ไลเพสของ *Pseudomonas cepacia* ตรึงรูปบนเม็ดพอลิสไตรีนชนิดขยายตัวได้

เพื่อการผลิตไบโอดีเซล

นางสาวจิตรานุช จิรปฐมกุล

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Pseudomonas cepacia LIPASE IMMOBILIZED ONTO EXPANDABLE POLYSTYRENE BEADS

FOR BIODIESEL PRODUCTION

Miss Jittranuch Jirapathomkul

A Thesis Submitted in Partial Fulfillment of the Requirements

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Thesis Title	*Pseudomonas cepacia* LIPASE IMMOBILIZED ONTO EXPANDABLE POLYSTYRENE BEADS FOR BIODIESEL PRODUCTION
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จิตรานุช จิรปฐมกุล : ไลเพสของ *Pseudomonas cepacia* ตรึงรูปบนเม็ดพอลิสไตรีนชนิด
ขยายตัวได้เพื่อการผลิตไบโอดีเซล (*Pseudomonas cepacia* LIPASE IMMOBILIZED ONTO
EXPANDABLE POLYSTYRENE BEADS FOR BIODIESEL PRODUCTION) อ.ที่ปรึกษา
วิทยานิพนธ์หลัก: รศ. ดร.สุรชัย พรภคกุล, 92 หน้า.

จากงานวิจัยของกลุ่มวิจัยที่ผ่านมาได้ทำการตรึงไลเพสของ *Pseudomonas cepacia* บนเม็ดพอ
ลิสไตรีนที่เคลือบด้วยพอลิกลูตารัลดีไฮด์-สไตรีนโคพอลิเมอร์ ซึ่งมีเพียงขนาดเดียว มีการรายงานว่าอัตราการ
เกิดปฏิกิริยาสูงสุดของเอนไซม์มีค่าสูงขึ้นเมื่อไลเพสถูกตรึงบนตัวค้ำจุนที่มีขนาดอนุภาคเล็กลง เพื่อเป็นการ
เพิ่มประสิทธิภาพของตัวเร่งปฏิกิริยาเอนไซม์ตรึงรูปสำหรับผลิตไบโอดีเซล ดังนั้น เม็ดพอลิสไตรีนที่เคลือบ
ด้วยพอลิกลูตารัลดีไฮด์-สไตรีนที่มีขนาดเล็กกว่าจึงถูกเตรียมขึ้น โดยการเปลี่ยนแปลงอุณหภูมิการเคลือบ
อย่างไรก็ตาม เม็ดพอลิสไตรีนที่ถูกเตรียมขึ้นนั้นยังไม่เหมาะสมสำหรับการตรึงไลเพส ดังนั้น เม็ดพอลิสไตรีน
ชนิดขยายตัวได้ 2 ขนาด ประกอบด้วย เม็ดพอลิสไตรีนชนิดขยายตัวได้เกรด 291 แอล ขนาด 1.00 ถึง
1.60 มิลลิเมตร และเม็ดพอลิสไตรีนชนิดขยายตัวได้เกรด 321 เอฟ ขนาด 0.63 ถึง 1.12 มิลลิเมตร จึงถูก
นำมาใช้สำหรับการเคลือบพอลิกลูตารัลดีไฮด์-สไตรีนโคพอลิเมอร์ จากนั้นตัวเร่งปฏิกิริยาเอนไซม์ตรึงรูปที่
เตรียมได้ ถูกนำไปประเมินค่ากิจกรรมเอนไซม์ตรึงรูปและประสิทธิภาพสำหรับผลิตไบโอดีเซลจากน้ำมันถั่ว
เหลือง ในระหว่างกระบวนการเคลือบ พบว่า เม็ดพอลิสไตรีนชนิดขยายตัวได้ที่เคลือบด้วยพอลิกลูตารัลดี
ไฮด์-สไตรีน ไม่เพียงแต่เตรียมได้จากการใช้พอลิกลูตารัลดีไฮด์ชนิดสารละลายตามที่เปิดเผยในงานวิจัยต่างๆ
แต่ยังเตรียมได้จากการใช้สารแขวนตะกอนพอลิกลูตารัลดีไฮด์ทั้งหมดทั้งที่อยู่ในสภาพสารละลายและไม่
ละลาย ซึ่งเตรียมจากสารละลายกลูตารัลดีไฮด์ที่ความเข้มข้นนั้นๆ การใช้สารแขวนตะกอนพอลิกลูตารัลดี
ไฮด์ทั้งหมดทั้งที่อยู่ในสภาพสารละลายและไม่ละลายในทั้ง 2 ขนาดของเม็ดพอลิสไตรีนชนิดขยายตัวได้ให้
เปอร์เซ็นต์การเคลือบติดของพอลิกลูตารัลดีไฮด์-สไตรีนมากกว่าการใช้พอลิกลูตารัลดีไฮด์ชนิดสารละลาย
สำหรับกิจกรรมของเอนไซม์ตรึงรูป พบว่า ตัวเร่งปฏิกิริยาเอนไซม์ตรึงรูปทั้ง 2 ขนาดที่เตรียมขึ้นจากการใช้
พอลิกลูตารัลดีไฮด์ชนิดสารละลายให้ค่ากิจกรรมของเอนไซม์ตรึงรูปมากกว่าที่เตรียมจากการใช้สารแขวน
ตะกอนพอลิกลูตารัลดีไฮด์ทั้งหมดทั้งที่อยู่ในสภาพสารละลายและไม่ละลาย และกิจกรรมของเอนไซม์ที่ตรึง
รูปบนเม็ดพอลิสไตรีนชนิดขยายตัวได้เกรด 321 เอฟ ให้ค่ากิจกรรมสูงกว่าเม็ดพอลิสไตรีนชนิดขยายตัวได้
เกรด 291 แอล สำหรับประสิทธิภาพของตัวเร่งปฏิกิริยาเอนไซม์ตรึงรูปที่เตรียมได้ในการผลิตไบโอดีเซลจาก
น้ำมันถั่วเหลือง พบว่า มีประสิทธิภาพต่ำมาก แต่เมื่อมีการเติมน้ำลงไปในปฏิกิริยากลับพบว่า สามารถช่วย
เพิ่มการผลิตไบโอดีเซลขึ้นอย่างมาก นอกจากนี้ ตัวเร่งปฏิกิริยาเอนไซม์ตรึงรูปขนาดเล็กที่เตรียมจากการใช้
พอลิกลูตารัลดีไฮด์ทั้ง 2 ชนิด มีประสิทธิภาพดีกว่าตัวเร่งปฏิกิริยาเอนไซม์ตรึงรูปขนาดใหญ่

สาขาวิชา   ปิโตรเคมีและวิทยาศาสตร์พอลิเมอร์   ลายมือชื่อนิสิต ....

ปีการศึกษา 2558   ลายมือชื่อ อ.ที่ปรึกษาหลัก ....

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KEYWORDS: POLYGLUTARALDEHYDE, EPS BEADS, STYRENE

JITTRANUCH JIRAPATHOMKUL: *Pseudomonas cepacia* LIPASE IMMOBILIZED ONTO EXPANDABLE POLYSTYRENE BEADS FOR BIODIESEL PRODUCTION. ADVISOR: ASSOC. PROF. SURACHAI PORNPAKAKUL, Ph.D., 92 pp.

In previous results of our research group, the lipase from *Pseudomonas cepacia* was immobilized onto polyglutaraldehyde-styrene copolymer coated expandable polystyrene beads (PGlu-STY/EPS) with only one particle size. There was a report that the maximum enzymatic reaction rate was at a higher level by using lipase-immobilizing with smaller particle size. To improve the efficiency of lipase-immobilized catalyst for biodiesel production, the smaller particle sizes of PGlu-STY/EPS beads were prepared by varying coating temperature and the beads prepared however were unsuitable for immobilization of the lipase. Thus two sizes of expandable polystyrene bead (EPS) consisting of EPS bead grade 291L, with size of 1.00-1.60 mm, and EPS bead grade 321F, with size of 0.63-1.12 mm, were used for coating with PGlu-STY copolymer and the lipase-immobilized catalysts were then evaluated their lipase activity and efficiency for biodiesel production from soybean oil. During coating process, it was found that PGlu-STY/EPS beads not only could be prepared by using soluble polyglutaraldehyde as described in the literature but also the whole suspension of polyglutaraldehyde generated from the same original concentration of the glutaraldehyde. Using the whole suspension of polyglutaraldehyde gave the higher percentage of coating PGlu-STY on both sizes of EPS beads than that of soluble polyglutaraldehyde. In both sizes of EPS beads, the lipase-immobilized catalysts that was prepared by using the soluble polyglutaraldehyde provides higher activity than that was prepared by using the whole suspension of polyglutaraldehyde and the activity of lipase immobilized on 321FEPS bead was higher than 291LEPS bead. The efficiency of those immobilized catalysts for biodiesel production from soybean oil was notably low. Nevertheless, the addition of water to the reaction could significantly improve production of biodiesel. Furthermore, the lipase-immobilizing with small size which was prepared by using the soluble and the whole polyglutaraldehyde has better efficiency than the bigger one.

Field of Study:	Petrochemistry and Polymer Science	Student's Signature	....
		Advisor's Signature	....
Academic Year:	2015		

ACKNOWLEDGEMENTS

CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SCHEMES

LIST OF ABBREVIATIONS

PGlu	Polyglutaraldehyde
STY	Styrene
SPGlu	Soluble polyglutaraldehyde
WPGlu	The whole polyglutaraldehyde
EPS	Expandable polystyrene
FAEE	Free fatty acid ethyl ester
FAME	Free fatty acid methyl ester
μM	Micromolar
°C	Degree Celsius
^{1}H NMR	Proton nuclear magnetic resonance spectroscopy
ATR-FTIR	Attenuated Total reflectance-Fourier transformed infrared
EtOH	Ethanol
g	Gram (s)
h	Hour (s)
H_2O	Water
M	Molar
MeOH	Methanol
mg	Milligram (s)
mm	Millimeter (s)
mM	Millimolar (s)
mmol	Millimole (s)
U	Unit

CHAPTER I

INTRODUCTION

Diesel fuel is the major energy resource for machinery and engine which is widely used in production, transportation, industry, agriculture etc. Since the diesel oil obtains from fossil fuel which has limited and high diminishing rate from increasing population and industrialization resulting in price of the diesel oil has increased significantly. In addition, the diesel fuel emits exhaust gases and green-house gases such as CO, CO_2 and SO_x which causes environmental problems [1].

For this reasons, finding of renewable energy resource to replace the diesel fuel is important. Biodiesel is one of the most attractive alternative fuels and renewable energy. So renewable raw material for biodiesel production is obtained from agricultural products available in the country. There are four primary methods for biodiesel production; direct use and blending, microemulsions, thermal cracking (pyrolysis), transesterification [2].

The most common method to produce biodiesel is transesterification of vegetable oils or animal fats with low molecular weight alcohols [3] in the presence of alkaline, acid or enzymatic as catalyst or without catalyst under supercritical condition of alcohol [4] The transesterification reaction can be represented as

$$
\begin{array}{c}
\text{CH}_2\text{-OOC-R}_1 \\
| \\
\text{CH-OOC-R}_2 \quad + \quad 3\text{R}^{'}\text{OH} \quad \xrightarrow[\longleftarrow]{\text{Catalyst}} \\
| \\
\text{CH}_2\text{-OOC-R}_3
\end{array}
\qquad
\begin{array}{c}
\text{R}_1\text{-COO-R}^{'} \\
\text{R}_2\text{-COO-R}^{'} \quad + \\
\text{R}_3\text{-COO-R}^{'}
\end{array}
\qquad
\begin{array}{c}
\text{CH}_2\text{-OH} \\
| \\
\text{CH-OH} \\
| \\
\text{CH}_2\text{-OH}
\end{array}
$$

Triglyceride   Alcohol   Alkyl ester   Glycerol
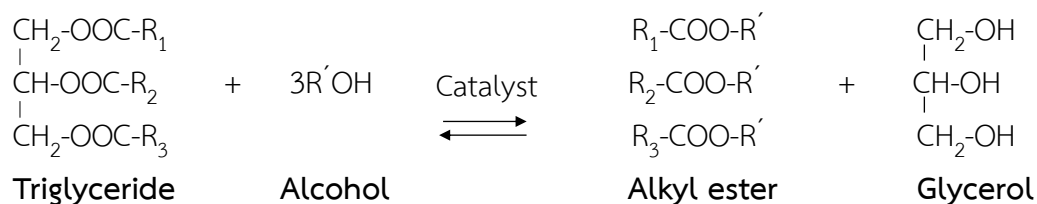
Scheme 1.1 Transsterification of triglyceride with alcohol.

Using chemical catalyst in the process gives high yields of biodiesel and short reaction time. However, pretreatment of the substrate is required when water is present and there are many drawbacks such as high energy requirements and

difficultly in recovery of catalysts and glycerol from the biodiesel product [5]. In addition, soap formed by saponification during the process, leads to an increase purification product cost. Recently, enzymatic catalyst has become more attractive to replace chemical catalyst (alkaline and acidic catalyst) because it can overcome these problems [6]. Thus the glycerol and catalysts can be removed from biodiesel product easily and results in less cost of glycerol purification. However, Using free enzyme catalyst has drawback for biodiesel process. One is the aggregation of free lipase results in decreasing stability and efficiency. The aggregated lipase is difficult to be removed from process for reusability. Moreover, the cost of free lipase catalyst is high in comparison with alkaline or acidic catalyst. Utilization of free lipase as catalyst in industrial is limited. Consequently, free lipase is developed by immobilization to lower the cost and increase the stability of lipase [7]. In previous preliminary research of RCBC group, using NOVOZYME 435 (immobilized on acrylic resin) as enzyme catalyst to produce biodiesel in stirred tank reactor found that the density of the biocatalyst was higher than oil (biodiesel) resulted in the catalyst sunk in the bottom of the reactor. The shear stress introduced mechanical damage to the biocatalyst, which can reduce the reusability of the catalyst [8]. In addition, by-product, glycerol, had the highest density which was at the bottom of the reactor, so it was easily adsorbed onto the surface of the NOVOZYME 435 also led to negative effect on lipase activity and operational stability [9].

The support is the key factor for immobilization and yield of biodiesel. So, our research group has an idea to immobilize lipase onto low density of support for solving these problems. Although our previous research, the lipase has ever immobilized onto low density of the support with only one particle size but the efficiency is still not satisfactory. There was a report that using lipase-immobilizing silica particles with smaller particle size and specific surface area, the maximum enzymatic reaction rate was at a higher level [10].

Consequently, this research focused on immobilizing *Pseudomonas cepacia* lipase onto expandable polystyrene beads (EPS beads) by covalent method in order to obtain smaller particle size of lipase-immobilized catalyst. Before immobilization,

EPS beads were activated surface by polyglutaraldehyde as cross-linking agent. It was expected that this process would increase the efficiency of immobilization and of biodiesel production.

1.1 Objectives of the research

- To prepare *Pseudomonas cepacia* lipase immobilized catalyst using different size of polyglutaraldehyde-styrene coated expandable polystyrene beads (PGlu-STY/EPS beads) as support.

- To study the efficiency of the immobilized lipase catalyst for biodiesel production from soybean oil.
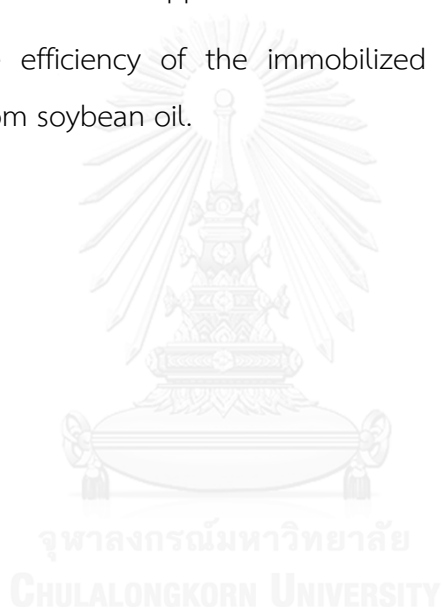
CHAPTER II
THEORY AND LITERATURE REVIEW

2.1 Biodiesel

Biodiesel has gained attractive to replace diesel fuel. The main advantage of using biodiesel is that the amounts of CO, SO_2, soot and halogens, which are major contributors to environmental pollution in the exhaust gas are much lower than those in diesel fuel [11], since raw materials for production of biodiesel (fatty acid alkyl ester) originating from vegetable oils and animal fats which can be renewable, biodegradable and non-toxic fuel [12]. Biodiesel can be produced by several processes and the some properties defined in the ASTM standard is similar to the diesel fuel as shown in Table 2.1 [13].

Table 2.1 Comparison properties of diesel and biodiesel [13]

Fuel properties	Diesel	Biodiesel
Fuel standard	ASTM D975	ASTM D6751
Lower heating value ,Btu/gal	~129,050	~118,170
Kinematic viscosity, at 40°c	1.3-4.1	4.0-6.0
Specific gravity, kg/l at 60°F	0.85	0.88
Density, lb/gal at 15°c	7.079	7.328
Water and sediment, vol%	0.05 max	0.05 max
Carbon, wt%	87	77
Hydrogen, wt%	13	12
Oxygen, by dif. wt%	0	11
Sulfur, wt%	0.55 max	0.0 to 0.0024
Boiling point, °c	180 to 340	315 to 350

Fuel properties	Diesel	Biodiesel
Flash point, °c	60 to 80	100 to 170
Cloud point, °c	-15 to 5	-3 to 12
Pour point, °c	-35 to -15	-15 to 10
Cetane Number	40-55	48-65
Lubricity SLBOCLE, grams	2000-5000	>7,000
Lubricity HFRR, microns	300-600	<300

2.1.1 Sources of raw materials

The main biodiesel feedstock is classified into three categories: 1) plant oil 2) animal fat 3) waste cooking oil and industrial waste oil. There are several oils from plants such as the oils from soybean [12], canola [14], sunflower [15]. Fat from animals such as beef tallow, lard have some disadvantages about quality because their compositions include a high saturated fatty acid content evidences poor cold temperature properties, which can cause some problems in winter operation. Moreover, non-edible oil and waste oil such as Jatropa and soybean soapstock have been used for as raw materials of biodiesel production [16].

2.1.2 The biodiesel production

Biodiesel is an alternative fuels that approximate the properties and performance of the hydrocarbon-based diesel fuel. The raw material of biodiesel is vegetable oil containing triglycerides which can be used as fuel for diesel engines but the main disadvantages of vegetable oils are lower volatility, the reactivity of unsaturated hydrocarbon chains and their viscosities are much higher than usual diesel fuel and require modification of engines. Consequently, there are various ways to reduce the viscosity of vegetable oils and produce biodiesel [17] as follows:

2.1.2.1. Dilution

Vegetable oil can be mixed with such materials as diesel fuels, a solvent or ethanol. The blending of sunflower oil with diesel fuel in the ratio 1:3 by volume has been studied and engine tests. The viscosity of this blend was 4.88 cSt at 40°C. They concluded that the blend could not be recommended for long-term use in the direct injection diesel engines because of severe injector nozzle coking and sticking. A comparable blend with high oleic safflower oil was also tested and it gave satisfactory results, but its use in the long term is not applicable as it leads to thickening of lubricant [2].

2.1.2.2 Microemulsion

Microemulsions are isotropic, clear, or translucent thermodynamically stable dispersions of oil, water, surfactant, and often a small amphiphilic molecule, called cosurfactant. The droplet diameters in microemulsions range from 100 to 1000 A [2]. To reduce the high viscosity of vegetable oil. Microemulsion can solve this problem by mixing alcohols such as ethanol or methanol. The use of 2-octanol as an effective amphiphile in themicellar solubilization of methanol in triolein and soya bean oil has been demonstrated. The viscosity was reduced to 11.2 cSt at 25°C [2].

2.1.2.3 Pyrolysis

Pyrolysis refers to a chemical change caused by the application of thermal energy in the presence of air or nitrogen sparge. The process is also known as thermal decomposition. The product of thermal decomposition of triglycerides including alkanes, alkenes, alkadienes, aromatics and carboxylic acids which results vegetable oil has low viscosity and a high cetane number compared to pure vegetable oils. The mechanisms for the thermal decomposition of triglycerides are shown in Scheme 2.1. Generally, thermal decomposition of these structures proceeds through either a free-radical or carbonium ion mechanism. Formation of homologous series of alkanes and alkenes is accountable from the generation of the

RCOO radical from 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 presence of unsaturation enhances cleavage at a position α, β to the unsaturation. Thermal positional isomerization and subsequent cleavage could account for the higher amounts of C_5 to C_{10} alkanes obtained from safflower compared with soya bean 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 [2].
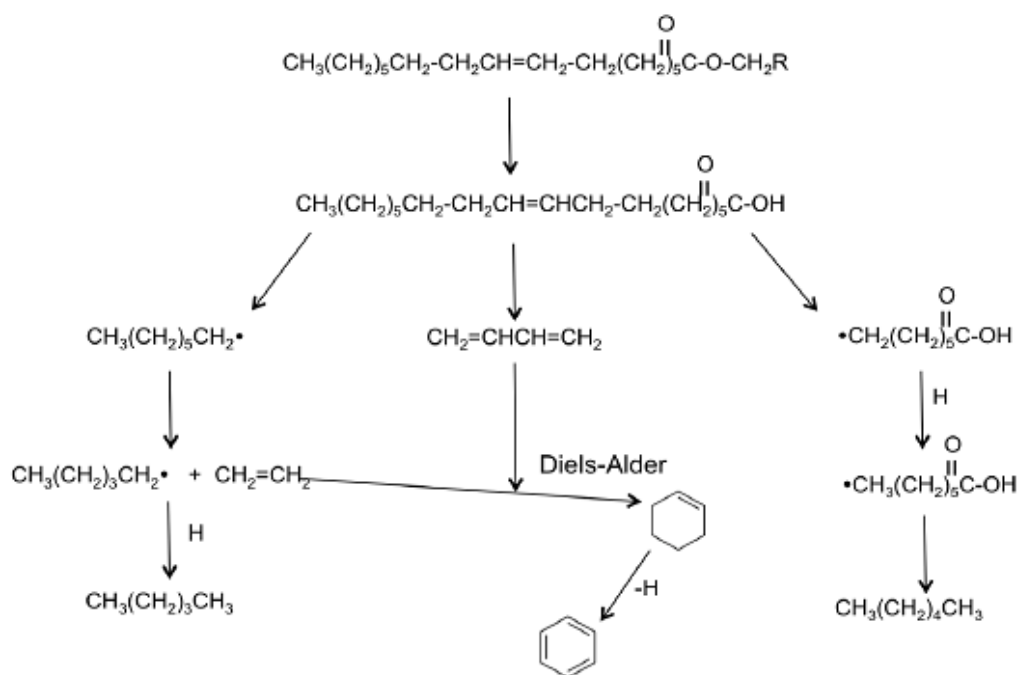
Scheme 2.1 Mechanism of thermal decomposition of triglyceride [2]

2.1.2.4 Transesterification

Transesterification is the most popular method for production of biodiesel. It is a chemical reaction between triglyceride and alcohol in the presence of catalyst. It consists of a sequence of three consecutive reversible reactions where triglycerides are converted to diglycerides and then diglycerides are converted to monoglycerides

followed by the conversion of monoglycerides to glycerol. In each step an ester is produced and thus three ester molecules are produced from one molecule of triglycerides [17]. The transesterification reaction requires a catalyst such as alkaline catalyst, acid catalyst, and biocatalyst or without catalyst under supercritical condition of alcohol. [1] In the alkaline process, NaOH, KOH, carbonates and corresponding sodium and potassium alkoxides are used as basic catalysts. The advantage of this process is react with alcohol easily, high conversion of ester under mild conditions and short time. However, there are risks of water contamination because water makes the reaction partially change to saponification, which produces soap leads to an increase purification product cost [18]. The mechanism of the alkaline catalyzed transesterification is shown in Scheme 2.2.

Scheme 2.2 Mechanism of the alkaline-catalyzed transesterification [19]

Acid catalyst is the second type of catalyst for biodiesel production. Sulfuric acid, sulfonic acids and hydrochloric acid are usually used as acid catalysts which is more suitable for glycerides that have relatively high free fatty acid contents and

more water [20]. Although these catalysts give very high yields in alkyl esters, but the reactions are slow, requiring temperatures above 100 °C and more than 3 h to reach complete conversion and the acidic is corrosive. The mechanism of the acid catalyzed transesterification is shown in Scheme 2.3.
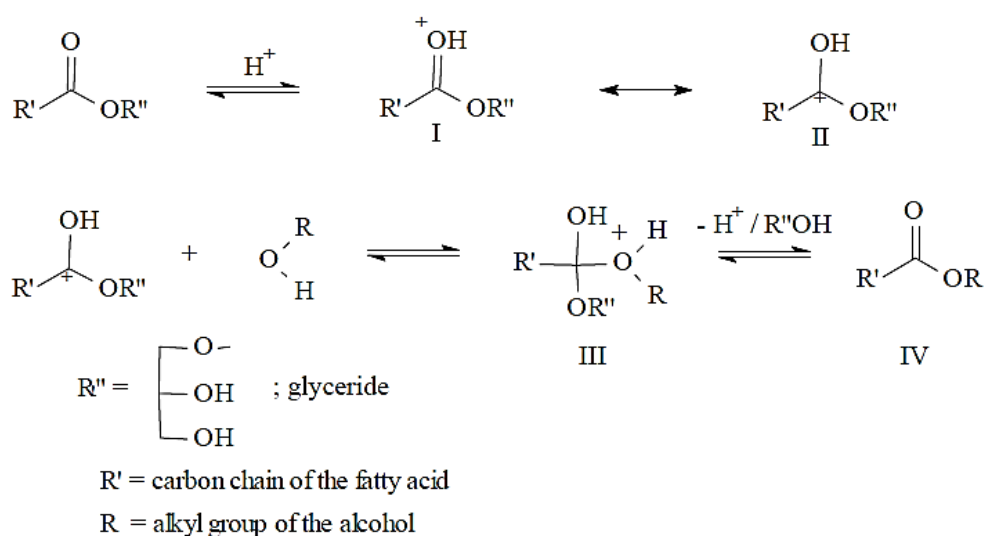
Scheme 2.3 Mechanism of the acid-catalyzed transesterification [21]

From these problems, so enzymatic catalyst has become more attractive to replace alkaline and acidic catalyst and can overcome these problems [6] above. In particular, using the enzymatic catalyst to produce biodiesel was found that by-product, glycerol, can be easily recovered without any complex process, and also that free fatty acids contained in waste oils and fats can be completely converted to methyl esters [20]. Recently, it has been found that lipase can be used as catalyst for transesterification process by immobilization lipase on support. The comparison between alkaline-catalyzed and lipase-catalyzed methods for biodiesel production is shown in Table 2.2.

Table 2.2 Comparison between alkaline-catalyzed and lipase-catalyzed methods for biodiesel production [20]

	Alkaline-catalyzed process	Lipase-catalyzed process
Reaction temperature	60-70°c	30-40°c
Free fatty acids in raw material	Saponified products	Methyl esters
Water in raw materials	Interference with the reaction	No influence
Yield of methyl esters	Normal	Higher
Recovery of glycerol	Difficult	Easy
Purification of methyl esters	Repeated washing	None
Production cost of catalyst	Cheap	Relatively expensive

2.2 Lipase

Lipases (triacylglycerol ester hydrolases, EC3.1.1.3.) are enzymes classified as hydrolases that catalyze the hydrolysis of fats and oils with subsequent release of free fatty acids, diacylglycerols, monoacylglycerols, and glycerol. In addition, lipases are also capable of catalyzing the reverse reaction, achieving esterification, transesterification (acidolysis, interesterification, alcoholysis.), aminolysis. The different of reactions catalyzed by lipase is shown Scheme 2.4 [22].
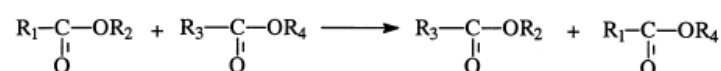
Hydrolysis :

$$R_1-\overset{\underset{\|}{O}}{C}-OR_2 \quad + H_2O \quad \longrightarrow \quad R_1-\overset{\underset{\|}{O}}{C}-OH \quad + \quad R_2-OH$$

Ester synthesis :

$$R_1-\overset{\underset{\|}{O}}{C}-OH \quad + \quad R_2-OH \quad \longrightarrow \quad R_1-\overset{\underset{\|}{O}}{C}-OR_2 \quad + H_2O$$

Acidolysis :

$$R_1-\overset{\underset{\|}{O}}{C}-OR_2 \quad + \quad R_3-\overset{\underset{\|}{O}}{C}-OH \quad \longrightarrow \quad R_3-\overset{\underset{\|}{O}}{C}-OR_2 \quad + \quad R_1-\overset{\underset{\|}{O}}{C}-OH$$

Interesterification :

$$R_1-\overset{\underset{\|}{O}}{C}-OR_2 \quad + \quad R_3-\overset{\underset{\|}{O}}{C}-OR_4 \quad \longrightarrow \quad R_3-\overset{\underset{\|}{O}}{C}-OR_2 \quad + \quad R_1-\overset{\underset{\|}{O}}{C}-OR_4$$

Alcoholysis :

$$R_1-\overset{\underset{\|}{O}}{C}-OR_2 \quad + \quad R_3-OH \quad \longrightarrow \quad R_1-\overset{\underset{\|}{O}}{C}-OR_3 \quad + \quad R_2-OH$$

Aminolysis :

$$R_1-\overset{\underset{\|}{O}}{C}-OR_2 \quad + \quad R_3-NH_2 \quad \longrightarrow \quad R_1-\overset{\underset{\|}{O}}{C}-NHR_3 \quad + \quad R_2-OH$$

Scheme 2.4 Different reactions catalyzed by lipase [22]

2.2.1 Source of lipase

Lipases can be produced by animals, microorganisms and plants. Microbial enzymes are often more useful than enzymes derived from plants or animals because of the great variety of catalytic activities available, the high yields conversion to product, ease of genetic manipulation, regular supply due to absence of seasonal fluctuations and rapid growth of microorganisms on inexpensive media. Microbial enzymes are also more stable than their corresponding plant and animal enzymes and their production is more convenient and safer [23]. There are many microorganisms having been reported in literature as lipase producers. The genera *Candida*, *Rhizopus,* and *Pseudomonas* are considered the main industrial sources of

lipases. The yeast *Candida rugosa* is the most employed microorganism for lipase production [24].

2.2.2 Enzymatic reaction of lipase

The reaction starts with an attack by the oxygen atom of hydroxyl group of the nucleophilic serine residue on the activated carbonyl carbon of the susceptible lipid ester bond. A transient tetrahedral intermediate is formed, with O⁻ stabilized by interactions with two peptide NH groups. The histidine donates a proton to the leaving alcohol component of the substrate, forming the covalent intermediate ("acyl enzyme"), in which the acid component of the substrate is esterified to the enzyme's serine residue. The incoming water molecule is activated by the neighboring histidine residue, resulting hydroxyl ion performs a nucleophilic attack on the carbonyl carbon atom of the covalent intermediate. Later, the histidine residue donates a proton to the oxygen atom of the active serine residue, the ester bond between serine and acyl component is broken, releasing acyl product and lipase regenerated. The stages of reaction catalyzed by lipase are shown in Scheme 2.5 [25].
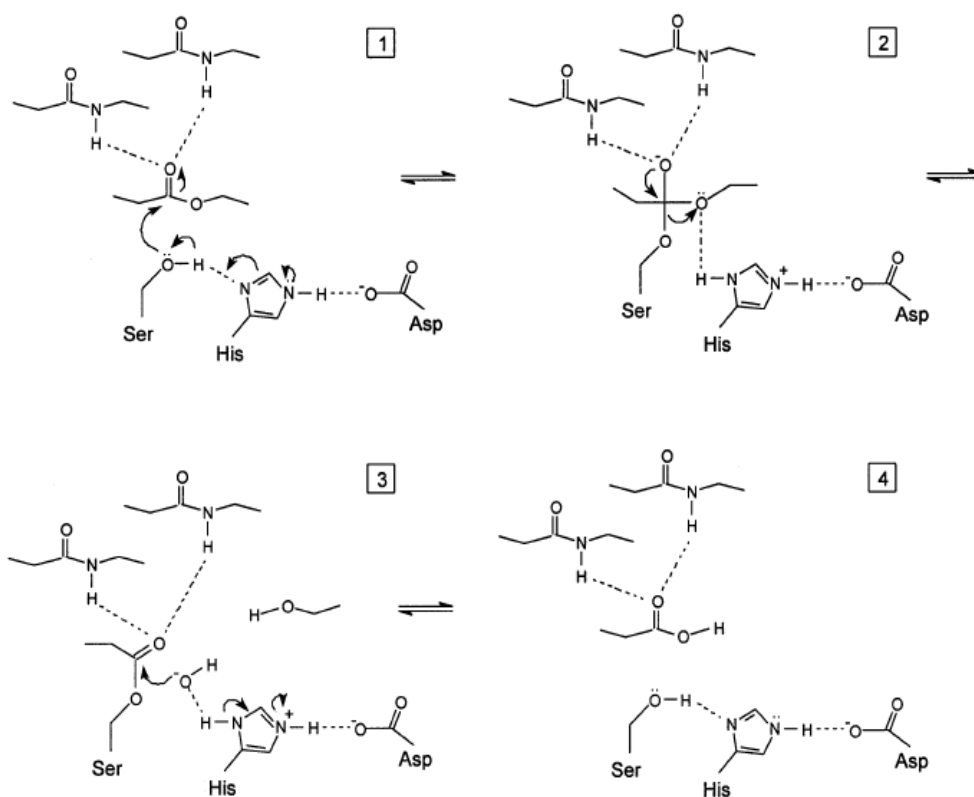
Scheme 2.5 Reaction mechanism of lipase [25]

2.2.3 Applications of lipases

Microbial lipase are widely used in industrial applications because of the versatility of their applied properties and ease of mass production and the lipases are chosen for potential applications in the food, detergent, pharmaceutical, leather, textile, cosmetic, paper industries and biodiesel fuel production [23]. There are many researches using lipase from different source as enzymatic catalyst for biodiesel production. The enzymatic transesterification of some fats and oils by various lipases is shown in Table 2.3 [24].

Table 2.3 Enzymatic transesterification of some fats and oils by various lipases [24]

Lipase source	Feedstock	Alcohol	Solvent	Yield (%)
C. antarctica	Rapeseed oil	Methanol	Hexane	98
C. antarctica	Tallow fat	Butanol	Hexane	84
P. cepacia	Tallow fat	Ethanol	-	95
P. cepacia	Palm Kernel oil	Methanol	-	15
P. fluoresces	Sunflower oil	Propanol	1,4-Dioxane	>95
T. lanuginosus	Soybean oil	Methanol	-	90
M. miehei	Soybean oil	Ethanol	Hexane	97
C. rugosa	Rapeseed oil	2-Ethyl-1-hexanol	-	97

2.3 Immobilization of lipases

Since using free enzyme has several drawback and limitation such as low enzyme stability, difficulty to recovery of free enzyme, inhibition by the medium or products easily and high cost. To solve some of the problems of free enzyme, techniques for their modification have been the subject of increased interest. The immobilization techniques are a very powerful tool to improve almost all enzyme properties, if properly designed: e.g., stability, activity, specificity and selectivity, reduction of inhibition [26]. These techniques can be classified into two main categories: chemical modification, physical modification.

2.3.1 Methods of enzyme immobilization

Modifications of lipase can conveniently be classified into two main categories: physical modification and chemical modification.

The physical modification of free enzyme is immobilization which involves attaching the free enzyme onto an insoluble solid support resulting in the catalysts that can be easily recovered at the end of the reaction and continuously recycled. Methods for immobilization of free enzymes can be classified in three groups consist of (i) adsorption on a carrier material (ii) covalent attachment to an activated matrix (iii) Entrapment and microencapsulation [22].

The chemical modification of lipases is improvement of the native properties of lipases, and to endow them with useful new functions proceeded by formation of a cross-linked lipase matrix using various bi-functional reagent such as crosslinking method.

1. Adsorption

This method is widely used for enzyme immobilization because of its simplification. The enzyme is immobilized onto a solid support by Van der Waals interactions, hydrophobic interactions, hydrogen bonds, and ionic bonds. The properties of support should be mechanical strength, chemical and physical stability, hydrophobic/hydrophilic character, enzyme load capacity and cost which are important for potential industrial applications However, this method may be desorption of free enzyme from the support [22]. The adsorption of enzyme onto the support is shown in Figure 2.1.

Figure 2.1 Enzyme immobilized by adsorption method [27]

The supports can be classified as inorganic and organic according to their chemical composition in Table 2.4. The organic supports can be subdivided into natural and synthetic polymers [28].

The size of the protein to be adsorbed, the specific area of the support and the nature of its surface (porosity and pore size) are important factor for the efficiency of immobilization. Using porous support is advantageous because the free enzyme will be absorbed at the outer surface and within the pores [22].

Table 2.4 Classification of supports [28]

Organic
Natural polymers
• Polysaccharides: cellulose, dextrans, agar, agarose, chitin, alginate
• Proteins: collagen, albumin
• Carbon
Synthetic polymers
• Polystyrene
• Other polymers: polyacrylate polymethacrylates, polyacrylamide, polyamides, vinyl, and alkyl-polymers
Inorganic
Natural minerals: bentonite, silica
Processed materials: glass (nonporous and controlled pore), metals, controlled pore metal oxides

2. Lipase attachment to a matrix by covalent bond

To avoid the desorption phenomenon between lipase and support, immobilization of lipase by covalent binding can solve these phenomenon. Covalent attachment of lipase to the support proceeds on side chain groups of the amino acids (e.g., primary amines for lysine, carboxylic functions for aspartic and glutamic

acids, hydroxyl groups for tyrosine, serine and threonine, thiol for cysteine. Many organic or mineral supports can be used, but some of the supports do not have functional group that can bind with free amino group of lipase and the surface of the supports thus has been activated. Polyglutaraldehyde is widely used as reagent for modified surface because it contains unsaturated aldehyde functions which can react with amino group. However, this method has the disadvantage because active site may be modified through the chemical reactions used to create covalent bonding resulting lose a part of their initial activity. As examples, the *C. cylindracea* and *Rhizopus oryzae* lipases were covalently attached to porous glass beads and alumina w78x, respectively, by treatment of these supports with g-aminopropyltriethoxysilane in acetone followed by reaction with glutaraldehyde solution [22]. The Covalent attachment of lipase to the support is shown in Figure 2.2.

Figure 2.2 Enzyme immobilized by covalent attachment method [27]

3. Entrapment and microencapsulation

This immobilization method as inclusion of free lipase in an insoluble polymer or in a micro-capsule or entrapped lipase by membrane which permits small molecules, e.g., substrates and products, can pass through it. The advantage of this method is the free lipase does not chemically interact with the polymer, and therefore the free lipase is not denaturation. However, mass transfer phenomena around the membrane are problematic [22].
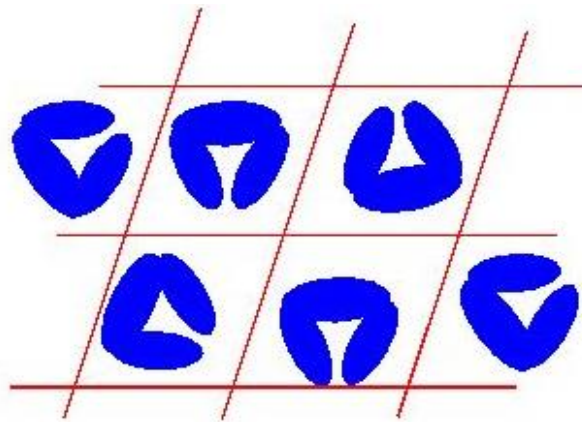
(a) Entrapment
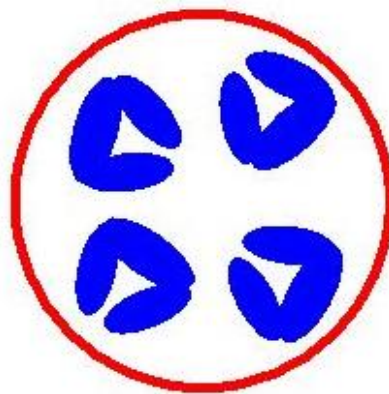
(b) Encapsulation

Figure 2.3 Enzyme immobilized by entrapment and encapsulation method [27]

4. Crosslinking

The immobilization of lipase by this method, free lipase molecule are chemically interacted with bi- or multifunctional reagent as called cross-linkers such as glutaraldehyde, hexamethylenediamine leading to three dimensional cross-linked aggregates [22]. However, the immobilization involves chemically bonding which may be cause significant changes in the active site of enzymes, leading to significant loss of activity. Crosslinking between molecules of free enzyme is shown Figure 2.4.
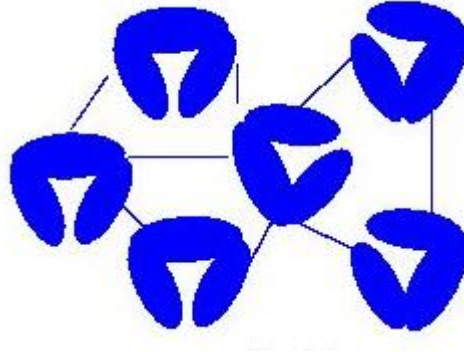
Figure 2.4 Enzyme immobilized by crosslinking method [27]

2.4 Literature reviews

In 2004, Lei *et al.* [26] immobilized lysozyme (LYZ) in mesoporous silicas (MPSs) with controlled morphologies. The effect of particle size of MPSs on immobilization of lysozyme was investigated. The lysozyme immobilized on rod-like SBA-15 with the length of ~1-2 μm gave amount of immobilization of lysozyme higher than conventional SBA-15 (~20 μm in length). These results suggested that the decreasing of particle size of MPSs resulted in increase of entrance amount of lysozyme and led to a significant improvement of immobilization ability.

In 2006, Park *et al.* [10] studied effect of particle size and specific surface area of silica particles on fatty acid methyl ester formation rate. Using 12 different kinds of silica particles with particle size of 1.6-700 μm and specific surface area of 14-620 m^2/g-support as support for immobilization of lipase QLM from *Alcaligenes*. They found that the lipase immobilized on P-707 with the particle size of 2.2 μm gave FAME formation rate higher than particle sizes of 200, 400, 700 μm. They suggested that the FAME formation rate was proportional to the interfacial surface area per unit volume of silica particle.

In 2008, Keskinler *et al.* [29] studied stability of *Thermomyces lanuginosus* and immobilized *Thermomyces lanuginosus* that was immobilized on STY-DVB-PGA

copolymer. Effect of pH and reaction temperature on enzymatic activity of free and immobilized lipase were investigated. They found that the optimum pH for free lipase was 7.0, whereas the optimum pH for immobilized lipase was between 6.0 and 7.0. Immobilization resulted in stable lipase so the immobilized lipase was more stable in lower and higher pH in comparison to free lipase. The optimum temperature for free lipase was 50°C and for immobilized lipase was at 40-50°C. This indicated that immobilized lipase was more stable at high temperatures.

In 2008, Dizge *et al.* [14] studied method for immobilization of lipase to produce biodiesel with transesterification between canola oil and methanol. *Thermomyces lanuginosus* lipase was immobilized within hydrophilic polyurethane foams and using polyglutaraldehyde as cross-linking reagent. The optimum pH for immobilization of lipase was 6 resulting in 80% immobilization yield. The optimum conditions for biodiesel production were found at 430 μg lipase, 1:6 oil/methanol molar ratio, 0.1 g water and 40°C reaction temperature. The maximum methyl esters yield was 90%.

In 2009, Dizge *et al.* [30] synthesized methyl esters or biodiesel by transesterification reaction of canola oil with methanol in the presence of immobilized lipase as catalyst. Lipase from *Thermomyces lanuginosus* was immobilized on styrene-divinylbenzene (STY-DVB) copolymer and polyglutaraldehyde activated styrene-divinylbenzene (STY-DVB-PGA) copolymer by physical adsorption and covalent attachment method, respectively. They found that loading yield of *T. lanuginosus* lipase immobilized on STY-DVB-PGA copolymer was higher than STY-DVB copolymer because it had functional group of aldehyde that can bind with free amino group of lipase. This immobilized lipase was used for biodiesel production. Under the optimized condition, the maximum biodiesel yield was 97%.

CHAPTER III

EXPERIMENTAL

3.1 Materials

1. Expandable polystyrene bead (EPS bead) size 1.00-1.60 mm (grade 291L) and size 0.63-1.12 mm (grade 321F); IRPC Public Company Limited, Thailand.

2. Styrene monomer; Thai Styrenics Company Limited, Thailand.

3. Glutaraldehyde (25% solution in water); Sigma-Aldrich.

4. Lipase from *Pseudomonas cepacia* (*P. cepacia*), activity 35 units/mg; Sigma-Aldrich.

3.2 Chemicals

1. Sodium hydroxide (NaOH); Merck.

2. Sorbitan monooleate (TWEEN$^®$80); Ajax Finechem.

3. Potassium persulphate ($K_2S_2O_8$); Carlo Erba Reagenti.

4. Sodium hydrogen phosphate (Na_2HPO_4); Merck.

5. Potassium dihydrogen phosphate (KH_2PO_4); Merck.

6. Sodium chloride (NaCl); Carlo Erba Reagenti.

7. Potassium chloride (KCl); Carlo Erba Reagenti.

8. *p*-nitrophenol (*p*-NP); Hopkin and Williams Limited.

9. *p*-nitrophenyl palmitate (*p*-NPP); Sigma-Aldrich.

10. Ethanol, absolute GR for analysis grade; Merck.

11. Sodium carbonate anhydrous (Na_2CO_3); Carlo Erba Reagenti.

12. Soybean oil

13. Methanol (MeOH), analytical grade; Merck

14. Ethanol (EtOH), absolute GR for analysis grade; Merck

15. Chloroform-d (CDCl$_3$), NMR spectroscopy grade; Merck

3.3 Methods

3.3.1 Preparation of solid support before immobilization

3.3.1.1 Preparation of polyglutaraldehyde (PGlu) [30]

A. After polyglutaraldehyde was generated by polymerizing glutaraldehyde solution (50 mL) at pH of which was 10.5 by adding NaOH solution (1M) at room temperature for 5 min, soluble polyglutaraldehyde (SPGlu) generated was used in the reaction for preparation of polyglutaraldehyde-styrene coated expandable polystyrene beads.

B. Polyglutaraldehyde suspension was generated by polymerizing glutaraldehyde solution (50 mL) at pH of which was 10.5 by adding NaOH solution (1M) at room temperature for 5 min, The whole suspension (WPGlu) generated was directly used for preparation of polyglutaraldehyde-styrene coated expandable polystyrene beads.

3.3.1.2 Preparation of polyglutaraldehyde-styrene coated expandable polystyrene beads (PGlu-STY/EPS beads)

Prior to use, the styrene monomer was purified by extracting with 12% sodium hydroxide solution in a separatory funnel for three times in order to remove the anti-polymerizer.

To a stirred mixture of 5%w/v of styrene monomer (2.5 g) and TWEEN®80 (2.2 g) in a 100 mL round bottom flask, polyglutaraldehyde suspension (soluble polyglutaraldehyde or the whole polyglutaraldehyde suspension, 50 mL) was added and stirred to form emulsion for 10 min. Potassium persulfate (1.75 g) as an initiator

was added. After stirring for 15 min, expandable polystyrene beads (12 g) were added and the mixture was heated to 60ºC for 30 min, continuously stirred at 60ºC for 2h and heated at 80ºC for polymerization time of 22h. Then PGlu-STY/EPS beads obtained was washed with deionized water and dried in desiccator for 24h.

3.3.1.3 Optimization of condition for preparing solid support (PGlu-STY/EPS beads)

There were many factors affecting preparation of PGlu-STY/EPS beads in order to obtain PGlu-STY/EPS beads that was suitable for immobilization. Thus effect of concentration of styrene monomer (2.5, 5 and 7.5%w/v) and types of polyglutaraldehyde suspension [the soluble or whole-suspension polyglutaraldehyde generated from glutaraldehyde (25, 20, 15, 10, 5%w/v)] and temperatures (60, 70, 80 ºC) were investigated.

3.3.1.4 Characterization of PGlu-STY/EPS beads

1. Attenuated total reflectance-Fourier transform infrared spectroscopic (ATR-FTIR)

EPS beads and PGlu-STY/EPS beads were characterized by ATR-FTIR (Nicolet 6700, USA). The solid sample was placed onto the Universal diamond ATR top-plate and then applying pressure to a solid sample on the Universal diamond ATR top-plate for characterization.

2. Loading estimation

Percentage of polyglutaraldehyde-styrene coated onto expandable polystyrene beads was calculated as %coating using following equation:

$$\%\text{coating} = \frac{\text{Weight of PGlu-STY coated on polystyrene beads}}{\text{Weight of polystyrene beads introduced}} \times 100$$

3. Size of polyglutaraldehyde-styrene coated expandable polystyrene beads (PGlu-STY/EPS beads)

Sizes of the PGlu-STY/EPS beads were measured by using digital microscope (AD 4113TL-Dino-Lite Pro2) at 60 magnifications. The average diameter of the beads was calculated by an image of one hundred beads from digital microscope photograph using dinocapture 2.0 software.

4. Scanning electron microscopy (SEM)

The morphology of PGlu-STY/EPS beads was determined by scanning electron microscope (SEM). The sample beads were placed on stub and coated with gold.

3.3.2 Immobilization of *Pseudomonas cepacia* lipase onto PGlu-STY/EPS beads by covalent linkage method

Immobilization of lipase was carried out by adding 1 gram of the PGlu-STY/EPS beads into a solution of lipase 12 mg in 0.025 M phosphate buffer (pH 6.70) 5 mL and immobilized at 37°C for 24h. After immobilization process, the immobilized lipase beads were filtered and dried in desiccator and then stored -20°C until use. After filtration, the residual enzyme solution was assayed for unbound protein (see 3.3.2.5). Since there were many factors affecting the activity of enzyme during immobilization process, effect of PGlu-STY ratios, lipase concentration (3.6, 9.6, 19.2 mg/mL) and immobilization time (3, 7, 12, 24, 48h) were investigated.

3.3.2.1 Measurement of activity of immobilized lipase beads [31]

Lipase activity was assayed using 0.5%w/v of p-nitrophenyl palmitate (p-NPP) in ethanol as substrate. The reaction mixture consisting of 1.1 mL of 0.025 M phosphate buffer (pH 6.70) and 20 mg of immobilized lipase beads were initiated by adding 1 mL of 0.5%w/v p-NPP solution and incubated for 5 min at 40ºC. The reaction was terminated by adding 2 mL of 0.5 N Na_2CO_3 followed by centrifuging for 10 min. The supernatant was diluted by stock dilution and then measured at 410 nm in a UV-VIS spectrophotometer (CARY 50 Probe). One unit (U) of enzyme activity was defined as the amount of enzyme which catalyzed the production of 1 μmol p-nitrophenol per minute under the experimental conditions. Percentage of relative specific activity (RSA) was calculated as the ratio of specific activity of immobilized enzyme to the specific activity at the optimum conditions. Each of the assays was performed in duplicate and mean values were presented.

3.3.2.2 Measurement of protein loading

Protein loading was estimated from enzyme activity of residual enzyme solution. To a reaction mixture consisting of 1 mL of 0.025 M phosphate buffer (pH 6.70) and 0.1 mL (about 1.75 unit/mL, see in appendix A) of residual enzyme solution was initiated by adding 1 mL of 0.5%w/v p-NPP solution in ethanol as substrate and incubated for 5 min at 40ºC. The reaction was terminated by adding 2 mL of 0.5 N Na_2CO_3 followed by centrifuging for 10 min. The variation of the absorbance at 410 nm of the assay against a blank was measured by spectrophometry. The increase in the absorbance caused by the release of p-nitrophenol in the hydrolysis of p-NPP [31]. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the production of 1 μmol p-nitrophenol per minute under experimental conditions.

The PGlu-STY/EPS bead was evaluated as described [30]

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

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

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

3.3.3 Biodiesel production by immobilized lipase catalyst

Lipase from *Pseudomonas cepacia* immobilized on PGlu-STY/EPS bead was used as catalyst for biodiesel production through reaction between soybean oil and alcohols (ethanol or methanol). The reaction parameters in term of optimal molar ratio of oil:alcohol, amount of water, type of immobilized catalyst were investigated.

3.3.3.1 Biodiesel production

The soybean oil (5 g) and alcohol (ethanol or methanol) were stirred to mixture in a round bottom. The immobilized lipase was added and the reaction mixture was heated in silicone oil bath at 40°C.

3.3.3.1.1 Optimization of the reaction parameters

The effect of molar ratio of oil:dried ethanol (1:2, 1:3, 1:4, 1:5, 1:6), amount of water (2, 10, 20 equivalents) and reaction time (2, 4, 6, 8, 10, 12, 24 and 194 hours) were studied.

CHAPTER IV
RESULTS AND DISCUSSION

Before immobilization of lipase, the surface of EPS bead was increased functional group of aldehyde. EPS bead was coated by radical polymerization of styrene monomer and polyglutaraldehyde suspension initiated with $K_2S_2O_8$. Previously our group research studied immobilization of lipase from *Pseudomonas cepacia* onto polyglutaraldehyde-styrene coated expandable polystyrene beads (EPS beads) and the lipase-immobilized catalyst prepared gave high degree of transesterification which resulted about 98% of fatty acid ethyl ester product [32]. Since smaller particle size of lipase-immobilized catalyst had more surface area, the research expected that the lipase-immobilized catalyst with smaller particle size might result in improving the efficiency of biodiesel production. Thus aims of this research were to prepare *Pseudomonas cepacia* lipase immobilized catalyst with small particle size and to evaluate the efficiency of the immobilized lipase catalyst for biodiesel production from soybean oil.

Since expandable polystyrene bead (EPS bead) had significant property that heating could expand it. This research expected that the support in different size could be prepared by varying temperature of polyglutaraldehyde-styrene coating. To prepare the smaller size of the support, the method of coating would be done followed by previous method of our group using amount of PGlu and STY and reaction temperature as shown in Table 4.1. The EPS bead coated with PGlu-STY copolymer at 60, 70, and 80°C was shown in Figure 4.1 and size of EPS and PGlu-STY/EPS bead and percent coating yield of copolymer at each temperature and different PGlu:STY ratios were presented in Table 4.1.

Heating at 60 and 70°C slightly expanded the uncoated expandable polystyrene bead [EPS bead, 1.00-1.60 mm (grade 291L)] while size of the beads was increased more than 2 fold of the original size (1.2 mm) when it was heated at 80°C. In Table 4.1 and Figure 4.1, it was found that PGlu:STY ratio affected the size of PGlu-STY/EPS beads and the increasing of temperature resulted in significant increasing of

percent coating of copolymer. Concentration of styrene monomer not only affected the size but also physical appearance of the PGlu-STY/EPS beads. When the amount of styrene monomer was increased at any reaction temperatures, the size of PGlu-STY/EPS beads was decreased from 2.53 to 1.77 mm. These results suggested that the increasing of styrene monomer in polymerization process resulted in the increase of conversion of polymerization [33]. So the polymerization rate of copolymer in condition 7.5%w/v of STY monomer, which coated on EPS beads, was the fastest and resulted in the size of PGlu-STY/EPS beads obtained from the highest STY monomer was the smallest. In Table 4.1, comparison of the size of PGlu-STY/EPS beads (1.77 mm) obtained from 7.5%w/v of STY monomer, 20%w/v of PGlu at reaction temperature 80°C with the size of uncoated EPS beads (2.67 mm) caused by heating at temperature 80°C (without PGlu-STY) also suggested that coating rate of PGlu-STY onto EPS beads should be faster than expansion rate of EPS beads.

Table 4.1 Effect of PGlu:STY ratio and reaction temperature on size of PGlu-STY/EPS beads and percent coating of copolymer

STY (%w/v)	PGlu (%w/v)	Average diameter of PGlu-STY/EPS[a] (mm)			Percent coating of copolymer		
		Temperature (℃)					
		60	70	80	60	70	80
2.5	20	1.46	2.08	2.53	47.80	52.60	62.40
5	20	1.96	1.99	2.02	66.8	74.8	81.9
7.5	20	1.77	1.81	1.77	69.1	76.8	91.5
Uncoated EPS[b]		1.35	1.48	2.67	-	-	-

[a] Average of diameter of original EPS beads 1.2 mm

[b] Average diameter of uncoated EPS bead (without PGlu-STY) after heat treatment for the same period of coating time (24h) measured by digital microscope.
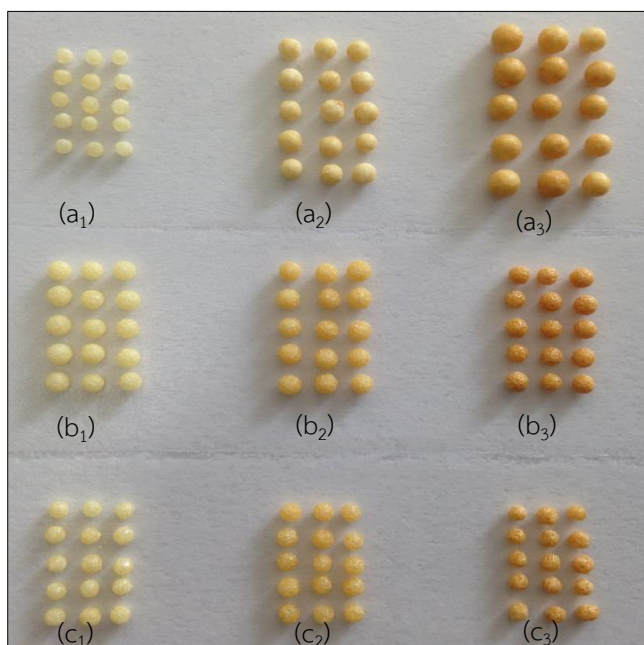
Figure 4.1 PGA-STY/EPS beads: (a) 2.5%w/v of STY at (a_1) 60°C, (a_2) 70°C, (a_3) 80°C (b) 5%w/v of STY at (b_1) 60°C, (b_2) 70°C, (b_3) 80°C (c) 7.5%w/v of STY at (c_1) 60°C, (c_2) 70°C, (c_3) 80°C

Although small particle sizes could be made by using various temperature of polyglutaraldehyde-styrene coating and PGlu:STY ratios, only the supports prepared at 80°C could be used for immobilization. Thus, smaller EPS bead [0.63-1.12 mm (grade 321F)] was used instead of EPS bead [1.00-1.60 mm (grade 291L)] for preparation of PGlu-STY/EPS bead. However it was found that using EPS bead [0.63-1.12 mm (grade 321F)] could not be performed. Because of higher volume of the smaller EPS bead made a difficulty in stirring the reaction, which resulted in nonuniform coating. Attempts to overcome this problem it fortunately found that both 321F and 291L EPS beads were well coated when using two-fold of total volume of the reaction. Thus this new method for preparation was further studied and both 291L EPS bead with size of 1.2 mm and 321F EPS bead with size of 0.875 mm were used in order to compare for immobilization of lipase and efficiency of biodiesel production. In addition effect of styrene monomer, reaction temperature and polyglutaraldehyde suspension (soluble polyglutaraldehyde, the whole suspension[a]) for preparation and surface modification were also investigated.

[a] the whole polyglutaraldehyde is both soluble and insoluble of polyglutaraldehyde suspension generated from various concentration of glutaraldehyde.

4.1 Preparation of solid support using modified coating method

4.1.1 Preparation of polyglutaraldehyde-styrene coated EPS beads by using soluble polyglutaraldehyde (SPGlu-STY/EPS), the whole polyglutaraldehyde suspension (WPGlu-STY/EPS)

4.1.1.1 Effect of concentration of styrene monomer on preparation of PGlu-STY/EPS beads

The effect of styrene monomer concentration was studied by using a fixed amount of polyglutaraldehyde (20%w/v, 50 mL), Tween 80[®] (2.2 g) and $K_2S_2O_8$ (1.75 g), and the reaction was carried out at temperature of 80°C for 24h. The amount of styrene monomer was varied from 2.5, 5 and 7.5%w/v and the PGlu-STY/EPS beads prepared were shown in Figure 4.2. The percent coating of PGlu-STY coated onto EPS bead using various concentration of styrene monomer was shown in Figure 4.3.
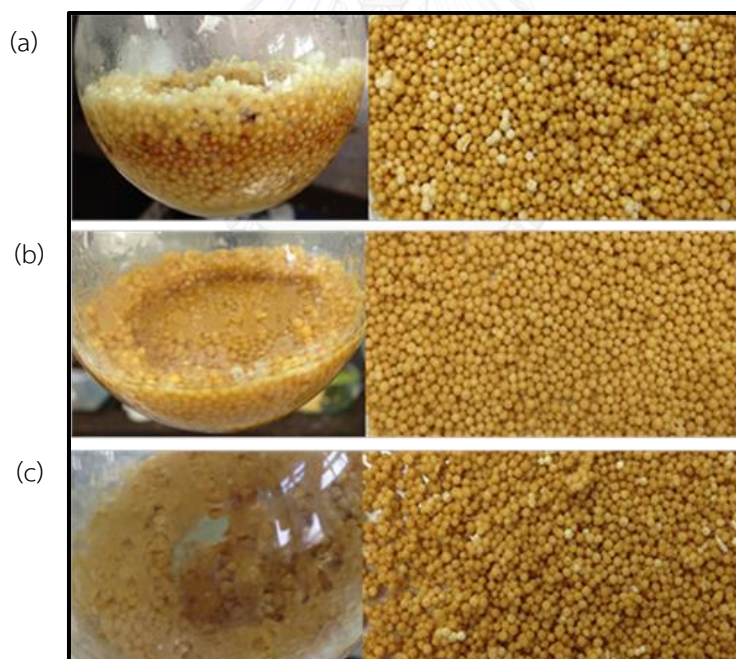
Figure 4.2 SPGlu-STY/EPS beads prepared by using (a) STY2.5% (b) STY5% (c) STY7.5%w/v at SPGlu 20%w/v, reaction temperature 80°C for 24h.
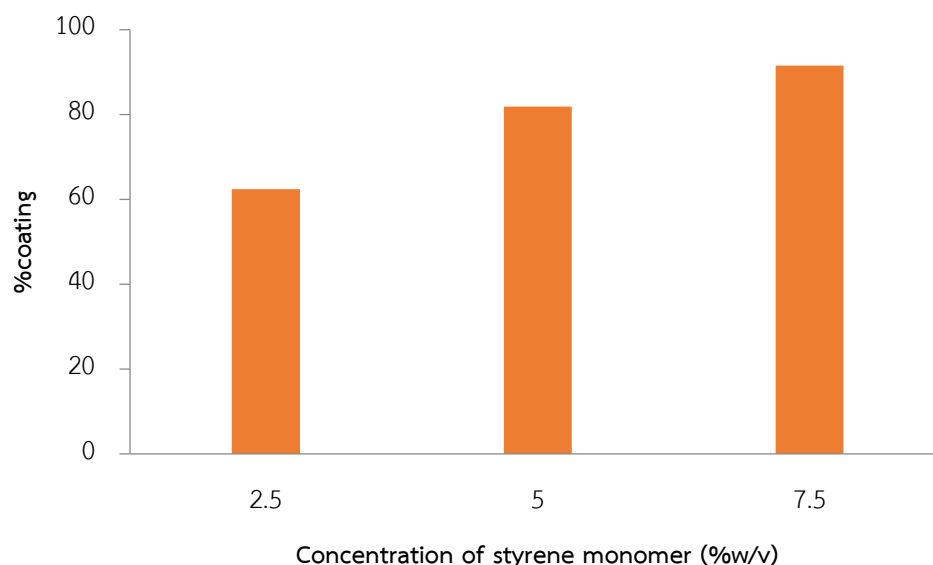
Figure 4.3 The percent coating of SPGlu-STY/EPS beads resulted by varying concentration of styrene monomer at 20%w/v of 80°C for 24h.

In Figure 4.3, the increase of styrene monomer resulted in significant increase of percent coating of copolymer. From Figure 4.2, it found that concentration of styrene monomer affected size and physical appearance of the PGlu-STY/EPS beads. Using 2.5%w/v of styrene monomer, the size of PGlu-STY/EPS bead was bigger than the beads when using 5% and 7.5%w/v. These results suggested that the decreasing of styrene monomer in polymerization process resulted in the decrease of conversion of polymerization [33]. So the expansion rate of EPS bead should be faster than the polymerization rate of copolymer caused by difficult stirring of the reaction. Since the PGlu-STY/EPS bead from using 5 and 7.5%w/v consisted of pale brown-yellow bead together with small amount of off-white beads, it indicated that the beads were partially uncoated. As using 5%w/v of styrene monomer, the increasing of styrene monomer in polymerization process resulted in coating of PGlu-STY on EPS bead should be faster than the expansion rate of EPS bead caused by stirring of the reaction was more easily and the PGlu-STY/EPS bead obtained was uniform. Using 7.5%w/v of styrene monomer gave the fastest polymerization rate

and the excessive styrene resulted in the bead has too much coating copolymer and partially agglomerate to lumps of beads. The PGlu-STY/EPS bead obtained has roughness surface. Therefore, 5%w/v of styrene monomer was suitable for preparation of the PGlu-STY/EPS bead.

Scanning electron microscopy (SEM) was employed to investigate the morphology of PGlu-STY/EPS bead obtained (Figure 4.4 and 4.5). It was clear that using the lowest styrene monomer, the expansion rate of EPS beads was faster than the polymerization rate of copolymer which resulted in the foam-like and the obtained PGlu-STY/EPS bead was the biggest size. When using 7.5%w/v of styrene monomer, the polymerization rate was faster than the expansion rate of EPS bead and the excessive styrene might penetrate into the beads that resulted in a distorted spherical shape and the obtained PGlu-STY/EPS bead was the smallest size.

| Average size = 2.53 mm | Average size = 2.02 mm | Average size = 1.77 mm |
| (a) | (b) | (c) |
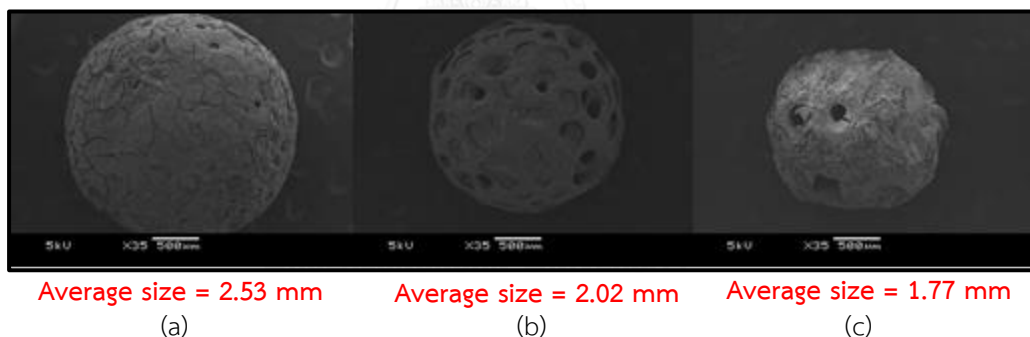
Figure 4.4 SEM micrographs of PGlu-STY/EPS prepared by using (a) 2.5%, (b) 5%, (c) 7.5%w/v of STY monomer and 20%w/v of PGlu at 80°C for 24h.
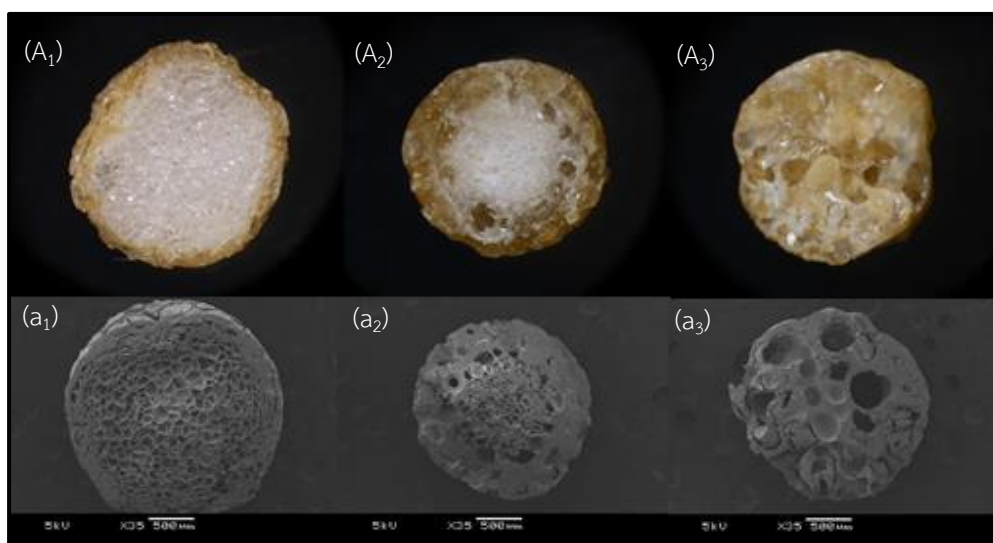
Figure 4.5 Pictures (A) and SEM micrographs (a) of cross-sectional PGlu-STY/EPS bead prepared by using at 2.5% (A_1, a_1), 5% (A_2, a_2), and 7.5%w/v (A_3, a_3) of STY monomer and 20%w/v of PGlu at 80°C for 24h.

4.1.1.2 Effect of reaction temperature on preparation of PGlu-STY/EPS beads

The effect reaction temperature was studied by using a fixed amount of 20%w/v polyglutaraldehyde, Tween 80$^{®}$, $K_2S_2O_8$ at 50 mL, 2.2 g and 1.75 g, respectively and reaction time of 24h. The reaction temperature was varied from 60°C, 70°C, and 80°C. The PGlu-STY/EPS beads prepared were shown in Figure 4.6 and effect of reaction temperature on percent coating yield shown in Figure 4.7
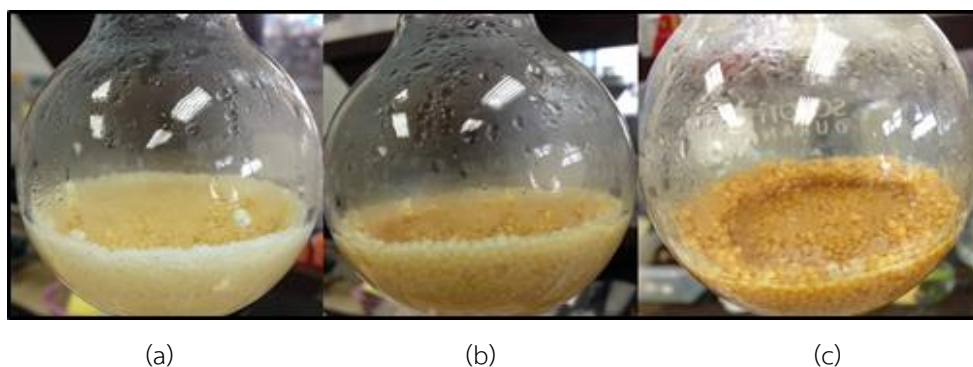
Figure 4.6 The PGlu-STY/EPS beads prepared by using 5%w/v of STY, 20%w/v of PGlu at (a) 60°C, (b) 70°C and (c) 80°C for 24h.
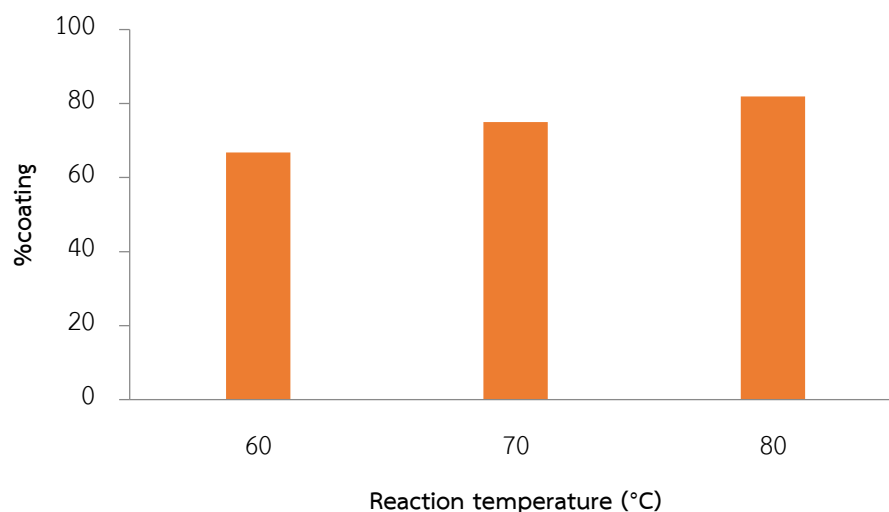
Figure 4.7 Effect of reaction temperature on percent coating yield

Generally, increasing of reaction temperature resulted in higher polymerization rate [33] because increasing temperature leads to increasing decomposition rate of initiator. The highest temperature (T=80°c) leads to higher coating of STY-PGlu on EPS bead than lower temperature and it was easy to distinguish how much coating of STY-PGlu by seeing color of the coated beads. The increase of brown color of the STY-PGlu copolymer related to increase of percent coating yield (see in Figure 4.6). In addition, Figure 4.8 was also the evidence that increase of brown color corresponded to increase of coating of STY-PGlu on EPS bead.

The ATR-FTIR spectra of EPS bead and PGlu-STY/EPS bead at reaction temperature 60, 70, and 80°C (Figure 4.8) showed absorption of aromatic and olefinic C-H stretching at 3081, 3052 and 3023 cm^{-1}, of aliphatic C-H stretching at 2924 and 2861 cm^{-1}, of C=O stretching at 1713 cm^{-1} for non-conjugated aldehyde and at 1675 cm^{-1} for conjugated aldehyde and C=C stretching at 1634 cm$^{-1.}$. These results indicated that coating styrene-polyglutaraldehyde onto EPS bead was successful. At high reaction temperature found that adsorption of C=O of conjugated aldehyde at

1675 cm^{-1} was increased. It indicated that the presence of a lot of STY-PGlu copolymer on EPS bead by polymerization process.
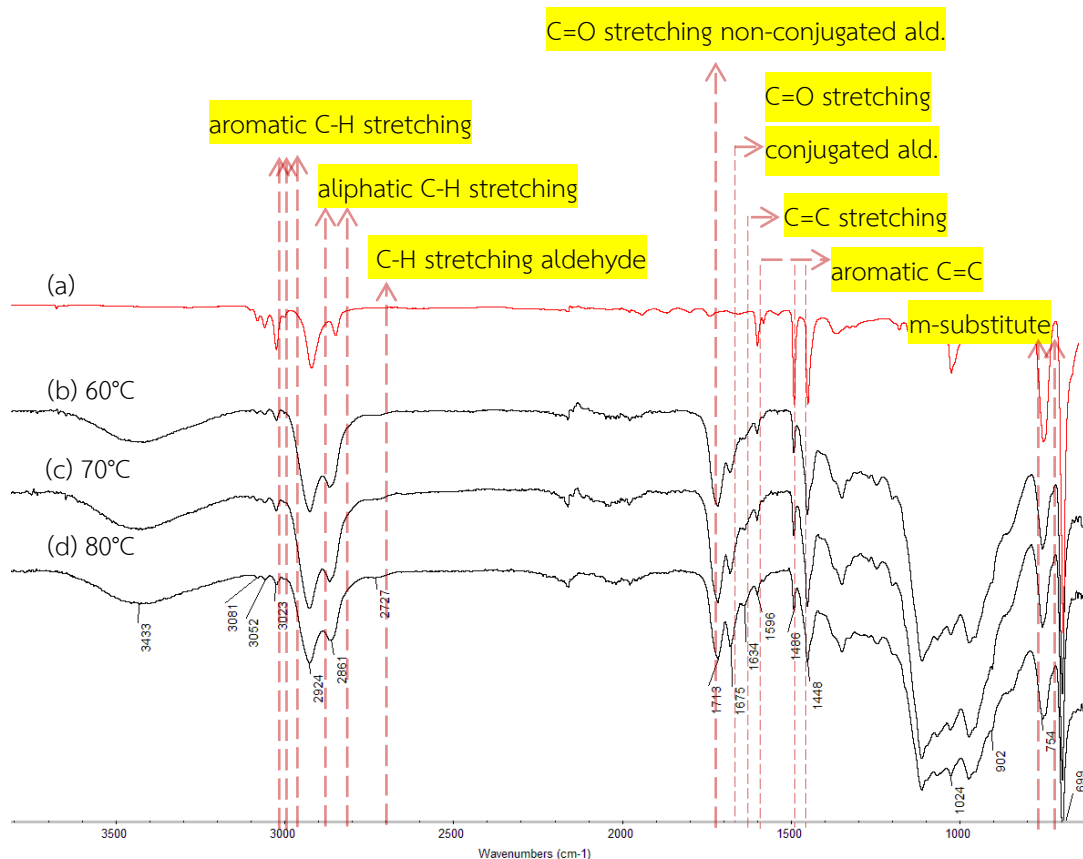
Figure 4.8 FT-IR spectra of (a) original EPS bead and PGlu-STY/EPS bead at reaction temperature: (b) 60°C, (c) 70°C, (d) 80°C and at 5%w/v of STY, 20%w/v of PGlu, 24h of reaction time.

4.1.1.3 Effect of soluble polyglutaraldehyde and the whole suspension on preparation of PGlu-STY/EPS beads

4.1.1.3.1 Effect of soluble polyglutaraldehyde on preparation of SPGlu-STY/EPS beads

The soluble polyglutaraldehyde was generated from 5-25%w/v of glutaradehyde solution and the soluble polyglutaraldehyde was then copolymerized with styrene in the coating process onto 291L EPS beads with original size of 1.2 mm

and 321F EPS beads with original size of 0.875 mm. The SPGlu-STY/291LEPS and SPGlu-STY/321FEPS beads prepared were shown in Figure 4.9 and 4.10, respectively.

Using the soluble polyglutaraldehyde as copolymer, it was claimed that there was a small amount of low molecular weight polyglutaraldehyde. According to results of the SPGlu-STY/291LEPS, prepared as seen in Figure 4.9, coating SPGlu-STY onto 291LEPS bead was successful by using SPGlu generated from 5% to 25% of glutaradehyde while coating SPGlu-STY onto 321FEPS bead was unsuccessful when SPGlu was generated from 25% of glutaradehyde. In case of SPGlu 25%w/v, the reaction was difficult in stirring because the volume of the 321FEPS bead was higher than of 291LEPS beads, and thus resulted in unperfected and unsuccessful preparation of the SPGlu-STY/321FEPS beads.

In Figure 4.11, percent coating yield of SPGlu-STY/291LEPS and SPGlu-STY/321FEPS depended on concentration of glutaraldehyde used. That was the higher concentration of glutaraldehyde, the higher percent coating yield. Also percent coating yield of both sizes was almost the same at the same concentration of glutaraldehyde.

The ATR-FTIR spectra of SPGlu-STY/EPS beads (Figure 4.12) showed that increasing concentration of glutaraldehyde used resulted in increasing the adsorption of C=O of non-conjugated at 1715 cm^{-1} and of conjugated aldehyde at 1675 cm^{-1}. This indicated the presence of a lot of STY-PGlu copolymer on EPS beads by copolymerization process at high concentration of soluble polyglutaraldehyde.

Figure 4.9 The SPGlu-STY/291LEPS beads prepared by using SPGlu generated from glutaraldehyde concentration of (a) 5%, (b) 10%, (c) 15%, (d) SPGlu 20% and (e) 25%w/v copolymerized with 5%w/v of STY at 80°C for 24h.

.

Figure 4.10 The SPGlu-STY/321FEPS beads prepared by using SPGlu generated from glutaraldehyde concentration of (a) 5%, (b) 10%, (c) 15%, (d) 20%, and (e) 25%w/v copolymerized with 5%w/v of STY at 80°C for 24h.

Figure 4.11 Percent coating of SPGlu-STY/291LEPS and SPGlu-STY/321FEPS

(%coating of SPGlu-STY/321FEPS was not applicable because the coated beads was attached together)

Figure 4.12 FT-IR spectra of (a) original EPS bead and SPGlu-STY/EPS beads prepared by using SPGlu generated from glutaraldehyde concentration of (b) 5%, (c) 10%, (d) 15%, (e) 20% and (d) 25%w/v copolymerized with 5%w/v of STY at 80°C for 24h.

4.1.1.3.2 Effect of the whole suspension on preparation of WPGlu-STY/EPS beads

Accidentally attempts to characterize of insoluble polyglutaradehyde by dissolving in solvents. It was found that the insoluble polyglutaradehyde was slow dispersed in surfactant-containing water. Thus the whole polyglutaradehyde suspension, generated from 7.5-20%w/v of glutaraldehyde, was directly used in the coating process. Both 291L and 321F EPS beads were also used in these studies. It found that The WPGlu-STY/291LEPS and WPGlu-STY/321FEPS beads were successfully made and appearances of those beads were shown in Figure 4.13 and 4.14, respectively.

(a)   (b)

(c)   (d)

Figure 4.13 The WPGlu-STY/291LEPS beads prepared by using WPGlu generated from glutaraldehyde concentration of (a) 7.5%, (b) 10%, (c) 15% and (d) 20%w/v copolymerized with 5%w/v of STY at 80°C for 24h.
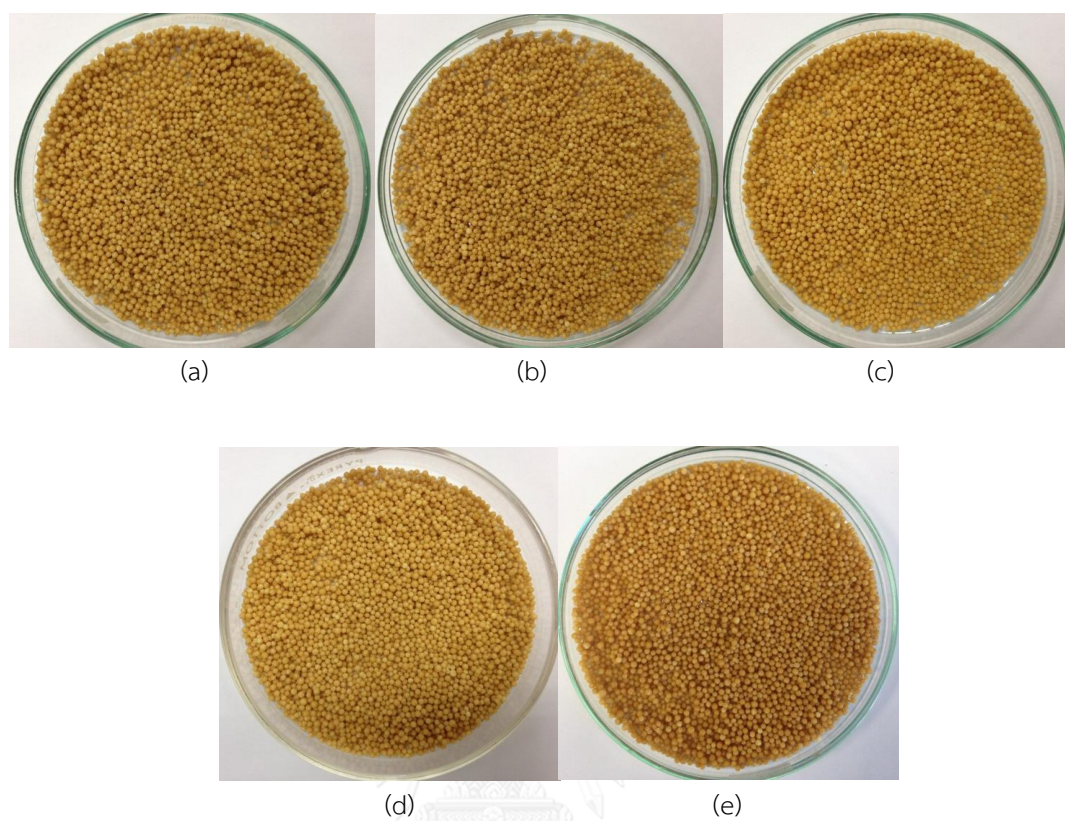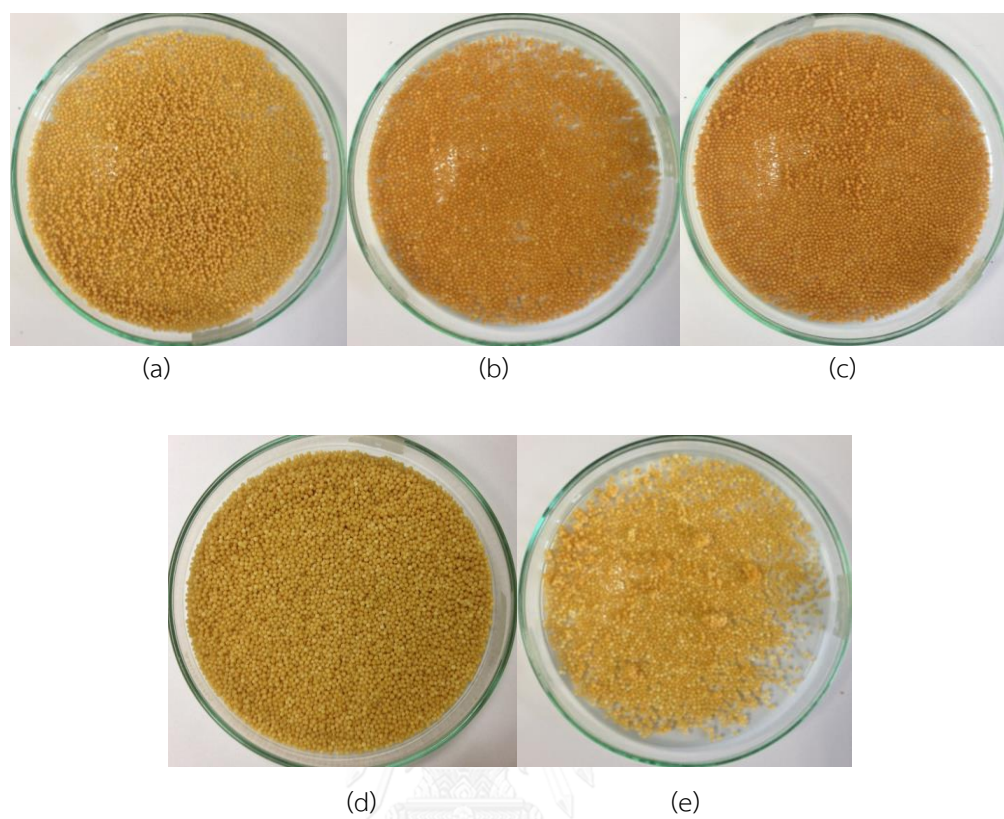
Figure 4.14 The WPGlu-STY/321FEPS bead prepared by using WPGlu generated from glutaraldehyde concentration of (a) 7.5%, (b) 10%, (c) 12.5% and (d) 15%w/v copolymerized with 5%w/v of STY at 80°C for 24h.
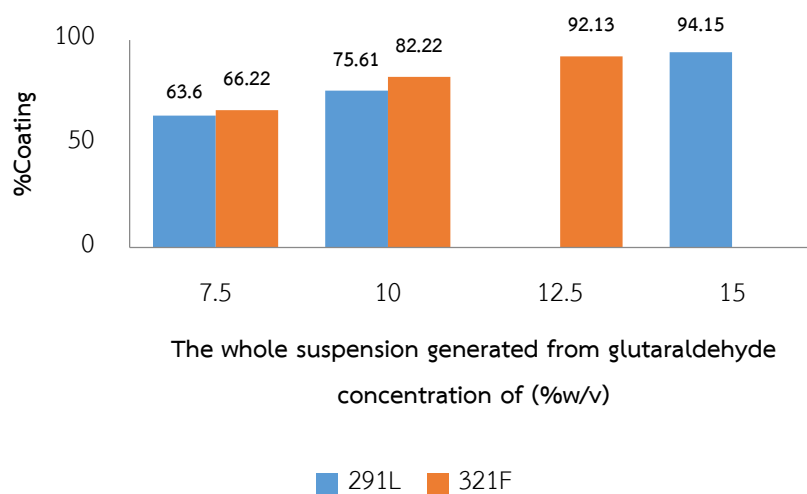
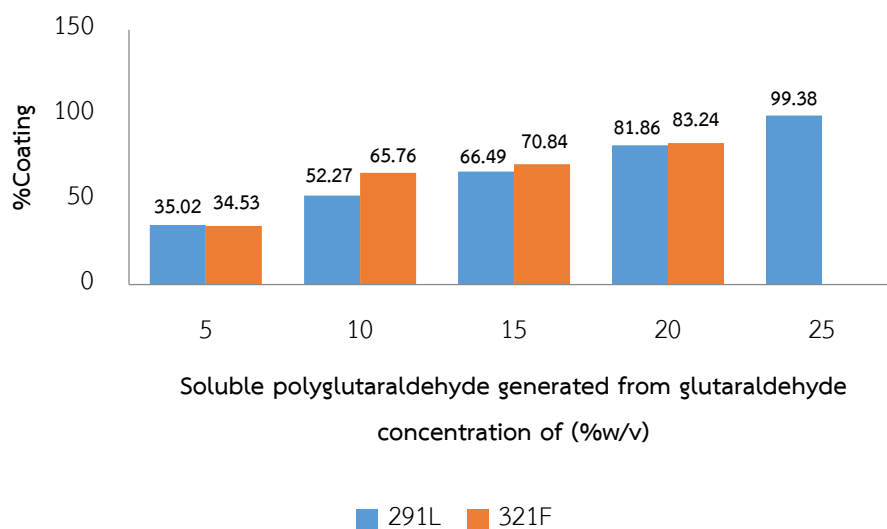**Figure 4.15** Percent coating of WPGlu-STY/291LEPS and WPGlu-STY/321FEPS

The WPGlu-STY/291LEPS and WPGlu-STY/321FEPS beads were successfully made when the whole polyglutaraldehyde suspension generated from less that 15% of glutaraldehyde was used while using the whole polyglutaraldehyde suspension generated from 20 and 25% of glutaraldehyde resulted in difficulty in stirring the reaction due to viscosity of the reaction mixture and the resulted WPGlu-STY/291LEPS and WPGlu-STY/321FEPS beads attached together to form lumps of the beads. These suggested that too much polyglutaraldehyde significantly affected coating process.

In Figure 4.15, percent coating yield of WPGlu-STY/291LEPS and WPGlu-STY/321FEPS depended on concentration of glutaraldehyde used. That was higher concentration of glutaraldehyde, higher percent coating yield. Also percent coating yield of both types was almost the same at the same concentration of glutaraldehyde similar to the above results. In comparison of coating yield of SPGlu-STY (52.27%) and WPGlu-STY (75.61%) on 291LEPS beads when using 10%w/v of glutaraldehyde, about 25% extra of copolymer, obtained from the whole polyglutaraldehyde suspension, should be caused by insoluble polyglutaraldehyde and higher polyglutaraldehyde in the WPGlu-STY/291LEPS and WPGlu-STY/321FEPS was also observed by ATR-FTIR spectra in Figure 4.16. The ATR-FTIR spectra of WPGlu-STY/EPS beads (Figure 4.17) showed that increasing concentration of glutaraldehyde used resulted in increasing the adsorption of C=O of non-conjugated at 1715 cm^{-1} and of conjugated aldehyde at 1675 cm^{-1}. This indicated the presence of a lot of STY-PGlu copolymer on EPS beads by copolymerization process at high concentration of the whole polyglutaraldehyde.
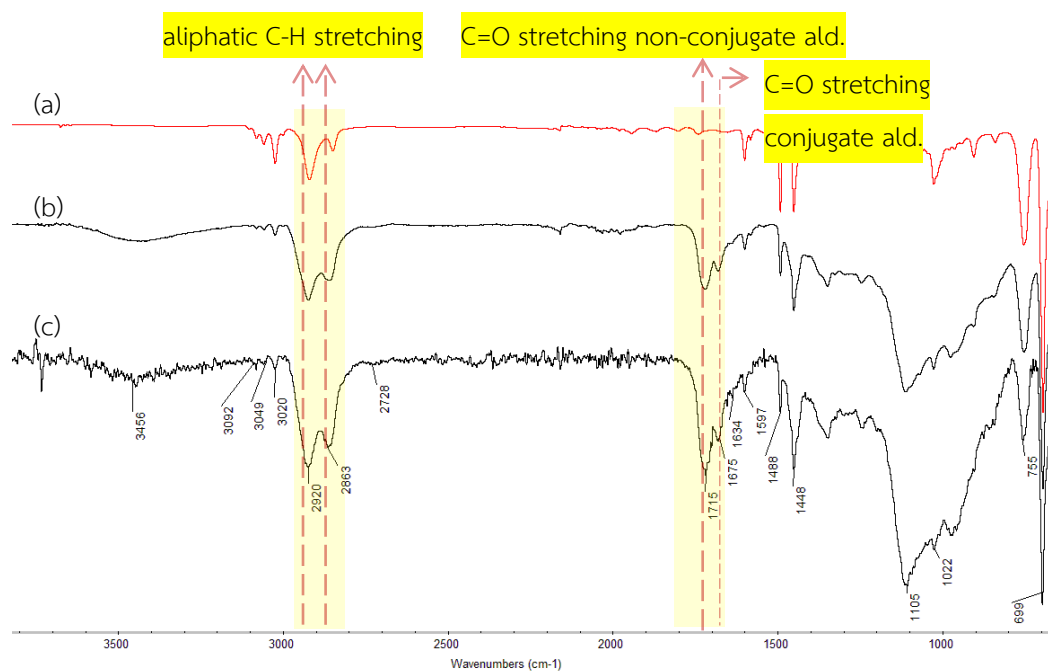
Figure 4.16 FT-IR spectra of (a) original EPS bead (b) SPGlu-STY/EPS and (c) WPGlu-STY/EPS beads prepared by using 10%w/v PGlu and 5%w/v of STY at 80°C for 24h.
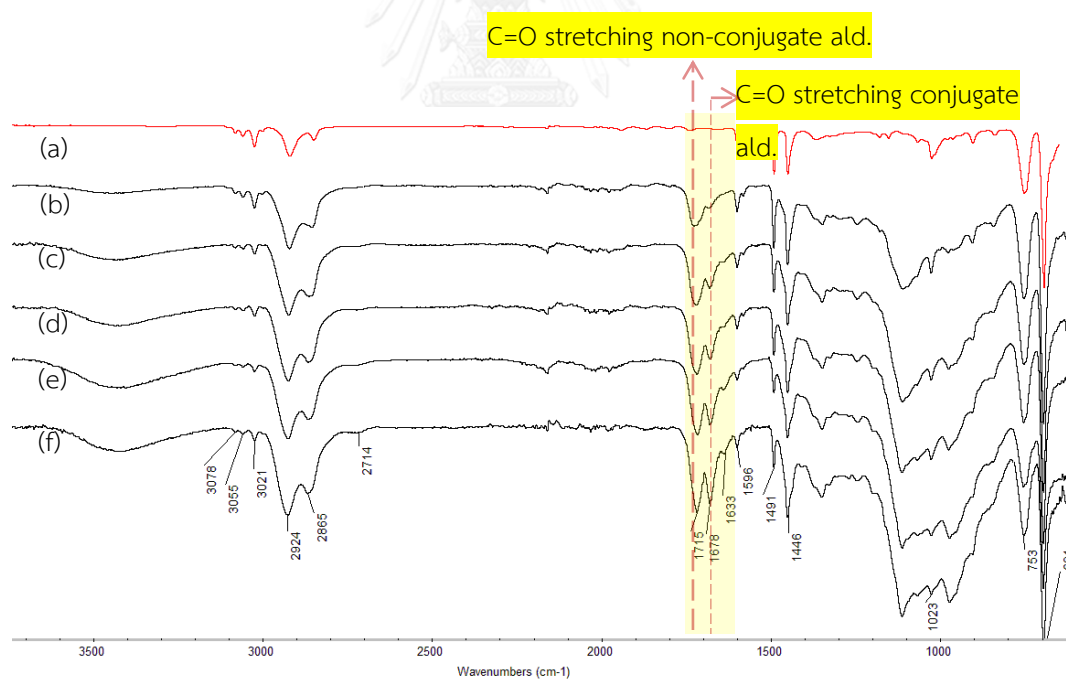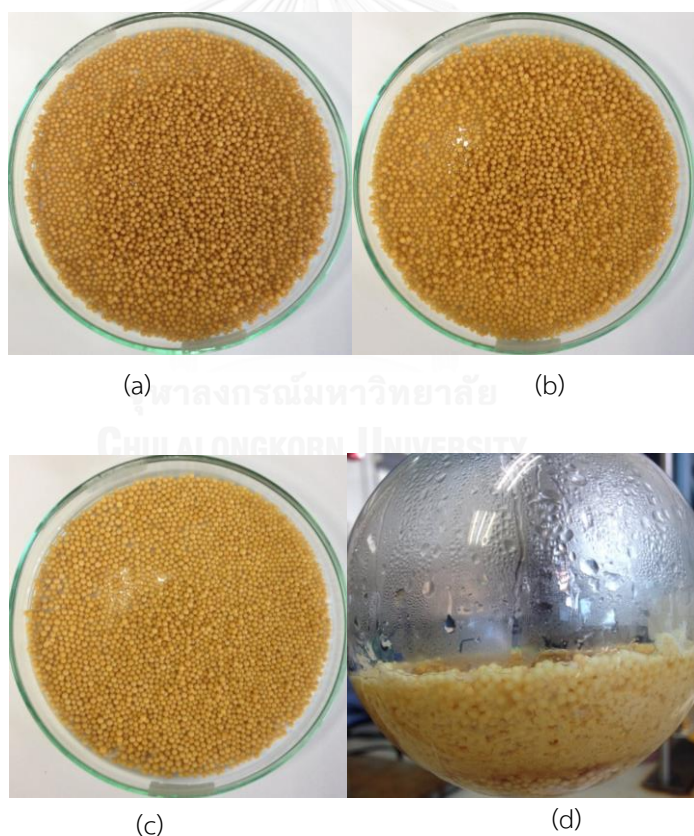
.
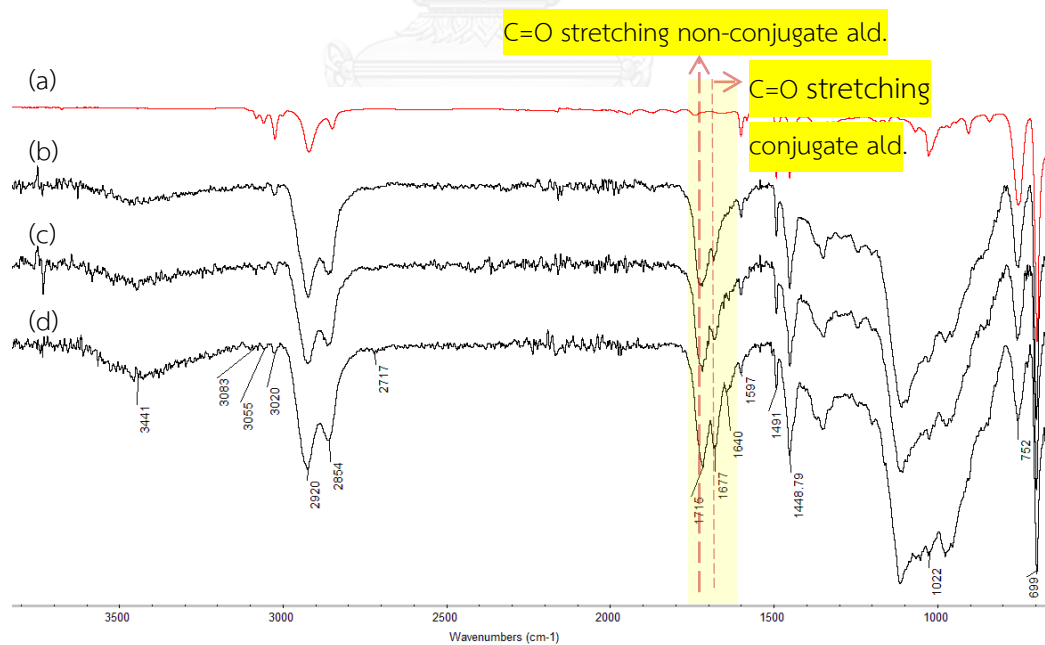
Figure 4.17 FT-IR spectra of (a) original EPS beads and WPGlu-STY/291LEPS beads prepared by using WPGlu generated from glutaraldehyde concentration of (b) 7.5%, (c) 10% and (d) 15%w/v copolymerized with 5%w/v of STY at 80°C for 24h.
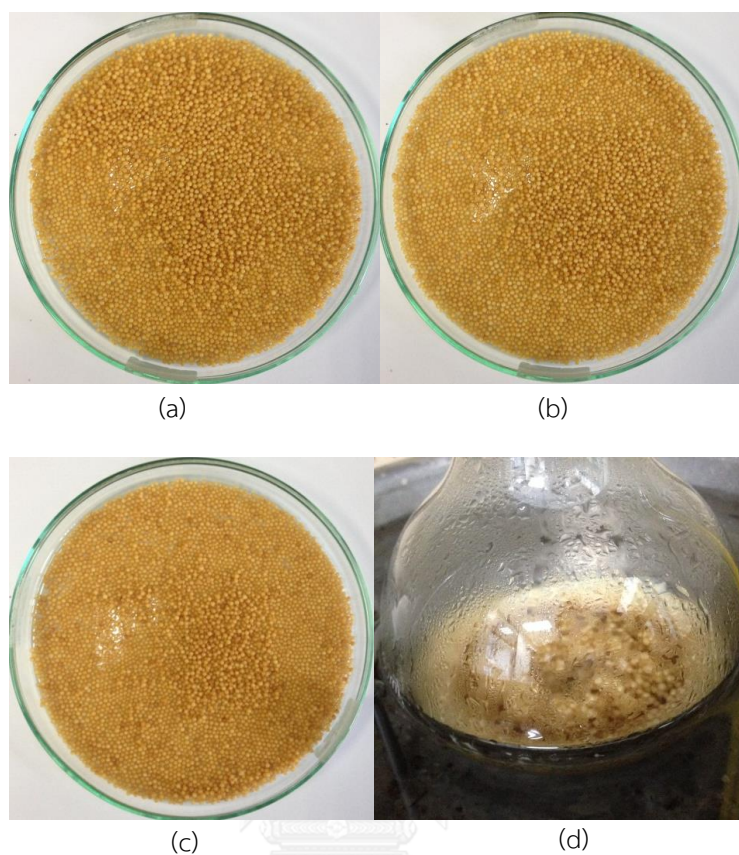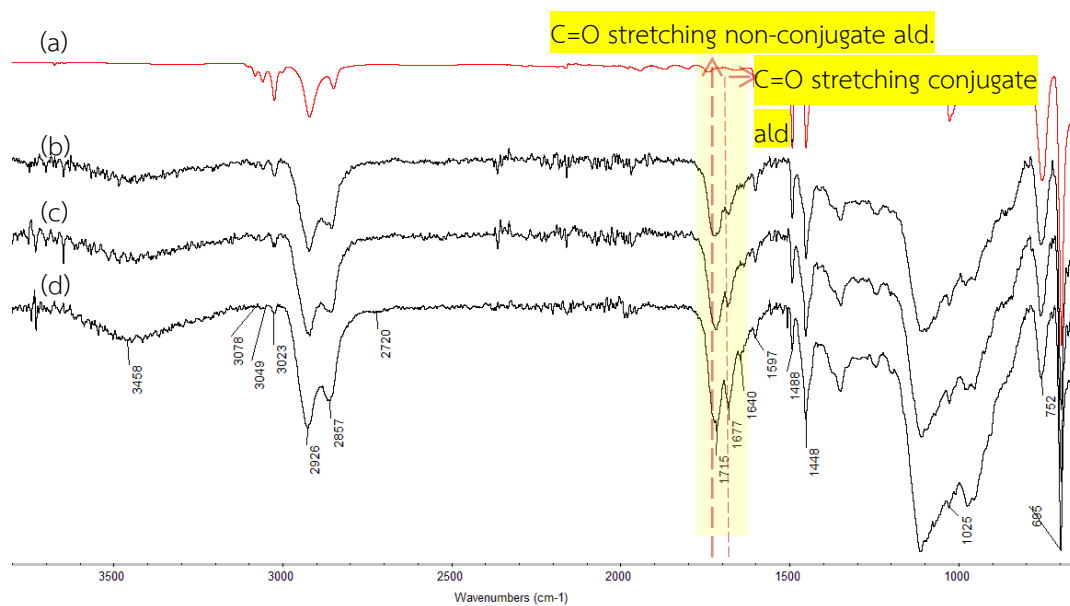
Figure 4.18 FT-IR spectra of (a) original EPS beads and WPGlu-STY/321FEPS beads prepared by using WPGlu generated from glutaraldehyde concentration of (b) 7.5%, (c) 10% and (d) 12.5%w/v copolymerized with 5%w/v of STY at 80°C for 24h.

4.2 Immobilization of lipase

From the preparation of PGlu-STY/EPS bead, there were 12 supports in total as shown in Table 4.2 consisting of the support obtained from soluble polyglutaraldehyde 6 supports and the whole polyglutaraldehyde 6 supports which could be used for immobilization. The supports were prepared using soluble polyglutaraldehyde, the whole suspension and size of support with 291L and 321F EPS beads, the prepared supports were designated by support code followed by S, W, L, F respectively.

Table 4.2 The support code for immobilization of lipase

Support		support code	
		291L	321F
STY (%w/v)	SPGlu (%w/v)		
5	5	LS5G5S	FS5G5S
	10	LS5G10S	FS5G10S
	15	LS5G15S	FS5G15S
STY (%w/v)	WPGlu (%w/v)		
5	7.5	LS5G7.5W	FS5G7.5W
	10	LS5G10W	FS5G10W
	12.5		FS5G12.5W
	15	LS5G15W	

There were many factors affecting the activity of lipase in immobilization process such as the support (PGlu:STY ratios copolymer coated on EPS bead), concentration of lipase and time of immobilization. Thus, these factors were investigated.

4.2.1 Effect of PGlu-STY ratios on activity of immobilized lipase

Pseudomonas cepacia lipase was immobilized on both WPGlu-STY/EPS beads and SPGlu-STY/EPS beads that there were 12 supports in total as shown in Table 4.2.

Lipase was immobilized onto the supports. Lipase activity and protein loading were measured by assay described in 3.3.2.1 and 3.3.2.2.

4.2.1.1 Effect of SPGlu-STY ratios on activity of immobilized lipase

Figure 4.19 Effect of SPGlu-STY ratios on activity of the immobilization of lipase on the SPGlu-STY/291L and 321F EPS beads, the support was immobilized by using 18 mg lipase at 37°C for 48h.
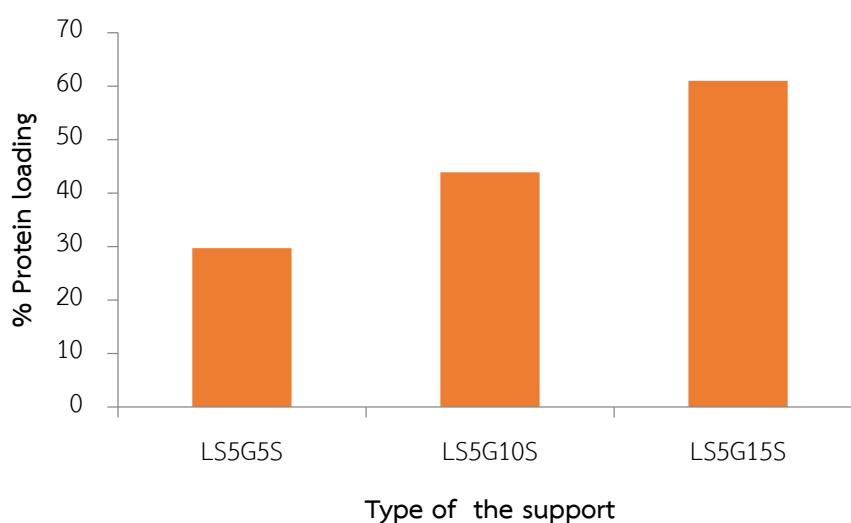
Figure 4.20 Effect of SPGlu-STY ratios on protein loading of lipase on the SPGlu-STY/291L, the support was immobilized by using 18 mg lipase at 37°C for 48h.

From Figure 4.19, it could be seen that the activity of both of size of support, which in type of SPGlu depended on concentration of glutaraldehyde used. When soluble glutaraldehyde was generated from higher concentration of glutaraldehyde, higher lipase activity of the catalyst was obtained. This was probably caused by more aldehyde functional group (see Figure 4.12) available for coupling with amino group of the lipase. When 10% and 15%w/v of glutaraldehyde were used to generate the soluble polyglutaraldehyde for making the support, the lipase-immobilized SPGlu-STY/321FEPS beads, which were smaller than SPGlu-STY/291L EPS beads, displayed a little higher lipase activity of the catalyst depended on the size of immobilized catalyst used. Since the smaller particle has higher surface area, more lipase could be immobilized onto the SPGlu-STY/321FEPS beads as described by percent coating (Figure 4.11) and protein loading in Figure 4.20 and availability of the lipase for the substrate was then higher than smaller surface area (291L). Thus the lipase-immobilized SPGlu-STY/321FEPS beads resulted in higher activity. When 5%w/v of glutaraldehyde was used to generate the soluble polyglutaraldehyde for making the support, the lipase-immobilized SPGlu-STY/321FEPS beads, which were smaller (or

higher surface area) than SPGlu-STY/291L EPS beads, displayed lower lipase activity of the catalyst. This was described by less lipase immobilization due to less availability of aldehyde functional group (less percent coating as shown in Figure 4.11). The lipase-immobilized SPGlu-STY/EPS beads with support code FS5G15S and LS5G15S gave the highest activity with lipase activity of 60.59 u/g-support and 57.34 u/g-support, respectively.

4.2.1.2 Effect of WPGlu-STY ratios on activity of immobilized lipase
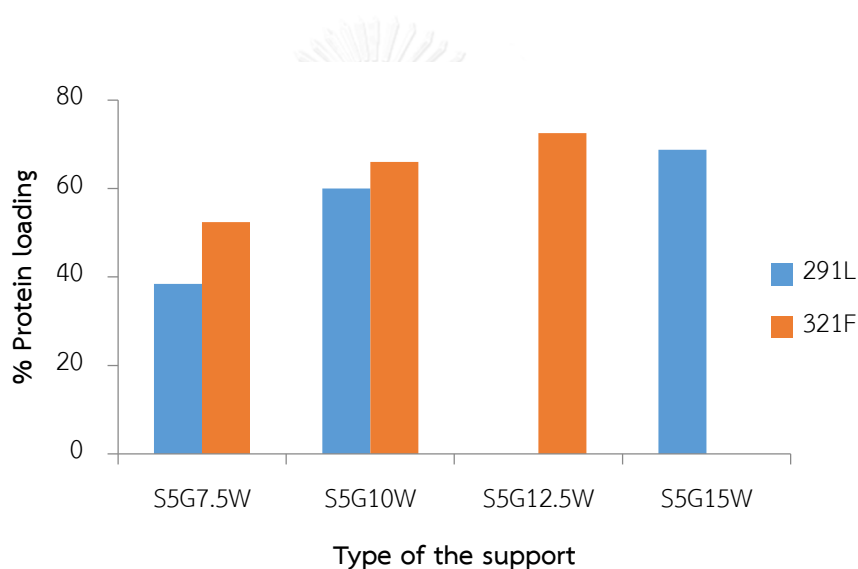
Figure 4.21 Effect of WPGlu-STY ratios on protein loading on the WPGlu-STY/291L and 321F EPS beads, the support was immobilized by using 12 mg lipase at 37°C for 24h.

From Figure 4.21, it found that percent protein loading of WPGlu-STY/291L and WPGlu-STY/321FEPS depended on concentration of glutaraldehyde used. That was the higher concentration of glutaraldehyde the higher percent protein loading because the concentration of glutaraldehyde was increased onto the support, the higher amount of amino group attached to the support. The comparison of percent protein loading of WPGlu-STY/291L and WPGlu-STY/321F found that the amount of

lipase, immobilized onto WPGlu-STY/321F, was higher than the WPGlu-STY/291L because of the higher surface, the higher percent protein loading.
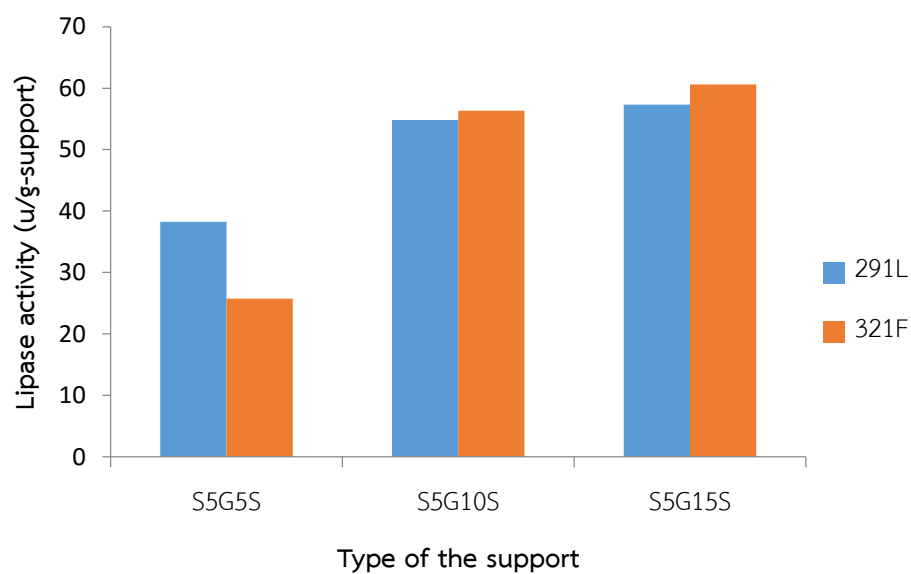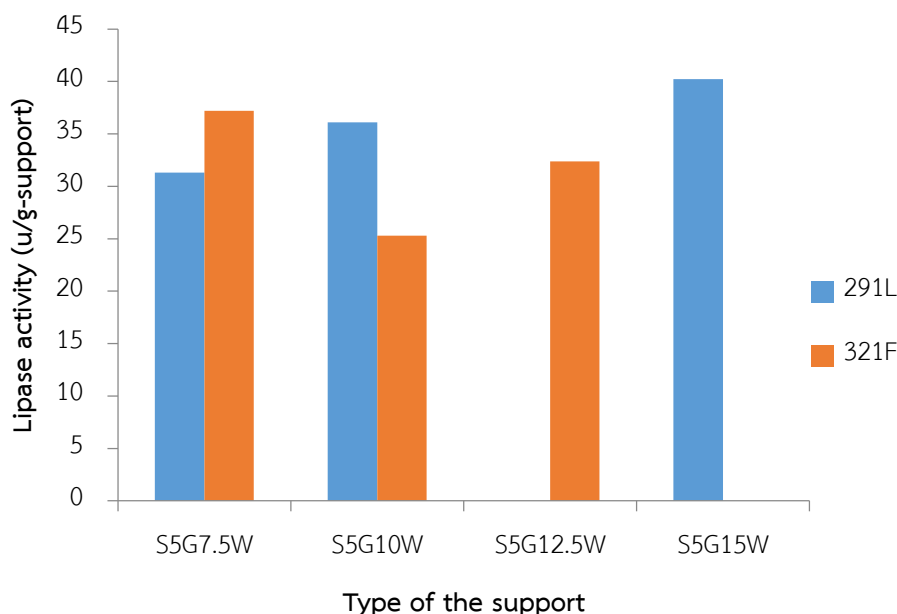

Figure 4.22 Effect of WPGlu-STY ratios on activity of the immobilization of lipase on the WPGlu-STY/EPS with 291L and 321F EPS beads, the support was immobilized by using 12 mg lipase at 37°C for 24h.

From Figure 4.22 it found that the higher activity of the immobilized WPGlu-STY/291LEPS beads was correlated with the higher concentration of glutaradehyde used to generate the whole polyglutaraldehyde suspension which the results were similar to the lipase-immobilized WPGlu-STY/321FEPS beads as described above. When lipase was immobilized onto the WPGlu-STY/321FEPS beads, lipase activity of the immobilized catalyst obtained from using the whole polyglutaraldehyde generated by using 7.5, 10 and 12.5%w/v of glutaraldehyde was 37, 25 and 32 u/g, respectively while the lipase loading (Figure 4.21) was 50, 60 and 70%, respectively. These suggested that when the whole polyglutaraldehyde was used, less concentration was needed for preparation of the WPGlu-STY/321FEPS beads. FTIR

spectrum in Figure 4.18 revealed that the WPGlu-STY/321FEPS beads, obtained from using 10%, 12.5%w/v of glutaraldehyde, contained higher conjugated aldehyde carbonyl when compared to FTIR spectra the WPGlu-STY/321FEPS beads, obtained from using 7.5%w/v of glutaraldehyde. It suggested that morphology of the WPGlu-STY/321FEPS beads, obtained from using 10%, 12.5%w/v of glutaraldehyde should be different from the WPGlu-STY/321FEPS beads, obtained from using 7.5%, w/v of glutaraldehyde and thus affected lipase activity of the immobilized catalyst. Since too much polyglutaraldehyde affected in decrease of lipase activity, the lipase-immobilized WPGlu-STY/321FEPS beads, obtained from using 10%, 12.5%w/v of glutaraldehyde displayed lower lipase activity than the catalyst obtained from using 7.5%w/v of glutaraldehyde. More evidence of too much polyglutaraldehyde that affected in decrease of lipase activity included the results of using the supports S5G15S/291LEPS and S5G15W/291LEPS (Figure 4.23).
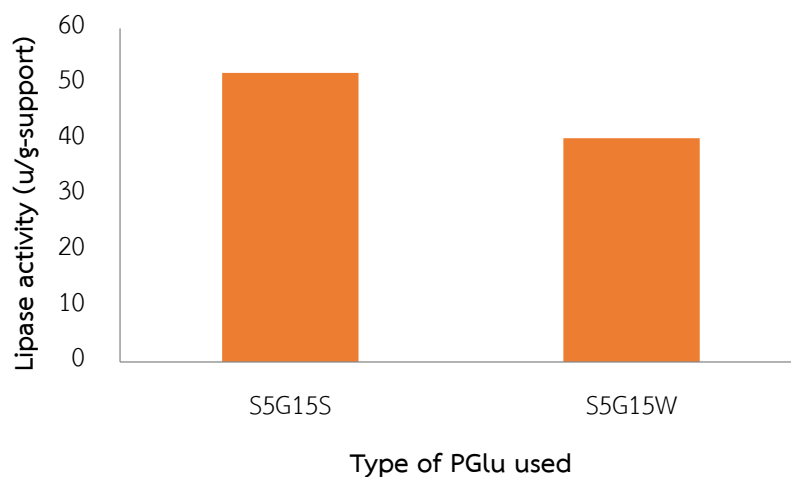
Figure 4.23 Effect of using the SPGlu-STY/291LEPS and WPGlu-STY/291LEPS on activity.

4.2.2 Effect of concentration of lipase on immobilization
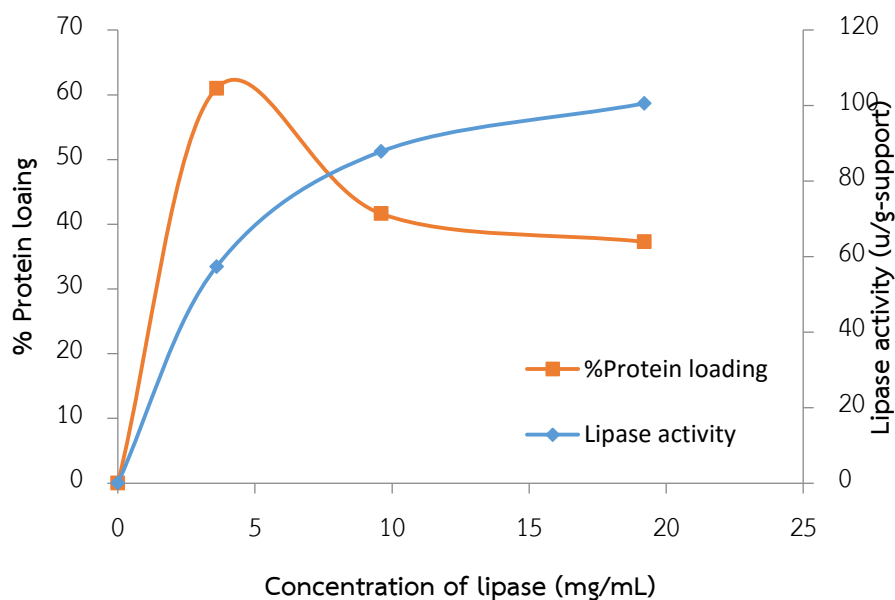
Figure 4.24 Effect of concentration of lipase on activity and percent protein loading of immobilized lipase.

In Figure 4.24, 3.6, 9.6, 19.2 mg/mL lipase solutions were immobilized onto 1 g of the SPGlu-STY/291EPS bead at 37℃ for 48h. Lipase activity of the immobilized catalyst was increased when the concentration of lipase while protein loading in 60% was obtained by using 3.6 mg/mL and about 40% protein loading was obtained when 12 and 19.2 mg/mL of lipase was used.

4.2.3 Effect of time of immobilization on activity of immobilized lipase
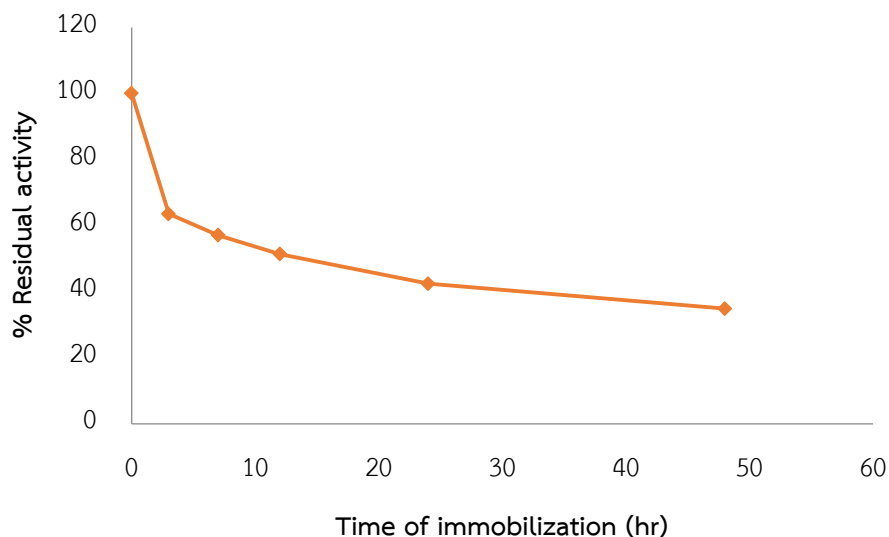
Figure 4.25 The effect of immobilization time on the residual activity of lipase by 2.4 mg/mL of *Pseudomonas cepacia* lipase solution were incubated at 37°C

Effect of time on immobilization of lipase was investigated by measuring lipase activity of the residual lipase. The results (Figure 4.25) showed that the activity of the residual lipase was sharply decreased from initial time to the incubation 3-12h and rather constant at about 42.40% of residual activity after 24h of the incubation.

4.3 Biodiesel production

The *Pseudomonas cepacia* lipase immobilized PGlu-STY/EPS bead was used as catalyst for biodiesel production through reaction between soybean oil and alcohols (ethanol or methanol). The reaction parameters in terms of optimal molar ratio of oil:alcohol, amount of water and the immobilized catalysts were investigated.

4.3.1 Effect of ratios of oil:alcohol on transesterification

The reactions were carried out by varying in a range of oil:ethanol 1:2 to 1:6 using 5 g of soybean oil and 158.5 mg (11.90 units) of imLS5G15W, 75.12 u/g as immobilized lipase at 40°C. Results (Table 4.3) showed that fatty acid ethyl esters (FAEEs) were produced when 1:2 and 1:3 ratios of oil:alcohol were used while the reaction could not be carry out by using ratios of oil:alcohol in a range of 1:4 to 1:6. These suggested that using ratios of oil:alcohol in a range of 1:4 to 1:6, the immobilized lipase could not catalyze the triglyceride (soybean oil) into FAEE product because the enzyme should be denatured by the excessive of alcohol. Using 1:2 ratio of oil:alcohol the FAEEs were produced in a slow rate of reaction which 3.38 %FAEEs were produced within 6h and 21.05 %FAEEs were produced within 149h. Although the ratio of oil:ethanol should be at least 1:3 to complete the reaction [12], results in Table 4.3 showed that the catalyst catalyzed triglyceride into FAEE product with a very slow rate of reaction. It suggested that partial lipase should be inactive when using 1:3 ratio of oil:alcohol. Since it was well known that lipases were also capable of catalyzing the reverse reaction, achieving hydrolysis and esterification [18], effect of water on the reaction was also investigated in order to improve enzymatic reaction.

Table 4.3 Effect of ratios of oil:ethanol on FAEE production*

Mole ratio oil: EtOH	%FAEE							
	2h	4h	6h	8h	10h	12h	24h	149h
1:2	-	-	2.93	3.29	4.73	5.00	7.89	21.00
1:3	-	-	-	-	-	-	-	7.26
1:4	-	-	-	-	-	-	-	-
1:5	-	-	-	-	-	-	-	-
1:6	-	-	-	-	-	-	-	-

* The reactions were carried out by using catalyst with the same lipase activity

(11.90 unit) and FAEE product was estimated by ^{1}H NMR analysis as shown in Appendix B.
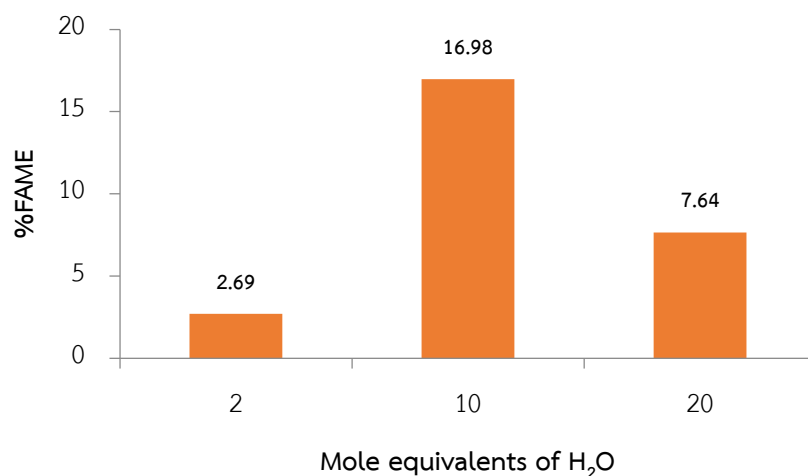
4.3.2 Effect of amount of water

Figure 4.26 Effect of amount of H_2O on FAEE production using 1:2 ratio of oil:ethanol, 40°C of reaction temperature, and 24h of reaction time

    The reactions were carried out by adding water in 2, 10 and 20 mole equivalents of oil into a mixture of 5 g of soybean oil, 1:2 oil:ethanol ratio and 158.5 mg (11.90 units) of imLS5G15W catalyst (75.12 u/g) at 40°C. In comparison between ^{1}H NMR data (Figure 4.28), published by Jin et al. [34], and ^{1}H NMR data (Figure 4.27) of the product from the reaction of soybean oil with ethanol in a presence of water (2, 10, 20 equivalents of oil) at 40°C for 24h, it revealed that the reactions slightly occurred when adding water in 2 equivalent of oils while adding water in 10 and 20 equivalent of oil resulted in hydrolysis and esterification to give monoglyceride, diglyceride and FAEE.

    The results (Figure 4.26) showed that adding water 10 equivalents in the reaction mixture resulted in the highest FAEE production, which was about 16.98%. In comparison with the absence of water from previous optimal condition, %FAEE was higher than the absence of water and the %FAEE was approximately 2 fold increased. Since ^{1}H NMR spectrum revealed that free fatty acid also presented as product, it suggested that the free fatty acid obtained from hydrolysis was continuously reacted with ethanol to result in forming free fatty acid ethyl ester.

These results also suggested that the prepared immobilized catalyst preferably catalyzed hydrolysis and esterification more than transesterification.
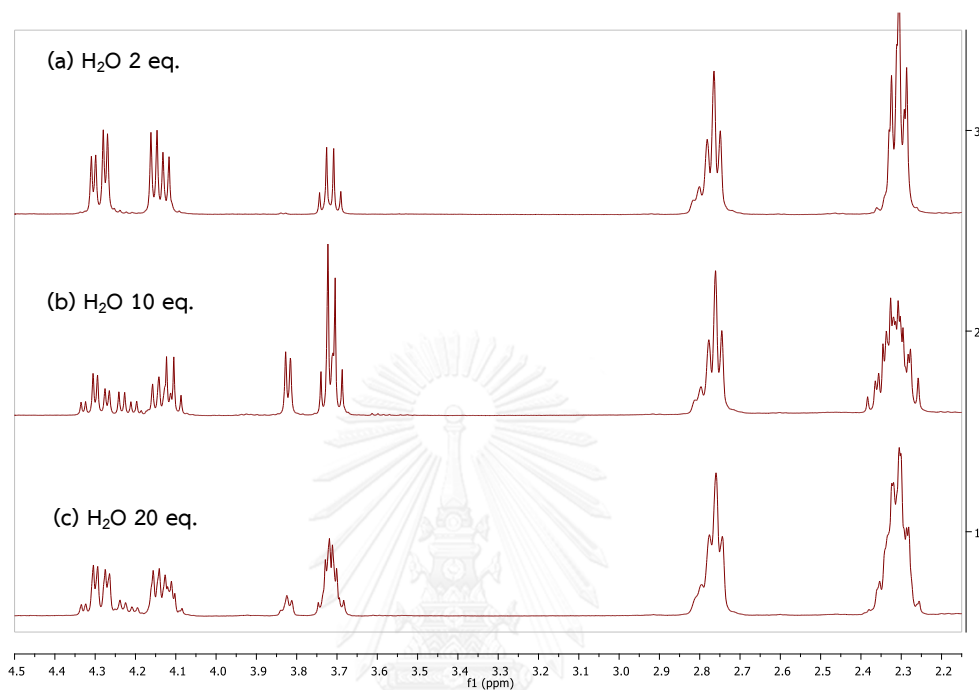
Figure 4.27 ^{1}H NMR of the product resulting from reaction of soybean oil with ethanol in a presence of water in a) 2, b) 10, and (c) 20 equivalents of oil at 40°C for 24h.
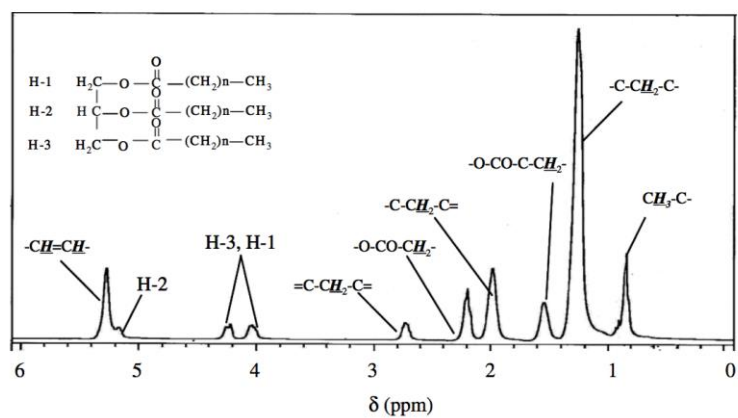
(a)
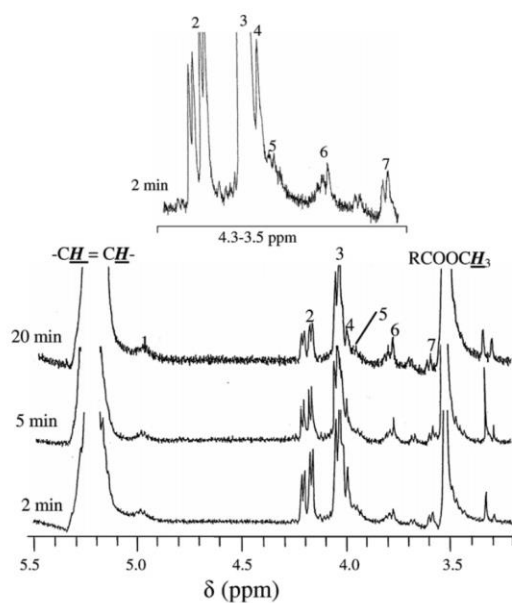
(b)

Figure 4.28 (a) ^{1}H NMR spectrum of a rapeseed oil. H-1, H-2 and H-3 labels indicate the protons attached to glycerol carbons (b) Expansion of 3.5-5.5 ppm region in the 1H NMR spectra of rapeseed oil after the methanolysis reaction at 20°c and with vary reaction ((1) H-2 of 1,2-DGs; (2) H-1(H-3) of TGs; (3) H-1(H-3) of TGs, sn-1,3-DGs, sn-1,2-DGs and sn-MGs; (4) H-2 of sn-1,3-DGs; (5) H-2 of sn-1-MGs; (6) H-3 of sn-1,2-GDs; (7) H-3 of sn-1-MGs. The description of H-1, H-2, H-3 is in (a) [34].

Table 4.4 Chemical shift and assignment of glycerides for ^{1}H-NMR

Comp.	Proton	References[a] (CDCl$_3$)	Authentic comp. (CDCl$_3$)
TGs	H-1(3), CH_2O–	4.10–4.20/4.4.2–4.4	4.15/4.39
	H-2, CHO–	5.15–5.22	5.25
sn-1,2-DGs	H-1, CH_2O–	4.13–4.18/4.29–4.34	4.15/4.39
	H-2, CHO–	5.06–5.2	5.07
	H-3, CH_2O	3.66–3.78	3.72
sn-1,3-DGs	H-1(3), CH_2O	3.99–4.10	4.10–4.25
	H-2, CHO–	4.0–4.06	5.07
sn-1-MGs	H-1, CH_2O–	4.10	
	H-2, CHO–	3.8	
	H-3, CH_2O	3.50–3.66	
sn-2-MGs	H-1(3), CH_2O–	3.9–4.0	
	H-2, CHO–	4.9–5.0	
	OH–		

From Refs [35-42]

4.3.3 Effect of using soluble polyglutaraldehyde and the whole polyglutaraldehyde suspension and particle size on the immobilized catalysts

To study effect of using soluble polyglutaraldehyde and the whole polyglutaraldehyde suspension and particle size of the support on the reaction between soybean oil and alcohol, the immobilized catalysts (Table 4.4), obtained from immobilization of lipase onto SPGlu-STY/291LEPS, WPGlu-STY/291LEPS, SPGlu-STY/321FEPS, and WPGlu-STY/E321F EPS, were used in the reaction between soybean oil and alcohol in a presence of water (10 eq). Efficiency of each catalyst was investigated by using the same unit of activity (about 11.90 units) and each reaction was carried out by using 5 g of soybean oil, 1:2 oil:methanol ratio and 11.90 units of immobilized lipase [imLS5G15S (229 mg), imLS5G15W (295.5 mg), imFS5G20S (227 mg), imFS5G7.5S (320.6 mg)] at 40°C for 4, 24, 312h. Since the reaction between soybean oil and ethanol resulted in a mixture of triglyceride, monoglyceride, diglyceride and FAEE as mentioned above and overlapping of the ^{1}H NMR data in some cases might interfere integral in the chemical region of the ethyl ester for estimation of %FAEE, this study was performed by using methanol instead of ethanol

in order to reduce the interference. Results (Figure 4.29) showed that the lipase, immobilized on the support prepared by using soluble polyglutaraldehyde, gave higher amount of FAME than using the whole polyglutaraldehyde suspension and the lipase immobilized onto the support with smaller size improved the efficiency and gave the higher enzymatic reaction rate.

Table 4.5 The support and immobilized code of immobilized lipase

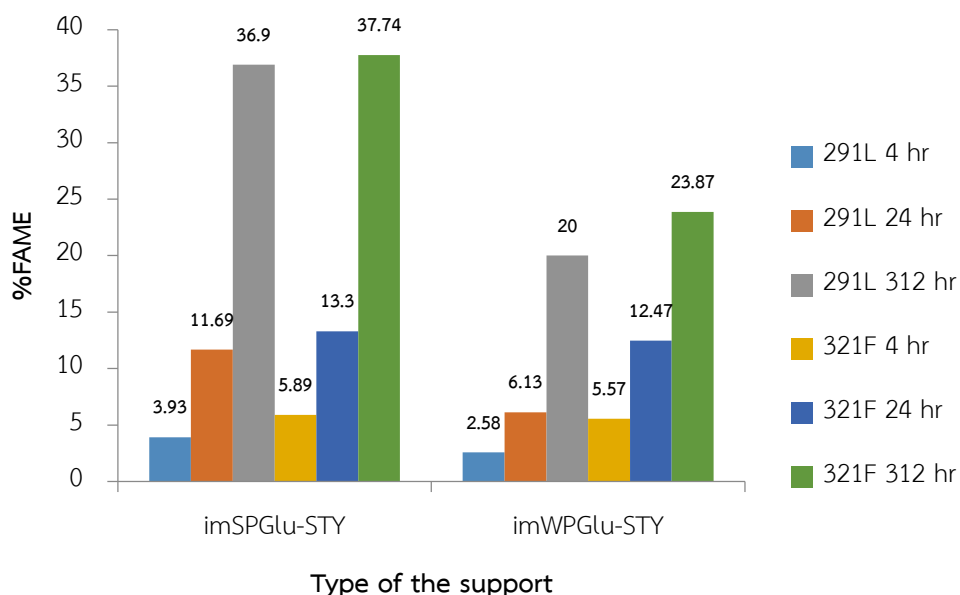| Support	Support code	Immobilized code	Lipase activity (u/g-support)
STY5%-SPGlu15%/291LEPS	LS5G15S	imLS5G15S	51.99
STY5%-WPGlu15%/291LEPS	LS5G15W	imLS5G15W	40.22
STY5%-SPGlu20%/321FEPS	FS5G20S	imFS5G20S	52.37
STY5%-WPGlu7.5%/321FEPS	FS5G7.5W	imFS5G7.5S	37.19

Figure 4.29 Effect of type of catalyst on %fatty acid methyl ester

CHAPTER V

Conclusion

In previous results of our research group, the lipase from *Pseudomonas cepacia* was immobilized onto polyglutaraldehyde-styrene copolymer coated expandable polystyrene beads (PGlu-STY/EPS) with only one particle size. There was a report that the maximum enzymatic reaction rate was at a higher level by using lipase-immobilizing with smaller particle size. To improve the efficiency of immobilized lipase catalyst for biodiesel production, the smaller particle sizes of PGlu-STY/EPS beads were prepared by varying coating temperature and the beads prepared however were unsuitable for immobilization of the lipase. Thus two sizes of expandable polystyrene bead (EPS) consisting of EPS bead grade 291L, with size of 1.00-1.60 mm and EPS bead grade 321F, with size of 0.63-1.12 mm were used for coating with PGlu-STY copolymer. The surface of EPS beads was coated with PGlu-STY copolymer in order to provide the aldehyde functional group for lipase-immobilization via covalent binding. EPS bead was coated by radical polymerization of styrene monomer and polyglutaraldehyde. During coating process, it was found that PGlu-STY/EPS beads not only could be prepared by using soluble polyglutaraldehyde as described in the literature but also the whole suspension of polyglutaraldehyde generated from the same original concentration of the glutaraldehyde. Using the whole suspension of polyglutaraldehyde gave the higher percentage of coating PGlu-STY on both sizes of EPS beads than that of soluble polyglutaraldehyde. In this study, 291LEPS and 321FEPS coated with the soluble polyglutaraldehyde-styrene copolymer (SPGlu-STY/291LEPS or 321FEPS beads) and coated with the whole polyglutaraldehyde-styrene copolymer (WPGlu-STY/291L or 321FEPS beads) were successful prepared by using two-fold of total volume of the reaction from previous method of our group. The SPGlu-STY/291LEPS and SPGlu-STY/321FEPS were successful made when used the soluble polyglutaraldehyde generated from 5-20%w/v of glutaraldehyde solution and the WPGlu-STY/291LEPS

and WPGlu-STY/321FEPS were successful made when used the whole suspension of polyglutaraldehyde generated from less 15%w/v of glutaraldehyde solution.

The immobilized lipase catalysts were evaluated their lipase activity and efficiency for biodiesel production from soybean oil. In both sizes of EPS beads, the lipase-immobilized catalysts that was prepared by using the soluble polyglutaraldehyde provides higher activity than that was prepared by using the whole suspension of polyglutaraldehyde and the activity of lipase immobilized on 321FEPS bead was higher than 291LEPS bead. For immobilization, lipase from *Pseudomonas cepacia* was successful immobilized on these supports. The efficiency of those immobilized catalysts for biodiesel production from soybean oil was notably low. Nevertheless, the addition of water to the reaction could significantly improve production of biodiesel. The prepared immobilized catalyst seemed to preferably catalyze hydrolysis and esterification more than transesterification. Furthermore, the lipase-immobilizing with small size which was prepared by using the soluble and the whole polyglutaraldehyde has better efficiency than the bigger one. The lipase-immobilized catalysts, obtained by using the soluble polyglutaraldehyde, gave higher amount of FAME than that using the whole polyglutaraldehyde and the lipase was immobilized on the support with smaller size improved the efficiency and gave the higher enzymatic reaction rate.

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        Biodiesel by 1H NMR Spectroscopy <u>energy & fuels</u> 23 (2009): 2273-2277.

APPENDIX

APPENDIX A

ENZYMATIC ASSAY

A-1 Lipase activity assay

1. Preparation calibration curve of p-nitrophenol [43]

The stock solution of p-nitrophenol (p-NP) was prepared by diluting the concentration of *p*-nitrophenol solution 10 µmol/mL with EtOH:PBS pH6.70:Na_2CO_3 in the ratio of 1:1.1:2 to p-nitrophenol solution concentration of 0.25 µmol/mL. Then, the stock solution was diluted by the same ratio of EtOH:PBS pH6.70:Na_2CO_3 in a range of 0-0.48 µmol/mL and after that the p-nitrophenol solution was measured at 410 nm which was shown in Figure A-1

Table A-1 The volume of p-NP and EtOH : PBS : Na_2CO_3 of the concentration *p*-nitrophenol solution in a range of 0-0.48 µmol/mL

p-NP (µmol/mL)	Volume of p-NP (mL)	Volume of EtOH : PBS : Na_2CO_3 (mL)
0.006	0.24	9.76
0.012	0.48	9.52
0.018	0.72	9.28
0.024	0.96	9.04
0.030	1.20	8.80
0.036	1.44	8.56
0.042	1.68	8.32
0.048	1.92	8.08

$$y = 20.567x - 0.0052$$
$$R^2 = 0.9996$$
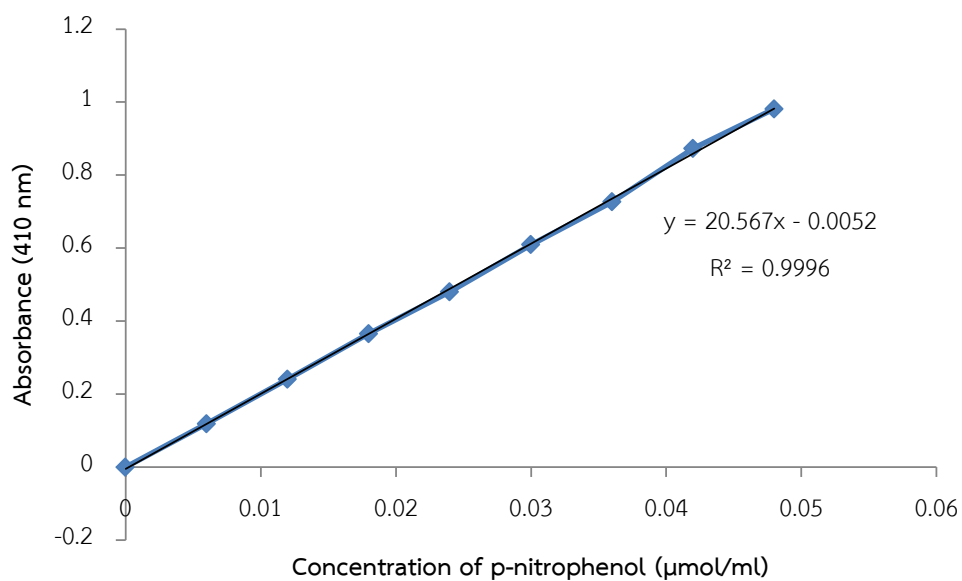
Figure A-1 The calibration curve of p-nitrophenol

2. Preparation of chemical for lipase activity assay

2.1 The concentration of p-nitrophenyl palmitate (p-NPP) 0.5%w/v was prepared by dissolving the p-NPP 10 mg in absolute ethanol 2 mL as substrate

2.2 Na_2CO_3 0.5M was prepared by dissolving Na_2CO_3 5.3 g in MilliQ water 100 mL.

2.3 The EtOH, PBS pH6.7 and Na_2CO_3 was mixed in the ratio of 1:1.1:2 as stock dilution.

3. Lipase activity assay
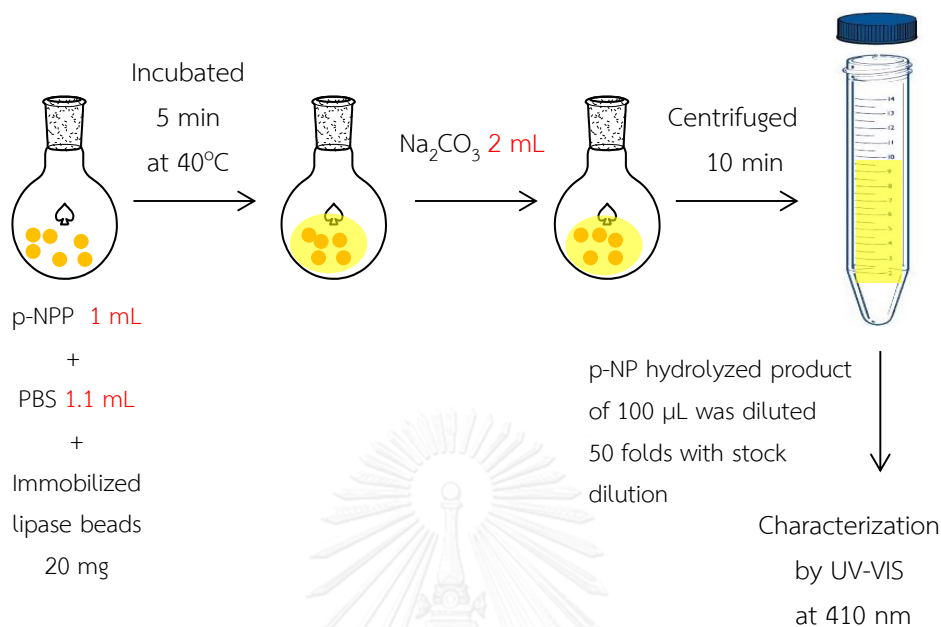
Figure A-2 The procedure of lipase activity assay

$$\text{Lipase activity (U/g-support)} = \frac{\text{Activity of immobilized lipase (U)}}{\text{Amount of support used (g-support)}}$$

One unit of lipase activity was defined as 1 micromole p-nitrophenol (p-NP) released per min from p-NPP by 1mL free enzyme or per gram of immobilized enzyme under standard conditions (in calcium acetate buffer at pH 6 and 40°C.

4. Calculation of lipase activity (U/g-support)

Lipase activity (U/g-support) was calculated as follows:

1. After hydrolysis, the absorbance value of diluted p-NP hydrolyzed product was compared to a calibration curve.

From

$$Y = 20.567X - 0.0052$$

Y = absorbance (nm)
X = concentration of p-NP

Absorbance of diluted p-NP hydrolyzed product = 0.40855

$$0.40855 = 20.567X - 0.0052$$
$$0.41375 = 20.567X$$
$$X = 0.0201 \ \mu mol/mL$$

So, Concentration of diluted p-NP hydrolyzed product = 0.0201 µmol/mL

2. To determine the actual concentration of p-NP hydrolyzed product. The concentration of diluted p-NP hydrolyzed product was multiplied by diluted factor

So, the actual concentration of p-NP hydrolyzed = 0.0201 µmol/mL x diluted factor (50X)
= 1.005 µmol/mL

3. To determine actual substance of p-NP hydrolyzed product (µmol) in the reaction of hydrolysis at the total volume was 4.1 mL.

So, the actual substance of p-NP hydrolyzed product (µmol) = 1.005 µmol/mL x 4.1 mL
= 4.1205 µmol

4. To determine activity of immobilized lipase (unit) based on the actual substance of p-NP hydrolyzed product (µmol).

So, the activity of immobilized lipase (unit) = $\dfrac{4.1205 \text{ µmol}}{5 \text{ min}}$

= 0.8241 unit

5. To determine lipase activity (u/g-support), g of support used = 20.5 mg

So, lipase activity (unit/g-support) = $\dfrac{0.8241 \text{ unit}}{0.0205 \text{ g-support}}$

= 40.2 unit/g-support

or calculated by equation below:

Lipase activity (U/g-support) = $\dfrac{\text{Con. of diluted } p\text{-NP hydrolyzed (µmol/mL) } \times \text{ total volume (mL) } \times \text{ dilute factor}}{\text{Incubated time (min) } \times \text{ amount of support used (g)}}$

A-2 Protein assay

Protein loading was estimated by measuring activity of the lipase. The ratio of substrate to unit of lipase used was significantly important for measuring the activity of lipase. Finding the optimal ratio of the substrate to unit of lipase used as well as the activity of lipase was still effective at 35 unit/mg was determined.

The stock solution of lipase (35unit/mL) was diluted by PBS pH6.70 to concentration of lipase solution in a range of 1.33–4.27 unit/mL. Then the several concentrations of lipase solution were reacted with the substrate (p-NPP) 13.25 µmol/mL by hydrolysis reaction. After that the p-nitrophenol solution was measured at 410 nm. The results shown in Table A-2, Figure A-3.

Table A-2 Effect of µmol of p-NPP/unit of lipase used on lipase activity

µmol of p-NPP (µmol/mL)	unit of lipase used (u/mL)	µmol of p-NPP/unit of lipase used (µmol/u)	Lipase activity (u/g-support)
13.25	4.27	3.1	26.99
13.25	3.43	3.86	28.39
13.25	2.59	5.12	31.47
13.25	1.75	7.57	34.81
13.25	1.33	9.96	34.68
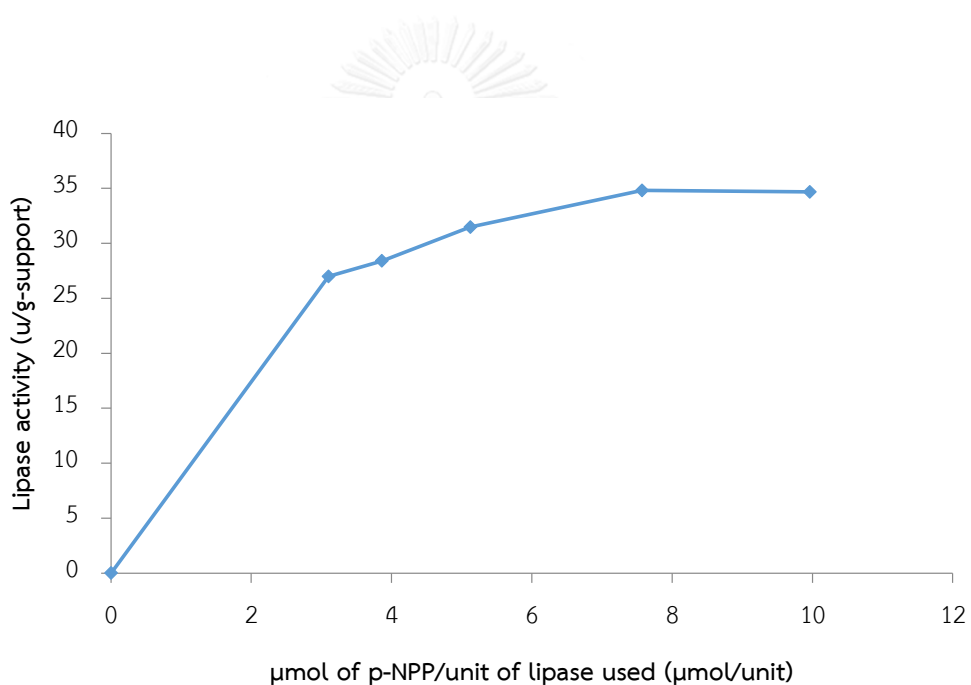
Figure A-3 Effect of µmol of p-NPP/unit of lipase used on lipase activity

1. Protein loading assay

Protein loading was estimated from enzyme activity of residual enzyme solution.
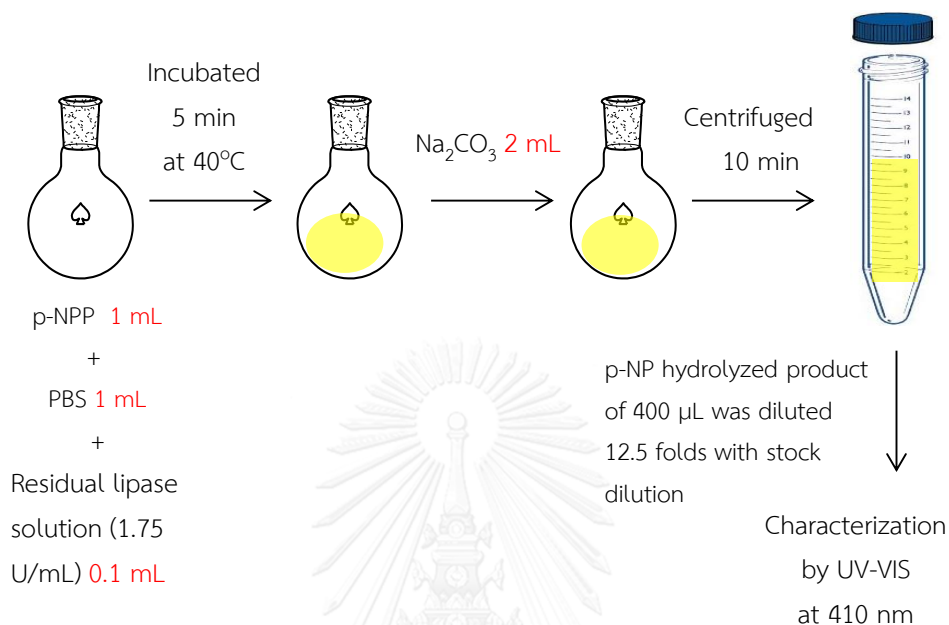
Figure A-4 The procedure of protein loading assay

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

2. Preparation of chemical for protein loading assay

2.1 The concentration of p-nitrophenyl palmitate (p-NPP) 0.5%w/v was prepared by dissolving the p-NPP 10 mg in absolute ethanol 2 mL as substrate

2.2 The residual lipase was diluted by PBS pH6.7 to the concentration of 1.75unit/mL

2.3 Na$_2$CO$_3$ 0.5M was prepared by dissolving Na$_2$CO$_3$ 5.3 g in MilliQ water 100 mL.

2.4 The EtOH, PBS pH6.7 and Na$_2$CO$_3$ was mixed in the ratio of 1:1.1:2 as stock dilution.

1. Calculation of protein loading

Protein loading was calculated as follows:

1. After hydrolysis, the absorbance value of diluted p-NP hydrolyzed product was compared to a calibration curve.

From

$$Y = 20.567X - 0.0052$$

Y = absorbance (nm)

X = concentration of p-NP

Absorbance of diluted p-NP hydrolyzed product = 0.36898

0.36898	=	20.567X - 0.0052
0.37418	=	20.567X
X	=	0.01819 µmol/mL

So, Concentration of diluted p-NP hydrolyzed product = 0.01819 µmol/mL

2. To determine the actual concentration of p-NP hydrolyzed product. The concentration of diluted p-NP hydrolyzed product was multiplied with dilute factor and diluted factor of residual lipase.

So, the actual concentration of p-NP hydrolyzed

= 0.01819 µmol/mL x diluted factor (12.5X) x diluted factor of res.lipase (144X)

= 32.742 µmol/mL

3. To determine actual substance of p-NP hydrolyzed product (µmol) in the reaction of hydrolysis at the total volume was 4.1 mL.

So, the actual substance of p-NP hydrolyzed product (µmol) = 32.742 µmol/mL x 4.1 mL

= 134.24 µmol of residual lipase solution

4. To determine total residual unit of lipase in total volume 5 mL of immobilization of lipase

So, total residual unit of lipase = $\dfrac{134.24 \text{ µmol x 5 mL}}{5 \text{ min}}$

= 134.24 unit

5. To convert total residual unit of lipase to mg-protein in total residual lipase

So, mg-protein in residual lipase = $\dfrac{134.24 \text{ unit}}{35 \text{ unit/mg}}$

= 3.84 mg

6. %protein loading

So, %protein loading

= $\dfrac{\text{amount of protein introduced - amount of res. lipase}}{\text{amount of protein introduced}}$ x 100

= $\dfrac{12 \text{ mg} - 3.84 \text{ mg}}{12 \text{ mg}}$ x 100

= 68.03%

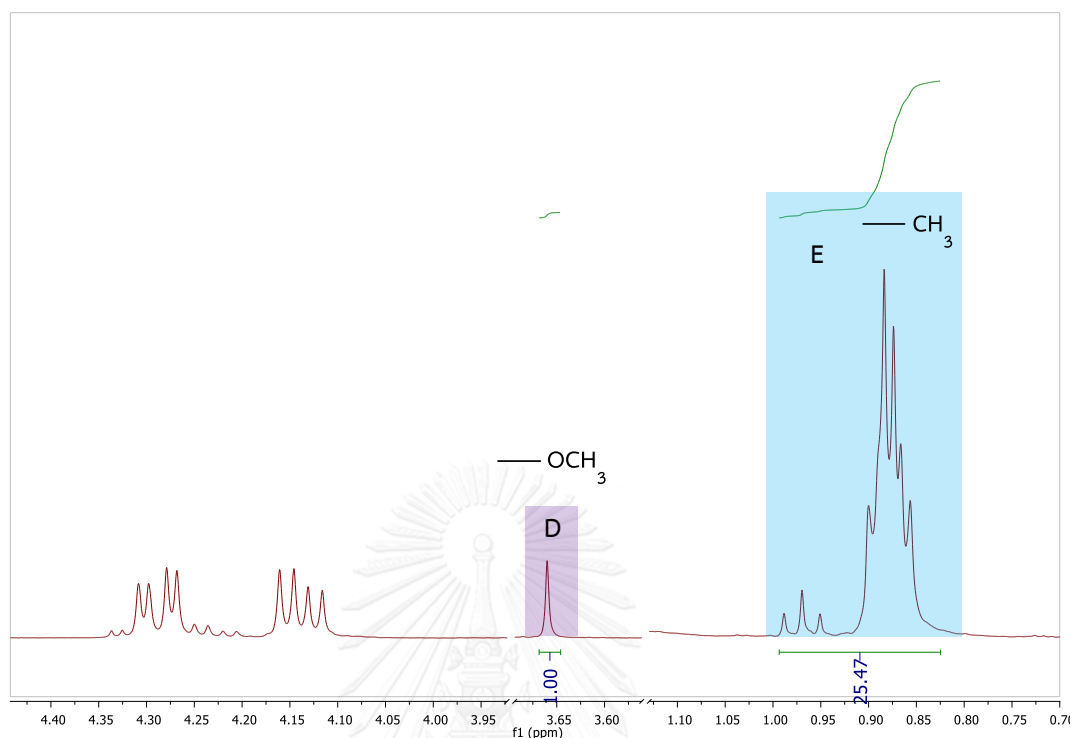# CALCULATIONS AND NMR DATA

B-1 Calculation of %FAEE
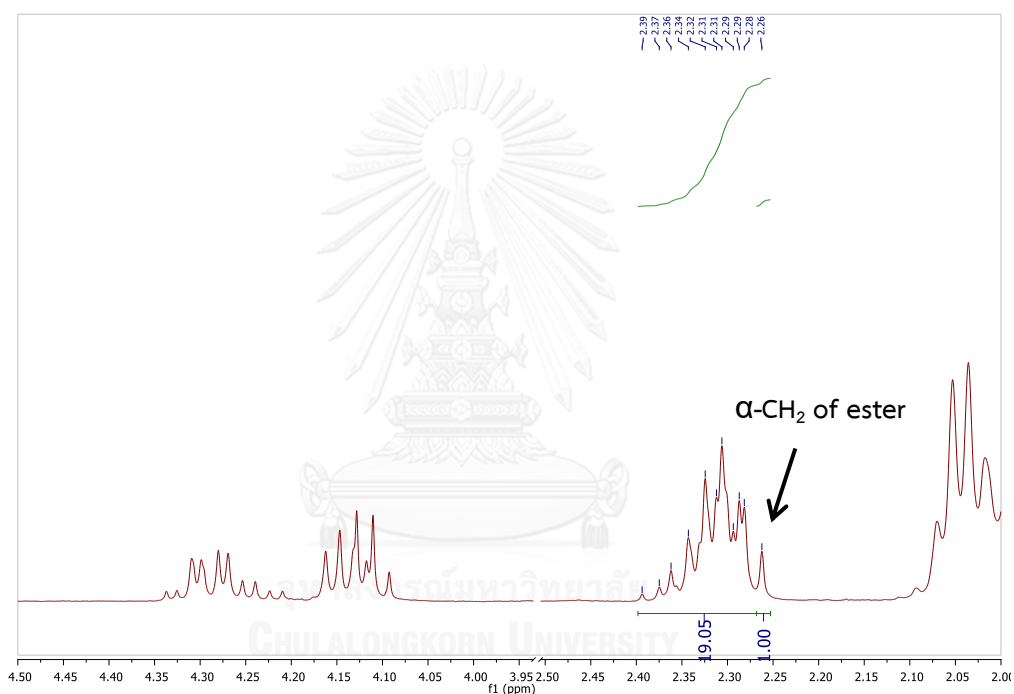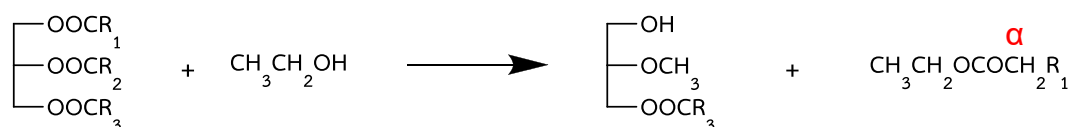
Figure B-1 ^{1}H NMR of biodiesel from soybean oil with ethanol using 1:2 ratio of oil:ethanol, imLS5G15W 11.90 units, 40℃ of reaction temperature, and 149h of reaction time.

%FAEE of the reaction was calculated as follows: [44]

$$\% \text{ of FAEE} = \frac{4 \times \text{area of unmerged peak of } \alpha\text{-CH}_2 \text{ of ester}}{\text{Total area of } \alpha\text{-CH}_2 \text{ of both FFA and ester}}$$

(a)

(b)
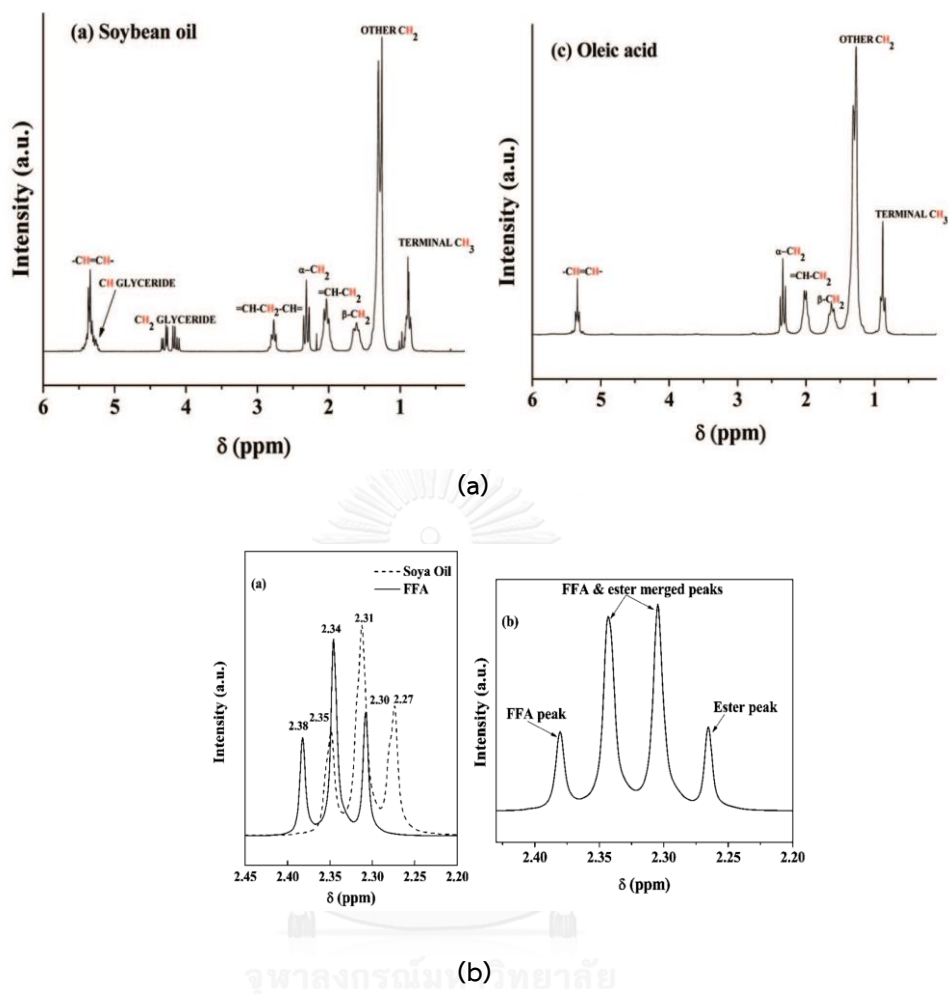
Figure B-2 ^{1}H NMR spectrum in R-CH$_2$ region: (a) soybean oil and oleic acid (FFA) and (b) mixture of oleic acid and its methyl ester [44].

Table B-1 Assignment of ^{1}H-NMR of FFA, vegetable oil, and biodiesel

proton(s)	functional group	compound/chemical shift, δ (ppm)		
		oleic acid	soybean oil	biodiesel
CH$_3$–C	terminal methyl group	0.88	0.80–1.01	0.8–1.0
–(CH$_2$)$_n$–	backbone CH$_2$	1.14–1.43	1.20–1.41	1.22–1.42
–CH$_2$CH$_2$COOH	β-methylene proton	1.54–1.69	1.53–1.70	1.55–1.69
=CH–CH$_2$–	α-methylene group to one double bond	1.92–2.11	1.94–2.11	1.93–2.10
–CH$_2$COOH	α-methylene group to acid	2.34	–	–
–CH$_2$COOR	α-methylene group to ester	–	2.31	2.31
=CH–CH$_2$–CH=	α-methylene group to two double bonds	–	2.76	2.77
–COOCH$_3$	methyl group of ester	–	–	3.67
–CH$_2$OCOR	methylene group (C1 and C3) of glyceride	–	4.09–4.34	–
–CHOCOR	methine proton at C2 of glyceride	–	5.25	–
–CH=CH–	olefinic protons	5.3–5.4	5.28–5.43	5.27–5.41

B-2 Calculation of %FAME

Figure B-3 ^{1}H NMR of biodiesel from soybean oil with methanol using 1:2 ratio of oil:ethanol, imLS5G15S 11.90 units, 40°C of reaction temperature, and 4h of reaction time.

%FAME of the reaction was calculated as follows:

$$\%FAME \quad = \quad \frac{D}{E} \quad \times \quad 100$$

Where, D was integration value of methylene protons of methyl ester resonated at 3.65 ppm, E was integration value of CH$_3$ protons in fatty acyl moiety resonated at around 0.8 – 1.0 ppm
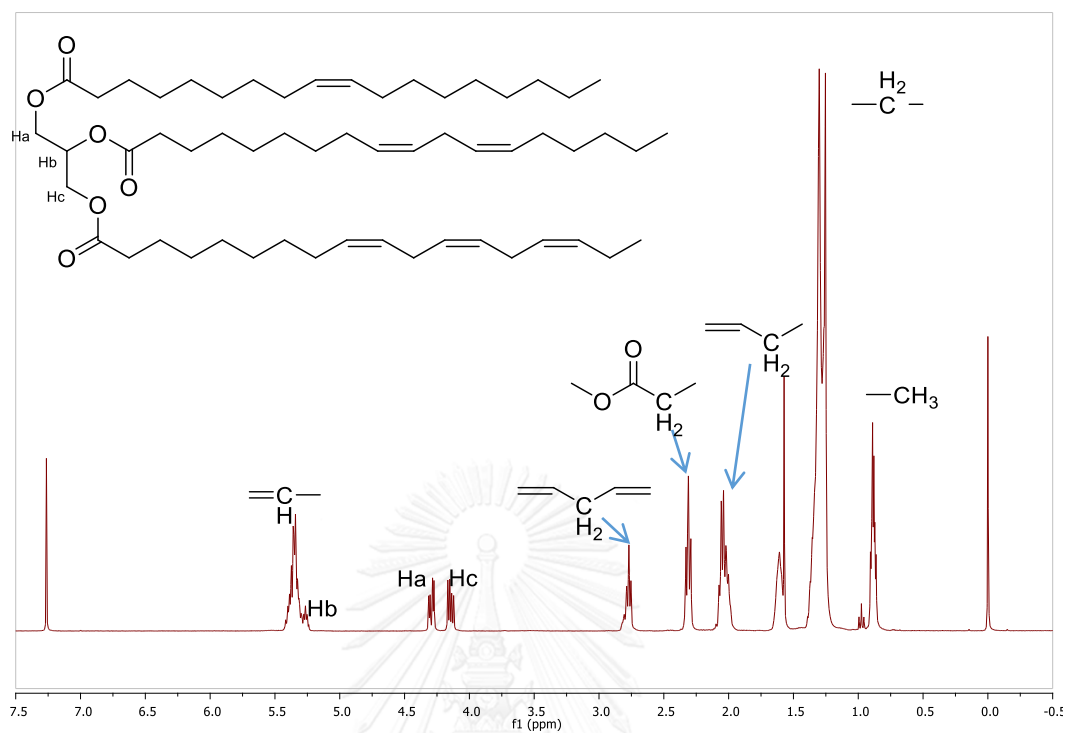
Figure B-4 ^{1}H NMR of soybean oil
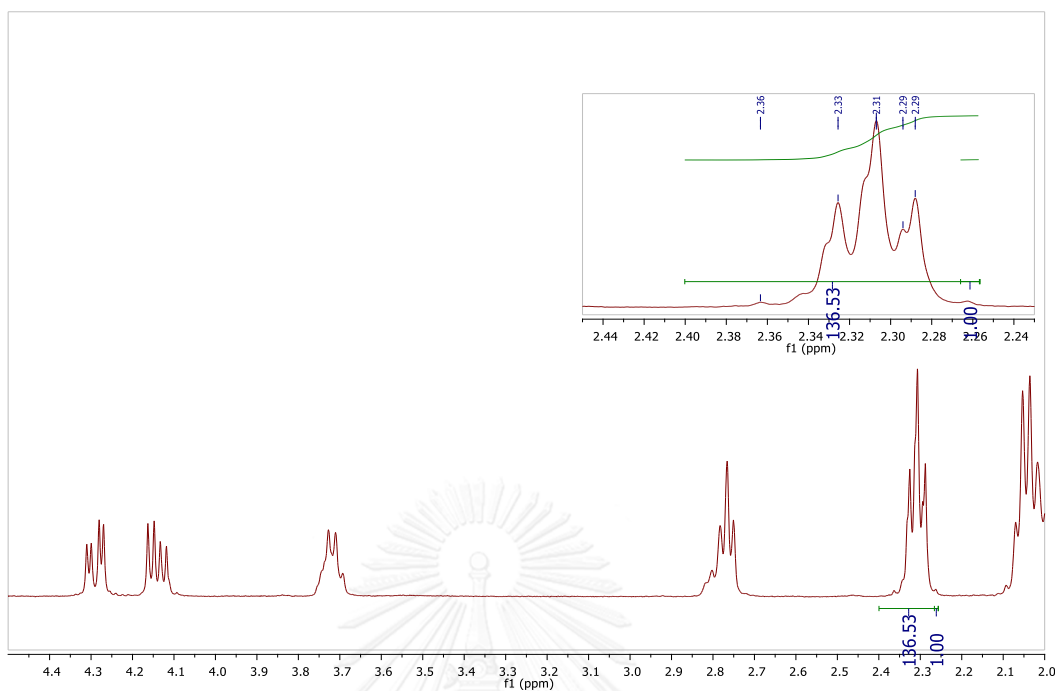
Figure B-5 ^{1}H NMR of biodiesel from soybean oil with ethanol using 1:2 ratio of oil:ethanol, 40°C of reaction temperature, and 6h of reaction time.
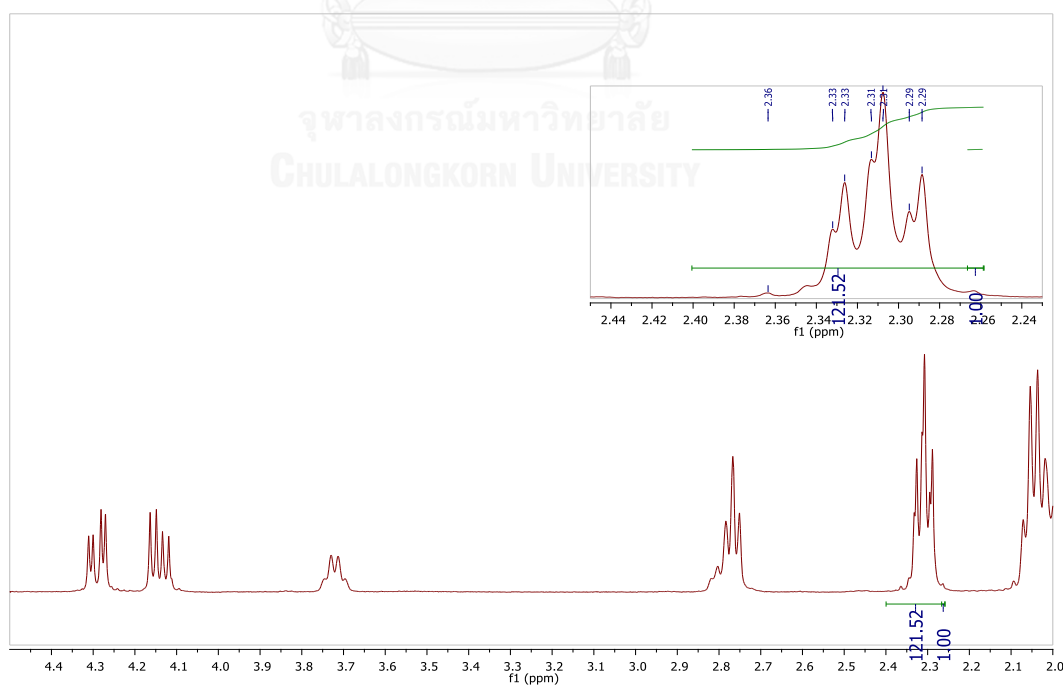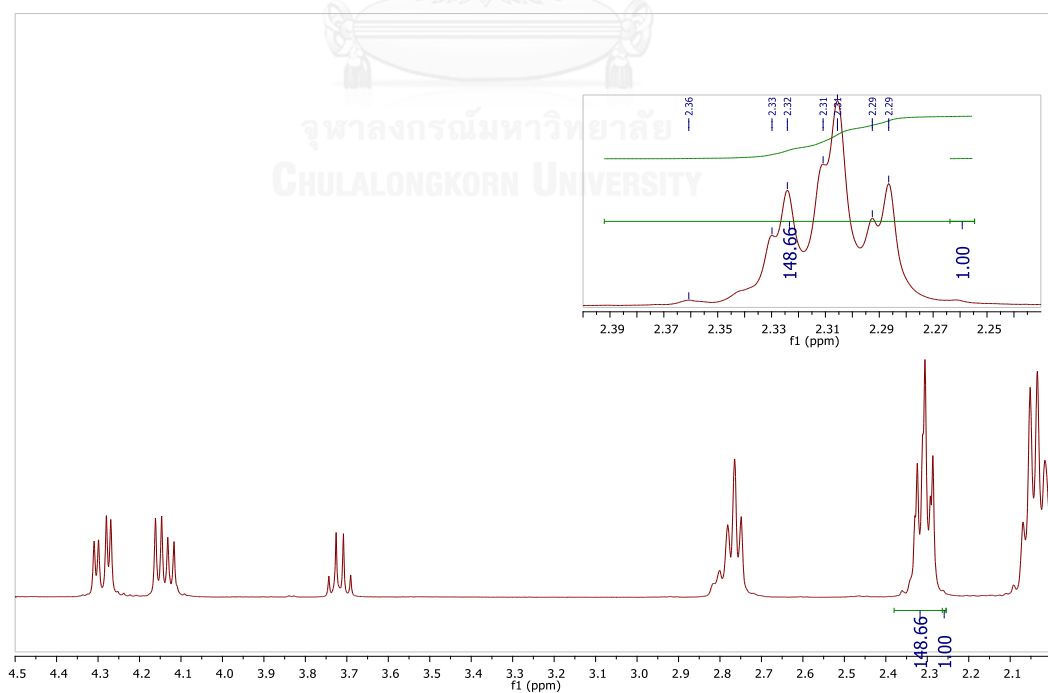

Figure B-6 ^{1}H NMR of biodiesel from soybean oil with ethanol using 1:2 ratio of oil:ethanol, 40°C of reaction temperature, and 8h of reaction time.
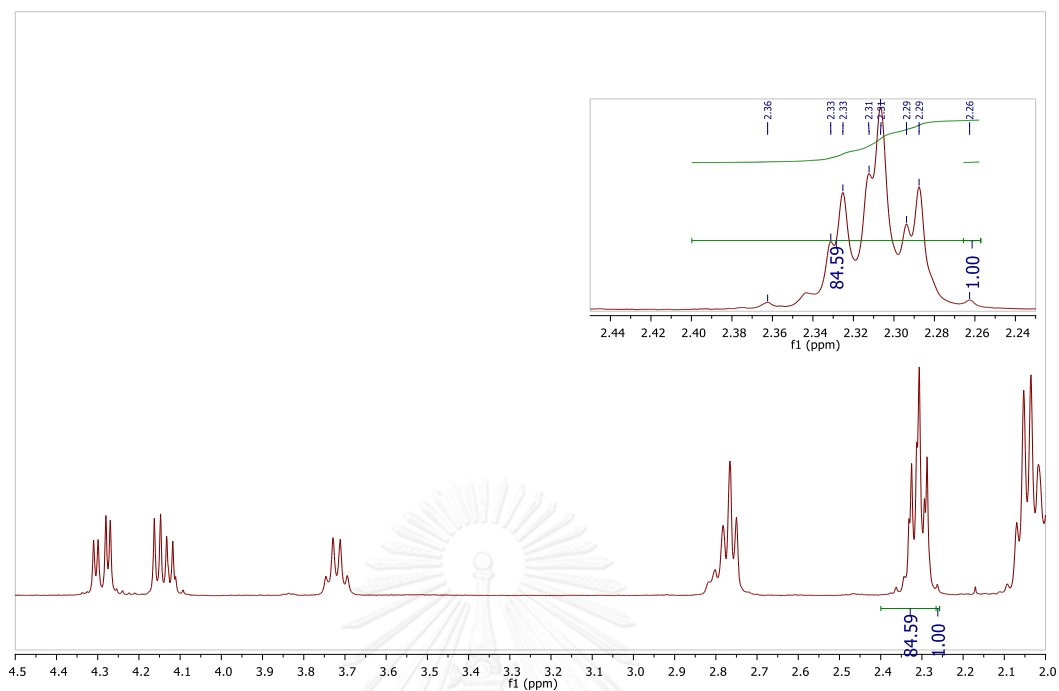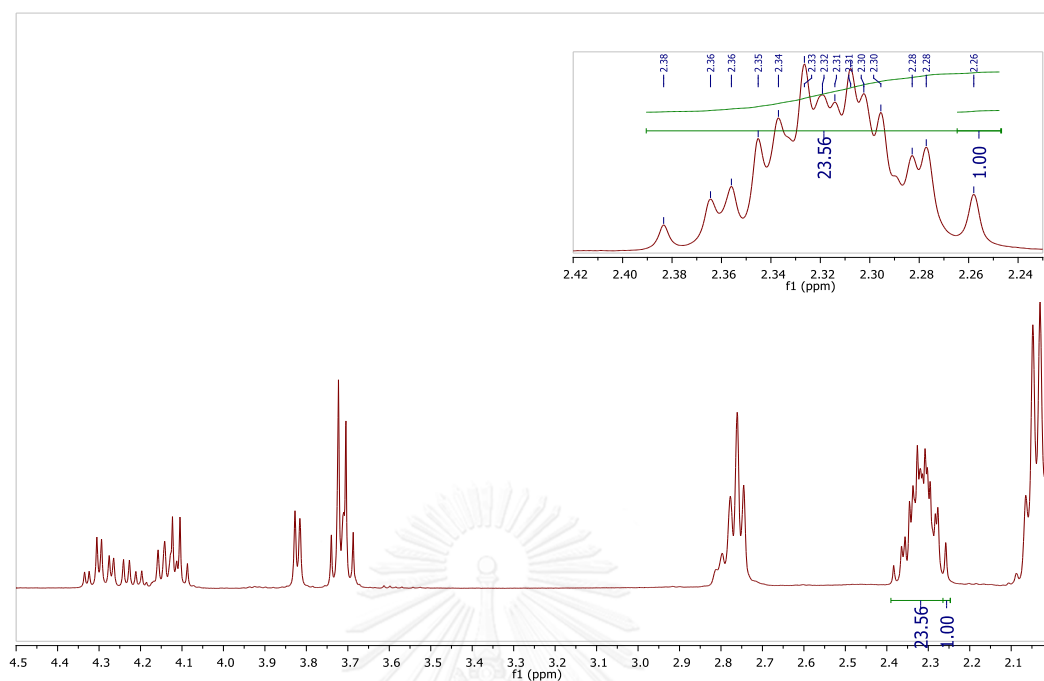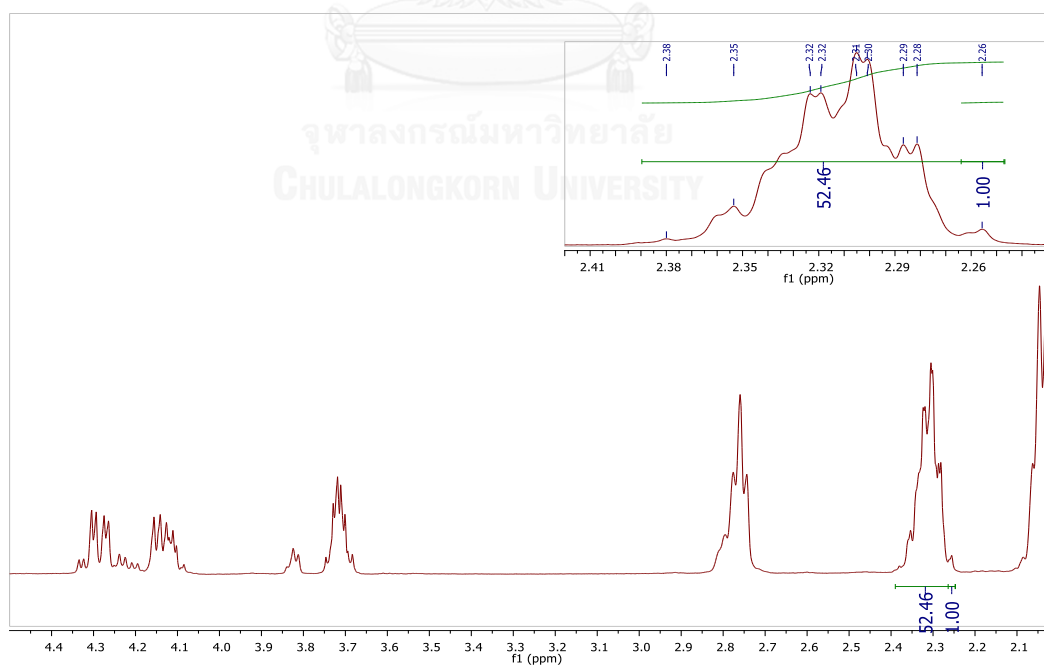
Figure B-7 ^{1}H NMR of biodiesel from soybean oil with ethanol using 1:2 ratio of oil:ethanol, 40℃ of reaction temperature, and 10h of reaction time.
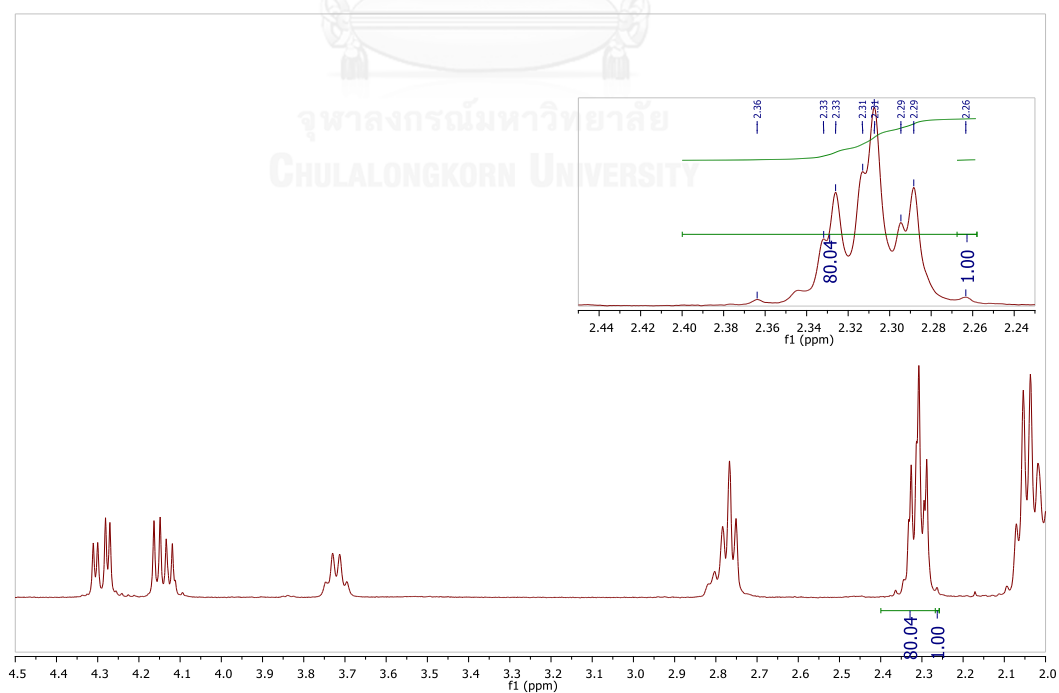
Figure B-8 ^{1}H NMR of biodiesel from soybean oil with ethanol using 1:2 ratio of oil:ethanol, 40℃ of reaction temperature, and 12h of reaction time.
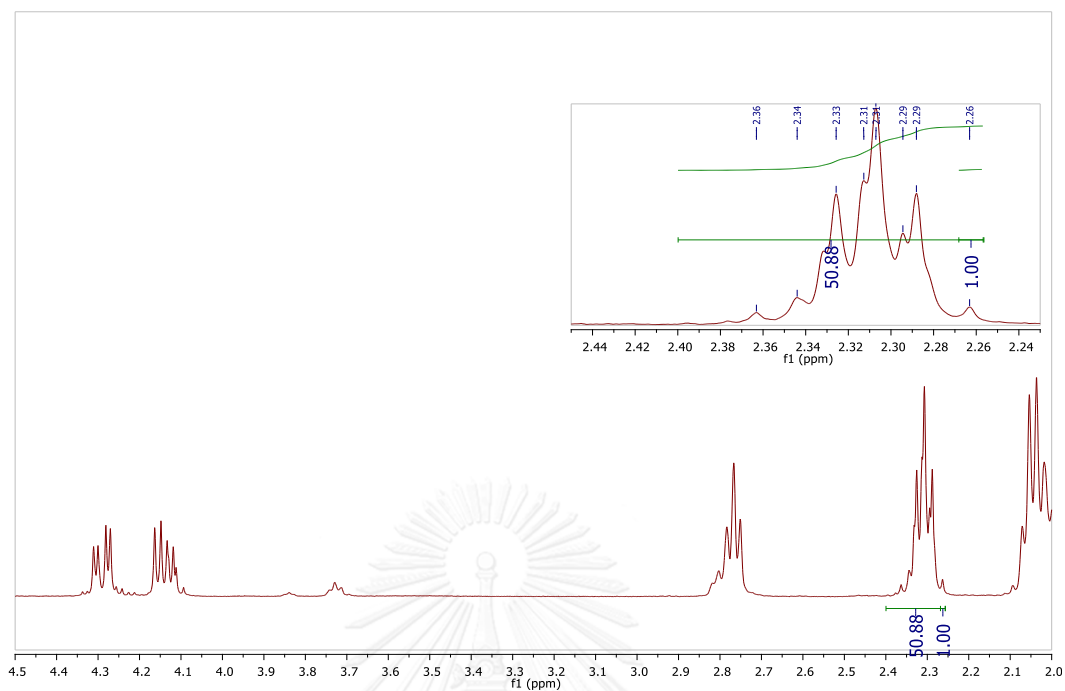
Figure B-9 ^{1}H NMR of biodiesel from soybean oil with ethanol using 1:2 ratio of oil:ethanol, 40℃ of reaction temperature, and 24h of reaction time.
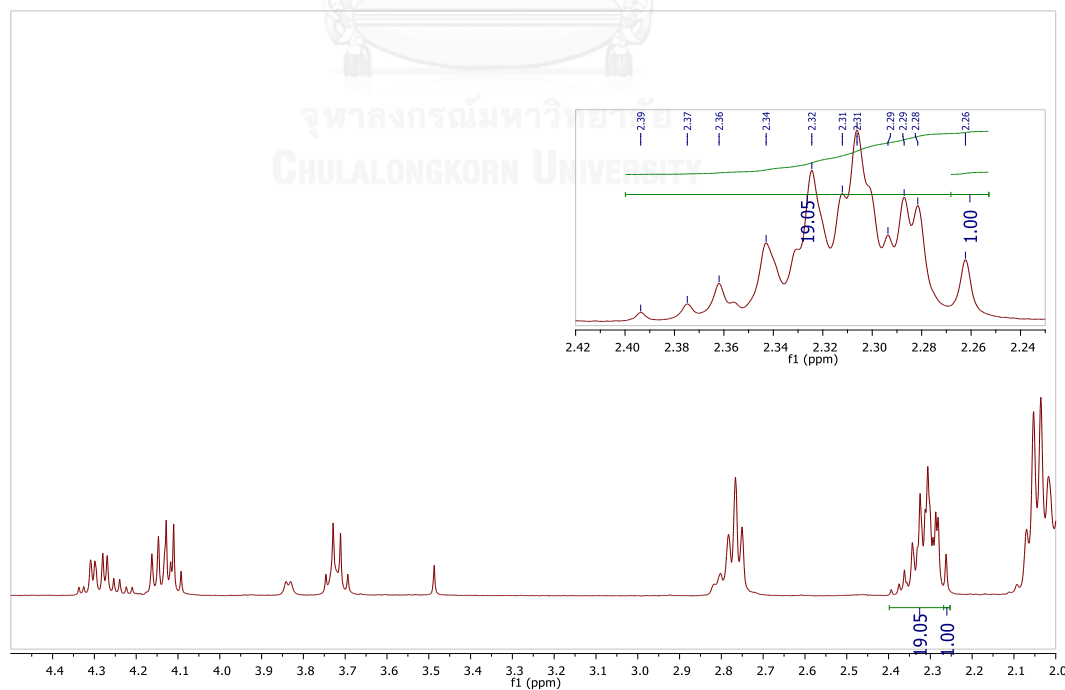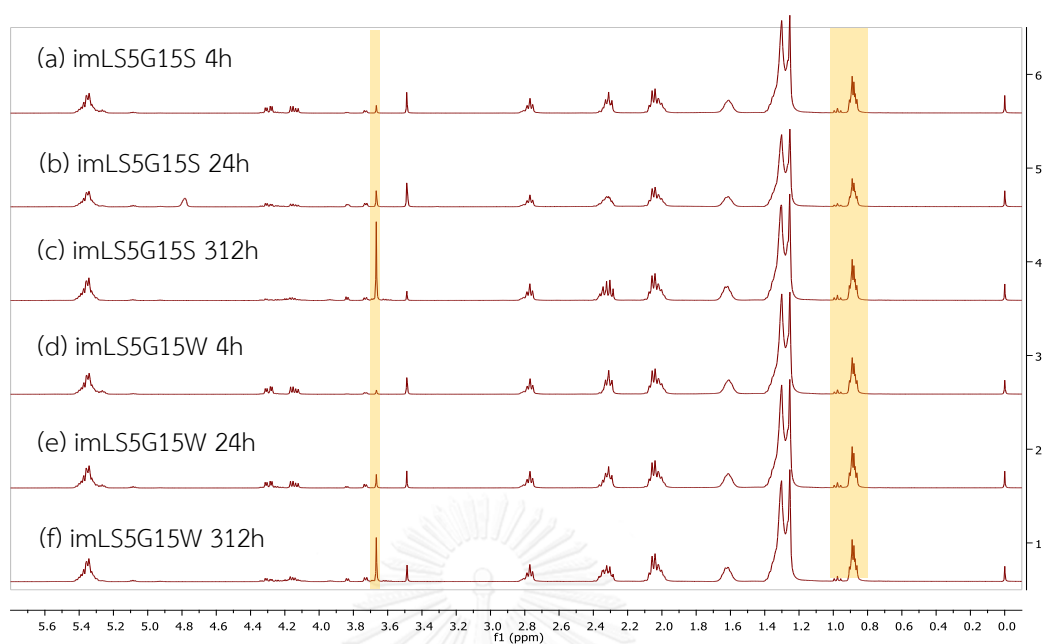

Figure B-10 ^{1}H NMR of biodiesel from soybean oil with ethanol using 1:2 ratio of oil:ethanol, 40℃ of reaction temperature, and 149h of reaction time.
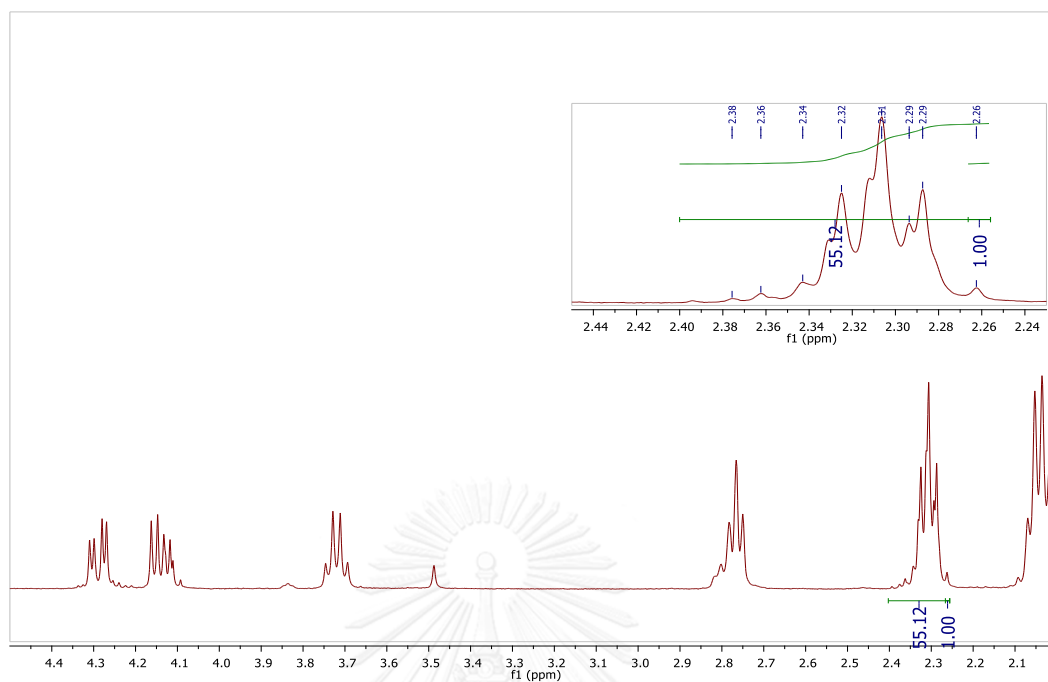
Figure B-11 ^{1}H NMR of biodiesel from soybean oil with ethanol using 1:3 ratio of oil:ethanol, 40°C of reaction temperature, and 149h of reaction time.

Figure B-12 ^{1}H NMR of biodiesel from soybean oil with ethanol using 1:2 ratio of oil:ethanol, water 2 eq., 40°C of reaction temperature and 24h of reaction time.
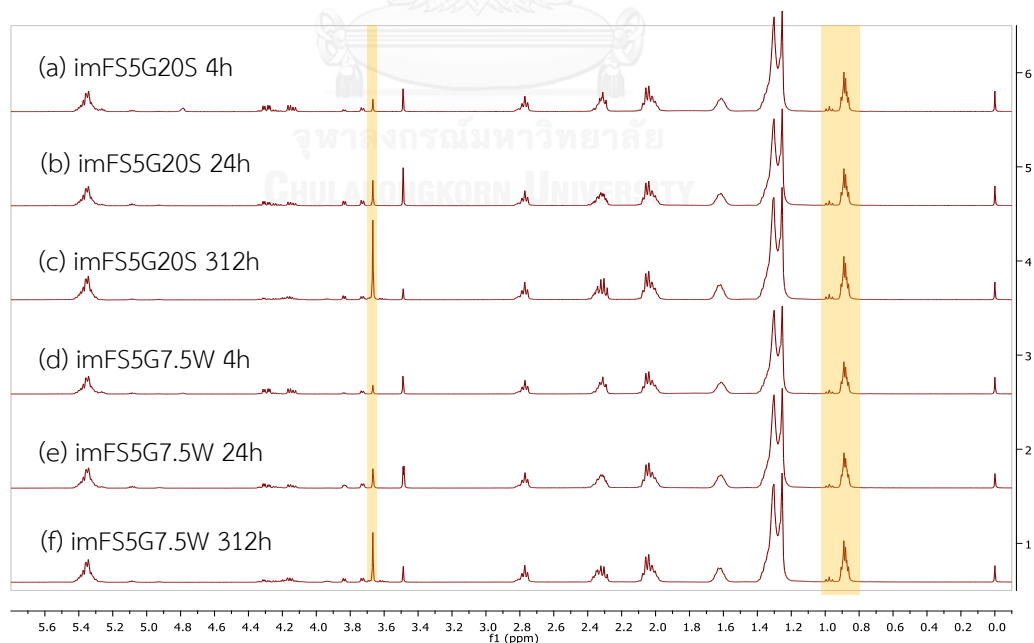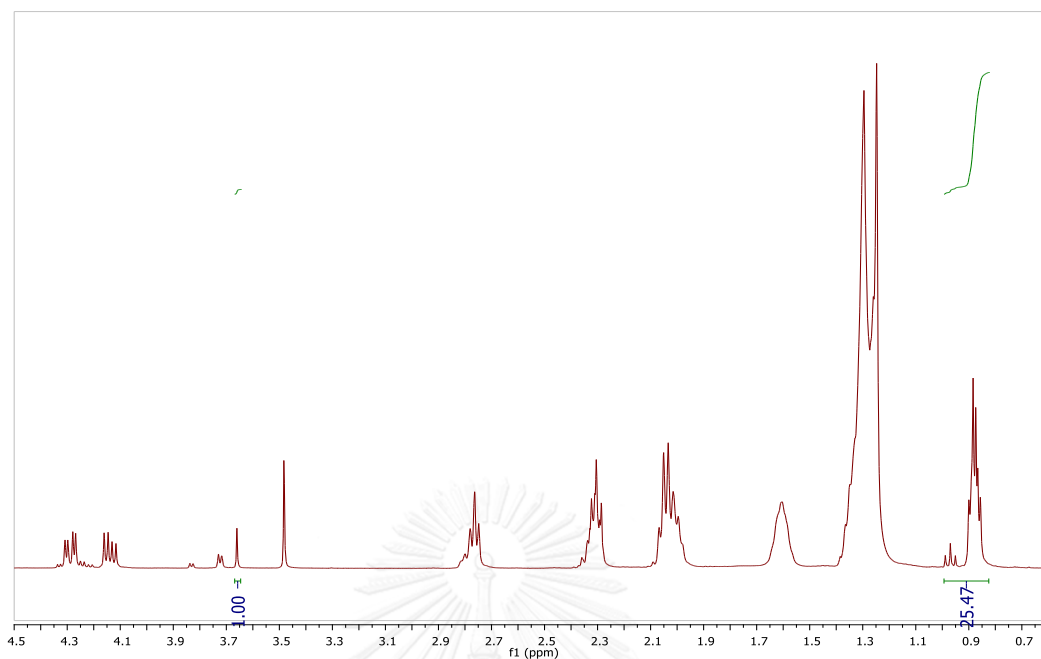
Figure B-13 ^{1}H NMR of biodiesel from soybean oil with ethanol using 1:2 ratio of oil:ethanol, water 10 eq., 40°C of reaction temperature and 24h of reaction time.

Figure B-14 ^{1}H NMR of biodiesel from soybean oil with ethanol using 1:2 ratio of oil:ethanol, water 20 eq., 40°C of reaction temperature and 24h of reaction time.
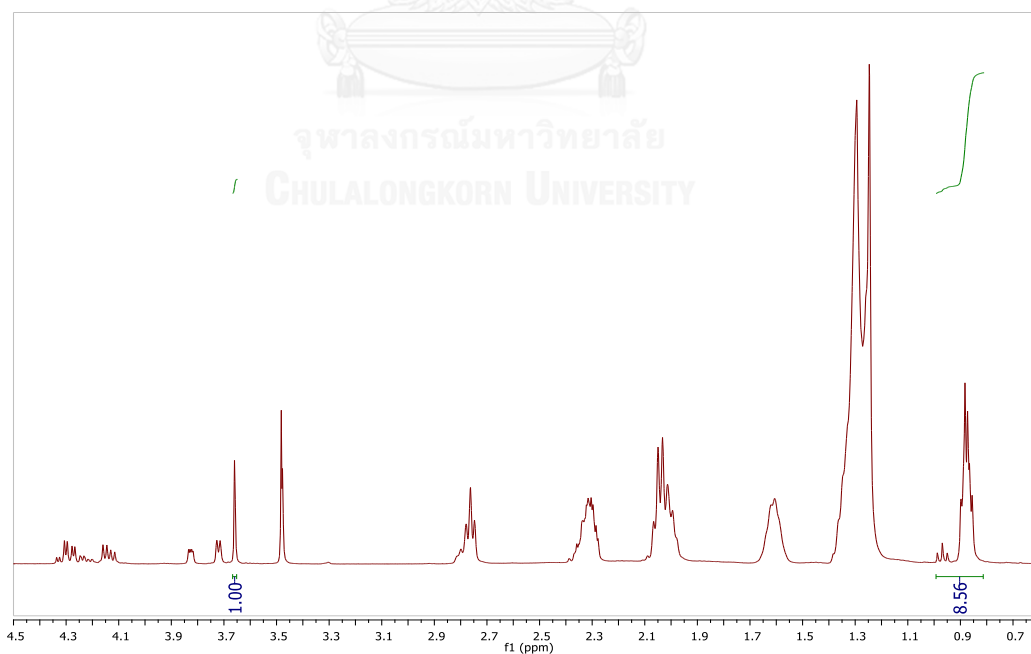
Figure B-15 ^{1}H NMR of biodiesel from soybean oil with methanol using 1:2 ratio of oil:methanol, water 10 equivalents, imLS5G15S at 40°C for reaction time, (a) 4h, (b) 24h, (c) 312h and imLS5G15W at 40°C for reaction time, (d) 4h, (e) 24h, (d) 312h.

Figure B-16 ^{1}H NMR of biodiesel from soybean oil with methanol using 1:2 ratio of oil:methanol, water 10 equivalents, imFS5G15S at 40°C for reaction time, (a) 4h, (b) 24h, (c) 312h and imFS5G15W at 40°C for reaction time, (d) 4h, (e) 24h, (d) 312h.

Figure B-17 ^{1}H NMR of biodiesel from soybean oil with methanol using 1:2 ratio of oil:methanol, water 10 equivalents, imLS5G15S at 40°C of reaction temperature and 4h of reaction time.
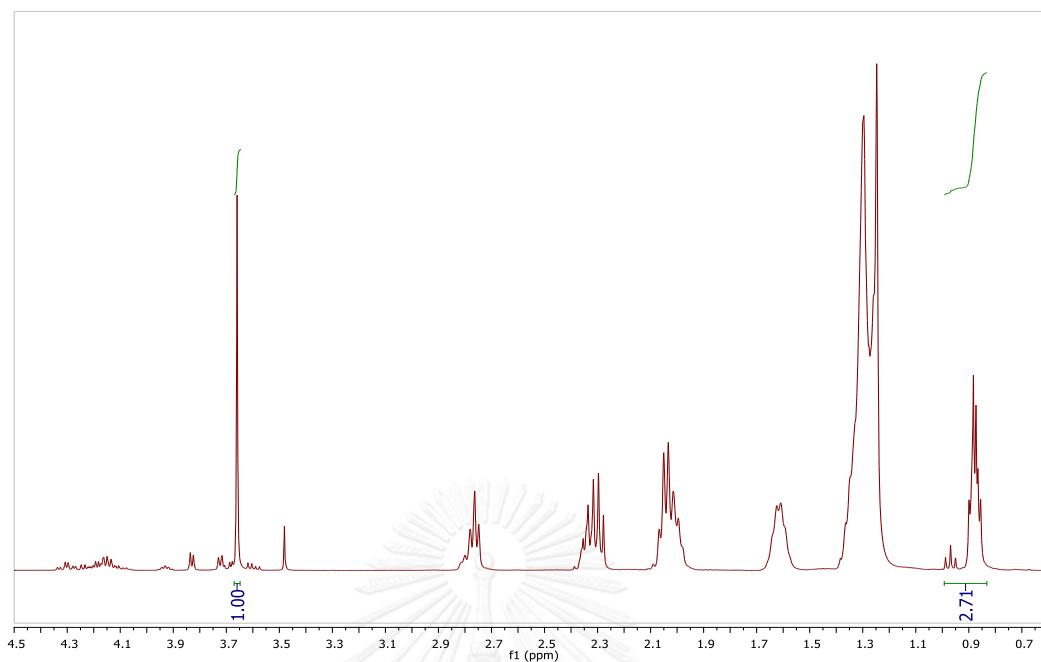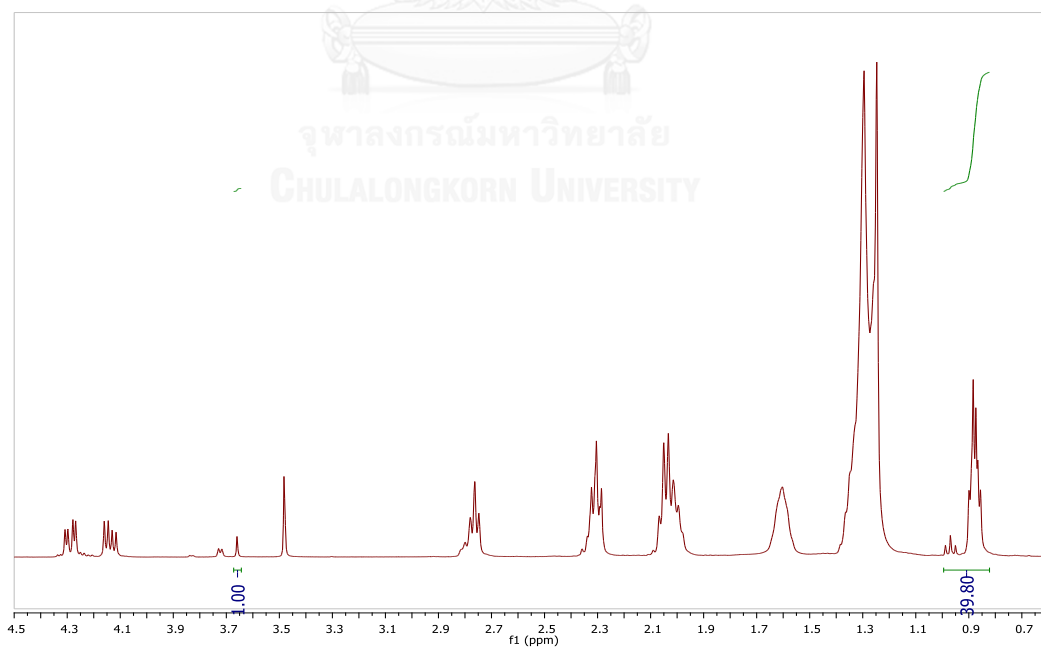

Figure B-18 ^{1}H NMR of biodiesel from soybean oil with methanol using 1:2 ratio of oil:methanol, water 10 equivalents, imLS5G15S at 40°C of reaction temperature and 24h of reaction time.
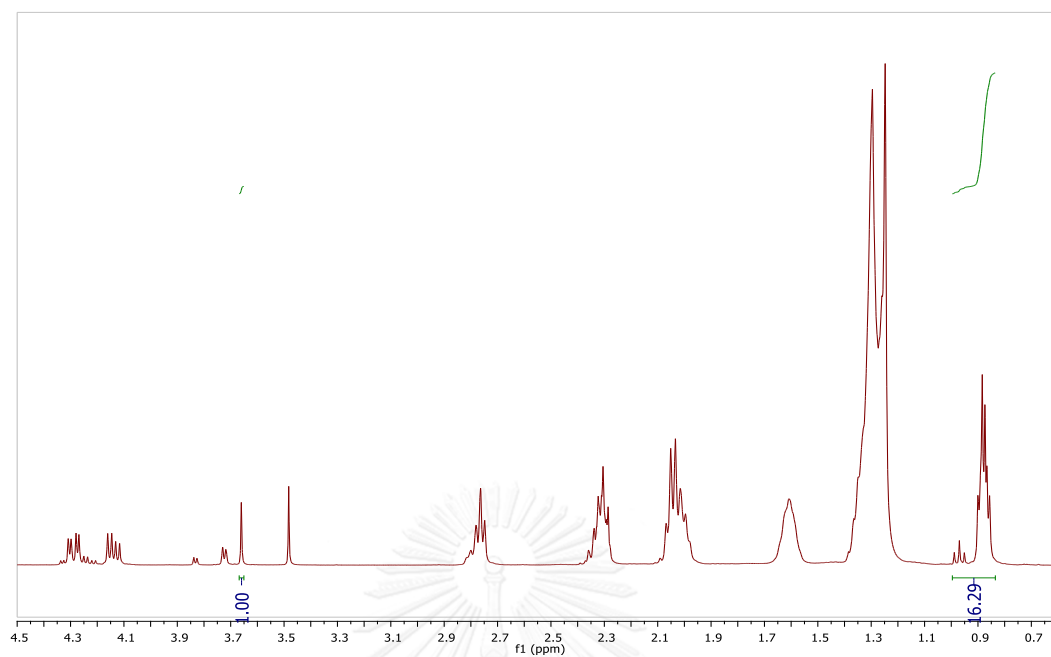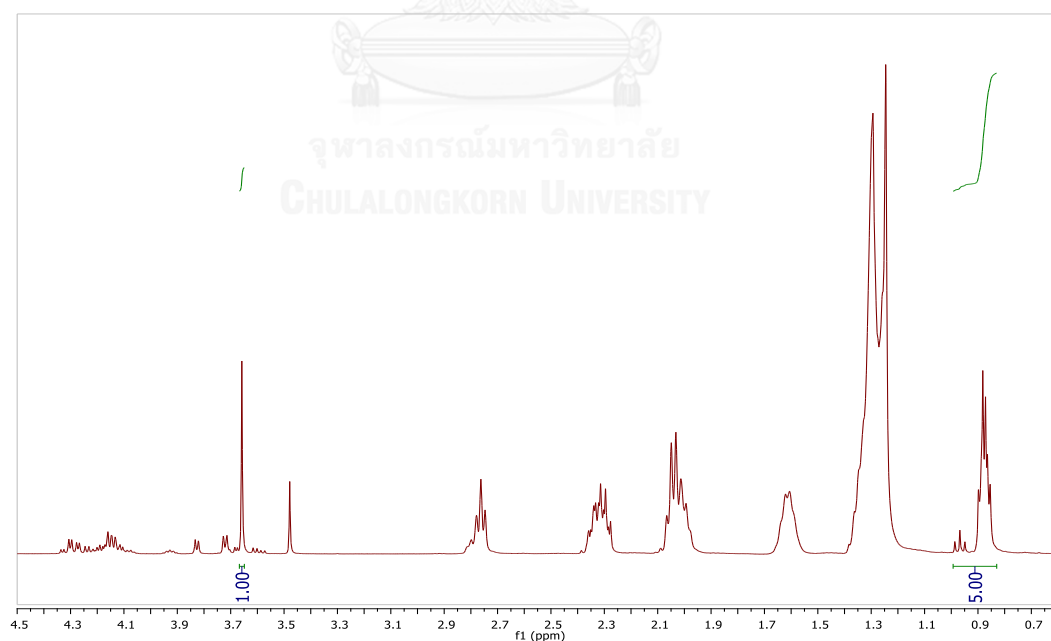
Figure B-19 ^{1}H NMR of biodiesel from soybean oil with methanol using 1:2 ratio of oil:methanol, water 10 equivalents, imLS5G15S at 40°C of reaction temperature and 312h of reaction time.

Figure B-20 ^{1}H NMR of biodiesel from soybean oil with methanol using 1:2 ratio of oil:methanol, water 10 equivalents, imLS5G15W at 40°C of reaction temperature and 4h of reaction time.

Figure B-21 ^{1}H NMR of biodiesel from soybean oil with methanol using 1:2 ratio of oil:methanol, water 10 equivalents, imLS5G15W at 40°C of reaction temperature and 24h of reaction time.
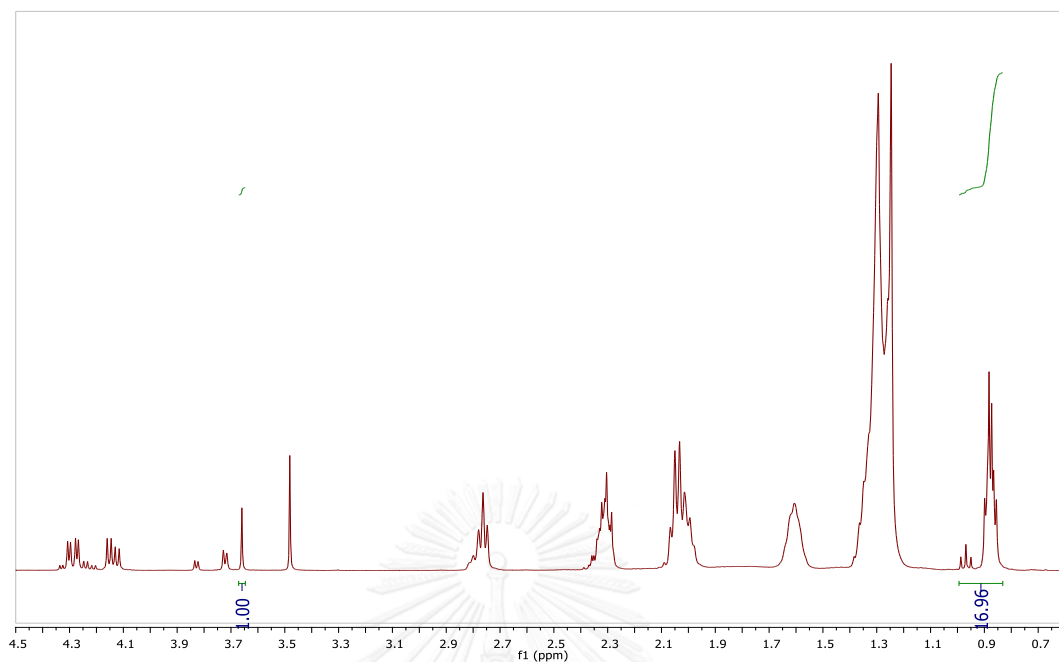

Figure B-22 ^{1}H NMR of biodiesel from soybean oil with methanol using 1:2 ratio of oil:methanol, water 10 equivalents, imLS5G15W at 40°C of reaction temperature and 312h of reaction time.

Figure B-23 [superscript 1]H NMR of biodiesel from soybean oil with methanol using 1:2 ratio of oil:methanol, water 10 equivalents, imFS5G15S at 40°C of reaction temperature and 4h of reaction time.
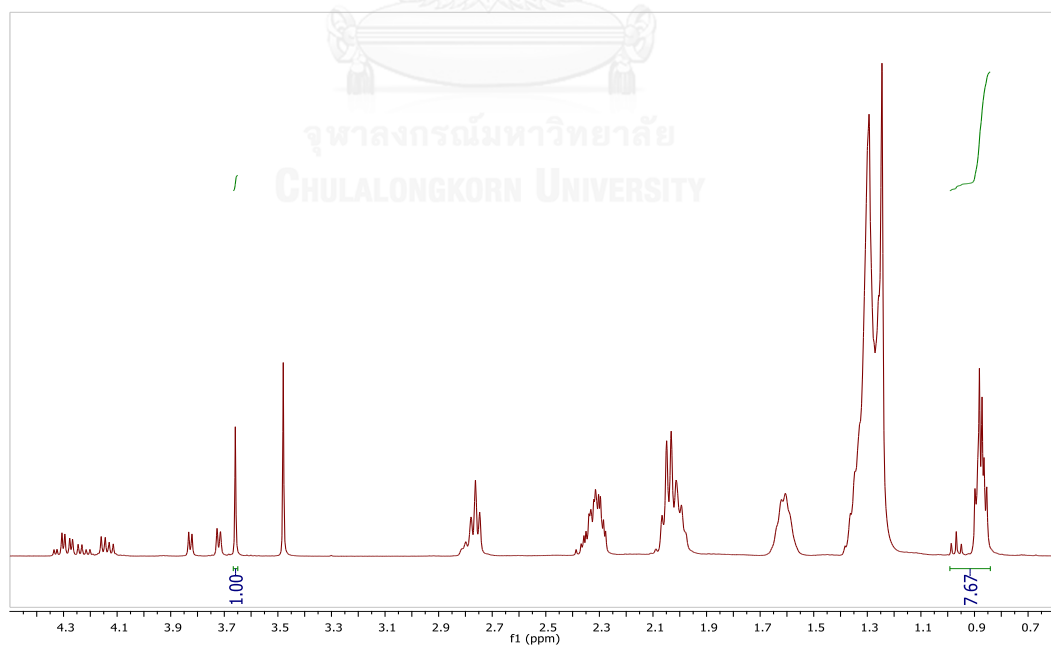

Figure B-24 [superscript 1]H NMR of biodiesel from soybean oil with methanol using 1:2 ratio of oil:methanol, water 10 equivalents, imFS5G15S at 40°C of reaction temperature and 24h of reaction time.
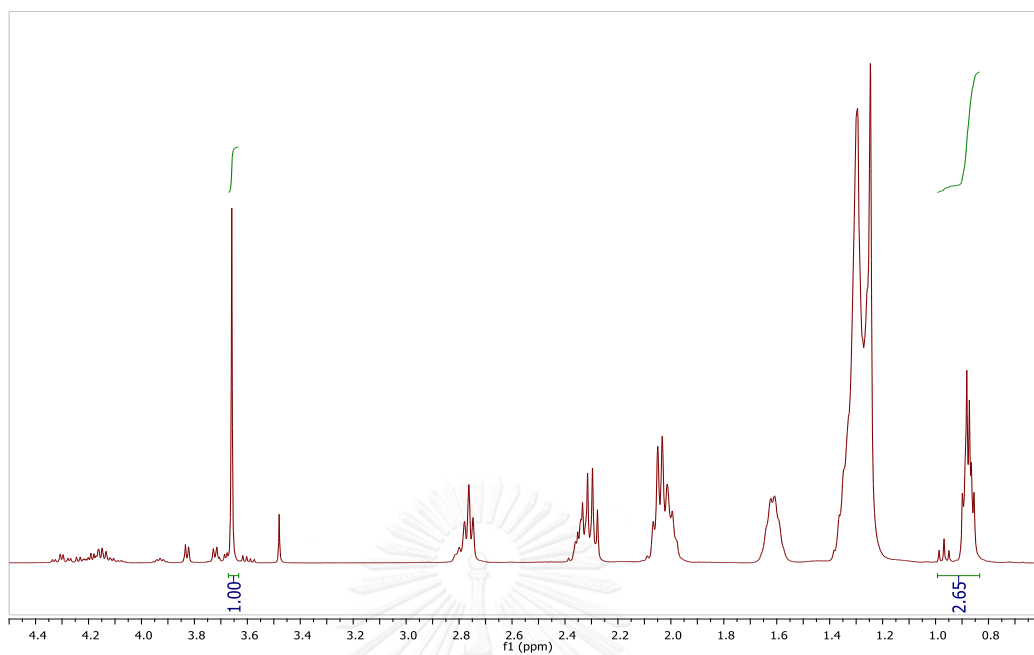
Figure B-25 ^{1}H NMR of biodiesel from soybean oil with methanol using 1:2 ratio of oil:methanol, water 10 equivalents, imFS5G15S at 40℃ of reaction temperature and 312h of reaction time.

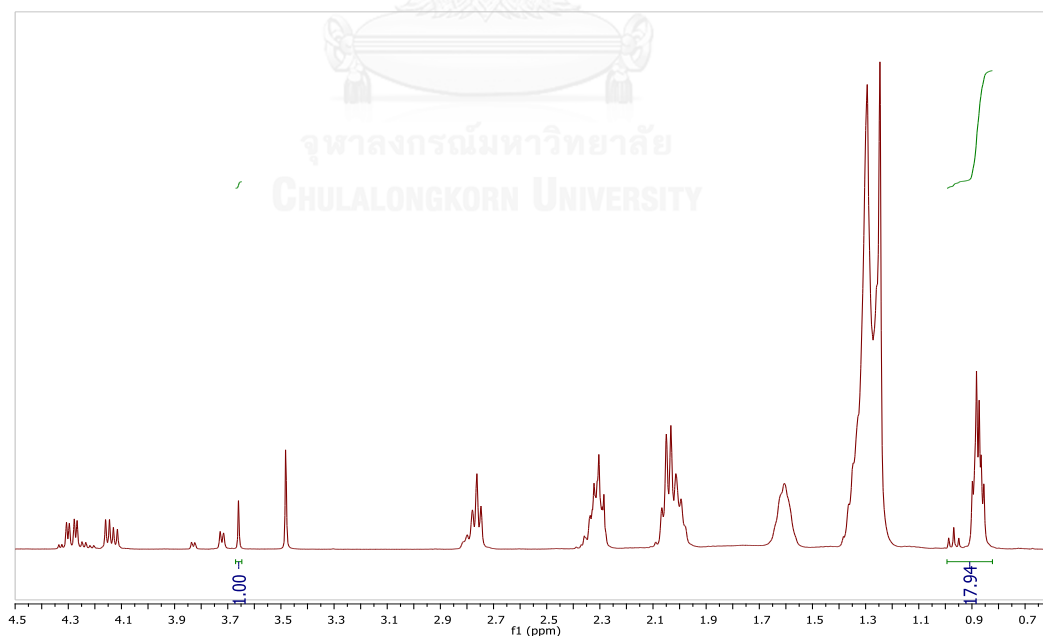

Figure B-26 ^{1}H NMR of biodiesel from soybean oil with methanol using 1:2 ratio of oil:methanol, water 10 equivalents, imFS5G15W at 40℃ of reaction temperature and 4h of reaction time.

Figure B-27 ^{1}H NMR of biodiesel from soybean oil with methanol using 1:2 ratio of oil:methanol, water 10 equivalents, imFS5G15W at 40°C of reaction temperature and 24h of reaction time.
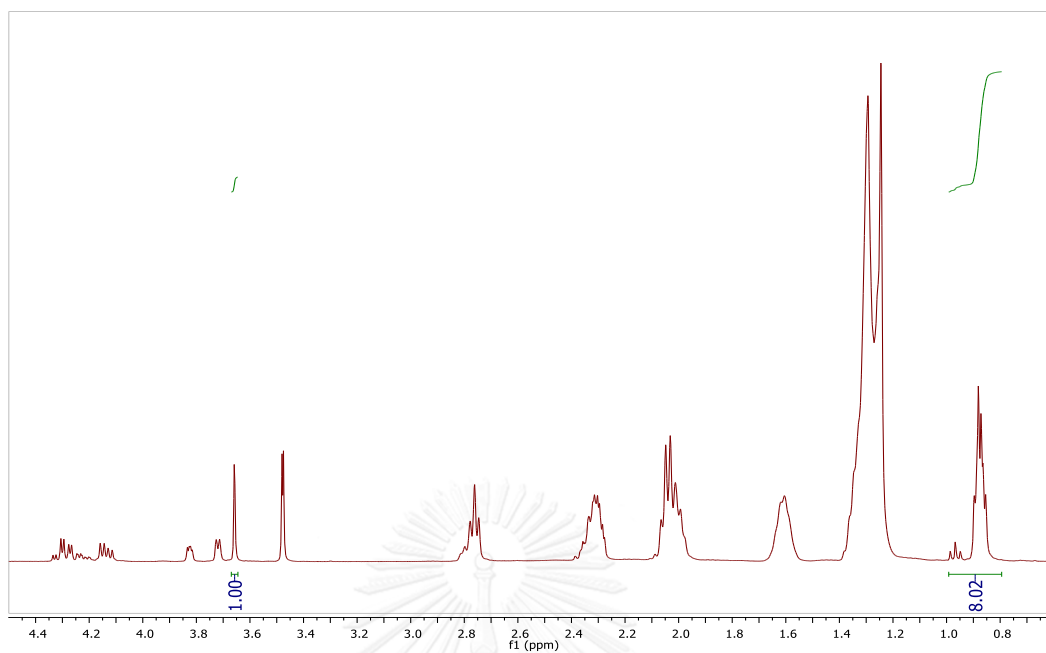

Figure B-28 ^{1}H NMR of biodiesel from soybean oil with methanol using 1:2 ratio of oil:methanol, water 10 equivalents, imFS5G15W at 40°C of reaction temperature and 312h of reaction time.
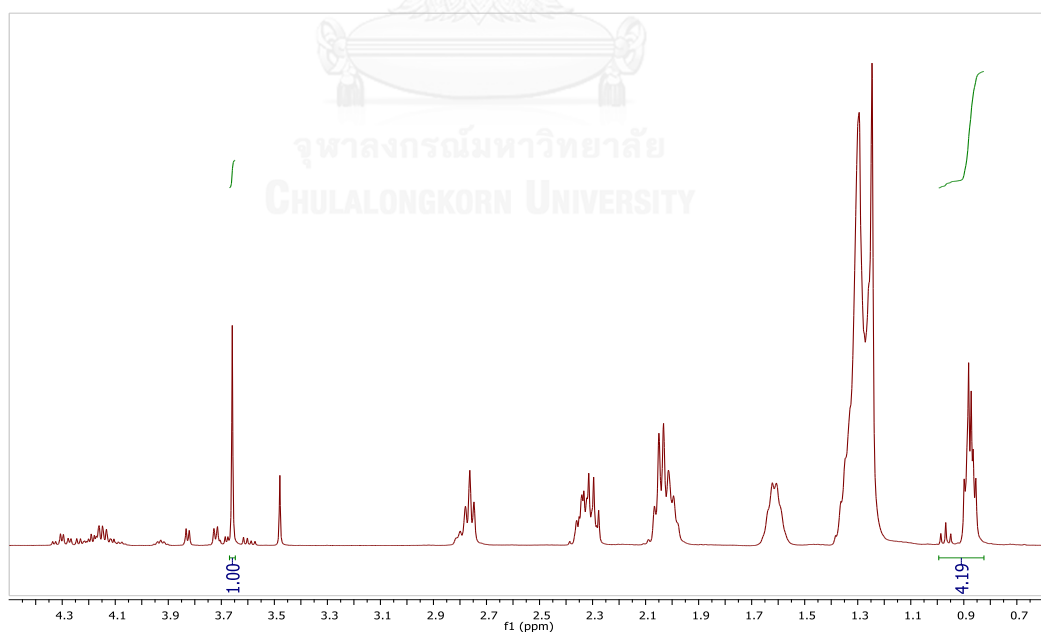
VITA

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Additionally, she presented the poster for International Polymer Conference of Thailand (PCT-5) in the topic of " Effect of PGA:STY Ratio and Reaction Temperature on Coating PGA-STY on EPS Beads" at Pathumwan Princess Hotel, Bangkok, Thailand on June, 2015