CHAPTER IV

RESULTS & DISCUSSION

In many enzymatic ester production, the use of free enzyme as a biocatalyst usually receives the higher ester yield than the use of immobilized enzyme. However, considering no reusability of the high cost lipase, the use of free enzyme is not cost effective for ethyl ester production. Thus, the immobilization techniques such as adsorption and entrapment are widely applied to develop immobilized enzyme with high enzymatic activities for ethyl ester production. Adsorption or entrapment technique is simple to apply with low material and energy costs, however, for long-term use of immobilized enzymes, the problems such as enzyme detachment, enzyme deactivation and diffusion limitations of substrate often occurred during the operation. In order to develop the immobilized enzyme for ethyl ester production from palm oil and palm fatty acid, *C. rugosa* lipase (CRL) immobilized by $CaCO_3$ doped alginate gel (CRLAE) was formed as a new carrier by the integration of adsorption and entrapment techniques. The immobilization procedure could be divided into two steps. Firstly, *C. rugosa* lipase was physically adsorbed onto the surface of $CaCO_3$. The second was the entrapment of $CaCO_3$-lipases in calcium alginate matrix.

This work attempts to develop CRLAE carrier for using in ethyl ester production. The experiments were divided into 3 parts. Firstly, the effects of enzyme quantity, adsorption time, enzyme/support ratio, bead diameter, sodium alginate concentration and temperature on hydrolysis activity were investigated. The results from the system with CRLAE were compared with that of the free enzyme, $CaCO_3$-lipase (CRLA) and lipase entrapped in calcium alginate (CRLE). Secondly, the effects of substrate molar ratio and shaking speed for the enzymatic ethyl ester production from purified palm oil and palm fatty acid were examined by using free *C. rugosa* lipase (CRL). After that the optimum condition from the study was applied for ethyl ester production using the substrate mixture of purified palm oil and palm fatty acid by the immobilized *C. rugosa* lipase on $CaCO_3$ (CRLA), immobilized *C. rugosa* lipase in Ca-alginate matrix (CRLE) and $CaCO_3$-lipases entrapped in alginate

(CRLAE). Lastly, the reusability of immobilized enzyme on ethyl ester yield was investigated.

4.1 Controlled parameters for lipase hydrolytic activity

4.1.1 Effect of lipase quantity

The effect of lipase quantity on the alcoholysis of purified palm oil was investigated by varying free lipase quantities at 1%, 3%, 5% and 10 % based on palm oil weight with a reaction temperature of 50°C. The results are presented in Figure 4.1 and 4.2. The ethyl ester content was increased by increasing lipase quantity up to 5%. At 5% of lipase, the hydrolytic activity and ethyl ester yield was 120 U and 83.4 %, respectively. According to Kose et al. (2001), the highest ethyl ester formation (82.6%) was obtained by using 30% *C. antarctica* lipase based on cotton seed oil weight with an operation temperature at 50°C. Awang et al. (2007) reported the highest conversion of 79.5% when 10% (w/w) of *C. rugosa* lipase concentration was used for esterification of oleic acid and oleyl alcohol in hexane. From the result in this study, *C. rugosa* lipase at 5% (by wt. of oil) was applied for the further study.

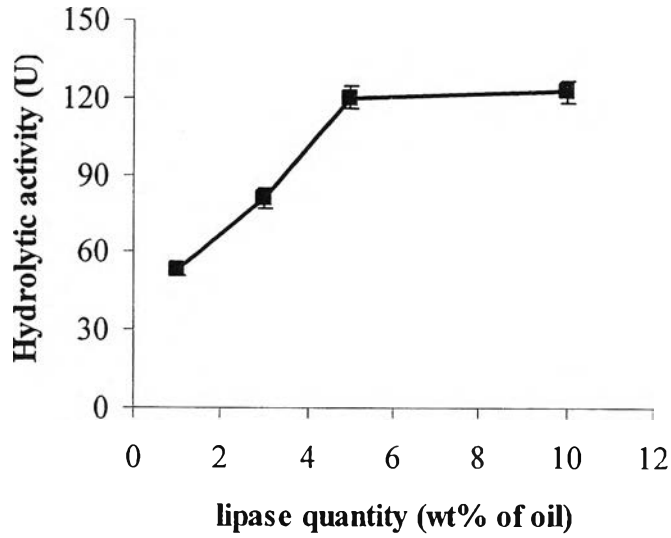


Figure 4.1 Effect of *C. rugosa* lipase quantity on hydrolytic activity, using the olive oil as the substrate, pH 7.0, 50°C, reaction time 12 h.

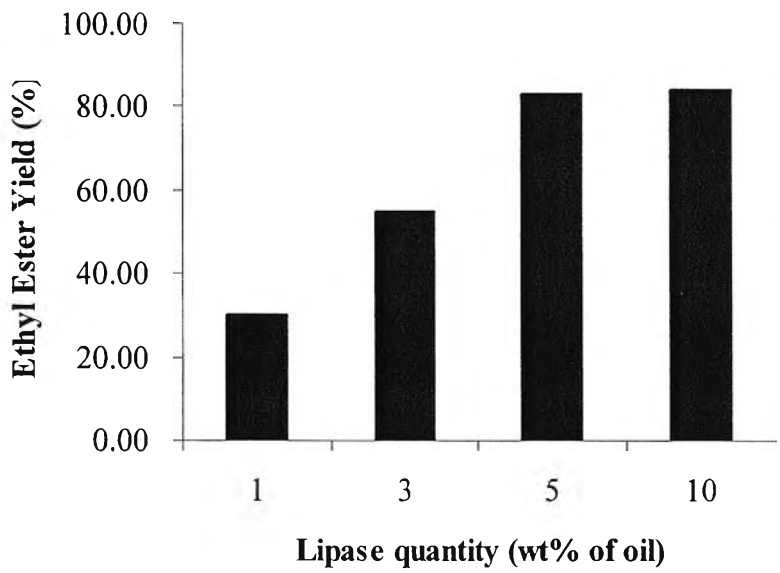


Figure 4.2 Effect of *C. rugosa* lipase quantity on ethyl ester yield, molar ratio of purified palm oil to ethanol 1:9, reaction temperature 50°C and reaction time of 24 h, 250 rpm.

4.1.2 Effect of adsorption time

To determine the optimum adsorption time, the effect of adsorption time on the amount of immobilized lipase was studied. The adsorption of *C. rugosa* lipase on calcium carbonate was prepared in the refrigerator at 4°C with mild stirring and varied the adsorption time from 30 minutes to 180 minutes. The activity yield of immobilized lipase increased as adsorption time increased up to 60 min and then was maintained constantly with further increases in the adsorption time from 60 min - 180 min as shown in Figure 4.3. The maximum of immobilized lipase activity yield was 88%. The result could indicate that the operating time for the dynamic balance between adsorption and desorption of immobilized lipase was about 60 min. This finding result was similar to the observation by Zhen-Gang et al. (2006) who reported that it would take about 90 minutes to make the *C. rugosa* lipase fully contact with nanofibrous membranes. Chang et al. (2007) also reported in the same behaviors that the activity of lipase AY on celite changed with the stirring time and reached the highest after 30-90 min. Their immobilization method showed 72.2% of protein loading yield of original lipase introduced (0.5 g). Therefore, to ensure the good adsorption of lipase onto calcium carbonate, the adsorption time at 90 minutes was applied for the following experimental studies.

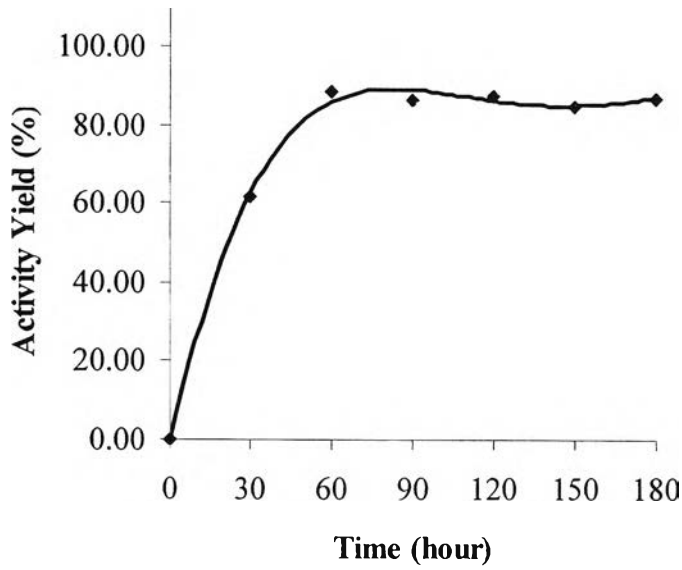


Figure 4.3 Effect of adsorption time of *C. rugosa* lipase on calcium carbonate ($CaCO_3$) on activity yield, using the olive oil as the substrate, pH 7.0, reaction time of 12 h, 50°C.

4.1.3 Effect of enzyme/support ratio

Adsorption is the most commonly used for enzyme immobilization because of its easy handing and its less expensive cost. The forces between a support and the enzymes include hydrogen bonding, Van der Waals forces and hydrophobic interactions (D'Souza, 1999; Abdul Rahman et al., 2005). In order to find the optimal amount of calcium carbonate ($CaCO_3$) for the adsorption, the immobilization activity of 0.25 g lipase on the various amounts of calcium carbonate was examined. As shown in Figure 4.4, the specific activity significantly increased when enzyme/support ratio was increased up to 1:2 (w/w), however, above that it significantly decreased. The highest specific activity at 15 U/mg proteins was achieved when enzyme/support ratio of 1:2 (or 0.5) was applied. At low concentration of $CaCO_3$, the amount of immobilized enzyme was related to the binding sites. Therefore, the enzyme specific activity increased when the enzyme/support ratio increased. However, at high $CaCO_3$ concentration, the free enzyme quantity became

limited substance. Thus, after that, the increasing of enzyme/support ratio might not lead to more lipase adsorption. Moreover, the addition of too high level of $CaCO_3$ could cause the negative effect on the enzyme activity from the increase of negative charge of the system. As being reported by Chang et al. in 2007 on the effect of *C. rugosa* lipase/support (celite), the maximum specific activity (14.9 U/mg-protein) of immobilized enzyme was achieved when the enzyme/support mass ratio was at 0.5 (w/w). Therefore, the enzyme/support ratio of 1:2 (w/w) was employed in the further studies.

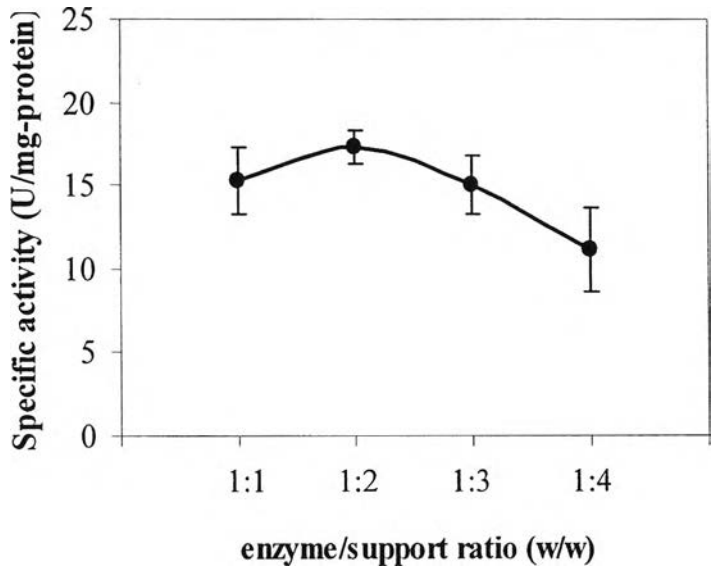


Figure 4.4 Effect of enzyme/support ratio on specific activity, using the olive oil as the substrate, pH 7.0, 50°C, reaction time of 12 h.

4.1.4 Effect of bead diameter

In immobilized enzyme system, diffusion resistance could be eliminated by using small particle, a high degree of turbulence around the particles and high substrate concentration. For large molecule of substrate such as vegetable oil or fat, for maximum conversion rate, the particle size should be as small as possible within

the constraints of particle integrity, mechanical strength and the nature of the particle recovery systems (Idris et al., 1994).

In order to determine the effect of bead diameter on the hydrolysis activity of immobilized lipase (CRLE and CRLAE), bead with diameter of 1.7, 2 and 4 mm were prepared with 1% of calcium alginate. The bead size was controlled by using a syringe with different needle diameters. The hydrolytic activity is determined using olive oil as a substrate (Yadav et al., 2005) under the temperature at 37°C and pH 7.0. As shown in Figure 4.5, the hydrolytic activity of 1.7 mm bead diameter system during 6-12 hours of incubation was higher than those of 2 mm and 4 mm bead diameter systems in the same period of time. Since the hydrolytic activity of immobilized lipase decreased with increasing of bead diameter, it is indicated that the diffusional interference in hydrolytic reaction over the calcium alginate bead intruded to slow the rate of reaction. Moreover, it was found that the hydrolytic activity of immobilized enzyme depended on the immobilization techniques; for this study, the order of hydrolytic activity was CRLE carrier > CRLAE carrier without filtration step > CRLAE carrier with filtration step.

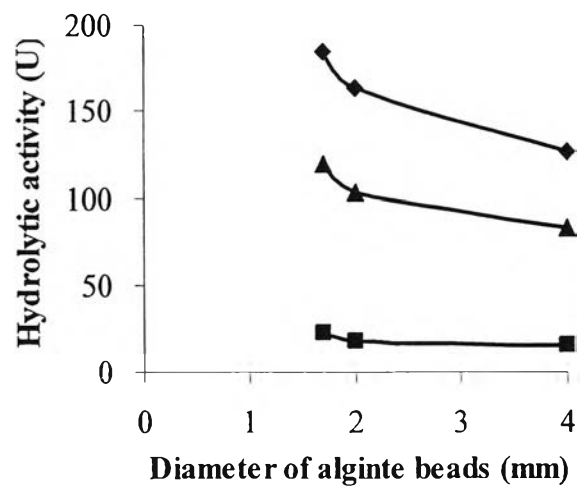


Figure 4.5 Effect of bead diameter on the hydrolytic activity of immobilized *C. rugosa* lipase by varied bead diameter at 1.7 mm, 2 mm and 4 mm. Using olive oil as the substrate, pH 7.0, 37°C, 12 h, and 1% Na-alginate concentration; (◆), CRLE

carrier;(▲), CRLAE carrier without filtration step;(■), CRLAE carrier with filtration step

The results revealed that the hydrolytic activity of immobilized enzyme of CRLAE prepared with filtration step was significantly lower than that of CRLE and CRLAE prepared without filtration step about 10 and 6 folds, respectively. It might be described that the lack of buffer solution in the bead could be the cause of the lower enzymatic activity. Therefore, in the further study, the CRLAE carriers were prepared without filtration step.

4.1.5 Effect of alginate concentration

Entrapment methods were based on the inclusion of enzyme within a rigid network to prevent the enzyme from diffusing into the surrounding substrate, while still allowing mass transfer of substrate. As the importance of diffusion limitation, the effect of alginate concentration on the hydrolytic activity of immobilized lipase was investigated in this study. The *C. rugosa* lipase was immobilized in 1.7 mm bead diameter of CRLE and CRLAE carrier that prepared from different concentration of alginate (1%, 1.5%, 2% w/v). The testing of hydrolysis activity of immobilized lipase was by using olive oil as the substrate (Yadav et al., 2005) under the temperature at 37°C and pH 7.0. The results of the hydrolytic activity are shown in Figure 4.6. The highest hydrolytic activity (184 U) was obtained when using the CRLE carrier with 1% alginate concentration. Hydrolytic activity decreased with increasing of alginate concentration. According to the previous works (Knezevic et al., 2002 and Fadnavis et. al., 2003), it was also found that the hydrolytic activity of immobilized enzyme decreases with increasing alginate concentration due to mass transfer resistance. This was due to conformational changes in the entrapped enzyme and/or limitation of substrate transfer from the bulk phase into the alginate beads as alginate concentration was increased (Knezevic et al., 2002).

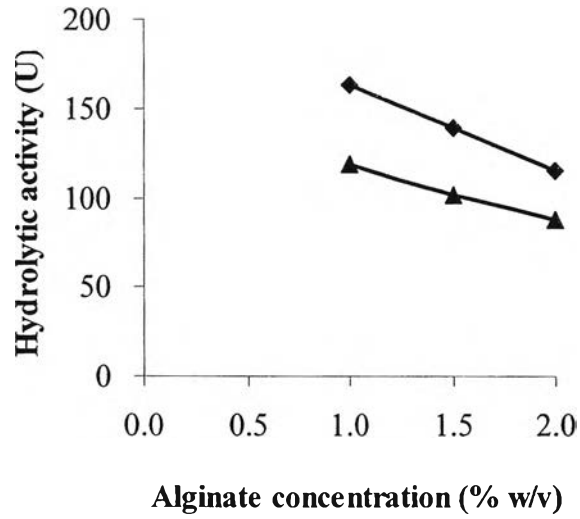


Figure 4.6 Effect of alginate concentration on the hydrolytic activity of immobilized *C. rugosa* lipase by varied Na-alginate concentration at 1%, 1.5% and 2%, using olive oil as the substrate, pH 7.0, 37˚C, 12 h of reaction time and 1.7 mm of bead diameter; (◆), CRLE carrier;(▲), CRLAE carrier without filtration step

4.1.6 Effect of temperature

The thermal stability of both free and immobilized lipase was determined by measuring the hydrolytic enzymatic activity using olive oil as substrate (Yadav et al., 2005). In this study, the different controlled temperatures of enzymatic activity assay were varied in ranging from 37°C to 60°C for 12 h of reaction time. The maximum activity of both free and immobilized enzymes appeared at 50°C as illustrated in Figure 4.7. With the increase of the operating temperature up to 50°C, the activity of the free lipase was increased. However, above 50°C, the enzyme activity of the free enzyme was significantly decreased with the increasing temperature, while the enzyme activity of the immobilized enzymes slightly decreased. Therefore, the immobilized *C. rugosa* lipase in Ca-alginate matrix (CRLE) and $CaCO_3$-lipase entrapped in Ca-alginate (CRLAE) carriers could provide good heat resistance.

In comparison with the previous work, Chen et al., (2006) reported that a reaction temperature above 40°C could result in loss of lipase activity for the

methanolysis of waste cooking oil using *R. oryzae* lipase. Jeong & Park (2008) reported in the similar way that the optimal reaction temperature for Novozym 435 was 40 °C, which allowed for sustained, high lipase activity and low reactant viscosity. Generally, the enzymatic transesterification is performed at a low temperature than the chemical reaction to prevent loss of lipase activity (Watanabe et al., 2001). A lower reaction temperature is desirable in order to reduce the energy cost inherent in biodiesel production (Jeong et al., 2004). However, increasing of temperature could enhance the reaction rate initially, which was mainly due to an increase in rate constants with temperature, and partly due to less mass transfer limitations (Al-Zuhair, 2007) in the solvent-free system. Moreover, since the transesterification is endothermic reaction, the equilibrium conversion therefore, increases with increasing of temperature.

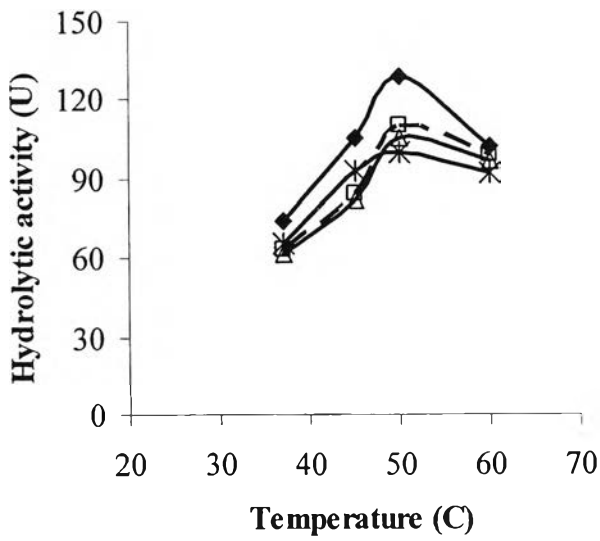


Figure 4.7 Effect of temperature on the hydrolytic activity of immobilized *C. rugosa* lipase at various temperatures between 37°C - 60°C, pH 7.0 and 12 h of reaction time by using olive oil as the substrate; ◆, free lipase; *, CRLA; Δ, CRLE carrier; □, CRLAE carrier (without filtration step).

4.2 The study of operating condition of enzymatic ethyl ester production

4.2.1 Effect of shaking speed

The limitation of diffusion of substrate could cause the major problem when the immobilized enzyme was used in the reaction. In the previous study, the factors that might affect the limitation diffusion of immobilized lipase such as temperature, bead diameter and alginate concentration were investigated. The optimum condition was found to be at 50°C, 1.7 mm and 1% w/v, respectively. In order to study the influence of external mass transfer phenomena on the ethyl ester production rate, the effect of shaking speed was varied at 150 rpm, 200 rpm, 250 rpm and 300 rpm. Figure 4.8 presents the effect of shaking speed on soluble lipase mediated tranesterification of purified palm oil at 50°C, ethanol:oil molar ratio at 9:1 with using 5% free lipase (by wt of oil). It was found that the reaction rate was accelerated with the increasing of shaking speed up to about 250 rpm. However, when using shaking speed at 250 rpm, the final ethyl ester yield of 67%-70% was obtained, which was comparable to that of shaking speed at 300 rpm.

Chulalaksananukul et al., (1990) studied the effect of mass transfer limitation on esterification using 30 mM oleic acid and 30 mM ethanol and suggested that the rotating speed might be varied from 100 rpm to 500 rpm. The plateau obtained for curves of initial velocity against rotating speed showed that external mass transfer limitations were not significant when the rotating speed was greater than 200 rpm. It could be explained that the reaction system was thoroughly emulsified by strongly shaking and the interfacial area was increased evidently. Consequently, the reaction was facilitated because the collision probability between lipase and substrate was improved greatly (Chen et al., 2008). Thus, from this study, the shaking speed at 250 rpm was used for the further study of optimum condition for ethyl ester production from purified palm oil and palm fatty acid.

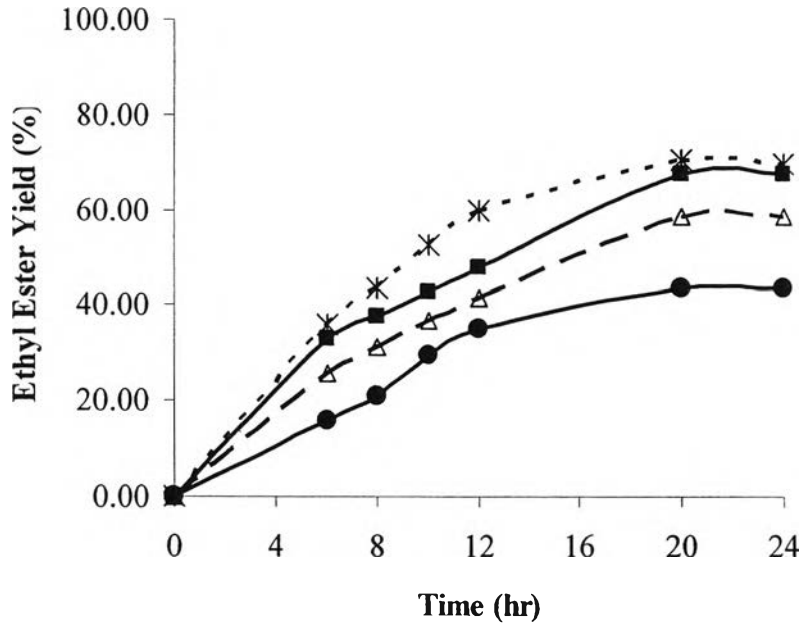


Figure 4.8 Effect of shaking speed on ethyl ester yield by using the free lipase (5% based on oil weight), molar ratio of purified palm oil to ethanol was 1:9, reaction temperature 50°C, 24 h; ●, 150; Δ, 200; ■, 250; *, 300 rpm.

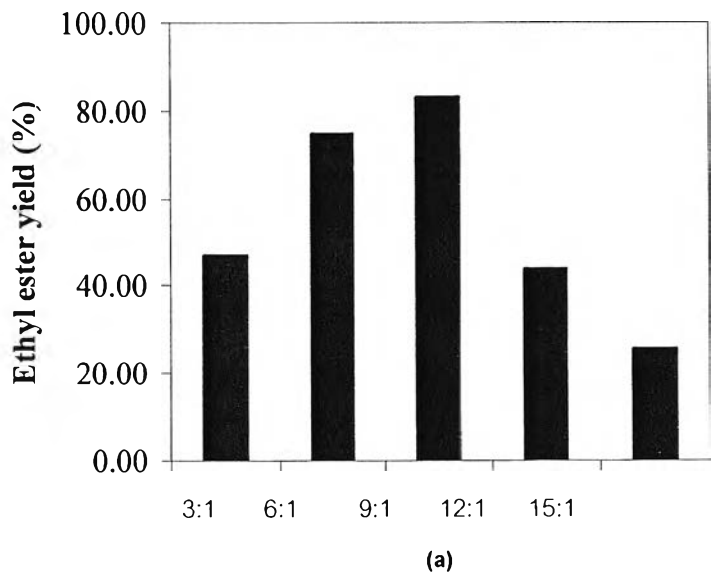
4.2.2 Effect of molar ratio of ethanol to reactant

One of the most important variables affecting the yield of ester is the molar ratio of alcohol to vegetable oil. The stoichiometric ratio for trans-esterification requires three moles of alcohol and one mole of glyceride to yield three moles of fatty acid ester and one mole of glycerol (Ma et al., 1999). For esterification, it requires one mole of both alcohol and fatty acid to yield one mole of fatty acid ester and water. The ethyl ester yield could be increased by using excess amount of alcohol to shift the equilibrium to the right-hand side.

In this work, the effect of molar ratio of ethanol to reactants on ethyl ester yield were determined in the range of molar ratio between 3:1-15:1 for purified palm oil and 1:1-9:1 for palm fatty acid. The lipase quantity was 5% (wt oil) and reaction temperature was 50°C. The ethyl ester yield showing in Figure 4.9 were obtained after the reactions were carried out for 24 hour of both transesterification and esterification.

Considering of the ethyl ester yield from the transesterification of purified palm oil, The ethyl ester yield was linearly increased when the ratio of ethanol to reactants were increased from 3:1 to 6:1 and 9:1 for purified palm oil. The ethyl ester yield increased from about 47 % to 83 % as the molar ratio of ethanol to palm oil increased from 3:1 to 9:1. However, the rise of ethanol to palm oil ratio to 12:1 and 15:1 significantly decreased ethyl ester yield. In similar with the result from transesterification, the ethyl ester yield from the esterification of palm fatty acid increased from 9 % to 16 % as the molar ratio of ethanol to reactant increased from 1:1 to 3:1 and then decreased when the ratio was above 3:1. Figure 4.9 (b) shows that the use of great excess of ethanol (molar ratio at 6:1-9:1) resulted in a significant reduction of ethyl ester yield. In the enzymatic catalysis in aqueous medium, the nature of the organic solvent influences the activity and the stability of the enzymes significantly. The most harmful solvents to the enzymes are those highly polar and more hydrophilic, because they are capable of solubilizing large amounts of water and to remove the layer of essential water from the enzymes, causing loss of the catalytic activity (A. P. et al., 2006).

Therefore, the optimum molar ratios of ethanol to reactants for catalytic transesterification of purified palm oil and catalytic esterification of palm fatty acid to biodiesel at 9:1 and 3:1, respectively, were used in the further studies.



(a)

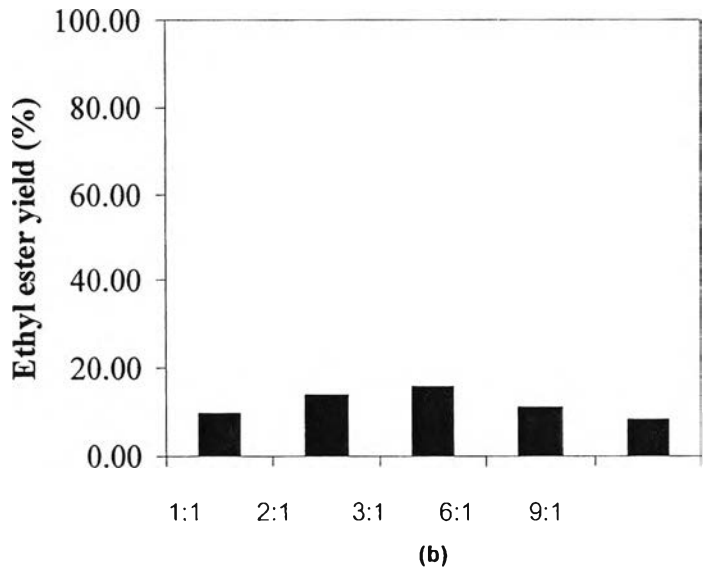


Figure 4.9 Effect of molar ratio of ethanol to reactants on ethyl ester yield by using free lipase at 5% by wt of oil, reaction temperature of 50°C and reaction time of 24 h; transesterification (a) and esterification (b)

4.2.3 Effect of mass ratio of substrate mixture

For long term, using of palm oil as the substrate for biodiesel production might not be appropriate as it is an edible oil widely used in many countries. On the other hand, a lot of palm fatty acid was obtained from the palm oil refinery. Not only its price is much cheaper than origin palm oil but it is also produced as the by product from palm oil refineries. Thus, the palm fatty acid has become more attractive to be applied as a substrate in the biodiesel production.

From the previous study, it was found that the ethyl ester yield from using purified palm oil as the substrate was 4 times higher than that from using palm fatty acid. It was found that, the highest ethyl ester yield was only 20% when using palm fatty acid as the substrate with the optimum molar ratio of ethanol to reactant of 3:1. This result indicated that the acidity of palm fatty acid could cause the violent condition that might inhibit the activity of lipase. Hence, the mixture of purified palm oil and palm fatty acid was suggested for the improved ethyl ester yield and to avoid

serious deactivation of lipase, as being observed when only palm fatty acid was used as a substrate. In this part, the optimum condition of mixed substrate mass ratio between purified palm oil and palm fatty acid was investigated by using the free *C. rugosa* lipase. The percentage of mass ratio of purified palm oil and palm fatty acid was varied at 90:10, 80:20, 70:30 and 50:50. The reaction was performed in the 50 ml Erlenmeyer flask, reaction temperature of 50°C, 5% lipase (wt of oil), and the molar ratio of ethanol to purified palm oil was 9:1,while the molar ratio of ethanol to palm fatty acid was 3:1. The result (Figure 4.10) shows that the highest ethyl ester yield (20%) was obtained when the percentage of mass ratio of purified palm oil and palm fatty acid was 70:30. Since the use of the mixture of purified palm oil and palm fatty acid as the substrate resulted in very low ethyl ester yield in comparison to that from the use of purified palm oil, it might be described that the addition of palm fatty acid at the initiation period of the reaction could cause the decrease in enzymatic activity from the high acidity in reaction medium.

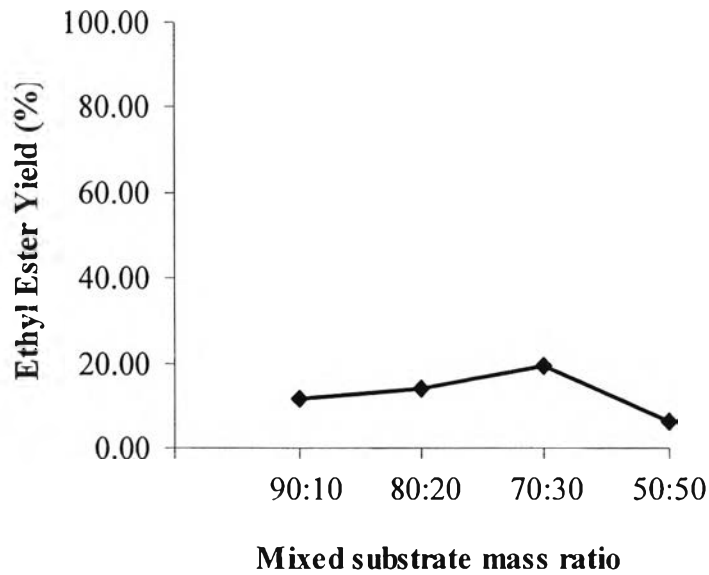


Figure 4.10 Effect of mass ratio of substrate mixture on ethyl ester yield by using free lipase, the mass ratio of mixed substrate was varied at 90:10, 80:20, 70:30 and 50:50; the molar ratio of purified palm oil to ethanol was 1:9 and the molar ratio of palm fatty acid to ethanol was 1:3, with 5% lipase (wt of oil), reaction temperature of 50°C and 24 h of reaction time.

4.2.4 Effect of addition time of palm fatty acid

As a consequence of the preliminary of mixed substrate mass ratio, there is considerable interest in exploring the optimum condition to the use of the mixture of purified palm oil and palm fatty acid as the substrate for biodiesel production. Although, the optimum mass ratio of purified palm oil and palm fatty acid was investigated with 70:30 and achieved the low ethyl ester yield (20%) but it was found that it could be possible to use the mixture of purified palm oil and palm fatty acid as the substrate for ethyl ester production by the adjusted addition time of palm fatty acid. The addition of palm fatty acid into the reaction medium at different times (6, 12 and 24 h) from the initiation point of the reaction was studied. The ethyl ester reaction was performed in batch system with the reaction temperature of 50°C, the total reaction time of 48 h, the amount of ethanol was calculated based on the purified palm oil only (molar ratio of ethanol to purified palm oil was 9:1).

In Figure 4.11, the ethyl ester yield, by using purified palm oil as the substrate, reached 34.9 % at 6 h. Then 30% of palm fatty acid (based on weight of total substrate) was added at 6 h of reaction time and the reaction was continued until 48 h. After 48 h of reaction time, the ethyl ester yield was reached 52.0 %. With the addition of palm fatty acid at 12 h, 58.1 % of ethyl ester yield was achieved. Maximum ethyl ester yield of 84.1% was obtained when 30% of palm fatty acid was added at the reaction time of 24 h. The experiment data shows that the addition time of palm fatty acid strongly affected the ethyl ester yield. It might be interpreted that the addition of palm fatty acid to the reaction after the end of the transesterification period could prolong the reaction. The amount of ethyl ester in the medium could create well mixing of palm fatty acid and the reaction medium. In addition, the ethyl ester in the medium could contribute to the diluted concentration of palm fatty acid in the reaction medium resulting in the lower acidity condition. The inhibition effect of free fatty acid on the soluble lipase activity was less at the low concentration (Chen et al., 2008). This finding leads to the success in the use of mixed substrates of purified palm oil and palm fatty acid as the substrate mixture for biodiesel production.

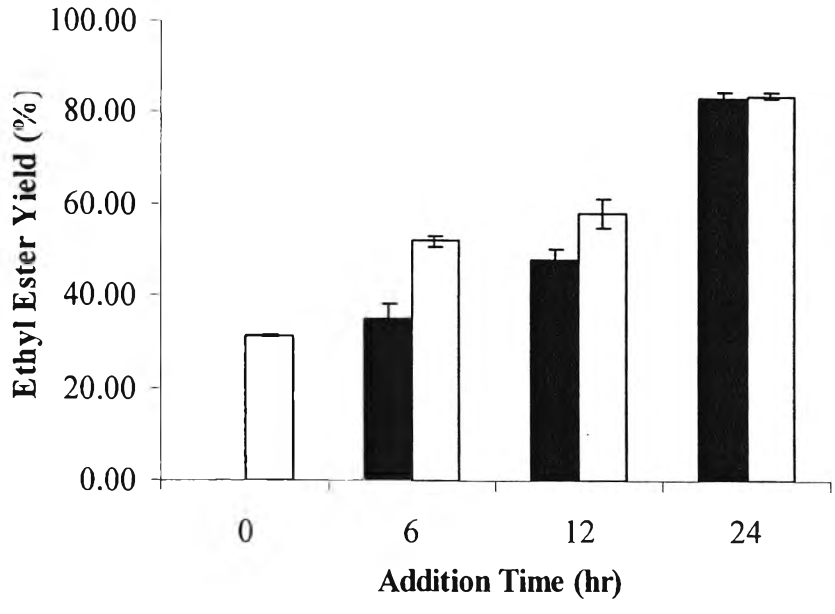


Figure 4.11 Effect of addition time of palm fatty acid on ethyl ester yield by using free lipase, addition time was varied at 6 h, 12 h and 24 h, molar ratio of purified palm oil to ethanol was 1:9 with 5% lipase (based on oil weight), reaction temperature of 50°C and total reaction time of 48 h; ■, before added palm fatty acid; □, at total reaction time (48 h).

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

In this study, the immobilized *C. rugosa* lipase in form of $CaCO_3$-lipase immobilized in calcium alginate beads (CRLAE) was applied for the ethyl ester production. The results was compared to that using free enzyme, immobilized lipase on $CaCO_3$ (CRLA), and immobilized enzyme in calcium alginate bead (CRLE). The reaction was carried out in batch mode using the mixture of purified palm oil and palm fatty acid in the ratio of 70:30 (by mass) as the substrate. The optimum reaction conditions from the previous study were employed: 50°C of reaction temperature, ethanol: reactant molar ratio of 9:1 (based on purified palm oil only), immobilized lipase of 700 mg (free lipase 0.5% based on oil weight), the addition time of palm fatty acid to the reaction mixture at 24 h and the total reaction time of 48 h.

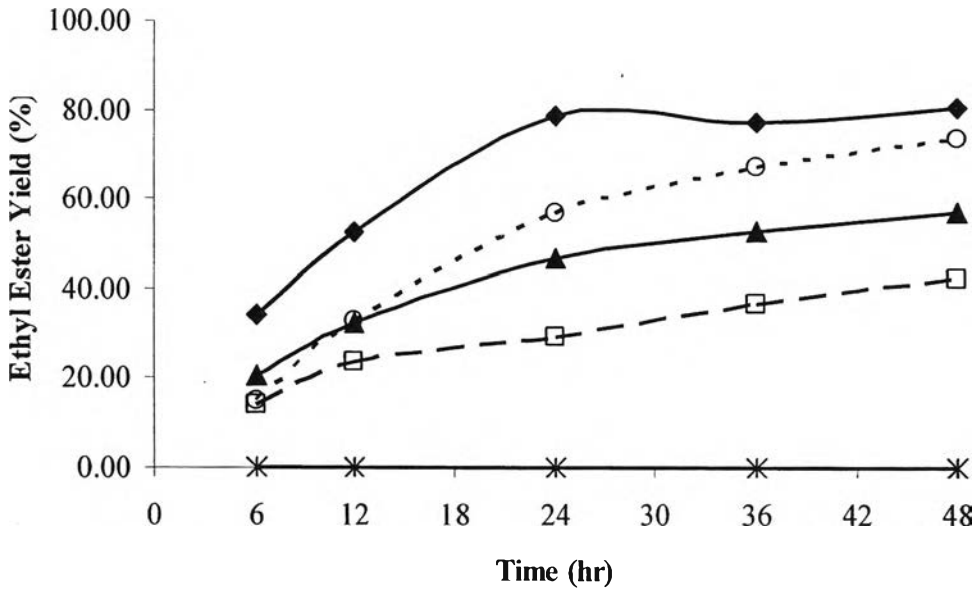


Figure 4.12 The ethyl ester yield by using the different techniques of immobilized lipase, molar ratio of purified palm oil to ethanol was 1:9, immobilized lipase of 700 mg (5% free lipase based on oil weight), reaction temperature of 50°C, the addition time of palm fatty acid to the reaction medium at 24 h and reaction time of 48 h; ♦, free lipase; ▲, CRLA; □, CRLAE; ○, CRLE; *, $CaCO_3$.

The ethyl ester yields of biodiesel by both catalytic transesterification of purified palm oil and catalytic esterification of palm fatty acid are illustrated in Figure 4.12. From the initial profiles of hydrolytic activity, it shows that the CRLA carrier was relatively more effective for the transesterification than the CRLE and CRLAE carriers. As can be seen from Figure 4.12 and Figure 4.13, after the reaction of 6 h, the ethyl ester yield of 21% was obtained from using CRLA carrier whereas, the yields of 15% and 14% were obtained from using CRLE and CRLAE, respectively. However, after 48 h, the overall ethyl ester production of the immobilized *C. rugosa* lipase in Ca-alginate matrix (CRLE) turned out the higher ethyl ester yield (74.2%) than that of the immobilization of *C. rugosa* lipase adsorbed on $CaCO_3$ (CRLA) (57.6 %) and $CaCO_3$-lipase entrapped in Ca-alginate (CRLAE) (42.7 %). The ethyl ester

production rate by using the immobilized lipase was significantly lower than that of the free lipase. The ethyl ester yield by the use of the free lipase remained constant at 79%-81% after 24 h of reaction time, whereas those from the use of the immobilized enzymes continually increased until 48 h.

4.2.6 Repeated use of the immobilized *C. rugosa* lipase

The main advantage of immobilization of an enzyme is that an expensive enzyme can be repeatedly used. However, it was usually observed that reaction behavior changes when an immobilized enzyme is used repeatedly (Ghamgui et al., 2004). In this study, the enzymatic activities for the repeated use of the immobilized *C. rugosa* lipase by CRLE, CRLA and CRLAE carriers was examined in the solvent free system. The biocatalyst was filtered off after each reaction, washed with ethanol and reused. As shown in Figure 4.13 and 4.14, when focusing on the hydrolytic activity, the repeatability of CRLE was better than CRLA and CRLAE. The hydrolytic activity of both CRLA and CRLAE rapidly reduced while the CRLE could be retained high activity until the second reuse. Analogous to the enzyme activity, for the 2^{nd} reuse, the ethyl ester yield about 60 % was achieved by using the CRLE carrier, whereas the ethyl ester yields of CRLA and CRLAE were 31.2% and 23.1%, respectively. The significantly decreased activity of the immobilized enzyme during each passing cycle was observed. The comparison between the carriers with and without $CaCO_3$ indicated that $CaCO_3$ might affect enzyme activity in the view of lipase deactivation.

The loss in activity of immobilized lipase could be due to leaching of enzyme from support and inactivation of enzyme when exposed to the violent condition. Although the entrapment technique was designed to prevent direct contact between the enzyme and surrounding environment, provide a suitable network around the enzyme and prevent enzyme from leaching from supporting material, the results from the reuse of the immobilized enzymes revealed the significantly reduction of hydrolytic activity and ethyl ester yield from the 1^{st} run to the 3^{rd} run.

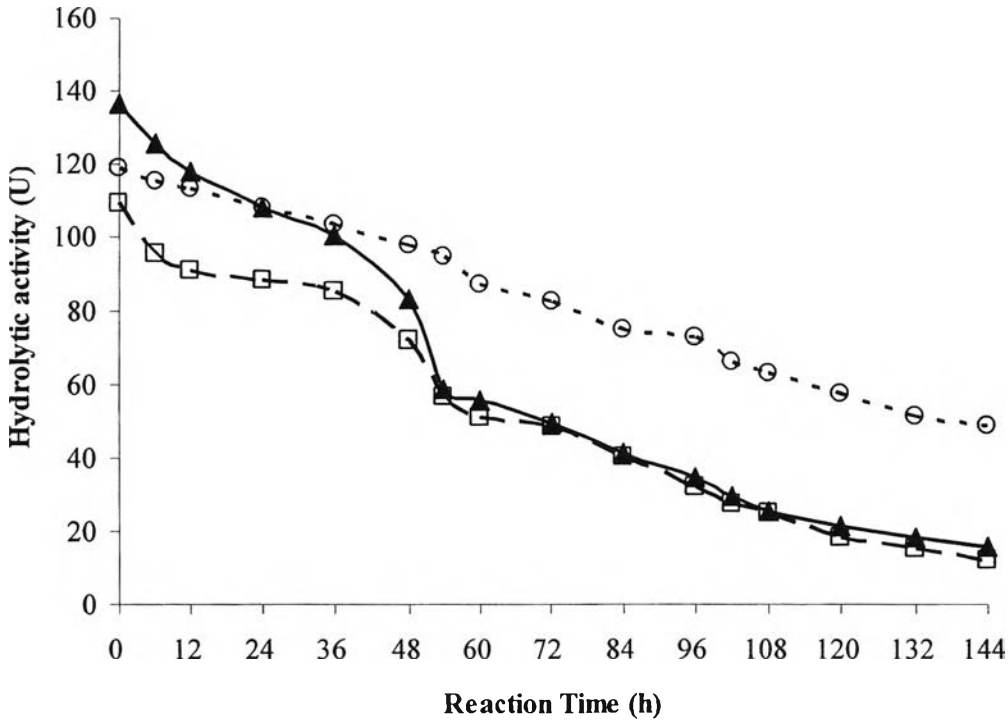


Figure 4.13 The hydrolytic activity of reusability of various type of immobilized lipase on ethyl ester yield, molar ratio of purified palm oil to ethanol was 1:9, immobilized lipase 700 mg (5% free lipase based on oil weight), reaction temperature at 50°C, addition time of palm fatty acid to the reaction medium at 24 h and total reaction time of 48 h for each cycle; ▲, CRLA; □, CRLAE; ○, CRLE.

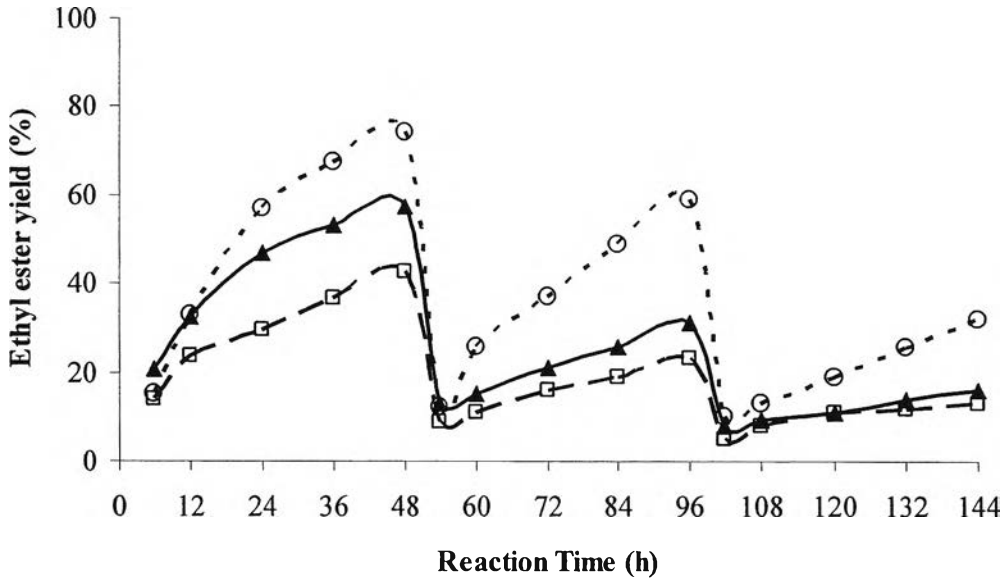


Figure 4.14 The reusability of various type of immobilized lipase on ethyl ester yield , molar ratio of purified palm oil to ethanol was 1:9, immobilized lipase 700 mg (5% free lipase based on oil weight), reaction temperature at 50°C, addition time of palm fatty acid to the reaction medium at 24 h and total reaction time of 48 h for each cycle; ▲, CRLA; □, CRLAE; ○, CRLE.