การผลิตเอทิลเอสเทอร์จากน้ำมันปาล์มและกรดไขมันปาล์ม
ซึ่งถูกเร่งปฏิกิริยาด้วยไลเปสจาก *Candida rugosa* ที่ถูกตรึง

นายชนุดม เมืองฉิม

ETHYL ESTER PRODUCTION FROM PALM OIL/PALM FATTY ACID
CATALYZED BY IMMOBILIZED *CANDIDA RUGOSA* LIPASE

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A Thesis Submitted in Partial Fulfillment of the Requirements
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CANDIDA RUGOSA LIPASE

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ชนุดม เมืองฉิม : การผลิตเอทิลเอสเทอร์จากน้ำมันปาล์มและกรดไขมันปาล์มซึ่งถูกเร่ง ปฏิกิริยาด้วยไลเปสจาก *Candida rugosa* ที่ถูกตรึง (ETHYL ESTER PROCUCTION FROM PALM OIL/PALM FATTY ACID CATALYZED BY IMMOBILIZED *CANDIDA RUGOSA* LIPASE) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร. เหมือนเดือน พิศาลพงศ์, 85 หน้า.

แบคทีเรียเซลลูโลส(BC)เป็นวัสดุที่มีศักยภาพในการนำมาประยุกต์ใช้เป็นตัวพยุงในการตรึง เอนไซม์เนื่องจากสมบัติที่โดดเด่นทั้งทางกายภาพและทางชีวภาพ ในงานวิจัยนี้ ได้นำทั้งวิธีการดูด ซับและการห่อหุ้มมาร่วมกันสำหรับการตรึงเอนไซม์ไลเปส ในขั้นตอนแรกเป็นการดูดซับทาง กายภาพของสารละลายไลเปสจาก*Candida rugosa* (CARL) บนตัวพยุงที่ผ่านการทำแห้งเยือก แข็งของแบคทีเรียเซลลูโลสผสมอัลจิเนต ในขั้นตอนที่สอง ไลเปสที่ถูกตรึงบนตัวพยุงของแบคทีเรีย เซลลูโลสผสมอัลจิเนตจะถูกนำไปห่อหุ้มภายในเมทริกซ์ของแคลเซียมอัลจิเนต (CARLE) สภาวะ ที่เหมาะสมของกระบวนการตรึงเอนไซม์ประกอบด้วย เอนไซม์ไลเปสเท่ากับ 10% (น้ำหนักเอนไซม์ ต่อน้ำมัน) ขนาดเส้นผ่าศูนย์กลางตัวพยุงเท่ากับ 1.2 เซนติเมตร ที่ความหนาของแผ่นตัวพยุงเท่ากับ 3 มิลลิเมตร สารละลายโซเดียมอัลจิเนตที่ความเข้มข้นเท่ากับ 2เปอร์เซ็นต์(น้ำหนักต่อปริมาตร)ถูก ใช้ในการทำเป็นชั้นเจลโดยใช้สารสร้างพันธะคือแคลเซียมคลอไรด์ที่ความเข้มข้นเท่ากับ 120 มิลลิ โมลาร์ เอนไซม์ที่ถูกตรึงถูกใช้เป็นตัวเร่งปฏิกิริยาสำหรับการผลิตไบโอดีเซลจากน้ำมันปาล์มและ กรดไขมันปาล์มกับเอทานอล ในงานวิจัยนี้สภาวะที่มีความเหมาะสมในการผลิตไบโอดีเซลโดย ปฏิกิริยาทรานส์เอสเทอริฟิเคชันด้วยเอนไซม์คือ สัดส่วนโมลาร์ของน้ำมันต่อ 95%เอทานอลเท่ากับ 1:9 อุณหภูมิในการเกิดปฏิกิริยาคือ 45 °C ความเร็วในการปั่นกวนเท่ากับ 250 รอบต่อนาที เวลา ในการเกิดปฏิกิริยาเท่ากับ 36 ชั่วโมง และจำนวนชั้นของแคลเซียมอัลจิเนตซึ่งหุ้มทับบน CARLE เท่ากับ 3 ชั้น (CARLE-3L) สัดส่วนผลได้ของเอสเทอร์ที่ได้จากการใช้เอนไซม์อิสระ(92.61%) สูง กว่าการใช้เอนไซม์ที่ถูกตรึงและห่อหุ้มด้วยแคลเซียมอัลจิเนตจำนวน 3 ชั้น(82.82%) 4   ชั้น (70.60%)และ 2 ชั้น(66.94%)ตามลำดับ อย่างไรก็ตาม เอนไซม์ที่ถูกตรึงมีสมบัติความต้านทานต่อ ความร้อน แรงทางกลและสภาวะความเป็นกรด สูงกว่าเอนไซม์อิสระ นอกจากนี้การเพิ่มจำนวน ชั้นในการห่อหุ้มด้วยแคลเซียมอัลจิเนตสามารถช่วยลดการหลุดออกของเอนไซม์และปกป้อง เอนไซม์ต่อสภาวะที่รุนแรง โดยพบว่า วิธีการเติมกรดไขมันอิสระในช่วงเวลาต่างๆสามารถช่วยลด การเสื่อมสภาพของตัวเร่งปฏิกิริยาทางชีวภาพจากความเป็นกรดของกรดไขมันอิสระ

ภาควิชา	วิศวกรรมเคมี	ลายมือชื่อนิสิต
สาขาวิชา	วิศวกรรมเคมี	ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก………………
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CHANUDOM MUANGCHIM: ETHYL ESTER PROCUCTION FROM
PALM OIL/PALM FATTY ACID CATALYZED BY IMMOBILIZED
CANDIDA RUGOSA LIPASE. ADVISOR: ASSOC.PROF.MUENDUEN
PHISALAPHONG, Ph.D., 85 pp.

Bacterial cellulose (BC) is a promising material to be applied as an enzyme-immobilizing support because of its unique physical and biological properties. In this study, the integration method of adsorption and entrapment was applied for lipase immobilization. Firstly, physical adsorption of soluble *C. rugosa* (CARL) on freeze dried BC-alginate support was performed. Secondly, the immobilized lipase on the BC-alginate support was entrapped within the matrix of calcium alginate (CARLE). The suitable conditions of the immobilization process were as follows: 10% (w/w) CARL to oil; the diameter of circular freeze dried BC-alginate piece of 1.20 cm with the thickness of 3 mm. Sodium alginate solution at 2% (w/v) was used to form gelling layer with a cross linking agent of 120 mM calcium chloride. The immobilized enzyme was applied to catalyze the reaction for biodiesel production from palm oil/ palm fatty acid with ethanol. In this research, the optimal conditions based on the enzymatic transesterification were at the molar ratio of palm oil to 95% (v/v) ethanol of 1:9, reaction temperature of 45 °C, shaking speed at 250 rpm, reaction time of 36 hours and 3 layers of calcium alginate coated on CARLE (CARLE-3L). The yield of the ester obtained by using free lipase (92.61%) was higher than that of CARLE-3L (82.82%), CARLE-4L (70.60%) and CARLE-2L (66.94%), respectively. However, the immobilized enzyme showed higher thermal, mechanical and acid resistance properties than the free enzyme. Besides, increasing of the entrapped layers with calcium alginate could prevent leaching and protect enzyme against violent conditions. It was found that the method of adding free fatty acid with interval time could be applied to decrease the deactivation of the biocatalyst from the acidity of the free fatty acid.

Department :   Chemical engineering   Student's Signature ....

Field of Study :   Chemical engineering   Advisor's Signature ....

Academic Year : 2010 ....

ACKNOWLEDGEMENTS

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LIST OF ABBREVATIONS

BC	Bacterial cellulose
$CaCl_2$	Calcium chloride
CARL	*Candida rugosa* lipase
CARLE	Soluble *C. rugosa* was adsorbed on freeze dried BC-alginate plate and entrapped within the matrix of calcium alginate
$(C_6H_7O_2Na)_n$	Sodium alginate
CH_3COOH	Acetic acid glacial
C_6H_{14}	n-Hexane
$C_{18}H_{36}O_2$	Ethyl palmitate
$C_{20}H_{38}O_2$	Ethyl oleate
$C_{20}H_{36}O_2$	Ethyl linoleate
$C_{20}H_{40}O_2$	Ethyl stearate
CPD	Critical point drying
DG	Diglyceride
EtOH	Ethanol (C_2H_5OH)
FAEE	Fatty acid ethyl ester
FAME	Fatty acid methyl ester
FFA	Free fatty acid
H_2SO_4	Sulfuric acid
KOH	Potassium hydroxide
MeOH	Methanol
MG	Monoglyceride
NaOH	Sodium hydroxide
Novozym® 435	*Candida antarctica* lipase immobilized within acrylic resin
RBDPO	Refined bleached deodorized palm oil
SA	Sodium alginate
SEM	Scanning electron microscope
TAG	Triglyceride
WCO, WCPO	Waste cooking oil, Waste cooking palm oil

CHAPTER I

INTRODUCTION

1.1 Motivation

According to the increment of the global human population and industrialization all around the world, the demand of the energy from the fossil fuel which is non-renewable energy also increases. It is not replaced as soon as we use it. Therefore, the petroleum reserves have been reducing for long time ago. On the other hand, the renewable energy such as biodiesel is a promising alternative energy source to the fossil fuel. The most important benefit of biodiesel is the reduction of environmental pollution. This is because it is clean and environmental safe. There is between 50% and 95% of hydrocarbon in crude oil. About 98% of carbon emission effect is from fossil combustion (Demirbas, 2009).

Basically, biodiesel is an alternative energy obtained from vegetable oils by transesterification with short chain alcohol to form esters or bioenergy. The name of the esters depends on each type of alcohols, for example; fatty acid methyl ester (FAME) is the product obtained from methanol. The advantages of using biodiesel over fossil fuel are higher cetane number, biodegradability (Krawczyk, 1996; Ma and Hanna, 1999; Demirbas, 2009; Zhang and Jiang, 2008), renewability, higher combustion efficiency, lower sulfur and aromatic content (Ma and Hanna, 1999). Its domestic origin would reduce dependency on imported fuel. Moreover, biodiesel increases lubricity which extends lifetime of diesel engine (Lotero et al., 2005). Increasing of this fuel would considerably reduce the amount of producing carbon dioxide. The comparison between biodiesel and the petroleum-derived diesel shows that biodiesel significantly reduces emission lower than diesel with its oxygen content of 10-11 % (Lotero et al., 2005).

However, there are some major disadvantages of biodiesel such as its higher viscosity, lower energy content, higher cloud point and pour point, higher nitrogen oxide emission rates, lower engine speed and power, injector coking, engine compatibility, higher engine wear and higher price (Demirbas, 2009). Biodiesel

production are still rather high cost compared to the cost of fossil fuel(price for biodiesel not counting tax breaks is about $2.02/gallon depending on the feedstock used for its preparation). The price for petroleum-based diesel is about $1.87/gallon (Goodwin et al., 2004; Lotero et al., 2005). Therefore, there are many research studies for the development of a novel method to lower the cost with accepted quality. There are two main factors that affect the cost of biodiesel; the cost of raw material and the cost of processing (Ma and Hanna, 1999). The cost of biodiesel production is mainly about the feedstock which affects the cost of the finished product up to 60-75 %(Yuan et al.,2008; Merve and Filiz, 2004; Xue et al.,2008). Waste fat of non-edible oil type could be used as the low cost feed stock of the raw material. This is because wastes of non-edible oils are less expensive than virgin oils (Fjerbaek et al., 2009; Srivastava and Prasad, 2000). In addition, to lower production cost, simplifying the operations and eliminating waste stream are considered.

In order to improve the reaction rate and yield, homogeneous and heterogenous catalysts, as well as free or immobilized enzymes are used in the reaction. Recently, transesterification of triglycerides and refined/edible type oils using methanol and ethanol with alkaline catalyst is the most considerable reaction of producing biodiesel (Fjerbaek et al., 2009). Chemical catalysts provide the yield of transesterification close to 99%; however, there are drawbacks of refined plant oil related to recovery of pure glycerol and formation of soap and pigments at the downstream process. Unless, the concentration of free fatty acid used for biodiesel production is lower than 0.5%, the yield of transesterificantion is below 99% (Antczak et al., 2009). Although, there is no soap formed with acid catalyst, higher temperature and higher substrate molar ratios are needed (Casimir et al., 2007). Consequently, the pretreatment is needed of the raw material with high water and free fatty acid (FFA) in order to esterify FFA to reduce soap formation and alleviate the separation of biodiesel and glycerol. However, the removal of catalyst and alkaline from waste water in the wastewater treatment is concerned to devouring of energy and environmental problem (Canakci and Van Gerpen, 2001; Yuan et al., 2008; Fjerbaek et al., 2009).

Interestingly, lipases are widespread enzymatic transesterification applied to refined plant oils or other waste fats and oils with various alcohols such as methanol,

ethanol and so on (Antczak et al., 2009). There are many advantages of using lipase-catalyzed enzyme (Kumeri et al., 2007; Akhor et al., 2008; Antczak et al., 2009; Fjerbaek et al., 2009). Particularly, enzymes are highly selective under mind conditions (Kreiner et al., 2005). These do not form soaps and can properly esterify both free fatty acid (FFA) and triglycerides (TAG) in one step. The glycerol produced as a by-product easily recovered and purified ester to accomplish the target (Fukuda et al., 2001). Hence, they are interested in an industrial-scale production, especially in the case of high FFA such as waste oil with high acid value (Li et al., 2009; Rao et al., 1992; Lai et al., 2005; Kumari et al., 2007). However, one of the common drawbacks of using enzyme is expensive. Therefore, there are different strategies trying to modify the enzyme by immobilizing to be more reusable enzyme. Normally, immobilized enzymes are more stable than free enzyme. These beneficial biocatalysts are easy to handle. Recovery and recycling from the biodiesel process make the process lower the cost (Bajaj et al., 2010).

Technique of biocatalyst's entrapment by physical restriction is very simply. To entrap the biocatalyst by making matrix of polymer gel is one of the famous methods due to mild gelling properties and non-toxicity (Won K et al., 2005). This study aims to develop the immobilized lipase by using bacterial cellulose (BC) as a supporter. There are many advantages of BC from its chemical and physical properties such as high mechanical strength, permeability, high crystallinity, high hydrophilicity, and ultrafine network structure. Besides, BC materials have high surface areas and good capacities for the adsorption liquid. Therefore BC is a promising material to be applied as a biocatalyst support in biodiesel production. Besides, there are plenty of agricultural raw-materials, palm oil and palm fatty acid in Thailand.

1.2 Objectives

To examine the use of immobilized lipase in BC as a biocatalyst for biodiesel production from palm oil/ palm fatty acid and ethanol.

1.3 Working scopes

Candida rugosa lipase is used as a biocatalyst in this research. Bacterial cellulose (BC) is chosen as an immobilized support material. Freeze dried BC is from the ratio of BC: alginate at 70:30 by weight. The optimal conditions for biodiesel production from palm oil/ palm fatty acid in solvent-free system are investigated; molar ratio of substrate (triglycerides: ethanol) at 1:9, reaction time at 12, 24 and 36 h, with amount of catalyst which relates to vegetable oil (5% and 10% w/w). The immobilized lipase was also examined for its stability and reusability for biodiesel production.

1.4 Expected benefits

This investigation will provide beneficial information for biodiesel production using immobilized lipase. Biodiesel is a renewable energy source to replace diminishing fossil fuels. Ethanol and biodiesel show positive picture to the environment. The benefits of this technology are not only the reduction of dependency on imported diesel fuel but also shortening of global warming pollution. Moreover, using of palm raw material which renewable resources in local area also reinforces agriculture in our country.

CHAPTER II

BACKGROUND AND LITERATURE REVIEW

2.1 Fats and Oils

Fats and oils are ester of fatty acid and glycerine. The prominent property of the fats and oils is immiscible with water. Both fats and oils compose of fatty ester of glycerol called "triglyceride" as the Figure 2.1 below. Three fatty acids are linked to glycerol with ester linkage (Ali and Hanna, 1994). Table 2.1 shows the compositions of fatty acid from carbon amount 16 atoms to 24 atoms. There are saturated and unsaturated fatty acids in carbon chain. For example, 16:0 denotes that there are carbon amount 16 atoms with no double bond; 22:1 denotes that there are carbon amount 22 atoms with one double bond. Normally, saturated fatty acid is solid state at room temperature. On the other hand, unsaturated fatty acid is liquid. At the high temperature, however, the unsaturated fatty acid starts changing to form gum which is the polymerization of unsaturated fatty acids. Table 2.2 shows fatty acid compositions of common oil sources.
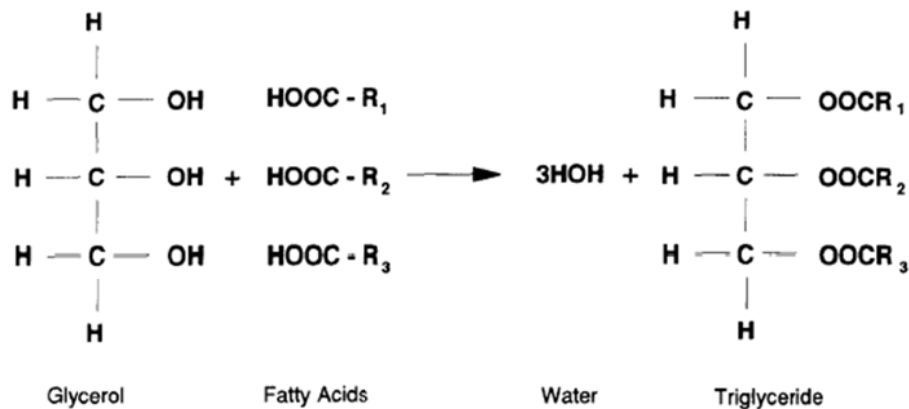
Figure 2.1 Chemical structure of vegetable oil.

Table 2.1 Chemical properties of vegetable oil (Goering et al., 1982).

Vegetable oil	Fatty acid composition, % wt									Acid[a] value	Phos[b] ppm	Peroxide[c] value
	16:0	18:0	20:0	22:0	24:0	18:1	22:1	18:2	18:3			
Corn	11.67	1.85	0.24	0.00	0.00	25.16	0.00	60.60	0.48	0.11	7.00	18.4
Cottonseed	28.33	0.89	0.00	0.00	0.00	13.27	0.00	51.51	0.00	0.07	8.00	64.8
Crambe	2.07	0.70	2.09	0.80	1.12	18.86	58.51	9.00	6.85	0.36	12.00	26.5
Peanut	11.38	2.39	1.32	2.52	1.23	48.28	0.00	31.95	0.93	0.20	9.00	82.7
Rapeseed	3.49	0.85	0.00	0.00	0.00	64.40	0.00	22.30	8.23	1.14	18.00	30.2
Soybean	11.75	3.15	0.00	0.00	0.00	23.26	0.00	55.53	6.31	0.20	32.00	44.5
Sunflower	6.08	3.26	0.00	0.00	0.00	16.93	0.00	73.73	0.00	0.15	15.00	10.7

[a] Acid values are milligrams of KOH necessary to neutralize the FFA in 1 g of oil sample.

[b] Phosphatide (gum) content varies in direct proportion to phosphorus value

[c] Peroxide values are milliquivalents of peroxide per 1,000 g of oil sample, which oxidize potassium iodide under conditions of the test.

Table 2.2 Fatty acid composition-common oil source (Kincs, 1985).

	Fatty acid composition, %wt					
Fatty acid	**Soybean**	**Cottonseed**	**Palm**	**Lard**	**Tallow**	**Coconut**
Lauric (12:0)	0.1	0.1	0.1	0.1	0.1	46.5
Myristic (14:0)	0.1	0.7	1.0	1.4	2.8	19.2
Palmitic (16:0)	10.2	20.1	42.8	23.6	23.3	9.8
Stearic (18:0)	3.7	2.6	4.5	14.2	19.4	3.0
Oleic (18:1)	22.8	19.2	40.5	44.2	42.4	6.9
Linoleic (18:2)	53.7	55.2	10.1	10.7	2.9	2.2
Linolenic (18:3)	8.6	0.6	0.2	0.4	0.9	0.0

2.2 Palm oil

The original of palm oil tree comes from Africa. Palm oil (*Elaeis guineensis*) is an edible oil containing high amount of beta-carotene, Pro vitamin A and vitamin E. Beside, it is also has a very high saturated fats when compares to other vegetable oils. Thus, its state at the room temperature is semi-solid. Yield of palm fruit can be exact oil from palm oil and palm kernel oil.

2.2.1 Process of palm oil production

The palm oil product made by milling and refining process. At the separation process, palm oil will be separated to sterin and olelin fraction which are solid and liquid, respectively. Then, the impurity will be removed by melting and degumming. Removing smell and coloration is the next step to produce refined bleached deodorized palm oil (RBDPO). The byproduct is free fatty acid, mainly raw material in manufacture of producing soap and detergent. The primary product, RBDPO, can be fractionated further into palm olein for cooking (Palm oil, 2011: online).

2.2.2 Using of palm 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 and the smell is accepted. Therefore, it is used for cooking. The products 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 in the form non-edible oil. 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 (Palm oil, 2011: online).

2.2.3 Palm oil in Thailand

Normally, palm grows in humid tropic area with rainfall of 1800- 5000 mm. per year. The oil from palm gives the highest yield per unit area. Amount of oil from freshly mesocarp is 45-56% and kernel palm oil is 40-50%. Thailand produce palm oil more than others vegetable oils. The total plant is about 300,000 ha and it will be extended to 1.6 million hectare by 2012 (Winayanuwattikun et al., 2008).

2.2.4 Free fatty acid

It is fatty acids which are not attached to others molecules. The uncombined fatty acids or free fatty acids may come from the breakdown of a triglyceride into its components. In the palm oil industry, it is the byproduct in the process of production palm oil. In addition, high free fatty acids occur from the moisture content during seed collection (Akbar et al., 2009). To produce biodiesel by alkaline catalyst, free fatty acid should less than 1%wt. Otherwise, there is saponification problem during transesterification reaction causing lower yield of ester (Prateepchaikul et al., 2009).

2.3 Ethanol

Ethanol or ethyl alcohol is organic chemical which the chemical formula is C_2H_5OH. The molecular weight is 46.07 g mol^{-1} and normal boiling point is $78^{o}C$. It is volatile, flammable and colorless liquid. Ethanol is wildly used as solvent and feedstock of other products. For example, it has been used for raw material of biodiesel production to produce ethyl ester.

Ethanol is more attractive use in biodiesel production instead of methanol. This is because it is less toxic and safer to handle (Marchetti and Errazu, 2010).

2.4 Diesel oil

Diesel fuel is hydrocarbon in the group of paraffin oil obtained from refining crude oil. It composes of 75% saturated hydrocarbons and 2% aromatic hydrocarbons. The ranging of chemical structure is from $C_{10}H_{20}$ to $C_{15}H_{28}$. Diesel is immiscible with water. It is generally used in diesel engine. However, diesel fuel causes high emission of greenhouse gas.

2.5 Crisis of fossil oil

The volume of global oil tends to be lower. Figure 2.2 shows global oil production scenarios based on today's production. A peak in global oil production may occur between 2015 and 2030. For the global energy sources, the Middle East is the prevailing oil region of the world, accounting for 63% of global reserves (Demirbas, 2009). Therefore, human try to find a new source of energy to replace petroleum oil.

In our country, Thailand cannot produce fossil oil to support our demand enough. Therefore, we have to pay too much to import crude oil per year. Figure 2.3 shows import value of the crude oil in Thailand from year 2001 to 2004. The consumption tends to be rapidly increased from 2003 to 2004.
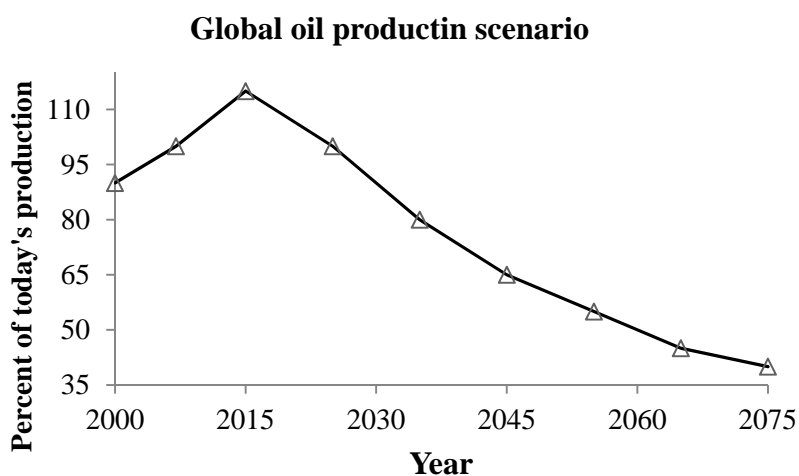
Figure 2.2 Global oil production scenarios based on current production (Demirbas, 2008).

Import value of the crude oil in Thailand
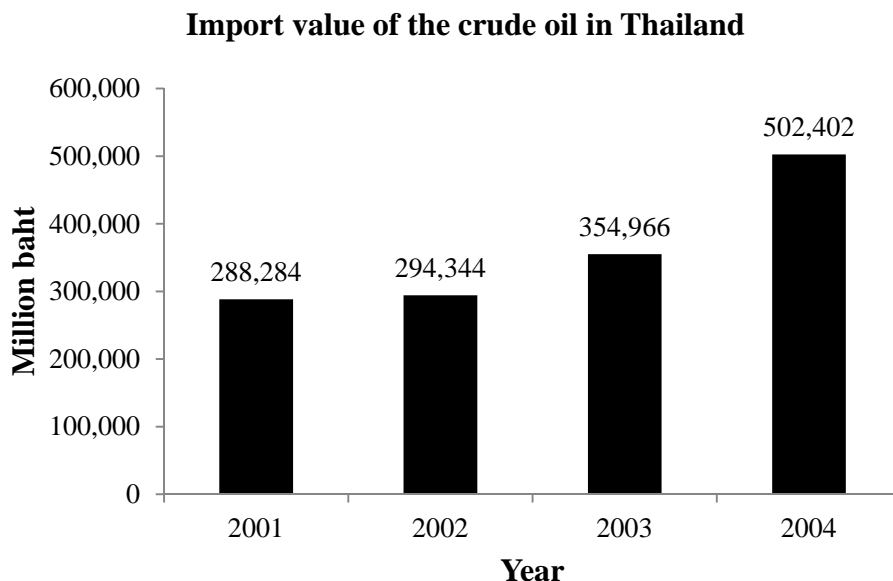
Figure 2.3 Import value of the crude oil in Thailand (Intania forum, 6th, Pisamai Janewanitchakul, Alternative energy: bioethanol and biodiesel).

2.6 Biodiesel

Biodiesel is a novel alternative energy obtained from vegetable oil and animal fat with alcohol by transesterification reaction. Table 2.3 shows comparison of fuel characteristics (Lujan et al., 2009). Although the heating value of biodiesel is lower than diesel fuel because of oxygen in molecule, the overall properties of biodiesel are nearly to diesel fuel. Recently, biodiesel is more attractive because of its eco-friendly fuel and it is made from renewable resource. Using of biodiesel can be overcome the problem from gas emission of greenhouse effect. Table 2.4 shows that the typical emission profiles of biodiesel and one of its blends, B20, which consists of 20% biodiesel and 80% diesel, using conventional diesel emissions as the reference. The information in the Table 2.4 proves how biodiesel significantly reduces emissions compared to diesel even when used as the minor component of a fuel blend. In addition, the amount of sulfur in biodiesel is quite low, which can significantly contribute to helping meet current sulfur emission standards in diesel vehicles (Goering et al., 1982; Lotero et al., 2005).

Table 2.3 Comparison of fuel characteristics.

	Diesel fuel	Biodiesel fuel (B100)
Summarized formula	$C_{15.27}H_{27.33}$	$C_{18.61}H_{35.65}O_2$
Molecular weight [g.mol^{-1}]	210.96	293.26
Oxygen content [%wt]	0	10.91
Cetane number	51.52	54.50
Density @ 15 ^{o}C [kg.m^{-3}]	843.0	881.4
Viscosity @ 40 ^{o}C [mm^{2}s^{-1}]	2.847	4.173
Sulphur content [mg.kg^{-1}]	27.9	0.2
H/C ratio	1.79	1.91
Lower heating value	42.834	35.910
CFPP (^{o}C)	-7	-3

Table 2.4 Average B100 and B20 Emission (in %) compared to normal diesel (Goering et al., 1982 and Lotero et al., 2005).

Emission	B100	B20
Carbon monoxide	-48	-12
Total unburned hydrocarbons	-67	-20
Particulate matter	-47	-12
Nitrogen oxides	+10	+2
Sulfates	-100	-20
Air toxics	-60 to -90	-12 to -20
Mutagennicity	-80 to -90	-20.0

The fuel in the form of pure or blend biodiesel can overcome the production of carbon dioxide (Sharmer et al., 1993). Consequently, the level of the pollutants will

be decreased and the alternative energy also produces lower levels of greenhouse gases compared with the fossil fuel source. In other words, reducing carbon dioxide from using biodiesel will stop global warming problem.

2.7 The production of biodiesel

2.7.1 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 fuel and the blend of 75:25 ester/diesel gave the best performance while the 50:50 blends consistently resulted in the lowest amounts of emissions over the whole speed range tested.

According to large molecular weight of triglyceride, this vegetable oil is very high viscosity and there are many problems occur after using vegetable oil direct to diesel engine, for example; carbon deposit, injection nozzle failure, lower volatile, poor fuel atomization, rings sticking (Murugesan et al., 2009). These problems, causes and solution are shown in Table 2.5; Micro-emulsification, pyrolysis, direct use of vegetable oils and/or the use of blends of the oils have generally been considered to be not satisfactory and impractical for both direct and indirect diesel engines. The high viscosity, free fatty acid content, as well as gum formation due to oxidation and polymerization during storage and combustion together with carbon deposits and lubricating oil thickening are obvious problems.

Table 2.5 Problems, causes and solution from direct using of vegetable oil (Ma and Hann, 1999).

Problem	Probable cause	Potential solution
Short-term		
1. Cold weather starting	High viscosity, low cetane, and low flash point of vegetable oils.	Preheat fuel prior to injection. Chemically alter fuel to an ester.
2. Plugging and gumming of filters, lines and Other ash microns.	Natural gums in vegetable oil.	Partially refine to the oil to remove gums. Filter to 4-injectors .
3. Engine knocking	Very low cetane of some oils. Improper injection timing.	Adjust injection timing. Use higher compression engine. Chemically alter fuel to an ester. Preheat fuel prior to injection.
Long-term		
4. Coking of injectors on piston and head of engine	High viscosity of vegetable oil, incomplete combustion of fuel. Poor combustion at part load with vegetable oils.	Heat fuel prior to injection. Switch engine to diesel fuel when operation at part load. Chemically alter the vegetable oil to an ester.
5. Carbon deposits on piston and head of engine	High viscosity of vegetable oil. incomplete combustion of fuel. Poor combustion at part load with vegetable oils.	Switch engine to diesel fuel when operation at part load. Chemically alter the vegetable oil to an ester.
6. Excessive engine wear	High viscosity of vegetable oil, incomplete combustion of fuel. Poor combustion at part load with vegetable oils. Possibly free fatty acids in vegetable oils.	Heat fuel prior to injection. Switch engine to diesel fuel when operating at part load. Chemically alter the vegetable oil to an ester. Increase motor oil changes.
7. Failure of engine Lubricating oil due to polymerization	Collection of polyunsaturated vegetable oil in crank-case.	Heat fuel prior to injection. Switch engine to diesel fuel when operating at part load.

2.7.2 Pyrolysis

Pyrolysis or thermal cracking is chemical decomposition of organic substance by heating in the absence of air or oxygen. Then, the large molecules are split into small molecules (Ma and Hann, 1999), and thus viscosity is reduced. The cetane number is also improved. It occurs at the high temperature from 450°C to $850\ ^{o}$C (Sonntag, 1979). Thermal decomposition of triglycerides produces the compounds of classess including alkanes, alkenes, alkadienes, aromatics and carboxylic acid. Different types of vegetable oils produce large differences in composition of the thermally decomposed oil. Mechanisms for the thermal decomposition of triglycerides are likely to be complex because of many structures and multiplicity of possible reactions of mixed triglyceride. In case of pyrolysis of fat, it has been investigated for more than 100 years, especially in the country which is lower volume of petroleum. The product is similar to diesel fuel. In cracking of petroleum, the standard catalyst is SiO_2/Al_2O_3. The compositions of pyrolyzed oil are listed in Table 2.6.

Disadvantages of this process include high equipment cost and need for separate distillation equipment for separation of various fractions. Also the product obtained was similar to gasoline containing sulfur which makes it less eco-friendly (Ma and Hanna, 1999; Srivathsan, 2008).

Table 2.6 Composition of pyrolysis of vegetable oil (Ma and Hann, 1999).

	Percent by weight			
	High oleic safflower		**Soybean**	
	N_2 sparge	**Air**	**N_2 sparge**	**Air**
Alkane	37.5	40.9	31.1	29.9
Alkene	22.2	22.0	28.3	24.9
Alkadienes	8.1	13.0	9.4	10.9
Aromatics	2.3	2.2	2.3	1.9
Unresolved unsaturates	9.7	10.1	5.5	5.1
Carboxylic acids	11.5	16.1	12.2	9.6
Unidentified	8.7	12.7	10.9	12.6

2.7.3 Microemulsion

A microemulsion is a system consisting of a liquid dispersed, 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 but prolong usage causes problems like injector needle sticking, carbon deposit formation and incomplete combustion (Ma and Hanna, 1999; Srivathsan, 2008).

2.7.4 Esterification

It is the formation of ester (R-COOR', where R and R' are either alkyl or aryl groups), which occurs though a condensation reaction that require two reactants, carboxylic acid (fatty acid) and alcohol. Esterification is a reversible reaction. Esterification reaction can be acid catalyzed and proceed slowly in the absence of strong acid such as sulfuric acid, phosphoric acid, organic sulfuric acids and hydrochloric acid.

2.7.5 Transesterification

Transesterification (also called alcoholysis) is the reaction of vegetable oil and simple alcohol to transform ester and glycerol. Methanol is widely used because of

physical and chemical nature. The transesterification reaction as shown in Figure 2.4 is reversible. Therefore, alcohol should be excess in order to increase yield of ester.

Triglyceride   Ethanol   Catalyst   Ethyl ester   Glycerol
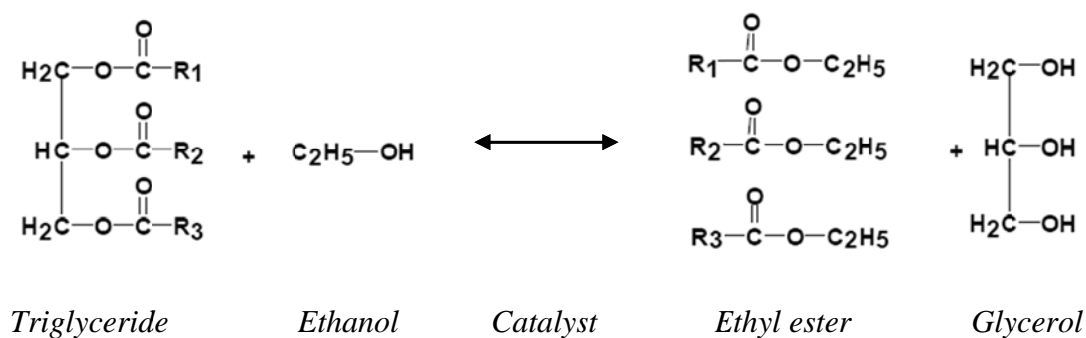
Figure 2.4 Transesterification reaction.

There are three steps to convert triglycerides to esters as shown in Figure 2.5 First, triglycerides are converted to diglycerides. Next, the conversions of diglycerides are followed to monoglycerides. Finally, monoglycerides are transformed to esters and glycerol (Fukuda et al., 2001).

$Catalyst$

Step1. **Triglyceride (TG) + R'OH** $\longleftrightarrow$ **Diglyceride (DG) + R'COOR$_1$**

$Catalyst$

Step2. **Diglyceride (DG) + R'OH** $\longleftrightarrow$ **Monoglyceride(MG)+ R'COOR$_2$**

$Catalyst$

Step3. **Monoglyceride(MG) + R'OH** $\longleftrightarrow$ **Glycerol(GL)+ R'COOR$_3$**
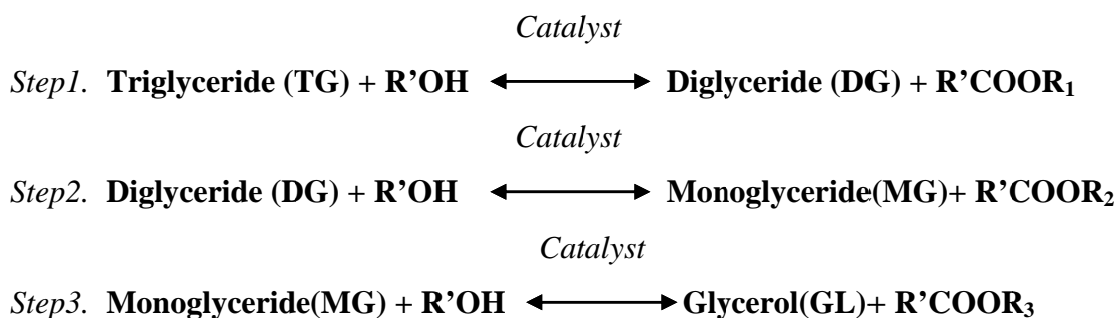
Where R$_1$, R$_2$ and R' are alkyl groups.

Figure 2.5 Three consecutive steps of transesterification reactions .

2.7.5.1 Chemical catalyst

The common method to produce biodiesel is transformation of vegetable oil and alcohol by using alkaline catalyst to speed up the reaction. These include based-catalyst; sodium hydroxide, potassium hydroxide, carbonates, and alkoxides such as

sodium methoxide, sodium ethoxide, sodium propoxide, and sodium butoxide. Sodium hydroxide is commonly used, due to economical reasons and availability (Khan, 2002). Additionally, alkaline catalysts are used more often than acid catalysts because reactions are faster (Freedman et al., 1984).

Alkali-catalyzed transesterification proceeds approximately 4000 times faster than that catalyzed by the same amount of an acidic catalyst (Fukuda et al., 2001). This process is accomplished by mixing alcohol with sodium hydroxide or potassium hydroxide to make sodium methoxide. Then, the sodium methoxide is added to vegetable oil in a reactor at the best molar ratio of oil and alcohol at 6:1. The mixture is stirred and heated at 60-63°C. After complete reaction, mixture is allowed to cool to room temperature. After that, the ester and glycerol are separated. The biodiesel is left on the top level, and the glycerol is left on the bottom (Freedman et al., 1984).

Alkali catalysts are less corrosive to industrial equipment than acid catalysts (Ali and Hanna, 1994). However, there are drawbacks from using acid oil (acid value higher than 1%) and water content more than 0.3% in the reaction (Herawan, 2004; Haas, 2004). This is because the saponification will be obtained from high FFA can make separation of biodiesel and alcohol difficult, and cause low yield of biodiesel. Pretreatment is necessary to reduce soap formation during the reaction and ease the extensive handling for separation of biodiesel and glycerol together with removal of catalyst and alkaline wastewater (Fjerbaek et al., 2009). Figure 2.6 shows the mechanism of the alkali-catalyzed trasesterification of vegetable oils. The first step is the reaction of the base with alcohol, producing alkoxide and the protonated catalyst. The nucleophilic attack of the alkoxide at the carbonyl group of the triglyceride generates a tetrahedral intermediate in the next step from which the alkyl ester and the corresponding anion of the diglyceride formed in the third step. The latter deprotonates the catalyst, thus regenating the active species in the next equilibrium which is able to react with a second molecule of the alcohol staring another catalyst cycle. Diglycerides and monoglycerides are converted by the same mechanism to form a mixture of alkyl ester and glycerol.

$$ROH + B \rightleftharpoons RO^- + BH^+$$

R'COO—CH₂
R"COO—CH   +   ⁻OR   ⟶   (I)
H₂C—OCR'''
        ‖
        O

R'COO—CH₂
R"COO—CH   OR
H₂C—O—C—R'''
        |
        O⁻

R'COO—CH₂
R"COO—CH   OR   ⟶
H₂C—O—C—R'''
        ‖
        O

R'COO—CH₂
R"COO—CH   +   ROOCR'''   (II)
H₂C—O⁻

R'COO—CH₂
R"COO—CH   +   BH⁺   ⟶   R'COO—CH₂   +   B   (III)
H₂C—O⁻   R"COO—CH
                             H₂C—OH
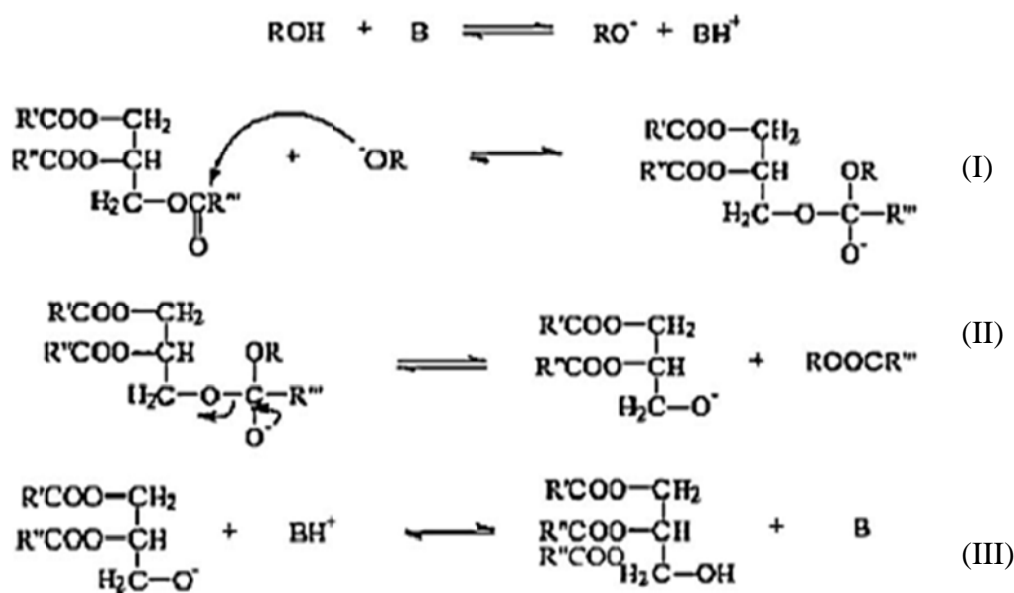
Figure 2.6 Mechanism of the alkali-catalyzed transesterification of vegetable oils (Sridharan and Mathai, 1974).

Acid-catalyzed transesterification reaction includes sulfuric, phosphoric, hydrochloric, and organic sulfonic acids. Acid-catalyzed process can reduce production cost because of using low cost feedstock. Used oil or waste frying oil with high free fatty acid can be used as raw materials. This process provides very high yield in alkyl ester, however, the reaction time is commonly slow. The mechanism of the acid-catalyzed trasesterification is shown in the Figure 2.7 The protonation of the carbonyl group of the ester leads to the carbocation II which, after a nucleophilic attack of the alcohol, produces the tetrahedral intermediateIII which eliminates to form the new ester IV, and to regenerate the catalyst H^+. According to this mechanism, carboxylic acids can be formed by reaction of the carbocation II with water present in the reaction mixture. This suggests that an acid-catalyzed transesterification should be carried out in the absence of water, in order to avoid the competitive formation of carboxylic acids which reduce the yields of alkyl esters (Schuchardt et al., 1998).
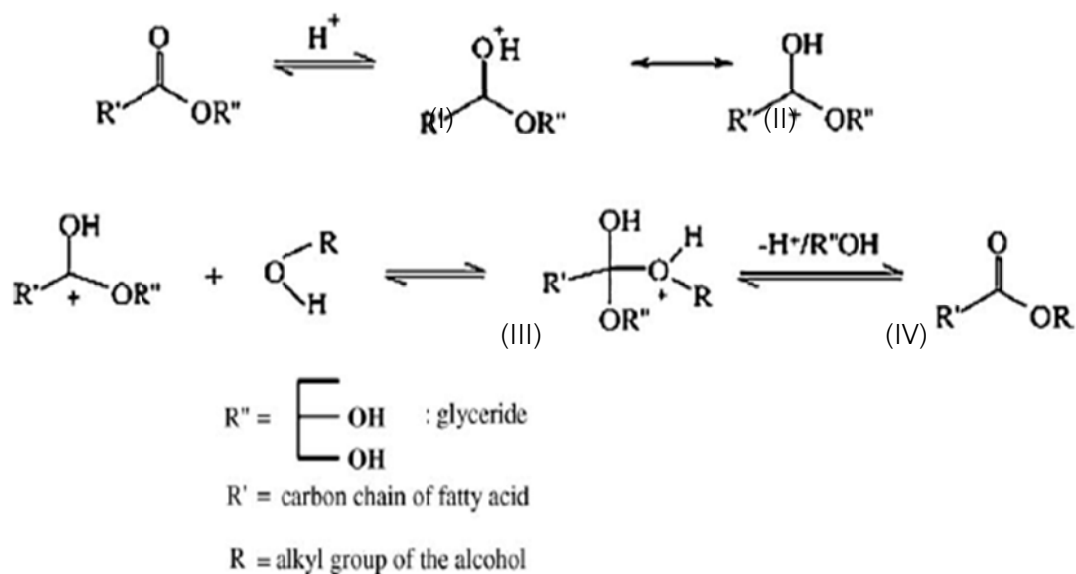
Figure 2.7 Mechanism of the acid-catalyzed trasesterification.

Two step transterification in which the first acid catalyzed step was followed by the second alkali catalyzed step was developed for the production of biodiesel from oil with high free fatty acid content. Initially, acid catalyst can be used to convert FFA to the ester and to decrease the FFA level. Next, alkali catalyst can be performed for the transesterification of oil (two-step). This technique overcomes the problem of a slow reaction rate with acid catalyst and the formation of soap with an alkali catalyst and increase the ester yield. However, the problem with the removal of the catalysts is still a big issue for the two-step method (Kulkarni et al., 2006).

The homogenous catalyzed biodiesel processes are fast and gives high conversion, however, there are several problem such as the catalyst cannot be recovered and must be neutralized at the end of the reaction.

At present, heterogeneous catalyst process is being considered as a suitable process replace to homogenous reaction. A solid catalyst would alleviate both the environmental aspects of dealing with sodium methoxide as well as the issues concerning the separation of the catalyst from the reactant for the later reuse.

The main advantage of a solid catalyst is that the catalyst is easily separated from the reactants and products. This process could potentially lead to cheaper production cost

because it is possible to reuse the catalysts and to carry out both transesterification and esterification simultaneously (Goodwin et al., 2005; Lopez et al., 2005).

2.7.5.2 Enzymatic catalyst

There are many disadvantages of using alkaline catalysts as shown in Table 2.7 the reaction temperature is very high and it has to purify the ester by repeated washing. Thus, it is energy intensive. The others drawbacks are recovery of glycerol are difficult because the alkaline or acid catalyst has to be removed from the product; therefore, it needs to treatment alkaline waste water. Moreover, the interference of free fatty acid and water on the reaction is observed.

In contrast to the chemical catalysts, enzyme catalysts do not form soap. Hence, there is no washing step. Although the general production cost of biodiesel catalyzed by lipase is greater than alkaline process, the production can overcome the several drawbacks of alkaline process. At present, enzymatic biodiesel production is a promising process to alkaline process. Enzymatic process is higher cost than chemical process. However, if consider to the pollution of natural environment, this comparable cost is accepted (Antczak et al., 2009).

Table 2.7 Comparison between alkali-catalysis and lipase-catalysis methods for biodiesel fuel production (Fukuda et al., 2001).

Items	Alkali-catalysis process	Lipase-catalysis process
Reaction temperature (^{o}C)	60-70	30-40
Free fatty acids in raw materials	Saponified products	Esters
Water in raw materials	Interference with the reaction	No interference
Yield of esters	Normal	Higher
Recovery of glycerol	Difficult	Easy
Purification of esters	Repeated washing	None
Production cost of catalyst	Cheap	Expensive

The cost of biodiesel production depends on feedstock of fats and oils up to 65-75% of total cost (Merve and Filiz, 2004). Cheaper cost of fats and oils is a good choice for biodiesel production, for example; used cooking oil or waste fats and oils with high free fatty acid is promising alternative to virgin oil due to its low price as raw material (Mengyu et al., 2009). Edible oil or refined oil was not profitable due to high price. The study of Antczak et al. (2009) emphasized in biodiesel synthesis that the use of useless or waste fats was found to be economically viable.

The acid oil which has high free fatty acid is not suitable to produce biodiesel by using alkaline catalyst due to the saponification as shown in Figure 2.8. Biocatalysts can be used to solve this problem.

$$R-\overset{\overset{\displaystyle O}{\|}}{C}-OH \ + \ NaOH \ \xrightarrow{Heat} \ R-\overset{\overset{\displaystyle O}{\|}}{C}-O^-Na^+ \ + \ H_2O$$

Free fatty acid   Alkaline   Salt   water
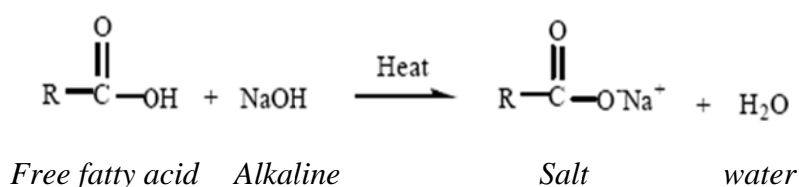
Figure 2.8 Saponification reaction.

When vegetable oil is free fatty acid, the reaction is direct esterification reaction. Esterification is the reaction that requires fatty acid and alcohol to form esters which can be seen in Figure 2.9 below.

$$\overset{Catalyst}{\text{R-COOH} \ + \ \text{ROH} \ \xleftrightarrow{\hspace{2cm}} \ H_2O \ + \ \text{R-CO-OH}_2\text{C-R}}$$

Free fatty acid   Alcohol   Water   Ester
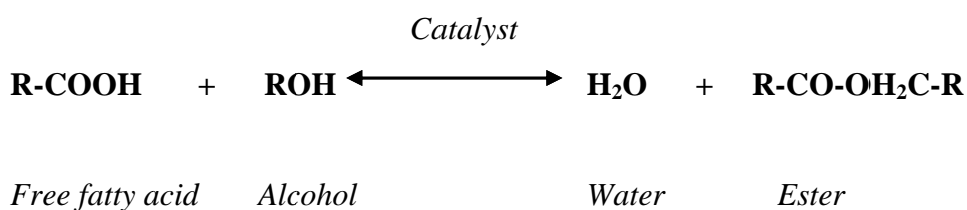
Where R is carbon chain

Figure 2.9 Esterification reaction.

Fats containing triacylglycerols (TG) and FFA can be enzymatically converted to biodiesel in a one-step process because lipases catalyze both transesterification and esterification reactions as shown in Figure 2.10 (Antczak et al., 2009).

.

$$n \begin{matrix} CH_2\text{-O-CO-}R_1 \\ | \\ CH\text{-O-CO-}R_2 \\ | \\ CH_2\text{-O-CO-}R_3 \end{matrix} + m\ R_4\text{-COOH} + (3n+m)\ \text{alkyl-OH} \rightarrow (3n+m)\ \text{alkyl-O-CO}R_n + n \begin{matrix} CH_2\text{-OH} \\ | \\ CH\text{-OH} \\ | \\ CH_2\text{-OH} \end{matrix} + m\ H_2O$$
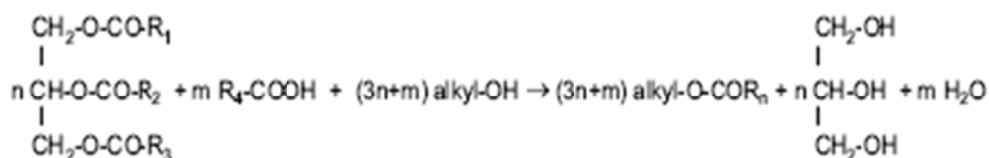
Figure 2.10 Enzymatic, one-step (trans)esterification of fats containing free fatty acids with short aliphatic alcohols.

2.7.5.3 Selection of alcohol

Ester which obtained from biodiesel production always comprises of short chain alcohol, for example, methanol, ethanol, propanol, n-butanol, isobutanol. Today, methanol and ethanol are commonly used as acyl acceptor. 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 (Shimada et al., 2002; Salis et al., 2005). Therefore, enzymes seem to prefer ethanol rather than methanol. Beside, ethanol is renewable source from plant feedstock.

The stability of enzyme depends on structure and concentration of alcohol. Higher molar ratio of alcohol higher yield of fatty acid ethyl ester. Nevertheless, it is negative effect to enzyme because of inactivation.

2.8 Lipase

Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) are produced from mammalian, fungal and bacteria (Antczak et al., 2009). Table 2.8 shows source of lipases. Lipases can be used in the wide range of pH and temperature. Application of lipase can be found in pharmaceutical, cosmetic, dairy, detergent, oleochemical and so on. Commercially, lipases are mostly extracellular and derived from microbials sources. Lipases can be used as catalysts to catalyze the hydrolysis, esterification and transesterification to synthesis esters in low water environment. It is high activity in organic solvent (Gupta N, 2004). The enzymatic reaction is mild condition making it possible to obtain products of very high purity (Herawan, 2004). Beside, their beneficial includes substrate specificity and regiospecificity.

Biodiesel systhesis can be found in both organic solvent and solvent-free system. For regiospecificity of biodiesel production, it respects to the length hydrocarbon chains of fatty acid. Figure 2.11 shows the mechanism of enzymatic alcoholysis of synthesis of ethyl ester by lipase analysis. Some lipases have selectivity towards the length of the fatty acids or the number and location of fatty acids. Lipases have been divided into three types (Antczak et al., 2009); hydrolyze ester bonds in positions R1 or R3 of TG, hydrolyze ester bond in position R2 of TG, nonspecific positions of ester bonds to be cleaved. It is to note that the position of R is based on Figure 2.4.
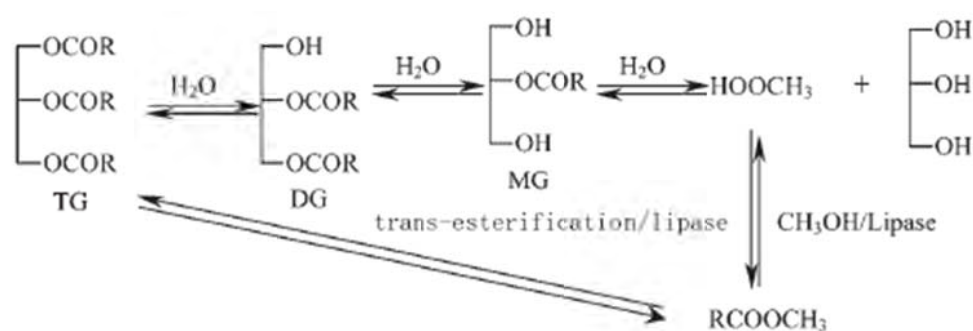
Figure 2.11 Mechanism of enzymatic transesterification catalyzed by lipase (Tan et al., 2006).

Table 2.8 Source of lipases (*Candida rugosa* lipase, 2011: online).

Source of lipases	Name
Mammalian	Human pancreatic lipase
	Horse Pancreatic lipase
	Pig Pancreatic Lipase
	Guinea Pig Pancreatic Lipase
Fungal	*Rhizomucor meihei*
	Pencillium cambertii
	Humicola lanuginosa
	Rhizopus oryzae
	Candida rugosa
	Candida antarctica Lipase A
	Candida antarctica Lipase B
	Aspergillus niger
	Geotrichium candidum
Bacteria	*Chromobacterium viscosum*
	Pseudomonas cepacia
	Pseudomonas aeruginosa
	Pseudomonas fluorescens
	Pseudomonas fragi
	Bacillus thermocate nulatus
	Staphylococcus hyicus
	Staphylococcus aereus
	Staphylococcus epidermidis

Candida rugosa **lipases**
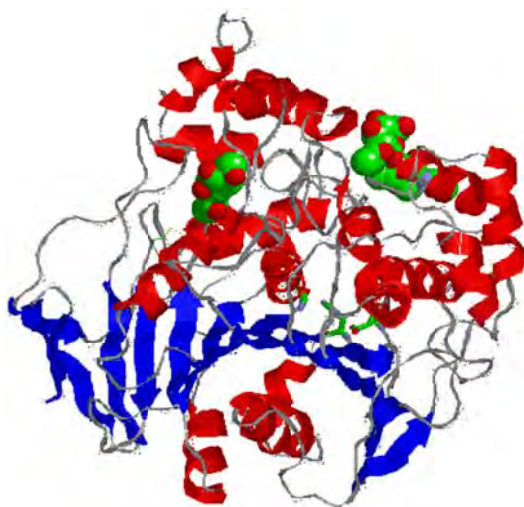
Figure 2.12 Illustration of *Candida rugosa* lipase.

C. rugosa comes from the genus *Candida*, which includes around 154 species. It is a spherical-shaped unicellular microfungi that is more beneficial than it is harmful (*Candida rugosa*, 2005: online). Isolating yeast from natural soil is the powerful lipase production capacity. Today it is reported that there is at least seven genes of *Candida rugosa*. The nomenclatures are Lip1 to Lip7 (Yamada et al., 1963). *Candida rugosa* lipase, an enzyme frequently employed in organic synthesis (Lalonde et al., 1995; Persichetti et al., 1996) or in aqueous media (Faber, 2004). It has been studied that very pure preparations do not always show high reactivity. Figure 2.12 shows the structure of *Candida rugosa* lipase. It has been published that all the isoenzymes are the same number of aminoacids with a high homology. The α-helix structure composes of amino acid with amphiphilic properties. The inactive form occurs when a lid form cover the active site.

2.9 Bacterial Cellulose

Cellulose, a linear polymer, is naturally basic material in plant which the chemical formula is $(C_6H_{10}O_5)_n$ and the chemical structure shown in the Figure 2.13 below. The hydrogen bonds between hydroxyl groups were located inter- and intra-molecular to form Van der Waals interactions. Bacterial cellulose (BC) is nearly

purified cellulose synthesized into small size of fibrils by the bacteria *Acetobactor xylinum*. BC has outstanding properties, both physical and chemical dimension, such as high mechanical strength, high crystallinity, high hydrophobicity and ultra-fine network structure. BC has found many applications in papers, textile and food industry and medicine (Bielecki et al., 2005).
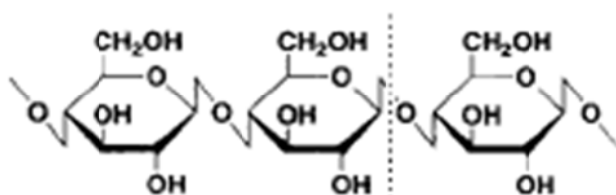
Figure 2.13 The chemical structure of Cellulose (Samejima M. et al., 1998).

2.10 Variables affecting transesterification and esterification

There are many factors that affect the transesterification and esterification reaction depending upon the reaction conditions used.

2.10.1 Effect of organic solvent

Organic solvents provide a better miscibility of a mixture of alcohol and oil. The advantages of organic solvent are to improve solubility of substrate and to increase operational stability of enzyme. Normally, organic solvents are used for reducing the denaturation of enzyme caused by alcohol (Antczak et al., 2009). Soumanou (2003) found that there was no need to add organic solvent for raw propanol and butanol. This is because solubility of propanol and butanol in oil is higher than methanol and ethanol.

In addition, the organic solvents are difficult to remove from the system. The volatility, flammability and toxicity are also drawbacks. Therefore, the biodiesel production with solvent-free is more interesting.

From the studied by Krisnangkura and Simamaharnnop (1992), the process of methanolysis and ethanolysis comprise solubilizing oil or fat in methanol or ethanol by addition of toluene as co-solvent in order to form a one phase reaction mixture, and addition an esterification catalyst. The processes proceed quickly, usually in less than 20 min, at ambient temperature, atmospheric pressure, and without agitation. The co-solvent increases the rate of reaction by making the oil soluble in methanol, thus increasing contact of the reactant. The lower alkyl fatty acid monoesters produced by the process can be used as biofuels and are suitable as diesel replacement or additives.

2.10.2 Effect of water content

Water content in enzymatic biodiesel is added to increase catalytic efficiency of enzyme. There are many researches explained that the rate of esters increase with a small addition of water (Antczak et al., 2009; Shah and Gupta, 2007; Kaieda et al., 2001). The biodiesel productions need to optimize the water content to keep minimum level of hydrolysis of esters with highest yield of product (Shimada et al., 2002; Antczak et al., 2009). However, water can consume the catalyst and reduce catalyst efficiency and it is believed that the presence of water has greater negative effect than that of the free fatty acids. Therefore, it is generally recommended that for typical transesterification of vegetable oil, the water content should be kept below 0.06% (Ma et al., 1998).

2.10.3 Ratio of alcohol to oil or fatty acids

This is one of the most important variables affecting the yield of ester in transesterification. From 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 esters.

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.

2.10.4 Reaction temperature

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 – 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).

2.10.5 Reaction time

The conversion increases with increasing of reaction time. For example, Freedman et al. (1984) studied the transesterification of peanut, cotton-seed, sunflower and soybean oil under the condition of methanol to oil molar ratio of 6:1, 0.5% sodium methoxide catalyst, and at 60 ^{o}C. An approximately yield of 80% was observed after 1 min for soybean and sunflower oils. After 1 h, the conversion was almost the same (93-98%) for all four oils.

2.10.6 Purity of reactant

Impurities present in the oil also affect conversion levels. The free fatty acids in the original oil interfere with catalyst, however, under conditions of high temperature and pressure this problem can be overcome (Freedman et al., 1984).

2.11 Literature review

In the past decade, several methods for biodiesel production are developed. There are variety of oils, different catalysts, alcohols and reaction conditions. Most of all concepts are composed of three reasons; increasing yield in short reaction time, low investment cost and environmental friendly. The cost of biodiesel production mostly depends on feedstock of fats and oils. High free fatty acid (High FFA) such as non-edible oil, used cooking oil or waste fats and oils are promising alternative to virgin oil due to its low price as raw material (Mengyu et al., 2009). Edible oil or refined oil was not profitable due to high price. However, the acid oil which has high free fatty acid is not suitable to produce biodiesel by using alkaline catalyst. This is because the soap will be obtained from the saponification. It will inhibit the separation of ester and glycerin. Pretreatment was a method used to solve this problem as shown in Table 2.9.

Veljkovic et al. (2006) investigated fatty acid methyl ester (FAME) from high fatty acid of tobacco seed oil (TSO) by two-steps process. The first step was reduced 35% 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 under the same temperature at 60 ^{o}C. The maximum yield of FAME was 91% in about 30 minute. The tobacco biodiesel obtained had the fuel properties within the limits prescribed by the latest American (ASTM D 6751-02) and European (DIN EN14214) standards, except for a somewhat higher acid value than that prescribed by the latter standard (<0.5). Niak et al. (2008) studied the production of biodiesel from high free fatty acid *Karaja (Pongamia pinnata) oil*, the Indian non-edible oil with high FFA up to 20%. Dual step process was used to produce methyl ester. The first step was acid catalyzed esterification by H_2SO_4 at 0.5% w/w of oil. The reaction was conducted at 65°C with Methanol/Oil at molar ratio of 6:1. The next step was transesterification with the aid of KOH. They found that the yield of dual step was observed to be 96.6-97%. Ramadhas et al. (2005) produced biodiesel from high FFA rubber seed oil using two step transesterification processes. The first step, acid-catalyzed esterification reduced the high FFA to less than 2%. And then, it will be transesterized by alkali catalyst to convert the product of first step to mono alkyl esters. Zhang and Jiang (2008)

developed the process of acid-catalyzed esterification of *Zanthoxylum bungeanum seed oil* (ZSO) which was high free fatty acid oil. The acid value of ZSO was reduced from 45.51 mg KOH/g to 1.16 mg KOH/g by one step acid catalyzed esterification. They found that during acid catalyzed esterification, FFA was converted to fatty acid methyl ester which was confirmed by ^{1}H NMR spectrum and can be confirmed with others two step pretreatment.

To reduce the problem of soap formation from alkali process in producing of biodiesel from high FFA oil, there are many researches used two step pretreatment for their works. The yield after the second step is very high; however, it is not simple to handle both acid and base catalysts. Beside, the supply energy will be high from the working temperature of the dual process.

On the other hand, we are recognizing in the dimension of eco-friendly in today. Because of supercritical transesterification was high cost of industrial production. Therefore, there are many researches tried to develop process of biodiesel production with low cost production and care for environment especially using biocatalysts. There are many advantages of using biocatalysts over chemical catalysts such as mild reaction conditions and specificity. Enzymes or whole cells can be reused by immobilization and can be genetically engineered to improve their efficiency, accept new substrates, are more thermostable, and are considered natural (Casimir et al., 2007). The various lipases and raw materials studied in biodiesel production are shown in Table 2.10.

Table 2.9 Biodiesel production from high free fatty acid oil.

Method	Alcoh-ol	Oil, %FFA	Molar ratio (Alcohol:oil)	Condition	Yield	References
Two step transesterification: First- acid catalyzed esterification	MeOH	Crude of Tobacco seed oil (TSO), 35%	18:1	60 ^{o}C, 25 min, catalyst 1.0% or 2.0% w/w of oil, 400rpm	FAME 91%	Veljkovic et al.,2006
Second- based transesterification	MeOH		6:1	60 ^{o}C,30 min ,catalyst 1.0% w/w of oil, 400rpm		
Dual step process: First- Esterification by sulphuric acid	MeOH	Karanja (Pongamia pinnata) , 20%	6:1	65°C, catalyst 0.5% w/w of oil	FAME 96.6-97%	Naik et al.,2008
Second- Transesterification by KOH	MeOH		6:1	65°C, catalyst 1.0% w/w of oil		
Two step transesterification: First- Esterification by sulphuric acid	MeOH		6:1	45$\pm$5 ^{o}C, 0.5% weight of sulfuric acid/weight of oil, 30 min	FAME ~ 98%	Ramadhas et al.,2005
Second-Transesterification by NaOH	MeOH	Rubber seed oil, 17%	9:1	45$\pm$5 ^{o}C , 0.5% weight of NaOH/weight of oil, 30 min		

| Acid-catalyzed esterification | MeOH | Zanthoxylum bungeanum seed oil, FFA 45.51 mg KOH/g | 24:1 | 60 ^{o}C, 80 min, catalyst 2% of sulfuric acid, FFA 1.16 mg KOH/g | FAME 98% | Zhang and Jiang ,2008 |
| Transesterification | MeOH | FFA 1.16 mg KOH/g | 6.5:1 | 60°C, 90 min, 600 rpm | | |

Table 2.10 Enzymatic of biodiesel production with various lipases and raw materials.

Enzyme	Alcohol	Oil	Molar ratio (Alcohol:oil)	Condition	Yield	References
Pseudomonas fluorescens(Free) *Pseudomonas cepacia(Free)* *Candida rugosa(Free)*	MeOH	Soy bean	3:1	Three-step transesterification, 35°C, 150 rpm	90% >80% 90%	Kaieda et al.,2001
Novozym 435(immobilized *Candida antarctica* Lipase B)	Ethyl acetate	Jatropha Karanj Sunflower	11:1	Interesterification, 12 h, 50°C, 10% w/w Novozym 435 based on weight oil, 150 rpm, solvent-free	91.3% 90% 92.7%	Modi et al.,2007
T. lanuginosus(Free) *T. lanuginosus* immobilized in polyurethane foams *T. lanuginosus(Immobilized+ three step addition of MeOH)*	MeOH	Canola	6:1	20 g refined canola oil; 430 ug lipase, 0.1 g water, 40 ^{o}C, 24 h	51% 80% 90%	Dizge and Keskinler ,2008

Pseudomonas cepacia	EtOH	Jathopha (FFA 2.7%)	4:1	50°C, 200 rpm, 24 h, 4-5% w/w (water), 10% enzyme based on oil weight	98%	Shah and Gupta, 2007
Psuedomonas cepacia entrapped in hydrophobic sol-gel support	MeOH	Soybean	7.5:1	10 g soybean oil: 35°C, 0.5 g water, 475 mg lipase	67%	Noureddini et al.,2005
			15:1	10 g soybean oil: 35°C, 0.3 g water 475 mg lipase	65%	

There are many researches tried to modify lipase by immobilization on support. Yasin U (2010) immobilized lipase by covalent binding onto olive pomace. FAME from using immobilized lipase was added a three-step addition of methanol. Under the optimized condition, the maximum yield of biodiesel was 93% at 25°C in 24 h reaction. Table 2.11 shows the enzymatic biodiesel production from used oil, non-edible oil, and second-hand oil. Nie et al. (2006) used salad oil and waste oil which high FFA at 46.75% catalyzed by immobilized *Candida* sp.99-125 onto textile membrane in methanolysis. The process was three-step transesterification reaction. Each steps were added 1/3 molar equivalent of methanol against total fatty acid in the oil. A hydrocyclone was used in order to on-line separate the by-product glycerol after every 1/3 molar equivalent of methanol was added. The petroleum ether was used as solvent. They found that biodiesel production in a fixed bed reactor also have been investigated, the final conversion ratio to FAME from plant oil and waste oil was 93% and 92%, respectively, under optimal conditions.

Halim and Kamaruddin (2008) studied of lipase on FAME production from waste cooking palm oil (WCPO), cheap feedstock, in tert-butanol system. They tried to eliminate the negative effect of solubilization of methanol and byproduct glycerol by using tert-butanol. tert-Butanol is a moderately hydrophilic solvent can solubilize oil, methanol and glycerol. They found that WCPO was successfully carried out in tert-butanol with the aid of Novozym $^{®}$435. Under the optimal condition, the yield of FAME was obtained at 88%.

Hajar et al. (2008) studied on reaction parameters in lipase-catalyzed methanolysis of plant oil in solvent-free system. There were three steps in addition of methanol used to solve the problem of inhibitory effect of undissolved methanol on lipase activity. They found that the optimal conditions for the reaction were as follows: enzyme amount 4%, molar ratio of methanol to oil 3:1, and temperature 35 °C. The maximum methyl ester yield of 84.4% was obtained after 72 h of reaction at optimum conditions. Three-step transesterification with solvent used to increase the yield of alkyl ester from cheap oil.

Chen et al. (2009) conducted the research in the topic of synthesis of biodiesel from waste cooking oil (WCO) using immobilized lipase in fixed bed reactor. Because of high FFA in WCO, the process of synthesizing biodiesel was different

from the virgin oil. The study shows that WCO can be efficiently converted to biodiesel in a three-step fixed bed transesterification by using immobilized *Candida* lipase. At the optimal condition of lipase/hexane/water/WCO weight ratio of 25:15:10:100 and the temperature was 45 $^{\circ}$C with reactant flow of 1.2 ml. min^{-1} provided the maximum yield at 91.08%.

This research deals with enzymatic biocatalysts of *Candida rugosa* lipase in BC-alginate matrix for biodiesel production. The reactions contained palm oil/ palm fatty acid as the substrate with 95% ethanol. The reaction is enzymatic transesterification for palm oil and enzymatic esterification for palm fatty acid.

Table 2.11 Enzymatic biodiesel production from used oil, non-edible oil, second-hand oil.

Process	Lipase	Form of lipase	Oil or fat sources(%FFA)	Alcohol	Conditions					References
					Alcohol: Oil	Solvent system	Water content	Reaction temp.(^{o}C)	Product&Reaction time(h)	
Three-step transesterification	*Candida sp.*99-125	Absorbing onto a textile membrane	Salad oil (46.75%) Waste oil (46.75%)	MeOH	3:1	Petroleum ether	15%	40	FAME 90% FAME 92%	Nie et al.,2006
Transesterification	*Lipozyme TL IM, Lipozyme RMIM, Novozyme 435*	Commercially immobilized lipase	Waste cooking palm oil Refined palm oil	MeOH	3:1	Tert-Butanol	4	40	FAME 88% (12) FAME 96% (12)	Halim and Kamaruddin, 2008
Three-step transesterification	*Candida antarcticar*	Commercial immobilized(Novozym 435)	Refined canola oil	MeOH	3:1	Free	Not mention	35	FAME 84.4%	Hajar et al.
Three-step fixed bed reactor	*Candida sp.*99-125	Absorbed on text cloth	Waste cooking oil(WCO)	MeOH	1:1	Hexane	10%	45	FAME 91.08%	Chen et al.,2009

CHAPTER III

METHODOLOGY

The experimental systems and procedures used in this research were separated into three parts.

1. Materials
2. Equipments
3. Methods and Characterization

3.1 Materials

Purified palm oil samples used in this study was manufactured and distributed by Lamsoong (Thailand) Public Co., Ltd. Oleic fatty acid was purchase from Panreac Quimica Sau (Bacelona, Spain). Bacterial cellulose pellicles were purchase from local market (Bangkok, Thailand). Enzymatic lipase from *Candida rugosa* (CARL) (Type VII $\geq$ 700 unit/mg solid, 1,187 U/mg) was used as biocatalyst purchased from Sigma Aldrich Chemical Co. Ltd. (St. Louis, USA). All other chemicals as shown in the Table 3.1 below were of analytical grade.

Table 3.1 Analytical grade of all chemicals used in this work.

Chemicals	Grade	Suppliers
Calcium chloride($CaCl_2$)	Analytical	Italmar Co.,Ltd. France
Sodium alginate ($C_6H_7O_2Na)_n$	Analytical	Carlo Erba reagents, Italy
Sodium hydroxide($NaOH$)	Analytical	Merck KGaA, Germany
Acetic acid glacial(CH_3COOH)	Analytical	Brightchem, China
n-Hexane(C_6H_{14})	Analytical	Fisher sciencetific, UK
Ethyl palmitate($C_{18}H_{36}O_2$)	Analytical	SAFC supply solution, USA

Ethyl oleate ($C_{20}H_{38}O_2$)	Analytical	Sigma-Aldrich, USA
Ethyl linoleate($C_{20}H_{36}O_2$)	Analytical	Sigma-Aldrich, USA
Ethyl stearate ($C_{20}H_{40}O_2$)	Analytical	Sigma-Aldrich, USA
Ethyl alcohol (C_2H_6O)	Analytical	Merck KGaA, Germany
Methyl alcohol (CH_4O)	Analytical	Malinkrodt Chemical, USA

3.2 Equipments

1. Blender (Philip Cucina)
2. Centrifuge

Figure 3.1 Centrifuges (Left: Labofuge 200, Right: Kubota 5100).

3. Micropipette

Figure 3.2 Auto micropipette (10 ml, 1 ml, 200 ul).

4. Vacuum oven

5. Sciencetific balance

Figure 3.3 Sciencetific balance (METTLER TOLEDO).

6. Scanning Electron Microscopy, SEM (JOEL JSM-5410LV, Japan)

7. Circular cutters

Top side   *Bottom side*

Figure 3.4 Circular cutters (Carla cart).

7. Incubator shaker

Figure 3.5 Incubator shaker (Innova 4000).

8. Freeze drier

Figure 3.6 Freeze drier (Labconco).

9. GC (Shimadzu GC-2010)

3.3 Methods and Characterization

There are 3 steps of biocatalyst preparation. Firstly, it was the preparation of homogenous BC by changing pieces of wet BC pellicles to homogeneous form. Secondly, the homogenous BC was mixed with Sodium alginate (SA) and cross-linked by calcium chloride. Then, it was frozen and dried to form the freeze dried BC-alginate. The sponge plate was cut to become circular pieces. Thirdly, the support was absorbed by soluble lipase. After that, it was dipped into SA and cross-linked by CaCl$_2$.

3.3.1 Preparation of homogenized bacterial cellulose

BC pellicles from the fermentation of coconut water were washed by water for 30 min and then, immersed in 1 %(w/v) NaOH to deproteinate for 24h. After that, the materials were dipped in 1% (v/v) acetic acid for 2h. Later, the materials were rinsed several times with distilled water, until a neutral pH was attained in the drained liquid. Next, the BC pellicles were homogenized to obtain slurry by a kitchen blender at room temperature. Finally, the materials were kept in the refrigerator prior to use.

3.3.2 Preparation of freeze dried BC-polymeric plate

Sodium alginate (SA) at 2% (w/v) was mixed with the BC slurry to produce the mixture having a weight ratio of 30 SA: 70 BC (w/w). Then, the mixtures were completely stirred at room temperature to form the gel solution. After that, the solution was spread over a plastic plate. The thickness of the solutions was manually controlled. Then, the casting solution was cross-linked by 0.12 M CaCl$_2$ aqueous solution for 12 h, followed by washing with distilled water until a neutral pH at room temperature. The fresh BC-alginate which the size was 12 x 17 x 0.5 cm was shown in the Figure 3.7. Then, it was frozen at -40 ^{o}C for 24 h before freeze-drying. Finally, it was cut by circular cutter to get circular pieces at the diameter of 1.20 cm and the thickness of 3 mm. The freeze dried BC-alginate and ready-to-immobilize piece were shown in the Figure 3.8 and Figure 3.9, respectively.

Figure 3.7 Form of the fresh BC-alginate after cross-linked by 0.12 M calcium chloride for 24 h.

Figure 3.8 Form of the freeze dried BC-alginate after freeze drying at -40°C for 48 h under vacuum condition.

Figure 3.9 The circular piece of freeze dried BC-alginate at the diameter of 1.20 cm and the thickness of 3 mm.

3.3.3 Immobilization of *Candida rugosa* lipase.

An integration method of adsorption and entrapment was applied in this study. Firstly, the immobilized lipase on freeze dried BC-polymeric material was performed in a simply way. The powder of *Candida rugosa* lipase (10% w/w of CARL to vegetable oil) was suspended in 3 ml of 0.001M phosphate buffer (pH 7.0). The adsorption of CARL on the sponge pieces was performed by soaking the pieces into the enzymatic solution for 30 min like the Figure 3.10. Secondly, the adsorbed pieces were dipped into 2% (w/v) SA and cross-linked by 0.12M $CaCl_2$ aqueous solution to form the entrapped pieces. The pieces were allowed to be hardened in the $CaCl_2$ solution for half and hour. The schematic and ready-to-use biocatalysts were shown in the Figure 3.11 and Figure 3.12 below. To increase the layer, the entrapped pieces were done with the same method as stated above.

Figure 3.10 Fifteen circular pieces of freeze dried BC-alginate was soaked in 3ml of enzymatic solution for 30 min.

Figure 3.11 One layer with a diameter of 1.40 cm. of the ready-to-use pieces of entrapped biocatalysts.

Figure 3.12 Enzyme immobilized by double layers entrapment of calcium alginate with a diameter of 1.60 cm.

Ca-alginate (outer layer)   *Ca-alginate (inner layer)*

Soluble CARL on a freeze dried BC-alginate
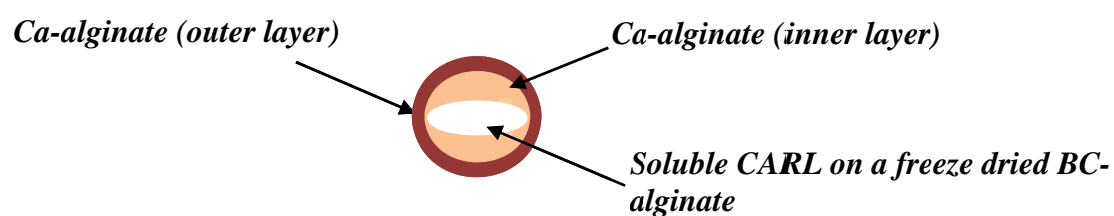
Figure 3.13 Illustration of a schematic biocatalyst which was double layers entrapment.

Figure 3.14 The immobilized lipase during the enzymatic alcoholysis with purified palm oil and 95 %(v/v) ethanol.

3.3.4 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 specimens were given alcohol at concentration equal to 30%, 50%, and 70% and absolute respectively. After that the material was dried by the method of critical point drying (CPD). Finally, 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.

3.3.5 Enzymatic transesterification

The reaction containing of 15 g of palm oil/palm fatty acid and 95% (v/v) ethanol was taken at a molar ratio of 1:9 in 250 erlenmayer flask with 10% lipase (base on weight of oil) in immobilized supports. The mixture was incubated at 45 °C with a constant shaking rate at 250 rpm. Samples of 5 ml were withdrawn at every 12 h during 36 h reaction period. The ethanol was dried out of the samples followed by centrifuging to separate the glycerol as shown in the Figure 3.15 below. The yield of ethyl ester formation by using free and immobilized CARL was analyzed by GC.

Fatty acid ethyl ester (FAEE)

Glycerol

Water
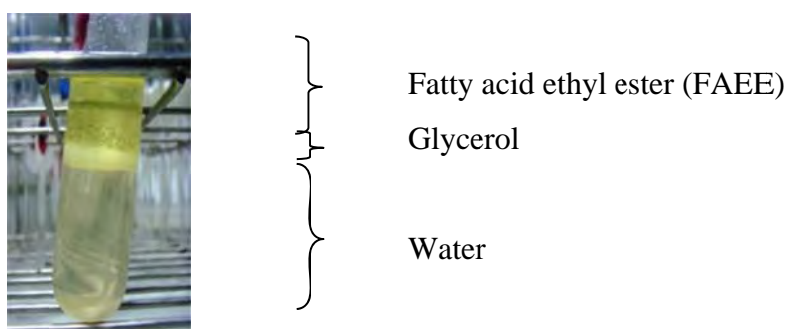
Figure 3.15 Show fatty acid ethyl ester after treating in the step of the glycerol separation.

3.3.6 Fatty Acid Ethyl Ester (FAEE) Analysis

FAEE compositions were analyzed by using gas chromatographic instrument (GC) (Shimadzu 2010 model) equipped by flame ionization detector (FID) in which one microliter of sample was injected into column. The GC consists of a capillary column (DB-WAX, Carbowax 20 M, 30m, 0.32mm ID, 0.25 µm). The injector, detector and column temperatures were at 250, 260 and 200 ^{o}C, respectively. Pressure was 64.1 kPa and linear velocity was 25 cm/sec. The carrier gas was helium (He) and the make-up gas was nitrogen (N_2). The samples were prepared by adding 0.1 ml of sample to 4.9 ml of n-hexane. The FAEE yield was estimated from the ratio of the quantity of FAMEs to that of reactants (purified palm oil or palm fatty acids):

% yield of ethyl ester = (W_{EE} / W_F) x 100

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

CHAPTER IV

RESULTS AND DISCUSSIONS

Generally, the cost of free enzyme is drawback of enzymatic process. To increase reusability and operational stability, enzymatic immobilization is a strategy used in the process dealing with changing state of temperature, shearing force and so on. The immobilized enzymes were easily separated from the product. Furthermore, it could be reused in many types of the reactors. This research attempted to develop novel support materials and immobilization techniques for lipase by using freeze dried BC-alginate.

Some key aspects of ester synthesis by enzymatic transesterification such as the effect of lipase quantity, the effect of the temperature, the influence of shaking speed, the effect of purified palm oil and fatty acid, and the effect of the layers of calcium alginate were also investigated. In addition, the optimal conditions from the previous study (Sawanpanya, N. 2009), such as the molar ratio of ethanol to palm oil of 9:1 and sodium alginate concentration at 2% (w/v) were applied in this study. According to Bhushan et al. (2008), the similar pattern of increasing of the concentration of sodium alginate and calcium chloride was limited, and the optimum concentration of sodium alginate was not over 2% (w/v). Won K et al. (2005) found that calcium chloride concentration was small effect in the range of 0.05 to 3 M.

This chapter presents the results of the biodiesel production from the raw-material of palm oil/palm fatty acid and ethanol by using free and immobilized lipase as biocatalysts. Some of the key aspects as stated above were discussed by determining the yield of fatty acid ethyl ester (FAEE) from enzymatic transesterification. The immobilized lipase was also examined for its stability and reusability in the ester synthesis. The results were compared between different types of immobilized lipase and free lipase. Scanning electron microscope (SEM) used to support some of the experimental data.

4.1 Effect of lipase quantity

Studying on the influence of lipase quantity on the ester yield in enzymatic transesterification of purified palm oil and 95% ethanol were carried using 5 and 10 % (wt) free lipase based on palm oil weight with the condition as follow: shaking speed at 250 rpm, reaction temperature at 45 $^{\circ}$C and the ratio of ethanol to oil at 9:1. The results are presented in Figure 4.1. The ethyl ester yield was enhanced by increasing of hydrolysis reaction with increasing of *C. rugosa* lipase quantity from 5% to 10%, in which the maximum ethyl ester yield at 93.3 % was obtained at around 36 h. Sawanpanya, N. (2009), reported the increase of hydrolysis reaction and ethyl ester yield with increasing of *C. rugosa* lipase concentration from 1.0 to 10.0% wt. However, no enhancement in ester yield or hydrolysis rate was observed with the increase of amount of lipase more than 10% wt. Moreover, the use of excessive lipase concentration could significantly raise the operating cost. Therefore, *C. rugosa* at 10% (by weight of oil) was applied to further studies.
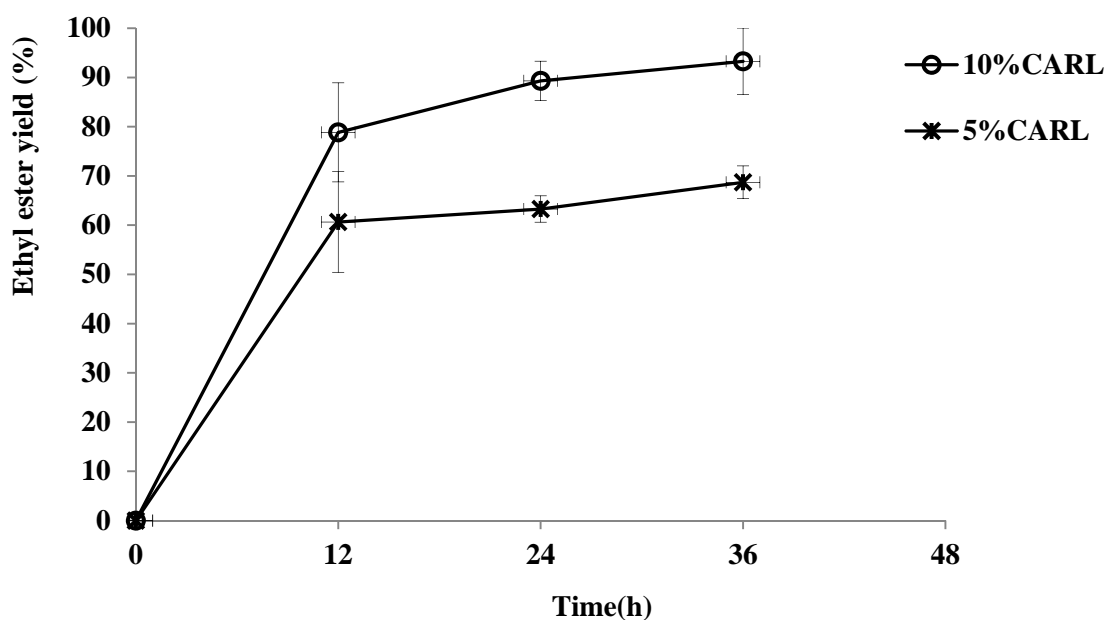
Figure 4.1 Effect of lipase quantity on the yield of ester at 45°C, 95% ethanol, 250 rpm and ethanol to palm oil ratio of 9:1.

4.2 Effect of temperature
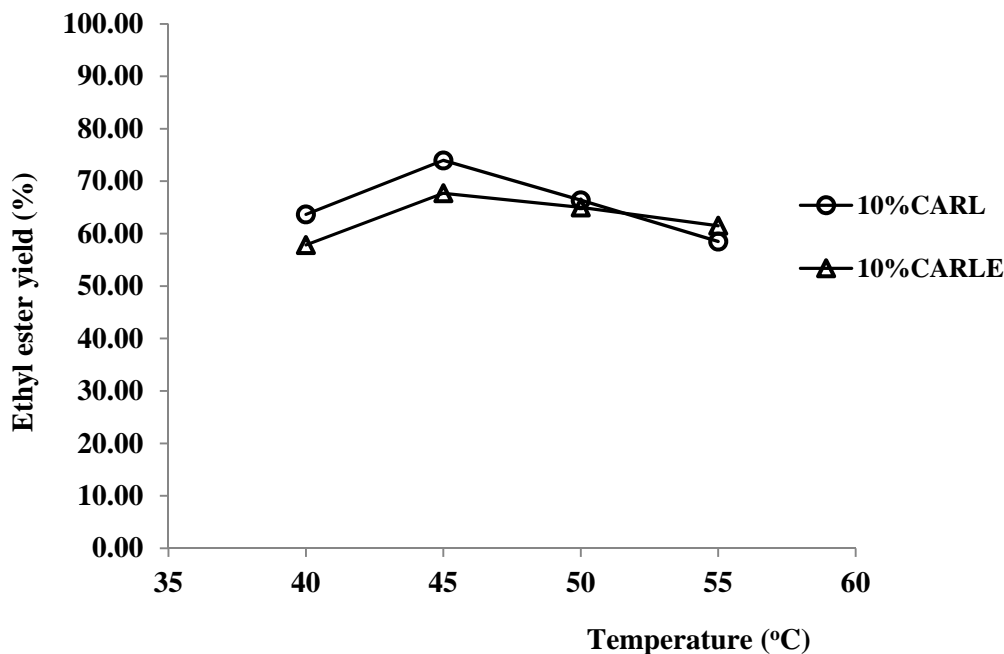
Figure 4.2 Effect of temperature on the yield of FAEE compared between 10% wt of free and immobilized lipase at the molar ratio of ethanol to palm oil ratio 9:1, 95% ethanol, 250 rpm and reaction time of 12 h.

The effect of the temperature on the yield of FAEE by using CARL and CARLE as biocatalysts was investigated. To search the optimal temperature, in the Figure 4.2, the reaction temperature was varied from 40 °C to 55 °C by using purified palm oil and 95% ethanol as a substrate. The FAEE's yield obtained by using CARL and CARLE increased with increasing of the operating temperature up to 45°C. The maximum ester yield of both free and immobilized enzymes appeared at the temperature of 45 °C. Therefore, the optimal temperature at 45°C was employed in further studies. High temperature increase miscibility between ethanol and oil (Shimada et al., 2002). However, at the temperature higher than 45°C, the ethyl ester by using free enzyme was considerably decreased, while the FAEE's yield of the immobilized enzymes was slightly decreased. Higher temperature gives rise to greater deactivation of enzyme. The immobilized lipase in BC-alginate support shows better

stability at high temperature than the free lipase. According to Awang et al. (2007), the reaction condition by using immobilized *C. rugosa* obtained the best conversion above 80% at the temperature of 40 $^{\circ}$C, which was not significantly different to this study.

4.3 Effect of shaking speed
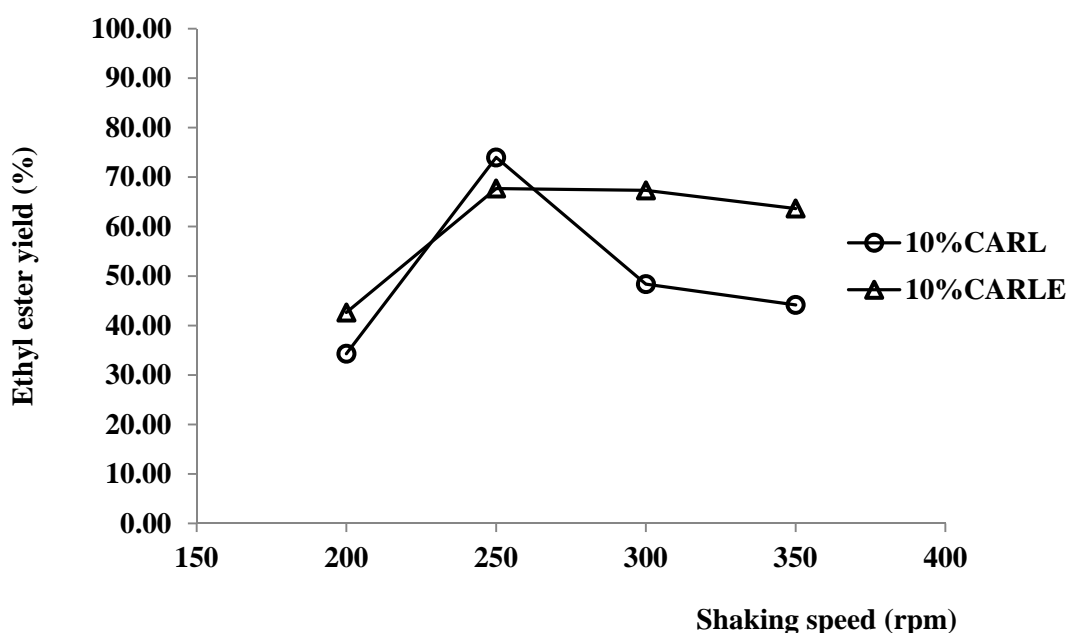
Figure 4.3 Effect of shaking speed on the yield of FAEE compared between 10% free and immobilized lipases with the molar ratio of ethanol to palm oil of 9:1, 95% ethanol, reaction temperature of 45°C and reaction time of 12 h.

The Figure 4.3 illustrated the influence of shaking speed on the ester yield by using CARL and CARLE as biocatalysts in transesterification of purified palm oil and 95% ethanol at the reaction temperature of 45°C, ethanol: oil molar ratio (w/w) of 9:1 with 10% free lipase (by wt of oil). It was found that the maximum ester yield was obtained at the shaking speed of 250 rpm. The mass transfer resistance was gradually decreased and the reaction rate was accelerated with increasing of shaking speed up to 250 rpm. At the shaking speed of 250 rpm, the ethyl ester yield by using free enzyme was 73.98%, whereas the immobilized lipase within freeze dried BC-alginate

exhibited the ethyl ester yield of 67.70%. However, at the shaking speed higher than 250 rpm, the shearing force could be the important factor causing considerable loss of free enzyme activity. The FAEE's yield by using CARLE as biocatalyst was slightly decreased with the increase of shaking speed from 250 – 350 rpm due to higher resistance to shearing force. According to Elias and Joshi (1998), the high hydrodynamic shear forces could cause damage to protein resulting in denaturation and inactivation of enzymes. Hence, in further studies, the shaking speed of 250 rpm was allowed to use in the enzymatic alcoholysis.

4.4 Surface morphology

The micrographs of cross-sectional surface are shown in the Figure 4.4 (a-b). The characterization of freeze dried material of BC-alginate was illustrated in the Figure 4.4(a). After freeze drying, the water-insoluble of cellulose fibrous without enzyme immobilization was becomes fibrous sponge. There were enzyme powders observed at the surface of BC-alginate after adsorption of soluble lipase as shown in the Figure 4.4(b). During the enzymatic reaction, immobilized enzyme could be deactivated or leached from the gelling beads. Tanaka et al. (1984) studied the diffusion characteristics of various substrates in calcium-alginate and expressed that compound with lower molecular weight than 2×10^4 could freely diffuse in/out gel beads. In order to increase an operational stability, Won et al. (2005) overcame this problem by coating silicate or chitosan on the gel surface. In this research, entrapment of the triple layer design of alginate gel was applied to prevent leaching and increase the operational stability of *C. rugosa* lipase which adsorbed on the freeze dried BC-alginate pieces.

a)
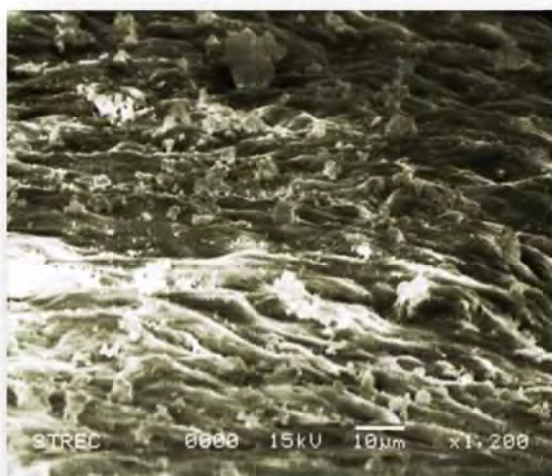
b)
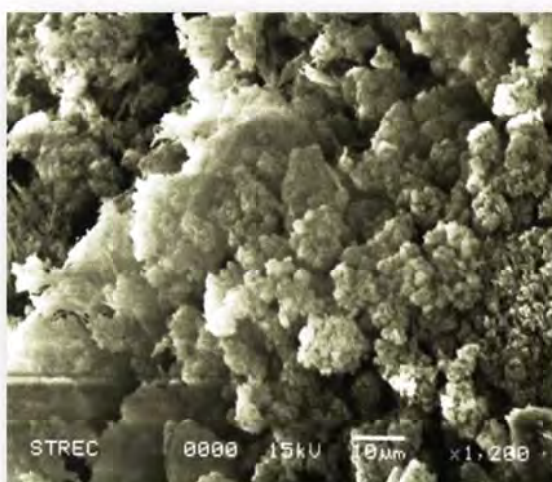
Figure 4.4 SEM micrographs of cross-sectional surface

a) Crossectional area of freeze dried BC-alginate without enzyme entrapped within double layers of Ca-alginate

b) Crossectional area of immobilized enzyme on freeze dried BC-alginate and entrapped within Ca-alginate (CARLE-3L) before using in enzymatic transesterification

4.5 Reusability and effect of number of the layer of Ca-alginate layers on BC-alginate support
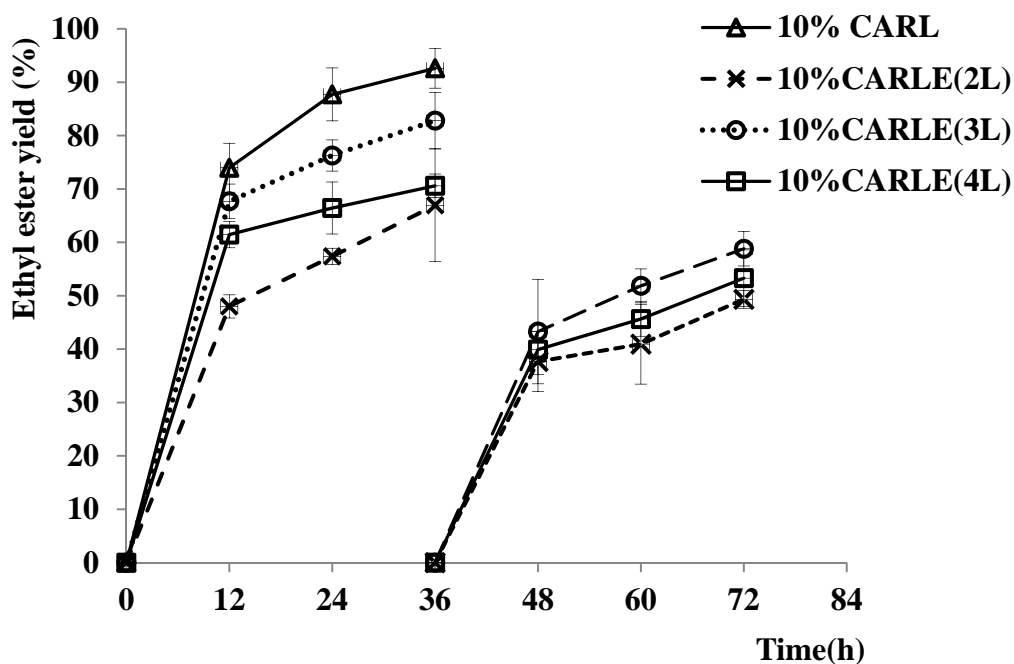
Figure 4.5 Reusability and effect of number of Ca-alginate layers on ethyl ester yield by using 10% lipase by wt of oil, 95% ethanol, and reaction temperature of 45°C, shaking speed 250 rpm and ethanol to palm oil ratio 9:1.

For improving cost-effective production, immobilized enzyme should able to be reused. The yields of biodiesel obtained by using different types of biocatalysts were compared as shown in Figure 4.5, when focusing on the Ca-alginate entrapped layer, the reusability of the immobilized lipase with triple Ca-alginate entrapped layers (CARLE-3L) was better than those with CARLE-4L and CARLE-2L, respectively. The maximum yield of CARLE-3L was 82.82% at 36 h. However, the significant decreases in FAEE yield after the second reuse of all of the immobilized enzymes are observed, which might owing to some leakages and/or the deactivation of enzymes when exposed to the violent condition. At the second reuse, the ethyl ester yield about 58.81 % was attained by using CARLE-3L, whereas the FAEEs at 53.31 and 49.32% were attained by using CARLE-4L and CARLE-2L, respectively.

According to Won et al. (2005), the reused biocatalyst immobilized in Ca-alginate matrix was inactivated due to activity loss. Therefore, CARLE-3L would be employed in further studies.

4.6 Effect of free fatty acid on transesterification

The influence of fatty acid on the yield of FAEE was investigated by using 10% (w/w) oleic acid mixed with purified palm oil as substrate. Figure 4.6(a) and Figure 4.6(b) present the ester's yield by using 95% ethanol and 99.9% ethanol as ethyl alcohol, respectively. Higher ethyl ester yields of 80-90% were observed in the system using purified palm oil. The maximum yield of purified palm oil was 92.61% at 36h by using 10% free lipase (by weight of oil), whereas 83.40% of the yield of FAEE was obtained when applying with CARLE-3L. On the other hand, system using with the mixture of 10% wt of oleic acid, the ethyl ester yields were severely reduced. It might be described that the acidity of palm fatty acid could cause negative effect to the enzymatic transesterification. However, when focusing on the substrate with 10% oleic acid, it was shown that ester yield obtained by using triple layer of calcium alginate was higher than using free lipase as biocatalyst. This was due to the benefit of the immobilizing support, which potentially prevented enzyme from harsh environments or toxic compounds.
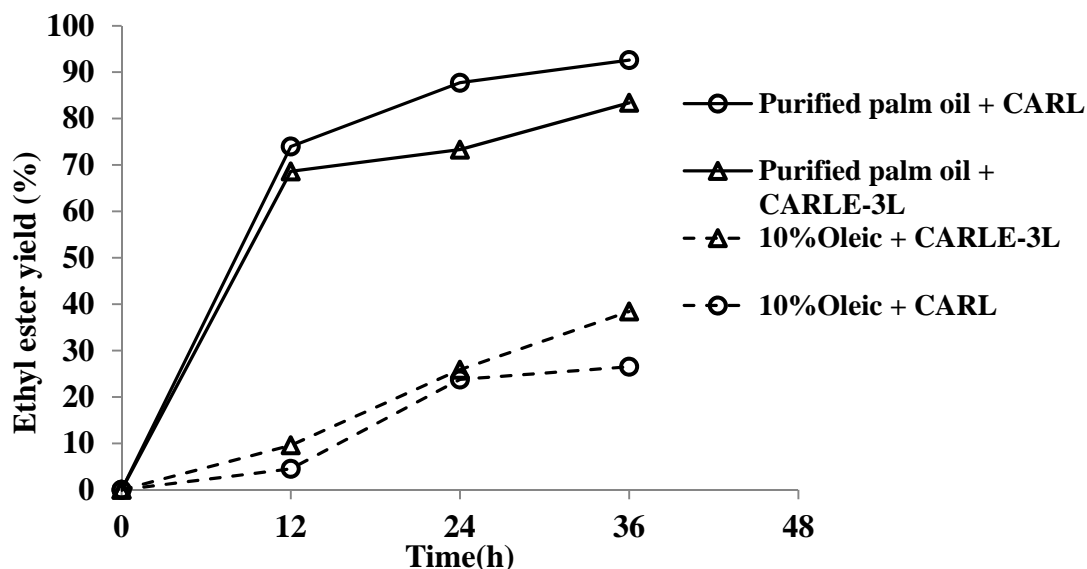
Figure 4.6(a) Effect of free fatty acid (10% wt in oil) on the transesterification of palm oil using 95% ethanol under the following condition: 10% by wt of lipase to oil, molar ratio of ethanol to palm oil ratio was 9:1, reaction temperature of 45°C and shaking speed of 250 rpm.
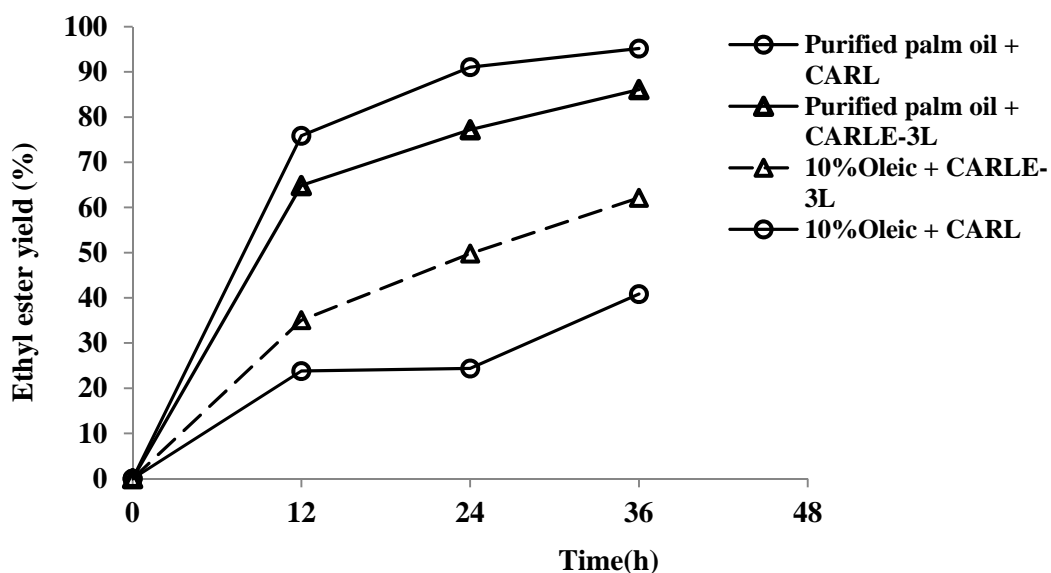
Figure 4.6(b) Effect of free fatty acid (10% wt in oil) on the transesterification of palm oil using 99.9% ethanol under the following conditions: 10% by wt of lipase to oil, molar ratio of ethanol to palm oil ratio was 9:1, reaction temperature of 45°C and shaking speed of 250 rpm.

4.7 Effect of water content in ethanol
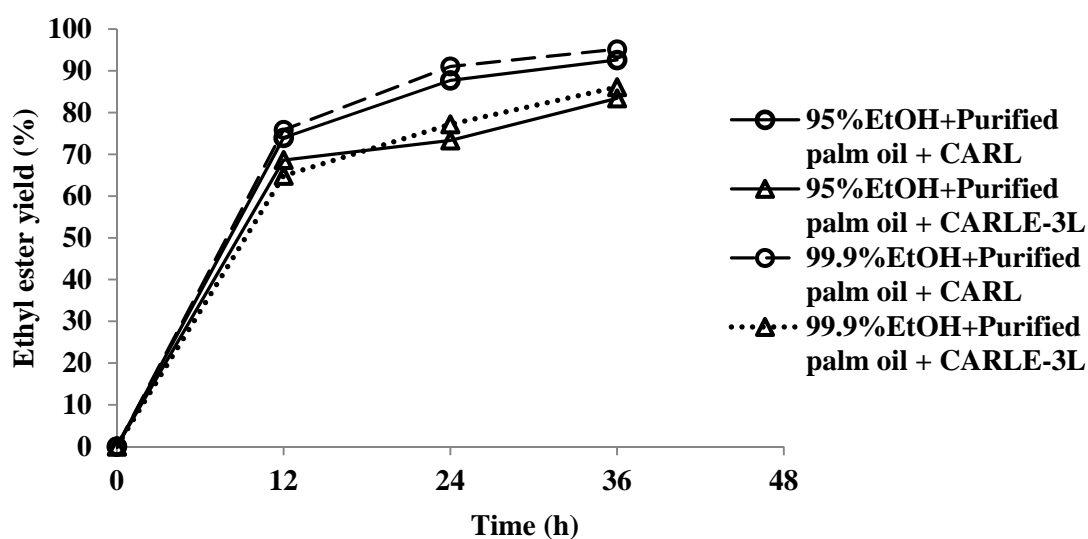
Figure 4.7 Effect of water content in ethanol (99.9% and 95.0% ethanol) on the yield of fatty acid ethyl ester under the following conditions: 10% by wt of lipase to oil, molar ratio of ethanol to palm oil ratio of 9:1, reaction temperature of 45°C and shaking speed of 250 rpm.

In order to evaluate the effect of water content in ethanol, the transesterification using 99.9% ethanol was carried out by compared with that of 95.0% ethanol base-biodiesel fuel was compared between 95% and 99.9% ethanol. Kwanchareon et al. (2006) has been reported that the miscibility of ethanol-oil component was increased when the concentration of ethanol and temperature were high. In the case of 95% ethanol, the high polarity of water could increase polar in an ethanol molecule. On the contrary, 99.9% ethanol was lower water concentration, therefore higher miscibility between ethanol and oil than 95% ethanol was obtained. Slightly higher ethyl ester yields were obtained by using 99.9% ethanol in comparison to that of 95.0% ethanol. According to Shimada et al. (1999), droplet of immiscible acyl acceptor in oil could cause inactivation of lipase. The low miscibility of oil-alcohol could cause the high inhibitory effect resulting in low ester yield. Instead of alcoholysis, hydrolysis reaction was preferred at high water concentration, and it made low ester conversion yield (Adlercreutz 2000; Chattopadhyay et al., 2011).

However, 95% ethanol is much cheaper than 99.9% ethanol and it can be easily produced domestically in our country. For economic consideration, the increasing of ester yield by using high cost of highly purified ethanol should be concerned whether it was appropriate for effective operating cost.

4.8 Influence of additional time of fatty acid (oleic acid)
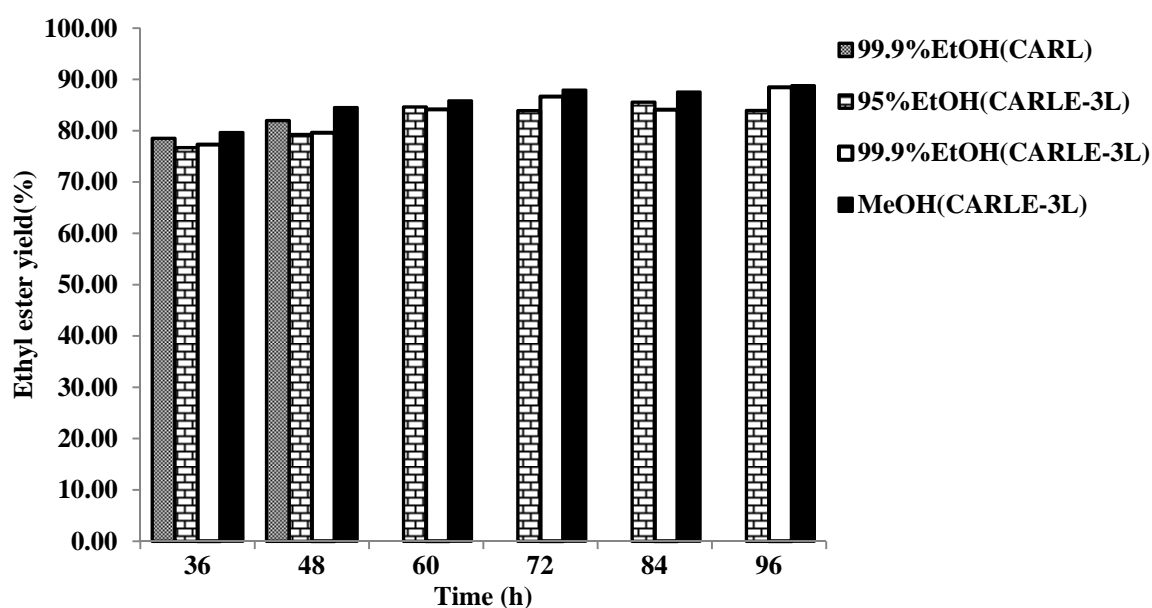
Figure 4.8 Influence of additional time of fatty acid on ethyl ester yield by using free and immobilized lipase with different alcohols, 95.0% EtOH, 99.9% EtOH and 99.9% MeOH, as reactants.

To reduce the deactivation of biocatalysts from the effect of free fatty acid, addition of fatty acid at the interval time was applied. According to the report by Sawangpanya, N. (2009), the optimal mass ratio of purified palm oil to fatty acid was at 70:30. Firstly, it was started dealing with alcoholysis by using purified palm oil under the optimal condition: reaction temperature of 45°C, shaking speed at 250 rpm, molar ratio of ethanol to palm oil at 9:1 and 10% by weight of lipase to oil. At 24 h, the reaction medium was added oleic acid at 30 %(w/w) and ethanol into the batch system. After that, the time interval of adding substrates and sampling the sample was every 12 h.

In the Figure 4.8, at 36h, the ester yields obtained by purified palm oil with various types of alcohols and enzyme as followed: 99.0% EtOH with CARL, 95.0% EtOH with CARLE-3L, 99.9% EtOH with CARLE-3L and 99.9% MeOH with CARLE-3L were 78.52%, 76.73, 77.31 and 79.66%, respectively. Then, after 36h, 30% oleic acid (base on weight of total substrate) was subsequently replaced to the mixture of products. The reaction should combine between transesterification and esterification. Interestingly, the ester yield at every 12h provided the nearly same level during 76% to 88.79%. The ester yield by using 99.9% MeOH was highest at every interval time. In addition, the ester yield by using of 95.0% and 99.9% EtOH was not significantly different. These data could be indicated that adding of fatty acid at interval time would reduce the effect of acidity in the system. This is because the biodiesel is more miscible with fatty acid and/or alcolhol than pure vegetable oil. According to Sawanpanya, N., 2009, and Chen et al., 2008, the mixture after filling fatty acid was gradually well-mix of palm fatty acid and reaction medium. The well-mixed environment of palm fatty acid and reaction medium could also enhance mass transfer coefficients between phases, resulting in improved reaction rate. Consequently, this modified method could overcome the deactivation problem from acid condition.

CHAPTER V

CONCLUSION AND RECOMMENDATIONS

5.1 CONCLUSION

The main objective of this ongoing work was to develop the immobilizing support for *C. rugosa* by using bacterial cellulose (BC) for ester synthesis of palm oil/ free fatty acid and ethanol. BC and other raw materials in this study could be produced from local areas in Thailand. The integration method of adsorption and entrapment was applied for the immobilization. Firstly, it was physical adsorption of soluble *C. rugosa* (CARL) on freeze dried BC-alginate. Secondly, the immobilized lipase on BC-alginate was entrapped within the matrix of calcium alginate (CARLE). The appropriate concentration of CARL for the ester synthesis was at 10% (w/w) based on oil weight. The diameter of circular freeze dried BC-alginate pieces was 1.20 cm, with the thickness about 3 mm. Sodium alginate solution at 2%(w/v) was used to form gelling layer with a cross linking agent of 120 mM calcium chloride. Comparison to the free lipase, the immobilized lipase showed enhancement of thermal, mechanical and acid resistance. Increasing of the entrapped layers with calcium alginate could prevent leaching and protect enzyme against violent conditions. The optimal conditions based on transesterification of purified palm oil with 95.0%(v/v) ethanol were at the molar ratio of ethanol to palm oil of 9:1, reaction temperature of 45 °C, shaking speed of 250 rpm, reaction time of 36 hours and 3 layers of calcium alginate coated on BC-alginate support (CARLE-3L). The ester yield of using the free lipase (92.61%) was higher than that of CARLE-3L (82.81%), CARLE-4L (70.60%) and CARLE-2L (66.94%), respectively. Upon the first recycling of the immobilized enzyme, the ester yield about 58.81 % was attained by using CARLE-3L, whereas the ester yields at 53.31% and 49.32% were attained by using CARLE-4L and CARLE-2L, respectively. Furthermore, it was found that the method of adding free fatty acid with interval time could be applied to diminish the deactivation of the biocatalyst from the acidity of the free fatty acid. The immobilized lipase can be reused for at least five times without loss of enzyme activity. By using

99.9% ethanol, 95.0% ethanol and 99.9% methanol as raw material under semi-continuous feeding of free fatty acid, the average ester yields at 84.60%, 83.48% and 86.92% were obtained, respectively. Ester yield by using 99.9% ethanol was not considerably higher than that of 95.0% ethanol, however, 95% ethanol is much cheaper than 99.9% ethanol. From this point of view, 95.0% ethanol was preferred to be used as raw material in enzymatic transesterification.

5.2 RECOMMENDATIONS

5.2.1 In order to lower the biodiesel production cost, the use of cheap raw materials available in Thailand that can potentially be used for biodiesel production, such as non-eatable oil, used cooking oil, waste oil and free fatty acids should be studied. In addition, using of non-eatable oil does not compete with food supply.

5.2.2 The reused immobilized enzyme samples on each batch should be taken to investigate enzyme leakage and deactivation.

5.2.3 According to the benefit of the method of semi-continuous feeding of free fatty acid in this study, further studies on pilot scale should be carried out to demonstrate the feasibility and performance of this method.

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APPENDICES

APPENDIX A

CALCULATION OF PERCENT YIELD OF ETHYL ESTER

A-1 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 A-1.1.

Table A-1.1 Composition and molecular weight of key components in of palm fatty acids.

Palm fatty acids	% Weight Fraction	Molecular weight
Palmitic acid	42.80	256.43
Oleic acid	40.50	282.47
Stearic acid	4.50	284.50
Linoleic acid	10.10	268.00
Linolenic acid	2.10	278.43

The data in the Table A-1.1 above can be used to find the molecular weight of palm fatty acids as shown below:

$$M_w = \sum(M_{Fa} \; x \; \% \; Weight \; of \; fraction \; fatty \; acid) \qquad \text{.................A-1.1}$$

Where,

M_w = Molecular weight of fatty acids

M_{Fa} = Molecular weight of each fatty acids

Apply the formula A-1.1 and the Table A-1.1 to compute M_{Fa}

M_{Fa} = (0.428 x 256.43) + (0.405 x 282.47) + (0.045 x 284.5) + (0.101 x 268) + (0.021 x 278. 43) = 269.85 (~270)

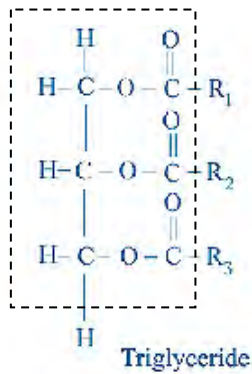**A-2** Calculation of molecular weight of purified palm oil

Figure A-2.1 Triglyceride structure.

R1, R2, R3: carbon chain of the fatty acids

Table A-2.1 Composition and molecular weight of key components of purified palm oil

Palm fatty acid	% Weight fraction	Molecular weight
Lauric	0.1	200
Myristic	1.0	228
Palmitic acid	42.8	256
Oleic acid	40.5	282
Stearic acid	4.5	284
Linoleic acid	10.1	280
Linolenic acid	0.2	278

The molecular weight of triglyceride is defined as:

$$Mw_{TG} = 3R_{aver} + 173 \qquad \text{... A-2.1}$$

$$R_{aver} = \sum \left(\frac{\%Fa_n}{100} \times Mw_n \right) \dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots \textbf{A-2.2}$$

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

From Table A-2.1, an equation A-2.1 and A-2.2 above

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

$$= 267.08$$

$Mw_{TG} = 3(267.08) + 173 = 974.23$

A-3 Calculation weight of biocatalyst

Example Base on volume of purified palm oil is 15.0 g. The mass ratio of lipase to purified palm oil is 5%.

   Weight of biocatalyst =   15 (5 / 100) = 0.75 g

A-4 Calculation of reactants

Molecular ratio of ethanol to reactants: $N_{EtOH} / N_{reactant}$

Example Base on molecular weight of purified palm oil and ethanol is 974.23 and 46.07 g/mol, respectively. The density of purified palm oil and ethanol is 0.92 and 0.79 g/ml. The weight of purified palm oil is 15 g. The molar ratio of oil to ethanol is 1:9.

$N_{EtOH} = 9N_{OIL}$

$N_{OIL} \quad = 15/974.23 = 0.0154$ mol

$N_{EtOH} \quad = 9(0.0154) = 0.1386$ mol

Volume of EtOH $= (0.138 \times 46.07) / 0.79 = 8.08$ ml

A-5 Calculation weight of each ethyl ester

The weight of ethyl ester is defined as:

$$W_{EE} = \left\{ \frac{C \times V_{TD}}{V_S} \right\} \times V_P \quad \dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots \textbf{A-5.1}$$

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

$C \quad$ = Concentration of each methyl ester from calibration curve (g/ml)

$V_{TD} \quad$ = Total diluted volume (ml)

$V_s \quad$ = Volume product dilute (ml)

$V_p \quad$ = Total volume of product (ml)

From this research,

$V_s \quad = 0.1$ ml,

$V_{TD} \quad = 5.0$ ml

A-6 Calculation of the percent ethyl esters yield

The percent ethyl esters yield is defined as:

$$\% \text{ Yield of ethyl esters} = \frac{W_{EE}}{W_{Fa} \times (x_i)} \times 100 \quad \dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots \textbf{A-5.2}$$

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

Where,

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

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

$W_{EP} \quad$ = 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)

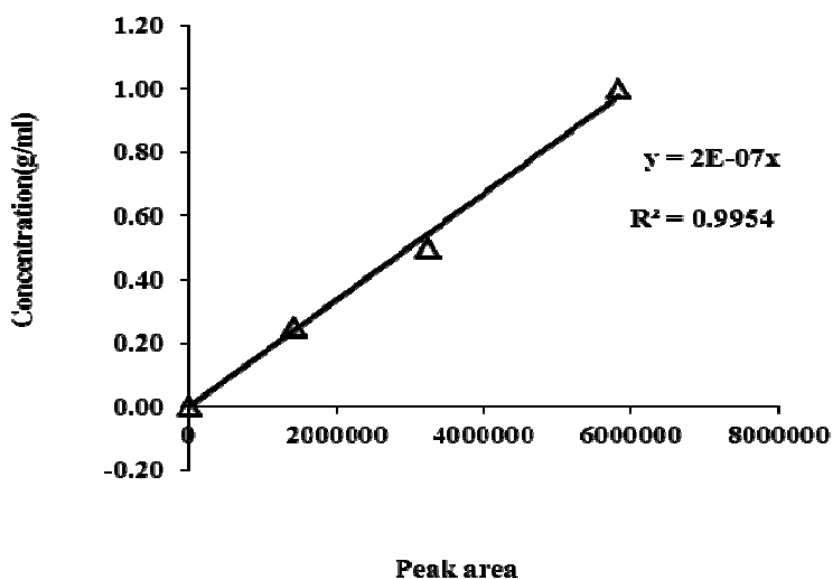x_i = Weight fraction of fatty acid

From this study, W_{Fa} = 15.0 g

A-7 Calibration

Table A-7.1 Data of the peak area of ethyl palmitate component in fatty acid ethyl ester

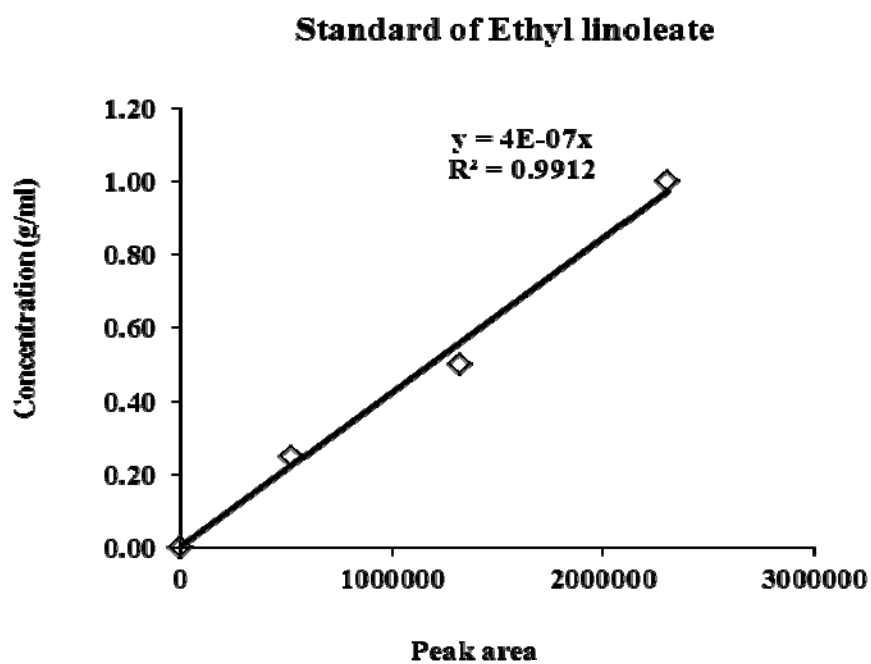| No.	Retention time	Area	Conc.(g/ml)
1	7.680	0	0.00
2	7.680	1416554	0.25
3	7.671	3241237	0.50
4	7.691	5831107	1.00

Standard of Ethyl palmitate

$y = 2E-07x$

$R^2 = 0.9954$

FigureA-7.1 Standard calibration curve of ethyl palmitate.

Table A-7.2 Data of the peak area of ethyl linoleate component in fatty acid ethyl ester

No.	Retention time	Area	Conc.(g/ml)
1	10.912	0	0.00
2	10.912	527525	0.25
3	10.919	1320068	0.50
4	10.932	2301771	1.00

FigureA-7.2 Standard calibration curve of ethyl linoleate.

Table A-7.3 Data of the peak area of ethyl oleate component in fatty acid ethyl ester

No.	Retention time	Area	Conc.(g/ml)
1	11.049	0	0.00
2	11.049	1187431	0.25
3	11.079	2291151	0.50
4	11.128	5690606	1.00

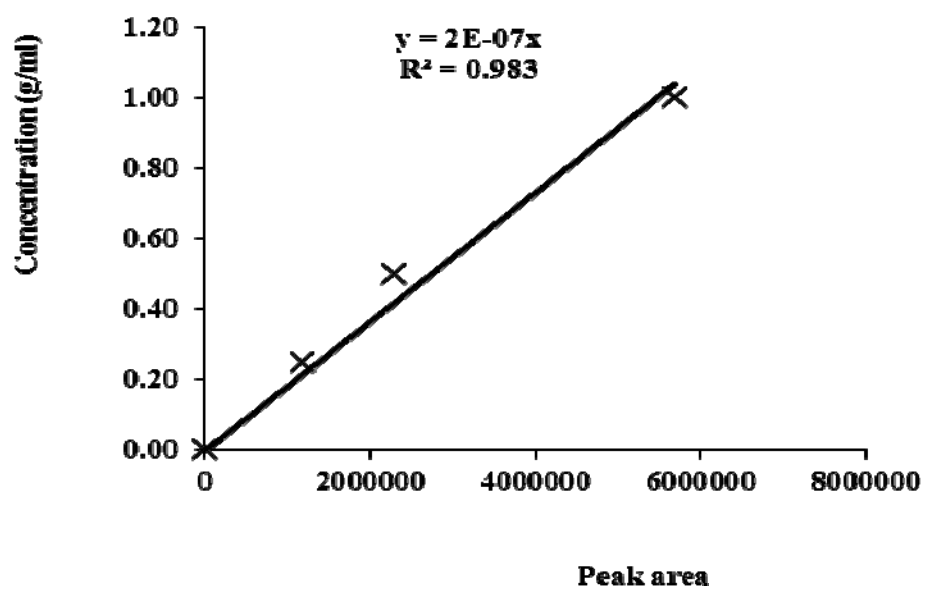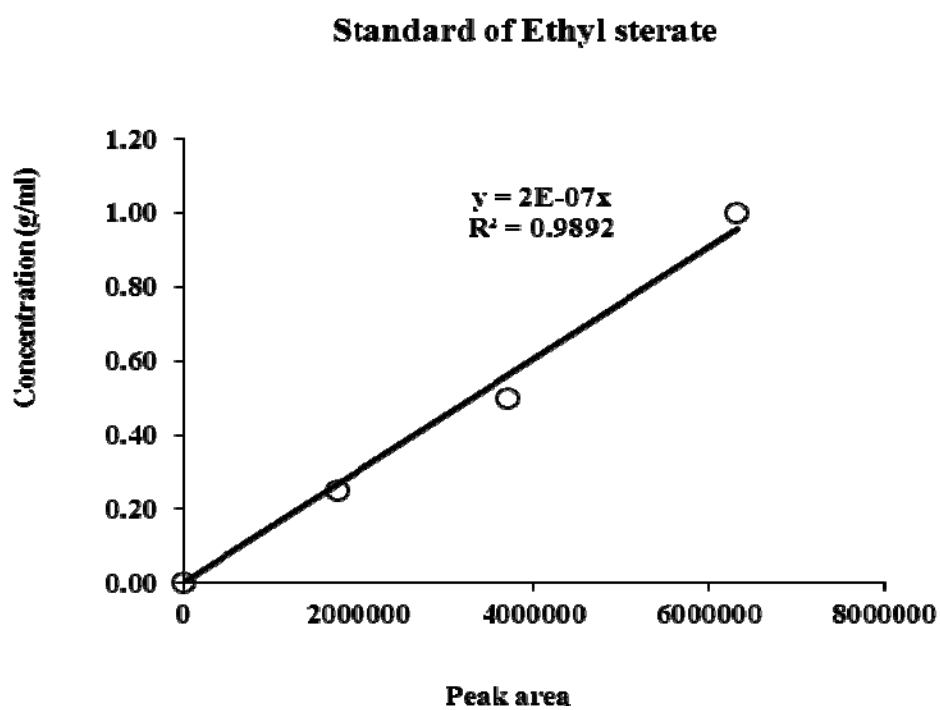**FigureA-7.3** Standard calibration curve of ethyl oleate.

Table A-7.4 Data of the peak area of ethyl stearate component in fatty acid ethyl ester

No.	Retention time	Area	Conc.(g/ml)
1	11.625	0	0.00
2	11.625	1763090	0.25
3	11.668	3705230	0.50
4	11.717	6316127	1.00

FigureA-7.4 Standard calibration curve of ethyl stearate.

APPENDIX B

EXPERIMENTAL DATA FOR ANALYSIS

B-1 Experimental data of enzymatic transesterification reaction of purified palm oil and enzymatic esterification reaction of palm fatty acid

Table B-1.1 Effect of lipase quantity on the yield of ester.

Time(h)	Average of %Yield of ethyl ester	
	5%CARL	**10%CARL**
0	0.00	0.00
12	60.64	78.85
24	63.28	89.31
36	68.71	93.28

Table B-1.2 The effect of shaking speed on the yield of fatty acid ethyl ester.

Shaking speed(rpm)	Average of %Yield of Ethyl ester	
	10%CARL	**10%CARLE**
200	34.30	42.63
250	73.98	67.70
300	48.37	67.32
350	44.17	63.65

Table B-1.3 The effect of temperature on the yield of fatty acid ethyl ester.

Temp.(^{o}C)	% Yield of ethyl ester	
	10%CARL	10%CARLE
40	63.67	57.82
45	73.98	67.70
50	66.37	65.00
55	58.50	61.50

Table B-1.4 Reusability and effect of number of the layer of Ca-alginate layers on BC-alginate support.

	Time(h)	10% CARL	10%CARLE-2L	10%CARLE-3L	10%CARLE-4L
Adding of EtOH round no.1	0	0.00	0.00	0.00	0.00
	12	73.98	48.00	67.70	61.45
	24	87.72	57.37	76.28	66.43
	36	92.61	66.95	82.82	70.60
Adding of EtOH round no.2	Time(h)	10% CARL	10%CARLE-2L	10%CARLE-3L	10%CARLE-4L
	36		0.00	0.00	0.00
	48		37.68	43.28	39.92
	60		40.93	51.86	45.64
	72		49.32	58.81	53.31

Table B-1.5 Standard deviation of reusability and effect of number of Ca-alginate layer on BC-alginate support.

Round	Time(h)	Error			
		10% CARL	10% CARLE-2L	10% CARLE(3L)	10% CARLE-4L
No.1	0.0	0.00	0.00	0.00	0.00
	12.0	4.58	2.17	3.23	2.46
	24.0	4.97	1.52	2.91	4.87
	36.0	3.74	10.55	5.28	2.20
No.2	36.0	0.00	0.00	0.00	0.00
	48.0		5.65	9.79	4.70
	60.0		7.48	3.18	3.25
	72.0		1.68	3.24	5.28

Table B-1.6 Effect of free fatty acid (10% wt in oil) on the transesterification of palm oil using 95% ethanol.

Time(h)	95%EtOH			
	10%Oleic + CARL	10%Oleic + CARLE-3L	Purified palm oil + CARL	Purified palm oil + CARLE-3L
0	0	0	0	0
12	4.53	9.60	73.98	68.62
24	23.79	25.89	87.72	73.32
36	26.52	38.43	92.61	83.40

Table B-1.7 Effect of free fatty acid (10% wt in oil) on the transesterification of palm oil using 99.9% ethanol.

Time(h)	99.9% EtOH			
	10%Oleic + CARL	10%Oleic + CARLE-3L	Purified palm oil + CARL	Purified palm oil + CARLE-3L
0	0	0	0	0
12	23.81	35.05	75.87	64.85
24	24.38	49.79	91.05	77.20
36	40.85	62.13	95.17	86.08

Table B-1.8 Effect of water content in ethanol (99.9% and 95.0% ethanol) on the yield of fatty acid ethyl.

Time(h)	95%EtOH +Purified palm oil + CARL	95%EtOH+ Purified palm oil + CARLE-3L	99.9%EtOH+ Purified palm oil + CARL	99.9%EtOH+Purif- ied palm oil + CARLE-3L
0	0	0	0	0
12	73.98	68.62	75.87	64.85
24	87.72	73.32	91.05	77.20
36	92.61	83.40	95.17	86.08

Table B-1.9 Influence of additional time of fatty acid on ethyl ester yield.

Time (h)	95%EtOH (CARL)	95%EtOH (CARLE-3L)	99.9%EtOH (CARLE-3L)	MeOH(CARLE-3L)
36	78.52	76.73	77.31	79.66
48	81.99	79.28	79.61	84.51
60		84.65	84.15	85.83
72		83.93	86.66	87.91
84		85.58	84.09	87.56
96		83.95	88.48	88.79

BIOGRAPHY

Mr.Chanudom Muangchim was born in Suratthani province, South of Thailand on March 3rd, 1981. He obtained a Bachelor's degree of Sciene from the Department of Chemistry, Faculty of Science, Prince of Songkla University, Amphur Hat Yai, Songkhla, Thailand, in 2004. He started working as a R&D supervisor-chemist at Thai Mitsui Specialty Chemical Company, Chachoengsao, Thailand, in August 2005. After that, he had worked as a QA and process improvement engineer for Siam Furukawa Co., Ltd., Amphur Nong-Kae, Saraburi, Thailand, since March 2005 to May 2008. Then, he subsequently fulfilled the requirement for a Master's degree of engineering, at the Department of Chemical Engineering, Faculty of engineering, Chulalongkorn University, Bangkok, Thailand, in 2011.

His academic publications are following.

1. Muangchim, C., and Phisalaphong, M. Ethyl ester production from palm oil catalyzed by immobilized *Candida rugosa* lipase in bacterial cellulose-alginate support. In Svasti, J., et al. (eds*.), The 3rd International Conference on Biochemistry and Molecular Biology(BMB 2011): From Basic to Translational Researches for a Better Life, Proceeding of international conference, The Empress Convention Center, Chiang Mai, Thailand, 6-8 April 2011*, pp.318-321.

2. Sawangpanya, N., Muangchim, C., and Phisalaphong, M. Immobilization of lipase on CaCO$_3$ and entrapment in calcium alginate bead for biodiesel production. *Science Jounal Ubon Ratchathani University* July-December, 2010; 1(2): 46-51.

3. Sawangpanya, N., Muangchim, C., and Phisalaphong, M. Immobilization of lipase on CaCO$_3$ and entrapment in calcium alginate bead for biodiesel production. In The Thailand Research Fund(TRF*), TRF-Master Research Congress V: Presentation of progressing or closing project, Jomtien Palm Beach Hotel& Resort, Pattaya, Chonburi, Thailand, March 30th-April 1st, 2011*, pp.217, Bangkok:Vista interprint co.,ltd., March 2011.