CHAPTER IV

RESULTS

4.1 Support selection

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. Lipases were adsorbed on each type of support under the same conditions and the activity was measured by the method described in 3.4.1 and 3.4.5.

Supports were prepared by suspending 1 g of support powder in 3 ml methanol. The suspension was kept stirred at 350 rpm at room temperature. After 30 min, methanol was removed from the reaction and supports were washed with 20 mM phosphate buffer pH 7.5 and kept stirred at 350 rpm at room temperature for 30 min 3 times. After that, the supports were separated into 2 groups. The first group was pretreated by drying at 45°C and the other was immediately used for immobilization. Then, crude *Candida rugosa* lipase was dissolved in 20 mM phosphate buffer solution, pH 7.5 and the solution was centrifuged to remove insoluble components. The supernatant was then brought in contact with 1 g of support and magnetically stirred at 350 rpm for 6 hours at room temperature. After incubation, the solution was removed from immobilized enzyme and washed with buffer. Then, the immobilized lipases were dried at room temperature in desiccator and the enzyme was finally assayed for activities as described in section 3.4.5

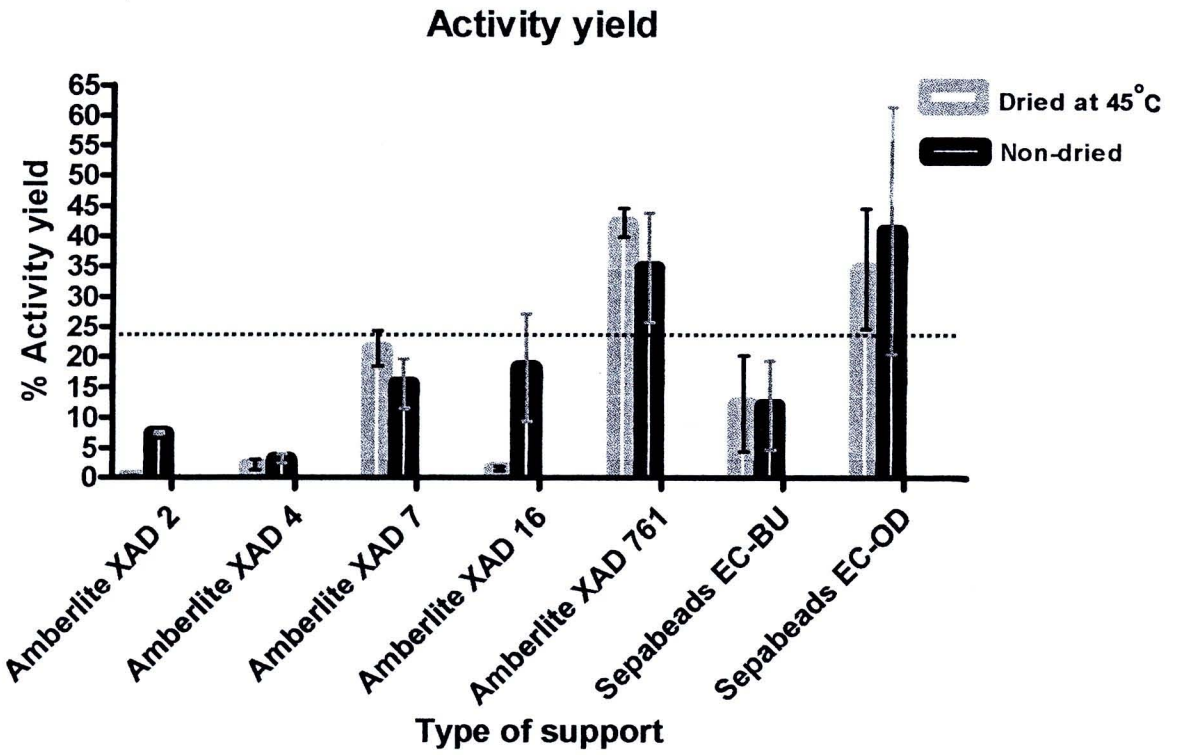


Figure 4-1. Yield of activity of the immobilization of lipase on hydrophobic supports. The reactions were carried out in a mixture of 3 mg/ml lipase solution prepared by dissolving crude lipase in 20 mM phosphate buffer solution pH7.5 and added with 1 g of support and magnetically stirred for 6 hr at room temperature (25°C).

Figure 4-1 showed the activity yields of the lipase from 7 types of hydrophobic supports. From the graph, it was found that even the same support gave different results if they were prepared from different methods. When the dried supports were used, the result showed that Amberlite XAD 7, Amberlite XAD 761 and Sepabeads EC-BU gave higher activity yield than the wet supports. When the wet supports were used, the resulted showed that Amberlite XAD 2, Amberlite XAD 4, Amberlite XAD 16 and Sepabeads EC-OD gave higher activity yield than the dried supports. So, from these results, 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 selected for subsequent experiment.

Next, 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 °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, wet Sepabeads EC-OD was selected as the optimal support for the subsequent immobilization.

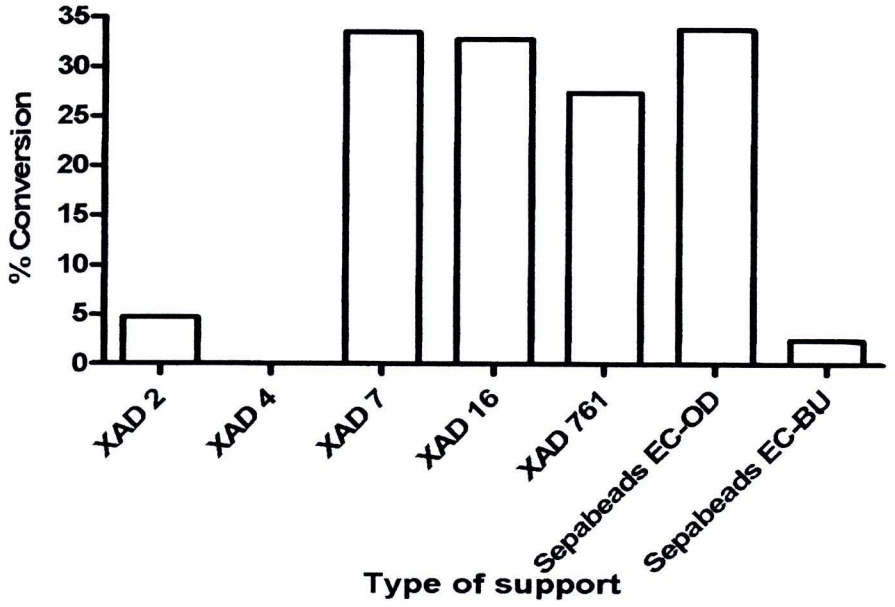


Figure 4-2. Support screening on transesterification of palm oil, a loading of 0.20 g immobilized lipase, 1 g oil, ratio of oil to methanol was one to three and added into reaction using 3 steps and continuously stirring for 24 hr at 40°C

4.2 Optimization of immobilization of lipase

The effect of pH, ionic strength, protein loading, immobilization time, temperature and adjuvants for the immobilization of *Candida rugosa* lipase onto Sepabeads EC-OD were investigated. The result for each factor was illustrated in Figure 4-3 to Figure 4-9.

4.2.1 Effect of pH on immobilization

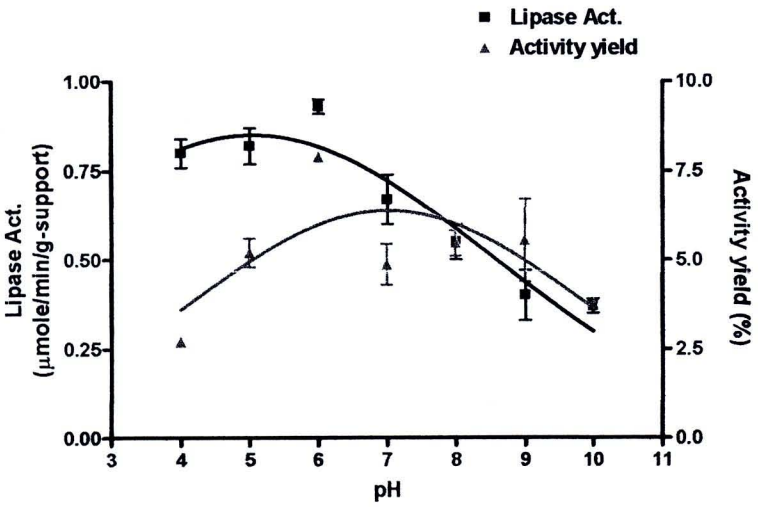


Figure 4-3. The effect of pH on the lipase activity and activity yield (%) of lipase immobilization. The enzyme solution was prepared by dissolving crude *Candida rugosa* lipase in 3 ml 20 mM buffer solution at various pH. Then, 2.5 ml lipase solution (3mg/ml) was added to 1 g of Sepabeads EC-OD and magnetically stirred for 5 hours at room temperature (25°C). Activities shown on the y-axis are the means ± SD of three individual experiments.

The activities of lipase and the activity yields at various reaction pH were studied and the results were shown in Figure 4-3. It can be seen that maximal activity was obtained when pH of the system reached pH 6 and started to decrease by nearly 3 folds when the pH subsequently rose to 10 since denaturation tend to increase under high pH values. The highest activity of lipase (0.93 (±0.02) umol/min/g-support) and activity yield (7.5 (±0.06) percent) were obtained at pH 6 in phosphate buffer (20 mM). As can be seen from the figure, the activity of lipase and activity yield increased from 0.80 (±0.04) to 0.93 (±0.02) umol/min/g-support and 2.71 (±0.14) to 7.5 (±0.06) percent when pH equaled to 4 and 6 respectively. From pH 6 to pH 10, the activity of lipase and activity yield dramatically decreased from 0.93 (±0.02) to 0.37 (±0.02) umol/min/g-support and 7.5 (±0.06) to 3.81 (±0.01)

percent respectively. The results indicated that immobilized lipase from *Candida rugosa* appeared more stable in acidic environments. The optimum pH for the maximum of activity of lipase was therefore fixed as 6.

4.2.2 Effect of ionic strength on immobilization

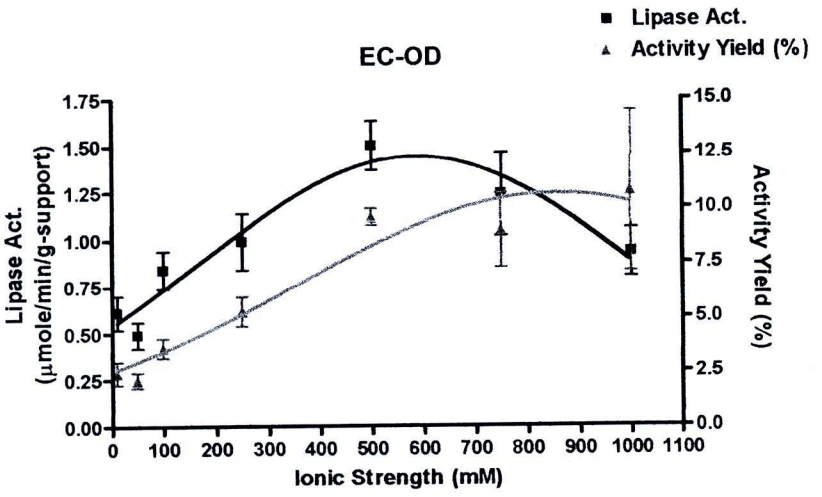


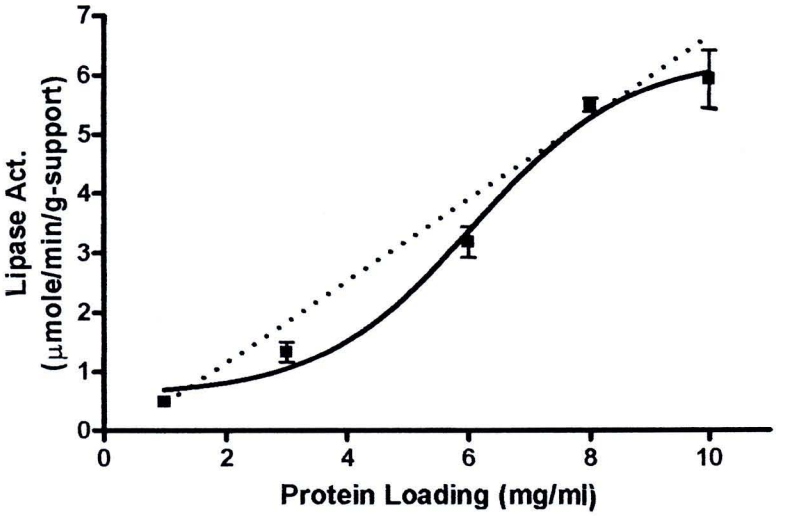
Figure 4-4. The effect of ionic strength on the lipase activity and activity yield (%) of lipase immobilization. 3 mg/ml lipase solution prepared by dissolving crude lipase in buffer solution pH6 at various concentrations was added to 1 g of Sepabeads EC-OD and magnetically stirred for 5 hr at room temperature (25°C). Activities shown on the y-axis are the means ± SD of three individual experiments.

When the optimal pH for immobilization was obtained at 6, the phosphate buffer, pH 6 at various concentrations from 10 mM to 1 M were therefore prepared to study the effect of ionic strength on activity of immobilized *Candida rugosa* lipase. The effect of the ionic strength of enzyme solution on the adsorbed amount of protein was investigated and these results were shown in Figure 4-4. It can be seen that the activity of lipase increased from 0.61 (±0.09) to 1.50 (±0.13) umol/min/g-support with increasing ionic strength from 10 mM to 500 mM and percentage of activity yield increased from 2.46 (±0.53) to 9.62 (±0.38). On the other hand, when the ionic strength was elevated from 500 mM to 1 M, the activity of

lipase was decreased from 1.50 (±0.13) umol/min/g-support to 0.93 (±0.13) umol/min/g-support and percentage of activity yield was rather stabilized at approximately 10 percents. Therefore, the optimal concentration (500mM) of phosphate buffer pH 6 was used as immobilization of *Candida rugosa* lipase to study the effect of protein loading on activity of immobilized lipase.

4.2.3 Effect of protein loading on immobilization

(a)



(b)

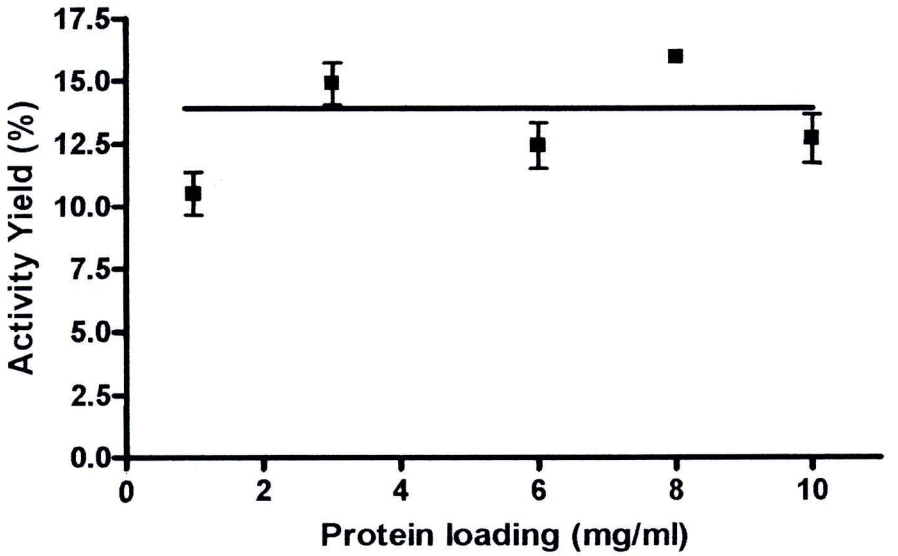


Figure 4-5. The effect of protein loading on (a) lipase activity and (b) % activity yield of lipase immobilization. Crude lipase was dissolved in 500 mM phosphate buffer, pH 6.0. The various quantities of enzyme were added to 1 g of Sepabeads EC-OD and magnetically stirred for 5 hr at room temperature (25°C). Activities shown on the y-axis are the means ± SD of three individual experiments.

When the suitable pH and ionic strength (500 mM phosphate buffer, pH 6.0) were obtained, the effect of protein loading was later studied. In this study, different quantities of enzyme were immobilized on the support from 1 to 10 mg/ml and the results were shown in Figure 4-5. It can be seen that the activity significantly increased from 0.49 (±0.01) to 3.18 (±0.26) umol/min/g-support when the protein loading was increased from 1 to 6 mg/ml and the maximal activity (5.93 (±0.48) umol/min/g-support) was obtained at approximately 10 mg/ml of protein (20 folds higher compared to 1 mg/ml of enzyme). However, when the activity of enzyme reached 8 mg/ml, the activity was not much different from 10 mg/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. Hence, the optimal condition was selected as described in section 4.2.1-4.2.3 to study the effect of time of immobilization.

4.2.4 Effect of immobilization time

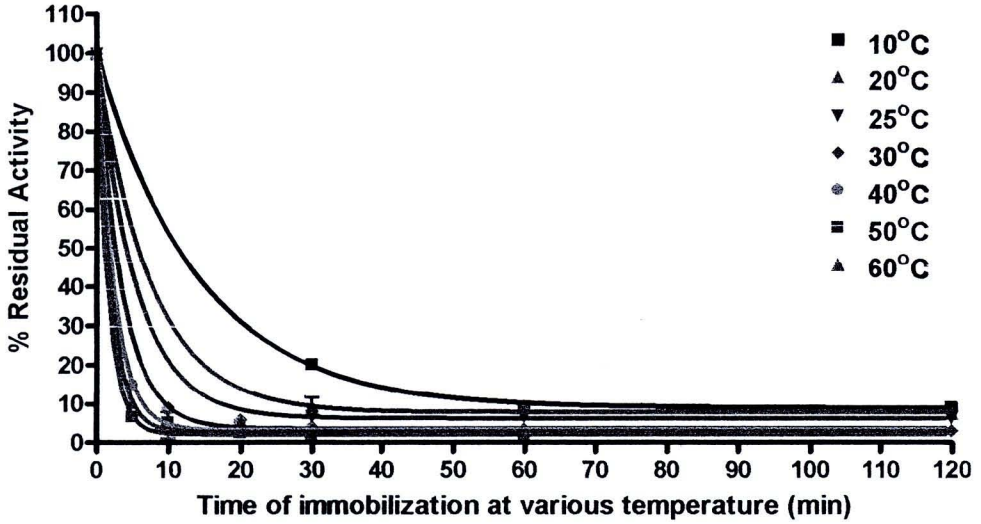


Figure 4-6. The effect of immobilization time on the residual activity of lipase. 8 mg/ml of crude *Candida rugosa* lipase solution were incubated at various temperatures. Residual activities on the y-axis are the means ± SD of three individual experiments.

The effect of time of immobilization on activity of immobilized *Candida rugosa* lipase was studied by using optimal conditions from the results described above. The residual activity of lipase solution was checked for each time of immobilization at various temperatures as described in 3.4.5.1. The relationship of the residual activity with immobilization time at various temperatures was shown in Figure 4-6. The results were expressed as the percentage of the residual activity at room temperature (25°C). When lipase was incubated at 10°C for 180 min, the residual activity of lipase appeared unchanged at 10.12 (±0.03) %. Furthermore, when incubation of lipase solution at 20°C and room temperature (around 25 °C), the results showed that the residual activity leveled off in both temperatures at 6.50 (±0.11) and 7.67 (±0.16) % respectively until 120 min. 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. Therefore, the optimal time for immobilization was 3 hr, 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.

4.2.5 Effect of temperature on immobilization

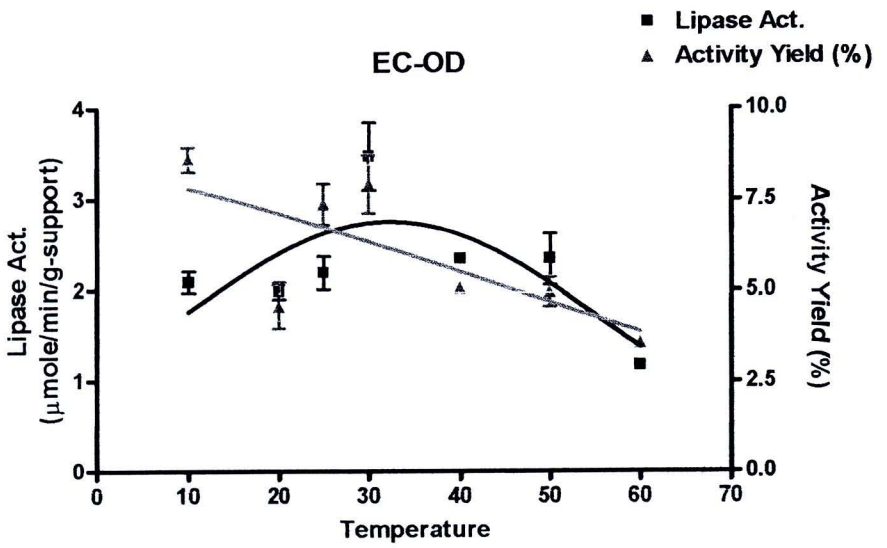


Figure 4-7. The effect of temperature on the lipase activity and % activity yield of lipase immobilization. The immobilization was performed in 500 mM phosphate buffer pH 6.0 and 8 mg/ml lipase solution at specific time for each temperature. Activities shown on the y-axis are the means ± SD of three individual experiments.

Lipase was immobilized on Sepabeads EC-OD at different temperatures from 10 to 60°C.The immobilization reaction was carried out under the optimal conditions obtained from all 4 previous sections. In this experiment, the immobilization of *Candida rugosa* lipase was determined by checking the activity of immobilized lipase. From Figure 4-7, it was shown that when 10°C was used for immobilization, the activity of lipase and activity yield were 2.09 (±0.12) umol/min/g-support and 6.62 (±0.34) respectively. They considerably increased to 3.47 (±0.37)

umol/min/g-support and 7.93 % (±0.79) respectively, when temperature rose to 30°C. However, when temperature was increased to 40, 50 and 60 °C, the lipase activity and activity yield decreased dramatically from 2.34 (±0.01) to 1.17 (±0.04) umol/min/g-support and 5.07 (±0.15) to 3.57 (±0.01) percent, respectively. From the above 4 optimal conditions, the activities of the immobilized lipase was increased approximately 3 folds higher from 0.93 (±0.02) to 3.47 (±0.37) umol/min/g-support. From these results, 30 °C was the optimal temperature for *Candida rugosa* lipase immobilization. Therefore, the optimal conditions from described 4.2.1 to 4.2.5 were subsequently selected for the next experiment namely, adjuvant.

4.2.6 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 was examined by using two types of adjuvants as described in section 3.4.3.5.

4.2.6.1 Effect of concentration of adjuvant

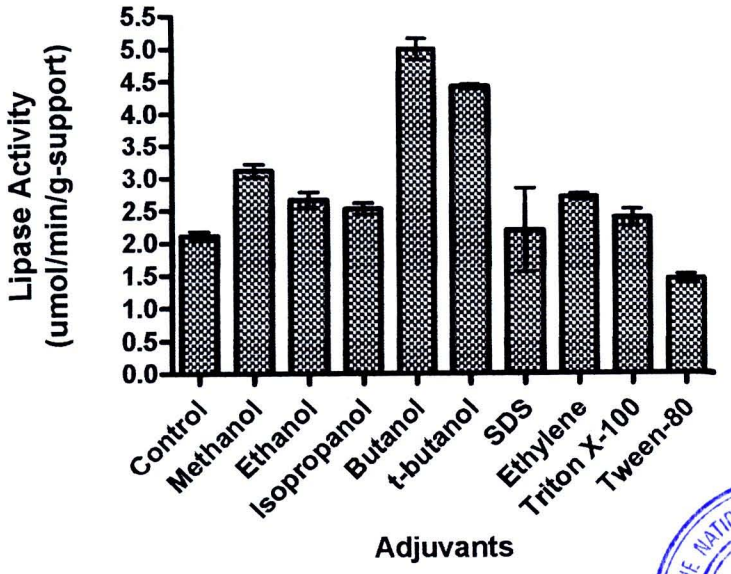
Table 4-1. The concentrations of various adjuvants with the highest activities of *Candida rugosa* lipase solution.

Type of adjuvants	Concentration of adjuvants (%v/v)
Methanol	5
Ethanol	5
Isopropanol	20
Butanol	5
t-butanol	5
SDS	0.75
Tween- 80	2.50
Ethylene glycol	2.50
Triton X-100	1

From Table 4-1, the effect of concentration of adjuvant on activity of *Candida rugosa* lipase solution was studied by using optimal conditions from the results described above. The activities of lipase solution were examined by using two types of adjuvants with various different concentrations as described section 3.4.3.5.1. The results were expressed as the percentage of the activity of untreated lipase solution. Then, the various concentrations of each adjuvants were selected from the highest activity of lipase. Table 4-1 showed the highest activity of each type of adjuvant. Therefore, the concentration of each adjuvant was used for the immobilization of lipase to further select the type of adjuvant.

4.2.6.2 Effect of the type of adjuvant

(a)



(b)

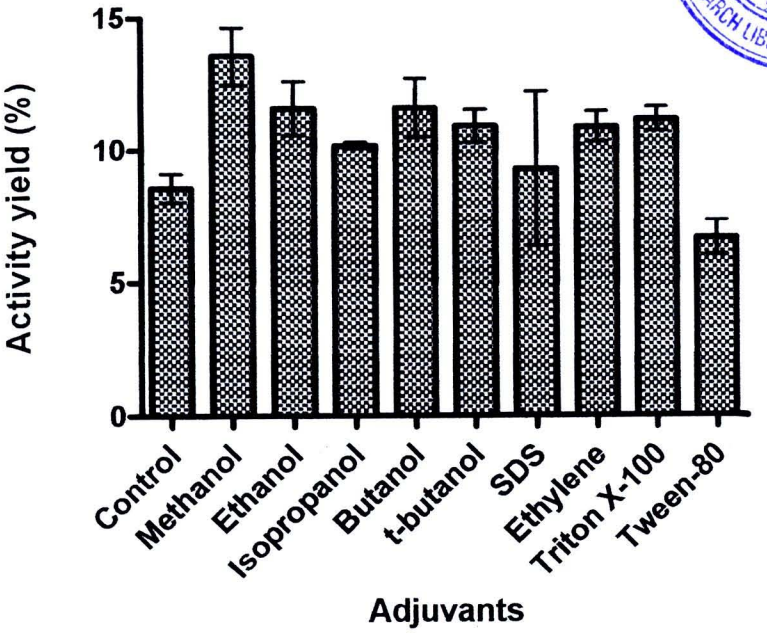


Figure 4-8. The effect of adjuvant types on the lipase activity (a) and % activity yield (b) of lipase immobilization. The suitable concentration of each

adjuvant was added in to the enzyme solution (8 mg/ml) for 2 min before contact with support. The reaction was magnetically stirred for 30 min at 30°C.

When the proper concentration of each adjuvant from the method described above was obtained, the effect of the type of adjuvant on the immobilization of *Candida rugosa* lipase was then determined as described in 3.4.5.1. The relationship between the lipase activity and the % activity yield with type of adjuvant were shown in Figure 4-8a and Figure 4-8b.

From Figure 4-8a, the result showed that the highest lipase activity (4.99 (±0.16) umol/min/g-support) was achieved when butanol was used as adjuvant. However, from Figure 4-8b, the result showed that the highest % activity yield (13.55 % (±0.16)) was obtained when methanol was used. Since both results were inconsistent, the immobilized lipases were therefore tested again for transesterification to determine the selection of the optimal type of adjuvant by the method described in section 3.4.7

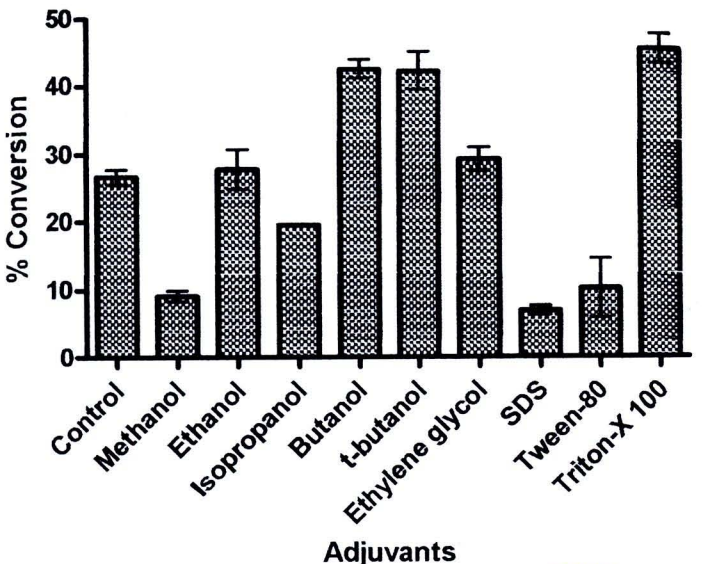


Figure 4-9. The effect of immobilized lipase with added various adjuvants on transesterification reaction. The reactions were carried out in a mixture of 0.5 g of palm oil, 1:3 oil:methanol ratio, 3 step addition mode of methanol and 20% (w/w of oil) immobilized lipase magnetically stirred in a water bath for 24 hr at 40°C. Percent conversion shown on the y-axis are the means ± SD of three individual experiments.

From Figure4-9, the results showed that %conversion was highest when butanol, t-butanol and triton-X 100 were used as adjuvants. So, t-butanol was selected as the optimal adjuvant for the *Candida rugosa* lipase immobilization and subsequently selected for the next experiment.

From overall results, the optimal conditions of lipase immobilization were obtained and summarized in Table 4-2. Under these conditions, the lipase activity of the immobilized lipase was 4.41 umol/min/g-support, specific activity was 0.15 umol/min/mg-protein, activity yield was 10.89 percent and with a protein loading of 83.81 percent.

Table 4-2. Optimal conditions of lipase immobilization

pH	6
Ionic strength (mM)	500
Protein loading (mg/ml)	8
Time (min)	30
Temperature (°C)	30
Type of adjuvant	t-butanol

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

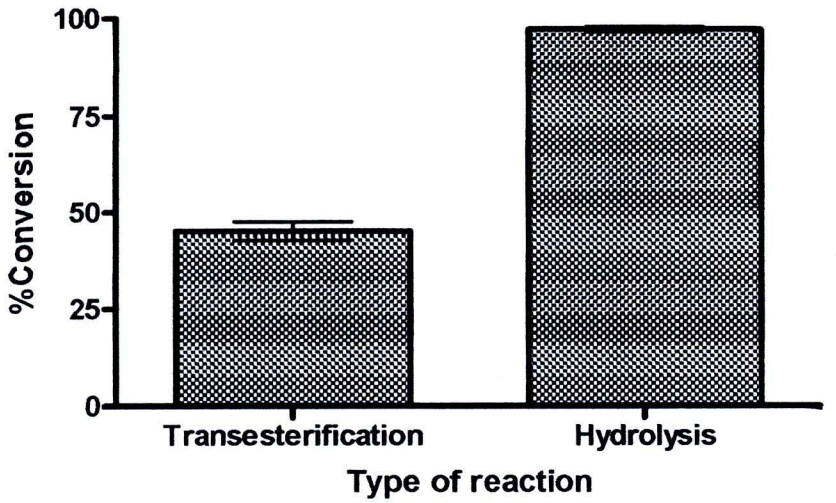


Figure 4-10. Transesterification and hydrolysis catalyzed by immobilized lipase. The transesterification reactions were carried out in a mixture of 0.5 g of palm oil, 1:3 oil:methanol ratio, 3 step addition mode of methanol and 20% (w/w of oil) immobilized lipase and magnetically stirred for 24 hr at 40°C. The hydrolysis reaction was carried out in a mixture of 20 % (w/w of oil) enzyme and 100 % (w/w of oil) water were magnetically stirred at 40 °C for 9 hours at 40°C.Percent conversion shown on the y-axis are the means ± SD of three individual experiments.

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 reactions were carried out in a mixture of 0.5 g of palm oil, 1:3 oil:methanol ratio, 3 step addition mode of methanol and 20% (w/w of oil) immobilized lipase magnetically stirred in a water bath for 24 hr at 40°C. The hydrolysis reactions were carried out in a mixture of 20 % (w/w of oil) enzyme and 100 % (w/w of oil) water and mixed well at 40 °C for 9 hours by magnetic stirrer in a water bath. From Figure 4-10, it was shown that 45.23 % (±2.43) biodiesel and 97.21% (±0.69) free fatty acid were obtained from transesterification and hydrolysis, respectively.

4.4 Screening of raw materials for feedstock

Biodiesel can be produced from any vegetable oil such as rapeseed, soybean, cottonseed, physic nut, rubber seed and others. Oils are primarily composed of triacylglycerols (TAGs), a form of lipid comprised of three fatty acid molecules attached to a glycerol backbone.

4.4.1 Extraction of seed oil

Table 4-3. Oil content of non-edible and waste plant seeds

Common name	Scientific Name	% oil content
Papaya	*Carica papaya* Linn.	25.00
Physic nut	*Jatropha curcas* L.	43.75
Pomelo	*Citrus maxima* (Burm.) Merr.	42.78
Pumpkin	*Cucurbita moschata* Duchesne	38.12
Rambutan	*Nephelium lappaceum* L.	41.25
Rubber	*Dipterocarpus alatus Roxb.ex G.Don*	38.77
Tangerine	*Citrus reticulate Blan co*	29.10
White silk cotton	*Ceiba pentandra* (L.)Gaertn.	29.21
Wild almond	*Irvingia malayana* Oliv. ex A.W. Benn.	72.06

The oil of non-edible and waste plant seeds was extracted using soxhlet 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 in papaya seeds to approximately 72 dry weight in wild almond seeds.

4.4.2 Fatty acid composition analysis

Seed oil from nine types of non-edible and waste plants commonly found in Thailand with oil contents ≥ 30% (dry weight) were analyzed for their fatty acid composition using chemical transesterification. One gram of oil sample was added with 1:3 molar ratio of methanol. The reaction was catalyzed with 10% by oil weight of NaOH at 55°C. Then, the fatty acid composition of obtained methyl esters was quantitated by GC.

The composition for various types of waste and non-edible oils is shown in Table 4-4. From this table, it was observed that the major oil composition in both non-edible and waste plant oils is generally similar. The major fatty acids content in both non-edible and waste plant oils are oleic, linoleic and palmitic acid, while the latter include lauric, myristic, palmitoleic, stearic, linolenic, arachidic and behenic acid. Noticeably, the major content in wild almond were found to be saturated fatty acids, lauric and myristic acids. White silk cotton and pomelo had high content of linolenic acid with ≥ 40% while physic nut, papaya, rambutan, and pumpkin contained similar high content of oleic acid. The obtained fatty acid composition of each non-edible and waste plant oils was then later used to calculate their saponification number (SN), iodine value (IV), cetane number (CN) and viscosity (η).

4.4.3 Characterization of oil

The quality of oil is expressed in terms of the physicochemical properties such as saponification value, iodine value, cetane number and viscosity (η) by equation shown in Appendix E. These values established their suitability for used as

substrates as shown in Table 4-4. From the results, 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. 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 such as 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.

Table **4-4**. Fatty acid composition, Saponification number (SN), Iodine value (IV), Cetane number (CN) and viscosity (η) of fatty acid methyl ester of non-edible and waste plant oils

No.	Common Name	Scientific Name	SN	IV	CN	η	Fatty Acid Composition (%)
1	Wild almond	*Irvingia malayana* Oliv. ex A.W. Benn	204	3.60	66.13	2.29	12:0(55.55), 14:0(38.19), 16:0(2.55), 16:1(0.44), 18:0(0.22), 18:1(2.63), 18:2(0.44)
2	White Silk Cotton	*Ceiba pentandra* (L.) Gaertn.	204	104.70	49.52	3.41	12:0(0.06), 14:0(0.16), 16:0(24.19), 16:1(0.21), 18:0(4.15), 18:1(26.30), 18:2(42.03), 18:3(1.73), 20:0(0.39), 22:0(0.69)
3	Rubber	*Dipterocarpus alatus* Roxb. ex G.Don	202	146.90	40.29	3.24	12:0(0.30), 14:0(0.42), 16:0(9.87), 16:1(0.22), 18:0(5.28), 18:1(21.76), 18:2(45.82), 18:3(16.12), 20:0(0.14), 22:0(0.09)
4	Physic Nut	*Jatropha curcas* L.	202	108.40	48.91	3.46	12:0(0.14), 14:0(0.17), 16:0(14.82), 16:1(0.81), 18:0(4.15), 18:1(40.98), 18:2(38.61), 18:3(0.27), 20:0(0.06)
5	Pomelo	*Citrus maxima* (Burm.) Merr.	203	106.20	49.29	3.40	12:0(0.01), 14:0(0.14), 16:0(25.24), 16:1(0.17), 18:0(4.25), 18:1(24.53), 18:2(43.32), 18:3(2.00), 20:0(0.27), 22:0(0.11)
6	Papaya	*Carica papaya* Linn.	202	75.60	56.27	3.69	12:0(0.26), 14:0(0.46), 16:0(17.12), 16:1(0.45), 18:0(2.98), 18:1(72.91), 18:2(4.83), 18:3(0.29), 20:0(0.67), 22:0(0.07)
7	Rambutan	*Nephelium lappaceum* L.	195	58.13	61.17	3.95	12:0(0.08), 14:0(0.11), 16:0(8.77), 16:1(0.96), 18:0(7.25), 18:1(55.25), 18:2(3.72), 18:3(0.26), 20:0(22.05), 22:0(1.34)
8	Pumpkin	*Cucurbita moschata* Duchesne	202	95.19	51.87	3.52	12:0(0.01), 14:0(0.18), 16:0(20.53), 16:1(0.07), 18:0(6.51), 18:1(38.68), 18:2(32.96), 18:3(0.22), 20:0(0.28), 22:0(0.60)
9	Tangerine	*Citrus reticulate Blan co*	203	118.40	46.48	3.34	12:0(0.01), 14:0(0.03), 16:0(21.67), 16:1(0.39), 18:0(4.03), 18:1(20.99), 18:2(48.00), 18:3(4.45), 20:0(0.24), 22:0(0.05)

4.5 Optimization of the transesterification reaction

This work purported to study the ability of the immobilized *Candida rugosa* lipase preparation to catalyze the synthesis of fatty acid methyl ester (biodisel). The following six main variables: addition mode of methanol, oil:methanol molar ratio, amount of lipase, water content, time and temperature were investigated.

4.5.1 Effect of addition mode of methanol on transesterification

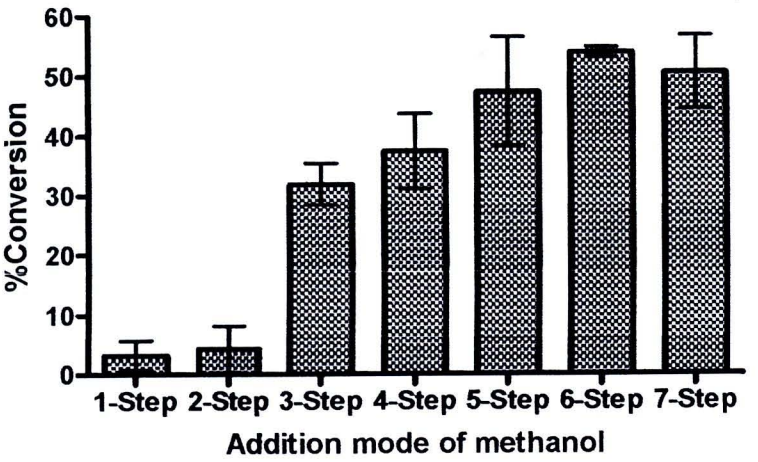


Figure 4-11. The effect of addition mode on percentage of conversion to biodiesel. The reactions were carried out in a mixture of 0.5 g of palm oil, 1:3 oil:methanol ratio with 20% (w/w of oil) immobilized lipase and continuously stirred at 40°C for 24 hr. Percent conversion shown on the y-axis are the means ± SD of three individual experiments.

The effect of addition mode of methanol from once to seven steps on transesterification was studied using the conditions as follows; 0.5 g of oil, 20% (w/w of oil) immobilized lipase and 1:3 mole ratio of oil:methanol. From Figure 4-11, it was found that when three mole of methanol were added at once and two steps, 3.16 % (±2.46) and 4.12 % (±3.95) biodiesel were obtained, 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 and declined lightly to 50% at 7 steps. The optimal addition mode of methanol for the maximum yield of biodiesel was therefore fixed at 6 steps and subsequently selected for the next experiment namely: optimal condition for the oil to methanol molar ratio.

4.5.2 Effect of oil to methanol molar ratio on transesterification

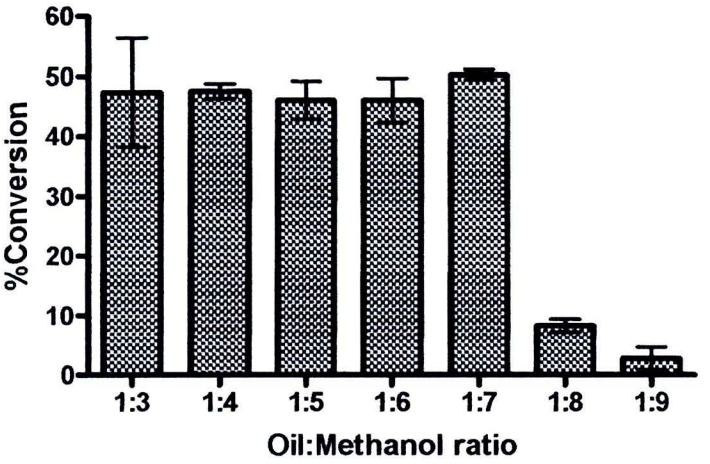


Figure 4-12. The effect of oil to methanol ratio on percentage of conversion to biodiesel. The reactions were carried out with 0.5 g of palm oil, 20% (w/w of oil) immobilized lipase and 6 steps addition mode of methanol and continuously stirred at 40°C for 24 hr. Percent conversion shown on the y-axis are the means ± SD of three individual experiments.

The effect of oil to methanol molar ratio on the conversion was performed at 40 °C, immobilized lipase concentration of 20 % (w/w of oil), and varying the oil to methanol molar ratio at the values of 1:3, 1:4, 1:5, 1:6, 1:7,1:8 and 1:9. Figure 4-12 showed the fatty acid methyl esters conversion obtained. As can be seen from this figure, the highest conversion (50.24%) was achieved at the molar ratio of 1 to 7. From the figure, the percent conversion from 1:3 to 1:7 was approximately 48% which was not much different. However, when the ratio was increased to 1:8, the yield of biodiesel was dramatically decreased nearly 6 folds to approximately 8%.

Moreover, when the ratio was increased to 1:9, the yield of biodiesel was continuously decreased for 24 folds to 2%. Therefore, in order to reduce the cost for further applications, 1 to 3 ratio was subsequently used as optimal oil to methanol ratio from this study. As a result, 6 addition mode of methanol and 1 to 3 molar ratio of oil to methanol were used as optimal conditions for transesterification to further study the effect of amount of enzyme on conversion.

4.5.3 Effect of amount of enzyme on transesterification

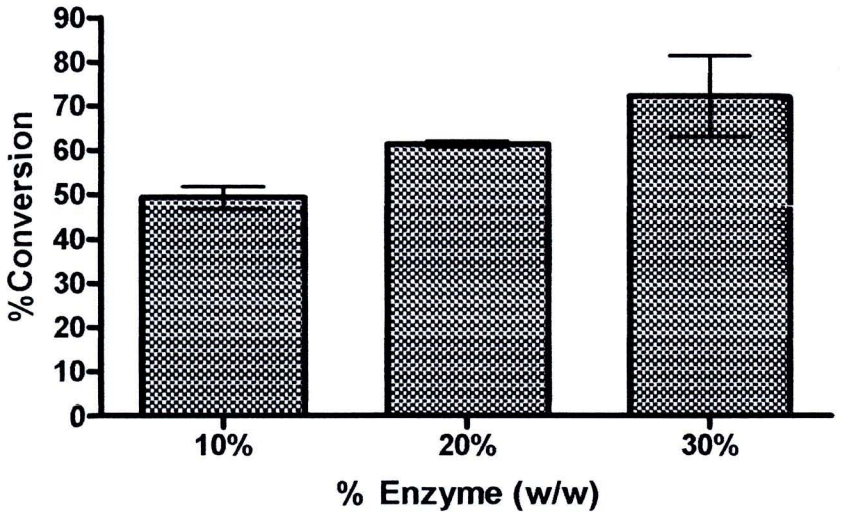


Figure 4-13. The effect of amount of enzyme on percentage of conversion to biodiesel. The reactions were carried out in a mixture of 0.5 g of palm oil, 1:3 oil:methanol ratio, 6 step addition mode of methanol and magnetically stirred in a water bath at 40°C for 24 hr. Percent conversion shown on the y-axis are the means ± SD of three individual experiments.

Since the optimal mode of methanol addition and the ratio of oil to methanol were 6 steps and 1 to 3 respectively, the effect of immobilized lipase concentration on conversion was performed with enzyme concentrations of 10%,

20% and 30% w/w of oil at 40 °C. Figure 4-13 illustrated the conversion of biodiesel when various amounts of immobilized lipase were used. From this Figure, it can be observed that the maximal yield of FAME at 72.3% was achieved from 30% immobilized lipase. When 10 and 20 % immobilized enzyme were used, the % conversion of fatty acid methyl ester increased from 50 - 60%. Therefore, the optimal conditions were as follows: 6 step of addition mode of methanol, 1 to 3 oil to methanol ratio and 30% amount of enzyme were subsequently selected for the next optimal condition namely; water content.

4.5.4 Effect of water content on transesterification

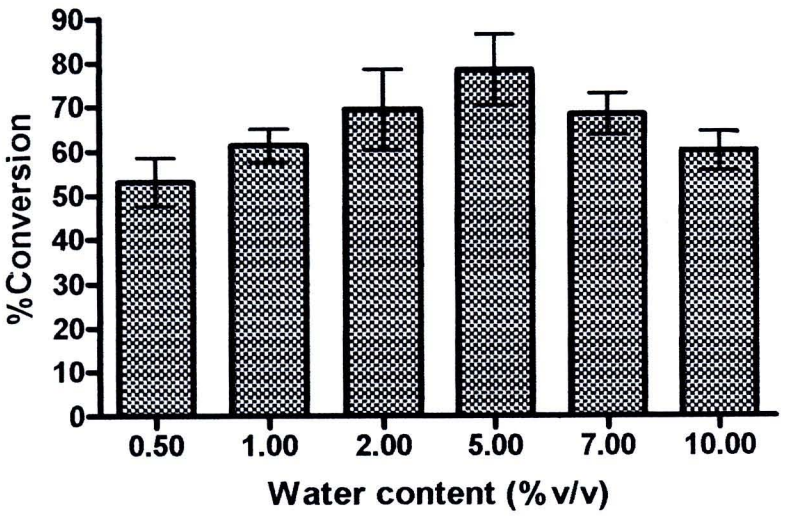


Figure 4-14. The effect of water content on percentage of conversion to biodiesel. The reactions were carried out in a mixture of 0.5 g of palm oil, 30% (w/w of oil) immobilized lipase, 1:3 oil:methanol ratio, 6 steps addition mode of methanol and continuously stirred at 40°C for 24 hr. Percent conversion shown on the y-axis are the means ± SD of three individual experiments.

The reaction was carried out by adding water ranging from 0.5% to 10% (%v/w) of the oil with 30% (w/w of oil) immobilized *Candida rugosa* lipase. The

reactions were carried out in mixtures of 0.5 g palm oil, 0.15 g of immobilized lipase, 71 µl of methanol (added at six steps, each 11.9 µl) and magnetically stirred in a water bath for 24 hr at 40°C. The results were shown in Figure 4-14.

As indicated in Figure 4-14, the fatty acid methyl ester content gradually rose from 52- 78% as water content increased from 0.5% to 5.0% (v/w) of oil, and then declined from 78% to 60% as water content rose from 5% to 10%. It can be seen that the FAME content reached its maximum at water content of 5% (v/w of oil) which was about 78.50% conversion higher than that in absence of water. From previous optimal conditions, the % conversion was increased from 50% to 80%, approximately 30 % higher.

4.5.5 Effect of time and temperature on transesterification

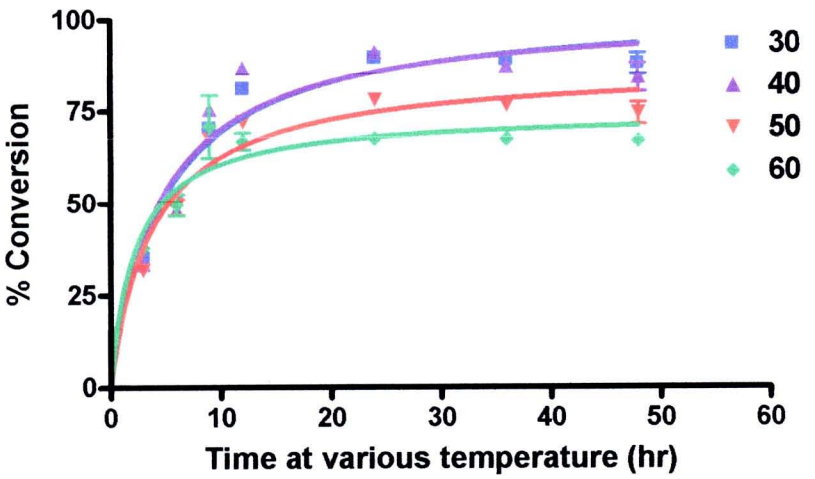


Figure 4-15. The effect of time and temperature on percentage of conversion to biodiesel. The reactions were carried out in a mixture of 2 g of palm oil, 30% (w/w of oil) immobilized lipase, 1:3 oil:methanol ratio, 6 steps addition mode of methanol, 5% (v/w of oil) water and magnetically stirred in a water bath at each

temperature for 48 hr. Percent conversion shown on the y-axis are the means ± SD of three individual experiments.

To study the effect of reaction temperature on fatty acid methyl esters formation, the transesterification reaction was carried out under the optimal conditions obtained from all 4 previous sections. The experiments were conducted at temperature ranging from 30 to 60 °C shown in Figure 4-15.

Experimental results showed that the transesterification reaction could proceed within the temperature range studied but the reaction time to complete the reaction varied significantly with reaction temperature. It can be seen that highest yield of 91% of biodiesel was obtained at 40 °C for the period of 24 hours whereas 87% conversion at 12 hours. The results in Figure 4-15 showed that the percentage yield of biodiesel suddenly rose when the reaction time was increased from 0 to 12 hours. After 12 hours, it was seen that increase in the reaction time did not have the effect on the production of biodiesel. When the reaction temperature increased to 50 °C and 60°C, it was found that the product yield started to decrease since the enzyme lost its activity dramatically. From previous optimal conditions, the % conversion was increased from 50 to 90%.

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

The immobilized *Candida rugosa* lipase was used to catalyze the transesterification at the optimal conditions using six non-edible and waste oils compared with 2 commercial lipases. Transesterifications were carried out as described in section 3.4.9. 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 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® RM

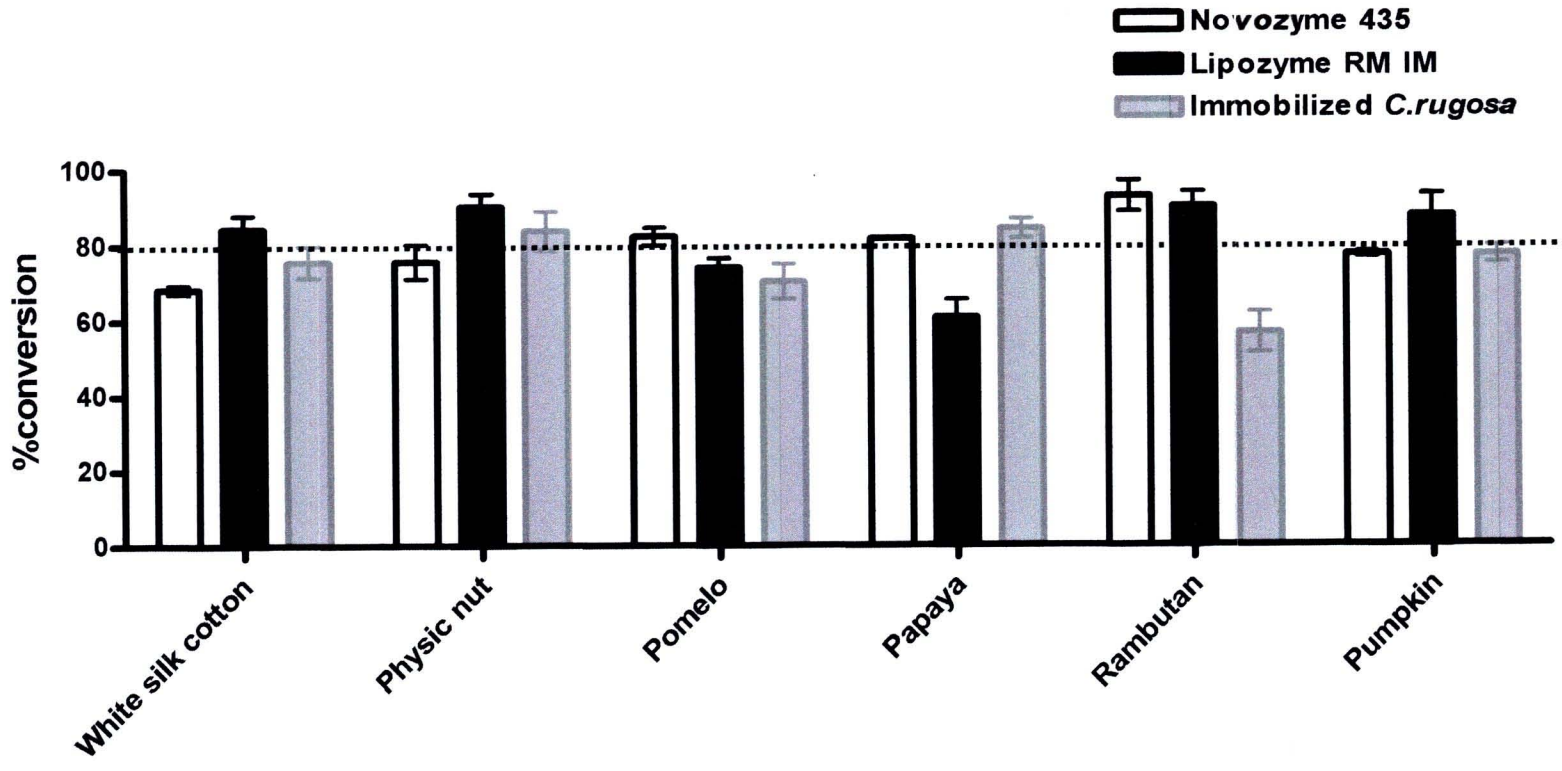


Figure 4-16. The percentage conversion of biodiesel from non-edible and waste oils catalyzed by 30% (w/w of oil) immobilized *Candida rugosa* lipase; grey column and later mixed with 1:3 mole ratio of methanol. The addition of methanol was performed using 6 steps at 0, 2, 4, 6, 6 and 10 hours by stirring the mixtures with magnetic stirrer for 12 hours at 40°C. The percentage conversion of biodiesel form non-edible and waste oils catalyzed by 20% (w/w of oil) Novozyme® 435; white column and Lipozyme® RM IM; black column and later mixed with 1:3 mole ratio of methanol. The addition of methanol was performed using 3 steps at 0, 8 and 16 hours by stirring the mixtures with magnetic stirrer for 24 hours at 55°C. Samples were taken from the reaction mixture and later analyzed for the products by high-performance liquid chromatography. The experiment was performed in triplicates, and the data are mean ± S.D.

4.7 Stability of immobilized *Candida rugosa* lipase

4.7.1 Thermal stability

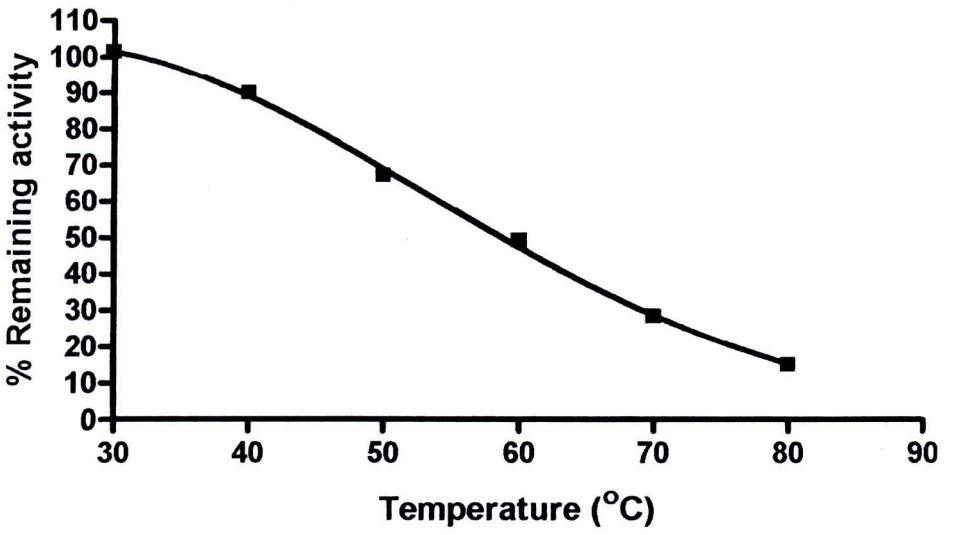


Figure 4-17. Effect of temperature on thermostability of immobilized *Candida rugosa* lipase. 2 mg of immobilized lipase were incubated at various temperatures from 30 to 80 °C in the temperature controlled heating block for 15 min. The results were average values of triplicate experiments.

500 M phosphate buffer, pH 6, 8 mg/ml of lipase solution, 30 min time of immobilization at 30 °C with t-butanol as adjuvant were selected as the optimal conditions for immobilization of *Candida rugosa* lipase to study the thermostability on residual activities of immobilized lipase using the method described in 3.4.10.1. The optimal temperature for thermostability study was screened by incubating 2 mg of immobilized lipase at various temperatures from 30 to 80 °C in the temperature controlled heating block for 15 min. Then, the residual activities were determined as percentage yield of activity at different temperatures compared to the activity at the optimal conditions. The results were shown in Figure 4-17.

Since the optimal temperature for the immobilization was found to be 30 °C, therefore, the selected temperature for this experiment was initially started at 30°C. From Figure 4-17, the results showed that the percentage of remaining activity significantly decreased from 100% to 15% when the temperature was increased from 30°C to 80°C and at 60 °C, 50% of the activity was retained. Hence, the optimal temperature to study the thermal stability of immobilized *Candida rugosa* lipase was selected at this temperature. 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, Novozyme 435 and Lipozyme RM IM at 60°C was 14.35, 316.67 and 59.45 min respectively.

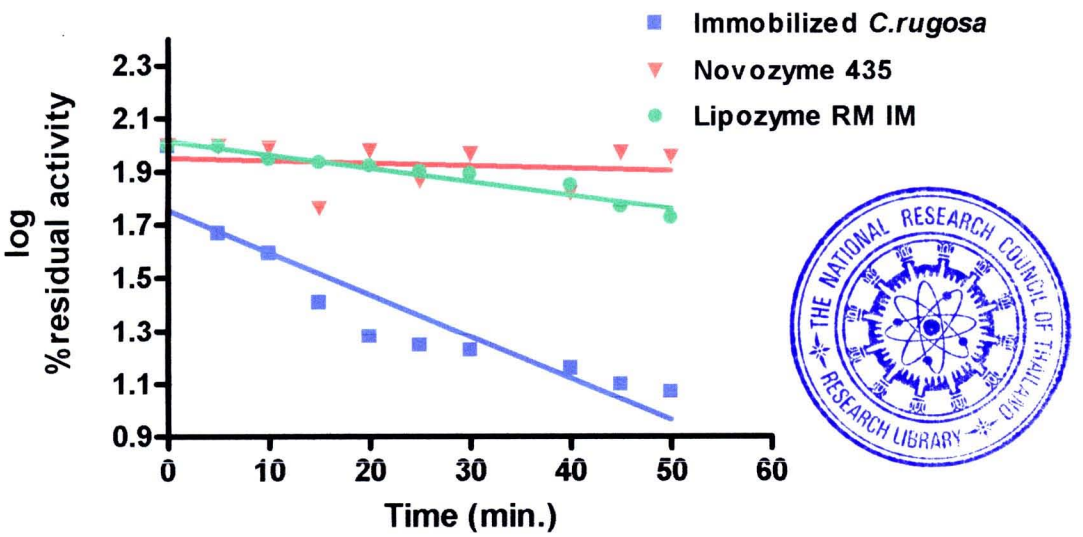


Figure 4-18. Half-life time ($t_{1/2}$) of immobilized *Candida rugosa* lipase, Novozyme 435 and Lipozyme RM IM. 2 mg of immobilized enzyme was incubated at 60 °C. The results were average values of triplicate experiments.

4.7.2 Repeated use of the immobilized *Candida rugosa* lipase

4.7.2.1 Repeated use on transesterification

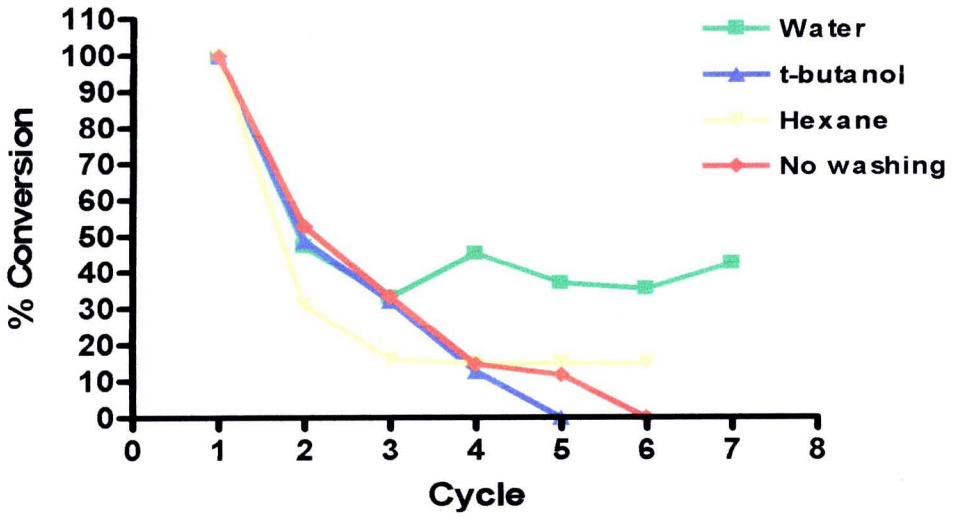


Figure 4-19. Operational stability of immobilized *Candida rugosa* lipase catalysis for transesterification. The reactions were carried out in a mixture of 2 g of palm oil, 1:3 oil:methanol ratio, six steps addition mode of methanol, 20% (w/w of oil) immobilized lipase, 5% (v/w of oil) water and continuously stirred at 40°C for 12 hr. The lipase was transferred into the same system for a new cycle after completion of the former reaction in 12 hours. Percent conversion shown on the y-axis are the means ± SD of three individual experiments.

The immobilized *Candida rugosa* lipases were used consecutively in a series for biodiesel production. The optimal conditions applied in this reusability study were 2 g of palm oil, 30% (w/w of oil) of immobilized lipase, 1:3 oil:methanol ratio and water content of 5% (v/w of oil) and magnetically stirred in a water bath at 12 hr. Under these conditions, approximately 90% of FAME content were obtained. The immobilized lipase was rinsed with water, t-butanol and hexane after each batch reaction to select the optimal washing solution and to remove glycerol and

oil in carriers. The dried immobilized lipase was dried in the desiccators and later used in the next batch reaction composed of new substrates. The results of each batch for the production of FAME content were graphically shown in Figure 4-19. The % conversion in every condition decreased in the second and third cycles of use with the first cycle set at 100%. After 3 uses, it can be seen that immobilized lipases washed with water could retain approximately 45% of its initial activity after 4 more cycles. The immobilized lipases washed with hexane could only retain about 15% of its initial activity after 3 more cycles. On the other hand, when the enzymes were washed with t-butanol, 15% of its initial activity was retained while the enzyme with no washing lost all of its activity after 5 and 6 cycles.

4.7.2.2 Repeated use on hydrolysis

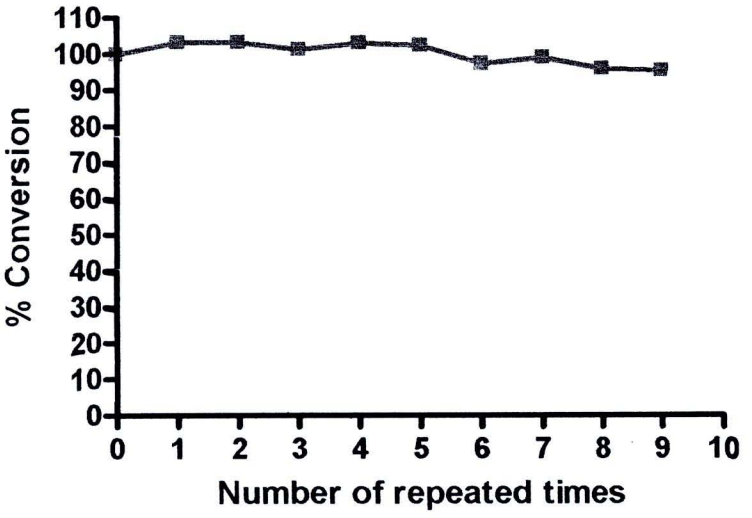


Figure 4-20. Operational stability of immobilized *Candida rugosa* lipase catalysis for hydrolysis. The reactions were carried out in a mixture of 3 g of palm oil, 20% (w/w of oil) immobilized lipase, 100% (v/w of oil) water and continuously stirred at 40°C for 9 hours. The lipase was transferred

into the same system for a new cycle after completion of the former reaction in 9 hours. Percent conversion shown on the y-axis is the mean ± SD of three individual experiments.

The immobilized *Candida rugosa* lipases were used consecutively in a series for hydrolysis. The optimal conditions applied in this reusability study were 3 g of palm oil, immobilized lipase content of 20% (w/w of oil) and water content of 100% (v/w of oil) and magnetically stirred in a water bath at 40°C for 9 hours. Under these conditions, approximately 97% of FFA content were obtained. The immobilized lipase was rinsed with water after each batch reaction and to remove glycerol and oil in carriers. The dried immobilized lipase was dried in the desiccators and later used in the next batch reaction composed of new substrates. The results of each batch for the production of FFA content were graphically shown in Figure 4-20. From the results, it was shown that no evident decrease of the lipase activity was observed during the first cycle. Additionally, immobilized lipase could be used at least 10 repeated times without important loss of activity.