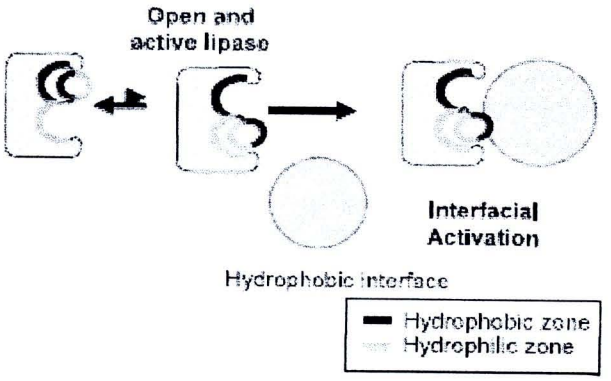
CHAPTER V
DISCUSSION

5.1 Selection of support

Lipases may exist in two different structural forms, the closed one where a polypeptide chain (lid or flat) isolates the active center from the medium, and the open form where this lid moves and the active center is exposed. This equilibrium is shifted towards the open form in the presence of hydrophobic surfaces, where the lipase becomes adsorbed by the large hydrophobic pocket around their active center and the internal face of the lid. Moreover, lipases may become adsorbed to other hydrophobic surfaces following a similar mechanism (Scheme1): droplets of oils, hydrophobic proteins, or on the surface of hydrophobic supports. The immobilization of lipases by their interfacial activation on hydrophobic supports may be suitable and simple method (Cabrera *et al.*, 2009).



Scheme1. Mechanism of lipase in aqueous medium (Palomo *et al.*, 2002)

Obviously, the characteristics of immobilized enzyme preparations are governed by the properties of both the enzyme and the carrier material. Numerous supports for the immobilization of lipases have been used. There are varieties of support materials such as chitosan (Feresti *et al.*, 2007, Ye *et al.*, 2007 and Amorim *et al.*, 2003), silica (Blanco *et al.*, 2004 and Blanco *et al.*, 2007) and $CaCO_3$ (Ghamgui *et al.*, 2004 and Rosu *et al.*, 1998).

In this study, *Candida rugosa* lipases were immobilized on 7 types of commercial hydrophobic supports namely Amberlite XAD 2, Amberlite XAD 4, Amberlite XAD 7, Amberlite XAD 16, Amberlite XAD 761, Sepabeads EC-BU and Sepabeads EC-OD. Comparative studies indicated that dramatic differences exist in the activity of lipases supported on different materials. Figure 4-1 showed lipase activity of the 7 types of hydrophobic supports. From the graph, more than 20% activity yield were obtained when dried Amberlite XAD 7 and Amberlite XAD 761 as well as wet Amberlite XAD 16 and Sepabeads EC-OD were used. The choice of supports is often limited by some other factors related to their structure, such as the specific surface area, pore shape and particle size (Lei *et al.*, 2004, Panzavolta *et al.*, 2005, Blanco *et al.*, 2007 and Ghiaci *et al.*, 2009). Supports used in this work with pore diameter of around 10-60 nm, allow only the immobilization of small enzymes within the pores. In contrast, the larger enzymes can only be adsorbed on the external surfaces of the particles. Bosley and Clayton studied the adsorption of lipase from *Mucor miehei* on controlled pore glass of eight different pore sizes. They concluded that the larger the pore diameter, the faster the adsorption rate (Bosley and Clayton, 1994). For instance, the internal surface may not be fully used to adsorb enzyme molecules, even when pore sizes are wide enough (Blanco *et al.*, 2007). Another key factor is the specific surface area. There is probably significant contribution of the micropore regions in the immobilization of the enzyme (Table A-1) and the external surface area of the support is also accessible to the enzyme molecules. The support can affect the partitioning of substrates, products, and water in the reaction mixture, and thereby, can influence the catalytic properties of the enzyme (Palomo *et al.*, 2002).

The immobilized enzymes with these supports were used to catalyze transesterification for the selection of the appropriate support. The conditions were 20 % (w/w of oil) immobilized lipase, the ratio of oil to methanol was one to three and three addition steps of methanol. The reactions were carried out at 40 $^{\circ}$C for 24 hours with continuous stirring by magnetic stirrer. The results were illustrated in Figure 4- 2 and the production of biodiesel obtained was expressed as percent conversion. It could be seen

that Sepabeads EC-OD gave the highest conversion approximately, 34%. Therefore, Sepabeads EC-OD was selected as the optimal support for the subsequent immobilization.

Sepabeads EC-OD which was selected as the carrier materials for optimal immobilization of *Candida rugosa* lipase are highly porous methacrylic polymer matrix spherical beads, with a high hydrophobicity for enzyme immobilization, low swelling tendency in high molar solutions. The support is resistant in common solvents and shows high resistance to microbial attack. The immobilization by physical adsorption on hydrophobic support, Sepabeads EC-OD is suitable to stabilize this enzyme. This result is consistent with other results concerning the immobilization of lipases on this kind of support. From the literature, immobilized lipase QL from *Alcaligenes* sp. by adsorption on octadecyl-sepabeads was studied. This immobilization technique improved the enzyme properties. The immobilized preparation exhibits a 135% of catalytic activity for hydrolysis of ρ-nitrophenyl propionate as compared to the soluble enzyme. The thermal stability of the immobilized enzyme is highly improved, a half-life time of 12 hr when incubated at 80°C and the optimal temperature was increased from 50°C (soluble enzyme) up to 70°C (immobilized enzyme) (Wilson *et al.*, 2006). Moreover, using this kind of carrier presents an additional advantage which is the possibility of reuse of the support due to the reversible adsorption of the enzyme on the support (Palomo *et al.*, 2003).

5.2 Optimization of immobilization of lipase

When the Sepabeads EC-OD were selected, they were then prepared for the studies of the optimized immobilization of *Candida rugosa* lipase. Previous studies have shown that many factors can affect the activity recovery and reusability of enzymes in immobilization process. Some of the most important factors are the properties of the enzyme molecule, concentration of enzyme, temperature, ionic strength, pH, water and solutes present. Hence, the effect of immobilization parameters, i.e. pH, ionic strength, protein loading, immobilization time, immobilization temperature and adjuvant were investigated.

5.2.1 Effect of pH on immobilization

The optimum pH for lipase activity varies with the enzyme species. From the results, the effect of changing pH and the percent lipase activity and activity yield of immobilized lipase were shown in Figure 4-3. It could be seen that the shape of the graph is bell-shaped curve which the maximum activity of immobilized lipase was obtained at pH 6.0. The activity of lipase increased with the increment of pH values. This result suggested that electrostatic forces are important for the adsorption; changes in pH over the isoelectric point of the protein will have a large impact on the protein binding constant. The protonation and deprotonation of the charged functional groups were dependent upon the pH of the solution (Lei *et al.*, 2009). Since isoelectric point of lipase from *Candida rugosa* is 4.6, overall net charge is close to 0. The lipase can be easily adsorbed to the nonionic or hydrophobic support by hydrophobic interaction. Moreover *Candida rugosa* is quite stable in acidic environments and the optimum pH values of *Candida rugosa* are between 6.0 and 7.0 which are correlated to the best pH during the enzyme immobilization process. It has been reported that the optimum of immobilized *Candida rugosa* lipase is slightly higher and lower than the free enzyme (Pereira *et al.*, 2001, Blanco *et al.*, 2004 and Yeşloğlu Y, 2005). However, the lipase activity started to decrease when the pH subsequently rose to more than 10. At more acidic or alkali pH, the denaturation of lipase tends to be increasing, like other proteins, which was in most cases greatly depended on the pH of the solution.

5.2.2 Effect of ionic strength on immobilization

The effect of ionic strength on immobilization was investigated and the results were shown in Figure 4-4. It could be seen that both lipase activity and activity yield of immobilized lipase were highest in 500 mM phosphate buffer pH 6.0. The activity of lipase and percentage of activity yield increased dependent on

increasing ionic strength from 10 mM to 500 mM. Conventionally, adsorption of proteins by hydrophobic interaction is stronger when the ionic strength is increased (Bastida *et al.*, 1998). Since at pH 6, lipase will exhibit net negative charge, therefore, the increase of ionic strength will gradually decrease the charges on enzyme molecules until the net charge reached near zero resulting in more pronounced hydrophobic interaction between enzyme and the support. In contrast, when the ionic strength was elevated from 500 mM to 1M, the activity of lipase and percentage of activity yield were decreased indicating that the higher ionic strength initiates more hydrophobic environment around the active sites. Accordingly, the numbers of active enzyme was further reduced.

5.2.3 Effect of protein loading on immobilization

The amount of enzyme loaded on the surface has a large effect on the performance of biocatalytic surface. In this study, different amount of enzymes were immobilized on the supports, by varying the protein loading (mg/ml) of enzyme solution from 1 to 10 mg/ml. The effect of protein loading on activities of lipase was shown in Figure 4-5. It could be seen that the activity of lipase significantly increased when the protein loading was increased. The highest activity of lipase was achieved when protein loading at 10 mg/ml was used. It is considered that the higher lipase loading makes the lipase form an intermolecular steric hindrance, which restrains the diffusion of the substrate and product. The similar phenomena were also observed in the previous studies. It was found that the activity of 12 g immobilized lipase/g ceramic from *Penicillium expansum* reached the maximum (Huang and Cheng, 2008). It is reasonable to conclude that the binding site on the surface areas of the support are limited (Jiang *et al.*, 2008 and Chang *et al.*, 2008) and the enzyme molecules need enough space for catalyzing the reaction of the substrate (Lei *et al.*, 2009). As could be seen from the figure, the activity of enzyme at 8 mg/ml was not much different from the activity obtained from 10mg/ml of the

enzyme. Therefore, in order to reduce the cost for further applications, 8 mg/ml was finally used as optimal amount of protein loading from this study.

5.2.4 Effect of immobilization times

The residual activity of lipase solution was checked for each time of immobilization at various temperatures as described in 3.4.3.4. The relationship of the residual activity with immobilization time at various temperatures was shown in Figure 4-6. The amount of soluble protein was rapidly decreased with the increment of the immobilization time. When lipase was incubated at 10°C for 180 min, the residual activity of lipase appeared unchanged. Furthermore, when lipase solutions were incubated at 20°C and room temperature (around 25 °C), the results showed that the residual activity leveled off until 120 min in both temperatures. At 30, 40, 50 and 60°C, the residual activity of lipase solution gave the similar pattern that they significantly decreased from initial time of the incubation to around 20-30 minutes and stayed unchanged to 60 minutes. An extra incubation time of the immobilization leads to a plateau value. As long as the immobilization process continues, the lipase activity will decline. The protein conformation of lipase might be denatured leading to the limitation of immobilization processes from the effect of longer coupling reaction time. This result was similar to the immobilization studied by Chang *et al.* The immobilization time of *Candida rugosa* lipase on poly (alpha-glutamic acid) was varied from 1 to 18 hours. They found that the optimal immobilization time was less significantly effective after more than 6 hours (Chang *et al.*, 2008). Similarly, Lei *et al.* studied the immobilization time of porcine pancreas lipase for 2 to 10 hours. The result indicated that the highest activity was obtained under immobilization time of 8 hours. After 8 hours of immobilization, the activity of immobilized enzyme was decreased (Lei *et al.*, 2009). Therefore, the optimal time for immobilization was 3hr, 2hr, 1hr and 20-30 min at 10, 20, 25 (RT) and 30-60 °C

respectively and subsequently selected for the next experiment namely, optimal condition for the temperature.

5.2.5 Effect of temperature on immobilization

Lipase was immobilized on Sepabeads EC-OD at different temperatures; 10, 20, 25, 30, 40, 50 and 60 °C. The results in Figure 4-7 showed that the activity of lipase was initially increased under the temperatures from 10 to 30 °C and the highest activity and activity yield were obtained when the temperature of immobilization reached 30 °C. As shown in Figure 4-7, this result is consistent with the optimal temperature of the free *Candida rugosa* lipases obtained from other previous studies (Hung *et al.*, 2003). Hung *et al.* reported that the optimum temperature of the lipase was not altered by immobilization. The activity of both free and immobilized lipases of *Candida rugosa* on chitosan were highest at 30°C. In contrast to Hung *et al* , Wilson *et al.* reported that the optimum temperatures of immobilized lipase QL from *Alcaligenes sp.* on octadecyl-sepabeads were altered from 50 to 70°C (Wilson *et al.*, 2006). The alteration of optimum temperature of immobilized enzyme might be depending on type of enzymes and nature of supports. On the other hand, when the temperatures were elevated from 40 to 60 °C, the activities were reduced. This can be simply explained that the excessive temperature provided to the immobilization system would inhibit the activity of the free lipase, because lipase might be inactivated by thermal denaturation. From the optimal immobilization time obtained in section 4.2.4, the activity of immobilized lipase was found to be suitable at 30 °C. Therefore, the optimal immobilization time was 30 minutes at 30 °C and subsequently selected for the next experiment.

5.2.6 Effect of adjuvant on immobilization

Once the optimal conditions of 500 mM phosphate buffer, pH 6, 8 mg/ml of *Candida rugosa* lipase solution for immobilization at 30 °C for 30 min were obtained, the effect of adjuvant on the activity of immobilized *Candida rugosa* lipase were examined by using two categories of adjuvants namely; alcohol and detergents.

5.2.6.1 Effect of concentration of adjuvant

The concentrations of each type of adjuvant necessary to obtain the desired effect of the highest concentration of each type of adjuvant with no damage of the enzyme activity had to be determined. From Table 4-1, it was shown that the concentrations of various adjuvants with the activities of *Candida rugosa* lipase solution. The highest concentration of each adjuvant with maximum of lipase activity was selected and later used for immobilization.

5.2.6.2 Effect of the type of adjuvant

The relationship between the lipase activity and adjuvant was shown in Figure 4-8A. The activity and the ability to be non-covalently immobilized on hydrophobic supports are known to be closely related to the hydrophobicity environment. Thus, the presence of hydrophobic adjuvant possibly contributes to a rearrangement of the tertiary structure involving a higher accessibility of some hydrophobic side chains of amino acids, which in such less polar medium become more to the surface of the protein. Only small-size of hydrophobic adjuvant necessary to obtain the desired effect of decreasing the hydrophobicity of the pore channels for the enzyme was adsorbed (Blanco *et al.*, 2007). Moreover, two forms of lipase show very different activity; closed form (inactive form) and open form (active form). If

the enzyme was able to fix with higher activity, the final immobilized preparation may be more active than the native one (Mateo *et al.*, 2007). Hydrophobic and small substrate such as detergent and short chain alcohol have been described to promote the interfacial activation of the lipase yielding the stabilization of the open form of the lipase (Fernândez-Lorente *et al.*, 2006). From these features, strategies to get immobilized lipases molecules with improved activity have been developed, trying to fix the open form of the lipase. This assumption has been supported by López-Serreno *et al.* that improvement of lipase activity was found when the enzyme was prepared in the presence of SDS (López-Serreno *et al.*, 2002). Furthermore, the stabilization of the fully open forms of lipases adsorbed to supports is extremely essential especially using immobilized lipase as catalyst in biodiesel production. To this goal, immobilized lipase was incubated in the presence of adjuvants such as methanol, ethanol, iso-propanol, butanol, t-butanol, SDS, ethylene glycol, tween 80 and triton X-100. The result showed that highest lipase activity with p-NPP was obtained when t-butanol was used that the activity of lipase was increased approximately 45%. In addition, increase of the conversion of biodiesel synthesis was about 43%, 44% and 46% when the lipase was immobilized in the presence of butanol, t-butanol and triton X-100, respectively (Figure 4-8B).

5.3 Transesterification and hydrolysis catalyzed by immobilized *Candida rugosa* lipase

After the optimal conditions for immobilization of *Candida rugosa* lipase from section 4.2.1-4.2.6 were obtained, the activity of immobilized lipase was investigated on transesterification and hydrolysis. The transesterification and hydrolysis reactions were conducted as described in section 3.4.4. From Figure 4-10, it was shown that 45.23 % (±2.43) biodiesel and 97.21% (±0.69) free fatty acids were obtained from transesterification and hydrolysis, respectively. From the results, it could be seen that hydrolysis may be more

preferable than transesterification for the catalytic properties of lipase. Obviously, lipase is generally categorized as hydrolases which catalyze the hydrolysis of triacylglycerols to glycerol and free fatty acids (Hung *et al.*, 2003). Consistent to this study, it was the hydrolytic activities of *Candida rugosa* which was higher than synthetic activity (Teng *et al.*, 2009).

5.4 Screening of raw materials for feedstock

5.4.1 Extraction of seed oil

The oil of non-edible and waste plant seeds was extracted by using soxhlet extraction method. 15 g of plant seeds were packed in a thimble and the oil was extracted with 250 ml *n*-hexane for 6 hr. The extracted oil was then measured to calculate the content of oil in the plant seeds shown in Table 4-3. The %oil content from non-edible and waste plant seed oils were in the range of 25 % to approximately 72 % dry weight seeds. The oil yield from the non-edible and waste plant oils itself is always the key factor to decide the suitability of a feedstock for biodiesel production. From Table 4-3, it can be seen that wild almond was the plant yielding the highest oil (72%). On the other hand, among the various non-edible oils shown in Table 4-3, physic nut was found to give the high yield (43%). However, the oil yield depends on many factors such as plantation and oil extraction techniques (Gui *et al.*, 2008).

5.4.2 Fatty acid composition analysis

Another important criterion to determine the suitability of oil as a raw material for the production of biodiesel is the composition of the oil itself. The composition of oil will subsequently determine the properties of the biodiesel obtained. The effect of oil composition on the properties of the biodiesel produced will be discussed in the

subsequent section. The composition for various types of non-edible and waste oils was shown in Table 4-4. From this table, the major oil compositions in both non-edible and waste plant oils were tabulated. The major fatty acids content in both non-edible and waste oils are oleic, linoleic and palmitic acid, while the latter include lauric, myristic, palmitoleic, stearic, linolenic, arachidic and behenic acid. Furthermore, the fatty acids in the oils are further categorized into saturated and unsaturated fatty acids which are also an important for the storage stability of biodiesel. In this concept, the oxidative stability is obtained based on the relative rates of oxidation of these positions in unsaturated fatty acid as well as their amounts. Bouaid *et al.* reported that the oxidative stability of the oil may be more strongly influenced by the presence of small amounts of highly unsaturated fatty. Two important factors affecting the degradation of biodiesel were also observed in their study, which are water content and air exposure (Bouaid *et al.*, 2007). However, the effect of the presence of unsaturated fatty acid on the storage stability of biodiesel can be avoided by taking proper precaution during the storage such as limiting contact to oxygen and exposure to light and moisture.

5.4.3 Characterization of oil

Apart from the oil yield of the raw materials, the properties of biodiesel vary accordingly to the fatty acid composition in the feedstock oil which is used to produce biodiesel. The properties include saponification number, iodine value, cetane number, and viscosity. Among all the properties listed in Table 4-4, it was shown that the calculated saponification number (SN), iodine value (IV) ranged from 195 to 204 and 3 to 147, respectively. Cetane number (CN) and viscosity (η) values among the species varied from 40 to 66 and 2.29 to 3.95, respectively.

CN is a significant expression of diesel fuel quality among a number of other measurements that determine overall diesel fuel quality. CN is actually a measure of a fuel's ignition delay, the time period between the start of injection and start of

combustion of the fuel. Fuels with higher CN, which have shorter ignition delays, provide more time for the fuel combustion process to be completed. Hence, higher speed diesels operate more effectively with higher CN fuels. This is one of the important parameters which are considered during the selection of FAMEs for use as biodiesel. The different countries or organizations have specified different minimal values. Biodiesel standards of Thailand (2007), USA (ASTM D6751-07a) and European Standards Organization (EN 14214:2003) have set value as 51, 47 and 51, respectively. Among the FAMEs of 9 species, 7 species (No.1–2, 4–8) have CN value higher than 51 (±2.5), which is the highest minimal value among the three biodiesel standards (Winayanuwattikun *et al.*, 2008).

The degree of unsaturation, which is measured as IV, is an important considerable factor for the selection of FAMEs. The unsaturated fatty acid component in FAMEs is required as it restricts the FAMEs from solidification. However, the higher degree of unsaturated FAMEs is not suitable for biodiesel. The unsaturated molecules react with atmospheric oxygen and are converted to peroxide, crosslinking with the other unsaturated molecules. The material may be come polymerized into a plastic like body. It makes an internal combustion engine quickly gummed up with the polymerized FAMEs. To avoid this situation, biodiesel standards have set a maximum limit of IV in their specifications. All of 7 species, which qualify the specification of CN, also meet the specification of IV. All of them have IV less than 120, the lowest maximum limit among the three biodiesel standards (Winayanuwattikun *et al.*, 2008).

Another important criterion for the selection of FAMEs is viscosity. The fuel viscosity significantly affects the atomization process that is the initial stage of combustion in a diesel engine. High viscosity interferes with injector operation, resulting in poorer atomization of the fuel spray, leading to the fuel injector operation problems such as injector coking, oil ring sticking and thickening and increased carbon deposits. The conversion of vegetable oil to their FAMEs results in a marked reduction in viscosity (Winayanuwattikun *et al.*, 2008).

The values obtained were used to predict the quality of oil for use as biodiesel. It was found that biodiesel obtained from 6 plant species; white silk cotton, physic nut, pomelo, papaya, rambutan and pumpkin met the major specification of biodiesel standards of USA, and European Standard Organization.

All of the non-edible and waste plant oils were then used as substrates for transesterification catalyzed by immobilized *Candida rugosa* lipase, Novozyme® 435 and Lipozyme® RM IM in comparison.

5.5 Optimization of the transesterification reaction

In this study, the effects of various factors on the transesterification catalyzed by *Candida rogosa* lipase immobilized onto Sepabeads EC-OD were investigated. Biodiesel yield was increased significantly by each sequential variable, namely, addition mode of methanol, molar ratio of oil:methanol, enzyme loading, water content , reaction time and temperature.

5.5.1 Effect of addition mode on transesterification

Watanabe *et al.* reported that more than 1/2 molar equivalent of methanol is insoluble in vegetable oils and immobilized lipases are easily inactivated by contacting with insoluble methanol (Watanabe *et al.*, 2002). It is also known that lipase from *Candida antarctica* are deactivated when exposed to high concentration of methanol >0.5 M equivalent of MeOH for the stoichiometric amount (Shimada *et al.*, 2002). Hence, addition mode has been suggested as a means of circumventing the deactivation problem. Lu *et al.* studied the number of added times for methanol ranging from 1 to 6 and reported that the conversion was about 88% by more than three successive additions of methanol. So three-step methanolysis was sufficient to convert lard to FAME (Lu *et al.*, 2007). Furthermore, Nie *et al.* also studied the effect of methanol addition to the reaction. Methanol addition was performed from 1 to 10

times and they reported that when the methanol was stepwisely added more than three times, the conversion could be increased to 95% (Nie *et al.*, 2006). The effect of addition mode of methanol from one to seven steps on transesterification was studied. From Figure 7, very small conversion of 3.16 % (±2.46) and 4.12 % (±3.95) % were obtained when three moles of methanol were added at once and two steps respectively. The yield suddenly rose from 4.12 % (±3.95) to 31.88 % (±3.48) when the adding step of methanol increased from two to three steps and increased further from 31.88 % (±3.48) to 53.90 % (±0.86) from three to six steps. Therefore, six step addition mode of methanol was most effective for the production of biodiesel, approximately 50% of fatty acid methyl ester were obtained.

5.5.2 Effect of oil: methanol molar ratio on transesterification

The molar excess of alcohol over fatty acids contained in TAG always increases transesterification yield but it can also inactivate the enzyme. In particular, when the alcohol is insoluble in reaction mixture, it forms emulsion and the size of droplets depends on intensity of stirring (Antczak *et al.*, 2009). However, at least three molar equivalents of methanol are required for the complete conversion of the oil to FAME. Dizge and Keskinler studied the oil:methanol ratio from 1:1 to 1:10. They reported that the highest methyl ester yield could be obtained at the oil:methanol molar ratio of 1:6 and the higher methanol concentration (1:10 molar ratio) would decrease the methyl ester yield (Dizge and Keskinler., 2008). In addition, Liu *et al.* reported that the biodiesel yield was decreased when the oil:methanol was over 1:15. Additionally, when the amount of oil:methanol ratio was excessive, the glycerol separation becomes more difficult (Liu *et al.*, 2007). Therefore, the effect of oil: methanol molar ratio was studied at different ratios from 1:3 to 1:9 and the results were shown in Figure 8. The yield obtained for FAME was approximately 48% when the oil: methanol molar ratio increased from 1:3 to 1:7. However, when the ratio was increased to 1:8 and 1:9, the FAME yield was

decreased to 8.27 and 2.75 %, respectively. Therefore, in order to reduce the cost for further applications, 1 to 3 oil:methanol ratio was subsequently used as optimal oil:methanol ratio from this study.

5.5.3 Effect of enzyme loading on transesterification

The effect of immobilized lipase concentration on conversion was performed with enzyme concentrations of 10%, 20% and 30% w/w of oil. From Figure 9, it was shown that the FAME yield increased rapidly to 72% when the amount of lipase was increased up to 30% (w/w of oil). Obviously, more lipase showed abundant activated sites and sufficient mass contact, consequently the FAME yields were higher (Ghamgui *et al.*, 2004, Rosa *et al.*, 2008, Chen *et al.*, 2009 and Dizge *et al.*, 2009). In addition, the FAME content increased along with the increase in enzyme content because the more lipase available, the more substrate molecules can be adsorbed onto the active center of the lipase (Chen *et al.*, 2009). Therefore, there exists an optimum enzyme loading leading to high conversion rate. The phenomenon has also been found by some other researchers. This result is in good agreement with results obtained by Lu *et at.* The synthesis of FAME from lard catalyzed by immobilized *Candida* sp. 99-125 was found to increase rapidly when the amount of lipase was increased to 20% (w/w) (Lu *et at.*, 2007). Furthermore, Köse *et al.* studied the effect of immobilized *Candida antarctica* lipase quantity on alcoholysis of cotton seed. It was found that the FAME content was increased by increasing lipase quantity up to 30%. It was also seen that the highest FAME formation (83.6%) was observed with the reaction using 30% lipase based on oil weight (Köse *et al.*, 2002). So from this study, the immobilized lipase at 30 % by weight of oil was selected for the optimal condition of transesterification.

5.5.4 Effect of water content on tansesterification

Water plays an important role in enzyme structure and function (Ghamgui *et al.*, 2004). It is well known that lipase, as a form of protein, requires the presence of water to maintain its active three dimensional structures. The activity of the enzyme in non-aqueous media is affected by the water content. In this study, the effect of water content on the transesterification of palm oil is presented in Figure10. From the result, it was shown that the FAME content rose gradually from 53 to 78% as water content increased from 0.5% to 5% (v/w) of the oil. Similar results had been reported in the previous studies. Lu *et al.* studied the effect of water on methanolysis of glycerol trioleate catalyzed by immobilized lipase *Candida* sp. 99–125. It was found that the biodiesel yield was increased from 35 to 85 % when the amount of water increased from 0 to 20 wt% (Lu *et al.*, 2009). Moreover, the effect of water content on transesterification catalyzed by immobilized lipase from *Candida* sp. 99-125 was tested with increasing water content to 15% (w/w) of oil. The conversion obtained was 98% (Yang *et al.*, 2006). This effect could strongly confirm the hypothesis that the necessary quantities of water to stabilize the hydrophilic groups located on the surface of the enzyme molecule. This results in the changing of topology of active site and lid leading to an active conformation of lipase. Furthermore, activation of the enzyme involves in unmasking and restructuring of the active site through conformational changes of the lipase molecules, which also requires the presence of oil-water interface. Lipase activity generally depends on the available interface area. With the increasing of additional water, the amount of water available for oil to form oil-water droplets were increased, consequently increasing the available interfacial areas (Noureddini *et al*, 2005). The results presented in Figure 10 indicated that the FAME content reached its maximum with the water content of 5 wt% which was about 78% higher than that in absence of water. This supports the fact that amount of water is required to activate the enzyme. However, the amount of FAME was decreased when more than 5% (v/w of oil) of

water was added. In this case, the yield reduction might be caused by the reduced homogeneity of substrate mixtures owing to immiscibility between water and oil compounds (Lu *et al.*, 2007). This result suggests that excessive water content affects the mass transfer of the oil phase of the reaction product, and inhibits transesterification (Hama *et al*, 2006 and Chen *et al.*, 2009).

5.5.5 Effect of times and temperatures on transesterification

Temperature can influence the reaction rate and biodiesel yield. Figure 11 showed the transesterification activity of the immobilized enzyme with variations in temperature at specific times. The experiments were conducted at temperature ranging from 30 to 60 °C illustrated in Figure. 11. The optimum temperature was 40°C after 12 hours and approximately 87.08 % of fatty acid methyl ester was obtained. This result is in agreement with results obtained by Nie *et al.* who conducted the methanolysis of salad oil using the immobilized *Candida* sp. 99-125 lipase from 27 °C to 50°C. The highest yield (87%) was observed at 40 °C when the reaction time was extended to 30 hours (Nie *et al.*, 2006). Furthermore, Lu *et al.* studied the transesterification of lard using the immobilized *Candida* sp. 99-125 lipase at various reaction temperatures from 40°C, 50°C and 60°C. It was found that the highest fatty acid methyl ester at 40°C was obtained and selected as the optimal reaction temperature. Devanesan *et al.* studied the effect of temperature on biodiesel production from *Jatropha* oil using immobilized cell of *P.fluorescens* at 30, 35, 40, 45 and 50°C. It was found that the maximum yield 70%of biodiesel at 40°C was obtained (Devanesan *et al.*, 2007). In addition, the effect of temperature on transesterification of canola oil catalyzed by immobilized *Thermomyces lanuginosus* lipase was studied and 40°C was found to be optimal temperature for biodiesel production (85.8%) (Dizge and Keskinler, 2008). From Figure 11, it was shown that when the reaction temperature increased to 50°C and 60°C, the product started to decrease, which is in agreement with the previous literature report (Dizge *et al.*,

2009 and Rashid *et al.*, 2008). A similar result was obtained by Yang *et al.* They studied the reaction temperature on transesterification of soybean oil catalyzed by *Candida* sp 99-125 and reported that the optimum temperature of the reaction was 40°C. Beyond 45°C, the relative conversion rate of biodiesel declined from 98% to 40% (Yang *et al.*, 2006). The advantage of higher temperature is a shorter reaction time. However, if the reaction temperature exceeds the boiling point of methanol (65°C), the methanol will vaporize from the reaction (Chen *et al.*, 2009) and high temperature certainly causes enzyme denaturation (Yang *et al.*, 2006, Devanesan *et al.*, 2007 and Dizge and Keskinler, 2009). Moreover, the results showed that when increasing the reaction time, the percentage yield of biodiesel was increased up to 12 hours. Thereafter, increase in the reaction time did not have the effect on the production of biodiesel. The phenomenon has also been observed by other researchers. Köse *et al.* studied the reaction time ranges from 2 to 24 hours on methanolysis of cotton seed oil. It was found that the methyl ester conversion was practically constant over reaction time ranges between 7 and 24 hours, indicating optimum reaction time could be 7 hours (Köse *et al.*, 2002).

5.6 Comparative studies of transesterification catalyzed by immobilized *Candida rugosa* lipase with Novozyme® 435 and Lipozyme® RM IM

It is well known that the ability of lipase is highly dependent on sources of lipase, substrates and reaction condition. Thus three different immobilized lipases and non-edible and waste oils were screened for FAME production. Transesterifications were carried out as described in section 3.4.7. The conversion of fatty acid methyl esters from these non-edible and waste plant oils were illustrated in Figure 4-16. From the results, it was shown that the efficiency of biodiesel production was different from the catalysis of Novozyme® 435, Lipozyme® RM IM and immobilized *Candida rugosa* depending on the types of feedstocks. The production of biodiesel obtained from catalysis of three types of immobilized lipases in 6 types of plant seeds were approximately at 70-80 %. However, rambutan oil gave 90% of

biodiesel when catalyzed by both commercial lipases but only 60% was obtained by the immobilized *Candida rugosa* lipase. On the other hand, 80% of biodiesel from papaya oil was obtained when catalyzed by the immobilized *Candida rugosa* lipase when only 60% was obtained from Lipozyme® RMIM.

Six out of nine plant oils being surveyed had suitable physical property as feedstock for biodiesel production catalyzed by lipase. Even though white silk cotton, pomelo and pumpkin seeds are agricultural waste but they could not be collected in large quantities. Hence, there is a limitation on the use of these oils for production of biodiesel. Under Thailand condition, physic nut, papaya and rambutan appear to be potential raw materials for the development of oils as diesel fuels. The cultivation and climatic conditions required for plantation of these four species are described here:

Physic nuts or *Jatropha curcas* produce non-edible oil in appreciable quantity and can be grown in large scale on non-cropped marginal lands and wastelands. It is well adapted in arid and semi-arid conditions and has low fertility and moisture demand. It can also grow on moderately sodic and saline, degraded and eroded soil. The ideal density of plants/hectare is 2500. It reaches maximum productivity by five years and can live up to 50 years. There are reports of oil yields of as high as 50% from the seed. Typically, the seed production would be 3.75 ton/ha, with oil yield of 30-35%, giving net oil yield of about 1.2 ton/ha. Although *Jatropha* oil seed is not yet cultivated on a large scale in Thailand, *Jatropha* oil is the major feedstock of the biodiesel program. The projection for plantation of this species was started in 2008 to supplement the utilized edible palm oil for production of biodiesel.

Papaya is a tropical or near tropical species, sensitive to frost and limited to the region between 32° north and 32° south of the equator. It needs plentiful rainfall or irrigation but must have good drainage. While doing best in light, porous soils rich in organic matter, the plant will grow in scarified limestone, marl, or various other soils if it is given adequate care. Optimum pH ranges from 5.5 to 6.7. Papaya plants bear well for 2 years and then productivity declines and commercial plantings are generally replaced after 3-4 years. The papaya black seeds contain 25-48% oil (w/w).

Rambutan is adapted to warm tropical climates and is sensitive to temperatures below 10 °C, and is grown commercially within 15° of the equator. Rambutan flourishes from sea-level to 1,600 or even 1,800 ft (500-600 m), in tropical, humid regions having well-distributed rainfall. The dry season should not last much over 3 months. The tree does best on deep, clay-loam or rich sandy loam rich in organic matter and thrive on hilly terrain as they require good drainage. Optimum pH ranges from 5.0 to 5.7. Rambutan trees may fruit after 2-3 years with optimum production occurring after 8-10 years. The seed kernel yield 37-43% oil (w/w). Both rambutan and papaya are food industrial waste products which can be collected in a large amount. Thus, they might be suitable for use as starting material for the production of biodiesel.

5.7 Stability of immobilized *Candida rugosa* lipase

5.7.1 Thermal stability

The resistance of immobilized lipase to temperature is an important potential advantage for practical applications of this enzyme. The residual activities of immobilized lipase on thermo stability were tested as described in 3.4.10.1. The optimal temperature for thermal stability was studied at various temperatures from 30 to 80 °C. Then, the residual activities were determined as percentage yield of activity at different temperatures compared to the activity at the optimal conditions. The results in Figure 4-17 revealed that the percentage of remaining activity significantly decreased from 100% to 15% when the temperature was increased from 30°C to 80°C. However, 50% of the activity was still retained at 60 °C. According to Hung *et al,* they reported that the inactivation of the enzyme occurred when they were treated at high temperature. Free lipase remained stable only up to 39 °C. At 60 °C, the residual activity of immobilized *Candida rugosa* lipase on chitosan was 23 % compared to 12 % for free lipase. Then, the thermal stability of immobilized lipase was carried out by incubating 2 mg of immobilized lipase at 60 °C. Later, the samples were periodically taken and the residual activities were

determined as the percentage yield of activity compared to the activity at the optimum conditions. Then, the half life time ($t_{1/2}$) were calculated as shown in Appendix D. The results were expressed as the percentage of relative of the residual activity and half life time as shown in Figure 4-18. It was shown that, half-lives of the immobilized *Candida rugosa* lipase at 60°C was 14.35 min. When an immobilization of *Candida rugosa* lipase by adsorption on bentonite was studied (Yeşiloğlu Y, 2005), the half –life of the immobilized enzyme was about 45 min, whereas for the soluble free lipase was 17 min at 50°C. Evidently, the immobilization has considerably increased the thermal stability of lipase. This result supported the fact that the strong hydrophobic interaction between the lipase and the hydrophobic carriers enhances the stability of the molecular conformation of the immobilized enzyme. This therefore will increase the thermal stability of the immobilized lipase (Huang and Cheng, 2008). The results indicated that immobilization helps preserve the enzyme structure from thermal inactivation.

5.7.2 Repeated use of the immobilized *Candida rugosa* lipase

The most important advantage of immobilization is the repetitive use of enzyme. The catalyst reusability was carried out to determine the stability of the immobilized lipase. The short operational life of the enzyme is normally caused by the negative effects of excessive methanol and by product glycerol. This phenomenon may be clarified by two explanations. Firstly, the glycerol adsorbed on the surface of immobilized lipase constrains the contact of substrate and enzyme molecules. This can be resolved by washing with a solvent such as acetone and t-butanol (Dizge *et al.*, 2008). Secondly, the lipase was inactivated or desorbed from carriers during repeated uses.

5.7.2.1 Repeated use on transesterification

It has been demonstrated that the cost of lipase accounts for a large part in the total cost of biodiesel production. One of the main advantages of an immobilized lipase is that it can be used repeatedly over an extended period of time (Ghamgui *et al.*, 2004 and Lu *et al.*, 2007). The byproduct; glycerol is the main problem of reusable of immobilized lipase in transesterification because it can deactivate enzymes, particularly in continuous and repeated-batch processes (Antczak *et al.*, 2009). The glycerol molecules were adsorbed on the surface of these carriers thereby forming the hydrophilic coating which made enzyme molecules inaccessible to substrates. Addition of another hydrophilic substance like acetone to the reaction system partially removed glycerol from the lipase environment (Dossat *et al.*, 1999). Du *et al.* also washed the immobilized lipase with iso-propanol for restoring its activity (Du *et al.*, 2003). In addition, to increase the operational stability of the lipases, washing of used *Candida antarctica* immobilized lipase with t-butanol or 2-propanol can be an efficient method of regeneration of immobilized lipase (Chen and Wu, 2003). To investigate the stability of the immobilized lipase, the optimal conditions for transesterification were conducted to obtain the production of biodiesel and repeated every 12 hours. Under these conditions, approximately 90% of FAME content was obtained. After completion of the reaction for 12 hours of each cycle, the immobilized lipase was rinsed with water, t-butanol, hexane and acetone in comparison .The purpose was to select the best washing solution for the removal of glycerol and oil from the carriers. The immobilized lipase was dried in the desiccator and later used in the next batch reaction composed of new substrates. The results demonstrated that the % production of FAME in each batch was reduced. After three or four times of uses, immobilized lipase retained approximately 45% of its initial activity

when washed with water. On the other hand, enzyme lost all of its activity after 5 cycles with t-butanol, hexane and acetone as the washing solutions. From the results, water is selected as the best washing solution for cleaning the lipase immobilized on hydrophobic support. It appears that the immobilized lipase on Sepabeads EC-OD was inactivated by methanol or desorbed from carriers during repeated use. Therefore, the reusability of immobilized lipase in hydrolysis of palm oil was examined.

5.7.2.2 Repeated use on hydrolysis

Repeated use of the immobilized lipase in batch hydrolysis of palm oil was tested as described in section 3.4.10.2.2. Under these conditions, approximately 97% of FFA content was obtained. The immobilized lipase was rinsed with water after each batch reaction to remove glycerol and oil in carriers. From the results, it was shown that stability of the lipase activity was evidently observed as in the first cycle. Additionally, immobilized lipase could be used at least 10 repeated times without significant loss of activity. From this result, it strongly confirmed that immobilized lipase on Sepabeads EC-OD was inactivated by methanol. This problem can be reduced by the modification of the enzyme with chemical and genetic techniques for more tolerance ability in the organic solvents. Otherwise, the alternative method is to apply a novel technology for the immobilization of the enzyme.

CONCLUSION

In this research, lipase from *Candida rogusa* was successfully immobilized on selected Sepabeads EC-OD by adsorption. The optimal conditions for the immobilization obtained were as follows: pH 6, 500 mM ionic strength, 8 mg/ml enzyme loading at 30 °C for 30 min and t-butanol as the adjuvant. When the immobilized lipase- catalyzed transesterification was carried out for the production of biodiesel, the maximal yield of 87% was finally achieved under the following optimal parameters: six step addition mode, 1to 3 molar ratio of oil: methanol, 30% (w/v) of oil enzyme loading at 40°C for 12 hours. Potential feedstocks from non-edible and waste plant seed oils including physic nut, papaya and rambutan could be highly converted to biodiesel when the reactions were comparatively catalyzed by immobilized *Candida rugosa* lipase and commercial lipases; Novozyme 435 and Lipozyme RMIM. Finally, the enzyme could be reused for three cycles for the production of biodiesel whereas approximately 95% relative hydrolytic activities could be well maintained over ten repeated cycles. Therefore, it can be concluded that the immobilized *Candida rugosa* lipase on Sepabeads EC-OD can catalyze the transesterification for the production of biodiesel as efficiently as the commercial enzymes.