CHAPTER V

RESULTS AND DISCUSSION

5.1 Selection of suitable lipase and acyl donors for menthyl acetate production

The aim of this section was to choose the suitable lipase and acyl donor for the transesterification of (±)menthol and an ester. The following lipases from three different sources were used:

1. *Candida cylindracea*
2. Porcine pancreas
3. Hog pancreas

Three types of acyl donors (the second substrate) were also tested for their suitability for the transesterification reaction. To obtain the product, menthyl acetate, acetate group from an acyl donor is substituted by the menthyl group from menthol. The following three types of acyl donors were used: ethyl acetate, butyl acetate, and hexyl acetate.

The experimental results shown in figure 5.1 are comparisons of transesterifications carried out with different acyl donor chain lengths, and sources of lipases in terms of initial reaction rate after 220 hours of reactions.

It is obviously noticed from figure 5.1 that lipase from *Candida cylindracea* was the best choice of catalysts among the three since the initial rates obtained from three various acyl donors were at least 26 times higher than those from *Porcine pancreas* which was the second best catalyst. Lipase from Hog pancreas was found to give the slowest initial rates. However, its catalytic capability was comparable to those of Porcine pancreas with the initial rates obtained from the reactions with butyl acetate as acyl donor were 0.57 and 0.93 μmole/lit-hr, respectively.

Our experimental results were in accordance with those of other researchers. Kamiya et al (1995) studied an esterification reaction of (-)menthol and lauric acid in a reaction catalyzed by lipases, and showed that lipase from *Candida cylindracea* gave an initial rate of 1.979 mM/hr, while those from *Porcine pancreas* was 0.052 mM/hr. In addition, Langrand et al (1988) studied the transesterification of isoamyl alcohol and ethyl acetate, and also found that lipase from *Candida cylindracea* showed higher initial rate than the enzyme from Porcine pancreas with the initial rates of 1.56 mM/hr and 0.36 mM/hr, respectively.

As for the type of acyl donors, hexyl acetate was found to be the best among the three with the initial rate of 26.46 μM/hr while those of butyl acetate and ethyl acetate were 24.08, and 22.61, respectively. The difference in initial rates obtained from various types of donors was probably due to the varied acyl donor chain lengths which resulted in different degree of covalent bond between acyl and acetate groups. It was found that the longer acyl chain length the lower the covalent bond. Therefore, hexyl group was most easily broken from the hexyl acetate molecule and substituted the OH group in menthol molecule easier than other acyl donors studied. Not only the acyl donors studied in this project, but also iso-butyl acetate, octyl acetate, triacetin, and etc. can be used in the reaction for producing menthyl acetate. This study focused only on the chainlength of acyl donors which did not include branch chain and triglyceride group. Therefore, the latter two groups of compounds were not investigated in this work. The time-course reactions and conversions of the transesterification by lipase from *Candida cylindracea* are shown in figures 5.2 and 5.3, respectively.

Figure 5.2 demonstrates that chemical equilibrium of transesterifications with various acyl donors was reached at approximately the same time which was around 150 hours after initiation. The final conversions were then calculated and the results in figure 5.3 show that hexyl acetate was, again, appeared to be the best acyl donor since it gave

the highest conversion of 17.23% base on racemic menthol, while butyl acetate and ethyl acetate gave 10.73 and 6.8%, respectively.

Similar results were reported by Langrand et al (1988) for transesterifications of isoamyl alcohol and two types of acyl donors (ethyl acetate and butyrate) by lipase from *Candida cylindracea*. Butyrate, higher number of carbon atoms, gave higher conversion (71%) than ethyl acetate (15%).

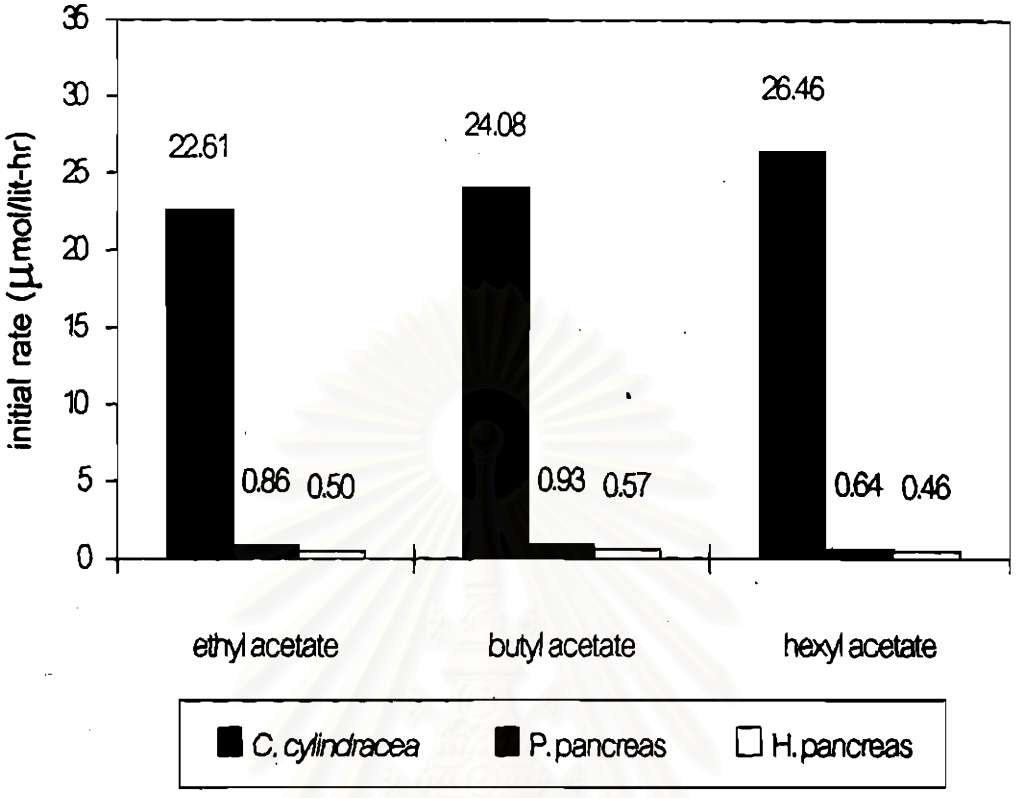


Figure 5.1 Comparison of types of lipases on menthyl acetate production from three types of acyl donors

Experimental conditions :

(±)menthol concentration	26 mM
acyl donors concentration	200 mM
lipase concentration	8 g/l
organic solvent	iso-octane
temperature	30 °C
stirring speed	175 rpm

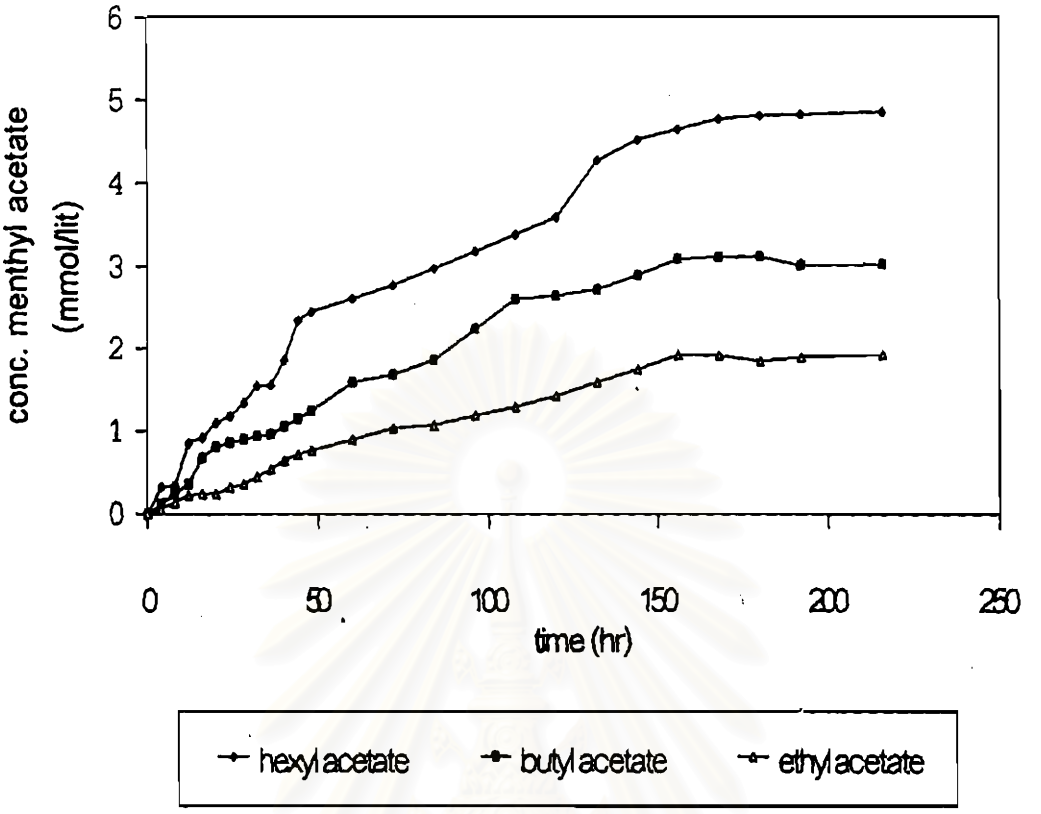


Figure 5.2 Time-course reaction of transesterification by lipases from *Candida cylindracea* with various acyl donors

Experimental conditions :

(±)menthol concentration	26 mM
acyl donors concentration	200 mM
lipase concentration	8 g/l
organic solvent	iso-octane
temperature	30 °C
stirring speed	175 rpm

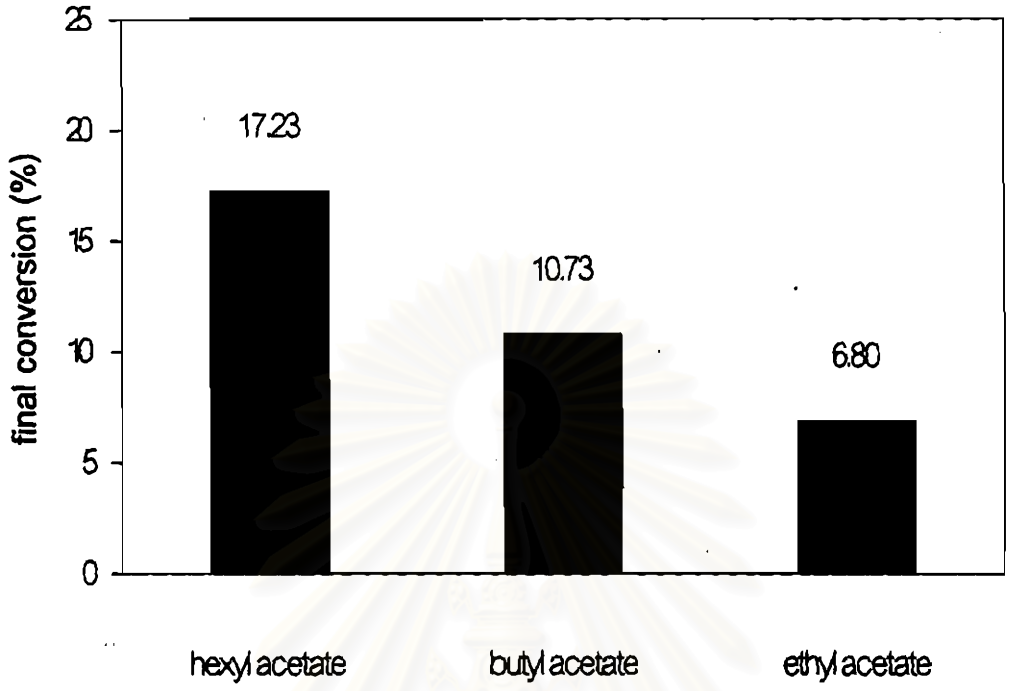


Figure 5.3 Comparison of final conversions (calculated based on (±)menthol) obtained from the transesterification with various acyl donors using lipase from *Candida cylindracea*

Experimental conditions :

(±)menthol concentration	26 mM
acyl donors concentration	200 mM
lipase concentration	8 g/l
organic solvent	iso-octane
temperature	30 °C
stirring speed	175 rpm

5.2 Selection of suitable organic solvent for menthyl acetate production

Enzymes can maintain its natural structure in organic solvent provided that essential-water layer is in its immediate surroundings. Loss of this essential-water layer from an enzyme could occur in certain organic solvents that appear to strip water off an enzyme. This work suggests that interactions between organic solvent and enzyme essential-water layer controls the activity of enzyme.

For the transesterification of racemic menthol and hexyl acetate we chose five kinds of organic solvents with various log P values and structures (straight chain, branched chain and cyclic ring). Hexane, heptane, iso-octane, cyclohexane, and benzene were chosen as reaction media for the transesterification by lipase from *Candida cylindracea*.

Figure 5.4 shows the relationship between log P value and initial rate for each organic solvent. Under the same experimental conditions, initial rate obtained from the reaction with iso-octane, the highest log P solvent, as reaction media is higher than those from heptane, cyclohexane, hexane and benzene, respectively. This implied that reaction rate tended to increase with log P value. In other words, the more hydrophobic the solvent, the better biocatalytic activity. This phenomenon could be explained as follows : water is bound to polar and charged residuals of proteins through mainly electrostatic forces. Increased solvent polarity weakens these electrostatic forces and enables water to desorb off enzymes into the bulk organic solvents. Hence, water-stripping from an enzyme into an nonaqueous media does occur and can be significant in hydrophilic solvents (Dordick, J. S., 1992). And this could result in enzyme denaturation and thus lower reaction rates.

In addition, figure 5.5 shows relative conversions of selected organic solvents from our experimental results compare with those from Goto et al 's (1994) and Kamiya et al 's (1995) who studied esterification reaction of lauric acid with benzyl alcohol by lipase from *Pseudomanas sp.* and lauric acid with (-)menthol by coated-lipase from *Candida*

cylindracea, respectively. Iso-octane was used as a reference since it gave the best conversion among other solvents, while benzene gave the lowest relative conversion for all the experiments. An interesting point was that cyclohexane, even of lower log P value than n-hexane, appeared to be a better solvent for the enzyme reaction. So it could be concluded that log P value could be used only as a rough guide for solvent selection. Iso-octane, consequently, was selected as the most appropriate organic solvent for our work and was used in all the following experiments.

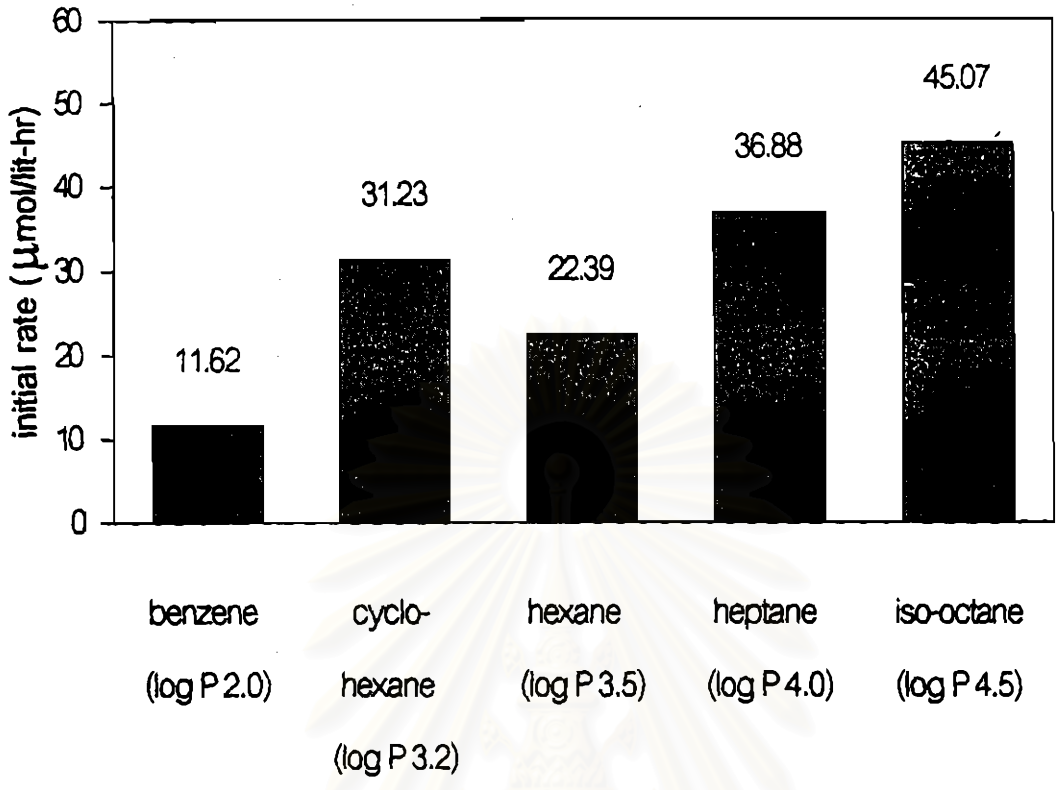


Figure 5.4 Comparison of types of organic solvents on menthyl acetate production

Experimental conditions :

(±)menthol concentration	26 mM
hexyl acetate concentration	256 mM
lipase concentration	8 g/l
temperature	30 °C
stirring speed	175 rpm

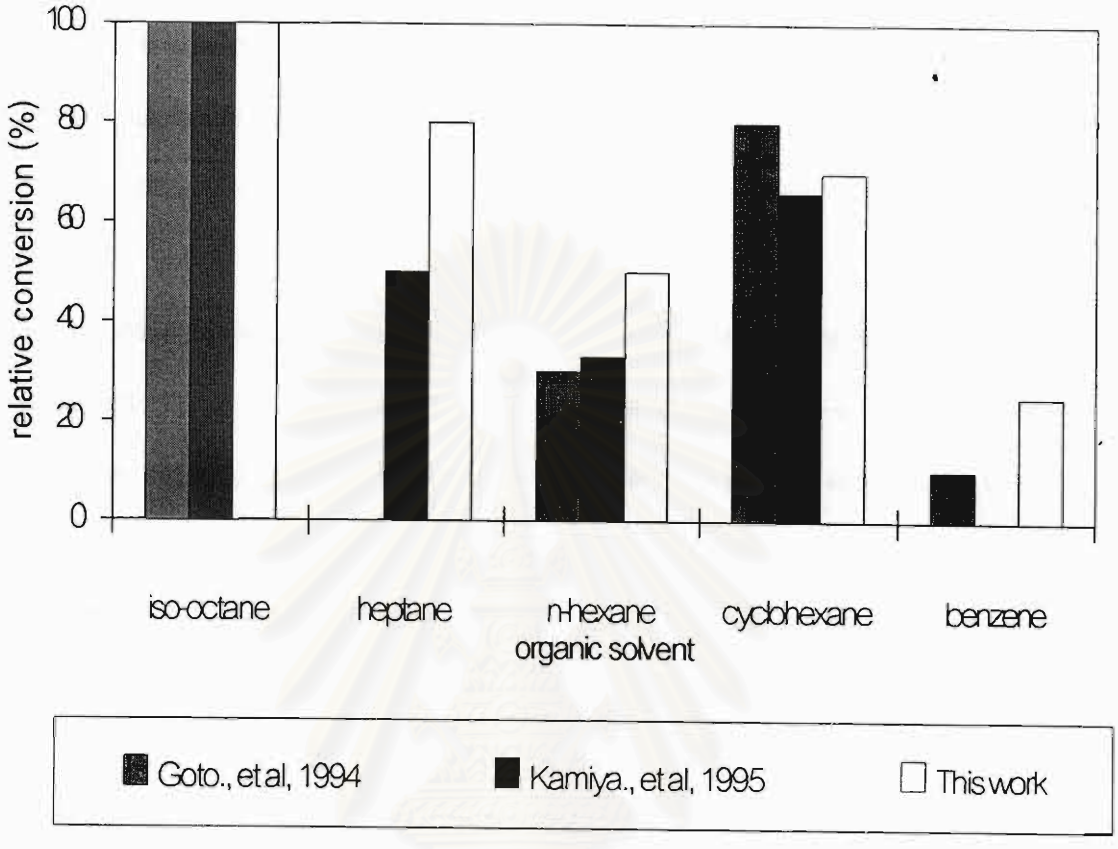


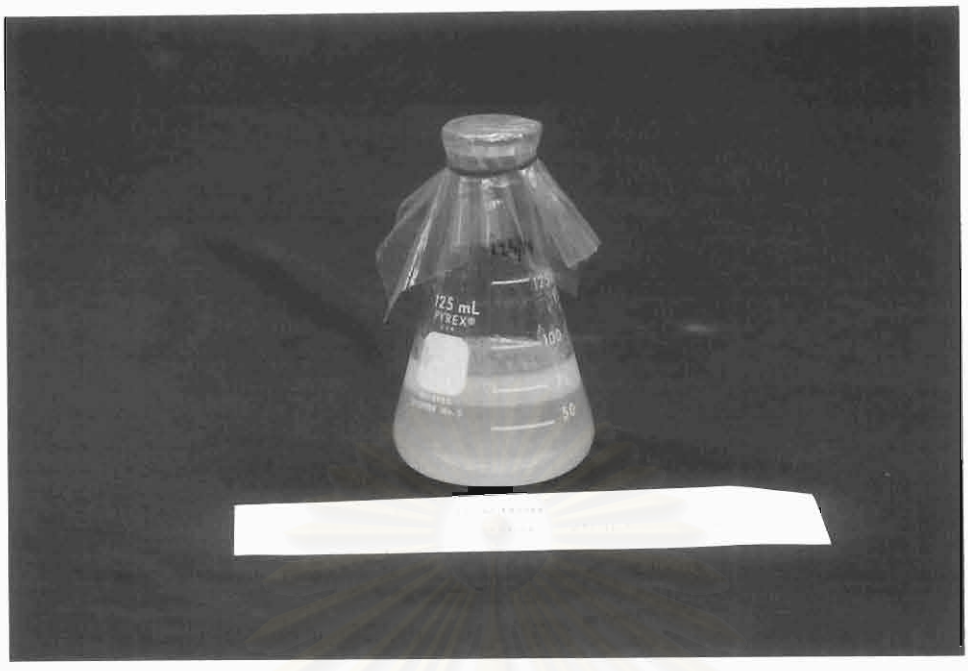
Figure 5.5 Comparison of relative conversions between our experimental results, and those of Goto., et al, 1994 and Kamiya., et al, 1995 of various organic solvents

5.3 Transesterifications in aqueous-organic two phase system

In aqueous-organic two phase system, substrates were dissolved in an organic solvent, while the enzyme was solubilised in a pH 7.0 buffer solution. The experiments were carried out in various volume ratios of iso-octane and buffer solution using the *Candida cylindracea* lipase, and hexyl acetate as the biocatalyst and acyl donor, respectively.

After vigorous mixing, the two phase system without lipase completely separated into the distinct phases in only a few minutes upon standing with iso-octane in the top phase. On the contrary, stable emulsions were formed in systems with lipase which suggested that the enzyme acted as a surface active agent in these systems. Figure 5.6 and 5.7 show characteristics of this aqueous-organic system with lipase. The emulsions formed were in the upper phase while the bottom phase was an aqueous buffer solution with the rest of the enzyme.

From the experiments we found that in all the two phase systems around approximately 5 ml of buffer solution was incorporated into the organic phase, and around 0.73 g of the lipase was left in the aqueous phase while the similar small amounts were in the organic phase (see figure 5.8).



(a)

(b)

Figure 5.6 The photographs of various aqueous-organic two phase systems with different volume ratios after mixing. a) organic phase 25%:buffer phase 75% b) organic phase 50%:buffer phase 50%

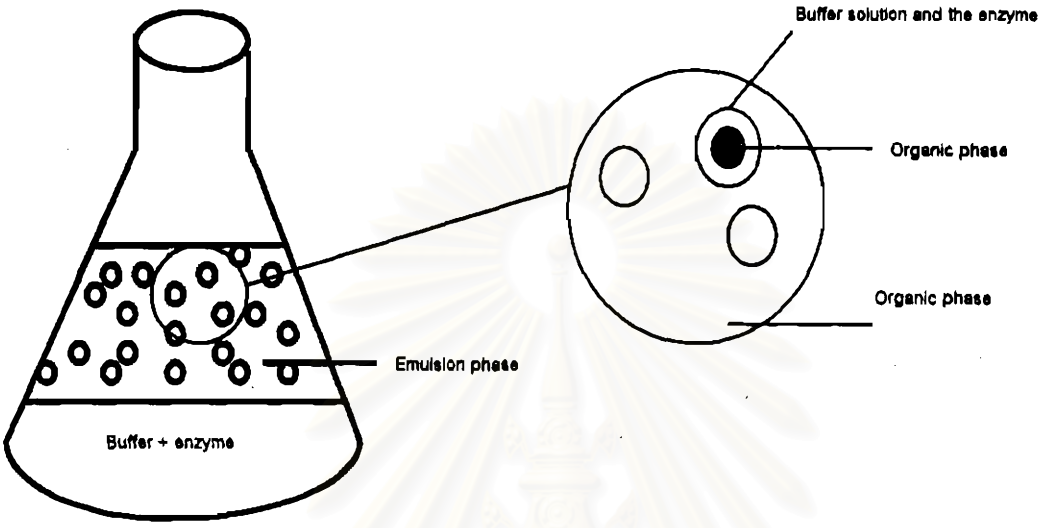


Figure 5.7 Demonstration of the characteristic of the two phase system

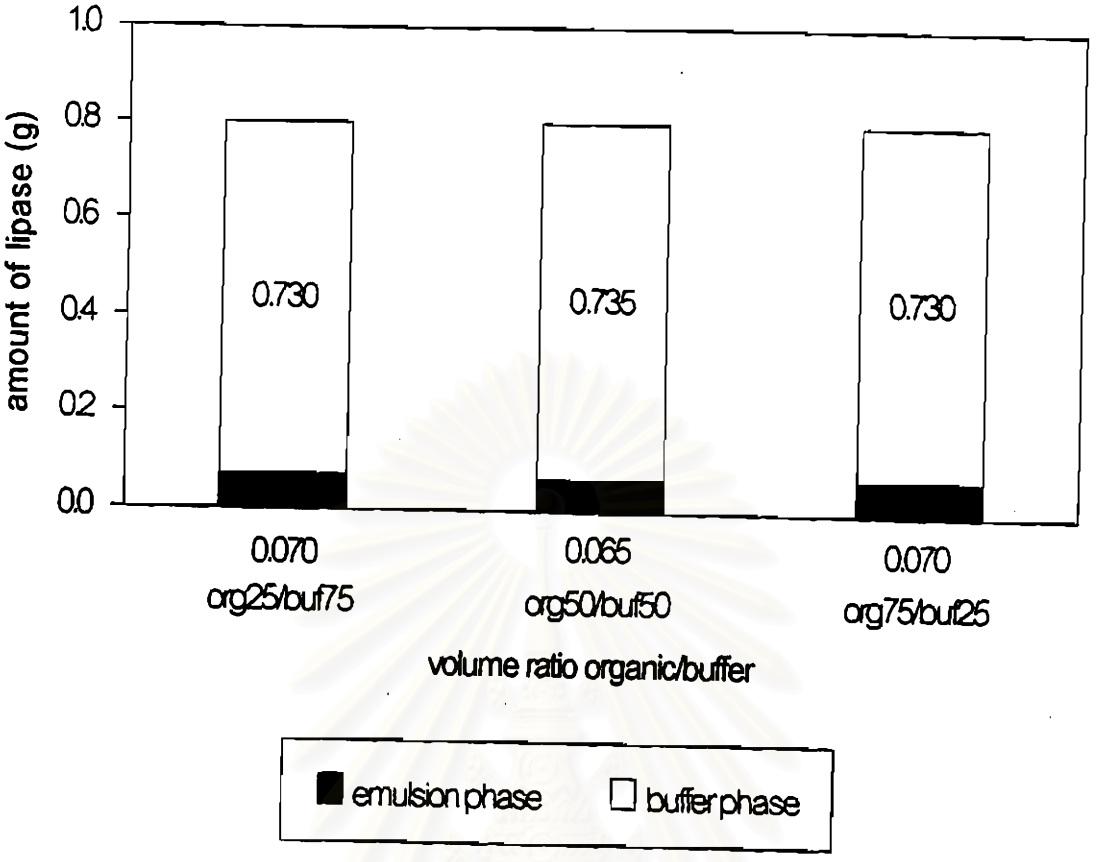


Figure 5.8 Distribution of lipase among the two phases in various organic/buffer systems

Experimental conditions :

(±)menthol concentration	26 mM (organic phase)
hexyl acetate concentration	256 mM (organic phase)
lipase concentration	8 g/l (both phase)
organic solvent	iso-octane
pH of buffer solution	7
temperature	30 °C
stirring speed	175 rpm

The transesterification in aqueous-organic two phase systems could occur in both phases where lipase resided. However, the production rate in the aqueous phase should be much lower than that in the organic phase due to an almost insolubility of hexyl acetate in buffer solution (lower than 0.025 mM which was the gas chromatography detection limit). Therefore, the results shown in figure 5.9 should result mainly from the transesterification in the organic phase.

Figure 5.9 demonstrates that the lower organic/aqueous volume ratios the higher the reaction rate. This was most likely due to the highest interfacial area obtained in the organic/aqueous volume ratio of 25:75 since the smallest dispersed droplets were found in the organic phase.

The emulsion in aqueous-organic two phase systems were found to be very stable, and thus complicates product purification process which then results in higher capital and operating costs. We, therefore, suggest that the transesterifications by lipase should be carried out in an organic solvent rather than in the aqueous-organic two phase system, and the experimental results obtained in the first system was compatible with the latter. In addition, the enzyme recovered from iso-octane after 3-week of catalysing was found to have approximately 95% catalytic activity of the fresh enzyme.

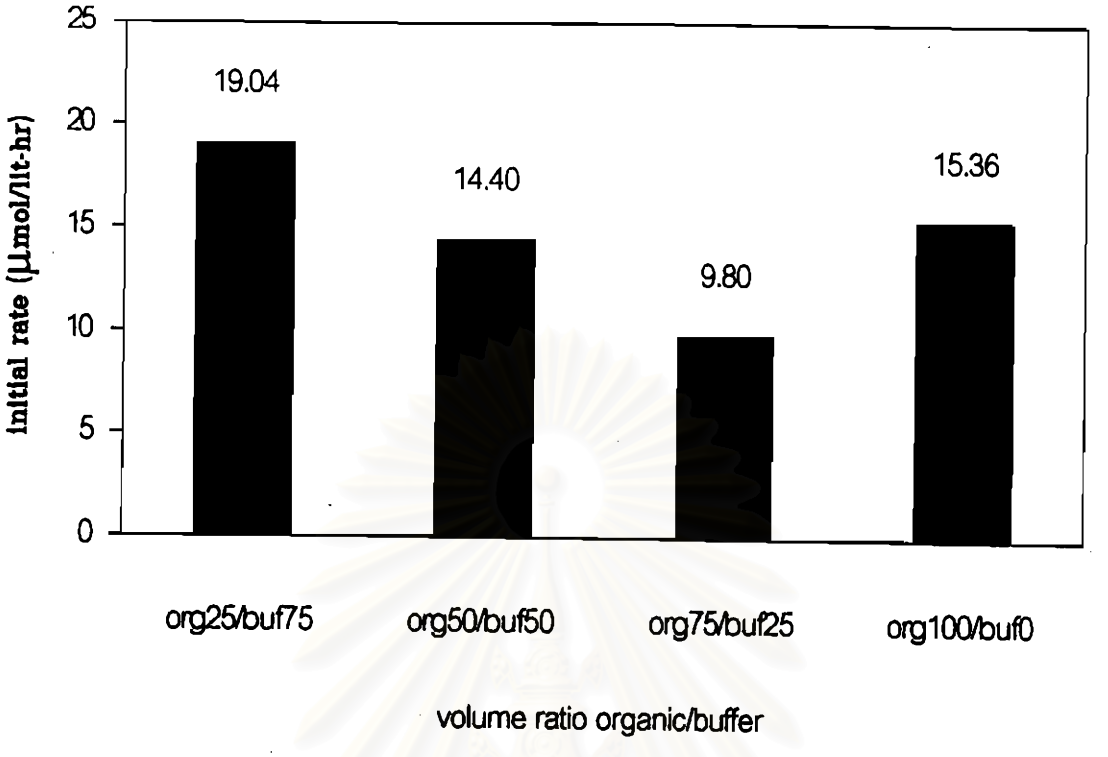


Figure 5.9 Initial rates obtained from various of aqueous-organic two phase systems with various organic/aqueous volume ratios

Experimental conditions :

(±)menthol concentration	26 mM (organic phase)
hexyl acetate concentration	256 mM (organic phase)
lipase concentration	8 g/l (both phase)
organic solvent	iso-octane
pH of buffer solution	7
temperature	30 °C
stirring speed	175 rpm

5.4 Experimental design for the optimization of the system

From the previous section, it was found that hexyl acetate and iso-octane are the most suitable acyl donor and organic solvent, respectively, for the *Candida cylindracea* lipase catalysed resolution of racemic menthol. Thus, they were used in succeeding experiments for condition optimisation of this specific system. Experimental design (Box et al., 1978) was applied to the reaction system to investigate influences of key parameters such as racemic menthol concentration (mM), hexyl acetate concentration (mM), temperature (°C), and stirring speed (rpm) on enzyme specific activity using 2^4 factorial design and central composite design (CCD), (see appendix A).

Advantages of the experimental design planning are as followed:

- A number of experiments can be reduced, thus time and money requirement is minimised.

- Main and interaction effects of key parameters on enzyme specific activity can be clearly distinguished and specified.

Results from designed experiments are shown in appendix A. It was found that key parameters which were main effects of the system were racemic menthol concentration, hexyl acetate concentration, and temperature, and interaction effects were between racemic menthol concentration and temperature, and hexyl acetate concentration and temperature. However, only interaction effects will be considered, and their response surfaces are plotted as shown in figure 5.13 and 5.14.

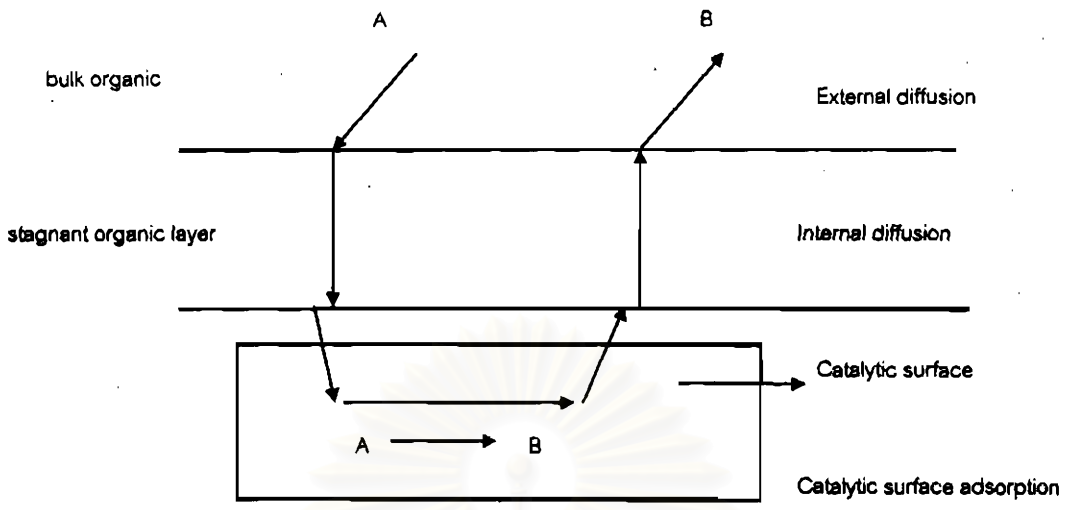
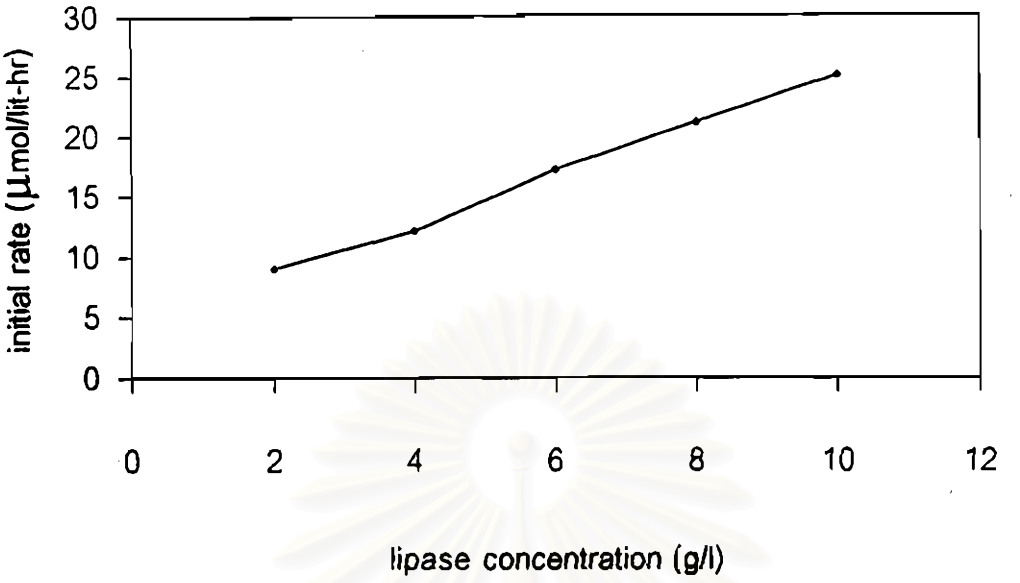


Figure 5.10 Steps in a heterogeneous catalytic reaction (Fogler, 1992)

The resolution of racemic menthol by *Candida cylindracea* lipase in iso-octane is a heterogeneous catalytic reaction since the enzyme is insoluble in the organic solvent. Therefore, the reaction mechanism can be divided into seven steps as shown in figure 5.10. The first step is diffusion of the reactant(s) (e.g., A) from bulk organic to the external surface of stagnant organic layer. And step 2 is diffusion of the reactant from external to internal surface of stagnant organic layer. Then, reactant A is adsorped onto the catalyst surface. Next, reaction occurs on the surface of the catalyst (e.g., A→B). And then, product(s) (e.g., B) is desorped from the surface of the catalyst. Step 6,product is diffused from internal to external surface of stagnant organic layer. The last step, product is diffused to bulk organic phase.

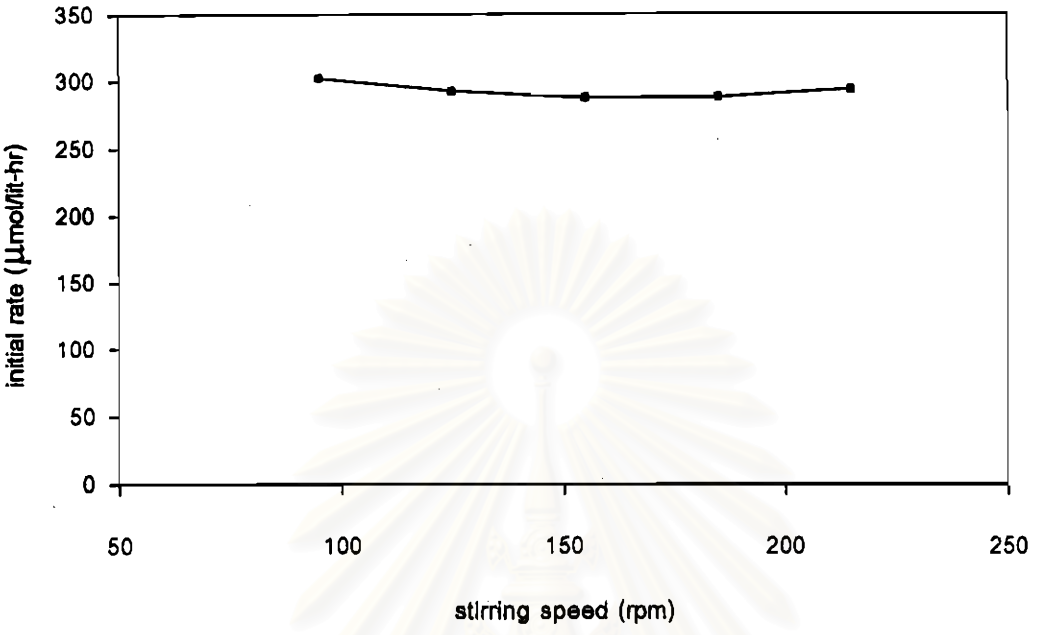


Experimental conditions:

(±)menthol concentration	12.8 mM
hexyl acetate concentration	192 mM
temperature	30 °C
stirring speed	175 rpm

Figure 5.11 The relationship between initial rate and lipase concentration

Figure 5.11 correlates a lipase concentration to initial reaction rate, the straight line indicates a catalytic controlled reaction at a stirring speed of 175 rpm. Results shown in figure 5.12, in addition, lead us to conclude that the reaction is catalytic controlled operating in the stirring speed range of 95 to 215 rpm since the change in initial rate due to stirring speed is in an experimental error boundary (10.32%).



Experimental conditions:

(±)menthol concentration	60 mM
hexyl acetate concentration	260 mM
temperature	60 °C

Figure 5.12 The relationship between initial rate and stirring speed

5.4.1 Effect of racemic menthol concentration and temperature

Figure 5.13 shows interaction effects between racemic menthol concentration and temperature. It was noticed that the initial rate of transesterification reaction increased with racemic menthol concentration at a fixed hexyl acetate concentration of 260 mM. Nevertheless, inhibition occured at high substrate concentration. It's worth noticing that substrate inhibition was more pronounced at higher temperature since the inhibition level reduced from 80 mM at 30 °C to 60 mM at 90 °C.

Temperature is another key parameter affecting initial rate of the reaction, since it increases the reaction rate constant (k), according to Arrhenius equation. However, at temperature beyond a certain point, initial rate was found to decrease with increasing temperature which was due to enzyme denaturation. In addition, it was found that the enzyme was more thermostable catalysing at low menthol concentration since its lost in activity started at the temperature higher than 60 °C with menthol concentration higher than 20 mM, while it was 75 °C at 20 mM. The optimum temperature for the transesterification by lipase from *Candida cylindracea* was optimum at 66 °C (see section 5.4.3).

From the experiments, the activation energy (E) of transesterification reaction of racemic menthol and hexyl acetate in organic system was determined at 47.124 kJ/mol.

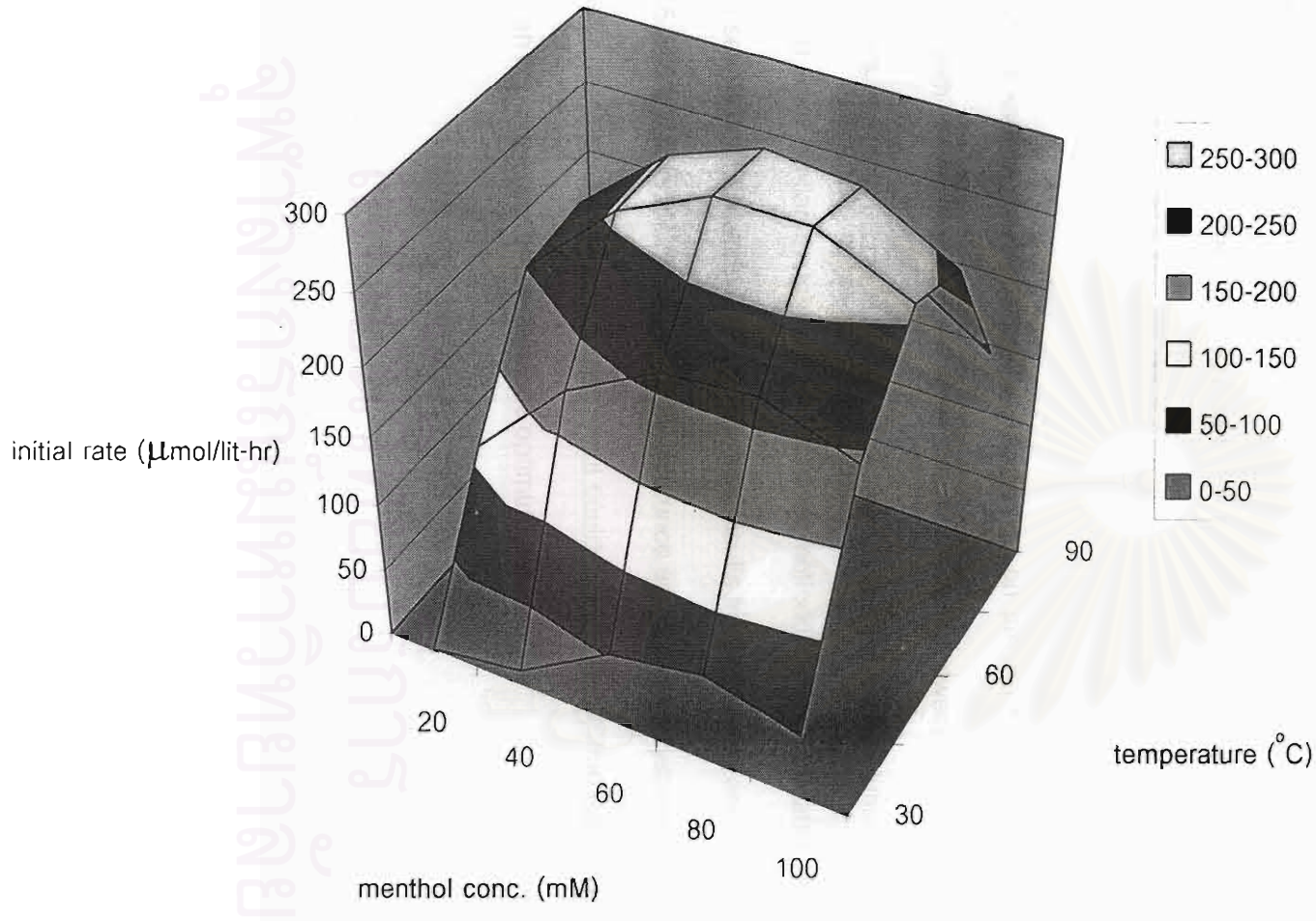


Figure 5.13 Response surface of interaction effect between menthol concentration and temperature

5.4.2 Effect of hexyl acetate concentration and temperature

The response surface in figure 5.14 shows interaction effect of hexyl acetate concentration and temperature. Similar to what was found in the case of menthol, hexyl acetate also inhibited the reaction at certain concentrations. It was found that hexyl acetate started to inhibit the reaction at the concentration of 380 mM under the whole temperature range from 30 to 90 °C. In contrast to what was found in case of menthol, hexyl acetate did not effect the enzyme thermostability since the loss of its activity was detected at operating temperatures higher than 60 °C under the whole concentration range of hexyl acetate studied.

To our knowledge, there has been no report on the transesterification of racemic menthol with hexyl acetate, therefore, direct comparison between results from this work and others could not be made. However, table 5.1 demonstrates optimum temperatures achieved from similar systems. It is obviously noticed that the suitable temperature range seems to lie between 25 to 45 °C. Only results from this work and Lokotsch's that are 65 and 90 °C, respectively. The only difference among these systems are type of the second substrate (acyl donor). Therefore, it could be concluded that type of acyl donor plays an important role determining the optimum temperature.

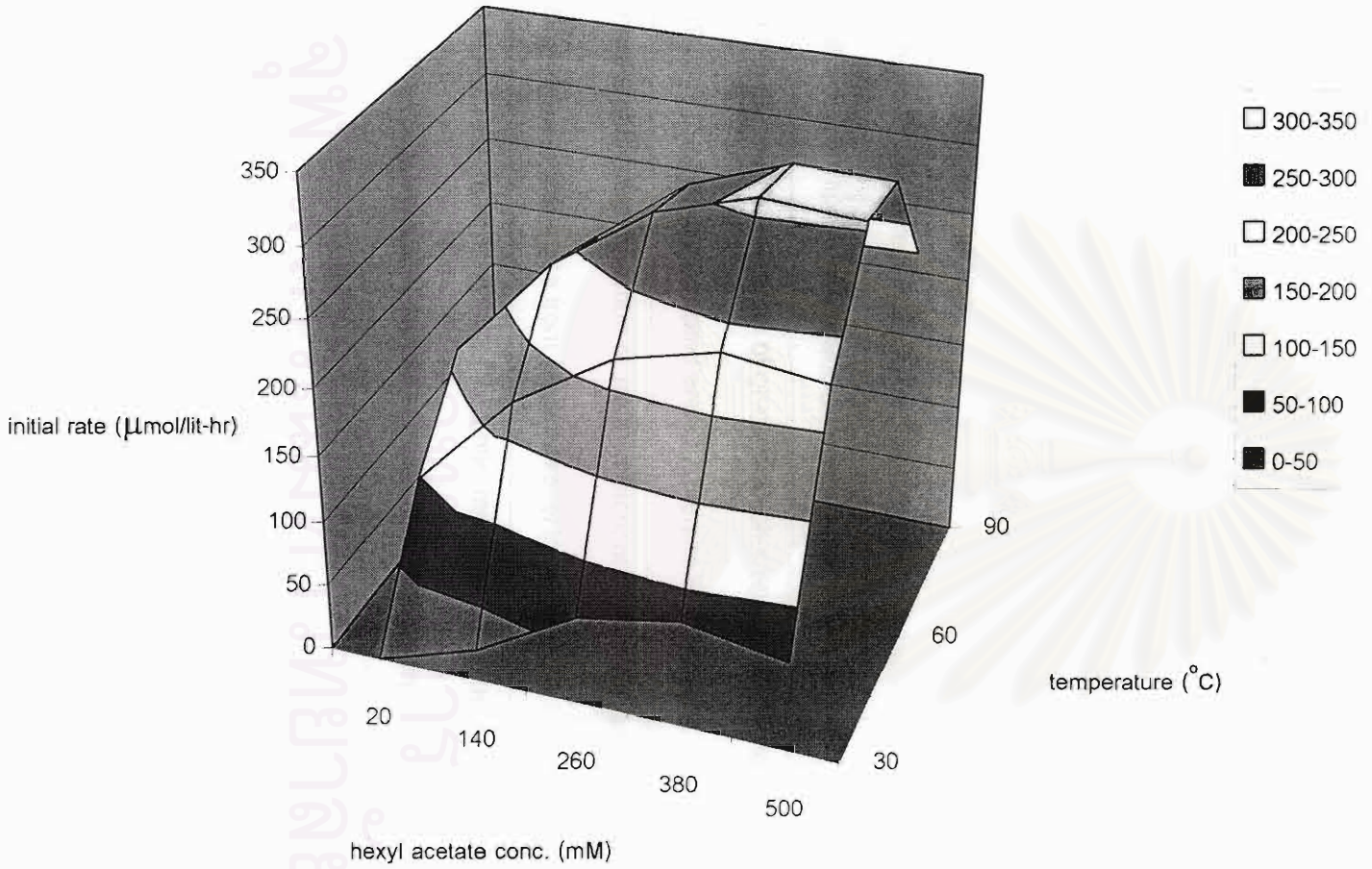


Figure 5.14 Response surface of interaction effect between hexyl acetate concentration and temperature

Table 5.1 Comparison of optimum temperatures obtained from various systems

Researchers	Reaction	Optimum Temperature
Derango et al., 1994	Transesterification of 2-hydroxyethylcarbamate and vinyl methacrylate in toluene/THF (3:1)	50 °C
Goto et al., 1994	Esterification of benzyl alcohol and lauric acid by lipase from *Pseudomonas sp.* in iso-octane	25 °C
Kamiya et al., 1995	Esterification of (-)menthol and lauric acid by lipase from *Candida cylindracea* in iso-octane	35 °C
Lokotsch et al., 1989	Interesterification of (-)menthol and triacetin by lipase from *Candida cylindracea* in iso-octane	90 °C
Martins et al., 1993	Esterification of glycidol alcohol and butyric acid by lipase from Porcine pancreas in chloroform	35 °C
Razafindralambo Et al., 1994	Esterification of isoamyl alcohol and acetic acid by lipase from *Mucor miehei* in n-heptane	45°C
Xu et al., 1995	Esterification of racemic menthol and propionic anhydride by lipase from *Candida cylindracea* in cyclohexane	30 °C
This work	Transesterification of racemic menthol and hexyl acetate by lipase from *Candida cylindracea* in iso-octane	66 °C

5.4.3 Optimum conditions

In order to determine the optimum conditions for the resolution of racemic menthol by *Candida cylindracea* lipase in iso-octane, Myers's (1979) method was used (see appendix A2). These conditions are:

concentration of racemic menthol	= 73	mM
concentration of hexyl acetate	= 360	mM
temperature	= 66	°C
stirring speed	= 110	rpm

The optimum specific reaction rate found was 79.5 mmol/hr-g.enz while the final conversion was 27.12% calculated base on racemic menthol.

5.4.4 Comparing results from this work with other researchers

In this section, the comparison between the results of transesterification of racemic menthol and hexyl acetate by lipase from *Candida cylindracea* operating under the optimum condition in iso-octane (this work) and those of Lokotsch et al (1989) for the interesterification of (-)menthol and triacetin are made. It was found that the optimum conditions for both system are quite different (see Table 5.2).

To consider the specific rate of transesterification reaction (at optimum conditions), this research is 79.5 μmol/hr-g.enz while Lokotsch et al, is 24.2 μmol/hr-g.enz. In both cases, (-)menthyl acetate is the main product. So, we can conclude that hexyl acetate as acyl donor gives higher specific rate than triacetin.

Table 5.2 Comparison results between experiment and Lokotsch et al (1989)

	This work	Lokotsch et al (1989)
Lipase from	*Candida cylindracea*	*Candida cylindracea*
Organic solvent	iso-octane	iso-octane
Menthol concentration	73 mM	12.8 mM
Acyl donor concentration	360 mM	9.1 mM
Optimum temperature	66 °C	90 °C
Specific rate	79.5 μmol/hr-g.enz	24.2 μmol/hr-g.enz

The remarkable difference in optimum conditions between the two works were substrate concentrations which were, in this work, more than six times higher than Lokotsch's (1989). The difference indicates that enzyme affinity to both substrates in Lokotsch's case was much higher than in this work.

5.5 Determination of kinetic parameters

The determination of the kinetic parameters of the transesterification reaction of racemic menthol and hexyl acetate was performed by initial rate analysis. Figure 5.15 shows the relationship of initial transesterification rate and concentration of substrates (racemic menthol and hexyl acetate) in iso-octane. It is discovered that initial rate increases with the increment of the concentrations of menthol and hexyl acetate and decreases if the concentrations of menthol and hexyl acetate were more than 60 and 320 mM, respectively. Therefore, it may be concluded that both substrates inhibit the reaction at concentrations higher than these certain values.

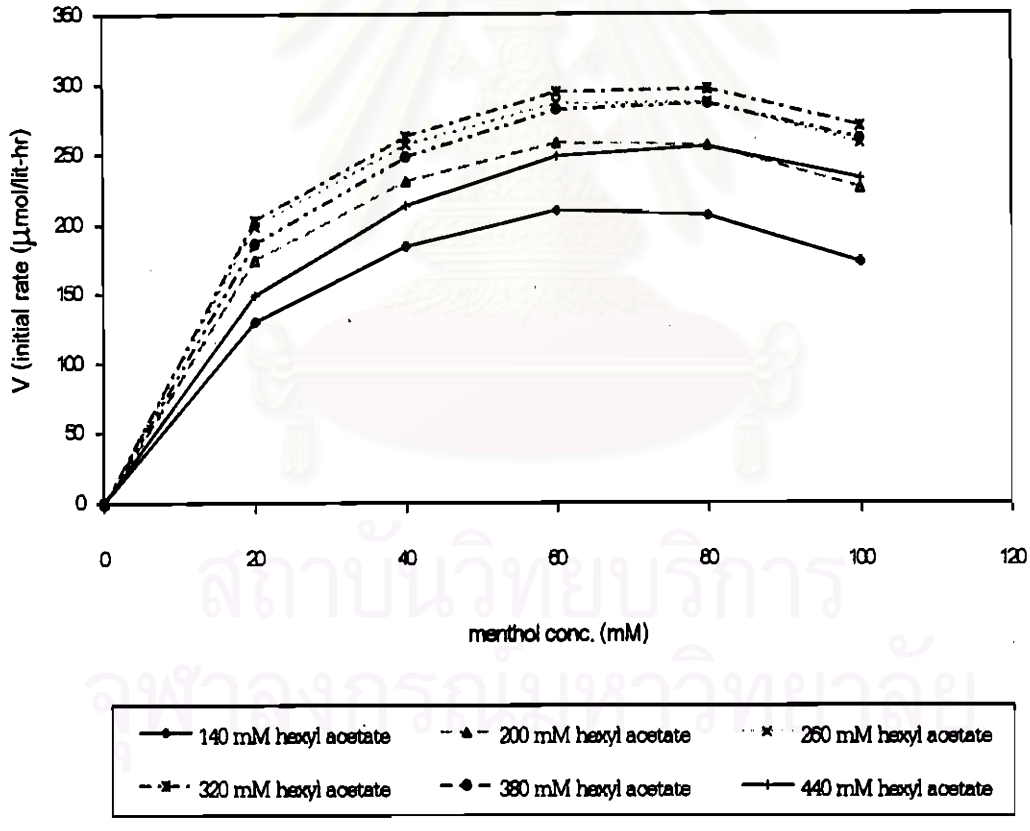


Figure 5.15 The relationship of initial reaction rate with menthol and hexyl acetate concentrations

Experimental conditions : temperature 66 °C and stirring speed 110 rpm

The reciprocal of initial rate and concentration of hexyl acetate are plotted for determination of kinetic parameters as shown in figure 5.16.

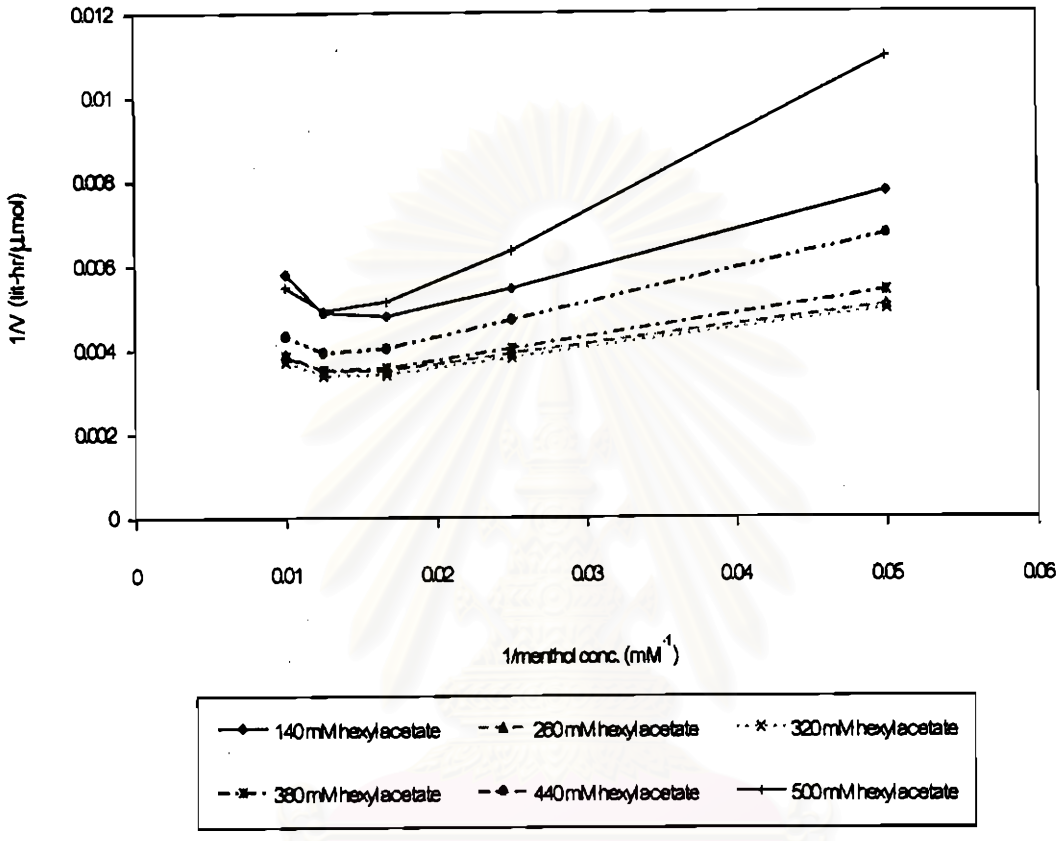


Figure 5.16 Double reciprocal plot of initial rate and menthol concentration at fixed hexyl acetate concentrations

From figure 5.16, it is noticed that the slope of the graph decreases with increasing hexyl acetate concentration until the concentration of 320 mM is reached. On the contrary, it increases with hexyl acetate concentration. This phenomenon indicates that the reaction studied follows random bi bi mechanism as was suggested by Segel (1975 ; See appendix B).

Kinetics of similar reactions were studied, and they were mostly identified to follow ping-pong bi bi mechanism. For example, an esterification of oleic acid and ethanol by

immobilized lipase of *Mucor miehei* (Chulalaksananukul et al, 1990), and an esterification of (-)menthol and lauric acid by *Penicillium simplicissimum* lipase in water-in-oil microemulsions (Stamatis et al, 1993). However, Lerktanakit (1997) found that the transesterification of (±)menthol and triacetin by *Candida cylindracea* lipase in AOT/iso-octane reverse micelles followed the random bi bi mechanism which corresponds to this study. The following equation represents the rate law corresponding to random bi bi mechanism with two substrate inhibitions.

$$\frac{V}{V_{max}} = \frac{[A][B]}{\alpha K_A K_B + \alpha K_B[A](1+\frac{[A]}{K_{i2}}) + \alpha K_A[B](1+\frac{[B]}{K_{i1}}) + [A][B]} \qquad (5.1)$$

It is, therefore, our task to determine all the kinetic parameters involved. Considering figure 5.16, it is concluded that menthol at concentrations ([B]) lower than a certain value did not inhibit the reaction. It, thus, can be assumed that $[B]/K_{i1} << 1$ and, as a result, can be neglected. Thus, equation 5.1 can be rewritten as:

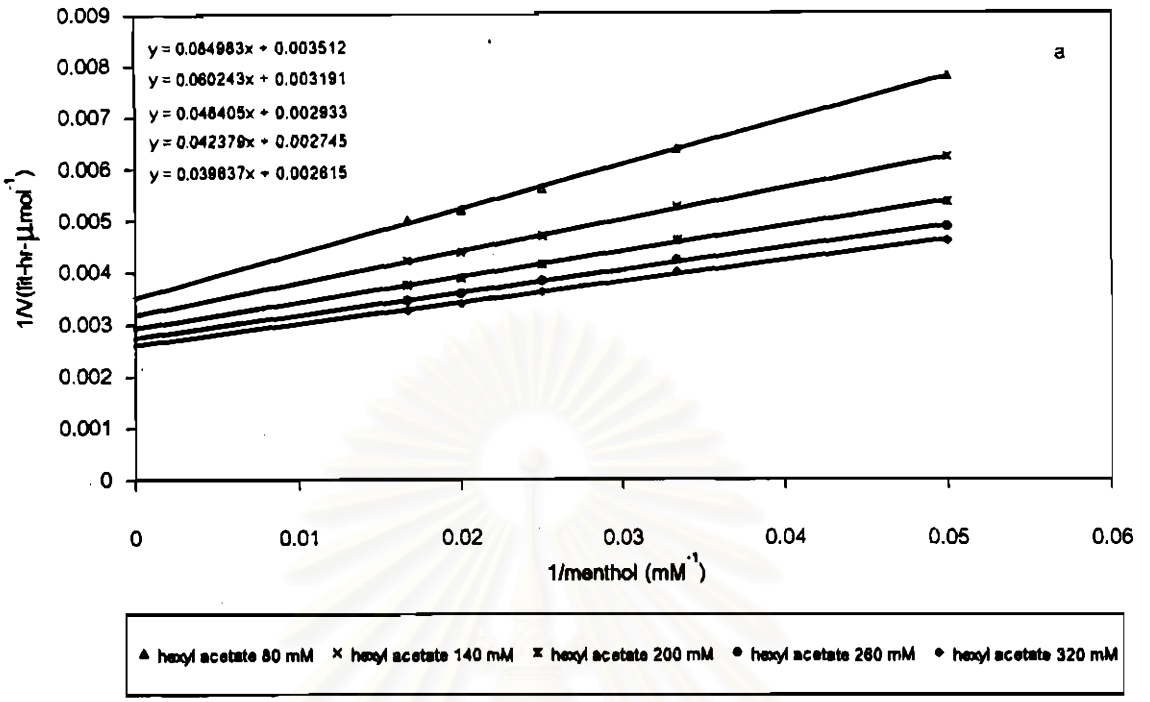
$$\frac{V}{V_{max}} = \frac{[A][B]}{\alpha K_A K_B + \alpha K_B[A](1+\frac{[A]}{K_{i2}}) + \alpha K_A[B] + [A][B]}$$

At a fixed hexyl acetate concentration ([A])

$$\frac{V}{V_{max}} = \frac{[B]}{\alpha K_B(1+\frac{[A]}{K_{i2}}+\frac{K_A}{[A]}) + [B](1+\frac{\alpha K_A}{[A]})} \qquad (5.2)$$

Then

$$\frac{1}{V} = \frac{1}{[B]}\frac{\alpha K_B}{V_{max}}(1+\frac{[A]}{K_{i2}}+\frac{K_A}{[A]}) + \frac{1}{V_{max}}(1+\frac{\alpha K_A}{[A]}) \qquad (5.3)$$



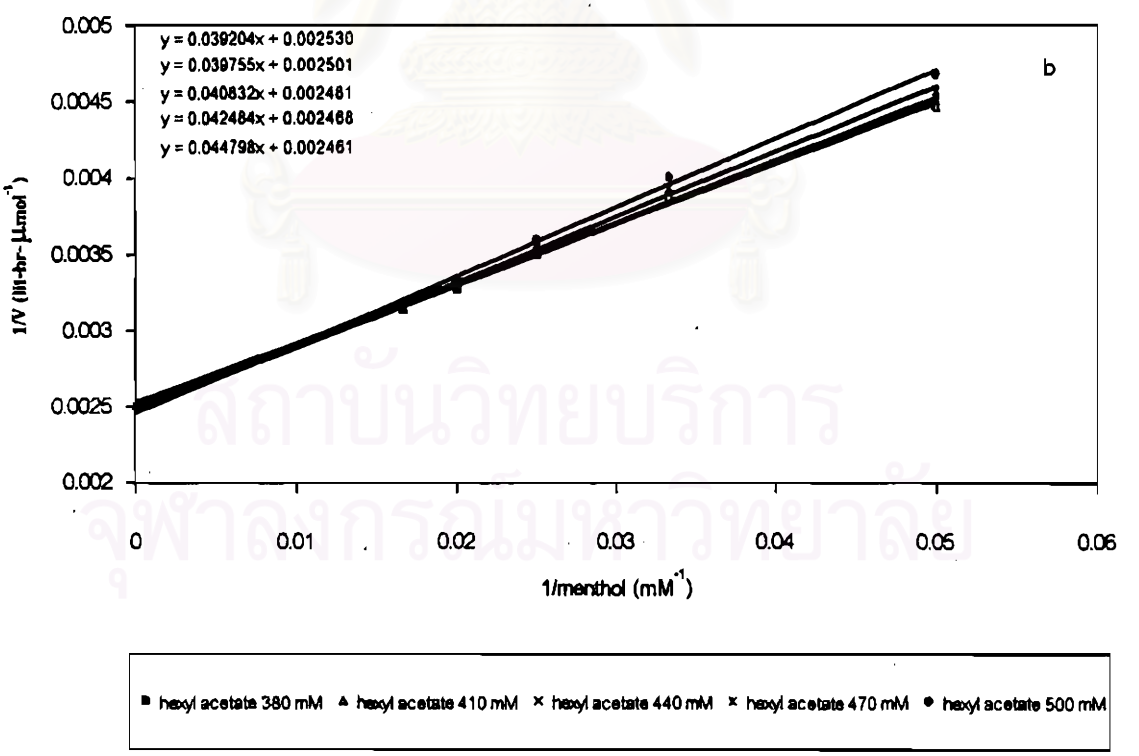


Figure 5.17 Double reciprocal plot of initial rate and menthol concentration (a) in the low hexyl acetate concentration range (b) in the high hexyl acetate concentration range

Figure 5.17 demonstrates the plot of 1/V and 1/[B] from equation 5.3 where the y-intercept is:

$$\text{y-intercept} = \frac{1}{V_{max}}\left(1 + \frac{\alpha K_A}{[A]}\right) \qquad (5.4)$$

Figure 5.17 shows only results obtained when menthol was used at concentrations lower than its inhibition level. This graph can be divided into two parts; the first one under low hexyl acetate concentrations, while the last one under the high hexyl acetate concentrations.

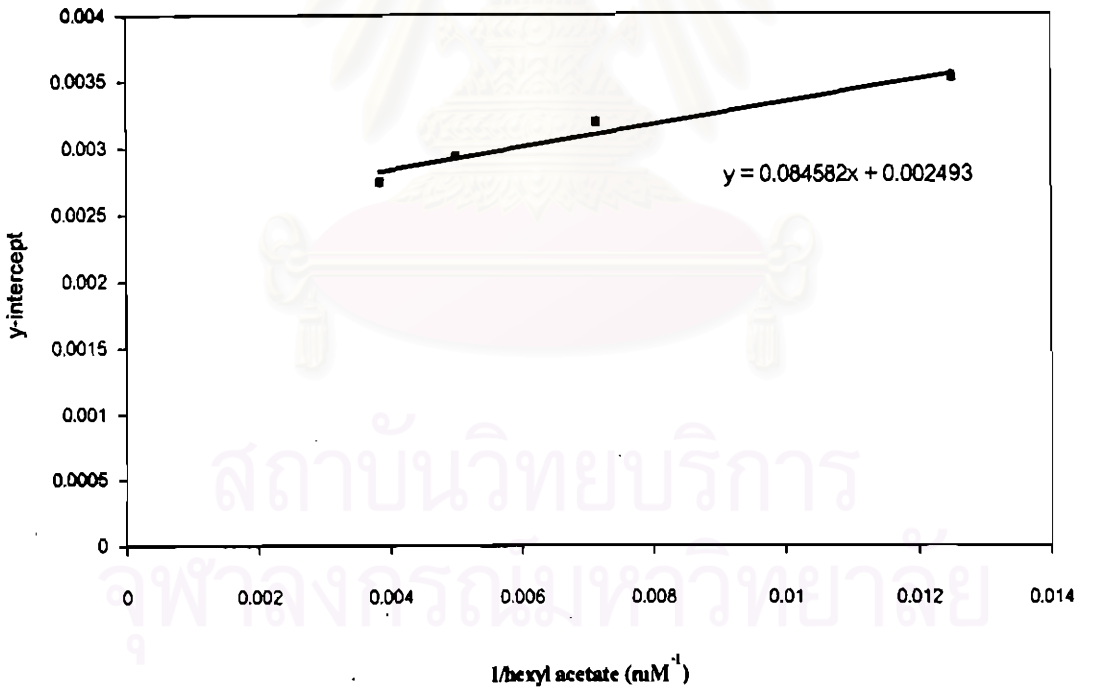


Figure 5.18 Plot of the y-intercept determined in figure 5.18 (a) and reciprocal of hexyl acetate concentration

From equation 5.4, it is demonstrated that the plot of y-intercept and reciprocal of concentration of hexyl acetate gives $1/V_{max}$ as the y-intercept, and $\alpha K_A/V_{max}$ as the slope.

$$\text{y-intercept} = \frac{1}{V\,max} = 0.002493$$

Therefore; $V_{max} = 401.12$ μmol/lit-hr

$$\text{slope} = \frac{\alpha K_A}{V\,max} = 0.084582$$

Therefore; $\alpha K_A = 33.93$ mM

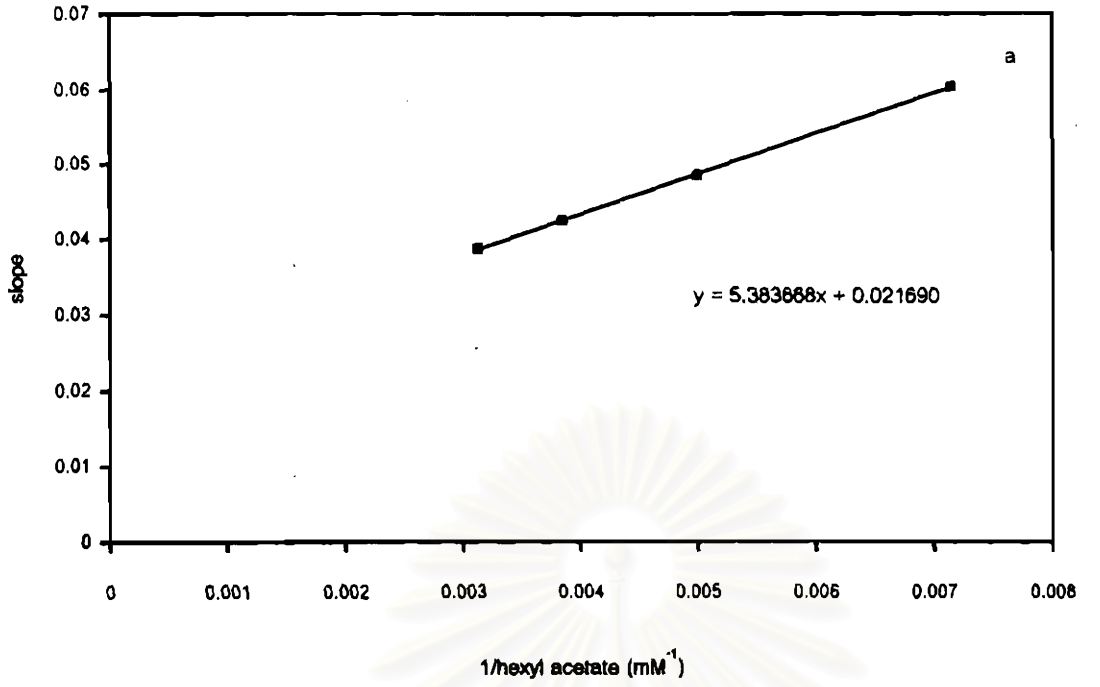
The slopes from equation 5.3 can be plotted double reciprocally between initial rate and concentration of menthol, and can be related to concentrations of hexyl acetate using the equation as follows:

$$\text{Slope}_{1/menthol} = \frac{\alpha K_B}{V\,max} + \frac{\alpha K_B}{V\,max\,K_{I2}}[A] + \frac{K_A(\alpha K_B)}{V\,max}\frac{1}{[A]} \qquad (5.5)$$

From equation 5.5, it is found that the relationship of the $\text{slope}_{1/menthol}$ and concentration of hexyl acetate can be divided into two cases.

Case 1 The concentration of hexyl acetate is lower than its inhibition level. So, it is assumed that $[A]/K_{I2} << K_A/[A]$ which results in the negligible $\alpha K_B[A]/V_{max}K_{I2}$ value. Consequently, equation 5.5 can be reduced to

$$\text{Slope}_{1/menthol} = \frac{\alpha K_B}{V\,max} + \frac{K_A(\alpha K_B)}{V\,max}\frac{1}{[A]} \qquad (5.6)$$



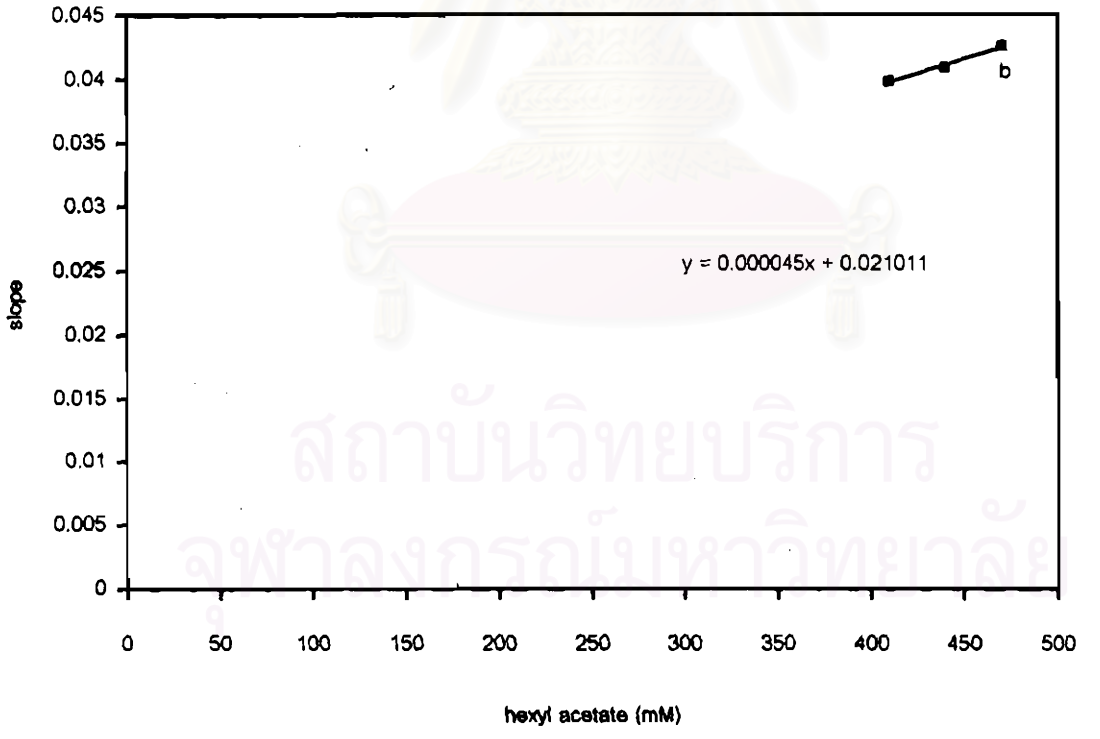


Figure 5.19 Plot of the slope determined in figure 5.17 (a) and (b) and

(a) reciprocal of low hexyl acetate concentration

(b) high hexyl acetate concentration

Plot of the $slope_{1/menthol}$ and reciprocal of concentration of hexyl acetate is demonstrated in figure 5.19(a).

$$\text{y-intercept} = \frac{\alpha K_B}{V_{max}} = 0.021690$$

Since V_{max} was determined at 401.12 μmol/lit-hr.

$$\alpha K_B = 8.7 \text{ mM}$$

$$\text{slope} = \frac{K_A(\alpha K_B)}{V_{max}} = 5.383668$$

Therefore; $K_A = 248.22$ mM

Case 2 The concentration of hexyl acetate is higher than its inhibition level. Therefore, it can be assumed that $[A]/K_{i2} >> K_A/[A]$ which results in the negligible $(K_A(\alpha K_B))/(V_{max}[A])$. Consequently, equation 5.5 can be reduced to

$$Slope_{1/menthol} = \frac{\alpha K_B}{V_{max}} + \frac{\alpha K_B}{V_{max} K_{i2}}[A] \qquad (5.7)$$

A Plot of the $slope_{1/menthol}$ and concentration of hexyl acetate is shown in figure 5.19 (b).

$$\text{slope} = \frac{\alpha K_B}{V_{max} K_{i2}} = 0.000045$$

Since αK_B , and V_{max} were determined at 8.7 mM and 401.12 μmol/lit-hr, respectively.

Therefore, $K_{I2} = 481.98$ mM

Similarly, the plot of reciprocal of initial rate with concentration of hexyl acetate less than its inhibition level can be obtained, and the term $[A]/K_{I2} << 1$ and, thus, can be neglected. Consequently, equation 5.1 can be reduced to

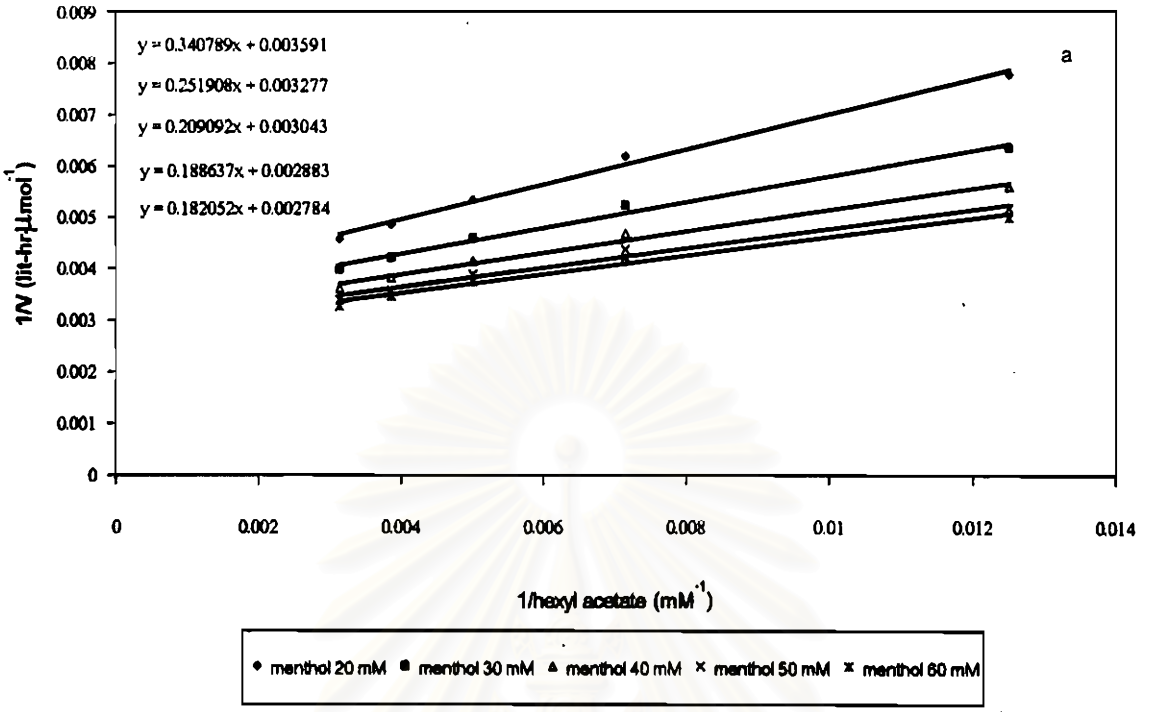
$$\frac{V}{V\,max} = \frac{[A][B]}{K_A\alpha K_B + \alpha K_B[A] + \alpha K_A[B](1 + \frac{[B]}{K_{I1}}) + [A][B]} \qquad (5.8)$$

At fixed [B]

$$\frac{V}{V\,max} = \frac{[A]}{\alpha K_A(1 + \frac{[B]}{K_{I1}} + \frac{K_B}{[B]}) + [A](1 + \frac{\alpha K_B}{[B]})}$$

$$\frac{1}{V} = \frac{1}{[A]}\frac{\alpha K_A}{V\,max}(1 + \frac{[B]}{K_{I1}} + \frac{K_B}{[B]}) + \frac{1}{V\,max}(1 + \frac{\alpha K_B}{[B]}) \qquad (5.9)$$

Figure 5.20(a) shows a relationship of double reciprocal of initial rate and concentration of hexyl acetate.



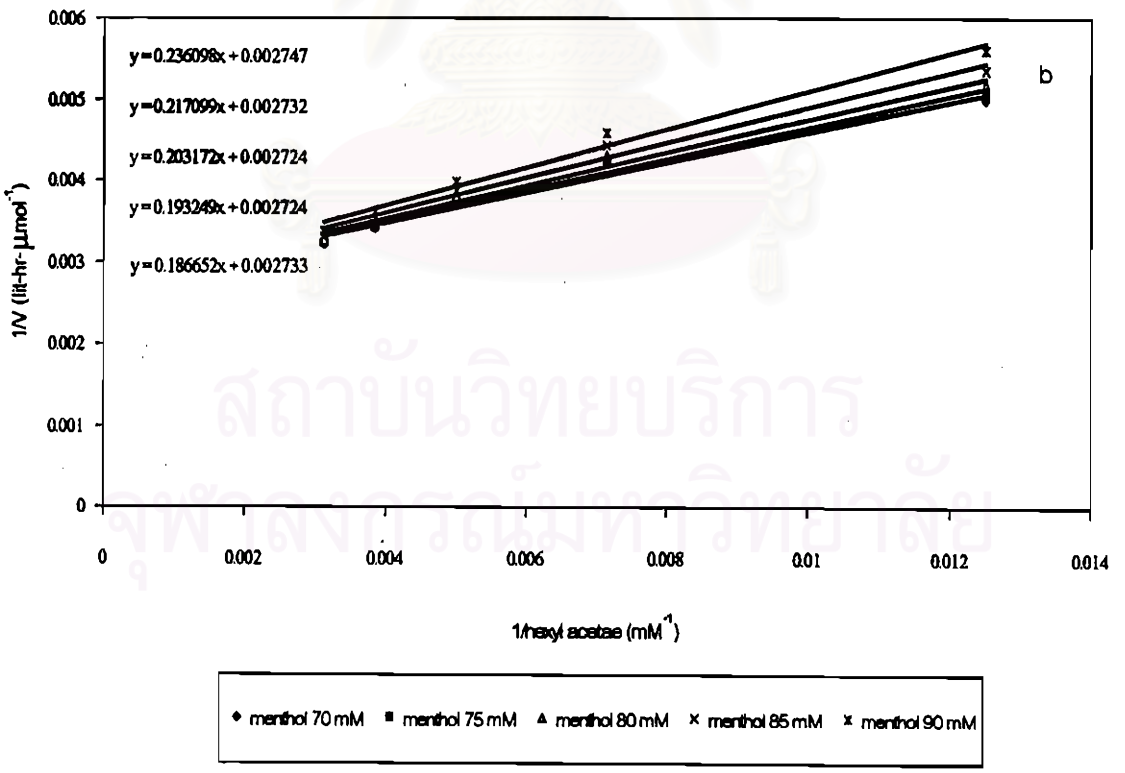


Figure 5.20 Double reciprocal plot of initial rate and hexyl acetate

Concentration (a) low menthol concentration (b) high menthol concentration

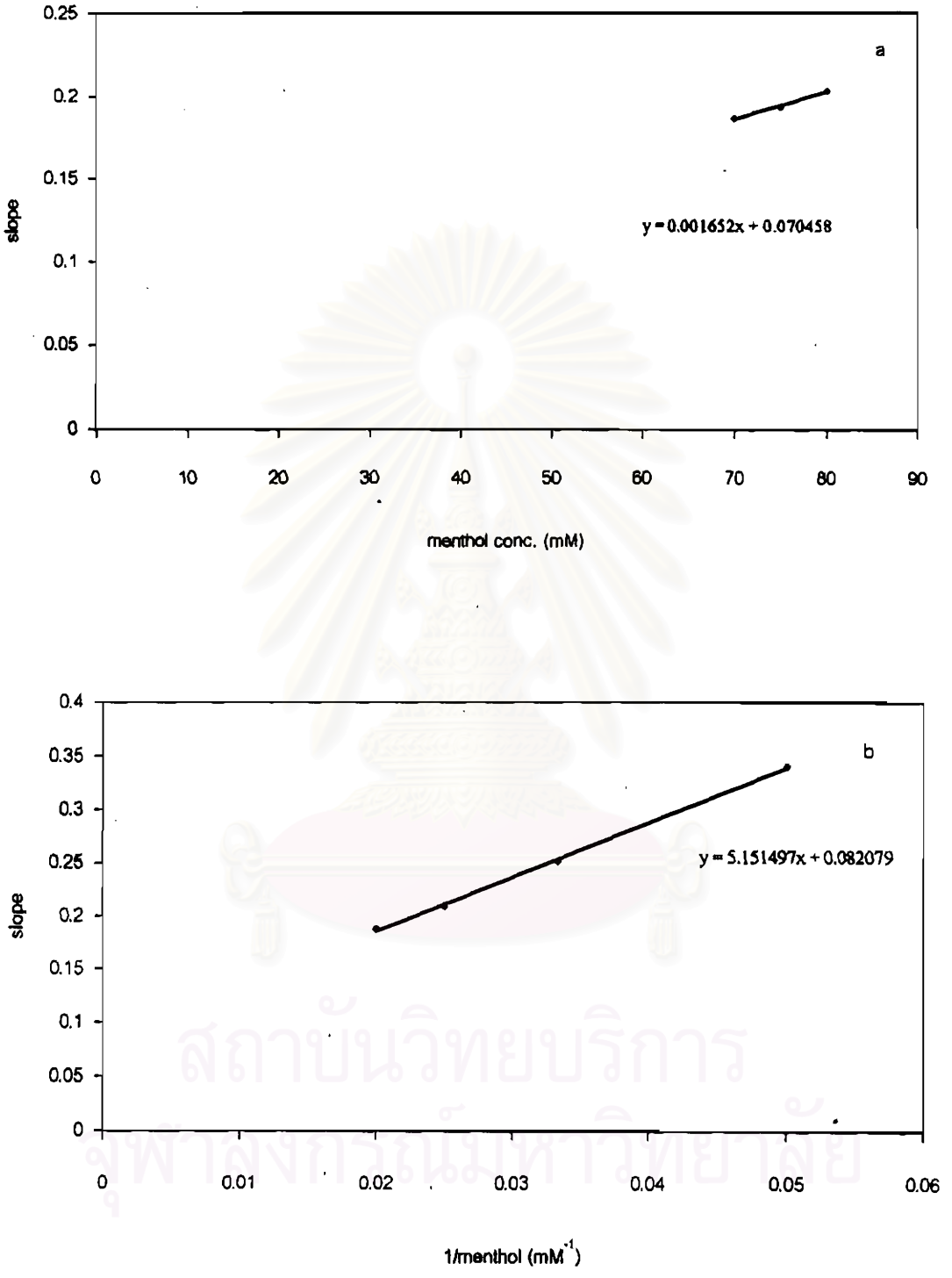


Figure 5.21 Plot of the slope determined in figure 5.20 (a) and (b) and

(a) low menthol concentration (b) reciprocal of high menthol concentration

From equation 5.9,

$$\text{Slope}_{\text{1/hexyl acetate}} = \frac{\alpha K_A}{V_{max} K_{I1}}[B] + \frac{(\alpha K_A)K_B}{V_{max}}\frac{1}{[B]} + \frac{\alpha K_A}{V_{max}} \qquad (5.10)$$

When concentration of menthol is higher than its inhibition level, it can be assumed that $[B]/K_{I1} >> K_B / [B]$. Therefore, equation 5.10 can be reduced to

$$\text{Slope}_{\text{1/hexyl acetate}} = \frac{(\alpha K_A)[B]}{V_{max} K_{I1}} + \frac{\alpha K_A}{V_{max}}$$

A Plot between the $\text{slope}_{\text{1/hexyl acetate}}$ with concentration of menthol in figure 5.21 (a) results in

$$\text{Slope} = \frac{\alpha K_A}{V_{max} K_{I1}} = 0.001652$$

Since αK_A, and V_{max} were determined at 33.92 mM and 401.12 μmol/lit-hr, respectively.

Thus, $K_{I1} = 51.19$ mM

When the concentration of menthol is less than its inhibition level, so it can be assumed that $K_B/[B] >> [B] / K_{I1}$. Consequently, equation 5.10 can be reduced to

$$\text{Slope}_{\text{1/hexyl acetate}} = \frac{(\alpha K_A)K_B}{V_{max}}\frac{1}{[B]} + \frac{\alpha K_A}{V_{max}}$$

A Plot between the $slope_{1/hexyl\ acetate}$ with reciprocal of concentration of menthol in figure 5.21 (b) results in

$$Slope = \frac{(\alpha K_A)K_B}{V\ max} = 5.151497$$

Since αK_A , and V_{max} were determined at 33.92 mM and 401.12 μmol/lit-hr, respectively.

Thus, $K_B = 60.92$ mM

In summary, kinetic parameters determined in this study were V_{max} = 401.12 μ mol/lit-hr , αK_A = 33.92 mM , αK_B = 8.42 mM , K_A = 248.22 mM , K_B = 60.92 mM , K_{I1} = 51.19 mM, and K_{I2} = 481.98 mM.

Therefore,

$$v = \frac{401.12[A][B]}{2066.4 + 8.42[A](1 + \frac{[A]}{481.98}) + 33.92[B](1 + \frac{[B]}{51.19}) + [A][B]} \quad \mu mol/lit\text{-}hr$$

or

$$v = \frac{100.28[A][B]}{2066.4 + 8.42[A](1 + \frac{[A]}{481.98}) + 33.92[B](1 + \frac{[B]}{51.19}) + [A][B]} \quad \mu mol/hr\text{-}g.enz$$