CHAPTER 1

INTRODUCTION

"Biodiesel fuel" comprises long-chain fatty acid methyl esters, which offer fuel properties that resemble diesel oil [1]. However, biodiesel does not produce carbon dioxide, carbon monoxide and sulfur oxide after combustion and it minimizes the hydrocarbons and soot particulates by one-third compared with the combustion of diesel oil [2]. Therefore, the property of biodiesel fuel is better than petroleum fuel [3]. Fatty acid methyl esters can be synthesized by methanolysis of triacylglycerols (TAGs) which are catalyzed by the chemical catalyst (acid or base) or biocatalyst (lipase) [4]. Acid- or alkali-catalyzed methanolysis produces methyl esters and glycerol [5]. Unfortunately, the reaction has some disadvantages, for instances, vigorous reaction condition (reaction temperature of 70-80^{o}C) [6], difficulty in the recovery of methyl esters (glycerol must be removed from the reaction) and the occurrence of soap from saponification, which reduces the yield of methyl esters [7]. As for lipases-catalyzed methanolysis, besides methyl ester is synthesized, other products such as glycerol, monoacylglycerols and diacylglycerols are obtained depending on the enzyme specificity [8]. The advantages of using lipases as biocatalysts, compared to acid and base are the milder reaction condition (reaction temperature of 30-40^{o}C), easier removal of glycerol, and better control over the amount of free fatty acids in the reaction by reacting with organic solvent and subsequent recovering of methyl esters [2].

Biodiesel can be synthesized by methanolysis that is catalyzed by microbial lipases such as immobilized lipases from *Rhizomucor miehei* [9], *Candida antarctica* [10] and free lipase from *Rhizopus oryzae* [11], which yield 98.5, 98 and 90% of methyl esters, respectively. However, there is no report on using lipase from *Carica papaya* latex in biodiesel fuel synthesis. As for the lipase from *C. papaya* latex, it is able to catalyze long-chain fatty acid alkyl esters synthesis by esterification of sitostanol with oleic acid [12], and alcoholysis of crambe and camelina oils [13]. Therefore, papaya lipase which is not only obtained from an economical plant throughout Thailand whole year round, but also cheaper than microbial lipase, is of particular interest in using as a biocatalyst for biodiesel fuel synthesis.

1.1 Biodiesel fuel

Biodiesel fuel (long chain fatty acid methyl esters) obtained by transesterification can be used as an alternative fuel for diesel engines [14-15]. The attractive features of biodiesel fuel are: (i) it is plant-derived, not petroleum-derived, and as such its combustion does not increase current net atmospheric levels of carbon dioxide, a "greenhouse" gas; (ii) it can be domestically produced, offering the possibility of reducing petroleum imports; (iii) it is biodegradable; (iv) relative to conventional diesel fuel, its combustion products have reduced levels of particulates, carbon monoxide, sulfur oxide, hydrocarbons, soot particulates, and under some conditions, nitrogen oxide [2]. Recently, it has been reported that a biodiesel fuel with good ignitability, such as one with a high methyl oleate content, gives lower levels of nitrogen monoxide, hydrocarbons, formaldehyde, acetaldehyde and formic acid, and also that soot particulate formation in suppressed, since biodiesel is an oxygenated fuel having an oxygen mass fraction of 10% [16]. As a consequence of its advantages, there is considerable interest in exploring and developing the use of biodiesel fuel.

Methyl esters of fatty acids can be produced by the methanolysis of TAGs such as animal fats, used edible oils, and plant oils [2,17] which are catalyzed either by the chemical catalyst (acid or alkali) or biocatalyst (lipase) [4].

1.2 Methanolysis

Methanolysis is an alcoholysis which is also called transesterification of alcohol. Alcoholysis is the displacement of alcohol from an ester by another in a process similar to hydrolysis, except that an alcohol is employed instead of water. The reaction represented by the general equation was shown in **Figure 1.1**.

Methanolysis reaction represented by general equation was also shown in **Figure 1.2**. The first step is the conversion of TAG to diacylglycerol, which is followed by the conversion of diacylglycerol to monoacylglycerol and of monoacylglycerol to glycerol, yielding one methyl ester molecule from individual glyceride at each step.

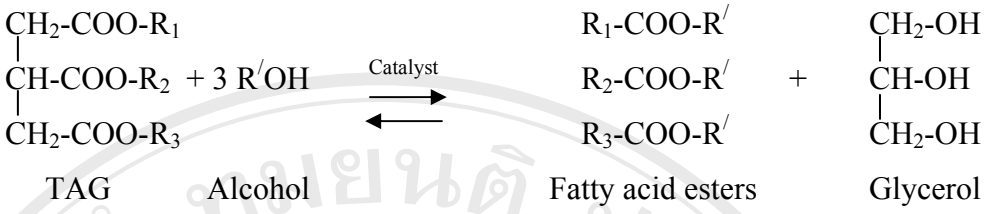

Figure 1.1 General equation of alcoholysis. R_1, R_2 and R_3 represent alkyl groups.

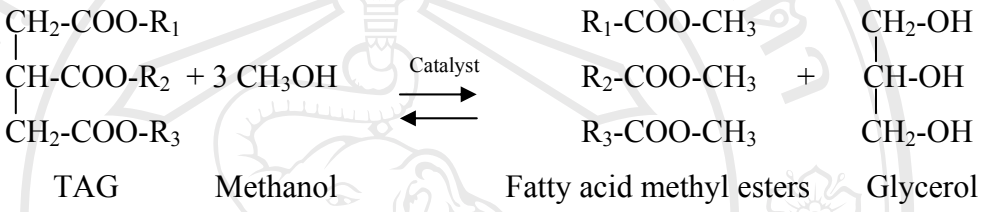

Figure 1.2 General equation of methanolysis of TAG. R_1, R_2 and R_3 represent alkyl groups.

1.2.1 Acid- or alkali-catalyzed alcoholysis

Acids used for alcoholysis include sulfuric, phosphoric, hydrochloric, and organic sulfonic acids. Although alcoholysis by acid catalysis is much slower than that by alkali catalysis [7,17-18], acid-catalyzed alcoholysis is more suitable for acylglycerols that have relatively high free fatty acid contents and more amounts of water [7,19]. When the oil component was a low grade material such as sulphur olive oil, it was necessary to perform alcoholysis under an acidic condition [19].

In situ alcoholysis differs from the conventional reaction in that the oil-bearing material contacts acidified alcohol directly instead of reacting with purified oil and alcohol. That is, extraction and alcoholysis proceed within the same process, the alcohol acting both as an extraction solvent and an esterification reagent. *In situ* alcoholysis of sunflower oil with acidified methanol produces fatty acid methyl esters in yields significantly greater than those obtained from the conventional reaction with pre-extracted seed oil [20-21].

Alkalis used for alcoholysis include sodium hydroxide, potassium hydroxide, carbonates, and alkoxides such as sodium methoxide, sodium ethoxide, sodium

propoxide, and sodium butoxide. Alkali-catalyzed alcoholysis proceeds approximately 4,000 times faster than that catalyzed by the same amount of an acidic catalyst [22], and is thus most often used commercially. Sodium methoxide has been found to be more effective than sodium hydroxide, presumably because a small amount of water is produced upon mixing sodium hydroxide and methanol [7,23]. However, sodium hydroxide and potassium hydroxide [24] are also able to catalyze alcoholysis. Especially, becaus e of their cheapness, they are widely used in industrial biodiesel production.

For alkali-catalyzed alcoholysis, the acylglycerols and alcohol must be substantially anhydrous because water by-products cause a partial reaction change to saponification, which eventually produces soap [25]. The soap consumes the catalyst and reduces the catalytic efficiency, as well as causes an increase in viscosity, the formation of gels and difficulty in achieving separation of glycerol. Free fatty acid contents of refined oil should be as low as possible, below 0.5% [26] and the oils should also be dry and free from free fatty acids [27].

The stoichiometry of alcoholysis reaction requires 3 moles of alcohol per mole of TAG to yield 3 moles of fatty acid esters and 1 mole of glycerol. Higher molar ratios result in greater ester conversion in a shorter time. Methanolysis of palm oil at 70^{o}C in an organic solvent with sodium methoxide as a catalyst, the conversion increased with increasing molar ratios of methanol to palm oil [28]. A molar ratio of 6:1 is normally used in industrial processes to obtain methyl ester yields higher than 98% on a weight basis [27,29]. In many countries, such as Europe, U.S.A. and Japan, strong alkalis have been used as catalysts in industrial processes for producing biodiesel. Such industrial process has production scale of hundreds of thousands of ton [30].

1.2.2 Enzymatic alcoholysis by lipase

Although chemical alcoholysis using an alkali-catalysis process gives high conversion levels of TAGs to their corresponding methyl esters in short reaction times, the reaction has several drawbacks: it is energy intensive, recovery of glycerol is difficult, the acidic or alkaline catalyst has to be removed from the product, alkaline waste water requires further treatment, and more importantly, free fatty acid and water

interfere with the reaction [2]. Therefore, a strong alkali process cannot smoothly handle rendered oils and fats which contain higher contents of moisture and free fatty acids.

Both extracellular and intracellular lipases are also able to effectively catalyze alcoholysis of TAGs in either aqueous or nonaqueous systems, as shown in **Table 1.1**, enzymatic alcoholysis methods can overcome the problems from alkali-catalysis process. Glycerol can be easily recovered without any complex process, and also that free fatty acids contained in waste oils and fats can be completely converted to methyl esters. In **Figure 1.3**, comparative flow diagrams for biodiesel fuel production by the alkali- and lipase-catalysis processes are presented.

Table 1.1 Comparison between alkali-catalysis and lipase-catalysis processes for biodiesel fuel production [2].

Comparison term	Alkali-catalysis process	Lipase-catalysis process
Reaction temperature	60-70^{o}C	30-40^{o}C
Free fatty acids in raw materials	Saponified products	Methyl esters
Water in raw materials	Interference with the reaction	No influence
Yield of methyl esters	Normal	Higher
Recovery of glycerol	Difficult	Easy
Purification of methyl esters	Repeated washing	None
Production cost of catalyst	Cheap	Relatively expensive

1.2.2.1 Alcoholysis using extracellular lipase

Various types of alcohols (primary, secondary, and straight- and branched-chains) can be employed in alcoholysis using lipases as catalysts (**Table 1.2**). In alcoholysis of rapeseed oil with 2-ethyl-1-hexanol, 97% conversions of esters was obtained using *Candida rugosa* lipase powder [31]. The conversion of fatty alcohol esters (C_4-$C_{18:1}$) in alcoholyis of oil from mowrah, mango, kernel and sal using immobilized *Mucor miehei* lipase (Lipozyme IM-20) in a solvent-free system ranged from 86.8 to 99.2% [32]. High yields of ethyl esters beyond 80% could be achieved

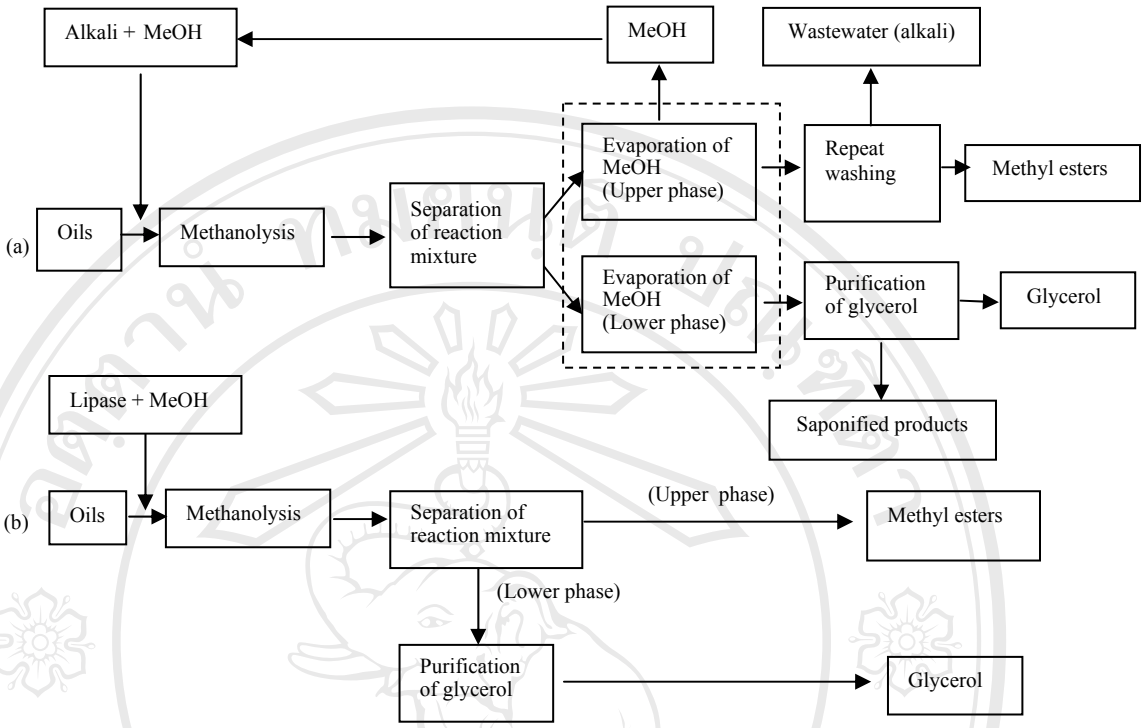

Figure 1.3 Flow diagrams comparing biodiesel production using (a) alkali-catalysis and (b) lipase-catalysis processes [2].

by using the lipase from *M. miehei* [33], *C. antarctica* [34], *Pseudomonas cepacia* [35] in ethanolysis of sunflower oil, fish oil and grease, respectively. The lipase from *M. miehei* was the most efficient for converting TAGs to their alkyl esters with primary alcohols whereas that from *C. antarctica* was the most efficient for alcoholysis of TAGs with secondary alcohols to give branched alkyl esters. Maximum conversion of 94.8-98.5% for the primary alcohols methanol, ethanol, butanol, and *i*-butanol, and of 61.2-83.8% for the secondary alcohols *i*-propanol and 2-butanol were obtained in the presence of hexane as a solvent. In solvent-free reactions, yields with methanol and ethanol were lower than those obtained with hexane; in particular, the yield with methanol decreased to 19.4% [9]. For alcoholysis of sunflower oil with methanol and ethanol, with and without petroleum ether as a solvent, the ester yields with ethanol were relatively high even in reaction without solvent while with methanol only traces of methyl esters were obtained [36]. The conversion of palm kernel oil to alkyl esters using *P. cepacia* lipase, ethanol gave the

Table 1.2 Enzymatic alcoholysis reaction using various types of alcohols and lipases.

Oil	Alcohol	Lipase	Conversion (%)	Solvent	Ref.
Rapeseed	2-Ethyl-1-hexanol	*C. rugosa*	97	None	31
Mowrah, mango, kernel, sal	C_4-$C_{18:1}$ alcohols	*M. miehei* (Lipozyme IM-20)	86.8-99.2	None	32
Sunflower	Ethanol	*M. miehei* (Lipozyme)	83	None	33
Fish	Ethanol	*C. antarctica*	100	None	34
Recycled restaurant grease	Ethanol	*P. cepacia* (Lipase Ps-30) + *C. antarctica* (Lipase SP435)	85.4	None	35
Tallow, soybean, rapeseed	Primary alcohols	*M. miehei* (Lipozyme IM60)	94.8-98.5	Hexane	9
	Secondary alcohols	*C. antarctica* (SP 435)	61.2-83.8	Hexane	
	Methanol	*M. miehei* (Lipozyme IM60)	19.4	None	
	Ethanol	*M. miehei* (Lipozyme IM60)	65.5	None	
Sunflower	Methanol	*P. fluorescens*	3	None	36
	Methanol		79	Petroleum ether	
	Ethanol		82	None	
Palm kernel	Methanol	*P. cepacia* (Lipase PS-30)	15	None	37
	Ethanol		72	None	

highest conversion of 72%, while only 15% of methyl esters was obtained with methanol [37]. The conversion results of these alcoholysis processes showed that the efficiency of methanolysis was likely to be very low compared to that with ethanolysis in both with and without solvent systems.

Recently, effective methanolysis reactions using extracellular lipase have been developed by numerous researchers, which were summarized in **Table 1.3**. The yields of methyl esters from repeated fed-batch methanolysis [38], and continuous three step flow methanolysis [39] of soybean oil catalyzed by immobilized lipase from *C.*

antarctica were 96-98% (48 h) and 92-94% (7 h), respectively. The pretreatment immobilized lipase from *C. antarctica* with methyl oleate, and subsequently with soybean oil before performing catalyzed stepwise methanolysis, gave 97% (3.5 h) of methyl esters [40]. Three-stepwise methanolysis of soybean oil could be catalyzed by the other non-specific microbial lipases such as free lipase from *C. rugosa, P. cepacia* and *P. fluorescens,* and yielded 80-100% of methyl ester content [41]. *R. oryzae* lipase, a 1(3)-specific enzyme also provided 80-90% of methyl esters [11].

Table 1.3 Effective methanolysis processes with extracellular lipases.

Lipase	Regiospecificity	Process and operation	ME content (%)	Reaction time (h)	Ref.
C. antarctica	None	Repeated fed-batch operation[a]	96-98	48	38
		Continuous operation[b]	92-94	7	39
		Fed-batch operation[a,c]	97	3.5	40
C. rugosa, *P. cepacia*, *P. fluorescence*	None	Fed-batch operation[a]	80-100	80-90	41
R. oryzae (F-AP15)	1(3)-Regiospecific	Fed-batch operation[a]	80-90	70	11

[a] Three-step addition of methanol; a reaction mixture of oil:methanol (1:1 mol/mol) was fed in each step.

[b] Continuous three-step flow reaction; a reaction mixture of oil:methanol (1:3 mol/mol) was fed into each column.

[c] Pretreatment of immobilized lipase with methyl oleate and soybean oil.

1.2.2.2 Methanolysis by intracellular lipase

Methanolysis can be carried out using either extracellular or intracellular enzymes, but extracellular enzymes require further purification that may be too complex for practical use. Furthermore, enzymes recovered through such process are generally unstable and expensive. Consequently, there has been considerable research into the direct use of whole cell as biocatalysts [42-44]. In **Figure 1.4**, the extracellular and intracellular (whole cells biocatalyst) lipase production processes are compared. Unlike in the case of extracellular lipase, no purification or immobilization

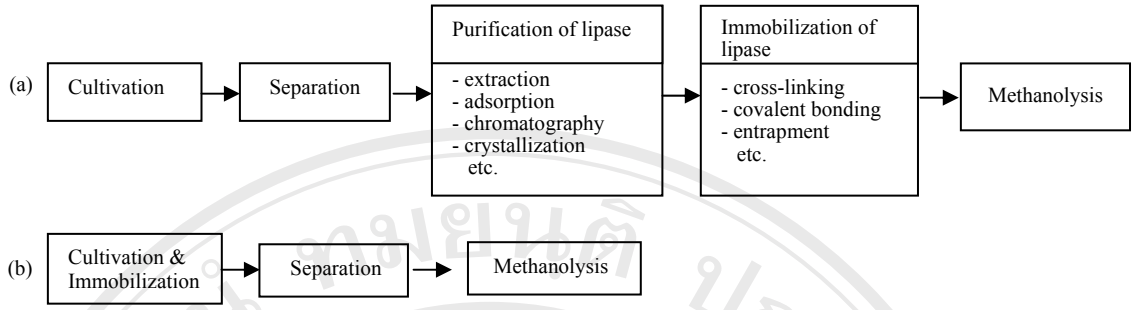

Figure 1.4 Comparison of microbial lipase production processes for methanolysis with (a) extracellular and (b) intracellular lipases [2].

processes are needed in preparing whole cell biocatalysts, since immobilization can spontaneously be achieved during batch cultivation.

To utilize whole cell biocatalysts in a convenient form, cells should be immobilized in such a way that they resemble ordinary solid-phase catalysts used conventionally in synthetic chemical reactions. Among many available immbilization methods, a technique using porous biomass support particles (BSPs) has several advantages over other methods in terms of industrial applications: (i) no chemical additives are required, (ii) there is no need for preproduction of cells, (iii) aseptic handling of particles is unnecessary, (iv) there is a large mass transfer rate of substrate production within BSPs, (v) the particles are reusable, (vi) the particles are durable against mechanical shear, (vii) bioreactor scale-up is easy, (viii) costs are low compared to other methods. The BSP technique has been applied successfully in a wide variety of microorganisms [45-59], animals [60-68], insects [69] and plant cells [70-75] systems. When methanolysis was carried out with stepwise additions of methanol using BSP-immobilized cells in the presence of 10-20% water, the methyl ester content in the reaction mixture reached 80-90% without any organic solvent pretreatment [76]. This level of methyl ester production is almost the same as that achieved using extracellular lipase [11].

1.2.2.3 Enhancing lipase activity in methanolysis

There are two major problems in using lipase for biodiesel production. The first one is that the activity of lipase is relatively low. Production of biodiesel

catalyzed by lipase takes time longer than that catalyzed by chemical catalyst due to low lipase activity. The other problem is that the immobilized enzyme is liable to be deactivated by methanol. There are, however, several methods to overcome these effects such as using hexane as a diluent to prevent the deactivation of enzyme by methanol [9] and maintaining a very low concentration of methanol during the reaction [40]. The other suitable method for enhancing the activity of lipase is washing lipase with alcohol having three or four of carbon atoms.

A. Methanolysis in the presence of organic solvent

The study on enzymatic alcoholysis with the aim of biodiesel production was summarized by Nelson *et al*. [9] that alcoholysis of TAGs with methanol and ethanol effectively proceeded in the presence of organic solvent. For example, methanolysis of beef tallow using immobilized *R. miehei* in the presence of hexane, 94.8% of the tallow was converted to methyl esters whereas only 65% conversion was obtained from the reaction without organic solvent.

For practical application, an organic solvent system is not suitable for production of biodiesel because of the dilution by organic solvent decreases the reaction rate and the requirement of the solvent removal. In case of using waste oil as starting TAGs, the system is risk to be explosed. From an economical point of view, a continuous reaction process without the use of any organic solvent is needed for the industrial production of biodiesel.

B. Stepwise addition of methanol

The three successive additions of methanol to the methanolysis of vegetable oil using immobilized *C. antarctica* were performed with the aim of increasing the yield of methyl ester [38]. The result of experiment was shown in **Figure 1.5**. The maximum conversion of the oil in the first-step reached 33.1% at 7 h. At the reaction time of 10 h, 1/3 molar equivalent of methanol was added, the conversion of the oil

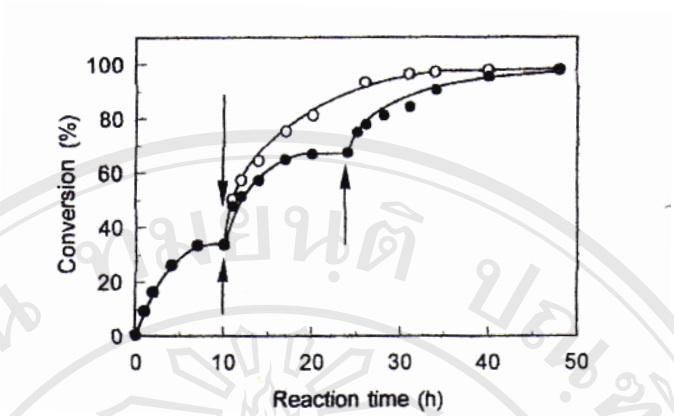

Figure 1.5 Time course of stepwise methanolysis of vegetable oil by immobilized *C. antarctica*; (●) three-step addition and (○) two-step addition [38].

was increased to constant at 66.4% at 24 h of reaction. The second addition of 1/3 molar equivalent of methanol conducted the conversion of oil to 97.3%.

Even though the stepwise addition of methanol is successful to prevent the deactivation of lipase, it has an inherent problem on precise control of very low concentration of methanol. Therefore, this method is not an appropriate approach for the production of biodiesel on a large scale.

C. Treatment of lipase with alcohol having three or four of carbon atoms

Base on the idea that decrease in lipase activity on alcoholysis of TAG and lower alcohol was mainly caused by physical factors, i.e. the immiscibility between methanol or ethanol and TAG. Wu and Chen [30] in 2002 disclosed an ideal solvent to wash a deactivated immobilized lipase. This solvent needs to be harmless to the lipase, and has good solubility to oil, grease, moisture and methanol or ethanol. For example, an alcohol with three or four of carbon atoms, preferably *i*-propanol, 2-butanol and *t*-butanol, can effectively regenerate a deactivated immobilized lipase. These two inventors also found that the activity of an immobilized lipase can be significantly increased when such ideal solvent is used to perform an immersion pretreatment on an immobilized lipase.

Table 1.4 shows the effect of pretreatment of the immobilized lipase, Novozyme 435 on enzyme activity in methanolysis of soybean oil. Novozyme 435 without any pretreatment exhibited very low activity and 2.5% (w/w) yield of methyl

Table 1.4 Effect of pretreatment on methanolysis of soybean oil by Novozyme 435 under different immersion conditions [77].

Type of pretreatment	Yield of methyl ester (% w/w)
None (soybean oil)	2.5
Immersion in *i*-propanol for 1 h and then in soybean oil for a further 1 h	16.8
Immersion in 2-butanol for 1 h and then in soybean oil for a further 1 h	17.6
Immersion in *t*-butanol for 1 h and then in soybean oil for a further 1 h	24.5

ester was obtained after 30 min of reaction. When the lipase was immersed in higher alcohols (e.g. *i*-propanol, 2-butanol, or *t*-butanol), the yield of methyl ester was about 7 to 10 times higher than that of the untreated immobilized lipase [77]. **Figure 1.6** demonstrates the initial reaction rate of the methanolysis with various concentrations of methanol and the same amount of the lipase and soybean oil. When the immobilized lipase was appropriately pretreated, not only the activity increased significantly, but also the ability of the lipase to resist deactivation by methanol was higher. At high concentrations of methanol, the immobilized lipase pretreated with alcohol exhibited higher activity particularly if the pretreatment was with *i*-propanol or 2-butanol [77].

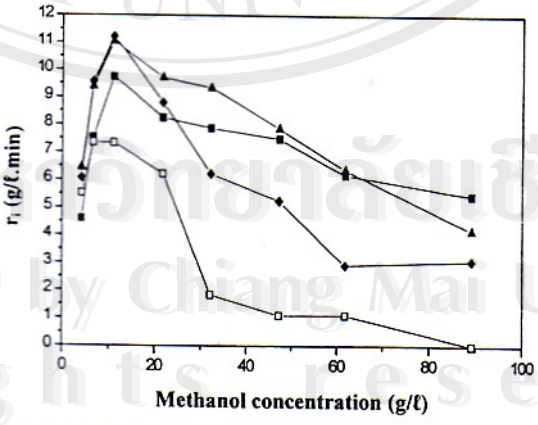

Figure 1.6 The effect of methanol concentrations on the initial reaction rate of methanolysis by pretreated Novozyme 435 with (■) *i*-propanol, (□) soybean oil, (▲) 2-butanol and (◆) *t*-butanol [77].

1.3 *Carica papaya* L.

C. papaya L. is a soft-stemmed and unbranched tree able to grow to 20 m in height. Its edible fruits and latex have been widely cultivated in tropical and subtropical regions around the world. The composition of the green fruit, ripe fruit and the leaves is reported in **Table 1.5** [78]. Leaves contain glycoside, carposide, and the alkaloid, carpaine. Fresh leaf latex contains 75% water, 4.5% caoutchouc-like substances, 7% pectinous matter and salt, 0.44% malic acid, 5.3% papain, 2.4% fat, and 2.9% resin. Per 100 g, the seeds are reported to contain 24.3 g protein, 25.3 g fatty oil, 32.5 g total carbohydrates, 17.0 g crude fiber, 8.8 g ash, 0.09 g volatile oil, a glycoside, caricin, and the enzyme, myrosin.

Table 1.5 Composition of *C. papaya* parts per 100 g [78].

Composition	Part of *C. papaya*		
	Green fruit	Ripe fruit	Leaves
Energy (calories)	26	32-45	74
Water (g)	92.1	87.1-90.8	77.5
Protein (g)	1.0	0.4-0.6	7.0
Fat (g)	0.1	0.1	2.0
Total carbohydrates (g)	6.2	8.3-11.8	11.3
Fiber (g)	0.9	0.5-0.9	1.8
Ash (g)	0.6	0.4-0.6	2.2
Ca (mg)	38	20-24	344
P (mg)	20	15-22	142
Fe (mg)	0.3	0.3-0.7	0.8
Na (mg)	7	3-4	16
K (mg)	215	221-234	652
Beta-carotene equivalent (μg)	15	710-1050	11565
Thiamine (mg)	0.02	0.03-0.04	0.09
Riboflavin (mg)	0.03	0.03-0.05	0.48
Niacin (mg)	0.3	0.3-0.4	2.1
Ascorbic acid (mg)	40	52-73	140
Vitamin E (mg)	-	-	136

1.3.1 *C. papaya* latex

Papaya latex is thixotropic fluid with a milky appearance that contains about 85% water. An insoluble particulate fraction whose composition is still practically unknown, make up 25% of the dry matter. The soluble fraction, on the other hand, contains the usual ingredients such as carbohydrate (~10%), salts (~10%) and lipid (~5%), representative biomolecules such as glutathione and cystein proteinases (~30%), and several other proteins (~10%). The papaya proteinases have been widely used for several decades in food (e.g. for meat tenderization and beer chill-proofing) and pharmaceutical industries [79].

Plant enzymes, such as lipases, may have advantages over animal or microbial enzymes because of their availability, their lower cost, their apparent relative ease of purification, and their particular specificities [80-81]. Lipase activities in plants have been mostly detected in cereal grains [82] and oil seeds [83]. These lipases are activated during germination and found in lipid storage tissue of seeds [84]. As early as 1935, a lipase activity was reported in latex of *C. papaya* [85]. Today papaya latex is one of the most important plant extracts exploited as papain using in several industries. Recently, properties and specificity of *C. papaya* lipase (CPL) have been reported [86].

1.3.2 Collection of latex

C. papaya latex is collected by making a series of longitudinal incisions depth 2-2.5 mm on the surface of unripe fruit (70-100 days). A stainless steel razor-blade is convenient but any cutting device which has a sharp cutting edge and does not expose the latex to contamination with elements reactive toward the sulfhydryl group (e.g. heavy metals) would be suitable [87]. Tapping should be done early in the morning in order to obtain a higher latex flow [88-89]. A wide range of tapping patterns and frequencies has been recommended: between 1-6 incisions per fruit [90] with a frequency of between 3-8 days [91]. The optimal method was 3 incisions per fruit with a 4-day interval of tapping [92]. However, latex yield varies considerably depending on cultivation techniques, collection procedure and environmental factors.

The latex which drips from the cuts is collected directly into a container or in a latex collecting equipment positioned below the fruit or rubber sheet positioned on the

floor. Typically the latex collection equipment consists of canvas or plastic attached to semicircular bows and clamped around the trunk [93] and as shown in **Figure 1.7**. After the latex ceases following, the latex collected in the canvas and coagulated on the skin of the fruit will be scraped off, transferred to a plastic container and then kept in a cool place avoiding the loss of enzyme activity.

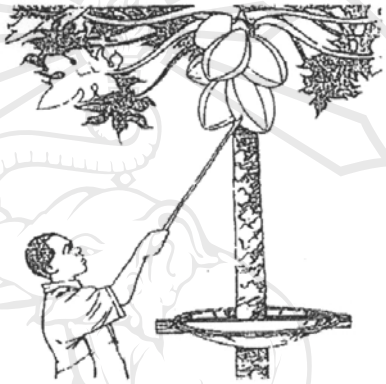

Figure 1.7 Tapping of papaya fruit using a latex collecting equipment [94].

1.3.3 Lipase from *C. papaya* latex

C. papaya latex is well known for containing protease, among which is papain, a thiol protease with many industrial applications, e.g., as a meat tenderizer, contact lens cleaner, digestive aid or bloodstain remover, in detergents. This plant exudate has not only proteolytic activity but also lipase activity. The lipase activity of different CPL preparations in crude papain was investigated using tributyrin as substrate [95]. Lipolytic activity was expressed as international units (IU) per gram of enzyme preparation. One IU corresponds to one μmol of butyric acid released per min. Lipolysis data given in **Table 1.6** indicates that none of the commercially available purified preparations of papain has any lipolytic activity, because the lipolytic enzymes contained in the crude *C. papaya* latex were eliminated during the purification process for the isolation of the protease. Only the crude latex was shown to efficiently catalyze lipolysis reactions. Therefore, papaya lipase should present in the particulate fraction of the crude latex. For each plant variety tested, lipolytic activity of CPL differed substantially depending on the frequency of fruit tapping.

Table 1.6 Lipolytic activity of crude CPL preparation from various plant varieties in comparison with commercially available crude or purified preparation of papain [95].

Enzyme preparation	Lipolytic activity (IU/g)
Refined papain	No activity
Water-soluble papain	No activity
Purified papain	No activity
Crude papain	1,567±35
Crude CPL-first tapping	
Variety MTQ2	587±18
Variety Deshaies	814±38
Variety Madagascar	145±17
Crude CPL-second tapping (day+5)	
Variety Deshaies	322±10
Variety Madagascar	68±5

The lipolytic activity of crude CPL preparations obtained by the second fruit tapping was always exhibited less (generally 50%) than the one collected during the first fruit tapping [95].

1.3.3.1 Preparation of *C. papaya* lipase

Lipase activity was reported in latex of *C. papaya* as early as 1935, however, the enzyme responsible for this activity was only characterized more recently [86]. Tributyroylglycerol hydrolase activity of spray-dried latex from *C. papaya* was investigated. **Table 1.7** shows that the homogenates of spray-dried latex have 2,500 IU of tributyroylglycerol hydrolase activity while the soluble fraction has no hydrolase activity. In contrast, the particulate fraction contained the entire tributyroylglycerol hydrolase activity. After delipidation of the pellet (step 4) the activity recovery yield was 72%.

No effect after treatment of *C. papaya* latex with proteolytic enzyme, anionic detergent such as SDS, cationic detergent such as alkyldimethylbenzylammonium

Table 1.7 Hydrolysis activity on tributyroylglycerol of lipase from *C. papaya* latex [86].

Step		Spray-dried *C. papaya* powder (mg)	Total activity (IU)
1. Homogenate in buffer*		1,000	2,500
2. Centrifugation (1,000×g, 30 min)	: Supernatant	800	0
	: Pellet	150	2,500
3. Centrifugation (1,000×g, 30 min)	: Supernatant	Traces	0
	: Pellet	150	2,500
4. Delipidation by extraction	: Supernatant	50	0
[Benzene-ethyl acetate (1:1, v/v)]	: Pellet	90	1,800

Tributyrin was used for detection of lipase activity

* 0.1 M PBS, 1 M NaCl, 10 mM DTT, 10 mM PMSF, pH 8.0

chloride, and neutral detergents such as Tween 80 and Triton X-100 was found which indicated a tight association of lipase with the particulate fraction of *C. papaya* latex [86]. So far, the particulate part of *C. papaya* latex was used as lipase without further purification.

1.3.3.2 Specificity of enzyme

Lipases (EC 3.1.1.3) are groups of enzymes which catalyze the hydrolysis in aqueous medium and also catalyze transesterification in nonaqueous medium. With respect to the acid moieties in their substrates, lipases exhibit specificity for both position of acid on the glycerol residue and for the structure of fatty acids [96]. Some enzymes are specific for acids on the outside (1,3) positions on glycerol. However, monoacylglycerols and diacylglycerols on the 2-position rearrange spontaneously so that complete hydrolysis of TAGs to fatty acids and glycerol ultimately occurs. In interesterification reaction there is no significant occurrence of partially hydrolyzed TAGs so that the rearrangement from the 2-position dose not occur. Hence, the 1,3-specific lipases are ineffective in exchanging the fatty acids at the 2-position [96].

The majority of lipases has a specificity for fatty acid residues which broadly parallels their occurrence in natural lipids with high activities on the predominant C_{16} and C_{18} acids and with slight differences in relative rates of hydrolysis depending on

source species. The substrate specificity of a lipase is defined by (A) positional specificity, (B) carboxylic acid selectivity, (C) alcohol selectivity and (D) stereospecificity [96].

A. Positional specificity

The positional specificity of lipase is defined as the ability to hydrolyze only the primary or both primary and secondary ester bonds of TAG and can be determined by using thin-layer chromatography (TLC) on silica gel impregnated with boric acid which separates 1,2- from 1,3-diacylglycerols and 1- and 2-monoacylglycerols. These products can be quantitated directly by gas-liquid chromatography (GLC) after conversion to trimethylsilyl ether derivatives. A mixed TAG with known positional distribution of fatty acids can be used as a substrate. After lipolysis, the reaction products are separated and analyzed. For example, from a column of silicic acid, the solvent mixture of hexane-ether (70:30) will elute TAGs, fatty acids and diacylglycerols; the monoacylglycerol can then be eluted with ether, and its fatty acid composition can be determined by GLC. By TLC on silica gel with petroleum ether-ether-acetic acid 70:30:2, all four products can be separated [96].

The CPL exhibits 1,3-positional selectivity in hydrolysis reaction. The breakdown of TAGs by CPL proceeds in **Figure 1.8**. The CPL releases fatty acids only from the outer 1- and 3-positions of the glycerol molecule, and TAGs (a) are hydrolysed to give free fatty acids, 1,2- and 2,3-diacylglycerols [(b) and (c)] and 2-monoacylglycerols (d).

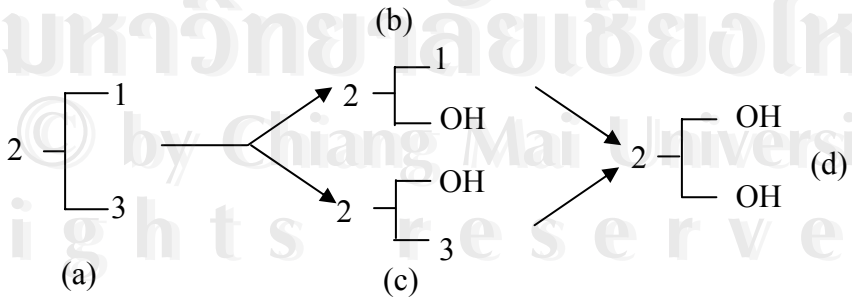

Figure 1.8 Hydrolysis of TAGs by CPL.

However, 1,2- or 2,3-diacylglycerols and 2-monoacylglycerols are chemically labile and easily undergo acyl migration which results in 1,3-diacylglycerols and 1-monoacylglycerols, respectively. Thus prolonged incubation would result in a complete breakdown of some fats into glycerol and free fatty acids.

B. Carboxylic acid selectivity

The effect of fatty acid chain-length in several TAGs on lipolysis of CPL was evaluated. Water-soluble substrates such as triacetin or partly water-soluble substrates such as tripropionin, tributyrin, trihexanoin and water-insoluble TAGs such as trioctanoin, tridodecanoin and triolein were used to titrimetrically test the hydrolytic activity of CPL [86]. **Figure 1.9** demonstratated that triacetin (short-chain TAGs) was poorly hydrolysed at 25, 37 and 55°C. On the other hand, an increasing temperature induces a relative increase in hydrolysis rate measured on long-chain TAGs. The TAG hydrolase activity of CPL was optimal for tributyrin as substrate at all temperatures tested.

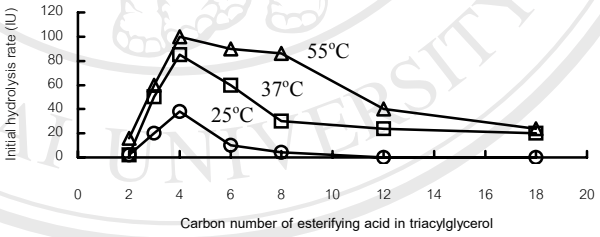

Figure 1.9 TAG hydrolase activity of CPL, measured at pH 8.0, with numerous TAGs [86].

The influence of fatty acid structure on esterification of various fatty acids with 1-butanol in the presence of myristic acid as the reference standard has been evaluated [97]. **Figure 1.10** showed the specificity constants obtained from the esterification of the individual fatty acids with 1-butanol catalyzed by CPL. For the effect of fatty acid unsaturation, low specificity constants were observed in the esterification of fatty acids having a *cis*-4 unsaturation, e.g. docosahexaenoic acid, *cis*-6

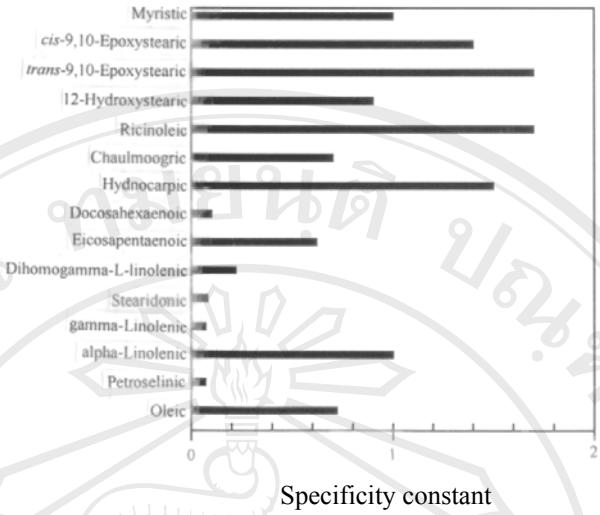

Figure 1.10 Specificity constant in the esterification of mixtures of myristic acid and individual unsaturated, hydroxy, epoxy and cyclopentenyl fatty acids with 1-butanol in hexane using CPL as a biocatalyst [97].

unsaturation, e.g. petroselinic, γ-linolenic, and stearidonic acids, and *cis*-8 unsaturation e.g. dihomo-γ-linolenic acid. In contrast, oleic and α-linolenic acids having a *cis*-9 unsaturation and eicosapentaenoic acid having a *cis*-5 unsaturation gave good specificity constants close to 1.

In esterification of fatty acids having hydroxy group, e.g. ricinoleic and 12-hydroxystearic acids, epoxy groups, e.g. *trans*-9,10-epoxystearic acid, and cyclopentenyl groups, e.g. hydnocarpic acid and chaulmoogric acid showed relatively high of the specificity constants compared to that of myristic acid. In conclusion, CPL catalyzed esterification reaction with 1-butanol preference for fatty acids having *cis*-5 and *cis*-9 unsaturations, hydroxy groups, epoxy groups and cyclopentenyl groups in esterification reaction.

C. Alcohol selectivity

Lipases have been shown to possess a high discriminating ability toward different alcohols depending upon their chain length and configuration. To study the role of alcohol on specificity of CPL, esterification reactions were carried out between

octanoic acid and equimolar mixtures of different combinations of alcohols using 1-hexanol as the reference [98]. A plot of the initial rates of esterification of individual alcohols relative to that of 1-hexyl octanoate synthesis (the reference reaction) was illustrated in **Figure 1.11**. Among the primary aliphatic alcohols, an optimal reaction rate for a chain length of C_8 was found. The highest relative reaction rate was obtained in the case of benzyl alcohol which was more than 9 times as reactive as 1-octanol. This could imply a high affinity of CPL for aromatic alcohols as compared to aliphatic ones. Among the terpene alcohols, both β-citronellol and geraniol exhibited higher rates of esterification than 1-decanol although they have the same carbon number. The order of reactivity was found to be β-citrolnellol >> geraniol > nerol >> terpineol. For secondary and tertiary alcohols, CPL showed the poor affinity owing probably to steric factors. Branching alcohol, *i*-amyl alcohol, has negligible effect on CPL.

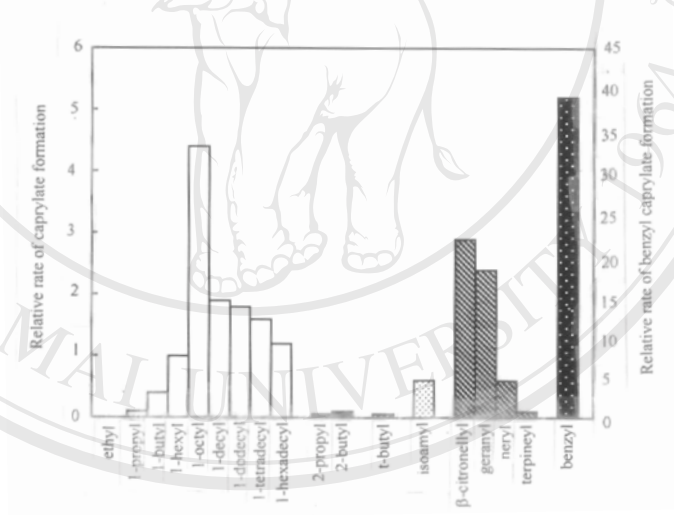

Figure 1.11 Formation rate of alkyl octanoates relative to formation rate of 1-hexyl octanoate during esterification of octanoic acid, catalyzed by CPL, with equimolar mixtures of various alcohols containing 1-hexanol as reference standard [98].

D. Stereospecificity

The stereospecificity is defined as the ability to hydrolyze only ester-1 or only ester-3 of the TAGs. The method to evaluate lipase specificity in interesterification reaction is that the use of a chiral TAG reacted with a homogeneous TAG whose constituent acid residues are different from the three acyl residues of the chiral *sn*-

TAG. The lipase then catalyzes one or more acyl exchanges between the two substrates. Thus, each acyl exchange between the *sn*-TAG and the homogeneous TAG is characterized by the formation of a new TAG molecule whose total equivalent carbon numbers (ECN) differ from the carbon number of the starting *sn*-TAG and homogeneous TAG.

The *sn*3 stereospecificity of CPL in hydrolysis reaction of selected TAGs was first reported in 1995 [99]. For interesterification, the acyl exchange reaction between the *sn*-TAG, 1-butyroyl-2-stearoyl-3-palmitoyl-*sn*-glycerol *(sn*-BSP, ECN 38) and trimyristin (MMM, ECN 42) catalyzed by CPL was studied [81]. The time course of this reaction was followed by GLC. Reduction in the GLC peak areas of the initial TAG's was accompanied by the appearance of newly formed TAG peaks, each of which was representative of a particular acyl exchange **(Figure 1.12).** For example, the peaks labeled BSM (ECN 36) and MMP (ECN 44) arise from an acyl exchange between the *sn*3 position of *sn*-BSP and a myristoyl residue of trimyristin. Similarly, the TAGs labeled BMM (ECN 32) and MSP (ECN 48) correspond to an acyl exchange at the *sn*1 position of *sn*-BSP. The least formed TAGs BMP (ECN 34) and MSM (ECN 46) most likely correspond to second generation TAGs that have already undergone an initial acyl transfer or less likely by acyl exchange at the 2-positions of *sn*-BSP and trimyristin. During the interesterification reaction the relation proportion of TAGs derived from *sn*3 interesterification (i.e. an exchange of acyl residues between the *sn*3 position of the *sn*-BSP and MMM) and *sn*1 interesterification with time were monitored. Over the first 3 h of this reaction, the sum of the TAGs BSM and

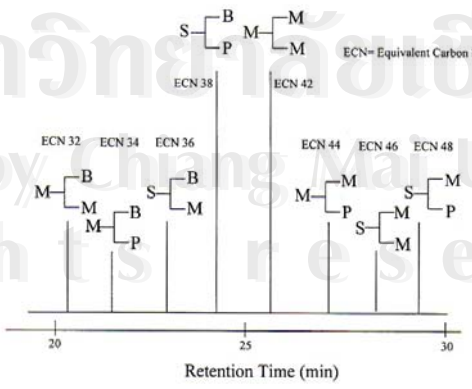

Figure 1.12 GLC identification of TAGs in the CPL-catalyzed interesterification of *sn*-BSP and MMM based on their equivalent carbon number (ECN).

MMP was higher than the sum of the TAGs BMM and MSP **(Table 1.8)**. It was concluded that acyl exchange at the *sn*3 position of *sn*-BSP was favored over acyl exchange at the *sn*1 position through the reaction. Therefore, it clearly demonstrated that CPL has a strong *sn*3 stereospecificity in TAG interesterification reactions. From these specificities, CPL can be a useful biocatalyst for synthesis of new structured TAGs to obtain new products with a predetermined composition and distribution of fatty acids such as synthesis of low-calorie triacylglycerol analogs [100] and structure a TAGs containing palmitic acid esterified at the *sn*2 position and short- or medium-chain fatty acid at the *sn*1,3 positions for infant nutrition and dietetics [101].

Table 1.8 Time course of the formations of new TAGs from the interesterification of 1-butyroyl-2-stearoyl-3-palmitoyl-*sn*-glycerol (*sn*-BSP) and trimyristin (MMM). TAGs formed by acyl exchange at the *sn*3 position of *sn*-BSP (BSM+MMP) and the *sn*1 position of *sn*-BSP (BMM+MSP).

TAG (Mol %)	Time (min)					
	0	30	60	90	120	180
*sn*3 (BSM+MMP)	0	6.0	12.8	21.8	26.2	28.3
*sn*1 (BMM+MSP)	0	2.1	6.3	11.0	14.4	16.0

1.4 Objectives

Papaya latex obtained by the incision of papaya fruit, which is not only an economical plant throughout Thailand, but also cheaper than microbial lipase, is of particular interest in using as a biocatalyst for biodiesel fuel synthesis. The objectives of this research are as followed:

(1) To study the specificity on pure TAGs and alcohols of CPL in alcoholysis.

(2) To establish the optimal conditions for synthesis methyl ester by methanolysis of pure TAGs catalyzed by CPL.

(3) To synthesize biodiesel fuel from methanolysis of palm oil catalyzed by CPL.