การผลิตเอทิลเอสเทอร์โดยใช้เอนไซม์ไลเปสที่ถูกตรึงบนตัวพยุงอัลจิเนตเสริมใยบวบ

นางสาวพุทธชาด จันทร์เมือง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิศวกรรมศาสตรมหาบัณฑิต

สาขาวิชาวิศวกรรมเคมี ภาควิชาวิศวกรรมเคมี

คณะวิศวกรรมศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

ปีการศึกษา 2553

ETHYL ESTER PRODUCTION USING IMMOBILIZED LIPASE ENTRAPPED IN LOOFA REINFORCED ALGINATE CARRIERS

Miss Puttachad Chanmuang

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Engineering Program in Chemical Engineering

Department of Chemical Engineering

Faculty of Engineering

Chulalongkorn University

Academic Year 2010

Thesis Title	ETHYL ESTER PRODUCTION USING IMMOBILIZED LIPASE ENTRAPPED IN LOOFA REINFORCED ALGINATE CARRIERS
By	Miss Puttachad Chanmuang
Field of Study	Chemical Engineering
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พุทธชาด จันทร์มือง : การผลิตเอทิลเอสเทอร์โดยใช้เอนไซม์ไลเปสที่ถูกตรึงบนตัวพยุงอัลจิเนตเสริมใยบวบ. (ETHYL ESTER PRODUCTION USING IMMOBILIZED LIPASE ENTRAPPED IN LOOFA REINFORCED ALGINATE CARRIERS) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: รศ.ดร. เหมือนเดือน พิศาลพงศ์, 67 หน้า.

งานวิจัยนี้เป็นการศึกษาการผลิตไบโอดีเซลจากน้ำมันปาล์มบริสุทธิ์และกรดไขมันจากปาล์มโดยใช้ตัวเร่งปฏิกิริยาทาง(ชีวภาพ)เอนไซม์ไลเปสอิสระและตรึงรูป ทำการศึกษาการตรึงไลเปส 2 วิธี ได้แก่ 1) ไลเปสตรึงรูปบนใยบวบ (CRLA) 2) ไลเปสตรึงรูปบนใยบวบและห่อหุ้มด้วยโครงข่ายแคลเซียมอัลจิเนต (CRLAE) ซึ่งเตรียมได้โดยการใช้ไลเปส 10 เปอร์เซ็นต์โดยน้ำหนักและ โซเดียมอัลจิเนต 2 เปอร์เซ็นต์โดยน้ำหนักต่อปริมาตร สภาวะที่เหมาะสมในการทำปฏิกิริยาคือที่ 45 °C โดยใช้ความเร็วรอบ 250 รอบต่อนาที และทำการห่อหุ้มด้วยโครงข่ายแคลเซียมอัลจิเนต 3 ชั้น สำหรับการผลิตไบโอดีเซล พบว่าสภาวะที่เหมาะสมสำหรับการใช้เอทานอลเป็นสารตั้งต้นคือที่ความเข้มข้นของเอทานอล 99.9%, สารละลายบัฟเฟอร์ฟอสเฟต 0.001 โมลต่อลิตรและใช้เวลาในการทำปฏิกิริยา 36 ชั่วโมง โดยผลได้เอทิลเอสเทอร์จากการใช้ไลเปสอิสระและ CRLAE มีค่าเท่ากับ 78.87 และ 96.18 เปอร์เซ็นต์ตามลำดับ ในขณะที่สภาวะที่เหมาะสมสำหรับการใช้เมทานอลเป็นสารตั้งต้นคือที่ความเข้มข้นของเมทานอล 99.9%, สารละลายบัฟเฟอร์ฟอสเฟต 0.001 โมลต่อลิตรและใช้เวลาในการทำปฏิกิริยา 12 ชั่วโมง โดยผลได้เมทิลเอสเทอร์จากเอนไซม์ไลเปสอิสระและจากตรึงไลเปสตรึงรูปใน CRLAE ที่ 96.97 และ 97.92 เปอร์เซ็นต์ตามลำดับ ส่วนผลจากการนำไลเปสตรึงรูปมาใช้ใหม่พบว่าร้อยละผลได้ของเอทิลเอสเทอร์ลดลงอย่างมีนัยสำคัญจากการใช้ครั้งที่ 1 ถึงครั้งที่ 2 และครั้งที่ 1 ถึงครั้งที่ 5 สำหรับเมทิลเอสเทอร์ ในขณะที่เมื่อใช้น้ำมันปาล์มบริสุทธิ์ผสมกรดไขมันจากปาล์มในอัตราส่วนเริ่มต้น 70:30 โดยน้ำหนัก เป็นสารตั้งต้น พบว่าภายใต้การเติมกรดไขมันปาล์มแบบกึ่งต่อเนื่องสามารถนำเอนไซม์ไลเปสตรึงรูปกลับมาใช้ซ้ำได้ถึงห้าครั้งโดยผลได้เอทิลเอสเทอร์ไม่ลดลง .

ภาควิชา.....วิศวกรรมเคมี..... ลายมือชื่อนิสิต.....พุทธชาด จันทร์เมือง.....

สาขาวิชา.....วิศวกรรมเคมี..... ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ปีการศึกษา.....2553.....

5270427421 : MAJOR CHEMICAL ENGINEERING

KEYWORDS : BIODIESEL / TRANSESTERIFICATION / IMMOBILIZED LIPASE

PUTTACHAD CHANMUANG: ETHYL ESTER PRODUCTION USING IMMOBILIZED LIPASE ENTRAPPED IN LOOFA REINFORCED ALGINATE CARRIERS. ADVISOR: ASSOC. PROF. MUENDUEN PHISALAPHONG, Ph.D., 67 pp.

This work investigated the biodiesel production from purified palm oil and palm fatty acids by using free and immobilized lipases as biocatalysts. Two methods of lipase immobilizations were studied: 1) adsorption of lipase onto loofa (CRLA) and 2) entrapment of loofa-lipase in Ca-alginate matrix (CRLAE). CRLAE was prepared by using 10% lipase based on oil weight and 2% w/v Na-alginate. The reaction conditions were at 45°C, 250 rpm and 3 layers of alginate coated on CRLAE. For the biodiesel production, the optimal condition for using ethanol was at 99.9% ethanol, 0.001 M phosphate buffer and 36 h of reaction time, yielded approximately 78.87% and 96.18% ethyl ester by the free lipase and CRLAE, whereas the optimal condition for using methanol were at 99.9% methanol, 0.001 M phosphate buffer and 12 h of reaction time, the biodiesel production by the free lipase and immobilized lipase in CRLAE resulted in methyl ester yields of 96.97% and 97.92%, respectively. The results from the reuse of the immobilized lipases revealed the significant reduction of ethyl ester yield from the 1st run to the 2nd run and from the 1st run to the 5th run for methyl ester yield. On the other hand, when using the mixture of purified palm oil and palm fatty acid in the initial mass ratio of 70:30 as substrate, the result showed that, under semi-continuous feeding of palm fatty acid, the ethyl ester yields obtained by using 95.0% ethanol, 99.9% ethanol and 99.9% methanol were relatively high and the enzymatic activity remained constant during the 5 repeated uses.

Department : Chemical Engineering Student's Signature พุทธชาด จันทร์เมือง

Field of Study : Chemical Engineering Advisor's Signature

Academic Year : 2010

ACKNOWLEDGEMENTS

The work presented in this thesis was meticulously conducted with the help and encouragements from many people who make sure work possible. I would like to take this opportunity to thank the following people for their contributions to this work.

Firstly, I would like to express my earnest gratitude to my advisor, Assoc. Prof. Muenduen Phisalaphong, Ph.D. for her encouragement, support, guidance and unfailing faith all the way through my thesis work and study.

Thanks to all of my thesis committee, Assoc. Prof. Tharathon MongKhonsi, Assoc.Prof. Bunjerd Jongsomjit and Assoc. Prof. Metta Chareonpanich for their kind advices and recommendations which are invaluable for improving my work.

Many thanks are also addressed to Mrs. Sunee Pakprapan (Scientific and Technological Research Equipment Centre, Chulalongkorn University) for their kind assistance in commencing Gas Chromatography (GC) and Scanning Electron Microscopy (SEM).

Finally, I would like to express my highest gratitude to my parents for their affectionate support, blessings, inspiration, and love which guide me all the way throughout my life and study.

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CHAPTER 1

INTRODUCTION

1.1 Motivation

In recent years, demand for fatty acid alkyl ester (biodiesel) being used as fuels in diesel engines and heating systems has increased due to the continuous rising of the petroleum oil price (Vicente et al., 2007). Biodiesel has drawn attention as a non-toxic, biodegradable and renewable source of fuel and energy with signficantly lower exhaust emissions of particulate matter and green-house gases such as CO, CO_2 and SO_x. Therefore biodiesel is environmentally friendly and shows great potential as an alternative liquid fuel and energy product (Lu et al., 2007).

Biodiesel fuel is produced by transesterification of oils and fats with short chain alcohols. The synthesis is classified as chemical or enzymatic production according to the catalysts employed in the process. Short time and high yields are obtained when chemical transesterification is applied (Tan et al., 2010). However, drawbacks such as high energy requirements, difficulties in the recovery of the catalyst and glycerol and potential pollution to the environment are major disadvantages in alkali or acid catalyzed process (Pizarro et al., 2003). On the other hand, lipases can be used to catalyze the reaction in mild conditions (Shimada et al., 2002). However, the high cost of the enzymes often makes the enzymatic process economically unattractive (Noureddini et al., 2005). Immobilization methods, such as adsorption entrapment and encapsulation have been introduced to improve lipase stability and allow for repeated utilization (Salis et al., 2008). Encapsulation in calcium alginate matrix offers a shield against the shear involved in the stirred reactor and also allows easy passage of reactant and products through the matrix (Ganapati et al., 2005). This study exploited this idea with the pre-immobilization of lipase on heterogeneous base by adsorption on loofa followed by

entrapment in calcium alginate beads to give an efficient reusable biocatalyst. Furthermore, the suitable condition for production of biodiesel by using this immobilized lipase was investigated.

1.2 Objectives

To develop immobilized enzyme system using loofa reinforced alginate carriers for ethyl ester production from palm oil/palm fatty acid and ethanol.

1.3 Working scopes

In this study, *Candida rugosa* lipase was used as a biocatalyst. The enzyme was adsorbed on loofa followed by entrapment in calcium alginate gel. The optimal conditions for biodiesel production from palm oil or palm fatty acid in solvent free system are investigated; reaction time at 12, 24, 36 and 48 h, a kind and concentration of buffer (Tris-HCl and phosphate buffer at 0.1 and 0.001 M), effect of the concentration of ethanol at 95% and 99.9%, stability and reusability of loofa-lipase entrapped in Ca-alginate.

1.4 Expected benefits

Useful information for a better understanding of immobilized enzyme technology for ethyl ester production.

CHAPTER 2

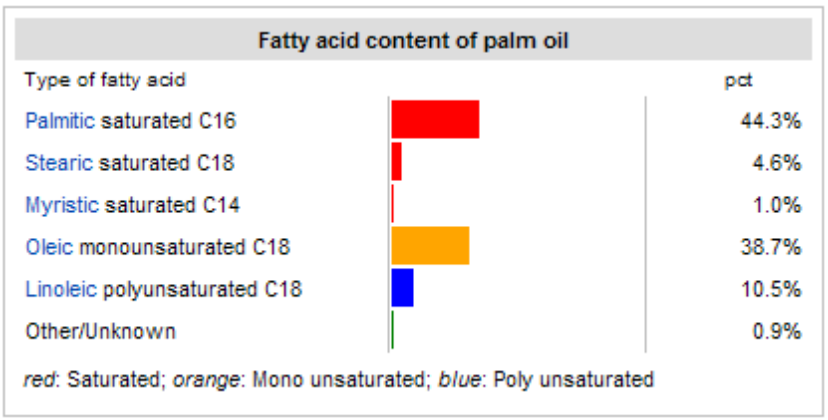
BACKGROUND AND LITERATURE REVIEW

2.1 Palm oil

The original of palm oil tree comes from Africa. Yield of palm fruit can be extracted oil from palm oil and palm kernel oil. Palm oil and palm kernel can be used in the food industry and oleochemical industry. For the food industry, there are many palm oil products, for example; cakes, sauces, powdered milk, margarine, coffee and ice-cream. The advantage of palm oil is resistance to high temperature with accepted smell. Therefore, it is used for cooking. The productions of palm oil and palm kernel oil in the oleochemical industry are such as fatty acids, fatty esters, fatty alcohols. The palm oil can be used for the production of non edible products, for example, soaps, candles, cosmetics, glue, printing ink and so on. Nowadays, palm oil and palm kernel oil have been increasingly used in biodiesel production (www.fedepalma.org).

Palm oil is composed of fatty acids, esterified with glycerol just like any ordinary fat. It is high in saturated fatty acids. The main fatty acid components of palm oil are the 16-carbon saturated fatty acid palmitic acid. Monounsaturated oleic acid is also another major constituent of palm oil. Unrefined palm oil is a large natural source of tocotrienol, part of the vitamin E family.

Table 2.1 The approximate concentration of fatty acids (FAs) in palm oil.



2.2 Ethanol

Ethanol also called ethyl alcohol, pure alcohol, grain alcohol, or drinking alcohol is organic chemical which the chemical formula is C_2H_5OH. The molecular weight is 46.07 g/mol and normal boiling point is 78°C. It is a volatile, flammable and colorless liquid. Ethanol has widespread use as a solvent of substances intended for human contact or consumption, including scents, flavorings, colorings, and medicines. In chemistry, it is both an essential solvent and a feedstock for the synthesis of other products.

2.3 *Candida rugosa* lipase

One of the most interesting and well-investigated class of enzymes in this field are lipases (triacyglycerol hydrolases, EC 3.1.1.3). Lipases are activated upon contact with a nonpolar substrate phase which leads to opening of a mobile element, the lid. This “interfacial activation” is triggered by adsorption to the water-lipid interfact, even though it was proposed that interfacial activation does not necessarily happen in all lipase (CRL) (Derewenda et al., 1992). CRL is a versatile biocatalyst which catalyzes hydrolysis, alcoholysis, esterification and transesterification of triacylglycerols and other hydrophobic ester. The crystal structure of this 57 kDa protein has been resolved at 2.06 and 2.1 Å resolutions with two different conformation, an open and a closed form. Like other microbial lipases, CRL is a member of the α/β-hydrolase fold family which consists of a central hydrophobic eight-stranded β-sheet packed between two layers of amphiphilic α-helices. A mobile lid region consist of two short α-helices linked to the body of the lipase by flexible element (Pandey et al., 1999). The lid consist of residues 62-92 and rotates by almost 90° from the closed to the open conformation. In the closed, inactive conformation, it covers the substrate site. In the open, active conformation, it extends away from the protein surface and makes the catalytic triad, Ser209, His449, and Glu341, accessible to substrate. Upon opening, the exposed substrate binding site and the inside of the lid form a large hydrophobic patch which is supposed to interact with the hydrophobic substrate interface.

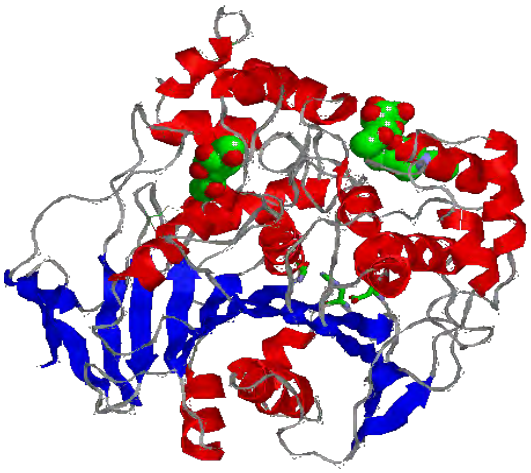


Figure 2.1 *Candida rugosa* lipase

2.4 Biodiesel

Biodiesel is an alternative fuel similar to conventional or 'fossil' diesel. Biodiesel can be produced from straight vegetable oil, animal oil/fats, tallow and waste cooking oil. The process used to convert these oils to Biodiesel is called transesterification. The largest possible source of suitable oil comes from oil crops such as rapeseed, palm or soybean. Most biodiesel produced at present is produced from waste vegetable oil sourced from restaurants, chip shops, industrial food producers such as Birdseye etc. Though oil straight from the agricultural industry represents the greatest potential source it is not being produced commercially simply because the raw oil is too expensive. After the cost of converting it to biodiesel has been added on, it is simply too expensive to compete with fossil diesel. Waste vegetable oil can often be sourced for free or sourced already treated for a small price. However, the waste oil must be treated before conversion to biodiesel to remove impurities. Biodiesel produced from waste vegetable oil can compete with fossil diesel.

The benefits of Biodiesel

Biodiesel has many environmentally beneficial properties. The main benefit of biodiesel is that it can be described as 'carbon neutral'. This means that the fuel produces no net output of carbon in the form of carbon dioxide (CO_2). This effect occurs because when the oil crop grows it absorbs the same amount of CO_2 as is released when the fuel is combusted. Biodiesel is rapidly biodegradable and completely non-toxic, meaning spillages represent far less of a risk than fossil diesel spillages. Biodiesel

has a higher flash point than fossil diesel and so is safer in the event of a crash (www.esru.strath.ac.uk).

2.5 The production of biodiesel

2.5.1 Direct use and Blending

Pure vegetable oils can be used or mixed with diesel petroleum without changing of chemical structure of substances (Ramadhas et al., 2005). Al-Widyan et al. (2002) studied the potential of ethyl ester used as biodiesel to substitute fossil diesel. Both blend and pure biodiesel were tested. The results showed that 100% ester blends consistently resulted in the lowest amounts of emissions over the whole speed range tested.

The advantages of vegetable oils as diesel fuel are liquid nature-portability, heat content (80% of diesel fuel), ready availability and renewability. The disadvantages are higher viscosity, lower volatility and the reactivity of unsaturated hydrocarbon chains (Pryde, 1983). Problems appear only after the engine has been operating on vegetable oils for longer periods of time, especially with direct-injection engines. The problems include coking and trumpet formation on the injectors to such an extent that fuel atomization does not occur properly or is even prevented as a result of plugged orifices, carbon deposits, oil ring sticking and thickening and gelling of the lubricating oil as a result of contamination by the vegetable oils.

Direct use of vegetable oils and/or the use of blends of the oils has generally been considered to be not satisfactory and impractical for both direct and indirect diesel engines. The high viscosity, acid composition, free fatty acid content, as well as gum formation due to oxidation and polymerization during storage and combustion, carbon deposits and lubricating oil thickening are obvious problems (Ma et al., 1999).

2.5.2 Pyrolysis

Pyrolysis is the conversion of substance into another by means of heat or by heat the aid of a catalyst (Sonntag, 1979b). It involves heating in the absence of air or oxygen (Sonntag, 1979b) for cleavage of chemical bonds to yield small molecules

(Weisz et al., 1979). Pyrolytic chemistry is difficult characterized because of the variety of reaction paths and the variety of reaction products that may be obtained from the reactions that occur. The pyrolyzed material can be vegetable oils, animal fats, natural fatty acids methyl esters of fatty acids. Figure 2 outlines a schematic that accounts for the formation of alkanes, alkenes, alkadienes, aromatics and carboxylic acids from pyrolysis of triglycerides (Schwab et al., 1988). Mechanisms for the thermal decomposition of triglyceride are likely to be complex because of many structures and multiplicity of possible reactions of mixed triglycerides.

The equipment for thermal cracking and pyrolysis is expensive for modest throughputs. In addition, while the products are chemically similar to petroleum-derived gasoline and diesel fuel, the removal of oxygen during the thermal processing also removes any environmental benefits of using an oxygenated fuel. It produced some low value materials and, sometimes, more gasoline than diesel fuel.

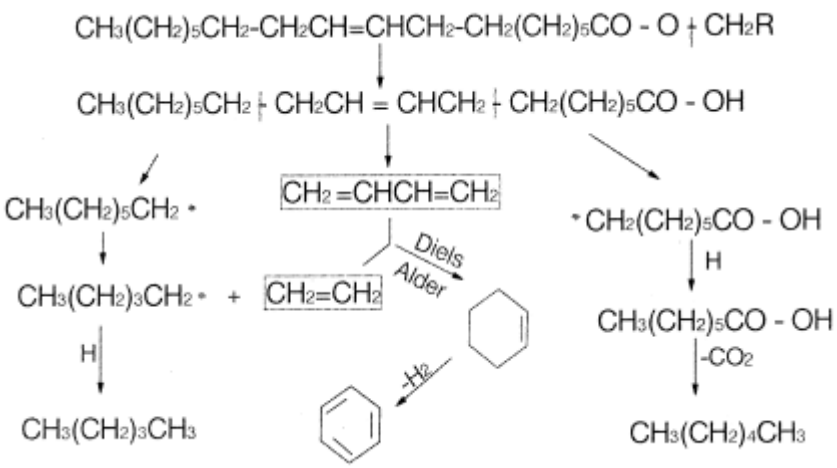


Figure 2.2 The mechanism of thermal decomposition of triglycerides (Schwab et al., 1988).

2.5.3 Microemulsion

A microemulsion is a system consisting of a dispersed liquid, with or without an emulsifier, in an immiscible liquid usually in droplets larger than colloidal size. It can be solved the problem of high viscosity of vegetable oils. A microemulsion is defined as a colloidal equilibrium dispersion of typically isotropic fluid microstructure. The specific droplet size needed for an emulsion to qualify as a microemulsion is not clear. The

mechanics for forming microemulsions can be different. In microemulsion formation, the stability of the emulsion is determined by the energy put into it and the type and amount of emulsifier. Microemulsion formation appears to be dependent upon interactions among the molecules of the constituents (Ali and Hanna, 1995).

It was found that microemulsion can improve spray characteristics by explosive vaporization of low boiling constituents in the micelles (Pryde, 1984). Viscosity reduction, increase in cetane number and good spray characters encourage the usage of microemulsions and prolong usage problems like injector needle sticking, carbon deposit formation and incomplete combustion (Ma and Hanna, 1999; Srivathsan, 2008).

2.5.4 Esterification

Esterification is the formation of ester through a condensation reaction that requires two reactants, carboxylic acids (fatty acid) and alcohol (Solomon et al., 1996). Esterification reactions are proceed slowly in the absence of strong acids such as sulphuric acid, phosphoric acid, organic sulfonic acids and hydrochloric acid. The equation of esterification reaction is show in Figure 3.

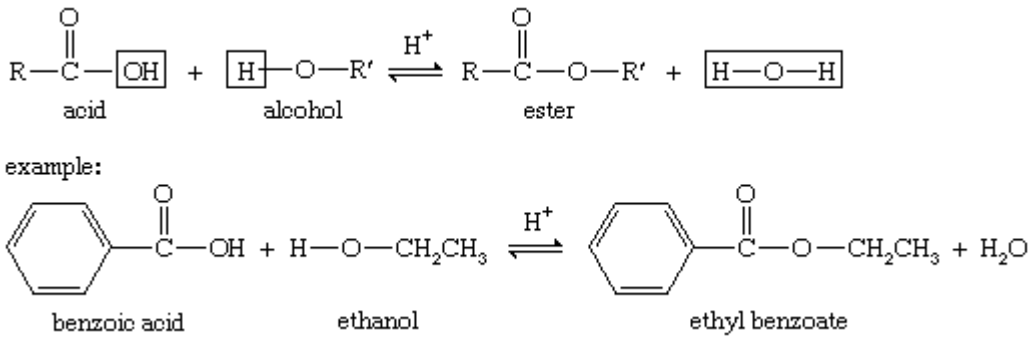


Figure 2.3 Esterification reaction.

2.5.5 Transesterification

Transesterication (also called alcoholysis) is the reaction of fat or oil with an alcohol to form esters and glycerol. The reaction is shown in Figure 4. Catalysts are usually used to improve the reaction rate and yield. Because the reaction is reversible, excess alcohol is used to shift the equilibrium to the products side.

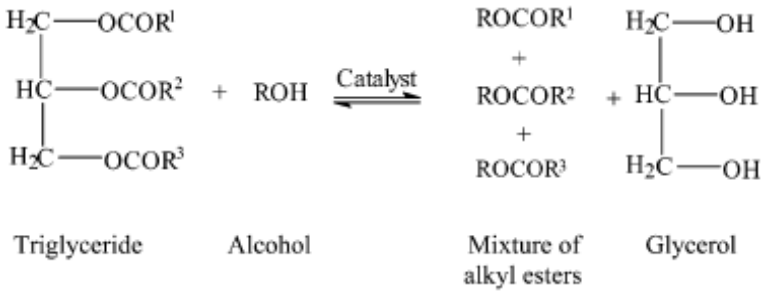


Figure 2.4 Transesterfication of triglycerides with alcohol (Fukuda et al., 2001).

Transesterication consists of a number of consecutive, reversible reactions (Schwab et al., 1987; Freedman et al., 1986). The triglyceride is converted stepwise to diglyceride, monoglyceride and finally glycerol (Figure 5). A mole of ester is liberated at each step. The reactions are reversible, although the equilibrium lies towards the production of fatty acid esters and glycerol.

1. Triglyceride (TG) + R'OH $\underset{\text{Catalyst}}{\rightleftharpoons}$ Diglyceride (DG) + $R'COOR_1$
2. Diglyceride (DG) + R'OH $\underset{\text{Catalyst}}{\rightleftharpoons}$ Monoglyceride (MG) + $R'COOR_2$
3. Monoglyceride (MG) + R'OH $\underset{\text{Catalyst}}{\rightleftharpoons}$ Glycerol (GL) + $R'COOR_3$

Figure 2.5 Three consecutive and reversible reactions. R_1, R_2, R_3 and R' represent alkyl groups (Fukuda et al., 2001). Catalyst

2.5.5.1 Chemical catalyst

Acid-catalyzed or In situ process

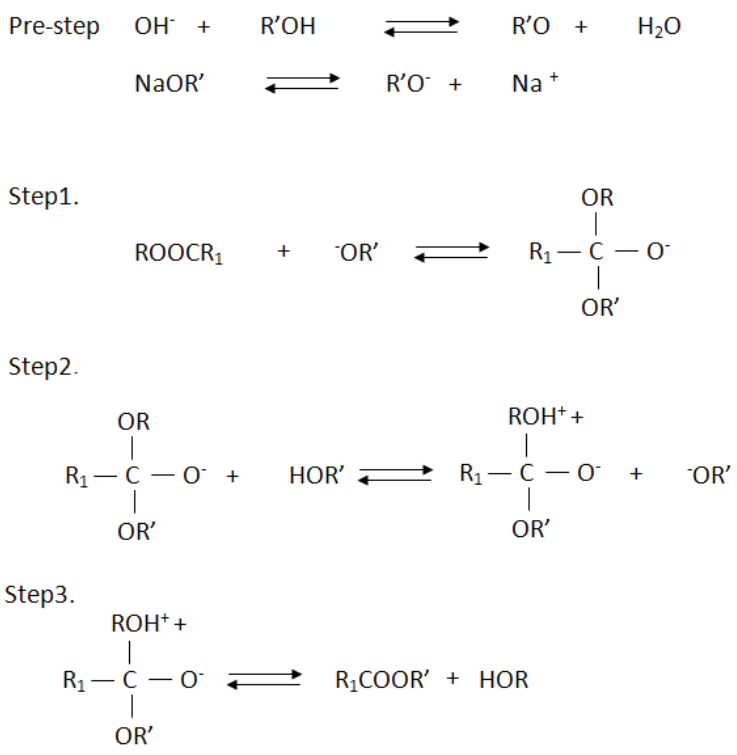
Acids used for transesterification include sulfuric, phosphoric, hydrochloric, and organic sulfonic acids. Although transesterification by acid catalysis is much slower than that by alkali catalysis, acid-catalyzed transesterification is more suitable for glycerides that have relatively high free fatty acid contents and more water. Aksoy et al, (1988) reported that it was necessary to perform transesterification under an acidic condition when the oil component was a low grade material such as sulphur olive oil. In general, the ethyl esters of monounsaturated or short-chain fatty acids with 2% sulfuric acid

should make good alternative fuels. *In situ* transesterification differs from the conventional reaction in that the oil-bearing material contacts acidified alcohol directly instead of reacting with purified oil and alcohol. That is, extraction and transesterification proceed within the same process, the alcohol acting both as an extraction solvent and an esterification reagent. *In situ* transesterification of sunflower oil with acidified methanol produces fatty acid methyl esters in yields significantly greater than those obtained from the conventional reaction with pre-extracted seed oil. Kildiran et al., (1993) have proposed *in situ* transesterification of soybean oil, while Ozgiil and Ttirkay (1993) reported the *in situ* esteritication of rice bran oil with different monohydric alcohols, exploiting the advantage of simultaneous easy extraction of neutral lipids from seeds and bran when *in situ* transesterification is carried out.

Alkali-catalyzed process

Alkalis used for transesterification include NaOH, KOH, carbonates, and alkoxides such as sodium methoxide, sodium ethoxide, sodium propoxide, and sodium butoxide. Alkali-catalyzed transesterification proceeds approximately 4000 times faster than that catalyzed by the same amount of an acidic catalyst and is thus most often used commercially.

The reaction mechanism for alkali-catalyzed transesterification was formulated as three steps (Eckey, 1956). The first step is an attack on the carbonyl carbon atom of the triglyceride molecule by the anion of the alcohol (methoxide ion) to form a tetrahedral intermediate. In the second step, the tetrahedral intermediate reacts with an alcohol (methanol) to regenerate the anion of the alcohol (methoxide ion). In the last step, rearrangement of the tetrahedral intermediate results in the formation of a fatty acid ester and a diglyceride. When NaOH, KOH, K_2CO_3 or other similar catalysts were mixed with alcohol, the actual catalyst, alkoxide group is formed (Sridharan and Mathai, 1974). A small amount of water, generated in the reaction, may cause soap formation during transesterification. Fig. 6 summarizes the mechanism of alkali-catalyzed transesterification.



Where R-OH diglyceride, R_1 long-chain alkyl group and R' short alkyl group

Figure 2.6 The mechanism of alkali-catalyzed transesterification of triglycerides with alcohol (Sridharan and Mathai, 1974; Eckey, 1956)

2.5.5.2 Enzymatic catalyst

Enzymatic conversion of triglycerides has been suggested as a realistic alternative to the conventional physiochemical methods. Because of difficulty removal of catalyst from biodiesel production that using an inorganic base or acid catalyst at or near the boiling point temperatures of the triglyceride/alcohol mixture, it has been recently found that enzyme such as lipase can be used to catalyze transesterification process by immobilizing them in a suitable support. Comparison between alkali-catalysis and acid-catalysis methods for biodiesel fuel production is show in Table 2 (Fukuda et al., 2001). Also, the operating temperature of the process is low (50°C) compared to other techniques (Nelson et al., 1996) and receive a higher quality of the products due to stereoselective and regioselective transformations of substrates. Although, the using of lipase as catalyst has some of the disadvantage such as the loss of initial activity due to volume of the oil molecule, the inconsistent of the support and the high cost of natural enzyme.

Table 2.2 Comparison between alkali-catalysis and acid-catalysis methods for biodiesel fuel production (Fukuda et al., 2001)

	Alkali-catalysis process	Lipase-catalysis 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

2.5.5.3 Selection of alcohol

Transesterification, also called alcoholysis, is the displacement of alcohol from an ester by another alcohol in a process similar to hydrolysis, except that an alcohol is employed instead of water. Suitable alcohols include methanol, ethanol, propanol, butanol, and amyl alcohol. Methanol and ethanol have lower molecular weight with lower density and temperature. However, these common two alcohols are denaturing and inactivating enzyme more than long chain alcohol (Antczak et al., 2009). The rate of lipase-catalyzed transesterification usually increases with the length of hydrocarbon chain of alcohol (Antczak et al., 2009). Haas et al., (2002) found that methanol and water can speed up enzyme denaturation while the presence of small water is importance for ethanol, propanol, butanol and isobutanol. Methanol is also known that it is more inactivation than ethanol. Therefore, ethanol is more readily accepted for use in a variety of industrial situations than methanol, which has also as constraint its toxicity. Beside, ethanol can easily be formed from renewable sources by fermentation (Freitas et al., 2009).

2.6 Variables affecting esterification and transesterification

2.6.1 Effect of organic solvent

Generally all immobilized lipase exhibited high conversion in non-polar solvents with only minor differences between them with respect to the maximum conversion. However, when a polar organic solvent such as acetone was used, significantly lower conversion was detected with only ~5% for *R. miehei* lipase. In such a medium, the solvent may alter the native conformation of the enzyme by disrupting hydrogen bonding and hydrophobic interactions, thereby leading to very low alcoholysis rate. Similarly, in dioxane another water-miscible solvent it has been reported to cause enzymes precipitation. Hexane appeared to be the best solvent for the alcoholysis reaction using *P. fluorescens*, whereas isooctane and petrol ether were the best ones using lipases from *T. lanuginosa* and *R. miehei*, respectively.

2.6.2 Effect of water content in transesterification

Although water is not involved as a reagent or a product in a transesterification reaction, its content is important since it favours the expression of the full enzymatic activity. Water acts as a 'lubricant' of polypeptide chains, thus conferring to the enzyme the necessary mobility to explicate the catalytic action. Since our reaction system is composed by different phases– the substrates (triglycerides and alcohol), the enzyme and the support – water is partitioned at a different extent among the components (Salis et al., 2008).

2.6.3 Ratio of alcohol to oil or fatty acids

This is one of the most important variables affecting the yield of ester in transesterification. Form stoichiometric ratio, there are three moles of alcohol to one mole of vegetable oil to form three moles of alkyl esters and one mole of glycerol. A large excess of alcohol requires driving the reaction to the right in transesterification because of equilibrium reaction, however, the high molar ratio of alcohol to vegetable oil interferes with separation of glycerin because of increasing of solubility. Consequently,

glycerin remains in solution and it drives equilibrium to the left side lowering the yield of ester.

For esterification, the molar ratio of alcohol to fatty acids is also importance. The stoichiometric ratio requires one mole of fatty acid and alcohol to form one mole of alkyl ester and water. Besides, no glycerol is produced. Hence, it is expected that lower alcohol to fatty acids molar ratio would be needed to compare with triglyceride transesterification.

2.6.4 Purity of reactant

Impurities present in the oil also affect conversion levels. Under the same condition, 67% to 84% conversion into ester using crude vegetable oils can be obtained from the tranesterification of oil by lipase compared with 94% to 97% when using refined oils. It was found that the free fatty acids in the original oils interfere with catalyst (Freedman et al., 1984).

2.6.5 Reaction time

The conversion increases with reaction time. For example, Freedman et al., (1984) studied the transesterification of peanut, cottonseed, sunflower and soybean oils under the condition of methanol to oil ratio of 6:1, 0.5% sodium methoxide catalyst and 60°C. An approximate yield of 80% was observed after 1 min for soybean and sunflower oils. After 1 h, the conversions were almost the same for all four oils (93±98%).

2.6.6 Reaction temperatures

The rate of reaction is strongly influenced by the reaction temperature. However, given enough time, the reaction will proceed to near completion even at room temperature. Generally, the catalytic reactions are conducted close to the boiling point of alcohol (60^{o}C to 70^{o}C), under atmospheric pressure. For supercritical conditions, the reaction is carried out under high pressure (9000 kPa) and high temperature (above 240^{o}C).

2.6.7 Effect of alginate and $CaCl_2$ concentration for entrapment technique

An increase in alginate concentration raised loading efficiency, but decreased immobilization yield (Won et al., 2005). $CaCl_2$ concentration was expected to have a similar effect on loading efficiency and immobilization yield, but the effect was small in the tested range of 50–300 mM. With increasing bead size, immobilization yield decreased due to mass transfer resistance, but loading efficiency was unchanged.

2.6.8 Effect of buffer

Buffer solutions are generally evaluated in studies designed to compare the effects of buffers with different features on the activity of the enzyme of interest. For example, sodium phosphate buffer is known to maintain enzymatic activity. Conversely, zwitterionic buffers such as 3-(N-morpholino) propane-1-sulfonic acid (MOPs) and 2-amino-2- hydroxymethyl-propane-1,3-diol (Tris) buffers contain both positive and negative ionizable groups, which were introduced by Zhao and Chasteen (2006). In zwitterionic buffers, secondary and tertiary amines provide the positive charges, while sulfonic acid and carboxylic acid groups provide the negative charges. In addition, these buffers are highly soluble in water and can maintain a stable pH in a physiological range of 6.5 and 8.0 over a wide range of temperature. However, the stability of zwitterionic buffers is not well defined and aggregation of buffer molecules may occur (Lee et al., 2009).

2.7 Literature review

In the past decade, several methods for biodiesel production are developed, There are variety of oils, different catalysts, alcohols and reaction conditions.

Peng et al., (2008) showed that the solid acid catalyst $SO_4^{2-}/TiO_2–SiO_2$ is inexpensive and environment friendly, has high catalytic activity, and is stable for biodiesel production from cheap raw feedstocks with high FFAs. Under the optimized conditions, reaction temperature 200 ^{o}C, molar ratio of methanol to oil 9:1 and catalyst concentration 3 wt.%. The proposed continuous process for the production of biodiesel has potential widespread applications on an industrial scale. The properties of the

biodiesel derived from the demonstration plant are comparable with those of diesel and biodiesel standards.

Although high purity and yield of biodiesel can be achieved in a short time with the alkali process, however, it is very sensitive to the purity of reactants and the amount of FFA water content causing soap formation, which results in the lower yield of ester and renders the separation of ester and glycerol by water washing difficult.

Zullaikah et al., (2005) studied the effect of temperature, moisture and storage time on the accumulation of free fatty acid in the rice bran, and investigated fatty acid methyl ester (FAME) from high fatty acid of rice bran by two-steps process. The first step was carried out at 60 ^{o}C reduced 49.8% FFA level to less than 2% by H_2SO_4 catalyzed esterification. The second step was converted the product of first step into FAME and glycerol at 100 ^{o}C. The maximum yield of FAME was 98% in less than 8 h. Ramadhas et al., (2005) produced biodiesel from the refined/edible type oil using two step transesterification processes. The first step, acid catalyzed esterification reduces the FFA content of the oil to less than 2%. The second step, alkaline catalyzed transesterification process converts the products of the first step to its mono-esters and glycerol.

Miao et al., (2009) investigated high effective acidic transesterification catalyzed by trifluoroacetic acid for biodiesel production. The results showed that the oil could be converted to biodiesel directly by one-step trifluoroacetic acid catalyze process without extreme temperature and pressure conditions. The optimum process combination was 2.0 M catalyst concentration with 20:1 M ratio of methanol to oil at temperature of 120 ^{o}C. It reduced product specific gravity from an initial value of 0.965 to a value of 0.878 in about 5 h of reaction time, and the methyl ester content reached as high as 98.4%.

The production of biodiesel using a biocatalyst such as lipase can eliminate the disadvantages of the alkali process by producing product of very high purity with less downstream operations (Fukuda et al., 2001). Ghamgui et al., (2007) used lipase from *Rhizopus oryzae* (ROL) was immobilized by physical adsorption onto $CaCO_3$. The immobilization yield was more than 95% during 30 min and corresponds to the loading of 2570 IU/g support. The optimum temperature for both free and immobilized lipase

activities was 37 °C. After 24 h of incubation at 50 °C, the immobilized ROL maintained 67% of its initial activity, while the free enzyme was completely inactivated.

Ting et al., (2008) developed an enzymatic/acid-catalyzed hybrid process for biodiesel production using soybean oil as feedstock. The soybean oil was hydrolyzed in the presence of lipase immobilized to chitosan beads using a binary method, and a higher degree of hydrolysis was achieved. The esterification of the feedstock by methanol at optimized conditions (50 °C; feedstock to methanol molar ratio of 1:15; 2.5% sulfuric acid) led to 99% conversion of biodiesel after 12 h.

Dizge and Keskinler (2008) studied of lipase on FAME production from canola oil in tert-butanol system. They tried to eliminate the negative effect of solubilization of methanol and by-product glycerol by using tert-butanol. tert-Butanol is a moderately hydrophilic solvent can solubilize oil, methanol and glycerol. The optimal conditions for processing 20 g of refined canola oil were: 430 μg lipase, 1:6 oil/methanol molar ratio, 0.1 g water and 40 °C for the reactions with methanol. Maximum methyl esters yield was 90% of which enzymatic activity remained after 10 batches, when tert-butanol was adopted to remove by-product glycerol during repeated use of the lipase. The immobilized lipase proved to be stable and lost little activity when was subjected to repeated uses.

Dizge et al., (2009) studied the effect of stepwise methanol addition on biodiesel yield. They have also observed the methanol inhibition when all the methanol (the molar ratio of methanol to oil 6:1) is added in one–step (20 g), the yield of biodiesel production was 42%. However, the yield was increased to 75% and 97% in two-step (10 g in each step) and three-step (6.67 g in each step) addition of methanol, respectively. Under the optimized conditions, 50 °C in 24 h reaction and the immobilized enzyme retained its activity during the 10 repeated batch reactions.

For the entrapment method, Won et al., (2005) studied the effects of immobilization conditions such as alginate concentration, $CaCl_2$ concentration, ratio by weight of enzyme to alginate (E/A) and bead size on loading efficiency and immobilization yield. They found that an increase in alginate concentration raised loading efficiency, but decreased immobilization yield. $CaCl_2$ concentration was

expected to have a similar effect on loading efficiency and immobilization yield, but the effect was small in the tested range of 50–300 mM. With increasing bead size, immobilization yield decreased due to mass transfer resistance, but loading efficiency was unchanged.

Table 2.3 Review studies production of biodiesel by heterogeneous, acid or alkali catalyzed methods.

References	Material	Method	Condition			Conversion	Objective
			Temp. (^{o}C)	Rx Time (hr)	Molar ratio(Alc:oil)		
Zullaikah et al., 2005	1. refined rice bran oil 2. methanol 3. H_2SO_4	two-step acid-catalyzed process (acid catalyst)	100	10	10:1	> 98%	To study effect of temperature, moisture and storage time on the free fatty acid content in storage bran and develop an efficient method for the production of biodiesel from rice bran oil with varying FFA content with the fewest adverse effects on the functionality of biologically active by-product.
Ramadhas et al., 2005	1. rubber seed oil 2. methanol 3. H_2SO_4 4. NaOH	two-step transesterification (acid-base catalyst)	45±5	0.5	6:1	>90%	To develop a method for esterification of high FFA vegetable oils.

Reference	Material	Method	Condition			Conversion	Objective
			Temp. (^{o}C)	Rx Time (hr)	Molar ratio(Alc:oil)		
Miao et al., 2009	1. refined soybean oil 2. methanol 3. trifluoroacetic acid	acid catalyst	120	5	20:1	98.4%	To develop a high effective acidic transesterification catalyzed by trifluoroacetic acid for biodiesel production.
Patil et al., 2009	1. jatropha oil 2. karanja oil 3. canola oil 4. corn oil 5. methanol 6. H_2SO_4 7. KOH	two-step and single-step transesterification process (acid-base catalyst)	40±5,	2	6:1	90-95%	To study optimization of biodiesel production for the edible and non-edible vegetable oils were done in detail with one-step alkali transesterification process and two-step acid esterification process, respectively with fuel property analysis of these oils.

References	Material	Method	Condition			Conversion	Objective
			Temp. (^{o}C)	Rx Time (hr)	Molar ratio(Alc:oil)		
Zhang et al., 2010	1. waste oils 2. methanol 3. SO_4^{2-}/TiO_2-SiO_2	solid acid catalyst	200	6	9:1	~ 92%	To study effect of various reaction parameters on biodiesel yield, such as catalyst concentration, reaction temperature, molar ratio of methanol to oil and amount of FFAs.
Jain et al., 2011	1. waste cooking oil 2. refined soybean oil 3. methanol 4. H_2SO_4 5. NaOH	acid-base catalyst	50	6	7:3	90.6%	To study of its kind which uses simple 2 step acid–base transesterification of high FFA waste cooking oil to convert it into biodiesel.

Table 2.4 Review studies production of biodiesel by enzymatic catalyzed methods.

References	Material	Source of lipase, method (Support)	Condition			Conversion	Objective
			Temp. (^{o}C)	Rx Time (hr)	Molar ratio (Alc:oil)		
Noureddini et al., 2005	1. soybean oil 2. methanol 3. ethanol	*Pseudomonas cepacia* Entrapment (sol-gel support)	35	1	7.5:1 15.2:1	67 % 65 %	To study the lipase PS from *Pseudomonas cepacia* was entrapped within a sol–gel polymer matrix for using in the transesterification of soybean oil with methanol and ethanol.
Salis et al., 2007	1. soybean oil 2. methanol	*Pseudomonas fluorescens* Adsorption (macroporous polypropylene)	30	49	8:1	96%	To study the characterization and the immobilization of eight commercial lipases onto macroporous polypropylene.

References	Material	Source of lipase, method (Support)	Condition			Conversion	Objective
			Temp. (^{o}C)	Rx Time (hr)	Molar ratio (Alc:oil)		
Yagiz et al., 2007	1.waste cooking oil 2. methanol	*Lipozyme-TL IM* Adsorption (hydrotalcite)	45	5	4:1	92.8%	To study the immobilization conditions of lipase enzyme on hydrotalcite prepared by coprecipitation and four different types of commercial zeolites.
Ghamgui et al., 2007	1. oleic acid 2. ethnaol	*Rhizopus oryzae* Adsorption ($CaCO_3$)	37	24	300 IU:1	83%	To study the best conditions for lipase immobilization on $CaCO_3$ and explain the difference between the immobilization yields obtained with two supports ($CaCO_3$ and celite 545).

References	Material	Source of lipase, method (Support)	Condition			Conversion	Objective
			Temp. (^{o}C)	Rx Time (hr)	Molar ratio (Alc:oil)		
Dizge et al., 2008	1. canola oil 2. methanol 3. tert-butanol	*Thermomyces lanuginosus* Cross-linking (hydrophilic polyurethane foams)	40	24	6:1	90%	To study the lipase from *Thermomyces lanuginosus* was immobilized into polyurethane foams and the immobilized enzyme was used in the transesterification of canola oil with methanol.
Ting et al., 2008	1. soybean oil 2. methanol 3. H_2SO_4	*Candida rugosa* Adsorption (chitosan beads)	50	12	15:1	99%	To develop an enzymatic/acid-catalyzed hybrid process for the production of biodiesel with a view to utilize edible and off quality soybean oils as feedstock.

References	Material	Source of lipase, method (Support)	Condition			Conversion	Objective
			Temp. (^{o}C)	Rx Time (hr)	Molar ratio (Alc:oil)		
Dizge et al.,2009	1. canola oil 2. methanol	*Thermomyces lanuginosus* Adsorption and covalent attachment (polyglutaraldehyde activated styrene–divinylbenzene copolymer)	50	24	6:1	97%	To present work describes the synthesis of a novel STY–DVB–PGA copolymer and its successful usage to immobilize lipase from *Thermomyces lanuginosus* to produce biodiesel from canola oil.
Zeng et al., 2009	1. refined rape oil 2. methanol	*Saccharomyces cerevisiae* Adsorption (Mg–Al hydrotalcite)	45	4.5	4:1	96%	To investigate kinetics of thermal activation/deactivation of the immobilized and free lipases and the optimum conditions for the immobilized lipase on Mg–Al hydrotalcite.

References	Material	Source of lipase, method (Support)	Condition			Conversion	Objective
			Temp. (^{o}C)	Rx Time (hr)	Molar ratio (Alc:oil)		
Sonare et al., 2010	1. sunflower oil 2. methanol 3. tert-butanol	*Lipozyme RM IM* and *Novozyme 435* Adsorption (macro-porous polyacrylic resin beads and anionic resin)	45	24	4.5:1	73%	To study the optimization of transesterification of used sunflower oil using immobilized lipase enzyme has been carried out and check the application of the immobilized enzyme for the transesterfication of used sunflower oil.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials and chemicals

Palm oil was purchased from a local supermarket (Bangkok, Thailand). Enzymatic lipase from *Candida rugosa* (CARL) (Type VII > 700 unit/mg solid, 1,187 U/mg) used as a biocatalyst was purchased from Sigma Aldrich Chemical Co. Ltd. (St. Louis, USA). Ethanol (95%) was used as a reactant in this work. All other chemicals used in this work were analytical grade, and were purchased from local suppliers in Thailand.

3.2 Lipase Immobilization

3.2.1 Immobilization of *C. rugosa* lipase on loofa

The enzyme immobilization was prepared using loofa sponges (10 x 10 x 2 mm^3) as the support. Support sheets of loofa sponges with the total area of 12 cm^2 were added into 3 mL of enzymatic solution (Tris-HCl and phosphate buffer, pH 7.0) and left for 30 min in order to obtain complete adsorption.

3.2.2 Calcium alginate entrapment of *C. rugosa* lipase immobilized on loofa

C. rugosa lipase was pre-immobilized by the adsorption onto loofa sponges. The calcium alginate entrapment was modified from the previous work as the following procedure: enzyme immobilized on loofa was dispersed uniformly in 2% (w/v) sodium alginate solution. Then, sodium alginate gel containing the immobilized enzyme on loofa was allowed to harden in 0.1 M calcium chloride solution for 30 min. To increase the layer, the entrapped sheets were done with the same method as stated above.

3.3 Enzymatic transesterification and esterification reaction

The reaction containing of 15 g of palm oil and ethanol in a molar ratio of 1:9 (or the molar ratio of palm fatty acid to ethanol 1:3) was carried out in 125 mL Erlenmayer flasks. The reaction was catalyzed by 10% (w/w of oil) lipase in 0.1 and 0.001 M

phosphate buffer in forms of free and immobilized CRLA. The mixtures were incubated at 45°C under a constant shaking at 250 rpm for 36 h. Samples of 5 mL were taken every 12 h during 36 h reaction period. The ethanol was dried from the samples followed by centrifuging to separate the glycerol. The yields of ethyl ester found by using free and immobilized CRLA were analyzed by GC.

The considerable variables on the ethyl ester production were including

- The ethyl ester production by using immobilized *Candida rugosa* lipase
- The effect of the concentration of buffer
- The effect of the concentration of ethanol
- The effect of the number of the layer of Ca-alginate on immobilized lipase
- Repeated use of the immobilized *Candida rugosa* lipase

3.4 Ethyl Ester Analysis

Ethyl ester compositions were analyzed by gas chromatographic instrument (GC) (Shimadzu 14B) equipped with flame ionization detector (FID) using a Rtx 5 column (50 m, 0.25 mm ID, 0.25 μm). The oven temperature was programmed from the beginning at 150 °C (2 min), ramp at 5°C/min, to 250°C (5min). Samples were prepared by adding 0.1 ml of sample to 4.9 ml of n-hexane. The injection volume was 2μL. The ethyl ester yield was estimated from the ratio of the quantity of FAEEs to that of the reactants:

$$\% = \frac{W_{ME}}{W_F} \times 100$$

Where W_{EE} and W_F are weights of ethyl ester (g) and the feed reactant (g), palm oil or palm fatty acid, respectively.

3.5 Characterization by Scanning electron microscope (SEM)

Scanning electron micrographs were taken with JEOL JSM-5410LV (Microscope at Scientific and Technological Research Equipment Center, Faculty of Pharmaceutical Science, Chulalongkorn University) was used to examine the surface morphologies of freeze dried BC-alginate before and after immobilization. The free surface was coated with gold, subsequently their surface were observed and photographed. The coated specimens were kept in dry place before experiment. SEM was obtained at 15 kV which is considered to be a suitable condition since too high energy can be burn the samples.

CHAPTER 4

RESULTS AND DISCUSSIONS

In this work, biodiesel was synthesized from the direct transesterification of palm oil with ethanol by *Candida rugosa* lipase. Compared to acidic and alkaline catalysts in biodiesel production, the use of lipase-catalyzed reaction can tolerate more water content of oil and increases biodiesel yield by avoiding the soap formation. Moreover, biodiesel and glycerin can be separated easily and the purification process is simpler. However, the high cost of enzymes often makes the enzymatic process economically unattractive. The enzyme immobilization is one of the best ways to reuse and improve its activity and stability. Thus, the immobilization techniques such as adsorption and entrapment are widely applied to develop immobilized enzyme with high enzymatic activities for ethyl ester production. Adsorption or entrapment technique is simple to apply with low material and energy costs. However, for long term use of immobilized enzymes, the problems such as enzyme detachment, enzyme deactivation and diffusion limitations of substrate often occurred during the operation. In order to develop the immobilized enzyme for ethyl ester production from palm oil and palm fatty acid, *C. rugosa* lipase (CRL) immobilized by loofa doped alginate matrix (CRLAE) was formed as a new carrier by the integration of adsorption and entrapment techniques. The immobilization procedure could be divided into two steps. Firstly, *C. rugosa* lipase was physically adsorbed onto the surface of loofa. The second was the entrapment of loofa-lipases in alginate matrix.

To optimize the condition, the biocatalysts efficiency was determined by the yield of methyl and ethyl ester from enzymatic transesterification. Scanning electron microscope (SEM) was used to characterize the biocatalysts to support the experimental data. The results were compared between the systems using immobilized and free lipase. After optimization of the system, the condition was applied for FAEE from palm oil/ palm fatty acid. The immobilized lipase was also examined for its stability and reusability for biodiesel production.

4.1 Ethyl ester production by using immobilized *C. rugosa* lipase

In this study, the immobilized *C. rugosa* lipase in form of loofa-lipase immobilized in calcium alginate matrix (CRLAE) was applied for the ethyl ester production. The results were compared to that using free lipase and immobilized lipase on loofa (CRLA). The reaction was carried out using purified palm oil as the substrate. The previous optimum conditions (Sawangpanya N, 2009) were applied as followed: reaction temperature at 45^{o}C, ethanol: reactant molar ratio of 9: 1 (based on purified palm oil), immobilized lipase of 1.5 g (10% based on oil weight), Na-alginate concentration at 1.0 -2.0 % and reaction time of 36 h.

The ethyl ester yields of biodiesel by catalytic transesterification of purified palm oil are presented in Figure 4.1. From the initial profiles of ethyl ester yield, it shows that the CRLAE-2% carrier was relatively more effective for the transesterification than CRLA and CRLAE-1% carriers. After the reaction of 36 h, the ethyl ester yield of 88.79 % was obtained from using CRLAE-2% whereas, the yields of 80.14 and 86.50 % were obtained from using CRLA and CRLAE-1%, respectively. Moreover, the ethyl ester yield remained constant after 36 h of reaction time. Therefore, the reaction time of 36 h was employed in the further study.

In this study, the ethyl ester yields for the repeated use of the immobilized lipase by CRLA, CRLAE-1% and CRLAE-2% carriers was examined in the free solvent system. As shown in Figure 4.1, when focusing on the ethyl ester yield, the repeatability of CRLAE-2% was better than that of CRLA and CRLAE-1%. The ethyl ester yield about 35.35 % was achieved by using the CRLAE-2%, whereas the ethyl ester yield of CRLA and CRLAE-1% were 27.03 and 26.01 % after 48 h, respectively.

The loss of ethyl ester yield of immobilized lipase could be due to leaching of enzyme from support and inactivation of enzyme when exposed to the violent conditions. Although the entrapment technique was designed to prevent direct contact between the enzyme and surrounding environment, provide a suitable network around the enzyme and prevent enzyme from leaching from supporting material, the results from the reuse of the immobilized enzyme revealed the significantly reduction of ethyl ester yield from the 1st run to the 2nd run.

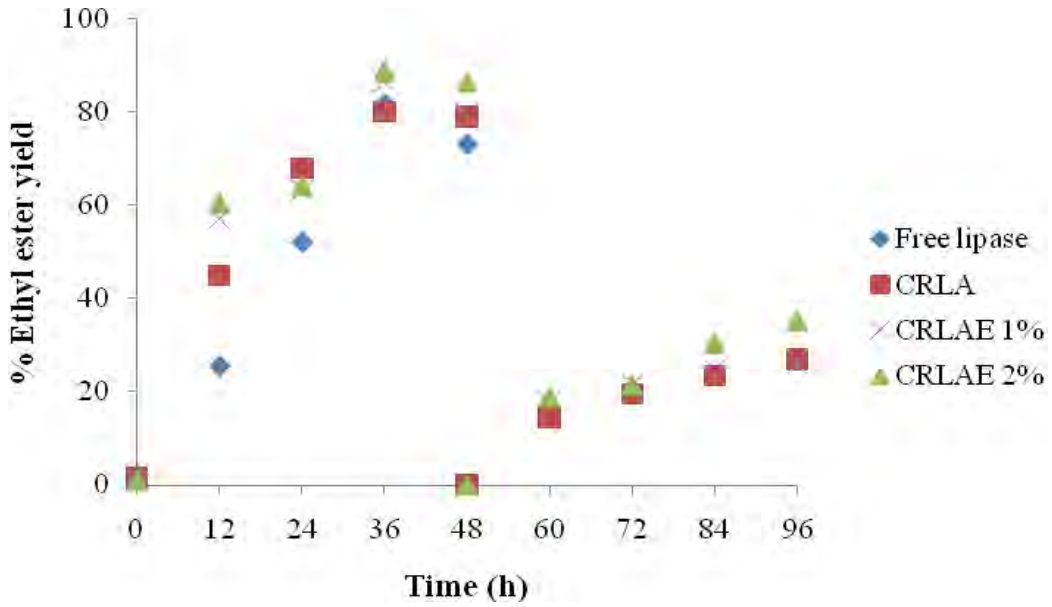


Figure 4.1 The ethyl ester yield by using the different techniques of immobilized lipase under the following conditions: 10% lipase by wt of oil, 95.0% ethanol, and reaction temperature of 45°C, shaking speed 250 rpm and ethanol to palm oil ratio 9:1.

4.2 Effect of buffer solution

Effect of buffer solutions was investigated in this study. Generally, phosphate buffer is known to maintain high enzymatic activity. However, phosphate buffer could affect on stability of immobilizing support based on alginate. Drury et al., in 2004 reported that the dissolution of alginate hydrogel leads to weakening of support based on alginate. The reduction in cross-linking is induced by phosphate ions contained in the buffer solution destabilizing the calcium-alginate gel. Conversely, zwitterionic buffers such as Tris-HCl buffer contain both positive and negative ionizable groups. These buffers are highly soluble in water and can maintain a stable pH in a physiological range of 6.5 and 8.0 over a wide range of temperature. However, the stability of lipase in zwitterionic buffers is not well defined and aggregation of buffer molecules may occur in an attempt to overcome (Zhao and Chasteen. 2006).

In this study, *C. rugosa* lipase was dissolved in each buffer with the same ionic strength at 0.1 M and the same pH of 7.0. Figure 4.2 shows the effect of buffers to the

ethyl ester yield. For all immobilize techniques, the ethyl ester yields obtained from the systems using 0.1 M phosphate buffer were significantly higher than those from 0.1 M Tris-HCl buffer. The maximum yield of 80.03% was obtained by using CRLAE-2% carrier. Similarly, for the second cycle (Figure 4.2), the enzymatic activity in the phosphate buffer was much better than that of the Tris-HCl buffer. The maximum ethyl ester yield at 39.25% was obtained by using CRLAE-2% carrier. Therefore, phosphate buffer was employed in the further study.

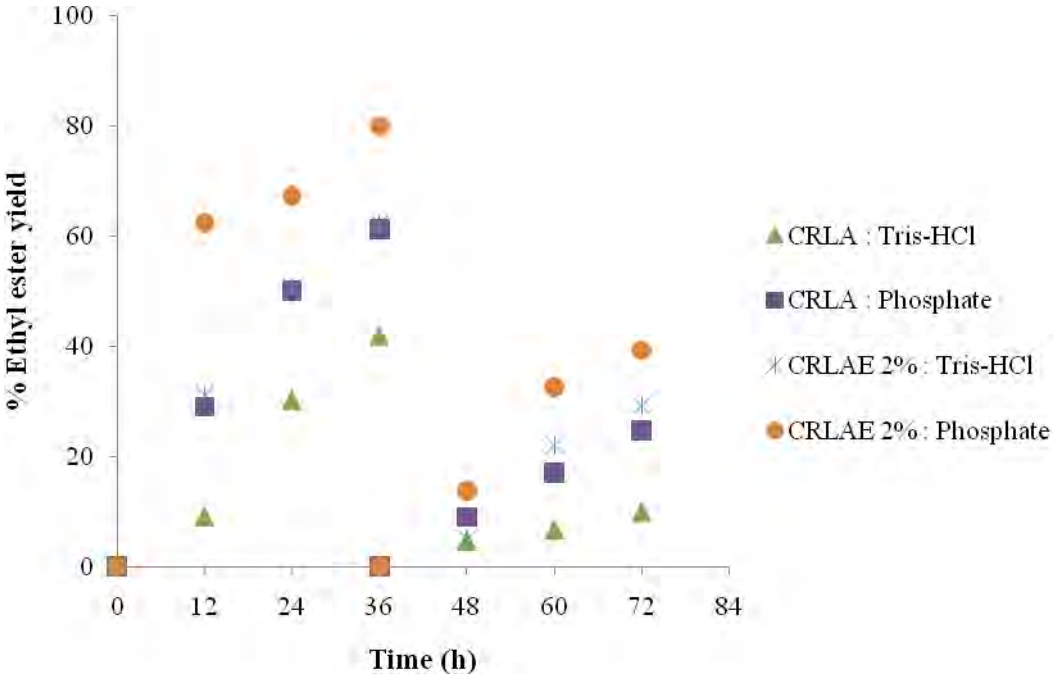


Figure 4.2 Reusability and effect of buffer solution on ethyl ester yield under the following conditions: 10% lipase by wt of oil, 95% ethanol, and reaction temperature of 45°C, shaking speed 250 rpm and ethanol to palm oil ratio 9:1 and 36 h.

4.3 Effect of phosphate buffer concentration

In order to improve the stability of immobilizing support based on alginate, the reduction of phosphate buffer concentration was required. As shown in Figure 4.3, when comparing the ethyl ester yield at the difference phosphate buffer concentration, it shows that the system using 0.001 M phosphate buffer exhibited relatively higher ester yields than those with 0.1 M. The maximum ethyl ester yield of 94.46% was obtained by using CRLAE-2% and 0.001 M phosphate buffer, whereas the ester yield of 66.43% was obtained by using CRLA at the same phosphate buffer concentration. Moreover, under the 2nd run of CRLAE-2%, the enzymatic activity with the use of 0.001 M phosphate buffer was also relatively higher than that of 0.1 M. Therefore, 0.001 M phosphate buffer was employed in the further study.

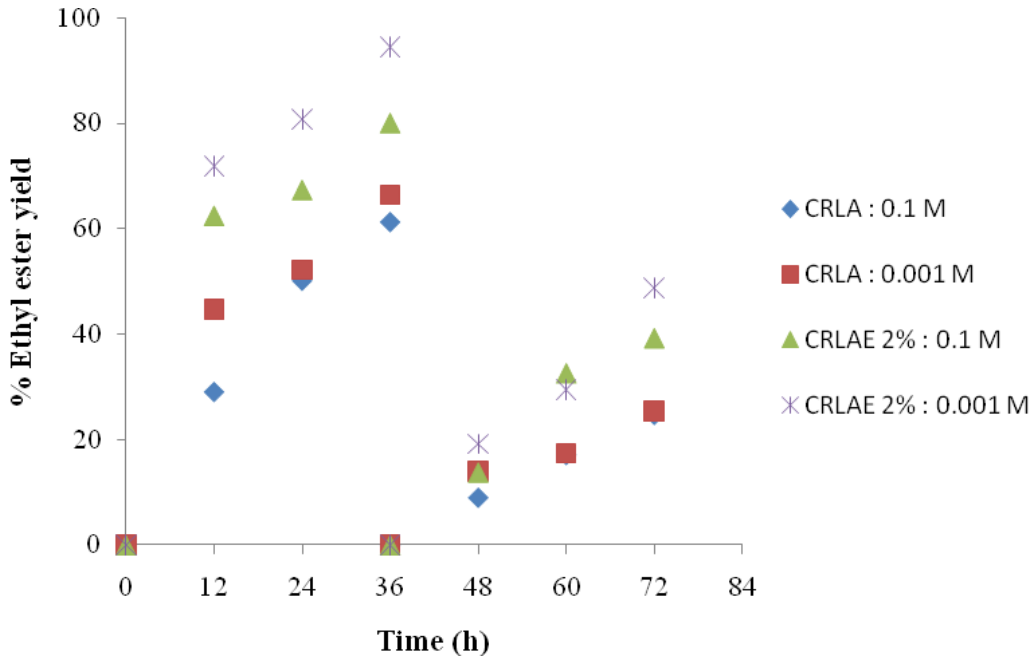


Figure 4.3 Reusability and effect of phosphate buffer concentration on ethyl ester yield under the following conditions: 10% lipase by wt of oil, 95% ethanol, and reaction temperature of 45°C, shaking speed 250 rpm, ethanol to palm oil ratio 9:1 and 36 h.

4.4 Effect of number of Ca-alginate layers

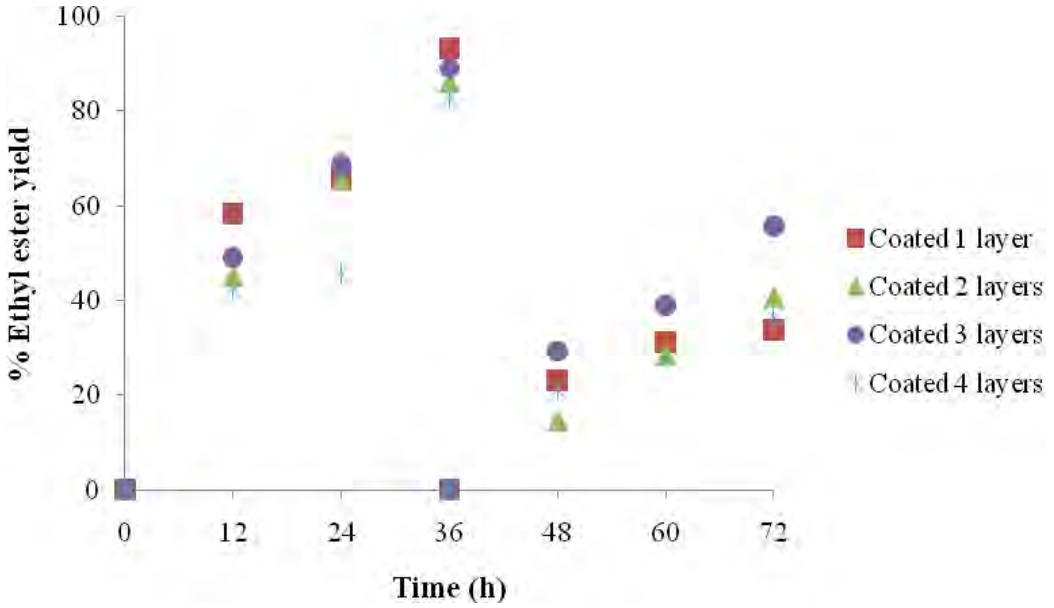


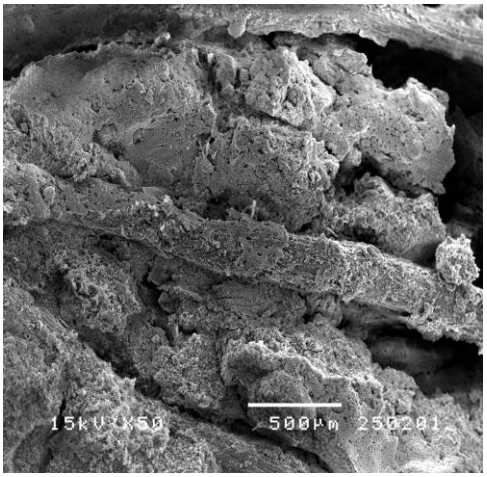
Figure 4.4 Reusability and effect of Ca-alginate layers on ethyl ester yield under the following conditions: 10% lipase by wt of oil, 95% ethanol, 45°C, 250 rpm, ethanol to palm oil ratio 9:1, 0.001 M phosphate buffer and 36 h.

In order to improve the mechanical strength and stability of Alginate-loofa support, the multiple coating of alginate film layers was studied. As shown in Figure 4.4, when focusing on the Ca-alginate entrapped layer, the system using the fresh immobilized lipase (the 1st run) of CRLAE coated with 1 alginate gel layer showed relatively better transesterification rate than those of CRLAE coated with 2,3 and 4 alginate gel layers, respectively. The maximum ester yield from CRLAE coated-1 layer was 93.28 % at 36 h, whereas CRLAE coated -2, -3 and -4 layers were 86.04, 89.06 and 82.88 % respectively. This could be explained by the effect of multicoating on mass transfer rate. However, at the 2nd run (Figure 4.4), the ethyl ester yield of 55.69 % was attained by using CRLAE coated-3 layers, whereas, the ester yields of 33.86, 40.77 and 36.41% were obtained from the use of CRLAE coated -1, -2 and -4 layers, respectively. Therefore, in order to produce ethyl ester, CARLE coated with 3 layers of alginate film exhibited the optimal configuration structure for lipase immobilization. This multi-

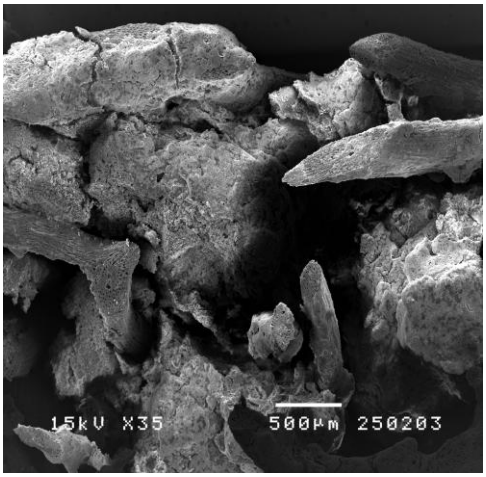
entrapment method was employed to the further study in order to prevent leakage of enzymes into the surrounding, while the mass transfer of the substrates was not significantly limited. However, the considerable decreases in ethyl ester after the 2nd run of all of the immobilized lipases were observed, which revealed the occurrence of enzyme leakages and/or deactivation when exposed to the violent condition.

4.5 Morphology by scanning electron micrograph (SEM)

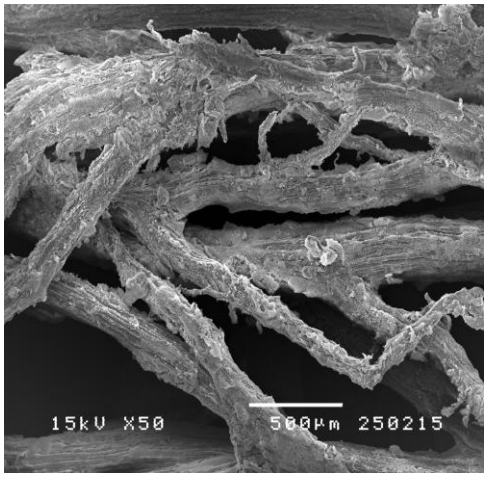
The surface of CRLAE structure coated with 3 alginate film layers was observed using a scanning electron microscope and the SEM images are shown in Fig. 4.5. It was found that there was different amount of lipase on loofa, depending on the reaction duration. The amount of lipase on loofa was slightly high before using in enzymatic transesterification (a), (b) but after the first (c), (d) and the second run (e), (f), the amount of lipase decreased significantly due to the leakage of lipase from the alginate gel network resulted in decreasing of ethyl ester yield.



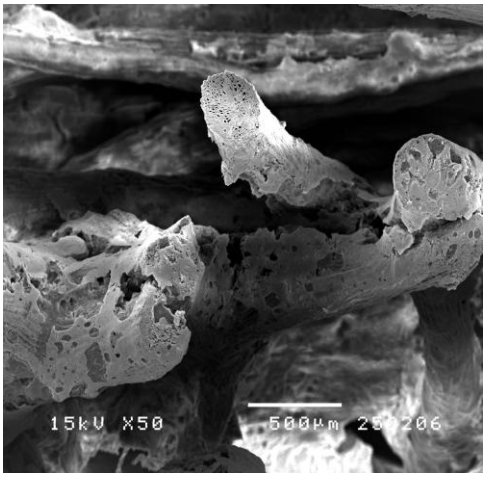
(a)



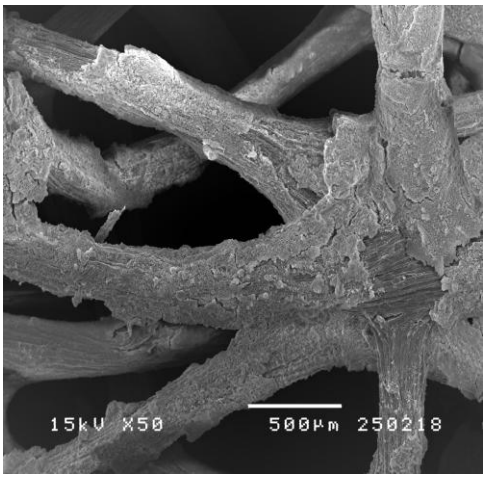
(b)



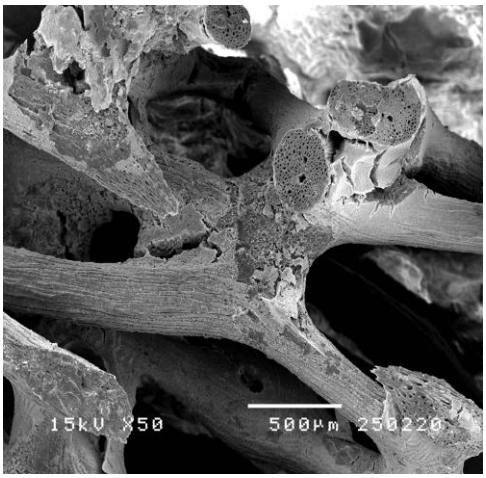
(c)



(d)



(e)



(f)

Figure 4.5 Scanning electron micrograph (surface view) (a) before using in enzymatic transesterification, (c) after the 1st run and (e) after the 2nd run, (cross-section) (b) before using in enzymatic transesterification, (d) after the 1st run and (f) after the 2nd run.

4.6 Effect of water content in ethanol (95.0 % vs. 99.9% ethanol)

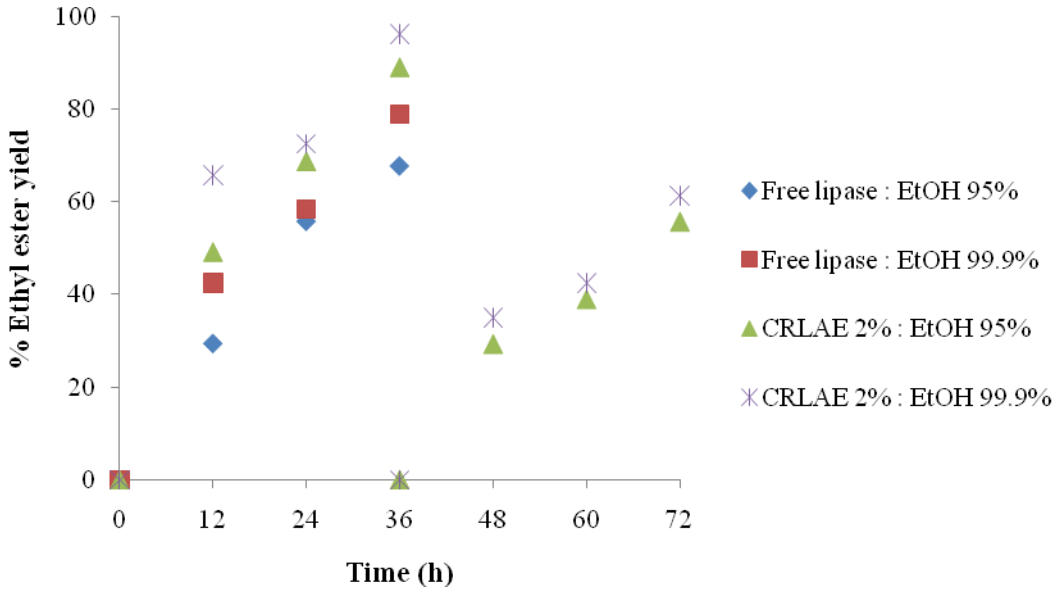


Figure 4.6 Reusability and effect of water content in ethanol (95.0 % vs. 99.9% ethanol) on the yield of ethyl ester under the following conditions:; 10% by wt of lipase to oil, molar ratio of ethanol to palm oil ratio was 9:1, reaction temperature of 45°C and shaking speed of 250 rpm, 0.001 M phosphate buffer, CRLAE-2% coated 3 layers and 36 h.

Although water is not involved as a reagent or a product in a transesterification reaction, its content is important since it favors the expression of the full enzymatic activity. Water acts as a 'lubricant' of polypeptide chains, thus conferring to the enzyme the necessary mobility to explicate the catalytic action. Since the reaction system was composed by different phases – the substrates (triglycerides and methanol), the enzyme and the support – water was partitioned at a different extent among the components (Salis et al., 2008). However, since lipases usually catalyze hydrolysis in aqueous media, excess water may also stimulate the competing hydrolysis reaction. The optimum water content is a compromise between minimizing hydrolysis and maximizing enzyme activity for the transesterification reaction (Noureddini et al., 2005).

In this work, the effect of water content in 95.0 % and 99.9 % ethanol solution was compared. The ethyl ester yields are presented in Figure 4.6. The result with 95%

ethanol shows slightly less ethyl ester yield than that of 99.9% ethanol due to the reasons previously mentioned. Since the price of 95.0% ethanol is much cheaper than that of 99.9% ethanol, the selection decision (95.0 % vs. 99.9% ethanol) is ultimately a trade-off between ethyl ester yield and cost of ethanol.

4.7 Reusability and effect of short chain alcohol (methanol)

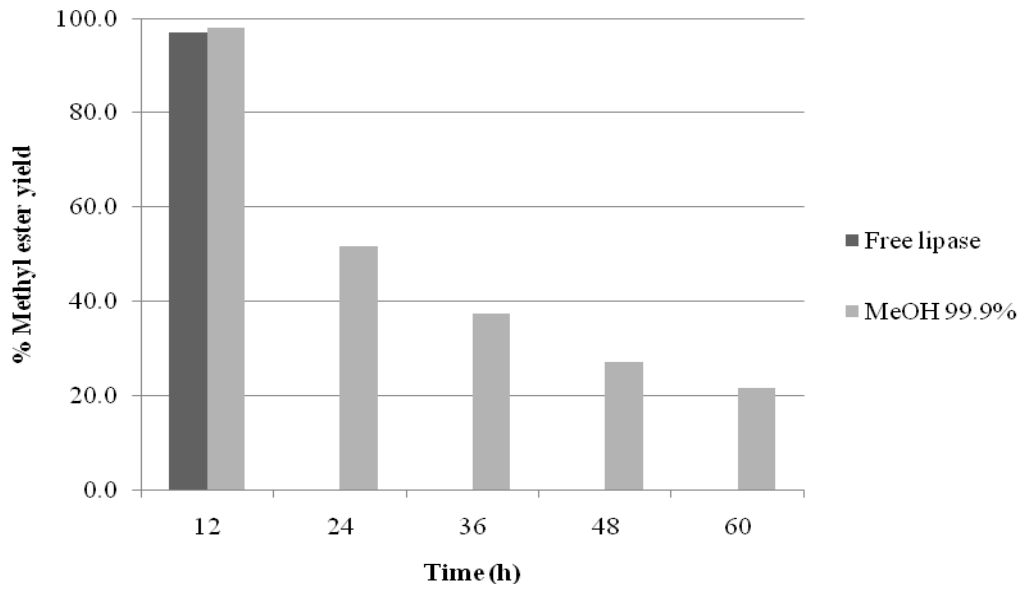


Figure 4.7 Reusability and effect of methanol (MeOH) to methyl ester yield under the following conditions: 10% by wt of lipase to oil, molar ratio of methanol to palm oil was 9:1, reaction temperature of 45°C and shaking speed of 250 rpm, 0.001 M phosphate buffer, 99.9 % methanol, CRLAE-2% coated 3 layers and reaction time of 12 h.

When comparing the performance of immobilized biocatalysts intended for preparative or industrial use, characterization of their operational stabilities is very important. To evaluate the reusability, the effect of repeated use on the methyl ester yield of the immobilized lipase is shown in Figure 4.7. After the 5^{th} run, methyl ester yield significantly decreased from 97.92 to 21.66 %. The similar behavior of immobilized

lipases was reported by Ye et al. (2006).This result is due to the inactivation of the enzyme caused by the denaturation of protein.

4.8 Effect of free fatty acid

For long term, using of palm oil as the substrate for biodiesel production might not be appropriate as it is edible oil widely used in many countries. On the other hand, a lot of palm fatty acid was obtained as a byproduct from the palm oil refinery. Its price is considered much cheaper as compared to palm oil. Thus, the palm fatty acid has become more attractive to be applied as a substrate in biodiesel production.

However, the study found that the acidity of palm fatty acid could cause the violent condition that might inhibit the activity of lipase. Hence the mixture of purified palm oil and palm fatty acid was suggested for the improved ethyl ester yield and to avoid serious deactivation of lipase as being observed when only palm fatty acid was used as a substrate (Sawangpanya N, 2009). In this study, the initial mass ratio of purified palm oil to palm fatty acid was 70:30 (Sawangpanya N, 2009). The semi-continuous addition of palm fatty acid into the reaction medium every 24 h from the initiation point of the reaction was studied. Figure 4.8 shows that with this strategy, the ethyl ester yields obtained by using 95.0% ethanol, 99.9% ethanol and 99.9% methanol were relatively high and the enzymatic activity remained constant during the 5 repeated uses. The combination of products (glycerol and biodiesel) and substrates in the mixture could help protect a *Candida rugosa* lipase against violent conditions.

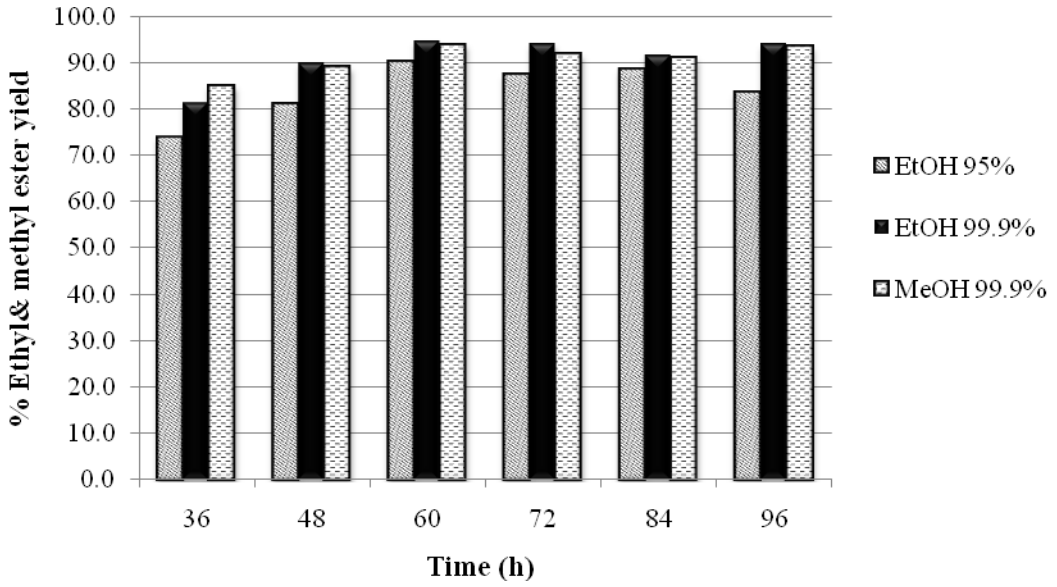


Figure 4.8 Effect of substrate mixture to reusability of *Candida rugosa* lipase under the following conditions: 10% by wt of lipase to oil, molar ratio of ethanol to palm oil was 9:1, molar ratio of ethanol to palm fatty acid was 3:1, reaction temperature of 45°C, shaking speed of 250 rpm, 0.001 M phosphate buffer, CRLAE 2% coated 3 layers and the addition time of palm fatty acid to the reaction medium at 24 h.

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

The main objectives of the present work were to develop a new lipase immobilizing method by adsorption of lipase on loofa followed by the entrapment in calcium alginate matrix (CRLAE), for using in biodiesel production. In this work, the suitable preparation condition of loofa-lipase (CRLA) was at 10% (wt) *Candida rugosa* lipase adsorbed on loofa. The CRLAE carrier preparation was by the entrapment of CRLA in calcium alginate gel layers. The condition for the carrier formation was by using 2% (w/v) Na-alginate. The condition of reaction was 45^{o}C, 250 rpm and 3 layers of alginate coating. From the repeated use of the immobilized lipase, methyl ester yield was decreased due to the inactivation of the enzyme caused by the denaturation of protein.

For the biodiesel production, the optimal condition for using ethanol were at 99.9% ethanol, 0.001 M phosphate buffer and 36 h of reaction time, yielded approximately 78.87% and 96.18% ethyl ester by the free lipase and CRLAE, whereas the optimal condition for using methanol were at 99.9% methanol, 0.001 M phosphate buffer and 12 h of reaction time, the biodiesel production by the free lipase and immobilized lipase in CRLAE resulted in methyl ester yields of about 96.97% and 97.92%, respectively. The results from the reuse of the immobilized lipases revealed the significantly reduction of ethyl ester yield from the 1st run to the 2nd run and from the 1st run to the 5th run for methyl ester yield.

On the other hand, when using the mixture of purified palm oil and palm fatty acid in the initial mass ratio of 70:30 as substrate, the result showed that, under semi-continuous feeding of palm fatty acid, the ethyl ester yields obtained by using 95.0% ethanol, 99.9% ethanol and 99.9% methanol were relatively high and the enzymatic activity remained constant during the 5 repeated uses.

5.2 Recommendations

i. Investigate the enzyme leakage by protein testing.

ii. Investigate the new alternative high activity and stability types of enzyme that appropriate for the reaction or crude enzyme for continuous reaction.

iii. To avoid serious degradation of lipase activity in the presence of high concentration of ethanol, the strategy for continuous ethanol and fatty acid feeding should be developed.

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APPENDICES

APPENDIX A

EXPERIMENTAL DATA FOR ANALYSIS

Experimental data of enzymatic transesterification reaction of purified palm oil and esterification reaction of palm fatty acid in batch system

Table A-1 The present to ethyl ester yield by using the different techniques of immobilized lipase, molar ratio of purified palm oil to ethanol was 1:9, 10% lipase based on oil weight, 45°C, 250 rpm, reaction time 48 h.

Reaction time (h)	% Ethyl ester yield						
	Free lipase	Cycle 1			Cycle 2		
		CRLA	CRLAE 1%	CRLAE 2%	CRLA	CRLAE 1%	CRLAE 2%
0	2.02	1.56	1.82	1.12	-	-	-
12	25.52	44.96	57.19	60.60	14.63	17.96	18.97
24	52.21	67.96	63.27	64.24	19.57	21.52	21.58
36	81.91	80.14	86.50	88.79	23.43	24.66	30.53
48	73.26	79.15	79.89	86.53	27.03	26.01	35.35

Table A-2 Effect of the buffer types to ethyl ester yield by using 10% lipase by wt of oil, 95% ethanol and reaction temperature of 45°C, shaking speed 250 rpm and ethanol to palm oil ratio 9:1 and 36h.

Reaction time (h)	% Ethyl ester yield									
	Free lipase		Cycle 1				Cycle 2			
			CRLA		CRLAE 2%		CRLA		CRLAE 2%	
	Tris-HCl	Phosphate	Tris-HCl	Phosphate	Tris-HCl	Phosphate	Tris-HCl	Phosphate	Tris-HCl	Phosphate
12	16.54	27.96	9.12	29.05	31.00	62.44	4.69	9.01	5.09	13.78
24	17.96	30.31	30.31	50.13	50.55	67.33	6.74	17.11	22.07	32.61
36	19.49	61.15	42.03	61.24	62.27	80.03	9.92	24.71	29.23	39.25

Table A-3 Effect of the concentration of buffer on ethyl ester yield by using 10% lipase by wt of oil, 95% ethanol and reaction temperature of 45°C, shaking speed 250 rpm and ethanol to palm oil ratio 9:1 and 36h.

Reaction time (h)	% Ethyl ester yield									
	Free lipase		CRLA				CRLAE 2%			
			Cycle 1		Cycle 2		Cycle 1		Cycle 2	
	0.1 M	0.001 M	0.1 M	0.001 M	0.1 M	0.001 M	0.1 M	0.001 M	0.1 M	0.001 M
12	27.96	41.42	29.05	44.69	9.01	13.88	62.44	71.88	13.78	19.10
24	30.31	56.13	50.13	52.15	17.11	17.38	67.33	80.71	32.61	29.48
36	61.15	63.28	61.24	66.43	24.71	25.42	80.03	94.46	39.25	48.79

Table A-4 Repeatability and effect of Ca-alginate layer on ethyl ester yield by using 10% lipase by wt of oil, 95% ethanol, and reaction temperature of 45°C, shaking speed 250 rpm and ethanol to palm oil ratio 9:1.

Reaction time (h)	% Ethyl ester yield								
	Free lipase	CRLAE 2%							
		Cycle 1				Cycle 2			
		C1*	C2*	C3*	C4*	C1*	C2*	C3*	C4*
12	29.38	58.35	45.03	49.15	42.29	23.08	14.58	29.30	21.16
24	55.77	65.75	65.45	68.73	45.73	31.21	28.60	38.94	28.04
36	67.70	93.28	86.04	89.06	82.88	33.86	40.77	55.69	36.41

*C1 = Coated 1 layer, C2 = Coated 2 layers,

C3 = Coated 3 layers, C4 = Coated 4 layers

Table A-5 Repeatability and effect of concentration of ethanol on the yield of fatty acid ethyl ester comparison between 95% ethanol and 99.9% ethanol when using the condition as follow; 10% by wt of lipase to oil, molar ratio of ethanol to palm oil ratio was 9:1, reaction temperature of 45°C and shaking speed of 250 rpm.

Reaction time (h)	% Ethyl ester yield					
	Free lipase		CRLAE 2%			
			Cycle 1		Cycle 2	
	95 %	99.9 %	95 %	99.9 %	95 %	99.9 %
12	29.38	42.46	49.15	65.80	29.30	35.01
24	55.77	58.39	68.73	72.55	38.94	42.44
36	67.70	78.87	89.06	96.18	55.69	61.27

Table A-6 Effect of methanol to methyl ester yield when using the condition as follow; 10% by wt of lipase to oil, molar ratio of methanol to palm oil was 9:1, reaction temperature of 45°C and shaking speed of 250 rpm, 0.001 M phosphate buffer, 99.9% methanol, CRLAE 2% coated 3 layers and 12 h.

Reaction time (h)	% Methyl ester yield	
	Free lipase	CRLAE 2%
4	28.46	49.24
8	57.40	83.08
12	96.97	97.92

Table A-7 Repeatability and effect of methanol to ethyl ester yield when using the condition as follow; 10% by wt of lipase to oil, molar ratio of methanol to palm oil was 9:1, reaction temperature of 45°C, shaking speed of 250 rpm, 0.001 M phosphate buffer, 99.9% methanol, CRLAE 2% coated 3 layers and 12 h.

Reaction time (h)	% Methyl ester yield				
	Cycle 1	Cycle 2	Cycle 3	Cycle 4	Cycle 5
12	97.92	51.70	37.26	27.20	21.66

Table A-8 Effect of substrate mixture to repeatability when using the condition as follow; 10% by wt of lipase to oil, molar ratio of ethanol to palm oil was 9:1, molar ratio of ethanol to palm fatty acid was 3:1, reaction temperature of 45°C, shaking speed of 250 rpm, 0.001 M phosphate buffer, CRLAE 2% coated 3 layers and the addition time of palm fatty acid to the reaction medium at 24 h.

Reaction time (h)	% Ester yield			
	Free lipase	Ethanol 95%	Ethanol 99.9%	Methanol 99.9%
36	78.5	73.8	81.1	84.8
48	82.0	81.1	89.8	88.9
60	-	89.9	94.6	93.7
72	-	87.2	93.9	91.6
84	-	88.5	91.6	90.9
96	-	83.3	93.8	93.3

APPENDIX B

CALCULATION ETHYL ESTER AND METHYL ESTER PERCENT YIELD

Calculation of molecular weight of palm fatty acids

The molecular weight of palm fatty acids is calculated from the weighted average of the molecular weight of the five key fatty acids: Palmitic acid, Oleic acid, Stearic acid, Linoleic acid and Linolenic acid. The compositions of palm fatty acids are shown in Table B-1

Table B-1 Composition and molecular waight of key components in palm fatty acids.

Palm fatty acids	%Weight Fraction	Molecular weight
Palmitic acid	42.8	256.43
Oleic acid	40.5	282.47
Stearic acid	4.5	284.5
Linoleic acid	10.1	280.45
Linolenic acid	2.1	278.43

The data in Table B-1 can be used to compute the molecular weight of palm fatty acids as shown below:

1 mole of palm fatty acids

$$M_w = \text{Sum } (M_{Fa} \times \%\text{Weight fraction fatty acids}) \text{.....B-1}$$

Where M_w = Molecular weight of fatty acids

M_{Fa} = Molecular weight of each fatty acids

Such as (Data in the table B-1) apply in equation B-1

$$= (0.428 \times 256.43) + (0.405 \times 282.47) + (0.045 \times 284.5) + (0.101 \times 280.45) + (0.021 \times 278.43)$$

$$= 271.13$$

Calculation molecular weight of purified palm oil

Table B-2 Fatty acid composition in purified palm oil sample

Fatty acid	wt%
Lauric	0.10
Myristic	1.00
Palmitic	42.8
Stearic	4.5
Oleic	40.5
Linoleic	10.10
Linolenic	0.20

Molecular weight of triglyceride

$$Mw_{TG} = 3R_{aver} + 173 \qquad \text{..... B-2}$$

$$R_{aver} = \sum\left(\frac{\%Fa_n}{100} \times Mw_n\right) \qquad \text{..... B-3}$$

Where

Mw_{TG} = molecular weight of triglyceride

R_{aver} = average molecular weight of fatty acid

$\%Fa_n$ = percent of fatty acid in vegetable oil

Mw_n = molecular weight of fatty acid

Example Find molecular weight of palm oil

Such as (Data in the Table B-2) apply in equation B-2 and B-3

$$R_{aver} = \left(\frac{0.1}{100} \times 200\right) + \left(\frac{1}{100} \times 228\right) + \left(\frac{42.8}{100} \times 256\right) + \left(\frac{4.5}{100} \times 284\right) + \left(\frac{40.5}{100} \times 282\right) + \left(\frac{10.1}{100} \times 280\right) + \left(\frac{0.2}{100} \times 278\right)$$

$$= 267.08$$

$$3R_{aver} = 3 \times 267.08 = 801.2$$

Mw_{TG}= 801.23 + 173 = 974.23

Calculation of reactants

Molar ratio of ethanol to reactants: $\frac{N_{EtOH}}{N_{reactant}}$

Volume of reactant: $\left(\frac{Mw_{reactant} \times N_{reactant}}{\rho reactant}\right) + \left(\frac{Mw_{EtOH} \times N_{EtOH}}{\rho EtOh}\right) = V_{reaction}$

Volume of ethanol: $V_{reaction} - V_{reactant} = V_{EtOH}$

Calculation of catalyst

Example

Base on volume of purified palm is 15 g. The lipase to purified palm oil mass ratio of 10%

Weight of catalyst = Weight of purified palm oil x Catalyst to purified palm oil mass ratio

= 15x10/100 = 1.5 g

Calculation of the percent ethyl esters yield

The percent ethyl ester yield is defined as

$$\%Yield\ of\ ethyl\ ester = \frac{W_{EE}}{W_{Fa}} \times 100 \qquad \text{..... B-4}$$

$$W_{EE} = W_{EP} + W_{ES} + W_{EO+}W_{EL}$$

Where

W_{EE}= Weight of ethyl ester (g)

W_{Fa}= Weight of fatty acid (g)

W_{EP}= Weight of ethyl palmitate (g)

W_{ES}= Weight of ethyl stearate (g)

W_{EO}= Weight of ethyl oleate (g)

W_{EL}= Weight of ethyl linoleate (g)

Calculation weight of each ethyl ester

$$W_{EE} = (\frac{C \times V_{TD}}{V_s}) \times V_P \qquad \text{..... B-5}$$

Where

W_{EE} = Weight of ethyl ester (g)

C = Concentration of each ethyl ester from calibration curve (g/ml)

V_{TD} = Total volume dilute (ml)

V_s = Volume product dilute (ml)

V_P = Total volume of product (ml)

The rate of ethyl esters for each fatty acid can be determined from GC data with corresponding calibration equation. Below are standard calibration curves for the key ethyl esters (Figure B-1-B-7). Then apply in the equation B-5

Table B-3 Data of area and concentration of ethyl ester standard

	area	conc.(g/ml)
EP	0	0
	2967730	0.25
	8295163	0.5
	14341159	1
EO	0	0
	1187431	0.25
	2291151	0.5
	5690606	1
EL	0	0
	1262521	0.25
	3052938	0.5
	6020661	1
ES	0	0
	2728225	0.25
	5853413	0.5
	10061759	1

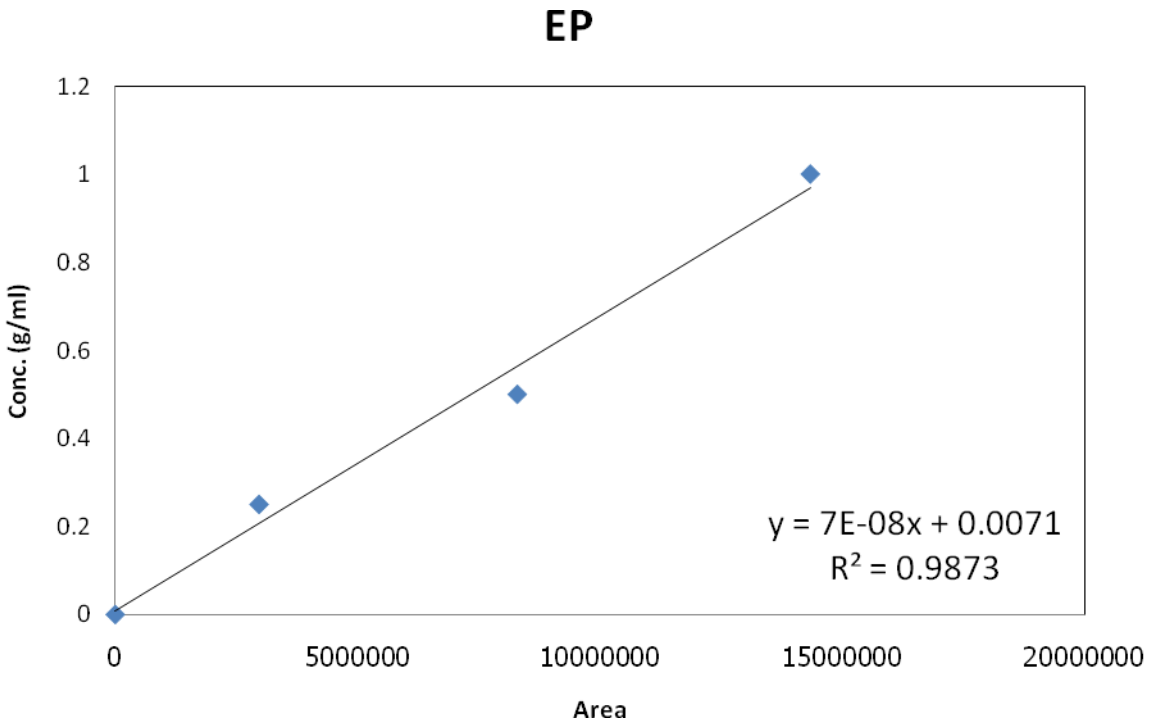

Figure B-1 Standard calibration curve for ethyl palmitate

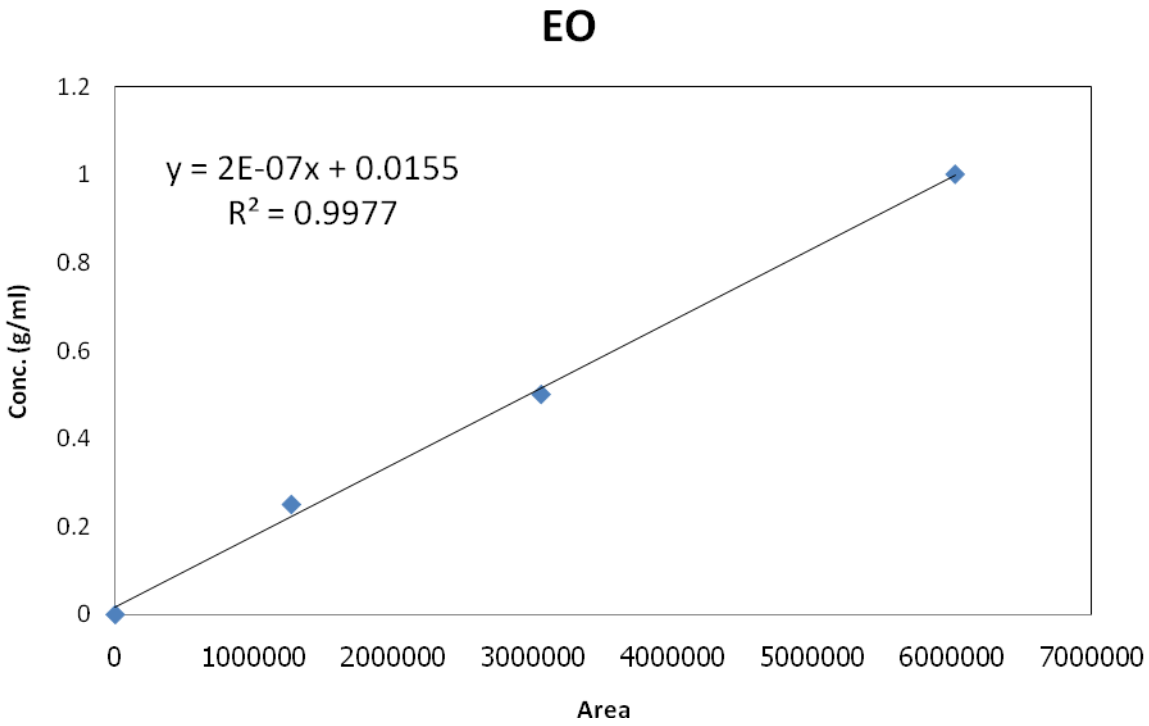

Figure B-2 Standard calibration curve for ethyl oleate

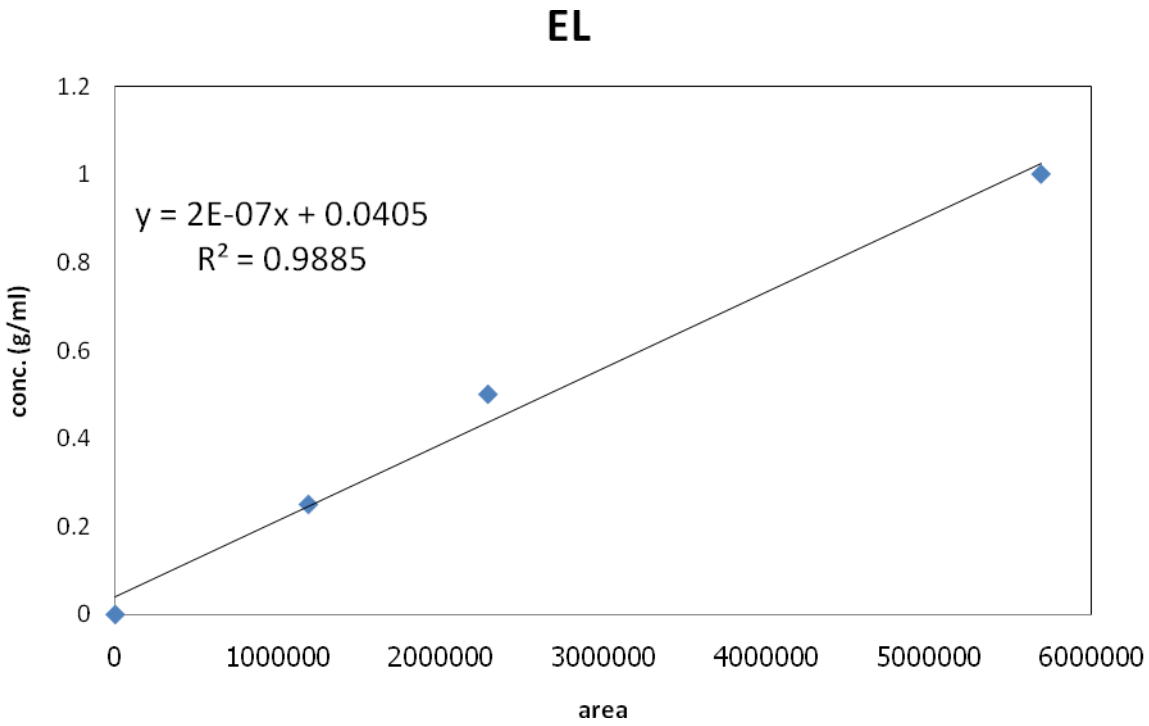

Figure B-3 Standard calibration curve for ethyl linoleate

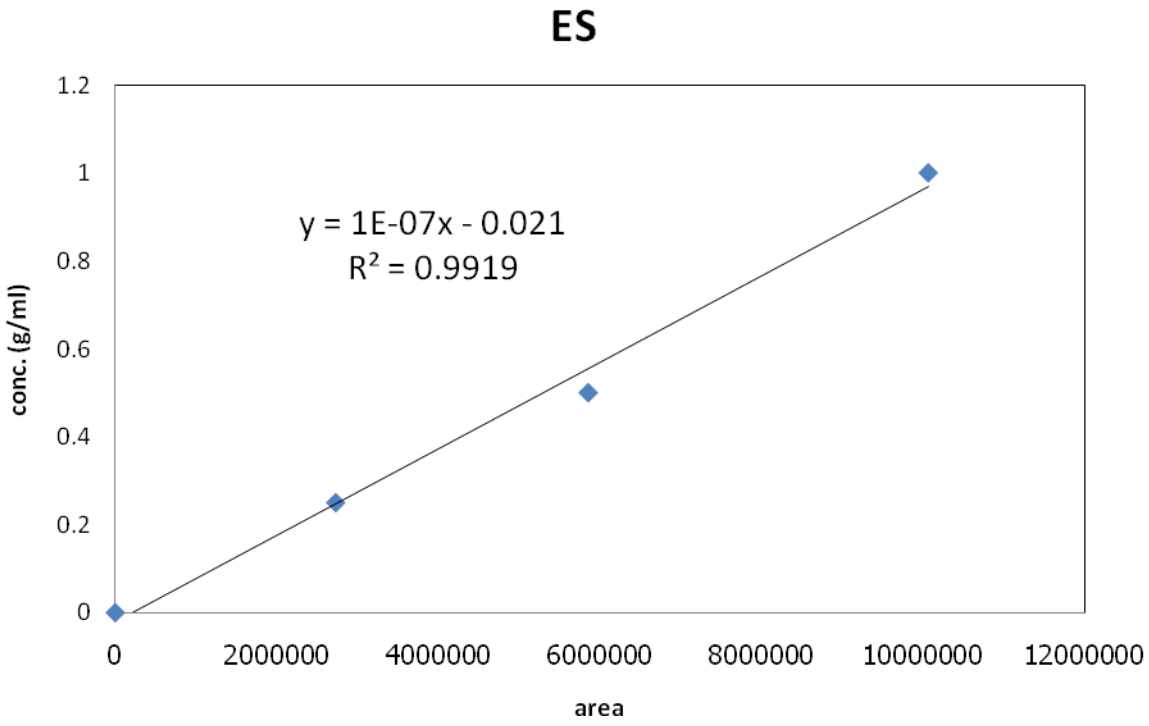

Figure B-4 Standard calibration curve for ethyl stearate

Table B-4 Data of area and concentration of methyl ester standard

	area	conc.(g/ml)
MP	0	0
	4926913	0.25
	10981567	0.5
	20708436	1
ML	0	0
	7113245	0.25
	12563343	0.5
	23917464	1
MO	0	0
	6803727	0.25
	12495207	0.5
	23393602	1

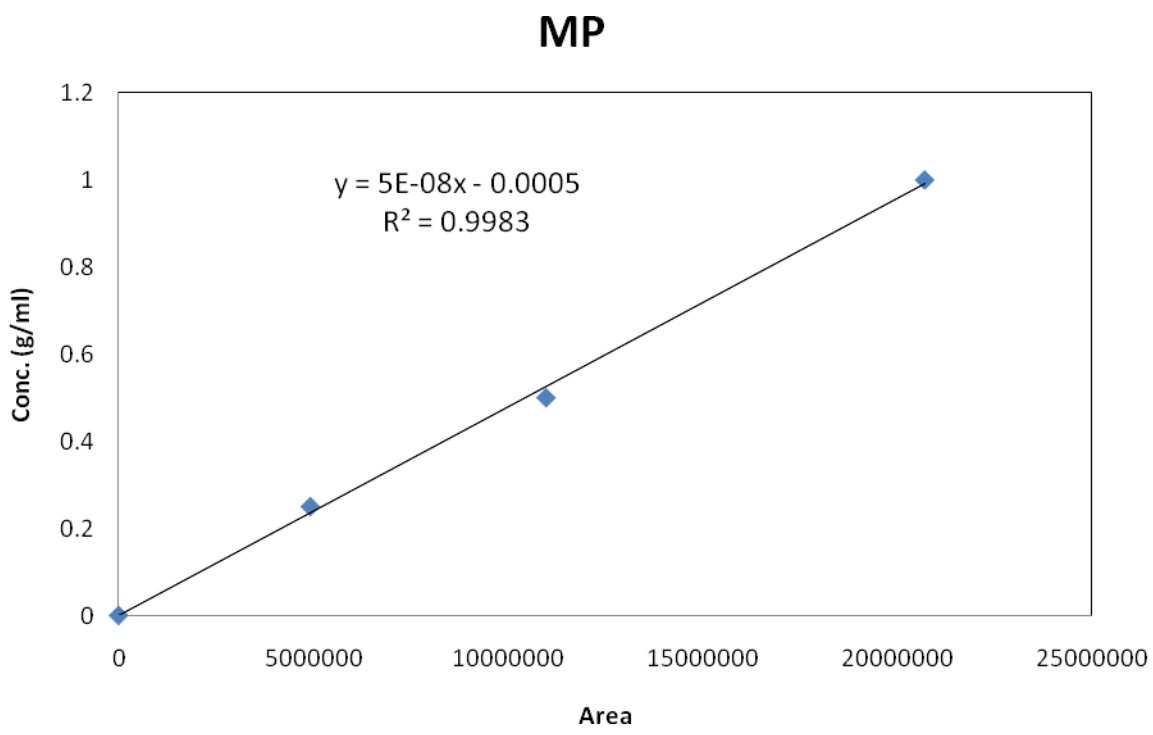

Figure B-5 Standard calibration curve for methyl palmitate

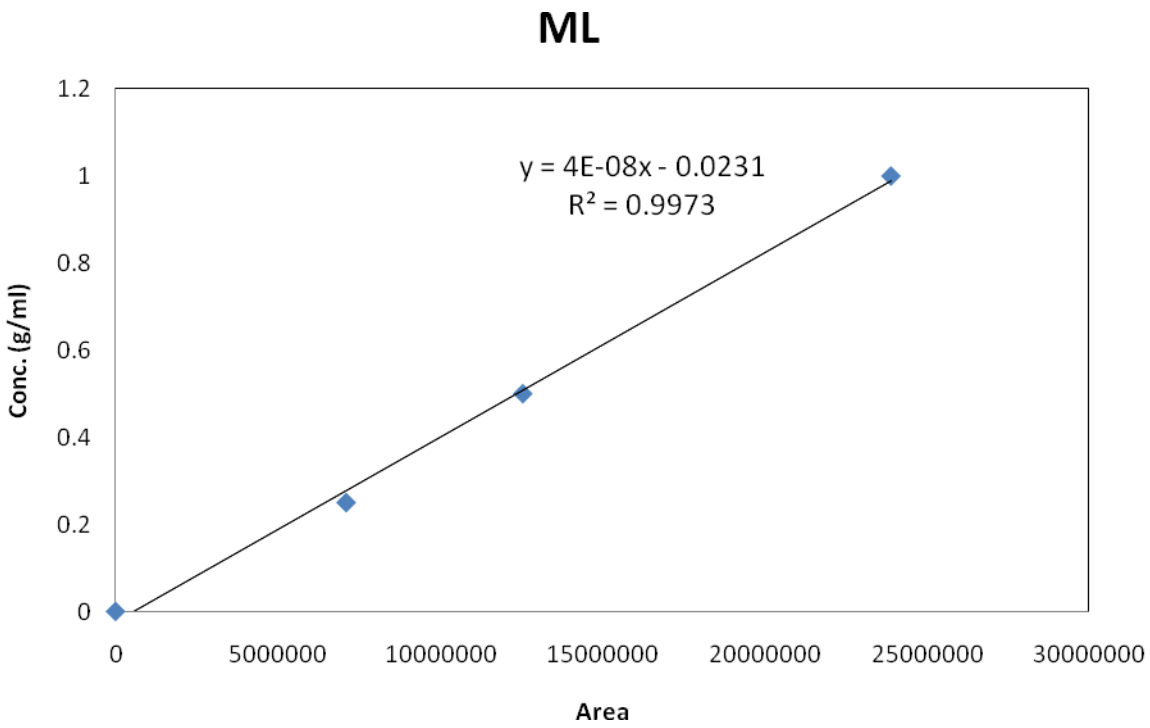

Figure B-6 Standard calibration curve for methyl linoleate

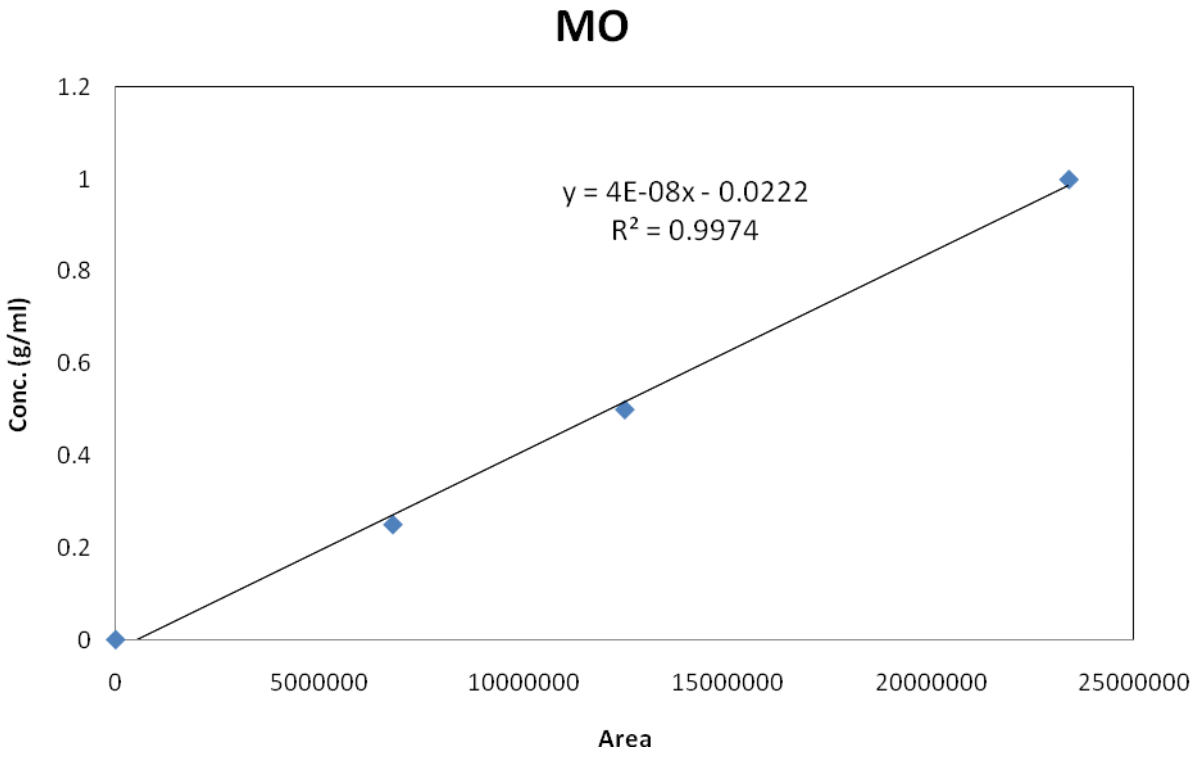

Figure B-7 Standard calibration curve for methyl oleate

VITA

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