Biodiesel Production from Algae through *In Situ* Transesterification Technology

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Dedication

This dissertation is dedicated to my parents Yanhe Ma and Jing Liu.

Abstract

Biodiesel, a promising renewable biofuels, is receiving increased attentions. Due to the high price of vegetable oils and the land use competition of biodiesel feedstock production and food production, it is necessary to find other ways to lower the biodiesel production cost and reduce the pressure on food and feed supplies. One possibility to overcome these problems is to produce biodiesel from microalgae feedstock using advanced conversion process. The conventional biodiesel production involves a two-step process in which oil is first extracted from oil feedstock and then subjected to transesterification step. Unfortunately, it is hard to extract oil from algae, making algae based biodiesel production very costly. In this thesis project, an innovative in situ direct transesterification method was investigated. In situ direct transesterification method combines the oil extraction and transesterification process into one step. In this project, microalgae (*Chlorella Vulgaris*) were used as the feedstock and several factors affecting the final lipid conversion rate were tested and optimized. At room temperature, the best conditions for the in situ transesterification process are: concentration of catalyst (KOH), 2% of the lipid content, reaction time, 10 h, and the methanol amount, 16.4 ml. At temperatures above 45 ℃, the optimal reaction time was 4 h. It was found that 60 ℃ was better than 45 and 75 ℃. Almost all the pretreatments tested were able to improve lipid conversion rate. The best pretreatment was the combination of methanol soaking and microwave irradiation, which increased the rate of conversion by 14.8%. The two-step traditional transesterification method was also tested for comparison purpose. The result suggested that in situ direct transesterification produced higher lipid conversion rate than

the conventional transesterification process, and could be an alternative, efficient and economical process for algal biodiesel production.

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Chapter 1 Introduction and literature review

1.1 Overview of energy

Currently, the three primary sources of global energy available are petroleum, coal, and natural gas. These non-renewable sources supply 90% of the world's energy needs (Dale 2008). Among them, petroleum oil supplies 40% of the energy needs (Sivakumar, Vail et al. 2010). Fossil fuels are formed from the geologic transformation of buried organic materials (i.e., dead plants and animals) over millions of years and are non-renewable. The continuously growing human population and the increase in industry and transportation lead to increasing energy demands all over the world. The consumption of fossil fuel is 10^5 times faster than nature can generate according to recent report (Satyanarayana, Mariano et al. 2011). Fossil fuels are about to reach their peak production and can be predicted to be exhausted in the future due to their limited and non-renewable nature. Therefore, the current use of fossil fuels is widely recognized to be unsustainable.

Another major concern with using fossil fuels is carbon dioxide (CO_2) emission. Since the beginning of the Industrial Revolution, the burning of fossil fuels has been contributing to the increasing atmospheric CO_2 concentration. Many scientists believe that fossil fuel derived CO_2 is one of the major factors for greenhouse effect, which increases temperatures of global temperature. According to the data from U.S. Energy Information Administration, over 80% of the greenhouse gas emissions come from energy-related CO_2 emissions in which petroleum is the largest fossil fuel source for

energy-related CO_2 emissions, contributing 42% of the total (U.S. Energy Information Administration. 2011).

Additionally, the high petroleum oil price is a big challenge for the 21st century, especially after the oil crisis in 1973 and then the Gulf War in 1991. Even though the price has fallen recently, petroleum fuel price will undoubtedly rise again because of the increasing energy consumption and limited availability. Worldwide concerns resulted in the search for new energy resources which would be renewable and sustainable. Bio-mass source fuel, or bio-fuels, may offer a promising alternative which have attracted increasing attention as evidenced by the growing research and development efforts found in the literature (Solomon 2010; Durrett, Benning et al. 2008).

The advantages of using bio-fuels can be summarized as three aspects: environment, energy security, and economy (Demirbas 2009). Bio-fuels have an environmentally friendly potential. Using bio-fuel could generate much less air pollution, carbon dioxide and greenhouse gas emissions compared to petroleum oil usage. Also biofuels are biodegradable, less or non-toxic. Bio-fuels can be converted from common biomass sources which contributes to sustainability and the reduction of fossil fuel consumption. For the economic aspect, the development and use of bio-fuel may help to create jobs related to bio-fuel generating industry and reduce the dependency on imported petroleum (Narasimharao, Lee et al. 2007).

Bio-fuels are produced from natural (biomass) materials, such as agricultural and forestry byproducts, grain and oil crops, used cooking fats or waste oils, animal fats, and microalgae. The term bio-fuel is referred to as solid (bio-coal), liquid (bioethanol,

vegetable oil, and biodiesel), or gaseous (biogas, biosyngas, and biohydrogen) fuel that is produced predominantly from biomass (Demirbas 2010). Liquid bio-fuels, like bioethanol and biodiesel, may offer a promising alternative because of similar characteristic as of the petroleum fuel (Antolín, Tinaut et al. 2002). It can be used directly as fuel although it may require some engine modifications, or blended with petroleum diesel and used in diesel engines with few or no modifications.

Biodiesel and bioethanol are the two potential renewable fuels that have attracted the most attentions. Despite the fact that they reduce both the consumption of crude oil and environmental pollution, they still have some disadvantages. Most traditional biofuels, such as ethanol from corn, wheat, or sugar beets, and biodiesel from oil seeds, are produced from classic agricultural food crops that require high-quality agricultural land for growth. The cost of biofuel production is still very high. Besides, bioethanol has additional complications, like lower energy density than gasoline, corrosiveness, low flame luminosity, lower vapor pressure, miscibility with water, and toxicity to ecosystems (Chisti 2008). Biodiesel from other feedstocks, like microalgae, appear to be the best option that has the potential to completely displace petroleum-derived transport fuels without adversely affecting supply of food and other crop products, since they do not require arable farm land for production.

1.2 Introduction to biodiesel

Chemically, biodiesel is a mixture of fatty acids methyl esters (FAMEs) produced from a reaction of triacylglycerols or TAGs (e.g., vegetable oils, animal fats, waste

cooking oil, algae lipids, or other fatty acids) with alcohols, termed transesterification in the presence of a catalyst (an acid, base, or an enzyme) (Shi, Valle-Rodr ́guez et al. 2011). Biodiesel has many merits as a renewable energy resource that includes being derived from a renewable, domestic resource, thereby relieving reliance on petroleum fuel, and it is biodegradable and non-toxic.

Biodiesel is better than petro-diesel because of several characteristics, including environmental friendliness, renewability, reduced emission, higher combustion efficiency, improved lubricity and higher safety etc. (Canakci and Sanli 2008). Biodiesel is considered a carbon neutral fuel. Other environmental advantages of biodiesel include no net emission of sulfur oxides. Compared with petro-diesel, biodiesel has a more favorable combustion emission profile, such as low emissions of carbon monoxide, particulate matter and unburned hydrocarbons. Biodiesel has near zero sulfide emission, lower carbon monoxide emissions (about 50%), reduces hydrocarbon, aldehydes, fume, and suspension particle by about 95%, 30%, 80%, and 30%, respectively (Kasim, Tsai et al. 2009). Biodiesel has a relatively high flash point (150 ℃), which makes it less volatile and safer to transport or handle than petroleum diesel. In addition, using biodiesel on a large scale will promote plantations of crops or algae used to produce its feedstock. This would result in more carbon dioxide recycling by photosynthesis, thereby minimizing the impact on the greenhouse effect (Al-Zuhair 2007). Table 1.1 shows a comparison of the chemical properties of biodiesel and petroleum diesel (Demirbas 2007).

Table 1.1 Chemical properties of biodiesel and petroleum diesel

Chemical property	**Biodiesel**	**Petroleum diesel**
Ash (wt%)	0.002-0.036	0.006-0.010
Sulfur (wt%)	0.006-0.020	0.020-0.050
Nitrogen (wt%)	0.002-0.007	0.0001-0.0003
Aromatics (vol%)	0	28-38
Iodine number	65-156	0
HHV(higher heating values) (MJ/kg)	39.2-40.6	45.1-45.6

In light of these advantages, the total world biodiesel demands and production has been constantly increasing, with a 16-fold increase over the past 10 years in production, and was estimated to amount to about 4 billion gallons in 2009, mainly produced in the European Union and the USA (Shi, Valle-Rodr ǵuez et al. 2011). Especially in the USA, biodiesel has been growing to be a vital alternative fuel to the United States (US) economy with a doubling production nationwide each year as shown in Figure 1.1. Furthermore, new plants or research centers for biodiesel production are being built in US at fast pace (National Biodiesel Board, 2011).

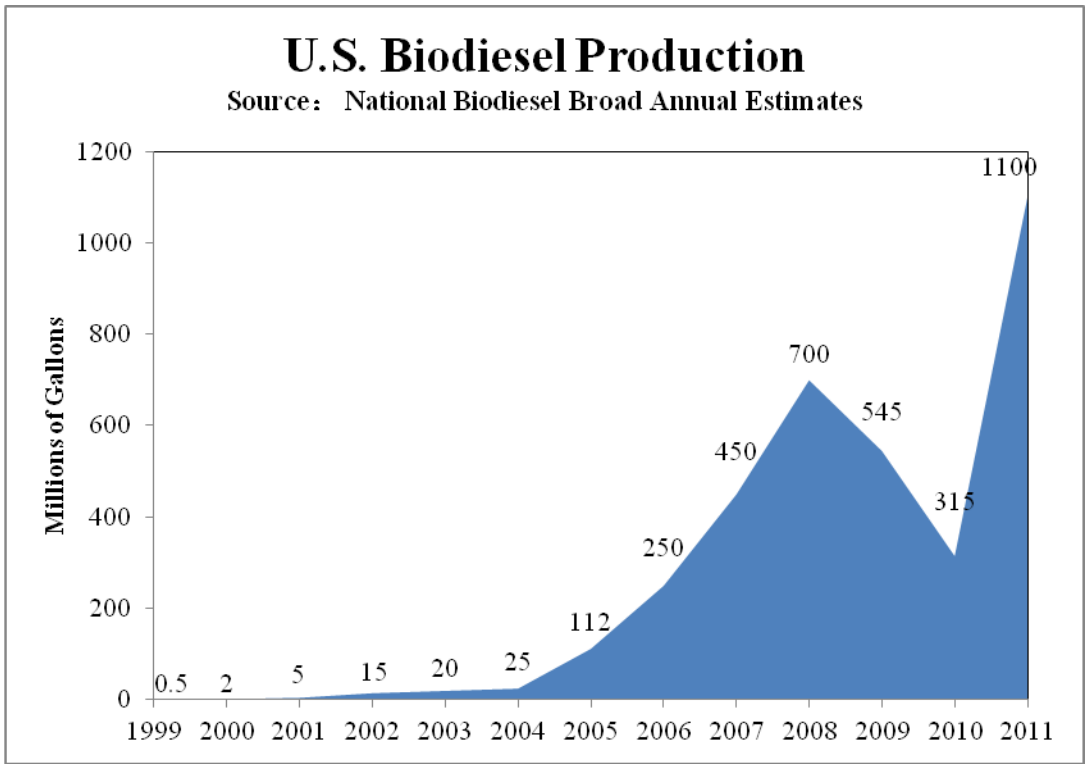

Figure 1.1 Biodiesel production per fiscal year in the United States (US National Biodiesel Board, 2011)

1.3 Feedstock for biodiesel production

As mentioned above, the feedstock for biodiesel includes vegetable oils, animal fats, waste cooking oil, and algae lipids. The main feedstock now is vegetable oil. Currently, about 84% the world biodiesel is produced by using rapeseed oil. The remaining portion is from sunflower oil (13%), palm oil (1%) and soybean oil and others (2%) (Gui, Lee et al. 2008). Due to the fact of more than 95% of the biodiesel is made from edible oil which may cause some other concerns and problems.

From 2001 to 2011, the consumption of biodiesel in USA increased from 5 million gallons to 1100 million gallons. Despite the large increase in its use, biodiesel

still represents a small percentage of total diesel consumption. In 2010, biodiesel contributed only about 1.84% of the diesel fuel consumption in the United States (U.S. Energy Information Administration 2011).

It is believed that large-scale production of biodiesel from edible oils may bring global imbalance to the food supply and demand market. In April 2008, United Nations officially claimed that the US and the EU took a criminal path by contributing to the global food crisis by using food crops for bio-fuel production (Xu and Mi 2011).

Recently, environmentalists have commented on the potential negative impact of biodiesel production from edible oils leading to deforestation and destruction of ecosystems. In some countries such as Indonesia, Brazil and Malaysia, forest has been cleared for agricultural plantations. The expansion of oil crop plantations for biodiesel production on a large scale could add to the deforestation already occurring in those countries. Furthermore, the line between food and fuel economies is blurred because both of the fields are competing for the same oil resources (Demirbas 2011). In other words, the limited arable land that has been used to grow food would instead be used to produce fuel and biodiesel crops. Although there is continuous increase in the production of vegetable oil; the ending stocks of vegetable oils are continuously decreasing due to increasing production of biodiesel (Ahmad, Khan et al). Eventually, with the implementation of biodiesel as a substitute fuel for petroleum-derived diesel oil, this may lead to the depletion of edible-oil supply worldwide (Gui, Lee et al. 2008).

Another related factor, production cost, has also contributed to the limited adoption of biodiesel. The biggest factor affecting the cost of biodiesel production is the

price of the feedstock. The feedstock cost constitutes approximately 70-95% of the overall cost of biodiesel production (Chhetri, Watts et al. 2008). For example, in the USA, it has been estimated that the soybean oil alone contributes 88% of the total cost of biodiesel (Behzadi and Farid 2007). As mentioned above, one of the reasons for the high price of the raw material input is competition from other commercial sectors, such as the food industry.

The vast amount of plant oil production is necessary to replace conventional diesel. Recently, the large increases in biodiesel production have caused an excess of glycerol supply over demand. The current limited worldwide supply of plant oils prevents biodiesel from replacing conventional diesel to a large extent. As discussed previously, devoting a greater proportion of plant oils to the production of biodiesel has already contributed to higher vegetable oil prices, not only making biodiesel production more expensive, but also having an impact on other sectors of the economy, such as food prices.

Due to the high price of edible plant and vegetable oils and the land use competition of biodiesel production and food production, it is necessary to find another way to lower the biodiesel production cost and solve the land use problem. One possibility to overcome these problems is using microalgae for biodiesel feedstock. Another way is improving the production process by using *in situ* transesterification method.

1.4 Introduction of algae as biodiesel feedstock

Algae are a very large and diverse group of plant-like and perform photosynthesis organisms. Based on their morphology and size, algae are typically subdivided into two major categories - macroalgae and microalgae. Microalgae are microscopic photosynthetic organisms, many of which are present in a unicellular manner and found in diverse environments (Chen, Wang et al. 2009). The best algae for biodiesel feedstock would be microalgae. Microalgae are an organism capable of photosynthesis that is less than 2 mm in diameter (most 2-50 μm in diameter). Macroalgae, like seaweed, are not as widely used in the production of biodiesel. Microalgae possess more oil than macroalgae and it is much faster and easier to grow (A. B.M. Sharif Hossain 2008). The following picture is the scanning electron micrograph of microalgae (*Chlorella*) (Satyanarayana, Mariano et al. 2011).

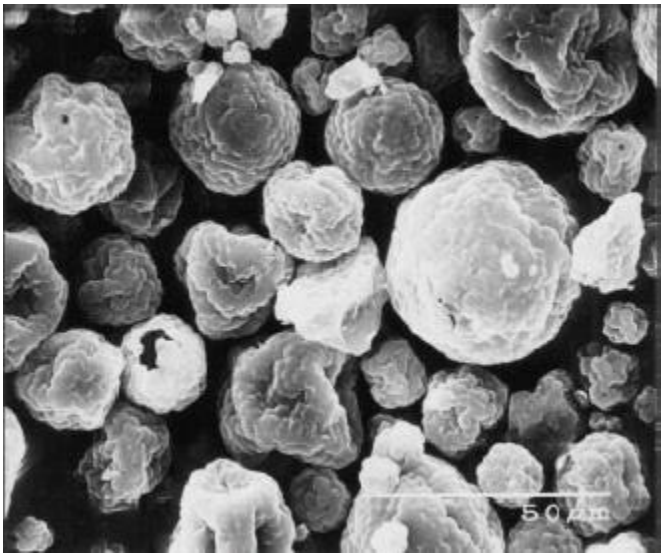

Figure 1.2 Scanning electron micrograph of microalgae (*Chlorella*)

Using microalgae as a biodiesel feedstock has many advantages. First, algae oils are renewable since microalgae produce oils from sunlight air, and water, and can do so all year round. Microalgae use carbon dioxide and other carbon sources for their energy

in addition to sun light and carbon supply. Algae, which assimilates CO_2 photoautotrophically or mixtrophically, is a perfect candidate for CO_2 fixation and reduction (Wang, Min et al. 2010). Microalgae, also have higher photosynthetic efficiency than terrestrial plants and are efficient CO_2 fixers. Additionally, high production of biomass and some metabolites are achieved by their heterotrophic growth (Burrell, Inniss et al. 1984). Therefore, higher biomass productions along with faster growth rate over other energy crops are observed. Microalgae require no cropland, and have high oil productivity per hectare also.

Due to its capability of high photosynthetic efficiency and other characteristics mentioned above, microalgae have another main advantage which is high oil content. Some algal species have 50–60% (dry biomass weight) of their total mass dedicated to lipids. The Aquatic Species Program sponsored by the US Department of Energy estimated that algal oil yield of over 5,000 to 10,000 gallons per acre per year is possible compared with 50 to 100 gallons per acre per year for traditional oil crops such as soybean (Kong, Li et al. 2010). Microalgae can produce up to 250 times the amount of oil per acre as soybeans, and 7 to 31 time greater oil than palm oil (A. B.M. Sharif Hossain 2008). In fact, algae are the highest yielding feedstock for biodiesel and producing biodiesel from algae may be the only way to produce enough automotive fuel to replace current gasoline and diesel.

Microalgae use photosynthesis to convert sun energy into chemical energy and complete an entire growth cycle only in every few days. Moreover they can easily grow in the place with sunlight and some simple nutrients, although the growth rates can be

accelerated by the addition of specific nutrients and sufficient aeration (Aslan and Kapdan 2006). Combined with algal ability to grow under harsher conditions, and their reduced needs for nutrients, they can be grown in areas unsuitable for agricultural purposes independently of the seasonal weather changes and can use wastewaters as the culture medium, not requiring the use of freshwater (Mata, Martins et al. 2010). The following chart compared the oil yield and land required of several biodiesel sources (Chen, Wang et al. 2009).

Table 1.2 Oil yield of biodiesel feedstock

Crops	**Oil Yield (gallon/acre/yr)**	**Land needed to produce 140.8 gallons biodiesel (million acre)**
Soybean	48	2933
Camelina	62	2270
Sunflower	102	1380
Jatropha	202	697
Oil palm	635	2217
Algae	1000-4000	140.8-35.5

1.5 Transesterification process

Transesterification (also called alcoholysis) is the reaction of a fat (organic esters) with an alcohol to form new esters and alcohols (e.g., glycerol in the case of methanol as

a reactant). This reaction is typically aided by the addition of catalysts to improve the reaction rate and yield (See Figure 1.3). The catalysts can be bases, acids, or even microbial enzymes (e.g., liptase). The bases include NaOH, KOH, carbonates and corresponding sodium and potassium alkoxides such as sodium methoxide, sodium ethoxide, sodium propoxide and sodium butoxide. Sulfuric acid, sulfonic acids and hydrochloric acid are usually used as acid catalysts. Alkali-catalyzed transesterification is much faster than acid-catalyzed transesterification and is most often the catalysts of choice for commercial scale operations. The alcohols which are used in the transesterification process include methanol, ethanol, propanol, butanol and amyl alcohol. Methanol and ethanol are used most frequently, especially methanol because of its low cost and its physical and chemical advantages (polar and shortest chain alcohol) (Ma, F. and M. A. Hanna. 1999).

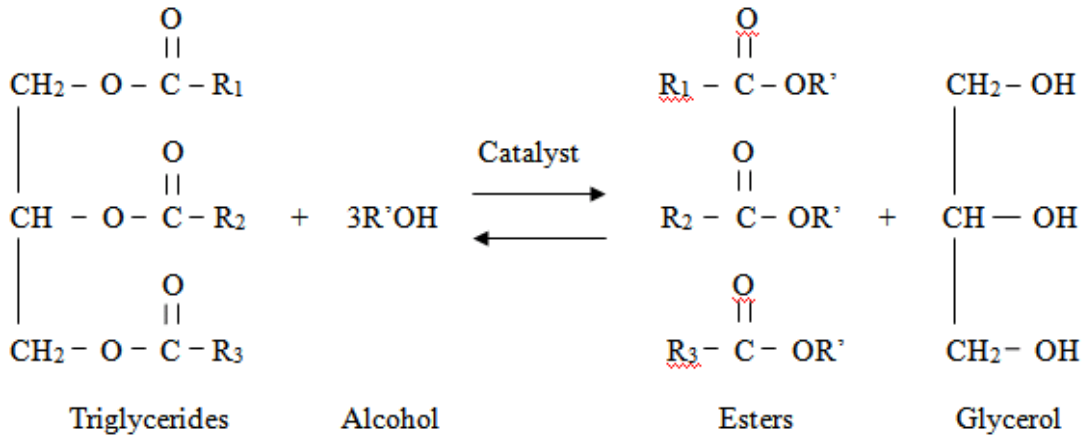

Figure 1.3 Transesterification of triglycerides (overall reaction)

From the above reactions in Fig.1.3, transesterification process includes three reversible steps in series: 1) triglycerides are converted to diglycerides, 2) diglycerides

are converted to monoglycerides, and 3) monoglycerides are then converted to esters (biodiesel) and glycerol (by-product). Thus, excess alcohol is used to shift the equilibrium toward the products. Stoichiometrically, a 3:1 molar ratio of alcohol to triglycerides is needed in transesterification (Mata, Martins et al. 2010). In practice, the ratio needs to be higher to maximumize ester yields. The relationship between the feedstock mass input and biodiesel mass output is about 1:1, which means that theoretically, 1 kg of oil results in about 1 kg of biodiesel (Sharma and Singh 2008).

The physical properties of the primary chemical products of transesterification are summarized in Tables 1.3 (Zhang 1994). The boiling points and melting points of the fatty acids, methyl esters, mono-, di- and triglycerides increase as the number of carbon atoms in the carbon chain increase, but decrease with increases in the number of double bonds. The melting points increase in the order of tri-, di- and monoglycerides due to the polarity of the molecules and hydrogen bonding.

Table 1.3 Physical properties of chemicals related to transesterification (Zhang 1994)

Name	**Specific gravity, g/ml (℃)**	**Melting point (℃)**	**Boiling point (℃)**	**Solubility (>10%)**
Methyl myristate	0.875 (75)	18.8	-	-
Methyl Palmitate	0.825 (75)	30.6	196.0	Acids, benzene, EtOH, Et2O
Methyl Stearate	0.850	38.0	215.0	Et2O, chloroform
Methyl Oleate	0.875	-19.8	190.0	EtOH, Et2O
Methanol	0.792	-97.0	64.7	H2O, ether, EtOH
Ethanol	0.789	-112.0	-	H2O, ether
Glycerol	1.260	17.9	290.0	H2O, EtOH

Biodiesel production from microalgae is through transesterification too. Many studies have used solvents to extract lipids from the cells. In the 1950s, Folch et al (Folch, Lees et al. 1957) and Bligh and Dyer (Bligh and Dyer 1959) used chloroform and

methanol to extract lipids. Solvent extractions from microalgae are typically based on those methods, then lipid are transesterified to produce biodiesel. However, solvent extraction often extracts lipids incompletely, particularly free fatty acids, and can extract significant quantities of non-nutritive, non-saponifiable material such as pigments. Some modifications in the protocol are needed to improve extraction efficiency. Instead of improving extraction methods, some investigators have been trying to eliminate the extraction completely by transesterifying lipids *in situ*.

1.6 Two-step versus one step *in situ* transesterification

Contemporarily, industrial biodiesel synthesis from oils involves isolation of oilseed glycerides by extrusion or solvent extraction, degumming and refining of the oil, and its alkali-catalyzed transesterification. This technology includes two steps at least --- lipid extraction and tranesterification (Figure 1.4). Hexane (or other solvents) extraction is the main technology for oil recovery in the United States. Although extraction plants achieve high levels of solvent recovery, there is about 1.25L solvent losing per metric ton of input in a typical soybean processing plant (Kemper 1997). However, in a large scale plant, discharge of solvent contributes to the production of atmospheric smog and to global warming and is classified as a hazardous air pollutant. Thus, simplification of the oil production or esterification processes could reduce the disadvantages of this attractive biobased fuel. Therefore, *in situ* transesterification, which takes place inside the biomass itself without the separate lipid extraction step, is receiving increasing interests.

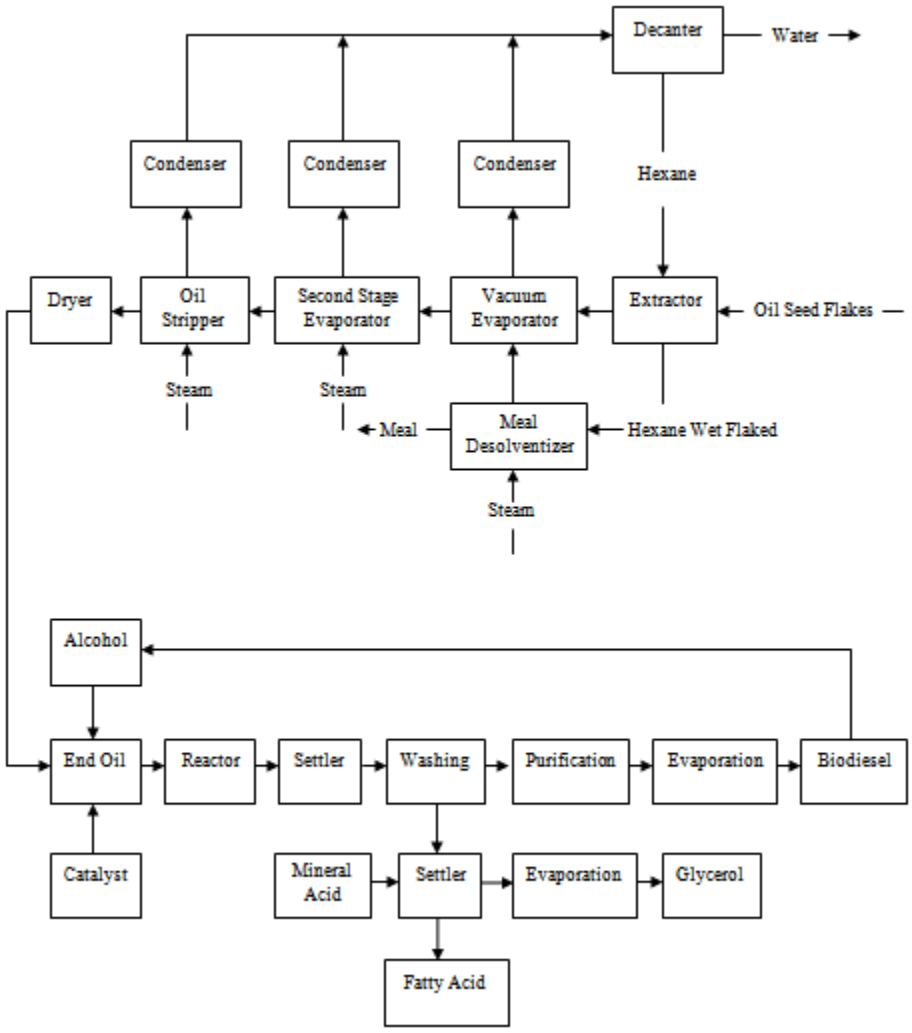

Figure 1.4 Conventional process for biodiesel production in the extraction-transesterification model (Xu and Mi 2011)

In situ transesterification differs from the conventional reaction in that the oil-bearing material contacts with alcohol directly instead of reacting with pre-extracted oil and alcohol. That is, extraction and transesterification proceed in one step, the alcohol acting both as an extraction solvent and an esterification reagent which enhances the porosity of the cell membrane, and would eliminate the need for extraction: yields found

are higher than via the conventional route, and waste is also reduced (Stavarache, C., M. Vinatoru, et al. 2005).

Chapter 2 Cultivation and characterization of algal lipid

2.1 Preparation of algal biomass

A wild-type algae strain of *Chlorella sp*., was screened from local lake freshwater in Minnesota, USA, and then cultivated in a pilot-scale 1300 L photobioreactor filled with Tris–Acetate-Phosphorus (TAP) media (See Table 2.1). The photobioreactor was set up in the greenhouse located on the Saint Paul campus at the University of Minnesota, Twin Cities, where the average daylight in May was 14-15 h per day and the greenhouse temperature fluctuated between 22 to 30 ℃, and stayed around 25 ℃ most of the time. When the biomass reached around 1 g/L, a semi-continuous harvesting regimen, in which 450 L of the culture volume was harvested followed by supplementing with the same volume of tap water enriched with TAP media, was carried out. Algae paste with a water content of 85–90% was obtained after flocculation and filtration, and then subjected to natural drying to constant weight. The air dried algae was dried to a constant weight (14 hours) at 70 ℃ in an incubator (Model 133000, Boekel Scientific, Feasterville, PA, USA).

Table 2.1 TAP media reagents and concentrations

Reagent	**Formula**	**Concentration (/L)**
Ammonium Chloride	NH_4Cl	0.400 g
Magnesium Sulfate	$MgSO_4 \cdot 7H_2O$	0.100 g
Calcium Chloride	$CaCl_2 \cdot 2H_2O$	0.050 g
Dipotassium Phosphate	K_2HPO_4	0.108 g
Monopotassium Phosphate	KH_2PO_4	0.056 g
Tris (hydroxymethyl aminomethane)	$C_4H_{11}NO_3$	2.420 g
Acetic Acid	CH_3COOH	1 ml
Trace elements solution		1 ml

The trace elements solution prepared by Hutner's method (Hutner et al., 1950). For 1 liter final mix, dissolve each compound in the volume of water indicated: EDTA disodium salt, 50g in 25ml water; $ZnSO_4 \cdot 7H_2O$, 22g in 100ml water; H_3BO_3, 11.4g in 200 ml water; $MnCl_2 \cdot 4H_2O$, 5.06g in 50ml water; $FeSO_4 \cdot 7H_2O$, 4.99g in 50ml water; $CoCl_2$ $6H_2O$, 1.61g in 50ml water; $CuSO_4$ $5H_2O$, 1.57g in 50ml water; and $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 1.10g in 50ml water. The EDTA should be dissolved in boiling water, and the $FeSO_4$ should be added last to avoid oxidation. Mix all solutions except EDTA. Next, the solution is heated until the start of boiling. At which time the EDTA solution is added. The solution turns green following this step. After the EDTA addition

and ensuring all the salts are dissolved, the mixture was allowed to cool to 70 ℃. The temperature is held at 70 ℃. Now, 85 ml of hot 20% KOH solution (20 g / 100 ml final volume) is added. This addition brings the final solution to 1 L total volume. The flask is sealed with a cotton plug (to allow air exchange) and is stored for 1-2 weeks, with daily shaking (Hutner et al., 1950).

2.2 Analysis of fatty acid and molecular mass of algae oil

The fatty acid analysis was determined using the American Oil Chemists' Society (AOCS) official method, Ce 1-62.

Table 2.2 Fatty acid composition

Fatty acid	**Molecular weight (g/mol)**	**Composition in the sample (%)**	**Molecular weight contribution (g/mol)**
C14:0	228.37	1.9	5.50
C14:1	226.37	2.0	5.75
C15:0	242.41	0.7	2.16
C16:0	256.42	18.8	61.26
C16:1	254.41	13.2	42.66
C18:0	284.48	2.4	8.68
C18:1	282.46	24.2	86.86
C18:2	280.45	6.6	23.53
C18:3	278.43	8.9	31.39

Average molecular weight of fatty acid is 267.89

According to the result, the lipid was mainly composed of mono-unsaturated fatty acid, with 24.2% of oleic acid (C18:1) and 13.2% of palmitoleic acid (C16:1). Another main component is palmitic acid (C16:0), which accounted 18.8% of total lipid. The result of the fatty acid composition was used to determine the average molecular weight of fatty acid which was taken as 267.9 g/mol.

Microalgae oil consists of different fatty acids, so the average molecular weight of fatty acid ($M_{fatty\ acid}$) was used to estimate the average molecular mass of microalgae oil (M_{oil}). Since the formation of the triglyceride molecule is facilitated by the combination of three fatty acid molecular and one molecular of glycerol with the condensation of three molecules of water, the average molecular mass of microalgae lipid can be estimated by using the following equation:

$$M_{oil} = 3M_{fatty\ acid} + M_{glycerol} - 3M_{water}$$

where the $M_{glycerol}$ and M_{water} is the molar weight of glycerol and water.

The mean molar mass of triglyceride was calculated to be 842 g/mol according to the Table 2.2 and this would determine the dosage of alcohol in the *in situ* transesterification reaction.

2.3 Determination of total lipid

The lipids were extracted using a one-step extraction method adapted from Folch (Folch et al., 1956). About 0.1 g dried algae powder were weighed into clean, 25 ml

screw-top glass tubes, in which 10 ml 2:1 chloroform–methanol (v/v) mixture was added. Extraction was carried out in 30 ℃ water bath (Cole-Parmer, USA) for 30 min with 100 rpm rotation speed. Upon completion of the reaction, the algal solid residues were removed by passing the suspension through a Whatman 934-AH glass fiber filter (Whatman Inc., USA). The filtrate was transferred to another clean, 25 ml screw-top glass tube containing 2 ml 0.9% NaCl solution to wash out water-soluble components. After centrifugation, a biphasic system without any interfacial fluff was obtained. The volume of the lower phase containing essentially all the algal lipids extracted was measured, and 3 ml of the lower phase was transferred into a weighed, clean, 5 ml glass tube, and then organic solvent was dried off under the N-EVAP Analytical Nitrogen Evaporator at room temperature, and the flow rate is 500 ml/min (Organomation Associates Inc., USA). Duplicate lipid determination was made for every sample. The lipid content of dry weight was calculated according to the following formula:

$$LC\ (g/g) = (m_2 - m_0) \times V / (3 \times m_1)$$

where LC stands for lipid content based on dry weight, m_1 is the weight of the dry algae, m_0 is the weight of the empty 5 ml glass tube, m_2 is the weight of the 5 ml tube with the dried lipids, and V is the total volume of the lower phase after washing.

The lipid content of microalgae biomass was 14.5% based on the dry weight.

2.4 Lipid conversion rate

According to the fatty acid composition analysis (Table 2.2), the fatty acid accounts for 78.7% of the total lipids, indicating that only 78.7% of the lipid could react

with alcohol to form biodiesel. Therefore, when calculating the lipid conversion rate, the part of lipids which cannot react with alcohol should be left out. After the *in situ* transesterification reaction was completed, chloroform was introduced to the mixer to transfer the biodiesel into the chloroform phase from alcohol phase. The crude biodiesel was purified by washing with sodium chloride solution (0.9 M). The mixture was then centrifuged at 2000 rpm in a centrifuge (International Equipment Co., Boston, MA, USA) for 10 min at room temperature to form a two-phase system. The chloroform layer in the tube was measured. Three ml biodiesel was collected for drying under a nitrogen stream. The lipid conversion rate was calculated according to the following equation:

$$BCR = (m_2 - m_0) \times V / (3 \times m_1 \times LC \times 0.787)$$

where BCR stands for lipid conversion rate based on the lipid content, LC is lipid content based on dry weight, m_1 is the weight of the dry algae, m_0 is the weight of the empty 5 ml glass tube, m_2 is the weight of the 5 ml tube with the dried lipids, V is the total volume of the lower phase after washing, and 0.787 is the proportion of fatty acids in the total algae lipid.

Chapter 3 Effects of catalyst, methanol, and reaction time on --- *in situ* transesterification process at room temperature

3.1 Introduction

As mentioned before, the use of *in situ* transesterification could simplify the biodiesel production process and save the energy and cost. However, there were few papers reported on biodiesel production from algae biomass. The first motivation for the research in this chapter was to explore a general process of biodiesel production from algae using *in situ* transesterification method. Another primary goal was to develop an efficient, lowest energy consumption process. Therefore, no heating and stirring were used in our process.

As many articles reported, there are four main factors that could affect the lipid conversion rate (Ehimen, Sun et al. 2010). They are catalyst concentration, molar ratio of alcohol to oil, reaction time, and reaction temperature. In this chapter, the first three factors will be studied at room temperature, and the effect of reaction temperature will be discussed in the next chapter.

3.1.1 Effect of type and concentration of catalyst on lipid conversion rate

Catalysts used for the transesterification process of triglycerides can be classified as alkali catalysts, acid catalysts, enzyme, and heterogeneous catalysts. The most common basic catalysts include potassium hydroxide (KOH), sodium hydroxide (NaOH), sodium methoxide ($NaOCH_3$), and potassium methoxide ($KOCH_3$). The common acid catalysts are sulphuric acid, hydrochloric acid, and sulphonic acid. Whether the catalyst

is alkaline or acidic, they are all homogeneous catalysts which mean the catalyst and the reactants are in the same phase.

In general, acid catalyzed transesterification is performed at high acid catalyst concentrations, high alcohol to oil molar ratios, and low to moderate temperatures and pressures. Acid-catalyzed reactions require high molar ratios of alcohol to oil in order to obtain the beast product yields within reasonable reaction time. For example, for sunflower oil transesterification, a 2OO:1 molar ratio and 4 h of heating in the presence of 100% acid catalyst (on oil basis) were reported to obtain a good conversion (96.5% based on the oil content) and quality of methylesters (Siler-Marinkovic and Tomasevic 1998). Acid catalysts is attractive because the insensitiveness to free fatty acids in the feedstock. However, acid-catalyzed reaction has been largely ignored mainly because of the relatively slow reaction rate.

In base-catalyzed transesterification, potassium hydroxide and sodium hydroxide are the two types of most commonly used catalysts. Both in concentration from 0.4 to 2% w/w of oil were reported (Freedman, Pryde et al. 1984). A known drawback with alkaline transesterification is that the oil containing significant amounts of free fatty acids could not be converted into biodiesels completely. The free fatty acids will react with base catalyst to produce soaps, and that inhibits the production and separation of biodiesel (Georgogianni, Kontominas et al. 2008). However, one of the greatest advantages of alkaline catalyst is that alkali-catalyzed transesterification proceeds approximately 4000 times faster than that catalyzed by the same amount of an acidic catalyst (Fukuda, Kondo

et al. 2001). Moreover, alkaline metal hydroxides (KOH and NaOH) are much cheaper than other catalyst. They are thus most often used in industrial biodiesel production.

The enzyme catalyzed transesterification is typically catalyzed by lipases from microorganisms, such as *Mucor miehei* and *Rhizopus oryzae* (Nelson, Foglia, et al. 1996; Shieh, Liao et al. 2003). The lipid conversion rate depends on the type of enzyme. The merit of biochemical catalysis method includes: (1) the process can proceed under milder conditions; (2) a greater selection of feedstock including waste oils with a high acidity can be used; (3) it is easier for subsequent separation and purification of biodiesel; and (4) there is less pollutants emission and it is therefore a more environment-friendly process. Since enzyme reactions are highly specific, the main problem of the lipase-catalyzed transesterification is the high cost of the lipases. Low stability and none reusability are also the problems of enzyme catalyst (Zhang, Weng et al. 2012).

Heterogeneous catalysts are solid acids or bases including immobilized enzymes, titanium-silicates, alkaline-earth metal compounds, anion exchange resins or guanadines heterogenized on organic polymers. The application of heterogeneous catalysts appears promising and growing. However, the use of homogeneous catalysts such as sodium or potassium hydroxide, sodium or potassium methoxide is still common in industries, because they are relatively cheap and quite efficient for the transesterification reaction (Helwani, Othman et al. 2009).

As mentioned above, alkali catalyzed transesterification proceeds approximately 4000 times faster than that catalyzed by the same amount of an acidic catalyst. In addition, the alkaline metal hydroxides are cheaper than other catalysts. Since the objective of this

research was to develop a general transesterification process and a rapid, efficient method to produce biodiesel, alkaline catalyst (potassium hydroxide) was used. To investigate the influence of concentration of catalyst, an excess methanol volume of 20 ml and enough reaction time of 8 h were used. Since the transesterification reaction is an equilibrium reaction, excess alcohol amount and reaction time are necessary to ensure complete reaction.

The concentration of KOH was calculated according to the lipid content in the algae. The concentration is expressed as the percentage of KOH and lipid (%, g KOH/g lipid). According to the literature, the optimum concentration of alkali catalyst for vegetable oil is 0.4%-2% (Georgogianni, Kontominas et al. 2008; Narasimharao, Lee et al 2007). Since the methanol does not contact the oil directly during the *in situ* transesterification process, a higher catalyst concentration might be needed to make the reaction happen. To study the effect of catalyst concentration, eight different concentrate levels were tested and they were 1.0%, 1.5%, 2.0%, 2.5%, 3.0%, 5.0%, 7.0%, and 10.0%. These eight concentration levels basically cover the concentrations reported in the literature with a high concentration.

3.1.2 Effect of reaction time on lipid conversion rate

In general, the lipid conversion rate increases with increasing reaction time. At the very beginning of the reaction, the reaction speed is very low because of the need for mixing and dispersion of alcohol into the oil. After this phase, for vegetable oil, 80% of transesterification reaction could complete in a short time, and almost completed in

another hour. However, with further increase in reaction time, the lipid conversion rate remained relatively constant (Freedman, Pryde et al. 1984). Moreover, excess reaction time will lead to a reduction in the product yield due to the reversible reaction of transesterification, resulting in the loss of esters as well as causing more fatty acids to form soaps (Leung, Wu et al. 2010).

For algae, the *in situ* transesterification reaction time will be longer than the vegetable oil, because that the alcohol contacts oil-bearing material directly instead of reacting with oil. It will take more time for the alcohol to diffuse into the algae cell and react with the oil. In this chapter, the study on the effect of reaction time on lipid conversion rate will be discussed.

To investigate the influence of reaction time, an excess methanol volume of 20 ml were used. According to the result in section 3.3.1, an optimum 2% KOH catalyst of the lipid content is used for examining the impacts of reaction time.

For algae, the *in situ* transesterification reaction time may be longer than the vegetable oil. Therefore, to account for this longer reaction time a wide range of ten reaction time intervals were selected: 10 min, 0.5 h, 1 h, 1.5 h, 2 h, 4 h, 6 h, 8 h, 10 h, and 12 h.

3.1.3 Effect of methanol volumes on lipid conversion rate

The amount of alcohol is also an important factor to the lipid conversion rate. As mentioned in Chapter 1, the stoichiometric ratio for transesterification reaction requires three moles of alcohol and one mole of triglyceride to yield three moles of fatty acid alkyl

esters and one mole of glycerol. However, transesterification is an equilibrium reaction, a large excess of alcohol is required to drive the reaction to the right. Therefore, higher molar ratio results in greater ester conversion in a shorter time. For maximum conversion to the ester, a molar ratio of 6:1 was used for many type of vegetable oil (Meher, Vidya Sagar et al. 2006).

The molar ratio has no effect on acid, peroxide, and iodine value of methyl esters. However, further increasing the alcohol amount beyond the optimal ratio will not increase the yield but will increase the cost for alcohol recovery. Additional, a part of the glycerol remains in the biodiesel phase which also decreased the yield of esters. Besides, the molar ratio also depends on the type of catalyst used and the molar ratio of alcohol to triglycerides. When the percentage of free fatty acids in the oils or fats is high, such as in the case of waste cooking oil, a molar ratio as high as 15:1 is needed when using acid-catalyzed transesterification (Leung, Wu et al. 2010).

For algal transesterification, methanol and lipid cannot be in direct contact, a relative large amount of methanol is needed. Additional, to ensure all the algae could be submerged in the methanol, a large volume of methanol is necessary. And for *in situ* transesterification, since oil extraction process combines with the transesterification process, more methanol will be needed for the two processes.

To investigate the influence of amount of methanol, 7 different volumes of methanol were used: 6.6 ml, 9.8 ml, 13.1 ml, 16.4 ml, 19.7 ml, 26.2 ml, and 32.8 ml. And according to the result in section 3.3.1 and section 3.3.2, a KOH catalyst of 2% of the lipid content and the 10 h reaction time were used.

3.2 Materials and methods

The algal biomass produced as described in Chapter 2 was dried at 70 ℃ for 14 hours in an incubator (Model 133000, Boekel Scientific, Feasterville, PA, USA) to constant weight.

Potassium hydroxide, sodium hydroxide, hydrochloric acid (36%, w/w), methanol (HPLC grade), chloroform (HPLC grade) and 0.9% sodium hydroxide solution were used in this chapter. Chemicals are from Fisher Scientific (Pittsburgh, PA, USA).

This set of experiments was designed to investigate the effects of three process variables, namely, amount of catalyst, reaction time, and methanol volumes on *in situ* transesterification carried out all at ambient temperature. The ranges of these three variables are described in the Results and Discussion section. This general in situ transesterification procedure was followed: One g oven dried algae was placed in a 50 ml conical polypropylene tube. A designated amount of methanol containing potassium hydroxide was added to the tube. The tube was stirred on a vortex mixer for 2 min, resulting in a monophasic solution. The mixture was kept at room temperature for a designated time. When the reaction was completed, hydrochloric acid (36%, w/w) was added to stop the reaction. Then, 10 ml chloroform was added and stirred for 2 min to transfer the biodiesel into the chloroform phase. The crude biodiesel was purified by washing with sodium chloride solution (0.9 M). The mixture was then centrifuged at 2000 rpm in a centrifuge (International Equipment Co., Boston, MA, USA) for 10 min at room temperature, which resulted in a two-phase system. The chloroform layer in the

tube was measured. Three ml chloroform phase was transferred into a weighed, clean, 5 ml glass tube, and then organic solvent was dried off under the N-EVAP Analytical Nitrogen Evaporator at room temperature, and the flow rate is 500 ml/min (Organomation Associates Inc., USA). This procedure was repeated three times.

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3.3 Result and discussion

3.3.1 Effect of catalyst concentration on lipid conversion rate

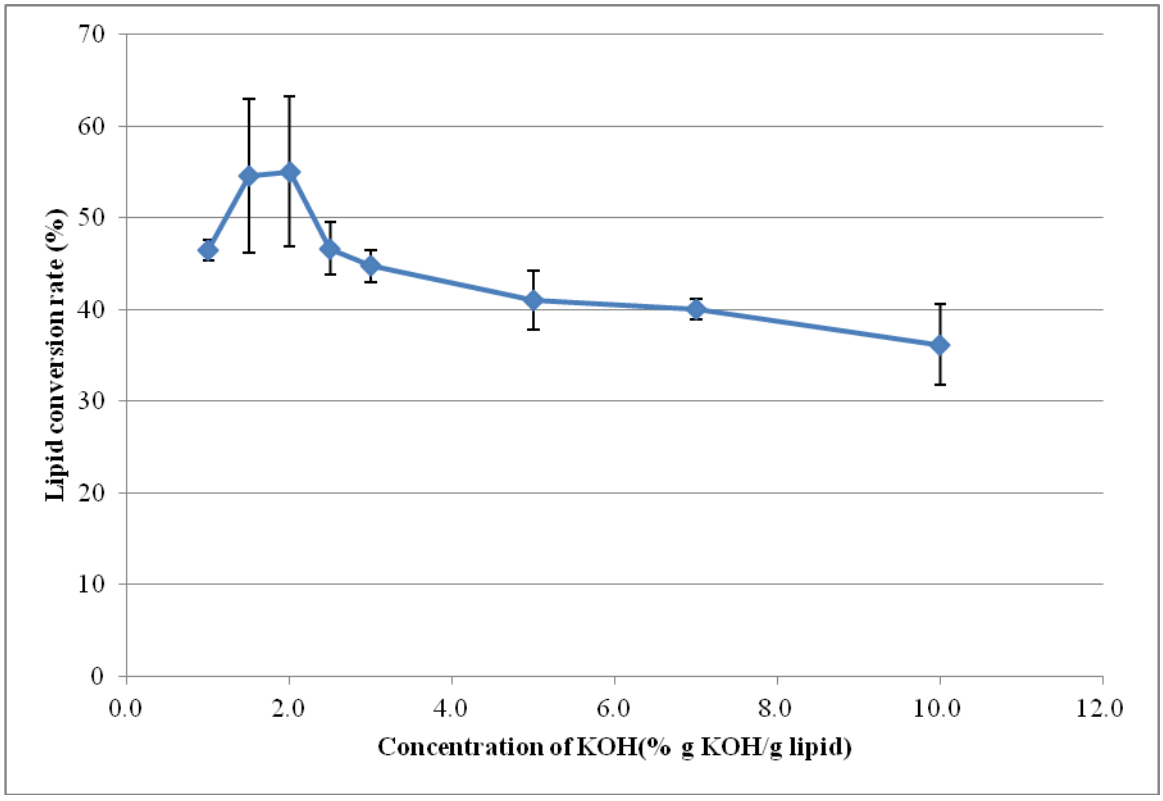

Figure 3.1 Influence of different concentration of catalyst (potassium hydroxide) on the lipid conversion rate

Figure 3.1 shows the relationship of catalyst concentration and lipid conversion rate. As can be seen, the highest lipid conversion rate (55.1%) is achieved when the

concentration of potassium hydroxide (KOH) is 2%. It is slightly higher than the lipid conversion rate (54.6%) when the catalyst concentration is 1.5%. The lipid conversion rate (46.7%) is decreased sharply when the concentration of KOH increased to 2.5% and then continued to decline with the increase of catalyst concentration.

From the figure, the transesterification reaction cannot be completed when the catalyst concentration is too low (1.0%). KOH may contain some water, because it is hygroscopic and it will absorb water from air during storage. Additional, KOH also form small amount of water when dissolved in the alcohol reactant which will affect the conversion rate. That is why that more catalyst needs to be added to complete the reaction. By using a higher concentration, the lipid conversion rate is higher which indicate that higher catalyst concentration is necessary to complete the transesterification reaction (Leung, Wu et al. 2010).

However, further increase in catalyst decreased the yield of fatty acid methyl ester. Under the high concentration of alkaline catalyst, KOH will react with fatty acid and form emulsion formation between soaps and water molecules (Georgogianni, Kontominas et al. 2008). As the catalyst concentration increased, more soap will be produced which consume the catalyst and reduces the catalytic efficiency. When the high concentration KOH catalyzed crude biodiesel was purified by washing with sodium chloride solution, the water phase solution is cloudier. Besides, saponification will also bring other problems, like increases in viscosity, the formation of gels, and difficulty in achieving separation of glycerol (Fukuda, Kondo et al. 2001).

This result is basically the same with the result of the concentration for vegetable oil. That means the catalyst could enter the algae cell membrane and make the reaction happened, if the reaction time is enough. The concentration of 1.5%-2.0% is enough for the *in situ* transesterification reaction. A higher concentration is not needed and it will lead to the decrease of lipid conversion rate.

3.3.2 Effect of reaction time on lipid conversion rate

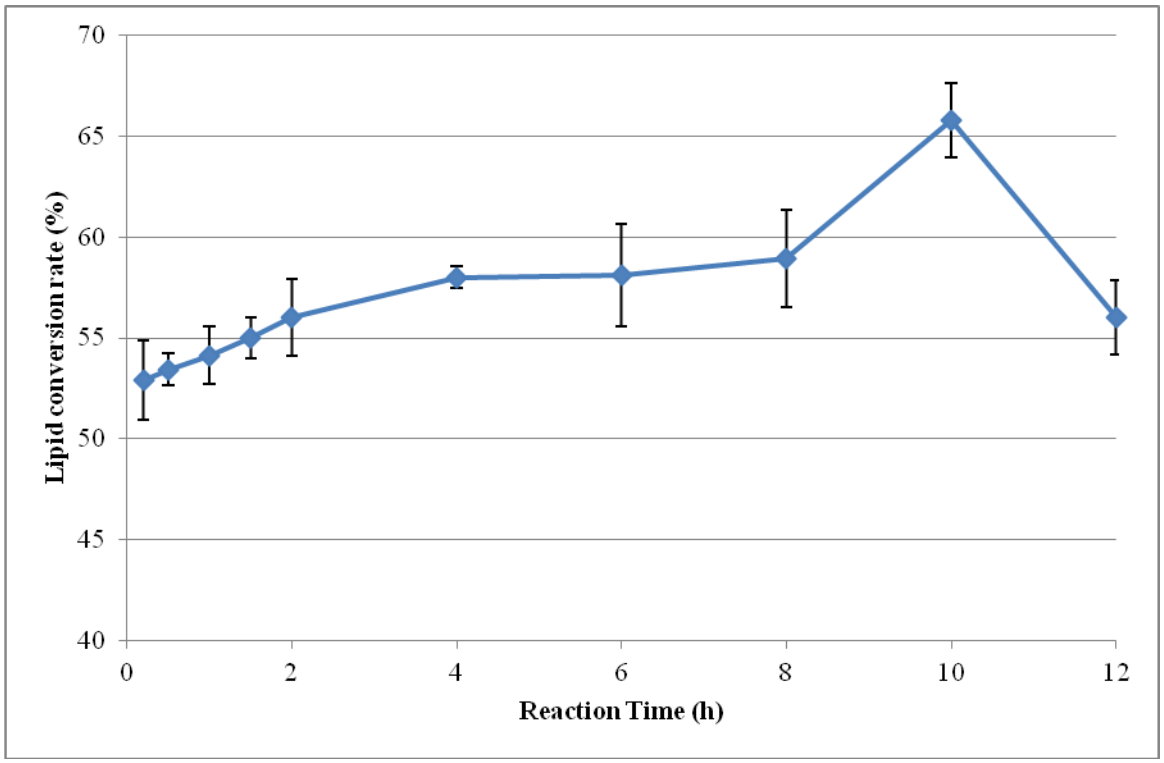

Figure 3.2 Influence of reaction time on the lipid conversion rate

Figure 3.2 shows that, the lipid conversion rate increased with the reaction time increasing until 10 h. The conversion rate (56.0%) decreased sharply when the reaction time was 12 h. The increase of conversion rate between 8 h (58.9%) and 10 h (65.8%)

was also sharp. The maximum lipid conversion rate was reached when the reaction time was 10 h.

At room temperature, the reaction rate is relatively low. There appears to be an eight hours lag phase before the reaction took off. The initial slow reaction may be attributed to the slow diffusion of alcohol and catalyst into algae cells, which may be the limiting step in this reaction. Once sufficient alcohol and catalyst entered the cells, reaction would proceed very quickly. That is why when the reaction time increased to 10 h, the conversion rate increased a lot.

However, a longer reaction time did not benefit the transesterification reaction. When almost all the methanol and catalyst could contact with the lipid in the algae cell, the reaction speed is should be faster. However, when the transesterification reaction was almost finished, few catalysts were used. The rest of the catalyst got a chance to react with the free fatty acid in the algae cell. That could be the reason that with increases in reaction time, the lipid conversion rate significantly decreased.

3.3.3 Effect of methanol volumes on lipid conversion rate

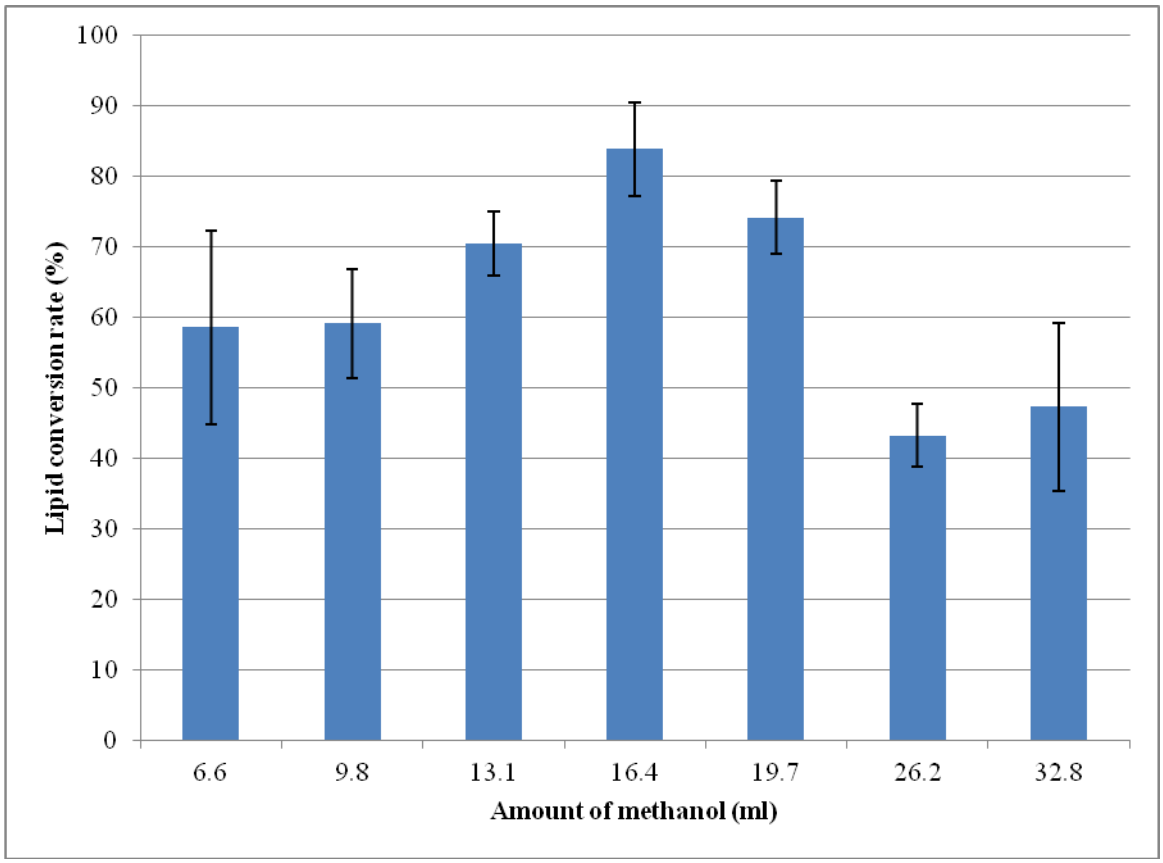

Figure 3.3 Influence of amount of methanol on the lipid conversion rate

Figure 3.3 shows that, the lipid conversion rate increased with increasing methanol volume up to 16.4 ml, and then decreased with further increase in methanol volume.

If the methanol volume is less than 6 ml, the algae cannot be completely immersed into methanol. Therefore, the 6.6 ml is the smallest volume for this study. The lipid conversion rate is about the same when the methanol volumes are 6.6 ml (58.6%) and 9.8 ml (59.2%) and the conversion rate is much lower than the volumes is 16.4 ml (84.0%). It demonstrates that these two methanol levels cannot make all the fatty acid

transfer to the biodiesel. The less volume of methanol will make the transesterification reaction reach equilibrium rapidly which inhibit the biodiesel production.

With the increase of methanol amount, the sufficient methanol drives the transesterification reaction to the biodiesel production direction. Also, a sufficient amount of methanol is helpful in destroying the association between lipids and cell constituents. When methanol volume increased to the 16.4 ml, the best lipid conversion rate was got. The lipid conversion rate (74.1%) was decrease a little when the volume increased to 19.7 ml, but it still maintained on a relatively high conversion rate.

However, with the further increase of methanol amount, the lipid conversion rate sharp declined which is even lower than the beginning methanol amount level. This result was not consistent with some report that no significant trends were observed with the alcohol volumes increasing. Ehimen reported that with the use of alcohol volumes over 60 ml for the in situ transesterification of 15 g of microalgae biomass, no significant trends were observed (Ehimen, Sun et al. 2010). The reason of the conversion decline may be that the catalyst concentration is relatively low when the methanol volumes are large. Therefore, the catalyst contacted with lipid is not sufficient for the transesterification reaction. And the rest KOH which did not participate in the reaction will react with free fatty acid and produce soap. Water can be produced in the system by the reaction of the KOH with lipid. The presence of water in the reaction cell offers the opportunity for hydrolysis reaction of some of the produced esters, resulting in soap formation. This undesirable saponification reaction reduces the ester yield and hampers the conversion efficiency (Schuchardt, Sercheli et al. 1998).

Chapter 4 Effort of reaction temperature on *in situ* transesterification process

4.1 Introduction

Transesterification and lipid conversion rate have been found to be influenced obviously by the reaction temperature and time depending on the type of the feedstocks and solvents used. Leung et al. (2010) observed that the oil viscosity is lower at high reaction temperature, which is a desired property for in situ transesterification and results in increased biodiesel yield. Some optimal reaction temperature levels were reported for different oil type, from room temperature to 90 ℃. Whether using vegetable oil or waste cooking oil, the optimal reaction temperature ranges from 50 to 70 ℃ (Antolín, Tinaut et al. 2002; Georgogianni, Kontominas et al. 2008; Johnson and Wen 2009).

In additional, the lipid conversion rate varies with different temperatures within a given reaction time. For example, FAME yields from a reaction involving refined oil, methanol, and NaOH were 94%, 87% and 64% at 60, 45 and 32 ℃, respectively, for the set reaction of 0.1 h. After 1 h, ester formation was identical for 60 ℃ and 45 ℃ runs and only slightly lower for the 32 ℃ run (Meher, Vidya Sagar et al. 2006). Compared with other transesterification reaction, biodiesel preparation from refined oil needs a relative short time. With increasing reaction times, biodiesel yield remained stable or decreased. However, in biodiesel production from microalgae, the in situ transesterification reaction time might be longer than the vegetable oil transesterification, because the alcohol contacts oil-bearing material directly instead of reacting with oil. It will take more time to let the alcohol into the algae cell and contact with the algae oil.

In the previous chapter, we reported that the highest lipid conversion rate of 84.0% was obtained when the reaction time was 10 h. At 25 ℃, this reaction time was very long compared with those for refined oil as feedstock. To determine an optimal combination of temperature and time for *in situ* transesterification of algae, we studied three reaction temperature levels: 45, 60, and 75 ℃, and six reaction time levels: 0.5 h, 1 h, 2 h, 3 h, 4 h, and 5 h. These experiments were conducted according the materials and procedures described in Ch 3 with the methanol volume and catalyst concentration fixed at 16.4 ml and 2% of the algae oil content, respectively.

4.2 Materials and methods

The algal biomass was produced as described in Chapter 2. Following production, the algal cells were dried at 70 ℃ to constant weight (14 hours) in an incubator (Model 133000, Boekel Scientific, Feasterville, PA, USA).

Potassium hydroxide, sodium hydroxide, hydrochloric acid (36%, w/w), methanol (HPLC grade), chloroform (HPLC grade) and 0.9% sodium hydroxide solution were used. Chemicals are from Fisher Scientific (Pittsburgh, PA, USA).

N-EVAP Analytical Nitrogen Evaporator (Organomation Associates Inc., USA) was used for drying off the organic solvent, and Water Bath (Cole-Parmer, USA) were used for pretreatment.

One g oven dried algae was placed in a glass pressure tube. Sixteen point forty ml methanol containing 0.002896 g potassium hydroxide was introduced to the tube, and stirred for 2 min to obtain a monophasic system on a vortex mixer. The mixtures were

heated through water bath and maintained at different temperature level for specific time. When the set reaction time has elapsed, hydrochloric acid (36%, w/w) was added to stop the reaction. Following, 10 ml chloroform was added and stirred for 2 min to transfer the biodiesel into the organic (chloroform) phase. The crude biodiesel was purified by washing with sodium chloride solution (0.9 M). The mixture was then centrifuged at 2000 rpm in a centrifuge (International Equipment Co., Boston, MA, USA) for 10 min at room temperature, which resulted in a two-phase system. The chloroform layer in the tube was measured. Three ml chloroform phase was transferred into a weighed, clean, 5 ml glass tube, and then organic solvent was dried off under the N-EVAP Analytical Nitrogen Evaporator at room temperature, and the flow rate is 500 ml/min (Organomation Associates Inc., USA). This procedure was repeated three times.

4.3 Results and discussion

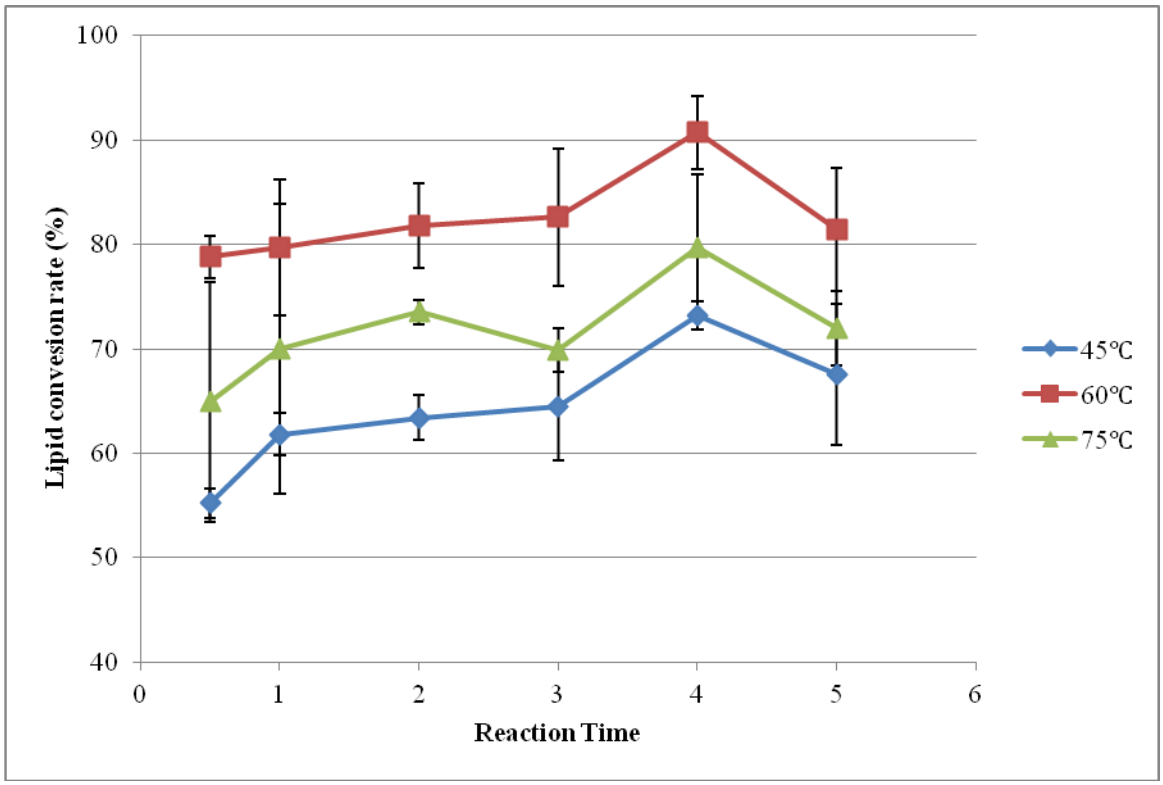

Figure 4.1: Influence of reaction time on lipid conversion rate at different temperature

The effect of the reaction time and reaction temperature on lipid conversion rate is shown in Figure 4.1. Four hour reaction time produced highest lipid conversion rate at all three temperature levels, and is much shorter than the 10 h required at the ambient temperature. The lipid conversion rate was negatively correlated with the reaction time.

With regard to reaction temperature, the lipid conversion rate increased as a function in the order of 45, 75, and 60 ℃ runs. The experiment results were not consistent with the work by Qian et al (Qian, Wang et al. 2008), who reported that reaction temperature had little influence on the extraction and conversion of vegetable oil

through *in situ* alkaline transesterification, and the conversion to methyl ester was almost the same at different temperatures.

A higher reaction temperature can increase reaction rate, including forward and reverse reaction. In this case, the reaction temperature 75 ℃ is higher than the boiling point of methanol(64.7 ℃) which causes the evaporation of methanol although the whole process was in a sealed system, thus the solvent is easy to evaporate, not to leak into the microalgae cell. The high reaction temperature might be in favor of reverse reaction.

The best reaction temperature 60 ℃ is below the boiling point of the methanol, indicating that transesterification reaction can take place without the alcohol vaporization and thus pressurized reaction equipment is not necessary. At 75 ℃, the lipid conversion rate decreased because a higher reaction temperature may also accelerates the saponification reaction of triglycerides. Although the temperature is higher which provides more energy, the result is not better.

From the figure, 4h is a critical point for the transesterification reaction which is enough to complete the reaction. Compared with the reaction at room temperature which lasts 10h to make the transesterification reaction complete, the reaction time of in situ transesterification in higher reaction temperature is much shorter. The overall trend is similar with the trend at room temperature. That indicates that high temperature can speed up the whole transesterification process, make the reaction toward the forward direction.

Chapter 5 Pretreatment of *in situ* transesterification

5.1 Introduction

From the previous two chapters, it is known that during the *in situ* transesterification reaction, the methanol cannot contact the lipid in the algae cells directly, and it takes time for methanol to diffuse into the algae cells and react with the lipids. Some physical and chemical disruption of the cellular structure of algae may increase the permeability of and liberate lipids in the cellular structure, and hence shorten the reaction time and improve the lipid conversion rate.

In this chapter, several pretreatment methods were studied, which include ultrasonic pretreatment, microwave, methanol soaking and heating. Ultrasonic wave and microwave were reported as an auxiliary step in biodiesel production and they were found to have a positive effect on the cell wall breaking (Sherbiny, Refaat et al 2010). As we all know, ultrasound energy produce physical and chemical effects that arise from the collapse of cavitation bubbles. During the process of ultrasonic jets which impinge one liquid in to the other, emulsification will formed because the collapse of cavitation bubbles disrupts the phase boundary in two-phase liquid system (Georgogianni, Kontominas et al. 2008). With a continuously changing electromagnetic field, microwave irradiation can activate the smallest degree of variance of polar molecules and ions like alcohol. The changing electrical field leads to interacts with the molecular dipoles and charged ion. Due to molecular friction, these ions or molecules will have a rapid rotation and the heat is generated. Very efficient heating can be obtained during this process (Azcan and Danisman 2007). In order to shorten the reaction time, a methanol soaking

pretreatment was tested in our studied. Methanol soaking may help the methanol to move into the algae cell and extract the lipid, and thus decrease the total reaction time of *in situ* transesterification. A heating pretreatment was also studied. Heat can cause protein denature and thus weaken the semi-permeability of cellular membranes. The heating temperature was the optimal reaction temperature determined in Ch 4.

To compare the effect of these pretreatment methods on lipid conversion rate, a 3 h reaction time was selected and a control with no pretreatment *in situ* transesterification was the control reference. For ultrasonic pretreatment, ultrasonicator has a low frequency, so pretreatment time is 1 h. In the microwave pretreatment section, three different pretreatment time levels were tested. The reaction time was minimized to prevent solvent evaporation, resulting in elevated pressures in the tube. During both the ultrasonic pretreatment and microwave pretreatment process, heat was generated. Therefore, a water bath heating pretreatment is studied in this chapter to compare the effect of different heating methods on lipid conversion rate. Also, heating time was 10 minutes after the temperature reached 60 ℃ to prevent the solvent evaporation which would result in high evaporating pressure.

5.2 Materials and methods

The algal biomass produced as described in Chapter 2 was dried at 70 ℃ for 14 hours in an incubator (Model 133000, Boekel Scientific, Feasterville, PA, USA) to constant weight.

Potassium hydroxide, sodium hydroxide, hydrochloric acid (36%, w/w), methanol (HPLC grade), chloroform (HPLC grade) and 0.9% sodium hydroxide solution were used in this chapter. Chemicals are from Fisher Scientific (Pittsburgh, PA, USA).

N-EVAP Analytical Nitrogen Evaporator (Organomation Associates Inc., USA) was used for drying off the organic solvent, 3510R-MTH Bransonic Ultrasonic Cleaner (Branson Ultrasonic Corporation, USA), Panasonic Microwave Oven (model NNSD787S, USA), and Water Bath (Cole-Parmer, USA) were used for pretreatment.

The dry algae samples obtained in Ch 2 were pretreated before they were subjected to the transesterification procedure described in Ch 3. All the materials and process parameters were similar to those used in Ch 3. The reaction time was set at 3 h, and the methanol volume and catalyst concentration fixed at 16.4 ml and 2% of the algae oil content, respectively. All reaction procedure was repeated three times. For ultrasonic and microwave pretreatments, algae samples and methanol were placed in the appropriate containers and then subjected to the pretreatments for a designated time period. Afterwards, KOH was added to the container to initiate the transesterificatio reaction. The reactions were carried out at ambient temperature. A control without any pretreatment was used.

For ultrasonic pretreatment, a low frequency ultrasonicator was used to process algae and methanol mixture for 1 h. In the microwave pretreatment, a glass test tube containing algae-methanol mixture was irradiated in a microwave oven for 5, 10, and 15 sec. It was assumed that no methanol evaporation occurred during these short time microwave treatments. During both the ultrasonic and microwave pretreatments, heat was

generated. To evaluate the thermal effect, algae-methanol mixture was heated in a water bath for 10 min when the mixture reached 60 ℃. For methanol soaking pretreatment, algae-methanol mixture was left in ambient temperature for 1 h before KOH was added. For the combined methanol soaking and microwave pretreatment, the algae-methanol mixture was left in ambient temperature for 1h before it was irradiated in microwave oven for 10 sec.

5.3 Result and Discussion

5.3.1 Microwave pretreatment

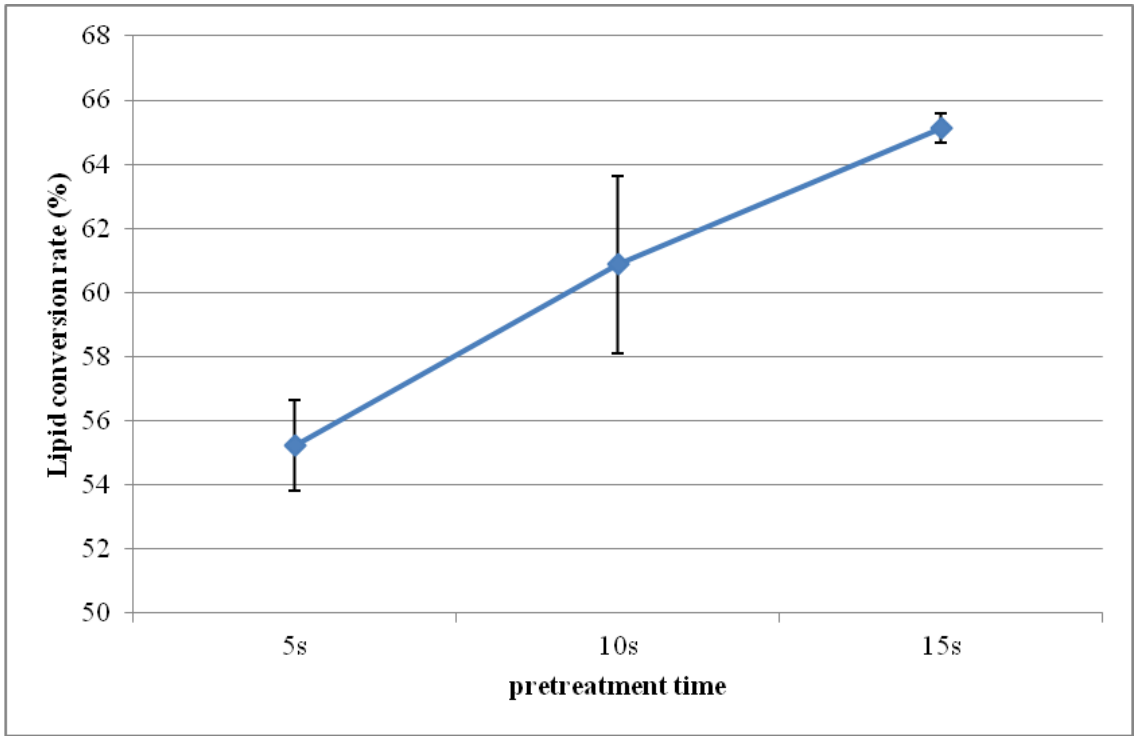

Figure 5.1 Lipid conversion rate of different microwave pretreatment time

Figure 5.1 shows that the lipid conversion rate increased linearly with microwave pretreatment time. The temperature after microwave pretreatment was also tested: 34 ℃

after 5 sec pretreatment, 59 ℃ after 10 sec pretreatment, and 60 ℃ after 15 sec pretreatment. There are two possible reasons for the improvement by microwave pretreatment. First, the heat efficiently generated by microwave accelerated the solvent (methanol) diffusion into the cells. Second, the microwave irradiation could help to disrupt the cellular structure and hence improved the solvent permeability (Marconi, Ruggeri et al. 2000). To analyze whether the high temperature is the reason for the conversion rate improvement, a water bath heating pretreatment is tested. A prolonged microwave treatment may overheat the solvent, causing too high a temperature and loss of solvent through evaporation. Therefore, 15 sec is the best microwave pretreatment time.

5.3.2 Comparison of pretreatment methods

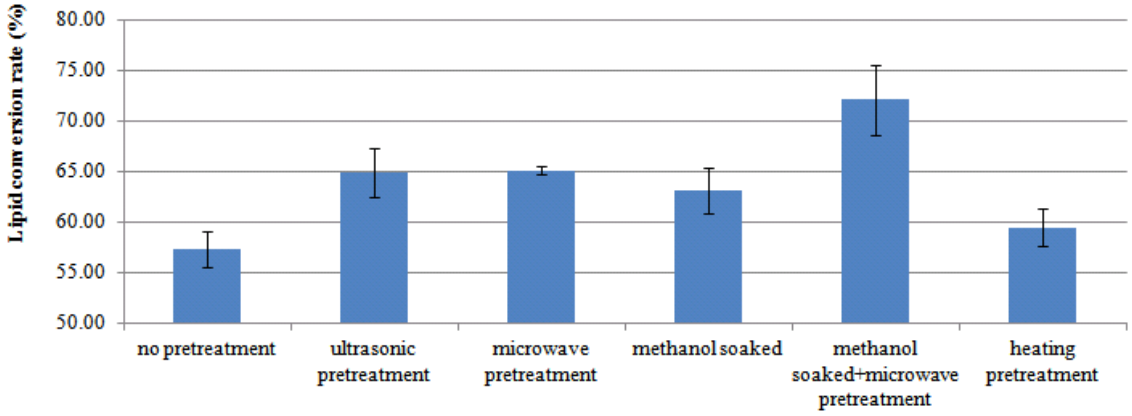

Figure 5.2 Comparison of pretreatment methods before *in situ* transesterification reaction

Almost all pretreatments were found to increase the lipid conversion rate (Figure 5.2). The greatest improvement (14.8% over control) was seen with combined methanol soaking and microwave treatment (72.1%). While ultrasonic (64.9%), microwave (65.1%), and methanol soaking (63.1%), showed a similar lipid conversion rate improvement. The heating pretreatment got a similar lipid conversion rate with the control (59.4%). Therefore, heat played a minor role in the microwave and ultrasonic pretreatments. However, the synergetic effect of heat and microwave or ultrasonic cannot be ruled out. If such synergy is indeed significant, microwave is a more efficient process than the ultrasonic pretreatment because the temperature rose to 65 ℃ in 15 sec in microwave pretreatment.

For the ultrasonic pretreatment, ultrasound energy produces chemical and physical effects that arise from the collapse of cavitation bubbles. The collapse of cavitation bubbles disrupts the phase boundary in a two-phase liquid system and causes emulsification by ultrasonic jets that impinge one liquid in to the other (Georgogianni, Kontominas et al. 2008). This process could help the methanol enter the algae cell, or break the cell. After the ultrasonic pretreatment, the beaker contained the mixture was warm. The temperature of the mixture increased a little. The increasing temperature may also have a positive effect for the cell breaking. In order to confirm the main reason for the lipid conversion rate improvement, water bath heating pretreatment is studied. However, the whole pretreatment took one hour. The long time pretreatment consumed a lot of energy compared to other pretreatment methods.

The microwave pretreatment only lasted 15 seconds and the temperature could increase to 60 ℃ rapidly during the process. In view of energy consumption, this method is more efficient than the ultrasonic pretreatment. Like ultrasonic wave, microwave could also help the algae cell break down. Microwave irradiation could change the electrical field of polar molecules and ions and make them rotate rapidly. Very efficient heating can be obtained due to molecular friction (Azcan and Danisman 2007). Pressure of liquid water vaporization will break down the cell membrane and cell wall, the formation of tiny holes, resulting in cracks. Also, in order to confirm if the heat is the main reason for the increase of lipid conversion rate, water bath heating pretreatment is studied following.

Using water bath heating pretreatment, the lipid conversion rate just increased 2.1%. The heating time is longer than that of the microwave pretreatment and the temperature is much higher than that of the ultrasonic pretreatment. However, the lipid conversion rate is lower than these two pretreatments. Therefore, heating might improve the lipid conversion rate observed by the ultrasonic wave and microwave pretreatments, but it is not the main reason of the increase the lipid conversion rate.

To shorten the reaction time, a methanol soaking pretreatment method was tested. The conversion rate of biodiesel is similar to that of the ultrasonic wave and microwave pretreatment. That means methanol soaking indeed improve the efficiency by extracting the lipid in advance. Since methanol soaking do not need any energy input, the method has a big advantage compared to other pretreatment methods.

In order to further investigate the effect of pretreatment, a method of combining methanol soaking and microwave was evaluated, Since methanol did not need heating and microwave pretreatment just lasted 15 sec, these two methods have the lowest energy consumption. From Figure 5.2, it indicated that the lipid conversion rate was almost 10% higher than other pretreatment method. The effect of the two pretreatment can be superimposed. Methanol soaking gave the time to let the methanol enter the algae cell. The following microwave pretreatment further increase the effect of the cell broken and penetration.

In terms of energy input and productivity, the ultrasonic pretreatment and the water bath heating require a relatively long process time and energy input. Since methanol soaking does not need any energy input, the method soaking pretreatment has a big advantage over other pretreatment methods. However, the microwave and methanol soaking treatments alone resulted in relatively low conversion rate. Our study demonstrated that combining methanol soaking with microwave pretreatment is the best approach.

Chapter 6 Comparison of *in situ* transesterification method and traditional two-step reaction process

6.1 Introduction

Industrial biodiesel synthesis from oils involves isolation of oilseed glycerides by extrusion or solvent extraction, degumming and refining of the oil, and its alkali-catalyzed transesterification. This technology includes two steps at least ---extraction and tranesterification. In a large scale plant, release of solvent contributes to the production of atmospheric smog and to global warming and is classified as a hazardous air pollutant. Thus, simplification of the oil production or esterification processes could reduce the disadvantages of this attractive biobased fuel.

In situ transesterification differs from the conventional reaction in that the oil-bearing material contacts with alcohol directly instead of reacting with pre-extracted oil and alcohol. In other words, the extraction and transesterification proceed in one step. In this combined process, the alcohol acts as both the extraction solvent and the esterification reagent. Methanol is helpful in dissolving polar lipids and destroying the association between lipids and cell constituents, and eliminates the need for separate extraction step (Smedes and Thomasen 1996). In our study or previous studies, this combined process also increases the conversion rate and decreases the amount of solvent waste over the conventional route.

The conventional extraction of algal cells for biodiesel production, the extraction procedure was adapted from the protocol described by Bligh and Dyer (Bligh EG, Dyer WJ.1959), in which two solvent are used. For the following alkali-catalyzed

transesterification, since the solvent directly contacts the oil, less solvent will be used compared to the *in situ* transesterification. The reaction condition of *in situ* transesterification is the same as the optimal reaction condition studied in chapter 3.

The optimum lipid conversion rate obtained in the present study was 90.7% under optimal process conditions as discussed above. To put this in perspective, the lipid conversion rate from the two-step traditional process was compared with those obtained in previous chapters. In this experiment, oil was extracted from algae and subjected to transesterification.

6.2 Two step traditional transesterification reaction

6.2.1 Oil extraction

One g oven dried algae was placed in a 50 ml conical polypropylene tube. A mixture of 4 ml water, 10 ml methanol and 5 ml chloroform was introduced to the tube, and stirred for 2 min to obtain a well-distributed system on a vortex mixer. Then the second 5 ml chloroform was added and stirred for 30 s on the vortex mixer. Finally, 5 ml water was added and stirred for 30 s on the vortex mixer. The homogenate was then centrifuged at 2,000 rpm in a centrifuge (International Equipment Co., Boston, MA, USA) for 10 min at room temperature to form a two-phase liquid. The chloroform layer was recovered and placed into a pressure tube. The microalgal oil was collected after organic solvent was dried off under the N-EVAP Analytical Nitrogen Evaporator at room temperature, and the flow rate is 500 ml/min (Organomation Associates Inc., USA). This procedure was repeated three times.

6.2.2 Alkali-catalyzed transesterification

Five ml methanol containing 2% KOH (of the algae lipid content) was added to the tube containing extracted lipids. The tube was immersed in a water bath at 90 ℃ for 1 h followed by addition of hydrochloric acid (36%, w/w) to stop the reaction. After the mixture cooling, added 5 ml chloroform and stirred for 2 min to transfer the biodiesel into the organic (chloroform) phase. The crude biodiesel was purified by washing with sodium chloride solution (0.9 M). The mixture was then centrifuged at 2000 rpm in a centrifuge (International Equipment Co., Boston, MA, USA) for 10 min at room temperature, which resulted in a two-phase system. The chloroform layer in the tube was measured. Three ml chloroform phase was transferred into a weighed, clean, 5 ml glass tube, and then organic solvent was dried off under the N-EVAP Analytical Nitrogen Evaporator at room temperature, and the flow rate is 500 ml/min (Organomation Associates Inc., USA). This procedure was repeated three times.

6.3 Result and discussion

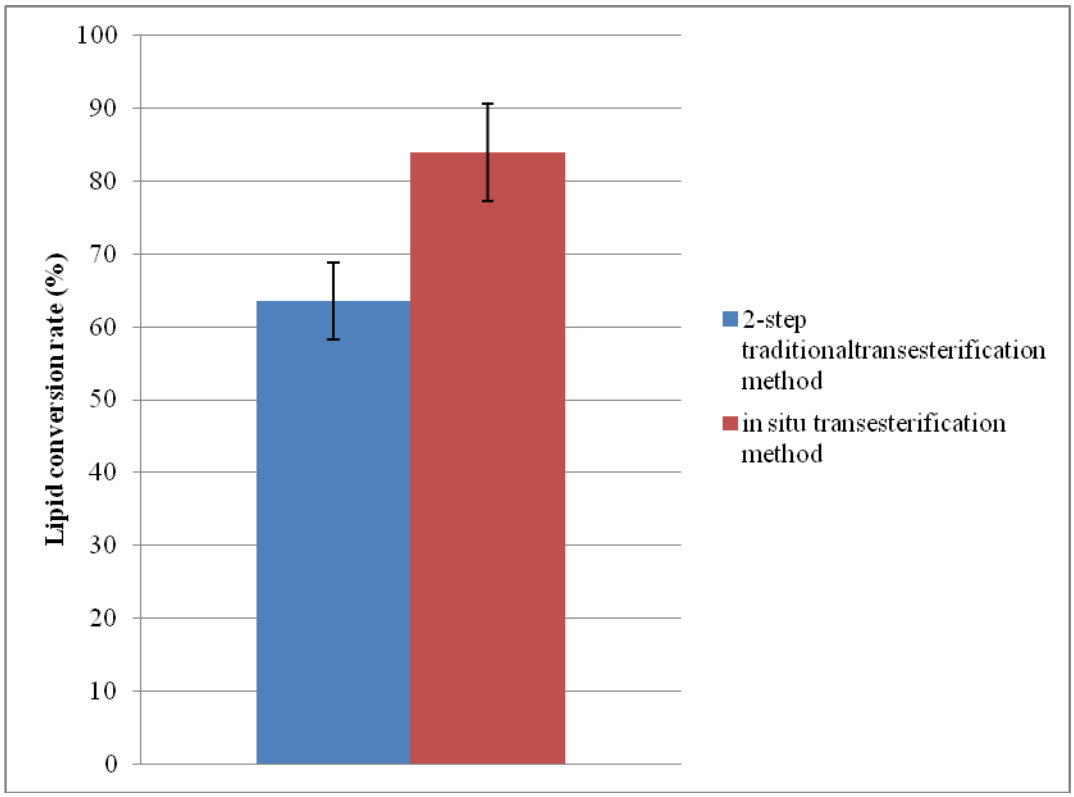

Figure 6.1 Lipid conversion rate of two-step conventional transesterification and *in situ* transesterification

The conventional way to produce biodiesel is to first extract the oil on the algae and then convert the oil to biodiesel through the transesterification process. In the 2-step transesterification process, the algal oil extraction procedure was adapted from the protocol described by Bligh and Dyer and followed by alkali-catalyzed transesterification. For both of the methods, 2% concentration of catalyst, and 1 g dry algae were used.

Figure 6.1 shows the lipid conversion rate from the two-step process and the optimum conversion rate from our *in situ* transesterification at ambient laboratory temperatures (25 ℃). The conversion for the two-step process was 63.5% compared with

84.0% for the *in situ* transesterification process. Therefore, from this data the *in situ* process was 20.5% more efficient than the traditional 2-step process. Furthermore, the conventional process uses 14% more solvents (methanol and chloroform) than the *in situ* process.

Additionally, water was used during the oil extraction step. Although the water and methanol phase were removed, there is a risk that some water may remain in the chloroform phase which contains oil. During the process of removing chloroform to produce oil, the residual water may not be completely removed by nitrogen stream. Therefore, the residual water may promote the saponification and hence reduce lipid conversion rate.

Our study demonstrated that the *in situ* transesterification can occur at room temperature, which decreases the energy input of heating. Most traditional transesterification requires heating during the process. The single-step *in situ* process eliminates the needs for extraction of oil from algal biomass and thus consumes less energy. Although the reaction time for *in situ* transesterification is relatively long, it--however-- saves substantial time by eliminating the oil extraction step. In addition, *in situ* transesterification method may reduce the potential lipid loss during the extraction step. Therefore, the in-situ transesterification has a great potential for maximum conversion of triglycerides into their corresponding fatty acid methyl esters and high lipid conversion rate.

Chapter 7 Conclusions

At room temperature, the best lipid conversion rate at room temperature is 84.0% and the best conditions for the *in situ* transesterification process are: concentration of catalyst (KOH) is 2% of the lipid amount, reaction time is 10h, and the methanol amount is 16.4 ml. At temperatures above 45 ℃, the optimal reaction time was 4 h. It was found that 60 ℃ was better than 45 and 75℃. And the best lipid conversion rate at 60 ℃ is 90.7%. Almost all pretreatment methods tested were able to improve lipid conversion rate. The best pretreatment was combination of methanol soaking and microwave irradiation, which increased the conversion rate by 14.8% compared with the control. The two-step traditional transesterification method was also tested for comparison purpose. The result suggested that *in situ* direct transesterification produced higher conversion rate than the conventional transesterification method, and could be an alternative, efficient and economical process for algal biodiesel production.

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