BIODIESEL PRODUCTION FROM SOYBEAN OIL USING *Pseudomonas cepacia*
LIPASE IMMOBILIZED ONTO POLYSTYRENE BEAD

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A Thesis Submitted in Partial Fulfillment of the Requirement
for the Degree of Master of Science Program in Petrochemistry and Polymer Science
Faculty of Science
Chulalongkorn University
Academic Year 2012
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การผลิตไบโอดีเซลจากน้ำมันถั่วเหลืองโดยใช้ไลเพส *Pseudomonas cepacia*
ตรึงรูปบนเม็ดพอลิสไตรีน

นางสาวธนพร จิตรสิงห์

Thesis Title   BIODIESEL PRODUCTION FROM SOYBEAN OIL
                    USING *Pseudomonas cepacia* LIPASE IMMOBILIZED
                    ONTO POLYSTYRENE BEAD

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Accepted by Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

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ธนพร จิตรสิงห์ : การผลิตไบโอดีเซลจากน้ำมันถั่วเหลืองโดยใช้ไลเพส *Pseudomonas cepacia* ตรึงรูปบนเม็ดพอลิสไตรีน. (BIODIESEL PRODUCTION FROM SOYBEAN OIL USING *Pseudomonas cepacia* LIPASE IMMOBILIZED ONTO POLYSTYRENE BEAD) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ.ดร.สุรชัย พรภคกุล, 95 หน้า.

การพัฒนาตัวตรึงรูปชนิดเม็ดพอลิสไตรีนสำหรับตรึงเอนไซม์ไลเพสจาก *Pseudomonas cepacia* เพื่อใช้เป็นตัวเร่งปฏิกิริยาสำหรับผลิตไบโอดีเซลได้ถูกศึกษาขึ้น โดยตัวตรึงรูปชนิดเม็ดพอลิสไตรีนจะถูกปรับปรุงพื้นผิวด้วยพอลิกลูตารัลดิไฮด์-สไตรีน โคพอลิเมอร์ทำให้ได้คุณสมบัติสำคัญ ที่มีความหนาแน่นน้อยกว่าน้ำและน้ำมัน จึงลอยอยู่ส่วนบนของปฏิกรณ์ นอกจากนี้ตัวตรึงรูปพอลิสไตรีนเชื่อมขวางด้วยพอลิกลูตารัลดิไฮด์ได้ถูกสังเคราะห์ขึ้นซึ่งมีความหนาแน่นมากจึงจมอยู่ที่ก้นของปฏิกรณ์ จากนั้นไลเพสจาก *Pseudomonas cepacia* จะตรึงรูปบนตัวตรึงที่แตกต่างกันทั้งสองชนิดนี้ ความสามารถของไลเพสที่ถูกตรึงและประสิทธิภาพของตัวเร่งปฏิกิริยาได้ถูกวิเคราะห์ขึ้น พบว่า ความสามารถของไลเพสที่ตรึงบนพอลิกลูตารัลดิไฮด์-สไตรีน-เม็ดพอลิสไตรีนมีค่า 26.12 ยูนิตต่อกรัมของตัวตรึง ในขณะที่ความสามารถของไลเพสที่ตรึงบนพอลิกลูตารัลดิไฮด์-พอลิสไตรีนที่สังเคราะห์ขึ้นมีค่า 30.25 ยูนิตต่อกรัมของตัวตรึง สำหรับการผลิตไบโอดีเซลจะถูกสังเคราะห์ด้วยปฏิกิริยาทรานเอสเทอร์ริฟิเคชันจากน้ำมันถั่วเหลืองกับเอทานอล(เออาร์ เกรด) และเอทานอลบริสุทธ์พบว่าไลเพสตรึงรูปบนพอลิกลูตารัลดิไฮด์-สไตรีน-เม็ดพอลิสไตรีนเป็นตัวเร่งปฏิกิริยาที่ดีที่สุดสำหรับการใช้งานกับ เอทานอลบริสุทธ์ ภายใต้สภาวะที่เหมาะสมคือ อัตราส่วน 1:5 ของน้ำมันต่อเอทานอลบริสุทธ์ ที่อุณหภูมิ 40 อาศาเซลเซียส เป็นเวลา 24 ชั่วโมง ผลิตไบโอดีเซลได้สูงถึง 98.8 เปอร์เซนต์ และตัวเร่งปฏิกิริยานี้ยังสามารถใช้งานได้นานมากกว่า 10 ครั้งและมีประสิทธิภาพในการเร่งปฏิกิริยาที่ดีกว่าตัวเร่งปฏิกิริยาที่ได้จากไลเพสตรึงรูปบนพอลิกลูตารัลดิไฮด์-พอลิสไตรีนที่สังเคราะห์ขึ้น

สาขาวิชา.ปิโตรเคมีและวิทยาศาสตร์พอลิเมอร์. ลายมือชื่อนิสิต...

ปีการศึกษา.................2555.........................… ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก................

5272326023: MAJOR   PETROCHEMISTRY AND POLYMER SCIENCE

KEYWORDS: BIODIESEL / IMMOBILIZATION / LIPASE / *Pseudomonas cepacia*

THANAPORN JITRASING: BIODIESEL PRODUCTION FROM SOYBEAN OIL USING *Pseudomonas cepacia* LIPASE IMMOBILIZED ONTO POLYSTYRENE BEAD: ASSOC. PROF. SURACHAI PORNPAKAKUL, Ph.D., 95 pp.

The improvement of expandable polystyrene bead support for immobilization of *Pseudomonas cepacia* lipase, which used as catalyst for biodiesel production, was studied. The surface of expanded polystyrene bead support was modified by polyglutaraldehyde-styrene copolymer (PGlu-STY-EPS bead) and gave the important property, which had lower density than water and oil thus it can float on the top of the reactor. Moreover, the polystyrene crosslinked with polyglutaraldehyde support was synthesized (synthesized-PGlu-polystyrene support), which gave a high density support for comparison the efficiency of catalyst. Lipase from *Pseudomonas cepacia* was immobilized on two different supports and its activity and efficiency of catalysis were analyzed. Results that the activity of lipase immobilized onto PGlu-STY-EPS bead support was 26.12 U/g-support whereas the activity of lipase immobilized onto synthesized-PGlu-polystyrene support was 30.25 U/g-support. For biodiesel production was synthesized by transesterification reaction of soybean oil with ethanol (AR grade) and absolute ethanol. The immobilized lipase onto PGlu-STY-EPS bead support was an excellent catalyst for using absolute alcohol. The optimal conditions were 1:5 molar ratio of oil/absolute ethanol at 40 ˚C for 24 hours. It gave maximal conversion at 98.8% and enzymatic reusability can be repeating over 10 batches and more efficiency than catalyst of immobilized lipase onto synthesized- PGlu-polystyrene copolymer.

Field of study: Petrochemistry and Polymer ScienceStudent's Signature.......................

Academic Year: 2012   Advisor's Signature……………...

ACKNOWLEDGEMENTS

I would like to express my deep and sincere gratitude to my advisor, Associate Professor Dr. Surachai Pornpakakul, for his excellent suggestion, his understanding, encouraging and a good guidance throughout the entire period of conducting this thesis.

I would also like to extend to Professor Dr. Pattarapan Prasassarakich, Associate Professor Dr. Wimonrat Trakarnpruk and Dr. Prapas Khorphueng, attending as the chairman and members of my thesis committee, respectively, for their kind guidance, helpful discussions and valuable suggestions throughout my study.

I would like to special thank to all staff at IRPC Public Company Limited Petrochemical Business for kind suggestion and helpful in providing facilities and materials for my thesis experiment.

In addition, I am grateful to Miss Sunisa Suwanjaroen, Miss Chotika Laksana and all members on Research Center for Bioorganic Chemistry (RCBC) for their kind gratitude of finding me the information and I thank Mr. Supongpan Srisurichan and Miss Premika Leelangamwongsa for the beautiful drawing, NMR information and their cooperation for my thesis experiment.

Finally, I most gratefully thank my family for their care and support to make my study successful. Thanks are also due to everyone who has contributed suggestions and supports throughout my research.

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LIST OF ABBREVIATIONS

CO_2	=	Carbondioxide
°C	=	Degree of Celsius
No.	=	Number
% wt	=	Percent by weight
wt	=	weight
%w/v	=	Percent weight by volume
g	=	gram
NO_x	=	Nitrogen oxides
SO_2	=	Sulphur dioxide
h	=	Hour
FAME	=	Fatty acid methyl ester
PGlu	=	Polyglutaraldehyde
STY	=	Styrene
P.cepacia	=	*Pseudomonas cepacia*
U/g-support	=	Unit per gram of support
TLC	=	Thin layer chromatography
% Loading	=	Percent loading
% Conversion	=	Percent conversion
^{1}H-NMR	=	Proton Nuclear Magnetic Resonance

CHAPTER 1

INTRODUCTION

The economic development can be found in the increasing energy requirement in the country. Therefore, the growths of human population and industrialization energy have been continuously increasing. Common sources of energy are petroleum, natural gas and coal from fossil fuels. This growing consumption of energy has been affect atmospheric pollution and environmental issues. The increasing environmental pressure is major problem of air pollutants. Fossil fuels emissions are majority to produce the global warming. The greenhouse gases such as CO, NO_x and SO_x are increased by the combustion from fossil fuels. Therefore, biodiesel is becoming the important topic in the almost country and is the best alternative energy to replace diesel engines without modification, make the vehicle perform better, can make your car last longer, and reduce both energy dependence on petroleum and air pollution. For this reason, the encouragements of researches are important to find an alternative source of renewable energy (Ching, J. J. et al., 2010).

Fatty acid alkyl esters (FAAEs) known as biodiesel are better than diesel fuel in terms of sulfur content, flash point, aromatic content. The main advantages are its renewability, better-quality exhaust gas emissions and its biodegradability (Moreira, A. et al., 2007).Preparations of biodiesel are four primary methods; direct use and blending, microemulsions, thermal cracking (pyrolysis) and transesterification (Srivastava, A. et al., 2000). The most commonly used method is transesterification of animal fats and vegetable oils with short chain alcohols. Methanol is mostly used because of its lower cost compared with other alcohols, so FAAEs most commonly refers to fatty acid methyl esters (FAMEs)(Tan, T. et al., 2010). The catalytic in the process is classified as chemical or enzymatic production. Chemical catalysts such as alkali (NaOH) or acid (H_2SO_4) are used to produce biodiesel in short time reaction and give high conversion level of oils to methyl esters. On the other hand, major of disadvantage in this catalyst are high-energy

requirements, difficulty of recycling glycerol and potential pollution to the environment (Pizarro, L. and Park, 2003).

The researches of enzymatic production have yearly increased. Specifically, lipase can be used to catalyze the hydrolysis of esters such as glyceride under mild conditions, produce the surfactants and prepare the enantioselective pharmaceutics(Medina, R. A. et al., 2009), (Song, S. Y. et al., 2009). The enzyme catalysts are mostly used in term of immobilization with several methods such as adsorption, covalent bonding, entrapment, encapsulation, and cross-linking for development in order to improve their activity. The immobilizations of enzyme are widely used for their industrial applications such as used in food, pharmaceutical, and agrochemical. These have been specifically studied due to their enhanced stability, easy separation, and reusability(Tan, T. et al., 2010).

Lipase derived from different sources such as *Pseudomonas fluorescens, Pseudomonas capacia, Mucor javanicus* and *Candida antarctica,* were used as catalyst for biodiesel production (Iso, M. et al., 2001).There are many supporting materials used for immobilization such as fumed silica(Cruz, C. J. et al., 2009), chitosan beads (Yi, S. et al., 2009), porous kaolin(Fernandez, L. R. et al., 1998) and styrene-divinylbenzene copolymer (Dizge, N. et al., 2009).Hydrophobic polymers such as styrene-divinylbenzene copolymer have been extensively used for lipase immobilization because these are effects on the activity/stability properties of lipase (Lafuente, R. et al., 1998).

Before lipase was generally immobilized, the supports were treated by polyglutaraldehyde to get an intense multi-function crosslinking between the lipase and the support for increasing stability of the lipase (Lorente, G. F. et al., 2006). The general applications of polyglutaraldehyde are widely used as a protein linker agent (Tanriseven, A. et al., 2008),for fixation of living cell (Sandholm, M. et al., 1976)or tissue (Pieraccini, G. et al., 2002) and found to serve as a productive binding agent of antibodies to microsphere (Molday, R. et al., 1984).

In this research the polyglutaraldehyde was used as protein linker agent for improving efficiency of polymer support. Also, the polystyrene bead was used as a core for encapsulation of polyglutaraldehyde-styrene copolymer (PGlu-STY) to provide various density of polymer support for immobilization of lipase.

1.1 Objectives of the research

- To improve efficiency of polystyrene bead support forimmobilization of *Pseudomonas cepacia*lipase

- To produce biodiesel from soybean oil using immobilized lipase by transesterification reaction

CHAPTER II

THEORY AND LITERATURE REVIEWS

2.1 Background

Nowadays, total energy outputs in the world are mainly supplied through petrochemical sources, coal and natural gases and professional have notified about the depletion of this actual source in the near future. Diesel fuels have a necessity factor in the industrial economy of a developing country and used for transportation of industrial and agricultural goods. Furthermore, the increasing environmental impact care has defined restrictions on fuel combustion emissions. These facts have stimulated recent interest in alternative sources for fossil fuel development. One of the most confidence sources is biodiesel, which is an alternative diesel fuel derivate from renewable sources with high quality and used as diesel fuel without engine modifications. Biodiesel shows a favorable combustion emission profile which is producing much less carbon monoxide, sulfur dioxide and unburned hydrocarbons than petroleum-based diesel fuel(Borges, M, E. et al., 2012).

2.1.1 Biodiesel production

There are several methods to make biodiesel: dilution, microemulsions, thermal cracking (pyrolysis) and transesterification. Direct use of vegetable oil (dilution) is not applicable to most of actual diesel fuels because the high viscosity of biodiesel in this method damaged the engine. Other methods of biodiesel production are microemulsion and thermal cracking would lead to incomplete combustion because a low cetane number. Transesterificatin method has been widely studied and is generally used for converting vegetable oil into biodiesel due to its simplicity (Srivastava, A. et al., 2000).

1. Dilution

Dilution of vegetable oils can be completed with petroleum diesel fuels and a solvent or ethanol to run the engine. The dilution method are generally used at ratio of sunflower oil with diesel fuels is 1:3 to produce biodiesel and bring to test property with engine (Schwab, A. W. et al., 1987). The viscosity of this fuel is 4.88 cSt at 40°C. Results that the fuel could not be recommended in the direct injection diesel engines because of severed injector nozzle coking and sticking. A comparable fuel with high oleic sunflower oil was also tested and it gave pleasing results, but it is not appropriate as it leads to thickening of lubricant.

2. Microemulsion

Microemulsions are a colloidal dispersion of colloidal particles in equilibrium of optically isotropic fluid microstructures with dimensions of spontaneous form in the range of 1 to150 nm from two immiscible liquids and ionic amphiphiles (Schwab, A. W. et al., 1988).The problem of general vegetable oils is high viscosity. Therefore, microemulsions can be reducing the viscosity of oils by adding solvent such as methanol, ethanol and 1-buthanol.

The microemulsions have similarly performances to diesel fuel. They are also improved spray characteristics by explosive vaporization of the low boiling elements in micelles. Disadvantage of this methods are decreasing a performance of engine such as significant injector needle sticking, carbon deposits, incomplete combustion and increasing viscosity of lubricating oils.

3. Pyrolysis

Pyrolysis is a method for a chemical change caused by the application of thermal energy. The advantages of this process are simple, wasteless, less pollution and effective compared with other cracking processes. Pyrolysis can be converting one substance into another compound by using heat or by using heat with catalyst and without air or oxygen. Many researchers have investigated the pyrolysis of triglycerides to produce the

appropriate products for diesel engines. The effect of temperature on the type of products obtained, the use of catalysts, largely metallic salts, similar paraffins and olefins obtained to those present in hydrocarbon-based diesel fuels and the characterization of the thermal decomposition products were studied.

Thermal decomposition of triglycerides produces the compounds of classes including alkanes, alkenes, alkadienes, aromatics and carboxylic acids (Srivastava, A. et al., 2000). The thermal decomposition is shown in Figure 2.1. The thermal decomposition mechanisms of triglyceride are likely to be complex because of many structures and multiplicity of reactions of mixed triglycerides. Normally, thermal decomposition of triglyceride molecule can be occurring though either a free radical or carbonium ion mechanism. For this reason, the formation of homogeneous series of alkanes and alkenes is accountable from the generation of the RCOO radical of the triglyceride cleavage and subsequent loss of carbon dioxide. The R radical, upon disproportionation and ethylene elimination, gives the odd-numbered carbon alkanes and alkenes. The unsaturation enhances cleavage at a position α, β to the unsatulation. Thermal positional of unsatulation enhances cleavage could account for the higher amounts of C_5, to C_{10} alkanes obtained from safflower compared with soybean oil. The formation of aromatics is supported by a Diels-Alder addition of ethylene to a conjugated diene formed in the pyrolysis reaction. Carboxylic acids formed during the pyrolysis of vegetable oils probably result from cleavage of the glyceride moiety (Srivastava, A. et. al., 2000).

$$CH_3(CH_2)_5CH_2\text{-}CH_2CH\text{=}CH_2\text{-}CH_2(CH_2)_5\overset{O}{\overset{||}{C}}\text{-}O\text{-}CH_2R$$

$$CH_3(CH_2)_5CH_2\text{-}CH_2CH\text{=}CHCH_2\text{-}CH_2(CH_2)_5\overset{O}{\overset{||}{C}}\text{-}OH$$

$CH_3(CH_2)_5CH_2\bullet$

$CH_2\text{=}CHCH\text{=}CH_2$

$\bullet CH_2(CH_2)_5\overset{O}{\overset{||}{C}}\text{-}OH$

$CH_3(CH_2)_3CH_2\bullet + CH_2\text{=}CH_2$

Diels-Alder

$\bullet CH_3(CH_2)_5\overset{O}{\overset{||}{C}}\text{-}OH$

$CH_3(CH_2)_3CH_3$

-H

$CH_3(CH_2)_4CH_3$
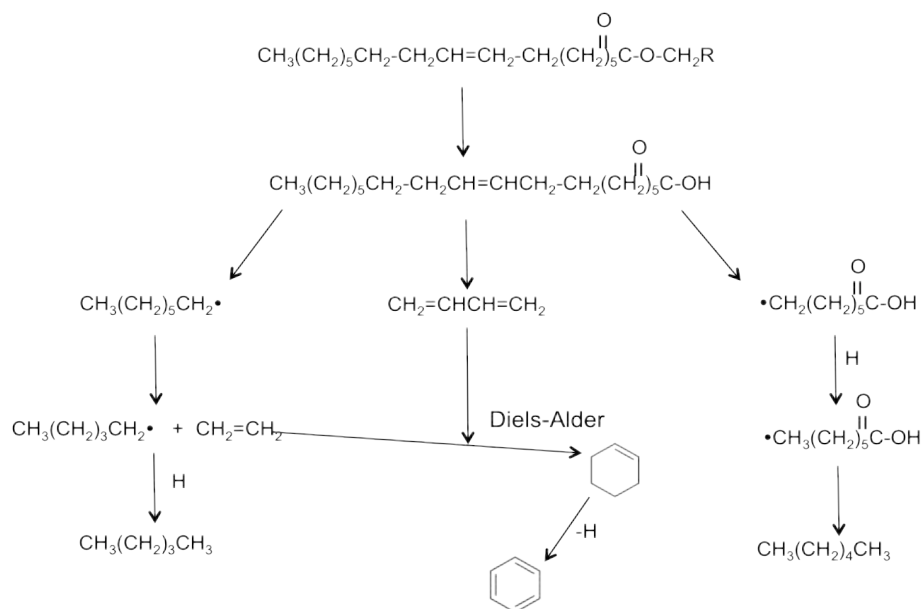
Scheme 2.1 Mechanism of thermal decomposition of triglyceride

4. Transesterification

Transesterification (also called alcoholysis) is the reaction process of triglyceride molecules present in animal fats or vegetable oils with short chain alcohol using a catalyst for improving the reaction rate to form esters and glycerol (by-product). This process has been widely used to reduce the viscosity of triglycerides and has been performed well in long-term diesel engine (Jaichandar, S. et al 2011). The transesterification reaction of triglyceride with alcohol is shown in Figure 2.2.
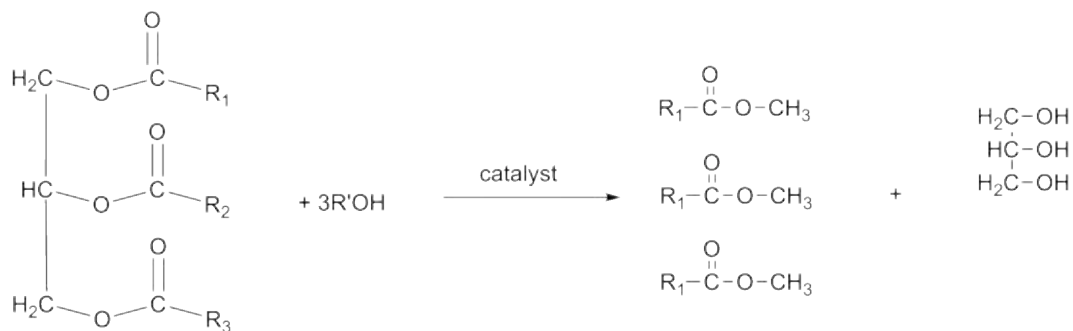
Scheme 2.2 Triglycerides transesterification reaction with alcohol

2.1.2 Advantages of biodiesel

Biodiesel as an alternative fuel for diesel engines are widely available from variety vegetable oils. There are many advantages of biodiesel including:

1. Biodiesel can be operated on diesel engines without modification of engines.

2. Biodiesel usually used pure oil or mixture with hydrocarbon-based diesel fuels.

3. Biodiesel is not harmful to the environment because of reduction of soot, unburnt hydrocarbons, and also of carbon monoxide.

4. No evaporation of low-boiling components takes place.

5. Biodiesel reduces the environmental effect because without exhaust gas of SO2 and halogens.

6. The engine setting is no change from NO_x emissions of combustion of engine.

7. Good performance in auto-ignition of fatty esters results in a smooth running diesel engine.

8. Biodiesel consumption resembles that of hydrocarbon-based diesel fuels.

2.2 Source of biodiesel

The biodiesel production mainly made from natural or renewable sources such as vegetable oil and fats. There are consisted of soybean, Jatropha, palm, sunflower, rapeseed, canola and cottonseed (Singh, S, P. et al., 2010). Generally, sources of biodiesel are depends on regional climate. Soybean oil is mostly feedstock in the United State, whereas rapeseed oil and palm oil are widely used in Europe and tropical countries respectively (Hanna, N. A. et al., 2005). Variation of feedstock is listed in Table 2.1.

A suitable source for biodiesel production more than 95% of feedstock comes from edible oil. However, there are many concerns of edible oil such as fuel crisis, increasing price of vegetable oil plants that an affect to economical viability of biodiesel industry. The solutions can be achieved to reduce the edible oil for biodiesel production is by exploiting non-edible oils (Atabani, A. E. et al., 2012).

Table 2.1 Source of oil(Singh, S, P. et al., 2010).

Vegetable oils	Animal Fats	Other sources
Soybeans	Lard	Bacteria
Rapeseed	Tallow	Algae
Canola	Poultry Fat	Fungi
Safflower	Fishoil	Micro algae
Barley		Tarpenes
Coconut		Latexes
Copra		Cooking Oil (Yellow Grease)
Cotton seed		Microalgae (Chlorellavulgaris)
Groundnut		

2.2.1The chemical composition of biodiesel

Generally, the different sources of oils or fats have different fatty acid composition. The fatty acids compositions depend on the number of chain length and number of unsaturated bonds. Three moles of fatty acids and one mole of glycerol are commonly referred to triglycerides molecule (Fats and oils).The generally amount of triglycerides in oils or fats molecule is 90–98% and others consist of small amount of mono and di-glycerides (Shal, E. G. et al., 1993).Biodiesel is made from transesterification reaction, where derived from oils (vegetable oils, animal fats and waste oil) combine with alcohol (methanol and ethanol etc.). These can be produce alkyl ester molecule. The structure depends on chain of fatty acids. Chemical structures of oil, biodiesel and petroleum diesel are given Table 2.2.

Table 2.2 Chemical structures of oil, ester and diesel.

Chemical structure of Monoglyceride	Chemical structure of Diglyceride	Chemical structure of Fat & Oil	Chemical structure of Ester	Chemical structure of Diesel
H_2C-OCR_1 $HC-OH$ H_2C-OH	$H_2C-O-OCR_1$ $HC-O-OCR_2$ H_2C-OH			$C_{12}H_{23}$

Biodiesel and diesel fuels have different chemical structures that contain with carbon, hydrogen and oxygen atoms in the structure. The arrangement of diesel structures is straight chain or branch chain configuration (Hoekman, S. K. et al., 2012). The structures straight chainis better combustion quality than branched chain. Diesel fuel can contain both saturated and unsaturated hydrocarbons but the latter are not present in large enough amounts to make fuel oxidation problem. Generally,diesel from petroleum is composed of about 75% saturated hydrocarbons (primarily paraffins including n-paraffins, iso-paraffins, and cycloparaffins), and 25% aromatic hydrocarbons (including naphthalenes and alkylbenzenes). The average chemical formula for common diesel fuel is $C_{12}H_{23}$, ranging from approximately $C_{10}H_{20}$ to $C_{15}H_{28}$(Singh, S, P. et al., 2010).

Structure of biodiesel or fatty acid alkyl ester is according to structure of vegetable oils that contain fatty acids, free fatty acids (generally 1-5%), phospholipids, phosphatides, carotenes, tocopherols, sulfur compound and traces of water. The fatty acids are commonly found in vegetable oils, which include stearic, palmitic, oleic, linoleic and linolenic (Abreu, F. R. et al., 2004). Table 2.3 summarizes the fatty acids composition of oils.

Table 2.3 Names and chemical structures of common fatty acids composition(Singh, S, P. et al., 2010).

Name of fatty acid	Chemical name of fatty acids	Structure (xx:y)	Formula
Lauric	Dodecanoic	12:0	$C_{12}H_{24}O_2$
Myristic	Tetradecanoic	14:0	$C_{14}H_{28}O_2$
Palmitic	Hexadecanoic	16:0	$C_{16}H_{32}O_2$
Stearic	Octadecanoic	18:0	$C_{18}H_{36}O_2$
Oleic cis-9-	Octadecanoic	18:1	$C_{18}H_{34}O_2$
Linoleic cis-9, cis-12-	Octadecadienoic	18:2	$C_{18}H_{32}O_2$
Linolenic	cis-9,cis-l2,cis-15-Octadecatrienoic	18:3	$C_{18}H_{30}O_2$
Arachidic	Eicosanoic	20:0	$C_{20}H_{40}O_2$
Behenic	Docosanoic	22:0	$C_{22}H_{44}O_2$
Erucle	cis-13-Docosenoic	22:1	$C_{32}H_{42}O_2$
Lignoceric	Tetracosanoic	24:0	$C_{24}H_{48}O_2$

2.2.2Fatty acids on biodiesel production(Demirbaş, A. 2003)

Oils usually contain fatty acids, free fatty acid (FFA), water, sterols, phospholipids, odorants and other impurities (Markley, K. 1960). The fatty acids, which found in vegetable oils are different such as stearic, palmitic, oleic, linoleic and linolenic, which a variety of these have an affect on molecule of triglyceride. The molecular weight

of triglycerideis among 800 to 900 (Goering, C. E. 1988).In Table 2.4shows different fatty acids composition, which respective number of carbon present in the structure of oils and alkyl ester.

Table 2.4 Free fatty acid composition for different oils (Demirbaş, A. et al., 2003)

Vegetable oil	Fatty acid composition (%w/w)							
	16:0	**16:1**	**18:0**	**18:1**	**18:2**	**18:3**	**20:0**	**22:0**
Cottonseed	28.7	0	0.9	13.05	57.4	0	0	0
Rapeseed	3.5	0	0.9	64.1	22.3	8.2	0	0
Sunflower	9	-	2	17	74	0	0	0
Linseed	5.1	0.3	2.5	18.9	18.1	55.1	0	0
Palm	42.6	0.3	4.4	40.5	10.1	0.2	-	-
Corn	12	-	2	25	6	1	0	0
Tallow	23.3	0.1	19.3	42.4	2.9	0.9	-	-
Soybean	13.9	0.3	2.1	23.2	56.2	4.3	-	-
Mahua	16-28	-	20-25	4.4	0.8	0	0-3.3	0
Castor	1.1	0	3.1	4.9	1.3	0	-	-
Coconut	9.7	0.1	3.0	6.9	2.2	0	-	-
Peanut	11.4	0	2.4	48.3	32.0	0.9	1	2

2.3 Catalyst for biodiesel production

2.3.1 Base catalyst

The reaction mechanism for alkali-catalyzed transesterification was produced in three steps as shown in Scheme 2.3. The first step is an attack on the carbonyl carbon atom of the triglycerides molecule by the anion of the alcohol. After that, tetrahedral form intermediate reacts with an alcohol (methanol) to regenerate the anion of alcohol. Last step, rearrangement of tetrahedral intermediate produces the formation of a fatty acid ester and a di-glyceride.The alkaline catalysts are widely used such as NaOH, KOH, K_2CO_3 or other similar catalysts. For alkali catalyzed transesterification, the triglyceride must contain the lower FFAs (free fatty acids) and the alcohol used is anhydrous due to occurrence of water has an affect to reaction, which produces soap through saponification (Rashid, U. et al., 2008).Advantages of alkali catalyst including easily react with alcohol to form the alkoxide group lead to complete the reaction, high yields of ester under mild conditions and short time for completion of the reaction.
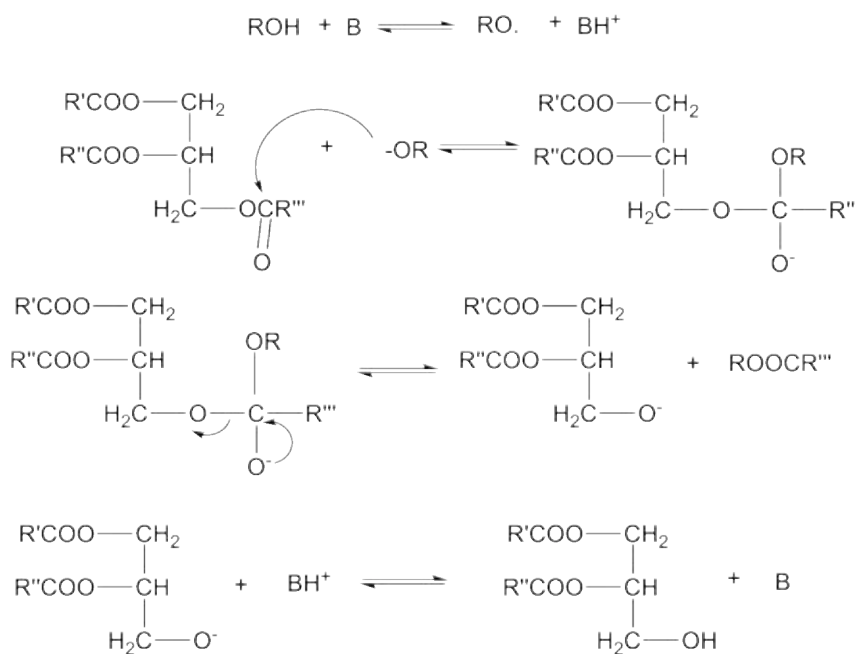
Scheme 2.3 Mechanism of the base-catalyzed transesterification of vegetable oils (Lee, D. W. et al., 2003)

2.3.2 Acid catalyst

An alternative process in acid catalyst for transesterification reaction of biodiesel production can be produce very high yields in alkyl esters, but disadvantage of the reactions are slow, temperatures are above 100 °C and reaction must be over 3 h to reach complete conversion. The mechanism of acid catalyzed transesterification of vegetable oil is shown in Scheme 2.4. However, it can be extended to di- and triglycerides. In the first step, the protonation of carbonyl group of the ester is change to the carbocation (II) and a nucleophilic attack of the alcohol, which produces a tetrahedral intermediate (III). This intermediate eliminates glycerol to form a new ester (IV) and to regenerate the catalyst (H+) (Schuchardt, U. et al., 1998).
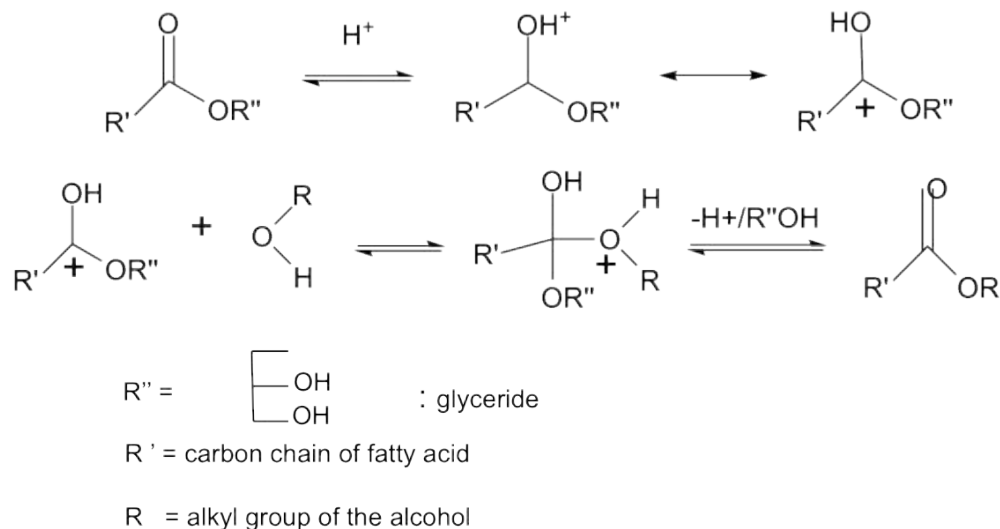
Scheme 2.4 Mechanism of the acid-catalyzed transesterification of vegetable oils (Schuchardt, U. et al., 1998).

2.3.3 Enzymatic catalyst

Transesterification process by using lipase as a catalyst is rather like an alkali catalyst in the term of ratio of catalyst and solvent. The process is using lipases as biocatalysts comparatively to alkali for biodiesel production are summarized

in Table 2.5.Enzymes catalyzed transesterificationdo not form soaps and can esterify both FFA and TAG through one step without washing step. Generally, the best enzymes can catalyze triglyceride to produce ester conversion over 90% and optimum temperature varies between 30 and 50°C (Fjerbaek, L. et al., 2009).

Table 2.5 Comparison of enzymatic technology versus chemical (alkaline) technology for biodiesel production (Casimir, C. A. et al., 2007).

Parameter	Enzymatic process	Alkaline process
FFA content in the raw material	FFA are converted to biodiesel	Soaps formation
Water content in the raw material	It is not deleterious for lipase	Soaps formation. Oil hydrolysis resulting more soaps
Biodiesel yield	High, usually around 90%	High, usually >96%
Reaction rate	Low	High
Glycerol recovery	Easy, high grade glycerol	Complex, low grade glycerol
Catalyst recovery and reuse	-Easy -Reusability proved but not sufficiently studied.	-Difficult; neutralized by an acid Partially lost in post-processing steps
Energy costs	Low, Temperature: 20-50 °C	Medium, Temperature: 60-80 °C
Catalyst cost	High	Low
Environmental impact	Low; wastewater treatment not needed	High; wastewater treatment needed

Lipases (triacylglycerol hydrolase, EC 3.1.1.3) are used for catalysis of the hydrolysis of carboxylic ester link in the triacylglycerol molecule to form free fatty acids, di- and monoglycerides and glycerol. For this reason, they can catalyze hydrolysis, alcoholysis, esterification and transesterification and widely used in spectrum of biotechnological applications. These are occurrence in all-living organisms and can be classified in intracellular and extracellular. The classification of lipase on the basis of the sources obtained from microorganism, animal, and plant. Lipases can be produced in high yields from microorganisms such as bacteria and fungi. For industry, microbial lipases are mostly used. The selection of a lipase for lipid modification is based on the nature of modification sought, for instance, position-specific modification of triacylglycerol, fatty acids specific modification, modification by hydrolysis, and modification by synthesis (direct synthesis and transesterification). The literature survey showed the use of lipases from some of the following sources. Microbial lipases are derived from *Aspergillus niger, Bacillus thermoleoVorans, Candida cylindracea, Candida rugosa, Chromobacterium* Viscosum, *Geotrichum candidum, Fusarium heterosporum, Fusarium oxysporum, Humicola lanuginose, Mucor miehei, Oospora lactis, Penicillium cyclopium, Penicillium roqueforti, Pseudomonas aeruginosa, Pseudomonas cepacia, Pseudomonas fluorescens, Pseudomonas putida, Rhizopus arrhizus, Rhizopus boreas, Rhizopus thermosus, Rhizopus usamii, Rhizopus stolonifer, Rhizopus fusiformis, Rhizopus circinans, Rhizopus delemar, Rhizopus chin- ensis, Rhizopus japonicus* NR400, *Rhizopus microsporus, Rhizomucor miehei, Rhizopus nigricans, Rhizopus niveus, Rhizopus oryzae, Rhizopus rhizopodiformis, Rhizopus stolonifer* NRRL 1478, *Rhodotorula rubra,* and *Staphylococcus hyicus* (Ghaly, A. E. et al., 2010).

A widely number of lipases from different sources has been benefited for biodiesel synthesis with various oils and acyl acceptors (alcohols or esters) as shown in Table 2.6.*Candida antarctica*lipase Bhas been the most studied enzyme for biodiesel production, which shows broad substrate specificity and high stability in organic solvents. However, the lipase from *Pseudomonas* has also many advantages as same as

C. Antarctica considering enzyme stability in a water-containing system in the presence of methanol. Many research reported that the lipase from *P. cepacia* has much higher methanol resistance than lipase from *Rhizopus oryzae*, *P. roqueferoti*, *C. lipolytica* and even *C. antarctica*, making it more attractive for use as a biocatalyst in methanolysis reaction processes and a rather high yield of biodiesel (>90%). Other lipases also have been reported to be an efficient biocatalyst for the methanolysis reaction in the solvent-free system. The main characteristic of lipases for biodiesel production from triacylglycerols is that they should be nonstereospecific so that all tri-, di- and mono-glycerides can convert to fatty acid alkyl esters. It is also an imperative that they should catalyze the esterification of free fatty acids. In addition, commercially available lipases (Lipozyme, Novozyme) appear to be more suitable catalysts for large-scale application, since the use of free *Pseudomonas* or other lipases requires the additional immobilization step.

Table 2.6 Enzymatic production of biodiesel with various lipases.

Lipase	Oil	Acyl acceptor	Solvent	Time	Yield	Authors
Candida antarctica B	Sunflower oil	Methanol	Propanol	24h	93.2%	Deng, L. et al., 2005
Pseudomonas cepacia	Jatropha oil	Ethanol	Solvent-free	8h	~98%	Shah, S. et al., 2007
Candida antarctica	Cotton seed oil	Methanol	Tert-butanol	7h	91.5%	Kose, O. et al., 2002
Pseudomonas cepacia	Soybean oil	Methanol	Solvent-free	90h	>80%	Noureddini,H. et al., 2003
Thermomyces lanuginosa	Soybean oil	Ethanol	solvent-free	24h	96%	Costa, R. R. et al., 2008
Rhizomucor miehei	Sunflower oil	Methanol	n-hexane	30h	>80%	Soumanou, M. M. et al., 2003
Pseudomonas cepacia	Mahua oil	Ethanol	Solvent-free	12h	>98%	Kumari, V. et al., 2007
Pseudomonas cepacia	Sapium sebiferum oil	Methanol	Solvent-free	12h	97%	Li, Q. et al., 2010
Pseudomonas fluoresces	Sunflower oil	Iso-butanol	Solvent-free	24h	45.3%	Deng, L. et al., 2005

2.4 Lipase from *Pseudomonas cepacia*

The structures of lipases from many sources are ranging from microbial (including fungi, yeast and bacteria) to enzymes. These conform to the α/β-hydrolase fold and show point of serine hydrolases with catalytic triads similar those of serine proteases. The first three-dimensional structures of lipases, which the surface loops act to be a lid cover the active sites and clearly indicated the essential of a conformational change to show up the catalytic site.

The active site of this lipase is comprised of Ser87, His286 and Asp264, which form a number of hydrogen bonds (Schrag, J. D. et al., 1996). Figure 2.1 shows a ribbon diagram of *Pseudomonas cepacia* along with a schematic of the topology of the protein. Many of the features of the α/β-hydrolase fold are maintained. The central β sheet in the core of the molecule conforms to strands β3–β8 of the α/β-hydrolase fold. Throughout this report the β strands will be numbered to be consistent with the numbering of the consensus α/β-hydrolase fold, so the first strand is named β3. The active site Ser87 lies at the C-terminal end of strand β5 in the strand-turn-helix.

Figure2.1 A ribbon diagram of *Pseudomonas cepacia*(Schrag, J. D. et al., 1996).

This lipase is called *Burkholderia cepacia* lipase, also known under its commercial trade name as lipase PS). This has been recently proven to be among the most versatile lipases (Yun, L. et al., 2010). This lipase was used for immobilized onto various supports with different methods such as encapsulation within hydrophobic sol-gel support (Noureddini, H. et al., 2005), adsorption on celite (Shah, S. et al., 2007) or crosslink with PAN nanofibrous (Sheng, F. L. et al., 2011). However, Lipase PS catalyst can produced high yield of biodiesel through transesterification reaction and was able to use in range of temperature is 30 to 50 °C (Qin, L. et al., 2010). Furthermore, can tolerate the inhibition of alcohols (Ranganathan, S. V. et al., 2008) and was able to use in high free fatty acid of oil content (Bajaj, A. et al., 2010).

2.5 Immobilized enzyme

The immobilization of lipase by binding them in some way onto substance (inert polymer or insoluble polymer was studied by the forefront scientists (Medina, A. R. et al., 2009). Of the several method methods in used for the immobilization of biological molecules, all have advantage and drawbacks in any given case. The properties of immobilized lipase consisted of increasing thermo-stability, activity, recovery the biocatalyst and reusability. For this reason, immobilized lipase can be reducing the cost of the production. The several methods for the immobilization can be classified in different way, which depended on the kind of interaction causing the immobilization, on the nature of the support or on the characterization o the result of aggregate (Roig, M, G. et al., 1986).

The physical immobilization procedures involve group of enzyme immobilization depends on the action of physical forces (electrostatic interactions), or on entrapment of enzymes inside microcapsule or on the inclusion of the enzyme within the special membrane. These methods are reusable enzyme in its active state. But disadvantage of method are incompletely recovered and some inactivation does take place (Mateo, C. et al., 2007).

Chemical immobilization procedures employed involve the formation of covalent bond between enzyme molecules by means of bi- or multifunctional reagent, leading to three dimensional crosslink aggregates. However, these methods are irreversible coupling due to the limited nature of the chemical reactions used in immobilization and cannot being regenerate the original enzyme. At the some case, the enzyme immobilization can be reversible because they may be achieved by coupling enzymes to aldehyde or sulfhydryl containing water insoluble polymers(Mateo, C. et al., 2007).

The main advantages of immobilized enzymes are including:

(1) Multiple or repeated use of the enzyme,

(2) Easy to separate the enzyme from the product,

(3) Increased operational stability of the enzyme,

(4) Product without contamination with the enzyme(Gog, A. et al., 2012)

2.5.1 Method for immobilization

Many methods of immobilization concern both chemical and physical treatments, and the nature of the interaction forces for the immobilizations are several varieties or not easily categorized. However, there are commonly four methods for immobilizing enzyme: adsorption (physical methods) crosslinking, entrapment (in a polymer matrix, in microcapsules, in droplets) and chemical bonding (crosslinking methods).

1. Adsorption

Adsorption is the simplest and cheap method of immobilization. The enzymes are adsorbed onto a support such as polymer beads and film material. Commonly, interactions between the support and the enzyme are based on physical adsorption or ionic binding such as hydrophobic interactions, ionic, hydrogen bonding and van der waals forces. All of these interactions are noncovalent. However, interactions between the enzyme and the support material are depended on the basic surface chemistry of the support and kind of amino acids appear on the surface of the enzyme molecule. Adsorption between enzymes and supports process is shown in Figure 2.2.
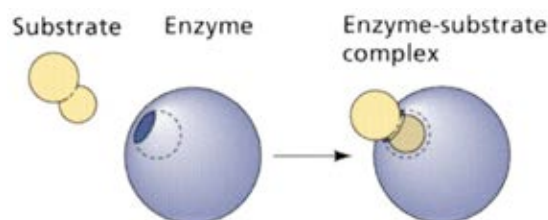
Figure 2.2 Biocatalysts bound to a support by adsorption (Roig, M, G. et al., 1986).

The support materials include inorganic supports, organic synthesis supports and natural organic supports such as ceramic, alumina, nylon, polystyrene and chitosan etc. Adsorption method for immobilization is more advantages, which including simplicity to process, easy to perform process, and possibility of regenerating inactive enzyme by addition of fresh enzyme. Furthermore, main disadvantage of this method was desorption of the biocatalyst from the support because of the weak interactions were established. The enzyme desorption can easily occur by changes in the environment medium such as pH, temperature, solvent, and ionic strength or in the case of extended reactions.

2. Entrapment

The entrapment method for immobilization consists of the physically entrapped within the matrix, film, gel or microencapsulation and cannot escape by permeation (see in Figure 2.3) (Roig, M, G. et al., 1986). This method can be achieved by entrapping one or more molecules of enzyme inside a cross-linked gel matrix. The polymers as a matrix are formed in an aqueous solution and then produce a lattice structure. Molecules of enzyme are entrapped by spherical semi permeable of polymer membranes, which formed to microencapsulation with controlled porosity.
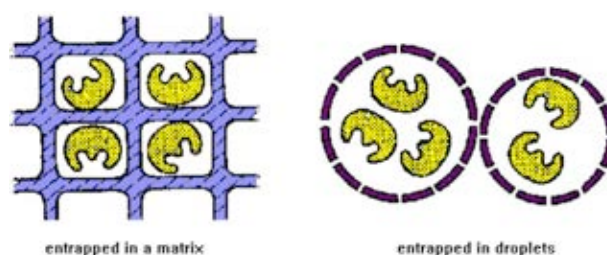
Figure 2.3 Enzyme encapsulation in (a) a matrix and (c) capsule.

Since the encapsulation of dyes, drugs, and other chemicals has been known for long time until 1960s the enzymes have been successfully immobilized by this method. The main advantages are the extremely large surface area between the substrate and the enzyme, small volume in the encapsulation, and the real possibility of simultaneous immobilization. The major disadvantages of this method include the infrequent inactivation of enzyme during microencapsulation and the high enzyme concentration required (Costa, S. A. et al., 2004).

3.Crosslinking

This method is based on the formation of covalent bonds between enzyme molecules. There are bi- or multifunctional reagent such as glutardialdehyde, glutaraldehyde, glyoxal, diisocyanates, hexamethylene diisocyanate, toluene diisocyanate, etc, which use for help enzyme can be attach to the support. Enzyme crosslinking involves normally the amino groups of the lysine but, in occasional cases, the sulfhydryl groups of cysteine, phenolic OH groups of tyrosine, or the imidazol group of histidine can also be used for binding. Figure 2.4 shows crosslinking method between molecules of enzyme.

Cross-linked enzyme
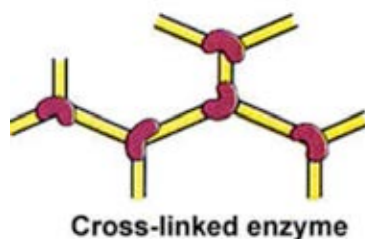
Figure 2.4 Crosslinking between molecules of enzyme(Roig, M, G. et al., 1986).

The main advantage is the simplicity of the process. The disadvantages are the particles can be fragile in some cases and the particles have limited to diffusion. However, the immobilized biocatalysts in this way frequently undergo changes in conformation with a resultant loss of activity. Therefore, glutaraldehyde or

epochlorohydrin used as linker agent to crosslink with biocatalyst. This linker can be producing a treatment of bacterial cell masses with glutaraldehyde that have formed fine particles in the large-scale.

2.6 Properties of biodiesel (Singh, S, P. et al., 2010)

Since biodiesel is a green energy and characteristics are similar to diesel fuels and it also becomes an attention fuels to replace the diesel fuels. Transesterification process is the reaction of triglycerides as oils is converted to alkyl esters (biodiesel) that have more advantage including clean fuel (cause it has no sulphur), increase higher cetane number(cause no aromatics and has oxygen about 10%). The cetane number of biodiesel is around 50 (See in Table 2.7).

Table 2.7 Physical properties of biodiesel obtained from some vegetable oils (Jaichandar, S. et al., 2011).

Vegetable oil methyl ester	Kinematic viscosity at 38 °C (cSt)	Cetane no.	Lower heating value (MJ/kg)	Cloud point (°C)	Pour point (°C)	Flash point (°C)	Density (kg/l)
Peanut	4.9 (37.8 °C)	54	33.5	5	-	176	0.883
Cottonseed	33.5	41.8	39.5	1.7	-15.0	234	0.915
Soybean	32.6	37.9	39.6	-3.9	-12.2	254	0.914
Palm	39.6	42.0	33.5	31.0	-	267	0.918
Sunflower	33.9	37.1	39.6	7.2	-15.0	274	0.916
Rapeseed	37.0	37.6	39.7	-3.9	-31.7	246	0.912
Diesel	3.06	50	43.8	-	-16	76	0.855

2.7 Literature review

2.7.1 Enzymaticreaction for biodiesel

In 2005, Noureddini and coworkers studied enzymatic transesterification of soybean oil with methanol and ethanol. Lipase PS from *Pseudomonas cepacia* as catalyst was immobilized by entrapment within chemically inert, hydrophobic sol–gel support, which was prepared by polycondensation of hydrolyzed tetramethoxysilane and iso-butyltrimethoxysilane. This lipase resulted in the highest yield of alkyl esters. The optimal conditions for processing 10g of soybean oil were: 1:7.5 oil/methanol molar ratio, 0.5g water and 475mg lipase at 35°C, and 1:15.2 oil/ethanol molar ratio, 0.3g water, 475mg lipase at 35°C. This reaction can be produce methyl and ethyl esters formation of 67 and 65mol% in 1h of reaction. The immobilized lipase so prepared was consistently more active than the free lipase toward the transesterification of soybean oil. The immobilized lipase also proved to be stable and lost little activity when was subjected to repeated uses.

In 2007Kumari and coworkers studiedimmobilization of lipase from *Pseudomonas cepacia* coated on polypropylene support, which was used as a catalyst for converting Mahua oil into ethyl esters. The crosslink agent for this reaction is 25 mM of glutaraldehyde, which appropriate for used to produce good modified support. Solvent-free systems were used for this process. The process optimization consisted of varying parameters, such as water content in the reaction medium and salt present during the drying of lipase prior to its use as powder. The lipase immobilized on this support gave 96% conversion in 6 hours. The best results were obtained using modified biocatalyst formulations, which are called cross-linked enzyme aggregates (CLEAs) and protein-coated microcrystals (PCMCs). The optimum condition for biodiesel production including molar ratio of oil: ethanol is 1:4, immobilized lipase 50 mg as catalyst at 40 °C with continuous stirring.

In 2007, Shah and coworkers studied alcoholysis of Jatropha oil, a non-edible oil, by a lipase for biodiesel production. The process of screening of lipases from *Pseudomonas cepacia, Pseudomonas fluorescens, Mucor javanicus, and Candida rugosa* for transesterification activity were studied. *Pseudomonas cepacia* lipase was the best-immobilized enzyme, which used as catalyst. This lipase was immobilized by physical adsorption method on celite supporter in phosphate buffer pH 7.0 via transesterification reaction with Jatropha oil: EtOH 1 : 4 molar ratio at 50 °C in the presence of 4–5% (w/w) water in 8 h. The best yield of biodiesel was 98% conversion. It was found that yields were not affected if analytical grade alcohol was replaced by commercial grade alcohol. This biocatalyst could be used four times without loss of any activity.

In 2008, Halim and coworkers studied transesterification reaction to produce fatty acid methyl ester (FAME) using waste cooking palm oil (WCPO) and methanol. *Candida antarctica* lipase B immobilized on acrylic resin (Novozyme 435) was used as biocatalyst. The optimum conditions for this reaction were the molar ratio 4: 1, 4% Novozyme 435 based on oil weight, 12h reaction time at 40 °C with a constant shaking. *Tert*-butanol was used as the reaction medium, which eliminated both negative effect caused by excessive methanol and glycerol as the byproduct. The FAME yield can reach up to 88%.

In 2010, Li and Yan studied transesterification of biodiesel production from *Sapium sebiferum* oil with methanol catalyzed by immobilized lipase from *Pseudomonas cepacia* G63. The optimal conditions for biodiesel preparation were: 4:1 methanol/oil molar ratio, 2.7% (w/w) lipase at 41 °C, and gave biodiesel yield at 97.07% under the optimal conditions, and R^2 = 98.19% show the model was considered to be accurate and availability of predicting the yield of biodiesel. There was no loss lipase activity after being repeatedly used for 20 cycles at the optimal reaction condition. The results suggested that using this immobilized lipase, as catalyst for biodiesel production from *S.Sebiferum* oil could be a promising way.

2.7.2 Immobilization of enzyme

In 2008, Dizge and coworkers studied method for immobilization of lipase within hydrophilic polyurethane foams using polyglutaraldehyde was developed for the immobilization of *Thermomyces lanuginosus* lipase to produce biodiesel with canola oil and methanol. The enzyme optimum conditions were not affected by immobilization and the optimum pH for free and immobilized enzyme were 6, resulting in 80% immobilization yield. 430 μg of immobilized lipase *T. lanuginosus* catalyzed transesterification of 20 g of canola oil with methanol. Reaction parameters consisted of 1:6 oil/methanol molar ratios, 0.1 g of water at 40 °C. It gave maximum methyl esters yield at 90%. The activity of enzyme remained after 10 batches.

In 2009, Dizge and coworkers studied methanolysis of canola oil with methanol and using immobilization of lipase from *Thermomyces lanuginosus* as catalyst. This lipase was immobilized by both physical adsorption and covalent attachment onto polyglutaraldehyde-activated styrene–divinylbenzene (STY–DVB) copolymer, which is synthesized by using high internal phase emulsion (polyHIPE). Lipase from *T. lanuginosus* was immobilized with 60% and 85% yield on the hydrophobic microporous STY–DVB copolymer and STY–DVB–PGA copolymer, respectively. Biodiesel production using the latter lipase preparation was realized by a three-step addition of methanol to avoid strong substrate inhibition. Under the optimized conditions, the maximum biodiesel yield was 97% at 50°C in 24 h reaction. The immobilized enzyme retained its activity during the 10 repeated batch reactions.

In 2009, Miletic′ and coworkers studied immobilization of *Candida antarctica* lipase B (Cal-B) on crosslinked macroporous hydrophilic poly(glycidyl methacrylate- co-ethylene glycol dimethacrylate) [abbreviated poly(GMA-co- EGDMA)] with good results. The supporters were synthesized by modifications of poly(GMA-co-EGDMA) with various diamines, 2-fluoroethylamine, glutaraldehyde and cyanuric chloride. *Candida Antarctica* lipase B (Cal-B) immobilized at 30 °C and pH 6.8 was leading to increased activities between enzyme and matrix beads. Physical adsorption method for

immobilization enzyme and copolymer of the supporters in which amine or fluorine groups were introduced into the supporter structure by modification with various diamines or 2-fluoroethylamine and these can be produce decrease activity. In contrary, modification of the supporters with glutaraldehyde and cyanuric chloride results in a covalent bonding between enzyme and supporters. The obtained results show a significant increase in Cal-B activity. Glutaraldehyde and cyanuric chloride used for modification resulted in an increase of the enzyme loading and activity of immobilized *C. antarctica* lipase B. These are showing the highest value for 0.66% and 0.050% w/w, respectively.

In 2009, Song and coworkers studied immobilization of lipase from *Candida rugosa* on modified and unmodified chitosan beads (CBs). Amino acid used as coupling for modification of chitosan beads, which were prepared by activation of a chitosan backbone with epichlorohydrin followed by amino acid coupling. The immobilized lipase on unmodified chitosan beads showed the highest immobilization yield (92.7%), but its activity was relatively low (10.4%). However, in spite of low immobilization yields (15–50%), the immobilized lipases on the amino acid modified chitosan beads showed activities higher than that of the unmodified chitosan beads. Amino acids are Ala or Leu, which modified chitosan beads (Ala-CB or Leu-CB) with 49% activity for Ala-CB and 51% for Leu-CB. The immobilized lipases on Ala-CB improved thermal stability at 55°C, compared to free and immobilized lipases on unmodified chitosan beads and the activity of immobilized lipase on Ala-CB retained 93%.After use for 10 times, the activity of the immobilized lipase on Ala-CB retained 77%.

In 2010, Li and coworkers studied a new method for immobilization of enzyme on different polymer films. In the process, a polystyrene-based diazonium salt (PS-DAS) used as molecular adhesive to immobilize β-glucosidase on the polymeric supports such as films of polyethylene, polypropylene and poly(ethylene terephthalate).The interaction between PS-DAS layer and β-glucosidase can be attributed to the covalent bonding, which is formed through the azo-coupling reaction between the diazonium salts and the

amine groups of the enzyme. The techniques for test surface modification were X-ray photoelectron spectroscopy (XPS), water contact angle measurement, and atomic force microscopy (AFM). The activity of immobilized enzyme was measured to be 26.4 U/mg, 39.8 U/mg, and 41.4 U/mg for those immobilized on the PE, PP and PET films, respectively. The immobilized enzyme shows changes in the optimized pH and temperature for the hydrolysis reaction catalyzed by β-glucosidase. The kinetic parameter (K_m) of the immobilized β-glucosidase is lower than that of its free enzyme. The immobilized enzyme shows significant enhancement in the thermal stability and reasonable reusability.

CHAPTER III

EXPERIMANTAL

3.1 Materials and equipments

3.1.1 Chemicals

1. Methanol; analytical grade; Merck

2. Ethanol; absolute GR for analysis grade; Merck

3. *tert*-Butanol; for synthesis grade; Merck

4. Hexane; analytical grade; Merck

5. Ethyl acetate; analytical grade; Fisher scientific

6. Glutaraldehyde solution; Sigma

7. Sodium hydroxide; Merck

8. Styrene monomer; Merck

9. Disodium hydrogen phosphate; Carlo erba

10. Potassium dihydrogen phosphate; Carlo erba

11. Sodium chloride; Carlo erba

12. Potassium chloride; Carlo erba

13. Pre-expanded polystyrene bead or expandable polystyrene bead (EPS bead) (for preparation of the expanded polystyrene foam; IRPC Co., Ltd.

14. Sodium cabonate; Merck

15. Potassium persulphate; Merck

16. Sorbitanmonooleate (Tween 80); Ajax Finechem Pty., Ltd.

17. Lipase from *Pseudomonas cepacia (P.cepacia)*, activity = 37.8 U/mg; Sigma

18. Vanillin solution (containing vanillin (1%) and conc.H_2SO_4(4.5%) in ethanol)

19. Chloroform-d (CDCl$_3$); NMR spectroscopy grade; Merck KGaA Darmstadt, Germany

20. *p*-nitrophenolpalmitate; Sigma

21. *p*-nitophenol; Hopkin and Williams Ltd.

3.1.2 Equipments

1. Hotplate stirrer with magnetic bar

2. Thermometer

3. Vessel vial, round bottom flask, volumetric flask and Erlenmeyer flask

4. Beaker

5. Filter paper

6. Centrifuge

7. Rotary evaporator

8. Buchner funnel

9. UV spectrometer; HP 8453

10. FT-IR spectrometer; Nicolet6700. Attenuated Total Reflection (ATR) formally examined solid samples. In ATR, a highly refracting prism (diamond probe) is used to contact to the sample. The absorption band was analyzed.

11. NMR spectrometer;[1]NMR was recorded on Varian Model Mercury +400 nuclear magnetic resonance spectrometer operating at 100 MHz for [1]H. Chemical shifts are reported in part per million (ppm) relative to tetramethylsilane (TMS) or using the residual protonated solvent signal as a reference (for ^{1}H-NMR; CDCl$_3$ 7.26 ppm, MeOD 3.31 ppm, DMSO-d$_6$ 2.50 ppm and D$_2$O 4.79 ppm).

3.2 Preparation of solid supports

3.2.1 Preparation of polyglutaraldehyde (PGlu) solution

Glutaraldehyde solution (25%w/v) was diluted to give concentration of 20%, 15% and 10%. Each concentration of glutaraldehyde was basified to pH 10.5 by 1 M NaOH solution at room temperature. After 15 mins, this polyglutaraldehyde (PGlu) suspension

was ready for preparation of polyglutaraldehyde-styrene copolymer coated expandable polystyrene bead for immobilization of enzyme.

3.2.2 Coating polyglutaraldehyde-styrene copolymer onto expandable polystyrene bead (PGlu-STY/EPS bead)

To a mixture of styrene monomer (1.00g, 28.88 mmol) and Tween 80 (0.88 g) in 250 ml round bottom flask a potassium persulphate ($K_2S_2O_8$) (0.35g, 2.59 mmol) and polyglutaraldehyde suspension were added (Dizge,N. et al., 2009). After stirring for 15 min, expandable polystyrene bead (4.55 g) was gradually added into the mixture with continuously stirring at 60 ℃. After 2 hours, the mixtures were heated to 80 ℃ and stirring was continued for 22 hours. The reaction mixture was then neutralized by adding 1M HCl solution. The resulting bead was filtered and washed with distilled water to obtain polyglutaraldehyde-styrene copolymer coated expandable polystyrene bead (PGlu-STY/EPS bead). The PGlu-STY/EPS bead obtained was dried in desiccators.

```
                    ┌─────────────────────────┐
                    │ Polymerization of styrene │
                    └─────────────────────────┘
┌─────────────────────┐             │
│ Polyglutaraldehyde   │ ──────→     │
│ (PGlu) suspension    │             ▼
└─────────────────────┘   ┌──────────────────┐
                          │ Crosslink process │
                          └──────────────────┘
┌─────────────────────┐             │
│ Polystyrene beads    │ ──────→     │
└─────────────────────┘             ▼
                          PGlu-STY-polystyrene
                                beads
                                   │
                                   │   Washing &
                                   │   Filtrate
                                   ▼
                          PGlu-STY-polystyrene
                                beads
```
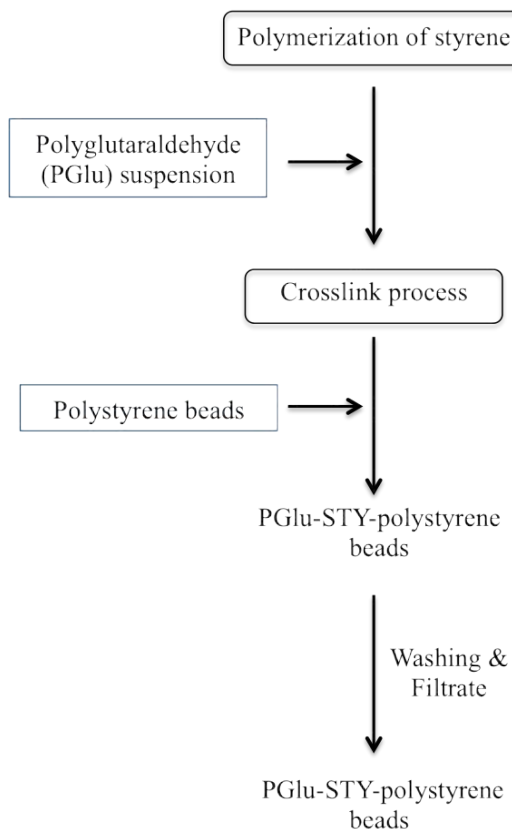
Scheme 3.1 Process of preparation of PGlu-STY/expandable polystyrene bead in the laboratory scale

3.2.2.1 Optimization of conditions for preparation of PGlu-STY/expandable polystyrene bead

In order to obtain the suitable PGlu-STY/EPS bead for immobilazation, the optimum conditions for preparation of PGlu-STY/EPS bead was determined by changing individually the parameters (concentration of styrene monomer and amount of polyglutaraldehyde-styrene copolymer).

1. Effect of concentration of styrene monomer

In order to reduce a brittleness of PGlu-STY copolymer on surface of the PGlu-STY/EPS bead (from preparation in 3.2.1) 20%, 15%, 10% and 5% of styrene monomer

was used during coating on expandable polystyrene bead.

2. Effect of copolymer coating time

The preparation of polyglutaraldehyde-styrene copolymer coated onto expandable polystyrene bead (PGlu-STY/EPS bead) was investigated on variation of time at 4, 6, 8, 12 and 24 hours, respectively. This experiment was performed to determine the optimal time of copolymer yield, that coating on PGlu-STY/EPS bead.

3.2.3 Synthesis of polyglutaraldehyde-styrene copolymer (PGlu-STY copolymer)

Synthesis of polyglutaraldehyde-styrene copolymer was carried out by emulsion polymerization as described by Sandler (Sandler, R. S. et al., 1998). To a mixture of styrene monomer (3.01g, 28.88 mmol) and Tween 80 (1.75 g) in the 250 ml two-neck round bottom flask with condenser, potassium persulfate (0.7g, 2.59 mmol) and distilled water (24 ml) were added for continuous stirring at 60 °C for 2 hours, polyglutaraldehyde (20ml) was added into the mixture and the temperature was raised to 80 °C for 24 hours. The resulting copolymer was filtered and washed with distilled water to obtain polyglutaraldehyde-styrene copolymer. The styrene-polyglutaraldehyde copolymer was subjected to FTIR analysis. The flow diagram for making crosslinked the poly (styrene-glutaraldegyde) support in the laboratory scale was showed in Scheme 3.2.

```
┌─────────────────────────────┐
│  Polymerization of styrene  │
└─────────────────────────────┘
              │
              ▼
┌──────────────────────┐
│  Polyglutaraldehyde  │ ─────▶
│   (PGlu) suspension  │
└──────────────────────┘
              │
              ▼
┌─────────────────────────┐
│    Crosslink process    │
└─────────────────────────┘
              │
              ▼
     styrene-polyglutaraldehyde
            copolymer
              │
              │   Washing &
              │    Filtrate
              ▼
     Poly (styrene-glutaraldehyde)
            support
```
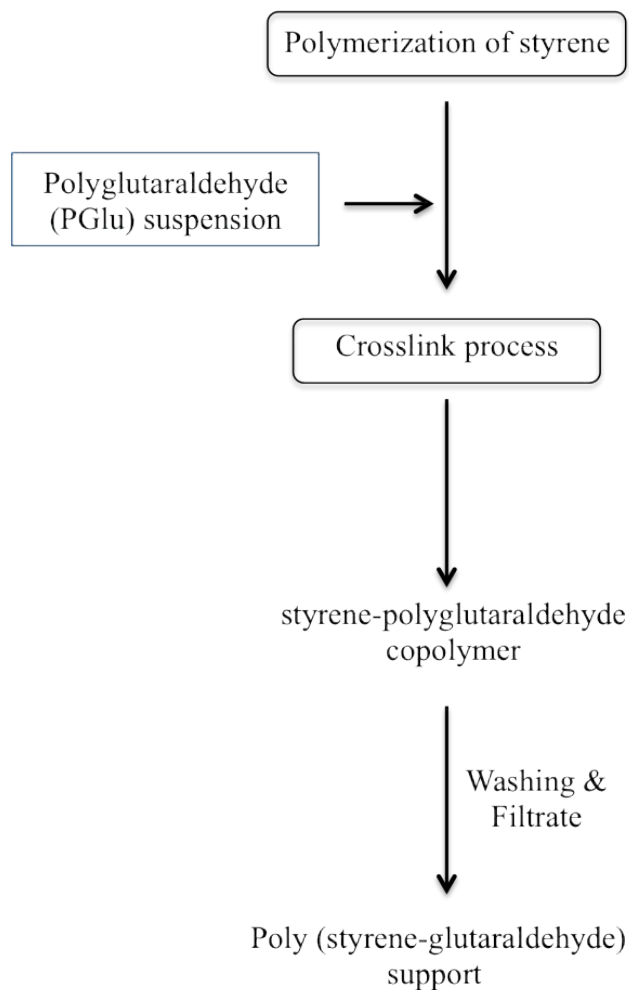
Scheme 3.2 Synthesis of polyglutaraldehyde-styrene copolymer as support in the laboratory scale

3.3 Immobilization of lipase

3.3.1 Immobilization of *Pseudomonas cepacia* (*P.cepacia*)

Lipase from *Pseudomonas cepacia* was immobilized on PGlu-STY/EPS bead and synthesized styrene-polyglutaraldehyde copolymer. 1 gram of the support was added into a solution of lipase 10 mg (37.8 U/g-support) in phosphate buffer (25mM, pH 7.0) 5 mL, and stirred at room temperature for 72 hour. The resulting suspension was filtered and assayed for unbound protein (see 3.3.1.1).

3.3.1.1 Estimation of immobilization

After immobilization process, a solution of lipase 0.01 ml was collected and put into sample cuvette with phosphate buffer 4.99 ml. This solution was measured at 322 nm by UV-spectrophotometer (HP 8453) and the protein-loading yield was calculated. Lipase immobilization onto polyglutaraldehyde-styrene (PGlu-STY) copolymer modified expandable polystyrene bead was evaluated as described by Dizge, N. et al., 2009.

$$\text{Lipase activity (U/g-support)} \quad = \quad \frac{\text{activity of immobilized lipase}}{\text{amount of support used}} \quad \text{x } 100$$

$$\text{Protein loading yield (\%)} \quad = \quad \frac{\text{amount of protein loaded}}{\text{amount of protein introduced}} \quad \text{x } 100$$

$$\text{Specific activity (U/mg-protein)} \quad = \quad \frac{\text{activity of immobilized lipase}}{\text{amount of protein loaded}} \quad \text{x } 100$$

3.3.1.2 Determination of lipase activity

Lipase activity was measured using 0.1 g of *p*-nitrophenyl palmitate (*p*-NPP) dissolved in 10 ml of ethanol as substrate. The increase in absorbance at 410 nm caused by the release of *p*-nitrophenol in the hydrolysis of *p*-NPP was measured by spectrophometrically. 200 mg of immobilized lipase was added to a mixture of 1 ml *p*-NPP solution and 1 ml of 0.25 M phosphate buffer (pH 7.0) and incubated for 5 min at room temperature. The reaction was terminated by adding 2 ml of 0.5 N of Na2CO3 followed by centrifuging for 10 min (Shao, H. et al., 2004).One unit (U) of enzyme activity was defined as the amount of enzyme, which catalyzed the production of 1 mmol *p*-nitrophenol perminute under the experimental conditions.(Shao, H. et al., 2004).

3.4 Enzymatic transesterification for biodiesel production

The immobilized lipase onto PGlu-STY/EPS bead and onto synthesized PGlu-polystyrene copolymer was used as catalyst for biodiesel production through transeserification reaction of soybean oil and anhydrous ethanol (dry EtOH) without co-solvent. The reaction parameters were investigated in term of the optimal molar ratio of alcohol: oil, catalyst dosage and temperature.

3.4.1 Preparation of anhydrous alcohol

Ethanol of a high degree of purity (~99.8 percentage purity) was prepared from alcohol (AR grade). To 10 ml of ethanol in round-bottomed flask 5 gram of clean dry magnesium turning and 0.5 gram of iodine were added. The mixture was warm until the iodine had disappeared (if a lively evolution of hydrogen did not occur, added a further 0.5gram of iodine. The reaction mixtures were heated until all magnesium was converted into ethanolate. Then added 10 ml of commercial ethanol and continuously reflux for 30 minutes. After that, the ethanol was distilled off into the vessel in which it was to be stored. However, using an apparatus similar to that investigated for preparation of the anhydrous methanol. The absolute alcohol was ready for used in transesterification reaction (Furniss, B. S. et al., 1989).

3.4.2 Transesterification reaction

The molar ratio of dry EtOH to oil was 5:1 and dry EtOH was added three-steps for biodiesel production. The reaction mixture was carried out by reacting immobilized lipase(0.46g) with soybean oil in the screw-capped vials and dry EtOH was added in three-step (0.56 ml in each addition). The mixture was continuously stirred at 40 °C with for 24 hours. After that, the mixtures were filtered to separate the catalyst and centrifuged to obtain the upper layer. This fatty acid ethyl ester (FAEEs) in upper layer was measured by NMR spectroscopy.

3.4.2.1 Optimization of the reaction parameter

1. Effect of molar ratio of ethanol/oil

The transesterification reaction was performed to determine the optimal molar ratio of soybean oil to dry EtOH. The different dry EtOH/oil ratios at 1:2, 1:3, 1:4, 1:5 and 1:6, respectively were investigated at 40 °C for 24 hours to produce ethyl ester production.

2. Effect of temperature

The variation of immobilized lipase was used an excellent catalyst in different temperature. The effect of temperature on transesterification for biodiesel production was performed at 25, 30, 40 and 50 °C.

In addition, the transesterification of soybean oil and anhydrous methanol (dry MeOH) was investigated. Dry MeOH was added three-steps for biodiesel production. 0.46 g of immobilized lipase and soybean oil (5g) were added in the screw-capped vials and dry MtOH was added in three-step (0.35 ml in each addition) with continuous stirring. The optimal conditions were molar ratio of dry MeOH to oil was 4:1at 40 °C with for 24 hours. After that, the mixtures were filtered to separate the catalyst and centrifuged to obtain the upper layer. This fatty acid methyl ester (FAEEs) in upper layer was measured by NMR spectroscopy.

3.5 Stability and reusability of the immobilized lipase

Generally, the tranesterification reaction was used to produce biodiesel and glycerol (by-product), which had a negative effect to enzyme catalyst. For this reason, lipase activity and stability were decreased (Wang, F. et al., 2006). The two different supports (PGlu-STY/EPS bead and synthesized PGlu-polystyrene copolymer) were immobilized by *Pseudomonas cepacia* lipase in phosphate buffer (25mM, pH 7.0) at

room temperature. This catalyst of transesterification reaction of soybean oil with three-step dry EtOH was investigated at 40°C for 24 hours (see 3.4.2). After that filtered the catalyst, centrifuged and kept the upper layer. The immobilized lipase on two different supports was used in the next batch reaction.

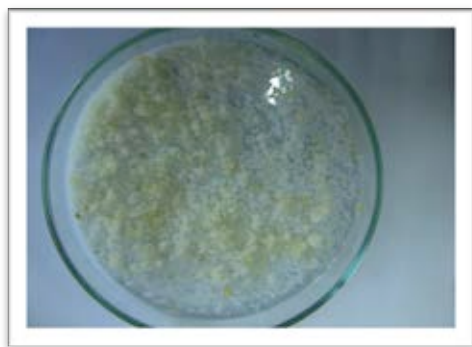# CHAPTER IV

RESULTS AND DISCUSSION

The process of expandable polystyrene bead support was coated by polyglutaraldehyde as a linker. The concentration of glutaraldehyde solution (Glu 25%w/v) was diluted by distill water to concentration at 20%, 15% and 10% and then coated on expanded polystyrene bead at 60ºC for 24 hours as shown in Figure 4.1.
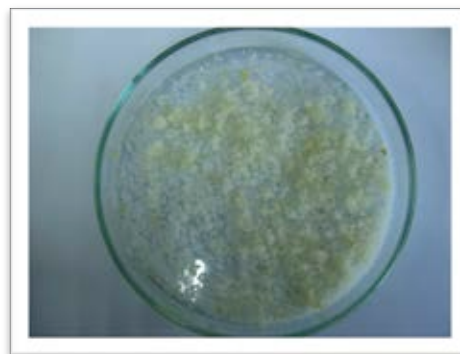

(a)   (b)

(c)   (d)

Figure 4.1 Variation of concentration of glutaraldehyde solution coated onto expandable polystyrene bead support (EPS bead) (a) 25%Glu coated on EPS bead (b) 20%Glu coated on EPS bead (c) 15%Glu coated on EPS bead and (d) 10%Glu coated on EPS bead

The results of the every concentration of glutaraldehyde solution (25%, 20%, 15% and 10%) were incompletely coated onto expandable polystyrene bead in each batch reaction because of glutaraldehyde solution could be polymerize by itself to give mass polymer and partial polyglutaraldehyde was brittle. For this reason, polyglutaraldehyde was crosslinked with styrene monomer, which used as linker for coating on expandable polystyrene bead.

4.1 The optimization factor of PGlu-STY-expandable polystyrene bead support

4.1.1 Effect of concentration of glutaraldehyde solution

Polyglutaraldehyde-styrene copolymers linker was used for coating on expandable polystyrene bead (PGlu-STY/EPS bead). This support was synthesized by emulsion polymerization. The effect of concentration of glutaraldehyde (GA) solution (the range from 10% to 25%) crosslinked with 10% of styrene monomer concentration for coating on expandable polystyrene bead was studied. The results as presented in Figure 4.2 that 10% glutaraldehyde (a) and 15% glutaraldehyde solution (b) crosslinked with 10% of styrene were incompletely coated on expandable polystyrene bead (To appear white color bead) because of low concentration of glutaraldehyde. At 25% glutaraldehyde solution crosslink with 10% of styrene were also incompletely coated on expandable polystyrene bead because of glutaraldegyde was polymerized by itself to gave mass polymer. For immobilization step, the modified support was tested lipase activity.
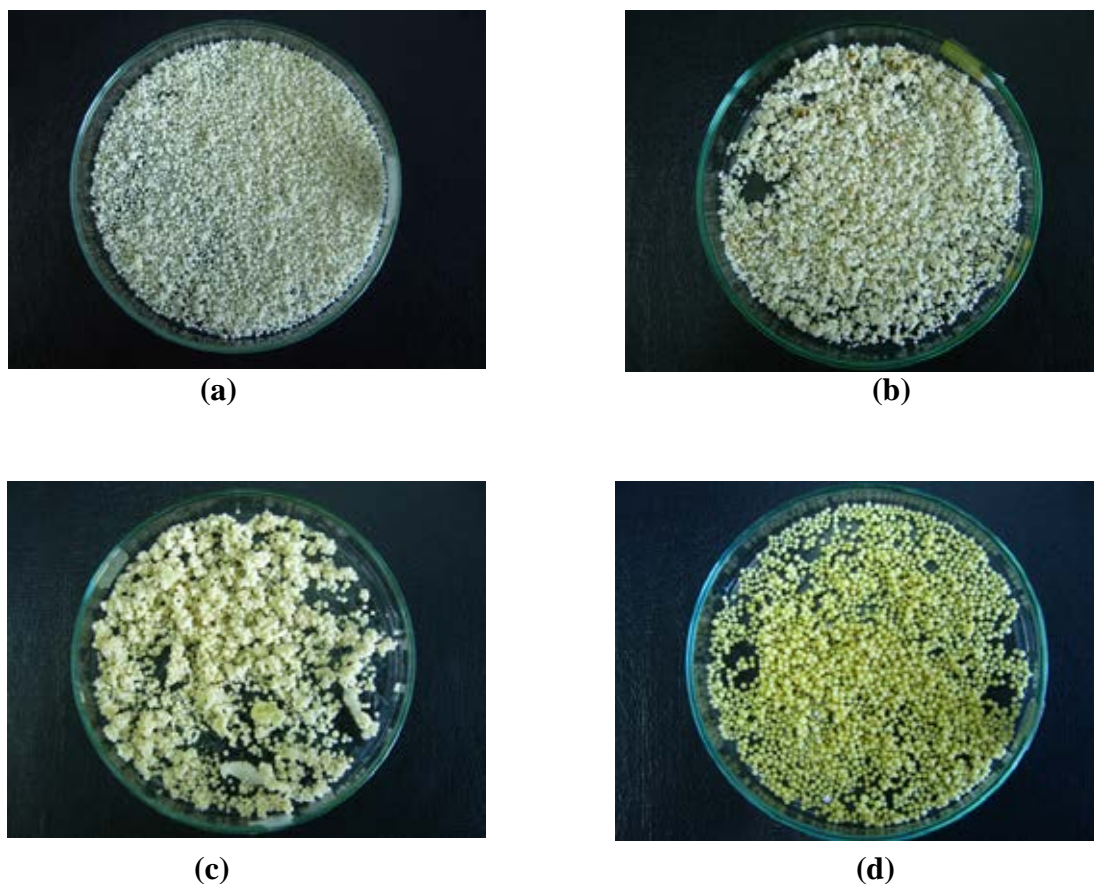
Figure 4.2 Comparison of glutaraldehyde concentration polymerized with 10%styrene monomer and then coated onto expandable polystyrene bead (a) 10% glutaraldehyge solution (b) 15% glutaraldehyge solution (c) 20% glutaraldehyge solution (d) 25% glutaraldehyge solution.

The concentration of 20% glutaraldehyde was successfully polymerized with 10% styrene monomer and then coated on expandable polystyrene bead because of this concentration hardly polymerized by it-self. The 20%Glu-10%STY/expandable polystyrene bead support was smooth surface. For immobilization test, lipase 1 mg immobilized onto four supports (25%Glu-STY/EPS bead, 20%Glu-STY-polystyrene bead, 15%Glu-STY/EPS bead and 10%Glu-STY-EPS bead) gave activity at 3.02,4.82, 3.75 and 2.51 U/g-support, respectively as shown in Figure 4.3. This lipase activity was

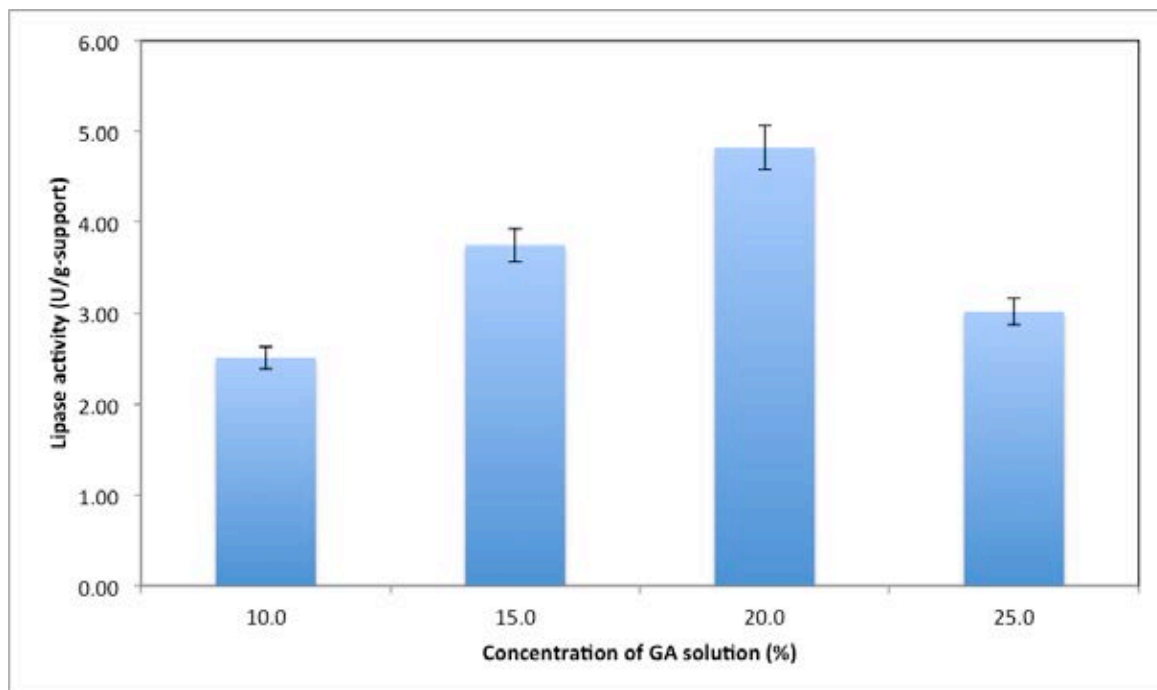performed to determine the optimal concentration of glutaraldehyde solution.

Figure 4.3 Effect of glutaraldehyde (Glu) concentration on activity of *P.cepacia*(1mg) immobilized onto PGlu-STY-expandable polystyrene beads (1g). The reaction parameter: 10%w/v styrene monomer at 60°C for 24hours.

4.1.2 Effect of styrene monomer concentration

The concentration of styrene monomer in the range 5 to 15% crosslinked with 20% glutaraldehyde and then coated on expandable polystyrene PGlu-STY/EPS bead was investigated. Using 10% of styrene monomer was successfully crosslinked with 20% glutaraldehyde. After that this copolymer already used to coat on expanded polystyrene bead. This support was smooth surface and hardly occur mass polymer of polymerization of linker. At 5% styrene monomer, was incompletely crosslinked with 20% glutaraldehyde because of polymerization of glutaraldehyde could be occurring mass polymer by itself as shown in Figure 4.4. This support was used for lipase immobilized 1 mg onto support 1 g for lipase activity test.
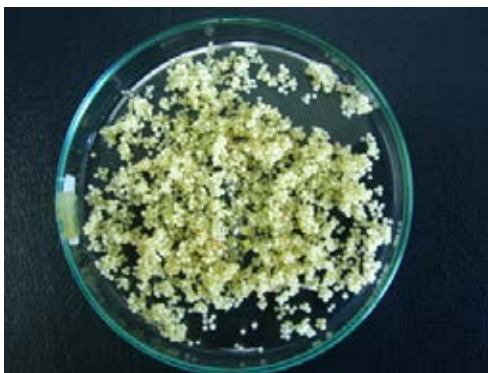
(a)
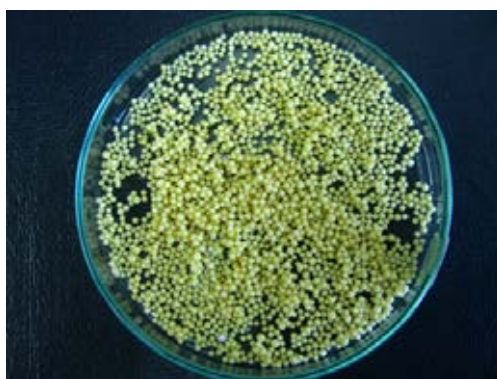
(b)

(c)

Figure 4.4 Comparison of styrene concentration crosslinked with 20%glutaraldehyde and then coated onto expandable polystyrene bead (a) 5%STY-20%Glu/EPS (b) 10%STY-20%Glu/EPS bead (c) 15%STY-20%Glu/EPS bead.

Support as 5%STY-20%Glu/EPS bead gave lower lipase activity (3.24 U/g-support) than 10%STY-20%Glu/EPS bead support (4.62 U/g-support) whereas 15%STY-20%Glu/EPS bead gave 4.60 U/g-support as shown in Figure 4.5.
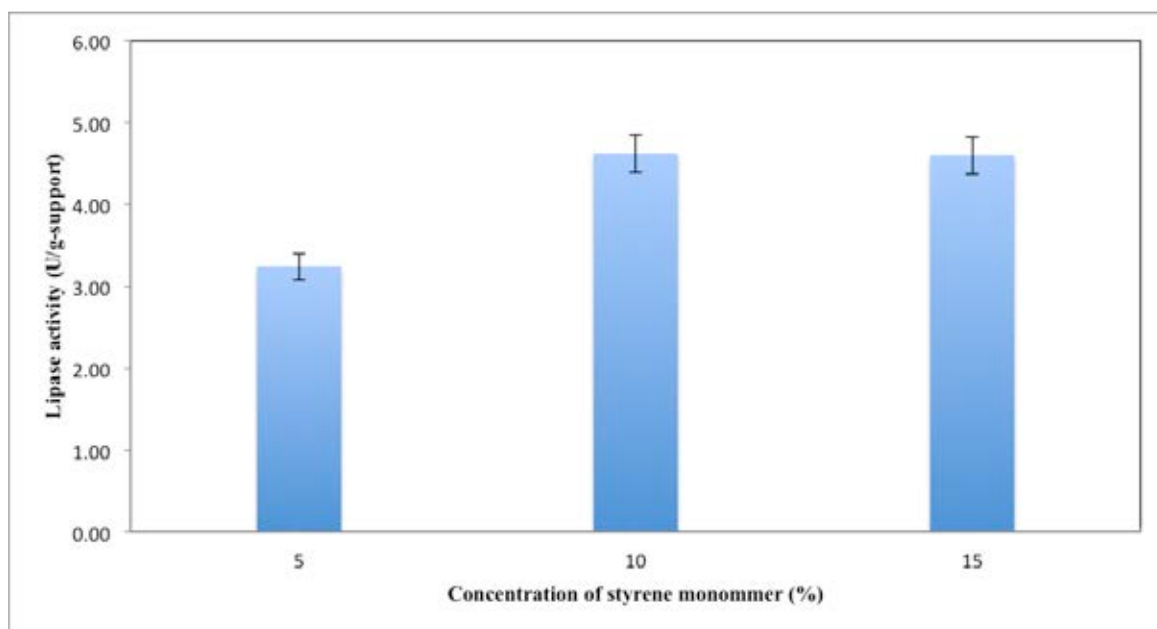

Figure 4.5 Effect of styrene monomer concentration on activity of immobilized *P.cepacia*on to PGlu-STY/EPS bead. The reaction parameter: 10%w/v styrene monomer at 60°C for 24hours.

4.1.3 Effect of copolymer coating

Expandable polystyrene bead was coated by 20%glutaraldehyde-10%styrene copolymer (PGlu-STY/EPS bead). Polymerization reaction of two polymers was carried out in the range of 4-24 hours to determine the optimal time of copolymer coating yield onto expandable polystyrene bead under optimal condition as presented in table 4.1.Results that increasing polymerization time of copolymer, yield of copolymer were increased. At 24 hours gave a maximal value at 8.95 gram because of ultimate ability of copolymer coat on expandable polystyrene bead.

Table 4.1 Variation of polymerization of time on yield of copolymer could be able to coat onto expandable polystyrene bead.

Time (hour)	Yield of copolymer coating
0	0
4	1.25
6	1.69
8	4.22
12	7.85
24	8.95
28	8.97

In the study, as yield of copolymer loading at 4 -6 hours were rarely increased. After 6 hours, copolymer was a sudden surge in copolymer coating and arrived at maximal value at 24 hours. The percentage of copolymer coating was 82.14 % as presented in Figure 4.6.
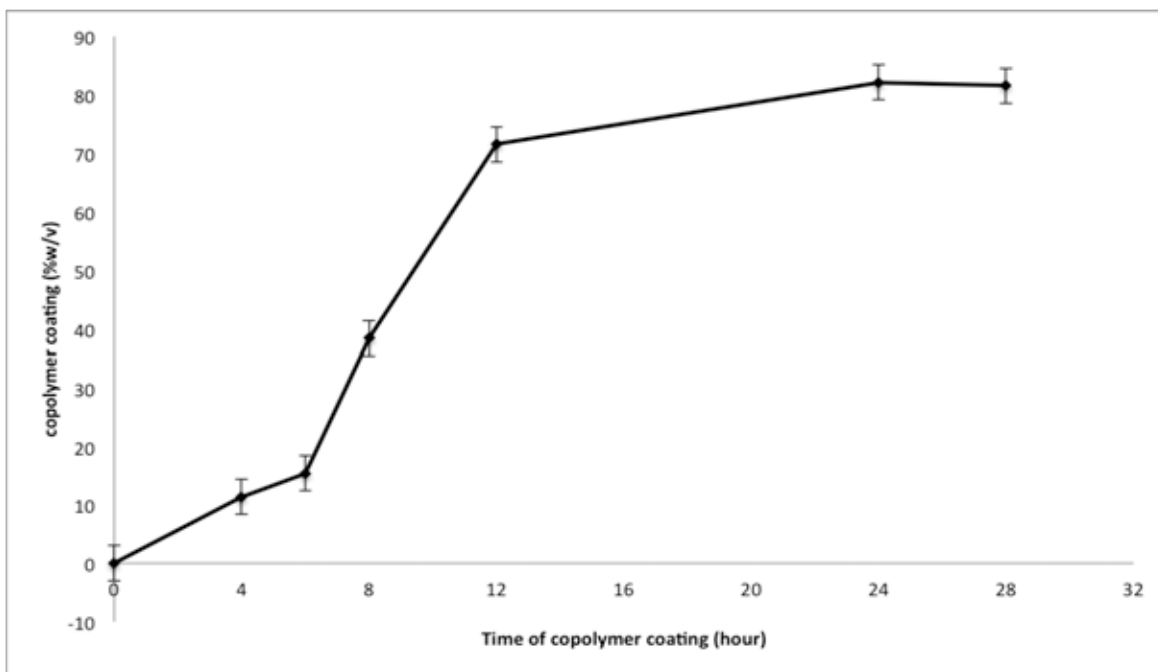

Figure 4.6 The effect of polymerization time on yield of copolymer coating. Reaction parameter: glutaraldehyde solution 20%w/v, styrene monomer 10%w/v at 60°C.

However, Polyglutaraldehyde-styrene copolymer coated on expandable polystyrene bead support was also investigated immobilization of lipase to confirm the optimal polymerization time of copolymer. 1 mg lipase immobilized onto 1 g of this support in variation of copolymer coating was shown in Figure 4.7. The maximal value of protein loading yield on this support at 24 hours was 14.77 %.
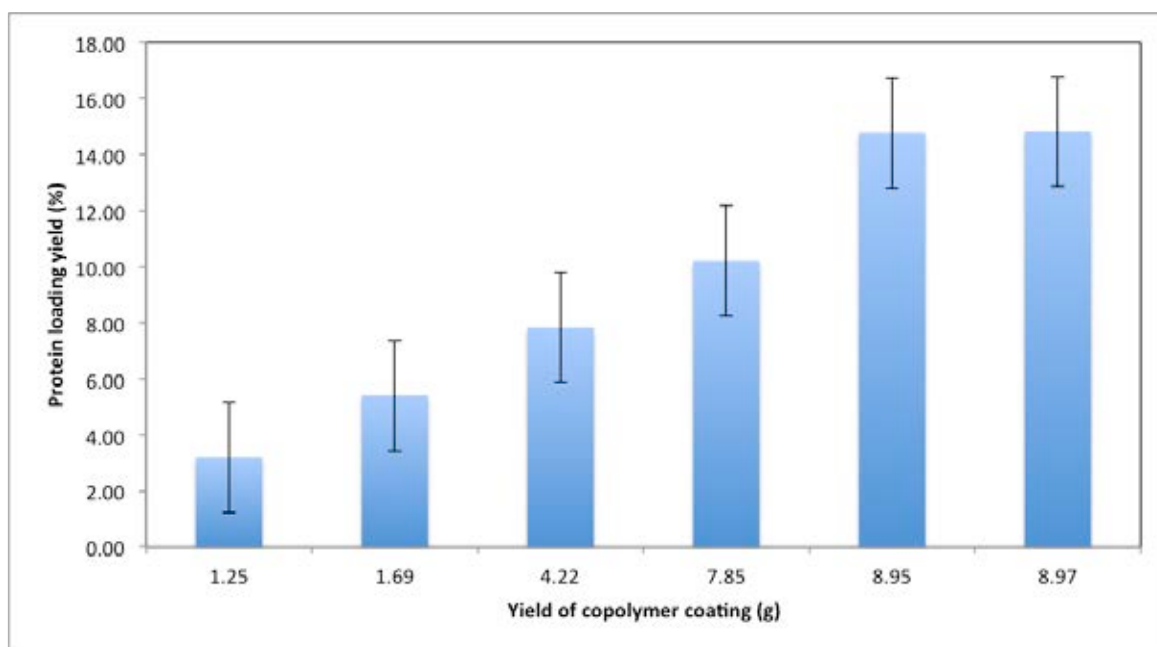

Figure 4.7 Effect of yield of copolymer coating onto PGlu-STY/expanded polystyrene bead support on protein loading yield (1 mg lipase immobilized onto 1 gram of support at room temperature for 72 hours).

4.2 The PGlu-polystyrene copolymer support

Synthesis of polyglutaraldehyde crosslinked with styrene copolymer through emulsion polymerization under suitable condition was studied. The successful polymerization of 30% styrene crosslinked with 20% glutaraldehyde was shown in Figure 4.8. This support was used to compare with PGlu-STY/EPS beads support. The particle size of PGlu-polystyrene copolymer (synthesized PGlu-polystyrene) was 100 µm.
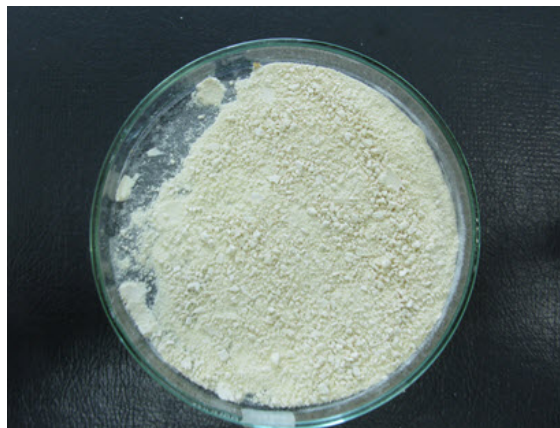
Figure 4.8 The copolymer of polyglutaraldehyde crosslinked with styrene monomer at 60°C for 2 hours and then reach temperature up to 80 °C for 2 hours.

The FT-IR spectra of two different supports were analyzed to determine the structure before and after modification. The FT-IR spectrum of expandable polystyrene bead coated by polyglutaraldehyde-styrene copolymer(PGlu-STY/EPS bead) (Figure 4.9) displayed absorption of O-H stretching vibration of the alcohol at 3402 cm^{-1}, absorption of C-H stretching vibration of aldehyde appeared at 2714 cm^{-1}, and absorption of aliphatic aldehyde at 1722 cm^{-1} for the C=O stretching and at 1079 cm^{-1}forconjugated C=O stretching. However, the FT-IR spectrum of the polystyrene-polyglutaraldehyde copolymer support was shown absorption as same as PGlu-STY/EPS bead(Figure 4.10).
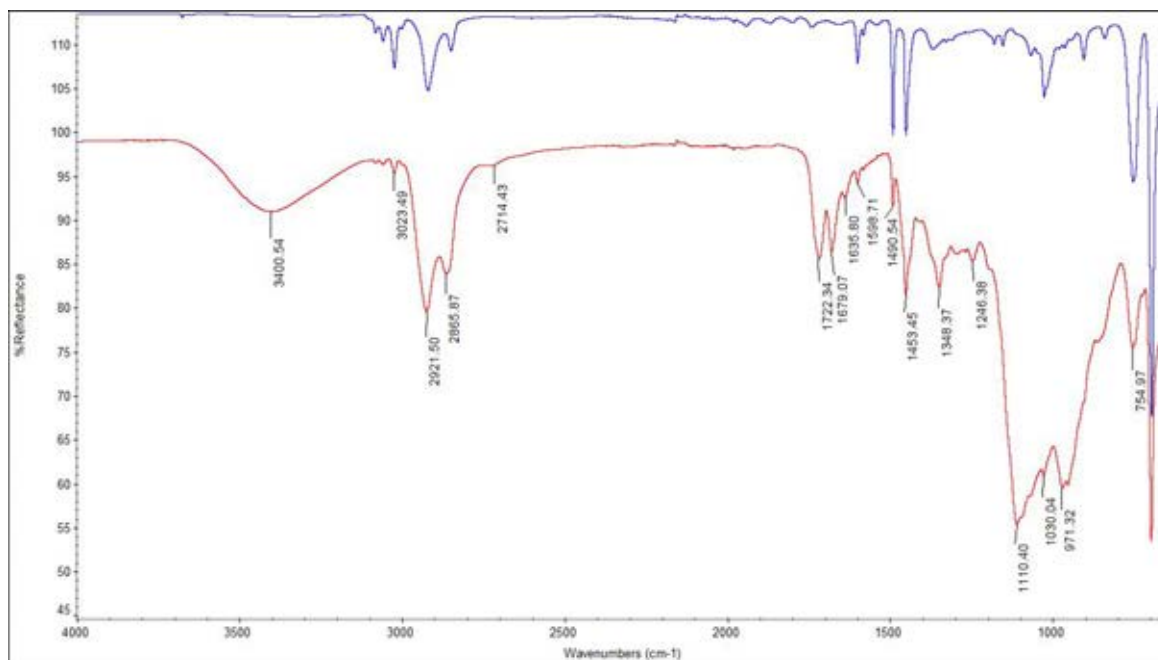
Figure 4.9 FT-IR spectrums of expandable polystyrene bead and PGlu-STY/EPS bead
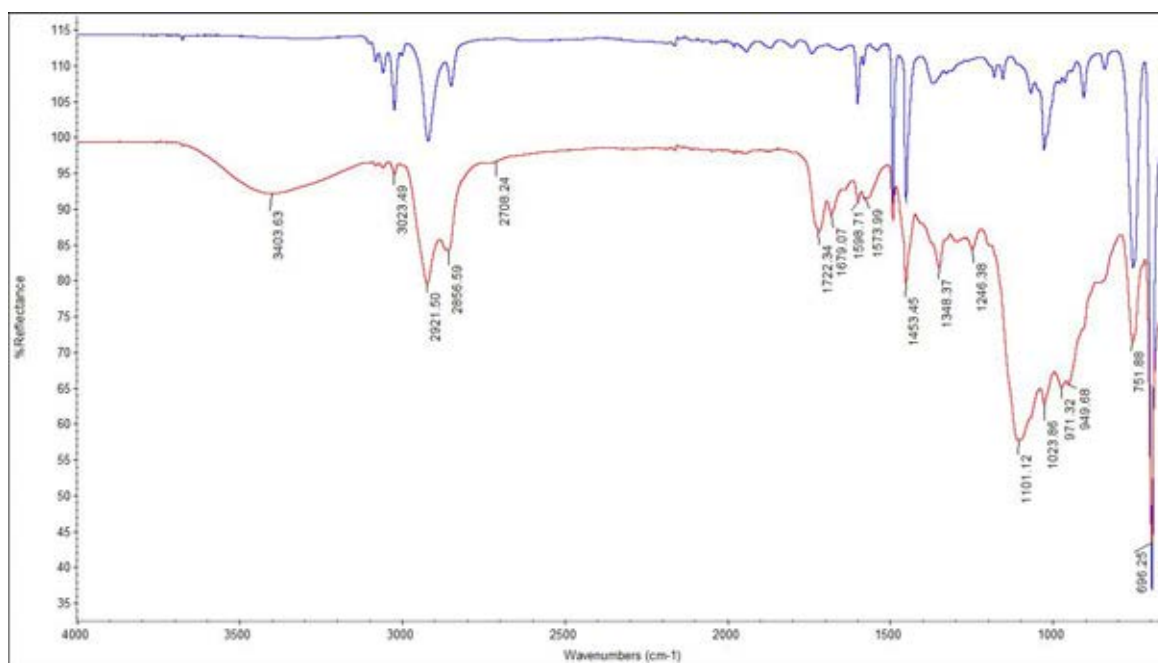

Figure 4.10 FT-IR spectrums of expandable polystyrene beads and synthesized PGlu-polystyrene copolymer

4.3 The optimization of immobilization lipase

4.3.1 Effect of immobilization on protein loading yield

1 mg, 2 mg, 5 mg, 7 mg, 8 mg and 10 mg of lipase from *Pseudomonas cepacia* was immobilized onto 1 g of PGlu-STY/EPS bead and then protein-loading yield was analyzed in phosphate buffer at room temperature. The results for the percentage of protein loading yield were present in Figure 4.11. 10 mg of lipase was successfully immobilized on PGlu-STY/EPS bead and it gave protein-loading yield at 71.81 %.
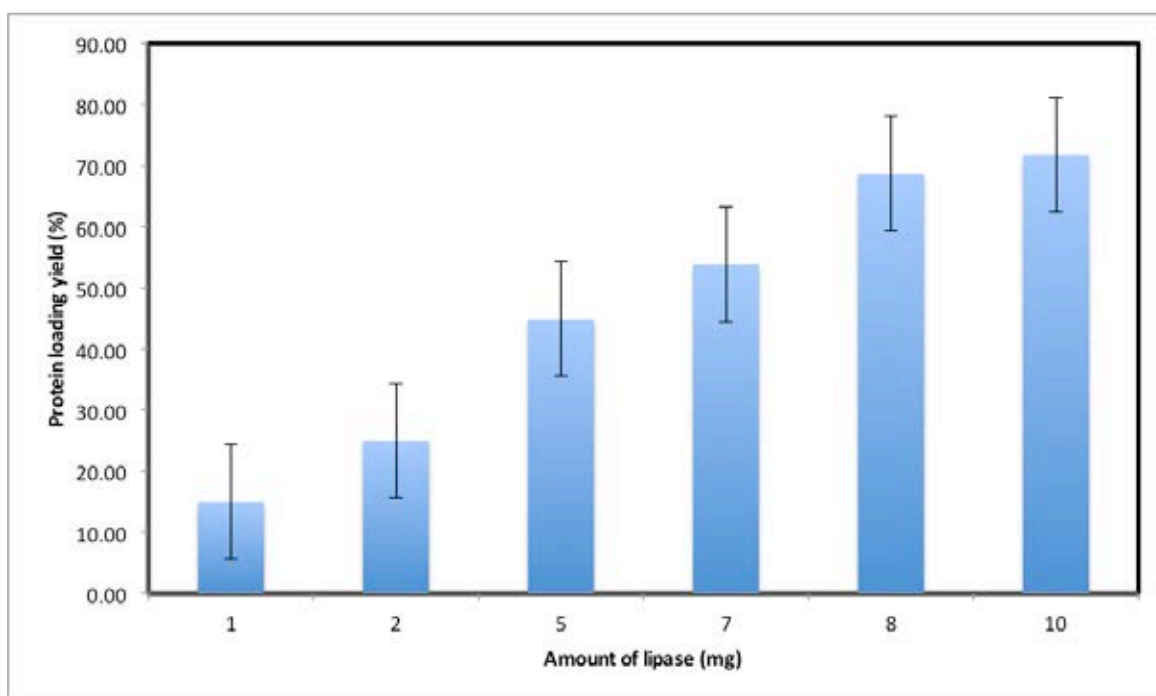

Figure 4.11 Variation of *P.cepacia* lipase was immobilized onto 1 gram of support on protein loading yield in phosphate buffer 25 mM at room temperature for 72 hours.

FT-IR spectra of immobilization lipase (10 mg) onto PGlu-STY/EPS bead were analyzed to confirm optimal amount of lipase that was appropriate reacted with active group of surface of the support as shown in Figure 4.12.The absorption of C=N bond at 1561 cm^{-1}and the absorption of C=O stretching of aliphatic aldehyde at1722 cm^{-1}reduced

because the amine group of lipase was reacted with carbonyl group of aldehyde on the surface of the support.
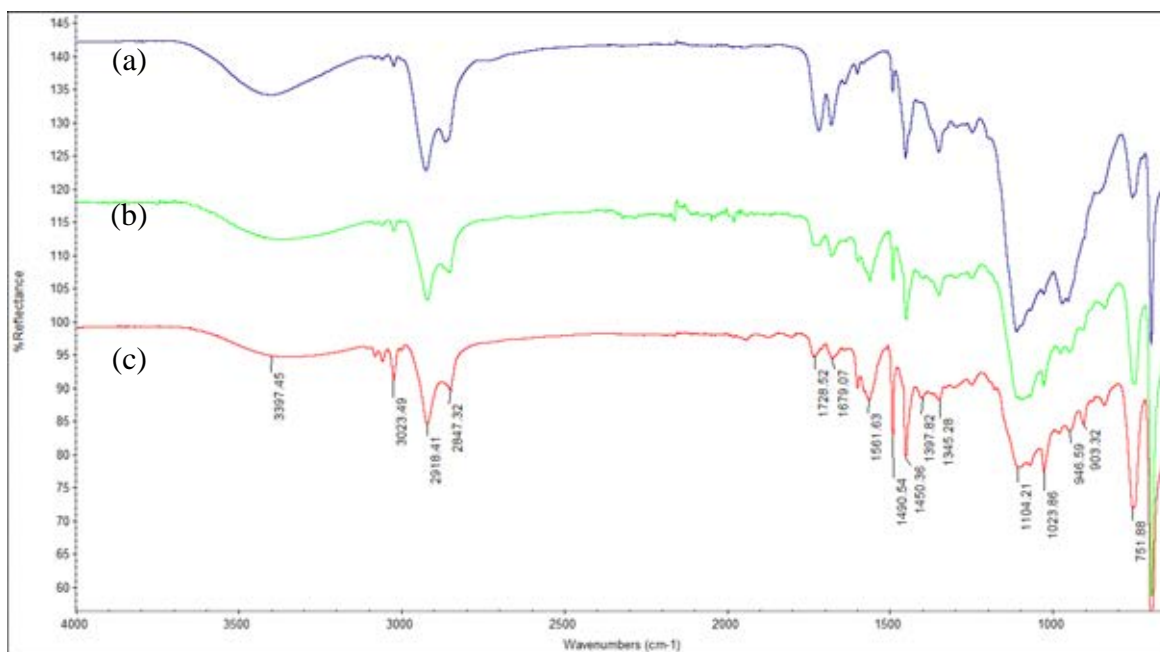

Figure 4.12 FT-IR spectrums of (a) PGlu-STY/EPS bead ,(b) 8mg of lipase immobilized onto PGlu-STY/EPS bead and (c) 10 mg of lipase immobilized onto PGlu-STY/EPS bead

4.3.2 Effect of immobilization time

The lipase from *P.cepacia* (10 mg) was immobilized onto PGlu-STY/EPS bead(1 g) with variation of immobilization time at12, 24, 48, 72 and 96 hours and then protein-loading yield was investigated. The results that protein loading yield at various time were 23.64, 28.26, 41.78, 70.35 and 70.41 %, respectively as shown in Figure 4.13. Therefore, the lipase was completely immobilized onto expandable polystyrene bead at 72 hours.
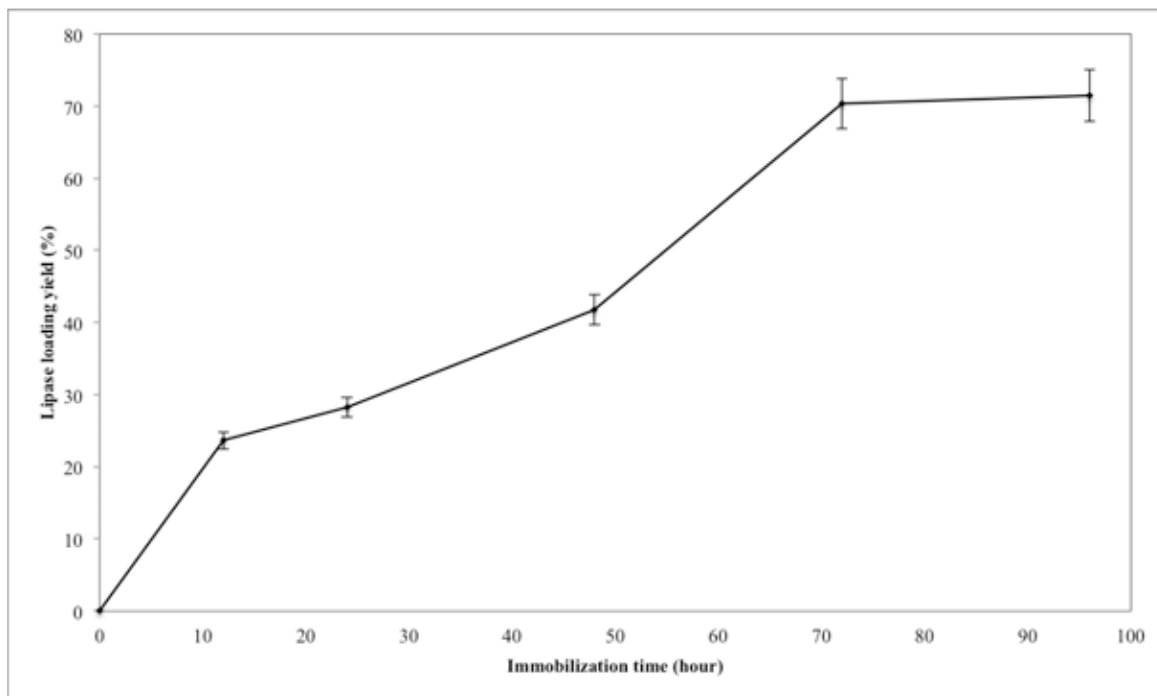
Figure 4.13 The variation of immobilization time using 10 mg of *P.cepacia* and 1 gram of support in 25 mM phosphate buffer at room temperature.

4.3.3 Effect of immobilization on lipase activity

Variation of amount of lipase from *Pseudomonas cepacia* (*P.cepacia*) (1 mg – 10 mg) immobilized onto PGlu-STY/EPS bead support was examined in phosphate buffer at room temperature for 72 hours. After that, the immobilized lipase on PGlu-STY/EPS bead was tested by lipase activity. Using 1, 2, 5 and 7 mg of lipase gave also lower activity, which there were 4.80, 6.72, 13.41, 17.28 U/g-support, respectively. At 8 mg of lipase was sufficiently immobilized on 1 gram of the PGlu-STY/EPS bead and it gave maximal value at 25.01 U/g-support of activity as shown in Figure 4.14.
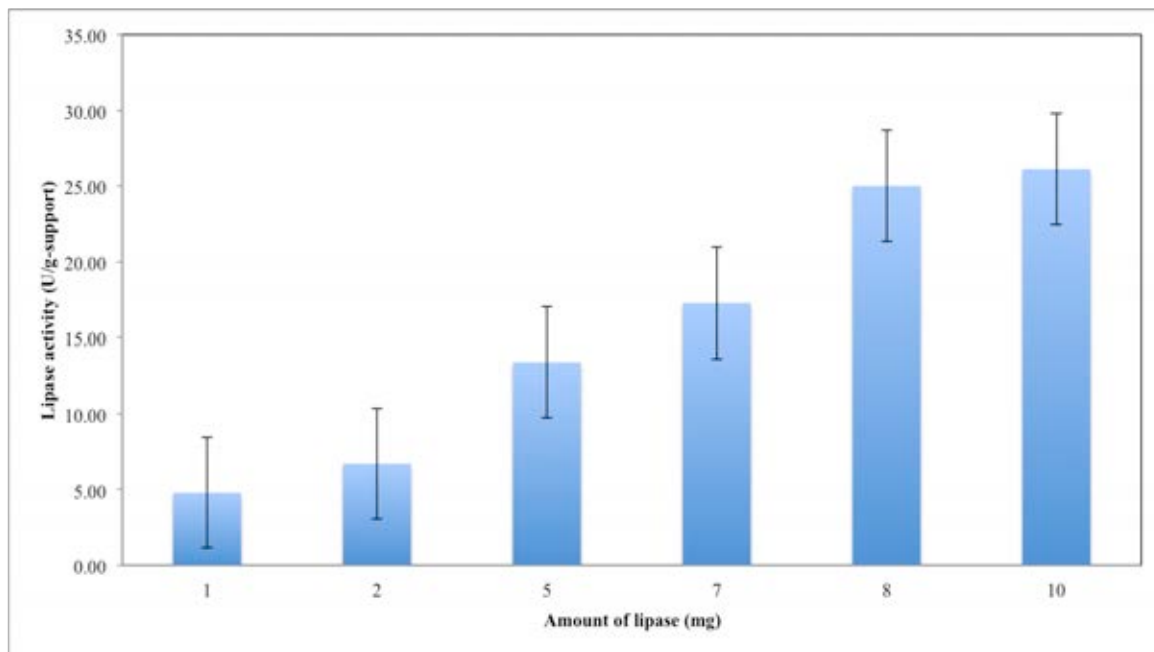
Figure 4.14 Effect of amount of lipase immobilized on PGlu-STY/EPS bead on activity
in 25mMphosphate buffer at room temperature for 72hours.

4.4 The optimization of immobilization

The immobilization efficiency of immobilized lipase (*P.cepacia*) onto PGlu-STY/EPS bead compared with synthesized PGlu-polystyrene copolymer was investigated in term of lipase activity, specific activity and protein loading yield. The effect of amount of protein immobilized onto PGlu-STY/expanded polystyrene bead gave 7.18 mg/g was whereas immobilized onto synthesized PGlu-polystyrene copolymer gave 8.00 mg/g. The percentage of protein loading yield onto PGlu-STY/EPS bead and synthesized PGlu-polystyrene copolymer was the maximal value at 71.8 and 80.02 %, respectively as shown in Table 4.2.

Table 4.2 Activity and protein loading yield of immobilized lipase onto PGlu-STY/EPS bead and synthesized PGlu-polystyrene copolymer.

Support	Protein loading mg/g-support	Protein loading yield %	Lipase activity U/g-support	Specific activity U/mg-protein
PGlu-STY/EPS bead	7.18	71.81	26.12	3.34
Synthesized PGlu-polystyrene copolymer	8.00	80.02	30.25	3.78

4.5 Enzymatic transesterification

Transesterification reaction of soybean oil with absolute ethanol (dry-EtOH) using immobilized lipase onto PGlu-STY/EPS bead support used as catalyst for biodiesel production was investigated. Property of this catalyst has lower density than water and oil. For this reason, catalyst was on the top of the screw-capped vials reactor and during the reaction; the catalyst was dispersed on the reaction mixture as shown in Figure 4.15.

(a)

(b)

Figure 4.15 Photographs of transesterification reaction using lipase immobilized on PGlu-STY/EPS bead (a) catalyst before stirring and (b) catalyst during the reaction.

The lipase immobilized ontosynthesized PGlu-polystyrene copolymer catalyst for transesterification reaction of soybean oil with absolute ethanol for biodiesel production as shown in Figure 4.16. This catalyst has high density, which also sink at the bottom of the screw-capped vials reactor.

(a)

(b)

Figure 4.16 Photographs of transesterification reaction using lipase immobilized on synthesized PGlu-polystyrene copolymer (a) catalyst before stirring and (b) catalyst during the reaction.

4.6 The optimal factor of transesterification reaction

4.6.1 Effect of co-solvent

Co-solvent was used in enzymatic transesterification reaction in order to increase reaction rate through improved mutual solubility of hydrophobic triglycerides and hydrophilic alcohols. In addition, co-solvent also protected enzymes from denaturation by high concentrations of alcohols (methanol and ethanol) (Antczak, M. S. et al., 2009).Hydrophilic co-solvent of this enzymatic synthesis was *tert*-butanol. Experiments were carried out in three batches reactor, including transesterification reaction of soybean oil and EtOH with *tert*-butanol (1), reaction of soybean oil and EtOH without *tert*-butanol (2) in addition, reaction of soybean oil and dry EtOH (3). Table 4.3 was shown ethyl ester conversion in three batches reaction.

Table 4.3 Transesterification reaction of soybean oil and alcohol (ethanol and methanol) in different three batches

Batch	Ethyl ester conversion (%)	Methyl ester conversion (%)
1	74.40	76.55
2	62.00	58.42
3	98.80	95.22

Therefore, the reaction of soybean oil and EtOH with *tert*-butanol gave higher conversion than the reaction of soybean oil and EtOH without *tert*-butanol. Especially, the reaction of soybean oil and absolute EtOH gave the maximal ethyl ester conversion because of reaction mixture including a small aliquot of water, which increased concentration of free fatty acids (Kieda, M. et al., 2001)(see ^{1}H NMR in Figure B-5). However, the experiments used the absolute alcohol for biodiesel synthesis. Transesterification of soybean oil and methanol in three batches reactor including (1) methanol with *tert*-butanol, (2) methanol without *tert*-butanol and (3) dry methanol were investigated. There were methyl ester conversion at 76.55, 58.42 and 95.22 %, respectively (see ^{1}H NMR in Figure B-9) as shown in Figure 4.17 Thus, the

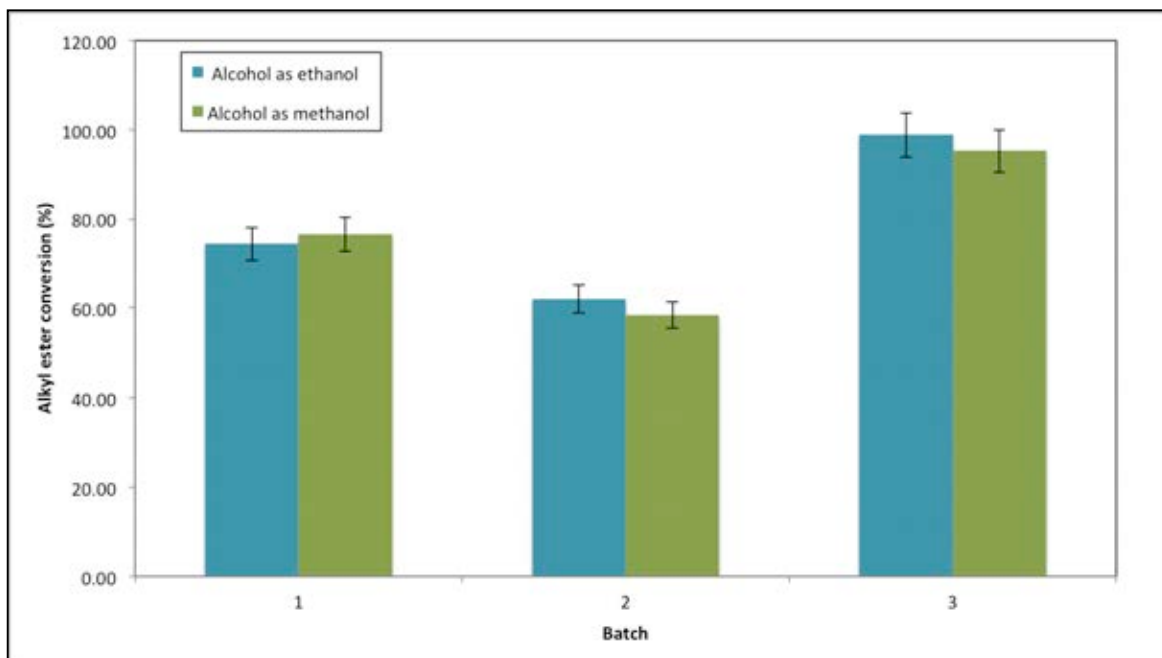transesterification reaction of soybean oil and dry EtOH under optimal condition was investigated.

Figure 4.17 Biodiesel conversions from three batches reactor of transesterification of soybean oil and (1) alcohol with *tert*-butanol, (2) alcohol without *tert*-butanol and (3) dry alcohol at 40°C for 12 hours

4.6.2 Effect of enzyme dosage

The effect of immobilized enzyme dosage on PGlu-STY/EPS bead compared with PGlu-polystyrene copolymer in the range of 0.125 - 0.75 gram on the transesterification of soybean oil with dry ethanol was presented in Figure 4.18.In the study, 0.46 gram of immobilized lipase onto PGlu-STY/EPS bead and immobilized lipase onto synthesized PGlu-polystyrene copolymer catalyzed the thransesterification of soybean oil and itgave the highest biodiesel conversion 94.02% and 86.81 %, respectively. Increasing the amount of enzyme, ethyl ester yield was a sudden surge, followed by a slower rate at higher enzyme dosage. After that the conversion was reached to maximum value. In the case of lipase that immobilized onto PGlu-polystyrene copolymer, it was higher density

than oil and sank to the bottom of the reactor so it swell with glycerol (by-product of transesterification reaction) and the activity of lipase was also decreased.
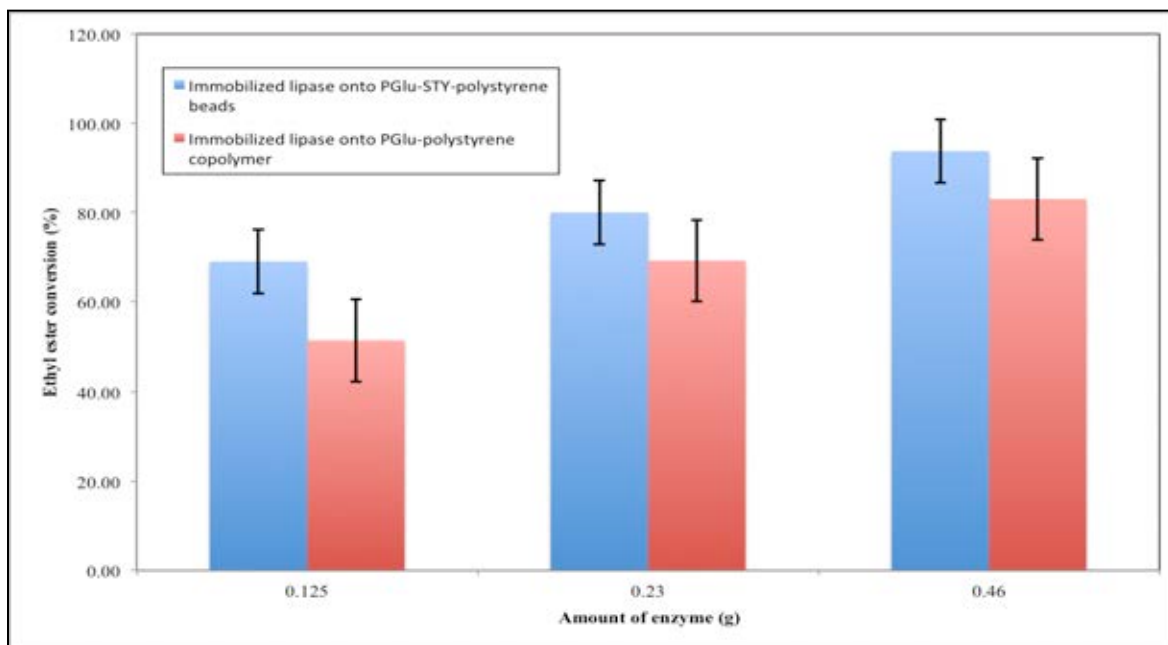

Figure4.18 The effect of enzyme dosage on the transesterification of soybean oil with dry EtOH at 40°C for 12 hours

The enzymatic transesterification reaction was investigated in term of unit equivalent of immobilized lipase on two different supports, which used as catalyst. Experiments were performed to confirm the efficiency of supports. The effect of enzyme dosage on temperature was presented in Figure 4.19. The maximal ethyl ester conversion was 98.52 %, which belong to immobilized lipase onto PGlu-STY/EPS bead whereas immobilized lipase onto synthesized PGlu-polystyrene copolymer gave 84.07 % conversion. For this reason, the support as PGlu-STY/EPS bead was more efficiency than synthesized PGlu-polystyrene copolymer support.
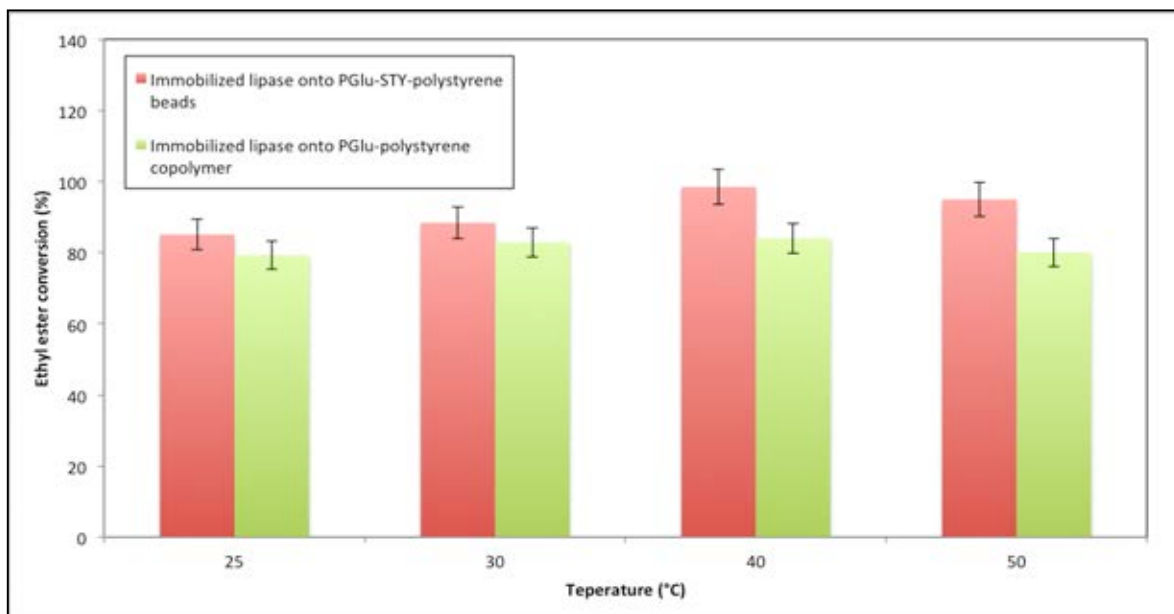
Figure 4.19 The effect of enzyme dosage on ethyl ester conversion of transesterification from soybean oil with ethanol for 12 hours.

4.6.3 Effect of ratios of alcohol to oil

The enzymatic transesterification is well known that immobilized lipase as catalyst with excessive short-chain alcohols (methanol or ethanol) had an affect to lipase activity was decreased. However, the optimal amount of alcohol at three molar equivalents was completed conversion of the oil to ethyl ester (Dizge, N. et al., 2008). The reaction was carried out in the range of 1:2 to 1:6.The results for the optimal ratio on alkyl ester conversion were presented in Figure. 4.20. The optimal ratio of ethanol to oil on biodiesel conversion of ethyl ester at 90.09 % (using immobilized lipase onto PGlu-STY/EPS bead) and 79.81% (using immobilized lipase onto synthesized PGlu-polystyrene copolymer) was obtained at 1:5.
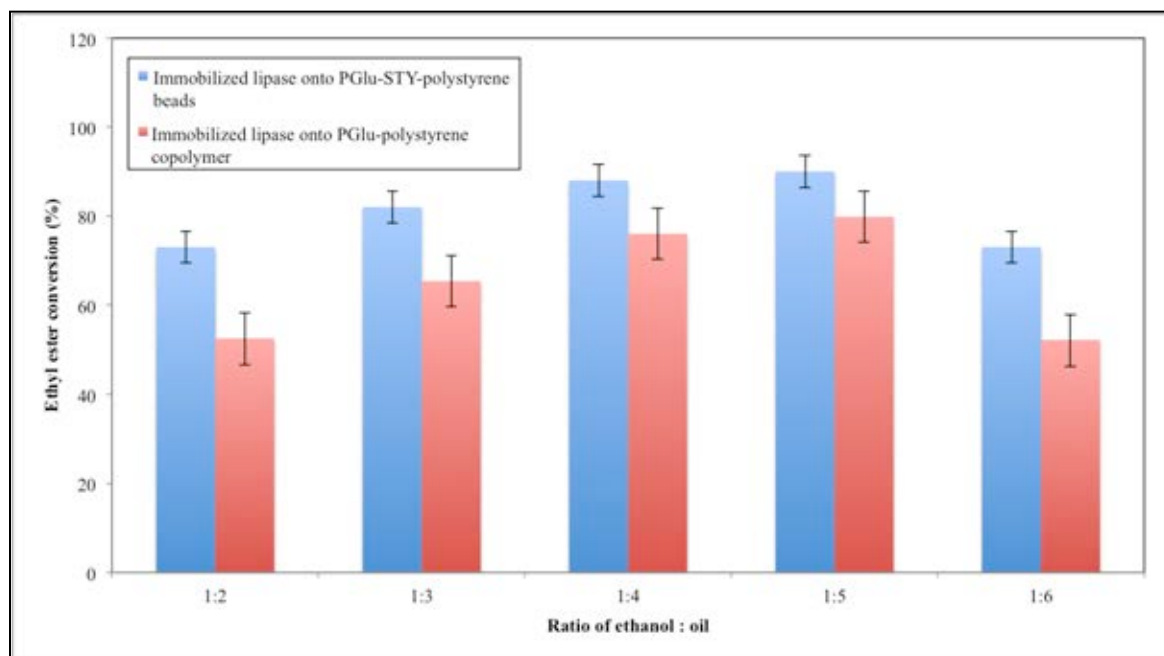
Figure 4.20 The effect of molar ratio of ethanol to oil on biodiesel conversion with 0.46 gram of enzyme dosage at 40°C for 12 hours

4.6.4 Effect of temperature

The effect of temperature on transesterification reaction of soybean oil catalyzed by the immobilized lipase was investigated in the range of 25 to 50 °C. The temperature at 40 °C was optimal value of immobilized lipase onto PGlu-STY/EPS bead and synthesized PGlu-polystyrene copolymer to produce maximal biodiesel conversion at 93.20% and 82.40%, respectively (Figure 4.21).At the higher temperature, the ethyl ester production was rarely alteration due to the hydrolysis reactions would happen and the lipase activity of immobilized lipase was lightly changed.
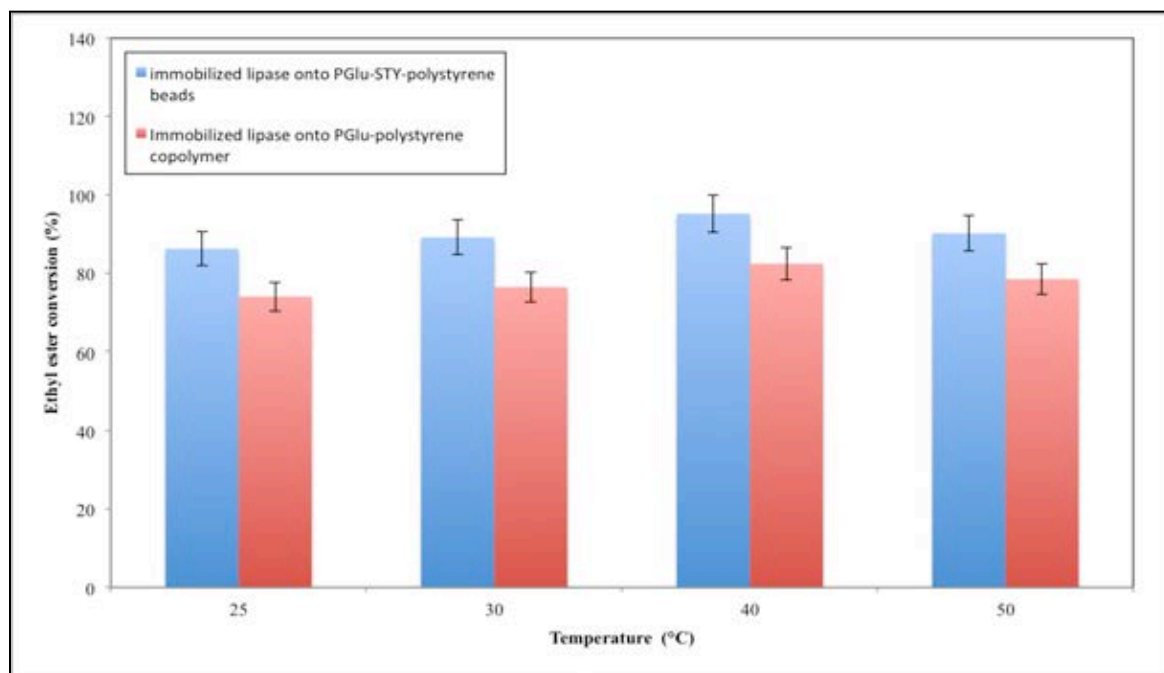
Figure 4.21 The effect of temperature on biodiesel conversion through transesterification reaction of soybean oil with 0.46 gram of immobilized lipase onto PGlu-STY/EPS bead and synthesized PGlu-polystyrene copolymer as catalyst.

4.6.5 Effect of reaction time

The reaction time of transesterification reaction from soybean oil catalyzed by immobilized lipase onto PGlu-STY/EPS bead and synthesized PGlu-polystyrene copolymer is an important parameter. Reactions were investigated in the range of 4 to 28 hours at 40 °C using 0.46 gram of immobilized lipase. The maximal value at 24 hours gave biodiesel conversion at 98.80% (immobilized lipase onto PGlu-STY/EPS bead) and 83.33% (immobilized lipase onto synthesized PGlu-polystyrene copolymer) as shown in Figure 4.22. There was confirmed the results by TLC chromatogram of transesterification using immobilized lipase onto PGlu-STY/EPS bead and onto synthesized PGlu-polystyrene copolymer that kept the sample at 4, 6, 8, 12, 22 and 24 hours as shown in Figure 4.23.
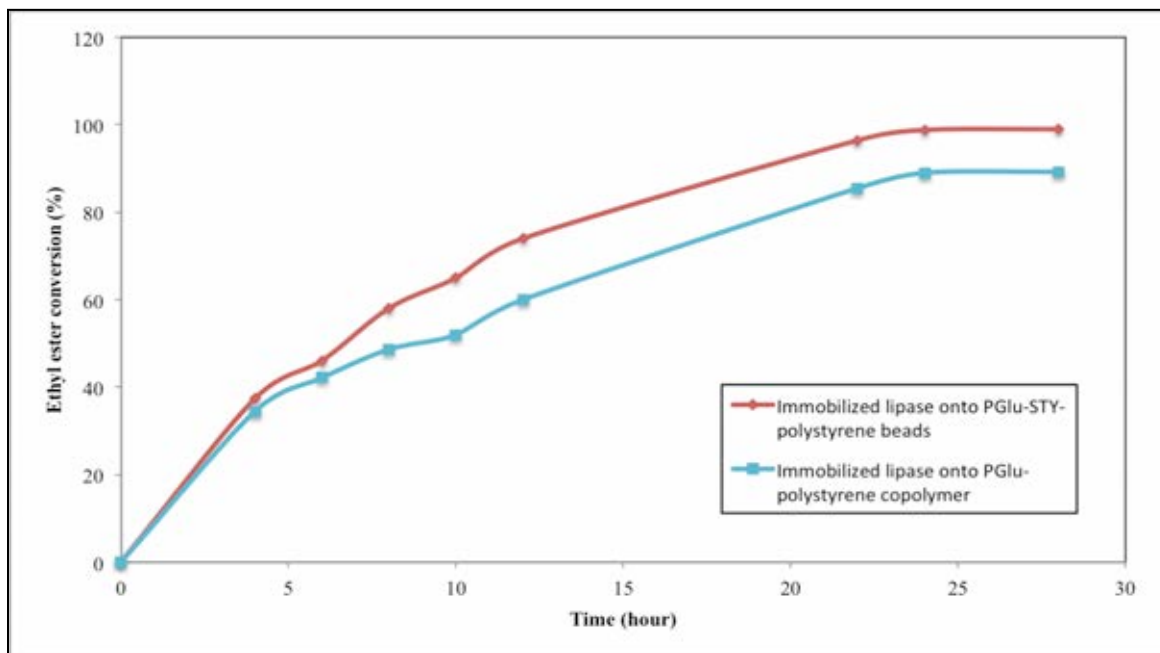
Figure 4.22 Variation of time on biodiesel conversion (%) with ethanol using 0.46 gram of enzyme dosage at 40 °C
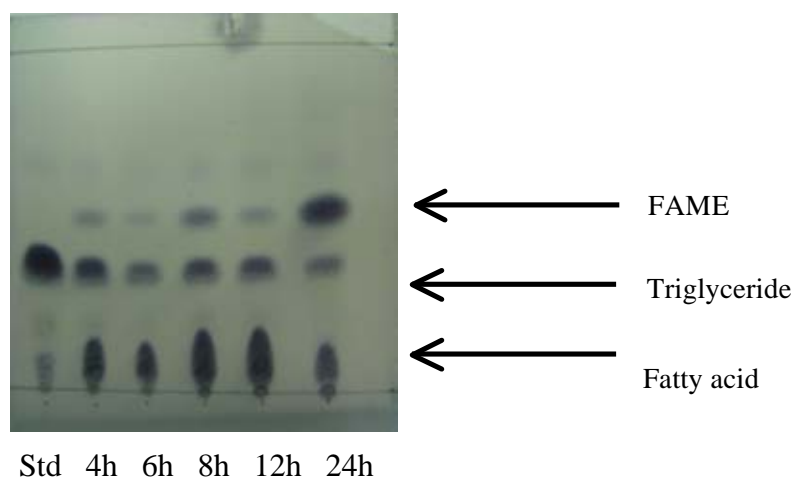

Std 4h 6h 8h 12h 24h

Figure 4.23 TLC chromatogram of ethyl ester production by 0.46 gram of immobilized lipase onto PGlu-STY/EPS bead, 1:5molar ratio of ethanol to soybean oil, reaction time 4, 6, 8, 12, 22 and 24hoursat 40°C.

4.7 Operational stability and reusability of the immobilized lipase

The most important characteristic of immobilized lipase was its stability and reusability in repeated of use. Biodiesel production through transesterification reaction of soybean oil and absolute ethanol (dry EtOH) under optimal condition using immobilized lipase onto different supports was investigated. The immobilized lipase from *Pseudomonas cepacia* (*P.cepacia*) onto PGlu-STY/EPS bead catalyzed transesterification was compared efficiency catalyst with immobilized lipase onto synthesized PGlu-polystyrene copolymer over an extended period of time. For example the research, in 2009 Dizge, N. et al. studied biodiesel production from canola oil and methanol using catalyst as immobilization of *Thermomyces lanuginosus* lipase onto styrene-divinylbenzene modified with polyglutaraldehyde at 50 °C for 24 hours. The maximal methyl ester was 97% of which lipase activity retained during the 10 batches.

In this study, low-density catalyst of immobilized lipase onto PGlu-STY/EPS bead could catalyze the transesterification of soybean oil and dry EtOH. It gave ethyl ester at 98.80%. The immobilized lipase retained its activity during ten batch reactions and biodiesel conversion was constant. Whereas, the immobilized lipase onto PGlu-polystyrene copolymer could catalyze the transesterification of soybean oil and dry EtOH and gave ethyl ester at 83.33%.Until seven-batches reaction, the ethyl ester was decreased due to this catalyst had high density as shown in Figure 4.24.
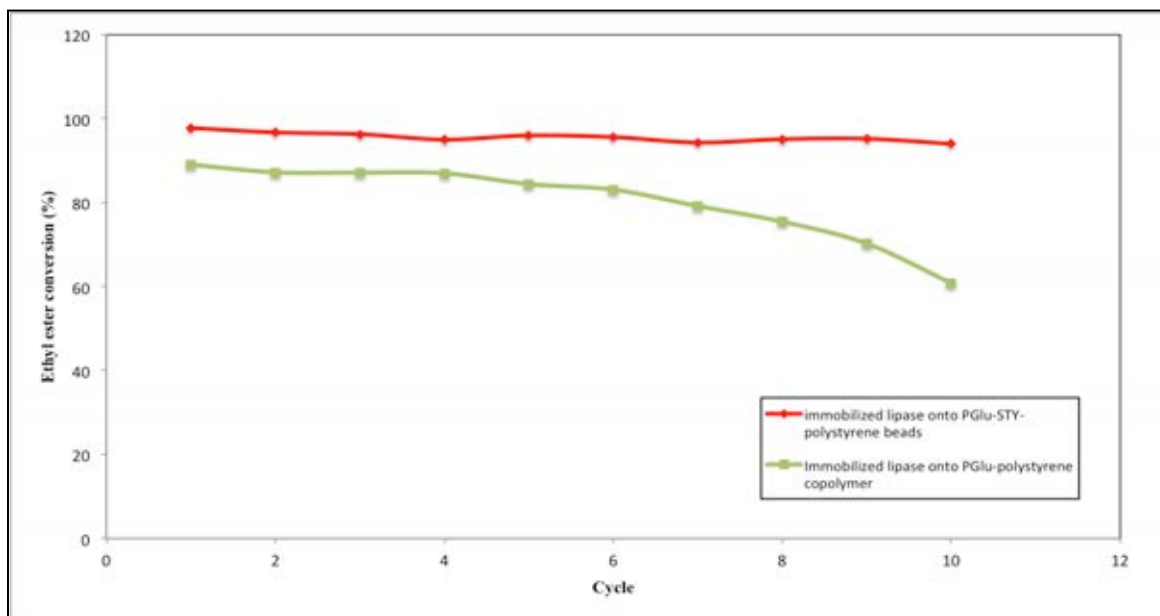
Figure 4.24 Operational stability and reusability of the immobilized lipase. (The reaction parameters: 5:1 molar ratio methanol to oil, 0.46 gram of enzyme dosage at40 °C, for 24 hours).

CHAPTER V

CONCLUSION AND SUGGESTION

5.1 Conclusion

The support as expandable polystyrene bead was coated by polyglutaraldehyde (PGlu), which crosslinked with styrene monomer. PGlu-STY/EPS bead support was an excellent support for lipase immobilization because polyglutaraldehyde had been used as a linker agent between free amine groups and this support and retained its lipase stability. The important property of this modified support was lower density than water and oil. Therefore, lipase immobilized onto this modified support was used catalyst for biodiesel production. Moreover, the polyglutaraldehyde crosslinked with polystyrene was synthesized to compare the efficiency of catalyst with previous catalyst.

In this study, lipase from *Pseudumonas cepacia* (*P.epacia*) was successfully immobilized on PGlu-STY/EPS bead and synthesized PGlu-polystyrene copolymer. The highest amount of lipase immobilized onto PGlu-STY/EPS bead and synthesized PGlu-polystyrene copolymer were 7.18 mg-protein/g-support and 8.00 mg-protein/g-support, respectively. For biodiesel production, the comparison ability of two different catalysts was investigated by transesterification reaction from soybean oil with ethanol or methanol using immobilized lipase onto PGlu-STY/EPS bead catalyst. This catalyst was an excellent catalyst for using ethanol and soybean oil and gave ethyl ester at 62.00%. Moreover, using absolute ethanol with this catalyst can produce a high ethyl ester conversion at 98.80%. Whereas the immobilized lipase onto synthesized poly(glutaraldehyde-styrene) catalyst gave ethyl ester conversion at 83.33% so immobilized lipase onto PGlu-STY/EPS bead has higher efficient catalyst than immobilized lipase onto synthesized PGlu-polystyrene catalyst.

5.2 Suggestion

      The low-density catalyst of immobilized lipase onto PGlu-STY/EPS bead support was used as catalyst for biodiesel synthesis with anhydrous ethanol as acceptor. This catalyst can retain its stability in the large scale and its reusability for a long time.

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APPENDICES

APPENDIX A

ENZYMATIC ASSAY

APPENDIX A

ENZYMATIC ASSAY

1. Preparation of *p*-nitrophenol standard curve

　　　　1.1.The concentration of *p*-nitrophenol solution (*p*-NP) 10 μmol/mL was diluted by 25 mM phosphate buffer at pH 7.0 to produce *p*-nitrophenol solution concentration 0.15 μmol/mL as stock solution. After that dilution of *p*-nitrophenol solution (0.25 μmol/mL) in the range of 0.00 - 0.06 μmol/mL was investigated.

Table A-1 Variation of concentration of *p*-nitrophenol solution.

Sample	*p*-NP (μmol/mL)	*p*-NP (mL)	Phosphate buffer (mL)
1	0.00	0	5.00
2	0.005	0.17	4.83
3	0.01	0.33	4.67
4	0.02	0.67	4.33
5	0.03	1.00	4.00
6	0.04	1.33	3.67
7	0.05	1.67	3.33
8	0.06	2.00	3.00

　　　　1.2. The *p*-NP solution was measured by colorimetric method at 410 nm. Results were presented on Figure A-1.
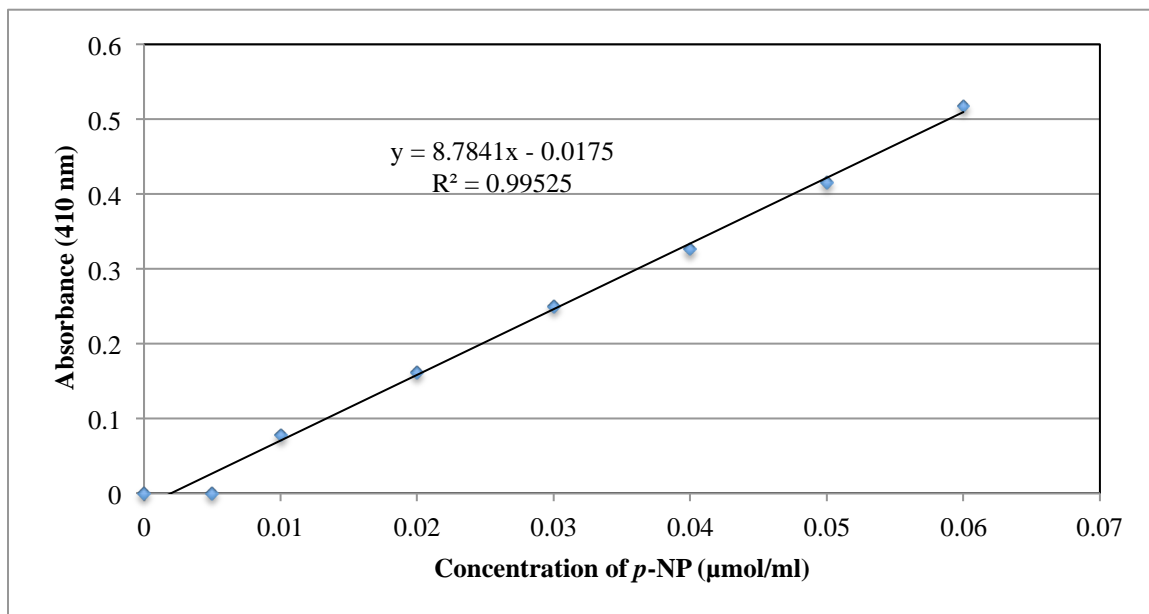
Figure A-1 Standard curve of *p*-nitrophenol concentration.

1.2 Calculation of lipase activity (One unit of lipase activity was defined as 1 μmol *p*-nitrophenol (*p*-NP) produced per min from *p*-NPP).

2. Analysis of enzyme dosage immobilized onto supports

2.1 Preparation of concentration of lipase as standard curve

The lipase from *Pseudomonas cepacia* (*p.cepacia*) concentration 1 mg/mL was diluted by 25 mM phosphate buffer (pH 7.0) to vary concentration in the range of 0.00 - 0.70 mg/mL. Results were presented in Table A-2.

Table A-2 Variation of lipase concentration.

Sample	P.cepacia (mg/mL)	P.cepacia (mL)	Phosphate buffer
1	0.00	0.00	5.00
2	0.10	0.50	4.50
3	0.20	1.00	4.00
4	0.40	2.00	3.00
5	0.60	3.00	2.00
6	0.80	4.00	1.00

2.2 The samples were measured by colorimetric method at 322 nm. The standard curve of lipase concentration was shown in Figure A-2.

Figure shows scatter plot with trendline: $y = 0.7849x - 0.0371$, $R^2 = 0.98158$
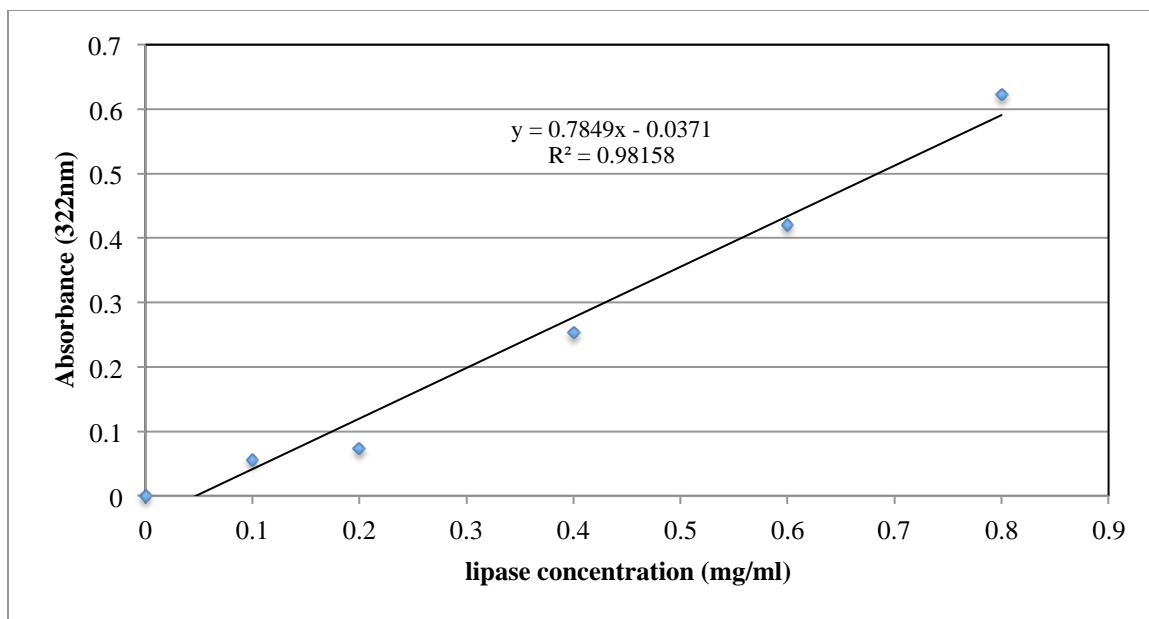
Figure A-2 Variation of lipase concentration in phosphate buffer pH 7.0.

2.3 Calculation of amount of lipase was immobilized onto PGlu-STY-polystyrene and PGlu-polystyrene copolymer.

APPENDIX B

CALCULATION

APPENDIX B

CALCULATION

1. Calculation of % biodiesel conversion

    1.1 Biodiesel production from soybean oil using immobilized lipase onto PGlu-STY/ EPS bead as catalyst

Table B-1 ^{1}H NMR results from biodiesel conversion of transesterification using immobilized lipase onto PGlu-STY/EPS bead.

Reaction	Feed stock	Acceptor	Solvent	Temp (°C)	Conversion (%)	Figure
1	Soybean oil	Ethanol	Tert-butanol	40	74.40	B-2
2	Soybean oil	Ethanol	Free-solvent	40	62.00	B-3
3	Soybean oil	Dry-ethanol	Free-solvent	40	98.80	B-4
4	Soybean oil	Methanol	Tert-butanol	40	76.55	B-6
5	Soybean oil	Methanol	Free-solvent	40	58.42	B-7
6	Soybean oil	Dry-methanol	Free-solvent	40	95.22	B-8
7	Palm oil	Dry-ethanol	Free-solvent	40	92.00	B-10
8	Palm oil	Dry-methanol	Free-solvent	40	94.50	B-11

$$\% \text{ Conversion} = \frac{\text{Integration of methoxy group per one mole equivalent}}{\text{Integration of methylene group per one mole equivalent}} \times 100$$
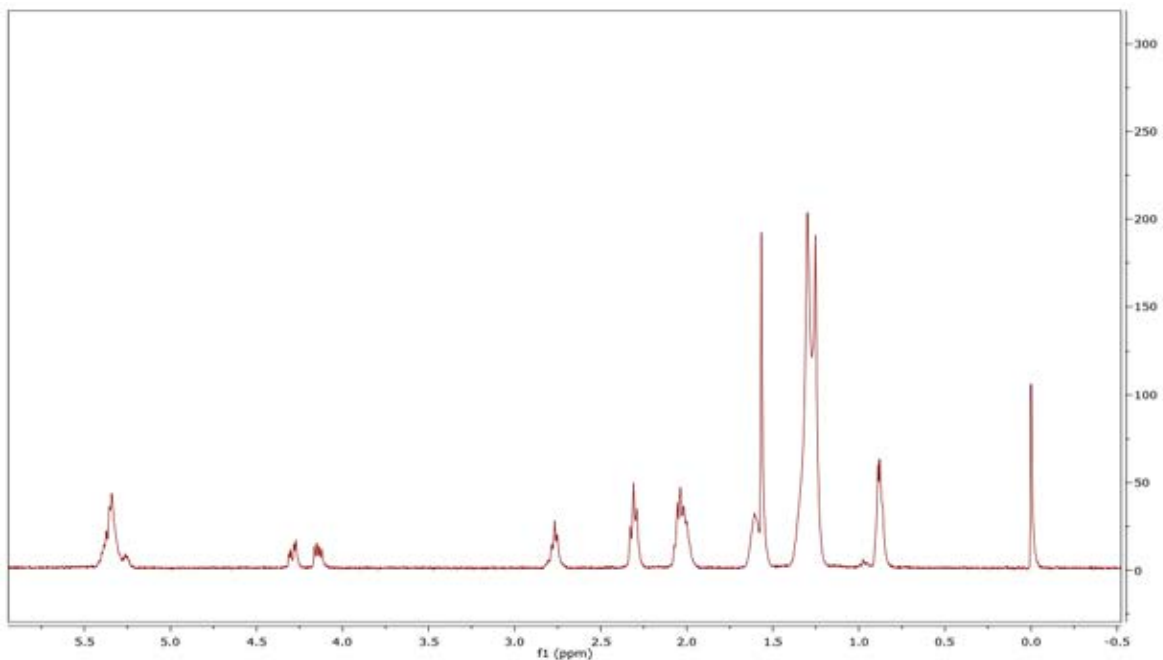
Figure B-1 ^{1}H NMR spectrum of soybean oil.
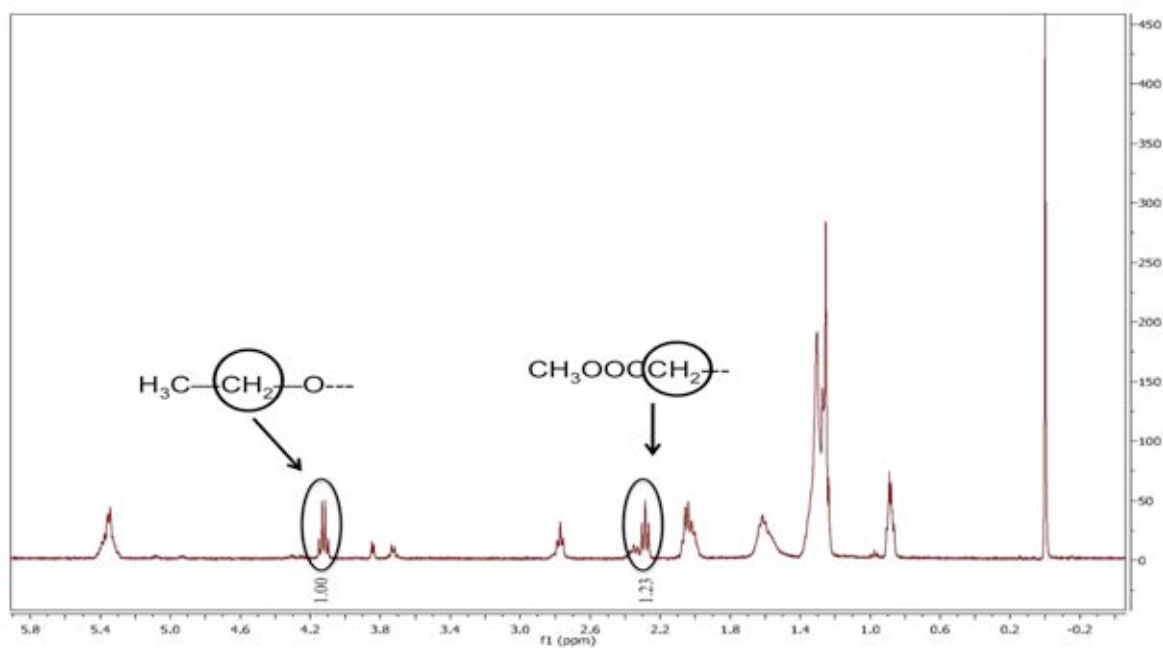
Figure B-2 ^{1}H NMR spectrum of biodiesel from soybean oil with ethanol in *tert*-butanol system.
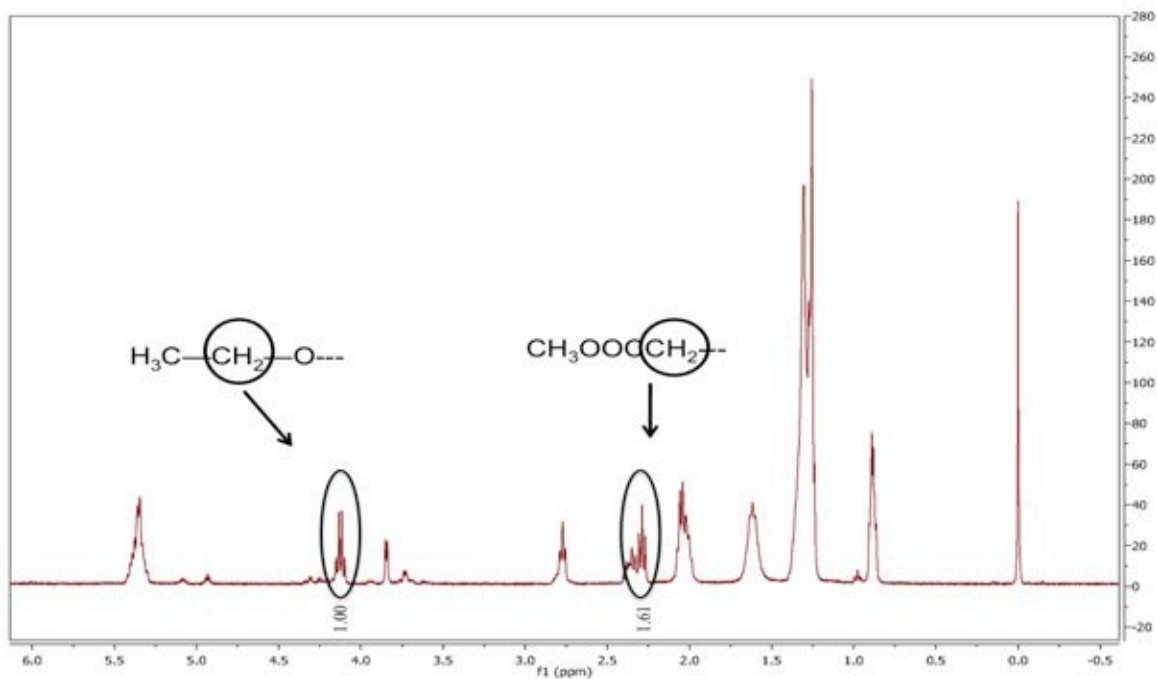
Figure B-3 ^{1}H NMR spectrum of biodiesel from soybean oil with ethanol.
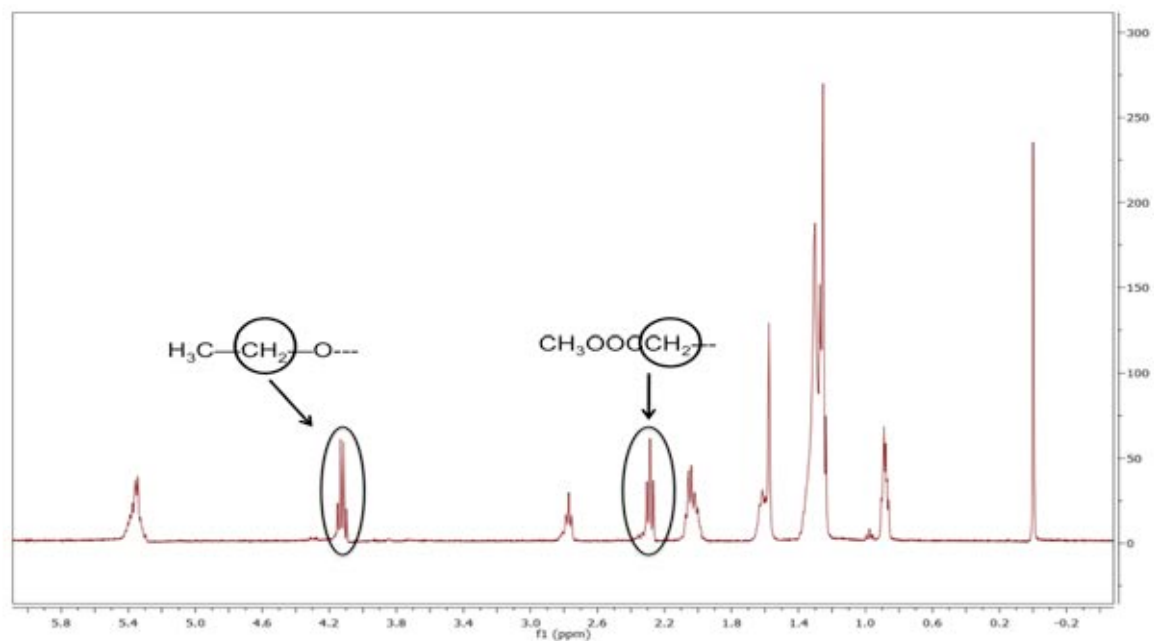
Figure B-4 ^{1}H NMR spectrum of biodiesel from soybean oil with dry ethanol.
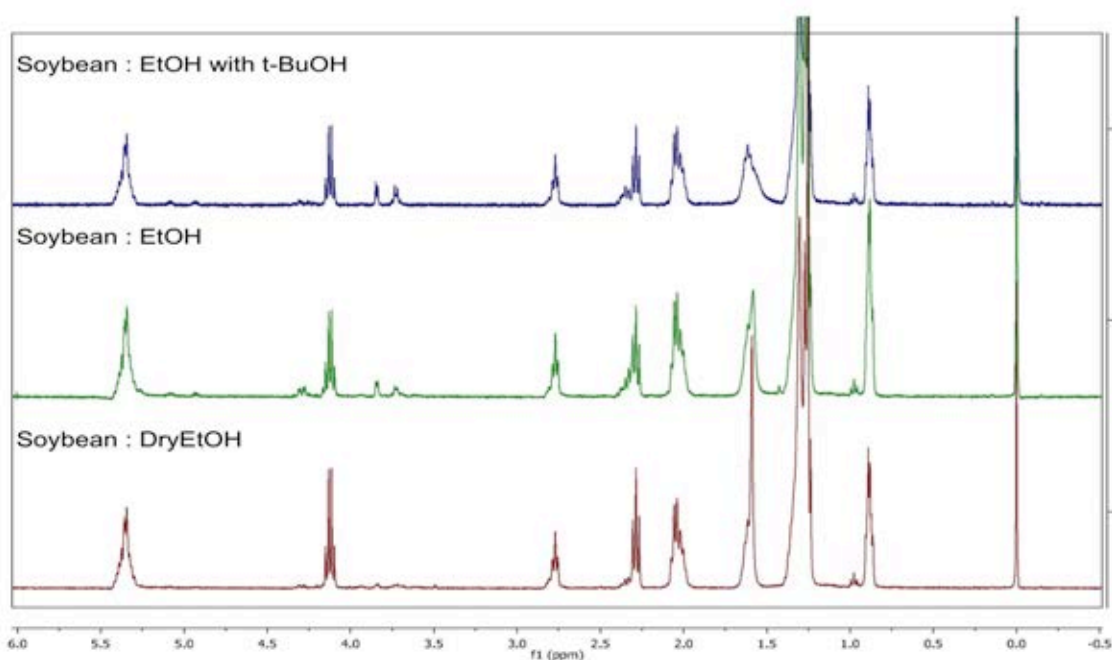
Figure B-5 ^{1}H NMR spectrums of comparison of biodiesel from soybean oil with ethanol (dry ethanol, ethanol, ethanol with organic solvent).
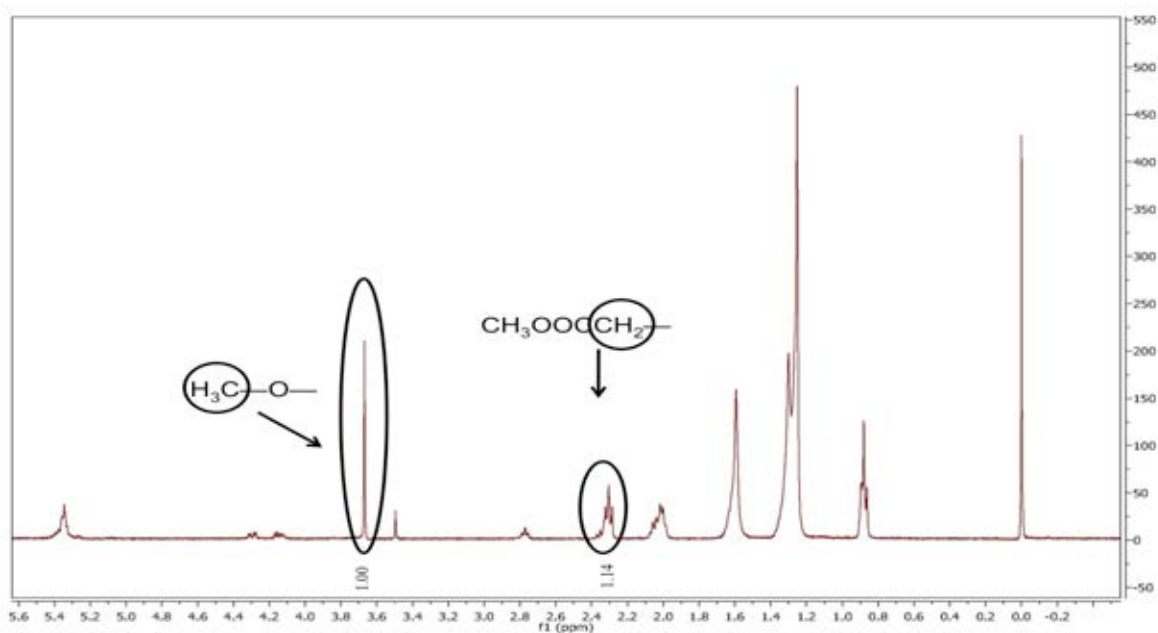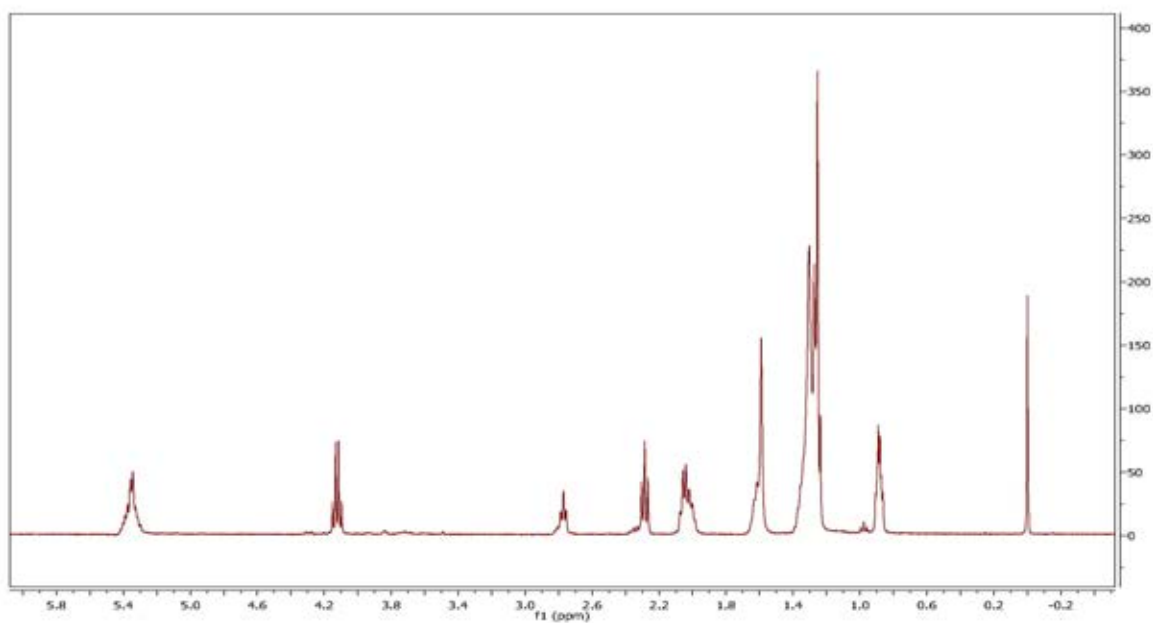

Figure B-6 ^{1}H NMR spectrum of biodiesel from soybean oil with methanol in *tert*-butanol system
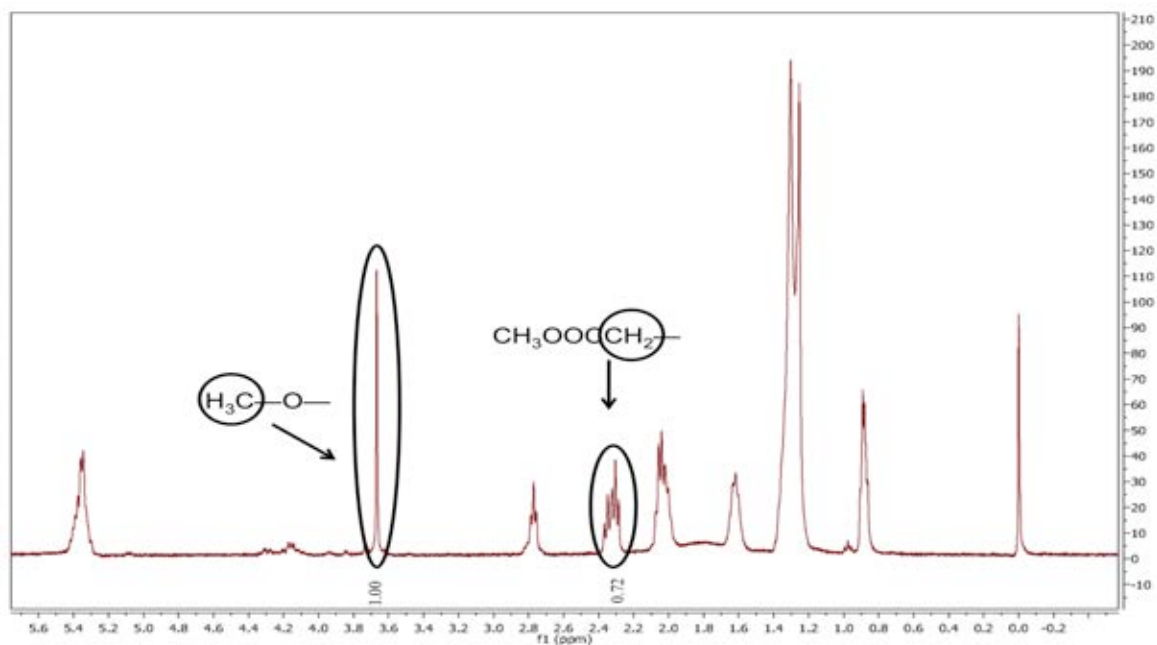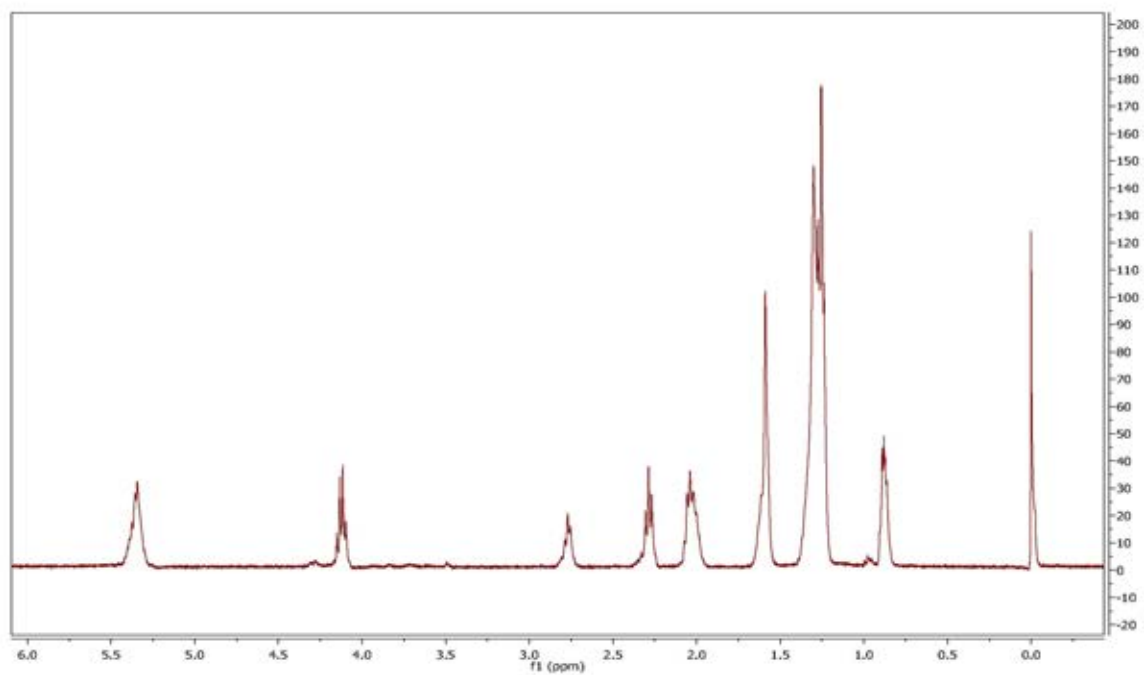
Figure B-7 ^{1}H NMR spectrum of biodiesel from soybean oil with methanol.
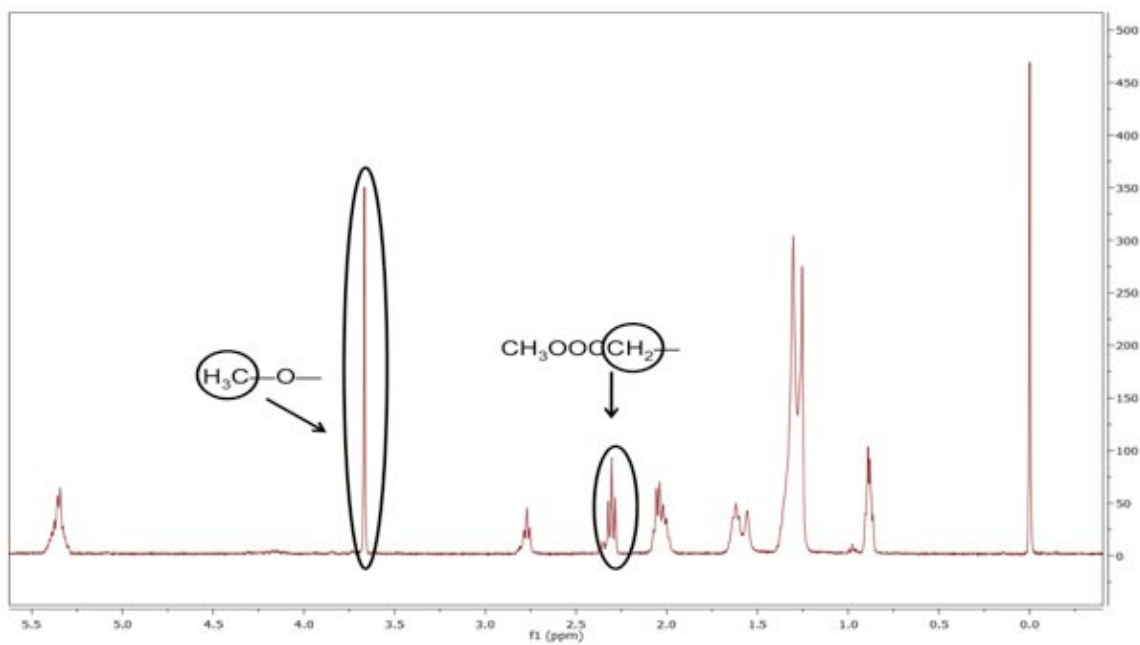
Figure B-8 ^{1}H NMR spectrum of biodiesel from soybean oil with dry methanol.
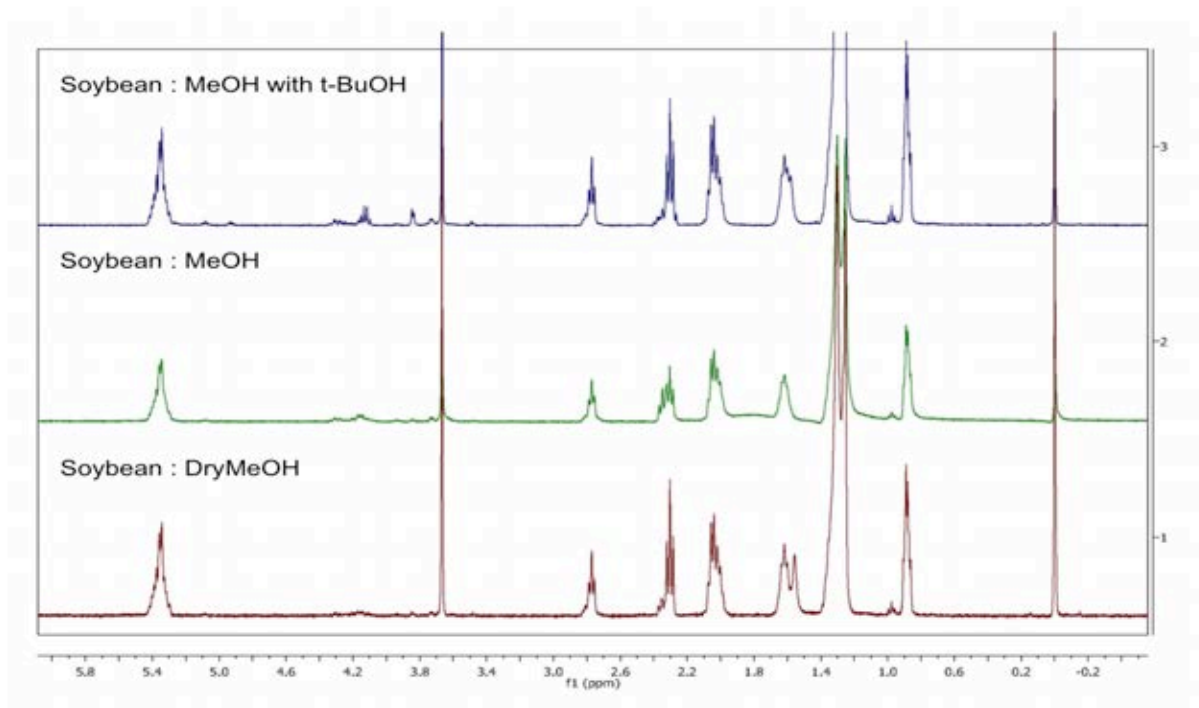
Figure B-9 ^{1}H NMR spectrums of biodiesel from soybean oil with methanol (dry methanol, methanol, methanol with organic solvent).
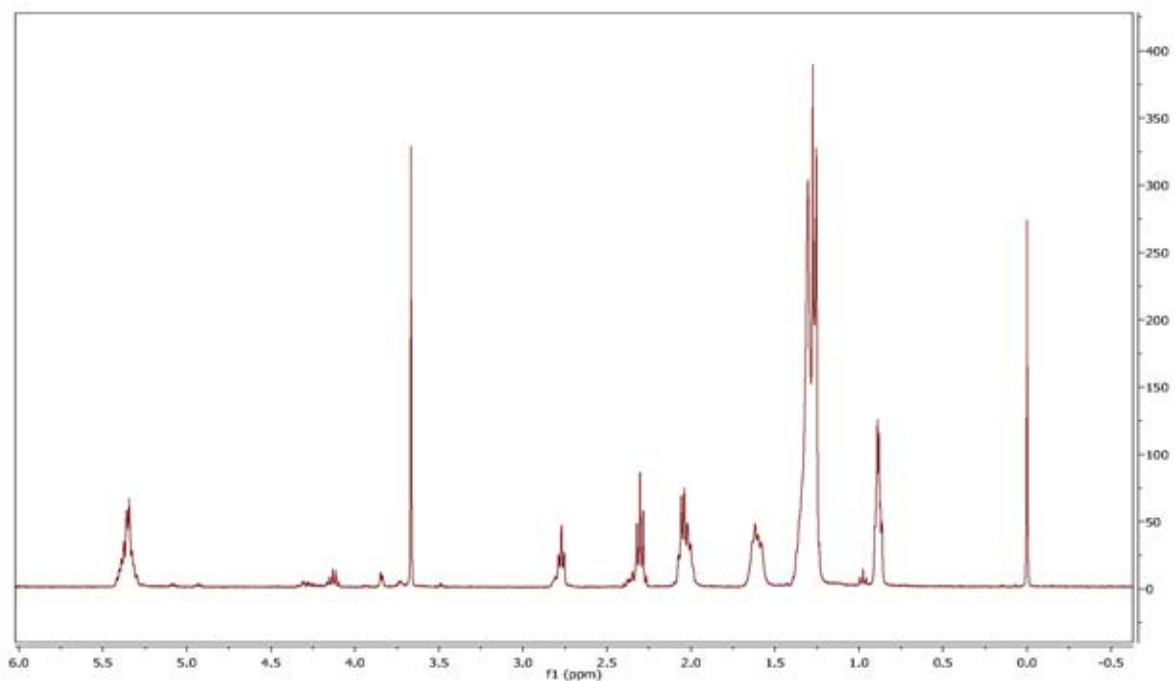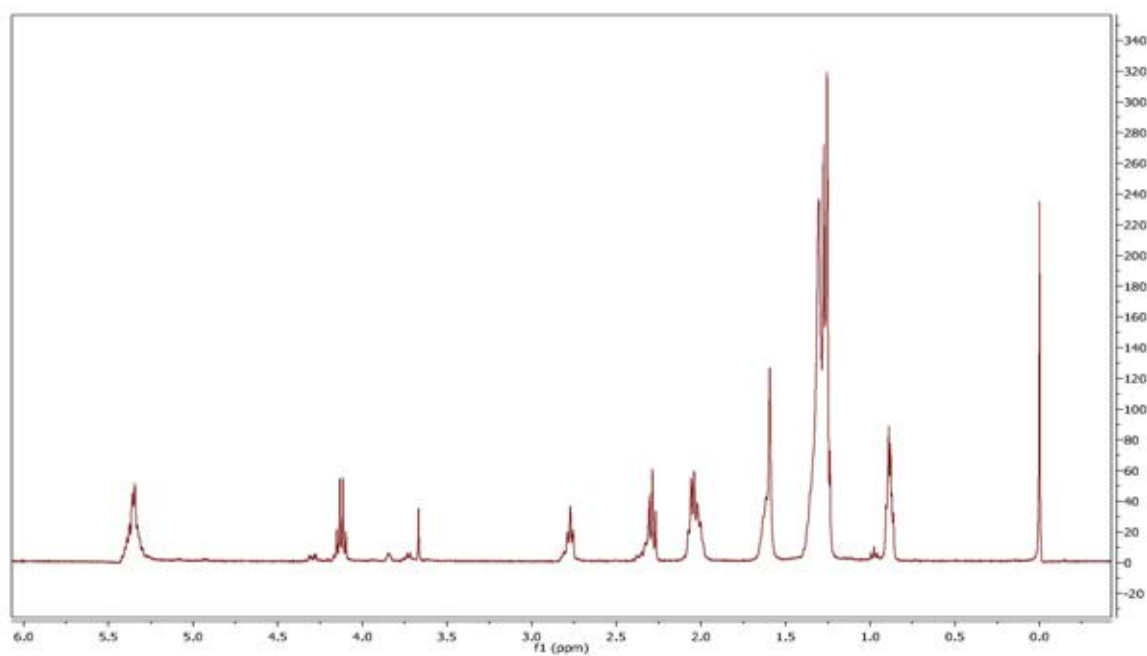

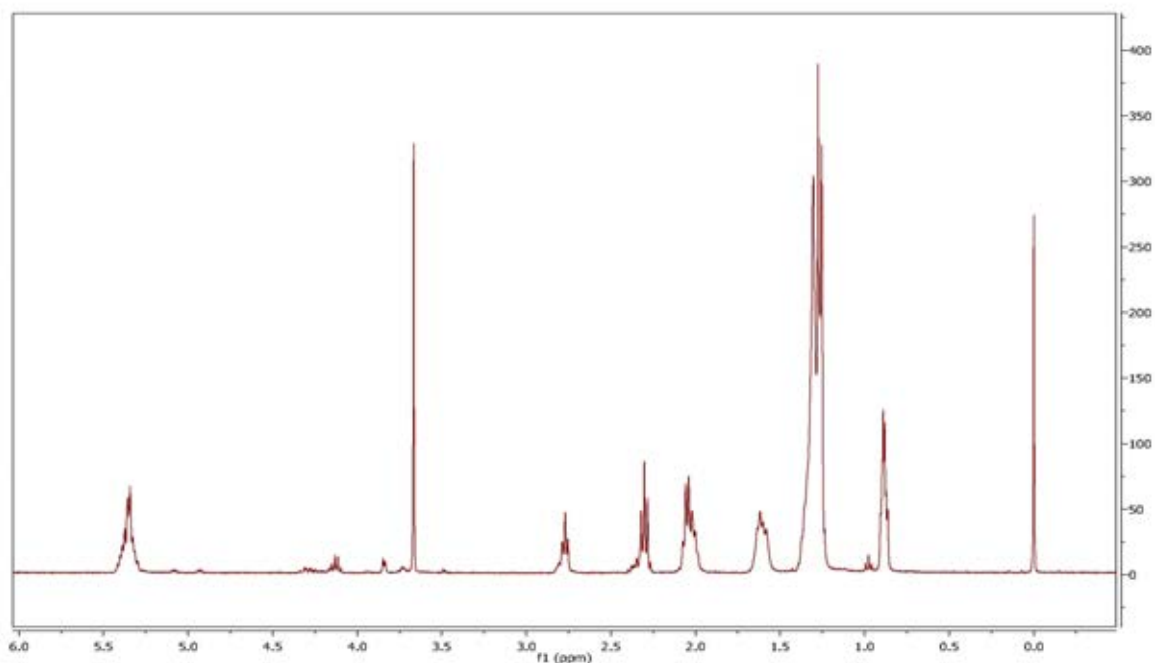**Figure B-10** ^{1}H NMR spectrum of biodiesel from palm oil with dry ethanol.
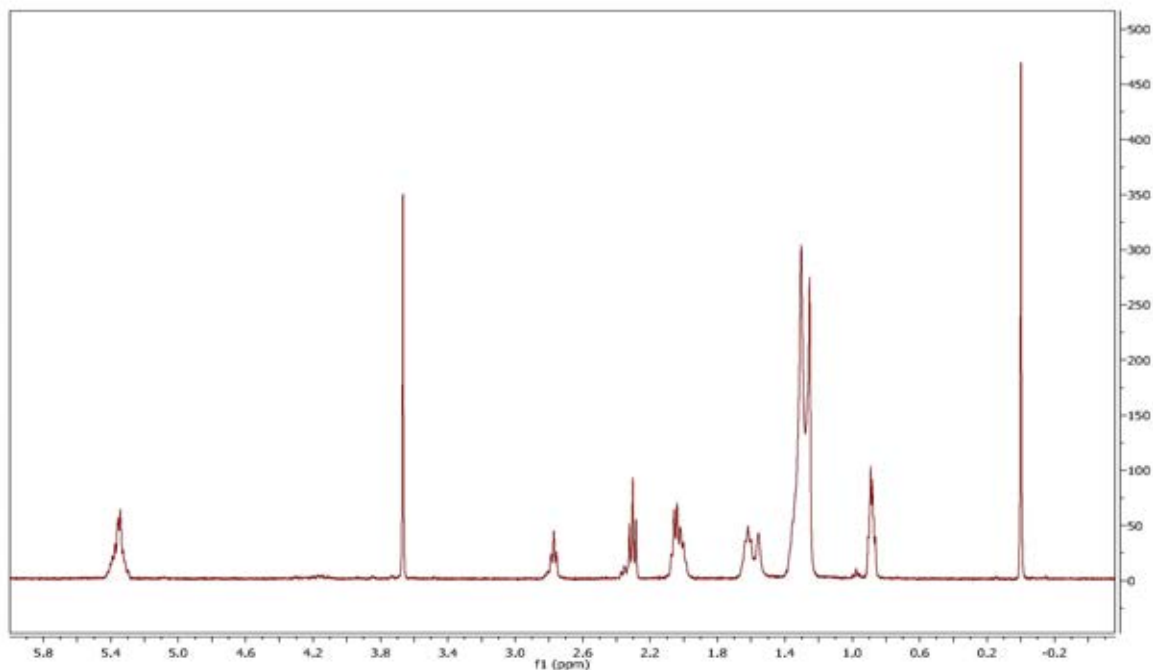
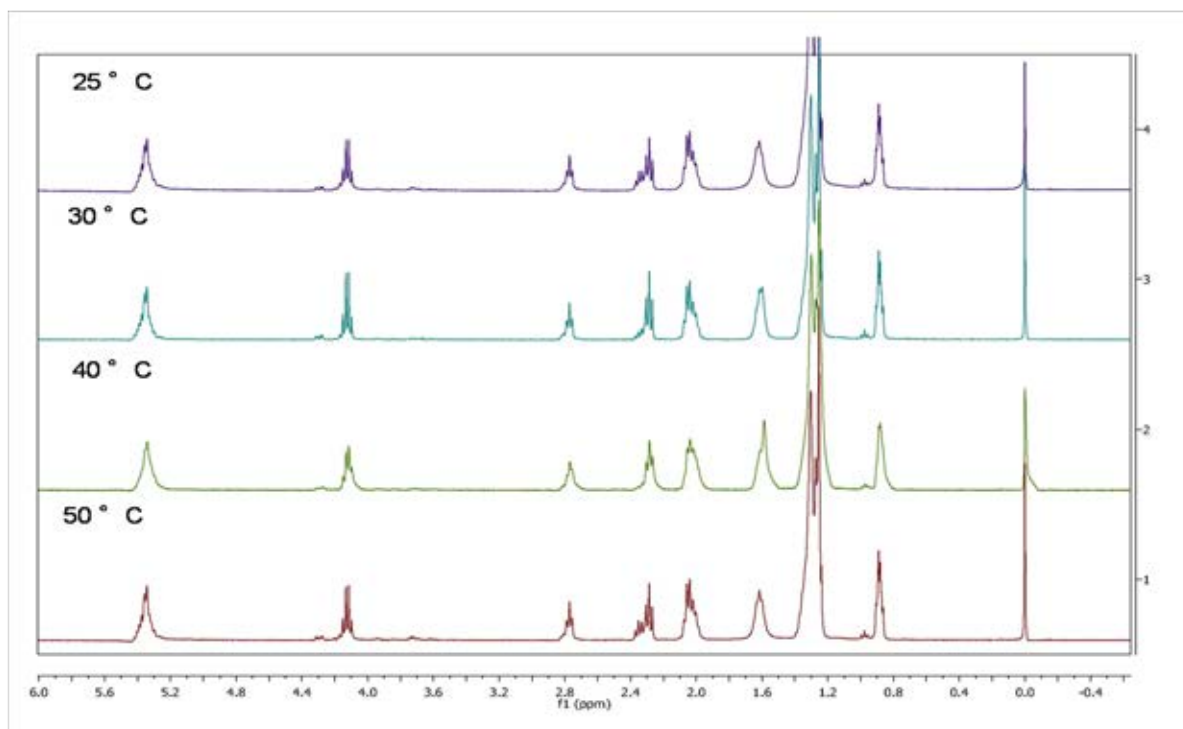**Figure B-11** ^{1}H NMR spectrum of biodiesel from palm oil with dry methanol.
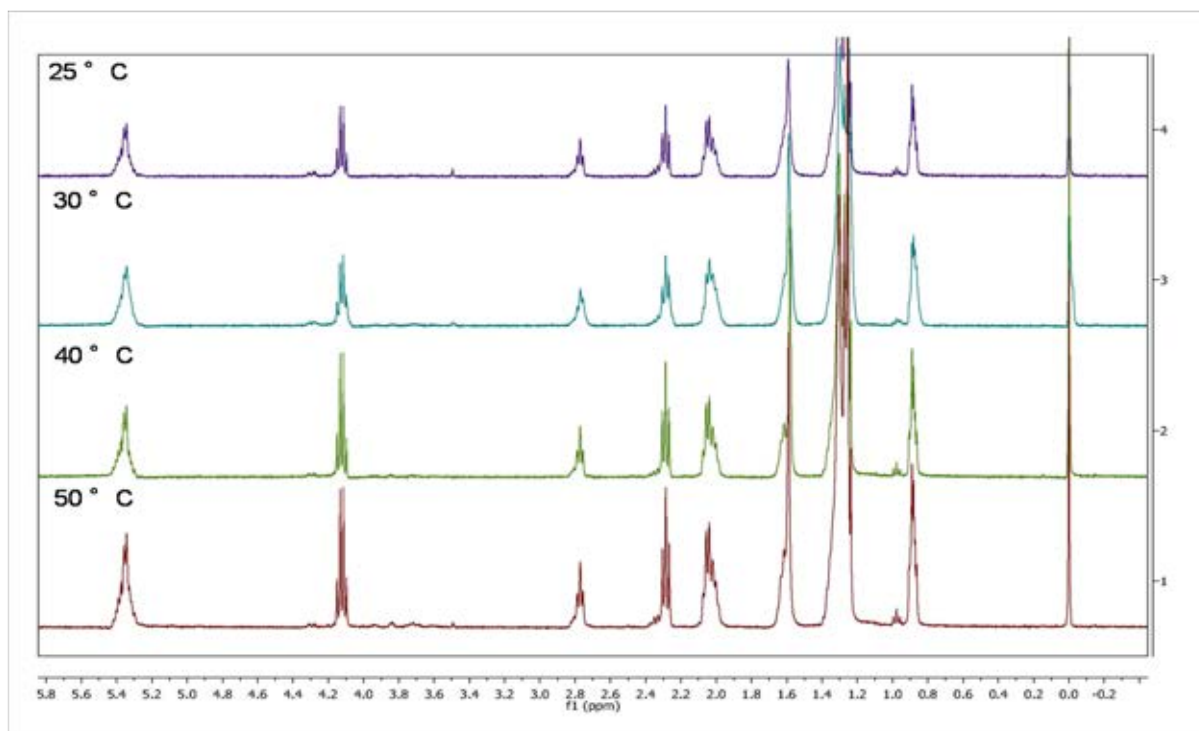
Figure B-12 ^{1}H NMR spectrums of variation of temperature on biodiesel conversion from soybean oil with dry ethanol.
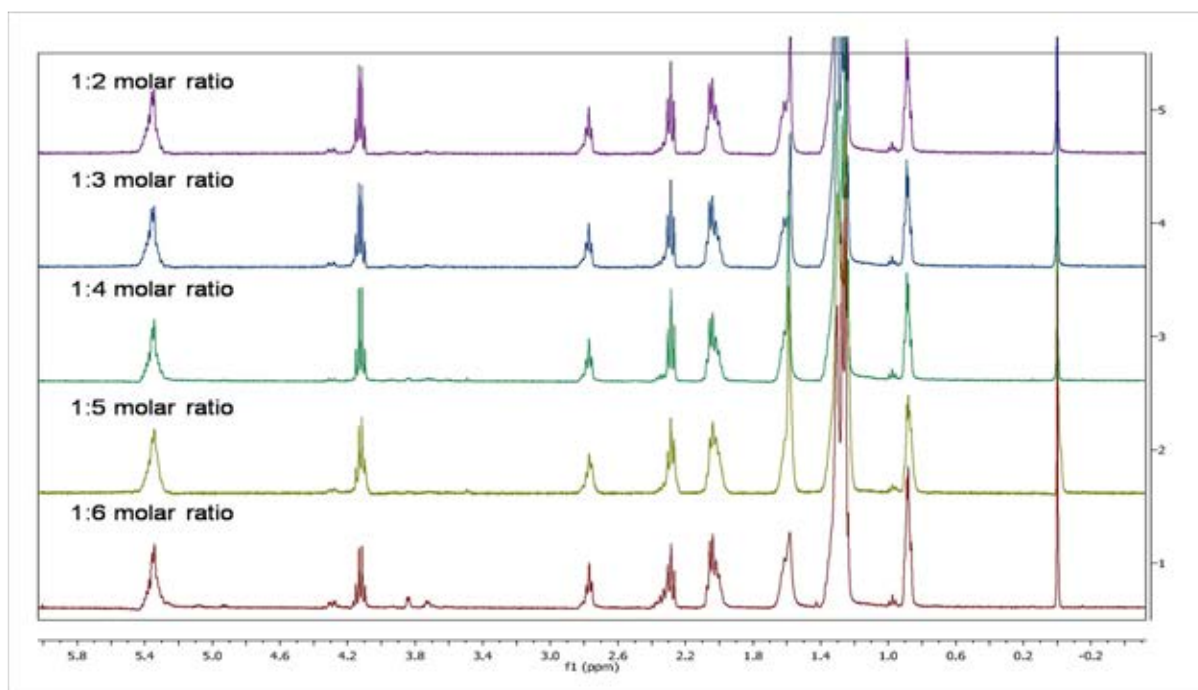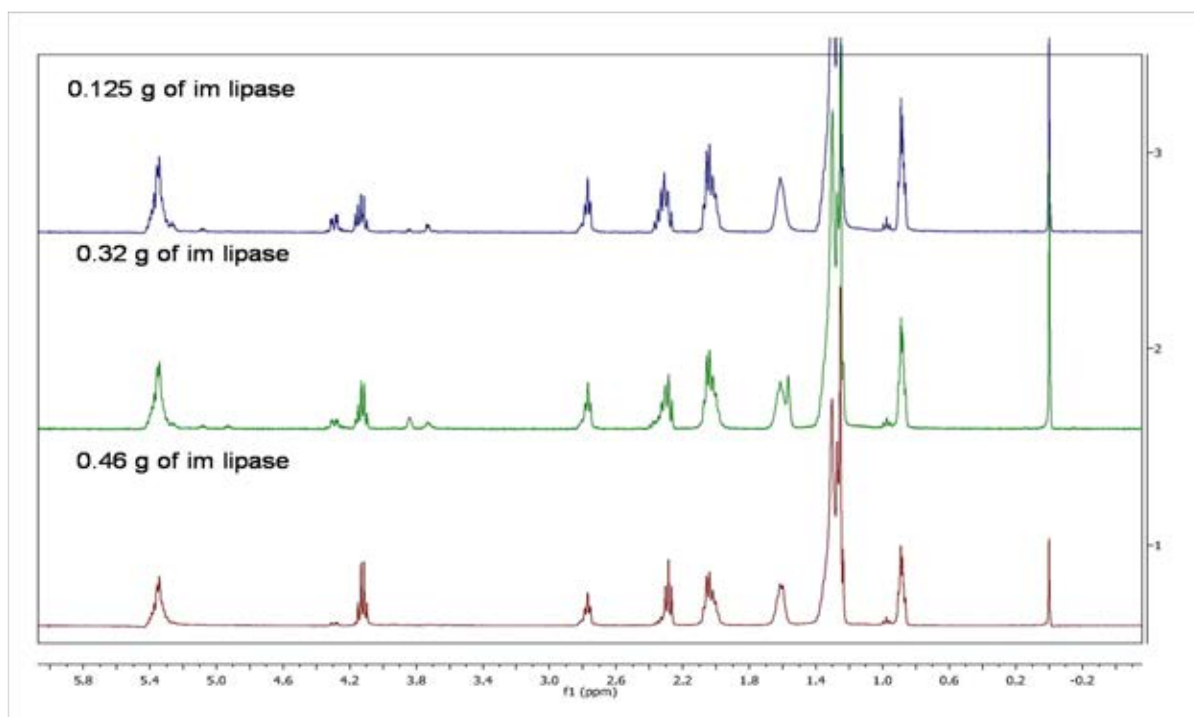
Figure B-13 ^{1}H NMR spectrums of variation of molar ratio on biodiesel conversion from soybean oil with dry ethanol
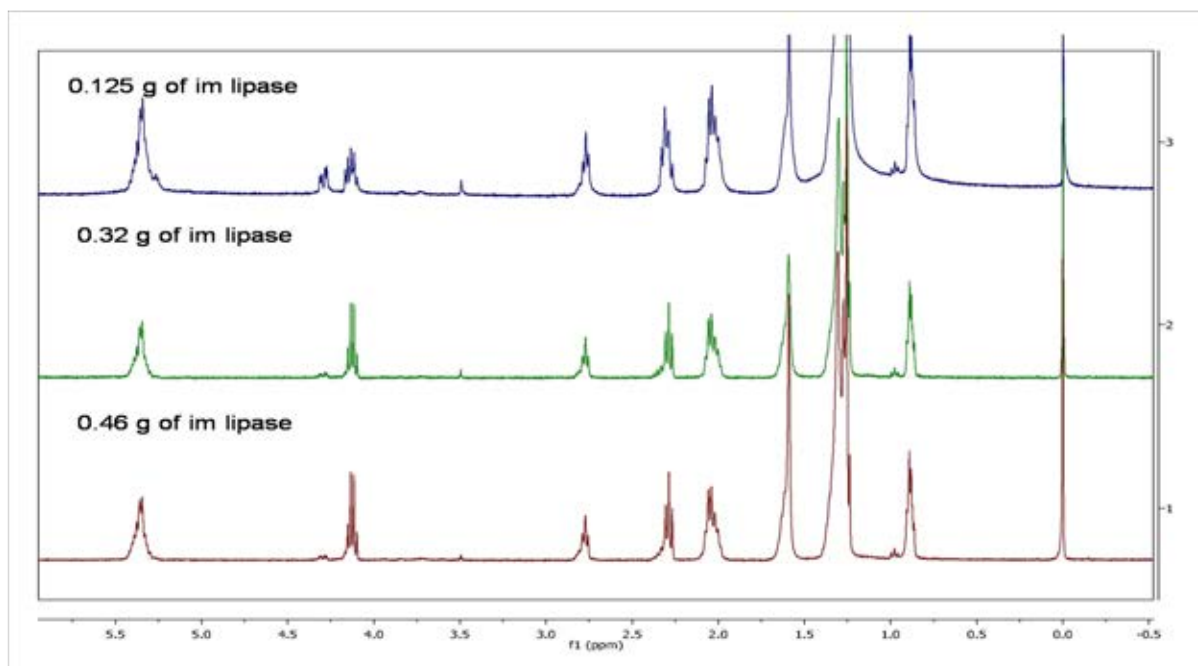
Figure B-14 ^{1}H NMR spectrums of variation enzyme dosage on biodiesel conversion from soybean oil with dry ethanol

1.2 Biodiesel production from soybean oil using immobilized lipase onto PGlu-polystyrene copolymer as catalyst

Table B-2 ^{1}H NMR results from biodiesel conversion of transesterification using immobilized lipase onto synthesized PGlu-polystyrene copolymer.

Reaction	Feed stock	Acceptor	Solvent	Temp (°C)	Conversion (%)	Figure
1	Soybean oil	Dry-ethanol	Free-solvent	40	90.00	B-15
2	Soybean oil	Dry-methanol	Free-solvent	40	87.43	B-16
3	Palm oil	Dry-ethanol	Free-solvent	40	85.00	B-17
4	Palm oil	Dry-methanol	Free-solvent	40	82.97	B-18

Figure B-15 ^{1}H NMR spectrum of biodiesel from soybean oil with dry ethanol

Figure B-16 ^{1}H NMR spectrum of biodiesel from soybean oil with dry methanol

Figure B-17 ^{1}H NMR spectrum of biodiesel from palm oil with dry ethanol

Figure B-18 ^{1}H NMR spectrum of biodiesel from palm oil with dry methanol.

Figure B-19 ^{1}H NMR spectrums of variation of temperature on biodiesel conversion from soybean oil with dry ethanol

Figure B-20 ^{1}H NMR spectrums of variation of enzyme dosage on biodiesel conversion from soybean oil with dry ethanol
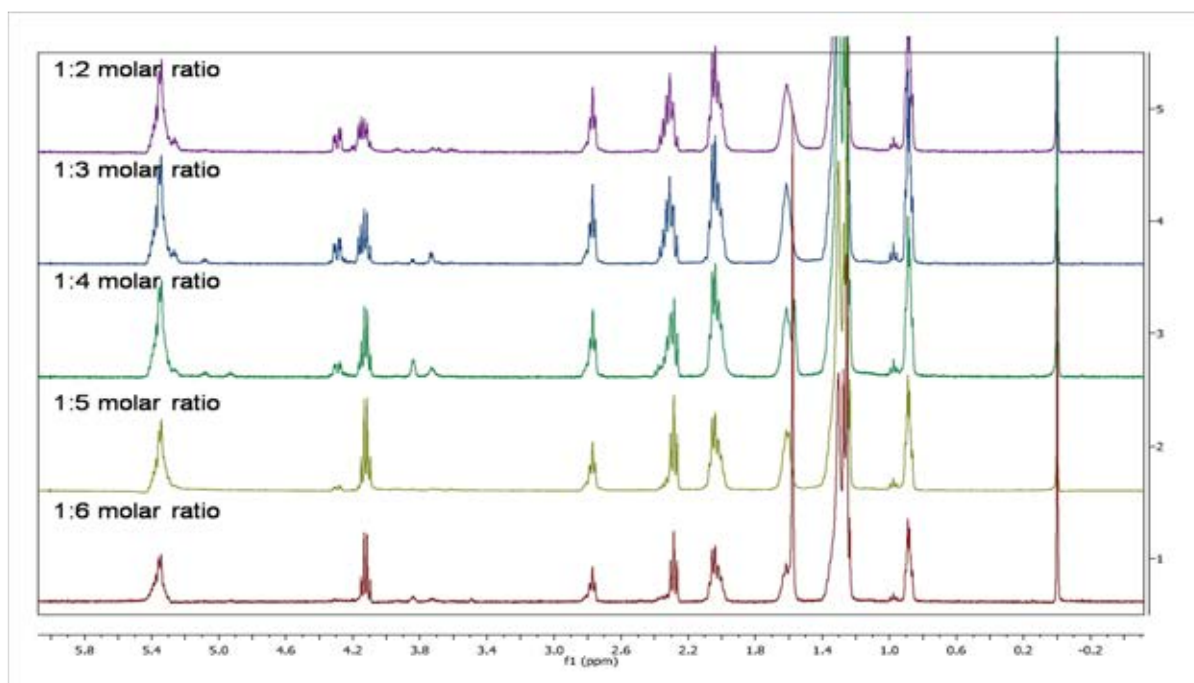
Figure B-21 ^{1}H NMR spectrums of variation of molar ratio on biodiesel conversion from soybean oil with dry ethanol

STRUCTURE
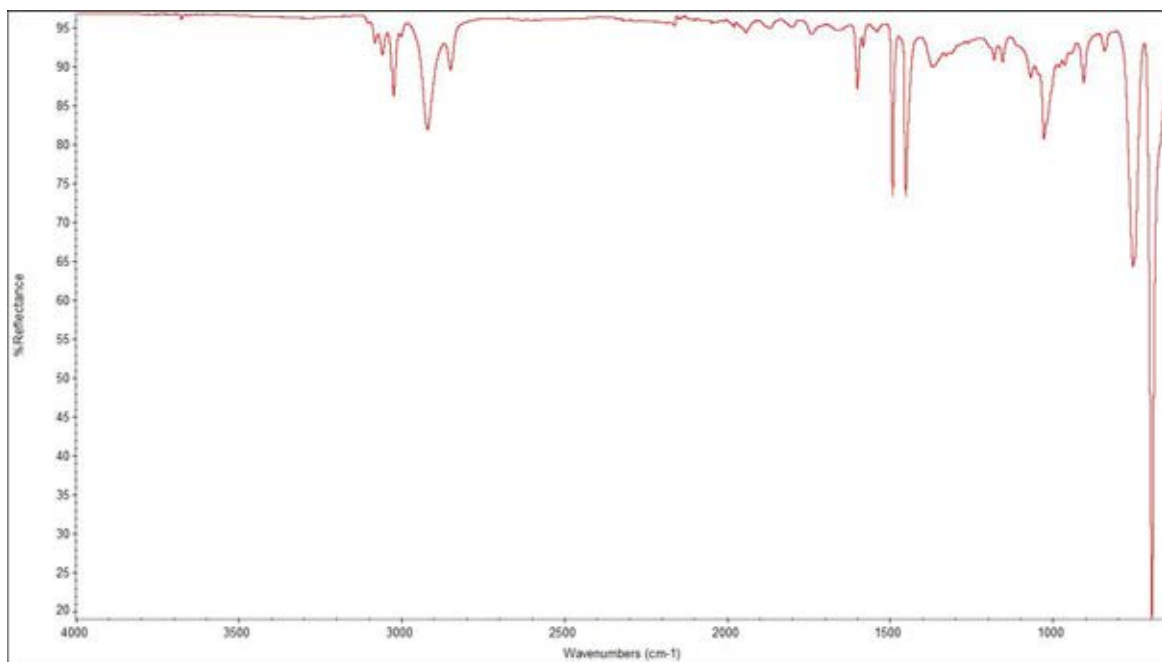
Figure B-22 FT-IR spectrum of polystyrene bead
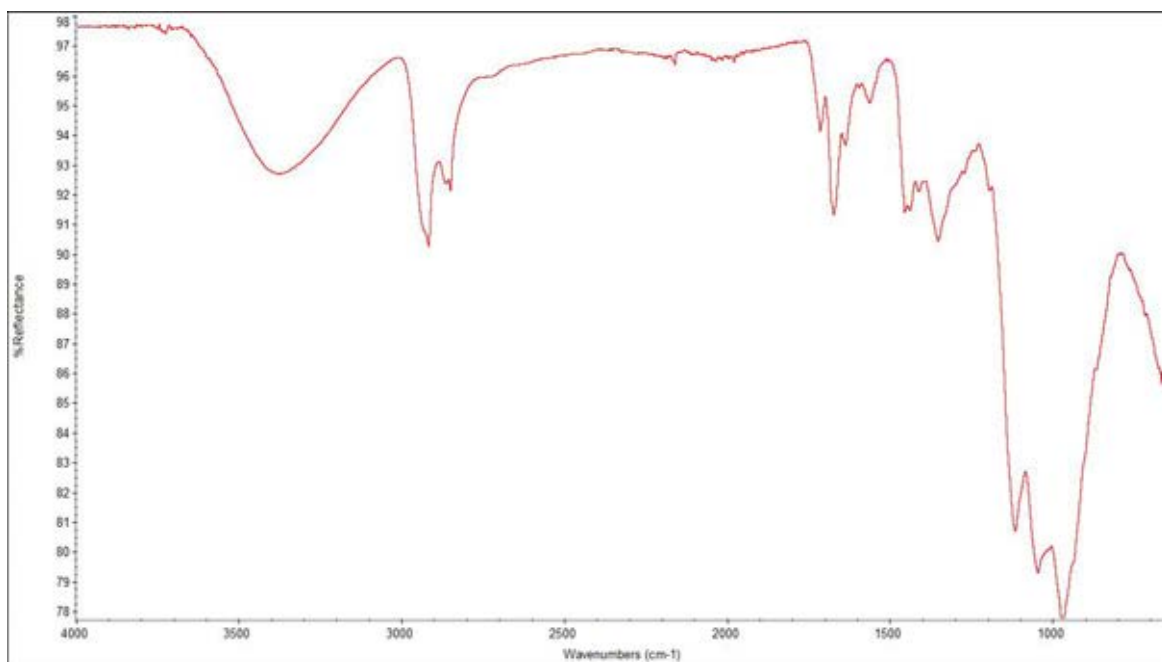
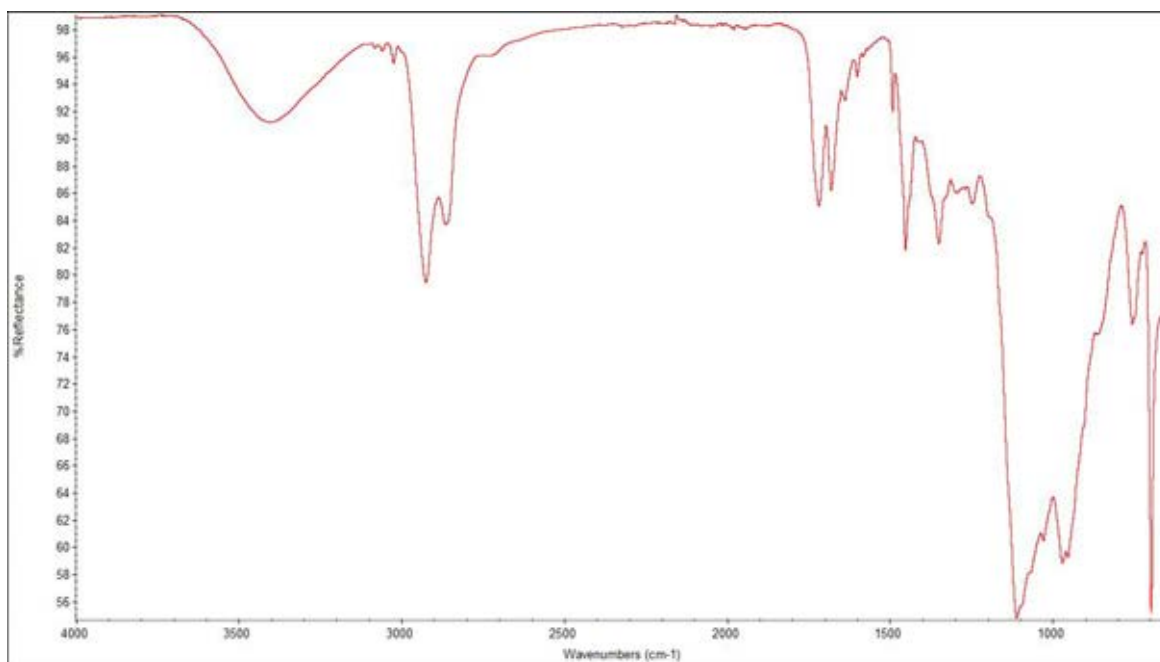**Figure B-23** FT-IR spectrum of polyglutaraldehyde

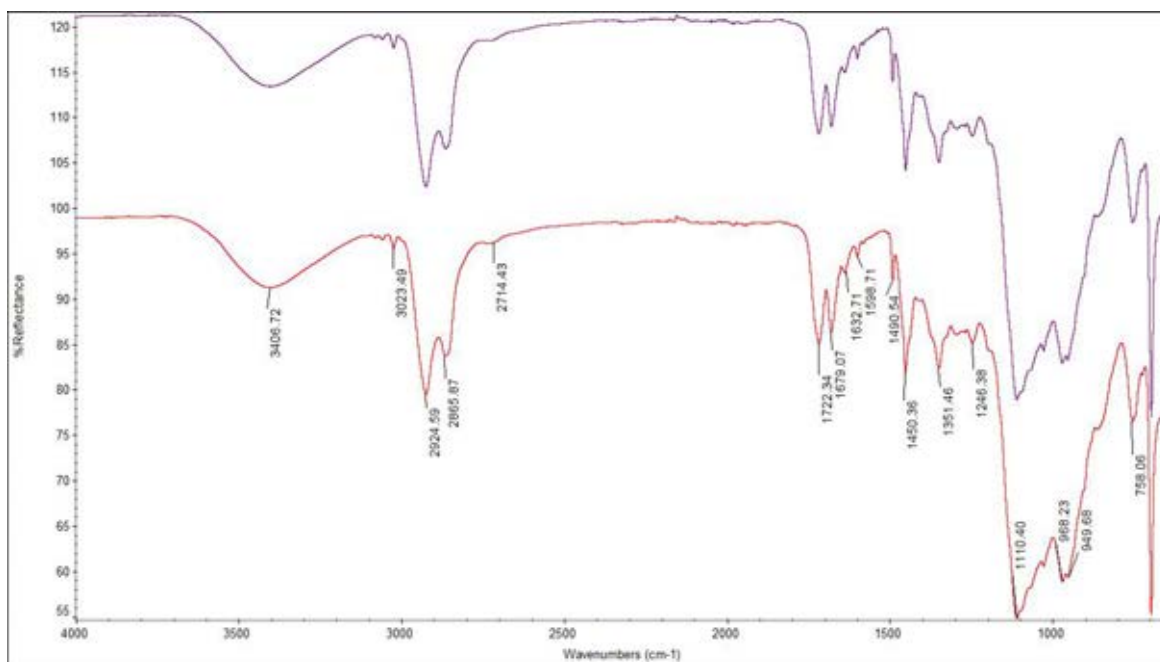**Figure B-24** FT-IR spectrum of PGlu-STY/EPS bead

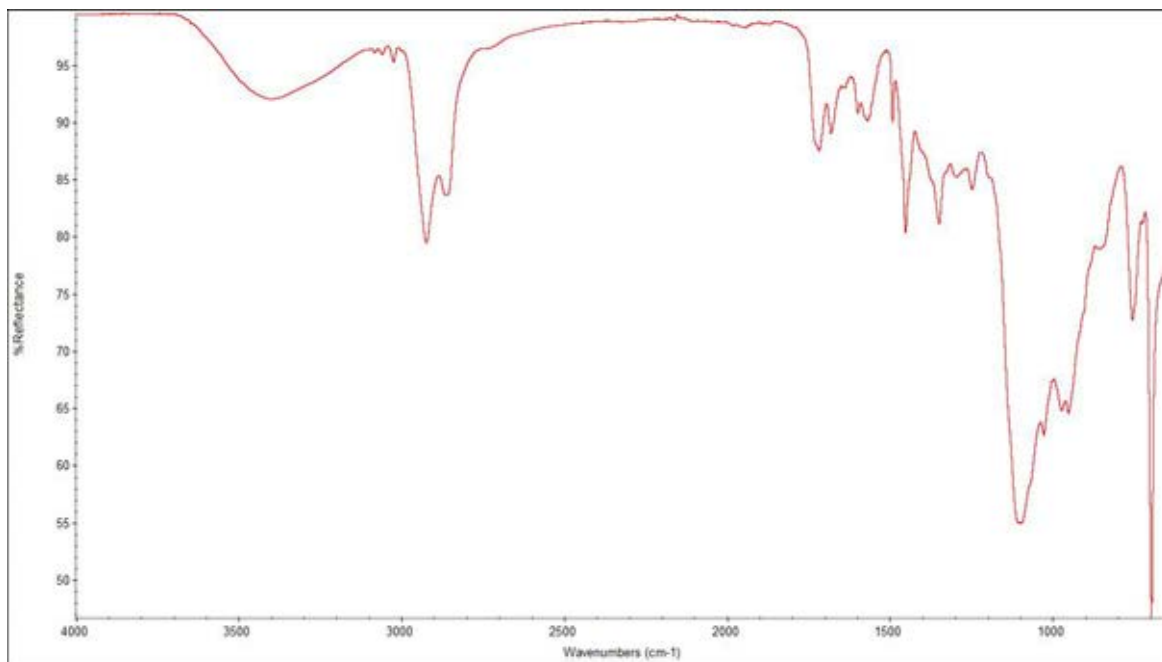**Figure B-25** FT-IR spectrums of two surfaces of PGlu-STY/EPS bead

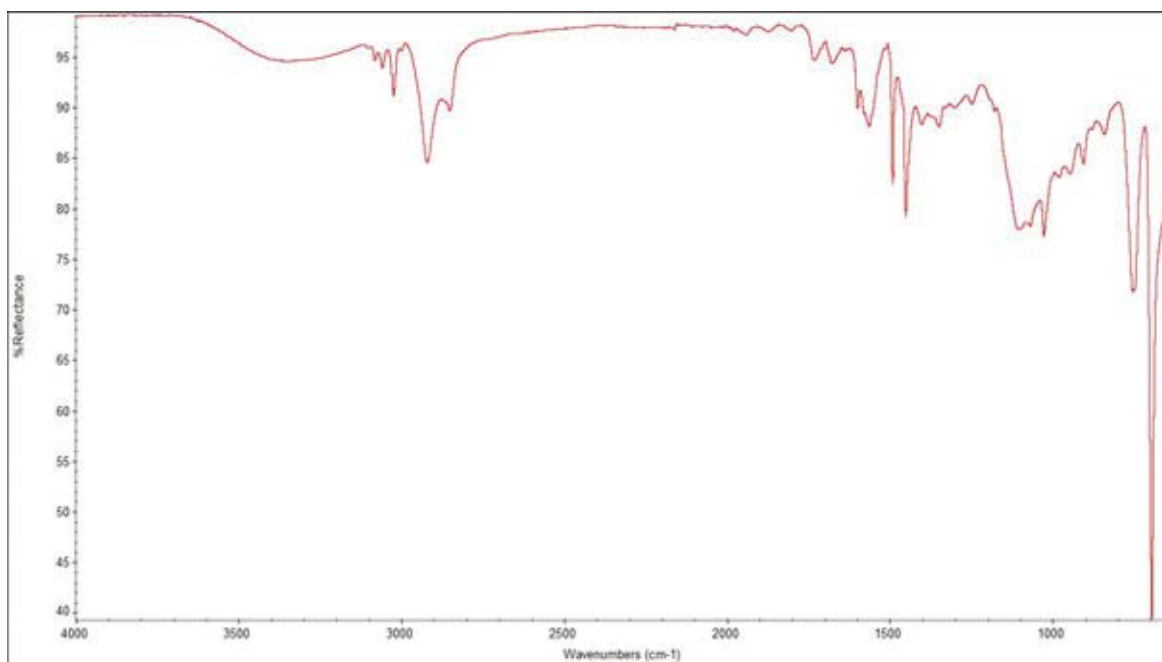**Figure B-26** FT-IR spectrum of synthesized PGlu-polystyrene copolymer

Figure B-27 FT-IR spectrum of immobilized lipase onto PGlu-STY/EPS bead

VITA

Miss Thanaporn Jitrasing was born on July 28, 1986 in Songkhla, Thailand. She got a Bachelor's Degree of chemistry in Faculty of Science at Prince of Songkhla University, Songkhla 2008. Then, she continued her Master Degree in program of Petrochemistry and Polymer Science, Faculty of Science, Chulalongkorn University, Bangkok in 2008 and completed the program in semester 1st, 2012.

Poster presentation for the 4th Science Research Conference (SRC 2012), which organized by Faculty of Science, Naresuan University in the topic of "Polyglutaraldehyde-styrene copolymer modified polystyrene beads for improvement of polymer supports" on 12-13 March 2012 at Naresuan University, Naresuan, Thailand.