BIODIESEL PRODUCTION FROM CEIBA PENTANDRA OIL USING ENZYMATIC REACTION

NORJANNAH BINTI BASAN

FACULTY OF ENGINEERING
UNIVERSITY OF MALAYA
KUALA LUMPUR

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NORJANNAH BINTI BASAN

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Name: DR. ONG HWAI CHYUAN

Designation: *Senior Lecturer*
Department of Mechanical Engineering
Faculty of Engineering
University of Malaya

BIODIESEL PRODUCTION FROM CEIBA PENTANDRA OIL USING ENZYMATIC REACTION

ABSTRACT

Biodiesel is a type of renewable fuel and a potential alternative for continuously consumed fossil resources. Despite the fact that biodiesel productions commonly use chemical-catalyzed reaction due to its easy steps and high yield, enzymatic transesterification is also able to generate high biodiesel yield with an even greener approach: no chemical involve (except methanol as its substrate), no saponification, and no wastewater generation. Nonetheless, there are some problems associated with enzymatic reaction: high cost of lipase enzyme and its deactivation. In this research, a commercial enzyme, *Candida Antarctica* lipase immobilized on acrylic resin (Novozym 435) was used to convert non-edible oil from tropical resources, *Ceiba pentandra* (kapok) to biodiesel using methanol as acyl acceptor. *C. pentandra* oil was obtained from its seeds that were usually thrown away after the cotton had been collected. Tests on methanol concentration, stepwise addition, and enzyme pretreatment were conducted to improve enzyme activity. Optimization process (using artificial neural network based program and genetic algorithm) and enzyme reusability test were performed as an effort to reduce the total biodiesel production cost. The results obtained showed that high methanol concentration would cause enzyme deactivation and this could be prevented by maintaining each addition of methanol to about 1 molar equivalent per step. Furthermore, biodiesel yield increased when using t-butanol but decreased when using sodium chloride solution as enzyme pretreatment. Optimization process demonstrated that the optimum condition was at 57.42 °C temperature, 3:1 methanol to oil molar ratio, and 71.89 h reaction time to produce a biodiesel yield of 80.75%. The reusability of enzyme was measured at 63.69% relative yield after three batches. The calculated biodiesel production costs were at $15.69/L and $0.97/L for enzyme price at $800/kg

(current enzyme cost) and $8/kg (enzyme cost in the future) respectively. To conclude, biodiesel production from *Ceiba pentandra* oil using biocatalyst is feasible and can be further improved for industrialization.

Keywords: *Ceiba pentandra*, enzyme, biodiesel, stepwise addition, optimization

PENGHASILAN BIODIESEL DARI MINYAK CEIBA PENTANDRA MENGGUNAKAN TINDAK BALAS ENZIM

ABSTRAK

Biodiesel adalah sejenis bahan bakar boleh diperbaharui dan berpotensi menjadi pengganti untuk sumber fosil yang digunakan tanpa henti. Walaupun pembuatan biodiesel selalunya menggunakan bahan kimia sebagai pemangkin kerana langkahnya yang mudah dan hasil produk yang tinggi, transesterifikasi enzim juga dapat menjana hasil biodiesel yang tinggi dengan pendekatan yang lebih mesra alam: tiada melibatkan bahan kimia (kecuali metanol sebagai bahan mentah), tiada saponifikasi (penghasilan sabun), dan tiada pembuangan air sisa. Namun begitu, terdapat beberapa masalah yang berkaitan dengan tindak balas enzim: kos enzim lipase yang tinggi dan penyahaktifannya. Dalam kajian ini, sejenis enzim komersial, *Candida Antarctica* lipase yang diletakkan pada resin akrilik (Novozym 435) telah digunakan untuk menukar sejenis minyak tidak boleh dimakan daripada sumber tropika, *Ceiba pentandra* (pokok kekabu atau kapok) kepada biodiesel menggunakan metanol sebagai penerima asil. Minyak *C. pentandra* telah diperolehi daripada biji benih yang biasanya dibuang selepas kapasnya telah dikumpulkan. Ujian ke atas kepekatan metanol, penambahan metanol langkah demi langkah, dan rawatan awal enzim telah dijalankan untuk meningkatkan aktiviti enzim. Proses optimisasi (menggunakan program buatan rangkaian neural bersama-sama algoritma genetik) dan ujian penggunaan semula enzim telah dilakukan sebagai satu usaha untuk mengurangkan jumlah kos pengeluaran biodiesel. Keputusan yang diperolehi menunjukkan bahawa kepekatan metanol yang tinggi akan menyebabkan penyahaktifan enzim dan ini boleh dicegah dengan mengekalkan setiap penambahan metanol kepada kira-kira 1 molar persamaan bagi setiap langkah. Tambahan pula, hasil biodiesel meningkat apabila menggunakan t-butanol tetapi menurun apabila menggunakan larutan natrium klorida sebagai rawatan awal enzim.

Proses optimisasi menunjukkan keadaan optimum adalah pada suhu 57.42 °C, 3:1 nisbah molar metanol kepada minyak, dan 71.89 jam masa tindak balas, untuk menghasilkan biodiesel sebanyak 80.75%. Kebolehgunaan enzim adalah sebanyak 63.69% hasil relatif selepas tiga kali penggunaan. Harga biodiesel hasil dari pengiraan adalah berjumlah $15.69/L dan $0.97/L, masing-masing berdasarkan harga enzim sekarang iaitu $800/kg dan harga enzim pada masa akan datang iaitu $8/kg. Kesimpulannya, pengeluaran biodiesel daripada minyak *Ceiba pentandra* menggunakan enzim sebagai pemangkin boleh dilaksanakan dan diperbaiki lagi untuk perindustrian.

Kata kunci: *Ceiba pentandra*, enzim, biodiesel, penambahan langkah demi angkah, optimisasi

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I love Allah

I love Mom & Dad

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LIST OF SYMBOLS AND ABBREVIATIONS

ANN	:	Artificial neural network
ASTM	:	American society for testing and material
BBD	:	Box-behnken design
DMC	:	Dimethyl carbonate
FAAE	:	Fatty acid alkyl ester
FAEE	:	Fatty acid ethyl ester
FAGC	:	Fatty acid glycerol carbonate
FAME	:	Fatty acid methyl ester
FFA	:	Free fatty acid
FTIR	:	Fourier transformed infrared
GA	:	Genetic algorithm
GC	:	Gas chromatography
MOC	:	Maintenance and operational cost
MSE	:	Mean square error
Mtoe	:	Million tonnes of oil equivalent
PBR	:	Packed bed reactor
R	:	Correlation coefficient
R^2	:	Coefficient of determination
RMSE	:	Root mean square error
RSM	:	Response surface methodology
STR	:	Stirred tank reactor
TAG	:	Triacylglyceride
TEC	:	Total equipment cost
TG	:	Triglyceride
Wt.%	:	Weight percent

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1 Background

Biofuel has been targeted as one of the alternatives for the non-renewable fossil fuel that keep on depleting each day. Biofuels are produced in three different states: solid (bio-char), liquid (bioethanol, biodiesel) and gaseous (biohydrogen, biogas) (Mubarak et al., 2015). As shown in **Figure 1.1**, biodiesel can be categorized into three generations: 1st generation which derived from edible vegetable oils; 2nd generation from non-edible vegetable oils (including *Ceiba Pentandra*) and waste cooking/frying oil; and 3rd generation from algae and other microorganisms (Mubarak et al., 2015; Singh et al., 2014).

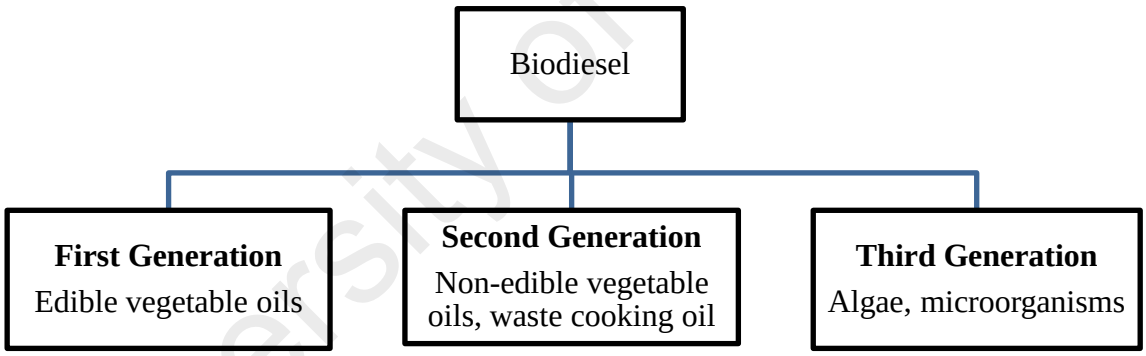

Figure 1.1: Classification of biodiesel

According to International Energy Agency (2015), 10.2% of world total primary energy supply in the year 2013 was contributed by biofuels and waste while 3.6% from other renewable sources such as hydro, geothermal, solar, wind, and heat (**Figure 1.2**). This data shows that biofuel has been used widely as energy source together with oil (31.1%), coal (28.9%) and natural gas (21.4%). Furthermore, data from BP Statistical Review of World Energy (2015) shows that the world total biofuel production in 2014 was 70.8 Mtoe (million tonnes of oil equivalent) and the largest producer was United

States at 30.1 Mtoe (**Figure 1.3**). About 10.6% of the biofuels were produced by Asia Pacific countries such as China, Indonesia, and Thailand.

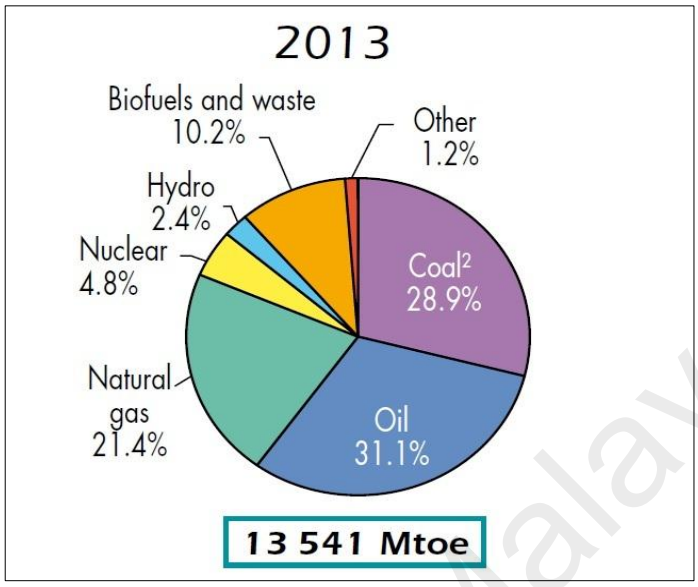

Figure 1.2: World total primary energy supply. Other (1.2%) include geothermal, solar, wind, heat, etc. (International Energy Agency, 2015)

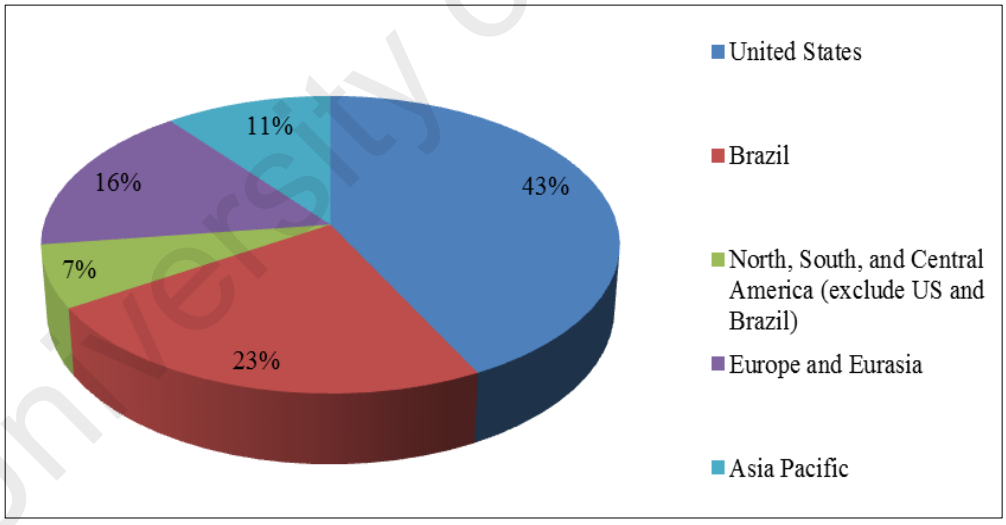

Figure 1.3: World biofuels production in 2014 (BP, 2015). Asia pacific includes Indonesia (3.5%), China (2.9%) and Thailand (2.0%).

Many countries, especially the major biofuel producing countries, have implemented biofuel policies to boost the growth of their biofuel sector. For example, in United States, Energy Policy Act of 2005 established a renewable fuel standard (RFS) that required the increase of renewable fuel usage from 9 billion per year in 2008 to 36

billion per year in 2022 (Y. Su et al., 2015). In 2012, the US president announced the establishment of "All-of-the-above energy" policy to make a long-term plan that uses every available sources of energy including wind, solar and biofuels. Other incentives such as tax credits of $1.01 per gallon and $1 per gallon were given to cellulosic biofuel and biodiesel productions respectively from December 2011 to December 2013.

Meanwhile in Brazil, invention of flexible fuel vehicle that can run on any gasoline-ethanol blend has increased the growth of its national ethanol market. Brazil also gives taxes exemption (PIS and CONFIN) for ethanol industries and provides low-interest loans and subsidies to sugarcane farmers for land expansion (Y. Su et al., 2015). The increasing proportion of biofuel blends in the market that is supported by government mandates also helps to sustain biofuel industry.

In Malaysia, its National Biofuel Policy has introduced biodiesel fuel blend in 2009 and the main feedstock for the biodiesel production is palm oil and its residues such as empty fruit bunches, shells and fibers (Ashnani et al., 2014). The current implementation of biodiesel in this country is at B7 (7% biodiesel in diesel) and it is expected to increase to B10 (10% biodiesel in diesel) at the end of year 2017.

Plant oil can be converted into alkyl ester through reactions called esterification or transesterification. There are three methods commonly used for biodiesel production: non-catalyzed reaction; chemical-catalyzed reaction, and enzyme-catalyzed reaction. Enzyme-catalyzed reaction will require the use of an enzyme called lipase to facilitate the conversion process. Immobilized lipase is much more preferred than free lipase as it can be reused for several cycles. As each type of enzyme is distinctive, many studies have been done to learn more about their specificity and reactivity. For biodiesel production process, the performance of a lipase is based on how efficiently it converts

oil that has different types of fatty acids and glycerides (tri-, di-, and monoglyceride) into fatty acid alkyl ester (FAAE).

1.2 Problem statement

The demand of fuel for transportation and industry has been increasing and causes the depletion of non-renewable energy such as petroleum and natural gas. In addition, the burning of fossil fuels contributes to carbon dioxide and methane gas emissions that have been associated with global warming and harming the Earth. These problems have become the reasons to find alternative sources of energy that are sustainable and also environmental friendly. One of the potential alternatives is by using plant oils as fuel.

The benefit of using biodiesel from plant is that its combustion will not increase the net atmospheric levels of carbon dioxide (E.-Z. Su et al., 2007). However, biofuel produced from edible plant oil has led to increase of food price and causes major controversy of food versus fuel. A possible solution is by using non-edible plant oils that is renewable, greener and free from any controversial issues. One of non-edible oils that can be used for biodiesel feedstock and is available in tropical areas including Malaysia and Indonesia is *Ceiba Pentandra* (kapok) oil. This tree is mainly grown for its fiber that is being used as stuffing material for mattresses and pillows. The oil is extracted from its seeds that were usually thrown away as waste.

Despite the fact that biodiesel productions commonly use chemical-catalyzed reaction due to its easy steps and high yield, it still has several drawbacks including saponification and generation of wastewater. Another method which uses enzyme as catalyst is also capable of producing high biodiesel yield with an even greener approach: use no chemical (except methanol as its substrate) and no generation of soap or wastewater. The high cost of lipase can be compensated by optimizing the reaction

and recycling of the enzyme. Enzyme performance may be enhanced by implementing methanol stepwise addition and enzyme pretreatment.

Research questions:

1. Usage of edible oil (palm oil) may cause food versus fuel controversy. Is there any other feedstock for biodiesel production using non-edible oil?
2. *C. pentandra* trees are grown for its fiber and the seeds are thrown away as waste. Could the seed oil be used as the source of non-edible oil?
3. Usage of chemical catalyst for biodiesel production may lead to saponification and production of wastewater. Could enzyme be used as the catalyst for *C. pentandra* biodiesel production?
4. Could biodiesel production form *C. pentandra* oil using enzyme catalyst be improved using pretreatment methods and optimization process?
5. What is the production cost of biodiesel using enzyme catalyst?

1.3 Research objectives

The objectives of this research are as follows:

1) To produce *Ceiba pentandra* biodiesel production using enzyme catalyst and analyze its characteristics.
2) To examine the effects of enzyme pretreatment, methanol concentration, and methanol stepwise addition to improve enzyme performance
3) To optimize the *Ceiba pentandra* biodiesel production process based on three parameters setting (methanol to oil molar ratio, temperature, and reaction time) using artificial neural network (ANN) and genetic algorithm (GA) to obtain a high biodiesel yield.
4) To measure the reusability of enzyme based on the biodiesel yield in *Ceiba pentandra* biodiesel production process.

5) To calculate biodiesel production cost per liter for *C. pentandra* biodiesel produced using biocatalyst.

1.4 Aim and scope of work

The aim of this study is to investigate and improve biodiesel production from non-edible *Ceiba pentandra* oil using enzyme as catalyst. The hypothesis for this research is *C. pentandra* biodiesel can be produced using enzyme catalyst and the process can be improved by using several methods including enzyme pretreatment, methanol stepwise addition, optimization, and enzyme reusability.

The enzyme used in this study is a commercially available and commonly used lipase called Novozym 435, a *Candida antarctica* lipase immobilized on acrylic resin. The biodiesel produced was mainly analyzed in terms of its fatty acid methyl ester (FAME) yield. The fuel properties were determined and compared with ASTM and EN international standards. Then, further experiments were carried out to tests several aspects of the enzyme-catalyzed biodiesel production which include effects of methanol concentration and its stepwise addition, enzyme pretreatment, optimization of the biodiesel production process, and enzyme reusability. An economic evaluation was also conducted to calculate biodiesel production cost per liter.

1.5 Thesis contributions

This thesis contains useful additional data on how certain conditions would affect FAME yield. The results were primarily related to how the enzyme reacts to its surrounding. For example, high concentration of methanol could decrease the yield thus stepwise addition of methanol should be incorporated in the biodiesel reactor (methanol ratio need to be maintained below 2 molar per addition). For oil feedstock with high free fatty acid content, lipase could not convert all the FFA to FAME thus oil pretreatment is needed to reduce the FFA amount. In addition, this study shows that combination of

ANN and GA software could be utilized for the optimization process of enzyme-catalyzed biodiesel production to gain high output.

1.6 Thesis outline

Chapter 1 will focus on the background of biodiesel and its current status. It will also contain problem statements and objectives of this research.

Chapter 2 is the literature review that contains information of different methods of biodiesel production, explains the important raw materials required for enzymatic transesterification, and describes the previous studies conducted on biodiesel production using enzymatic reaction including details on methanol concentration and stepwise addition, enzyme pretreatment, optimization, enzyme reusability, and biodiesel production cost.

Chapter 3 will explain the materials and research methodology in details.

Chapter 4 will describe the results obtained from the research works and provide critical analysis, discussion, and comparison with results from previous studies.

Chapter 5 will conclude what is obtained from this research, presents the key findings and also suggests some recommendations for future studies.

CHAPTER 2: LITERATURE REVIEW

2.1 Biodiesel production process

Generally, biodiesel is produced in form of fatty acid alkyl ester (FAAE) through esterification reaction of fatty acids with short chain alcohols or transesterification reaction of triglyceride (TG) with short chain alcohol that generate glycerol as byproduct (Röttig et al., 2010). Three methods commonly used for biodiesel process are: (i) non-catalyzed reaction; (ii) chemical-catalyzed reaction; and (iii) enzyme-catalyzed reaction (**Figure 2.1**).

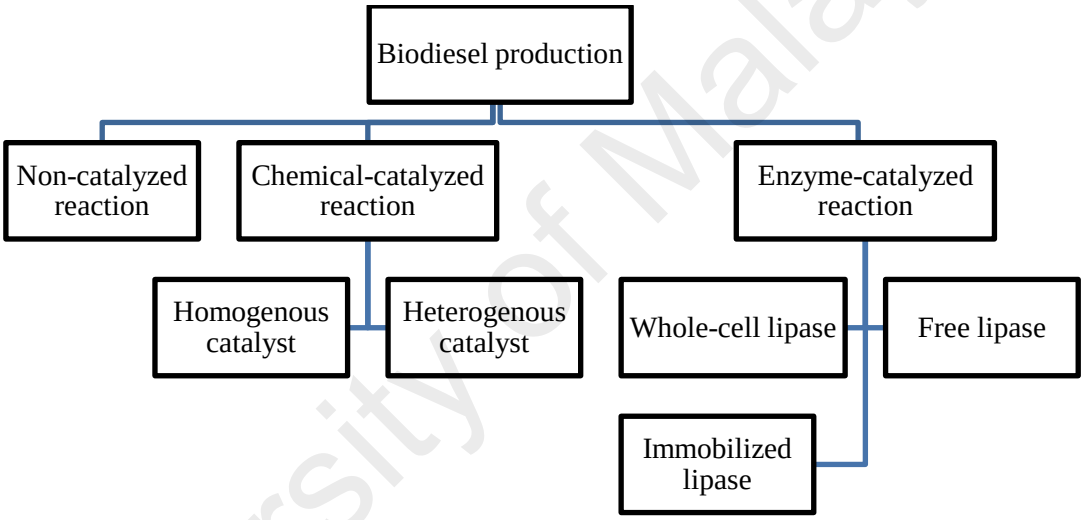

Figure 2.1: Various biodiesel production methods

Non-catalyzed reaction usually involved transesterification in supercritical conditions (methanol or ethanol). Non-catalyzed reaction has high reaction rate, easy separation of products and no waste generation (Stamenković et al., 2011). This reaction can complete in a short time as fast as 2 minutes but requires high temperature and pressure ranges from 280 to 400 °C and 10 to 30 MPa, consumes great energy, and involves high cost (Aransiola et al., 2014; Atabani et al., 2013; Madras et al., 2004).

Chemical-catalyzed reaction is divided into homogenous- and heterogenous-catalyzed reaction. Homogenous-catalyzed reactions involve the usage of acid or alkali catalysts in liquid form. The examples of homogenous acid catalysts are hydrochloric,

sulphuric, sulfonic and phosphoric acids, while for homogenous alkali catalysts are sodium hydroxide, sodium methoxide, potassium hydroxide, and potassium methoxide (Aransiola et al., 2014; Bharathiraja et al., 2014 ; Ong et al., 2014). Biodiesel productions from non-edible feedstocks such as *Jatropha curcas, Ceiba pentandra, Sterculia foetida,* and C*alophyllum inophyllum* using homogenous catalysts have been done previously together with the tests on fuel properties and engine performance (Ong et al., 2014; H. C. Ong et al., 2013 ; Ong et al., 2014).

Heterogenous-catalyzed reactions involve the usage of acid or alkali catalysts in solid form. Examples of heterogenous acid catalysts are sulphated zirconia, tungstated zirconia, heteropoly acids (HPAs), and Nafion-NR50 while for heterogenous alkali catalysts are calcium based mixed metal oxides (CaO-MgO), alkaline earth metal oxides, hydrotalcites, and basic zeolites (Aransiola et al., 2014; Bharathiraja et al., 2014; Taufiq-Yap et al., 2011). New heterogenous catalysts such as binary metal oxide CaO-La_2O_3 that has both acid and base properties and can catalyze esterification and transesterification simultaneously have also been synthesized (H. V. Lee et al., 2015).

The advantage of using acid catalyst, either in solid or liquid form is its capability to convert FFA. Alkali catalysts are not suitable for converting oil with high amount of FFA because it can lead to soap formation (saponification). There are many disadvantages associated with chemical-catalyzed method such as high energy consumption, high cost of recovery and purification of catalysts and glycerol, and the need of wastewater treatment (Christopher et al., 2014; Juan et al., 2011). Wastewater is mainly generated from the washing step to remove soap and glycerin impurities from biodiesel product that can cause engine and fuel storage problems (Wall, 2011).

Because of the mentioned problems, researchers have started to explore enzyme-catalyzed reaction. The main reason for choosing enzymatic reaction is due to its green

aspect: no usage of chemical catalyst and no generation of wastewater. Other advantages of biodiesel production using enzyme will include high specificity towards substrate, wide substrate variation, catalysis of free fatty acids, high quality of products, mild reaction temperatures, low alcohol to oil ratio, and no saponification (Christopher et al., 2014; Fjerbaek et al., 2009). The comparison of advantages and disadvantages between enzymatic reaction and other methods are listed in **Table 2.1**.

Table 2.1: Comparison of biodiesel production using enzymatic reaction, non-catalyzed supercritical condition, and chemical-catalyzed reactions (Aransiola et al., 2014; Atabani et al., 2013; Gog et al., 2012; Stamenković et al., 2011 ; Guldhe et al., 2015).

Methods	Advantages	Disadvantages
Enzymatic reaction (immobilized lipase)	Medium yield, can convert FFA, low energy usage, high product and by-product purity, reusable catalyst, no wastewater	Inhibition by alcohol or by-product, high cost of enzyme, slow reaction
Non-catalyzed reaction (supercritical alcohol)	Super-fast reaction, high yield, can convert FFA, no catalyst, easy product purification, no waste	High temperature and pressure, high cost of reactor, high alcohol to oil molar ratio
Chemical-catalyzed reaction (homogenous)	High yield, low cost Can convert FFA (acid catalyst)	Wastewater, need product purification steps, difficult catalyst recovery Saponification (alkali catalyst)
Chemical-catalyzed (heterogenous)	Fast reaction, high yield, reusable catalyst, medium cost, can be used in continuous process Can convert FFA (acid catalyst)	High energy, difficult catalyst preparation, catalyst leaching Saponification (alkali catalyst)

Industrial scale production of biodiesel using enzyme as catalyst is no longer conceptual. In recent years, enzyme manufacturers and biodiesel producers have collaborated with each other to develop new technology of enzymatic biodiesel production that is more feasible and economical. For example, Novozymes (an enzyme maker company from Denmark) has collaborated with many biodiesel producer companies such as Piedmont Biofuels, Blue Sun Biodiesel, WB services, Buster

Biofuels, including Viesel Fuel LLC that has a enzymatic biodiesel production line with a capacity of 5 million gallons output per year (Hobden, 2014; Kotrba, 2014).

There are already many biodiesel plants that produced biodiesel using enzymatic reaction presently. In 2007, Lvming Co. Ltd. built an enzymatic production line with capacity of 10,000 tons in Shanghai, China (Tan et al., 2010). The factory used immobilized lipase Candida sp. 99–125 as catalyst (0.4% to the weight of oil) and waste cooking oil as raw material. About 90% FAME yield was obtained under optimal condition. The process was conducted in a stirred tank reactor, and a centrifuge was used to separate glycerol and water. In 2012, Piedmont Biofuels, North Carolina, developed a new technology (FAeSTER) for a continuous biodiesel production using immobilized or liquid enzyme (Christopher et al., 2014). They established an enzymatic biodiesel process that can utilize high free fatty acids feedstocks, as high as 100% FFA (Piedmont Biofuels, n.d.). Another factory, Hainabaichuan Co. Ltd. in Hunan Province, China, applied the technology from Tsinghua University and used commercial Novozyme 435 as catalyst (Tan et al., 2010).

Nonetheless, enzyme-catalyzed biodiesel production is still not widely used compared to chemical-catalyzed due to its high cost, slow reaction rates, enzyme inhibition and loss of activity (Christopher et al., 2014; Fjerbaek et al., 2009). Therefore, further improvement to reduce the price, increase the reaction rate, or minimize enzyme deactivation will be revolutionary.

2.2 Enzymatic transesterification

There are several factors that will affect the yield of biodiesel produced using enzymatic reaction. The factors include lipase specificity and efficiency, lipase immobilization, substrate fatty acid composition and types of acyl acceptor used. Furthermore, different enzyme might need different operating conditions for its

optimum activity. The main parameters to be controlled for the operating condition include temperature, acyl acceptor to oil molar ratio, lipase amount, reaction time, and stirring speed. Other factors that could also affect enzyme activity are water content, pH and solvent.

The temperature for biodiesel production using enzyme ranges from 20°C to 60°C (Maceiras et al., 2011) and the optimum temperature in solvent-free system ranges from 30°C to 50°C (Szczęsna Antczak et al., 2009). Low temperature may cause the enzyme to be inactive while high temperature may cause denaturation of its molecular structure. Stirring speed need to be adjusted at an optimum rate so that the mechanical stress will not damage or harm the enzyme.

Optimum pH and water content is needed to maintain enzyme structure and keeping it active. The amount of water needed depends on the types of lipase, immobilized support, and the organic solvent used in the reaction system (Lu et al., 2009). Water content needs to be controlled because excessive water will cause hydrolysis reaction (production of fatty acids) being favored more than transesterification (production of FAAE) thus reduces the yield (Lu et al., 2008; E.-Z. Su et al., 2007). Besides, water also involves in several mechanisms that could cause lipase inactivation (Salis et al., 2005).

Biodiesel production through enzymatic reaction usually consumes long period of time. Many reactions need about 12 to 24 hours to achieve complete conversion and some may take up to 72 hours. A fast reaction (short reaction time) is better than a slow reaction because it will consume lesser energy (heat) per cycle and reduce mechanical stress acts upon the lipase. Although high amount of lipase is capable of shortening the reaction time, it is not advisable because enzyme is very costly. Moderate amount of lipase that able to produce optimum conversion yield is more preferred. Many tests have been done to reduce the reaction period of enzymatic reaction including lipase

pretreatment and adding of solvent. Furthermore, the configuration of reactor may also affect the reaction period and productivity.

Biodiesel is produced in a reactor in either batch or continuous system. There are many types of reactor that have been developed such as fluidized beds, expanding beds, recirculation, and membrane reactors (Gog et al., 2012). Among these, the common types of reactor used for biodiesel production are stirred tank reactor (STR) and packed bed reactor (PBR) (**Figure 2.2**). STR generally uses agitation/stirring to disperse the enzyme in the reaction mixture, while PBR contains packed enzyme in a column. The stability of immobilized lipase in term of mechanical and operational determines its suitability to be used in a reactor. For example, the immobilized support needs to have high resistance towards friction and shear stress in STR, and high resistance towards compression in high flow rates PBR (Poppe et al., (2015).

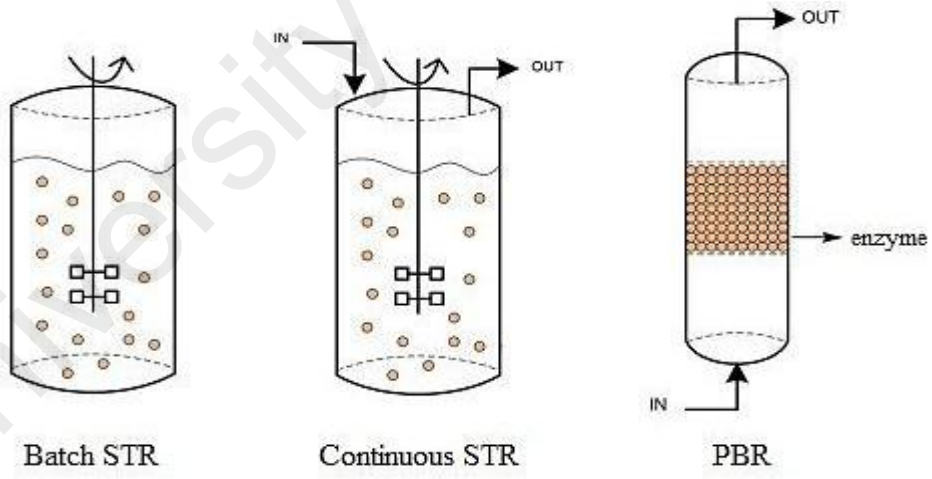

Figure 2.2: Reactor designs of batch STR (stirred tank reactor), continuous STR, and PBR (packed bed reactor) (Poppe et al., 2015)

The common problem associated with enzymatic production of biodiesel is inhibitory effect by alcohol and glycerol. Methanol is the most used acyl acceptor due to its cheaper price. However, it is toxic and may cause enzyme deactivation especially at higher concentration. To avoid enzyme deactivation, it is necessary to control the molar

ratio of acyl acceptor to oil (acyl acceptor : oil) used in the reaction. Glycerol is the by-product of transesterification reaction and could cause mass transfer limitation and reaction rate reduction (M. Lee et al., 2011). Glycerol is usually removed during biodiesel synthesis or separated from the product upper layer at the end of the reaction by mere standing (glycerol in bottom layer) (Shimada et al., 1999). Continuous biodiesel production will usually include a glycerol removal system to avoid accumulation of glycerol that may cause column clogging and pressure dropping (Tran et al., 2016).

Even though currently enzyme-catalyzed reaction is not the first choice for biodiesel production industry, it has a big potential to become one. One of the important tasks to do is to design a good enzymatic reaction, not only to reduce operational cost but also to get an optimum amount of biodiesel yield. High-yield enzymatic transesterification can be obtained by controlling the reaction conditions, manipulating the factors affecting the reaction, designing a good bioreactor, and also applying additional methods that can reduce enzyme inhibition or loss of activity during transesterification process. Above all else, it will depend on the selection of three major components of the process: lipase, oil and acyl acceptor.

2.2.1 Lipase

The type of enzyme that is used for biodiesel production is lipase (triacylglycerol acylhydrolase EC 3.1.1.3) and this enzyme will convert oil to biodiesel in the form of fatty acid alkyl ester and glycerol as its by-product. Lipases can be extracted from several sources such as fungi, bacteria and yeast (**Table 2.2**) and they possess different regioselectivity, specificity and catalytic activity.

Table 2.2: Different sources of lipase (Christopher et al., 2014)

Fungi	Bacteria	Yeasts
Alternaria brassicicola	*Achromobacter lipolyticum*	*Candida deformans*
Aspergillus niger	*Aeromonas hydrophilia*	*candida parapsilosis*
Candida antarctica	*Bacillus subtilis*	*Candida rugosa*
Mucor miehei	*Burkholderia glumae*	*Candida quercitrusa*
Rhizomucor miehei	*Chromobacterium viscosum*	*Pichia burtonii*
Rhizopus chinensis	*Pseudomonas aeruginosa*	*Pichia sivicola*
Rhizopus oryzae	*Pseudomonas cepacia*	*Pichia xylosa*
Streptomyces exfoliates	*Staphylococcus aureus*	*Saccharomyces lipolytica*
Thermomyces lanuginosus	*Staphylococcus canosus*	*Geotrichum candidum*

In terms of regioselectivity, lipases can be divided into four groups (Kapoor & Gupta, 2012; Poppe, Matte, et al., 2015; Szczęsna Antczak et al., 2009):

i. sn-1,3-specific: hydrolyze ester bonds at position sn-1 and sn-3

ii. sn-2-specific: hydrolyze ester bond at position sn-2

iii. fatty acid specific: hydrolyze ester bonds of long-chain fatty acids with double bonds in between C9 and C10

iv. non-specific: hydrolyze ester bonds at any positions

The product of the enzymatic reaction can be monoglyceride, and/or diglyceride or glycerol (complete breakdown). Among the four groups of lipase listed above, non-specific lipase is considered the best option and it is widely used for biodiesel transesterification due to its capability for a complete breakdown (hydrolysis) of triglyceride. Examples of non-specific lipases are lipases from *C. antarctica*, *C. rugosa, P. cepacia,* and *P. fluorescence* (Kaieda et al., 2001). Sn-1,3-specific lipases such as lipases from *R. oryzae, M. miehei* and *T. lanuginosa* are also good biocatalysts (Kaieda et al., 2001; L. Li et al., 2006; Nelson et al., 1996). Studies conducted using immobilized T. lanuginosa lipase obtained up to 100% conversion which is more than

its theoretical yield (66%) due to acyl migration from position (Du et al., 2005; R. C. Rodrigues et al., 2010; Tongboriboon et al., 2010).

Each lipase has different specificity towards its substrates, both triglyceride and alcohol. For triglyceride, the preferences include types of fatty acids, length of fatty acids, presence of double bonds and branching (Kapoor & Gupta, 2012; Szczęsna Antczak et al., 2009). For example, *C. antarctica* lipase prefers short- and medium-chain length fatty acids while *R. miehei* lipase prefers longer fatty acids (Poppe, Matte, et al., 2015). For alcohol, most lipases prefer primary alcohols compared to secondary and tertiary alcohols, with the tertiary as the least preferred (Kapoor & Gupta, 2012). For example, *P. cepacia* immobilized on diatomaceous earth reacts slower with 2-butanol compared to 1-butanol when converting triolein to oleic acid ester (Salis et al., 2005). Furthermore, different lipases show highest enzymatic activity with different alcohols or acyl acceptors. *C. antarctica* lipase immobilized on macroporous resin (Novozym 435) produced highest yield with methanol, *T. lanuginosus* lipase immobilized on acrylic resin (Lipozyme TL IM) reacted best with ethanol, while *R. miehei* lipase immobilized on anion-exchange resin (Lipozyme RM IM) preferred butanol (R. Rodrigues et al., 2008).

The mechanism for enzymatic transesterification follows ping-pong bi-bi mechanism (Fjerbaek et al., 2009; Gog et al., 2012). Ping-pong bi-bi mechanism can be described as two substrates react to produce two products through formation of enzyme-substrate intermediates (Guldhe et al., 2015). There are three kinetic pathways proposed in the literature: (1) direct alcoholysis of glycerides (triglycerides, diglycerides and monodiglycerides) into fatty acid alkyl esters ; (2) two consecutive steps which consist of hydrolysis (conversion of glycerides into free fatty acid) and followed by esterification (conversion of free fatty acids into esters) ; and (3) simultaneous reactions

of both alcoholysis and hydrolysis followed by esterification (Al-Zuhair et al., 2007; Canet et al., 2016; Cheirsilp et al., 2008; Y. Li et al., 2015; S. Liu et al., 2014).

Lipase has two different conformations: inactive closed form and active open form (Mateo et al., 2007). In aqueous medium, the equilibrium shift towards closed form, where the active center is blocked by a polypeptide chain called lid or flap (R. C. Rodrigues et al., 2013). Strategies that can be applied to immobilize lipase with open form include adsorption on hydrophobic support (**Figure 2.3**) and cross linking or lyophilization in the presence of detergent (Mateo et al., 2007; R. C. Rodrigues et al., 2013).

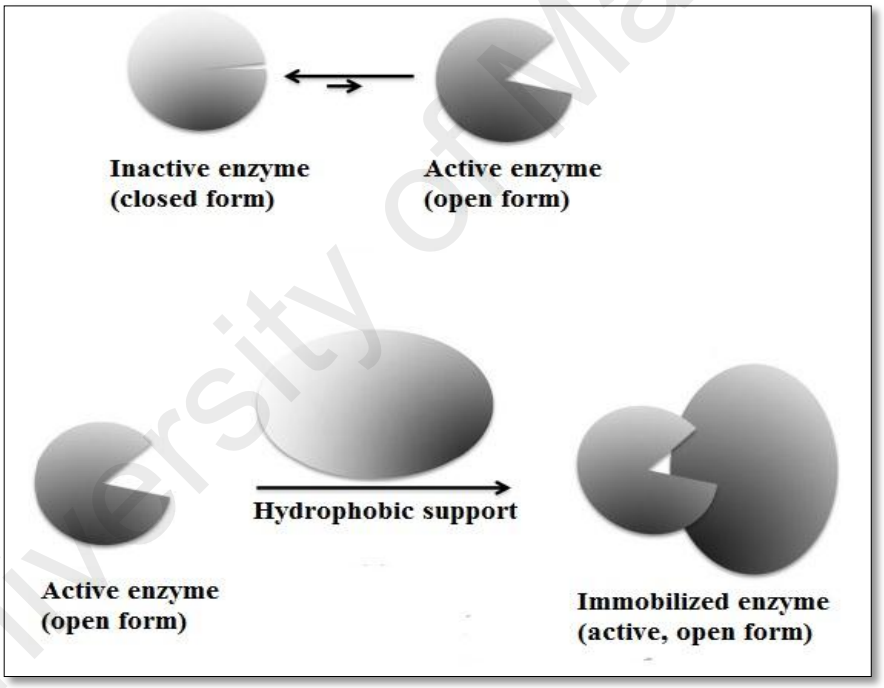

Figure 2.3: Immobilization of lipase enzyme on hydrophobic support (R. C. Rodrigues et al., 2013)

Immobilized lipase is much more preferred than free lipase because it promotes easy recovery and enables reuse of enzyme. It may also increase enzyme stability in the presence of organic solvents (Mohammadi et al., 2015) and improve enzyme relative activity (Maceiras et al., 2011). Immobilization of enzyme may affect enzyme activity, specificity and selectivity and also alter its structural form. These changes may not

always give positive effects to the enzyme properties. Some may cause improvement while some may lead to impoverishment. The improvement may be caused by stabilization of enzyme hyperactivated form, dispersion of enzyme on the support surface, protection against drastic conditions due to rigidification, and/or promotion of diffusional limitation and component partition by porous support (R. C. Rodrigues et al., 2013)

Immobilization method and support material may affect the enzymatic activity of lipase. For example, *P. cepacia* lipase immobilized on diatomaceous earth has faster reaction rate than *P. cepacia* lipase immobilized on ceramic particles or kaolinite (Salis et al., 2005). There are many types of supports that are good for lipase immobilization such as decaoctyl sepabeads, chitosan beads, glyoxyl activated agarose gels, green coconut fiber, mesoporous carbon beads, styrene-divinylbenzene beads, and periodic mesoporous organosilica (Gascon et al., 2014; Poppe et al.). There are four common methods for enzyme immobilization: adsorption, cross-linking, entrapment, and encapsulation (Ghaly, 2010). Other immobilization technologies invented are cross-linked enzyme aggregates (CLEA), protein-coated microcrystals (PCMC), cross-linked PCMC (CL-PCMC), magnetic particles carrier, and electrospun nanofibers (Guldhe, Singh, Mutanda, et al., 2015; Kumari et al., 2006).

Other than free lipase and immobilized lipase, there is also whole cell catalyst. The benefit of using whole cell catalyst is that there is no need for lipase extraction and purification steps, thus reduces its cost (Guldhe, Singh, Mutanda, et al., 2015). In addition, the immobilization process is not complicated since the *R. oryzae* cells immobilized spontaneously onto BSPs during its cultivation in air-lift bioreactor. Examples of whole-cell catalysts are whole-cell *R. chinensis* that produced 93% yield from soybean oil (He et al., 2008), whole-cell *A. nomius* with 95.3% yield from palm oil

(Talukder et al., 2013), and whole-cell *A. niger* with 90.82% yield from microalgal lipid (Guldhe et al., 2016).

One of the current topics in enzymatic reaction is genetic engineering, which includes the expression of different lipases in a single host organism. In recent years, there have been many studies conducted on recombinant lipases (Amoah et al., 2016; Duarte et al., 2015; Kuo et al., 2015). Yan et al. (2012) used whole-cell *Pichia pastoris* displaying both *C. antarctica* and *T. lanuginosus* lipases on its surface for converting soybean oil to biodiesel. They managed to get 95.4% conversion after 12.6 h, which is relatively short period of time. Furthermore, they found that the conversion percentage is about the same with the reaction combining same quantity of two immobilized lipases, Novozym 435 and Lipozyme TL IM (97.3%). This is believed to be able to lower the cost of buying different lipases separately. Another study was done by Guan et al. (2010) using *R. miehei* lipase (1,3-specific) and *P. cyclopium* lipase (non-specific) both expressed in and extracted from *Pichia pastoris*. They converted soybean oil to biodiesel and obtained 95.1% conversion after 12 h. Recombinant *Pichia pastoris* whole cell with intracellular overexpression of *T. lanuginosus* lipase was used as biocatalyst in biodiesel production from waste cooking oil and had produced 82% yield within 84 h (J. Yan et al., 2014).

The quest for the best lipase as biocatalyst in biodiesel production has never ended. Lipase with characteristics such as high tolerance in temperature, organic solvent, pH, and mechanical stress could promote enzymatic biodiesel production to a more feasible industry. Example of new type of lipase with desired properties is *Burkholderia ubonensis* SL-4 lipase that had good stability in non-ionic detergent and organic solvent, and maintained good activity at high temperature (50°C) and pH (pH 8.5) (Yang et al., 2016). Another example is lipase from *Bacillus safensis* DVL-43 which has great

stability in organic solvents as it able to retain 100% activity after 24 h incubation in xylene, DMSO, and toluene at 25% v/v (Kumar et al., 2014).

2.2.1.1 Novozym 435

In this research, *Candida antarctica* lipase immobilized on acrylic resin (commercial name: Novozym 435) was used. Novozym 435 is the isoform B of lipase from *Candida antarctica* (CAL-B) immobilized within a macroporous acrylic polymer resin which was most probably immobilized onto the material by hydrophobic interactions through undisclosed protocol (Poojari & Clarson, 2013). The resin has an average size of 315-1000 µm, pore diameter of about 150 Å, and surface area of 130 m^2/g (B. Chen et al., 2008). Novozym 435 has the ability to provide high regioselectivity during esterification and transesterification of sugars, showed high thermal stability up 100 °C in diphenyl ether, and able to maintain high catalytic activity when incubated in toluene at 80 °C for about a month (Poojari & Clarson, 2013; Sahoo et al., 2005).

Novozym 435 is commonly used due to its non-specificity, biocatalytic efficiency and availability. Several previous studies have shown that Novozym 435 produced the highest amount of yield or conversion when compared with other several lipases such as *Rhizopus delemar, Fusarium heterosporum, Aspergillus niger, Rhizomucor miehei* (Lipozyme RM IM and LipozymE IM60), and *Thermomyces lanuginosus* (Lipozyme TL IM) (Shimada et al., 1999; E.-Z. Su et al., 2007; Xu et al., 2003).

Water is usually needed in enzymatic transesterification to maintain lipase in active conformation (Salis et al., 2005). However, its amount needs to be controlled because excessive water will cause hydrolysis reaction (production of fatty acids) being favored more than transesterification (production of FAAE) thus reduces the yield (Lu et al., 2008; E.-Z. Su et al., 2007). The amount of water needed is different for each lipase. For example *P. cepacia* lipase immobilized on diatomaceous earth needs water activity (a_w)

at 0.4-0.6 for maximum enzymatic activity (Salis et al., 2005). Interestingly, unlike any other enzymes, there is no addition of water needed for the biodiesel production using Novozym 435. Previous research has found that Novozym 435 enzymatic reaction decreased with increasing water content (Shimada et al., 1999). This is beneficial since there will be no water removal step involved downstream that could increase the production cost.

Furthermore, Novozym 435 has already been used in biodiesel production industry. A factory named Hainabaichuan Co. Ltd. in Hunan Province, China, applied the technology from Tsinghua University and used commercial Novozyme 435 as catalyst (Tan et al., 2010). The enzyme maker company, Novozymes (from Denmark) has collaborated with many biodiesel producer companies such as Piedmont Biofuels, Blue Sun Biodiesel, WB services, Buster Biofuels, including Viesel Fuel LLC that has a enzymatic biodiesel production line with a capacity of 5 million gallons output per year (Hobden, 2014; Kotrba, 2014).

2.2.2 Oil feedstock

Oils that are currently used as sources of triglyceride (also known as triacylglyceride) for biodiesel production include edible vegetable oil, non-edible vegetable oil, algae oil, and waste frying/cooking oil. List of potential sources for edible oil, non-edible oil and algae oil for biodiesel production is tabulated in **Table 2.3**.

As mentioned previously, non-edible oil (second generation biodiesel) is usually chosen over edible oil (first generation biodiesel) to avoid food versus fuel controversy. Non-edible plants have better traits which include pest and disease resistant and able to grow at arid land, higher rainfall, or non-agricultural areas (Atabani et al., 2013). In addition, biodiesel production from non-edible oil could create jobs in rural places and

produce useful by-product (seed cakes) that can be used as fertilizers (Atabani et al., 2013)

Biodiesel productions from non-edible feedstocks such as *Jatropha curcas, Ceiba pentandra, Sterculia foetida,* and *Calophyllum inophyllum* have been done previously together with the tests on fuel properties and engine performance (Ong et al., 2014; H. C. Ong et al., 2013 ; Ong et al., 2014). The biodiesels showed good fuel properties and engine performance in term of engine torque, engine power, fuel consumption, and brake thermal efficiency. Modi et al. (2007) conducted biodiesel production of *Jatropha curcas* and *Pongamia pinnata* oils using ethyl acetate and obtained 91.3% and 90% yield respectively.

Table 2.3: Potential sources for edible oil, non-edible oil and algae oil for biodiesel production (Aransiola et al., 2014; Atabani et al., 2013; Demirbas & Fatih Demirbas, 2011; Gui et al., 2008; Noraini et al., 2014)

Non-edible oils	
Jatropha curcas L.	*Calophyllum inophyllum L.* (polanga)
*Ceiba pentandra (*kapok)	*Madhuca indica* (mahua)
Carton megalocarpus	*Nicotiana tabacum* (tobacco)
Sterfulia foetida (poon)	*Azadirachta indica* (Neem)
Oryza sativa (rice bran seed)	*Hevea brasiliensis* (Rubber seed)
Aleuriter moluccana (candle nut tree)	*Pongamia pinnata L.* (karanja)
Ricinus communis (castor)	*Simmondsia chinensis* (jojoba)
Sleichera triguga (kusum)	*Sapindus mukorossi* (soapnut)
Edible oils	
Glycine mas (soybean)	*Helianthus annuus* (sunflower)
Elaeis guineensis (palm)	*Gossypium spp.* (cottonseed)
Arachis hypogaea (groundnut)	*Zea mays* (corn)
Olea europaea (olive kernel)	*Cocos nucifera* (coconut)
Brassica campestris (canola/rapeseed)	*Sesamum indicum* (sesame seed)
Algae oils	
Chlorella protothecoides	*Botryococcus braunii*
Chlorella vulgaris	*Tetraselmis suecica*
Chlorella pyrenoidosa	*Nannochloris*
Dunaliella tertiolecta	*Scenedesmus* TR-84
Ankistrodesmus TR-87	*Phaeodactylum tricornutum*

Other than vegetable oil, waste oil has also been studied to be the substrate for biodiesel production. Other than its low price, using waste oil for biodiesel production may reduce the amount of waste thrown to the environment. It was estimated that countries such as United States and China generate large amount of waste cooking oil each year (about 10 million tonnes and 4.5 million tonnes respectively) (Lam et al., 2010). In addition, waste oil has different properties than that of refined or crude oils; waste oil usually has higher water content and free fatty acid (L. Li et al., 2006; Tongboriboon et al., 2010) which may affect biodiesel yield.

Other type of oil feedstock is oil extracted from microalgae, which is classified as the third generation of biodiesel. Examples of microalgae species used for biodiesel production are *Chlorella*, *Botryococcus*, *Scenedesmus*, *Dunuliell*, *Chlamydomonas*, and *Nannochloropsis* (Ho et al., 2014). High yield up to 98% was obtained using *Chlorella prototheco*ides, *Candida* sp. 99-125 lipase and methanol (Xiong et al., 2008). Algae is divided into two categories: (i) microalgae which is unicellular microscopic photosynthetic organism that are found in saltwater and freshwater environments; and (ii) macroalgae which is multicellular and form root, stem and leave structures of higher plants (Mubarak et al., 2015; Noraini et al., 2014). Both macro- and micro-algae can be used as raw material for biodiesel production. Microalgae have many advantages such as contains high oil content (25-75% of its dry weight), fast growth rate, high photosynthetic efficiency, high biomass production, and can grow on land unsuitable for agriculture (Halim et al., 2012; Mubarak et al., 2015).

Despite these advantages, microalgae oil is different than vegetable oil since it has high content of polyunsaturated fatty acids with four or more double bonds and higher content of phospholipid (more than 10%) (Noraini et al., 2014). Fatty acids composition could affect the physicochemical properties of biodiesel produced while high phospholipid can cause negative effect on the reaction system in terms of yield, reaction

rate and also biodiesel quality (Knothe, 2005; Noraini et al., 2014; Singh et al., 2014). Besides, biodiesel production from microalgae needs large quantity of algal biomass and its oil extraction process is still costly and energy intensive. These disadvantages make the second generation biofuel still become favorable.

Each oil feedstock will have different fatty acid composition. Both fatty acid composition of feedstock oil and alcohol moieties play important roles in determining biodiesel properties including cetane number, viscosity, lubricity, melting point, heat of combustion, oxidation stability, cold flow and also exhaust emission of the biofuel produced (Knothe, 2005; R. Rodrigues et al., 2008).

According to G. Knothe (2005), the fatty acid properties that affect biodiesel properties are unsaturation degree, chain length and branching of the chain. Cetane number, viscosity, heat of combustion and melting point will increase with increasing chain length and decrease with increasing degree of unsaturation (Knothe, 2005). For example, feedstock oil such as soybean oil, sunflower oil, and rice bran oil has low oxidation stability due to high amount of linoleic acid that has double bonds (R. Rodrigues et al., 2008). Therefore, choosing an oil feedstock with a good fatty acid composition can determine its suitability to become a fuel for engine.

2.2.2.1 Ceiba pentandra

Presently, there are many plant species that have been identified as potential sources of non-edible oil for biodiesel production such as *Jatropha curcas, Pongamia pinnata, Calophyllum inophyllum, Nicotiana tabacum, Azadirachta indica* and others (Atabani et al., 2013). One of the non-edible plants that is also a good source of non-edible oil is *Ceiba pentandra*. *C. pentandra* (kapok or silk-cotton) is a drought resistant tree under Malvaceae family and can be found in tropical America, west Africa, and Asia including Malaysia, Indonesia, Vietnam, Philippines, India and Pakistan (H. C. Ong et

al., 2013; Rashid et al., 2014). The pods are leathery, 10-25cm long, 3-6cm diameter, and have high fiber content (**Figure 2.4**) (Sivakumar et al., 2013). The fiber is commonly used as stuffing material for mattresses, pillows and cushions and has a potential to become a feedstock for bioethanol (Tye et al., 2012).

Adult *C. pentandra* tree produces 1000 to 4000 seed pods at a time, each with almost 250 seeds that contains 25-28% oil per seed (Senthil Kumar et al., 2015). Average oil yield for *C. pentandra* is about 1280 kg/ha annually (Yunus Khan et al., 2015) and has a relatively short harvesting time of 4 to 5 months (L. K. Ong et al., 2013). *C. pentandra* oil has high content of cyclopropene ring fatty acids that are known to cause physiological disorders in animals and thus make it not safe for consumption (Norazahar et al., 2012).

Figure 2.4: *C. pentandra* fiber, seeds and pods (Ring Organic, n.d.)

C. pentandra oil has been tested as raw material for biodiesel production and the biodiesel-diesel blends was proven to give good engine performance and reduced carbon monoxide and smoke density (Senthil Kumar et al., 2015; Silitonga, Masjuki, et al., 2013). These results show that *C. pentandra* oil is suitable for biodiesel production

and its methyl ester can be used in diesel engine. However, currently there is no research investigate *C. pentandra* using biocatalyst.

2.2.3 Acyl acceptor

Acyl acceptor is one of the substrates needed for biodiesel production; it reacts with oil to produce biodiesel. Acyl acceptors that can be used for biodiesel synthesis are esters, alcohols and dimethyl carbonate (DMC). The comparison between these acyl acceptors are tabulated in **Table 2.4**.

Table 2.4: The advantages and disadvantages of acyl acceptor in enzymatic reaction

Acyl acceptor	Advantages	Disadvantages
Methanol	Cheap, fast reaction, high maximum engine performance.	Cause enzyme deactivation, require stepwise addition, synthesized from fossil fuel
Ethanol	Synthesized from biomass (green), improve fuel properties, low harmful emission.	More expensive than methanol, FAEE has higher kinematic viscosity than FAME.
Other alcohols	Better miscibility with oil	Slow reaction.
Ester (methyl or ethyl acetate)	High yield even with unrefined oil, high reusability of enzyme, higher value by-product (triacetin).	High amount of ester and lipase needed for optimum reaction.
Dimethyl carbonate (DMC)	Non-toxic, can be used as both extraction solvent and transesterification substrate.	Expensive, high amount of DMC and lipase needed for optimum reaction.

Esters used for biodiesel production are methyl acetate and ethyl acetate. Methyl and ethyl acetate do not cause negative effect on lipase activity compared to methanol or ethanol and will produce higher value by-product called triacetin or triacetylglycerol (**Figure 2.5**) which has no negative effect on reaction (Du et al., 2004; Modi et al., 2007). In spite of these advantages, there are also several drawbacks involved. The reaction may require high acyl acceptor to oil molar ratio and high amount of lipase for

an optimum reaction (Du et al., 2004; Modi et al., 2007; E.-Z. Su et al., 2007; Xu et al., 2003).

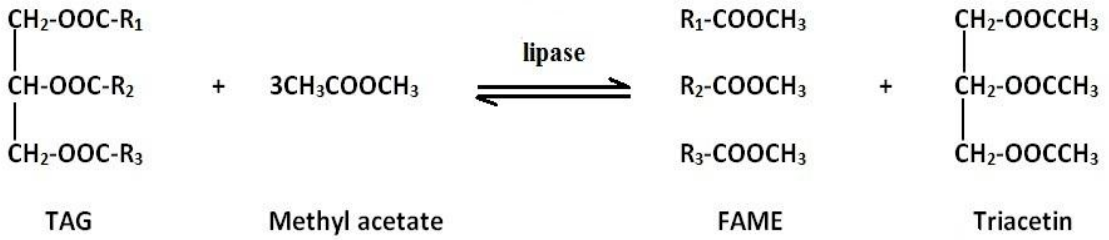

Figure 2.5: Reaction of TAG with methyl acetate producing FAME and triacetin as by-product (Du et al., 2004)

Other than ester, it was also discovered that dimethyl carbonate (DMC) can be a suitable acyl acceptor for biodiesel production. DMC is odorless, non-toxic, and heat-stable solvent which can be used as extraction solvent as well as substrate for interesterification reaction (O. K. Lee et al., 2013). Reaction between triglyceride and DMC will produce FAME and fatty acid glycerol carbonate (**Figure 2.6**) that will be further broken down into glycerol dicarbonate and glycerol carbonate. (Calero et al., 2015). Biodiesel production using DMC as acyl acceptor does not need multiple step addition (E.-Z. Su et al., 2007) but this solvent is expensive thus may increase the overall biodiesel production cost.

The common acyl acceptor used for biodiesel synthesis is alcohol due to its effectiveness and low price. The general equation for the synthesis of biodiesel or fatty acid alkyl ester (FAAE) using alcohol is shown in **Figure 2.7.** Types of alcohol that can be used will include primary, secondary, long chain, and branched alcohols. It was observed that secondary alcohols react slower than primary alcohols which might due to steric hindrance and also the specificity of lipase used (Salis et al., 2005). However, fatty acid esters of secondary or branched-chain alcohols have their own advantages.

Instead of adding additives like butyl oleate, adding of these esters can improve low temperature properties such as cloud point and pour point of the fuel (Salis et al., 2005).

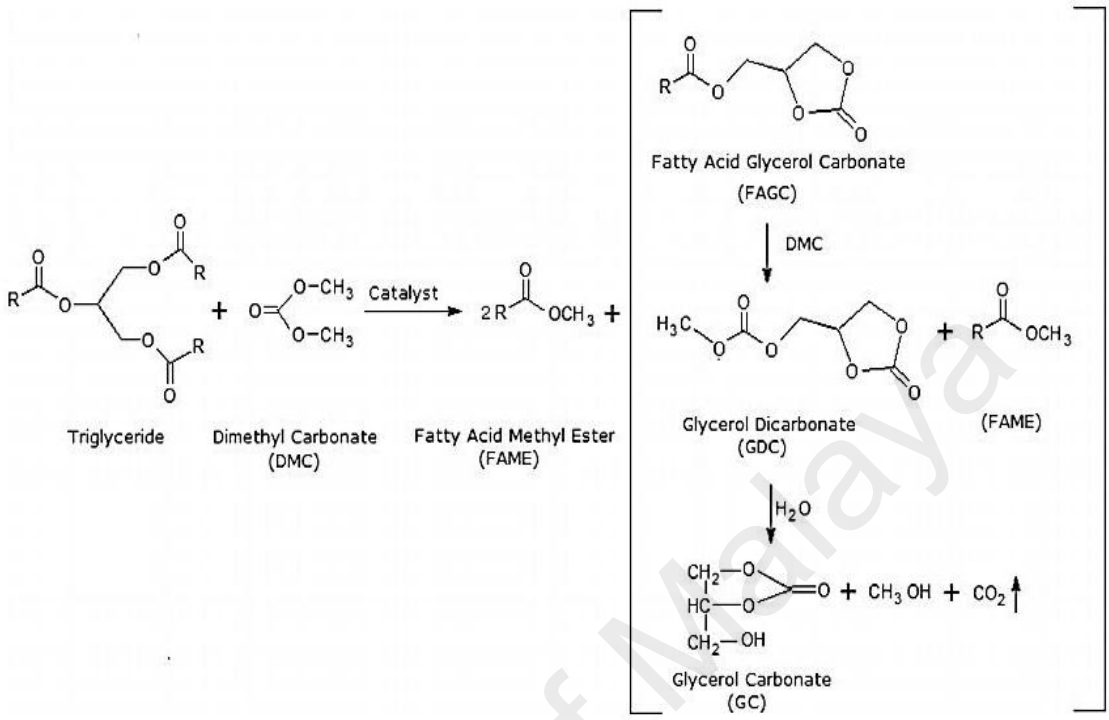

Figure 2.6: Reaction between triglyceride and dimethyl carbonate (DMC) producing FAME and Fatty Acid Glycerol Carbonate (FAGC) (Calero et al., 2015). FAGC will be further broken down into glycerol dicarbonate and glycerol carbonate.

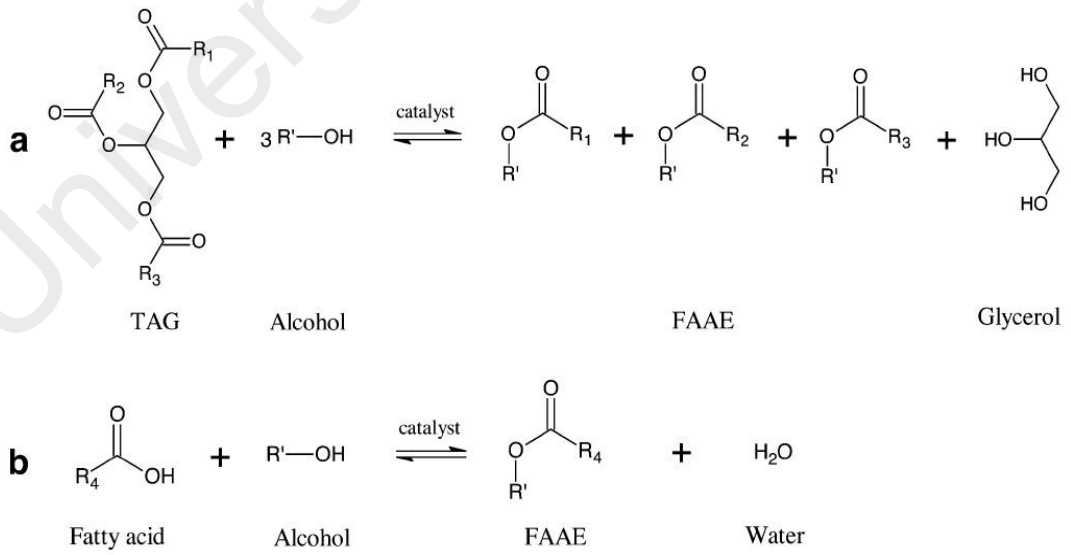

Figure 2.7: Reactions for synthesis of fatty acid alkyl ester (FAAE) (Röttig et al., 2010). (a) Transesterification of TAG (triacylglyceride) with alcohol (b) Esterification of fatty acid with alcohol. R_{1-4} are acyl residues, R' is alcohol moiety.

The widely used alcohols for this reaction are methanol and ethanol. The biodiesel product is fatty acid methyl ester (FAME) and fatty acid ethyl ester (FAEE) if methanol and ethanol is used, respectively. Even though ethanol is greener (synthesized from renewable sources), methanol's high polarity and short chain length make it the most efficient alcohol for transesterification reaction (Ko et al., 2012). Methanol is also much cheaper than ethanol. Despite these advantages, one of the problems of using methanol is that it can cause lipase deactivation. Nonetheless, applying stepwise addition instead of one-step addition of methanol into the system may reduce this effect.

2.2.4 Solvent

Biodiesel production using enzyme as catalyst can be done with or without solvent. Solvent is used as a way to decrease the effect of lipase inhibition or intoxication by methanol or glycerol. Other than increased production yield, there are many advantages of using solvent in reaction system. Solvent can help reduce viscosity and ensure homogeneity of reaction mixture due to immiscibility of alcohol and triglyceride (Cerveró et al., 2014; Fjerbaek et al., 2009). It also keeps the water around the enzyme which consequently helps increase water activity and enzyme stability (Fjerbaek et al., 2009).

There have been many studies conducted to gain more insights about the effect of solvent in enzymatic transesterification**.** Lu et al. (2008) have tested the conversion of glycerol trioleate to biodiesel using immobilized *Candida* sp. 99-125 with twelve different organic solvents. From this study, they have made several important points: (i) there might be a correlation between hydrophobicity (log *P*) value with yield obtained; (ii) hydrophilic solvents need less water while hydrophobic solvents need more water in the system to be effective; and (iii) solubility of methanol in reaction system does not affect production yield. The result obtained from their study was immobilized *Candida*

sp. 99-125 produced higher yield in hydrophobic solvents such as n-hexane, benzene, toluene, CCl_4, and cyclohexane.

This result is also supported by He et al. (2008) who tested nine kinds of solvents and found that organic solvents with log *P* between 4.0 and 4.5 produced better results than the others. Kojima et al. (2004) tested with eighteen solvents and found that *C. cylindracea* activity was stable in solvents with hydrophobicity index higher than 1.3 such as chloroform, toluene, tetrachloromethane, n-hexane, kerosene and diesel.

In addition, Su et al. (2007) obtained high conversion in non-polar organic solvent as compared to that of polar organic solvent. This is because polar solvent may interfere with lipase hydrogen bonding and hydrophobic interactions, and thus cause alteration of its molecular structure (E.-Z. Su et al., 2007). t-Butanol, an amphiphilic and moderately polar solvent is also known to give positive results. Several experiments conducted using immobilized lipase with and without t-butanol as solvent show that the yield or conversion increased when t-butanol was added (L. Li et al., 2006; Nasaruddin et al., 2014; Royon et al., 2007). Many researchers may argue that the t-butanol may participate in the transesterification as acyl acceptor but Royon et al. (2007) found that t-butanol was not a substrate in the reaction since there is no alcoholysis took place without methanol addition.

Another potential solvent is ionic liquid. Ionic liquid has unique properties such as low vapor pressure, high thermal stability, good solubility in both organic and inorganic materials, and its ability to form multiple phase systems (Mohammad Fauzi & Amin, 2012). Physical and chemical properties of ionic liquid such as melting point, acidity and basicity, viscosity, density and hydrophobicity can be tuned by altering the combination of cations and anions in it (Ha et al., 2007; Mohammad Fauzi & Amin,

2012). Despite all these advantages, ionic liquid is considered expensive and hazardous if contain hexafluorophosphate (PF_6) anion (Guldhe, Singh, Mutanda, et al., 2015).

Supercritical carbon dioxide has the advantage to be used as a solvent due to its non-toxic and non-flammable properties. Biodiesel production using this solvent is capable of producing high biodiesel yield in a short reaction time and the separation is much easier since the products do not dissolve in carbon dioxide at room conditions (Stamenković et al., 2011). Compared to non-catalyzed reaction that uses very high temperature, supercritical carbon dioxide is used in a moderate temperature thus make it suitable for enzyme reaction. By using this supercritical fluid, Gameiro et al. (2015) obtained 98.8% yield at 40 °C and 250 bar, and Colombo et al. (2015) obtained 94% yield at 70 °C and 200 bar.

Even though addition of solvent can improve production yield, the amount added into the reaction mixture need to be controlled. Li et al. (2006) conducted experiments using Lipozyme TL IM, rapeseed oil and t-butanol as solvent and discovered that the yield decreased with high volume of t-butanol due to excessive dilution. Furthermore, differences in lipase origin or immobilization method would affect how the enzymes will react in organic solvents (Lu et al., 2008). For example, n-hexane gave positive result to Candida sp. 99-125 (Lu et al., 2008) but it did not affect *P. cepacia* lipase. In research conducted by Kumari et al. (2006) on mahua oil using *P. cepacia* lipase and different solvents such as hexane, octane, and acetonitrile, only octane gave slightly higher conversion compared to solvent-free reaction. The other two solvents did not give any positive results.

Usage of solvent in biodiesel production also has several issues related to it. Some solvents are toxic, flammable, and volatile which makes them dangerous to human. Biodiesel production using solvent may also need elimination or recovery steps, larger

reactor volume and additional production cost (Cerveró et al., 2014; Fjerbaek et al., 2009; Shimada et al., 1999).

An economical assessment was done to compare enzyme-catalyzed production of biodiesel, with or without solvent (t-butanol), from rapeseed oil (Sotoft et al., 2010). The results obtained shows that the product price and total capital investment for production with solvent was much higher than the production with no solvent and concluded that co-solvent production process was too expensive and not a viable choice. Details on the price are discussed further in Chapter 2.2.5. After considering the above factors, this study was conducted with no solvent used.

2.2.5 Biodiesel production cost

When developing a biodiesel production process, one of the major concerns for enzymatic biodiesel production is its economical aspect. The higher cost of enzyme makes the enzyme-catalyzed reaction to be less favorable compared to chemical-catalyzed production. Nonetheless, this drawback can be minimized through repeatable use of enzyme, which directs to the application of immobilized lipase.

There have been a few studies that measured the economical aspect of enzymatic biodiesel production. For example, Jegannathan et al. (2011) conducted an economic assessment of biodiesel production between three catalysts: alkali, soluble enzyme, and immobilized enzyme. This assessment was calculated for batch mode (stirred tank) with a production capacity of 10^3 tonne. The price estimated for the lipase was \$150/kg. It was calculated that alkali catalysts had the lowest production cost (\$1166.67/tonne) compared to immobilized lipase catalyst (\$2414.63/tonne) and soluble lipase catalyst (\$7821.37/tonne). The higher production cost when using immobilized enzyme was due to higher cost of lipase and longer reaction time. However, it has to be mentioned that

this assessment included washing process in the production line which is not necessarily needed for enzyme catalyst.

Since the enzymatic production of biodiesel can be done with or without solvent, an economical comparison between these two processes had also been done. Sotoft et al. (2010) evaluated the production of 8 and 200 mio. kg biodiesel/year from rapeseed oil and methanol, and made a comparison between solvent free and cosolvent (t-butanol) production. They used two prices of enzyme that account for the current price (762.71€/kg enzyme) and estimated price in the future (7.63€/kg enzyme). The product price for solvent free production was estimated to 0.73–1.49€/kg biodiesel and 0.05–0.75€/kg biodiesel for enzyme price of 762.71€/kg enzyme and 7.63€/kg enzyme respectively. Meanwhile, the product price for cosolvent production was estimated as 1.50–2.38€/kg biodiesel. The total capital investment for cosolvent production was calculated to be higher due the installation costs of solvent recovery column, which was higher than the cost of extra number of reactors and decanters needed for solvent free operation.

An economic analysis of a biodiesel production plant from waste cooking oil (WCO) using supercritical carbon dioxide was done by Lisboa et al. (2014). It was estimated that the biodiesel cost was 1.64€/L and 0.75€/L (for a WCO price of 0.25€/kg and enzyme prices of 800€/kg and 8€/kg, respectively). This production cost was calculated based on conversion of 8000 ton WCO/year, using immobilized lipase *Thermomyces lanuginosus* (Lipozyme TL IM) and ethanol.

2.3 Physicochemical properties

To ensure satisfactory quality of biodiesel, its physicochemical properties should meet international standard of either ASTM D6751 or EN 14214. **Table 2.5** shows the properties and its limitations described in both standards.

Property	Unit	ASTM D 6751		EN 14214	
		Limit	Method	Limit	method
Density (15°C)	kg/m^3	880 max	D 1298	860-900	EN 3675
Kinematic viscosity (40° C)	mm^2/s	1.9 – 6.0	D 445	3.5 – 5.0	EN 3104/ EN 3105
Acid value	mg KOH /g	0.50 max	D 664	0.50 max	EN 14104
Oxidation stability (110 °C)	h	3 min	EN 14112	6 min	EN 14112
Flash point	°C	93 min	D93	101 min	EN 3679
Pour point	°C	-15 to -16	D 97	-	-
Cloud point	°C	-3 to -12	D 2500	-	-
Cloud filter plugging point	°C	19	+5 max	D 6371	EN 14214
Cetane no.	-	47 min	D 613	51 min	EN 5165
Iodine value	g I_2/100 g	-	-	120 max	EN 14111
Total contamination	mg/kg	-	-	24 max	EN 12662
Water and sediment	vol%	0.05 max	D 2709	0.05 max	EN 12937
Methanol	wt%	0.20 max	EN 14110	0.20 max	EN 14110
Sulfated ash	wt%	0.020 max	D 874	0.020 max	EN 3987
Total sulfur	ppm	15 max	D 5453	10	EN 20846
Phosphorus	ppm	10 max	D 4951	4	EN 14107
Group I metals Na^+ K	mg/kg	5.0 max	EN 14538	5.0 max	EN 14108
Group II metals Ca^+ Mg	mg/kg	5.0 max	EN 14538	5.0 max	EN 14538
Carbon residue	wt%	0.05 max	D 45.0	0.30 max	EN 10370
Ester content	wt%	-	-	96.5 min	EN 14103
Linolenic acid methyl ester	wt%	-	-	12 max	EN 14103
Polyunsaturated acid methyl ester	wt%	-	-	1.0 max	prEN 15799
Copper strip corrosion 3 h at 50°C	-	No. 3 max	D 130	No. 1 max	EN 2160
Free glycerin	wt%	0.02 max	D 6584	0.02 max	EN 14105
Total glycerin	wt%	0.24 max	D 6584	0.25 max	EN 14105
Monoglyceride	wt%	0.52	-	0.80	EN 14105
Diglyceride	wt%	-	-	0.20	EN 14105
Triglyceride	wt%	-	-	0.20	EN 14105

Table 2.5: US (ASTM D6751) and European (EN 14214) specifications for biodiesel (B100) (Atabani et al., 2012; Hoekman et al., 2012)

2.3.1 Density

Density is mass per unit volume and the unit commonly used is g/ml or kg/m^3. It greatly influences fuel injection process as the amount of fuel injected into engine is estimated by its volume, thus affecting the air-fuel ratio and energy content in the

combustion chamber (Hoekman et al., 2012). Biodiesel generally has higher density (860 – 890 kg/m^3) compared to diesel which is around 850 kg/m^3 (Sajjadi et al., 2016). Plant oils (before conversion into biodiesel) have higher density within 910 – 930 kg/m^3 thus make it difficult for direct application in the engine. Density is dependent on temperature where it will increase at cold temperature. Both ASTM and EN standards use temperature 15° C for the determination of biodiesel density.

2.3.2 Viscosity

Viscosity is the measure of resistance for a volume of liquid to flow through a calibrated glass capillary viscometer at 40° C. High viscosity would affect fuel injection where it causes decrease in injection volume, delay in start of injection, and increase of injection variability (Miers et al., 2007). Viscous fuel would lead to larger droplet size, poor atomization and vaporization, narrower injection angle spray, and increased in-cylinder penetration, which could cause weak combustion and increased emissions (Hoekman et al., 2012). Viscosity increases with increasing number of carbon atoms (chain length) and degree of saturation (Refaat, 2009). Same like density, viscosity is dependent on temperature and biodiesels are usually more viscous than diesel. Viscosity for plant oil is within the range of 27.2 – 53.6 mm^2/s which is much higher compared to biodiesel (2.8 – 6.0 mm^2/s) (Sajjadi et al., 2016).

2.3.3 Acid value

Acid value determines the amount of carboxylic acid groups (fatty acid) in the substance. It is measured by the amount of potassium hydroxide (KOH) needed to neutralize one gram of substance. The unit used is mg KOH/g. Using a fuel with high acid value would cause corrosion and engine deposits especially in the fuel injectors (Pullen & Saeed, 2012). Crude oil that has high acid value cannot be directly converted into biodiesel using alkali catalyst because it will cause the formation of soap. The oil is

usually pretreated with acid catalyst prior to alkali-catalyzed reaction to reduce the FFA amount.

2.3.4 Oxidation stability

Oxidation stability is the measure of the fuel stability towards degradation by oxidation process. Oxidation stability is influenced by the biodiesel's FAME composition and the storage condition. This would include its exposure components such as air, light, heat, metals, peroxides, and the type of storage container (Knothe, 2005). Oxidation stability would also depend on the unsaturation degree of fatty acid in which biodiesel containing high unsaturated fatty acid chains is more susceptible (Pullen & Saeed, 2012). It is common to add antioxidant in biodiesel to reduce the oxidation process and improve its oxidation stability. There are two types of antioxidant which are natural antioxidant such as tocopherol (vitamin E), and synthetic antioxidants which include tertiary butylhydroquinone (TBHQ), pyrogallol, and propyl gallate (Pullen & Saeed, 2012). Low oxidation stability causes increased viscosity and formation of contaminants such as sediment and gums (Hoekman et al., 2012), that would lead to poor engine performance.

2.3.5 Calorific value

Calorific value shows the energy content in a substance, by measuring the amount of heat produced by a specific amount of substance in a complete combustion. The unit used is joules per kilogram or megajoules per kilogram (MJ/kg). There is no specification for calorific value in both ASTM and EN standards however it is included in EN 14213 to be at least 35 MJ/kg (Rashid et al., 2009). Biodiesel has a calorific value within the range of 34.4 – 45.2 MJ/kg which is lower than diesel (42 – 45.9 MJ/kg) (Sajjadi et al., 2016).

2.4 Methanol concentration and stepwise addition

One of the major obstacles for enzyme-catalyzed biodiesel production is deactivation of enzyme. Deactivation of enzyme may be caused by the blocking of triglycerides

entry by lower linear alcohol such as methanol and ethanol, immiscibility between triglycerides and alcohol, or adsorption of alcohol onto polar immobilized material (acrylic resin, polyurethane foam) (J.-W. Chen & Wu, 2003; Ko et al., 2012; Maceiras et al., 2011). Despite the fact that three molars of alcohol are needed for complete transesterification, lipase will deactivate in the presence of more than one molar equivalent of methanol (Shimada et al., 1999). To solve this problem, several previous studies have suggested stepwise addition or continuous addition of methanol into the system (Christopher et al., 2014; Ko et al., 2012; Shimada et al., 1999).

The most common method used for methanol addition is three-step methanol addition which managed to obtain high conversion of more than 90% (**Table 2.6**). Shimada et al. (1999) gained a high conversion of 97.4% using Novozym 435 and mixture of soybean and rapeseed oil, while Watanabe et al. (2002) achieved 95.9% conversion with Novozym 435 and soybean oil. Further tests were done to compare three-step with one-step or two-step methanol addition. Cerveró et al. (2014) obtained 40%, 60%, and 90% conversions for one-step, two-step, and three-step addition respectively. Meanwhile Lu et al. (2010) obtained 74.4% yield for three-step and 2.44% yield for one-step addition. These results show that three-step methanol addition is better than one-step or two-step methanol addition.

Furthermore, methanol additions of more than 3 steps are also able to give high yield. Samukawa et al. (2000) used six-step and nine-step addition of methanol to maintain maximum initial reaction rate based on Michaelis-Menten equation for pretreated and non-treated lipase respectively and obtained over 97% methyl ester content for both methods. You et al. (2013) conducted methanol addition using 3 h, 5 h or 8 h intervals and obtained high yield up to 89%.

Table 2.6: Biodiesel production with different techniques of methanol addition

No	Lipase	Lipase weight% based on oil weigh	Substrate	Methanol to oil molar ratio	Reaction conditions	Methanol addition technique	Yield/ Conversion (%)	References
1	Novozym 435	4	Soybean and rapeseed oils	3:1	48h, 30°C, 130 oscillation/min	Three-step (1 molar equivalent added at 0h, 10h and 24h)	97.4	(Shimada et al., 1999)
2	Novozym 435	4	Soybean oil	1:1	48h, 30°C, 130 oscillations/min	Three-step (1/3 molar equivalent added at 0h, 10h and 24h)	95.9	(Watanabe et al., 2002)
3	Imm.[a] whole-cell *R. oryzae*	4	Jatropha oil	1:1	60h, 30°C Water 5% (v/v)	Three-step (1/3 molar equivalent added at 0h, 4h and 17h)	80	(Tamalampudi et al., 2008)
4	Novozym 435	5	Soybean oil	3:1	24h, 37°C	Three-step (1/3 at molar equivalent added at 0h, 7h and 14h)	90	(Cerveró et al., 2014)
						Two-step (1/3 molar equivalent added at 0h, 2/3 molar equivalent added at 7h)	60	
						One-step	40	
5	*Candida* sp.99-125	10	Soybean oil	1:1	12h, 40°C, 180 rev/min. Solvent n-hexane (2ml), Water 200 µl	Three-step (1/3 molar equivalent added at 0h, 4h and 8h)	74.4	(Lu et al., 2010)
						One-step	2.44	

Table 2.6, continued

No	Lipase	Lipase weight% as oil weigh	Substrate	Methanol to oil molar ratio	Reaction conditions	Methanol addition technique	Yield/ Conversion (%)	References
6	Novozym 435	4	Soybean oil	2.65:1	24h, 30°C, 150 oscillations/min	Multiple-step (1 molar equivalent at 0h, then 0.33 molar equivalent at 1h, 3h, 5h, 7h, and 9h to maintain methanol content at around 30g/l)	97	(Samukawa et al., 2000)
7	Imm. *B.cepacia*	8	Jatropha oil	6.6:1	30h, 30°C, 150 rpm Water content 7% (v/w)	3h intervals	89	(You et al., 2013)
						5h intervals	App.[b] 88	
						8h intervals	App. 82	
						One-step	App. 62	

[a] imm., immobilized [b] App., approximately

2.5 Enzyme pretreatment

It has been found that pretreatment can restore enzyme deactivation, improve methanol tolerance, increase biodiesel yield and enhance enzymatic activity (J.-W. Chen & Wu, 2003; Lu et al., 2010; Maceiras et al., 2011; Samukawa et al., 2000). Pretreatment usually involves immersion, incubation, or washing of lipase with substrates, organic solvents, salts, or enzyme lycoprotectants (Christopher et al., 2014; Lu et al., 2010) before using the lipase for enzymatic reaction.

Previous studies have conducted several tests on enzyme pretreatment using solutions such as its substrate (vegetable oil and ethyl acetate), product (methyl ester), and others such as hexane, glutaraldehyde, methyl oleate, salt solution, and water. Pretreatment with hexane, methyl ester and soybean oil did increase the yield of 30 min reactions using Novozym 435 (J.-W. Chen & Wu, 2003). Pretreatment with methyl oleate reduce the reaction period for Novozym 435 from 24 h to 3.5 h to obtain 97% methyl ester content (Samukawa et al., 2000) while immersion in ethyl acetate gave no improvement on enzyme activity (Modi et al., 2007). In addition, immersion of immobilized *Candida* sp. 99-125 in water increases the yield for one-step methanol addition (Lu et al., 2010). This might because water pretreatment has affected the water distribution in the immobilized lipase and thus improved lipase flexibility (Lu et al., 2010).

Studies by Ban et al. (2002) showed that glutaraldehyde-pretreatment of whole-cell *R. oryzae* immobilized on biomass support particles (BSPs) increased the stability of the lipase, protected it from the negative impact of high concentration of methyl ester, and also prevented lipase leakage from the cells. Residual activities are more than 70% with incubation in 0.05 -1.0 vol.% glutaraldehyde solution for 7 days compared to 16 % residual activity of untreated lipase. Furthermore, residual activities after 6 cycles were around 70 to 83% compared to 50% of untreated lipase.

Alcohols are frequently tested for lipase pretreatment. However, it is important to know that different lipases may react differently to different alcohol and not all alcohols are suitable as enzyme pretreatment. For example, t-butanol pretreatment increases the initial reaction rate of immobilized *C. Antarctica* lipase (J.-W. Chen & Wu, 2003), but it does not give any improvements on immobilized *Candida* sp.99-125 lipase (Lu et al., 2010). This result may be due to the distinct characteristic of the lipases, influence of the immobilized support or the presence of solvent in the system.

Example of pretreatment using alcohol was done by Chen and Wu (2003) using Novozyme 435 and soybean oil feedstock. They pretreated Novozym 435 with alcohol of 3 or 4 carbons: isopropanol, 2-butanol and t-butanol by immersing it in the alcohol for 1 h, and then immersed in soybean oil for another 1 h. They obtained highest yield of 24.5% (30 min reaction time) using t-butanol pretreatment with an increase of almost tenfold. Another study conducted by Maceiras et al. (2011) on Novozyme 435 and *C. Antarctica* lipase B (free lipase) using methanol and propanol pretreatments, but both resulted with decrease in relative activity.

Other pretreatment solution that has been proved to improve the yield of enzyme is salt solution. Lu et al. (2010) tested pretreatment of *Candida* sp. 99-125 with salt solution of low saturation salt solution: 1 mM of potassium chloride (KCl), calcium chloride ($CaCl_2$), magnesium chloride ($MgCl_2$), potassium sulfate (K_2SO_4) and ammonium sulfate ($(NH_4)_2SO_4$). These pretreatments gave slight increases for three-step methanol addition but significant impact to one-step addition. The best result was obtained using MgCl2 with an increase from 1.54% yield (control) to 74.5%, almost comparable with the yield when using three-step addition.

2.6 Oil pretreatment

Other name for oil pretreatment is oil esterification using acid catalyst (**Figure 2.8**). This process was usually conducted to effectively reduce free fatty acid level of crude oil. Some biodiesel production also used acid catalyst to directly produce biodiesel but it has several disadvantages which include high sensitivity towards the presence of water which causes inhibition of reaction, long reaction time, and high methanol to oil molar ratio (Aransiola et al., 2014). As the usage of alkali catalyst (transesterification process) for an oil feedstock with high FFA level (more than 2%) will cause saponification, acid catalyst was actually used in oil pretreatment reaction before the actual biodiesel production process (Patel & Sankhavara, 2017). This process is called two-step biodiesel production. Saponification need to be avoided because it could make the downstream purification and recovery very difficult.

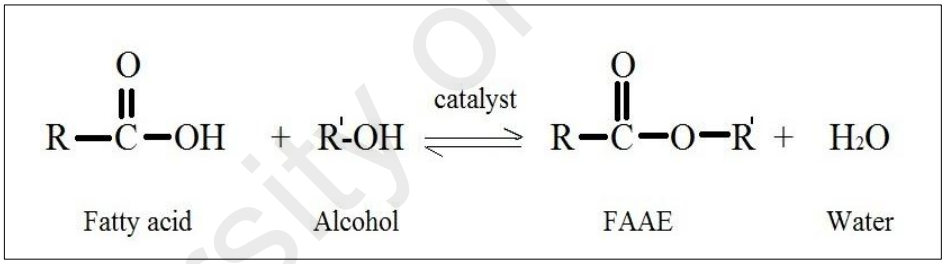

Figure 2.8: Esterification of fatty acid to fatty acid alkyl ester using acid catalyst

For the pretreatment reaction, an acid and an alcohol are needed. One of the common combination used are sulfuric acid and methanol. Hayyan et al. (2011) succeeded in reducing FFA of sludge palm oil from 23.2% to less than 2% using sulfuric acid and methanol and obtained 83.72% FAME yield after transesterification. Otadi et al. (2011) used 6:1 methanol to oil molar ratio, 1.5% silica sulfuric acid, at 60 °C and managed to reduce FFA to less than 1% and obtained methyl ester yield of 90%. Many other previous studies have done oil pretreatment or esterification in their biodiesel

production to reduce free fatty acid amount and obtained high FAME yield (Dharma et al., 2016; Gupta et al., 2016; Patil & Deng, 2009).

2.7 Optimization of biodiesel production

In biodiesel production process, several parameters could affect the FAME output. To minimize the cost of biodiesel production process and maximizing its efficiency, optimizing the parameters using mathematical methods is necessary (Avramović et al., 2015). In this study, artificial neural network (ANN) and genetic algorithm (GA) were used to optimize three parameters of biodiesel production (methanol to oil molar ratio, temperature and reaction time) to obtain the highest biodiesel yield (FAME yield percentage).

ANN is nonlinear computer algorithms modeled based on the functioning of the human brain which works by processing the data using interconnected neurons and gained knowledge by training, testing and validation (Javed et al., 2015). Once trained, ANN can be used to perform prediction of the output based on the input given to it. ANN has been applied in a wide range of field such as mechanical, chemical engineering, agriculture, medicine, finance, economics, and weather forecasting (Kundu et al., 2015). It also has several advantages over response surface methodology (RSM), a commonly used mathematical and statistical technique for modeling and optimization. While RSM can only be used for quadratic approximations, ANN able to approximate almost all kinds of non-linear functions (including quadratic functions), while requiring no prior specification of suitable fitting function (Kundu et al., 2015).

Nonetheless, ANN may not be able to solve all problems and one way to reduce this limitation is by merging this algorithm with another optimization technique, such as GA. GA is a method for solving optimization problem by applying the principle of Darwinian biological evolution where a population with randomly generated candidates

evolves towards better offspring (Yazdanmehr et al., 2009). This algorithm is favorable over traditional optimization techniques due to its capability to solve non-differentiable fitness functions efficiently (Fayyazi et al., 2015).

Various studies have proved that the combination of ANN and GA is a powerful technique to solve the optimization problems (Bahrami et al., 2005; Mousavi Anijdan & Bahrami, 2005). Rajendra et al. (2009) successfully utilized ANN-GA for predicting optimized process parameters required for reducing high free fatty acids (a pretreatment process before biodiesel production). Avramović et al. (2015) compared optimization of sunflower oil ethanolysis catalyzed by calcium oxide between ANN-GA and RSM and found that ANN model was more accurate. Taghavifar et al. (2014) also applied ANN-GA to predict and optimize diesel engine spray characteristics.

2.8 Reusability of enzyme

To enable easy recovery and reuse of enzyme, immobilized lipase is commonly chosen instead of free lipase. The major obstacle for reusing of enzyme is the enzyme loss of activity due to prolonged exposure to high temperature, physical stress, or methanol. Reusability of enzyme is commonly tested to determine how many times the enzyme can be reused in a biodiesel production process. Enzyme with high reusability may reduce the total production cost of biodiesel that is affected mostly by the high cost of lipase. The reusability test of enzyme catalyst was usually done by reusing the same enzyme for several batches of biodiesel production, and measuring the biodiesel yield produced from each batch.

Previous studies have shown that enzyme reutilization is viable. Rodrigues et al. (2008) reused three types of lipase (Novozym 435, Lipozyme TL-IM, and Lipozyme RM-IM) and found that all lipases retained about 60% relative conversion yield on the third batch. The relative conversion yield then dropped to below 10% on the fifth batch

for Novozym 435. *P. cepacia* lipase immobilized on magnetic nanoparticles retained about 70% on the third batch and dropped to 40% on the fifth batch (C.-Y. Yu et al., 2013). Meanwhile a mixture of *T. lanuginosus* and *R. meihei* immobilized lipases maintained about 60% relative yield conversion after seven batches (R. C. Rodrigues & Ayub, 2011).

CHAPTER 3: MATERIALS AND METHODS

3.1 Introduction

In this research, the first step was determining the biodiesel's composition and physicochemical properties before comparing it with ASTM and EN international standards. Properties tested include density, viscosity, acid value, oxidation stability, and calorific value. Further experiments were carried out to tests several aspects of the enzyme-catalyzed biodiesel production which include effects of methanol concentration and its stepwise addition, enzyme pretreatment, optimization of the biodiesel production process, and enzyme reusability. The parameters included in optimization process were temperature, methanol to oil molar ratio, and reaction time. Finally, an economic evaluation was conducted to calculate biodiesel production cost per liter. Overall methodology is shown in **Figure 3.1.**

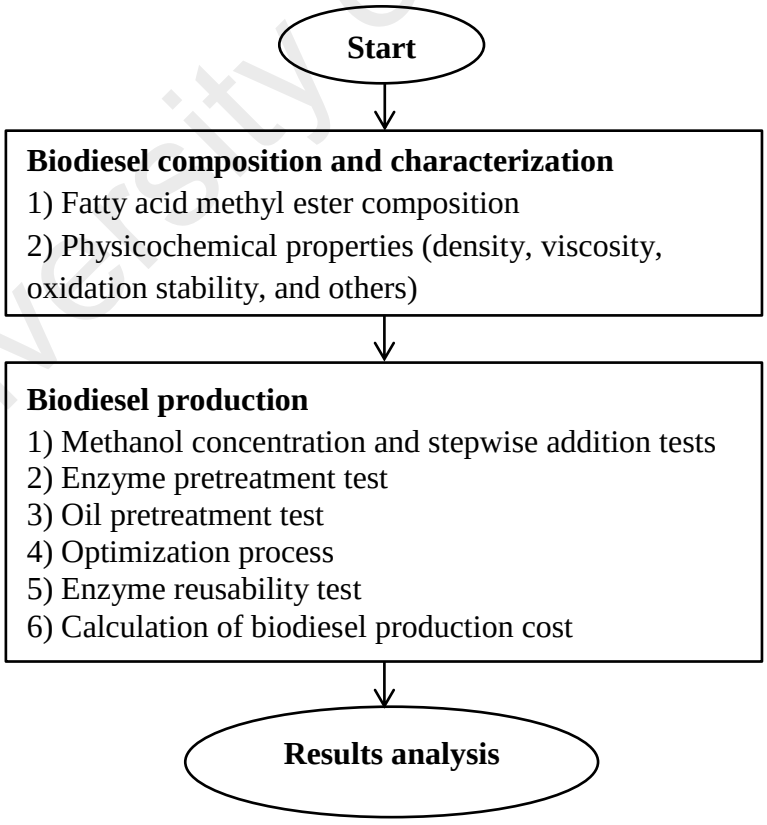

Figure 3.1: Flow chart of overall methodology

3.2 Materials

Crude *Ceiba pentandra, Jatropha curcas,* and rice bran oils oil were purchased from West Java, Indonesia. *Candida antarctica* lipase immobilized on macroporous acrylic resin (Novozym 435, recombinant, expressed in *Aspergillus niger*) from Sigma Aldrich was used as biocatalyst (**Figure 3.2**). All reagents of high purity including methanol, t-butanol, and sodium chloride were purchased from suppliers in Malaysia. For gas chromatography analysis, C8-C24 FAME Mix (Certified Reference Material) was used as reference. Methyl nonadecanoate analytical standard (C19) was purchased from Sigma Aldrich to be used as internal standard (IS).

Figure 3.2: Novozym 435

3.3 Biodiesel production

The method and parameters setting used for each sample for biodiesel production were the same as the following unless stated otherwise: crude oil (10 g) was mixed with 5 wt.% (weight of lipase/weight of oil) Novozym 435 and methanol in a 50-100 ml screw-capped glass vial (**Figure 3.3**); Samples were then incubated with shaking in an incubator-shaker (**Figure 3.4**) at 40 °C and 150 rpm for 72 h for enzymatic reaction to take place. Three-step methanol addition was applied to each sample. Three-step methanol addition means the total volume of methanol was divided equally into 3 portions, and each portion was added into the reaction system at 3 different times. For

example, if the total methanol calculated for the reaction is 1.4 ml, this amount will be divided by 3, thus 0.47 ml will be added in each step (**Appendix A**). For 72 h reaction time, the time of addition will be at 0^{th} h, 24^{th} h, and 48^{th} h.

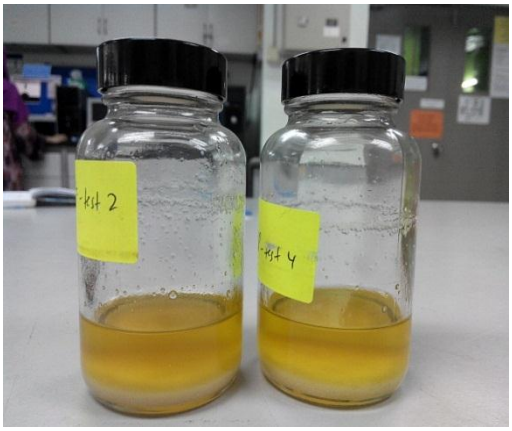

Figure 3.3: Sample mixture

Figure 3.4: Incubator-shaker

3.3.1 Methanol concentration and stepwise addition

To test the effect of methanol concentration to biodiesel yield, three different batches with molar ratio of 3:1, 6:1, and 9:1 (methanol to oil) were chosen. Three-step methanol addition was applied to all batches with time of addition at 0^{th} h, 24^{th} h, and 48^{th} h.

To investigate the effect of stepwise addition, three batches with the same molar ratio as above (3:1, 6:1, and 9:1) were analyzed with different number of steps of methanol stepwise addition. The number of steps was set to keep the methanol added at 1 molar per step. For 3:1 molar ratio sample, three-step addition was used. For 6:1 molar ratio, six-step addition was applied with time of addition at 0^{th} h, 6^{th} h, 24^{th} h, 30^{th} h, 48^{th} h, and 54^{th} h. For 9:1 molar ratio, nine-step addition was applied with time of addition at 0^{th} h, 3^{rd} h, 6^{th} h, 24^{th} h, 27^{th} h, 30^{th} h, 48^{th} h, 51^{st} h, and 54^{th} h.

3.3.2 Enzyme pretreatment

Before starting the biodiesel production process, 5 wt.% enzyme was immersed in t-butanol or salt solution (1 mM, 10 mM and 1000 mM concentrations) for 1 h. After that, the enzyme was filtered and let dry at room temperature. The pretreated enzyme

was then used in biodiesel production process. Another sample was prepared using untreated enzyme to act as control. Molar ratio of 3:1 (methanol to oil) was used and three-step methanol addition was applied where the equal amount of methanol was added at 0^{th} h, 24^{th} h, and 48^{th} h.

3.3.3 Optimization process using ANN-GA

In this study, artificial neural network (ANN) and genetic algorithm (GA) were used in optimizing three parameters of biodiesel production (methanol to oil molar ratio, temperature and reaction time) to obtain the highest biodiesel yield (FAME yield percentage). Firstly, Box–Behnken design (BBD) was used to develop the experimental design. Three variables (parameters) were chosen: alcohol to oil molar ratio, temperature, and reaction time. The settings of the variables are as follows: alcohol to oil molar ratio (3:1, 7:1, 11:1), temperature (40 °C, 50 °C, 60 °C), and reaction time (24h, 48h, 72h), as shown in **Table 3.1**.

Table 3.1: Independent variables for optimization process and their levels

Independent variable	**Units**	**Variable level**		
		-1	0	+1
Alcohol to oil molar ratio	mol/mol	3	7	11
Temperature	°C	40	50	60
Reaction time	h	24	48	72

The number of experiments (*N*) required for the development of BBD model is defined as:

$$N = 2k(k-1) + Nc \quad (3.1)$$

where *k* is the number of factors and *Nc* is the number of central points (Kundu et al., 2015). MATLAB with neural networks and genetic algorithm toolboxes (MATLAB 8.1.0.604) was applied for the formulation of artificial neural network modeling and the optimization of the FAME content. The learning algorithm used was a feed forward,

back-propagation algorithm. The Levenberg–Marquardt (LM) algorithm was used for training the algorithm with the hyperbolic tangent sigmoid (tansig) transfer function used from input to hidden layer. While the purelin transfer function applied from hidden layer to output. The selected ANN was trained until the minimum mean square error (MSE) was reached and average correlation coefficient (R) was close or equal to 1 using heuristic procedure. The set of input–output data experiments was divided into training (70%), testing (15%) and validating (15%). Then, the optimum set of the process variables was determined by applying GA over the ANN model to predict the FAME for biodiesel process at various combinations of independent parameters.

The performances and predictive capacity of ANN model was statistically measured by the coefficient of determination (R^2) and root mean square error (RMSE) using equations as follow:

$$R^2 = 1 - \sum_{i=1}^{n} \left(\frac{(y_{ei} - y_{pi})^2}{(y_m - y_{pi})^2} \right) \quad (3.2)$$

$$RMSE = \sqrt{\frac{1}{n} \sum_{i=1}^{n} (y_{pi} - y_{ei})^2} \quad (3.3)$$

Where n is number of experimental data; y_{ei} is experimental value of yield; y_{pi} is predicted value of yield and y_m is average of experimental value of yield. The lowest value of RMSE and the highest R^2 (is recommended that R^2 should not be less than 80%) were used to define the accuracy of the model (Stamenković et al., 2013).

3.3.4 Reusability of enzyme

10 g crude oil and 5 wt.% lipase were used for each batch. The parameters for biodiesel production for this test were set at 50 °C, 3:1 methanol to oil molar ratio, with a reaction time of 24 h. After each batch, the enzyme was filtered, let dry, and reused for the next batch. Three-step methanol addition was applied where equal amount of

methanol was added at 0^{th} h, 3^{rd} h, and 9^{th} h. The enzyme was reused until the fifth batch.

3.4 Physicochemical properties analysis

Physicochemical properties of *C. pentandra* biodiesel such as kinematic viscosity, dynamic viscosity, density, calorific value, acid value, and oxidation stability were determined and then compared with ASTM D6751 and EN 14214 standards. These standards are the common international standards referred to for the utilization of biodiesel in engine. Equipment and test method used are listed in **Table 3.2**. The figures of the equipment used are also shown (**Figure 3.5 – 3.9**).

Density is the relationship between mass and volume of a substance. The unit commonly used is g/ml or kg/m^3. Dynamic viscosity is the measurement of the force required to overcome internal resistance for a fluid to flow, with a unit of mPa.s. Kinematic viscosity also refers to the fluid resistance to flow, but dependent to the fluid density. It is actually a ratio of dynamic viscosity to density, using a unit of mm^2/s. All these three properties (density, dynamic viscosity and kinematic viscosity) indicate the flow of a fuel inside the engine and thus affect the operation of an engine.

Calorific value shows the energy content in a substance, by measuring the amount of heat produced by a specific amount of substance in a complete combustion. The unit used is joules per kilogram, or in this study, megajoules per kilogram (MJ/kg). Acid value determines the amount of carboxylic acid groups (fatty acid) in the substance. It is measured by the amount of potassium hydroxide (KOH) needed to neutralize one gram of substance. The unit used is mg KOH/g. Oxidation stability measures the stability of the substance towards air exposure.

Table 3.2: List of equipment for physicochemical properties tests

Properties	Equipment	Test Method	Accuracy
Kinematic viscosity	SVM 3000 viscometer (Anton Paar)	D 445	± 0.01 mm^2/s
Dynamic viscosity	SVM 3000 viscometer (Anton Paar)	D 445	± 0.01 mPa.s
Density	DM40 density meter (Mettler Toledo)	D 127	± 0.1 kg/m^3
Calorific value	6100 calorimeter (Parr)	D 240	± 0.001 MJ/kg
Acid value	G20 compact titrator (Mettler Toledo)	D 664	± 0.001 mg KOH/g
Oxidation stability	873 biodiesel rancimat (Metrohm)	EN 14112	± 0.01 h

Figure 3.5: SVM 3000 viscometer

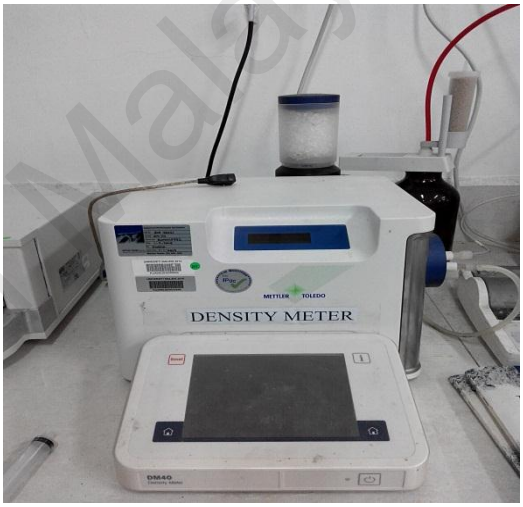

Figure 3.6: DM40 density meter

Figure 3.7: 6100 calorimeter

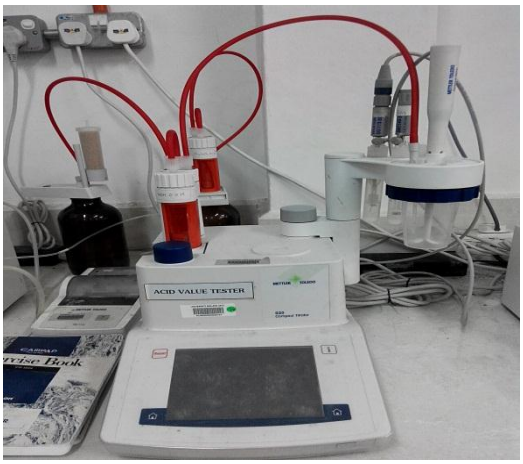

Figure 3.8: G20 compact titrator

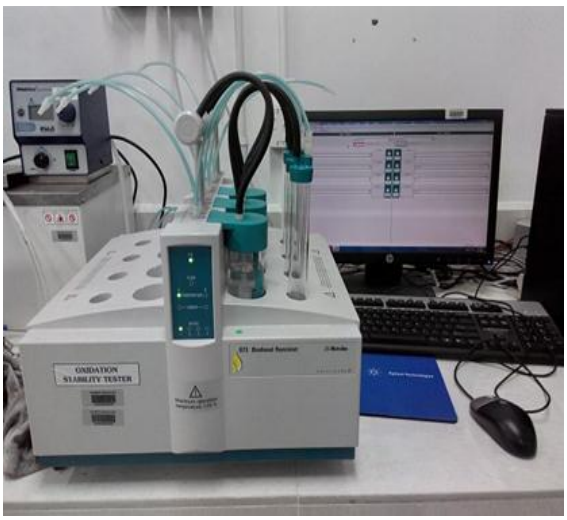

Figure 3.9: 873 biodiesel rancimat

3.5 Oil pretreatment

For oil pretreatment, 150 ml crude oil was added into a round-bottom glass reactor attached with a condenser. The reactor was placed inside a metal pot filled with cooking oil to ensure even heating of the crude oil inside the reactor (**Figure 3.10**). The crude oil was stirred and heated to 60 °C using a hot plate with magnetic stirrer. 75 ml methanol (50% of oil's volume) and 1.5 ml of sulfuric acid, H_2SO_4 (1% of oil's volume) were then added into the reactor. The stirring and heating were continued for 2 hours.

The mixture was then transferred into a separatory funnel and left overnight to form two layers. The top layer containing methanol, acid, and impurities was removed. The bottom layer (pretreated oil) was washed with warm water (60 °C) three times to remove remaining impurities, acid, and glycerol. The pretreated layer was then heated to 60 °C under vacuum condition using rotary evaporator to remove excess methanol and water.

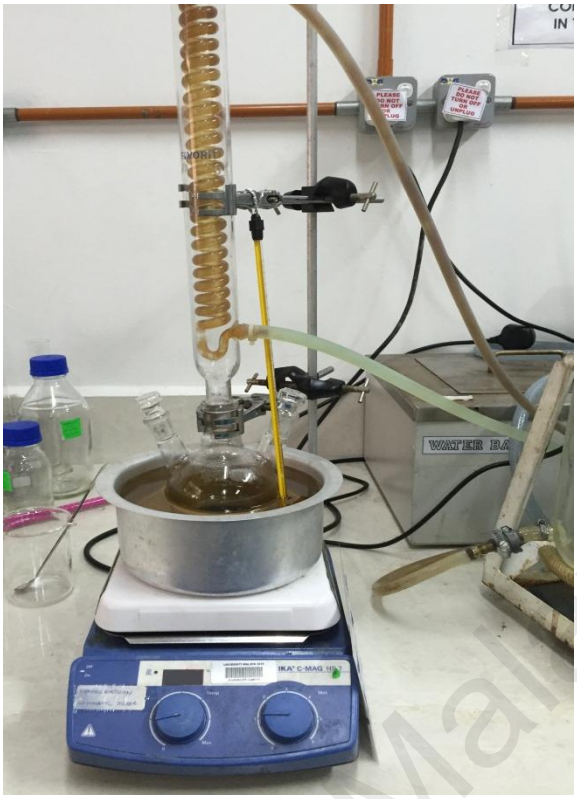

Figure 3.10: Oil pretreatment process

3.6 Gas Chromatography (GC) analysis

GC analysis was done to determine the FAME composition and yield percentage. After transesterification was complete, a portion of the biodiesel was taken and centrifuged. About 100 mg of the top layer was diluted and mixed with 10 ml toluene and 100 mg methyl nonadecanoate (internal standard). A volume of 1 µL of the mixture was injected to GC for analysis. Gas chromatography (Agilent 7890A, **Figure 3.11**) equipped with HP-INNOWAX capillary column (30m x 0.320mm x 0.25µm), FID detector, and injector (split and splitless) was used to determine the fatty acid methyl ester (FAME) content.

The column temperature was programmed according to EN 14103:2011 standard as follows: 60 °C hold for 2 min, 10°C/min to 200 °C, kept for 0 min, and 5°C/min to 240 °C, hold for 7 min. Both detector and injector were set at 250 °C. Helium was used as the carrier gas with a constant flow rate of 1.5 ml/min. The resulted chromatogram was compared with chromatogram obtained from C8-C24 FAME Mix to determine the

methyl ester peaks. FAME yield% was obtained by comparing the area of methyl esters peaks with internal standard peak using the following equation:

$$E = \frac{(\sum A) - A_{EI}}{A_{EI}} \times \frac{W_{EI}}{m} \times 100\% \quad (3.4)$$

In this equation, E represents the fatty acid methyl ester content (%), $\sum A$ is the sum of the peak areas of the fatty acid methyl ester content from C8:0 to C24:0, A_{EI} is the peak area of the internal standard, (methyl nonadecanoate), W_{EI} is the weight (milligrams) of internal standard being used, and m is the weight (milligrams) of the biodiesel sample.

Figure 3.11: Gas chromatography machine

3.7 Fourier transformed infrared (FTIR)

Biodiesel sample was analyzed by FTIR spectrophotometer (**Figure 3.12**) to determine its infrared spectrum of absorption. When an infrared beam is applied, molecule bonds will absorb the energy and perform vibrations such as stretching and bending.

FTIR enabled the identification of chemical bonds and functional groups presence in a sample by referring to the absorbance spectrum. The height of the peaks will show

different intensity which due to the change in dipole moment and also the molecule concentration in the sample. Beers's law as shown below,

$$A = \epsilon l c \tag{3.5}$$

where *A* represents the absorbance, ε is the absorptivity, *l* is the pathlength, and *c* is the concentration, shows the correlation between concentration and absorbance.

The FTIR spectrophotometer used in this study was TENSOR 27, Bruker Optics Inc, USA. It had a spectral range of 11,000-350 cm^{-1} and was equipped with a detector. The spectrum was then analyzed using OPUS Spectroscopy software.

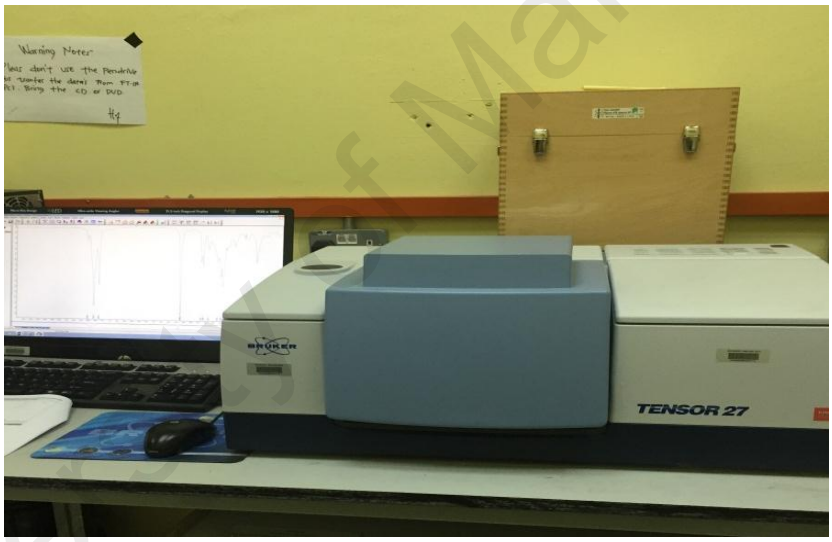

Figure 3.12: FTIR machine

3.8 Safety aspect

Safety is one of the important aspects to be taken into account when conducting any experiment in the laboratory. Safety guidelines need to be followed to reduce and avoid any risk of accidents. The list of guidelines and rules that took place while conducting this research was as follow:

1) Wear appropriate attire and lab coat. Wear safety shoes or closed toe shoes at all times.
2) Wear goggle and gloves (preferable nitrile) when dealing with liquid chemicals (for example methanol and t-butanol) and include face mask if dealing with chemicals that have harmful odor/gas.

3) Wear gloves and face mask when dealing with enzyme.
4) Chemicals with hazardous fumes or vapors should be handled in fume hood with extra care.
5) Check label on all chemical bottles before use. Take note of the material safety data sheet (msds) kept in the lab.
6) Take note of the location of first aid kit, fire extinguisher, emergency shower and eyewash station inside or near the lab.
7) Handle glassware and any equipment with care.
8) Do not eat or drink in the lab.
9) Dispose oil and chemical wastes properly in the designated waste container. Do not throw away wastes in the sink.
10) Wash skin or eyes immediately after any contact or accident with chemical. Report any accident for example spill, breakage or injury to lab technician immediately.
11) Never handle broken glass with bare hands. Use brush and dustpan to clean the broken glass and place it in the designated waste container.
12) Wash hands with soap and water after finishing all experiments.

CHAPTER 4: RESULTS AND DISCUSSION

The focus of this study is to investigate and improve biodiesel production from non-edible *Ceiba pentandra* oil using enzyme as catalyst. The biodiesel produced was mainly analyzed in terms of its fatty acid methyl ester (FAME) yield. In this research, biodiesel's composition and physicochemical properties were measured before comparing it with ASTM and EN international standards. Other experiments were then carried out which include effects of methanol concentration and its stepwise addition, enzyme pretreatment, optimization of the biodiesel production process, and enzyme reusability. Lastly, an economic evaluation was conducted to calculate biodiesel production cost per liter. This chapter will thoroughly describe the results obtained from the research works and provide critical analysis, discussion, and comparison with results from previous studies.

4.1 Biodiesels production from three oils and enzyme mechanism

Biodiesel production from *C. pentandra, J. curcas*, and rice bran oils using Novozym 435 and methanol was conducted and analyzed. The setting and condition used were 10 g crude oil, 5 wt.% enzyme, and 3:1 methanol to oil molar ratio, incubated with shaking at 40 °C and 150 rpm for 72 h. Three-step methanol addition was applied to each sample.

FAME contents measured for the biodiesels were 59.41%, 66.58% and 83.84% for *J. curcas*, *C. pentandra,* and rice bran oil respectively. This shows that the method used was valid and could produce more than 80% ester content. The difference in yield may due to the quality of the oils. Crude feedstock oil that is freshly extracted would produce higher biodiesel yield and exhibit better fuel properties compared to oil that has been stored for a long period. Other factors would be the oil's water content and fatty acid composition (Ko et al., 2012; E.-Z. Su et al., 2007). Impurities such as seed cake

particles and gum may also affect the yield especially when enzyme is used as the catalyst.

Previous studies have shown that reaction using the same operating conditions, lipase and acyl acceptor but different substrate could produce different yield%. Modi et al. (2007) used Novozym 435 and ethyl acetate, with three different oils: *Jatropha curcas*, *Pongamia pinnata*, and *Helianthus annuus* and obtained highest yield of 92.7% with *H. annuus* oil. Su et al. (2007) used *Candida* sp. lipase immobilized on cellulose fabric and dimethyl carbonate with variety of oil: olive oil, rapeseed oil, sunflower oil, soybean oil, corn oil, cottonseed oil, peanut oil, castor oil and sesame oil. All have different conversions with the highest using soybean oil (22.8%) and lowest with castor oil (0.13%).

The basic mechanism of an enzyme-catalyzed reaction is the binding of substrate to the active site of the enzyme. Active site of an enzyme is a region of an enzyme where the catalytic reaction takes place. As mentioned in Chapter 2.2.1, the mechanism for enzyme-catalyzed biodiesel production follows Ping Pong Bi Bi mechanism. This mechanism can be explained where each product is released between addition of substrates. One of the suggested reaction mechanism is the two-step mechanism (Fjerbaek et al., 2009).

$$E + S \leftrightarrow E \cdot S \leftrightarrow F \cdot S' \leftrightarrow F + S' \quad (4.1)$$

$$F + M \leftrightarrow F \cdot M \leftrightarrow E \cdot Es \leftrightarrow E + Es \quad (4.2)$$

In this equations, E = enzyme, S = substrate (tri-, di- or monoglyceride), F = fatty acid, S' = product with alcohol moiety (di- or monoglyceride or glycerol), M = methanol, and Es = FAME. The first step is the reaction between enzyme and substrate (oil) that released one fatty acid chain from the oil. The second step is the reaction between enzyme, fatty acid and methanol which produces methyl ester.

4.2 *C. pentandra* biodiesel composition and characteristics

Analysis of *C. pentandra* biodiesel was done to determine its composition. FTIR spectrum of *C. pentandra* biodiesel was presented in **Figure 4.1**. The strong absorbance peak at 1742 cm^{-1} was attributed by C=O bond which is an indicator for esters, and sharp peaks at 2923 and 2854 cm^{-1} were due to C-H stretching vibration (Yatish et al., 2016). Peaks ranging from 1196 to 1010 cm^{-1} were due to stretching vibration of C-O-C bond of esters (Dharma et al., 2016). This FTIR spectrum proved the strong presence of ester in the biodiesel. Other peaks are explained in **Table 4.1**.

Table 4.1: Wavenumber and functional group of FTIR absorbance peaks from *C. pentandra* biodiesel

Wavenumber (cm^{-1})	Functional groups	Absorption intensity
2923	=C-H	Strong
2854	$-CH_2$	Medium
1742	-C=O	Strong
1460, 1436	$-CH_2$ or $-CH_3$	Medium
1244	$-CH_3$	Medium
1196, 1169	C-O-C	Medium
1010	C-O-C	Weak
722	$-CH_2$	Medium

Different oil feedstock will have different fatty acid and FAME composition. **Figure 4.2** shows the GC result of *C. pentandra* biodiesel obtained using parameters setting at 40 °C, 150 rpm and reaction time of 72 h. The highest FAME presented in *C. pentandra* biodiesel was methyl linoleate (C18:2) at 41.8% (**Table 4.2**). This was followed by methyl palmitate (C16:0) at 22.78%, methyl oleate (C18:1) at 20.72%, and methyl octadecanoate (C18:0) at 11.22%. The methyl linolenate content of the biodiesel is at 1.63% which conformed to EN 14214 Standard (less than 12%). FAME composition obtained from using enzyme as catalyst was compared with the one obtained using chemical catalyst (conducted by Norazahar et al. (2012)) and similarities were observed. This shows that enzyme can be used to replace chemical catalyst in biodiesel production, as an option for a greener initiative.

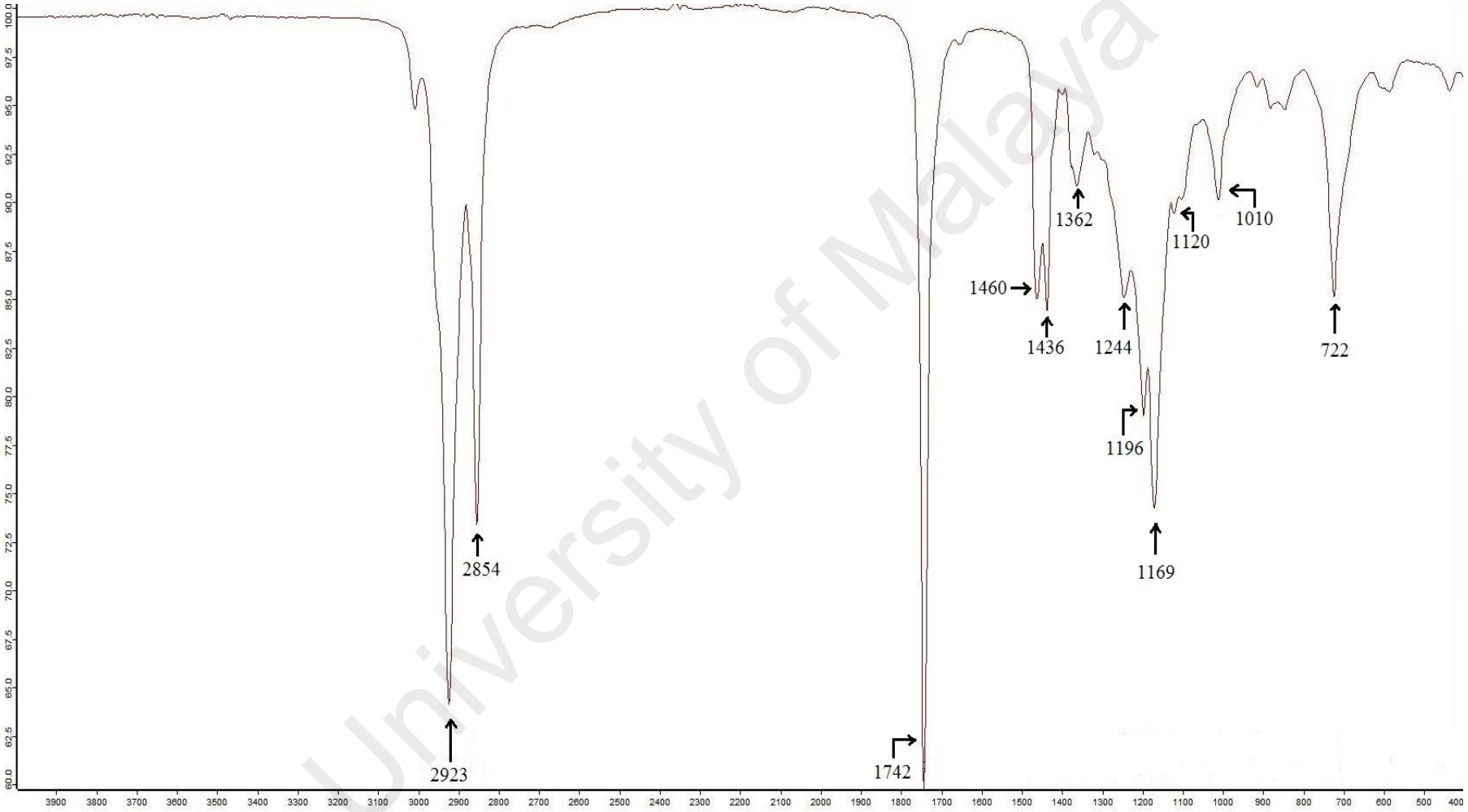

Figure 4.1: FTIR spectrum of *C. pentandra* biodiesel

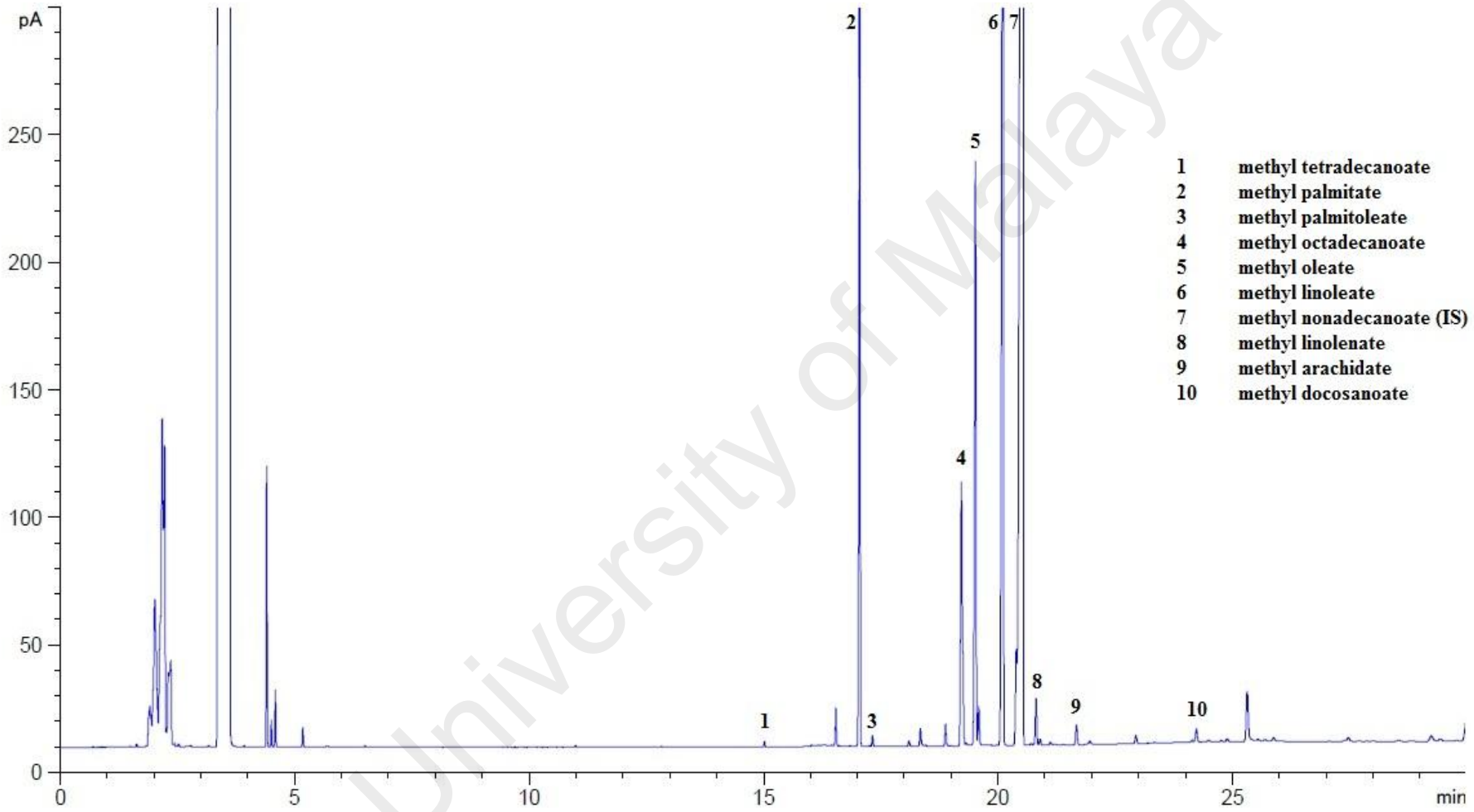

Figure 4.2: Chromatogram of *C. pentandra* biodiesel from GC analysis

Table 4.2: FAME composition of *C. pentandra* biodiesel produced using enzyme and chemical catalyst

FAME	Carbon	Percentage (%)	
		Enzyme	Chemical catalyst (Norazahar et al., 2012)
Methyl tetradecanoate	C14:0	0.12	-
Methyl palmitate	C16:0	22.78	23.17
Methyl palmitoleate	C16:1	0.29	-
Methyl octadecanoate	C18:0	11.22	4.73
Methyl oleate	C18:1	20.72	22.88
Methyl linoleate	C18:2	41.98	30.00
Methyl linolenate	C18:3	1.63	-
Methyl arachidate	C20:0	0.74	1.18
Methyl docosanoate	C22:0	0.52	-

It is common for non-edible oil to have higher percentage of C16:0 and C18 (C18:0, C18:1, and C18:2) (Atabani et al., 2013). Nitrogen oxides (NOx) emission is lower for C18:0 compared to shorter carbon chain fatty acids but increases with increasing degree of unsaturation (Pinzi et al., 2013). Generally, biodiesel gives higher NOx emissions compared to petrol fuel. It was also tested that peak pressure increased as unsaturation increased in which C18:2 gave higher peak pressure than C18:0 and C18:1 (Pinzi et al., 2013).

Physicochemical properties such as cetane number, viscosity, and melting point will increase with increasing chain length and decrease with increasing degree of unsaturation (double bond) (Knothe, 2005). The properties of *C. pentandra* crude oil, pretreated crude oil, and biodiesels (produced from crude oil and pretreated crude oil) were tabulated in **Table 4.3** together with limits of international standards for biodiesel: ASTM D6751 and EN 14214. The properties tested include kinematic viscosity, dynamic viscosity, density, calorific value, acid value, and oxidation stability.

.

Table 4.3: Properties of *C. pentandra* biodiesel with comparison to standards

Properties	**Unit**	**ASTM D6751**	**EN 14214**	**Crude *C. pentandra* oil**	**Pretreated crude *C. pentandra* oil**	***C. pentandra* biodiesel**	
						Without oil pretreatment	**With oil pretreatment**
Kinematic viscosity (40°C)	mm^2/s	1.9 – 6.0	3.5 – 5.0	34.9	27.0	5.94	5.62
Kinematic viscosity (100°C)	mm2/s	N/S	N/S	7.85	6.57	3.53	2.07
Density (15°C)	kg/m^3	880	860 – 900	922	918	889	890
Dynamic viscosity (40°C)	mPa.s	N/S	N/S	31.5	24.3	5.18	4.90
Dynamic viscosity (100°C)	mPa.s	N/S	N/S	6.78	5.65	2.99	1.71
Calorific value	MJ/kg	N/S	35	38.0	38.1	38.4	37.7
Linolenic acid methyl ester content	% (m/m)	N/S	12% max.	-	-	1.63	1.33
Acid value	mg KOH/g	0.5 max.	0.5 max.	22.2	0.34	7.72	0.16
Oxidation stability (110°C)	h	3 min.	6 min.	0.10	1.67	0.52	1.22

N/S = not specified

The definitions of these properties were explained in detail by Atabani et al. (2013). Kinematic and dynamic viscosity show how smooth the fuel will flow and it will affect the operation of fuel injection equipment. Density is the measure of mass per unit volume and could give an indication about the ignition quality and specific energy of the biodiesel. Calorific value is the energy content of the biodiesel and is usually lower than of diesel because of its higher content of oxygen. Meanwhile, oxidation stability shows the fuel stability against oxidative degradation. Acid value measures the amount of fatty acid in the biodiesel, where high value may cause corrosion in the engine.

It was observed that kinematic viscosity (40°C) and density of *C. pentandra* crude oil were quite high (34.9 mm^2/s and 922 kg/m^3 respectively) thus make it not suitable to be used directly in the engine. As expected, conversion of the plant oil to biodiesel reduced its viscosity and density (5.94 mm^2/s and 889 kg/m^3 respectively). Viscosities were also calculated under the temperature of 100°C as the normal operating temperature for engines were expected to be within 90 to 105 °C. The viscosities and density were considerably low enough for the better performance of the engine. The calorific value (38.4 MJ/kg) of the biodiesel also passed the minimum limit of 35 MJ/kg.

Further tests on the biodiesel showed that oxidation stability was really low, which was 0.52 h, lower than the minimum limits of 3h and 6h by ASTM D6751 and EN 14214 respectively. This may be caused by the high number of unsaturation level contributed by methyl linoleate and methyl oleate in the biodiesel. According to G. Knothe (2005), oxidation stability of biodiesel that is usually affected by the presence of air, heat, and traces of metals can also be affected by the number of double bond, where higher unsaturation degree will make the biodiesel susceptible to oxidation.

Another possible reason for this is that the crude oil obtained for this study was already old and had been stored for a long period of time before the purchase. In other words, it may have already been oxidized. This is supported by the data collected where the oxidation stability of the crude oil was only 0.10 h. This value is too low compared to oxidation stability measured in previous study which was 4.23 h (Yunus Khan et al., 2015). Hoekman et al. (2012) also stated that the age of biodiesel and the conditions it is stored does affect its oxidation stability. It is expected that if fresh oil was used, the oxidation stability of the biodiesel may have passed the biodiesel standard requirement. This is possible based on previous studies where *C. pentandra* biodiesel produced using chemical catalyst managed to obtained good oxidation stability at 9.22 h (Kusumo et al., 2017) and 4.42 h (Silitonga et al., 2013)

Nonetheless, oxidation stability of biodiesel tends to be lesser than petroleum diesel and is one of the major issues to be overcome before the biodiesel can be used in engines. Therefore, antioxidants such as chain breakers (peroxide radical quenchers) and hydro peroxide decomposers (reducing agents) are commonly used to increase biodiesel stability (Yaakob et al., 2014).

The oxidation stability of the crude oil may also affect its acid value number. During auto-oxidation process, decomposition of peroxides will produce aldehydes which are prone to oxidation, and causes the formation of more fatty acids with shorter chain length (Yaakob et al., 2014). This explains the high acid value of *C. pentandra* crude oil (22.2 mg KOH/g). Based on experimental results, it was observed that enzyme catalyst able to reduce acid value from 22.2 mg KOH/g (crude oil) to 7.72 mg KOH/g. This shows that the enzyme still able to convert free fatty acid, but not that effective.

To solve the problem of high acid value, an attempt was made by conducting a pretreatment reaction (using sulfuric acid) to the crude oil before biodiesel production

process. The acid value decreased from 7.72 mg KOH/g (without pretreatment) to 0.16 mg KOH/g (with pretreatment) which was below the maximum limit of 0.5 mg KOH/g. The oxidation stability of the biodiesel was also improved from 0.52 h to 1.22 h. There were not many differences in term of viscosities, densities and calorific values of the biodiesel between with and without oil pretreatment. For the comparison in term of FAME yield, the yield increased from 75.95% (no pretreatment) to 78.55% (pretreated).

It is expected that the physicochemical properties will be further improved after blending with diesel as proven by studies conducted by Ong et al. (2014) (**Table 4.4**). When diesel percentage in the blends increased, properties such as kinematic viscosity, density, and acid value decreased while other properties including oxidation stability and calorific value increased. Blending is also necessary as the current application of biodiesel in Malaysia is B10 (biodiesel mixed with 90% diesel) at the most.

Table 4.4: Properties of *C. pentandra* biodiesel-diesel blends (Ong et al., 2014)

Biodiesel percentage in diesel (%)	Viscosity (mm^2/s)	Density (kg/m^3)	Acid value (mg KOH/g)	Oxidation stability (h)	Calorific value (MJ/kg)
10	3.51	851	0.17	20.8	44.5
20	3.58	854	0.18	15.8	43.2
30	3.96	855	0.20	11.8	42.9
50	4.12	865	0.26	10.9	40.6

4.3 Effect of methanol concentration and stepwise addition

A set of samples with different methanol to oil molar ratio was used to determine the amount of methanol needed to produce high FAME yield. The FAME yields obtained for 3:1, 6:1, and 9:1 molar ratio were 72.99%, 75.95%, and 20.60% respectively (**Figure 4.3**). This shows that FAME yield decreased dramatically at high methanol concentration of 9:1. This happened may be due to the adsorption of alcohol onto polar immobilized material (acrylic resin), unfolding of enzyme, and/or immiscibility

between triglycerides and alcohol (J.-W. Chen & Wu, 2003; Ko et al., 2012; Korman et al., 2013; Maceiras et al., 2011).

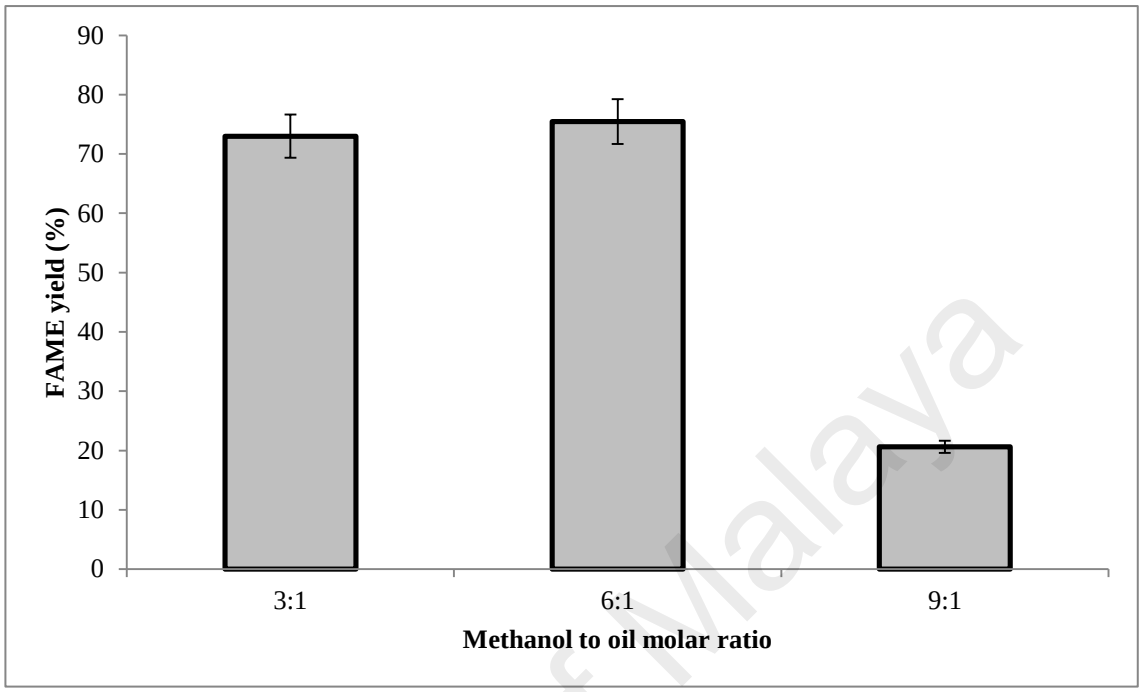

Figure 4.3: Effect of high concentration of methanol to FAME yield

Adsorption of alcohol by acrylic resin (material used to immobilize Novozym 435) may block the triglyceride entry of the enzyme, thus inhibiting the reaction (Maceiras et al., 2011). Immiscibility of the triglyceride and alcohol can be observed during the addition of methanol to the crude oil, as homogenous mixture was not formed and distinct layers were seen. This may hinder enzyme from executing an efficient reaction. Methanol, as a polar organic solvent, may disrupt the hydrophobic interaction of the protein structure and strip water off from enzyme thus causing enzyme instability and unfolding (Korman et al., 2013). Furthermore, it was found that alcohol adverse effect to enzyme was inversely proportional to the number of carbon atom of the alcohol when compared between methanol, ethanol, n-propanol, and n-butanol (J.-W. Chen & Wu, 2003), where methanol caused the most damage.

In term of mechanism, inhibition by methanol of an enzyme is considered as competitive inhibition. Competitive inhibition happens when the inhibitor substance (in this case, the methanol molecule) binds in the active site of the enzyme thus blocking the main reaction to happen. This will result in slowing down or even stopping of the catalytic reaction. The equation for this inhibition is as follow:

$$E + M \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E \cdot M \tag{4.3}$$

In the above equation, E = enzyme, M = methanol, E.M = the enzyme-methanol complex, k_1 and k_{-1} are the rate constants for the reversible formation of the enzyme-methanol complex. This equation is also supported by previous study by Al-Zuhair et al. (2007).

Taking note that three-step addition was applied to all three samples, the amount of methanol added for 9:1, 6:1 and 3:1 molar ratio were 3, 2 and 1 molar equivalents per step respectively. The results presented in **Figure 4.3** show that the enzyme able to maintain high yield in the 6:1 molar ratio (2 molar equivalents of methanol per step). Therefore, it can be proposed that Novozym 435 could tolerate up to 2 molar equivalents of methanol at a time in this reaction.

Another test was conducted to solve the problem of enzyme deactivation due to high concentration of methanol. The proposed solution was by increasing the methanol stepwise addition. The method used for 3:1, 6:1, and 9:1 molar ratio were three-step, six-step, and nine-step methanol addition respectively. The FAME yield obtained for 3:1, 6:1, and 9:1 molar ratio were 72.58%, 76.02% and 78.00% respectively (**Figure 4.4**). It can be seen that 9:1 molar ratio produced the highest yield, which contradicted from the previous result. The yield increased from 20.60% when using three-step addition to 78.00% when using nine-step addition. This high yield was achieved as the

volume of methanol added was kept at 1 molar equivalent per step. The low concentration of methanol in the system was tolerable by the enzyme thus preventing them from being deactivated.

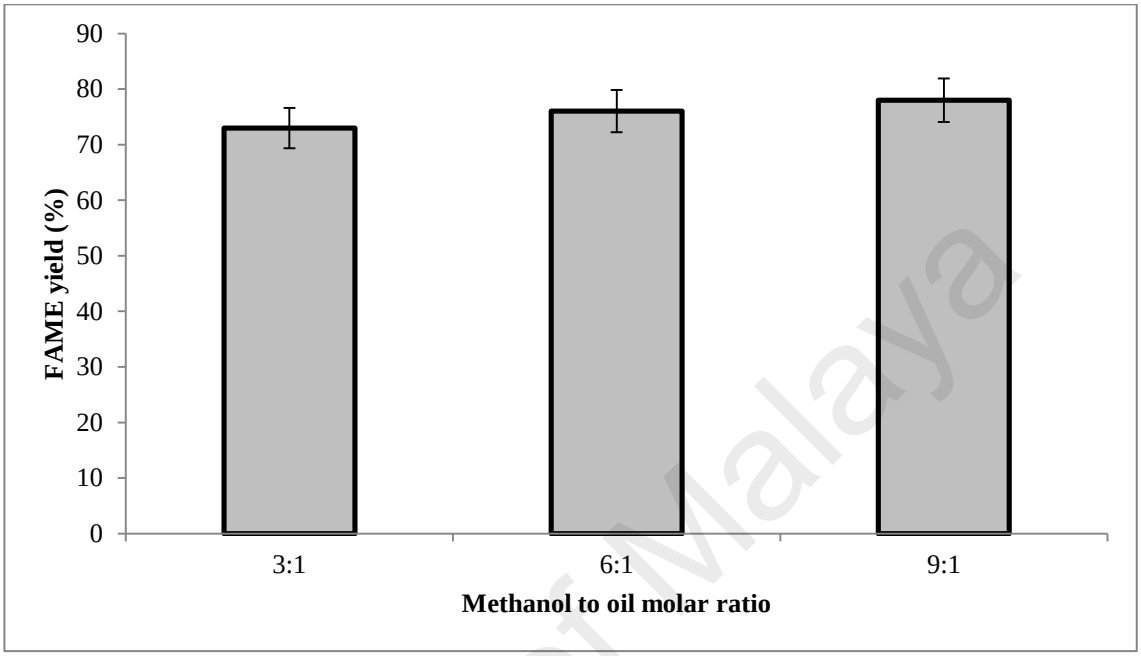

Figure 4.4: Effect of stepwise addition of methanol to FAME yield

The results obtained from this experiment are in agreement with previous studies. A research using soybean oil and 3:1 molar ratio (methanol to oil) to compare the biodiesel conversions between one-step, two-step, and three-step methanol addition demonstrated that the highest conversion was obtained using the three-step addition (one-step:40%; two-step:60%; three-step:90%) (Cerveró et al., 2014). Stepwise method was also used in recent biodiesel production studies and managed to attain good yields (Y. Liu et al., 2011; Yang et al., 2016). For example, Huang et al. (2012) conducted a study using recombinant *Pichia pastoris* whole cell displaying *Rhizomucor miehei* lipase, applying three-step methanol addition and obtained 83.14 % methyl esters yield after 72 h.

From this test, it can be observed that stepwise addition of methanol is beneficial to lipase enzyme. It is also expected that incorporation of continuous methanol addition in

biodiesel reactor may resulted in high biodiesel yield productivity. Many recent studies have applied stepwise or continuous addition of methanol in their biodiesel production to avoid lipase deactivation (Bonet-Ragel et al., 2015; Guldhe et al., 2016 ; Guldhe et al., 2015). This method can also be applied to the design of biodiesel reactor, both batch and continuous, for a large scale biodiesel production.

4.4 Enzyme pretreatment

To study the effect of enzyme pretreatment, lipase was pretreated with t-butanol and salt (sodium chloride, NaCl) solution before being used in biodiesel production process. The yield obtained with pretreated enzyme was compared with yield obtained from untreated enzyme (control) (**Figure 4.5**). In this experiment, it was observed that pretreatment using t-butanol resulted in increased of FAME yield from 66.58% to 67.29% but pretreatment using NaCl (low and high concentrations) caused decreased in yield. The yield was 58.06% at 1mM NaCl concentration and dropped to 50.05% at 1000 mM.

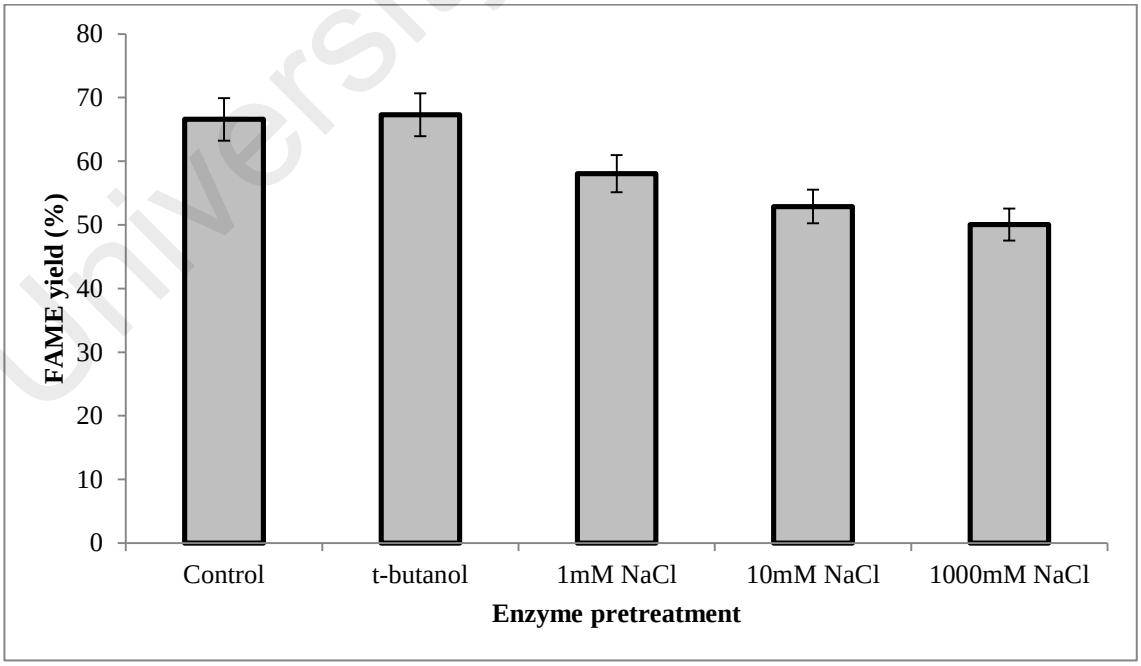

Figure 4.5: Effect of enzyme pretreatment using t-butanol and sodium chloride (NaCl) to FAME yield. Control is biodiesel produced using untreated enzyme (no pretreatment).

This result was contradicted with the one obtained when using immobilized *Candida sp. 99-125* lipase, where the yield increased after salt pretreatment (Lu et al., 2010). This can be explained as different lipase possesses different regioselectivity, specificity and catalytic activity, thus may react differently towards any substrate or solvent. For immobilized lipase, the immobilization method and material used may also affect enzyme reaction (R. C. Rodrigues et al., 2013). Nonetheless, it is believed that salt pretreatment process can be improved by developing a better method for the incorporation of salt or binding of ions to the lipase. One of the suggested method is lyophilization (freeze drying) to replace the normal drying method (H. W. Yu et al., 2005).

Immobilization of enzyme within porous support provides some advantages to enzyme activity. It may protect the enzyme from harmful conditions such as strong stirring and extreme pH. However, it may also cause diffusional or mass transfer limitation. In this study, pretreatment using t-butanol may have improved mass transfer on the surface layer and inside the porous support (**Figure 4.6**), thus increased the yield produced. Pretreatment with t-butanol gave positive effect to the yield may also due to its amphiphilic and moderately polar properties. Polar solvent may not be effective as it may interfere with lipase hydrogen bonding and hydrophobic interactions, and thus cause alteration of lipase molecular structure (E.-Z. Su et al., 2007).

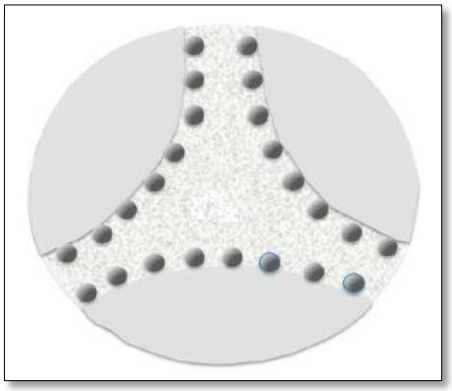

Figure 4.6: Enzyme immobilized inside porous support (R. C. Rodrigues et al., 2013)

4.5 Optimization

Methanol to oil molar ratio, temperature, and reaction time were chosen as the parameters in this experiment. **Table 4.5** shows the Box–Behnken design matrix of the experiment of 17 standard runs. Three input parameters (*i.e.* methanol to oil molar ratio, temperature, and reaction time), hidden layer, and a single output variable of FAME yield was analyzed using ANN.

Table 4.5: Experimental design for optimization process

Experimental run no.	Methanol to oil ratio (mol/mol)	Temperature (°C)	Time (h)	FAME yield%	FAME ANN prediction
1	7	40	72	54.15	54.35
2	3	50	72	71.31	69.18
3	7	50	48	16.86	16.42
4	11	50	24	11.93	10.05
5	7	60	24	10.45	10.44
6	3	40	48	56.70	55.42
7	7	50	48	16.07	16.42
8	7	50	48	16.44	16.42
9	7	50	48	15.63	16.42
10	11	60	48	7.126	6.645
11	7	60	72	11.90	17.02
12	7	50	48	18.15	16.42
13	3	50	24	64.52	64.55
14	7	40	24	20.37	20.07
15	3	60	48	68.35	63.62
16	11	50	72	12.85	7.500
17	11	40	48	24.93	25.10
				R^2	0.9906
				RMSE	2.3157

By using the heuristic procedure, the optimum number of hidden neurons was found to be 3-10-1 for biodiesel process (**Figure 4.7**). This was chosen due to the lowest values of mean square error (MSE = 5.363) (**Table 4.6**) and greatest value of R training (0.999), R validation (0.996), R test (0.997), and R all (0.995) (**Figure 4.8**). The values of the R^2 and RMSE (**Table 4.5**) were 0.9906 and 2.3157 respectively, which shows a

good agreement between ANN prediction values and experimental data. This indicates that this model had good performance and predictive capability.

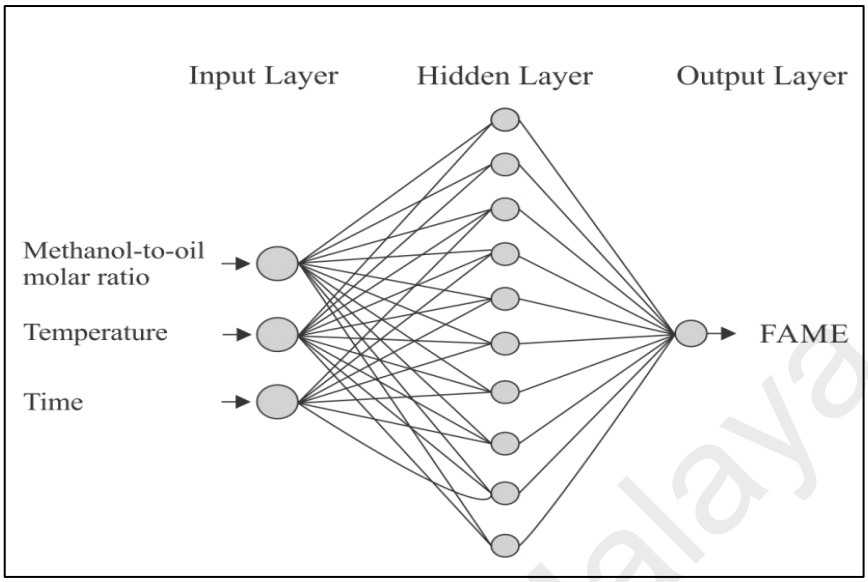

Figure 4.7: Architecture of the ANN model

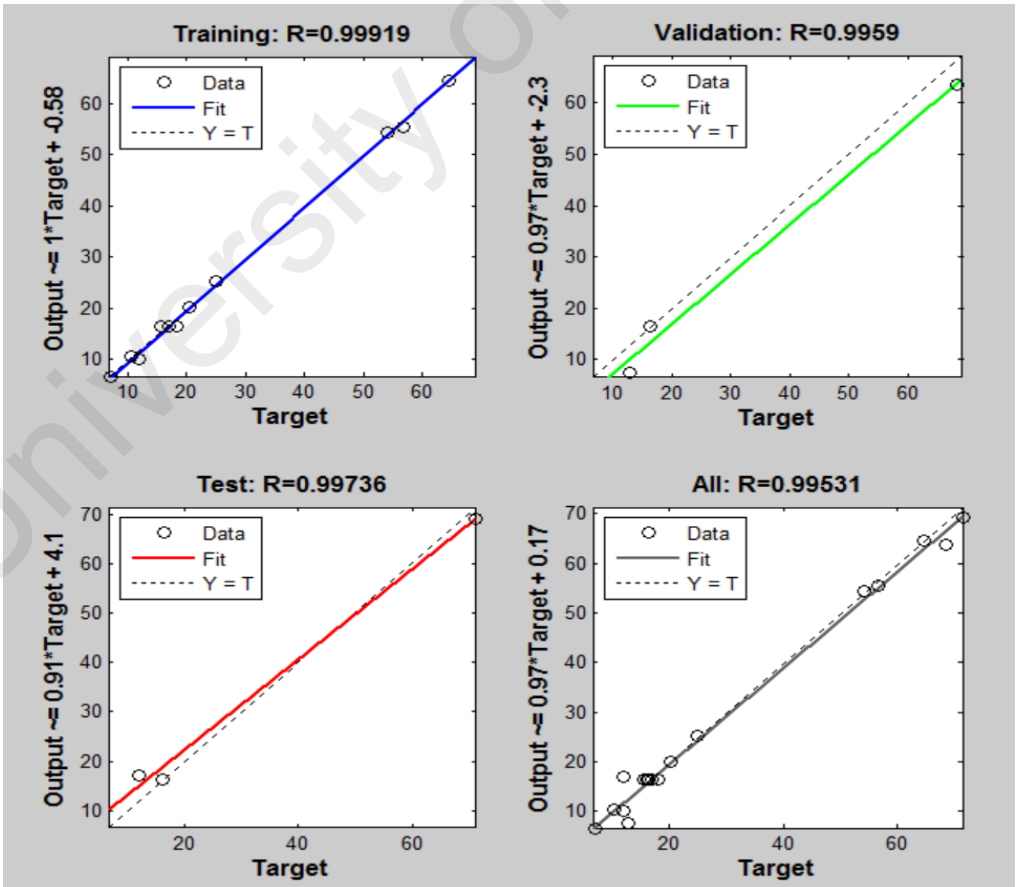

Figure 4.8: R values of training, validation, test data, and all

Table 4.6: Hidden neurons training

Number of hidden neuron	MSE	R
2	86.689	0.916
3	77.527	0.934
4	56.336	0.952
5	35.509	0.97
6	31.193	0.972
7	25.204	0.977
8	15.734	0.987
9	7.159	0.994
10	5.363	0.995
11	12.889	0.989
12	19.414	0.981

Furthermore, the obtained R^2 value of above 80% (R^2 = 99.06%) shows that the model was reliable in predicting the response (**Figure 4.9**). This implies that empirical models derived from ANN can be used to describe the input variables for biodiesel synthesis.

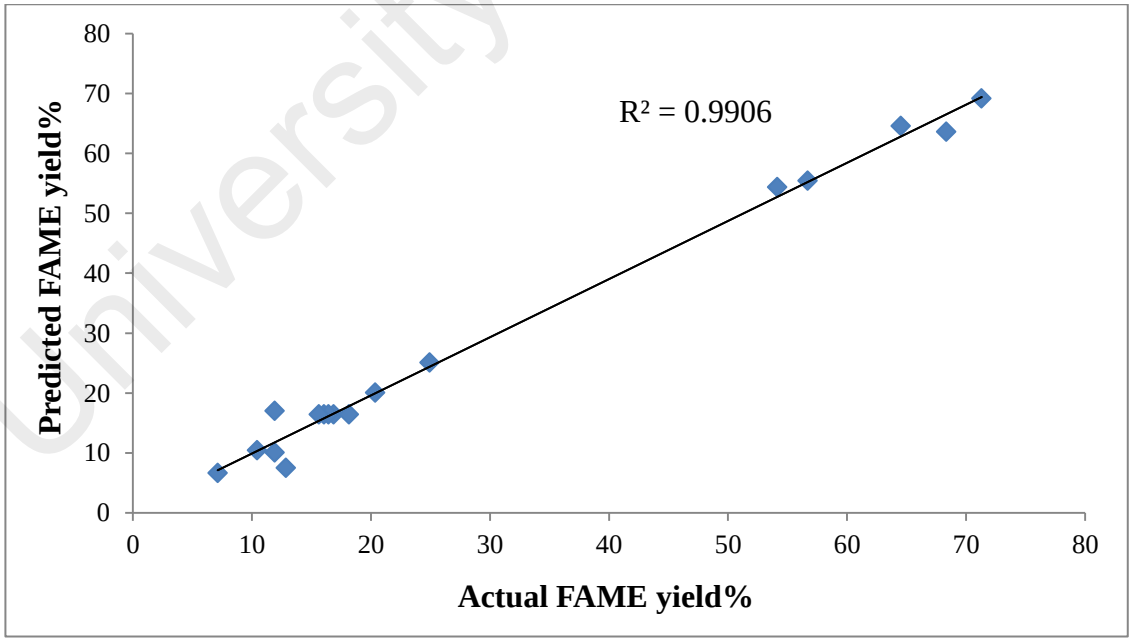

Figure 4.9: Comparison of actual and predicted FAME yield%

Further comparison between experimental results and ANN predictions is shown in **Figure 4.10**. The comparison shows that the model was linearly and closely fit with the

supplied target values. This indicates that the model was well suited for the biodiesel production prediction with high accuracy. The consistency of the network predicted values with the actual experiment for transesterification process of biodiesel could also be observed (**Figure 4.10)**. This suggests the inherent sensitivity of the network in its proficiency to map the transesterification process simultaneously with excellent accuracy.

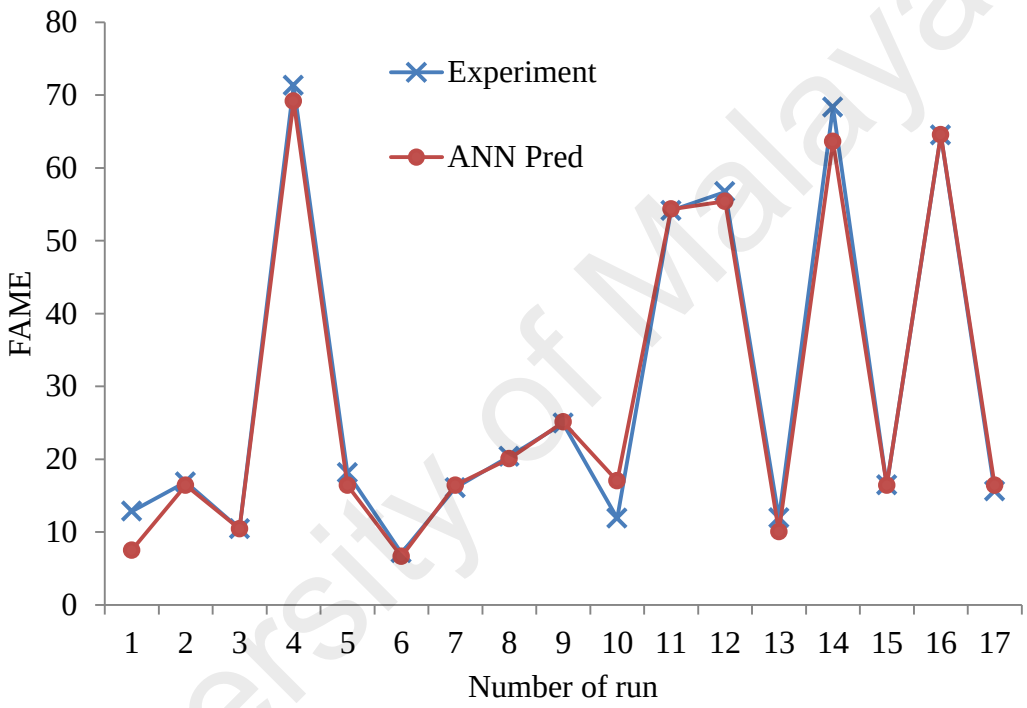

Figure 4.10: The experimental results versus ANN prediction

To predict the optimal condition for the biodiesel production synthesis using the network model, GA optimization algorithm was employed. The predicted optimum condition for transesterification process was temperature 57.42 °C, reaction time 71.89 h, methanol to oil molar ratio 3:1, to obtain a predicted methyl ester yield of 80.75%.

The obtained optimum temperature of 57.42 °C falls within the range of temperature for enzyme-catalyzed biodiesel production: 20 °C to 60 °C (Maceiras et al., 2011). The temperature is not too low for the enzyme to be inactive, and not too high that may

cause denaturation of enzyme molecular structure. Biodiesel production using enzyme catalyst usually takes up long reaction time. The long reaction time (71.89 h) and the low FAME yield (80.75%) may be due to the small amount of lipase used, which was only 5 wt.% (based on oil's weight). Previous studies have shown that increasing the lipase amount could increase the yield. Methyl esters yield increased from about 55% when using 5 wt.% Novozym 435 lipase to almost 90% with 10 wt.% lipase when converting waste frying oil to biodiesel (Maceiras et al., 2009). Another study shows that biodiesel yield increased from about 70% with 5 wt.% immobilized *Burkholderia cepacia* lipase to approximately 92% yield with 10 wt.% lipase using jatropha oil as feedstock and 30 h reaction time (You et al., 2013). Even though increasing the amount of lipase could shorten the reaction time and increase the yield, it may affect the total biodiesel production cost due to its high price. Nonetheless, it is expected that lipase price will drop over time when its usage is more common in the industry.

Methanol is the most common acyl acceptor (substrate) used for biodiesel production due to its cheap price, but its toxicity may result in enzyme deactivation especially at higher concentration. Thus, the expected optimum methanol to oil molar ratio of 3:1 is a good setting for enzyme activity.

4.5.1 Effects of reaction parameters on biodiesel yield

Methanol to oil molar ratio, reaction time, and temperature are three important parameters for biodiesel production using biocatalyst. The results of varying the values of each parameter were discussed for further understanding on how each parameter would affect FAME yield.

4.5.1.1 Effect of methanol to oil molar ratio

The ratio used for biodiesel production was varied at 3:1, 7:1, and 11:1 to observe the effect of methanol to oil molar ratio to biodiesel yield. Three dimensional plot of the

combined effect of methanol to oil molar ratio and time to FAME yield was shown in **Figure 4.11**. It can be observed that when the methanol concentration was increased, the yield decreased. This is similar with the results obtained previously in Chapter 4.3. This may due to toxic effect of methanol that causes enzyme deactivation. This result shows that 3:1 methanol to oil is enough to obtain high FAME yield, which agrees with theoretical value of 3 molar ratio needed for a complete conversion of triacylglyceride to biodiesel.

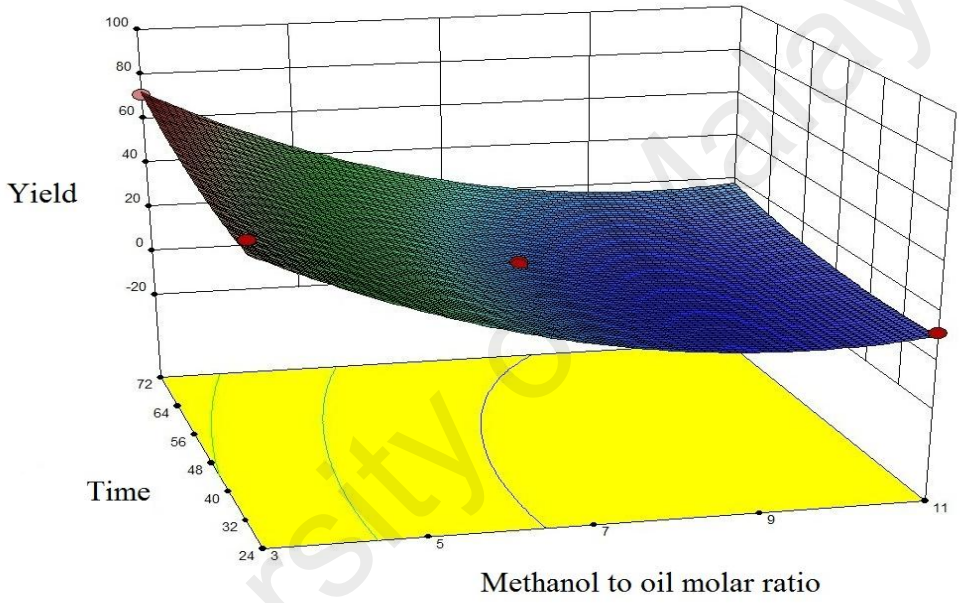

Figure 4.11: Surface plot for the combined effects of methanol to oil molar ratio and reaction time on biodiesel yield

4.5.1.2 Effect of reaction time

The time was varied at 24 h, 48 h, and 72 h to observe the effect of time to biodiesel yield. From **Figure 4.12**, it was observed that the longer the reaction time, the higher the yield. Generally, enzyme requires long reaction time compared to chemical catalyst. The long period gave the enzyme enough time to convert triacylglyceride and free fatty acid to methyl esters. In this experiment, the longer time of 72 h gave the best result for

high FAME yield. This result was similar with previous test done by Amini et al. (2017) that convert *Ocimum basilicum* (sweet basil) seed oil to biodiesel.

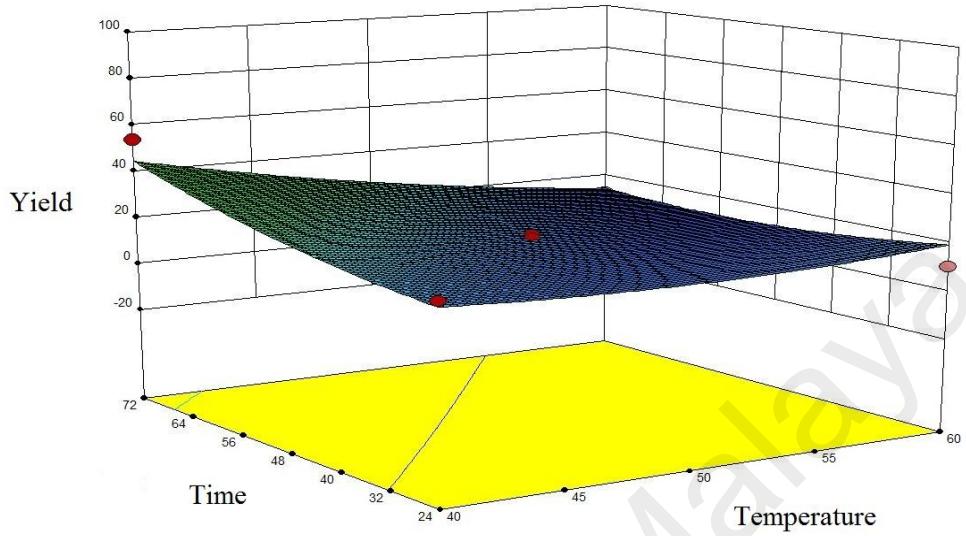

Figure 4.12: Surface plot for the combined effects of reaction time and temperature on biodiesel yield

4.5.1.3 Effect of temperature

Figure 4.13 shows the combined effects of temperature and methanol to oil molar ratio FAME yield. The temperature was varied at 40 °C, 50 °C and 60 °C for this test. Generally, enzyme will become inactive if the temperature of its surrounding is too cold, and will denature if the temperature is too hot. However, the results presented in **Figure 4.13** shows that the yields obtained were about the same even if the temperature were changed. The possible reason for this is Novozym 435 could perform enzyme activity effectively within temperature of 40 – 60 °C. The yield may have dropped if the biodiesel production was conducted at a temperature lower than 40°C or higher than 60°C.

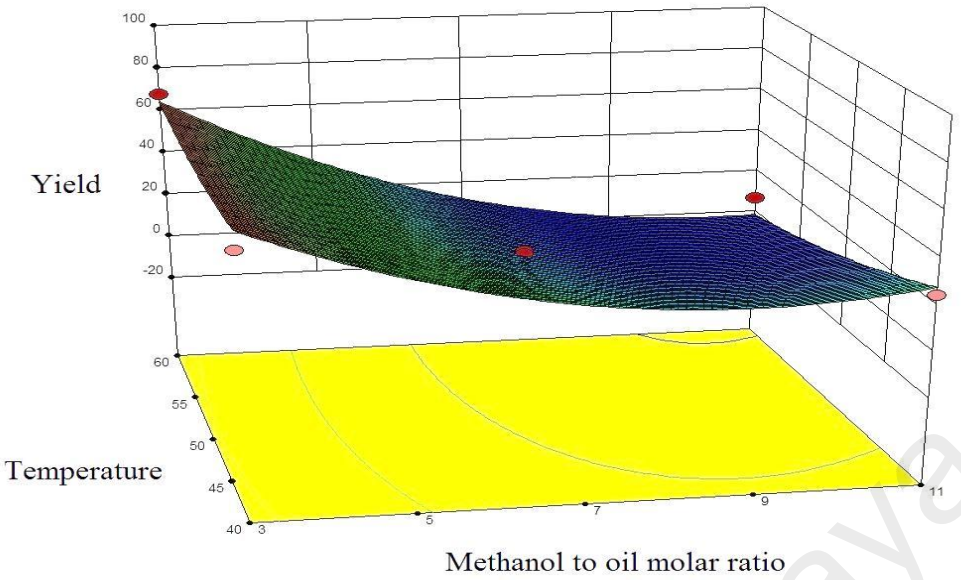

Figure 4.13: Surface plot for the combined effects of temperature and methanol to oil molar ratio on biodiesel yield

4.6 Enzyme reusability

Immobilized lipase is much more preferred compared to free lipase because it enables easy recovery of enzyme and maintenance of its thermal and pH stability (Tian et al., 2016). Reusability of enzyme is important to reduce the effect of enzyme high price on the total cost production of biodiesel. Hence, this study was carried out to determine the reusability of enzyme up to fifth batch.

The yields obtained from this test are presented in relative to the yield of the first batch (**Figure 4.14**). The third batch produced 63.69% relative FAME yield while the fifth batch produced only 27.70%. The decreased in yield may be the result of loss of enzyme activity over continuous exposure to temperature, mechanical stress, and substrates. Adsorption of glycerol and formation of layer containing heterogenous mixture of oil and biodiesel on enzyme surface during reaction may also block enzyme activity (Aguieiras et al., 2016; R. Rodrigues et al., 2008). To ensure high productivity in this biodiesel production, the enzyme may be reused up to three times.

Nonetheless, there are several methods proposed to minimize enzyme loss of activity. One of the suggested methods is post-treatment process such as enzyme washing after each reaction cycle. The most common solvent used for enzyme washing is hexane. Since the heterogenous layer formed is non-polar, non-polar hexane is believed to be effective in washing away the layer (R. Rodrigues et al., 2008).

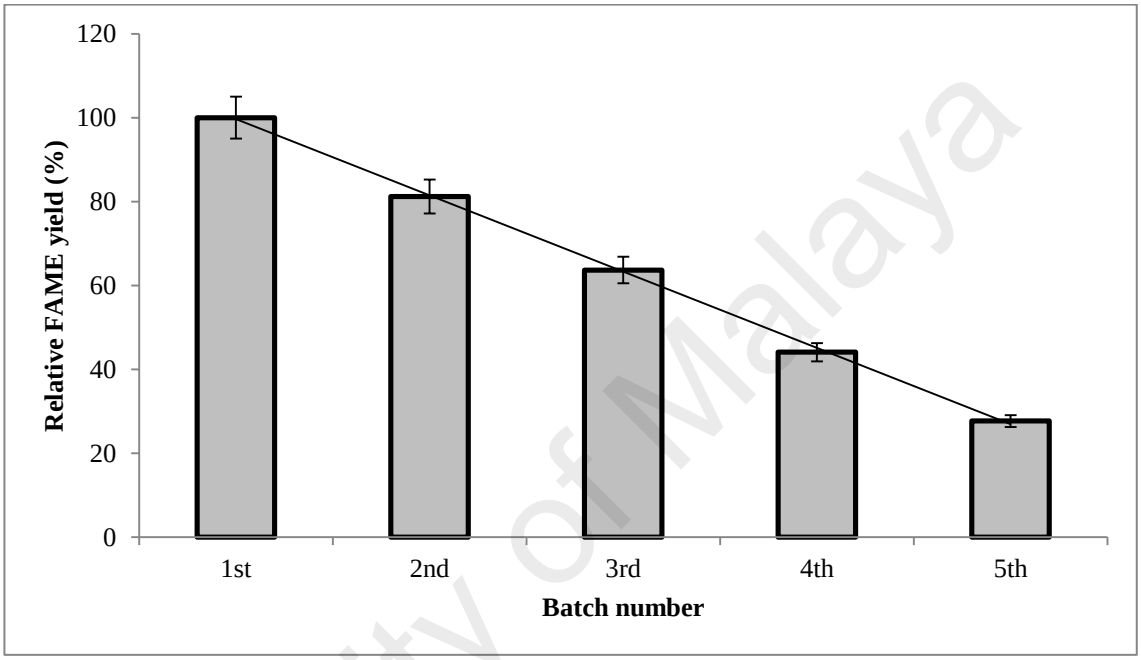

Figure 4.14: Reusability of enzyme (Novozym 435)

Other than hexane, alcohols also were used for enzyme washing. Yu et al. (2013) washed immobilized *P. cepacia* lipase with t-butanol after each cycle and the lipase retained about 80% of its initial conversion after three repeated uses (unwashed retained only about 70%). Chen and Wu (2003) reactivate completely deactivated Novozym 435 with 2-butanol and t-butanol to 56% and 75% of its original activity respectively.

There are several things that should be taken into account in choosing the solvent to be used. This include its effectiveness in removing the heterogenous layer and glycerol on the enzyme surface, its effect on the structure of lipase, as well as its effect on the immobilization support (Aguieiras et al., 2016). Wrong choice of solvent would cause adverse effects on the support. For example, solvent like hexane could dissolve

macroporous resin support, and polar solvent such as ethanol and butanol could change the morphology of a gel of granulated silica support (Aguieiras et al., 2016).

4.7 Biodiesel production cost

An assesment was made to calculate the cost of production of *C. pentandra* biodiesel using biocatalyst where the expected output of biodiesel was 8 kilotonne per year. The process flow diagram for biodiesel production using enzyme catalyst is shown in **Figure 4.15**. Three raw materials (oil, enzyme, and methanol) are mixed in the mixer and transferred to reactor for the reaction to take place. Centrifuge is then used to collect the enzyme for the next cycle. The main product (biodiesel) will be separated from the mixture of glycerol and excess methanol in the decanter. Lastly, distillation collumn is used to collect unused methanol and separate it from glycerol that can be sold for revenue.

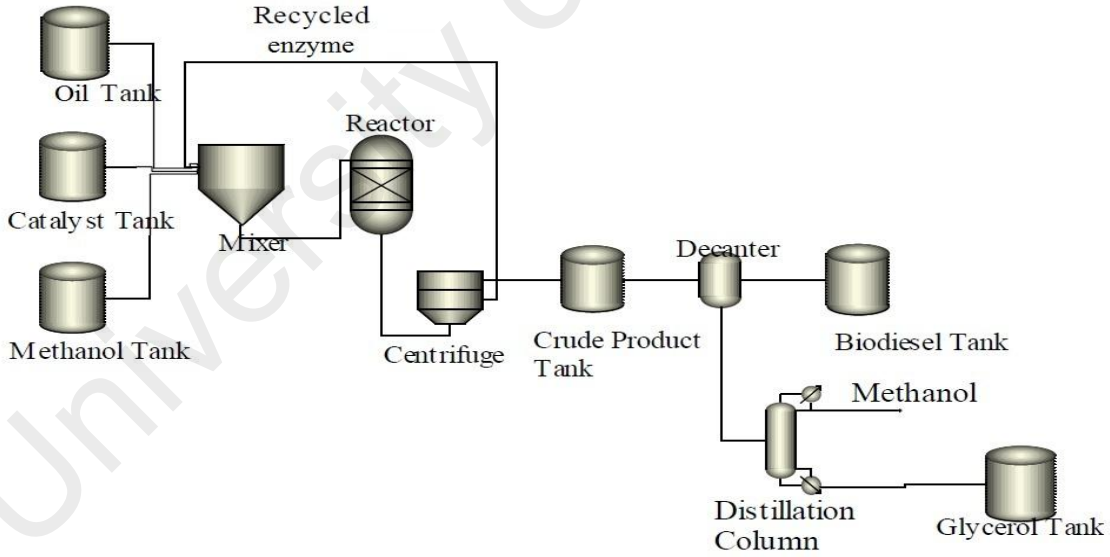

Figure 4.15: Process flow diagram for the production of C. pentandra biodiesel using enzyme catalyst (Karmee et al., 2015)

Total equipment cost (TEC) of the plant was expected to be $745,000 (**Table 4.7**). This value was obtained by referring the cost of each equipment to previous study done by Karmee et al. (2015). Meanwhile, the plant investment cost was calculated to be

around $1,974,250 as shown in **Table 4.8** (Jegannathan et al., 2011; Karmee et al., 2015). The plant investment cost or also called as total capital cost is the one-time expenses allocated for the purchase of equipment, land, building, and other additional costs that are required for the biodiesel plant to fully operate.

Table 4.7: Total equipment cost (TEC)

Equipment	Quantity	Total Cost (USD)
Tank (100 m^3)	6	390,000
Mixer	1	56,000
Reactor (15 m^3)	1	88,000
Centrifuge	1	15,000
Decanter	2	27,000
Distillation column (1 m diameter, 15 m height)	1	169,000
	Total	745,000

Table 4.8: Plant investment cost

Category	Percentage of TEC (%)	Cost (USD)
Total equipment cost (TEC)	100	745,000
Installation	15	111,750
Piping	20	149,000
Electric and instrumentation	30	223,500
Buildings, structure, and yard	60	447,000
Engineering and supervising	30	223,500
Land acquisition	10	74,500
Total	265	1,974,250

To calculate biodiesel production cost per liter, two price of lipase were used: $800/kg and $8/kg. $800/kg is the current enzyme price while $8/kg is the expected enzyme price in the future. Enzyme price is expected to be cheaper in the future once it becomes widely used in the industry. The same method of analysis was conducted by Sotoft et al. (2010) using both current and future prices of enzyme. The calculations were also based on parameter setting obtained from optimization results: 3:1 methanol to oil molar ratio, 57.4 °C, 72 h, and the enzyme to be recycled 3 times (assuming 80% yield for all three cycles). With a total biodiesel output of 8 kilotonne (8,000,000 kg)

per year, the consumption of raw materials per year were expected to be as follows: 10 kilotonne crude *C. pentandra* oil, 1 kilotonne methanol, and 167 tonne enzyme.

Biodiesel production cost is the sum of all expenses for the production of biodiesel including the cost of raw materials, electricity, labor, maintenance, and operational cost, and subtracts it with the income obtained from glycerol sales. It was expected that 10kg glycerol is produced for every 100kg biodiesel (Santibañez et al., 2011). The total biodiesel production cost is expected to be at \$15.69/L and \$0.97/L for enzyme price of \$800/kg and \$8/kg respectively (**Table 4.9**). From this data, it can be seen that enzyme price will significantly affect the product cost and the viability of the process. The current price of enzyme has become a hindrance for enzyme-catalyzed production to become widely employed. However, it is predicted to become more economically feasible once the lipase price become comparable with chemical catalysts in the future.

Table 4.9: Biodiesel production cost

Category	**Unit cost (USD)**	**Cost (USD)**	
		\$800/kg enzyme	**\$8/kg enzyme**
Enzyme catalyst	\$800/kg ; \$8/kg	133,600,000	1,336,000
Methanol	\$0.35/kg	350,000	
C. pentandra oil	\$0.73/kg	7,300,000	
Glycerol sales	\$1.04/kg	832,000	
Electricity (40 kWh/ton of biodiesel produced)	\$0.15/kWh	48,000	
Labor (10 employees)	\$20,000/employee/year	200,000	
Maintenance and operational cost (MOC)	10% of TEC	74,500	
Factory overhead	50% of labor and MOC	137,250	
General expenses	25% of labor and MOC	68, 625	
Property insurance	5% of TEC	37,250	
Contingency	10% of labor, MOC and factory overhead	41,175	
	Total cost for 8 kilotonne biodiesel	141,024,800	8,760,800
	Total cost per 1 liter biodiesel	15.69	0.97

CHAPTER 5: CONCLUSION

Enzymatic reaction is more advantageous than chemical methods in term of its mild reaction conditions, easy product recovery, no wastewater generation, and no saponification. Due to high cost of enzyme, slow reaction rate and enzyme inhibition, biocatalyst is not commonly used for biodiesel production as compared to chemical catalyst. In this study, immobilized lipase from *Candida antarctica* (Novozym 435) was used as biocatalyst for the production of biodiesel from non-edible *Ceiba pentandra* oil. Experiments were conducted based on research objectives and results obtained were analyzed and discussed.

Results from *C. pentandra* biodiesel's composition and physicochemical properties show that enzyme could be used as catalyst for *C. pentandra* biodiesel production. The biodiesel produced had high content of ester especially methyl linoleate (C18:2) and methyl palmitate (C16:0). Oil pretreatment that was conducted before transesterification process managed to reduce its acid value and improve its antioxidant stability.

For the test on the effects of methanol concentration and stepwise addition, it was observed that FAME yield decreased dramatically at high methanol concentration (9:1 methanol to oil molar ratio) but stepwise addition could reduce the effect. Enzyme was able to tolerate up to 2 molar equivalent of methanol at a time and 3:1 ratio was enough for biodiesel production. For the test on enzyme pretreatment, biodiesel yield increased when using t-butanol but decreased when using sodium chloride solution. This is due to the amphiphilic property of t-butanol.

Prediction of the optimized process parameters was done using artificial neural network (ANN) based program and genetic algorithm (GA). ANN predictions were compared with experimental results and obtained good agreement (coefficient of determination, R^2 of 0.9906). The optimum parameters setting is determined to be at

57.42 °C temperature, 3:1 methanol to oil molar ratio, and 71.89 h reaction time, to achieve a biodiesel yield of 80.75%.

For enzyme reusability test, enzyme activity decreased gradually after each batch and the reusability of the enzyme was measured at 63.69% relative yield after three batches. The calculated biodiesel production costs were at $15.69/L and $0.97/L for enzyme price at $800/kg (current enzyme cost) and $8/kg (enzyme cost in the future) respectively. It is predicted that biodiesel production from *C. pentandra* oil using biocatalyst will become more economically feasible once the lipase price become comparable with chemical catalysts in the future.

This research work has given many additional data on how certain conditions would affect FAME yield. The results were primarily related to how the enzyme reacts to its surrounding. For example, high concentration of methanol could decrease the yield thus stepwise addition of methanol should be incorporated in the biodiesel reactor (methanol ratio need to be maintained below 2 molar per addition). For oil feedstock with high free fatty acid content, lipase could not convert all the FFA to FAME thus oil pretreatment is needed to reduce the FFA amount. In addition, this study shows that combination of ANN and GA software could be utilized for the optimization process of enzyme-catalyzed biodiesel production to gain high output.

Recommendations

There will be a bright future for enzyme-catalyzed production of biodiesel if continuous research is done in this field. This is because there are still a lot of things needed to be learned about this enzyme called lipase. The attractive and desirable characteristics of the enzyme may not be as good as it expected to be. For example, many literatures stated that enzyme able to conduct complete catalysis of free fatty acid

to biodiesel. However, it is presented in this study that there may be some limitations to it.

Future research should include utilization of the *C. Pentandra* biodiesel produced using biocatalyst biodiesel in diesel engine. This would include engine performance tests in term of engine torque, engine power, fuel consumpton and brake thermal efficiency, and emission tests to measure the amount of carbon dioxide, carbon monoxide, nitrogen oxides (No_x), and hydrocarbon emitted to the surrounding. Experiments to determine the effects of reusable enzyme and stepwise methanol eddition on fuel properties could also be done.

Further tests on enzyme such as pre-treatment and post-treatment with other different types of solvent would also give additional information to improve the biodiesel process. Production of new type of immobilized enzyme that has enhanced traits such as better heat stability, good methanol tolerance, and high reusability rate would definitely help to promote the application of enzyme as the catalyst for biodiesel production. It is aspired that the results from this research could help the government in implementing its current biodiesel mandate and contribute towards a greener and environmental friendly biodiesel production globally.

REFERENCES

Aguieiras, E. C. G., Ribeiro, D. S., Couteiro, P. P., Bastos, C. M. B., Queiroz, D. S., Parreira, J. M., & Langone, M. A. P. (2016). Investigation of the Reuse of Immobilized Lipases in Biodiesel Synthesis: Influence of Different Solvents in Lipase Activity. [journal article]. *Applied Biochemistry and Biotechnology*, 1-12.

Al-Zuhair, S., Ling, F. W., & Jun, L. S. (2007). Proposed kinetic mechanism of the production of biodiesel from palm oil using lipase. *Process Biochemistry, 42*(6), 951-960.

Amini, Z., Ong, H. C., Harrison, M. D., Kusumo, F., Mazaheri, H., & Ilham, Z. (2017). Biodiesel production by lipase-catalyzed transesterification of Ocimum basilicum L. (sweet basil) seed oil. *Energy Conversion and Management, 132*, 82-90.

Amoah, J., Ho, S.-H., Hama, S., Yoshida, A., Nakanishi, A., Hasunuma, T., Ogino, C., & Kondo, A. (2016). Converting oils high in phospholipids to biodiesel using immobilized Aspergillus oryzae whole-cell biocatalysts expressing Fusarium heterosporum lipase. *Biochemical Engineering Journal, 105, Part A*, 10-15.

Aransiola, E. F., Ojumu, T. V., Oyekola, O. O., Madzimbamuto, T. F., & Ikhu-Omoregbe, D. I. O. (2014). A review of current technology for biodiesel production: State of the art. *Biomass and Bioenergy, 61*(0), 276-297.

Ashnani, M. H. M., Johari, A., Hashim, H., & Hasani, E. (2014). A source of renewable energy in Malaysia, why biodiesel? *Renewable and Sustainable Energy Reviews, 35*(0), 244-257.

Atabani, A. E., Silitonga, A. S., Badruddin, I. A., Mahlia, T. M. I., Masjuki, H. H., & Mekhilef, S. (2012). A comprehensive review on biodiesel as an alternative energy resource and its characteristics. *Renewable and Sustainable Energy Reviews, 16*(4), 2070-2093.

Atabani, A. E., Silitonga, A. S., Ong, H. C., Mahlia, T. M. I., Masjuki, H. H., Badruddin, I. A., & Fayaz, H. (2013). Non-edible vegetable oils: A critical evaluation of oil extraction, fatty acid compositions, biodiesel production, characteristics, engine performance and emissions production. *Renewable and Sustainable Energy Reviews, 18*(0), 211-245.

Avramović, J. M., Veličković, A. V., Stamenković, O. S., Rajković, K. M., Milić, P. S., & Veljković, V. B. (2015). Optimization of sunflower oil ethanolysis catalyzed by calcium oxide: RSM versus ANN-GA. *Energy Conversion and Management, 105*, 1149-1156.

Bahrami, A., Mousavi Anijdan, S. H., & Ekrami, A. (2005). Prediction of mechanical properties of DP steels using neural network model. *Journal of Alloys and Compounds, 392*(1–2), 177-182.

Ban, K., Hama, S., Nishizuka, K., Kaieda, M., Matsumoto, T., Kondo, A., Noda, H., & Fukuda, H. (2002). Repeated use of whole-cell biocatalysts immobilized within biomass support particles for biodiesel fuel production. *Journal of Molecular Catalysis B: Enzymatic, 17*(3–5), 157-165.

Bharathiraja, B., Chakravarthy, M., Kumar, R. R., Yuvaraj, D., Jayamuthunagai, J., Kumar, R. P., & Palani, S. (2014). Biodiesel production using chemical and biological methods – A review of process, catalyst, acyl acceptor, source and process variables. *Renewable and Sustainable Energy Reviews, 38*(0), 368-382.

Bonet-Ragel, K., Canet, A., Benaiges, M. D., & Valero, F. (2015). Synthesis of biodiesel from high FFA alperujo oil catalysed by immobilised lipase. *Fuel, 161*, 12-17.

BP. (2015). BP Statistical Review of World Energy, June 2015.

Calero, J., Luna, D., Sancho, E. D., Luna, C., Bautista, F. M., Romero, A. A., Posadillo, A., Berbel, J., & Verdugo-Escamilla, C. (2015). An overview on glycerol-free processes for the production of renewable liquid biofuels, applicable in diesel engines. *Renewable and Sustainable Energy Reviews, 42*(0), 1437-1452.

Canet, A., Bonet-Ragel, K., Benaiges, M. D., & Valero, F. (2016). Lipase-catalysed transesterification: Viewpoint of the mechanism and influence of free fatty acids. *Biomass and Bioenergy, 85*, 94-99.

Cerveró, J. M., Álvarez, J. R., & Luque, S. (2014). Novozym 435-catalyzed synthesis of fatty acid ethyl esters from soybean oil for biodiesel production. *Biomass and Bioenergy, 61*(0), 131-137.

Cheirsilp, B., H-Kittikun, A., & Limkatanyu, S. (2008). Impact of transesterification mechanisms on the kinetic modeling of biodiesel production by immobilized lipase. *Biochemical Engineering Journal, 42*(3), 261-269.

Chen, B., Hu, J., Miller, E. M., Xie, W., Cai, M., & Gross, R. A. (2008). Candida antarctica Lipase B Chemically Immobilized on Epoxy-Activated Micro- and Nanobeads: Catalysts for Polyester Synthesis. *Biomacromolecules, 9*(2), 463-471.

Chen, J.-W., & Wu, W.-T. (2003). Regeneration of immobilized Candida antarctica lipase for transesterification. *Journal of Bioscience and Bioengineering, 95*(5), 466-469.

Christopher, L. P., Hemanathan, K., & Zambare, V. P. (2014). Enzymatic biodiesel: Challenges and opportunities. *Applied Energy*, *119*(0), 497-520.

Colombo, T. S., Mazutti, M. A., Di Luccio, M., de Oliveira, D., & Oliveira, J. V. (2015). Enzymatic synthesis of soybean biodiesel using supercritical carbon dioxide as solvent in a continuous expanded-bed reactor. *The Journal of Supercritical Fluids, 97,* 16-21.

Demirbas, A., & Fatih Demirbas, M. (2011). Importance of algae oil as a source of biodiesel. *Energy Conversion and Management, 52*(1), 163-170.

Dharma, S., Masjuki, H. H., Ong, H. C., Sebayang, A. H., Silitonga, A. S., Kusumo, F., & Mahlia, T. M. I. (2016). Optimization of biodiesel production process for mixed Jatropha curcas–Ceiba pentandra biodiesel using response surface methodology. *Energy Conversion and Management, 115*, 178-190.

Du, W., Xu, Y.-Y., Liu, D.-H., & Li, Z.-B. (2005). Study on acyl migration in immobilized lipozyme TL-catalyzed transesterification of soybean oil for biodiesel production. *Journal of Molecular Catalysis B: Enzymatic*, *37*(1–6), 68-71.

Du, W., Xu, Y., Liu, D., & Zeng, J. (2004). Comparative study on lipase-catalyzed transformation of soybean oil for biodiesel production with different acyl acceptors. *Journal of Molecular Catalysis B: Enzymatic*, *30*(3–4), 125-129.

Duarte, S. H., del Peso Hernández, G. L., Canet, A., Benaiges, M. D., Maugeri, F., & Valero, F. (2015). Enzymatic biodiesel synthesis from yeast oil using immobilized recombinant Rhizopus oryzae lipase. *Bioresource Technology, 183*, 175-180.

Fayyazi, E., Ghobadian, B., Najafi, G., Hosseinzadeh, B., Mamat, R., & Hosseinzadeh, J. (2015). An ultrasound-assisted system for the optimization of biodiesel production from chicken fat oil using a genetic algorithm and response surface methodology. *Ultrasonics Sonochemistry, 26*, 312-320.

Fjerbaek, L., Christensen, K. V., & Norddahl, B. (2009). A review of the current state of biodiesel production using enzymatic transesterification. *Biotechnol Bioeng, 102*(5), 1298-1315.

Gameiro, M., Lisboa, P., Paiva, A., Barreiros, S., & Simões, P. (2015). Supercritical carbon dioxide-based integrated continuous extraction of oil from chicken

feather meal, and its conversion to biodiesel in a packed-bed enzymatic reactor, at pilot scale. *Fuel, 153*, 135-142.

Gascon, V., Diaz, I., Blanco, R. M., & Marquez-Alvarez, C. (2014). Hybrid periodic mesoporous organosilica designed to improve the properties of immobilized enzymes. [10.1039/C4RA05362A]. *RSC Advances, 4*(65), 34356-34368.

Ghaly, A. E., Dave, D., Brooks, M.S., and Budge, S. (2010). Production of Biodiesel by Enzymatic Transesterification: Review. *American Journal of Biochemistry and Biotechnology, 6*(2), 54-76.

Gog, A., Roman, M., Toşa, M., Paizs, C., & Irimie, F. D. (2012). Biodiesel production using enzymatic transesterification – Current state and perspectives. *Renewable Energy, 39*(1), 10-16.

Guan, F., Peng, P., Wang, G., Yin, T., Peng, Q., Huang, J., Guan, G., & Li, Y. (2010). Combination of two lipases more efficiently catalyzes methanolysis of soybean oil for biodiesel production in aqueous medium. *Process Biochemistry, 45*(10), 1677-1682.

Gui, M. M., Lee, K. T., & Bhatia, S. (2008). Feasibility of edible oil vs. non-edible oil vs. waste edible oil as biodiesel feedstock. *Energy, 33*(11), 1646-1653.

Guldhe, A., Singh, B., Mutanda, T., Permaul, K., & Bux, F. (2015). Advances in synthesis of biodiesel via enzyme catalysis: Novel and sustainable approaches. *Renewable and Sustainable Energy Reviews, 41*(0), 1447-1464.

Guldhe, A., Singh, B., Rawat, I., Permaul, K., & Bux, F. (2015). Biocatalytic conversion of lipids from microalgae Scenedesmus obliquus to biodiesel using Pseudomonas fluorescens lipase. *Fuel, 147*, 117-124.

Guldhe, A., Singh, P., Kumari, S., Rawat, I., Permaul, K., & Bux, F. (2016). Biodiesel synthesis from microalgae using immobilized Aspergillus niger whole cell lipase biocatalyst. *Renewable Energy, 85*, 1002-1010.

Gupta, J., Agarwal, M., & Dalai, A. K. (2016). Optimization of biodiesel production from mixture of edible and nonedible vegetable oils. *Biocatalysis and Agricultural Biotechnology, 8*, 112-120.

Ha, S. H., Lan, M. N., Lee, S. H., Hwang, S. M., & Koo, Y.-M. (2007). Lipase-catalyzed biodiesel production from soybean oil in ionic liquids. *Enzyme and Microbial Technology, 41*(4), 480-483.

Halim, R., Danquah, M. K., & Webley, P. A. (2012). Extraction of oil from microalgae for biodiesel production: A review. *Biotechnology Advances, 30*(3), 709-732.

Hayyan, A., Alam, M. Z., Mirghani, M. E. S., Kabbashi, N. A., Hakimi, N. I. N. M., Siran, Y. M., & Tahiruddin, S. (2011). Reduction of high content of free fatty acid in sludge palm oil via acid catalyst for biodiesel production. *Fuel Processing Technology, 92*(5), 920-924.

He, Q., Xu, Y., Teng, Y., & Wang, D. (2008). Biodiesel Production Catalyzed by Whole-Cell Lipase from Rhizopus chinensis. *Chinese Journal of Catalysis, 29*(1), 41-46.

Ho, S.-H., Ye, X., Hasunuma, T., Chang, J.-S., & Kondo, A. (2014). Perspectives on engineering strategies for improving biofuel production from microalgae — A critical review. *Biotechnology Advances, 32*(8), 1448-1459.

Hobden, R. (2014). Commercializing Enzymatic Biodiesel Production. *Biodiesel Magazine*. Retrieved from http://www.biodieselmagazine.com/articles/9481/commercializing-enzymatic-biodiesel-production

Hoekman, S. K., Broch, A., Robbins, C., Ceniceros, E., & Natarajan, M. (2012). Review of biodiesel composition, properties, and specifications. *Renewable and Sustainable Energy Reviews, 16*(1), 143-169.

Huang, D., Han, S., Han, Z., & Lin, Y. (2012). Biodiesel production catalyzed by Rhizomucor miehei lipase-displaying Pichia pastoris whole cells in an isooctane system. [Article]. *Biochemical Engineering Journal, 63*, 10-14.

International Energy Agency. (2015). Key World Energy Statistics, 2015.

Javed, S., Satyanarayana Murthy, Y. V. V., Baig, R. U., & Prasada Rao, D. (2015). Development of ANN model for prediction of performance and emission characteristics of hydrogen dual fueled diesel engine with Jatropha Methyl Ester biodiesel blends. *Journal of Natural Gas Science and Engineering, 26*, 549-557.

Jegannathan, K. R., Eng-Seng, C., & Ravindra, P. (2011). Economic assessment of biodiesel production: Comparison of alkali and biocatalyst processes. *Renewable and Sustainable Energy Reviews, 15*(1), 745-751.

Juan, J. C., Kartika, D. A., Wu, T. Y., & Hin, T.-Y. Y. (2011). Biodiesel production from jatropha oil by catalytic and non-catalytic approaches: An overview. *Bioresource Technology, 102*(2), 452-460.

Kaieda, M., Samukawa, T., Kondo, A., & Fukuda, H. (2001). Effect of Methanol and water contents on production of biodiesel fuel from plant oil catalyzed by various lipases in a solvent-free system. *Journal of Bioscience and Bioengineering, 91*(1), 12-15.

Kapoor, M., & Gupta, M. N. (2012). Lipase promiscuity and its biochemical applications. *Process Biochemistry, 47*(4), 555-569.

Karmee, S. K., Patria, R., & Lin, C. (2015). Techno-Economic Evaluation of Biodiesel Production from Waste Cooking Oil—A Case Study of Hong Kong. *International Journal of Molecular Sciences, 16*, 4362-4371.

Knothe, G. (2005). Dependence of biodiesel fuel properties on the structure of fatty acid alkyl esters. *Fuel Processing Technology, 86*(10), 1059-1070.

Ko, C.-H., Yeh, K.-W., Wang, Y.-N., Wu, C.-H., Chang, F.-C., Cheng, M.-H., & Liou, C.-S. (2012). Impact of methanol addition strategy on enzymatic transesterification of jatropha oil for biodiesel processing. *Energy, 48*(1), 375-379.

Kojima, S., Du, D., Sato, M., & Park, E. Y. (2004). Efficient production of fatty acid methyl ester from waste activated bleaching earth using diesel oil as organic solvent. *Journal of Bioscience and Bioengineering, 98*(6), 420-424.

Korman, T. P., Sahachartsiri, B., Charbonneau, D. M., Huang, G. L., Beauregard, M., & Bowie, J. U. (2013). Dieselzymes: development of a stable and methanol tolerant lipase for biodiesel production by directed evolution. *Biotechnology for Biofuels, 6*, 70-70.

Kotrba, R. (2014). Novozymes' Eversa Enzymatic Biodiesel Solution Ready for Market. *Biodiesel Magazine*. Retrieved from http://www.biodieselmagazine.com/articles/252323/novozymes-eversa-enzymatic-biodiesel-solution-ready-for-market

Kumar, D., Parshad, R., & Gupta, V. K. (2014). Application of a statistically enhanced, novel, organic solvent stable lipase from Bacillus safensis DVL-43. *International Journal of Biological Macromolecules, 66*, 97-107.

Kumari, V., Shah, S., & Gupta, M. N. (2006). Preparation of Biodiesel by Lipase-Catalyzed Transesterification of High Free Fatty Acid Containing Oil from Madhuca indica. *Energy & Fuels, 21*(1), 368-372.

Kundu, P., Paul, V., Kumar, V., & Mishra, I. M. (2015). Formulation development, modeling and optimization of emulsification process using evolving RSM

coupled hybrid ANN-GA framework. *Chemical Engineering Research and Design, 104*, 773-790.

Kuo, T.-C., Shaw, J.-F., & Lee, G.-C. (2015). Conversion of crude Jatropha curcas seed oil into biodiesel using liquid recombinant Candida rugosa lipase isozymes. *Bioresource Technology, 192*, 54-59.

Kusumo, F., Silitonga, A. S., Masjuki, H. H., Ong, H. C., Siswantoro, J., & Mahlia, T. M. I. (2017). Optimization of transesterification process for Ceiba pentandra oil: A comparative study between kernel-based extreme learning machine and artificial neural networks. *Energy, 134*, 24-34.

Lam, M. K., Lee, K. T., & Mohamed, A. R. (2010). Homogeneous, heterogeneous and enzymatic catalysis for transesterification of high free fatty acid oil (waste cooking oil) to biodiesel: A review. *Biotechnology Advances, 28*(4), 500-518.

Lee, H. V., Juan, J. C., & Taufiq-Yap, Y. H. (2015). Preparation and application of binary acid–base CaO–La2O3 catalyst for biodiesel production. *Renewable Energy, 74*(0), 124-132.

Lee, M., Lee, J., Lee, D., Cho, J., Kim, S., & Park, C. (2011). Improvement of enzymatic biodiesel production by controlled substrate feeding using silica gel in solvent free system. *Enzyme and Microbial Technology, 49*(4), 402-406.

Lee, O. K., Kim, Y. H., Na, J.-G., Oh, Y.-K., & Lee, E. Y. (2013). Highly efficient extraction and lipase-catalyzed transesterification of triglycerides from Chlorella sp. KR-1 for production of biodiesel. *Bioresource Technology, 147*(0), 240-245.

Li, L., Du, W., Liu, D., Wang, L., & Li, Z. (2006). Lipase-catalyzed transesterification of rapeseed oils for biodiesel production with a novel organic solvent as the reaction medium. *Journal of Molecular Catalysis B: Enzymatic, 43*(1–4), 58-62.

Li, Y., Du, W., Dai, L., & Liu, D. (2015). Kinetic study on free lipase NS81006-catalyzed biodiesel production from soybean oil. *Journal of Molecular Catalysis B: Enzymatic, 121*, 22-27.

Lisboa, P., Rodrigues, A. R., Martín, J. L., Simões, P., Barreiros, S., & Paiva, A. (2014). Economic analysis of a plant for biodiesel production from waste cooking oil via enzymatic transesterification using supercritical carbon dioxide. *The Journal of Supercritical Fluids, 85*, 31-40.

Liu, S., Nie, K., Zhang, X., Wang, M., Deng, L., Ye, X., Wang, F., & Tan, T. (2014). Kinetic study on lipase-catalyzed biodiesel production from waste cooking oil. *Journal of Molecular Catalysis B: Enzymatic, 99*, 43-50.

Liu, Y., Liu, T., Wang, X., Xu, L., & Yan, Y. (2011). Biodiesel synthesis catalyzed by burkholderia cenocepacia lipase supported on macroporous resin NKA in solvent-free and isooctane systems. [Article]. *Energy and Fuels, 25*(3), 1206-1212.

Lu, J., Chen, Y., Wang, F., & Tan, T. (2009). Effect of water on methanolysis of glycerol trioleate catalyzed by immobilized lipase Candida sp. 99–125 in organic solvent system. *Journal of Molecular Catalysis B: Enzymatic, 56*(2–3), 122-125.

Lu, J., Deng, L., Zhao, R., Zhang, R., Wang, F., & Tan, T. (2010). Pretreatment of immobilized Candida sp. 99-125 lipase to improve its methanol tolerance for biodiesel production. *Journal of Molecular Catalysis B: Enzymatic, 62*(1), 15-18.

Lu, J., Nie, K., Wang, F., & Tan, T. (2008). Immobilized lipase Candida sp. 99-125 catalyzed methanolysis of glycerol trioleate: Solvent effect. *Bioresource Technology, 99*(14), 6070-6074.

Maceiras, R., Vega, M., Costa, C., Ramos, P., & Márquez, M. C. (2009). Effect of methanol content on enzymatic production of biodiesel from waste frying oil. *Fuel, 88*(11), 2130-2134.

Maceiras, R., Vega, M., Costa, C., Ramos, P., & Márquez, M. C. (2011). Enzyme deactivation during biodiesel production. *Chemical Engineering Journal, 166*(1), 358-361.

Madras, G., Kolluru, C., & Kumar, R. (2004). Synthesis of biodiesel in supercritical fluids. *Fuel, 83*(14–15), 2029-2033.

Mateo, C., Palomo, J. M., Fernandez-Lorente, G., Guisan, J. M., & Fernandez-Lafuente, R. (2007). Improvement of enzyme activity, stability and selectivity via immobilization techniques. *Enzyme and Microbial Technology, 40*(6), 1451-1463.

Miers, S. A., Kastengren, A. L., El-Hannouny, E. M., & Longman, D. E. (2007). An Experimental Investigation of Biodiesel Injection Characteristics Using a Light-Duty Diesel Injector. (48116), 311-319.

Modi, M. K., Reddy, J. R. C., Rao, B. V. S. K., & Prasad, R. B. N. (2007). Lipase-mediated conversion of vegetable oils into biodiesel using ethyl acetate as acyl acceptor. *Bioresource Technology, 98*(6), 1260-1264.

Mohammad Fauzi, A. H., & Amin, N. A. S. (2012). An overview of ionic liquids as solvents in biodiesel synthesis. *Renewable and Sustainable Energy Reviews, 16*(8), 5770-5786.

Mohammadi, M., Ashjari, M., Dezvarei, S., Yousefi, M., Babaki, M., & Mohammadi, J. (2015). Rapid and high-density covalent immobilization of Rhizomucor miehei lipase using a multi component reaction: application in biodiesel production. [10.1039/C5RA03299G]. *RSC Advances, 5*(41), 32698-32705.

Mousavi Anijdan, S. H., & Bahrami, A. (2005). A new method in prediction of TCP phases formation in superalloys. *Materials Science and Engineering: A, 396*(1–2), 138-142.

Mubarak, M., Shaija, A., & Suchithra, T. V. (2015). A review on the extraction of lipid from microalgae for biodiesel production. *Algal Research, 7*(0), 117-123.

Nasaruddin, R. R., Alam, M. Z., & Jami, M. S. (2014). Evaluation of solvent system for the enzymatic synthesis of ethanol-based biodiesel from sludge palm oil (SPO). *Bioresource Technology, 154*, 155-161.

Nelson, L., Foglia, T., & Marmer, W. (1996). Lipase-catalyzed production of biodiesel. *Journal of the American Oil Chemists' Society, 73*(9), 1191-1195.

Noraini, M. Y., Ong, H. C., Badrul, M. J., & Chong, W. T. (2014). A review on potential enzymatic reaction for biofuel production from algae. *Renewable and Sustainable Energy Reviews, 39*(0), 24-34.

Norazahar, N., Yusup, S., Ahmad, M., Abu Bakar, S., & Ahmad, J. (2012). Parametric optimization of kapok (Ceiba pentandra) oil methyl ester production using Taguchi approach. *Int J Energy Environ, 6*(6), 541-548.

Ong, H. C., Masjuki, H. H., Mahlia, T. M. I., Silitonga, A. S., Chong, W. T., & Leong, K. Y. (2014). Optimization of biodiesel production and engine performance from high free fatty acid Calophyllum inophyllum oil in CI diesel engine. *Energy Conversion and Management, 81*(0), 30-40.

Ong, H. C., Masjuki, H. H., Mahlia, T. M. I., Silitonga, A. S., Chong, W. T., & Yusaf, T. (2014). Engine performance and emissions using Jatropha curcas, Ceiba pentandra and Calophyllum inophyllum biodiesel in a CI diesel engine. *Energy, 69*, 427-445.

Ong, H. C., Silitonga, A. S., Masjuki, H. H., Mahlia, T. M. I., Chong, W. T., & Boosroh, M. H. (2013). Production and comparative fuel properties of biodiesel from non-edible oils: Jatropha curcas, Sterculia foetida and Ceiba pentandra. *Energy Conversion and Management, 73*(0), 245-255.

Ong, L. K., Effendi, C., Kurniawan, A., Lin, C. X., Zhao, X. S., & Ismadji, S. (2013). Optimization of catalyst-free production of biodiesel from Ceiba pentandra (kapok) oil with high free fatty acid contents. *Energy, 57*, 615-623.

Otadi, M., Shahraki, A., Goharrokhi, M., & Bandarchian, F. (2011). Reduction of Free Fatty Acids of Waste Oil by Acid-Catalyzed Esterification. *Procedia Engineering, 18*, 168-174.

Patel, R. L., & Sankhavara, C. D. (2017). Biodiesel production from Karanja oil and its use in diesel engine: A review. *Renewable and Sustainable Energy Reviews, 71*, 464-474.

Patil, P. D., & Deng, S. (2009). Optimization of biodiesel production from edible and non-edible vegetable oils. *Fuel, 88*(7), 1302-1306.

Piedmont Biofuels. (n.d.). *Enzymatic Biodiesel*. Retrieved from http://www.biofuels.coop/enzymatic-biodiesel

Pinzi, S., Rounce, P., Herreros, J. M., Tsolakis, A., & Pilar Dorado, M. (2013). The effect of biodiesel fatty acid composition on combustion and diesel engine exhaust emissions. *Fuel, 104*, 170-182.

Poojari, Y., & Clarson, S. J. (2013). Thermal stability of Candida antarctica lipase B immobilized on macroporous acrylic resin particles in organic media. *Biocatalysis and Agricultural Biotechnology, 2*(1), 7-11.

Poppe, J. K., Fernandez-Lafuente, R., Rodrigues, R. C., & Ayub, M. A. Z. Enzymatic reactors for biodiesel synthesis: Present status and future prospects. *Biotechnology Advances*(0).

Poppe, J. K., Fernandez-Lafuente, R., Rodrigues, R. C., & Ayub, M. A. Z. (2015). Enzymatic reactors for biodiesel synthesis: Present status and future prospects. *Biotechnology Advances, 33*(5), 511-525.

Poppe, J. K., Matte, C. R., Peralba, M. d. C. R., Fernandez-Lafuente, R., Rodrigues, R. C., & Ayub, M. A. Z. (2015). Optimization of ethyl ester production from olive and palm oils using mixtures of immobilized lipases. *Applied Catalysis A: General, 490*(0), 50-56.

Pullen, J., & Saeed, K. (2012). An overview of biodiesel oxidation stability. *Renewable and Sustainable Energy Reviews, 16*, 5924–5950.

Rajendra, M., Jena, P. C., & Raheman, H. (2009). Prediction of optimized pretreatment process parameters for biodiesel production using ANN and GA. *Fuel, 88*(5), 868-875.

Rashid, U., Anwar, F., & Knothe, G. (2009). Evaluation of biodiesel obtained from cottonseed oil. *Fuel Processing Technology, 90*(9), 1157-1163.

Rashid, U., Knothe, G., Yunus, R., & Evangelista, R. L. (2014). Kapok oil methyl esters. *Biomass and Bioenergy, 66*, 419-425.

Refaat, A. A. (2009). Correlation between the chemical structure of biodiesel and its physical properties. *International Journal of Environmental Science & Technology, 6*(4), 677-694.

Ring Organic. (n.d.). *Kapok Seed Powder*. Retrieved from http://www.ringorganic.com/Kapok_Seed_Powder.html

Rodrigues, R., Volpato, G., Wada, K., & Ayub, M. (2008). Enzymatic Synthesis of Biodiesel from Transesterification Reactions of Vegetable Oils and Short Chain Alcohols. *Journal of the American Oil Chemists' Society, 85*(10), 925-930.

Rodrigues, R. C., & Ayub, M. A. Z. (2011). Effects of the combined use of Thermomyces lanuginosus and Rhizomucor miehei lipases for the transesterification and hydrolysis of soybean oil. *Process Biochemistry, 46*(3), 682-688.

Rodrigues, R. C., Ortiz, C., Berenguer-Murcia, A., Torres, R., & Fernandez-Lafuente, R. (2013). Modifying enzyme activity and selectivity by immobilization. [10.1039/C2CS35231A]. *Chemical Society Reviews, 42*(15), 6290-6307.

Rodrigues, R. C., Pessela, B. C. C., Volpato, G., Fernandez-Lafuente, R., Guisan, J. M., & Ayub, M. A. Z. (2010). Two step ethanolysis: A simple and efficient way to improve the enzymatic biodiesel synthesis catalyzed by an immobilized–stabilized lipase from Thermomyces lanuginosus. *Process Biochemistry, 45*(8), 1268-1273.

Röttig, A., Wenning, L., Bröker, D., & Steinbüchel, A. (2010). Fatty acid alkyl esters: perspectives for production of alternative biofuels. *Applied Microbiology and Biotechnology, 85*(6), 1713-1733.

Royon, D., Daz, M., Ellenrieder, G., & Locatelli, S. (2007). Enzymatic production of biodiesel from cotton seed oil using t-butanol as a solvent. *Bioresource Technology, 98*(3), 648-653.

Sahoo, B., Brandstadt, K. F., Lane, T. H., & Gross, R. A. (2005). "Sweet Silicones": Biocatalytic Reactions to Form Organosilicon Carbohydrate Macromers. *Organic Letters, 7*(18), 3857-3860.

Sajjadi, B., Aziz Abdul Raman, A., & Arandiyan, H. (2016). A comprehensive review on properties of edible and non-edible vegetable oil-based biodiesel: Composition, specifications and prediction models. *Renewable and Sustainable Energy Reviews, 63*, 62-92.

Salis, A., Pinna, M., Monduzzi, M., & Solinas, V. (2005). Biodiesel production from triolein and short chain alcohols through biocatalysis. *Journal of Biotechnology, 119*(3), 291-299.

Samukawa, T., Kaieda, M., Matsumoto, T., Ban, K., Kondo, A., Shimada, Y., Noda, H., & Fukuda, H. (2000). Pretreatment of immobilized Candida antarctica lipase for biodiesel fuel production from plant oil. *Journal of Bioscience and Bioengineering, 90*(2), 180-183.

Santibañez, C., María, T., Varnero, & Bustamante, M. (2011). Residual Glycerol from Biodiesel Manufacturing, Waste or Potential Source of Bioenergy: A Review. *Chilean Journal of Agricultural Research, 71*.

Senthil Kumar, T., Senthil Kumar, P., & Annamalai, K. (2015). Experimental study on the performance and emission measures of direct injection diesel engine with Kapok methyl ester and its blends. *Renewable Energy, 74*, 903-909.

Shimada, Y., Watanabe, Y., Samukawa, T., Sugihara, A., Noda, H., Fukuda, H., & Tominaga, Y. (1999). Conversion of vegetable oil to biodiesel using immobilized Candida antarctica lipase. *Journal of the American Oil Chemists' Society, 76*(7), 789-793.

Silitonga, A. S., Masjuki, H. H., Mahlia, T. M. I., Ong, H. C., & Chong, W. T. (2013). Experimental study on performance and exhaust emissions of a diesel engine fuelled with Ceiba pentandra biodiesel blends. *Energy Conversion and Management, 76*, 828-836.

Silitonga, A. S., Ong, H. C., Mahlia, T. M. I., Masjuki, H. H., & Chong, W. T. (2013). Characterization and production of Ceiba pentandra biodiesel and its blends. *Fuel, 108*, 855-858.

Singh, B., Guldhe, A., Rawat, I., & Bux, F. (2014). Towards a sustainable approach for development of biodiesel from plant and microalgae. *Renewable and Sustainable Energy Reviews, 29*(0), 216-245.

Sivakumar, P., Sindhanaiselvan, S., Gandhi, N. N., Devi, S. S., & Renganathan, S. (2013). Optimization and kinetic studies on biodiesel production from underutilized Ceiba Pentandra oil. *Fuel, 103*, 693-698.

Sotoft, L. F., Rong, B.-G., Christensen, K. V., & Norddahl, B. (2010). Process simulation and economical evaluation of enzymatic biodiesel production plant. *Bioresource Technology, 101*(14), 5266-5274.

Stamenković, O. S., Rajković, K., Veličković, A. V., Milić, P. S., & Veljković, V. B. (2013). Optimization of base-catalyzed ethanolysis of sunflower oil by regression and artificial neural network models. *Fuel Processing Technology, 114*, 101-108.

Stamenković, O. S., Veličković, A. V., & Veljković, V. B. (2011). The production of biodiesel from vegetable oils by ethanolysis: Current state and perspectives. *Fuel, 90*(11), 3141-3155.

Su, E.-Z., Zhang, M.-J., Zhang, J.-G., Gao, J.-F., & Wei, D.-Z. (2007). Lipase-catalyzed irreversible transesterification of vegetable oils for fatty acid methyl esters production with dimethyl carbonate as the acyl acceptor. *Biochemical Engineering Journal, 36*(2), 167-173.

Su, Y., Zhang, P., & Su, Y. (2015). An overview of biofuels policies and industrialization in the major biofuel producing countries. *Renewable and Sustainable Energy Reviews, 50*, 991-1003.

Szczęsna Antczak, M., Kubiak, A., Antczak, T., & Bielecki, S. (2009). Enzymatic biodiesel synthesis – Key factors affecting efficiency of the process. *Renewable Energy, 34*(5), 1185-1194.

Taghavifar, H., Khalilarya, S., & Jafarmadar, S. (2014). Diesel engine spray characteristics prediction with hybridized artificial neural network optimized by genetic algorithm. *Energy, 71*, 656-664.

Talukder, M. M. R., Lee, H. Z. S., Low, R. F., Pei-Lyn, L. C., Warzecha, D., & Wu, J. (2013). Potential use of whole cell lipase from a newly isolated Aspergillus nomius for methanolysis of palm oil to biodiesel. *Journal of Molecular Catalysis B: Enzymatic, 89*(0), 108-113.

Tamalampudi, S., Talukder, M. R., Hama, S., Numata, T., Kondo, A., & Fukuda, H. (2008). Enzymatic production of biodiesel from Jatropha oil: A comparative study of immobilized-whole cell and commercial lipases as a biocatalyst. *Biochemical Engineering Journal, 39*(1), 185-189.

Tan, T., Lu, J., Nie, K., Deng, L., & Wang, F. (2010). Biodiesel production with immobilized lipase: A review. *Biotechnology Advances, 28*(5), 628-634.

Taufiq-Yap, Y. H., Lee, H. V., Yunus, R., & Juan, J. C. (2011). Transesterification of non-edible Jatropha curcas oil to biodiesel using binary Ca–Mg mixed oxide catalyst: Effect of stoichiometric composition. *Chemical Engineering Journal, 178*(0), 342-347.

Tian, X., Dai, L., Liu, M., Liu, D., Du, W., & Wu, H. (2016). Lipase-catalyzed methanolysis of microalgae oil for biodiesel production and PUFAs concentration. *Catalysis Communications, 84*, 44-47.

Tongboriboon, K., Cheirsilp, B., & H-Kittikun, A. (2010). Mixed lipases for efficient enzymatic synthesis of biodiesel from used palm oil and ethanol in a solvent-free system. *Journal of Molecular Catalysis B: Enzymatic, 67*(1–2), 52-59.

Tran, D.-T., Chen, C.-L., & Chang, J.-S. (2016). Continuous biodiesel conversion via enzymatic transesterification catalyzed by immobilized Burkholderia lipase in a packed-bed bioreactor. *Applied Energy, 168*, 340-350.

Tye, Y. Y., Lee, K. T., Wan Abdullah, W. N., & Leh, C. P. (2012). Potential of Ceiba pentandra (L.) Gaertn. (kapok fiber) as a resource for second generation bioethanol: Effect of various simple pretreatment methods on sugar production. *Bioresource Technology, 116*, 536-539.

Wall, J., Gerpen, J.V., Thompson, J. (2011). Soap and Glycerin Removal from Biodiesel Using Waterless Processes. *American Society of Agricultural and Biological Engineers, 54*(2), 535-541.

Watanabe, Y., Shimada, Y., Sugihara, A., & Tominaga, Y. (2002). Conversion of degummed soybean oil to biodiesel fuel with immobilized Candida antarctica lipase. *Journal of Molecular Catalysis B: Enzymatic, 17*(3–5), 151-155.

Xiong, W., Li, X., Xiang, J., & Wu, Q. (2008). High-density fermentation of microalga Chlorella protothecoides in bioreactor for microbio-diesel production. *Appl Microbiol Biotechnol, 78*(1), 29-36.

Xu, Y., Du, W., Liu, D., & Zeng, J. (2003). A novel enzymatic route for biodiesel production from renewable oils in a solvent-free medium. *Biotechnology Letters, 25*(15), 1239-1241.

Yaakob, Z., Narayanan, B. N., Padikkaparambil, S., Unni K, S., & Akbar P, M. (2014). A review on the oxidation stability of biodiesel. *Renewable and Sustainable Energy Reviews, 35*, 136-153.

Yan, J., Zheng, X., & Li, S. (2014). A novel and robust recombinant Pichia pastoris yeast whole cell biocatalyst with intracellular overexpression of a Thermomyces lanuginosus lipase: Preparation, characterization and application in biodiesel production. *Bioresource Technology, 151*, 43-48.

Yan, Y., Xu, L., & Dai, M. (2012). A synergetic whole-cell biocatalyst for biodiesel production. [10.1039/C2RA20974H]. *RSC Advances, 2*(15), 6170-6173.

Yang, W., He, Y., Xu, L., Zhang, H., & Yan, Y. (2016). A new extracellular thermo-solvent-stable lipase from Burkholderia ubonensis SL-4: Identification, characterization and application for biodiesel production. *Journal of Molecular Catalysis B: Enzymatic, 126*, 76-89.

Yatish, K. V., Lalithamba, H. S., Suresh, R., Arun, S. B., & Kumar, P. V. (2016). Optimization of scum oil biodiesel production by using response surface methodology. *Process Safety and Environmental Protection, 102*, 667-672.

Yazdanmehr, M., Anijdan, S. H. M., & Bahrami, A. (2009). Using GA–ANN algorithm to optimize soft magnetic properties of nanocrystalline mechanically alloyed Fe–Si powders. *Computational Materials Science, 44*(4), 1218-1221.

You, Q., Yin, X., Zhao, Y., & Zhang, Y. (2013). Biodiesel production from jatropha oil catalyzed by immobilized Burkholderia cepacia lipase on modified attapulgite. *Bioresource Technology, 148*(0), 202-207.

Yu, C.-Y., Huang, L.-Y., Kuan, I. C., & Lee, S.-L. (2013). Optimized Production of Biodiesel from Waste Cooking Oil by Lipase Immobilized on Magnetic Nanoparticles. *International Journal of Molecular Sciences, 14*(12), 24074-24086.

Yu, H. W., Chen, H., Yang, Y. Y., & Ching, C. B. (2005). Effect of salts on activity, stability and enantioselectivity of Candida rugosa lipase in isooctane. [Article]. *Journal of Molecular Catalysis B: Enzymatic, 35*(1-3), 28-32.

Yunus Khan, T. M., Atabani, A. E., Badruddin, I. A., Ankalgi, R. F., Mainuddin Khan, T. K., & Badarudin, A. (2015). Ceiba pentandra, Nigella sativa and their blend as prospective feedstocks for biodiesel. *Industrial Crops and Products, 65*, 367-373.

LIST OF PUBLICATIONS

1) Norjannah, B., Ong, H. C., Masjuki, H. H., Juan, J. C., & Chong, W. T. (2016). Enzymatic transesterification for biodiesel production: a comprehensive review. [10.1039/C6RA08062F]. *RSC Advances, 6*(65), 60034-60055.
2) Norjannah, B., Ong, H. C., & Masjuki, H. H. (2017). Effects of methanol and enzyme pretreatment to *Ceiba pentandra* biodiesel production. [10.1080/15567036.2017.1344747]. *Energy Sources, Part A: Recovery, Utilization, and Environmental Effects*, 1-8.