CHAPTER 2

LITERATURE SURVEY

2.1 LIPIDS

2.1.1 SOURCES

Most lipids, substances ranging from the simple fatty acids to the complex sphingo lipids, from the sterols and steroids to certain pigments and vitamins, are naturally occurring in the forms of animal fat and vegetable oils. Furthermore, those lipids as a large and diverse group of important organic compounds in living organisms, play an important roles in biological membrane of mammals, plants and microorganisms. For example, glycerides serve as excellent sources of potentially chemical energy source and thermal insulators in mammals.

2.1.2 PHYSICAL PROPERTIES OF LIPIDS

Definition of lipids depends on physical properties than on chemical structure or function. Generally, lipids are insoluble in water but are soluble in organic solvents such as chloroform, benzene or ethanol. This distinctive properties of lipid depends on long hydrocarbon chains that are a main parts of their structure (Hardly, 1985). Melting points of each lipid are also determined by ratio of unsaturated fatty acids to saturated fatty acids.

2.1.3 GENERAL ASPECTS OF TRIGLYCERIDES

The neutral fats and phosphoglycerides are two biologically important groups of lipids, which have glycerol as a structural backbone. Acylglycerol or glyceride is fatty acid ester of glycerol and the most widespread of these, in which three of the hydroxyl groups of glycerol are esterified to fatty acids that are typically long chain and contain an even number of carbon atoms, are triacylglycerols or triglycerides. Besides monoglycerols and diacylglycerols, in which only one or two of the hydroxyl groups of glycerol are esterified, respectively, are also found but they are less abundant. The common structure of mono-, di-, and triglyceride is shown in **Figure 2.1, on page 26.**

Triacylglycerols occurs in the solid of liquid form that depends on the nature of constituent of fatty acids. Due to a large proportion of unsaturated fatty acids, i.e., oleic, linoleic or linolenic acids, triglycerides in most plants are found in the form of liquids at room temperature. However, triglycerides in animals, which contain a higher proportion of saturated fatty acids such as palmitic and steric acids, are semisolid or solid at room temperature. Moreover, the reactivity of ester bond and degree of unsaturation in hydrocarbon chain are determined by the chemical reactivities of triglycerides. Acid and base hydrolysis of triglycerides is differently occurred in ester linkage, i. e., difference of this is acid hydrolysis reaction is reversible while base hydrolysis is irreversible (Conn and Stumpf, 1967).

2.2 ENZYMATIC HYDROLYSIS OF TRIGLYCERIDES

2.2.1 GENERAL ASPECTS AND DEFINITION

In recent years, enzymes that degrade triglyceride lipids by hydrolysis of esters, especially esters of carboxylic acids, have been receiving much attention due to their potential use in industrial processes. During the past decades, many investigators have extensively studied the activity of lipolytic enzyme called **lipases** or **glycerol ester hydrolase** (E.C.* 3.1.1.3) in both academic and industrial viewpoints. Among the large amount of these published reports, the lipases produced by various kinds of microorganisms have optimum pH in alkaline region and have been called alkaline lipases produced by fungi, ***Mucor lipiticum***, and yeast, ***Candida paralypolytica*** (Nishio et. al., 1987). However, the lipolytic enzyme which showed high activity in acid region produced by fungi, ***Aspergillus niger*** has also been elucidated (Fukumoto, 1963).

Lipolytic enzymes compose of two major groups, the first groups called lipases which are triacylglycerol acylhydrolase and the other called the phospholipases A_1 (E.C. 3.1.1.32) and A_2 (E.C. 3.1.1.4) which are phosphoglyceride acylhydrolases (Wong, 1995). Triacylglycerol acylhydrolase is responsible for hydrolysis of the fatty acid ester bonds of triglycerides whereas the phospholipases A_1 and A_2 hydrolyze the phosphoglycerol ester bonds.

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*All abbreviations of any term used in the text were shown in ABBREVIATION, on page xvi

Lipases are defined as surface-active enzymes that breakdown the triglyceride ester bond to give free fatty acid, partial glycerides and glycerol (see **Figure 2.2,** on **page27**) because their hydrolytic activity compared with their activity toward dissolved substrates, distinctively increased by binding to emulsified triglyceride substrates (Verger, 1984; Kugimiya, 1986). Among enzymes that catalyze the hydrolysis of esters, the carboxyl ester hydrolases which specify for soluble esters are called esterases (E.C. 3.1.1.1) while lipases are specific for insoluble fatty acid esters (Stauffer, 1989). Each lipase, like the other enzymes, has its own optimum pH, ranging from acid to neutral and neutral to alkaline. Besides, thermostable lipases are also different considerably due to their sources (Yamane, 1987).

Many species of animals, plants and microorganisms can produce lipases, either alone or in combination with esterases. Especially, microbial lipases are important for practical purposes and used in the diary and other food processes (Aoyama and et al., 1988). However, very wide range of properties of various lipases is dependent upon its source, which relates to positional specificity, fatty acid specificity, pH optimum and thermalstability. These properties guide the appropriate application to the suitable lipases or event lipase producing organisms.

2.2.2 STRUCTURES OF LIPASES

Over the past two decades, several mammalian and microbial lipases whose crystal structures have been elucidated (Derewenda et. al.; Sama et. al.; Schrag et. al.; 1990). Generally, three dimensional structure of lipases contains alpha/beta (α/β) fold consisting of a central core of a mixed β sheet where all the residues in catalytic triad, histidine-serine-asparagine/glutamine (His-Ser-Asp/Glu), are located at active site like serine proteinase and a surface loop restricting access of the substrate to the active site (Weete, 1998). The three dimensional structures of lipases are shown in **Figure 2.3, on page 28.**

In addition, three dimensional structure of all lipases from animals, bacteria and fungi tend to be similar. Moreover, they all folded in the same ways and same catalytic sites. However, most lipases have large difference in amino acid sequences. Lipases are commonly folded into two domain, C-terminal domain and N-terminal domain, in which N-terminal domain contains the active site with a hydrophobic tunnel from the catalytic serine to the surface that long fatty acid chain is accommodated (Willis and Marangoni, 1998).

2.2.2.1. Bacterial Lipases

By crystallization including preliminarily X-ray crystallographic studies of bacterial lipases, several ***Pseudomonas*** species i. e., ***P. cepacia*** (Kim et. al., 1992), ***P. glumae*** (Cleasley et. al., 1992) and ***P. fluorescens*** (Larson et. al., 1991) have extensive sequence homology on molecular masses, which range in 30 to 35 kDa, to one another but little to those

other lipases. The conserved sequence in active site region of those is commonly glycine-any amino acids-serine-any amino acids-glycine (Gly-X-Ser-X-Gly). Lipases from ***Bacillus*** species, however, contain asparagine-any amino acids-serine-any amino acids glycine (Asp-X-Ser-X-Gly) sequence and have no interfacial activation (Weete, 1998). Recently, by cloning and sequencing the ***lip*** gene that codes for extracellular lipase in ***B. subtilis*** 168. Dortois et. al. (1992) reported that ***B. subtilis*** enzyme lacks the conserved sequence in active site, unlike most bacterial lipases.

2.2.2.2. Fungal Lipases

Among microbial lipases, the structures of fungal and yeast lipases have also been identified by crystallization, for instance, ***Humicola lanuginosa*** (Lawson et. al., 1994), ***Candida rugosa*** (Grochulski et. al., 1993) and ***Geotrichum candidum*** (Shrag and Cygler, 1993; Schrag et. al., 1991). Moreover, the structures of consensus sequence that is the Gly-X-Ser-X-Gly site region of fungal lipases are similar to pancreatic lipase (Bourne et. al., 1994; Antonian, 1988). Many fungal lipases also have the serine protease catalytic triad, Ser-His-Asp, in their active sites. Furthermore, a lid that prevents access of the substrate to the active site, is also found in fungal lipases (Weete, 1998).

2.2.2.3. Other Lipases

In comparison with microbial lipases, and three dimensional structures of lipases from different organs or tissues of mammals have

also been studied, especially, human and other pancreatic lipases which are thoroughly studied (Gubenator et. al.; Abergel et. al.; Cambillau and Bourne, 1990). Pancreatic lipases, are capable to hydrolyzing ester of triacylglycerols completely to fatty acids and glycerols. Human pancreatic lipases composed of two domains. One is a larger N-terminal domain containing the active site for glycosylation, calcium ion binding, and potentially haparin-binding. The other is a smaller C-terminal domain, which prepared for colipase binding. Unlike human pancreatic lipases, animal lipases may have different properties in detail, for example, pancreatic lipase from guinea pig shows phospholipase A activity (Hjorth et. al., 1993). On the contrary to other sources of lipases, plant lipases have been less investigated. However, Mukherjee and Hills (1994) have recently reviewed plant lipases. Oilseed lipases from various plants exhibit differences, such as their substrate specificity, optimum pH and hydrophobicity (Huang et. al., 1998). Furthermore, these properties are relatively specific for the native triglycerides of the species from which they were isolated (Weete, 1998).

2.2.3. EXTRACELLULAR MICROBIAL LIPASES

Owing to the versatility and high potential of microbial lipases used in biomedical and industrial applications, and also their inexpensive production, microbial lipases have been developed continuously. Most of the microorganisms, i. e., bacteria, fungi and yeasts that use neutral oils and fats as a carbon sources for their growth by breaking down the oils and fats prior to their digestion can excrete extracellular lipases through the external membrane into the circumstance. Because microbial lipases

are inducible and extracellular enzymes, the properties of the enzyme producer as well as ratio of extracellular to intracellular lipases depend on culture conditions, individually inducible substrates such as olive oil. In addition, Chen et. al. (1993) found that lipase production of fungi was also enhanced by hydrocarbons such as linear and n-alkanes.

Since lipases have a number of unique characteristics such as fatty acid specificity, stereospecificity, regiospecificity and positional specificity so the microbial lipases based on their specificity can be classified into three groups (**see Figure 2.4, on page 29**) which include the followings (Macrae, 1983):

2.2.3.1 Nonspecific Lipases

This group of lipolytic enzymes shows no marked specificity both as regards the position on the glycerol molecule, which is attacked and the nature of released fatty acid. In addition, these lipases hydrolyze the absolute breakdown of triglycerides to free fatty acid and glycerol whereas diglycerides and monoglycerides involve or act as intermediates in the reaction.

2.2.3.2 1,3-Specific Lipases

This lipolytic enzyme catalyzes the release of fatty acid specifically from the outer 1- and 3-positions of glyceride. Therefore, triglyceide is hydrolyzed to yield free fatty acids, 1,2-or2,3-diglycerides and 2-monoglycerides as reaction products.

2.2.3.3 Free Fatty Acid Specific Lipase

The last lipolytic enzyme catalyses the specific release of a particular type of fatty acid from glyceride molecules.

Among these groups, 1,3-specific lipase is commonly found in microbial lipases, while fatty acid specific lipase is little on extracellular microbial lipases. Because microbial lipases have received much attention for their potential use in biomedical and industrial applications, the properties of some extracellular microbial lipases have recently been characterized as shown in **Table 2.1,** on **page 30.** In addition, microbial lipases have properties that are generally similar to those of human pancreatic lipase. For example, some microbial lipases have various degrees of glycosylation.

2.3 CATALYTIC REACTION OF LIPASES

2.3.1 MECHANISM OF ACTION AT LIPID / WATER INTERFACE

Since triglycerides of long-chain fatty acids are nature substrates which are insoluble water. Thus the interfacially catalytic phenomenon between a monolayer of lipid and water is one of unique characteristics of most of the lipases (Wong, 1995). The activity of lipases depends on the concentration of micellar substrates at the interface, while the activity of esterases, which catalyze only on the water-soluble substrates is dependent of substrate concentration.

Generally, interfacial activation of lipases involves two equilibrium processes in which the overall reaction is illustrated in **Figure 2.5, on page 31** (Wong, 1995; Weete, 1998), i. e.,

1. Change in enzyme conformation

The lipolytic enzyme must pass through the interface to act so the adsorption of lipase to that site involves a conformation change in the enzyme resulting in an increase in activity.

2. Production of enzymatic products

When the enzyme-substrate complex is formed, the catalytic reaction occurs, the products are formed and the enzyme is generated as E^{*}_{a2} because of desorption of the enzyme from the micelle and repenetration to the interface for substrate binding in each catalytic turnover.

Furthermore, many investigators have studied on the mechanism of interfacial activation based on the three dimensional structural analysis of lipase from fungi such as ***Rhizomucor michei*** (Brady et. al., 1990; Derewenda et. al., 1992).

2.3.2 ACTIVATION AND INHIBITION OF LIPASES

By binding of calcium to the lipase enzyme resulting in conformational change, promoting adsorption of the lipase to the substrate-water interface and/or dissociating from the interface fatty acid products of hydrolysis that may reduce end-product inhibition of the

reaction, stimulation of lipase-catalyzed hydrolytic activity may be occurred. However, microbial lipase activity influenced by calcium may be different depending on the enzyme source and assay conditions. Nevertheless, inhibition of lipase activity is appeared by various substances, i. e., anionic surfactants, certain proteins, metal ions and phosphorus-containing compounds such as phenylmethyl sulfonylfluoride (Weete,1998). In addition, inhibition of lipase activity in the presence of anionic surfactant, was also conducted (Andree et. al., 1980).

2.3.3 BIOCHEMICALLY CATALYTIC REACTION OF TRIGLYCERIDE LIPASES

Although triglyceride lipases normally catalyze the reaction of lipolysis, they also catalyze the reverse hydrolysis in reaction media with low water content or in the presence of organic media. Therefore, biochemical reactions catalyzed by lipases involve with three types of reactions (see **Figure 2.6,** on **page 32**). In water soluble media, lipases catalyze hydrolysis of ester whereas in media with low water content, however, the lipases catalyze a wide variety of transesterification and interesterification reactions such as alcoholysis and aminolysis.

2.4 LIPOLYTIC BACTERIA

A group of bacteria that play an important role in lipid metabolism in the environments is called lipolytic bacteria. The excreted lipases are responsible for the breakdown of the lipids that are used as carbon and energy sources for their growth. Some lipolytic bacteria are able to

produce lipases, in which show high activities at extreme pH such as alkaline pH, then they are called alkaline lipase producing bacteria.

2.4.1 GENERAL ASPECTS OF LIPOLYTIC BACTERIA

2.4.1.1 Habitats

A large number of lipolytic bacteria are naturally found in the presence of oils and fats contaminated in the environments. Lipolytic activity usually involves in area close to soil surface occurring triglycerides of long chain fatty acids or hydrocarbon oils, even in the extreme environments such as hot springs where thermostable lipases, either alone or in combination with alkaline lipases, existed. Besides, lipolytic system also appears on surface of wastewater with oils from different sources such as restaurants and leather bleaching factory etc.

2.4.1.2 Culture Media

Because of cultivation of lipase producing microorganisms are usually not difficult. However, quite low yields of lipases are reported. Since then, medium and cultivation conditions for improvements in lipase fermentation yield are explained in many publications (Lawrence et. al., 1967; Iwai et. al., 1973; Yamaguchi et. al., 1973; Winkler and stuckmann, 1979).

Being excreted through the external membrane into the culture medium of extracellular microbial lipases, the quantities of lipase is

influenced by the environmental factors such as cultivation temperature, pH, nitrogen composition, carbon and lipid sources, concentration of inorganic salts and the availability of oxygen. Moreover, lipase production is enhanced by lipids such as olive oil, lard, butter and fatty acids (Suzuki et. al., 1988; Omar et. al., 1987). Formation of lipase from ***Pseudomonas mephitica***, which is stimulated effectively by triglycerides such as cotton seed oil, olive oil and fatty acids such as oleic acid, was conducted by Kosugi and Kamibayashi (1971).

2.4.1.3 Screening and Isolation Methods

A large number of lipases have mainly been screened for different applications as food additives, industrial reagents and stainremovers, as well as for medical applications such as digestive drugs (Aires–Barros et. al., 1994). Screening and isolation of microorganisms for lipase activity from soil and water samples or other biological materials is relatively easy. Therefore, lipases catalysed hydrolysis that is most frequently carried out using agar plated containing triglycerides, is detected by appearance of clear zones or precipitates of salts of fatty acids such as calcium salt (Wohlfarth and Winkler, 1988), even in screening systems making use of chromogenic substrates (Godtfredsen, 1990).

2.4.1.4 The Use of Fluorescent Dye Rhodamine B in Lipase Detection

The common used methods for detecting the lipolytic activity of bacteria is appearance of the clear zones around the bacterial colonies

on agar plates by using tributyrin (Lawrence et. al., 1967; Mourey and Kilbertus, 1976) or tween 80 (Sierra, 1957) as a substrate. In order to detect true lipases, however, these substrates are not suitable because they are also hydrolyzed by esterases. Nevertheless, time for verifying the existence of lipases is required. An idea substrate for true lipase is trioleoylglycerol. At present, the use of fluorescent dye rhodamine B in agar plate containing trioleoylglycerol has been used either in identifying lipolytic bacteria or in quantifying lipase activity in culture supernatants (Kouker and Jacker, 1987). Since the formation of fluorescent products is occurred from trioleoylglycerol hydrolysis by lipase but the molecular mechanism is still unknown. The suggested mechanism by Kouker and Jacker (1987) is that an orange fluorescent complex formation with an excitation wavelength of 350 nm is the reaction between cationic rhodamine B and the uranyl-fatty acid ion.

In addition, this rhodamine B plate method provides the basis of specific and sensitive assay for lipolytic bacteria, which formation of orange fluorescent halo around bacterial colonies visible upon UV irradiation at 350 nm is caused by hydrolyzed trioleoylglycerol. Unlike other plate methods, which used trioleoylglycerol as substrate with pH indicator such as Nile–blue sulfate (Collins et. al., 1934) or Victoria blue (Alford, 1967), the rhodamine B plate method is insensitive to pH changes. Moreover, it also allows reisolation of organisms, which show no growth inhibition or even change of physiological properties (Kouker and Jacker, 1987).

2.4.1.5 Known Lipolytic Bacteria

In the last twenty years, numerous papers relating to lipase producing microorganisms have been published (Kokusho et. al., 1982; Iwai et. al., 1973; Lotrakul and Dharmsthiti, 1997). Most of them were concerned with constitutive lipase produced by fungi and bacteria. With the recent increase in research on microbial lipases, many potent bacteria for lipase production have been isolated from different sources such as soil or water, even in diary products such as raw milk, as well as identified as various genera and species, i. e., mesophiles, psychrophiles or thermopiles. Examples of some isolated bacteria are illustrated in **Table 2.2,** on **page 33.** However, some of these lipolytic bacteria may be pathogens while they are able to produce high activity of lipase. It is worth characterization the enzymatic properties from lipase producing bacteria in order to apply the enzyme efficiently for the suitable industries.

2.4.1.6 Bacterial Metabolism of Lipids

Because the triglycerides as well as other lipids are main energy sources, which potentially produces greater yield of ATP than do other carbohydrates or proteins. Thus microorganisms produce extracellular lipases, which attack initially on both triglycerides and phosphoglycerides resulting in removal of the fatty acids from the glycerol molecule of the triglyceride by hydrolysis of the ester bond. The products of lipase including phospholipases are then penetrated through the cell membrane of microorganisms. Metabolism of glycerol is

occurred by conversion to dihydroxyacetone phosphate that is an intermediate in enol monophosphate (EMP) pathway in bacterial cell (see **Figure 2.7, on page 34**). On the contrary, the β–oxidation as the main pathway of fatty acids, occurs with repetition of a sequence of reaction, which results in the removal of two carbon atoms as acetyl Co-A with each repetition of sequence. The involved reactions is illustrated in **Fig 2.8, on page 35.**

2.4.2 GENETICS OF LIPOLYTIC ENZYMES

In the last few years, several publications on the molecular cloning of the microbial lipase gene have been reported and revealed the primary structure of their lipases, and even three dimensional structure of them were also elucidated by X-ray analyses. For instance, cDNA of fungal lipase gene was cloned from ***Geotrichum candidum*** (Shimada et. al., 1989; 1990), even cDNA of yeast lipase gene from Candiad cylindreceae was also cloned (Alberghina et. al., 1990).

2.4.2.1 Cloning and Sequencing of Bacterial Lipase Genes

As mention above, currently received much attention of microbial lipases because of their potential use in many industrial applications. The genes for several such lipases have been cloned. Moreover, bacterial lipase genes from a large number of species were cloned and sequenced in order to deduce the primary structure of their lipases from the nucleotide sequences of the cloned genes (see **Table 2.3-**

2.4, on page 36). Expression of these genes was mostly done in ***Escherichia coli*** after cloning by similar methods. In addition, the determination of the primary structure of lipases provides the molecular mechanism of the catalytic reaction and the relationships between the function and the structure of lipase (Aoyama et. al., 1988).

2.4.2.2 Genetic and Regulation of Bacterial Lipase Production

So far, regulation of lipase production in many bacterial species have been studied (Lesuisse et. al., 1993; Jorgensen et. al., 1991). Among various lipolytic bacteria, the mechanisms of lipase production including regulation of gene expression and the mechanisms of translation and secretion are quite different. However, the distinct gene responsible for extracellular bacterial lipases is defined as ***lip*** gene on chromosomal DNA. Recently, Mahler et. al. (2000) reported that the ***lip*** A gene encodes for the extracellular lipase of ***Acinetobacter calcoaceticus*** is highly similar to lipases of several ***Pseudomonas species*** and also, a ***Pseudomonas***–type specific lipase chaperone called ***lip*** B plays an important role in extracellular lipase production of ***Acinetobacter***. Besides, the mechanism of the formation and secretion of the extracellular lipase requires lipase translocation across the inner and outer membrane (Kok et. al., 1996; Kok et. al., 1995). Furthermore, the control over the rate of lipase production is enhanced by several post transcriptional steps, as well as the amount of active lipase in extracellular medium depends or the proteolytic degradation of mature lip A (Mahler et. al., 2000; Kok et. al., 1996). Over production of lipase, by

recombinants, ***Pseudomanas species***, was reported (Shimada et. al., 1994).

2.5 BIOTECHNOLOGICAL APPLICATIONS OF LIPASES

2.5.1 LIPASES IN VARIOUS INDUSTRIES

Since lipases are widely versatile and received as one of the most important class of industrial enzymes, considerable attention for industrial uses of lipases has currently developed. The utilization of both gene technology and new production technologies also offer several benefits to the enzyme industry such as improved properties of lipases (Falch, 1991). Recently, the potential of lipases for industrial application has been reviewed (Macrae and Hammond, 1985). Furthermore, the engineered lipases used in the lipids industry have also been reviewed (Yamane, 1987). Lipases are currently used in a wide range of application, for example, in diary industry for cheese flavor enhancement, in the oleochemical industry of hydrolysis, i. e., glycerolysis, alcoholysis of fats and oils and for synthesis the desired substances used in cosmatic industry, in pharmaceutical industry and agrochemical industry. However, the largest current use of industrial enzyme is in laundry detergents, which give friendly environmental impact because of biodegradability which litter consumed energy. Among other enzymes such as proteases, amylases and cellulases used in detergents, lipases are relatively new recommendation. Although lipases attack oily and greasy soils and promote to making the detergent separately effective at role washing temperature, few lipases are stable in alkaline condition

including anionic surfactants used in laundry detergents (Kojima et. al., 1994). A list of the important application areas of some microbial lipases in different industries is shown in **Table 2.5, on page 37.**

2.5.2 LIPASES IN IMMOBILIZED FORM

Although, lipases which biological function is to hydrolyze triacylglycerols or water-insoluble carboxylic esters, are capable to be active in reaction mixtures containing high proportions of organic solvents (Zaks and Klibanov, 1988). Moreover, other reaction, such as ester synthesis, alcoholysis, acidolysis and interesterification as well as hydrolysis, in which occurred in low-water system have relatively much commercial potential (Macrae and Hammond, 1985). Thus, immobilizing of lipases on innert support is one of the methods of reuse the lipases to make enzymes more stable and more suitable for desired purposes, for instance, production of free fatty acids and glycerol (Yang and Rhee, 1992). Furthermore, the interesterification of oils and fats to modify the composition of triglycerides mixture (Malcata et. al., 1990) and synthesis of esters for application in the food industry (Claon and Ashok, 1994) are also applied by this method. Many reports on immobilized lipase from microbes have been published. One of these examples is that immobilization of purified lipase from ***Pseudomonas cepacia*** was performed to study on enzymatic property in water and organic solvent (Pencrac et. al., 1997). Furthermore, the improved immobilization methods are recently being developed for many applications of biotechnology to new products.

2.6 THE FUTURE IMPACT OF INDUSTRIAL LIPASES

In comparison with major industrial enzymes such as protease and cellulases, the economic importance of lipases to the enzyme industry has been quite limited. At present, currently, many new potential applications of lipases have been proposed and become of increasing importance for such as the detergent and food industries.

By attempt to reduce the consumption of surfactants for environmental reasons and the trend towards using lower washing temperatures, the interest in developing lipases for use in detergent composition has been the main issue. However, the idea of using lipases practically in this field has previously been creased because of enzymes production costs and the strictly functional stability of detergent lipase. Recently, these problems have been solved due to the application of genetic-engineering techniques.

It is known that lipase technologies are likely not to give an impact on the commercial areas of industrial enzymes, but also to decrease environmental impact such as the safety of the products generated and the high safety of processes employed when compared with common chemical processes (Bjorkling et. al., 1991).

In this study, the screening and isolation technique were conducted at alkaline pH, 9.0 by using rhodamine B plate containing corn oil as the main substrate in order to receive the alkalophilic lipolytic bacteria with high activity of alkaline lipases. Moreover, some properties of those enzymes are also studied.

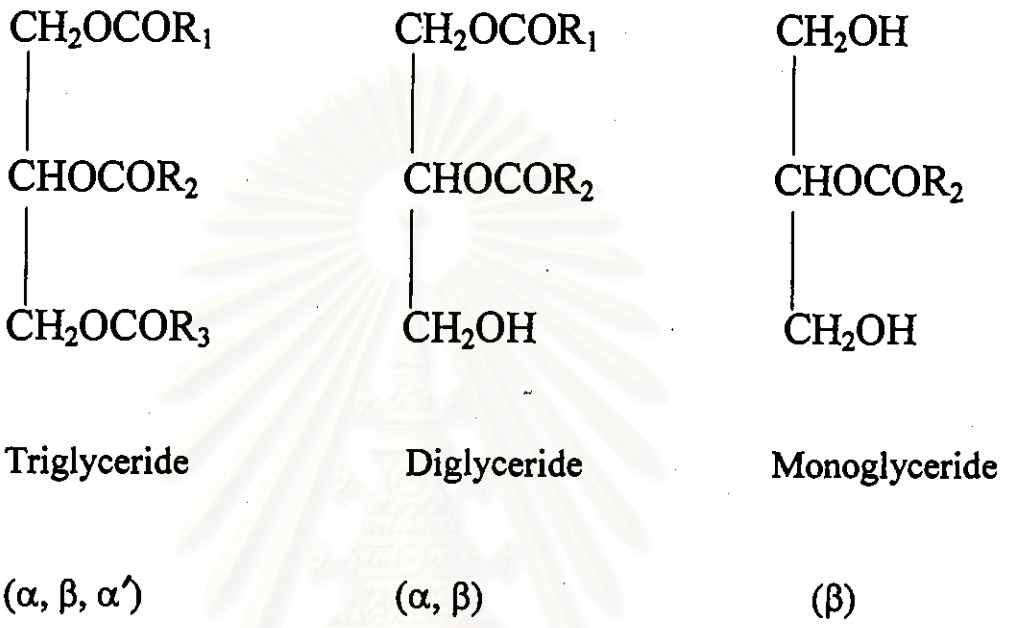


Figure 2.1 Model of mono-, di-, and triglyceride (Hanahan, 1960).

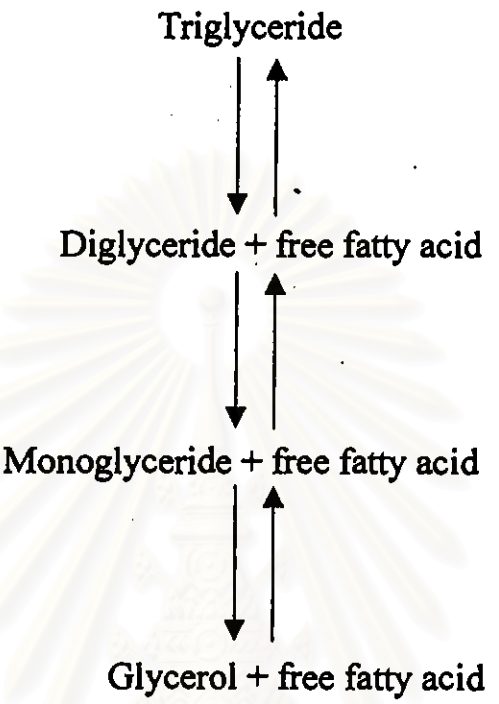


Figure 2.2 The lipase reaction (Macrae, 1983)

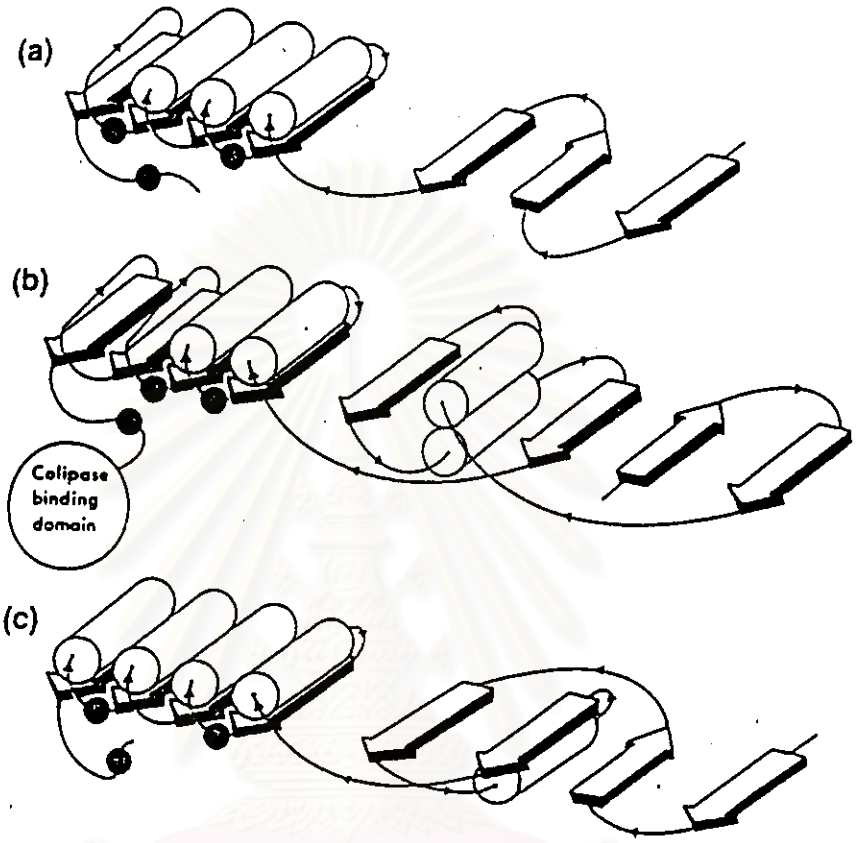
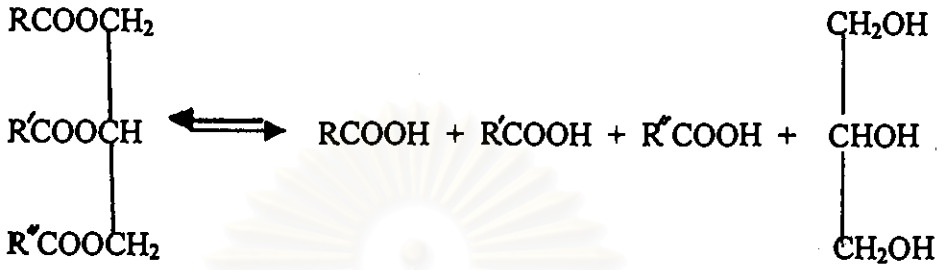
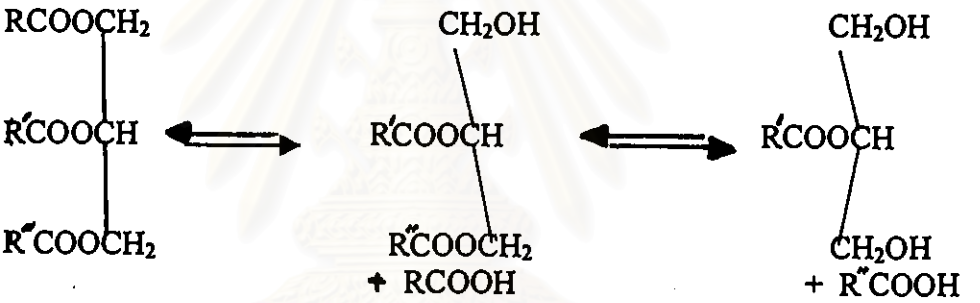


Figure 2.3 The common α/β fold of lipases : (a) ***Rhizomucor miechei***, (b) human pancreatic, (c) ***Greotrichum candidum***. All the contain central, predominantly parallel β sheets. The core consists of four parallel strands, which often have helical connections; it also contains the catalytic triad and constitutes the most invariant structural motif of the fold (cited by Wong, 1995).

1. Nonspecific Lipase:



2. 1,3-Specific Lipase:



3. Fatty Acid Specific Lipase:

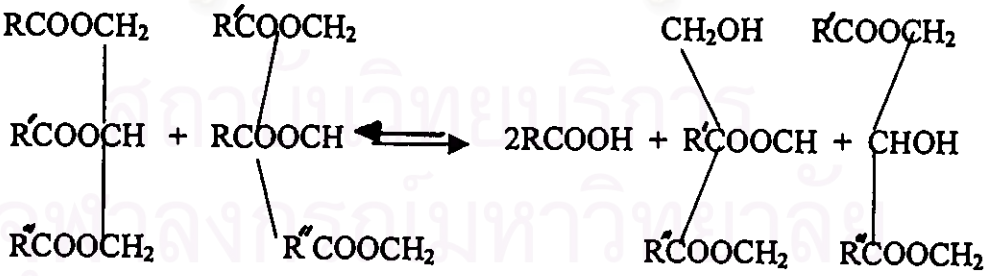
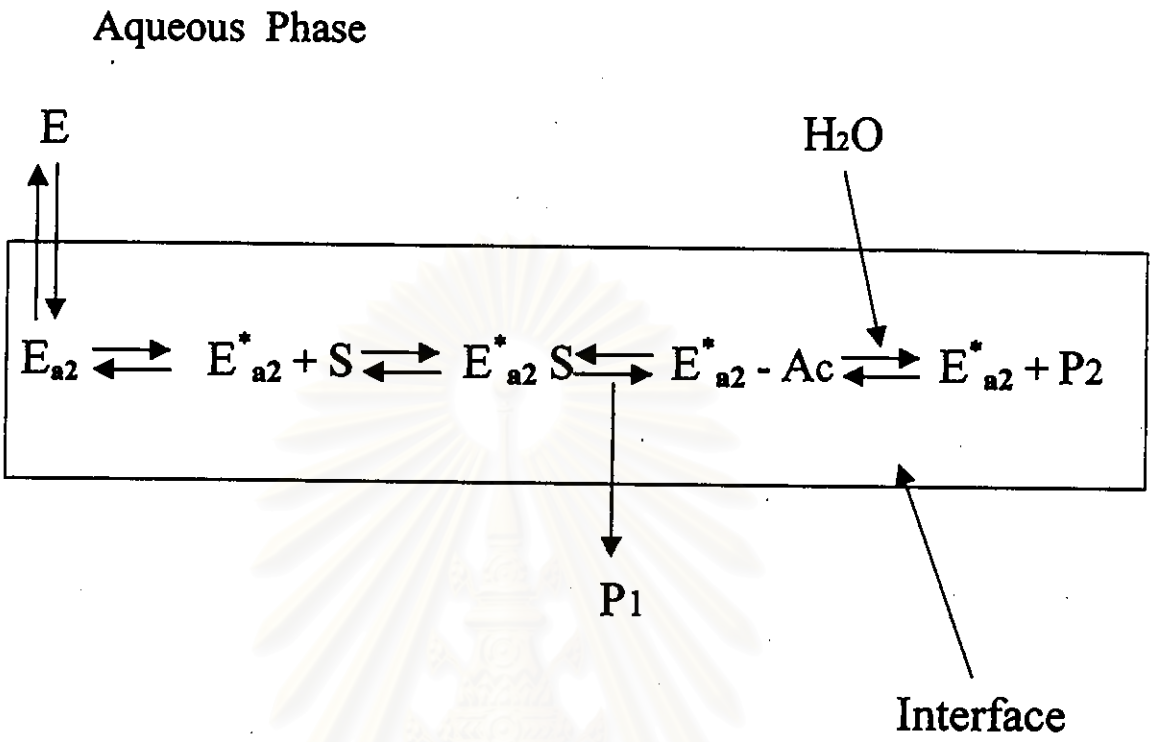


Figure 2.4 Products formed by lipase-catalyzed hydrolysis of triglycerides (Macrae, 1983).

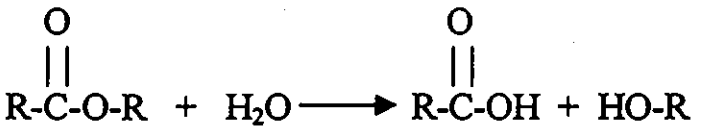
Table 2.1 Properties of some recently characterized extracellular microbial lipases (Weete, 1998).

Organism	Molecular Weight (Kda)	pH Optima	Optimum temperature (C)	Specific Activity (U/mg)	Specificity
Neurospora sp.TT-241	55	6.5	45	8203	No specificity but preferred 1-and 3-positions
Botrytis cinerea	60	6.0	38	2574	Highest activity with oleic acid esters
Pseudomonas fluorescens AK102	33	8-10	55	6200	Nonspecific for fatty acids
Candida parapsilosis	160	6.5	45	-	High specificity for long chain fatty acids, particularly polyunsaturated fatty acids
Propionibacterium acidi-propionici	6-8	7.0	30	-	Preferred substrates with high saturated fatty acids
Neurospora crassa	54	7.0	30	44	Preferred triglycerides with C16 and C18 acyl chains
Pythium ultimum#144	270	8.0	30	63	1,3-Specific, preferring substrates with higher unsaturation
Rhizopus delemar ATCC 34612	30.3	8.0-8.5	30	7638	-
Fusarium heterosporium	31	5.5-6.0	45-50	2010	1,3-Specific; preferred triacylglycerols with C6-C12 fatty acids
Penicillium roquefortil	25	6.0-7.0	30	4063	Preferred triacylglycerols with C4-C6 fatty acids
Penicillium sp.uzlm-4	27	7.0	25	1001	1,3-Specific, discriminates against diglycerides and active at low surface pressures



Figuer 2.5 Schematic representation of the adsorption and hydrolytic activities of lipases at the oil (substrate complex; E^*a_2-Ac, acylenzyme; P below the plan) and aqueous (above the plane) nterface: E, enzyme in the aqueous phase; E^*a_2 enzyme adsorbed at the interface; E^*a_2, activate enzyme; S, substrate; Ea*S, enzyme-substrate complex; E^*a_2-Ac, acylenzyme; P_1, product (diacylglycerol); P_2, product (fatty acid) (Weete, 1998).

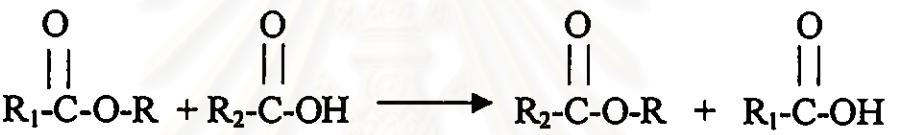
(1) Hydrolysis of Ester



(2) Synthesis of Ester

(3) Transesterification

(3.1) Acidolysis



(3.2) Alcoholysis

(3.3) Ester Exchange (Interesterification)

(3.4) Aminolysis

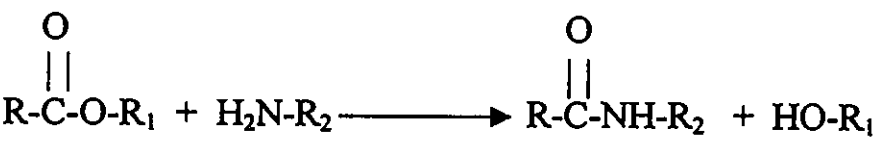


Figure 2.6 Types of reaction catalyzed by lipase (Yamane, 1987).

Table 2.2 Some known lipase producing bacteria

Organisms	Source	Properties of enzymes	References
Pseudomonas fragi	Soil	alkali with excellent thermostability	Nishio et al., 1987
P.sp KWI-56	Soil	extracellular thermostabilily	Iisumi,1990
P.areuginosa EF2	soil	extracellular thesmostability	Gilbert et. al.,1991
Bacillus sp.	soil	extracellular thesmostable	Sugihara et. al.,1991
B. strain A30-1(ATCC 53841)	sediment	extracellular thermostable alkaline	Wang et. al.,1995
P.pseudoalcalizaner F-111	soil	alkaline lipase	Lin et. al.,1996

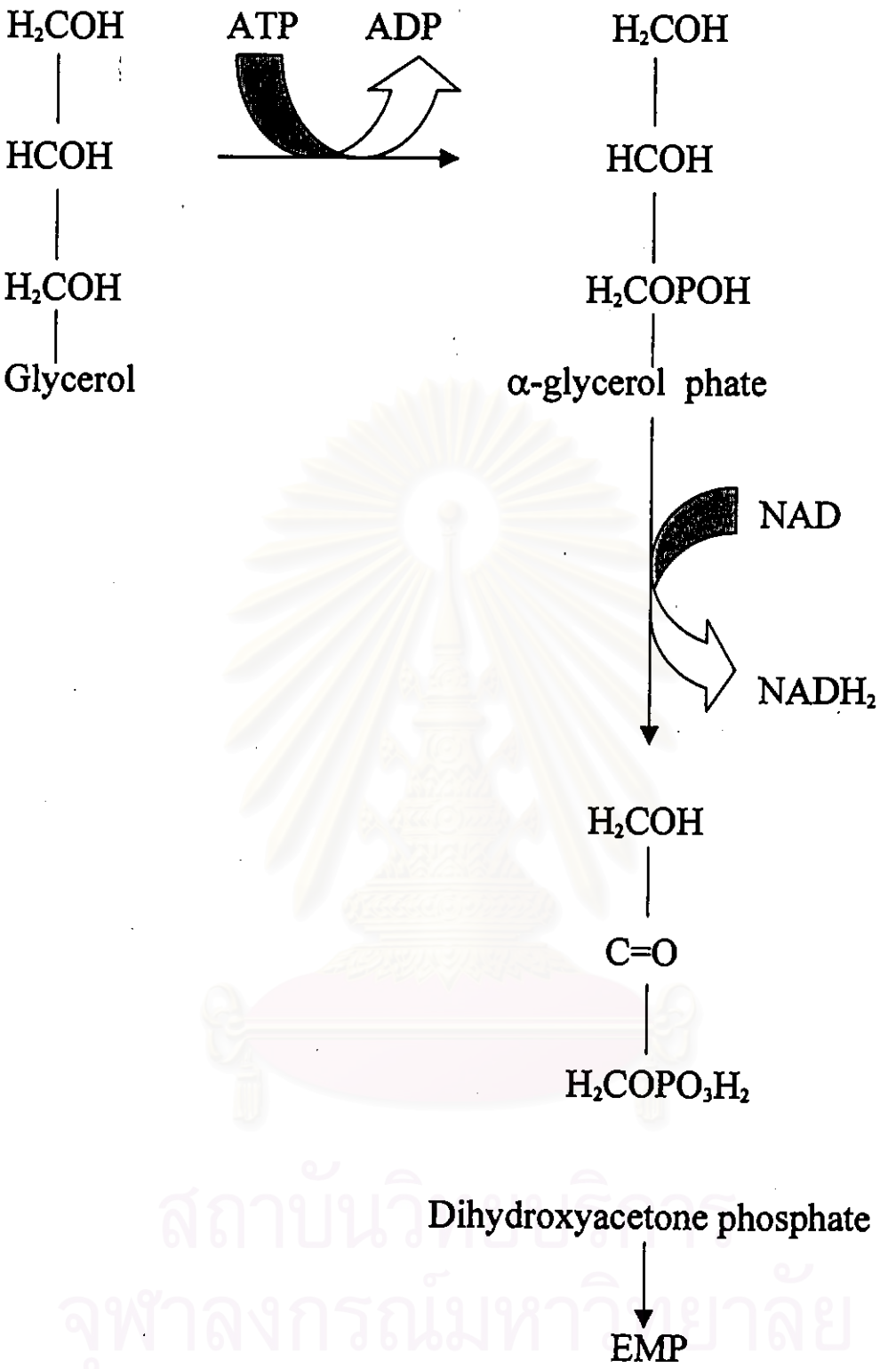


Figure 2.7 Degradative metabolism of glycerol (Kates, 1960).

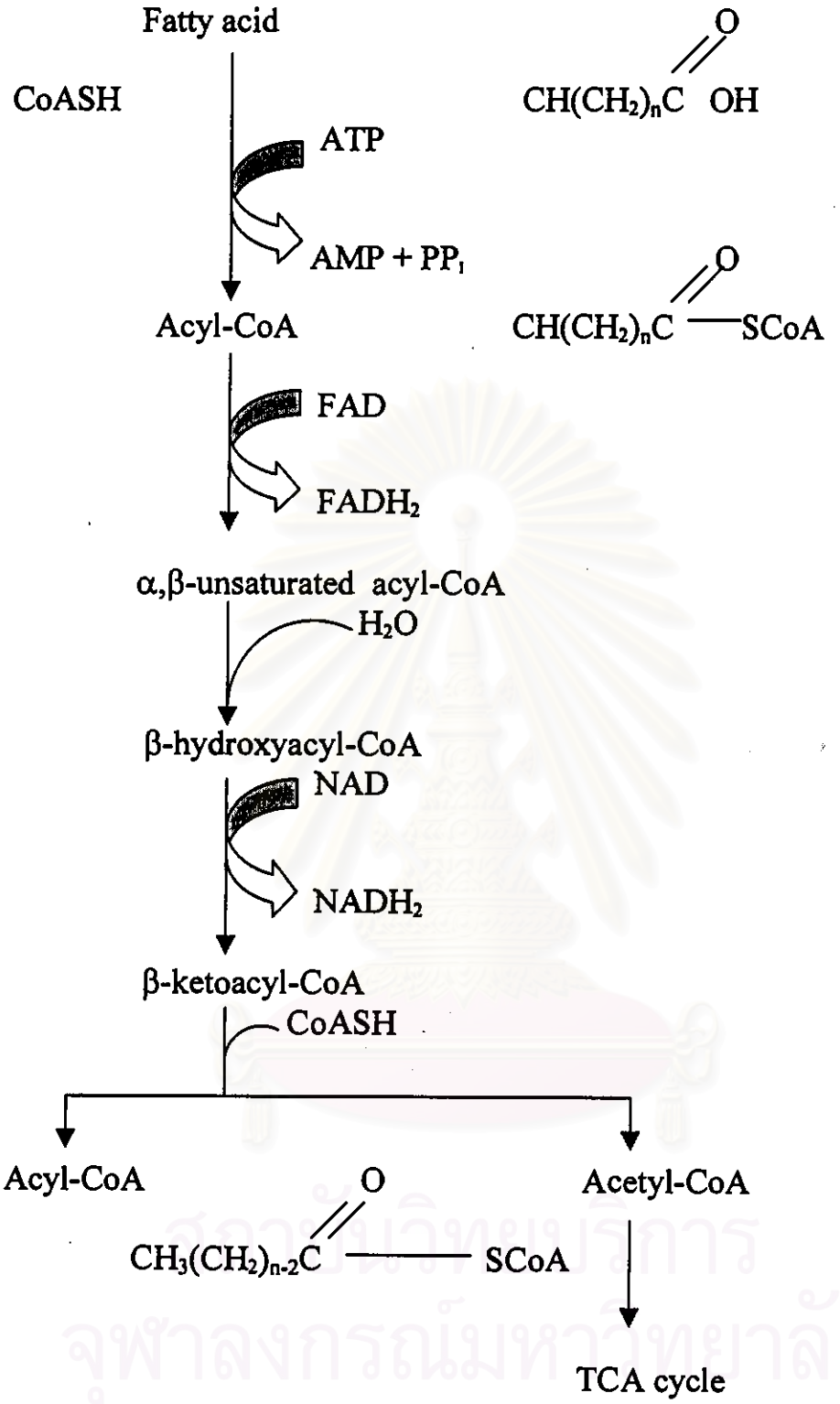


Figure 2.8 Degradation of a fatty acid by β-oxidation (Kates, 1960).

Table2.3 Some known lipolytic bacteria with cloned and sequenced lipase genes.

Organisms	References
Stephylococeus aereus	Lee and Iandolo, 1986
S. hyicus	Gotz et. al.,1985
Pseudomonas cepacia	Jorgensen et. al., 1991
P.fluorescences	Chung et. al., 1991
P. sp KWI-56	Iizumi et. al., 1991
P.fragi	Kugimiya et. al., 1986
Bacillus subtilis 168	Lesuisee et. al., 1993

Table 2.4 The amino acid sequences near the active serine of bacterial lipases (Shimada and et al., 1994).

Lipase producing bacteria	Amino acid sequence
Stephylococeus aureus	405 K V H L V G H S M G G
S.hyicus	362 P V H F I G H S M G G
Pseudomonas fragi	76 R V N L I G H S Q G A
P.cepacia	80 K V N L V G H S Q G G
P.fluorescens	199 D V L V S G H S L G G
P. sp.KWI-56	80 K V N L V G H S Q G G
P. sp.109	101 K V N L I Q H S H G G

Table 2.5 Industrial application areas for microbial lipases (cited by Godtfredsen, 1990).

Industry	Effects	Products
Dairy food	Hydrolysis of milk fat	Flavor agents
	Cheese ripening	Cheese
	Modification of butter fat	Butter
Bakery food	Flavor improvement and Shelf life prolongation	Bakery products
Food dressing	Quality improvement	Mayonnise, dressings and whippings
Health food	Transesterification	Health food
Meat and fish	Flavor development and fat removal	Meat and fish products
Fat and oils	Transesterification	Cocoa butter, margarine
	Hydrolysis	Fatty acids, glycerol, mono-and diglycerides
Chemical	Enantioselectivity	Chirale building blocks and chemicals
	Synthesis	Chemicals
Pharmaceutica	Transesterification	Specialty lipids
	Hydrolysis	Digestive aids,
Cosmetics	Synthesis	Emulsifiers, moisturing agents
Leather	Hydrolysis	Leather products
Paper	Hydrolysis	Paper products
Cleaning	Hydrolysis	Removal of cleaning Agents e.g. surfactants