to enhance the processing efficiency for viable algal biodiesel production.

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Chapter 4. Phospholipids Transesterification in Sub-/Super-critical Methanol with the Presence of Free Fatty Acids

4.1 Abstract

Phospholipids and free fatty acids (FFA), along with triglycerides, are naturally formed constituents in unrefined vegetable oils and other plant lipids. Presence of phospholipids and FFA in such oils can cause processing difficulties, such as saponification and decrease in catalytic efficiency, in the transesterification of such oils for biodiesel production, thus leading to adverse process efficiency. This phenomenon was also observed in our previous study on converting microalgal lipids to fatty acid methyl esters (FAME) via in situ transesterification. This study aimed at further exploring the transesterification of phospholipids and the effects of processing conditions and presence of FFA in biodiesel production from plant oils in sub- and/or super-critical methanol (SubCM/SCM).

To explore the effects of phospholipids and FFA in biodiesel production, experiments were carried out in a batch reactor in (SubCM/SCM) under various conditions. Pure chemicals of lecithin and stearic acid were used as the model compounds for phospholipids and FFA, respectively. The product yield (FAME mol%) of phospholipids after transesterification, as affected by the presence of FFA under different conditions, was selected as the respond factors to determine the process efficiency. Experimental results showed that the transesterification of phospholipids is largely affected by the interactive effect of operating temperature and reaction time. The increases of product yield is proportional to the increases of temperature and/or reaction time with the maximum product yield (68.1 mol%) appearing at 250°C and 120 min. The product yield started to level off once the system reached the super-critical methanol state due to more sediment at 290°C and 54.9 wt% of phosphorus compounds move to glycerin phase. Another phenomenon observed is that the presence of FFA enhanced the lipid conversion. However, the FFA enhancement became less significant when the system was operated for a longer period of time, which could be caused by a more completed reaction. Overall, experiment results proved phospholipids can be converted to FAME with a supercritical methanol without catalysts and be able to achieve a highest product yield of 93.9 mol% at 250°C for 120 min.

4.2 Introduction

Oleaginous microalgae are considered as a suitable resource for biodiesel production based on their ability of fast growing, high lipid productivity, low cultivation cost, and environmental friendliness. Microalgal lipids have similar a fatty acids profile to those of vegetable oils. However, microalgal lipids still have many differences in chemical composition from the conventional biodiesel feedstocks. The composition of microalgal lipids can be divided into four categories, i.e., saturated fatty acids, polyunsaturated fatty acids, free fatty acids and impurities (phospholipids and pigments).[1] The colorful appearance of algae is derived from three major classes of photosynthetic pigments, i.e., chlorophylls, carotenoids and phycobilins. Algal pigments are steroid lipids that cannot react readily with methanol via transesterification into methyl esters. Therefore, for algal biodiesel production, this portion of lipids is undesirable. In contrast to triglycerides, phospholipids are polar lipids and mainly exist in cell membranes. Most phospholipids contain a diglyceride, a phosphate group, and a simple organic molecule such as choline. Depending on the organic molecules, phospholipids can be classified as phosphatidyl choline ($-CH_2CH_2N(CH_3)_3$), phosphatidyl ethanolamine ($-CH_2CH_2NH_3$), phosphatidyl glycerol ($-CH_2CH(OH)CH_2OH$), phosphatidyl inositol ($-C_6H_{12}O_6$) and phosphatic acid (-H). The most commonly occurring phospholipids in eukaryotes are phosphatidylcholine, phosphatidyl-ethanolamine and phosphatidylinositol, where the relative amount of individual phospholipid type varies according to species and the imposition of environmental stress on the organism. In this study, phosphatidylcholine (i.e. lecithin) was selected as a model for phospholipid conversion as it is the most abundant of all phospholipids found in eukaryotes.[2] According to the ASTM standard (ASTM D6751:2012), phosphorus concentration in final biodiesel must be reduced to less than 10 ppm. For biodiesel production, a high content of phospholipids in the raw feedstocks means a concomitant loss of yield in FAME production, as the fatty acids enclosed into the phospholipid molecules are not accessible to the catalysts for transesterification.[3] Van Gerpen et al.[4] claimed that the phospholipids present in the oil were not carried over into the methyl esters, and the yield of FAME was reduced by 3-5 wt% if the phosphorus content in the oil was above 50 ppm. In the traditional techniques of biodiesel production from plant oils, phospholipids are commonly

eliminated before transesterification process through degumming, which is typically carried out by mixing the crude oil with an aqueous solution of phosphoric acid or sulfuric acid and chemically converting the phospholipids into hydratable compounds. Hot water alone can also extract a fraction of so called hydratable phospholipids. The phase separation after mixing the oil with hot water requires centrifugation. In many cases the initial cost of a centrifuge and its operation cost are too high for small producers. Therefore, people pay attention on how to produce biodiesel without degumming phospholipids.

Phospholipids still contain the valuable components of fatty acids which potentially are able to be converted to biodiesel. Successful conversion of phospholipids will increase the efficient use of the valuable feedstock and productivity. However, converting phospholipids possesses serious challenges. In order to convert phospholipids via transesterification, both biological and chemical catalysts can be used to accelerate the reaction efficiency. Enzymatic transesterification was frequently studied for phospholipids conversion due to its many characteristics.[3] Enzymatic reaction on un-degummed oil for biodiesel production allows mild reaction conditions and easy recovery of glycerol, without the need for further purification or chemical waste production. In addition, the enzymatic process tolerates a certain water content in oil and avoids the typical soap formation due to alkaline transesterification, thus increasing the biodiesel yield.[5] Different types of microbial phospholipases are able to hydrolyze phospholipids depending on the acyl ester bond.[6] The main phospholipase types are A1, A2, C, and D with their target sites different at a general representation of a phospholipid (figure 4-1), where the X residue may vary from hydrogen to more complex entities like choline, ethanolamine, serine, inositol etc. Each phospholipase type focuses on one particular site of bond that provides high selectivity on transesterification using various substrates. To date, high lipase activity and stability, with great conversion rates in short reaction times, have been reported.[7–9] In 2004, a research treated non-degummed soybean oil with *Novozym 435*, a type A phospholipase, to breach the barrier of phospholipid transesterification.[10] A FAME yield of 92 wt% was achieved when methyl acetate was used as the acyl acceptor with a molar ratio of methyl acetate to oil of 12:1 and methyl acetate showed no negative effect on enzymatic

activity. Another similar work achieved a 94 wt% of FAME yield by immersing lipase in crude oil (phospholipid content of 0.36 wt%, water content of 430 ppm, and acid value of 2) at 40°C for 120 h.[11] A similar FAME yield was obtained for refined oils. This research proved that immersing pretreatment of lipase in oils could improve both the reaction rate and methyl ester yield significantly. Watanabe et al. used a lipase-catalyze transesterification method (*Candida antarctica*) to convert crude soybean oil with 0.7 wt% FFAs, 0.08 wt% phospholipids and 0.12 wt% water.[7] A three-step methanolysis method was used by adding methanol three times at 30°C and a 94 wt% of oil conversion rate was achieved. Besides the study showed that the lipase could be reused for 25 cycles without any loss of the activity.

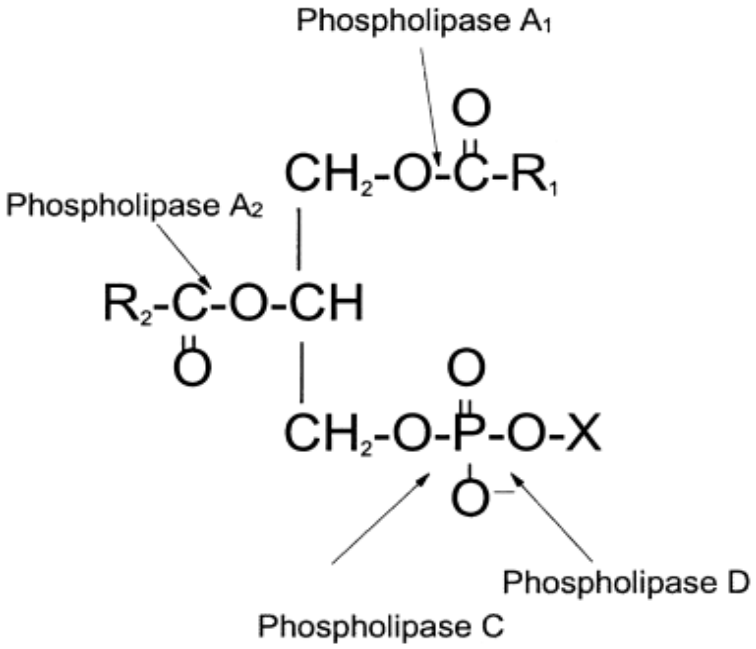

Figure 4-1. Depiction of phospholipase hydrolysis sites on a phospholipid of the various phospholipase types A1, A2, C and D, where X=H, choline, ethanolamine, inositol, etc.[6]

Other than enzymatic transesterification, acid/ base catalysts also work on oil sample with high phospholipid content to remove phospholipids from microalgal lipids.[12] The principle of acid/ base catalytic transesterification is also to hydrolyze phospholipid at acyl ester bond to produce FAME. Unfortunately, a high content of phospholipids in the

raw feedstocks will lead a concomitant loss of yield in FAME production, as the fatty acids enclosed into the phospholipid molecules are not accessible to the catalysts for transesterification.[3] Chen et al. used a two-step catalytic conversion (i.e., degumming and acid catalytic transesterification) to produce microalgal biodiesel with high phospholipid content.[13] They experimented on three types of microalgal oils (*Scenedesmus* sp., *Nannochloropsis* sp., and Dinoflagellate oil). All three oils were degummed by stirring with 1% phosphoric acid and 10 wt% water at 85˚C for 1h to remove most of phospholipids and non-lipid impurities then followed by acid catalytic transesterification. While *Scenedesmus* sp. oil with a highest FFA content of 15 wt% oil weight and phospholipids content of 37 wt% oil weight gave a highest FAME yield of 56 wt%. Moreover, the phosphorus concentrations in crude biodiesel from *Scenedesmus* sp. was as high as 296 ppm while the ASTM limits it to 10 ppm.[13] This study proved phospholipids could be converted to FAME under appropriate conditions; however, the incomplete reaction of phospholipids can result in loss of the product yield by as much as 45 wt% due to the phospholipid precipitation and saponification.[13] Furthermore, base catalysts (i.e. KOH, calcium methoxide, and calcium oxide) were also studied in order to successfully perform phospholipids transesterification. The work of Balasubramanian and Obbard[2] concluded that KOH, calcium methoxide and calcium oxide catalyzed transesterification of phospholipids and soybean oil mixture resulted a FAME yield in excess of 90 wt% at a reaction condition of 60˚C, 250 min with an oil to methanol molar ratio of 1:12 (catalyst concentration of 3 wt%). However, removal of catalysts from the FAME layer was challenging. The phosphorous content in FAME remained at a high level (0.081 wt% P/FAME).

EN 14214 and ASTM D6751 standards establish a maximum phosphorus content in final biodiesel at 10 ppm. Therefore, it is important to know the composition of each stream in the biodiesel process regarding to phosphorus content. Recently, a study of phosphorus balance through a biodiesel production process from non-degummed vegetable oil.[14] A non-degummed soybean oil and coconut oil containing FFA, triglycerides, and phospholipids were used as the feedstock in a base catalyzed transesterification process and was followed by an acid wash to acquire FAME. The mass balance of phosphorus compounds showed that only 1 wt% of the initial

phosphorus compounds remained in the biodiesel phase and 97 wt% of initial phosphorus stayed in the glycerin phase.

Although scientific research has proved that phospholipids can be eliminated or converted by pretreatment or catalytic transesterification, their presence increases the complications of final product purification and the cost of production. In addition, phospholipids are abundant component in some feedstocks, such as microalgal lipids, and have the chemical property that does not favor the transesterification reaction and thus causes phospholipids saponification.[5] However, under a critical thermal condition (SCM condition), the miscibility of lipids and alcohol is reinforced. Thus there is a potential that phospholipids are able to be converted to FAME. Besides, FFA is an organic acid that can be considered as an acid catalyst. The chemical polarity of methanol is enhanced, the chemical activity boosted and the acidity of FFA is increased, especially when methanol was heated up to a higher temperature (i.e. 250˚C). Therefore, the presence of FFA in the system of phospholipids and methanol for transesterification reaction could promote the overall reaction yield. Hence, the ultimate goal of this study was to explore the transesterification of phospholipids and the effects of processing conditions and presence of FFA in sub- and/or supercritical methanol for biodiesel production.

4.3 Experiments

4.3.1 Materials

L-α-lecithin (3-sn-Phoshatidylcholine) in powder form derived from egg yolk was purchased from Acros Organics (Geel, Belgium). Fatty acid profile of lecithin was determined and listed by the manufacture as approximately 33 wt% of palmitic acid (C16:0), 13 wt% of stearic acid (C18:0), 31wt% of oleic acid (C18: 1) and 15 wt % linoleic acid (C18: 2) which would give an average molecular weight of approximately 768 g/ mol. Stearic acid (95%), FFC (food chemical codex) grade, was obtained from Sigma-Aldrich (St. Louis, MO). Both lecithin and stearic acids were stored in a cold dry environment during the experiment period.

Transesterification reagent, methanol, was purchased from Macron Inc Chemicals (Center Valley, Pa.) in HPLC grade. High purity gas, carbon dioxide (99.9999%) was provided by Oxarc (Lewiston, ID).

4.3.2 Experimental procedures

All experiments were carried out in a batch reactor (Pressure Reactor 4560, 300 mL; Par Instrument Co., Moline, IL) controlled by a PC-based 4857 Reactor Controller on temperature and agitation speed. A computer connected to the controller displays the operating temperature, pressure, and agitation speed (constant at 500 rpm). The reactor system was hosted in a metal-framed chamber and vented out through a duct to ensure safe operation of the system.

Stearic acid was added in the reactant mixture of lecithin and methanol as a treatment. The quantity of stearic acid was chosen according to the free fatty acid content (16.6 wt% of total lipid basis) of microalgae *S. limacinu*m as used in previous studies. The phosphorus content of *S. limacinum* lipids was determined by ICP (Inductively Coupled Plasma) at Analytical Sciences Laboratory, University of Idaho (Moscow, ID). The phosphorus content of *S. limacinum* lipids was determined as 155 μg/g and equivalent to 0.38 wt% on the total lipids basis as the existed phospholipids are all lecithin. Phospholipid to methanol molar ratio (sRatio) was fixed as 1:75 and reacted at 210˚C, 250˚C and 290˚C for 30 min and 120 min, respectively. The product mixture from the phospholipid transesterification was in slurry form. Diluted FAME was separated from the solid residue by centrifuge at 3000 rpm for 15 min. FAME liquid product was collected and concentrated by evaporating excess methanol. Finally, concentrated FAME was analyzed by GC-MS to determine the product yield (FAME mol%) of total FAME. The effect of free fatty acid (stearic acid) was determined by comparing the product yields from experiments with/ without stearic acid involved. Mass balance of each experiments was kept to provide a thorough check on the investigation.

4.3.3 Analytical procedures

FAME content in the transesterified phospholipid product was analyzed by a gas chromatography-mass spectrometry (GC-MS) (PolarisQ instrument, ThermoFinnigan, West Palm Beach, Fla.) in the electron impact mode. A sample of the transesterified product was transferred into a 2 mL GC glass vial to which a solution of anthracene

(0.3-0.5 mg/mL) in dichloromethane (1 mL) was added as an internal standard. Gas separation was achieved by a ZB-1 (30 m x 0.25 mm dia. Phenomenex) capillary column with a helium carrier gas at 0.8 mL/min. The temperature was programed at 5°C/min from 40°C to 250°C while the injector and MS-transfer line were kept constant at 255°C. An Xcalibur software package (ThermoFinnigan, West Palm Beach, Fla.) was employed in data processing. Compound identification was performed by comparing the retention times of the components as identified by MS with the standards of fatty acids and methyl esters.

Molar yield of FAME was used to reflect the effects of various operation conditions and FFA on the phospholipid transesterification process. The total product yield (mol%) without FFA involvement was defined as the percentage of total phospholipids converted into FAME. The total product yield (mol%) is defined as (Eq. 1).

$$\text{Product yield (mol\%)} = \left[\frac{mol\ of\ total\ FAME\ in\ product}{2 \times mol\ of\ phospholipids\ in\ feedstock}\right] \times 100\% \qquad \text{(Eq. 1)}$$

When FFA involved in the reaction system, the product yield (mol%) of total FAME is defined as the percentage of total phospholipid converted into FAME while assuming all FFA were converted to FAME via esterification. Therefore, the formula used to calculate the product yield (mol%) of the phospholipid transesterification with FFA is as (Eq. 2)

$$\text{Product yield (mol\%)} = \left[\frac{mol\ of\ total\ FAME\ in\ product}{2 \times mol\ of\ phospholipids\ in\ feedstock + mol\ of\ FFA\ in\ feedstock}\right] \times 100\% \qquad \text{(Eq. 2)}$$

The quantity of phosphorus compounds was determined by spectroscopic analysis of Fourier Transform Infrared (FTIR). It was performed on an Avatar 370 spectrophotometer (Thermo Nicolet) with an attenuated total reflection (ATR) probe (ZnSe crystal). The spectra were ATR and baseline corrected using Omnic v9.0 software. Moreover, phosphorus content of mass balances in each stream of an integrated process includes esterification/transesterification, and glycerin separation. Both concentrated FAME products and glycerin residues were sent to Analytical Sciences Laboratory, University of Idaho (Moscow, ID) for phosphorous content determined by ICP.

4.4 Results and Discussion

4.4.1 Phospholipid transesterification without free fatty acids

Phospholipid transesterification was performed by reacting lecithin, the model chemical, and methanol at sRatio of 1:75 and under the operation conditions of 170˚C, 210 ˚C, 250 ˚C and 290 ˚C for 30 min and 120 min, respectively. The product yields (mol%) of total FAME under various operation conditions are compared to reveal the process effect on phospholipid transesterification (figure 4-2).

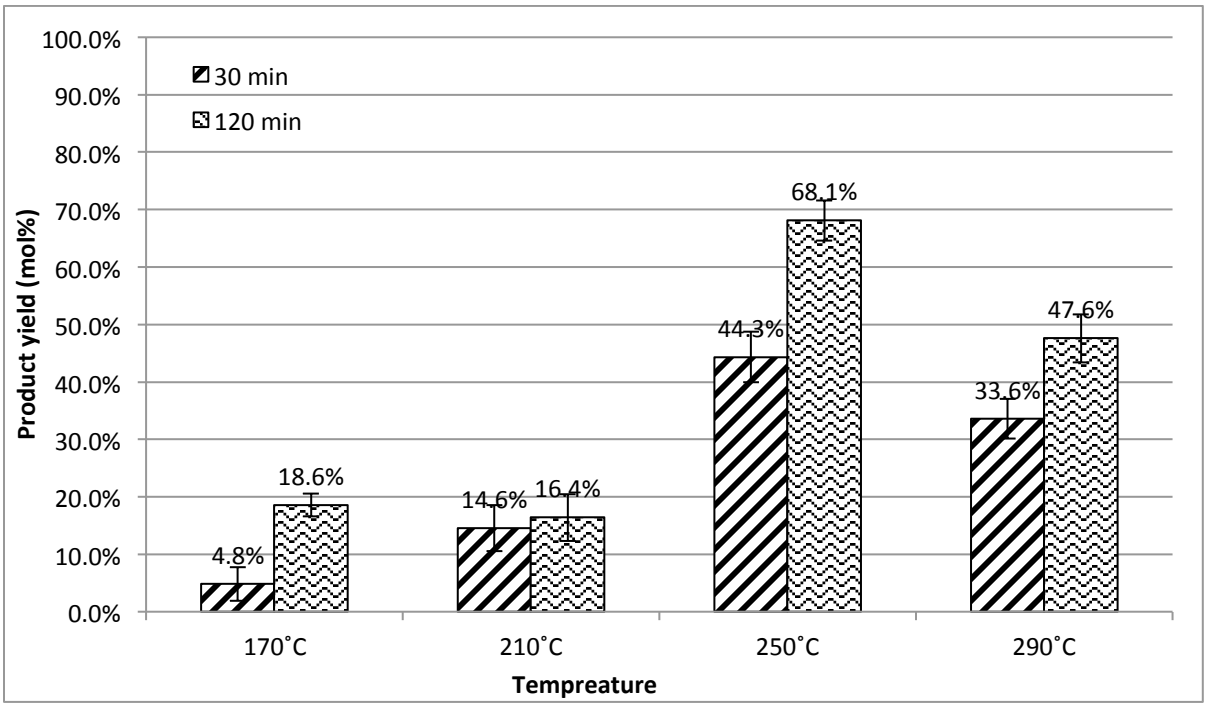

Figure 4-2. Product yield (FAME mol%) of phospholipid transesterification without FFA at sRatio of 1:75.

The experimental results presented in figure 4-2 were determined by analyzing the concentrated liquid product from FAME rich phase through GC-MS. All product yields in this graph represent the percentages of total phospholipid converted into FAME in this process. The FAME yield presents a trend of parabola curve with the highest product yield at 250˚C and 120 min, which indicates an optimal operation condition for phospholipid transesterification at 250°C. The effect of reaction time also presents a trend as expected that a longer reaction period leads to a higher product yield since the most significantly product yield elevation occurred at 250˚C. However, temperature

effect appeared an unusual behavior. A higher operation temperature (290°C) did not give a higher product yield rather a significant reduction.

The reaction mechanism of phospholipid transesterification is similar to that of triglyceride transesterification. But chemically they are slightly different. In Phospholipids, one phosphatidylcholine group combines with alcohol to forms amino alcohol instead of glycerin. For biodiesel production, a high content of phospholipids in the raw feedstocks means a concomitant loss of yield in FAME production, as the fatty acids enclosed into the phospholipid molecules are not accessible to the catalysts for transesterification.[3] However, at 250°C, methanol is at the state of supercritical fluid that possesses a decrease in dielectric constant and the two phase oil/methanol mixture becomes a single phase.[15,16] Therefore, a rational explanation of product yield spick at 250°C may be due to the change of methanol physical properties in nature that overcome the barrier of phospholipid molecules inaccessibility.

Another phenomenon related to the results, which was illustrated by previous studies, elaborated that high content of phospholipid in feedstock will cause saponification which will reduce FAME yield.[16] Therefore, the significant reduction in product yield at 290°C would indicate that the greater loss in productivity at a higher operation temperature may be caused by saponification so that a larger portion of phosphorus compounds precipitated in glycerin phase. In order to determine the quantity of phosphorus content in the residues, solid samples collected from glycerin phase were analyzed by ICP and the result supports greatly our previous speculation. In the original phospholipid feedstock, or lecithin, phosphorus content was determined as 40.4 mg/g equivalent to 1.01 g. per batch This value was used as the reference and compared to the phosphorus contents in solid residues from experiment implemented at 250°C and 290°C for 120 min. The concentrations of phosphorus compounds in the solid residues were 51 mg/g and 32 mg/g under the operation conditions of 250°C and 290°C, 120 min, respectively, which are equivalent to 40.6 wt% and 54.9 wt% of the original phosphorus compounds in the feedstock. These findings indicated that more phosphorus compounds moved to glycerin phase at 290°C. Correlated to the results in figure 4-3, the replacement of phosphatidylcholine group by carboxyl group from methanol might be inhibited at a higher temperature of 290˚C; herein phosphorous

compound exacerbated saponification reaction and caused the reduction of product yields.[4] A FTIR spectroscopic analysis reinsured the hypothesis that phosphorous compounds deposited in the transesterification residues (figure 4-3).

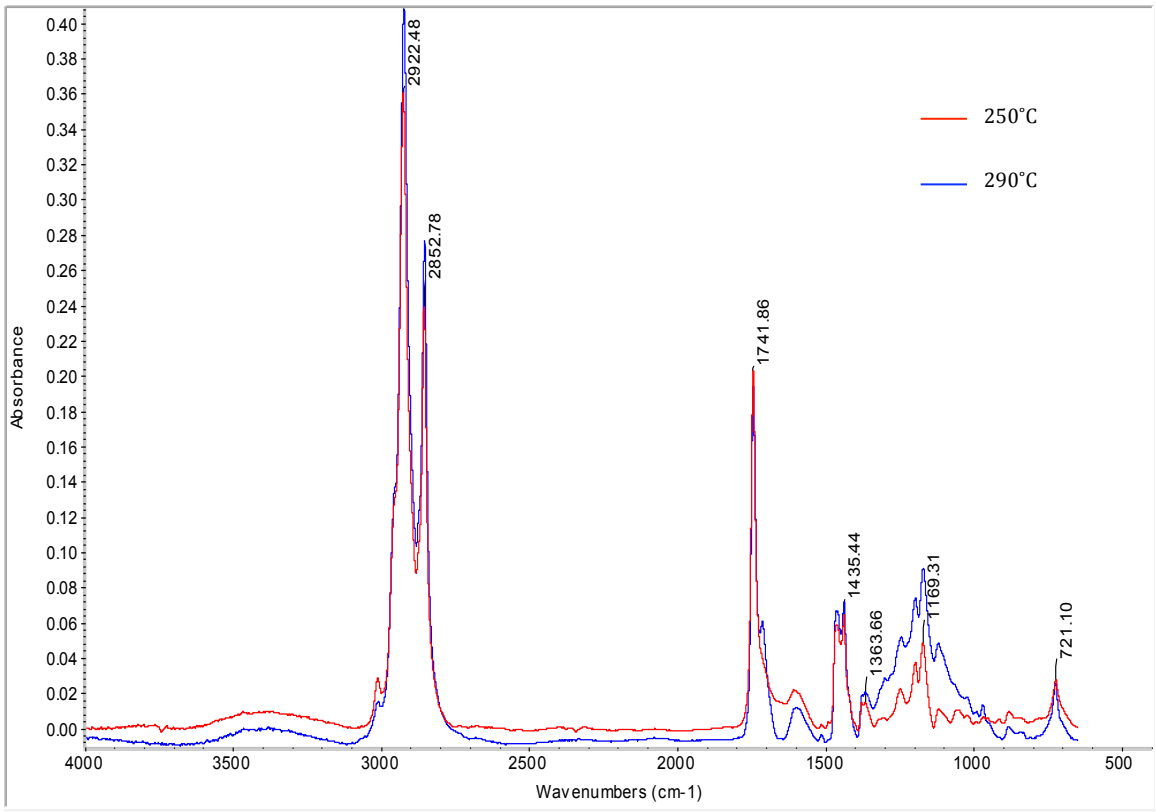

Figure 4-3. FTIR spectra of solid residues from phospholipid transesterification at 250˚C and 290˚C for 120 min.

FTIR spectra in figure 4-3 illustrate the chemical function groups in the residues after phospholipid transesterification under different operation temperatures. A significant increase in absorption at 1169 cm^{-1} belongs to the P=O stretching. On the other hand, P-OR ester band absorption (900-1050 cm^{-1}) did not show in both spectra which indicate that phosphate-choline group did not react through the transesterification to form esters. This phenomenon also proves that lecithin (phosphatidylcholine) is in favor of forming FAME via non-catalyzed transesterification at 250˚C.

4.4.2 Phospholipid transesterification with free fatty acid aid

Organic acids which are weak acids abundant in microalgal lipids, can act as a role of acid catalysts in the transesterification. Especially, when methanol was heated up to a higher temperature (i.e. 250˚C), its chemical polarity is enhanced, and its chemical

activity boosted. Therefore, one hypothesis is that methanol under supercritical condition is a more aggressive reagent that could vigorously react with lipids. Besides, esterification reaction has a higher reaction rate than transesterification reaction at the same temperature. Therefore, FFA is easier to react with methanol and form FAME and water. Water could further react on phospholipids and generate FFA through oil hydrolysis reaction. Therefore, FFA would continuously served in sub-/supercritical methanol transesterification as acid catalyst. In our previous study of *in situ* transesterification, microalgal lipids of *S. limacinum* contained 16.6 wt% of free fatty acids. To simulate a similar condition, 16.6 wt% of stearic acids was added in this study as a treatment in order to discover the effect of FFA in phospholipid transesterification. Experiments results are summarized in figure 4-4.

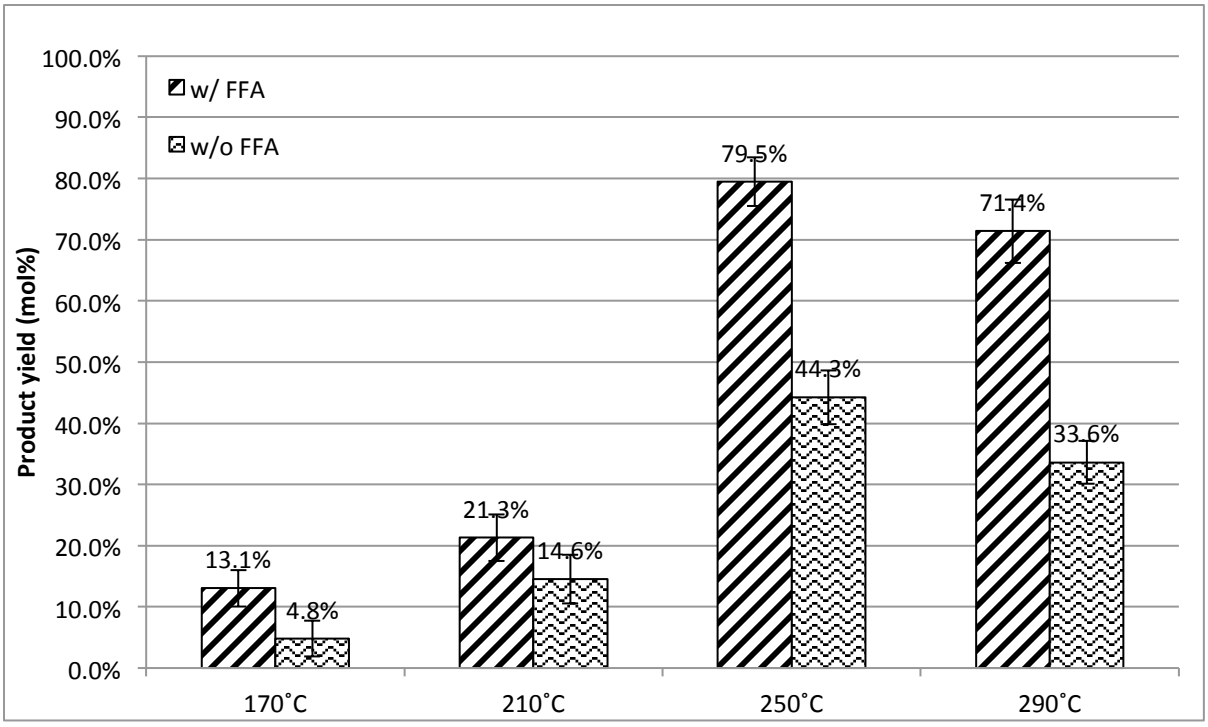

Figure 4-4. Product yield (FAME mol%) of phospholipid transesterification with/ without FFA at sRatio of 1:75 and reaction time of 30 min**.**

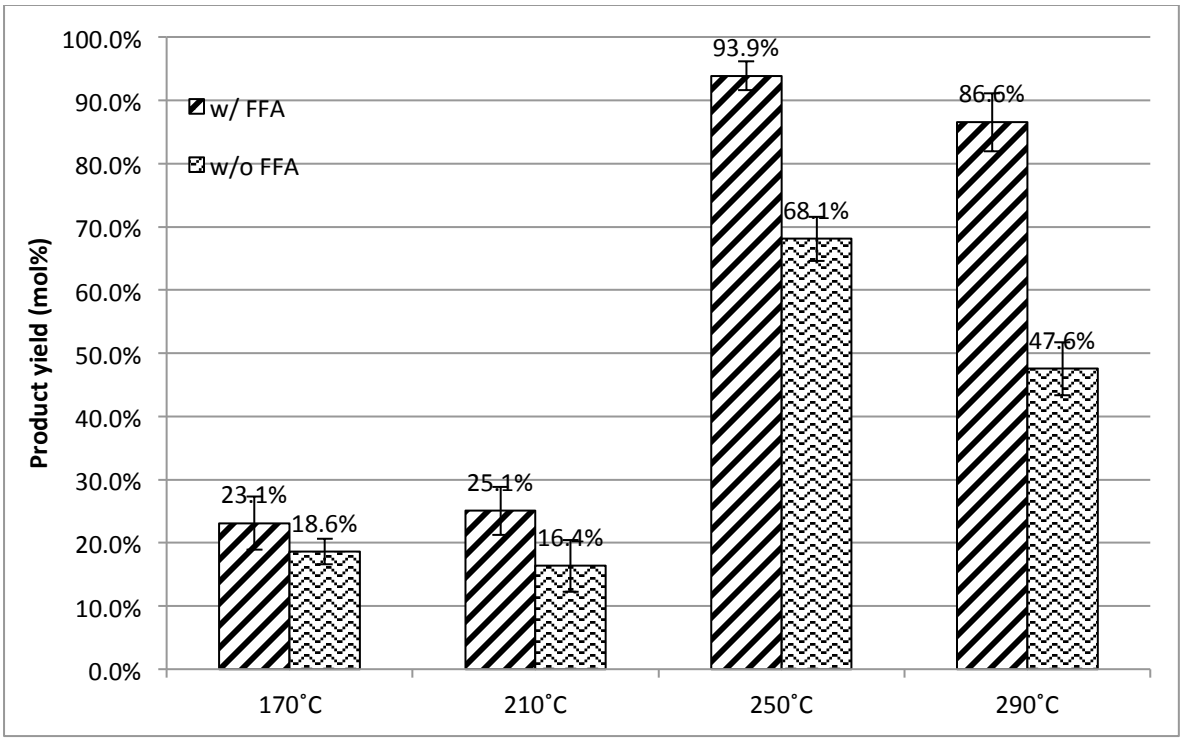

Figure 4-5. Product yield (FAME mol%) of phospholipid transesterification with/ without FFA at sRatio of 1:75 and reaction time of 120 min.

Both figure 4-4 and figure 4-5 show the same trend of product yields. A longer reaction time leads to higher product yields with or without free fatty acids. Free fatty acid has an impact on enhancing phospholipid transesterification at 290˚C or lower. This result indicates that phospholipid transesterification reaches its maximum efficiency at operation temperature of 250˚C in which acid catalyst (i.e. free fatty acid) does not boost up the reaction.

4.5 Conclusion

The experimental results show that phospholipids can be converted into esters through non-catalyzed transesterification. The preferred reaction condition for phospholipid transesterification with a sRatio of 1:75 is 250˚C and 120 min reaction time. Process efficiency was surprisingly declined when operation temperature rose up beyond 250˚C. A FTIR spectroscopic analysis showed that more phosphorous compounds ended up in the solid residues at 290˚C. On the other hand, free fatty acid has an effect on increasing the product yield to a maximum 93.9 wt% at 250˚C for 120 min. In conclusion, non-

catalyzed transesterification is able to convert phospholipids to FAME and the optimum result of total FAME yield (93.3 mol%) happened at 250˚C operation temperature for 120 min.

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Chapter 5. Effects of Free Fatty Acids and Water Content in Transesterification with Sub/ Super-critical Methanol

5.1 Abstract

Free fatty acids (FFA) and water are the two common interferences in conventional catalyzed transesterification process. Consequently, high quality or refined oil feedstocks need to be utilized to avoid side reactions phenomenon that would reduce the product yield. In situ transesterification was previously studied to directly convert microalgal lipids into fatty acid methyl esters (FAME) and developed successful achievement. Therefore, the process effectiveness of in situ transesterification on FFA content and water content need to further investigate individually. Three levels of FFA content were tested to figure out the FFA effect on sub-/ supercritical methanol transesterification. A higher product yield was given by feedstock contained higher FFA content and the highest product yield happened (60.3 mol%) at 290°C 30 min with 25 wt% FFA at an oil to methanol molar ratio of 1:75. Besides, supercritical methanol state was proved to be the critical factor of non-catalyzed transesterification with high FFA content. In the part of water effect on microalgae transesterification deliver an idea that product yield will decrease with water content increase which is consistently with other studies. The most significant reduction of product yield happened when water content added up from 1.15 wt% to 20 wt%, whereas production yield reduction difference became decelerated when water content exceed 20 wt%. This phenomenon also can be explained as that water content less than 20 wt% would give a significant impact on production yield.

5.2 Introduction

Along with the critical position of renewable energy has been revealed, biodiesel attracted a lot of attention for a valuable alternative to petroleum fuels due to its non-toxicity, biodegradability and low emission.[1–3] The conational biodiesel production process based on the use of alkaline catalyst, such as sodium hydroxide or sodium methoxide, for transesterification of triglyceride posed serious separation problem.[1] The present of water and free fatty acids (FFA) in the reaction system causes saponification[4], leads to lower yield of methyl ester[5] and increased difficulty in product separation.[6] Therefore, high quality oil feedstock requires a prior refinery in industrial

process. The refined feedstock requires a limited quality with less FFA content (≤ 3 wt %) and water content (≤ 0.06 wt %) for alkali catalyzed transesterification.[5,7]

High FFA content feedstock of biodiesel production is commonly found in animal fat, waste cooking oil and microalgae lipids. The FFA levels of vegetable oils are generally lower than 1% while the FFA level of the rendering plant feedstock is generally between 5% and 25%.[8] Most of high FFA feedstock was processed by two-step catalytic transesterification, first carrying out the acid catalyzed pre-esterification of the FFA prior the alkaline catalyzed triglyceride transesterification. The researchers have suggested that the FFA level of the feedstock should be reduce to less than 1% before used in alkaline catalyst transesterification.[9] Acid catalyzed transesterification alone also make successful conversion on high FFA feedstock such as microalgae. *Chlorella protothecoides* were used to have achieved a biodiesel yield higher than 70 % wt. in 5 h with sulfuric acid.[10] However, acid-catalyzed system is not always a good choice for commercial applications due to longer reaction time, higher reaction temperature, high molar ratio of alcohol to oil, and serious environmental and corrosion related problem.[11,12]

Water is another major factor interfering the transesterification reaction. During transesterification reaction, presence of water reduces the conversion of triglycerides to biodiesel fuel. Therefore, it is essential to minimize the water content in the feedstocks prior to transesterification process. In some works, the negative effects of water on transesterification reaction also present as triglycerides hydrolysis that increase the FFA content and cause saponification in alkaline catalyzed transesterification.[13] Canakci and Van Gerpen suggested that the presence of water higher than 1 % wt. during transesterification reaction will reduce the formation of FAME.[14] Microalgae, aquatic organisms, will catch-up enormous amount of water when directly harvested from cultural. It requires a great deal of energy to remove the water from the algal cells during the period of pretreatment. Moreover, water in biodiesel feedstcks can strongly affect catalytic transesterification. Water also has influence on alkali-catalyst transesterification by inhibiting its activity. The reaction requires high-quality feedstock with water content less than 0.06 wt%.[7] Thus alkali-catalyzed transesterification are greatly affected by the presence of water, which makes the

reaction partially change to saponification, leading to soaps formation. Hass noted that water inhibits transesterification reaction since it competes with the alcohol, thereby the major reaction shifted from ester transformation to hydrolysis into FFA.[15] The FFA formation favors saponification that inhibited the transesterification reaction. Therefore, high water content feedstocks require a prior refinery in industrial process. The refined feedstock requires a limited quality with less FFAs content (≤ 3 wt %) and water content (≤ 0.06 wt %) for alkali-catalyzed transesterification.[5,7]

Herein, the transesterification of triglycerides via supercritical fluid (SCF) has great strength comparing to catalysis transesterification on producing biodiesel. In this non-catalyzed process, only reactants (oil feedstock and alcohol) are added in the reaction mixture and heated to supercritical alcohol condition to produce biodiesel, which makes the process relatively simple and cost-effective by eliminating biodiesel purification and refinery processes. The primary obstacle of triglycerides transesterification is that they are immiscible fluids. During supercritical fluid condition, the solubility parameter of alcohol is reduced substantially to a value near to triglycerides, which form a homogeneous mixture, and leads to higher reaction rate in contrast to catalytic reaction due to the alcohol dielectric constant decreases at the supercritical state.[16–18] Besides, FFA is an organic acid that can be considered as an acid catalyst. Especially, when methanol was heated up to a higher temperature (i.e. 250˚C), the chemical polarity of methanol is enhanced, the chemical activity boosted and the acidity of FFA is increased. Therefore, the presence of FFA in the system of triglycerides and methanol for transesterification reaction could promote the overall reaction yield. In this study, the process effect of non-catalyzed transesterification via subcritical/ supercritical methanol on vegetable oil with various FFA content levels was investigated. When whole biomass used in non-catalyzed transesterification biodiesel production, other operations such as supercritical, ultrasonic, and microwave processes to improve process efficiency.

Previous study has proved FAME conversion can be implemented via transesterification with SubCM/ SCM from microalgal lipids. In current stage of research, the main purpose is to investigate the effects of FFA and water contents on transesterification process of biodiesel production from vegetable oil and wet microalgae biomass. Vegetable oil

feedstock (canola oil) was reacted with methanol to perform a transesterification reaction under different temperature that the effect of free fatty acids will be able to determine. Water content effect was examined by determine total FAME yield from *S. limacinum* microalgae biomass with various water contents through subcritical and supercritical methanol transesterification process. Both sub critical and supercritical methanol were used to transesterificate microalgal lipids into FAMEs. For FFA effect investigation, a relatively higher FAME yield would be expected in the system has FFA involved and under a higher temperature and reaction. On the other hand, total FAME yield from various water contents microalgae biomass should present decline trend while increase the water content in biomass. The FAME yield was tested and determined by GC-MS technique. All mathematical results were calculated and presented in a graphic form via Excel.

5.3 Experiments

5.3.1 Materials

5.3.1.1.1 FFA effect on transesterification from vegetable oil

Commercial canola oil was purchased in grocery store produced by Crisco® (Smucker Foods of Canada Crop. Markham, ON, Canada). Fatty acid profile was determined by GC-MS technique. GC-MS results indicated the fatty acid contents of this canola oil are 4.2 % of palmitic acid (C16:0), 2.0 % of stearic acid (C18:0), 64.6 % of oleic acid (C18: 1), 17.7 % of linoleic acid (C18: 2), 7.5 % of linolenic acid (C18: 3), and 1.3 % of eicosanoic acid (C20: 1) which give an approximately molecular weight of 876.6 g/ mole. 10 kg of stearic acid (95%), FFC (food chemical codex) grade was bought from Sigma-Aldrich (St. Louis, Mo) in powder form and both of lecithin and stearic acids were storage in a cold dry environment.

5.3.1.1.2 Effect of water content on microalgae *in situ* transesterification

A lipid-rich green microalga *Schizochytrium limacinum* (*S. limacinum*) was obtained from ENN Energy Service Co., Ltd (Langfang, China) as a dry powder biomass with 55 wt% of lipid content, and used in all *in situ* transesterification experiments in this study. The microalgae biomass was manually ground in mortar to a relative uniformity in particle size (10-20 μm) beforehand. Characterization was performed on the *S.*

limacinum biomass, including proximate analysis, ultimate analysis and fatty acid composition, before the experiments. Fatty acid profile of the lipids was analyzed by gas chromatography-mass chromatography (GC-MS) as described by Hammond.24 It was determined that the microalgal biomass contains 1.6 wt % of moisture, 78.5 wt% of volatile matters, 8.9 wt% of fixed carbon and 11.0 wt% of ash content on dry basis. Elementally, the *S. lmacinum* biomass consists of 61.4 wt% carbon, 8.9 wt% of hydrogen, 19.5 wt% of oxygen, 4.0 wt% of nitrogen, and 0.6 wt% of sulfur on dry basis. The gross heating value was calculated as 29.7 MJ/kg, based on the formula proposed by Channiwala and Parikh. 25 Moreover, the FA profile of the *S. limacinum* lipids, as determined via GC-MS after solvent extraction (i.e., hexane, methanol, and chloroform/ methanol separately), was 1.5 wt% of myristic acid (C14:0), 22.3 wt% of palmitic acid (C16:0), 0.7 wt% of stearic acid (C18:0), 3.0 wt% of oleic acid (C18:1), 7.2 wt% of linoleic acid (C18:2), 1.0 wt% of linolenic acid (C18:3), 3.6 wt% of clupandonic acid (C22:5), and 15.1 wt% of docosahexaenoic acid (C22:6) on dry basis. Furthermore, the analysis, according to AOCS official method Ca 5a-40 26, indicated that the extracted microalgal lipids contained 16.6 wt% of FFA.

All chemicals used in this experiment are HPLC grade. Methanol was purchased from Macron ine Chemicals (Center Valley, Pa.). High purity gases, carbon dioxide (99.9999%) was used for the reaction and was provided by Oxarc (Lewiston, Id). De-ionized water used in the experiment of water effect in microalgae biomass on transesterification.

5.3.2 Experiment procedures

All experiments were carried out in a batch reactor (Pressure Reactor 4560, 300 mL; Par Instrument Co., Moline, Ill.) controlled by a PC-based 4857 Reactor Controller on temperature and agitation speed. A computer connected to the controller displays the operating temperature, pressure, and agitation speed (constant at 500 rpm). The reactor system was hosted in a metal-framed chamber and vented out through a duct to ensure safe operation of the system.

5.3.2.1.1 FFA effect on transesterification from vegetable oil

Stearic acid (C18:0) was added in the reactant mixture of canola oil and methanol as a treatment. The quantity of stearic acid was chose according to the free fatty acid

content of microalgae *S. limacinu*m which is 16.6 wt% of the whole biomass weight. Therefore, stearic acid was added as 16.6 wt% of canola oil weight. Besides, two other FFA content levels (5 wt% and 25 wt%) were used to further determine the FFA effect in this non-catalyzed transesterification process with sub-/ supercritical methanol. Canola oil to methanol molar ratio (sRatio) were fixed at 1:75 and reacted at temperature 170˚C, 210˚C, 250˚C and 290˚C for 30 min respectively. The effect of free fatty acid (stearic acid) was determined by comparing the product yields (FAME mol%) from experiments with/ without stearic acid involved.

5.3.2.1.2 Effect of water content in microalgae *in situ* transesterification

To investigate the effect of water content, dry microalgal biomass (moisture content = 1.15 wt%)has been mixed with de-ionized water for overnight at various water content (wt%). Four levels of water content in microalgal biomass were used in this investigation including the original water content in microalgal biomass. They are 1.15 wt%, 20 wt% 50 wt%, and 80 wt% on dry mass basis. The wet biomass was processed by *in situ* transesterification under both sub- and supercritical methanol conditions. The sRatio was set at the ratio (1:75) since previous experimental results indicated no significant product yield changes after sRatio beyond 1:75. Moreover, operation temperature and reaction time were both key parameters that could interactively effect on product yield. Therefore, the investigation of water content on microalgae *in situ* transesterification was operated at 210˚C 120 min, 250˚C 30 min, and 290˚C for 30 min respectively.

5.3.3 Analytical method

The FAME was content in the phospholipid transesterified product and analyzed by a gas chromatography-mass spectrometry (GC-MS) (PolarisQ instrument, ThermoFinnigan, West Palm Beach, Fla.) in the electron impact mode. A sample of the transesterified product was transferred into a 2 mL GC glass vial to which a solution of anthracene (0.3-0.5 mg/mL) in dichloromethane (1 mL) was added as an internal standard. Gas separation was achieved by a ZB-1 (30 m x 0.25 mm dia. Phenomenex) capillary column with a helium carrier gas at 0.8 mL/min. The temperature was programed at 5°C/min from 40°C to 250°C while the injector and MS-transfer line were

kept at 255°C. An Xcalibur software package (ThermoFinnigan, West Palm Beach, Fla.) was employed in data processing. Compound identification was performed by comparing the retention times of the MS components with the standards of fatty acids and methyl esters.

Molar yield of FAME was used to reflect the effects of various operation conditions and FFA on the non-catalyzed transesterification process. The total product yield (mol%) was defined as the percentage of total phospholipid converted into FAME. All data from GC-MS were collected and calculated by Excel and the total product yield (mol%) was calculated as following equation (Eq.1).

$$\text{Product yield (mol\%)} = \left[\frac{mol\ of\ total\ FAME\ in\ product}{3\times mol\ of\ triglycerides\ in\ feedstock + mol\ of\ free\ fatty\ acids\ in\ feedstock}\right] \times 100\% \quad \text{(Eq. 1)}$$

5.4 Results and discussion

5.4.1 Investigation of FFA effect on transesterification from canola oil with sub/ super critical methanol

Non-catalyzed transesterification on canola oil with sub/ super critical methanol was performed to obtain a base line of process efficiency. Canola oil and methanol were reacted at sRatio of 1:75 under the operation temperature of 170˚C, 210 ˚C, 250 ˚C and 290 ˚C for 30 min respectively via transesterification reaction. Numbers listed in figure 5-1 represent the product yield (mol%) of total FAME at each reaction condition. Both temperature and reaction time are distinguished as the key operation parameters of transesterification reaction. According to previous study, FAME has been converted through transesterification by the reaction time of 30 min and the conversion efficiency got boost up by temperature elevation. The results appear an increase of product yield along with a temperature ascent. Conventional transesterification for biodiesel production from vegetable oil can achieve approximate 98 % product yield from a process at 60˚C through 2 h reaction time with alkaline catalysts assistant.[2,11,19] The best product yield for the non-catalyzed transesterification was obtained as 40.8 mol% at 250˚C, 30 min with 16.6 wt% of FFA added (figure 5-1). In order to discover the effect of FFA on transesterification reaction, stearic acids (16.6 wt% of canola oil content) were added with canola oil reacted with methanol at sRatio of 1:75 for various

operation conditions. The weight proportion of stearic acid was decided according to microalgale, *S. limacinu*m, FFA content of total lipids. FFA was used as a treatment in transesterification reaction, hence FFA effect on transesterification can be concluded by comparing and contrasting product yield of experiments with/ without FFA. According to the product yields listed in figure 5-1, FFA presents positive effect on transesterification conversion. The highest product yield (53.8 mol%) was given by non-catalyzed transesterification with FFA at 250°C. Product yield increased at each operation condition while FFA added in the oil feedstock. However, the increment speed of product yield at adding FFA got sudden rising at 250°C and 290°C. This huge growth of product yield happened when methanol became supercritical fluid. Thus indicate the physical state of methanol fluid is more critical than operation temperature and time. This phenomenon proved our hypothesis stated previously that organic acid can react as acid catalysts in transesterification with subcritical and supercritical methanol and performed a higher efficiency on FAME production. Besides, the increasing of product yield of total FAME at an elevated temperature has slowed down while operation temperature exceeded 250˚C, which revealed a fact that the effectiveness of operation temperature reaches maximum level at 250˚C. On the other hand, this phenomenon also indicates physical state of methanol fluid is more significantly affecting factor than operation temperature.

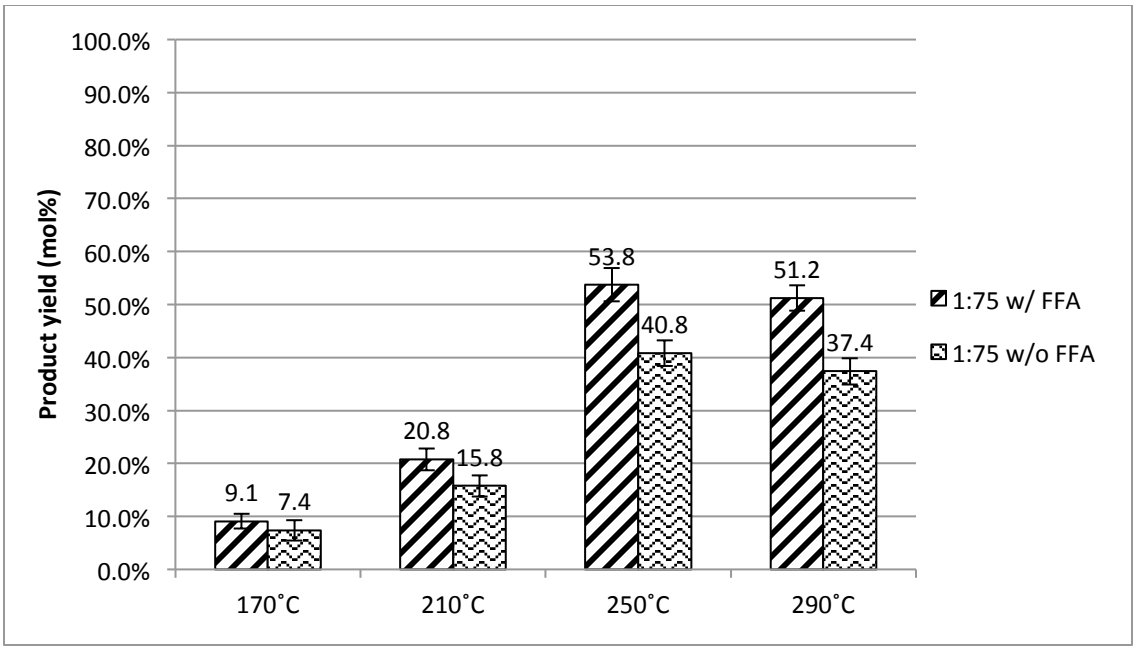

Figure 5-1. Product yield (FAME mol%) of transesterification from canola oil with/ without FFA with sRatio of 1:75 at operation temperature of 170˚C, 210˚C, 250˚C and 290˚C and reaction time of 30 min respectively.

Acid catalyst commonly involved in transesterification process for a high FFA content feedstock to eliminate saponification reaction due to a prior esterification reaction. Stearic acid is organic acid that presents weak acidity in the ambient environment. However, its acidity increases under supercritical fluid state due to the elongation of acid hydrogen bond that put hydrogen proton in a more radical state and behavior as a Lewis acid. Lewis acid catalysts were proved to be active for both esterification and transesterification reaction, but the reaction is very slow due to the limits of mass-transfer between methanol and oil phase in a conventional process. Herein, supercritical methanol would help resolve the problem of mass-transfer barrier between methanol and oil phase in the ambient atmosphere. Besides, FFA will react with methanol through esterification in a relatively faster rate than transesterification and form FAME and water; and then water will continuously hydrolyze oil into fatty acids. There has been literatures concluded that fatty acid has an important role as acid catalyst in hydrolysis reaction of oil will leads results of higher yield of FAME.[20–22] In order to further investigate the effect of FFA content in this non-catalyzed transesterification process, two other FFA content levels (5 wt% and 25 wt%) were

introduced in this study. In figure 5-2, the product yields of canola non-catalyzed transesterification with three different levels of FFA content were compared and contrasted. A consistent increment of product yield presented in each operation condition while increases the FFA content in oil feedstock. This result have been found out consistently with previous study conducted by Tan et al. that a steady increase in yield with the increment of FFA content in reaction mixture.[21] Moreover, significant rises of product yield also occurred when operation temperature reached the state of supercritical methanol that also proves the idea that physical state of methanol is a more critical requirement for a non-catalyzed transesterification with high FFA content feedstocks.

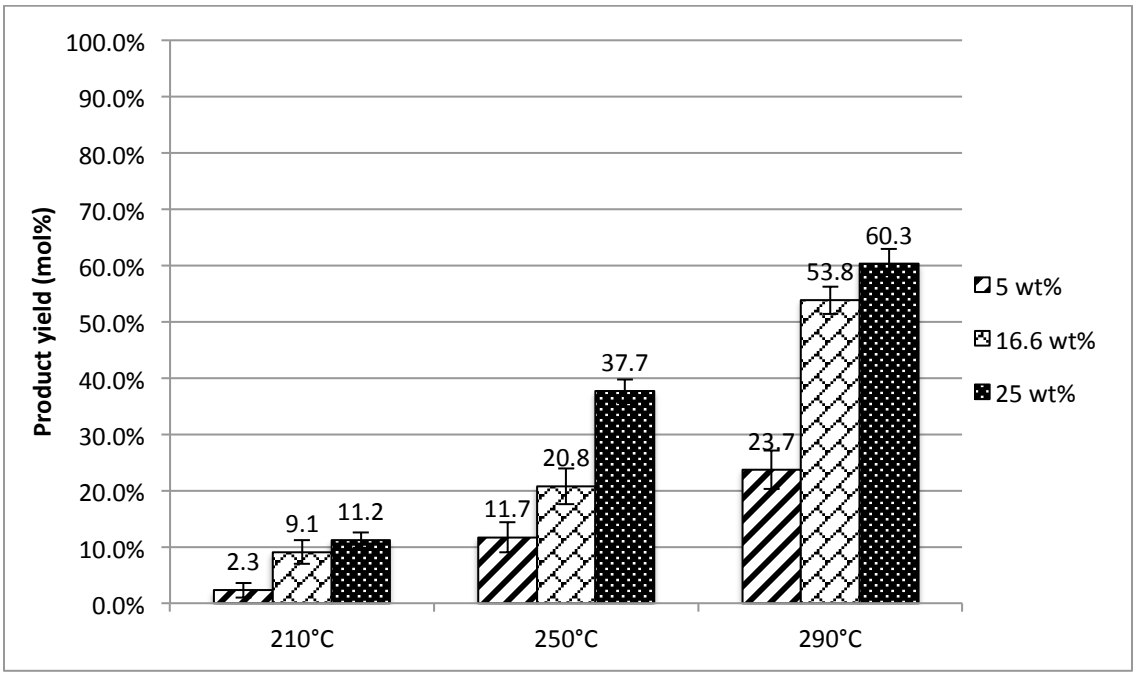

Figure 5-2. Product yield (FAME mol%) of transesterification from canola oil with FFA at level of 5 wt%, 16.6 wt% and 25 wt% at sRatio of 1:75 at operation temperature of, 210˚C, 250˚C and 290˚C and 30 min respectively.

5.4.2 Investigation of water content effect on in situ transesterification from microalgae with sub/ super critical methanol

Water content of the feedstock for biodiesel production is suggested to be kept below 0.06% in order to prevent deterioration of the catalyst, which adversely affects the transesterification.[23] Naturally, microalgae biomass directly harvested from medium contains nearly 80% of water which is not suitable for biodiesel production via

conventional transesterification. Therefore, numbers of researches focused on convert microalgal lipids into biodiesel without drying process.[24] In a review written by Park et al., recent studies of direct transesterification from wet microalgae were summarized. In this paper, the highest water content microalga has been studied was *Nannochloropsis* sp. with 90% of water content and achieved 85.8% of biodiesel conversion yield by supercritical methanol transesterification.[25] Moreover, another similar study illustrated a direct transesterification with supercritical ethanol on *Chlorella vulgaris* (80% water content) and obtained 100% biodiesel conversion yield.[25] Previous study has developed a direct process procedure of microalgal biodiesel production via transesterification and the water content of feedstock is 1.15 wt%. In this section, microalgae, *S. limacinum,* were mixed with de-ionized water at various levels and then reacted with methanol to perform a biodiesel production through *in situ* transesterification. Wet microalgae with water content of 20 wt%, 50 wt% and 80 wt% reacted with methanol at operation conditions of 210˚C for 120 min, 250˚C and 290˚C for 30min respectively. Product yields of total FAME from experiment of each water content at every operation condition are presented in a series of curves (figure 5-3).

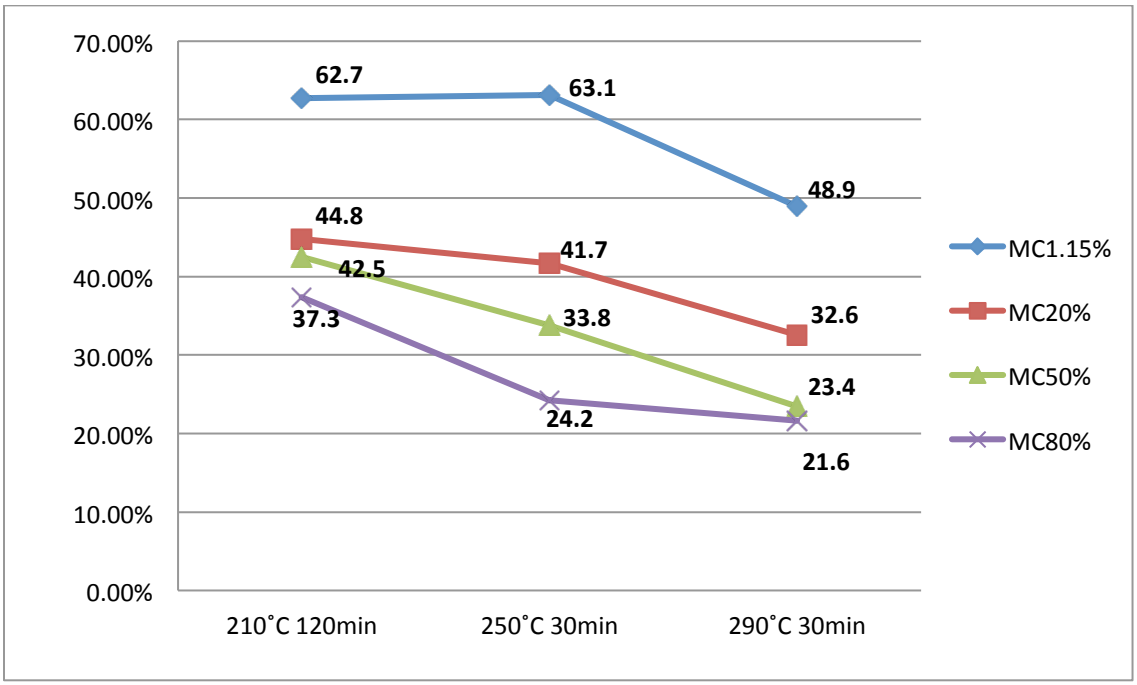

Figure 5-3 Product yield (FAME mol%) of *in situ* transesterification from wet microalgae with water content of 1.15 wt%, 20 wt%, 50 wt% and 80 wt% at sRatio of

1:75 at operation temperature of 210˚C 120 min, 250˚C 30 min and 290˚C 30 min respectively.

First of all, a lower product yield of total FAME occurred along with increasing water content in the feedstock at each operation condition. Besides, higher operation temperature did not help on preventing product yield drop. Whereas, the reduce of product yield got slow down at 290˚C 30 min when water content increased from 50% to 80% which indicate the effect of water content got resisted by a higher operation temperature (290˚C). Furthermore, the significant drop of product yield causing by water content was mainly happened from MC 1.15 wt% to MC 20 wt%. There is no obvious change of product yield at 210˚C 120 min when water content beyond 20 wt%. Operation condition of 210˚C 120 min on *in situ* transesterification has been proved to be the optimal reaction condition for biodiesel production from microalgae *S. limacinum*. However, the significant reduction of product yield happened when water content exceed 20 wt% with both subcritical and supercritical methanol. Within a higher operation temperature (250˚C), the reduction of production yield got elevated for feedstock with water content of 20 wt% , 50 wt% and 80 wt%. As the numbers shown in figure 5-3, the lowest product yield occurred at 290˚C 30 min reaction time with water content of 80 wt%. In contrast, the highest production yield remained at operation condition of 210˚C 120min with water content of 1.15 wt%.

5.5 Conclusion

Previous studies presented an overall picture of process efficiency of *in situ* transesterification on microalgae *S. limacinum*. In this paper, a more detail investigation has conducted on two potential factors in order to further understand the process effectiveness of *in situ* transesterification. Free fatty acid and water content have been selected to be the effective factors. In the part of FFA effect, the results indicated an impact of FFA increment could lead to a product yield increase in both subcritical and supercritical methanol transesterification which proved the hypothesis of organic acid reacted as acid catalysts in transesterification reaction. The sudden jump of product yield at operation condition of 250˚C 30 min supported the skeptical of the acid level of organic acid would enhanced by the condition of supercritical methanol and the

character of microalgal lipid processes high content of FFA would increase transesterification efficiency.

In the second part of water effect on microalgae transesterification deliver an idea that product yield will decrease with water content increase which is consistently with other studies. The most significant reduction of product yield happened when water content added up from 1.15 wt% to 20 wt%, whereas production yield reduction difference became decelerated when water content exceed 20 wt%. This phenomenon also can be explained as that water content less than 20 wt% would give a significant impact on production yield.

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Chapter 6. Conclusion

Microalgae process, even after being harvested, requires extensive research and development before the technology can be efficiently and economically applied for biofuels production. In the past few years, researchers in this area have done tremendous work and vast information has been published. Conventionally, algal biodiesel is produced from wet algal biomass in a series of steps including preparation of dry algae powder, extraction of algal oils with chemical solvents, and conversion of the algal oil to biodiesel with a catalytic transesterification. However, drying the biomass and extraction of algal oils by conventional methods is both energy- and cost-intensive. The *in situ* transesterification method developed in this study is a catalyst free process and showed positive effect on microalgal biodiesel production via single step. In this chapter, research findings are concluded individually.

The experimental results showed that the direct conversion of algal lipids of microalga *Schizochytrium limacinum* can be achieved in one step. The sub-/ supercritical *in situ* transesterification without catalyst application has significant results with respecting to product yield and selectivity. The actual effects on product yield are collectively contributed by the operating temperature, which determines the fluid status of methanol, and sRatio. The effect of reaction time was determined not significant but affected the system by its interaction with operating temperature. Another important response factor is the selectivity of the targeted microalgal methyl esters or FAME. Experimental data on product selectivities lead to similar conclusions as those observed on product yield. Generally, the product selectivity increases as the operating temperature increases in the range of 170-250˚C. A clear trend was noticed that when the operating temperature was at 290˚C, the selectivities were generally lower than those at lower temperatures. The FTIR spectroscopy analysis proved the deduction of the statistical analysis and also gave an idea that the formed FAME may decompose and reacte with other components at higher temperature (i.e., 250˚C and 290˚C) and longer reaction time (i.e., 90 and 120 min). Overall, an optimum operation condition for *in situ* transesterification was determined at 210˚C, 120 min with sRatio 1:75 and the highest product yield was 64.5 mo% and product selectivity of 33.1 wt%. The advantage of such

a proposed one-step, *in situ* transesterification without addition of catalysts is to simplify the microalgae processing process and overcome the technological challenges in traditional processes thus to enhance the processing efficiency for viable algal biodiesel production.

Microalgal lipids are composed of triglycerides, free fatty acids, phospholipids and possible water. Free fatty acids, phospholipids and water are considered as undesired compounds and cause problems in the conventional transesterification process. Therefore, the study was further moved on to individual investigation on fatty acids effect, phospholipids effect and water effect on *in situ* transesterification process. In the experimental of phospholipids effect on *in situ* transesterification, results show that phospholipids can be converted into esters through non-catalyzed transesterification. The preferred reaction condition for phospholipid transesterification with a sRatio of 1:75 is 250˚C and 120 min reaction time. Process efficiency was surprisingly declined when operation temperature rose up beyond 250˚C. A FTIR spectroscopic analysis showed that more phosphorous compounds ended up in the solid residues at 290˚C. On the other hand, free fatty acid has an effect on increasing the product yield to a maximum 93.9 wt% at 250˚C for 120 min. In conclusion, non-catalyzed transesterification is able to convert phospholipids to FAME and the optimum result of total FAME yield (93.3 mol%) happened at 250˚C operation temperature for 120 min. In the chapter of free fatty acids and water content effect, a more detail investigation has conducted in order to further understand the process effectiveness of *in situ* transesterification. In the part of FFA effect, the results indicated an impact of FFA increment could lead to a product yield increase in both subcritical and supercritical methanol transesterification which proved the hypothesis of organic acid reacted as acid catalysts in transesterification reaction. The sudden jump of product yield at operation condition of 250˚C 30 min supported the skeptical of the acid level of organic acid would enhanced by the condition of supercritical methanol and the character of microalgal lipid processes high content of FFA would increase transesterification efficiency. Besides, a higher FFA content intended to lead with a higher conversion yield, which also proved the idea of FFA playing a role of acid catalyst. The highest

product yield obtained with FFA aided was 60.3 mol% for a 25 wt% FFA content in a sRatio of 1:75 at 290˚C 30 min.

In the part of water effect on microalgae *in situ* transesterification, experiment results delivered an idea that product yield decrease with water content increase. The most significant reduction of product yield happened when water content added up from 1.15 wt% to 20 wt%, whereas production yield reduction difference became decelerated when water content exceed 20 wt%. According to previous works on water effect on transesterification by other researchers, water can work on triglycerides through hydrolysis reaction and form FFA under subcritical fluid condition. And this behavior can lead to a higher conversion rate due to the rapid reaction of esterification. However, our results proved water does not promote reaction in this system when the content exceed 20 wt%. This phenomenon also can be explained as that water can also reacts on lingocellulosic matters and lost the effect of oil hydroxylation.