CHAPTER III

THEORY

This chapter will explain topics theoretically related to the system studied in this work which include various types of reaction media for lipase biocatalysis, criteria of solvent selection, and finally, enzyme kinetics.

3.1 Lipase in aqueous/organic two phase media

This section emphasises on lipase catalysis in aqueous/organic two phase systems. Lipases are soluble in aqueous solution. Brockman (1987) found that lipases are more active at the interfaces. Thus, he proposed a schematic model of this system for a lipolysis reaction as shown in figure 3.1. The reaction mechanism involves adsorption of lipase and transportation of the lipid substrate to the interface. The rate of lipolysis is not determined by the overall concentration of the substrate, but by the interfacial concentration. Other amphiphilic molecules that compete for interfacial sites reduce observed reaction rates by decreasing enzyme or substrate interfacial concentrations.

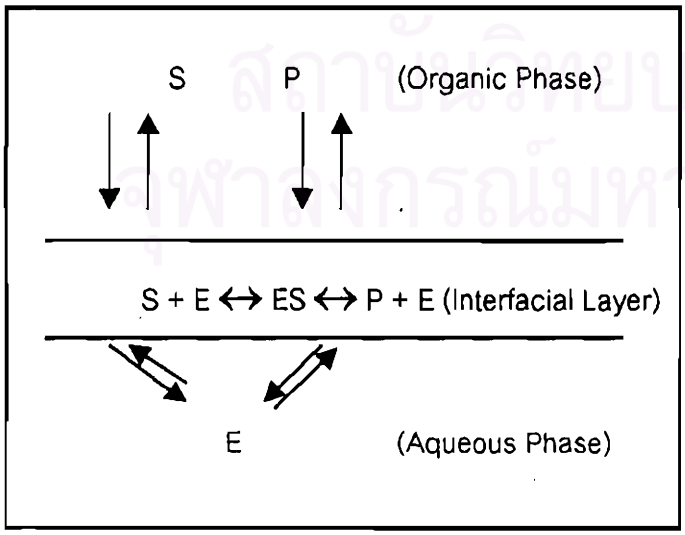

Figure 3.1 A schematic model for lipase-catalyzed hydrolysis

3.2 Lipase in nonaqueous media

It's well known that enzymes are generally denatured when it is in contact with some organic solvents. This is because enzymes loose their essential water layers (hydration layers) which help preventing their destructuring (see fig 3.2). Enzyme in an organic solvent normally suspends in bulk organic phase, and surrounded by the hydration layer. Substrates dissolved in the organic phase will diffuse through hydration layer before reaching an active site, products will then diffuse back to the bulk solvent. As mentioned in Klibanov's (1986) study, water is essential to maintain enzyme conformation, but the real question is the amount of water needed. Two modes of substrate-enzyme contact in limited-water environments have been studied intensively. The first is the concept of providing just sufficient water to hydrate the enzyme and allow it to stay locked in an active conformation, therefore, relatively nonpolar organic solvents must be used. This is the concept of an enzymatic catalysis in monophasic organic solvents. The second mode is that of an enzyme encapsulation in water-in-oil microemulsions which is called reversed micelles.

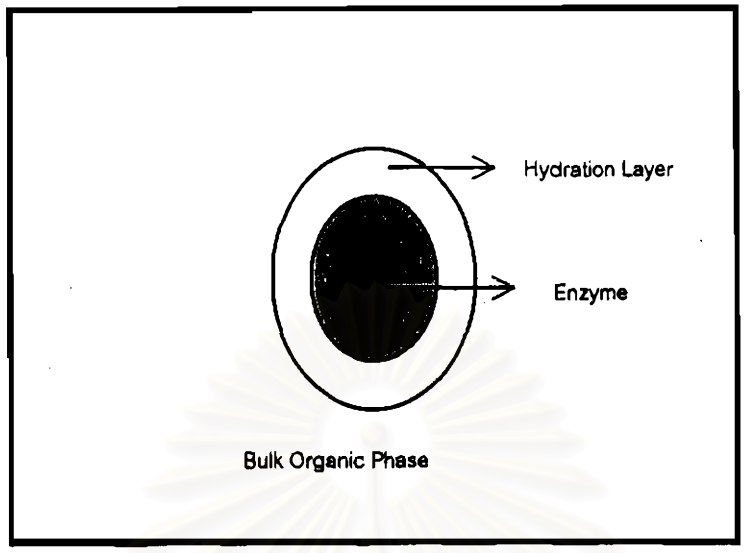

Figure 3.2 Enzymes in organic solvents

3.2.1 Lipase in organic solvents

The reaction of lipase in organic solvents can involve either the synthesis of stereoselective esters, or the resolution of racemic alcohol mixtures. For a specific example (see figure 3.3), the reaction of racemic menthol (1) with 5-phenylvaleric acid (2) was found to take place with conversion of only l-menthol to l-menthyl-5-phenylvalerate (3), leaving d-menthol (4) essentially unreacted. The lipase from *Candida cylindracea* was found to be effective in carrying out the reaction with high enantioselectivity (John and Abraham, 1991).

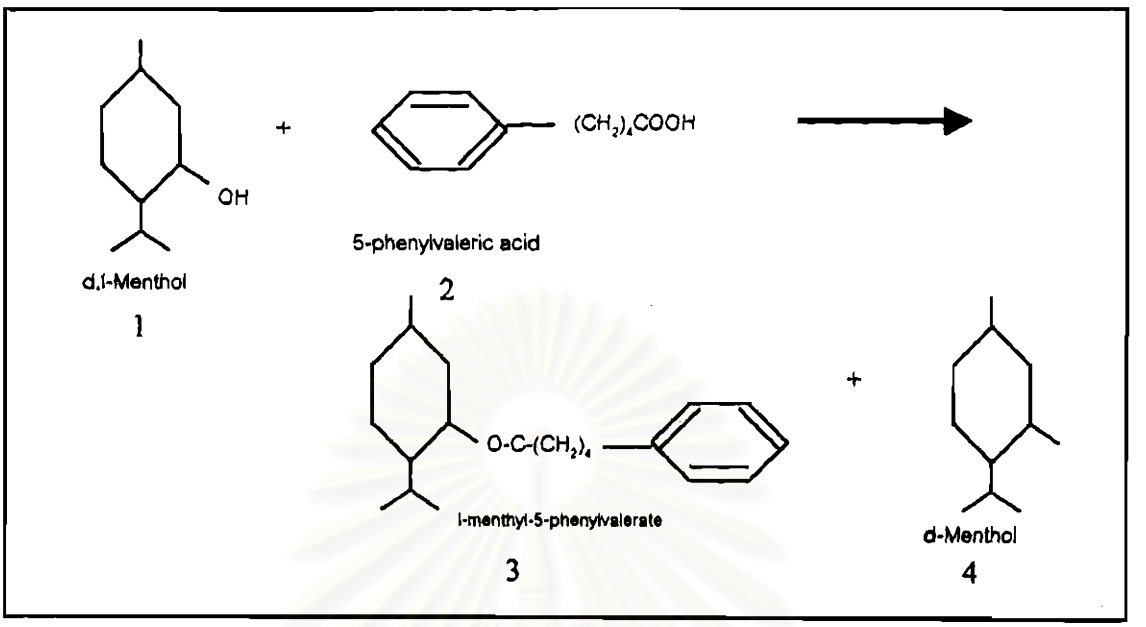

Figure 3.3 The reaction of racemic menthol using acyl donor as phenylvaleric acid

In addition to obtaining an enantiomeric ester with high optical purity, lipases can also be used to catalyse resolutions of racemic alcohols such as menthol. The schemes are: (John and Abraham, 1991)

a. esterification between:

(±)menthol + acid → (+)menthol + (-)menthyl ester + water

b. transesterification between (±)-menthol and an ester:

(±)menthol + ester → (+)menthol + (-)menthyl ester + alcohol

Separation of (-)menthol from the (-)menthyl ester can be achieved by further hydrolysis (chemical or enzymatic) of the (-)menthyl ester to (-)menthol.

c. transesterification between racemic menthyl ester and alcohol:

(±)menthyl ester + alcohol → (-)menthol + (+)menthyl ester + ester

followed by chemical hydrolysis of the (+)menthyl ester.

d. hydrolysis of racemic menthyl esters:

(±)menthyl ester + water → (-)menthol + (+)menthyl ester + acid

and further chemical hydrolysis of the (+)menthyl ester after separation from (-)menthol. Chiral terpene alcohols and derived esters have applications in pharmaceuticals, food flavor chemistry, and cosmetics industry such as fragrance constituents and enhancers.

3.2.2 Lipase in reverse micelles

In recent years, reverse micelles had been extensively investigated [Han and Rhee (1986), Rhee et al (1987), Barbaric and Luisi (1981), and Luisi et al (1983)]. Reverse micelles are formed by amphiphilic molecules (surfactants) in organic solvents : the polar groups (heads) of the surfactant molecules are directed towards the internal of the spherical aggregate, forming a polar core and the surfactant aliphatic chains direct towards the bulk organic solvent. Water can be solubilized in the polar core of reverse micelles, forming the water pool. Reverse micelles can be envisaged as droplets of water maintained in solution in apolar solvents by the action of the surfactant. Enzyme is contained in the water pool of reverse micelles. Substrates and products are dissolved in the bulk organic solvent. Reaction occurs at an interface between the water pool and bulk organic phase. Products partition back into the bulk organic phase after reaction proceeds. Reverse micelles are of large interfacial area which is the benefit of this system. However, the main problem is the difficulty in downstream separation (Luisi and Laane ,1986).

3.3 Selection of a suitable organic solvent

It has become apparent in the past few years that many biocatalysts can work well in various organic media. This is especially true for rather hydrophobic solvents. To date the general consensus seems to be that high biocatalytic activity is favoured in relatively hydrophobic solvents and that none or low activity is observed in relatively hydrophilic solvents. This fact seems to be biocatalyst and system independent, since it holds for whole (bacterial) cells, free or immobilized enzymes, as well as for two-liquid-phase systems, pure (dry) organic solvents, or aqueous media saturated with organic solvents. Log P can be used as an indicator to organic solvent hydrophobicity ; the higher log P value the more hydrophobic the solvent. Log P is defined as the logarithm of the partition coefficient in a standard octanol-water two-phase system:

$$P = \frac{[\text{solute}]_{\text{octanol}}}{[\text{solute}]_{\text{water}}} \qquad (3.1)$$

In analogy with other indicators, Brink and Tramper (1985) plotted the activity-retention data against log P-values of various organic solvents (Figure 3.4). Activities were found to be low in relatively hydrophilic solvents having log P < 2, were quite high in solvents having log P between 2 and 4, and highest in hydrophobic solvents having log P > 4. Similar correlations were found for completely different biocatalytic systems in organic media i.e. anaerobic cells suspended in aqueous media saturated with water-immiscible organic solvents, and two different lipases in dry solvents. Laane et al (1987) showed that the activity of enzymes such as cholesterol oxidase, xanthine oxidase, and enoate reductase also follow the same trend.

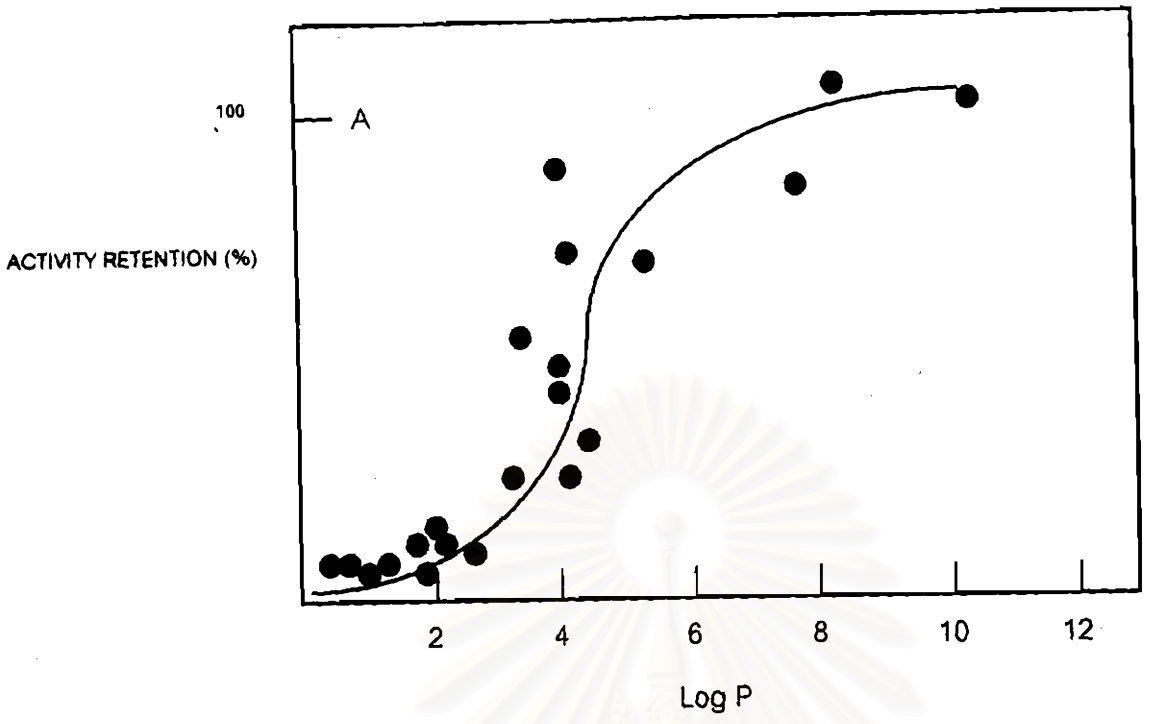

Figure 3.4 Activity retentions of epoxidizing cells exposed to organic solvents versus log P

What is so special about log P > 4 solvents? As discussed earlier, a reasonable answer to this question is that log P > 4 solvents do not distort the essential water layer around biocatalysts, thereby leaving the biocatalyst in an active state. On the other hand, solvents having a log P < 2 are in general not suitable in biocatalytic systems since they strongly distort the essential water-biocatalyst interactions, thereby inactivating or denaturating the biocatalyst. Between these extremes there are solvents (2 < log P < 4) which are weak water-distorters, and will affect biological activity to an extent which is yet rather unpredictable. In Table 3.1 the most commonly used basic organic solvents (and their corresponding log P-value) are listed . It can be seen that many solvents (> 50%) have a log P < 2 and are therefore not suitable for bioorganic synthesis. Only about 20% of the solvents listed are applicable for this purpose.

Table 3.1

Log P values of commonly used organic solvents. Log P values were calculated from hydrophobic fragmental constants according to Laane et al (1987)

Solvent	log P	Solvent	log P
1.diethylsulfoxide	-1.3	2.dioxane	-1.1
3.N,N-dimethylformamide	-1.0	4.methanol	-0.75
5.acetonitrile	-0.33	6.ethanol	-0.24
7.acetone	-0.23	8.acetic acid	-0.23
9.ethoxyethanol	-0.22	10.methylacetate	0.16
11.propanol	0.28	12.propionic acid	0.29
13.butanone	0.29	14.hydroxybenzylethanol	0.40
15.tetrahydrofuran	0.49	16.diethyamine	0.64
17.ethylacetate	0.68	18.pyridine	0.71
19.butanol	0.80	20.pentanone	0.80
21.butyric acid	0.81	22.diethylether	0.85
23.benzylethanol	0.90	24.cyclohexanone	0.96
25.methylpropionate	0.97	26.dihydroxybenzene	1.0
27.methylbutylamine	1.2	28.propylacetate	1.2
29.ethylchloride	1.3	30.pentanol	1.3
31.hexanone	1.3	32.benzylformate	1.3

Solvent	log P	Solvent	log P
33.phenylethanol	1.4	34.cyclohexanol	1.4
35.methylcyclohexanone	1.5	36.phenol	1.5
37.m-phthalic acid	1.5	38.triethylamine	1.6
39.benzylacetate	1.6	40.butylacetate	1.7
41.chloropropane	1.8	42.acetophenone	1.8
43.hexanol	1.8	43.nitrobenzene	1.8
45.heptanone	1.8	46.benzoic acid	1.9
47.dipropylether	1.9	48.hexanoic acid	1.9
49.chloroform	2.0	50.benzene	2.0
51.methylcyclohexanol	2.0	52.methoxybenzene	2.1
53.methylbenzoate	2.2	54.propylbutylamine	2.2
55.pentylacetate	2.2	56.dimethylphthalate	2.3
57.octanone	2.4	58.heptanol	2.4
59.toluene	2.5	60.ethylbenzoate	2.6
61.ethoxybenzene	2.6	62.dibutylamine	2.7
63.pentylpropionate	2.7	64.chlorobenzene	2.8
65.octanol	2.9	66.nonanone	2.9
67.dibutylether	2.9	68.styrene	3.0
69.tetrachloromethane	3.0	70.pentane	3.0
71.ethylbenzene	3.1	72.xylene	3.1
73.cyclohexane	3.2	74.benzophenone	3.2
75.propoxybenzene	3.2	76.diethylphthalate	3.3
77.nonanol	3.4	78.decanone	3.4

Solvent	log P	Solvent	log P
79.hexane	3.5	80.propylbenzene	3.6
81.butylbenzoate	3.7	82.methylcyclohexane	3.7
83.ethyloctanoate	3.8	84.dipentylether	3.9
85.benzylbenzoate	3.9	86.decanol	4.0
87.heptane	4.0	88.cymene	4.1
89.pentylbenzoate	4.2	90.dephenylether	4.3
91.iso-octane	4.5	92.undecanol	4.5
93.ethyldecanoate	4.9	94.dodecanol	5.0
95.nonane	5.1	96.dibutylphthalate	5.4
97.decane	5.6	98.undecane	6.1
99.dipentylphthalate	6.5	100.dodecane	6.6
101.dihexylphthalate	7.5	102.tetradecane	7.6
103.hexadecane	8.8	104.dioctylphthalate	8.8
105.butyloleate	9.6	106.didecylphthalate	11.7

3.4 Enzyme kinetics

Enzymes are biological catalysts. Therefore, their major function is to catalyse the making and breaking of chemical bonds. Similar to any other catalysts, they increase the rate of reaction without themselves undergoing permanent chemical changes.

The advantages of using biocatalysts over chemical catalysts are (Lee, 1992):

1. Enzymes are highly specific catalysts.
2. Reaction rates of enzyme reactions are mostly higher than those of other chemical catalysed reactions under mild operating conditions.
3. The reaction conditions (temperature, pressure, pH, and so on) for the enzyme reactions are very mild.

In this work, we are interested in two substrate enzyme kinetics which corresponses to the reaction studied. Mechanisms of the two substrate enzyme reactions are demonstrated as follows (Segel, 1975).

3.4.1 Rapid equilibrium random bi bi

Both substrates (A and B) bind with enzyme before the two products (P and Q) are formed. Both substrates randomly bind with the enzyme. In the same way, both products have the same chances to be released from enzyme. The binding of substrates and the release of products are in random fashion and can be shown by the scheme as follows:

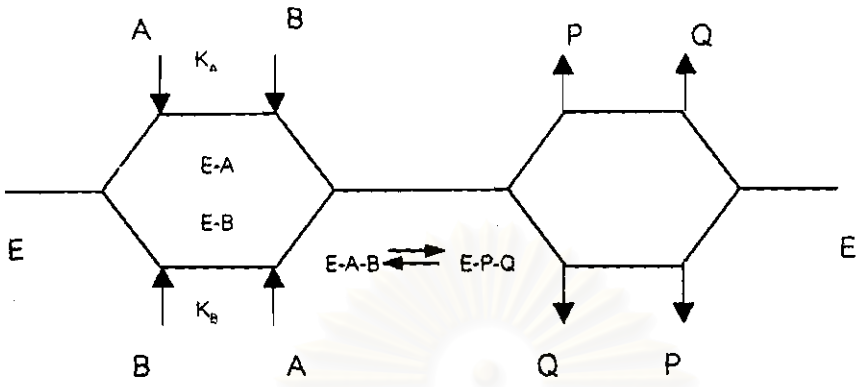

Figure 3.5 Schematic of rapid equilibrium random bi bi mechanism

3.4.2 Ordered bi bi

Both substrates (A and B) bind with enzyme before the two products (P and Q) are formed. The substrate A bind with enzyme before substrate B and the product P is released from enzyme before product Q. The binding of substrates and the release of products are in order and can be illustrated by the scheme as follows:

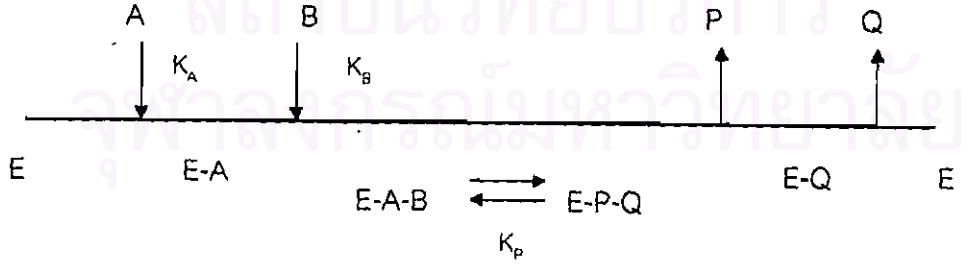

Figure 3.6 Schematic of ordered bi bi mechanism

3.4.3 Ping pong bi bi

The mechanism that substrate can bind with enzyme one by one and produce one kind of product which is called "one substrate one product" or ping pong bi bi mechanism. Enzyme is changed to two stable forms E and F. Steps of reaction is shown as follows:

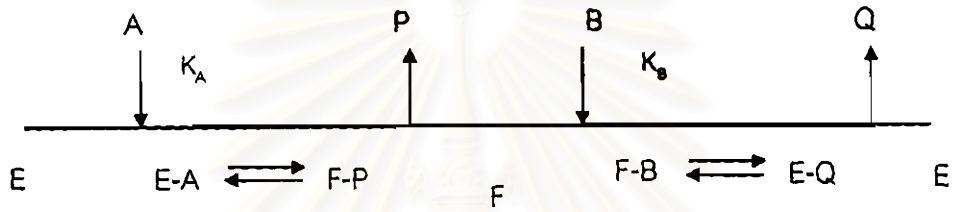

Figure 3.7 Schematic of ping pong bi bi mechanism

3.5 The influences on enzyme activity

Many factors can influence the catalytic activity of enzymes, presumably by affecting the enzyme's structural or chemical state. Included among these factors are:

3.5.1 Concentration of substrates

By using constant amount of enzyme and increasing concentration of substrate it was found that initial rate of the reaction increases rapidly in the low initial substrate concentration range. Rates of reaction increase along with concentration of substrate until they reach the one point that rate of reaction can not increase even the system still has an increasing of concentration of substrate. The concentration of substrate that resulted in the maximum rate of reaction (V_{max}) is saturated concentration of substrate.

3.5.2 Effect of pH

pH of solution in the reaction has a strong effect on the rate of reaction that used enzymes as a catalyst. Suitable pH of reaction is varied depends on the types of enzymes used such as pepsin has suitable pH at 2 to 3 or amylase has suitable pH at 6.8. Effect of pH on rate of reaction can be explained as follows:

- Enzyme is the protein that comprise amino acid residues (amino acids residues have no water molecules).

- Amino acid residue has some groups that can be breaking into ions. The amount of anion and cation in the solution had an effect on the breaking into ions in the active sites of enzyme.

- Enzyme can stimulate the occurance of the reaction when the amino acid residue in the active site had a specific ion. As a result, the changing of pH can affect the catalytic activity of the enzyme.

3.5.3 Effect of temperature

The rate of a reaction depends on the temperature according to Arrhenius equation as shown in equation 3.2.

$$k = A_o e^{-E/RT} \qquad (3.2)$$

The temperature dependence of many enzyme-catalysed reactions can be described by the arrhenius equation. An increase in the temperature increases the rate of reaction, since the atoms in the enzyme molecule have greater energies and a greater tendency to move. However, the temperature is limited to the usual biological range. As the temperature rises, denaturation processes progressively destroy the activity of enzyme molecules. This is due to the unfolding of the protein chain after the breakage of weak (for example, hydrogen) bonds, so that the overall reaction velocity drops. For many protein, denaturation begins to occur at 45 to 50 °C. Some enzymes are very resistant to denaturation by high temperature, especially the enzymes isolated from thermophilic organisms found in certain hot environments.