

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Microemulsion Studies

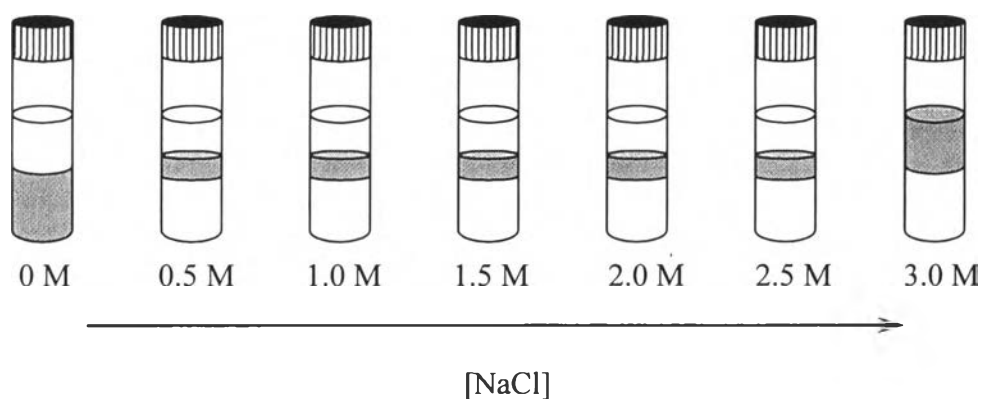
The NaDEHP/isooctane/NaCl microemulsion system was prepared by mixing appropriate amount of hexanol as a co-surfactant in isooctane containing 100mM HDEHP and 100mM NaOH in aqueous buffer (pH 7.6) with varying concentrations of NaCl from 0-3.0 M. NaDEHP was formed during the mixing of organic and aqueous phases. The reaction can be presented as:



4.1.1 Phase Behavior

Figure 4.1 shows the effects of salt and hexanol as a co-surfactant on NaDEHP W/O microemulsion formation. The results showed that reverse micelles were not formed without adding NaCl or hexanol to the system. Increasing NaCl and hexanol concentration was found to promote the formation of microemulsion. When NaCl concentration was further increased, the system changed from Winsor III microemulsion (bicontinuous structure) to Winsor II (water in oil) microemulsion. When hexanol was added to the system, water in oil microemulsion was formed at lower NaCl concentration.

0 mM Hexanol



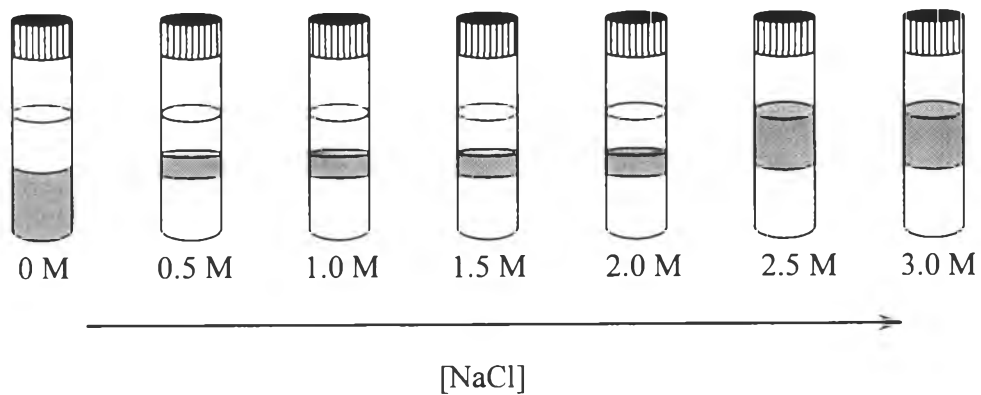
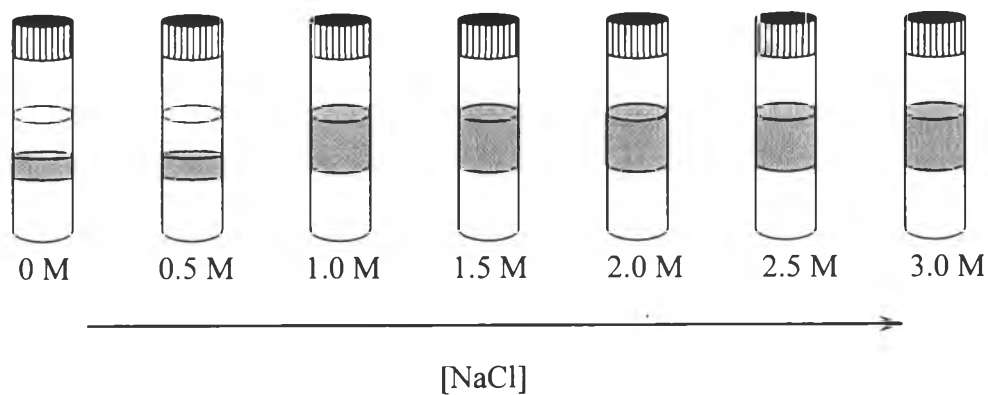
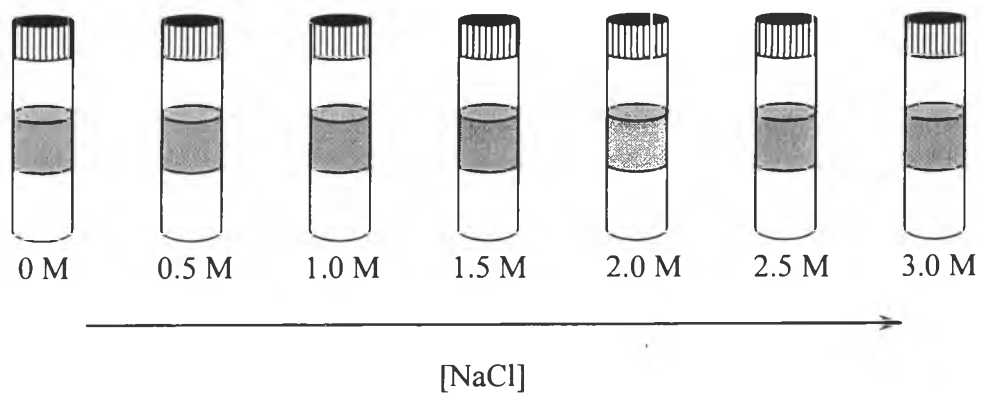
50 mM Hexanol100 mM Hexanol150 mM Hexanol

Figure 4.1 Phase behavior of NaDEHP/isooctane/NaCl aqueous solution with hexanol as a co-surfactant.

4.1.2 Water-to-surfactant Molar Ratio (W_o)

After W/O microemulsion was formed, the water content in the microemulsion (expressed in terms of the water-to-surfactant molar ratio, W_o) of NaDEHP/isooctane/NaCl(aq) microemulsion system was measured by coulometer at various NaCl, hexanol, types of substrate and substrate concentrations.

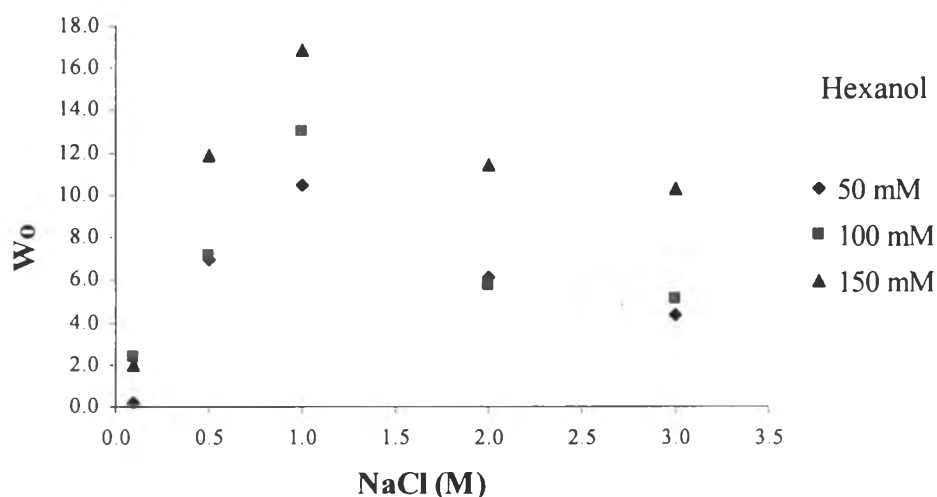


Figure 4.2 Effect of NaCl and hexanol on water-to-surfactant molar ratio of 100mM NaDEHP/50-150 mM hexanol in isooctane/0.5-3.0 M NaCl(aq).

The results showed that the water-to-surfactant molar ratio (W_o) increased as NaCl concentration increased, but started to decrease at higher NaCl concentration. When increasing concentration of hexanol from 50 mM to 150 mM resulted in increasing water content. The decrease in water content might be due to the effect of NaCl, which decreases ionic repulsion between the surfactant head groups, promoting the closer packing of hydrophilic head groups of the surfactant molecules forming the reverse micelle.

Water content was found to depend not only on types of substrate but also substrate concentrations. Table 4.1 shows effect of substrate on the water content of W/O microemulsion.

Table 4.1 Effect of caprylic acid and oleic acid on water content of W/O microemulsion of 100 mM NaDEHP/100 mM Hexanol/1.0 M NaCl

Substrate Concentration, mM	Wo
No substrate	12.01
50 mM caprylic acid	8.61
50 mM oleic acid	7.02
75 mM oleic acid	6.29
100 mM oleic acid	5.88

Increasing in the amount of substrate in W/O microemulsion led to a decrease in water content of the reverse micelles. From the results, oleic acid has a much stronger effect on W_o than caprylic acid because the longer chain of oleic acid assists in closer packing of surfactant head groups.

4.2 Esterification Reaction

4.2.1 Effect of Lipase Concentration on Catalytic Activity

The W/O microemulsion system of 100 mM NaDEHP/100 mM hexanol/100 mM caprylic acid in isooctane/1.0 M NaCl(aq) was prepared for the catalytic activity of lipase. The *Candida antarctica* lipase B (CALB) concentrations of 20.0, 40.0 and 80.0 $\mu\text{g/ml}$ in the aqueous phases were used. Encapsulation of lipase in the reverse micelles was measured from the lipase in the aqueous phase before and after encapsulation by UV-VIS absorption at 280 nm. The results shown in Table 4.1 indicate a decrease of the lipase concentration in the aqueous phase after encapsulation. Most of lipases (90%) were encapsulated inside the water pool of microemulsions.

Table 4.2 CALB concentrations in the aqueous phase before and after encapsulation in 100 mM NaDEHP/100 mM hexanol/100 mM caprylic acid in isooctane/1.0 M NaCl(aq) microemulsion system

Lipase concentration ($\mu\text{g/ml}$)	Lipase concentration measured ($\mu\text{g/ml}$)	
	Before encapsulation	After encapsulation
20.00	19.12	2.11
40.00	37.40	3.98
80.00	74.92	6.59

The rate of esterification was measured at various concentrations of *Candida antarctica* lipase B (CALB) and *Thermomyces lanuginosa* lipase (TLL) by measuring the depletion of caprylic acid as a function of time as shown in Figures 4.3 and 4.4. The rate of esterification was not significantly increased when increasing lipase concentrations. Therefore, 0.04 mg/ml lipase was selected for further study in catalytic activity of esterification.

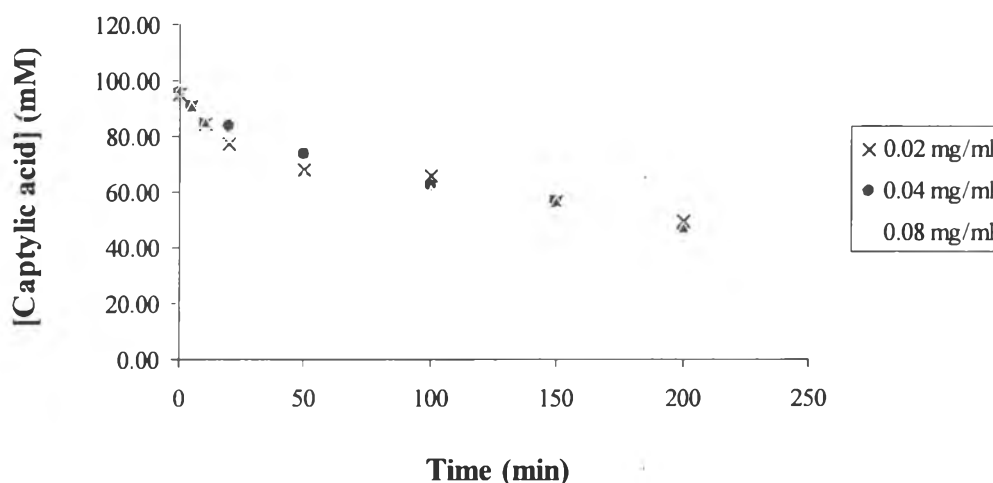


Figure 4.3 Effect of CALB concentration (0.02-0.08 mg/ml) on the catalytic activity in 100 mM NaDEHP/ 100mM hexanol/100mM caprylic acid/isooctane/1.0 M NaCl(aq) microemulsion system.

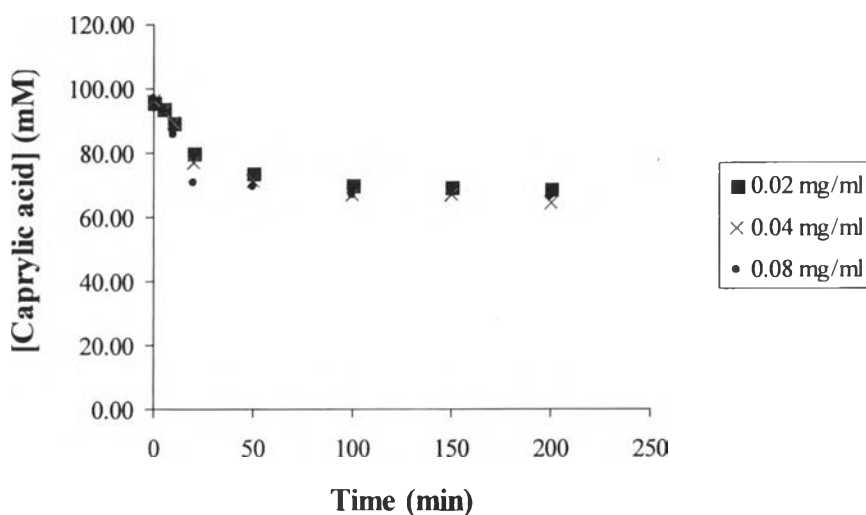


Figure 4.4 Effect of TLL concentration (0.02-0.08 mg/ml) on the catalytic activity in 100 mM NaDEHP/100 mM hexanol/100 mM caprylic acid/isooctane/1.0 M NaCl(aq) microemulsion system.

4.2.2 Effect of Water Content on Catalytic Activity

The rate of esterification of oleic acid or caprylic acid with hexanol with varying water content and the W/O microemulsion of 25-150 mM NaDEHP/100 mM hexanol/100 mM oleic acid or caprylic acid/ isooctane are shown in Figures 4.5-4.8. The initial W_o values were ranged 1.23-9.28 for CALB and 1.11-13.67 for TLL in the 100 mM oleic acid system. CALB and TLL showed the highest catalytic activity (the most depletion of oleic acid) when encapsulated in 100mM NaDEHP reverse micelles corresponding to the initial W_o of 6.67 for CALB and 8.44 for TLL.

For the 100 mM caprylic acid system, the decrease in the caprylic acid concentrations in esterification of CALB (Figure 4.7) and TLL (Figure 4.8) corresponded to the highest catalytic activity when CALB encapsulated in 125 mM NaDEHP reverse micelles (initial $W_o = 9.31$) but TLL showed the highest catalytic activity in 100 mM NaDEHP reverse micellar (initial $W_o = 8.44$) system. Mostly, CALB showed higher catalytic activity than TLL.

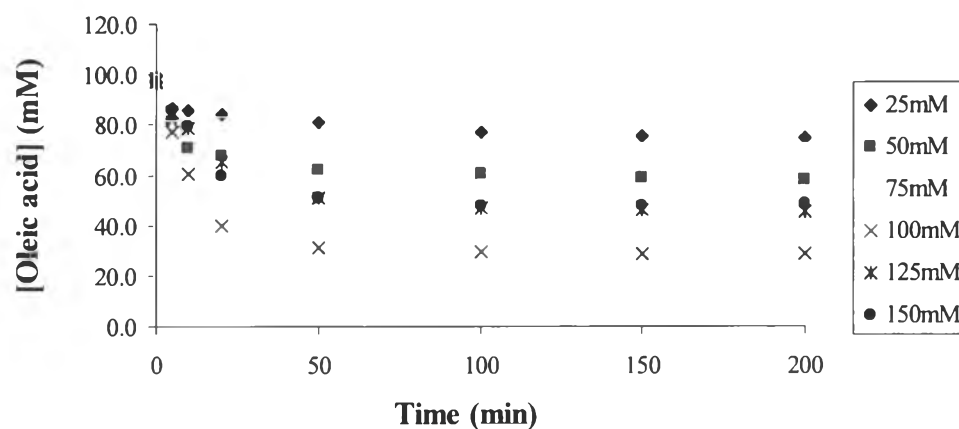


Figure 4.5 Effect of water content on the catalytic activity of 0.04 mg/ml CALB in 25-150 mM NaDEHP/100 mM hexanol/100 mM oleic acid in isooctane/1.0 M NaCl(aq) microemulsion system.

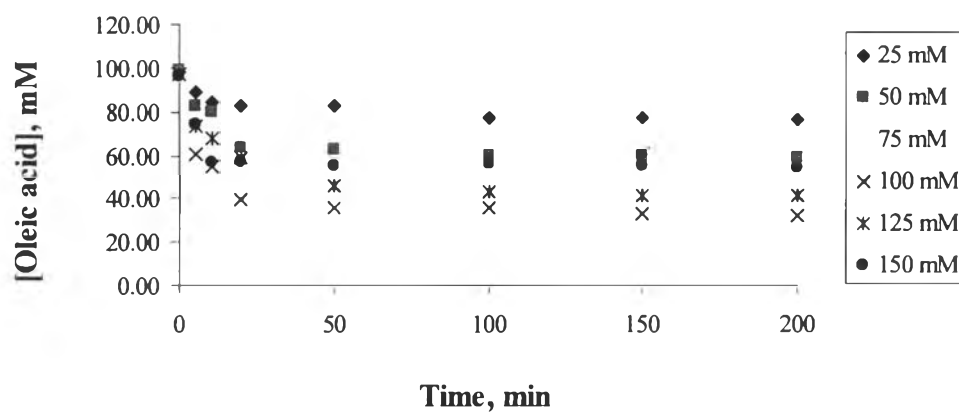


Figure 4.6 Effect of water content on the catalytic activity of 0.04 mg/ml TLL in 25-150 mM NaDEHP/100 mM hexanol/100 mM oleic acid in isooctane/1.0 M NaCl(aq) microemulsion system.

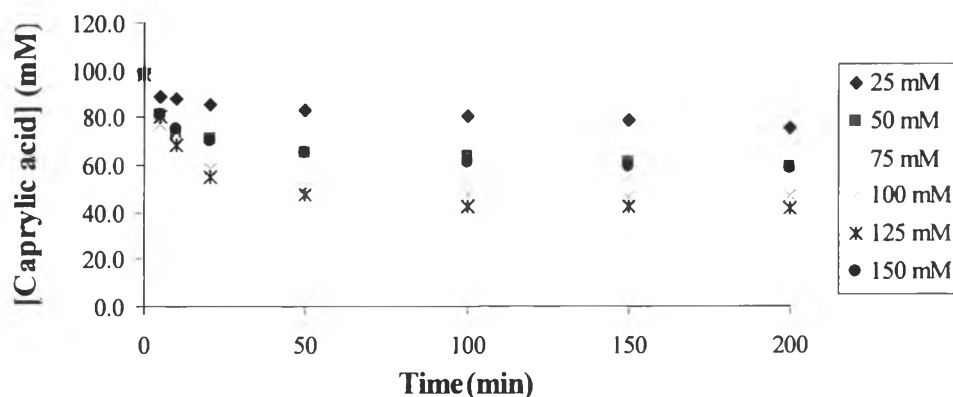


Figure 4.7 Effect of water content on the catalytic activity of 0.04 mg/ml CALB in 25-150 mM NaDEHP/100 mM hexanol/100 mM caprylic acid in isooctane/1.0 M NaCl(aq) microemulsion system.

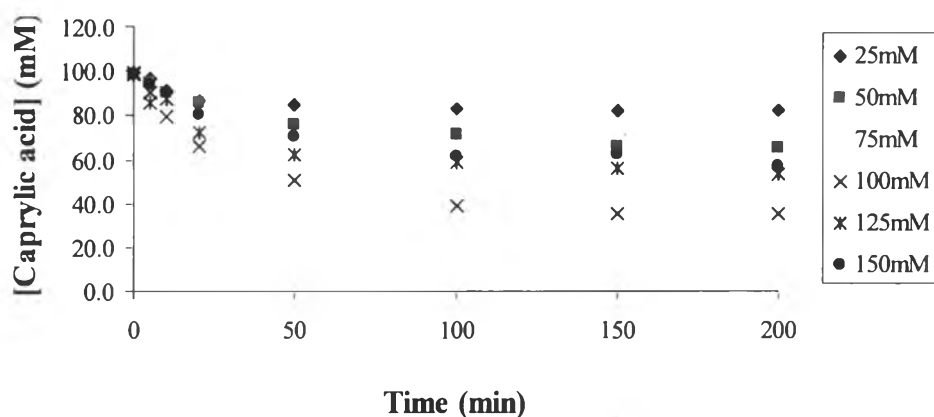


Figure 4.8 Effect of water content on the catalytic activity of 0.04 mg/ml TLL in 25-150 mM NaDEHP/100 mM hexanol/100mM caprylic acid in isooctane/1.0 M NaCl(aq) microemulsion system.

Figure 4.9 shows the effect of water content on the specific activity in CALB and TLL catalyzing esterification of oleic acid and caprylic acid with hexanol. The highest specific activity of CALB catalyzing esterification of oleic acid was $51.32 \text{ mM min}^{-1} \text{ mg lipase}^{-1}$ with the initial W_o of 6.67 and caprylic acid was $45.18 \text{ mM min}^{-1} \text{ mg lipase}^{-1}$ (initial $W_o=9.31$), while TLL shows the highest specific

activity for oleic acid at $45.17 \text{ mM min}^{-1} \text{ mg lipase}^{-1}$ with the initial W_o of 8.44 and for caprylic acid at $21.32 \text{ mM min}^{-1} \text{ mg lipase}^{-1}$ and the initial W_o 7.00.

The conversions of fatty acids in the esterification of CALB at the optimum initial W_o and the maximum specific activity is shown in Figure 4.10. The highest conversion of fatty acids catalyzed by CALB were 68.53% ($W_o=6.67$) and 51.70% ($W_o=9.31$) for oleic acid and caprylic acid, respectively, while the highest conversions by TLL were 63.17% ($W_o=8.44$) and 48.72% ($W_o=7.00$) for oleic acid and caprylic acid, respectively.

According to the esterification reaction, water is one of the products formed during the reaction. All the results showed the increase in the water content after the reaction. Moreover, it was found that the amount of water produced (observed as a difference of the water content before and the increase amount of water was agreed with the depletion amount of fatty acid used in the esterification after 200 minutes the reaction) depended on the reaction conversion. At the optimum W_o CALB gave higher specific activity than TLL for both oleic acid and caprylic acid.

The optimum W_o value appears to be specific for each type of lipase. It has been proposed that the optimum enzyme activity occurs around a value of W_o at which the size of the droplet is equal to the size of the enzyme molecule (Martinek, 1986). For this study, CALB and TLL encapsulated in NaDEHP reverse micelles might be located in close contact with organic substrates at W_o is 6-9 and 7-11, respectively. The initial rate increased with increasing water content and reached the maximum at the optimum initial water content. Moreover, each lipase requires the different water content to obtain the highest catalytic activity. The initial rate calculation based on the optimum water contents were 4.56 mM min^{-1} for CALB and 1.89 mM min^{-1} for TLL.

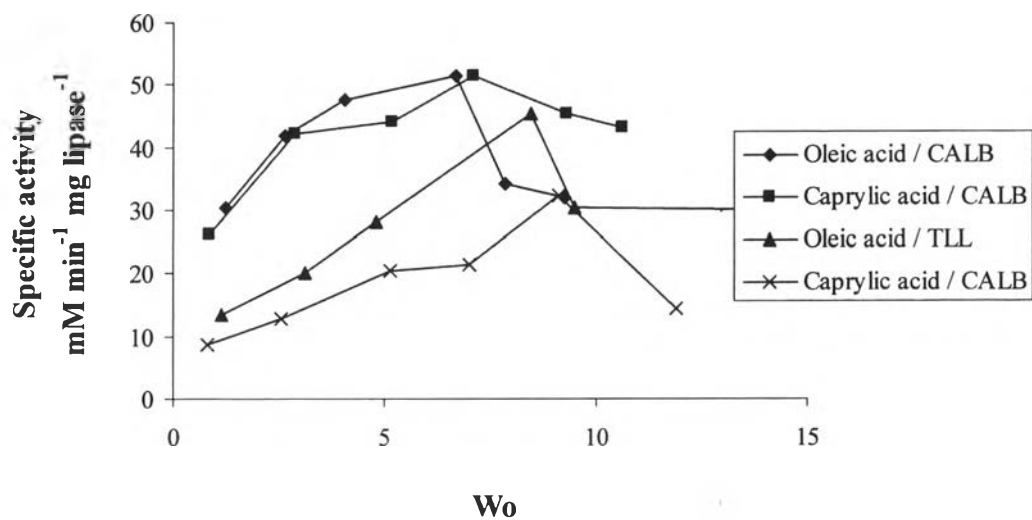


Figure 4.9 Effect of water content on the specific activity in the esterification of CALB and TLL in 25-150 mM NaDEHP/100 mM hexanol/100 mM oleic acid or 100 mM caprylic acid in isooctane/1.0 M NaCl(aq).

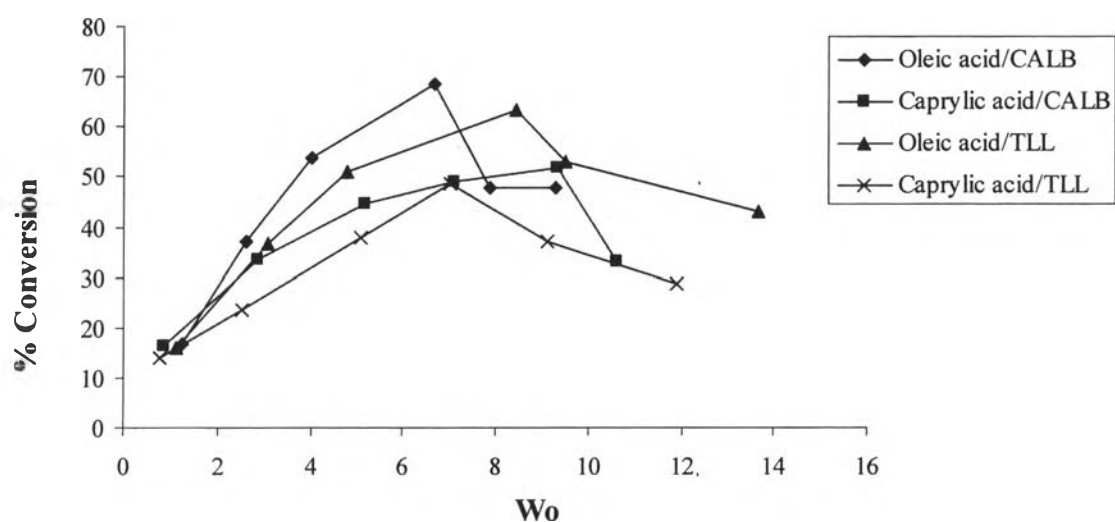


Figure 4.10 Effect of water content on the fatty acid conversion in the esterification of CALB and TLL in 25-150 mM NaDEHP/100 mM hexanol/100 mM oleic acid or 100 mM caprylic acid in isooctane/1.0 M NaCl(aq).

4.2.3 Effect of Types of Substrate and Substrate Concentrations on Catalytic Activity

To study the effect of types of substrate on the catalytic activity, caprylic acid and oleic acid were used as short chain and long chain substrates, respectively and the fatty acid concentration were varied from 25mM to 150mM.

The rates of oleic acid (or caprylic acid) depletion using CALB and TLL as biocatalysts in 100mM NaDEHP/100mM hexanol/oleic acid (or caprylic acid)/ isooctane microemulsion system are shown in Figures 4.11 to 4.14. CALB and TLL showed the most depletion of oleic acid (or caprylic acid) when catalyzing low fatty acid concentrations and decreasing the depletion at higher fatty acid concentrations.

Figure 4.15 shows the effect of concentrations of oleic acid and caprylic acid on the specific activity of CALB in esterification reaction of fatty acid with hexanol in NaDEHP reverse micellar system. The results show that the maximum specific activity of CALB is $40.12 \text{ mM min}^{-1} \text{ mg lipase}^{-1}$ at 50 mM oleic acid and $31.52 \text{ mM min}^{-1} \text{ mg lipase}^{-1}$ at 100 mM caprylic acid while TLL shows the maximum specific activity at $29.59 \text{ mM min}^{-1} \text{ mg lipase}^{-1}$ and $20.80 \text{ mM min}^{-1} \text{ mg lipase}^{-1}$ at 100mM oleic acid and caprylic acid, respectively.

From the results, the concentration of fatty acid increased, the catalytic activity of lipase increased up to the maximum value. However, the catalytic activity decrease when fatty acid concentration was further increased. Decreasing of the catalytic activity at high concentration of fatty acid could be related to a possible instability of lipase in the microemulsion system and an inhibitory effect of high substrate concentration to lipase. Moreover, the reduction of water content of the reverse micellar system leads to the decrease in the catalytic activity of lipase. In this study, CALB and TLL better catalyzed oleic acid with hexanol than caprylic acid in NaDEHP reverse micellar system. Therefore, CALB and TLL prefer to long chain fatty acid than the short one. This selectivity was related to the localization of lipase from different sources within the reverse micelles due to the degree of hydrophobicity of the enzyme. On the other hand, CALB and TLL are the surface active enzymes which can react with the fatty acid at the oil and water interface.

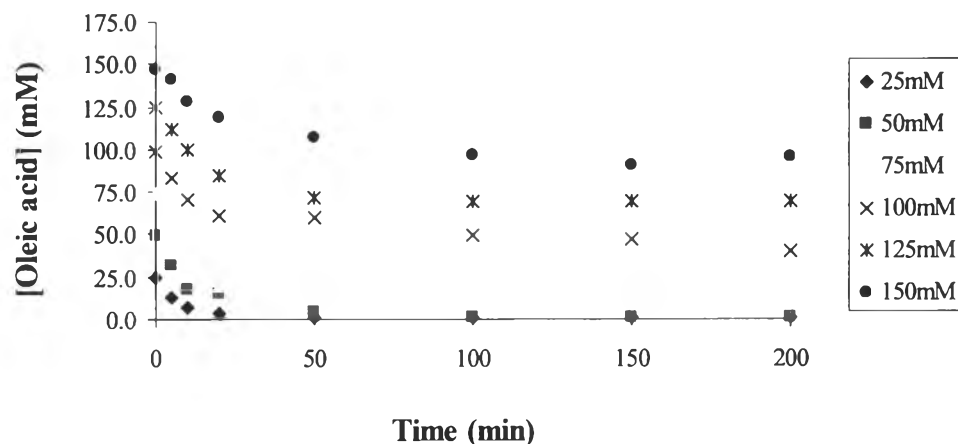


Figure 4.11 Effect of concentrations of oleic acid on the catalytic activity in 100 mM NaDEHP/100 mM hexanol/25-150 mM oleic acid in isooctane/1.0 M NaCl(aq)/0.04 mg/ml CALB microemulsion system.

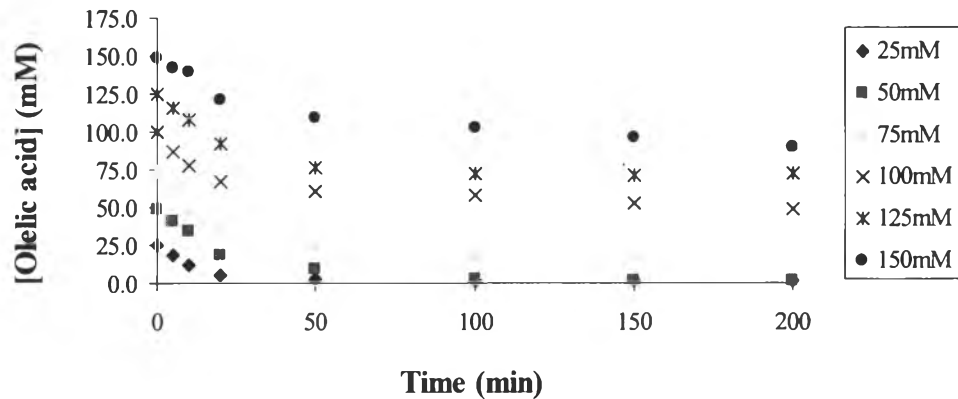


Figure 4.12 Effect of concentrations of oleic acid on the catalytic activity in 100 mM NaDEHP/100 mM hexanol/25-150 mM oleic acid in isooctane/1.0 M NaCl(aq)/0.04 mg/ml TLL microemulsion system.

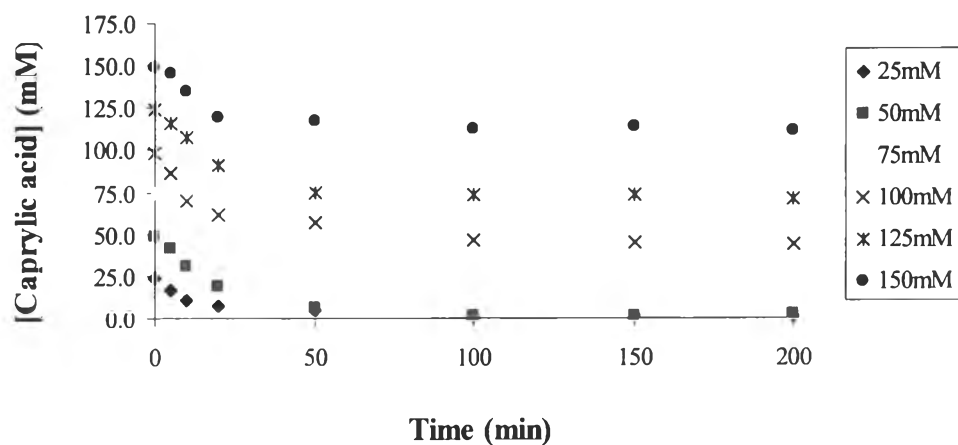


Figure 4.13 Effect of concentrations of caprylic acid on the catalytic activity in 100 mM NaDEHP/100 mM hexanol/25-150 mM caprylic acid in isooctane/1.0 M NaCl(aq)/CALB microemulsion system.

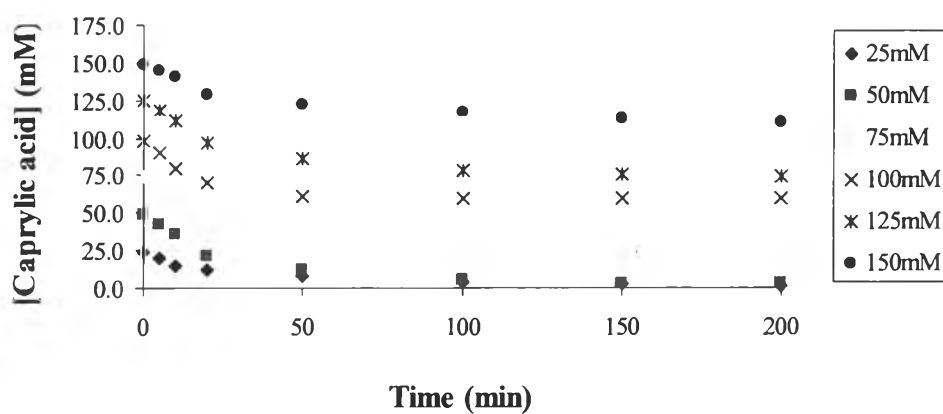


Figure 4.14 Effect of concentrations of caprylic acid on the catalytic activity in 100 mM NaDEHP/100 mM hexanol/25-150 mM caprylic acid in isooctane/1.0 M NaCl(aq)/TLL microemulsion system.

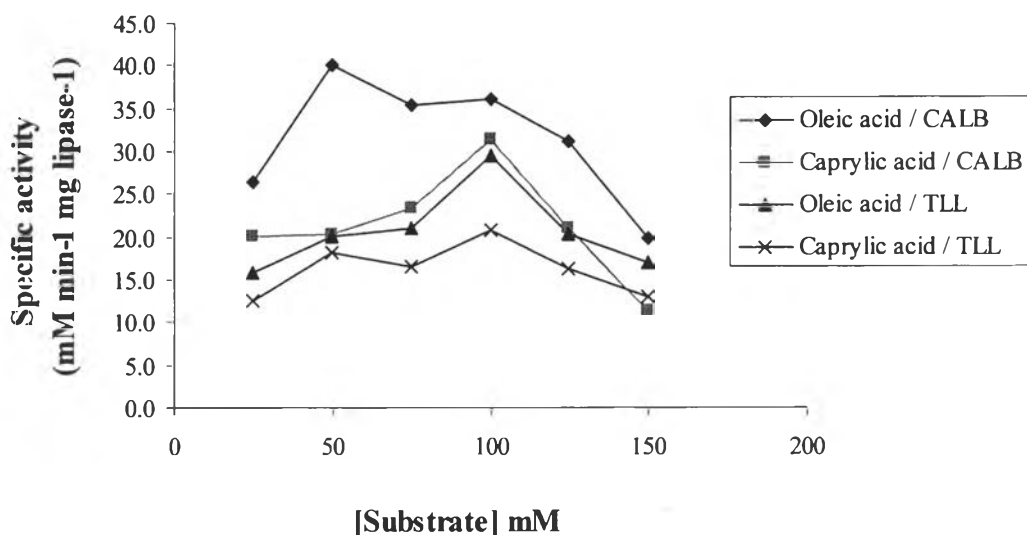


Figure 4.15 Effect of concentrations of oleic acid on the specific activity of 100 mM NaDEHP/100 mM hexanol/25-150 mM oleic acid and 25-150 mM caprylic acid in isoctane/1.0 M NaCl(aq)/CALB and TLL microemulsion system.

4.3 Chemical Analyses

The samples from upper phase of microemulsion were taken before and after 200 minutes reaction time to identify the production of ester by FTIR. The interested range is between 1600 and 1800 cm^{-1} , the same absorbance peak of carbonyl of the ester band is at 1740 cm^{-1} and fatty acid band at 1720 cm^{-1} .

The peak observed from the esterification of 100 and 150mM oleic acid with hexanol by CALB (Figures 4.17 and 4.18) show the shift of the carbonyl of fatty acid at 1720 cm^{-1} to the ester peak at 1740 cm^{-1} . For the esterification of caprylic acid and oleic acid with hexanol by TLL (Figure 4.19), the peak of ester after reaction did not appear. It might be due to the concentration of ester product from the esterification was too low to be observed. However, the intensity of fatty acid peak was obviously decreased in all experiments.

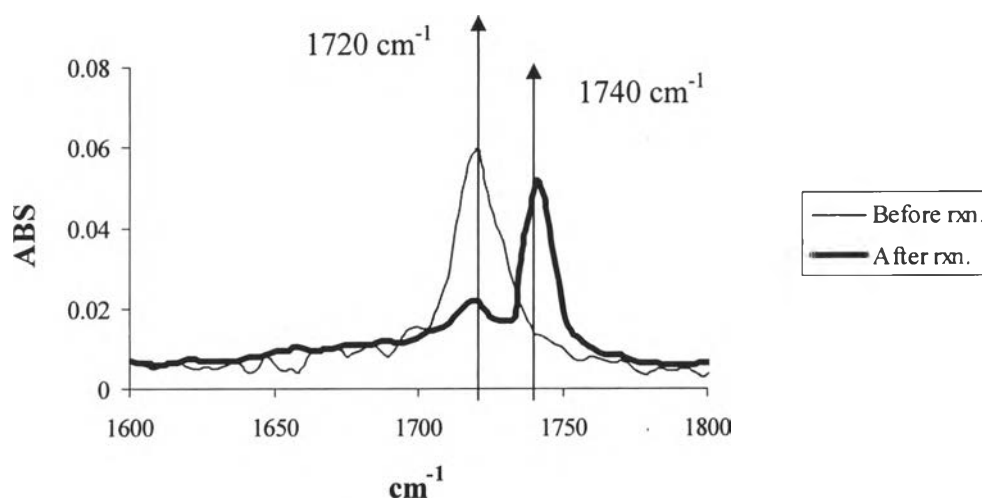


Figure 4.16 Spectra of before and after esterification of oleic acid with hexanol in 100mM NaDEHP/100mM Hexanol/100mM oleic acid in isooctane/1.0M NaCl/CALB microemulsion system.

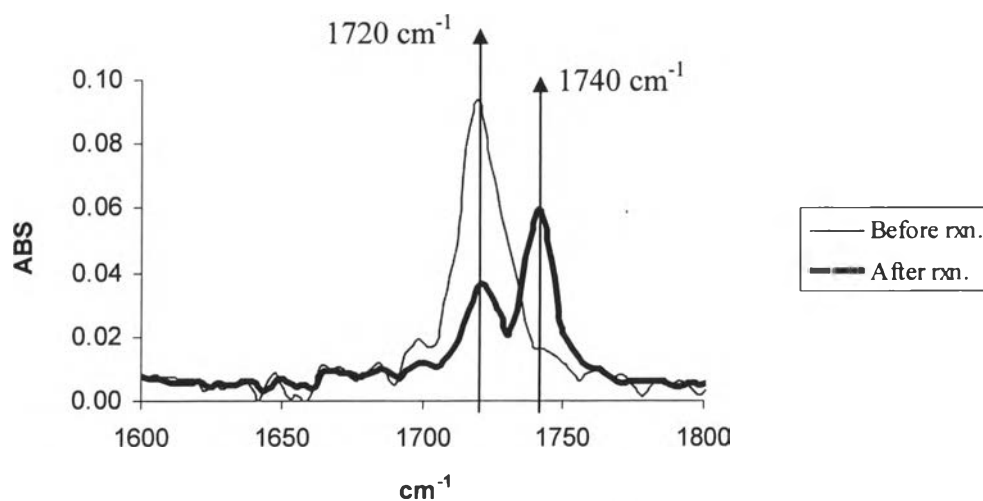


Figure 4.17 Spectra of before and after esterification of oleic acid with hexanol in 100mM NaDEHP/100mM Hexanol/150mM oleic acid in isooctane/1.0M NaCl/CALB microemulsion system.

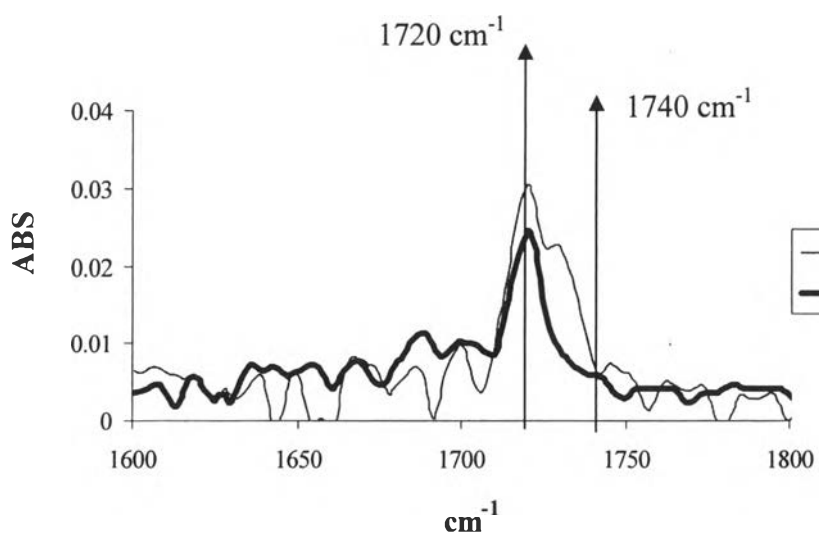


Figure 4.18 Spectra of before and after esterification of oleic acid with hexanol in 100mM NaDEHP/100mM Hexanol/150mM oleic acid in isooctane/1.0M NaCl/TLL microemulsion system.