CHAPTER II
BACKGROUND AND LITERATURE SURVEY

2.1 Enzymes and Their Catalytic Functions

Enzymes are known as the biological catalyst controlling biochemical reactions at the molecular level, where they play an important role in inducing and storing the energy for living cells. In general, enzymes are classified into six main classes based on the reactions that they catalyze. Moreover, each main class can be subdivided as shown in Table 2.1.

Table 2.1 Classification of enzymes and their catalyzed reactions (Laskowski *et al.*, 2000).

Main Class	Subclass	Type of Catalyzed Reaction
Oxidoreductase	Oxidase	Oxidation of a substrate
	Reductase	Reduction of a substrate
	Dehydrogenase	Induction of double bond (C-C or C-O)
Transferase	Transaminase	Transfering amino groups
	Kinase	Transfering a phosphate group
Hydrolyase	Lipase	Hydrolyzing ester group of lipids
	Protease	Hydrolyzing amide bonds of proteins
	Nuclease	Hydrolyzing phosphate esters in nucleic acids
Lyase	Dehydrase	Loss of water from a substrate
	Decarboxylase	Loss of carbon dioxide from a substrate
Lipase	Synthetase	Formation of a new C-C bond from two water substrates
	Carboxylase	Formation of a new C-C bond with carbon dioxide
Isomerase	Epimerase	Isomerization of a chiral carbon center

2.2 Lipase and Lipase-Catalyzed Reactions

2.2.1 Lipase

The terms Lipase are used interchangeably as these enzymes catalyze the hydrolysis of carboxylic ester bonds and are classified among the hydrolyase. Microorganisms are the most common sources of lipase followed by mammalian cells and plants with the molecular weights range 20,000-60,000. The properties of some purified microbial lipases are shown in Table 2.2. *Rhizomucor miehei* is an example of lipase from plant, which can be extracted from Thai rice bran (Phraephrewngarm, 1999). Lipase can be categorized into three groups based on their specificity. Nonspecific lipases break down akylglycerol molecule at random positions, producing free fatty acids and glycerol with monoacylglycerols and diacylglycerols as intermediates.

Table 2.2 Properties of purified microbial lipases (Wiseman, A., 1995).

Species	Relative Molecular mass	Isoelectric Point	Specific activity of purifed enzyme (lipase units per mg of protein)	Average Hydropho-bicity
Aspergillus niger	25,000	4.6	2,400	1,058
Candida cylindracae	120,000	4.2	1,140	1,150
Humicola lanuginosa	27,500	-	1,490	1,079
Rhizopus arrhizus	43,000	6.3	9,300	1,097
Rhizopus delemar	41,300	4.2	4,000	1,270

2.2.2 Lipase-Catalyzed Reactions

The catalytic reaction of lipase normally occurs at the water-lipid interface, which is the interface between hydrophobic and hydrophilic regions at room temperature by encapsulation the substrate on the specific site. The reaction is known as a metabolism of lipid and can be simplified as shown in Scheme 2.1

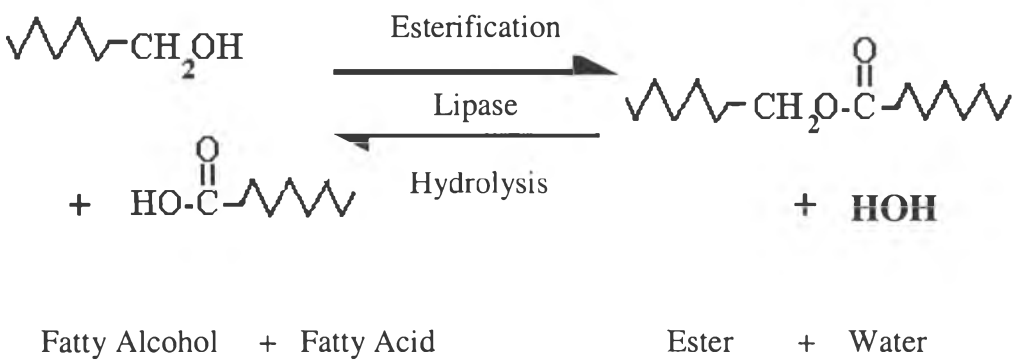
Lipid + water $\longrightarrow$ carboxylic acid + alcohol

Scheme 2.1 Lipase acts as a catalyst in lipid metabolism.

In addition to the lipid metabolism, lipase also catalyzes enantioselective derivatization of chiral compounds and a host of esterification, transesterification, and interesterification reactions.

2.2.2.1 Lipase-catalyzed esterification and ester hydrolysis reactions

In general, fatty acid and alcohol are used as substrates for esterification reactions catalyzed by lipase, as shown in Scheme 2.2. For example, Claon and Akoh (1994) have focused on the esterification of acetic acid and geraniol in *n*-hexane with 98% conversion after 14 h at 20°C. On the other hands, Egri and his group (2000) used lipase to catalyze the enantiotope selective acetylation of 2-acyloxypropane-1,3-diols.

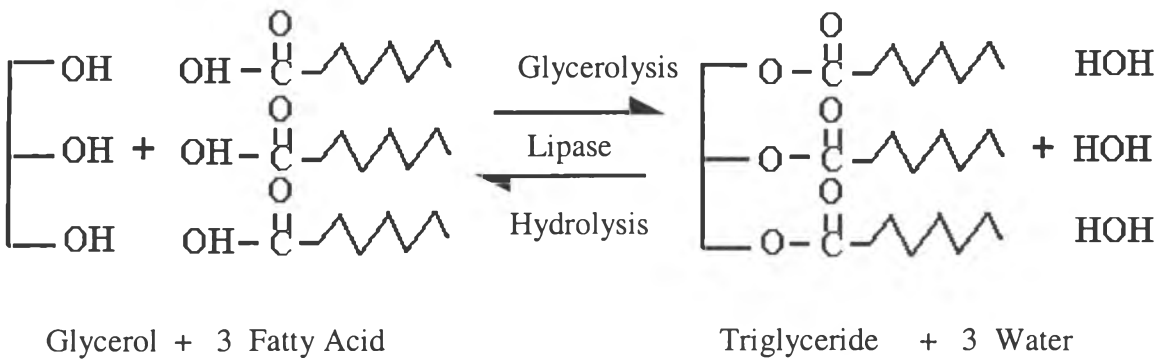


Scheme 2.2 Lipase-catalyzed esterification of fatty acid-alcohol.

The hydrolysis of esters, which is the reverse of esterification reactions, has been focused in lipase-catalyzed hydrolysis of triglyceride oil in W/O microemulsions to obtain monoglycerides. By using a 1,3-specific enzyme the hydrolysis takes place in a fairly regioselectivity, conversion into 2-monoglyceride being completed in 2-3 h at 37 °C. Prolonged reaction time results in a decrease in conversion while maintaining the regioselectivity (Holmberg and Österberg, 1988). Also there is an interest in 2 steps of biocatalytic conversion of an ester to an aldehyde in W/O microemulsion, which two reactions were carried out in reverse micelles (Yang and Russell, 1994).

2.2.1.1 Lipase-catalyzed glycerolysis

Lipid conversion in water-free microemulsions with glycerol as a component can be performed through catalytic glycerolysis instead of hydrolysis as shown in Scheme 2.3.



Scheme 2.3 Lipase-catalyzed glycerolysis of triglyceride.

Lipase-catalyzed glycerolysis of triglyceride would yield a mixture of 1- and 2-monoglycerides, which the first one is an important emulsifier in food industry. But low enzyme activity is usually observed in the completely non-aqueous system because the solvolytic agent in the reaction cannot be fully replaced in its role as an activator of enzyme (Holmberg *et al.*, 1989).

2.2.2.3 Lipase catalyzed transesterification

Lipase-catalyzed transesterification, i.e., replacement of one acyl group in a triglyceride by another group, can be performed in microemulsions of low water content, which low cost substrates such as palm oil can be converted to more valuable products (Holmberb and Österberg, 1988).

Lipase-catalyzed esterification and transesterification in anhydrous media (i.e., praganic solvent and supercritical fluid) has been an area of major research activities in the past decade, where the absence of water eliminates the competing hydrolysis reaction. In contrast, the presence of water in transesterification reaction system will lead to a hydrolytic side reaction (Yahya *et al.*, 1998). Moreover, varying the reaction medium can control substrate specificity, regioselectivity, and stereoselectivity of enzymes. Although organic solvents, which

are generally used for lipase-catalyzed reactions, are nearly anhydrous, they still contain water in tracer quantities. This water content can be controlled over a range, which has a profound effect on the activity of lipase.

2.3 Lipase-Catalyzed Reactions in Low Water Systems

As mentioned earlier, the formation of water presents a problem in the esterification reactions by promoting reverse reactions (hydrolysis of ester). The early approach used to conduct the reaction in low water content media was oil-water emulsion systems for lipase-catalyzed esterification. Still, the results revealed the low activity of lipase, which has led to the new concepts of low water system for lipase-catalyzed reactions. This can be done in a number of ways but there are three promising methods which will be discussed in this section, namely immobilized enzyme on support, microemulsion-based gels, and encapsulated enzymes in microemulsions (reverse micelles).

2.3.1 Immobilized Enzyme on Support

There are many types of support that can be used to immobilize enzymes such as lipase. In general, polymer or solid supports with strong hydrophobic and electrostatic interactions are used to immobilize enzyme. They can be used with inorganic solvent to immobilize enzyme as show by Yang and his group (1994) who used Ca-alginate gel bead coated with polyetheneimine and glutaraldehyde to immobilize *Expansum penicillium* lipase. In 1998, Knezevic *et al.* used zeolite type Y to immobilize *Candida cylindracea* lipase for palm oil hydrolysis. In addition to synthesis via reactions, purification by immobilized enzyme was also studied. Shimada *et al.* (1998) used immobolized *Rhizomucor miehei* lipase for purification of ethyl docosahexanoate by selective alcoholysis of fatty acid ethyl ester. However low activity problem was observed in some systems, where denature of the enzyme after immobilization occurred.

2.3.2 Microemulsion-Based Gels

Microemulsions are also extended their uses in the preparation of microemulsion-based gels (MBGs) as reaction media. The MGBs are made by mixing W/O microemulsions with aqueous gelatin solutions to form gel under the proper conditions. The gel can be viewed as immobilized enzyme-containing microemulsions, which is resistant to hydrocarbon solvents. By charging a hydrocarbon solution of acid and alcohol on the top of MGBs column, the ester can be produced and recovered. This is an elegant way to avoid the problems of product separation.

2.3.3 Encapsulated Enzyme in Microemulsions System

Water in oil microemulsions (reverse micelles), nanometer-sized water droplets dispersed in organic media by action of surfactants, amphiphilic molecules, arrange themselves in such a way that their hydrophobic parts are in contact with the bulk solution of organic solvent, while the polar (hydrophilic) head group surround a water core, water pool. In these water containing microdroplets enzyme molecules can be entrapped, avoiding direct contact with the unfavorable organic medium and retaining their catalytic ability. The substrates are solubilized in the aqueous or organic phase, depending on their nature, undergo an enzymatic conversion, and the products diffuse to the phase corresponding to their polarity.

Microemulsions have been studied in a variety of research areas in the past decade because of their unique properties, enhancing their utility. It has been shown that many proteins can be solubilized in microemulsions based on non-polar solvents such as aliphatic hydrocarbon without denaturation or loss of functions. The first use of microemulsions as a medium for enzyme-catalyzed reactions was reported in 1997 and it brought about the idea of immobilization of proteins in bioseparations (Holmberg *et al.*, 1997). Using microemulsions to encapsulate enzyme provides not only low water environment to maintain high activity lipase but also large interfacial area of water and oil catalyze esterification reactions.

2.3 Catalyzed Esterification Reactions by Lipase Encapsulated in Microemulsions

2.4.1 Microemulsion

Microemulsion is a single phase of optically clear and thermodynamically stable solutions, macroscopically homogeneous but microscopically heterogeneous, which can be formed by adding two immiscible liquids (i.e., oil and water) with an appropriate surfactant or surfactant mixture. It attracts much of scientific and technological interest over the past decade because of its unique properties, such as ultralow interfacial tension, large interface area, and high solubilization capacity for both oil- and water-soluble compounds. Schulman *et al.* (1959) was the first group who introduced the term microemulsion in publication.

Microemulsions form under a wide range of surfactant concentrations, water to oil ratios, and temperatures. In addition, formation of microemulsion can be occurred if the interfacial tension is low enough, thus balancing of hydrophilic to lypophilic balance (HLB). The effect of adding cosurfactant, increasing salanity, or temperature can also be employed to form different types of microemulsions depending on the system of interest.

There are three types of microemulsions. The first type is spherical of oil droplets dispersed in water (O/W) with range between 100 to 1000 Å, which is called Winsor I (W1). Second type is reverse of Winsor I, or water in oil microemulsions (W/O), which is called reverse micelles or Winsor II (W2). The third type is the bicontinuous structure or Winsor III (W3) in which the surfactant forms interfaces of rapidly fluctuating curvature and both oil and water domains are continuous. The schematic of ternary phase diagram for a typical water/nonionic surfactant/oil system is shown in Fig. 2.1.

As mentioned before, due to their unique properties, i.e. the ultralow interfacial tension, microemulsions can be applied in many applications and in several phenomena such as in oil recovery and soil decontamination.

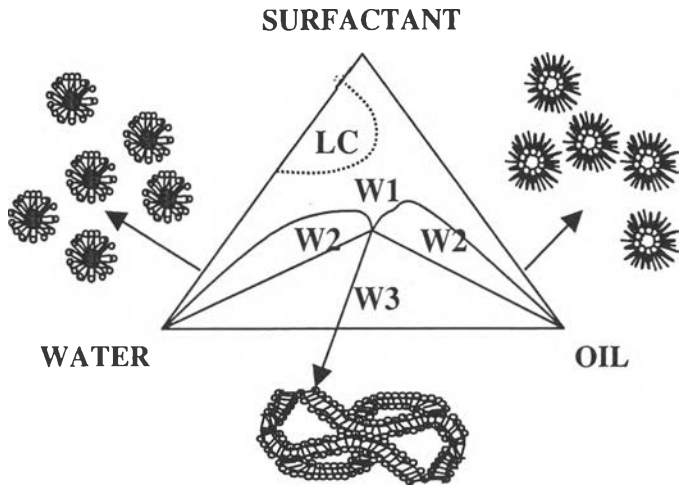


Figure 2.1 Schematic of ternary phase diagram for a typical water/nonionic surfactant/oil system at HLB temperature. Microemulsion structure is shown in the normal regions of occurrence; left to rigth: O/W globular microemulsions (W1), bicontinuous microemulsion (W3), and W/O globular microemulsions (W2) (Solans *et al.*, 1997).

2.4.2 Protein Solubilization or Extraction into Microemulsion

High solubilization capacity and selectivity is the important property of microemulsions, which is used to enhance protein extractions and purification. The proteins are extracted or encapsulated under the proper conditions into reverse micelles with high solubility and selectivity. For example, Adachi and his co-workers (1997) studied the bioaffinity separation of trypsin using trypsin inhibitor immobilized in L2 of nonionic surfactant. The results showed high recoveries of trypsin from a mixture, no loss of activity of trypsin in L2. In general, backward extraction of proteins or recovery step can be done by increasing ionic strength via mixing with high salt or high pH of another aqueous solution to microemulsions system. In 1999, Jarudilokkul *et al.* reported the new backward extraction using mild condition by addition of a counterionic surfactant, which the rate of back-extraction is 3 times faster than the forward rate.

2.4.1 Lipase-Catalyzed Esterifications in Microemulsions

Recently W/O microemulsion or reverse micelle has been applied for lipase-catalyzed esterifications, Bello and Thomas (1987) was the first group who investigated the synthesis of triglycerides from esterification of glycerol and fatty acid in reverse micelle, instead of emulsion system. They discovered that lipase in this system maintained its regioselectivity, resulting in the high yield of reaction. In addition, Singh and Shah (1993) studied lipase catalyzed esterification in monolayer system comparing with microemulsion system, focusing on the effect of long chain hydrocabon on the system (Oh *et al.*, 1996). There are other esterification reactions that also have been investigated. For example, esterification of glucose and mantose with fatty acid was also studied by Hayes and Gulari (1990). Formation of poly-fatty acid ester by lipases was also investigated by the same group. Kermasha and his group (1995) carried out the inter-esterification of fatty butter with lipase. Glucoside ester synthesis was also studied (Skagerlind *et al.*, 1997), and more recently, the lipase-catalyzed esterifications in microemulsion has been reviewed (Gandhi *et al.*, 2000).

The most frequently used reverse micellar system in a number of study was sodium bis (2-ethylhexyl) sulfosuccinate (AOT) with different solvents. In 1989, Hayes and Gulari investigated esterification reaction of *Canadida cylindra* and *Rhizopus delemar* lipase in water/AOT/iso-octane in revere micelles microemulsions with various fatty acids and alcohols. They observed different enzyme activity for different types of alcohol and fatty acids. It is likely that the small polar molecules such as methanol or propanol cannot penetrate into the interface such that small rate of reaction was observed in this system. They also suggested that the different initial velocities and diameters of micelles (R_h) observed between *C. cylindracea* and *R. delemar* lipase could be attributed to the differences in enzyme localization in the microstructure media. In addition, they found that the water content (Wo) had no effect on the kinetic rate for the value up to 1.15 M. However, it has been shown that the activity of lipase dramatically decreases after being encapsulated in microemulsions. Stamatis *et al.* (1993) investigated the role of enzyme localization in relation to its selectivity in AOT/iso-octane/water with *P. simplicissimum, R. delemar, and R. arrhizus* lipases. They found that lipase selectivity was related to

the localization of enzyme molecule within the micelles structure due to hydrophobic and hydrophilic ratio of enzyme. The structure of reverse micelle characteristics as well as localization of enzyme were examined by fluorescence quenching measurement and spectroscopic studies. They offered the explanations that *R. delemar, and R. arrhizus* lipases, which prefered to localize in water pool of reverse micelles more than at the interface, could not catalyze the tertiary alcohol. It is evident that apolar substrates cannot be catalyzed with enzymes that localize mainly in the water pool. On the other hand, the ability to catalyze the tertiary and secondary alcohols of *P. simplicissimum* can be improved due to the preference of localization near the interface of oil and water, which tertiary and secondary alcohols prefer to be. Not only the anionic sufractant systems, the nonionic systems also have been investigated, such as the catalytic behavior of *Pseudomonas cepacia* lipase in reverse micelles of Neodol 91-2.5/AOT system (Stamatis *et al.*, 1994).