MICROWAVE ASSISTED ENZYMATIC TRANSESTERIFICATION OF WASTE COOKING OIL & RUBBER SEED OIL

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Thesis submitted in fulfilment of the requirements
for the award of the degree of
Master of Engineering (Bioprocess)

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SUPERVISORS' DECLARATION

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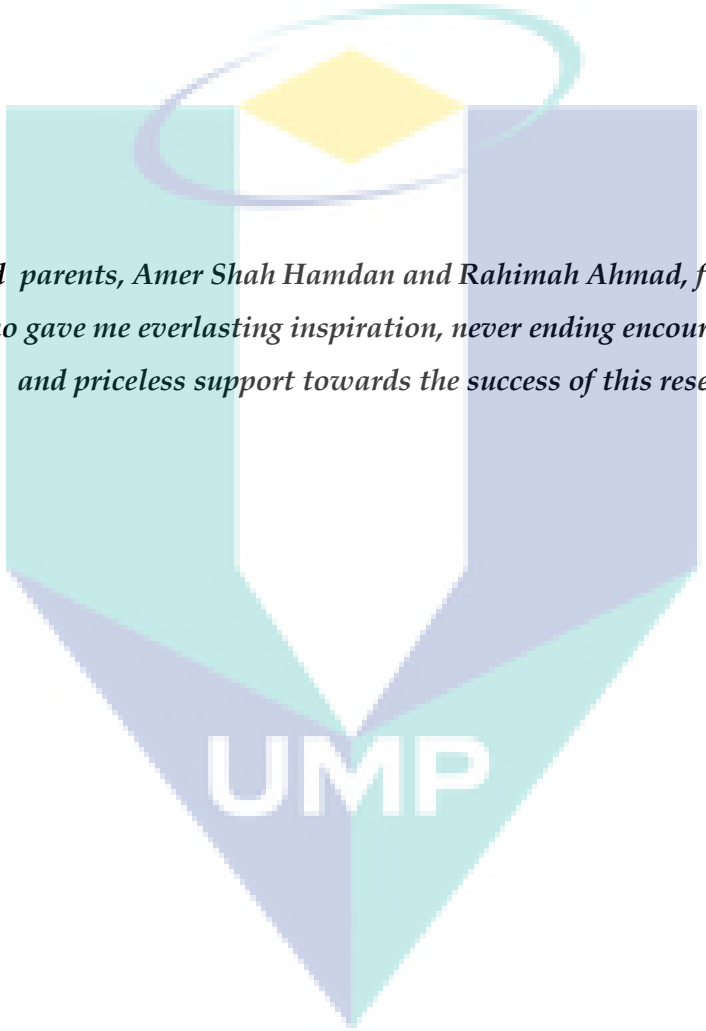
To my beloved parents, Amer Shah Hamdan and Rahimah Ahmad, families, and friends,
who gave me everlasting inspiration, never ending encouragements
and priceless support towards the success of this research



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ABSTRACT

This thesis presents biodiesel production from rubber seed oil via enzymatic process using Immobilized *Lipase Candida Rugosa*. The objectives of this study is to produce biodiesel fuels from waste cooking oil and rubber seed oil that comply with ASTM standard under optimum conditions of transesterification process such methanol to oil ratio, reaction temperature, mixing speed and microwave power using commercial and purpose made Immobilized *Lipase Candida Rugoas* enzyme in microwave reactor. This research also focus on production of biodiesel economically from non-edible renewable resources which using rubber seed oil and waste cooking oil as raw material instead of edible oil that competed with food sources. From the screening of three type of reactors, which were batch, microwave and ultrasonic, microwave reactor produced highest methyl ester in enzymatic transesterification. Microwave irradiation was applied in immobilization process. of *Lipase Candida Rugosa* enzyme was immobilized onto silica gel by cross-linking immobilization. The enyzme loading of using cross-linking method in this study was 47 % which is lower than reported by other researchers but the enzyme activity of purpose made immobilized lipase enzyme was 33 % higher than commercial immobilized lipase enzyme. From the parameter study of enzymatic transesterification process of waste cooking oil in microwave reactor, the optimum conditions were 6:1 methanol to oil ratio, 40°C reaction temperature, 150 rpm of mixing speed and 100 W microwave power. Enzyme leaching and enzyme recyclability has also been studied. Microwave irradiation increased the methyl ester production but, 25 % of this work immobilized enzyme was leached from silica support and reduced 21 % of its activity after 8 cycles. Compared with commercial immobilized lipase enzyme, purpose made immobilzied lipase enzyme showed higher stability. The purpose made immobilized enzyme than was been tested in transestrification process of palm oil, rubber seed oil and waste cooking oil. The resuls shows that the palm oil produced highest methyl ester, followed by rubber seed oil and waste cooking oil. 99 % of methyl ester was successfully produced using crude rubber seed oil, 1 wt. % purpose made immobilized lipase enzyme with 6:1 methanol to oil ratio, 40°C reaction temperature, 150 rpm of mixing and 100W of microwave power irradiation. The results also complied with ASTM standard and produced significant data.

ABSTRAK

Tesis ini berkenaan dengan pengeluaran biodiesel daripada minyak biji getah secara enzimatik menggunakan enzim pegun *Lipase Candida Rugosa*. Objektif tesis ini adalah menghasilkan minyak biodiesel daripada minyak buah getah dan minyak masak terpakai yang mematuhi piawaian ASTM dalam keadaan tindak balas yang optimum dalam penghasilan biodiesel seperti kadar nisbah methanol kepada minyak, suhu kadar tindak balas, kelajuan pengacau, dan juga tenaga mikrogelombang dengan menggunakan enzim pegun komersial dan juga enzim pegun yang dihasilkan sendiri di dalam reaktor mikrogelombang. Melalui proses penyaringan tiga reaktor yang berbeza iaitu reaktor kelompok, reaktor ultrasonik dan reaktor mikrogelombang, reaktor mikrogelombang menunjukan pretasi yang paling bagus dalam penghasilan metil ester perbanding yang lain. Reaktor mikrogelombang juga digunakan dalam penghasilan ezim pegun secara perangkaian silang. Kaedah ini menghasilkan kadar serapan sebanyak 47% enzim ke dalam gel silika. Walaupun kadar serapan ini adalah lebih rendah berbanding yang pernah direkodkan oleh pengkaji yang lain, kadar aktiviti enzim pegun ini lebih tinggi daripada enzim pegun komersial. Walau bagaimanapun kaedah ini menghasilan enzim pegun yang lebih baik daripada enzim pegun komersial yang digunakan dalam tesis ini. Ujian pengoptimuman paremeter dibuat menggunakan reaktor mikrogelombang dan parameter yang paling optimum adalah kadar nisbah metanol kepada minyak 6:1, suhu tindak balas 40°C, 150 rpm kelajuan pengacau dan 100 W tenaga mikrogelombang.. Enzim ini juga memiliki kadar melarut resap sebanyak 25 % dan ini menyebakan kadar aktivity enzim pegun menurun sebanyak 21 % selepas 8 kali digunakan. Enzim pegun yang telah berjaya dihasilkan itu kemudiannya diuji dalam proses penghasilan biodiesel menggunakan minyak buah getah, minyak masak terpakai dan minyak kelapa sawit mentah dalam keadaan kadar tidak balas yang paling optimum. Minyak kelapa sawit mentah menghasilkan metil ester yang paling tinggi diikuti oleh minyak biji getah dan minyak masak terpakai. Sebanyak 99 % metil ester telah terhasil daripada minyak buah getah dengan menggunakan 1 % berat enzim pegun yang dihasilkan daripada kajian ini dalam keadaan kadar tindak balas yang optimum iaitu kadar nisbah methanol kepda minyak 6:1, suhu tindak balas 40°C, 150 rpm kadar kelajuan pengacau dan 100 W tenaga reaktor mikrogelombang. Biodiesel yang terhasil jugak mematuhi piawaian ASTM dan memilki data yang signifikan.

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LIST OF ABBREVIATIONS

ASTM	America Standard for Testing & Materials
CO_2	Carbon Dioxide Gas
FAME	Fatty Acid Methyl Ester
CH_3ONa	Sodium Methoxide
NaOH	Sodium Hydroxide
KOH	Potassium Hydroxide
FFA	Free Fatty Acid
H_2SO_4	Sulphuric Acid
APTES	3-aminopropyltriethoxysilane
GC	Gas Chromatography
BSA	Brovine Serum Albumin
MOPS	3-(N-morpholino) propane sulfonic acid
FTIR	Fourier Transform Infrared Spectrophotometer
TGA	Thermal Gravimetric Analyzer
SEM	Scanning Electron Microscope
ANOVA	Analysis of Variance

CHAPTER 1

INTRODUCTION

1.1 MOTIVATION AND STATEMENT OF PROBLEM

Biodiesel is a mixture of various fatty acid methyl ester oils produced from vegetable oil or animal fat for use as fuel in diesel engine and in compliance with American Society for Testing and Materials (ASTM) standards. Biodiesel was first introduced by Rudolph Diesel (Akoh et al., 2007) in 1900. Biodiesel is considered as the best candidate for to substitute petrodiesel after the world oil crisis in 1970's. Biodiesel can be used alone or mixed with petroleum-based diesel as an alternative efficient fuel and can be used in any diesel engine without modification (Demirbas, 2009a). The most common biodiesel blends are B2 (2% biodiesel and 98% petroleum diesel), B5 (5% biodiesel and 95% petroleum diesel), and B20 (20% biodiesel and 80% petroleum diesel) (Balat & Balat, 2010). Besides, the world oil crisis which urged the researcher to find the alternative for petroleum based fuel biodiesel also has several advantages such as portability, ready availability, renewability, higher combustion efficiency, lower sulfur and aromatic content, higher cetane number and higher biodegradability which contributed to greener fuel. The higher the blending ratio of biodiesel means the fewer the carbon emission to the environment (Fukuda et al, 2001). But biodiesel also has several disadvantages such as high viscosity of vegetable oils as compared to petroleum-based diesel (Demirbas, 2007). Consequently, it causes poor fuel atomization which yielded incomplete combustion and engine fouling (Chauha et al., 2010).

Despite of the Europian Union and United States enforcement on biodiesel use on vehicles due to it green advantages, Malaysia's biodiesel industry continued to show lack of progress until the fifth Fuel Policy was announced under the Malaysia Plan (2001–2005). This policy was revised from the earlier fourth Fuel Diversification Policy in 1981 that aims at preventing overdependence on oil as the main energy resource. The new policy among other stated to supplement the conventional supply of energy and new sources such as renewable energy will be encouraged. In this regard, the fuel diversification policy which comprises oil, gas, hydro and coal will be extended to include renewable energy as the fifth fuel, particularly biomass, and mini-hydro. Of these, biogas, municipal waste, solar biomass resources such as oil palm and wood waste as well as rice husks, on a wider basis mainly for will be used potential sources of energy electricity generation. Other will include palm diesel and hydrogen fuel (eib.ptm.org.my). Thereafter, to encourage utilization of renewable resources greater efforts had being undertaken and this had developed renewed interest in biodiesel.

Although interest in biodiesel is rapidly increasing, the process by which it is synthesized has not substantially changed in recent years. The primary commercial process used today for biodiesel production is chemical catalyzed transesterification. Catalytic reactions can use alkali catalyst, acid catalyst or enzymatic transesterification (Chen et al., 2012). Enzymatic method can be used to produce better quality biodiesel with little or no by product. Lipases are one of the biotechnologically most relevant enzymes for biodiesel production. Lipases are group of enzyme that catalyzes hydrolysis, esterification and transesterification reactions. They act on several ester compounds and their natural substrates are acylglycerols (Sayari et al., 2005). The reasons for these enzymes great biotechnological potential, besides the different types of reaction that they can catalyze are their high stability in the presence of organic solvents, the lack of need for cofactors and their ability to catalyze reactions with chemo-, region- and enantioselectivity (Akoh et al., 2007). The conventional enzymatic transesterification is not efficient and time consuming. This work aims to overcome these issues by employing a microwave irradiationand ultrasonication to improve enzymatic transesterification process. Recently, the European Union has voted against a biofuel production using first generation biofuel sources (edible

oils) such as palm oil, corn, soy bean and maize, which are also consumed as food. This had open a new avenue of producing a biodiesel using a non-food source crop such as forest seeds (i.e. *Callophyluminnophylum L.*, *Elateriospermumtapos*, *HeveaBrasiliensis*) which was used in this work.

The industry has favoured chemical catalyst because of the high convention obtained in a short time and the low cost of the catalysts. However, there are some drawbacks in the process that have encouraged researchers to look into different biodiesel production methods. The use of enzymes (lipases) as catalysts in biodiesel production overcomes the problems inherent to alkali catalysts. However, biodiesel plants using lipases are not yet industrialized because there are some challenges that are yet to be overcome before biocatalysts can be made feasible for biodiesel production such as their higher cost, biodiesel productivity and inhibition by reactants and products (Al-Zuhair et al., 2011).

Even though the commercialization of biodiesel production from biocatalyst is uncertain, the world demands for biodiesel keep increasing year by year and this attracted researcher to search for the new feedstock with optimum parameter to obtain high yield, high quality and comparable biodiesel fuel with petroleum-based diesel. This study was optimizing the transesterification process of biodiesel production from waste cooking oil and rubber seeds oil using microwave irradiation. Besides the new feedstock and optimum reaction parameter, enzyme modification is also one of the interests in this study. Lipases enzyme was chemically modified by cross linking method before immobilize into silica support.

1.2 OBJECTIVES

The goal of this research is to produce biodiesel from waste cooking oil and rubber seed oil using immobilized *Lipase Candida Rugosa* enyzme and microwave reactor. In order to archieved this goal, this reasearch aims to succed at the following objectives:

1. To Immobilize the *Lipase Candida Rugosa* enzyme into silica gel support using cross-linking technique.
2. To study the effect of parameters such as reaction time, methanol to oil ratio, reaction temperature, mixing, microwave power effect and Immobilized enzyme recyclability in microwave assisted enzymatic transesterification.
3. To produce biodiesel from rubber seed oil using immobilized *Lipase Candida Rugosa* under microwave assisted enzymatic transesterification process.

1.3 RESEARCH SCOPES

To achieve the objectives of this research, several scopes have been identified
:

1. Reactor screening between batch, ultrasonic and microwave reactor in order to choose the reactor that produce the highest methyl ester in enzymatic biodiesel production. The experiment conditions such reaction time, reaction temperature, methanol to oil ratio and mixing speed was set as constant in all reactors except the microwave power and ultrasonic power. All the parameters were taken from the literature review.
2. Rubber seed oil extraction in microwave extractor using the optimum parameters from literature review. The extracted RSO use as one of the feedstock in transesterification process.
3. Immobilization of *Lipase Candida Rugosa* enzyme into silica gel support using cross linking techniques to increase the activity of the enzyme. Enzyme loading and enzyme activity was measured using specific methods while the immobilized enzyme was characterized using Scanning Electron Microscope, Thermal Gravimetric Analyzer, Fourier Transform Infrared Spectrophotometer and Wet Laser Diffraction.
4. Microwave assisted enzymatic transesterification of waste cooking oil and extracted rubber seed oil using purpose made immobilized enzyme and commercial immobilized enzyme. The parameters are study using OFAT (one factor at one

time) method which are reaction time, reaction temperature, methanol to oil ratio, mixing speed, microwave power, enzyme leaching and enzyme recyclability.

5. The methyl ester produce then analyze using gas chromatography for methyl ester content and characterize using American Society for Testing and Materials Standard (ASTM).

1.4 SIGNIFICANT OF RESEARCH

The combination of enzymatic transesterification and microwave irradiation can produce higher methyl ester in shorter time compare to conventional enzymatic transesterification process that ususally need >70 hours to produce 80% of methyl ester. Currently, the microwave assisted enzymatic transesterification was studied statistically only by Noguire et al (2010) that use Macauba oil with immobilized novozyme 435 and Perez et al., (2014) that use Babasu oil with immobilized *Lipase Burkholderia Cepacia* enzyme. None had studied using waste cooking oil with immobilized *Lipase Candida Rugosa* enzyme in. This study utilized the use of green catalyst that discharge less waste water compared to chemical catalyst since the purification method was easier due to the easy glycerol separation. This reserach also use waste to wealth concept by using the waste cooking oil and rubber seed oil to produce biodiesel. Instead of dumping into the sewage the waste cooking oil was collected and use as feedstock while the rubber seed oil had no other application since it was non-edible. Cross linking immobilization technique was great to increase the enyme activity and stability compared to other techniques.

1.5 ORGANIZATION OF THESIS CHAPTERS

The structures of the reminder thesis are given as follows:

Chapter 2 gives a review on enzymatic biodiesel production. Detail discussion on the past researches and current researches in biodiesel production, current problems in biodiesel production and potential solution that become the objectives of study and comply with the reserach scopes. Chapter 3 gave brief explanation on materials and methods used in the study. Chapter 3 was divided into overview, materials, methodology, statistical analysis and summary. All the chemicals and enzyme used in the study was listed in materials section while methodology divided into seven subchapters which explained all the methods used in the whole experiment including analysis. Chapter 4 focuses on result and discussion of the whole study finding. Chapter 4 was divided into 8 subchapters. Subchapter 4.1 was overview of chapter 4 which a brief introduction on chapter 4. Subchapter 4.2 was discussed on comparative study of immobilized and free lipase enzyme and three different reactors which are batch, ultrasonic and microwave reactors in biodiesel production. *Lipase from Candida rugosa* enzyme microwave assisted Immobilization into silica gel by cross linking technique finding was discussed in subchapter 4.3. Subchapter 4.4 was discussed on microwave assisted transesterification of waste cooking oil using immobilized lipase enzyme study. This sub chapter focused on finding the optimum parameters in biodiesel production using immobilized lipase enzyme. Subchapter 4.5 discussed the microwave irradiation influence on immobilized lipase enzyme in biodiesel production and subchapter 4.6 was comparing the biodiesel produced from waste cooking oil and rubber seeds oil using commercial using purposed made immobilized lipase enzyme under optimum MAET reaction conditions. ASTM analysis od biodiesel produced was discussed in sub chapter 4.7 and the last sub chapter which is 4.8 was a summary of chapter 4.

CHAPTER 2

LITERATURE REVIEW

2.1 OVERVIEW

This chapter presents the literature of biodiesel production including the world biodiesel trend and Malaysia biodiesel trend from year 1980 to 2012. Chapter 2.3 dicussed the biodiesel transesterification and divided into 5 subchapters according to the factors that affect the biodiesel transesterification which are catalyts, feedstock, reactor, solvent and reaction conditions. Subchapter 2.3.1 discussed the type of catalyst used in biodiesel production which are chemical catalyst, biocatalyst and including the biocatalyt immobilization techniques. Subchapter 2.3.2 discussed the feedstock used in biodiesel production which can be choose between edible and non-edible oil. While subchapter 2.3.3 disccused the reactor used in biodiesel production such microwave reactor, ultrasonic reactor and batch reactor. The solvent used in biodiesel transesterification was disccused in subchapter 2.3.4 and the last subchapter which is subchapter 2.3.5 disccused briefly the reaction conditions used in biodiesel production. All the finding from the literaure review then being summarized in the last chapter which is chapter 2.4.

2.2 INTRODUCTION

World climate changes are worsening day by day. There were a lot of pollutions worldwide that going out of the control despite the efforts from such as European countries to reduce the CO_2emissions using green technologies and policies. From the U. S. Energy Information Administration statistic, the world CO_2 emission increased 43.42% from 1980 to 2011. This resulted from the high energy consumption worldwide. The major CO_2 emission came from the transportation sector that used petroleum & diesel fuels. However the world petroleum depletion was an ignition source to biodiesel mass researches and productions. According to the BP Annual Statistic Review of World Energy, it was estimated that the world petroleum reserves was enough to produce petroleum in another 42 years (Balat & Balat, 2010).

Biodiesel was introduced to the world a century ago by Rudolf Diesel in year 1900 at Paris World Exhibition (Sahoo & Das, 2009).Rudolf demonstrated the used of peanut oil directly to the diesel engine(Panwar et al., 2010). At that time, lack of biodiesel technology and information make it less interesting compared to this day. Direct use of vegetable oil in diesel engine cause several problems to the engine such as poor atomization of fuels, incomplete combustion and engine coking (Karmakar et al., 2010) thus, transesterification of vegetable oil was one of the methods to reduce the viscosity of the oil and improved the quality of biodiesel and make its more suitable to use in diesel engine.

Biodiesel has several advantages over petroleum based diesel fuels such renewable, high combustion than vegetable oil, less toxic by producing low sulfur and aromatic content, biodegradable and high flash point (Balat & Balat, 2010) (Ramezani et al., 2010). However, from the ASTM analysis shows that the quality of biodiesel is still low compared to petroleum based diesel fuels thus, the biodiesel was blend with diesel to produce high qualityof biodiesel that can increase engine performance (Balat & Balat, 2010). Currently the marketed blend biodiesel was B5 and B20. B5 means 5% of biodiesel was blended with another 95% of diesel from petroleum based while B20 consisted of 20% of biodiesel and another 80% of petroleum based diesel.

2.2.1World Biodiesel Trend

World petroleum demand greatly increase over the past decades due to the increase in population and vehicles. World population recorded in year 2011 is 6.94 billion and in year 1980 it is just 4.4 billion. The population increase lead to the increase of the human basic needs such foods and energy. From the U. S. Energy Information Administration statistic data that show in figure 2.1, the petroleum demand increased year by year and the recorded from year 1980 to 2012 shows that the petroleum demand reached 89million barrels per day at 2012 despite the petroleum reserve depletion.

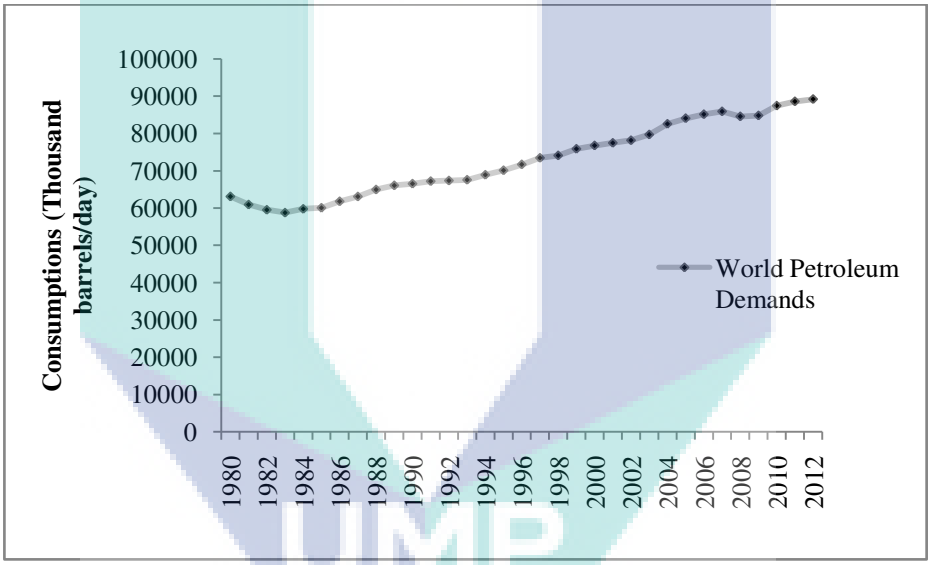


Figure 2.1: World Petroleum Demands by EIA statistic graph. (http://www.eia.gov)

These make the research, production and policies on biodiesel imperative. However the world biodiesel production and demands are still far behind the petroleum demand. Figure 2.2 shows that in year 2011, only 1.9 million barrels per day of biodiesel had been produced compared to 89 million barrels per day of petroleum production, it was 97% of margin. The exponential graph also clearly shows the result from biodiesel enforcement efforts. In year 2000 there was only 300 thousand barrel per day of biodiesel being produced and in just 10 years the value increased to 1.9 million barrel per day. The

consumption of biodiesel shows the similar pattern. That exponential growth resulted from a lot of enforcement worldwide especially from European countries.

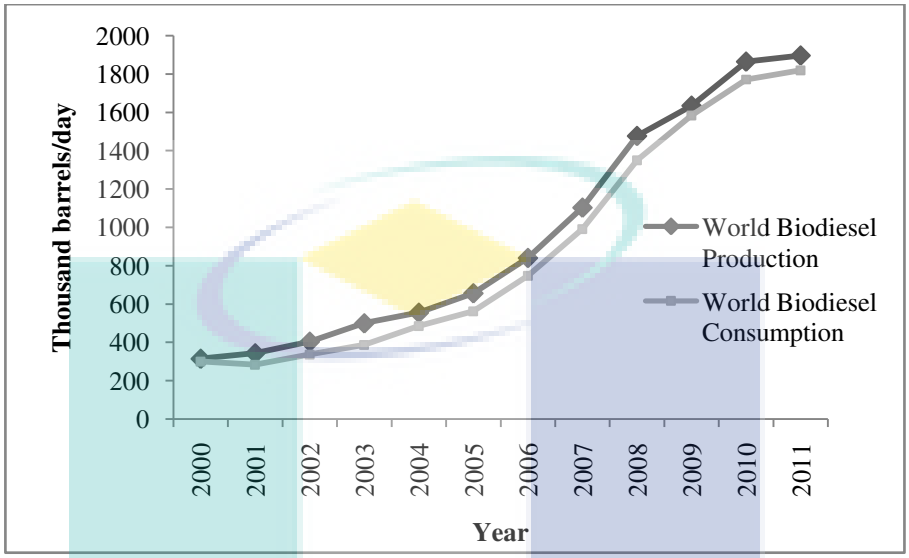


Figure 2.2: World Biodiesel Production and Consumption graph. (http://www.eia.gov)

European core biodiesel producers are Germany and France. The European countries supplied and consumed two over third of world biodiesel (ECOFYS report, 2011). Europe also plays an important role in biodiesel implementation as in April 2009; European Union endorsed a minimum binding target of 10% for biofuels use in transportation to be achieved by 2020, as a part of EU Directive 2009/28/EC on Renewable Energy. This Directive was a consequence on other Directives such as Directive 2003/96/EC on Energy Taxation and Directive 2003/30/EC. Direactive 2003/96/EC on Energy taxation allowed the tax exemption on promoting while Directive 2003/30/EC set 5.57% target of biofuels market penetration by 2010 (Sorda, Banse, & Kemfert, 2010).

2.2.2 Malaysia Biodiesel Trend

Malaysia has different scenario than Europe countries in biodiesel production and consumption. Despite the fact that Malaysia is one of the crude palm oil producers in the world, Malaysia tries to play a role in palm oil biodieselproduction. From European Biodiesel Board statistic data (www.ebb-eu.org), Malaysia produced 80 million tonnes of biodiesel in 2010 and exported 90 million tonnes of biodiesel to European Union and also United State of America countries. From this data it shows that Malaysia had successfully produced biodiesel for the export purpose but not for other local purpose like stated in Malaysia National Biofuel Policy. Malaysia launched National Biofuel Policy in 2006 that containts five strategic thrusts which are biofuel for transport, biofuel for industry, biofuel for technologies, biofuel for export and biofuel for cleaner environment. Followed after was a Malaysia Biofuel Industry Act 2007 that allowed the incentives for biodiesel production and consumption however, this act was not fully implemented and cause a lot of biodiesel industries when to bankruptcy as the authorities failed to monitor and support the industries. Beside, the palm oil biodiesel cannot compete with crude palm oil as Malaysia had a lot of attractive incentives on palm oil production and consumption, it suit to the name asone of the word palm oil producercountry (Biofuels in Malaysia, 2011).

2.3 BIODIESEL TRANSESTERIFICATION

Biodiesel production method was first patented in 1977 by Expedito Parente. Biodiesel is a clear yellowish liquid with almost similar viscosity as petroleum based diesel however biodiesel is non-flammable and non-explosive compared to diesel since it has high flash point than diesel. Biodiesel is produce from a reaction of triglycerides with alcohol. Figure 2.3 shows the biodiesel production chemical reaction. One mole triglyceride need three mole of alcohol to produce three mole fatty acid methyl ester (FAME) and one mole glycerol with the aid of catalyst.

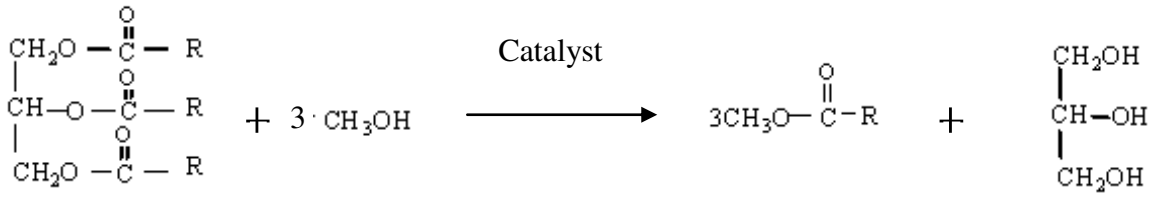


Figure 2.3: Biodiesel (FAME) chemical reaction mechanism

To date, there are a lot of technologies and researches on biodiesel. This includes any others study on catalyst, feedstock, reactor and reaction conditions.

2.3.1 Catalyst

Catalyst use to increase the reaction rate without being consume by the reaction. Catalyst can be divided to chemical catalyst and biocatalyst or also known as enzyme. Basically, chemical catalyst are acid and base catalsyts.

Chemical Catalyst

Chemical catalysts are the common catalysts used in biodiesel production. Industry sector favors chemical catalyst over biocatalyst because of it high yield and less production time. Chemical catalyst can be divided into acid and base catalysts. Leung & Guo (2006) studied on transesterification of biodiesel using sodium hydroxide (NaOH), potassium hydroxide (KOH) and sodium methoxide (CH_3ONa) found that among the three, CH_3ONa produced highest yield which is 89%. NaOH and KOH are conventional or primary alkali compared to CH_3ONa. Sodium methoxide was a secondary product of NaOH by deprotonation of methanol into NaOH.

At 70°C, 30 min reaction, methanol to oil molar ratio 7.5:1 and 600 rpm of mixing CH3ONa produced 3% higher biodiesel yield compared to NaOH and KOH using 1.3 wt %

of catalyst (Leung & Guo, 2006). The almost similar result was also reported by Rashid & Anwar (2008) that used KOH in transesterification of rapeseed oil. The optimum conditions that produced 96% of biodiesel was at 65°C, 6:1 methanol to oil molar ratio, 1 wt % of catalyst and 600 rpm of mixing in 70 minutes of reaction. Using NaOH alkali catalyst, Keera, Sabagh, & Taman (2011) managed to produce 98% of biodiesel in 1 hour of reaction with reaction conditions 60°C, 6:1 methanol to oil molar ratio, 1 wt % catalyst and 400 rpm mixing. Under high free fatty acid (FFA) and water, the alkali catalysts produces soap as the by product and make it difficult for separation process (Lam, Lee, & Mohamed, 2010).

Acid catalyst was one of the catalysts used in biodiesel production however, using acid catalyst, fatty acid was easily denatured due to high acidity conditions and need excess methanol to drive the transesterificationreaction for high yield production of biodiesel (Wu et al. 2008). Acid catalyst is less sensitive to FFA and water content that produced less glycerol but has slow reaction compared to alkali catalyst. Therefore, a combination of acid and alkali transesterification that was called acid-base catalysis was studied. Berchmans & Hirata (2008) reported that by using two-steps transesterification process, the high free fatty acid content (15%) in crude jatropha oil was reduced to less than 1% and produced 90% of methyl ester in 2 hours. 1 % w/w of Sulphuric acid (H_2SO_4) was used in first step for 1 hour at 50°C and at the second step 1.4% w/w of NaOH was used to produce biodiesel at 65°C.

Biocatalyst/Enzyme

Some drawbacks in the use of chemical catalyst in biodiesel production had drawn researcher's attention. Chemical catalysts producea large amount of by product which is glycerol. Glycerol is difficult to remove in separation process and generate wastewater problem (Lam et al., 2010). The use of enzyme in biodiesel production presents an alternative to the chemical catalyst. Enzyme has no or less by-product, easy glycerol separation, less sensitive to FFA content, work in mild reaction condition and recyclable (Kulkarni & Dalai, 2006). Enzyme also can overcome the high acid value level in biodiesel production (Li, Zong, & Wu, 2009). Lipase enzymes are the hydrolytic enzyme that

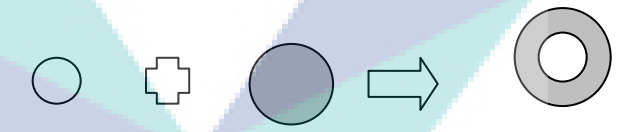
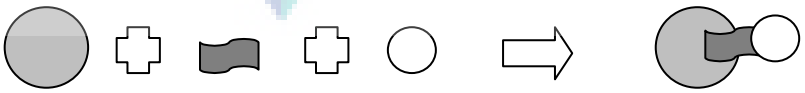
specialized in wide range reactions including transesterification reaction which is important to convert vegetable oil into fatty acid methyl ester in biodiesel making process (Noureddini et al., 2005). Wang et al. (2010) reported that Immobilized *R. Oryzae Lipase* successfully produced 90.5% biodiesel yield under optimum conditions which are methanol to oil molar ratio 4.8:1, 24 U/g enzyme activity, 37°C reaction temperature and 160 rpm for 12 hours. Using Immobilized Lipase from *Penicillium Expansum* Zong et al. (2009) produced 92.8% of methyl ester after 7 hours and the enzyme activity retained 68.4% of enzyme activity after 10 cycles. Immobilized enzyme has bright future in biodiesel production since the non-immobilized usually take 70 hours to reach reaction equilibrium.

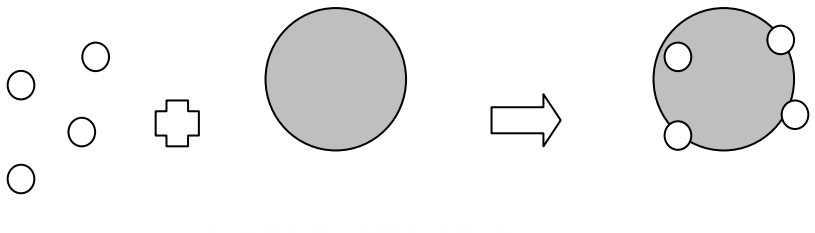
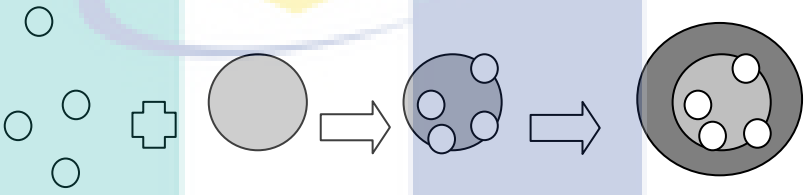
Lipase enzyme can be produced from wide range of plants and organisms. Among the four types of lipase enzyme tested which are Lipase from *MucorMiehi*, Lipase from *Candida Antartica*, Lipase from *Pseudomonas Cepacia* and Lipase from *Candida Rugosa,* Pirajàn & Giraldo (2011) had reported that Lipase from *Candida Rugosa* produced highest yield of mono alkyl esters. 85% of methyl and ethyl esters were produced after 1 hour of reaction using 500 mg of Immobilized Lipase from *Candida Rugosa*with 50 g of palm oil, 14.5:1 ethanol to oil molar ratio and 1.0 g of water at 35°C by flow microcalorimetry.While Noureddini et al., (2005) investigated nine types of lipase enzymes including Lipase from *Candida Rugosa* using soybean oil and stated that the Lipase from *Pseudomonas Cepacia* resulted in highest yield of alkyl esters. 475 mg of Immobilized Lipase from *Pseudomonas Cepacia* mixed with 10 g of soybean oil, 17:5 methanol to oil molar ratio, 0.5 g of water at 35°C for 1 hour produced 67 mol % of alkyl esters. Both Pirajan & Giraldo (2011) and Noureddini et al. (2005) produced the almost similar optimum parameters and methyl ester yield. Compared to chemical catalyst, enzyme also can produce high quality of biodiesel in short time. However enzyme have some drawbacks which are expensive and has slow reaction than chemical catalyst. To overcome this drawback, enzyme immobilization was introduced.

Enzyme Immobilization

Immobilization is a method to attach, entrap or chemically modified the enzyme. The earliest immobilization method was reported by Nelson & Griffin in 1916 that used adsorption technique (Crouch et al., 1990). Immobilization resulted in higher enzyme reaction and stability than conventional enzyme during biodiesel production Bhushan et al. (2008) stated that the immobilization of enzyme enhance the pH, thermal and storage stability. Heterogeneous Immobilized enzyme is easy to separate from the biodiesel final product and recyclable. Immobilization technique can be divided into four types which are Entrapment, Covalent binding, Adsorption, and Encapsulation. Table 2.1 shows the mechanism of immobilization for every types.

Table 2.1: Mechanism of Immobilization Techniques Table

Technique	**Mechanism**
Entrapment	 Enzyme support Enzyme entraped inside support
Covalent Binding	 support Linker Enzyme Enzyme linked/binded with support

Adsorption	 Enzyme support Enzyme adsorpted into the support
Encapsulation	 Enzyme Support Enzyme adsorpted into support The immobilized enzyme then encapsulated

From table 2.1, entrapment technique use support to entrap enzyme inside the support. This technique need the support in liquid state before the enzyme is immobilize with the support. The liquid support being changed to gel and entrap the enzyme inside. Covalent binding use biofunctional linker such glutraldehyde to bind the enzyme with support using the covalent bond. Adsorption is the simplest immobilization technique that need only the mixing to naturally force the enzyme to being adsorp inside the pore's support. But this method produced weak bonding of enzyme to support. Encapsulation technique modified the adsorption technique by adding the encapsulation step at the end of enzyme immobilization. Table 2.2 shows the summary of literature review on enzyme immobilization technqiue.

Table 2.2: Literature Review summary on Enzyme Immobilization Techniques Table

Immobilization Technique	Enzyme	Support	Enzyme Activity	Efficiency (Loading %)	Recyclability	References
Entrapment	*Lipase Candida Rugosa*	Alginate & Chitosan bead	1150 U/ml	50	-	(Betigeri & Neau, 2002)
Covalent binding	*Lipase Candida Rugosa*	Glass bead	92.53 U/g support	79.42	Retained 25% conversion ratio after 5 cycles	(Yilmaz et al., 2011)
Adsorption	*Lipase Candida Rugosa*	Natural Kaolin	-	77	Stable up to 12 days suspended with n-hexane	(Abdul Rahman et al., 2005)
Encapsulation	*Lipase Candida Antarctica*	Hexagonal mesoporous silica	304 U/min	62	Retained 96% activity after 4 cycles of transesterification	(Yadav & Jadhav, 2005)
Covalent Binding	*Lipase Candida Lipolytica*	palygorskite	3300U/g support		Retained 97% activity after 8 cycles	(Huang, Liu, & Wang, 2009)
Covalent Binding	*Lipase Rizhopus Oryzae*	Silica Gel	-	90	Retained 80% activity after 20 cycles	(Kim et al., 2006)

From the table 2.2, Betigeri & Neau (2002) used Alginate and chitosan bead to entrap the *Lipase Candida Rugosa*. The chitosan solution in 1% (w/v) acetic acid was mixed with 10.20 mg lipase in 2 ml of 0.5% (v/v) acetic acid. This mixture then injected using the syringe into the 0.136 M of sodium tripolyphosphate solution. This procedure formed the droplet or bead of enzyme and chitosan mixture.

Covalent binding method is different from entrapment method. In the covalent binding method study by Yilmaz et al. (2011), the glass bead used was activated using 2M NaOH solution before silanization using (3-aminopropyl)-triethoxysilane (APTES) and dry toluene for 8 hours with nitrogen gas. APTES remove hydroxyl functional group from glass bead surface The modified glass bead then been suspended into glutaraldehyde and phosphate buffer solutions for 6 hours to obtain the aldehyde functional group at the glass bead surface. This aldehdye functional group acts as the linkage to the enzyme and glass bead. Finally the lipase enzyme was covalently bind to the glass bead surface by mixing the 1.5 g of modified glass bead with lipase enzyme solution that content 0.45 g of *lipase candida rugosa* powder in phosphate buffer. Eventhough, the enzyme activity was the lowest among the other in table 2.4 due to the low enzyme concentration used which is 0.45 g but, yilmaz et al. (2011) managed to obtained 79.42% of protein loading into the support. Huang, Liu & Wang (2009) also reported on covalent binding of *lipase candida lipolytica* onto palygorskite using APTES and glutaraldehyde at 12 hours of enzyme immobilization process after silanization. Kim et al. (2006) produced the highest percentage of protein binding to the support which is 90% and the immobilized enzyme also retained 80% of it activity even after 20 reuse.

Compared to entrapment and covalent binding method, the adsorption method is the simplest method in immobilization. From Abdul Rahman et al. (2005), 15 ml of *Lipase Candida Rugosa* solution was mixed with 2 g of kaolin for 1 hour with 100 rpm of mixing at room temperature in adsorption immobilization process. The encapsulation procedures was an extended from adsorption method. Yadav & Jadhav (2005) reported that Immobilization of lipase from *candida Antarctica* on Hexagonal Mesoporous Silica was carried out by encapsulation technique. 200 mg of immobilized enzyme was suspended with 4% sodium alginate solution and 0.1 M calcium chloride. The sodium alginate solution that contained the immobilized enzyme then been injected through a syringe into 0.1 calcium chloride solution. The beads are formed in calcium chloride solution and allowed to harden for 1 hour. From the literature review it shows that cross linking technique has the highest percent of protein binding and the most stable compared with other techniques. To this date, researcher even combined the cross linking technique with

physical treatment such microwave irradiation to increase the enzyme activity. Wang et al., (2011) had reported that the immobilized Aldolase enzyme activity increase to 157% higher than non-microwave assisted microwave immobilized enzyme. Thus, microwave irradiation on cross linking technique is an interesting option in immobilization.

Enzyme Cross linking Immobilization Mechanism

Cross linking immobilization technique use the chemical modification that use covalent bond to immobilize the eznyme to silica support. Covalent cross linking methods may result in decreasing of enzyme activity because of it harsh chemical treatment but it provided a great deal of binding stability. Figure 2.4 show the mechanism of cross linking immobilization process.

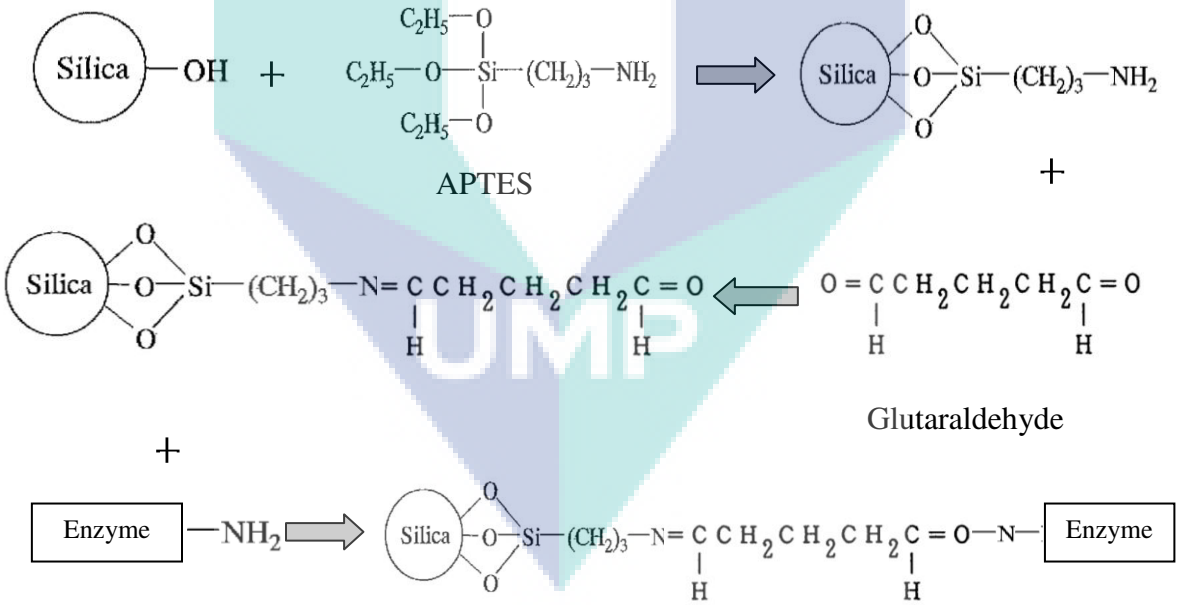


Figure 2.4: Mechanism of Enzyme Cross Linking Immobilization Into Silica Gel.

From figure 2.4, it shows that the hydroxyl group (OH) of silica gel was replaced by amine group (NH_2) using APTES as silanization agent in the process called silanization process. The modified silica was attached with glutaraldehyde biofunctional linker before

linked with amine group of enzyme. Glutaraldehyde acted as cross linking agent to link the amino group of silica gel and amino group of enzyme in immobilization through amine-aldehyde Schiff reactions (Huang et al., 2009) & (Kim et al., 2006).

2.3.2 Feedstock

Biodiesel can be produced from wide range of edible and non-edible oils. Currently, edible oil is the major feedstock in biodiesel production. The increasing demand of biodiesel increased the needs of edible oil and this overlap with the increasing demands of edible oil for foods as the world population had also increased. These competing demands raise the edible oil price and turn the researcher's attention to non-edible oil to produce biodiesel which has no competing issue with foods and cheaper price (Gui, Lee, & Bhatia, 2008).

Edible Oil

Edible oil is produced from the non-poison crop such as sunflower, soybean, and palm oil. The edible crude oils and its biodiesel properties are listed in table 2.2. From the table 2.1 it shows that the crude soybean oil has the lowest acid value, density and viscosity than crude palm oil however the palm oil show more balanced fatty acids composition between saturated and non-saturated fatty acids.

From the table 2.2, Encinar et al. (2012) used soybean oil to produce high quality methyl ester by microwave flow system. This system produced 99.7% of methyl ester in 10 min of reaction with methanol to oil molar ratio 12:1, 1 wt % of KOH catalyst, 70°C and 200W with 2 min interval.Rosset et al. (2011) also studied the transesterification process using soybean oil and Immobilized Lipase B from *Candida Antarctica*. It reported that 82.9% of biodiesel was produced after 24 hours reaction at 32°C with 5% w/w of Immobilized enzyme, 50 g of soybean oil, 150 ml of ethanol and 130 rpm of mixing.

Table 2.3: Summary on edible Crude oils and Biodiesel oils properties: Soybean oil, Sunflower oil & Palm oil Table

Reference	Encinar et al. (2012), Guerreiro et al. (2006)	Encinar et al. (2012)	Demirbaş, (2003)	Rashid et al. (2008)	Suzana & Khan (2010), Raita et al. (2010)	Soetaredjo et al. (2011)
Property	**Soybean Oil**	**B100 soybean**	**Sunflower Oil**	**B100 sunflower**	**Palm Oil**	**B100 Palm**
Density (kg/m^{-3})	930.7	0.877		0.88	0.92	0.88
Viscosity (cSt)	33.3	4.22	34.4	4.9	38.1	4.2
Water Content (%)	0.07	0.02	-	<0.01	-	-
Acid Value (mg KOH/g)	1.79	0.14	-	0.24	18.5	-
Iodine Value (mg.I_2/g)	115.4	115.3	132.32	-	-	-
Flash Point (°C)	-	173	-	130	-	170
Methyl Ester (%)	-	99.7	-	97.1		90.7%
Palmitic Acid (16:0)	11		6.4		43.5	
Strearic Acid (18:0)	5		2.9		4.3	
Oleic Acid (18:1)	22		17.7		39.8	
Linoleic Acid (18:2)	52		72.9		10.2	
Linolenic Acid (18:3)	10		0		-	

Sunflower oil transesterification was studied by Rashid et al. (2008) using NaOH catalyst with reaction conditions 6:1 methanol to oil molar ratio, 60°C reaction temperature, 1% w/w catalyst loading, 600 rpm of mixing and 120 min (2 hours) reaction time. Rashid et

al. (2008) obtained 97.1% of methyl ester in conventionally. On the other hand Dizge et al. (2009) reported that sunflower oil was successfully obtained 97% methyl ester using Lipase Immobilized onto monolithic microporous polymeric biocatalyst. Dizge et al. (2009) mixed 1 g of Immobilized lipase with 92 g of sunflower oil, 20 g of methanol in batch reactor with 250 rpm, 65°C reaction temperature for 24 hours of reaction and also reported that the immobilized enzyme retained its activity after 10 cycles.

Soetaredjo et al. (2011) had studied the biodiesel production from palm oil using KOH/bentonite as catalyst. It stated that with KOH to bentonite ratio 1:4, 3% catalyst loading, methanol to oil ratio 6:1, 500 rpm of mixing, 60°C reaction temperature and 6 hours reaction produced 90.7% of biodiesel yield. Palm oil methyl ester was also been produced using microcrystalline lipase in *tert*-butanol system. The commercial recombinant *Aspergillus* strain expressing *ThermomycesLanuginosus Lipase* was immobilized onto protein coated microcrystals and it was reported that the optimum conditions were 45°C reaction temperature, tert-butanol to triacylglycerol molar ratio 1:1, 20% (w/w) Immobilized enzyme loading, ethanol to oil molar ratio 4:1, 500 mg palm oil and at 24 hours reaction time, it can produced 82.2% biodiesel yield.

From the comparison in the table 2.1 it shows that the soybean oil, sunflower oil and palm oil can producehigh quality of biodiesel. Sunflower biodiesel has the lowest flash point value that indicated the flash point than two other biodiesels. The viscosities of the oils reduced greatly after transesterification process using either chemical catalysts or biocatalysts and the methyl ester percentage showed that the soybean oil produced highest methyl ester however, this percentage is differ according to the reaction conditions such as catalysts, parameters, and reactors.

Non-Edible Oil

Non-edible oil refers to the oil that cannot be used for foods. Non-edible oil can be extracted from Jatropha and rubber seed kernels. Rubber seed oil and jatropha oil are potential non-edible new feedstcok in biodiesel production. The biodiesel produced from

both oil shows a good quality of biodiesel but, among the two rubber seed oil was more attractive than jatropha oil. Rubber seed kernels can be harvested at readily available rubber plantation but, the jatropha tree need a special plantation only for biodiesel production since jatrapha did not produce other commercial products from jatropha tree itself. This prevents the other rural area and jungle to be clear for plantation. Some reports that waste cooking oil was used in soap manufacturing, energy production via anaerobic digestion and biodiesel production(Sabudak & Yildiz, 2010). Moreover, waste cooking oil contains about 54.35% of linoleic acid, which is comparable with sunflower and soybean oil (Dizge et al., 2009). The ever increasing of world population increases the demand of cooking oils but there are only a few solutions to manage the waste cooking oil which has become the matter to waste water plant thus, environment (Araujo, Hamacher, & Scavarda, 2010).

Rubber tree (HaveaBrasiliensis) is one of the abundant sources in South East Asia countries such Indonesia, Thailand and Malaysia. Rubber tree was mainly use to produced rubber latex. It was listed in the Malaysia Rubber Board Official Website that Malaysia had 37 Rubber Plantation which cover 1 million ha area. Beside rubber latex and rubber trunk to made Heveawood products there was no other use of rubber tree had been reported in Malaysia. Average rubber seed oil content from its kernel was reported between 40%-50% (Suzana& Khan, 2010) (Ramadhas et al., 2005). From table 2.3, it shows that non-edible oil is suitable to use in biodiesl production.

From the table 2.3, Ramadhas et al. (2005) reported that the rubber seed oil biodiesel was produced using two-step acid base transesterification process. Acid esterification used 0.5% of sulphuric acid, 200 ml methanol at 50°C reaction temperature for 30 minutes with stirring. At second step alkaline esterification, 5 g of NaOH was dissolved in 300 ml of methanol which is 6:1 of methanol to oil molar ratio at 45°C for 30 minutes. This process reduced FFA content to < 2% and produce >98% of biodiesel. Gimbun et al. (2013) was successfully produced biodiesel from high FFA rubber seed oil (RSO) using cement clinker. 96.9% of biodiesel was produced using 5 wt% catalyst loading, 4:1 methanol to oil molar ratio, 65°C reaction temperature for 4 hours of reaction. Deng et al., (2010) reported that 96.4% of biodiesel produced from *jatrophacurcas* oil

using two-step alkali base transesterification process. The optimum conditions obtained was 6:1 methanol to oil molar ratio, 1 wt % NaOH, 4 wt % of H_2SO_4, 100 ml Jatropha oil and 1.5 hours with ultrasonic irradiation.

Table 2.4: Summary on non-edible Crude oils and Biodiesel oils properties: *Jatropha Curcas* oil and Rubber Seed oil Table

Reference	**Deng et al. (2010)**	**Deng et al. (2010)**	**Ramadhas et al. (2005)**	**Ramadhas et al. (2005)**	**Jain et al. (2011)**	**Jain et al. (2011)**	**Gimbun et al. (2013)**
Property	**Jatropha Oil (JO)**	**B100 (JO)**	**Rubber Seed Oil (RSO)**	**B100 (RSO)**	**Waste Cooking Oil (WCO)**	**B100 (WCO)**	**B100 Rubber Seed**
Density (kg/m^{-3})	0.912	0.882	0.91	0.874	0.937	0.892	0.87
Viscosity (cSt)	8.72	3.96	66.2	5.81	50	4.2	4.64
Acid Value (mg KOH/g)	10.47	0.32	34	0.118	21.84	0.38	0.07
Flash Point (°C)	125	133	198	130	235	178	154.6
Methyl Ester (%)		96.4%	-	>98%		90.6%	96.9%
Palmitic Acid (16:0)	13.5		10.2		4.1		
Strearic Acid (18:0)	6.1		8.7		1.4		
Oleic Acid (18:1)	21.8		24.6		40.5		
Linoleic Acid (18:2)	47.4		39.6		38.0		
Linolenic Acid (18:3)	-		16.3		10.6		

Jain et al. (2011) reported that waste cooking oil produced 90.6% of methyl ester after 6 hours of reaction using two-steps acid base transesterification process. Purified waste cooking oil has the same quality as the crude oil as shows from the table 2.2 thus, instead being dump into the sewage waste cooking oil has the potential to recycle and use as biodiesel feedstock.

Rubber seed oil extraction

Gimbun et al. (2013) reported that using the microwave extractor the rubber seed oil extracted yield achieved 40% at 15 min compared to conventional soxhlet extration method that needed 6 hours to achieved 36% of extracted oil yield. Gimbun et al. (2013) used 250 g of crushed rubber seed, 500 ml n-hexane at 15 min of reaction time, 64 °C reaction temperature and 200 W of microwave power for microwave extraction.

2.3.3 Reactor

Another factor that plays an important role in biodiesel production is a reactor. Reactor can provide suitable or optimum conditions for transesterification of biodiesel. Table 2.4 summarize some of the reactors used in biodiesel production.

From the table 2.4, the highest biodiesel yield produced using microwave continuous with flow and supercritical reactor which were 99%, followed by microwave assisted batch, ultrasonic and batch with stirrer reactor. Demirbas (2009) studied the transesterification of biodiesel from waste cooking oil using KOH and methanol in supercritical reactor. Supercritical method offer great solution to high fatty acid waste cooking oil and eliminates the pre-treatment stage. However this reactor run at high temperature and pressure which are 100-300 °C and pressure up to 100 MPa.

Table 2.5: Literature Review Summary on Reactors used in Biodiesel Production Table

Reactor	Feedstock	Catalyst	Solvent	Reaction Conditions	Yield (%)	References
Ultrasonic	Jatropha oil	KOH (1.4wt%)	Methanol (6:1)	1.5 h, 60 °C, 210 W, 600 rpm	96.4	**(Deng et al., 2010)**
Microwaves assisted-batch	Safflower oil	NaOH (1wt %)	Methanol (10:1)	6 min, 60 °C, 300 W	98.4	**(Duz et al., 2011)**
Microwaves-continuous flow	Soybean oil	KOH (1wt %)	Methanol (12:1)	15 min, 70 °C, 200 W	99	**(Encinar et al., 2012)**
Supercritical	Waste cooking oil	KOH (6wt%)	Methanol (90%)	290 °C, 900s	99	**(Demirbas, 2009b)**
Batch with stirrer	Palm oil	Immobilized *Lipase Candida Rugosa*	Ethanol (3:1)	45°C, 500 rpm, 12 hours	>80%	**(Cheirsilp et al., 2010)**

Microwave reactor can produce higher biodiesel than ultrasonic reactor. Encinar et al. (2012) investigated the transeterificationof soybean oil in microwave flow system. 99% of methyl ester was produced within 30 minutes using microwave flow system at methanol to oil ratio 12:1, 1 wt% KOH, 70°C reaction temperature and 200W microwave power with 2 min interval. On the other hand, Duz et al, (2011) produced 98.4% of biodiesel from safflower oil under 300 W microwave irradiations with reaction conditions of 10:1 methanol to oil molar ratio, 1 wt% NaOH catalyst, 60 °C reaction temperature and stirring. Deng et al. (2010) had reported that *jatrophacurcas* oil produced 96.4% of methyl ester using two-steps acid base esterification under ultrasonic irradiation. The optimum conditions obtained were 6:1 methanol to oil molar ratio, 1 wt % NaOH, 4 wt % of H_2SO_4, 100 ml Jatropha oil and 1.5 hours with ultrasonic irradiation.

The conventional batch reactor produced lowest methyl ester compared to others. Cheirsilp et al. (2010) had studied the transesterification of palm oil in batch system using immobilized lipase from *candida rugosa* enzyme. The transesterification produced more than 80% of methyl ester after 12 hours of reaction at 45 °C, 500 rpm of mixing and 3:1 ethanol to oil ratio. Supercritical reactor used a lot of energy since it operated at high temperature and pressure eventhough it can produce up to 99% of methyl ester yield. Ultrasonic and microwave reactor can produced high methyl ester yield in short time compare to other type of reactors.

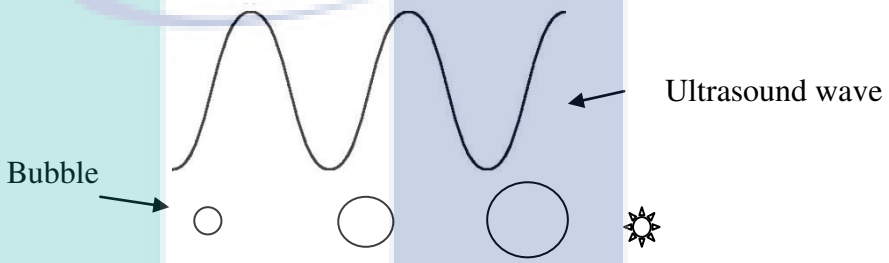


Figure 2.5: Ultrasonic mechanism in water

Ultrasonic work based on ultrasound frequency. Ultrasound frequency create a low and high pressure condition in ultrasonic water tank. Figure 2.5 shows that the small bubble produced by low pressure condition continue to grows under ultasound frequency and rupture due to the different in pressure. This process create shear stress that acts to enhance the bulk pysical mixing and reaction rate in transesterification process (Yu et al., 2010).

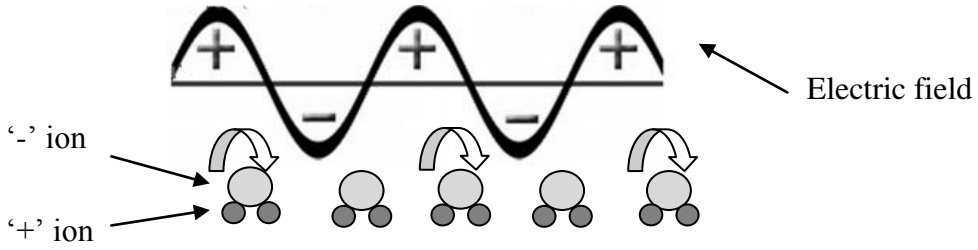


Figure 2.6: Microwave alternating electric field mechanism

Microwave radiation is an alternating electric field that interven with dipolar molecules. Figure 2.6 shows that the opposite charge of ion rotate to allign with the changing electric field. This molecules rotation create friction among molecules that increase the reaction temperature rapidly (Azcan & Danisman, 2007), enhance the bulk pysical mixing and reaction rate. The microwave reactor capability to produce higher methyl ester in shorter time compare to ultrasonic is probably due to it molecular level friction. This make the microave reactor an interesting reactor to use in biodiesel production. The study of microwave assisted in enzymatic biodiesel production is new in the field. It only had being study statistically by Perez et al. (2014) and Noguiera et al. (2010).

2.3.4 Solvent

Solvent played an important role in biodiesel production. Common solvent use in biodiesel production is alcohol. The transesterification process is reversible reaction thus; excess alcohol was required to shift the equilibrium to product side and raise the product yield and allow the phase separation from the glycerol to be formed. The minimum alcohol to oil ratio is 3:1 because 1 mole fatty acid need to 3 mole of alcohol theoretically in transesterification reaction.

Commonlly used alcohol is methanol and ethanol because of their low cost and has shortest chain alcohol. Among alcohol tested, methanol is most active for production of

biodiesel from jatropha oil and the activities of lipase decrease with the increase in alcohol chain length (Akihiko et al., 2001). The ratio of methanol to oil at 6:1 or higher generally gives the maximum yield (higher than 98 wt %). Excess alcohol levels may inhibit the enzyme activity and thereby decrease it catalytic activity toward the transesterification reaction (Moreno & Giraldo, 2010).

Lower ratios required a longer time to complete the reaction.

2.3.5 Reaction Conditions

Reaction conditions of transesterification reaction play an important role in biodiesel production. Every research has it own optimum parameters due to the feedstock, enyzme and solvent used. Nogueira et al. (2010) that statistically studied the microwave activation of enzymatic catalyst for biodiesel production reported that the most important variable for biocatalyst activity was reaction time and enzyme loading. However Perez et al. (2014) that also statistically studied the characterization of the product obtained by enzymatic route accelerated by microwave irradiation reported that the alcohol to oil molar ratio strongly influenced the biodiesel production and the interaction of alcohol to oil molar ratio and reaction temperature. As shows in table 2.6, the reaction conditions for enzymatic transesterification in biodiesel production are vary in every research and only reaction temperature has small range.

All research in table 2.6 used batch reactor. Rogers at el. (2013) had studied the comparison between transesterification of coconut oil using NaOH and commercial Immobilized lipase enzyme from *Candida Antartica*. Jiang et al. (2010) use the purposed made of immobilized recombinant *Rhizopus Oryzae Lipase* on anion resin in biodiesel production from soybean oil. While Shimada et al. (2007) use acid oil which is the by-product of vegetable oil refining as feedstock in biodiesel production. Perez et al. (2014) also use purposed made immobilized *Burkholderia Cepacia lipase* enzyme onto SiO_2-PV.

Table 2.6: Literature review summary on biodiesel production reaction conditions Table

Reference	Rogers et al., 2013	Jiang et al., 2010	Shimada et al., 2007	Perez et al., 2014
Oil	Coconut oil	Soybean oil	Acid oil	Babasu oil
Temperature (°C)	30	40	30	50
Alcohol	Ethanol	Methanol	Methanol	Ethanol
Alcohol to oil ratio	3:1	5:1	51%	12:1
Enzyme	Novozyme 435	Immobilized Recombinant *Rhizopus Oryzae Lipase*	Novozym 435	Immobilized *Burkholderia Cepacia Lipase*
Enzyme Loading	1 wt %	24U/g	1.5 wt%	20 vol %
Mixing (rpm)	350	180	100	150
Reaction Time	50 h	48 h	24 h	48 h
Biodisel yield	>80%	88%	>98%	>98%

The study on enzymatic transesterification is wide in range since researcher tend to use the new feedstock, new transesterification method, new immobilized enzyme and new combination of slovent thus, the optimum parameters are varied. From table 2.6, it shows that immobilized enzyme from any kind of lipase has an optimum temperature range from 30 °C to 50 °C. 1 wt % of enzyme loading and 100 rpm of mixing is enough for transesterification process. While solvent to oil ratio need at least 3:1 of vegetable oil.

2.4 SUMMARY

As a conclusion from literature review study, biodiesel should be investigated more since the diesel petroleum based now day become more expensive day by day and that non-renewable source is facing the depletion issues. Biodiesel have several advantages over diesel such as less carbon emissions, less toxic and biodegradable. To produce biodiesel, non-edible oil was much suitable to use it had no issues with food supply such as waste cooking oil and rubber seed oil. Among the reactor discussed in literature review, microwave was a highly efficient reactor compared to batch reactor. Microwave reactor use in biodiesel production need to be investigates more. Biocatalyst or enzyme was anattractive substitute to chemical catalyst in biodiesel production since it was reported as environmental friendly, biodegradable, less glycerol produced, easy separation process, and workin mild reaction conditions. Enzyme also produced almost same quality of biodiesel with biodiesel produced from edible oil. Enzyamtic transesterification process are 2 magnitude slower than chemcial catalyst but, it produce cleaner biodiesel. To overcome this slow process, microwave assisted transestrification is a better choice and the enzyme immobilization technology will increase the enzyme activity and stability over conventional enzyme thus, increase the transesterification reaction rate. Overall, there were none of the studies done on microwave assisted enzymatic transesterification from waste cooking oil and rubber seed oil using Immobilized *lipase Candida Rugosa* enzyme.

CHAPTER 3

METHODOLOGY

3.1 OVERVIEW

This chapter presents materials, methods procedure and analytical instruments used in this work. Materials part was divided into two sections which is feedstock and chemicals. Feedstock refers to biodiesel feedstock which is oil use to produce biodiesel. Chemicals refer to all chemicals used in either experiment or analysis. In Methods section, it is divided into experiment's method and analysis methods to give clear information on methods. Methods section also provides some information on conditions used during analysis or experiment.

3.2 MATERIALS

3.2.1 Feedstocks

The palm waste cooking oil (WCO) was collected from a local restaurant in Gambang, Pahang, Malaysia. The collected oil was filtered to remove visible impurity and treated with fuller earth for an hour in stirred vessel to reduce moisture content and other impurities. The oil was then centrifuged at 8000 rpm for 3 minutes to separate the oil from fuller earth and other particulate matter. Rubber Seeds oil (RSO) was extracted from rubber seeds kernel by microwave assisted extraction in hexane. A 27 kilogram rubber seed with kernel and coat was purchased from local rubber plantation at RM 3.00 per kilogram. Table 3.1 list the properties of crude rubber seed oil and waste cooking oil.

Table 3.1: Crude Rubber Seed oil & Waste Cooking oil properties Table

Properties	*Unit*	*This work CRSO*	*This work WCO*
Kinematic Viscosity@40°C	*Mm^2/s*	*42*	*40*
Specific Gravity	*-*	*0.9*	*0.9*
Acid Value	*Mg KOH/g*	*30*	*41*
Flash Point	*°C*	*200*	*232*

From table 3.1, it shows that waste cooking oil has higher acid value than crude rubber seed oil due to the impurities contain in waste cooking oil. Both crude rubber seed oil and waste cooking oil has high flash point and kinematic value while having similar specific gravity. These value is expect to reduce after the fatty acid of the oils convert to methyl ester and comply with American Society for Testing & Materials (ASTM) range.

3.2.2 Chemicals

Lipase from *Candida Rugosa*immobilized on immobead 150 Enzyme was obtained from Sigma-Aldrich Netherlands while lipase yellow-brown powder was obtained from Sigma-Aldrich Japan (Enzyme No 3.1.1.3). Silica Gel 100-200 mesh sizes which comply with the ASTM D1319 standard was brought from Fisher Chemicals. Silica gel used in this experiment was type 30Å pore with 60-200 mesh which means the average pore diameter of silica gel was 3nm and it size was 200µm until 75µm. Glycerol Tributyrate used in this experiment was brought from SAFC (Sigma-Aldrich) Italy with 97% purity and CAS number 60-01-5, Bovine Serum Albumin (BSA) biotechnology grade at purity >98% was brought from Amresco with CAS 9048-46-8. This BSA PH was 5.2 when 5% diluted in water. Methyl Palmitate GC Standard (CAS: 112-39-0), Methyl Linoleate GC standard (CAS: 112-63-0) & Methyl Oleate GC standard (CAS: 112-62-9) was obtained from Sigma USA. Analytical grade methanol with purity 99.9% and analytical grade acetone with 99%

purity was brought from Fisher Scientific, UK. Industrial Grade Hexane with purity of 60% was taken from Faculty of Chemical Engineering & Natural Resources Storage while GC grade Hexane with purity of 98% was brought from Merck, Germany together with Ethanol 99.9% purity, Sodium Phosphate Monobasic Monohydrate 99% purity (PH 4.1-4.5) and Sodium Hydroxide pellet 99% purity, Sodium Phosphate Dibasic Dihydrate ($Na_2HPO_4.2H_2O$) powder at purity of 99.5% (PH9) and Lowry Reagent 1 (modified) was brought from R & M Chemicals, UK. 3-(N-Morpholino) propane sulfuric acid MOPS ≥99.5% (CAS: 1132-61-2), 3-Aminopropyltriethoxysilane (APTES)≥98% , Grade II Glutaraldehyde 25% in water, Folin Ciocalteu's Phenol Reagent (Lowry Reagent 2) at 2 N, Fuller Earth (Kaolinite) with 100-200 mesh size (CAS: 8031-18-3) and Florisil (Activated Magnesium Silicate) with size ≤200 mesh (CAS: 1343-88-0) was brought from Sigma Aldrich USA.

3.3 METHODOLOGY

The overall methodology of experiment was presented in figure 3.1. As shows in figure 3.1, the experiment start by rubber seed oil (RSO) microwave assisted extraction as RSO use as one of the feedstock in this experiment beside waste cooking oil. After RSO extraction, the screening was done to batch, ultrasonic and microwave reactor. There were none of research reported on the comparison of batch, ultrasonic and microwave reactor in enzymatic transesterification. Therefore, there is a need to investigate which reactor among ultrasonic and microwave reactors can produce highest methyl ester in shorter time in enyzmatic transesterification and compare with batch reactor.

Besides using only the commercial immobilized *Lipase Candida Rugosa* enzyme in this experiment, the *Lipase Candida Rugosa* enzyme was immobilized into silica gel using cross-linking technique. The immobilized enzyme then being characterized using several method. The immobilized enzyme was characterized using Thermal Gravimetric Analysis, Fourier Transform Infrared Spectrophometer, Scanning Electron Microscope and Wet Laser Diffraction. Enzyme loading into silica gel and enzyme activity of immobilized enzyme was also measured. The enzymatic transesterification was studied using microwave

reactor. The optimum parameter was studied using waste cooking oil and commercial immobilized enzyme before being used on rubber seed oil and purposed made immobilized enzyme beacuse the extracted rubber seed oil and lipase enzyme powder was limited in amount. The parameters studied are reaction time, reaction temperature, methanol to oil ratio, mixing and microwave power. Enzyme leaching and recyclability was also studied. Methyl ester produced then being purified before analyze with ASTM standard testing and gas chromatography. Unpurified methyl ester effect the gas chromatography and ASTM result.

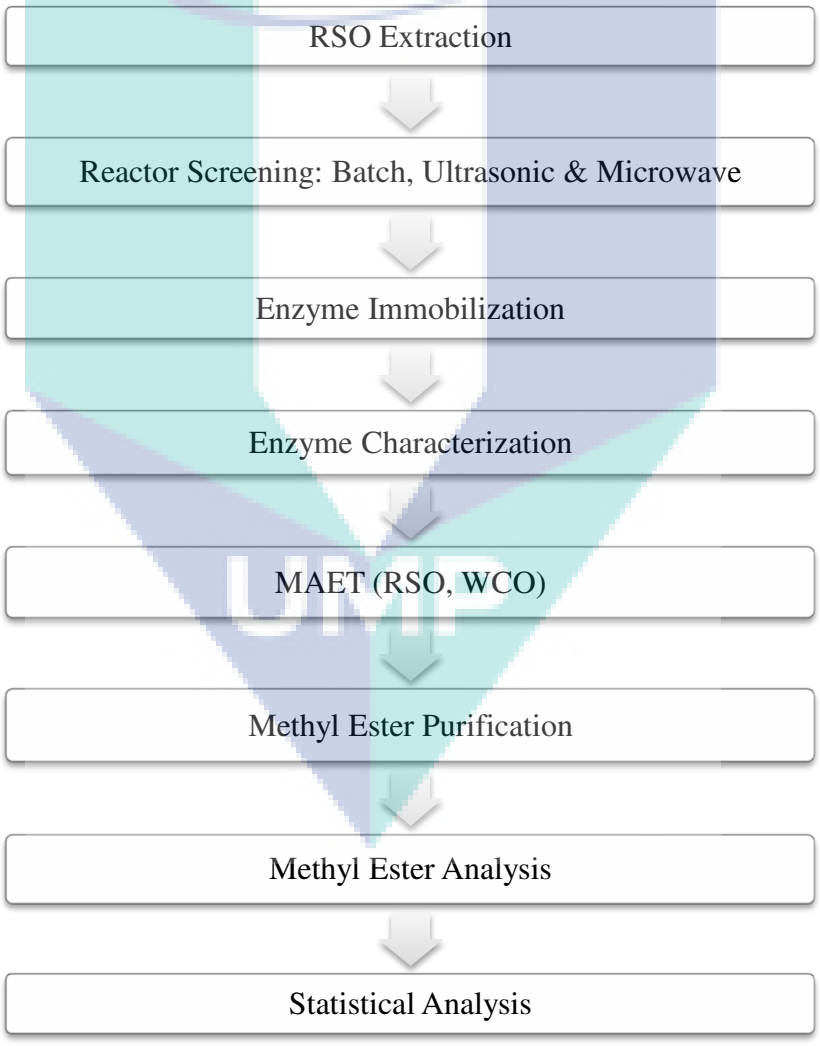


Figure 3.1: Overall methodology flow chart

Statistical analysis was done on enzyme loading, enzyme activity, enzyme leaching enzyme recyclability study and ASTM results. The detail experiment methods is explain in the next sub-chapters.

3.3.1 Rubber Seed Oil Microwave Assisted Extraction

Oil extraction from rubber seeds oil was done using microwaves extractor as it is produced higher yield in shorter time than soxhlet extractor method as reported by Gimbun et al. (2013).The extratcion perfomed using a Milestone Micro synth ATC-FO 300 microwave extractor. Figure 3.2 shows the microwave extractor diagram. Microwave extractor equiped with condenser and controller that able to control the microwave temperature, microwave power and operation time.

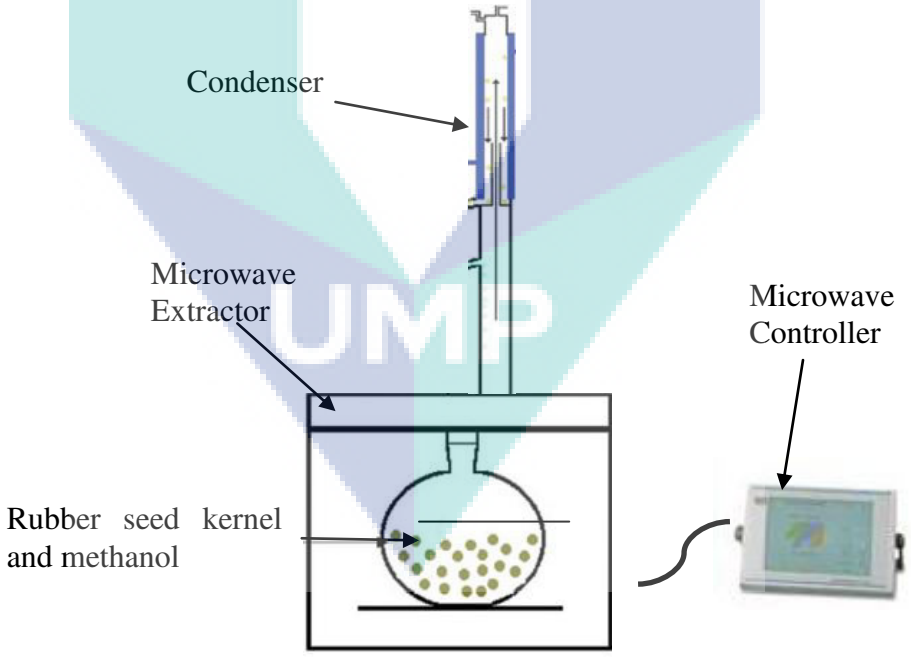


Figure 3.2: Microwave Extractor Diagram

The seeds kernel was taken from rubber seeds after it coat was crushed and the kernel was weighted before dried at 80°C in oven for 24 hour to reduce the moisture

content to 5%. The kernel was dried in dryer untill the weight become constant and the mositure content reached 5% of total moisture. Grinding was done using a mechanical grinder. The total amount of oil available to extract depend on the surface area. Small particle provided higher surface area to volume ratio thus; more oil will be sticking to the surface area instead inside particles. 50 gram of grinded rubber seeds kernel was dispersed in 150 gram of hexane which is 3:1 seeds to solvent ratio.

The mixture then placed in the microwaves extractor for 10 minutes at 200 W of power and 64°C. The temperature used must lower than solvent boiling point to prevent it from vaporizes completely into the air. The microwaves extractor was provided with the condenser to condense the vaporized solvent and prevent it from released into the open air. The extracted seed was separate from the solution using the soxhlettimble to get the clear solution containing excess solvent and oil. The solution then purified using rotary evaporator at 70°C until the weight of the oil was constant to remove excess hexane from rubber seeds oil. Table 3.2 shows the ASTM properties of the crude rubber seed oil. All the properties was comparable with Ramadhas et al. (2005) and Maksudur et al. (2011) that also studied the biodiesel production from rubber seed oil.

Table 3.2: Crude Rubber Seed Oil ASTM Properties Table

Properties	*Unit*	*This work CRSO*	*Ramadhas et al., (2005)*	*Maksudur et al., (2011)*
Calorific Value	*MJ/kg*	-	*37.5*	-
Kinematic Viscosity@40°C	*Mm^2/s*	*42*	*66.2*	*33*
Specific Gravity	-	*0.9*	*0.91*	*0.88*
Acid Value	*Mg KOH/g*	*30*	*34*	*45*
Flash Point	*°C*	*200*	*198*	-
Cetane Number	-	-	-	-

3.3.2 Reactor Secreening

All the experiment in reactor screening use purified waste cooking oil, methanol and commercial immobilized lipase from *Candida Rugosa* enzyme.

Batch Transesterification

Batch transesterification was done in water bath as the temperature of water bath can be controlled to the desired reaction temperature. 50 gram of oil was added into the conical flask and mixed with methanol at 6:1 of molar ratio. 1 wt % of immobilized lipase enzyme was measured to the oil weight and added to the solution mixture. The reaction temperature was set at 40°C and overhead stirrer was used to mix the solution at 200 rpm.

The conical flask was covered with aluminium foil and silicone tape besides, a small hole was made to allow the stirrer. Even though, the methanol boiling point is higher than 40°C as a precaution, the reaction was done near the suction area to make sure no methanol vapor was released to the environment.

Ultrasonic Assisted Transesterification

Ultrasonic assisted transesterification was done inside ultrasonic bath model. 50 gram of oil was placed inside the conical flask and 6:1 molar ratio of methanol was added. 1 wt % of immobilized enzyme was measured and added to the solution mixture. The reaction conditions was 40°C, 35 kHz, 100 W, 40% of ultrasonic total power and been run up to 5 hour with 30 minute interval. The stirrer was placed to mix the solution mixture at 200 rpm.

Microwave Assisted Transesterification

The transesterification process was carried out in a glass beaker using 50 g of WCO, methanol to oil molar ratio 6:1, 1 wt. % of immobilized enzyme with respect to WCO, reaction time of 5 hours with 30 minute interval, mixing at 200 rpm and temperatures at 40 °C. MAET was performed using a Milestone Micro synth ATC-FO 300 microwave extractor.

3.3.3 Lipase Enzyme Immobilization using Cross Linking Method

This study use the Lipase from *Candida Rugosa* enzyme which is the same type with the commercial immobilized enzyme. This immobilization method was adapted from (Kim et al., 2006) as it produced the highest eznyme loading in literaure review. Overall process of enzyme immobilization presented in figure 3.3. As shows in figure 3.3, 1 gram of Lipase was dispersed in 100 ml of 0.25 M 3-(N-morpholino) propane sulfonic acid (MOPS) – Sodium phosphate buffer at pH 6.5 to be treated with 1% rubber seeds oil or waste cooking oil at 40°C for 45 minutes. This stage introduced the enzyme in the oil environment before the immobilization process.

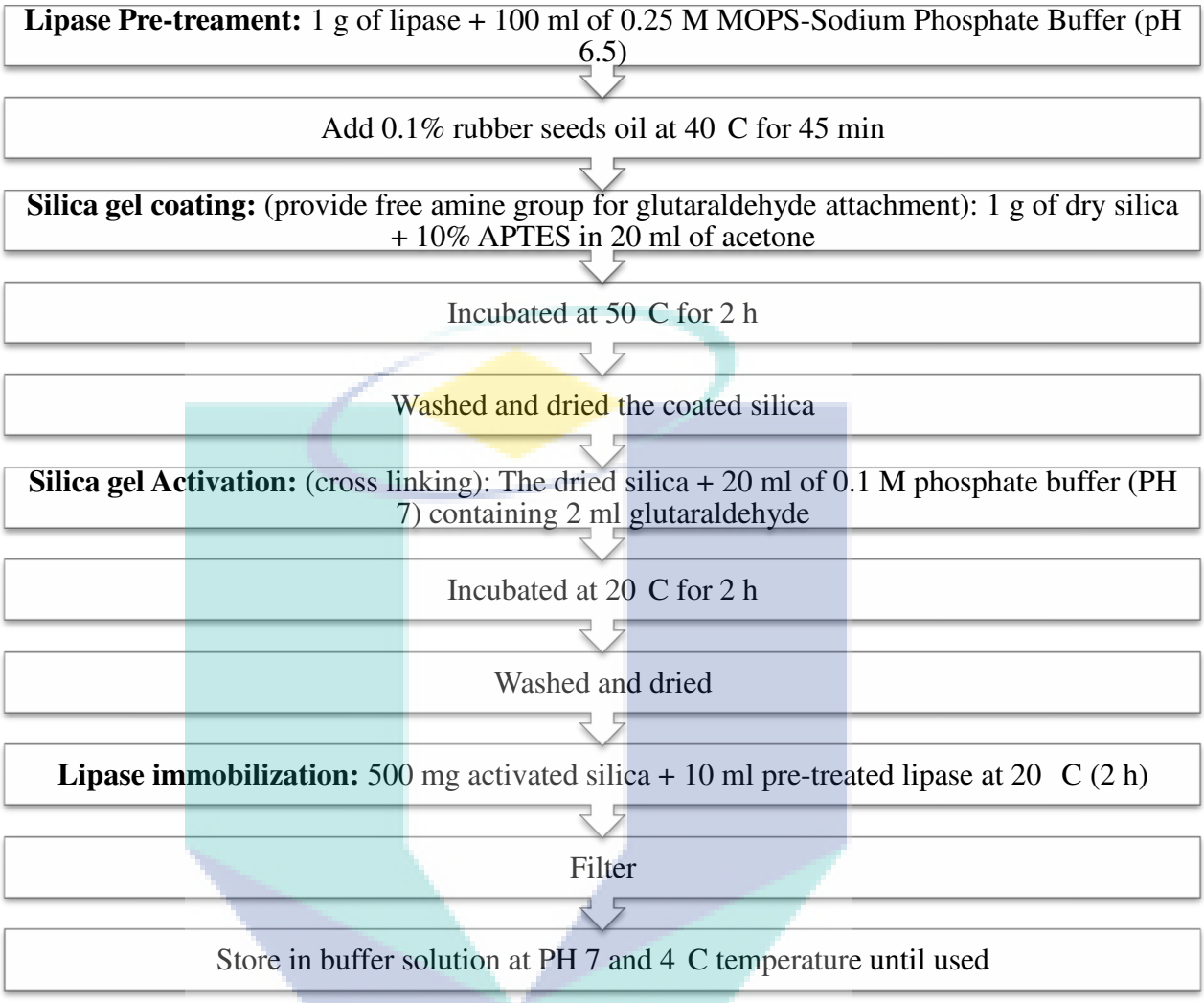


Figure 3.3: Cross Linking Immobilization Overall Step Flow Chart

Immobilization process continues by activated the immobilization support which in this study is silica gel. 1 gram of silica gel was mixed 10% of 3-aminopropyltriethoxysilane (APTES) and 20 ml of acetone and incubated at 50°C for 2 hours to chemically modify the silica gel structured by providing the amine group for activator attachment. The modified silica gel then had been washed and dried in the oven at 50°C. APTES was added in the solution to replace the hydroxyl group on silica gel surface with the amino group. The dried silica gel then was mixed with 20 ml of 0.1 M phosphate buffer at pH 7.0 containing 2 ml of glutaraldehyde to activate the silica gel and incubated at 20°C for 2 hours.

The activated amine group was attached with glutaraldehyde to obtain aldehyde functional group. The activated silica gel was washed and dried and stored in buffer solution at 4°C until used. The aldehyde functional group was acted as the linker agent to link the amino group of silica gel with amino group of enzyme in immobilization process. Lipase immobilization was performed using 500 mg of activated silica mixed with 10 ml of pretreated lipase at 20°C and gently stirred for 30 minutes. After 30 minutes the immobilized lipase was filtred using watman filter paper and stored in buffer solution until used.

Figure 3.4. show the picture of silica gel before and after immobilization process. The white powder silica gel change colour to brownish after immobilization process.

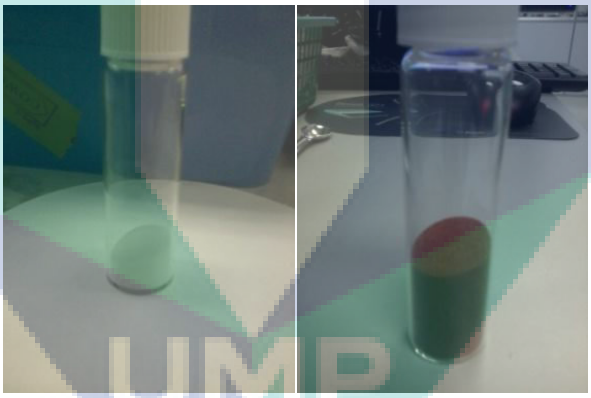


Figure 3.4: Silica Gel before and after immobilization Image

To study the microwave irradiation influence on cross linking technique, the lipase immobilization part in cross linking method was exposed to microwave irradiation for only 1 min and the exposure continue until 120 min with 10 min interval. The immobilization temperature was maintained by cooling system. This process ensures that the microwave irradiation does not affect the immobilization temperature.

Buffer Preparation

Buffer is water based salt solution that can help to maintain a constant pH of solution. The buffer is commonly prepared at pH 7 using monosodium phosphate and it conjugate disodium phosphate. It can be prepared at different pH by adjusting the salt concentration or by adding some acid or alkaline into the salt mixture solution. This experiment used two type of buffer which are 0.1 M phosphate buffer and MOPS-phosphate buffer.

To prepare the 0.1 M phosphate buffer stock solution of sodium phosphate monobasic monohydrate was mix with stock solution of sodium phosphate dibasic dihydrate. From the calculation using Molecular weight, 13.8 gram of sodium phosphate monobasic monohydrate was diluted into 1 liter of deionized water while sodium phosphate dibasic dihydrate needed 17.8 gram in 1 liter of deionized water to make a stock solution. 45 ml stock solution of sodium phosphate monobasic monohydrate was mix with 55 ml stock solution of sodium phosphate dibasic dihyrate to archived pH 7 of buffer solution. A slightly different in buffer pH can be adjusted either using the hydrochloric acid or sodium hydroxide solution. The weight needed to dilute in 1 liter of deionized water was calculated using the equation 3.1.

$$Weight\ Needed\ (gram) = Desired\ Molarity\ \left(\frac{mole}{liter}\right) \times Molecular\ Weight\ \left(\frac{gram}{mole}\right) \times Desired\ Vol.\ (liter) \quad \textbf{(3.1)}$$

To prepare MOPS-Phosphate buffer, 52.315 gram of MOPS was diluted in 1 liter deionized water to make a MOPS stock solution while sodium phosphate monobasic monohydrate needed 34.5 gram in 1 liter of deionized water. Sodium phosphate monobasic monohydrate was choose to mix with the MOPS because of it contradict pH values. 0.25M MOPS stock solution pH reading was 4 meanwhile the sodium phosphate monobasic monohydrate pH was 8. MOPS-Phosphate buffer was prepared at pH 6.5.

3.3.4 Immobilized Enzyme Analysis

The immobilized enzyme was analyzed using several methods. Immobilized enzyme was characterized using Thermal Gravimetric Analysis (TGA), Fourier Transform Infrared Spectrophotometer (FTIR), Scanning Electron Microscope (SEM) and Wet Laser Diffraction.

Immobilized Enzyme Characterization

The TGA study was important to know the thermal stability of the materials The analysis was done using TA instrument TGA Q500 with sensitivity up to 0.1 µg and temperature range from ambient to 1000°C. Approximately, 5 mg of sample needed in TGA analysis. The material was heated in dry nitrogen gas from 30°C to 800°C at 10°C/min heating rate. The analysis was done in 78 minute.

FTIR analysis was performed using Thermo Scientific Nicolet Avatar 370 with infrared range from 400 to 4000 cm^{-1}. FTIR fundamental used infrared light beam to measure the spectrum. Different fucntional groups in sample has different spectrum since it absorb infrared beam at different wavelength depends on it internal energy. The detector record the vibration energy produced versus wavelength and translate it into % transmittance versus wavelength. Transmittance defined as fraction of infrared light at specific wavelength that passed thru the sample. The samples were prepared by mixing the spectroscopy grade potassium Bromide with sample at ratio 1:1 and hydraulically pressed to a 12 mm diameter disk. The FTIR were recorded at spectral resolution 4 cm^{-1}.

Scanning Electron of Microscope is an electron microscope that uses electron beam instead of light beam to scan the sample and resulted in morphology of sample. Primary electron was focused on the sample and secondary electron that passed thru the solid was collected to form an image of the sample. A modern SEM provided an image resolution between 1 nm and 10 nm. Prior to the SEM analysis, the sample was coated by carbon to make sure that electron emitted can be transfered effectively and also to prevent charge

built-up on sample. SEM analysis was perfomed using Carl Zeiss EVO 50. The EHT was set to 10 kV and samples image was recorded from several magnifications.

The main idea of particle size laser diffraction is that a particle will scattered the light that emitted by red light beam laser according to the particle angle and produced light scattering patterns that determined the particle size. Larger particle will scatter at small angle will the small particle scatter at wide angle. Wet Laser Diffraction was used because silica gel was not dissolve in water. Water was used as dispersant. The samples were analyzed on laser diffraction (Malvern Mastersizer MS2000, 5.6 version software) with wet analysis using the Hydro S dispersion unit (capacity 100-500 ml). The refraction index used for silica gel is 1.45, it was determine from the reference text. The MS2000 has a particle size distribution range of 0.02 – 2000µm. An absorption index of 0.1 was used since it gave the best fit to the data.

Enzyme Loading

Protein loading was study using Lowry's Method by measuring the amount of protein bound to activate silica gel. 0.01 g of lipase powder was diluted in 10 ml of 0.1 M Phosphate Buffer to get the concentration of 1000 µg/ml. 10 ml of lipase solution was pre-treated with 1 % of waste cooking oil before added with 0.5 g of activated silica gel. Protein concentration before and after added with activated silica gel was determined using Lowry's Method and UV-Visible spectrometer at 700 nm wavelength. The different determined the protein loaded into activated silica gel in immobilization process.

Protein Estimation by Lowry's Method

Protein loaded into silica gel was measured using Lowry;s method. Protein Estimation is a widely used method for enzyme and highly sensitive procedure that can detect proteins in the range of 0.1-1.0 mg/ml concentration. This method used Lowry reagent (Lowry Reagent 1) with addition of Folin Ciocalteu reagent (Lowry Reagent 2) that produced blue color solution. Lowry Reagent 1 contains copper sulphate, potassium

tartrate, sodium hydroxide, sodium carbonate and sodium larylsulphate. Absorbance of colored solution can be read at a suitable wavelength 500 nm and 800 nm. The protein concentration was determined from a calibration curve. 1 ml of sample solution was added with Lowry Reagent and leave for 20 minute at room temperature. After 20 minute, with rapid and immediate mixing 0.5 ml of FolinCiocalteu reagent was added. The solution was allowed to develop for 30 minute. The solution was slowly change to the blue color. Transfer the solution into the cuvet and the absorbance was measured at 700 nm using UV-visible spectrometer. Protein standard curve was constructed from the concentration of 400 ug/ml, 300 ug/ml, 200 ug/ml, 100 ug/ml and 50 ug/ml with the R^2=0.997. Bovine Serum Albumin was use as a standard protein. The concentration of samples was determined by comparing the absorbtivity of the sample with standard curve. Figure 3.5 shows the BSA standard curve constructed.

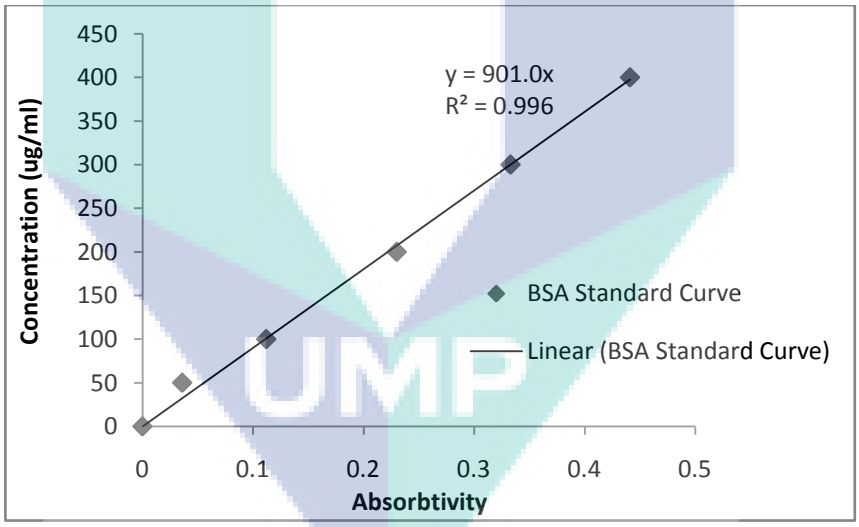


Figure 3.5: Bovine Serum Albumin (BSA) Standard Curve

Enzyme Activity Measurement

Lipase activity was measured with glycerol tributyrate as substrate by titration with NaOH 0.05 M using a pH-stat. This method employ from Wang et al. (2010). 3 ml of glycerol tributyrate was mixed with 47 ml of deionized water in a flask and the temperature

was controlled at 37°C. The solution pH was adjusted at 6.3 using NaOH solution. The 0.1 gram of immobilized lipase or free lipase enzyme was added and stirred with moderate stirring speed. In the same time the NaOH was titrated into the solution continuously to keep the pH constant for 30 minutes. The temperature was kept constant at 37°C. Lipase activity was defined as the µmol number of acetic acid produced per gram protein, based on the volume of NaOH solution consumed as shows in the equation 3.2. The commercial immobilized lipase from *Candida Rugosa* enzyme enzyme activity was 83.33 U/ g.

$$Lipase\ Activity = \frac{Vol.of\ NaOH\ consume\ (ml) \times Molarity\ of\ NaOH\ (M)}{Time\ of\ incubation\ (min) \times Amount\ of\ Lipase\ added\ (g)} \quad \textbf{(3.2)}$$

3.3.5 Microwave Assisted Transesterification Parameter Study

The microwave assisted enzymatic transesterification was study by one factor at one time method. MAET was performed using a Milestone Micro synth ATC-FO 300 microwave. This microwave has a temperature controlled. The transesterification process was carried out in a glass beaker using 50 g of WCO and 1 wt. % of commercial immobilized *Lipase Candida Rugosa* enzyme. The first parameter was studied was methanol to oil ratio beacuse methanol to oil ratio was a critical parameter that affect the methyl ester production as reported by perez et al. (2014) that studied the microwave enzymatic transesterification of babasu oil statistically. Methanol to oil ratio was varied from 3:1, 4:1, 5:1, 6:1 and 7:1. In methanol to oil ratio study, other parameter were set as constant which are 200 rpm of mixing, 40 °C reaction of temperature, 100 W of microwave power and 5 hours of reaction time. Using the optimum methanol to oil ratio value the study then proceed to reaction temperature study. Reaction temperature is second important parameter in biodiesel production (Perez et al., 2014) (Nogueira et al., 2010). But the reaction temperature has small range since it directly effect by enzyme. All type of enzyme either it immobilized or not, has a limit in temperature between 30 °C and 50 °C thus, the reaction temperature range was varied from 30 °C, 35 °C, 40 °C, 45 ° and 50 °C. Physical mixing of reaction solution was study at 0 rpm, 150 rpm and 200 rpm. Microwave power study range from 75 W, 100 W and 200 W. The reaction time was studied from 30 min, 1

h, 2 h, 3 h, 4 h and 5 h. This value was estimated from the literaure review. The feedstock volume and enzyme amount were constant parameters. The optimum parameters obtained then used to study the comparison of microwave assisted enzymatic transeterification of waste cooking oil and rubber seed oil using both commercial and purposed made immobilized *Lipase Candida Rugosa* enzyme.

Enzyme recyclability study was studied using optimum conditions of transesterification in this experiment. Enzyme recyclability will reduce the production cost since enzyme is more costly than chemical catalyst. After transesterification process, the immobilized enzyme was filtered using filter paper and washed with 0.1 M Phosphate buffer before dried at room temperature to prevent the enzyme deactivation because of alcohol and high temperature effect. The immobilized enzyme then been used in the next cycle until 8 cycles of transesterification.

Leaching is one of the important factors that affect the reusability of Immobilized enzyme Enzyme leached from support will reduced the enzyme activity. 0.1 g of Immobilized enzyme was added into 100 ml of 0.1 M Phosphate buffer before mixed at 200 rpm on hot plate stirrer. The protein content in solution mixture was determined using Lowry's Method at 30 minutes interval for 3 hours. Enzyme leaching percent was calculated from the amount the of leaching enzyme over loaded enzyme and multiple by 100.

3.3.6 Biodiesel Purification

After transesterification process and filtration using filter paper to recover the immobilized enzyme, biodiesel produced was washed with warm deionized water (60°C) to remove excess methanol and other impurities such as glycerol. Biodiesel was washed several times until the washing water color become clear and the pH reached 7. Rotary evaporator was used to totally remove the methanol and water. After that the biodiesel was mixed with magnesium silicate to dry the biodiesel oil from moisture. The mixture then been separated by centrifuge.

3.3.7 Methyl Ester Analysis

Methyl Ester Determination Using Gas Chromatography

The fatty acid methyl ester (FAME) composition of waste cooking oil was determined using gas chromatography flame ionization detector (GC-FID) according to ASTM D6584 by analyzed the oil produced using Agilent Technologies 6890 N Network GC system equipped with a flame ionization detector and HP-INNOWax capillary column (30 m x 0.25 mm x 0.25 µm). This GC method was adapted from The Essential Chromatography & Spectroscopy Catalog 2011-2012 Edition by Agilent Technologies (page 630-Free Fatty Acids). The oven temperature was set at 120°C for 1 min and 250°C for 5 min with 10°C/min heating rate. For the sample preparation,20 µl of oil produced was diluted in 2000 µl of HPLC grade hexane and filtered using 0.2 µm nylon filter and put into GC vials for GC analysis. 1 µl of diluted sample was injected into GC in split mode and it split ratio 40:1. Helium was used as the carrier gas with total flow 1.8 ml/min. The sample injected in GC was flow inside the column by carrier gas and ignited in the flame detector with the mixture of air and hydrogen. The burned sample that contain high carbon produced ions and electrons which conduct electricity in the flame detector. The flame detector detects mass of ions and electrons produced. The FAME composition was determined as a relative percentage of the total peak area. Methyl Palmitate, Methyl Oleate and Methyl Linoleate GC standard was used to build a calibration curve using concentration range 10,000 ppm to 500 ppm for quantitative measures. This 3 of standards was choosen prior to the top 3 highest fatty acid content in feedstock. Identification of peaks of all methyl ester produced was performed by comparing the retention times with the library standards under the same conditions. Figure 3.6 shows the Methyl Palmitate standard curve while figure 3.7 shows the Methyl Oleate standard curve and figure 3.8 shows the Mehtyl Linoleate standard curve constructed.

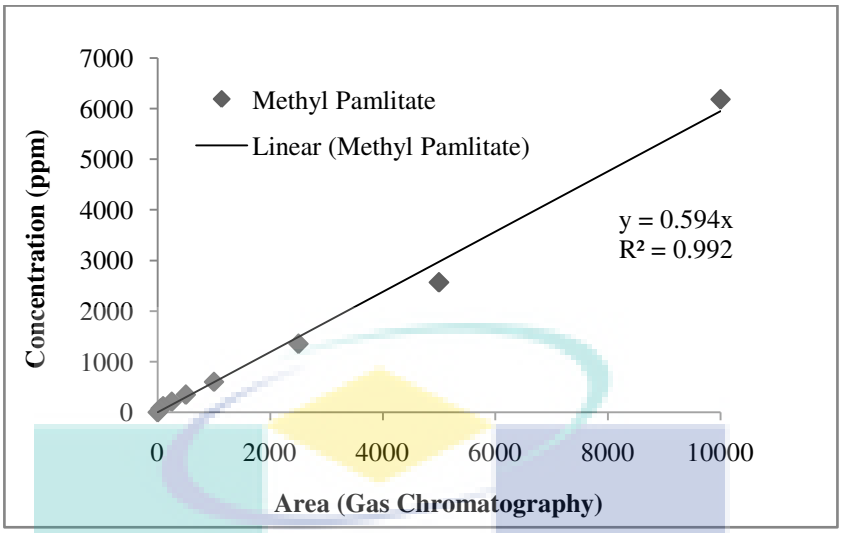


Figure 3.6: Methyl Palmitate Standard Curve

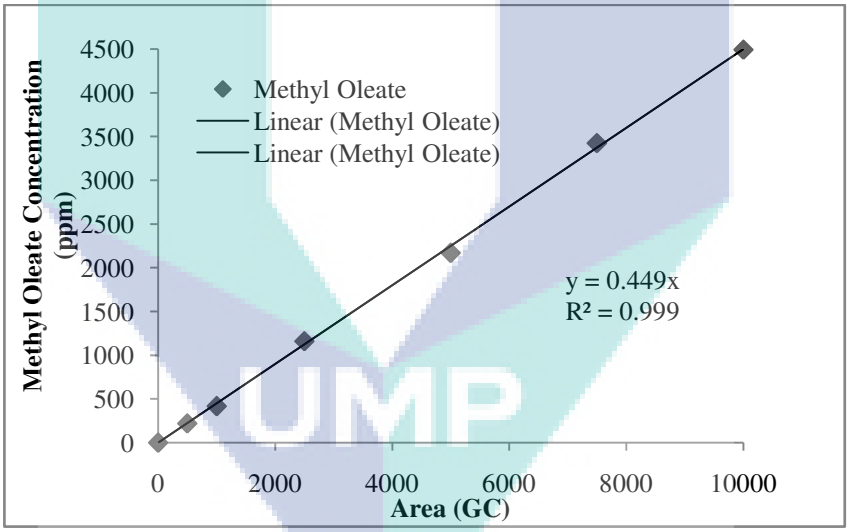


Figure 3.7: Methyl Oleate Standard Curve

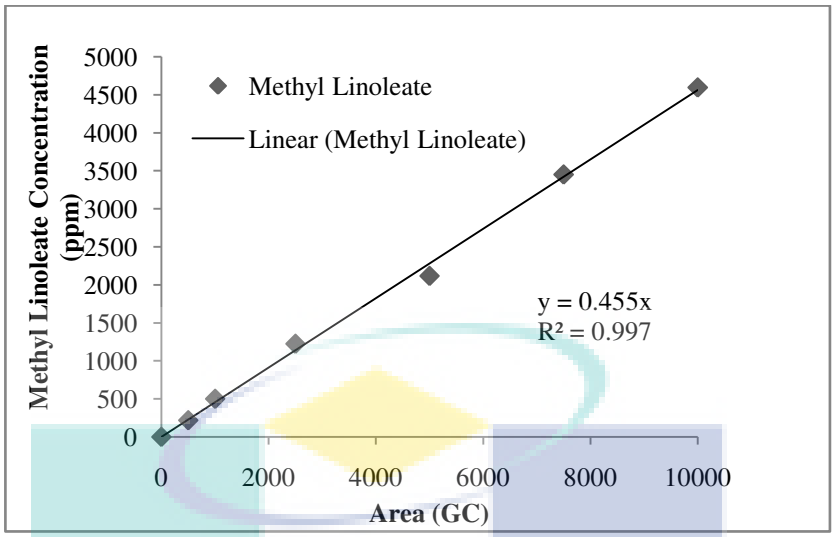


Figure 3.8: Methyl Linoleate Standard Curve

All the constructed graph has an intercept at (0, 0) and has high regression value. The equations (Y= 594X, 0.449X & 0.455X) in the graphs used to calculate the methyl ester concentration from the gas chromatography area.

Methyl Ester Characterization using ASTM Standard

The oil produced was being analyzed according to biodiesel ASTM standards (ASTM D6751). Selected ASTM 6751 standard methods were being use to find, acid value (ASTM D664), calorific value (ASTM D240), kinematic viscosity (ASTM D445), flash point (ASTM D93) and specific gravity (ASTM D287).

Acid value defined as amount of Potassium Hydroxide (KOH) needed to neutralize the acid in 1 g of oil. High acid number of oil increases the corrosiveness effect of oil. Automatic Titrator 785 DMP TitrinoMetrohm model was used according to the ASTM D664 standard to test the acid value in every samples of biodiesel. Ethanol was used as a solvent with ratio 5:1 to oil and Potassium Hydroxide was used as titrator. The weight of 1 ml volume of oil used to test the acid value and acid value number was calculatedbase on KOH consumed.

Kinematic viscosity is a resistance of fluid to flow because of the shear stress between fluid molecules and density of fluid. Lower kinematic oil decreased fuel consumption but the heat generated was increased. It is important to balance the kinematic viscosity needed in engine. Kinematic viscosity test was done in oil bath which the temperature controller. The Cole-Palmer temperature controller was set at 40°C as required by ASTM D445. The viscometer number 300 was chosen as the oil was viscous compared to water. The higher the viscometer number means the larger the diameter of the viscometer tube. 5 ml oil was put inside the tube and allows it to reach the temperature of 40°C inside the oil bath. After a few minute, the vacuum pump was attached at the end of the tube and allowed the pressure to suck the oil higher than the first mark on the tube. Detached the pump and allow the oil to flow back down tube. When it past the first mark on the tube, start counting the time and stop it at the second mark immediately. Time consumed when the oil past through the first mark to the second mark was used to calculate the kinematic viscosity by multiply the time consumed in second unit with the suitable viscometer constant that given by the suppler.

Flash point is the lowest temperature of oil that can vaporize and form an ignitable air mixture.Flash point analysis was done using Petrotest PM 4 model according to ASTM D93 standard. The oil was placed inside the close system heating camber with ignition source.The flash point determined by first ignition of vapor inside the chamber.

Calorific value of fuel determines the quantity of the heat produced when the fuel is completely burned. Parr 1341 Plain Oxygen Bomb Calorimeter was used to determine the heat of combustion of biodiesel. Approximately, 0.5 gram of sample was weighted and placed on the combustion plate that attached with 10 cm fuse wire and filled with oxygen. The ignition take placed in water bucket inside the bomb calorimeter.The temperature different in every one minute was recorded until it constant. The fuse wire balance from the combustion was measured in cm. The heat of combustion was calculated from the balanced of fuse wire.

Specific density of sample was a comparison between sample and water density. The specific gravities was measured using Thermo Fisher scientific brand hydrometer with density range from 0.8 to 0.9 the measurement done at room temperature.

3.4 STATISTICAL ANALYSIS

Statistical analysis was done using a single factor ANOVA in Microsoft Excel to statistically prove the significant of result gained from the experiment. The alpha level or signifant level of ANOVA was set at 0.05. This means that the confidence level is 95% or the probability to reject the null hypothesis when the hypothesis is true is 5%. From the ANOVA analysis, the significant of datas can be determined from the P value. If the P value is less than 0.05 ($P < 0.05$), the null hypothesis was rejected. It is also determined that the repeated data mean has significant difference. If the P value is more than 0.05 ($P > 0.05$), the null hypothesis was accepted. It is determined that the repeated data mean does not has a significant difference.

3.5 SUMMARY

This chapter explained the detailed procedures and methods used in this experiment. The experiment start with the rubber seed oil extraction then, the reactor screening, lipase immobilization, microwave assisted enzymatic transesterification, biodiesel prurification, methyl ester analysis and statistical analysis. The screening done with batch, ultrasonic and microwave reactor. The batch reactor used glass beaker that had been placed inside the water bath for temperature control with the installation of head stirrer for mixing and the ultrasonic reactor used glass beaker that had been placed inside the ultrasonic bath and installed with head stirrer for mixing purpose while microwave used special glass beaker for microwave purpose only and the top of the microwave was installed with head stirrer for mixing. Lipase Candida Rugosa was immobilized using cross-linking method by employ the Kim et al., (2006) method. The enzyme was immobilized into silica support using 3-aminopropyltriethoxysilane (APTES) and glutaraldehyde. The immobilized enzyme was analyzed analytically and physically using various equipments such Ultraviolet Visible Spectrometer (UV-Vis), Thermal Gravimetric Analyzer (TGA), Scanning Electron Microscope (SEM), Wet Laser Diffraction. Protein estimation employsthe popular Lowry's Method while enzyme activity measurement employ from Wang et al., (2010). The transesterification reaction parameter was studied by varies the parameters. The reaction time range was from 30 min to 300 min, reaction temperature range from 30°C to 50°C, methanol to oil ratio range was 3:1 to 7:1, mixing speed range from 0 rpm, 150 rpm and 200 rpm while microwave power range was 75W, 100W and 200W. The methyl ester produced then being analyzed using ASTM standard. The ASTM standard analyzed in this study was kinematic viscosity, specific gravity, calorific value, acid value, cetane number, flash point and methyl ester percentage using specific equipments. Gas chromatography was used to analyze the methyl ester produced. The ANOVA use as a tool to validate the results.

CHAPTER 4

RESULTS & DISCUSSIONS

4.1 OVERVIEW

Chapter 4 presented all the finding from the whole experiments. Figure 4.1 summarized all the results in flow chart. From figure 4.1, this chapter start by subchapter 4.2 by presenting the results from the comparative study of reactors screening and comparative study between free and immobilized *Lipase Candida Rugosa* enzyme in biodiesel production. This subchapter gave information on which reactors can produce highest methyl ester in equal conditions and also the information on how much methyl ester can be produce from free and immobilized enzyme for the equal reaction time in microwave reactor. Subchapter 4.3 presented the results of Cross linking enyzme immobilization. The enzyme loading and enzyme activity was measured to determined the immobilization effeciency. The Immobilized enzyme thermal stability, fuctional group, morphology and particle size distribution was also characterized using specific equipments. Subchapter 4.4 present the finding from the one factor at one time study of microwave assisted transesterification using waste cooking oil and immobilized enzyme. The parameter studied was methanol to oil ratio, reaction temperature, reaction time, mixing and microwave power. All the optimum parameters from subchapter 4.4 then being used in subchapter 4.5 to study the enzymes recyclability and leaching. Subchapter 4.6 present the comparison of methyl ester conversion from extracted rubber seed oil and waste cooking oil under optimum MAET reaction conditions and purposed made immobilized enzyme.

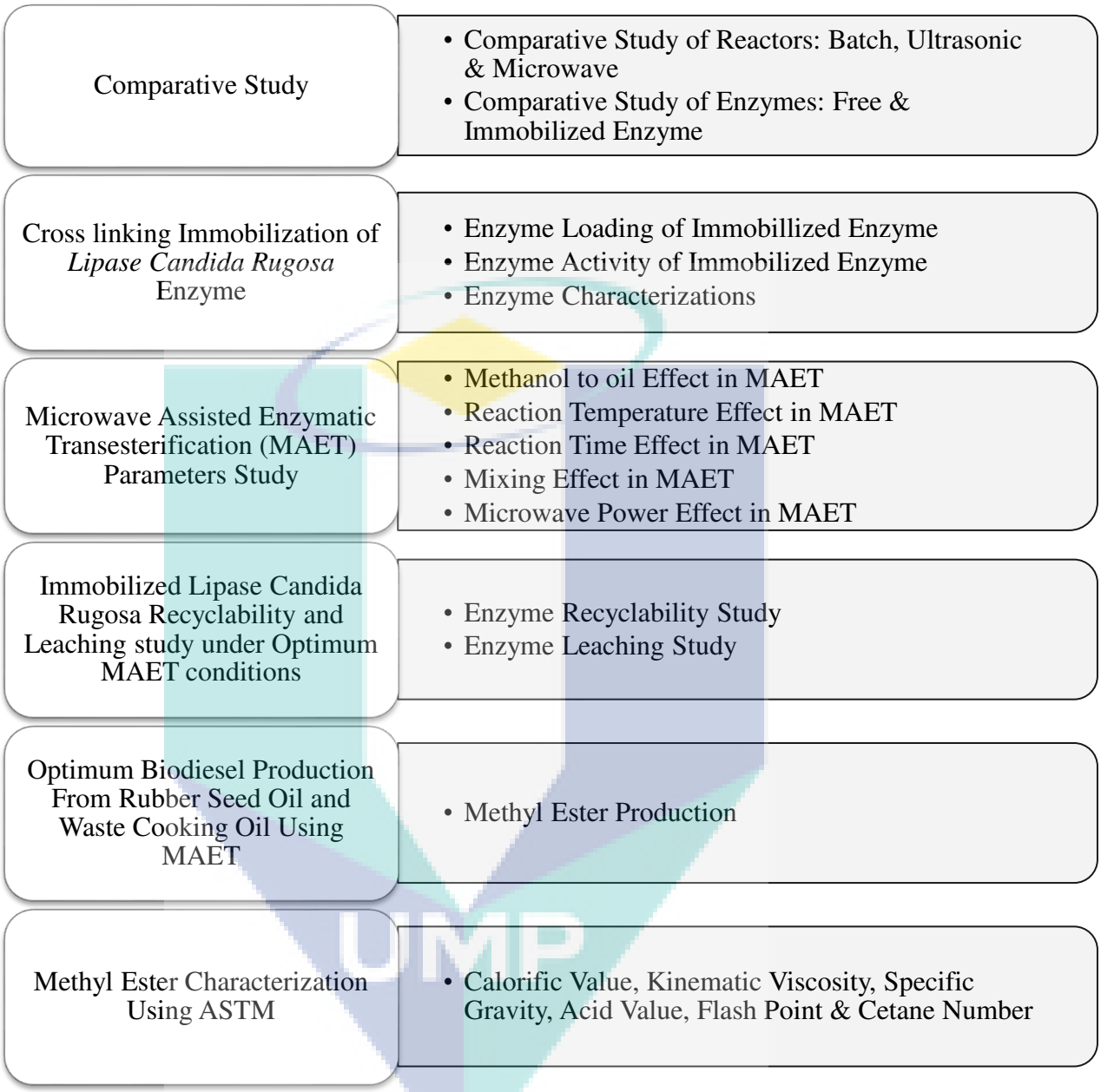


Figure 4.1: Whole Experiment Results Flow Chart

The purified biodiesel produced in this experiment then being analyzed using ASTM. The ASTM that used to analyzed the purified biodiesel were calorific value, kinematic viscosity, specific gravity, acid value, flash point and cetane number.

4.2. THE COMPARATIVE STUDY OF REACTORS & ENZYMES

4.2.1 The Comparative study of Microwave, Ultrasonic and Batch Reactors

A comparative study of microwave, ultrasonic and batch was done in this experiment to verify which reactor produces highest yield of methyl ester. According to the literature review, microwave produced the highest yield in biodiesel production however there were none of the research reported on comparison between these two reactor using immobilized enzyme in biodiesel production. Biodiesel was produced from 50 g of waste cooking oil using 1 wt. % commercial immobilized *Lipase Candida Rugosa* enzyme and methanol as solvent.

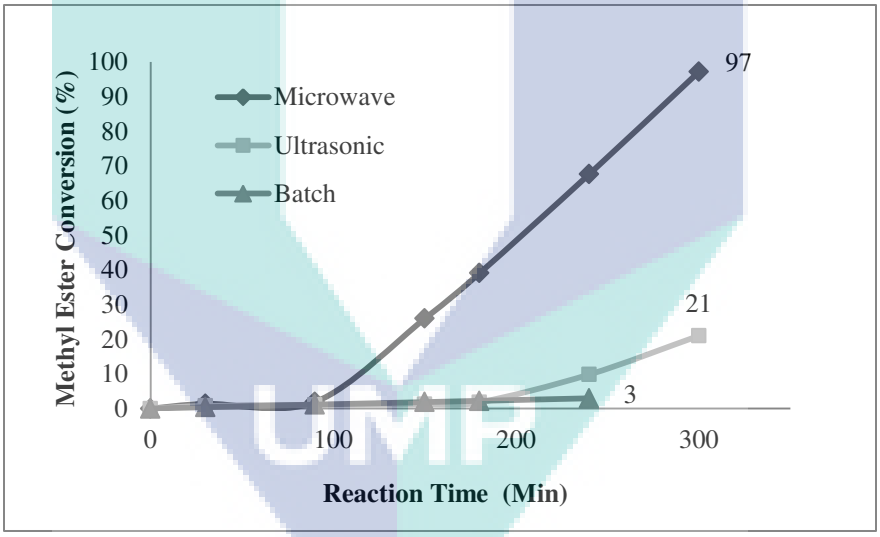


Figure 4.2: Methyl Ester conversion percentage using Ultrasonic, Microwave and batch reactors

Biodiesel was produced under the same amount of irradiation power (100 W) in ultrasonic and microwave reactor for 5 hours and mixed with methanol at 5:1 to oil ratio using the stirrer at 150 rpm and 40°C using commercial Immobilized lipase enzyme. Figure 4.2 shows that the microwave has the highest methyl ester percentage which is 97 % over ultrasonic and batch reactors. Microwave produces 76 % higher methyl ester after 5 hours than ultrasonic while batch reactor show slow reaction at 5 hours which is only 3 % of

methyl ester conversion. The conventional batch reactor enzymatic transesterification often needs about 70 hours to achieve conversion above 70 % (Lin & Liu, 1995). Figure 4.3 shows the batch transesterification of waste cooking oil using the same commercial immobilized enzyme and reaction conditions.

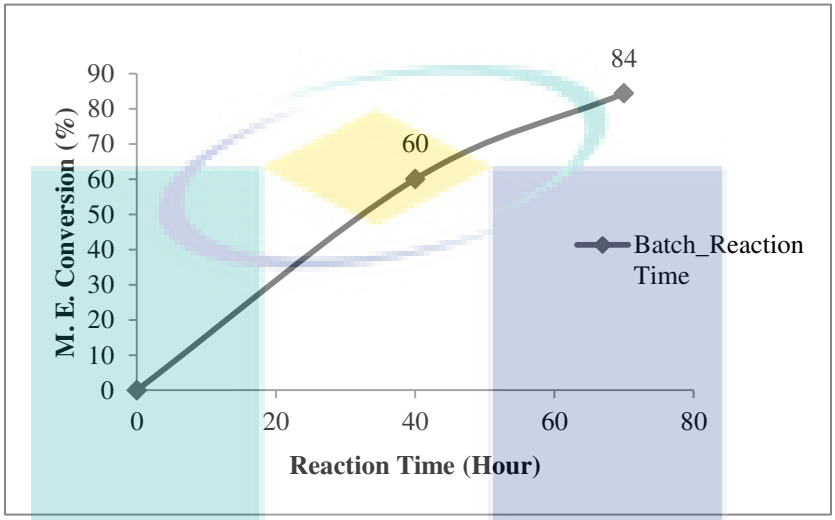


Figure 4.3: Batch Transesterification of Waste Cooking Oil using Commercial Immobilized *Lipase Candida Rugosa* Enyzme

Result from figure 4.3 was similar as stated by Lin & Liu (1995). After 70 hours of reaction 84 % of fatty acid in waste cooking oil was converted to methyl ester. The different in results was due to the different mechanism of the reactors. Microwave irradiation activates the smallest degree of variance of polar molecules and ions with the continuously changing magnetic field (Azcan & Danisman, 2007). The changing electrical field, which interacts with the molecular dipoles and charged ion, causes these molecules or ions to have a rapid rotation and heat is generated due to molecular friction (Azcan & Danisman, 2007). Ultrasonic waves are energy application of sound waves which is vibrated more than 20,000 per second. Vigorous mixing increases the contact area between oil and alcohol phases with producing smaller droplets than conventional stirring (Stavarache, Vinatoru, & Maeda, 2006). The comparison of methyl ester concentration and reaction time showed that microwave irradiation is more efficient than ultrasonic irradiation

because the energy interacts with the sample on a molecular level, very efficient and rapid heating can be obtained in microwave heating.

4.2.2 Comparative Study of Immobilized Lipase Enzyme and Free Lipase Enzyme in Microwave Reactor.

Microwave assisted transesterification using enzyme was not yet being widely study. At the time, only Perez et al. (2014) and Noguiere et al. (2010) had studied the microwave assisted transesterification statistically from Babasu oil and Macauba oil respectively but none of them had done the comparative study between immobilized lipase and free lipase enzyme under microwave irradiation. The comparative study of immobilized lipase enzyme and free lipase enzyme was studied using the same reaction conditions in subchapter 4.2.1. The 50 g of waste cooking was mixed with 5:1 methanol to oil ratio, 1 wt. % of immobilized enzyme or free enzyme, 150 rpm mixing, 100 W microwave power, 40°C reaction temperature for 5 hours reaction time.

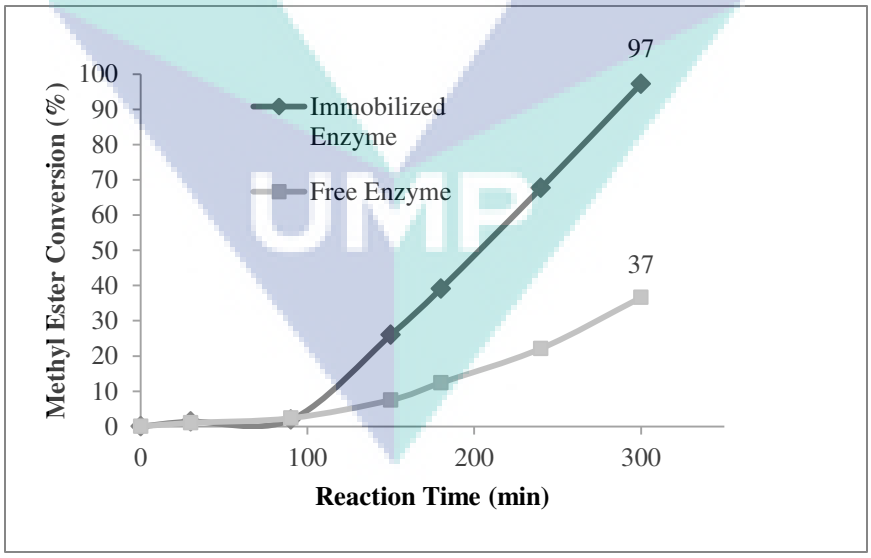


Figure 4.4: Comparison between Immobilized lipase enzyme and free lipase enzyme transesterification in microwave reactors

Figure 4.4 shows the increasing line for both immobilized and soluble enzyme but at different rate. Immobilized lipase enzyme shows higher methyl ester conversion which is 60 % higher than free enzyme methyl ester conversion. This shows that lipase immobilized enzyme was more efficient and suitable to use even under microwave irradiation than free lipase enzyme. Even though free lipase enzyme supposedly to have higher enzyme activity than Immobilized lipase enzyme but under the microwave irradiation it might inhibit free lipase enzyme activity as protein is known sensitive toward reaction conditions such temperature and alcohol. Immobilization of enzyme also makes it more stable and resistant to the reaction conditions such temperature, solvent and physical mixing or irradiation but in the same time increase the enzyme activity (Wang et al., 2008).

4.3 *LIPASE CANDIDA RUGOSA* ENZYME IMMOBILIZATION INTO SILICA GEL BY CROSS-LINKING

4.3.1 Enzyme Loading Study in Cross-Linking Immobilization under Varies Microwave Power.

The immobilization efficiency was measured from the amount or percentage of enzyme loaded into the silica support. 1 g of lipase candida rugosa enzyme was dissolved in 100 ml M MOPS-Sodium Phosphate Buffer solution before being immobilized with activated silica gel. The protein content in enyme solution was measured before and after the immobilization process using lowry method. The protein content was analyzed for every 30 min until 120 min.

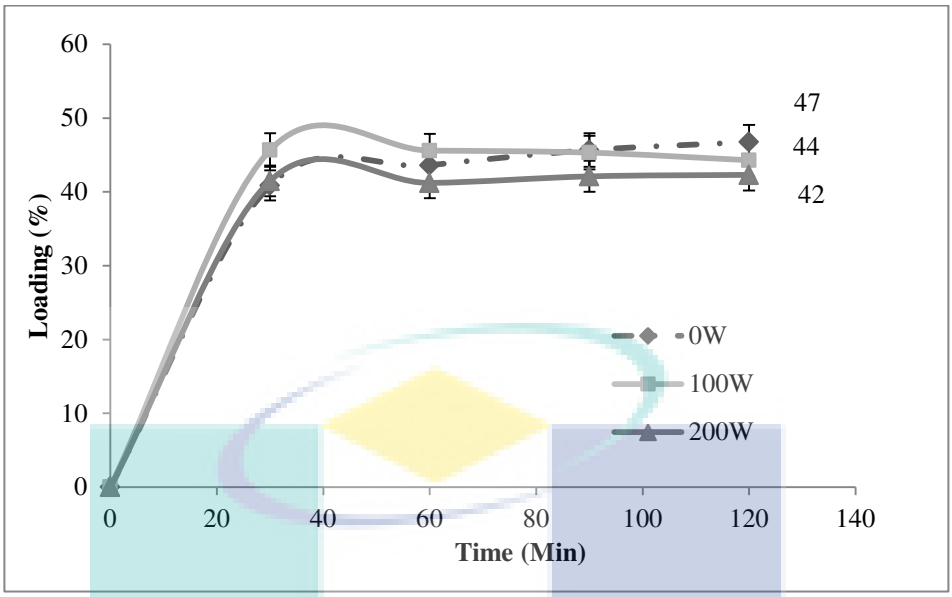


Figure 4.5: Enzyme loading in immobilization graph

Figure 4.5 shows that the enzyme loading percentage decreased in immobilization when the microwave irradiation power increase. Non-assisted microwave irradiation immobilization shows the higher loading percentage than microwave assisted immobilization at 47 % of loading. Non-assisted microwave irradiation microwave or 0 W irradiation was slightly higher than 100 W and 200 W irradiation. Enzyme loading shows linear pattern after 30 min of immobilization probably because the enzyme active site was already full. Enzyme loading was effected by several factors such as support pore size. It had been reported that the ratio of support pore size to enzyme size is a crucial factor in immobilization. Small pore size support may absorb enzyme but the resulting in a low loading while the larger pore size will lead to the enzyme leaching in immobilization process which count for the decrease of enzyme loading (Wang et al., 2010). Serra et al. (2008) was reported that the ideal size for support pore size was 3 to 5 times larger than enzyme size and the total pore volume did not play any important role in immobilization. In this study, the ezyme loading was 47 % which is lower than reported by Wang et al. (2010) that reached 57 % and the report had stated that the best support pore size for *lipase candida rugosa* enzyme immobilization was 15.6 nm. From the MSDS of the product was reported that the support pore size was 3-5 nm which is too small compared with the 15.6

nm. Small support pore size will lead to lower enzyme loading because enzyme molecules access to the pore was limited, the pore is the active site of the binding between support and enzyme.

Other factor that affecting the enzyme loading was the excess aldehyde group that may attacked the immobilized enzyme active site (Kim et al., 2006). Excess aldehyde from the silanization process may attack the immobilized enzyme active site if the excess aldehyde was not properly removed. Kim et al. (2006) was adding a low molecular weight materials such glycine to remove the excess aldehyde. In this study there is no low molecular weight material was added after immobilization process. Wang et al. (2011) was reported that the microwave assisted immobilization of Adolase enzyme was success at 88 % of enzyme loading for 3 min of 30 W microwave irradiations exposure. The Adolase thermal and storage stability was also increase significantly. Immobilization of Papain enzyme into mesocellular siliceous foams increased at 20 % times higher than conventional method at 300 W of microwave irradiation (Shen et al., 2008). Microwave irradiations accelerated the immobilization process (Galinada et al., 2007). Immobilization temperature plays an important role in immobilization process. At 100 W the reaction temperature reached 30°C after 3 min and the reaction temperature for 200 W after 3 min was approximately 45°C. The suitable immobilization temperature was 20°C (Kim et al., 2006) and at 30°C the enzyme activity was reduced 38 % from the higher peak of enzyme activity at 20°C at 120 min. However, under microwave irradiation the enzyme was loaded slighlyt faster than non-irradiated immobilization.

Table 4.1: Enzyme Loading Percentage Table for 0 W & 100 W

	Loading (%)				
Time (Min)	**30**	**60**	**90**	**120**	***P-value***
100 W	45.66±0.87	45.59±0	45.32±0.53	44.29±0.73	0.501905
0 W	40.87±3.77	43.61±2.03	45.66±1.26	46.75±1.16	0.425467

Table 4.1 shows the the enzyme loading percentage in immobilization under 0 W and 100 W microwave irradiation. Under 100 W, 45.7 % of enyzme was loaded into silica

support after 30 min while without microwave irradiation the immobilization take 90 min to reached 45.7 % of enzyme loading. The analysis was repeated three times. P >0.05, shows that the result has insignificant different within the group means with confident level 95 %. However, the P < 0.05 (6.556E-06) if the 100 W & 0 W was compared between each other. This means that the reaction time in immobilization process play insignificant role and the microwave irradiation power gave significant results. Microwave irradiation was also believe to increased the immobilization reaction between lipase enzyme and activated silica gel.

4.3.2 Enzyme Activity Study of Immobilized Enzyme under Microwave irradiation Immobilization

The enzyme activity was measured using the specific method adapted from kim et al. (2010). Lipase activity was measured with glycerol tributyrate as substrate by titration with NaOH 0.05 M using a pH-stat. Lipase activity was defined as the µmol number of acetic acid produced per gram protein, based on the volume of NaOH solution consumed. Glycerol tributyrate hydrolyzed acetic acid using the immobilized *Lipase Candida Rugosa* enzyme as catalyst. One mole acetic acid neutralized one mole NaOH thus, the amount of NaOH solution consumed was equal to the amount of acetic acid produced.

Figure 4.6 shows that the enzyme activity was reduced when the microwave irradiation power increase. The enzyme activity was reduced 46 % when exposed to 100 W of microwave irradiation and reduced 70 % when exposed to 200 W of microwave irradiation during immobilization process. Since the enzyme loading was reducedthe enzyme activity was also reduced according to the several factors discussed previously.

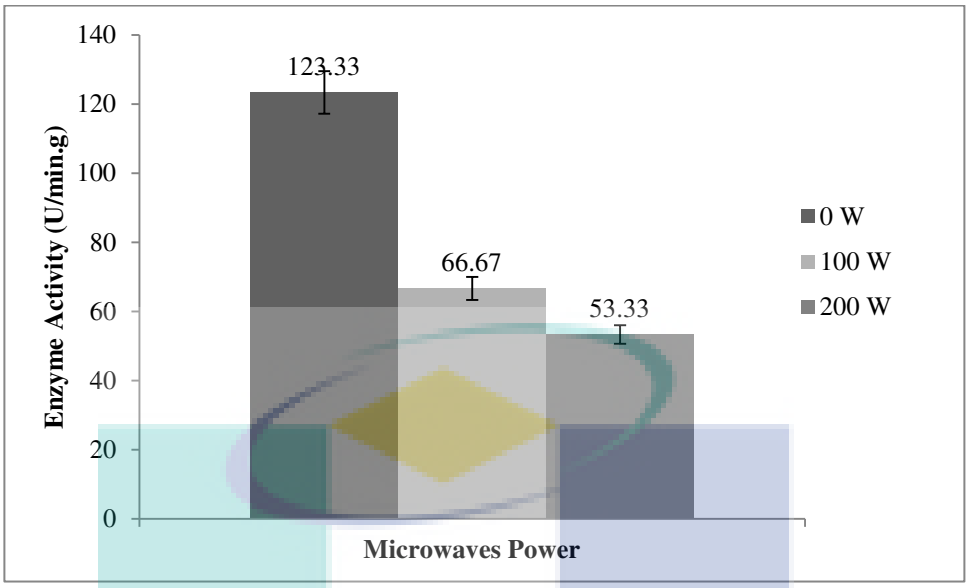


Figure 4.6: Enzyme Activity of Microwave Assisted Immobilized enzyme

High microwave irradiation power in immobilization did not help to increase the enzyme activity because under the high microwave irradiation exposure the enzyme's protein was denatured at the time free enzyme was going to be immobilized with the support. But, the microwave irradiation was indeed helping to increase the enzyme activity at the low microwave irradiation power (Wang et al., 2011). Free lipase enzyme was known very sensitive to the high temperature. The free enzyme lipase activity decrease after 40°C of reactions temperature (Yiğitoğlu & Temoçin, 2010). It is very important to control the immobilization temperature since immobilization process need 20°C reaction temperature condition for the optimum immobilization.

Table 4.2: Enzyme Activity After Immobilization with Varies Microwave Power Table

Power (W)	0	100	200	P	F
Enzyme Activity (U/g)	121.67 ± 2.4	65.00 ± 2.4	55.00 ± 2.4	0.000182135	465.33

Table 4.2 shows the enzyme activity after being immobilized under varies microwave irradiation power. The analysis was repeated three times. $P < 0.05$, shows that the result has significant different between the group means with confident level 95 %. The standard deviation of mean between groups were also small.

4.3.3 Characterizations of Immobilized *Lipase Candida Rugosa* Enzyme

The cross linking Immobilized lipase candida rugosa was characterize using TGA, FTIR, SEM and particle size distributor to study the immobilized enzyme physical characteristics. Only the non-microwave irradiated cross linking immobilized enzyme was study in this subchapter since it has highest enzyme activity.

Thermal Stability by Thermal Gravimetric Analysis (TGA)

Figure 4.7 present the thermo diagram of immobilized silica gel using silica gel as support. The microwave assisted immobilized enzyme total loss of mass was 24 %. It was observed that the first degradation stage occurred in the temperature range from 30°C to 110°C with the weight loss of 16 % due to the loss of water content and enzyme degradation. Second stage occurred from 110°C to 550°C with 8 % of weight loss. This was probably because of the degradation of the modified silica support.

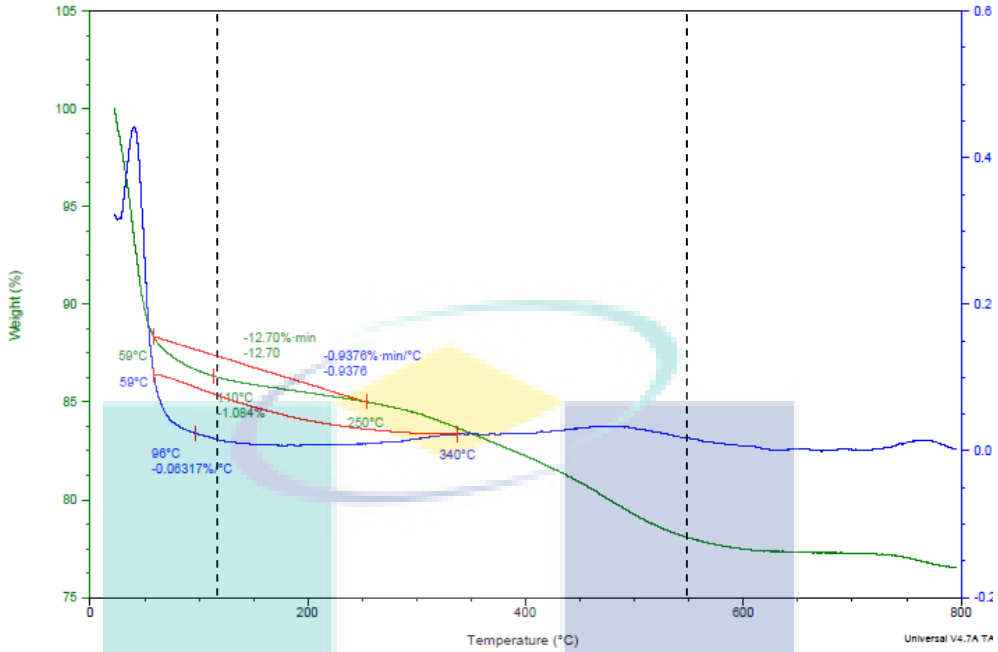


Figure 4.7: TGA Graph for Cross-linking Immobilized enzyme

Modification on silica support by cross linking had decreased it thermal stability due to the change of it functional group and structure (Poppe et al., 2013). Pure silica support had higher degradation temperature than modified silica. Zhou et al. (1997) reported that silica weight loss was only 2 % below the 1000°C temperature. These significant change was a proved of modification on silica support and enzyme immobilization had occurred.

Functional Group analysis by Fourier Transform Infrared Spectrophotometer (FTIR)

Figure 4.8 shows the FTIR characterization of cross linking Immobilized enzyme under 100 W of microwave irradiation (after) and unmodified silica support (before).

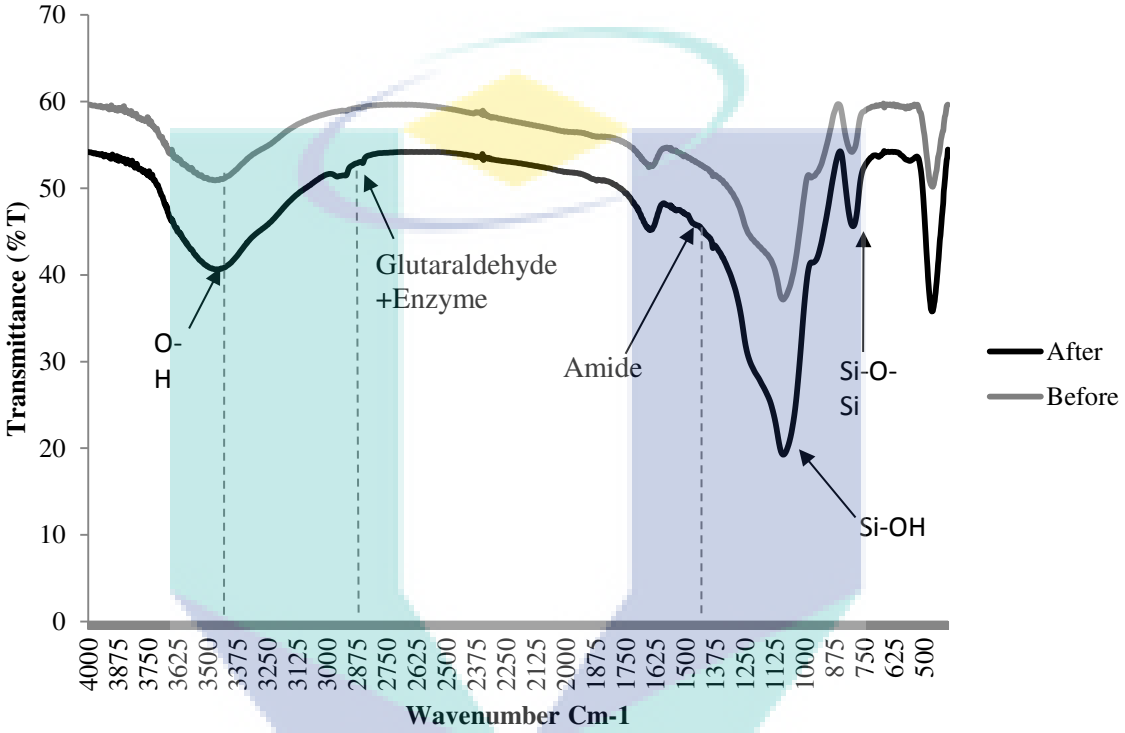


Figure 4.8: FTIR Spectra for Cross-linking Immobilized Enzyme

Band 810^{cm-1} shows the vibration of Si-O-Si group. The increase of Si-O-Si group indicated the occurring of silanization reaction by aminopropyltriethoxysilane (APTES). Lipase has two characteristic bands at 1470^{cm-1} and 700^{cm-1} (primary and secondary amino groups) that shows the existing of lipase (NH) in silica support. FTIR band 480^{cm-1} and 1056^{cm-1} shows the higher different between transmittance percentage. The bands are associated with the bending vibrations of OH and Si-O-Si groups. Both bands show the increasing transmittance that indicated the silica gel was graft with APTES and lipase enzyme. The C-H stretching vibration at band 2900^{cm-1} was contributed from the APTES, glutaraldehyde and enzyme. While band 3455^{cm-1} shows the present of hydroxyl groups.

Morphology by Scanning Electron Microscope (SEM)

The immobilized silica and untreated silica gel morphology was studied by Scanning Electron Microscope (SEM). Figure 4.9 & Figure 4.10 shows the morphology of silica gel before immobilization while Figure 11 & figure 4.12 shows the morphology of silica gel after immobilization process.

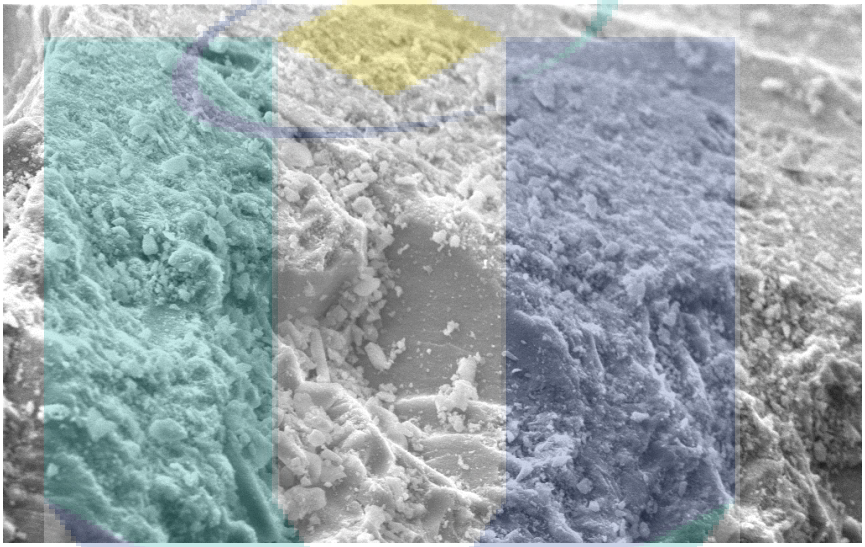


Figure 4.9: SEM Image before Immobilization (2.5 K x)

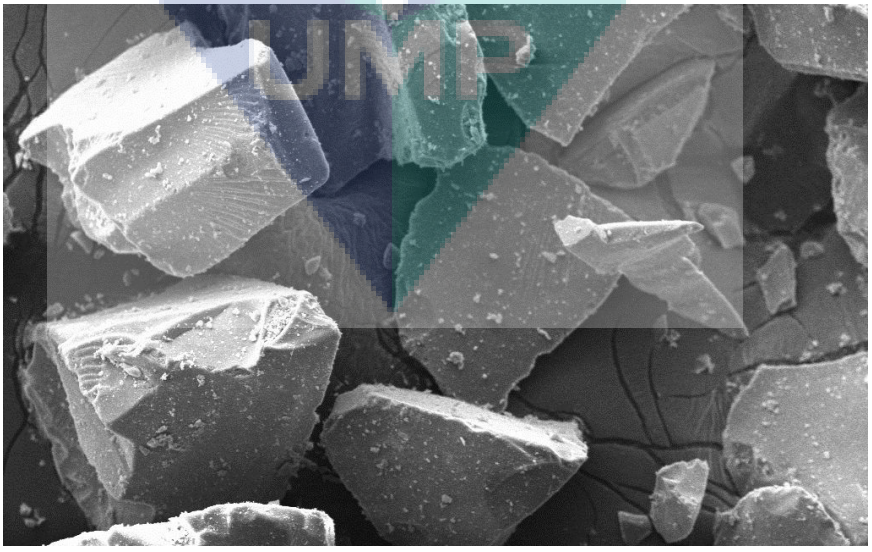


Figure 4.10: SEM image before immobilization (250 x)

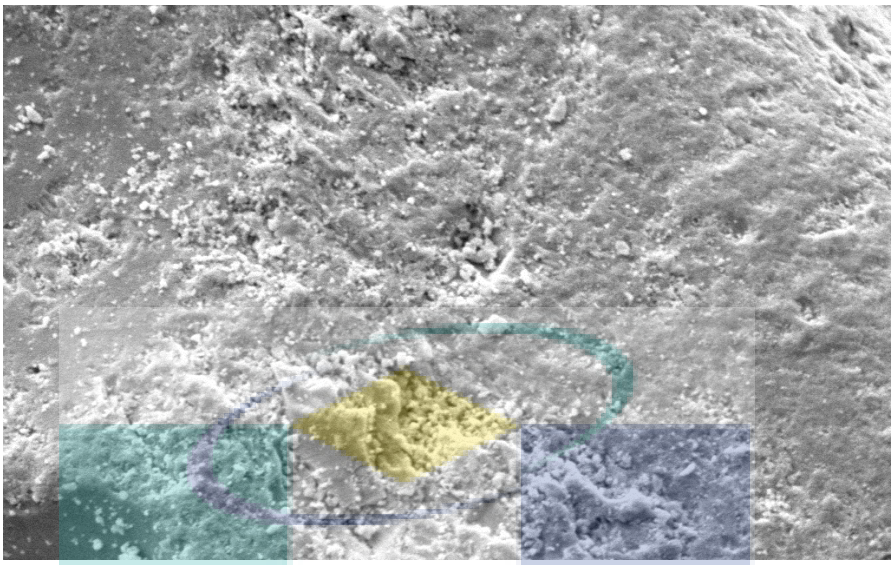


Figure 4.11: SEM image after immobilization (2.5 K x)

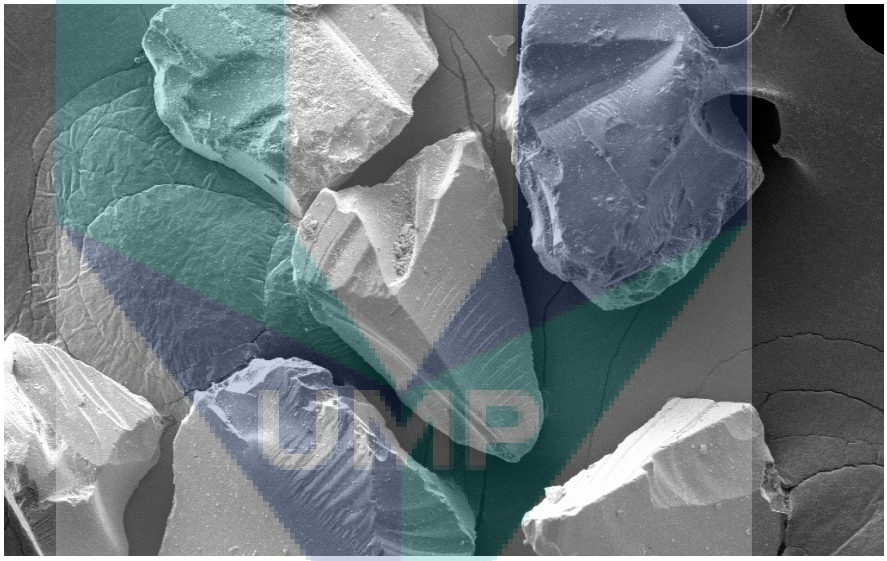


Figure 4.12: SEM image after immobilization (250 x)

Figure 4.9 & Figure 4.10 shows the morphology of silica gel before immobilization while Figure 11 & figure 4.12 shows the morphology after immobilization process. After immobilization process the silica surface had become more porous and clean than before immobilization. Chemical modification on surface changed the morphology of silica gel.

Particle Size Distribution analysis by Wet Laser Diffraction

The size of silica gel before and after immobilization that obtained from wet laser diffraction analysis was complied with SEM result. Figure 4.13 shows the binomial graph from the analysis that indicated the changed of molecules size distribution before and after immobilization. Before immobilization there was a lot of molecules size at approximately between 50 µm to 250 µm according to the volume percent shows. After the immobilization the size range was wider from 50 µm to 300 µm with the volume percent had reduced. Silica gel size of molecules before and after immobilization was majority approximately 110 µm according to the highest peak of binomial which is slightly different from SEM since the efficient of equipmentsmay cause the different result. This slightly changed of molecules size was because of additional attachment of molecules at silica gel surface after immobilization compared to untreated silica gel. The binomial wide range was a resulted from wide range mesh size of original silica gel purchased which is from 60-200 mesh size. This result was consistent with SEM result.

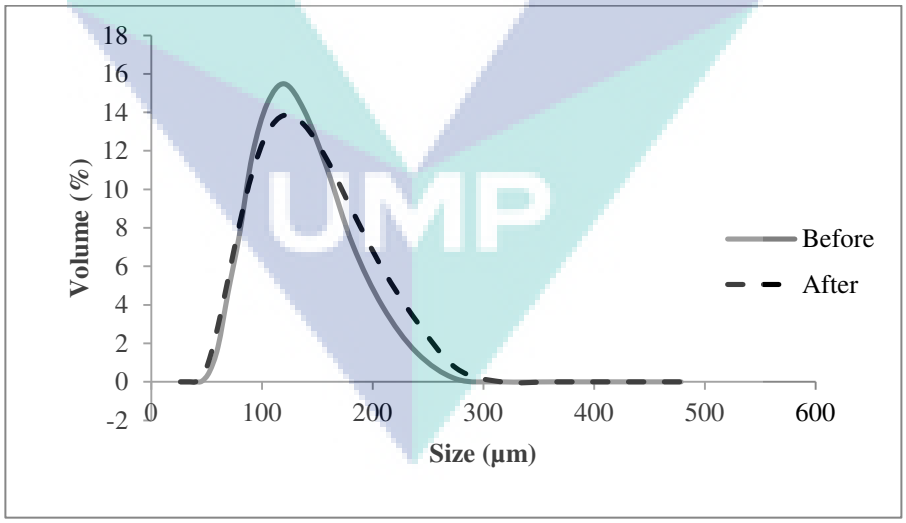


Figure 4.13: Binomial graph of silica gel after and before immobilization by wet laser diffraction analysis

4.4: MICROWAVE ASSISTED ENYMATIC TRANSESTERIFICATION (MAET) FROM WASTE COOKING OIL (WCO) USING COMMERCIAL IMMOBILIZED *LIPASE CANDIDA RUGOSA* ENZYME PARAMETERS STUDY

The microwave assisted enzymatic transesterification (MAET) was performed using a Milestone Micro synth ATC-FO 300 microwave that had the temperature, time and microwave power controller. Waste cooking oil (WCO) was used as a feedstock in this parameters study because of the abundant of source.

4.4.1 Methanol to Oil Ratio Effect in MAET

Methanol to oil ratio played an important role in biodiesel synthesis. Thus, a different methanol to oil ratio was employed to MAET to study their effect to methyl ester conversion.

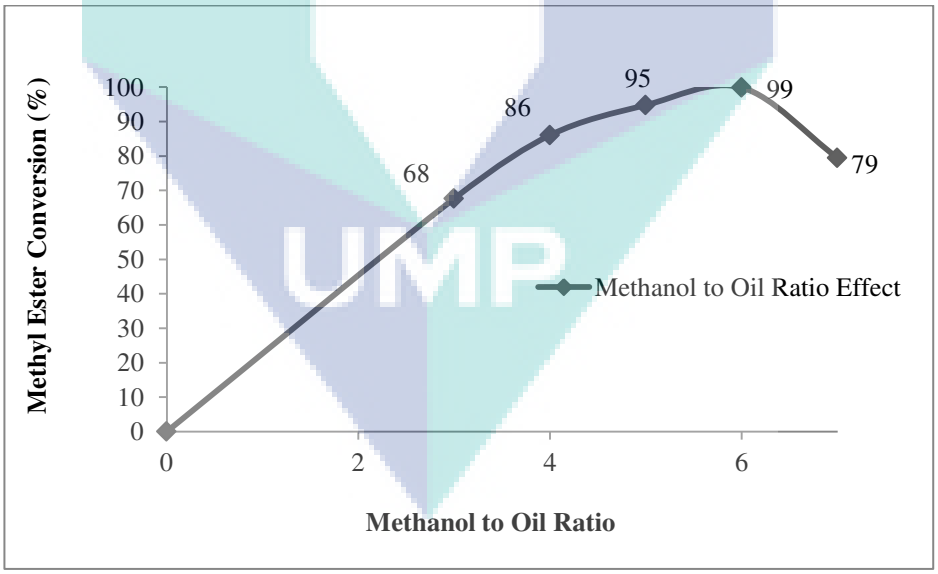


Figure 4.14: Methanol to Oil Ratio Effect in MAET

The 1 wt. % of immobilized *lipase candida rugosa* was added to 50 g of WCO and varies methanol to oil ratio under constant reaction temperature at 40 °C with microwave

power set at 100 W, and stirred at 150 rpm. Theoretically 1 mole triglyceride required 3 mole of alcohol to produce 3 mole of methyl ester and 1 mole of glycerol as the side product. However, this stoichiometry is not always possible since the reaction produced different result from different source and conditions. Figure 4.14 shows that the methyl ester production increased with the methanol to oil molar ratio increase until 6:1 molar ratio. Further increased of the molar ratio decreased the methyl ester production because the excess methanol increase the solubility of the by-product and initiate the reversible reaction which reduced the reaction rate. The optimum methanol to oil molar ratio in this study is 6:1. Yu et al. (2010) produced similar optimum methanol to oil ratio in ultrasonic assisted transesterification of soybean oil using Immobilized Novozym 435.

4.4.2 Reaction Temperature Effect in MAET

Generally, the rate of reaction increase as the reaction temperature increases because the elevated temperature helps the substrate molecules gained energy to pass over the energy barrier and enhance the reaction rate.

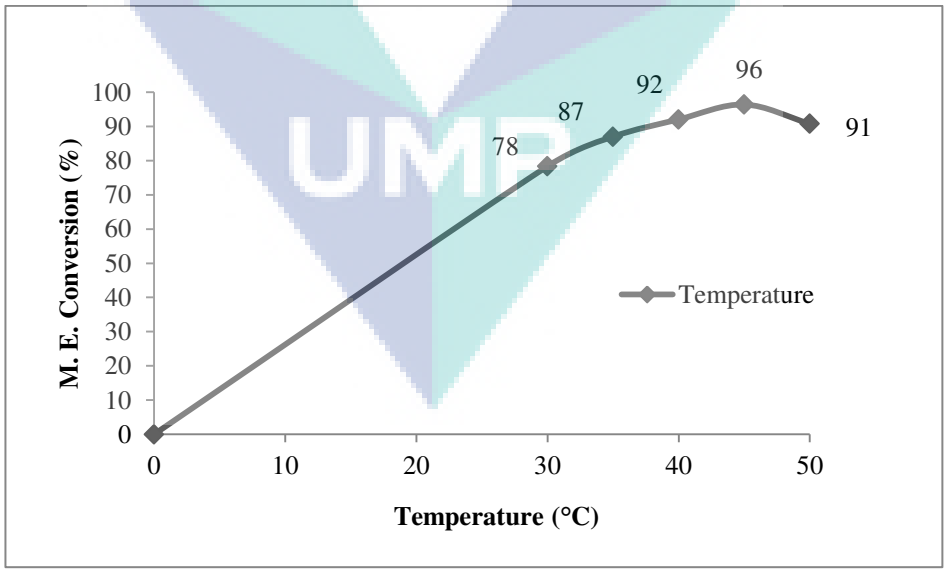


Figure 4.15: Reaction Temperature Effect in MAET

The energy also helps the enzyme-substrate formations that increase the enzyme activity Yu et al. (2010). The 1 wt. % of immobilized *lipase candida rugosa* was added to 6:1 methanol to WCO ratio, temperature ranged from 30 to 50 °C, microwave power was set at 100 W and stirred at 150 rpm. Figure 4.15 shows the increment in methyl ester production from 30°C to 40°C. Further increase of temperature decreases the methyl ester production because the enzyme was a temperature-depended and can be easily deactivated or degraded at a higher temperature. Lower temperature below 30 °C is also associated to enzyme deactivation and hence not examined in this work. Optimum temperature for MAET in this work is 45°C. However due to the fast heating from the microwave irradiation, reaction temperature 40°C was choose instead of 45°C. At 50°C methyl ester convension show the deceased pattern. This result is similar with (Yiğitoğlu & Temoçin, 2010) that reported 40°C as optimum reaction temperature. The optimum temperature of immobilized lipase was identical with optimum temperature of free lipase which is 40°C and the enzyme activity reduced after optimum temperature (Monier et al., 2010). The microwave irradiation did not affect the immobilized enzyme activity as long as the temperature of reaction was controlled effectively. Nogueira et al. (2010) also reported that the effect of temperature is minor under microwave irradiation if controlled.

4.4.3 Reaction Time Effect in MAET

Influence of reaction time to MAET was studied by extending the reaction time up to 5 hours 30 min. Reaction time was an important factor in biodiesel production. Enough time given for the transesterification reaction makes sure that the reaction receives enough energy to brake the moleculer bonding. MAET was set to run with methanol to WCO ratio of 6:1, temperature of 40 °C, microwave power at 100 W, agitated at 150 rpm and 1 wt. % of immobilized *lipase candida rugosa*.

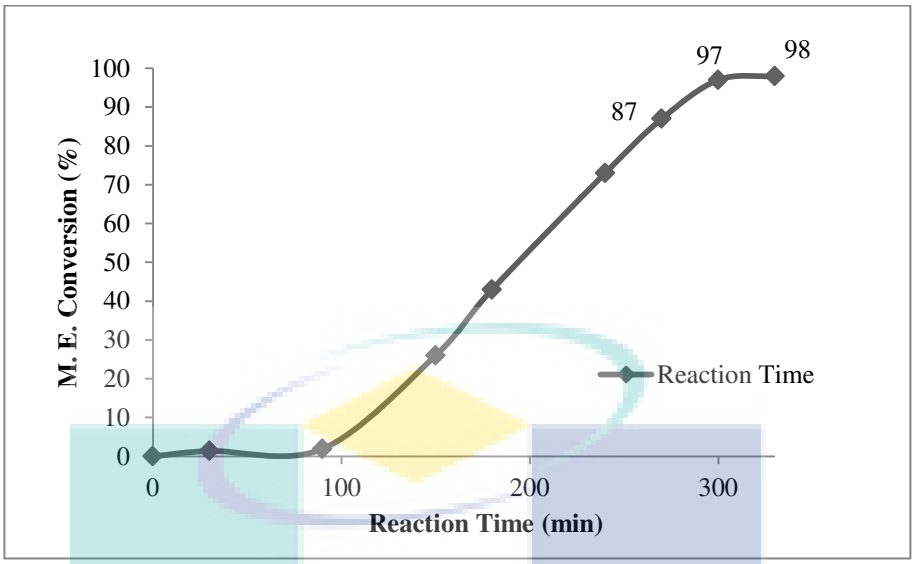


Figure 4.16: Reaction Time Effect in MAET

As shown in Figure 4.16 the methyl ester production increases as the reaction time increases and its keep increase after 5 h (300 min). The highest methyl ester content of 98 % was achieved after 5 h 30 min. The methyl ester conversion reached equilibrium after 5 h. After 5 h the methyl ester conversion increased only 1 %. This reaction is faster than reported by Da Ros et al. (2014) which produced 100 % yield after 10 hours of reaction using *Burkholderia cepacia lipase* immobilized on SiO2-PVA as catalyst in microwave system. The different type of enzyme used and reaction conditions might affect the result difference. Da Ros et al. (2014) also reported that the microwave assisted in biodiesel production using Immobilized lipase was 79 % faster than conventional process that need 48 hours to archived 100 % yield.

4.4.4 Reaction Mixing Effect in MAET

Mixing is one of the important parameters in biodiesel production because reaction cannot take place without proper contact between enzyme and substrate. For instance, Yu et al. (2010) reported that without physical mixing even under the ultrasonic assisted transesterification that base on cavitation the reaction may not initiate a significant enzymatic transesterification.

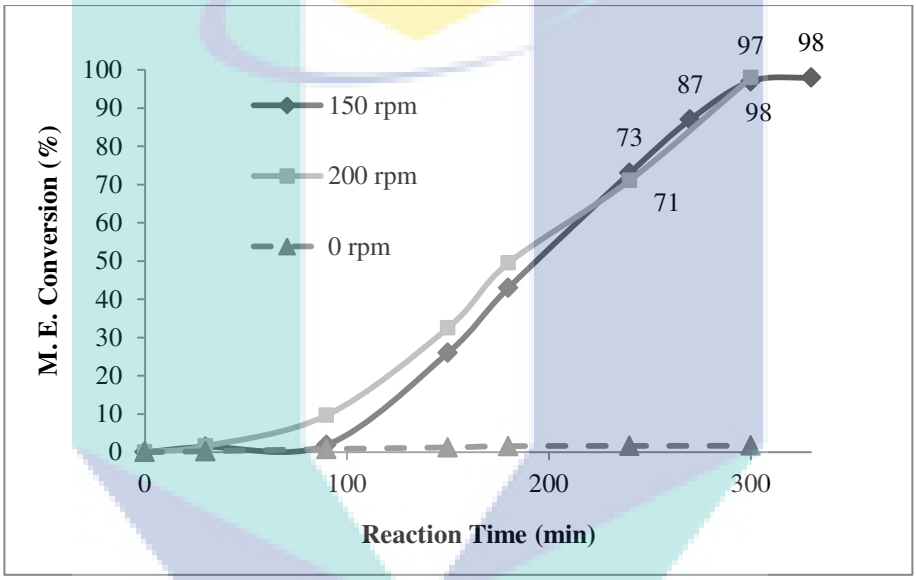


Figure 4.17: Reaction Mixing Effect in MAET

Different agitation levels from 0 rpm to 200 rpm were set for MAET with methanol to WCO ratio of 6:1, temperature of 40 °C, time of 5 hours, microwave power set at 100 W and 1 wt. % of immobilized *lipase candida rugosa*.Results obtained from this work as presented in Figure 4.17 shows that at 150 rpm and 200 rpm of mixing 97 % and 98 % of methyl ester was produced respectively while without any mixing only 2 % methyl ester was produced after 5 hours. Figure 4.17 also suggest a similar finding, i.e. physical mixing is needed for MAET. This is due to the fact that microwave irradiation can only initiate a molecular level movement but not in the bulk region mixing. Furthermore, agitation reduces the size of boundary layer hence promoting higher reaction rate. There is an

observable increment of methyl ester production with increasing agitation from 100 rpm to 200 rpm as the thickness of boundary layer decreases with increasing agitation. The result suggests that, without agitation, the substrate (WCO) transport from the bulk region to the immobilized enzyme is largely prohibited by the thick boundary layer on the enzyme surface and hence limiting the reaction.

4.4.5 Microwave Power Effect in MAET

Microwave power was one of the important parameters to study in this work since microwave irradiation can enhance the chemical reaction by deliver the energy directly to the molecules (Motasemi & Ani, 2012).

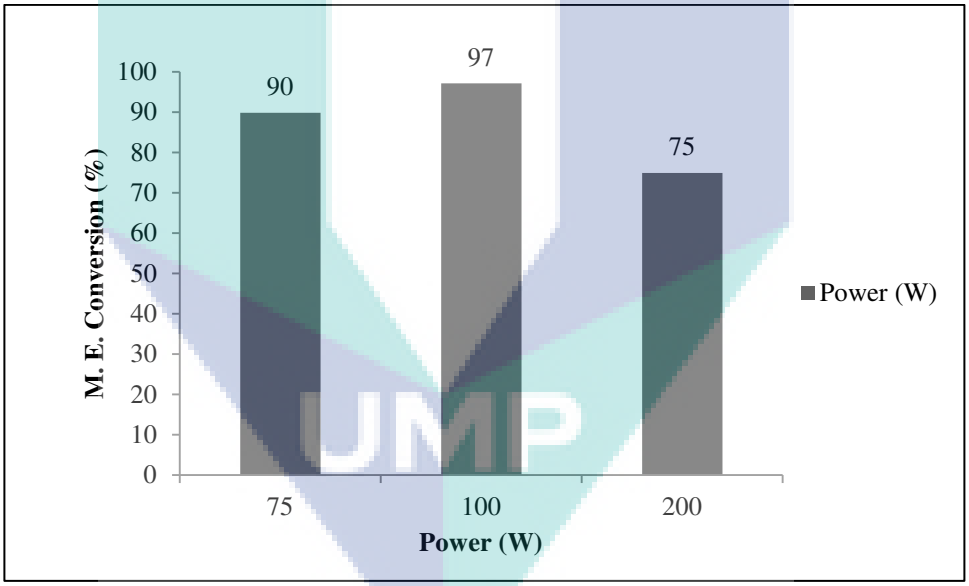


Figure 4.18: Microwave Power Effect in MAET

Using the same reaction conditions which are 50 g of waste cooking oil, 1 wt. % of commercial immobilized lipase enzyme, 150 rpm, 40°C of reaction temperature and 6:1 methanol to oil ratio for 5 hours under varies microwave irradiations power 75 W, 100 W & 200 W. Figure 4.18 shows that the highest methyl ester conversion was at 100 W microwave irradiation which is 97 %. At 75 W, methyl ester conversion was 90 % and

increase 7 % to 97 % when the microwave irradiation was increase to 100 W and greatly decreases 22 % at 200 W of microwave irradiation. This graph clearly shows that higher microwave irradiation or more than 100 W the methyl ester conversion was reduced. Heat generated during reaction that cause by microwave irradiation was the main factor that effect the methyl ester production. Using some simple experiment it shows that at 200 W the reaction temperature was easily increase up to 65°C within 5 min. There was a need to properly control the reaction temperature during the microwave irradiation transesterification. High reaction temperature or more than 40°C will denature the protein enzyme thus, reduced it activity. From this study 100 W was the optimum microwave irradiation in methyl ester production using the immobilized lipase enzyme.

4.5 IMMOBILIZED *LIPASE CANDIDA RUGOSA* ENZYME RECYCLABILITY & LEACHING STUDY UNDER OPTIMUM MAET CONDITIONS

4.5.1 Enzyme Recyclability Study under Optimum MAET Conditions.

Enzyme recyclability play an important role in biodiesel production. Higher recyclability of enzyme reduced the production cost since enzyme is expensive. The recyclability study use the optimum parameters of MAET obtained in this study. The MAET process was repeated until eight times. Figure 4.19 shows the recyclability of both commercial Immobilized *Lipase Candida Rugosa* and Covalent Cross linking Immobilized *Lipase Candida Rugosa* that been immobilized in the previous study. Commercial enzyme had reduced it enzyme activity for about 40 % after had been used at first cycle while this work covalent cross linking immobilized enzyme was reduced it enzyme activity at 11 %. Both enzyme shows stable decreasing pattern for each cycle until 8 cycles. At 8 cycles commercial immobilized enzyme reduced 66 % of it activity while this work immobilized enzyme reduced 21 % of it activity. It proved that the covalent cross linking immobilization technique increase the stability of immobilized enzyme.

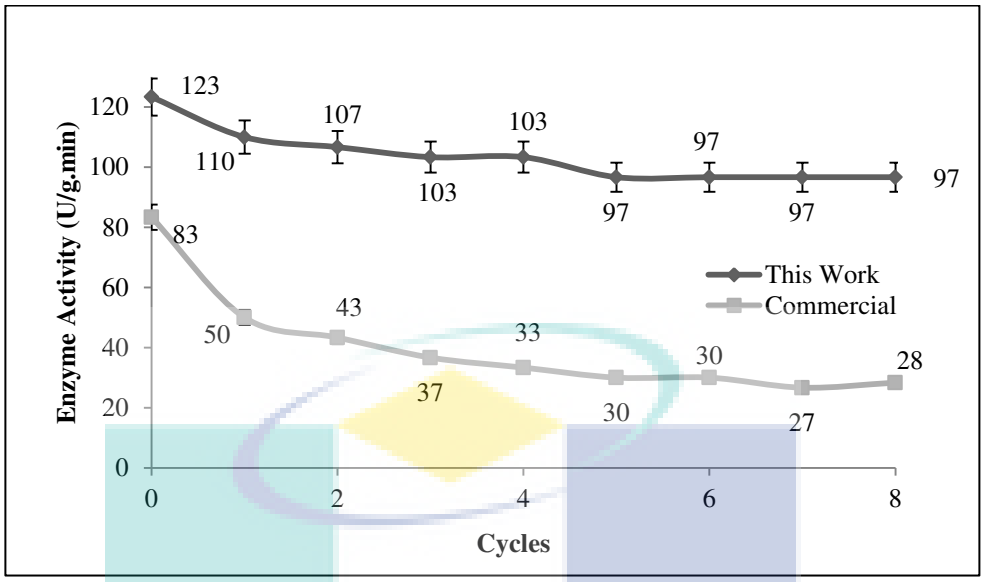


Figure 4.19: Immobilized Enzyme Recyclability Study in Optimum Conditions of MAET

Wang et al. (2011) reported that the covalent cross linking immobilization help to improved enzyme thermal and storage stability. Immobilized enzyme activity reduced about 5 % after 7 cycles due to the immobilization technique that hold and protect the enzyme in the support (Varma & Madras, 2010). Cao et al. (2007) reported that Novozyme 435 (Immobilized *lipase candida antartica*) enzyme activity reduced slightly which is 8 % after 5 cycles under microwave irradiation. One of the factors that reduced the enzyme activity was due to the glycerols that block the immobilized enzyme active site and enzyme poisoning by methanol (Lam et al., 2010). The temperature was less effect to the immobilized enzyme in this study since the microwave power used was 100 W and with that irradiation power it increased the temperature of the reaction solution to about 40°C after 5 min of continuous irradiation and was controlled automatically. The reaction solution was exposed to irradiation power at the interval of 30 min and it was enough for the temperature of solution to cool down with the aid of the mixing. This was proven in the figure 4.18 that shows the different patterns of both enzyme activities that reduced at first cycle. Enzyme activity of for both immobilized enzyme keep decreasing was probably because of the amount of glycerol produced in biodiesel production that keep blocking the active site of the immobilized enzyme. The glycerol amount that attached at the

immobilized enzymes increase ineachcyclesand thus reduced the enzyme activity ineachcycles. The analysis was repeat at least three times and analyzed using ANOVA. The means between this work and commercial immobilized enzymes group shows significant different ($P > 0.05$)

4.5.2 Enzyme Leaching Study under Optimum MAET Conditions.

Microwave irradiation created the molecules friction due to its electric charge that rotated the dipolar ion. This molecular level friction and physical bulk mixing may cause enzyme leaching that reduced the enzyme activity. To make sure this, leaching study was first done using the commercial immobilized lipase enzyme under 100 W microwave irradiation and without microwave irradiation with 150 rpm of mixing in 100 ml of 0.1 M Phosphate buffer and 0.2 g of immobilized enzyme (0.05 wt. %). The enzyme leached into the buffer was measured using Lowry method for every 30 min. Figure 4.20 shows the result of microwave irradiation effect

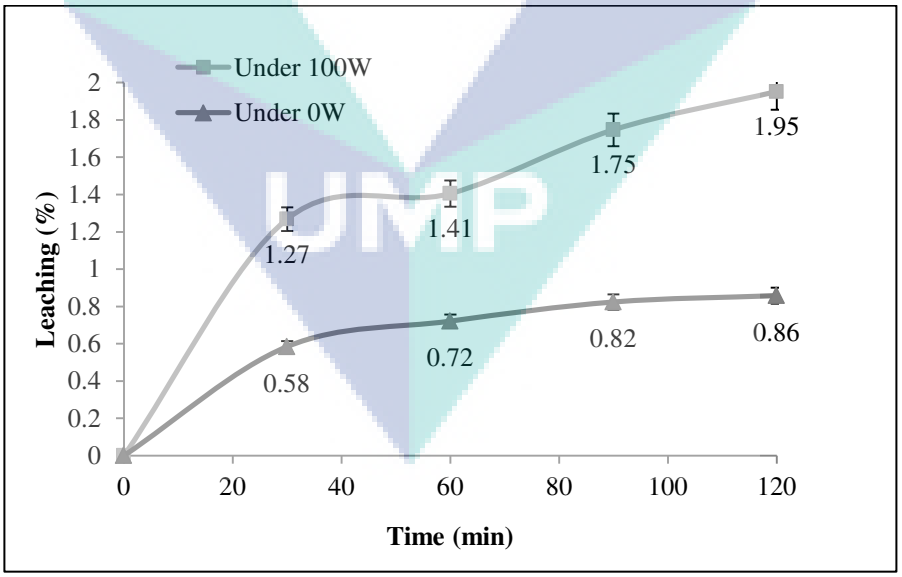


Figure 4.20: Leaching Study of Commercial Immobilized Enzyme with and without Microwave Irradiation

The enzyme keep leaching for both conditions which are with or without microwave irradiation but, the scale of leaching was totally different. Under 100 W of microwave irradiation, the leaching was higher 1 % than without microwave irradiation after 120 min. It shows higher stability due to the cross linking technique used. The results proved that, the microwave irradiation did affect the immobilized enzyme-support bonding that make the enzyme leached from the support thus reduced the enzyme activity. It is consisted with Nogueira et al. (2010) that stated, under the microwave irradiation the reaction conversion increase significantly but decrease in Enzyme Activity. With the fact that under 100 W microwave irradiation, the enzyme leaching was higher than the conventional method the cross-linking immobilized *Lipase Candida Rugosa* enzyme leaching was compared with commercial immobilized *Lipase Candida Rugosa* enzyme. Figure 4.21 shows the comparison between purposed made cross-linking immobilized enzyme and commercial immobilized enzyme leaching in optimum conditions of MAET (100 W).

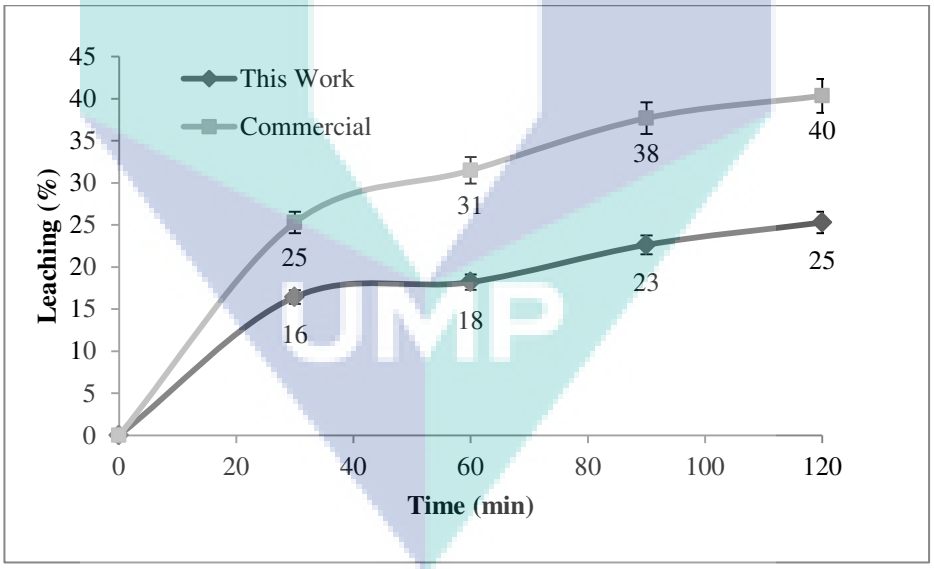


Figure 4.21: Immobilized Enzyme Leaching Study in Optimum Conditions of MAET at 100 W Microwave Irradiation

From figure 4.21, the leaching of commercial immobilized enzyme was higher than this work immobilized enzyme which is about 9 % after 30 min. After 120 min, this work immobilized leached 25 % of enzyme from the silica support while commercial

immobilized enzyme shows higher leaching which is 40 % of leaching. This shows that the cross-linking immobilization technique did improved the stability of enzyme attachenment inside silica gel pore. The oscillating microwave field tends to oscillate polar ends of molecules or ions continuously (Marra et al., 2010) that increase the collision and friction between molecules.Since the enzyme attached to the silica support active site was not too strong due to the small silica pore size, the friction from microwave irradiation probably break the enzyme-support bond. Even though, microwave irradiationis good in generate energy and speed up the reaction rate but it will also leach out the enzyme from it support faster because of the immobilization conditions such pore size matter. The analysis was repeat at least three times and analyzed using ANOVA. The means between this work and commercial immobilized enzymes group leaching shows significant result ($P > 0.05$).

4.6 OPTIMUM BIODIESEL PRODUCTION FROM RUBBER SEEDS OIL & WASTE COOKING OIL

4.6.1 Biodiesel Production From Rubber Seed Oil & Waste Cooking oil using Purposed Made Immobilized *Lipase Candida Rugosa* Enzyme under Optimum conditions of MAET.

This study used the optimum reaction conditions of MAET for both rubber seed oil and waste cooking oil. The 50 g of extracted rubber seed oil and purified waste cooking oil was mix with methanol at 6:1 methanol to oil ratio at 40°C reaction temperature, 1 wt. % of this work immobilized enzyme with 150 rpm of mixing and 100 W of microwave power. The reaction was run for 5 hours. From the figure 4.22 it shows that the rubber seeds oil was indeed a competitive oil substitute. After 3 hours of reaction 71 % of biodiesel was produced from rubber seed oil, which is 6 % higher than biodiesel produced from waste cooking oil. After 5 h (300 min) of reaction both oils reached equilibrium transesterification. Both reaction are faster 30 min than using the commercial immobilized lipase enzyme under optimum MAET conditions in subchapter 4.4 study, this happened due to the higher enzyme activity of purposed made cross-linking immobilized enzyme.

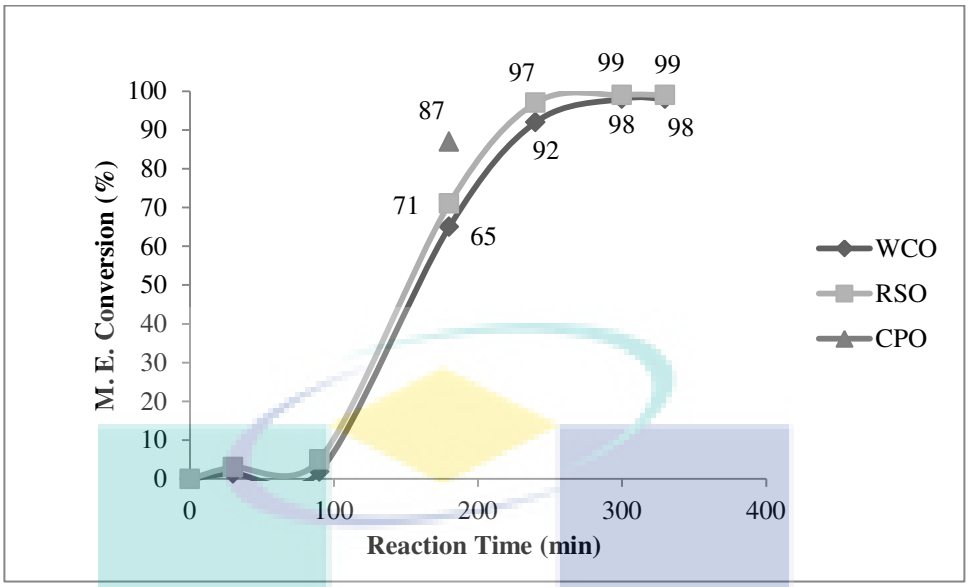


Figure 4.22: Comparison of Methyl Ester Conversion (%) from Rubber Seed Oil & Waste Cooking Oil under Optimum MAET Conditions.

Rubber seed kernel contained 40 %-50 % of oil (Ramadhas et al., 2005) while palm kernel contained 30-40 wt. % of oil (Suzana& Khan, 2010) however the amount of oil content in both kernels are varies depending on researchers experiment conditions. The impurities contains in this palm oil waste cooking oil may contributed to the lower methyl ester conversion compared with crude rubber seed oil. From the study, purified palm oil produced 87 % of methyl ester after 3 hours reaction using the same reaction conditions in this study which is optimum MAET conditions as marked in figure 4.22. Crude rubber seed oil is a competitive feedstock in biodiesel production.

4.7 ASTM ANALYSIS

The properties of FAME produced from this study were compared with Ramadhas et al. (2010), who studied rubber seed oiltransesterification using a two-step method and Maksudur et al. (2011) who studied the rubber seed oil transesterification using acid catalyst.

Table 4.3: Biodiesel from Rubber Seed Oil, & Waste Cooking Oil ASTM

Properties	*Unit*	*ASTM 6751*	*Limits*	*This work BPO*	*This work BWCO*	*This work BRSO*	*Ramadhas et al., (2005) Acid cat. BRSO*	*Maksudur et al., (2011) BRSO*
Calorific Value	*MJ/kg*			*37.85*	*41.0326*	*-*	*36.5*	*-*
Kinematic Viscosity@40°C	*Mm^2/s*	*D445*	*1.9-6.0*	*4.6*	*5.72*	*7.2*	*5.81*	*4.5*
Specific Gravity	*-*	*D287*	*0.82-0.9*	*0.88*	*0.88*	*0.9*	*0.874*	*0.85*
Acid Value	*Mg KOH/g*	*D664*	*<0.5*	*0.23*	*0.35*	*0.42*	*0.118*	*0.12*
Flash Point	*°C*	*D 93*	*100-170*	*128*	*165*	*195*	*130*	*120*
Cetane Number	*-*	*D613*	*>47*	*73*	*68*	*-*	*-*	*-*
Methyl Ester (3 hours)	*%*	*D6584*	*>96.5*	*87*	*65*	*71*	*>97*	*98*

As shown in Table 4.3, all the properties are within the biodiesel specification described by ASTM D6751. Biodiesel from purified palm oil show finest quality than biodiesel from rubber seed oil and waste cooking oi. Biodiesel from rubber seed oil content higher acid value 0.42 mg.KOH/g compared with the biodiesel from waste cooking oil and crude palm which are 0.35 mg.KOH/g oil 0.23 mg.KOH/mg respectively. This result also shows similar pattern for kinematic viscosity and flash point. Kinematic viscosity is one of the important factors in biodiesel since it will be use in engine. Viscosity is a resistance of the fluid to flow. Higher viscosity will lead to poor engine atomization since the fuel cannot be spray effectively in the atomization chamber. Biodiesel from rubber seed oil flash point

and kinematic viscosity values exceeded the ASTM range limitseven though; the methyl ester conversion form rubber seed oil was higher than waste cooking oil. Higher viscosity reduces the cetane number thus increase the flash point (Muthukumar et al., 2008). The rubber seed oil viscosity is affecting by many factors such as molecular weight and unsaturated fatty acids (Igwe, 2004). Waste cooking oil collected was palm cooking oil but the values are varies from crude palm oil since the waste cooking oil content oligomeric compounds after being used. This compounds increase molecular mass thus reduce the volatility of the biodiesel (Sharma et al., 2012).

4.8 SUMMARY

High methyl ester conversion was produced using Immobilized *lipase candida rugosa* enzyme in microwave, ultrasonic and batch reactor. Among the three reactors, microwave produced the highest methyl ester while immobilized lipase enzyme produced higher and faster methyl ester than free lipase enzyme. Using the microwave reactor and immobilized lipase enzyme the optimum parameters in transesterification process found to be at 6:1 methanol to oil ratio, 1 wt. % of immobilized lipase enzyme, 5 hours reaction time, 40°C of reaction temperature, 150 rpm of mixing and 100 W of microwave power. To study further on immobilized lipase enzyme, the *lipase candida rugosa* enzyme was immobilized using cross-linking method into silica gel. The lipase enzyme was successfully immobilized into silica gel using the cross linking technique with 47 % of enzyme loading and produced 123 U/min.g of enzyme activity, this was also supported with physical characteristic study. This work immobilized enzyme then had been compared with commercial Immobilized lipase enzyme in biodiesel production. As the result this work Immobilized lipase enzyme enhanced the transesterification reaction by reduced the reaction time at 30 min faster than commercial Immobilized lipase enzyme. However, high microwave irradiation reduced the enzyme activity. The next chapter then studied the microwave irradiation effect on Immobilized lipase enzyme in biodiesel production. The microwave irradiation did increase the methyl ester production but it also the main factor that decay the immobilized enzyme and reduced the recyclability of the immobilized enzyme. After 8 cycles commercial immobilized enzyme reduced 66 % of it activity while this work immobilized enzyme reduced 21 % of it activity. After 120 min under microwave irradiation and in biodiesel production, this work immobilized leached 25 % of enzyme from the silica support while commercial immobilized enzyme shows higher leaching which is 40 % of leaching. Despite the fact that the microwave irradiation can decay the Immobilized enzyme the methyl ester was successfully being produced from new feedstock which was rubber seed oil. Biodiesel from rubber seed oil was competitive methyl ester feedstock as it can produced higher methyl ester than waste cooking oil. The most important was all the data was successfully validated using ANOVA. The statistical analysis shows that the data are significant.

CHAPTER 5

CONCLUSION & FUTURE WORKS

5.1 CONCLUSION

Biodiesel was successfully produced from extracted rubber seed oil, waste cooking oil and crude palm oil that comply with ASTM standard under optimum transesterification conditions studied using commercial and purpose made cross linking immobilized lipase candida rugosa enzyme in microwave reactor. This research also was successfully produced high methyl ester yield up to 99 % and optimized the green technology using the enzymatic transesterification. Thru the screening, it shows that the microwave was the competitive reactors compared with ultrasonic and batch reactor in this study. The optimum parameter in this study was 6:1 methanol to oil ratio, 1 wt. % of immobilized lipase enzyme, 150 rpm of agitation during reaction, 40°C of reaction temperature, 5 hours reaction time and 100 W of microwave power. Purpose made Immobilized *Lipase Candida Rugosa* enzyme was successfully immobilized using cross-linking method into silica gel support and enhanced the enzyme activity by produced 30 min faster methyl ester than commercial Immobilized *Lipase Candida Rugosa* enzyme. However, under microwave irradiation 4 % of purposed made immobilized lipase enzyme leached from silica support and reduced 25 % the enzyme activity after 8 cycles. The new feedstock Rubber seed oil produced higher methyl ester than waste cooking oil. The data was validated using ANOVA statistical analysis and it stated that the data are significance besides, the methyl ester kinematic viscosity, calorific value, specific gravity, acid number, cetane number and flash point was analyzed using ASTM standard.

5.2 FUTURE WORKS

Using the successfully purposed made immobilized lipase enzyme, high conversion of methyl ester been produced under optimum parameter studied however, there were a lot of new concerned arise at the end of this study. Crosslinking immobilization method indeed was a good immobilization method but, the cost for this method was expensive since it used a lot of special chemicals such coupling reagent. To reduce the operation cost there was a need to reduce the biocatalyst cost. In future, the crosslinking method should use low cost chemicals or the method itself should be modified. Another factor was the immobilization support, the support can be investigate further especially on the degradability and toxicity of the support. Instead of using microwave irradiation in batch reactor, the microwave reactor in biodiesel production should be investigating further in the aspect of reactor design.

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APPENDIX A

LIST OF PUBLICATIONS

Journals:

- ***Biodiesel Production from Rubber Seed Oil Using A Limestone Based Catalyst***
  Advances in Material Physics and Chemistry, 2(4):138-141, October 2012
  Authors: Chin Kui Cheng, Jolius Gimbun, Shahid Ali, Chitra Charan Suri, Nurul Hidayah Muhammad, Liyana Amer Shah, Said Nurdin

- ***Biodiesel Production from Rubber Seed Oil Using Activated Cement Clinker as Catalyst***
  Procedia Engineering 53(2013): 13-19. (Elsevier), 2013
  Authors: Chin Kui Cheng, Jolius Gimbun, Shahid Ali, Chitra Charan Suri, Nurul Hidayah Muhammad, Liyana Amer Shah, Said Nurdin

- ***Fuel Physical Characteristics of Biodiesel Blend Fules with Alcohol as Additives***
  Procedia Engineering 53(2013): 701-706 (Elsevier), 2013
  Authors: Rizalman Mamat, Ahmad Fitri Yusop, Rafidah Rahim, Amir Aziz, Liyana Amer Shah

APPENDIX B

LIST OF HONORS & AWARDS

Honors & Awards

Gold Medal & Honor of Invention,
World International Intellectual Property Association (WIIPA), 38th International Invention Show (INOVA) 2013, Zagreb, Croatia. (12-16 November 2013)
Members: Liyana Amer Shah, Mohd Faiz A. Razak, Nurul Hidayah Muhammad@Ghazali, Jolius Gimbun & Said Nurdin

Gold Medal & Sir Anthony Leggett Nobel Award: Ultrasonic Assisted Transesterification of Waste Cooking Oil Catalysed by Cement Clinker Based Catalyst
Universiti Malaysia Perlis (April 2013)
International Engineering Invention & Innovation Exhibition (i-ENVEX) and Malaysia International Young Inventors Olympiad (MIYIO) 2013
Members: Liyana Amer Shah, Mohd Faiz A. Razak, Nurul Hidayah Muhammad@Ghazali, Jolius Gimbun & Said Nurdin

Gold Medal: Ultrasonic Assisted Transesterification for Biodiesel Production from Waste Cooking Oil using KOH Supported Alkaline Catalyst
Universiti Malaysia Pahang (Mac 2013)
Creation, Innovation, Technology & Research Exposition (CITREX), 26-28 March 2013, Universiti Malaysia Pahang.
Members: Liyana Amer Shah, Mohd Faiz A. Razak, Nurul Hidayah Muhammad@Ghazali, Jolius Gimbun & Said Nurdin

APPENDIX C

LIST OF SEMINAR/CONFERENCE/EXHIBITION

Seminar/Conferences/Exhibition:

1 Jolius Gimbun, Shahid Ali, Chitra Charan Suri Charan Kanwal, Liyana Amer Shah, Nurul Hidayah Muhamad Ghazali, Chin Kui Cheng, Said Nurdin, Biodiesel Production From Rubber Seed Oil Using A Limestone Based Catalyst, World Congress on Engineering and Technology Oct. 26 -28, 2012 in Beijing, Beijing China

2 Jolius Gimbun, Shahid Ali, Chitra Charan Suri Charan Kanwal, Liyana Amer Shah, Nurul Hidayah, Muhamad Ghazali, Chin Kui Cheng, Said Nurdin, Enhancement of Biodiesel Yield from High FFA Malaysian Rubber Seed Oil with Sodium Methoxide Treated Limestone, Int. Conf. Biomass & Value Added Product, 22-23 Oct 2012, Kuala Lumpur

3 Said Nurdin, Jolius Gimbun, Rozaini Abdullah, Nur Syazwani Ghazali, Liyana Amer Shah, Nur Farzana Omar, Biodiesel Synthesis from Castor Oil Using Egg Shell Waste as Solid catalyst, Int. Conf. Biomass & Value Added Product, 22-23 Oct 2012, Kuala Lumpur

4 Jolius Gimbun, Shahid Ali, Chitra Charan Suri Charan Kanwal, Liyana Amer Shah, Nurul Hidayah Muhamad @ Ghazali, Chin Kui Cheng, Said Nurdin, Biodiesel production from rubber seed oil using activated cement clinker as catalyst, MUCET 2012, Kangar Perlis, 20-21 Nov 2012

5 Liyana Amer Shah, Jolius Gimbun and Said Nurdin, Microwave assisted transesterification of waste cooking oil using immobilized lipase enzyme, ICCBPE-SOMChE 2012, 21-23 Nov 2012, K. Kinabalu Sabah

6 Liyana Amer Shah, S. Nurdin and J. Gimbun, A Comparative Study of Immobilized and Soluble Lipase Enzyme in Three Different Reactors: Ultrasonic, Microwaves and Batch, NCON-PGR 2012, Universiti Malaysia Pahang, Kuantan, 8th– 9th September 2012

7 Liyana Amer Shah, Said Nurdin and Jolius Gimbun, Production of Biodiesel from *Jatropha C*. Oil using KOH and *Lipase Candida R*. in airlift Reactor, ICCBPE_SOMChE 2011, 28th Nov-1st Dec 2011, Kuantan, Pahang.

8 Liyana Amer Shah, Jolius Gimbun, Said Nurdina and Nurul Hidayah Muhamd@Ghazali, Influence of Microwave Irradiation on Immobilized Lipase Enyzme in Biodiesel Production, 2nd POCER 2013, Awana Genting Highland, Pahang, 28-29 June 2013.

APPENDIX A

ENZYME ACTIVITY DATA & CALCULATION EXAMPLE

Commercial Immobilized Enzyme

U=µmol acetic acid/min.g
Mole=MV/100
1 mole= 1000000 µmole

	Min	ml				U/g-sprt
Rec	Time	NaOH	Mole	µmole	Min x g	Activity
0	0	2.5	0.00025	250	3	83.33333
1	30	1.5	0.00015	150	3	50
2	60	1.3	0.00013	130	3	43.33333
3	90	1.1	0.00011	110	3	36.66667
4	120	1	0.0001	100	3	33.33333
5	150	0.9	0.00009	90	3	30
6	180	0.9	0.00009	90	3	30
7	210	0.8	0.00008	80	3	26.66667
8	240	0.85	0.000085	85	3	28.33333

Purposed Made Immobilized Enzyme

	Min	ml				U/g-sprt
Rec	Time	NaOH	Mole	µmole	Min x g	Activity
0	0	3.7	0.00037	370	3	123.3333
1	30	3.3	0.00033	330	3	110
2	60	3.2	0.00032	320	3	106.6667
3	90	3.1	0.00031	310	3	103.3333
4	120	3.1	0.00031	310	3	103.3333
5	150	2.9	0.00029	290	3	96.66667
6	180	2.9	0.00029	290	3	96.66667
7	210	2.9	0.00029	290	3	96.66667
8	240	2.9	0.00029	290	3	96.66667

Enzyme Activity Measurement (Purposed made Immobilized Enzyme)

ml					U/g-sprt
Power	NaOH	Mole	µmole	Min x g	Activity
0	3.7	0.00037	370	3	123.3333
100	2	0.0002	200	3	66.66667
200	1.6	0.00016	160	3	53.33333

Enzyme Activity Calculation

$$= \frac{\mu mole}{min \times g}$$

$$= \left(\frac{MV}{100}\right)(1000000)\left(\frac{1}{min \times g}\right)$$

MASTERSIZER 2000

Result Analysis Report

Sample Name:
SILICA 2

Sample Source & type:

Sample bulk lot ref:

SOP Name:

Measured by:
BP80-50

Result Source:
Measurement

Measured:
Tuesday, 18 June, 2013 5:02:13 PM

Analysed:
Tuesday, 18 June, 2013 5:02:14 PM

Particle Name:
Silica 1.45

Particle RI:
1.450

Dispersant Name:
Water

Accessory Name:
Hydro 2000MU (A)

Absorption:
0.1

Dispersant RI:
1.330

Analysis model:
General purpose

Size range:
0.020 to 2000.000 um

Weighted Residual:
1.069 %

Sensitivity:
Normal

Obscuration:
15.46 %

Result Emulation:
Off

Concentration:
0.2821 %Vol

Specific Surface Area:
0.0504 m²/g

Span :
0.988

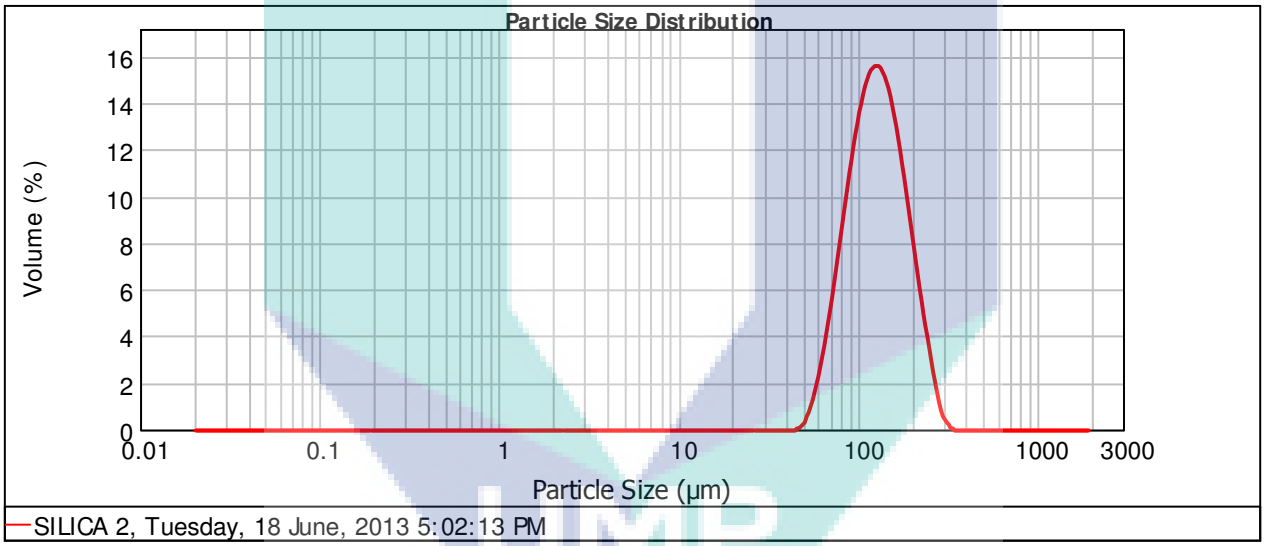
Surface Weighted Mean D[3,2]:
118.933 um

Uniformity:
0.307

Vol. Weighted Mean D[4,3]:
135.312 um

Result units:
Volume

d(0.1): 78.729 um **d(0.5):** 127.095 um **d(0.9):** 204.344 um



Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %
0.010	0.00	0.105	0.00	1.096	0.00	11.482	0.00	120.226	14.10	1258.925	0.00
0.011	0.00	0.120	0.00	1.259	0.00	13.183	0.00	138.038	13.24	1445.440	0.00
0.013	0.00	0.138	0.00	1.445	0.00	15.136	0.00	158.489	11.17	1659.587	0.00
0.015	0.00	0.158	0.00	1.660	0.00	17.378	0.00	181.970	8.30	1905.461	0.00
0.017	0.00	0.182	0.00	1.905	0.00	19.953	0.00	208.930	5.37	2187.762	0.00
0.020	0.00	0.209	0.00	2.188	0.00	22.909	0.00	239.883	2.81	2511.886	0.00
0.023	0.00	0.240	0.00	2.512	0.00	26.303	0.00	275.423	0.69	2884.032	0.00
0.026	0.00	0.275	0.00	2.884	0.00	30.200	0.00	316.228	0.00	3311.311	0.00
0.030	0.00	0.316	0.00	3.311	0.00	34.674	0.00	363.078	0.00	3801.894	0.00
0.035	0.00	0.363	0.00	3.802	0.00	39.811	0.00	416.869	0.00	4365.158	0.00
0.040	0.00	0.417	0.00	4.365	0.00	45.709	0.17	478.630	0.00	5011.872	0.00
0.046	0.00	0.479	0.00	5.012	0.00	52.481	1.26	549.541	0.00	5754.399	0.00
0.052	0.00	0.550	0.00	5.754	0.00	60.256	3.20	630.957	0.00	6606.934	0.00
0.060	0.00	0.631	0.00	6.607	0.00	69.183	5.83	724.436	0.00	7585.776	0.00
0.069	0.00	0.724	0.00	7.586	0.00	79.433	8.82	831.764	0.00	8709.636	0.00
0.079	0.00	0.832	0.00	8.710	0.00	91.201	11.56	954.993	0.00	10000.000	
0.091	0.00	0.955	0.00	10.000	0.00	104.713	13.49	1096.478	0.00		
0.105		1.096		11.482		120.226		1258.925			

Operator notes:

MASTERSIZER 2000

Result Analysis Report

Sample Name:
SILICA 2

SOP Name:

Measured:
Tuesday, 18 June, 2013 5:11:50 PM

Sample Source & type:

Measured by:
BP80-50

Analysed:
Tuesday, 18 June, 2013 5:11:51 PM

Sample bulk lot ref:

Result Source:
Measurement

Particle Name:
Silica 1.45

Accessory Name:
Hydro 2000MU (A)

Analysis model:
General purpose

Sensitivity:
Normal

Particle RI:
1.450

Absorption:
0.1

Size range:
0.020 to 2000.000 um

Obscuration:
14.74 %

Dispersant Name:
Water

Dispersant RI:
1.330

Weighted Residual:
0.861 %

Result Emulation:
Off

Concentration:
0.1824 %Vol

Span :
0.943

Uniformity:
0.295

Result units:
Volume

Specific Surface Area:
0.0731 m²/g

Surface Weighted Mean D[3,2]:
82.099 um

Vol. Weighted Mean D[4,3]:
129.401 um

d(0.1): 77.167 um **d(0.5):** 123.069 um **d(0.9):** 193.184 um

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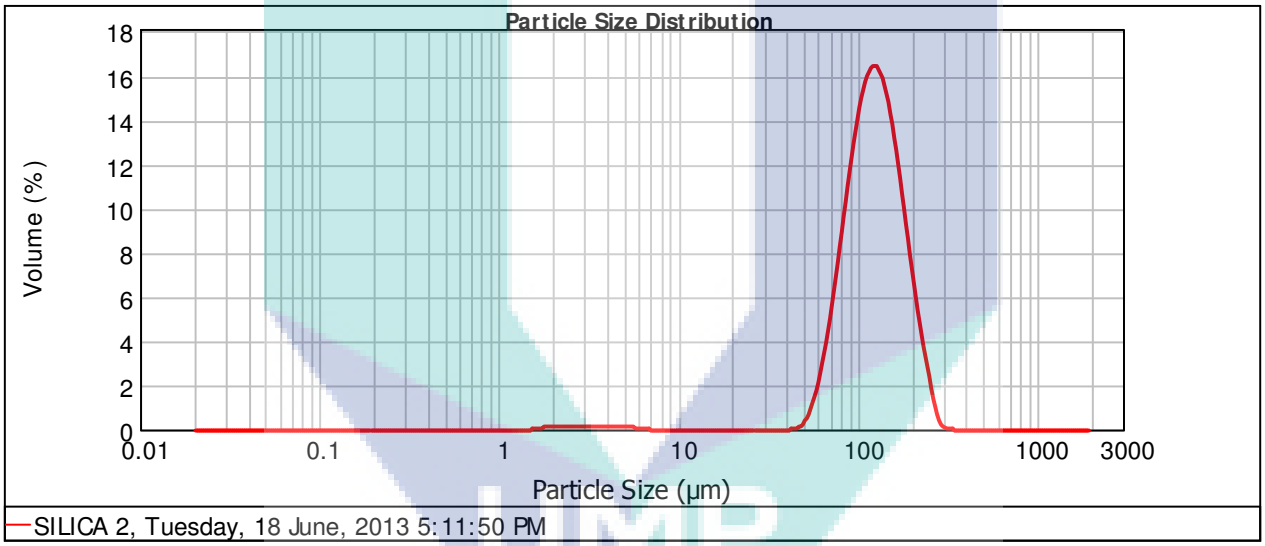

Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %
0.010	0.00	0.105	0.00	1.096	0.00	11.482	0.00	120.226	14.82	1258.925	0.00
0.011	0.00	0.120	0.00	1.259	0.00	13.183	0.00	138.038	13.43	1445.440	0.00
0.013	0.00	0.138	0.00	1.445	0.01	15.136	0.00	158.489	10.70	1659.587	0.00
0.015	0.00	0.158	0.00	1.660	0.08	17.378	0.00	181.970	7.33	1905.461	0.00
0.017	0.00	0.182	0.00	1.905	0.10	19.953	0.00	208.930	4.24	2187.762	0.00
0.020	0.00	0.209	0.00	2.188	0.12	22.909	0.00	239.883	1.84	2511.886	0.00
0.023	0.00	0.240	0.00	2.512	0.13	26.303	0.00	275.423	0.17	2884.032	0.00
0.026	0.00	0.275	0.00	2.884	0.14	30.200	0.00	316.228	0.00	3311.311	0.00
0.030	0.00	0.316	0.00	3.311	0.15	34.674	0.00	363.078	0.00	3801.894	0.00
0.035	0.00	0.363	0.00	3.802	0.14	39.811	0.00	416.869	0.00	4365.158	0.00
0.040	0.00	0.417	0.00	4.365	0.12	45.709	0.23	478.630	0.00	5011.872	0.00
0.046	0.00	0.479	0.00	5.012	0.10	52.481	1.19	549.541	0.00	5754.399	0.00
0.052	0.00	0.550	0.00	5.754	0.04	60.256	3.09	630.957	0.00	6606.934	0.00
0.060	0.00	0.631	0.00	6.607	0.00	69.183	5.89	724.436	0.00	7585.776	0.00
0.069	0.00	0.724	0.00	7.586	0.00	79.433	9.23	831.764	0.00	8709.636	0.00
0.079	0.00	0.832	0.00	8.710	0.00	91.201	12.33	954.993	0.00	10000.000	
0.091	0.00	0.955	0.00	10.000	0.00	104.713	14.41	1096.478	0.00		
0.105		1.096		11.482		120.226		1258.925			

Operator notes:

MASTERSIZER 2000

Result Analysis Report

Sample Name:

SOP Name:

Measured:
Tuesday, 18 June, 2013 4:25:29 PM

Sample Source & type:

Measured by:
BP80-50

Analysed:
Tuesday, 18 June, 2013 4:25:31 PM

Sample bulk lot ref:

Result Source:
Measurement

Particle Name:
Silica 1.45

Accessory Name:
Hydro 2000MU (A)

Analysis model:
General purpose

Sensitivity:
Normal

Particle RI:
1.450

Absorption:
0.1

Size range:
0.020 to 2000.000 um

Obscuration:
17.04 %

Dispersant Name:
Water

Dispersant RI:
1.330

Weighted Residual:
4.057 %

Result Emulation:
Off

Concentration:
0.2214 %Vol

Span :
1.002

Uniformity:
0.316

Result units:
Volume

Specific Surface Area:
0.0705 m²/g

Surface Weighted Mean D[3,2]:
85.102 um

Vol. Weighted Mean D[4,3]:
136.458 um

d(0.1): 78.177 um **d(0.5):** 129.354 um **d(0.9):** 207.790 um

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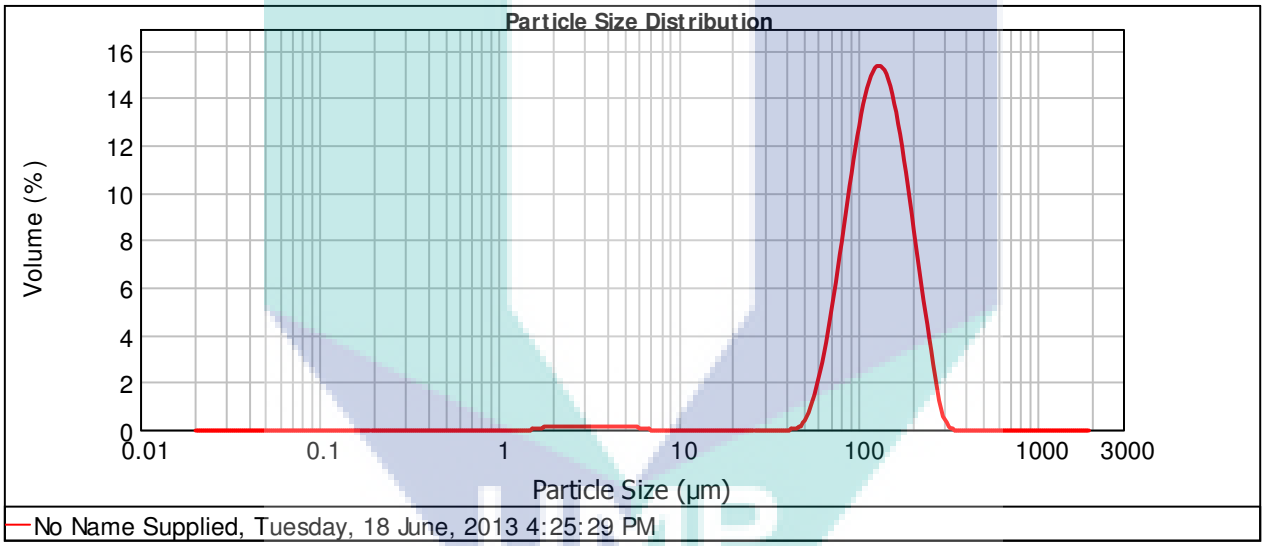

Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %
0.010	0.00	0.105	0.00	1.096	0.00	11.482	0.00	120.226	13.85	1258.925	0.00
0.011	0.00	0.120	0.00	1.259	0.00	13.183	0.00	138.038	13.35	1445.440	0.00
0.013	0.00	0.138	0.00	1.445	0.01	15.136	0.00	158.489	11.56	1659.587	0.00
0.015	0.00	0.158	0.00	1.660	0.08	17.378	0.00	181.970	8.84	1905.461	0.00
0.017	0.00	0.182	0.00	1.905	0.09	19.953	0.00	208.930	5.87	2187.762	0.00
0.020	0.00	0.209	0.00	2.188	0.11	22.909	0.00	239.883	3.13	2511.886	0.00
0.023	0.00	0.240	0.00	2.512	0.13	26.303	0.00	275.423	0.71	2884.032	0.00
0.026	0.00	0.275	0.00	2.884	0.14	30.200	0.00	316.228	0.00	3311.311	0.00
0.030	0.00	0.316	0.00	3.311	0.14	34.674	0.00	363.078	0.00	3801.894	0.00
0.035	0.00	0.363	0.00	3.802	0.14	39.811	0.00	416.869	0.00	4365.158	0.00
0.040	0.00	0.417	0.00	4.365	0.12	45.709	0.22	478.630	0.00	5011.872	0.00
0.046	0.00	0.479	0.00	5.012	0.10	52.481	1.19	549.541	0.00	5754.399	0.00
0.052	0.00	0.550	0.00	5.754	0.05	60.256	2.92	630.957	0.00	6606.934	0.00
0.060	0.00	0.631	0.00	6.607	0.00	69.183	5.31	724.436	0.00	7585.776	0.00
0.069	0.00	0.724	0.00	7.586	0.00	79.433	8.14	831.764	0.00	8709.636	0.00
0.079	0.00	0.832	0.00	8.710	0.00	91.201	10.86	954.993	0.00	10000.000	
0.091	0.00	0.955	0.00	10.000	0.00	104.713	12.94	1096.478	0.00		
0.105		1.096		11.482		120.226		1258.925			

Operator notes:

MASTERSIZER 2000

Result Analysis Report

Sample Name:

SOP Name:

Measured:
Tuesday, 18 June, 2013 4:39:42 PM

Sample Source & type:

Measured by:
BP80-50

Analysed:
Tuesday, 18 June, 2013 4:39:44 PM

Sample bulk lot ref:

Result Source:
Measurement

Particle Name:
Silica 1.45

Accessory Name:
Hydro 2000MU (A)

Analysis model:
General purpose

Sensitivity:
Normal

Particle RI:
1.450

Absorption:
0.1

Size range:
0.020 to 2000.000 um

Obscuration:
14.34 %

Dispersant Name:
Water

Dispersant RI:
1.330

Weighted Residual:
3.687 %

Result Emulation:
Off

Concentration:
0.1219 %Vol

Span :
0.965

Uniformity:
0.316

Result units:
Volume

Specific Surface Area:
0.105 m²/g

Surface Weighted Mean D[3,2]:
57.273 um

Vol. Weighted Mean D[4,3]:
126.820 um

d(0.1): 73.480 um **d(0.5):** 123.723 um **d(0.9):** 192.909 um

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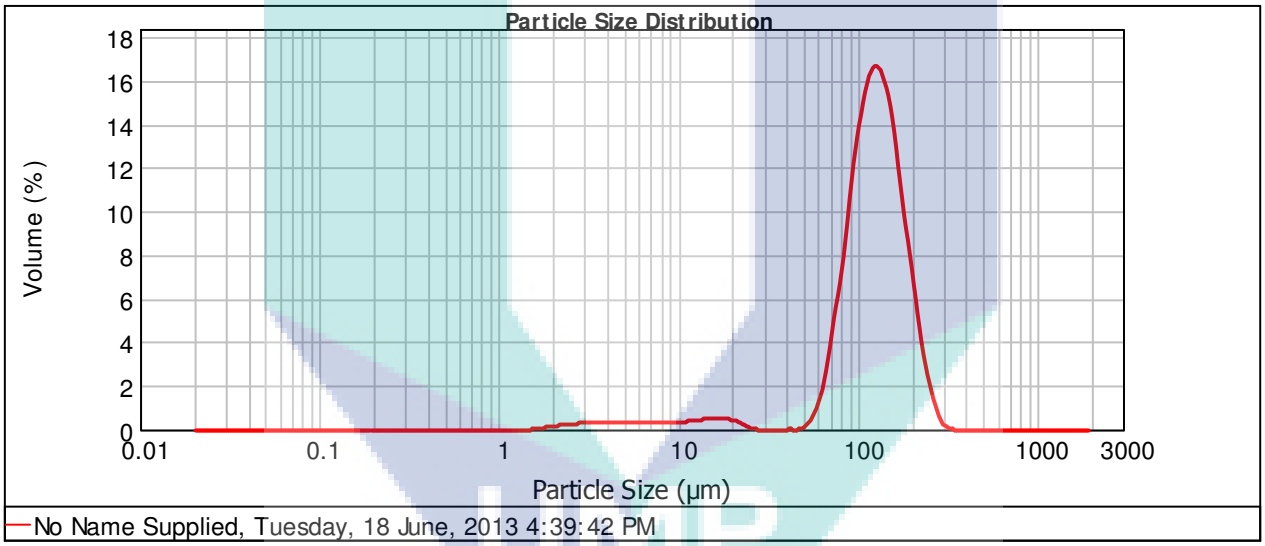

Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %
0.010	0.00	0.105	0.00	1.096	0.00	11.482	0.37	120.226	15.07	1258.925	0.00
0.011	0.00	0.120	0.00	1.259	0.00	13.183	0.43	138.038	13.85	1445.440	0.00
0.013	0.00	0.138	0.00	1.445	0.01	15.136	0.46	158.489	10.73	1659.587	0.00
0.015	0.00	0.158	0.00	1.660	0.07	17.378	0.43	181.970	7.42	1905.461	0.00
0.017	0.00	0.182	0.00	1.905	0.13	19.953	0.34	208.930	4.05	2187.762	0.00
0.020	0.00	0.209	0.00	2.188	0.20	22.909	0.11	239.883	1.69	2511.886	0.00
0.023	0.00	0.240	0.00	2.512	0.25	26.303	0.00	275.423	0.33	2884.032	0.00
0.026	0.00	0.275	0.00	2.884	0.29	30.200	0.00	316.228	0.00	3311.311	0.00
0.030	0.00	0.316	0.00	3.311	0.31	34.674	0.00	363.078	0.00	3801.894	0.00
0.035	0.00	0.363	0.00	3.802	0.31	39.811	0.00	416.869	0.00	4365.158	0.00
0.040	0.00	0.417	0.00	4.365	0.30	45.709	0.05	478.630	0.00	5011.872	0.00
0.046	0.00	0.479	0.00	5.012	0.29	52.481	0.60	549.541	0.00	5754.399	0.00
0.052	0.00	0.550	0.00	5.754	0.27	60.256	1.97	630.957	0.00	6606.934	0.00
0.060	0.00	0.631	0.00	6.607	0.25	69.183	4.79	724.436	0.00	7585.776	0.00
0.069	0.00	0.724	0.00	7.586	0.25	79.433	7.78	831.764	0.00	8709.636	0.00
0.079	0.00	0.832	0.00	8.710	0.27	91.201	11.76	954.993	0.00	10000.000	
0.091	0.00	0.955	0.00	10.000	0.31	104.713	14.25	1096.478	0.00		
0.105		1.096		11.482		120.226		1258.925			

Operator notes: