ISOLATION AND IDENTIFICATION OF LIPASE PRODUCING SOIL FUNGUS; CLONING, SEQUENCING AND PARTIAL CHARACTERIZATION OF ITS LIPASE

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ABSTRACT

ISOLATION AND IDENTIFICATION OF LIPASE PRODUCING SOIL FUNGUS; CLONING, SEQUENCING AND PARTIAL CHARACTERIZATION OF ITS LIPASE

Lipases are well known enzymes which catalyze the hydrolysis of long chain triglycerides. Contrary to many other enzymes, lipases show a wide range of substrate specificity and remarkable levels of activity and stability in non-aqueous environments. Therefore, they have a great potential in many industrial applications such as detergent industry, paper and food technology, as biocatalysts for the synthesis of organic intermediates. Lipases can be obtained from animals, plants as well as from natural and recombinant microorganisms in good yields.

The aim of this thesis was isolation and identification of a lipolytic fungus and purification and characterization of its lipase enzyme. For this purpose, a lipolytic fungus was isolated from soil sample collected from Kula. Lipase activity of this fungus was detected rapidly by Rhodamin B - olive oil plate assay. The lipolytic fungus was identified by 28S rRNA gene sequence analysis and determined to be a strain of *Rhizopus stolonifer*. Because this lypolytic fungus was isolated from soil sample collected from Kula, it was named as *R. stolonifer K45*. This fungus showed best growth at 25°C and did not grow above 35°C. In the second part of the study, lipase enzyme of the fungus was partially purified but previously, optimum time and carbon source for lipase production was determined. According to this, optimum lipase production was obtained at 7^{th} day of growth in the media including only olive oil as carbon source. Glucose when included in the growth media was observed to reduce the amount of lipase. In order to purify fungal lipase, aceton precipitation (30 %) and ultrafiltration methods were used. Lipase activity assay was performed spectrophotometrically. The chain length specificity of this lipase was detected and highest activity was observed towards *p*-NP laurate. The effect of different temperature and pH values on lipase activity and stability was also determined and optimum temperature and pH were found 45°C and pH 8, respectively. Furhermore, different organic solvents and metal ions were tested on lipase activity. The lypolytic enzyme was inhibited by n-hexane. However, methanol and DMSO were detected to enhance the lipase activity.

ÖZET

LİPAZ ÜRETEN TOPRAK KAYNAKLI KÜFÜN İZOLASYONU VE TANIMLANMASI; LİPAZIN KLONLANMASI, SEKANS ANALİZİNİN YAPILMASI VE KISMİ KARAKTERİZASYONU

Lipazlar, uzun zincirli trigliseridlerin hidrolizini katalizleyen iyi bilinen enzimlerdir. Diğer birçok enzime kıyasla, susuz ortamlarda dikkat çekici seviyelerde aktivite ve stabilite ve geniş bir substrat özgünlüğü gösterir. Bu nedenle, deterjan endüstrisi, kağıt ve gıda teknolojisinde ve organik ara ürünlerin sentezi için biyokatalistler olarak birçok endüstriyel uygulamada büyük bir potansiyele sahiptirler. Lipazlar hayvanlardan, bitkilerden ve aynı şekilde mikroorganizmalardan oldukça iyi miktarlarda elde edilebilir.

Bu tezin amacı, lipaz üreten küfün izolasonu ve tanımlanması ve lipaz enziminin saflaştırılması ve karakterizasyonudur. Bu amaçla, lipaz üreten bir küf Kula'dan toplanan toprak örneğinden izole edilmiştir. Bu küfün lipaz aktivitesi Rhodamin B – zeytinyağı petri metodu ile hızlı bir şekilde belirlenmiştir. Lipolitik küf, 28S rRNA gen sekans analizi ile tanımlanmış ve *Rhizopus stolonifer* türünün bir straini olduğu belirlenmiştir. Bu lipolitik küf Kula dan toplanan toprak örneğinden izole edildiği için *R. stolonifer K45* olarak adlandırılmıştır. En iyi 25°C'de büyüme göstermiş 35°C' nin üzerindeki sıcaklıklarda büyüme gözlenmemiştir. Çalışmanın ikinci kısmında, lipaz enzimi küften kısmen izole edilmiştir fakat bunun öncesinde, lipaz enziminin üretilmesi için optimum inkübasyon zamanı ve karbon kaynağı belirlenmiştir. Buna göre, optimum lipaz üretimi büyümenin 7. gününde ve karbon kaynağı olarak sadece olive oil içeren minimal besiyerinde gerçekleşmiştir. Büyüme ortamında glukoz bulunduğunda ise üretilen lipaz miktarının azaldığı görülmüştür. Lipazın saflaştırılması için aseton presipitasyonu (30%) ve ultrafiltrasyon metodları kullanılmıştır. Lipaz aktivite testi spektrofotometrik olarak gerçekleştirilmiştir. Lipazın substrat özgünlüğü belirlenmiş ve en yüksek aktiviteyi *p*-NP laurat için gösterdiği izlenmiştir. Aynı zamanda farklı sıcaklık ve pH değerlerinin lipaz aktivitesi üzerine etkisi analiz edilmiş ve optimum sıcaklık ve pH sırasıyla 45°C ve pH 8 olarak bulunmuştur. Bundan başka, farklı organik çözücüler ve metal iyonlarının lipaz aktivitesi üzerine etkileri test edilmiştir. Lipazın n-hexan ile inhibe edildiği görülmüştür. Bunun aksine, metanol ve DMSO nun ise lipaz aktivitesini arttırdığı tespit edilmiştir.

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LIST OF ABBREVIATIONS

LB	Luria-Bertani
UV	Ultraviolet
Rpm	Revolutions per minute
CTAB	Cetyl trimethylammonium bromide
PCR	Polymerase chain reaction
rRNA	Ribosomal ribonucleic acid
cDNA	complementary deoxyribonucleic acid
dNTP	Deoxyribonucleosidetriphosphate
ng	Nanogram
µg	Microgram
Taq	Thermus aquaticus (DNA polymerase)
µl	Microliter
ml	Milliliter
µM	Micromolar
mM	Millimolar
BLASTn	Basic local alignment search tool for nucleic acids
min	Minute
sec	Second
w/v	Weight per volume
v/v	Volume per volume
µm	Micrometer
nm	Nanometer
sp.	species (singular)
bp	Base pair
kb	Kilobase pairs
kDa	Kilodalton
U	Unit
DMSO	Dimethyl sulfoxide

CHAPTER 1

INTRODUCTION

1.1. Lipases

Lipases or triacylglycerol acylhydrolases are ubiquitous enzymes that catalyse the hydrolysis of relatively long chain triacylglycerides (with acyl chain lengths of > 10 carbon atoms) at fat-water interface with the formation of diacylglyceride, monoacylglyceride, glycerol and free fatty acids (Verger 1997). It is well known that the reaction is reversible and lipases can also catalyse the synthesis of esters in the environments containing low water concentrations (Saxena, et al. 1999; Sharma, et al. 2001).

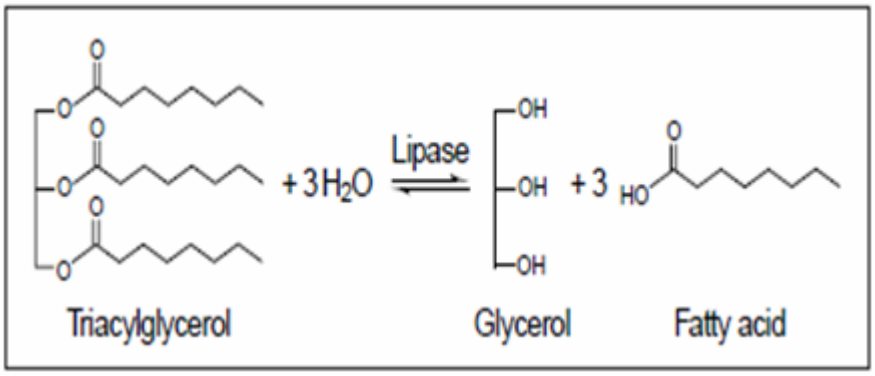


Figure 1.1. The hydrolytic and synthetic action of lipases.
(Source: Jaeger and Reetz 1998)

The ability to catalyse hydrolysis of insoluble long chain fatty acid esters distinguishes lipases from other esterases which catalyse the hydrolysis of soluble esters (Sharma, et al. 2001). Lipases stand amongst the most important biocatalyts primarily due to their properties such as broad substrate specificity, wide range of pH optimum, thermostability, positional specificity etc. (Saxena, et al. 1999). Apart from their natural substrates, lipases can catalyse chemo-, regio- and enantioselective hydrolysis and synthesis of natural and non-natural esters (Reetz 2002). Nonspecific lipases that catalyse the reactions at all positions in triacylglycerols have limited value whereas

regioselective, e.g., 1,3-specific, and enantioselective lipases offer greatest value in industrial applications, such as the production of structured lipids with unique functional properties, synthesis of cocoa butter substituents, the resolution of chiral drugs, biofuels, and for synthesis of personal care products and flavour enhancers (Saxena, et al. 1999).

1.2. Structure and Mechanism of Lipases

In 1958, Sarda and Desnuelle defined lipases in kinetic terms, based on the phenomenon of 'interfacial activation'. This phenomenon describes the fact that activity of lipases is increased towards insoluble substrates (supersubstrate) that form an emulsion (Schmid and Verger 1998). In 1990, the structures of first two lipases (*Rhizomucor miehei* lipase and human pancreatic lipase) have been solved by X ray crystallography and their three-dimensional structures suggested that interfacial activation might be due to the presence of a short α helix (lid or flap) covering the active center of enzyme (Schmid and Verger 1998; Verger 1997). The determination of three dimensional structure of lipases provided a strong evidence that upon binding to the interface, this lid structure mowes away, turnes the 'closed ' form of enzyme into 'open' form and thus makes the enzyme accessible to the substrate (Peters, et al. 2001). In other words, in the absence of substrate the lid is closed and enzyme is inactive whereas in the presence of substrate, water-oil interface exist, lid is opened and enzyme becomes active (Winkler, et al. 1989; Rahman, et al. 2006). Interfacial activation distinguishes lipases from other hydrolases such as serine proteases or esterases. However, structural and numerous biochemical data on highly purified lipases exhibited that interfacial activation is not observed with all lipases and some lipases do not have a lid structure controlling acces to the active center. Lipases from *Pseudomonas glumae* and *Candida antarctica* (type B) both have lid structure covering the active site of enzyme but do not exhibit interfacial activation. Therefore, neither the phenomenon of interfacial activation nor the presence of a lid structure can be suitable criteria to define an esterase as a lipase. Lipases can be defined as carboxylesterases acting on long-chain acylglycerols (Schmid and Verger 1998; Beisson, et al. 2000).

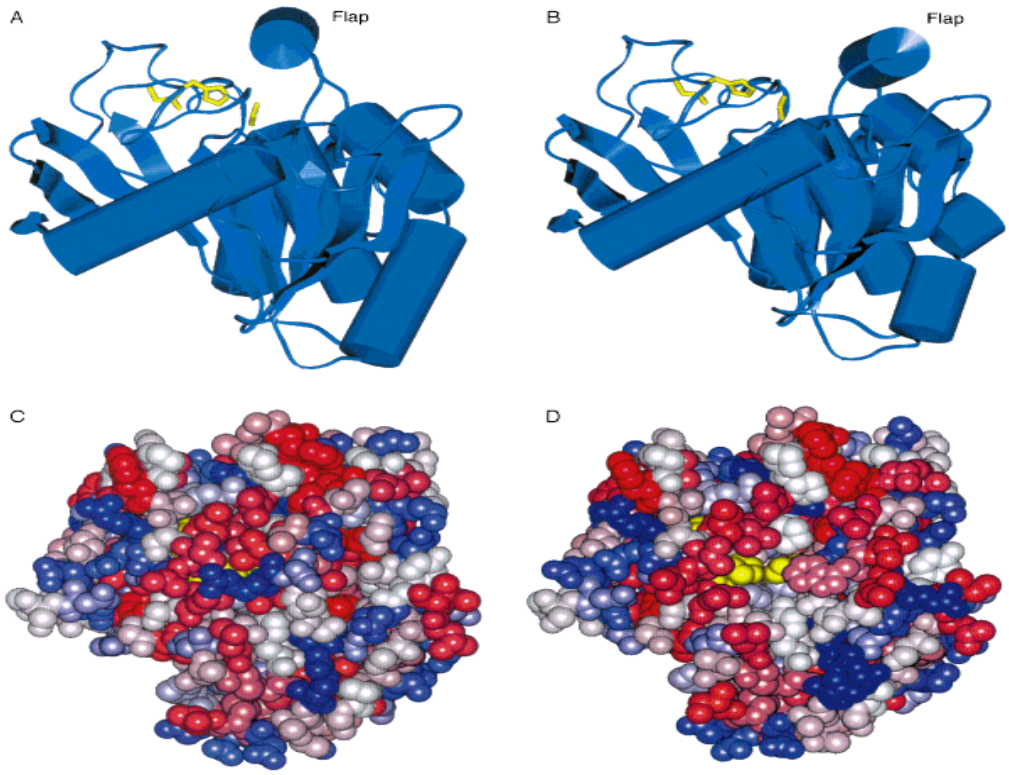


Figure 1.2. Structure of Mucor miehei lipase in closed (A, C) and open form (B, D). A and B (side view): the catalytic triad (yellow) and secondary structure elements showing the α/β-hydrolase fold common to all lipases (Source: Schmid and Verger, 1998).

Lipase structures that have been solved so far, exhibit a characteristic folding pattern known as α/β hydrolase fold. The lipase core consists of up to eight different β strands (β1-β8) and six α helices (A-F). The active site of enzyme composed of amino acids serine, aspartic (or glutamic) acid and histidine which forms catalytic triad; the nucleophilic Ser residue is located at the C-terminal end of strand β5 in a highly conserved pentapeptide GXSXG, forming a characteristic β–turn–α motif named the 'nucleophilic elbow' (Figure 3) (Cygler, et al. 1993; Nardini and Dijkstra 2000)

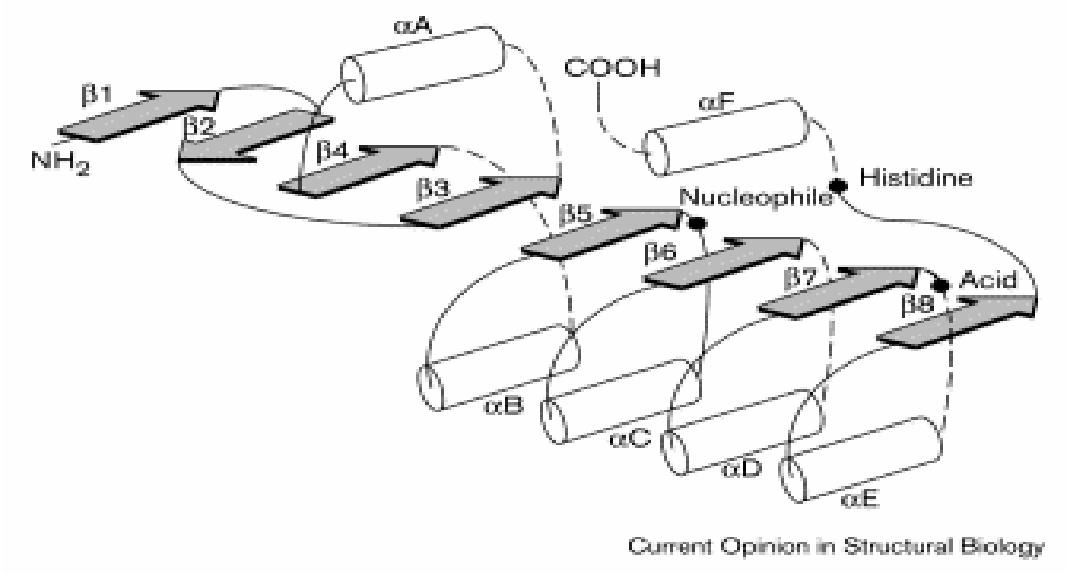


Figure 1.3. Secondary structure diagram of the 'canonical' α/β hydrolase fold. α helices and β strands are represented by white cylinders and gray arrows, respectively. The location of the catalytic triad is indicated by black dots. Dashed lines indicate the location of possible insertions (Source: Nardini and Dijkstra 2000).

Substrate hydrolysis starts with a nucleophilic attack by the catalytic-site- Ser oxygen on the carbonyl carbon atom of the ester bond, leading to the formation of a tetrahedral intermediate stabilized by hydrogen bonding to nitrogen atoms of main-chain residues that belong to the socalled 'oxyanion hole'. An alcohol is liberated, leaving behind an acyl–lipase complex, which is finally hydrolysed with liberation of the fatty acid and regeneration of the enzyme (Figure 1.4) (Jaeger and Reetz 1998).

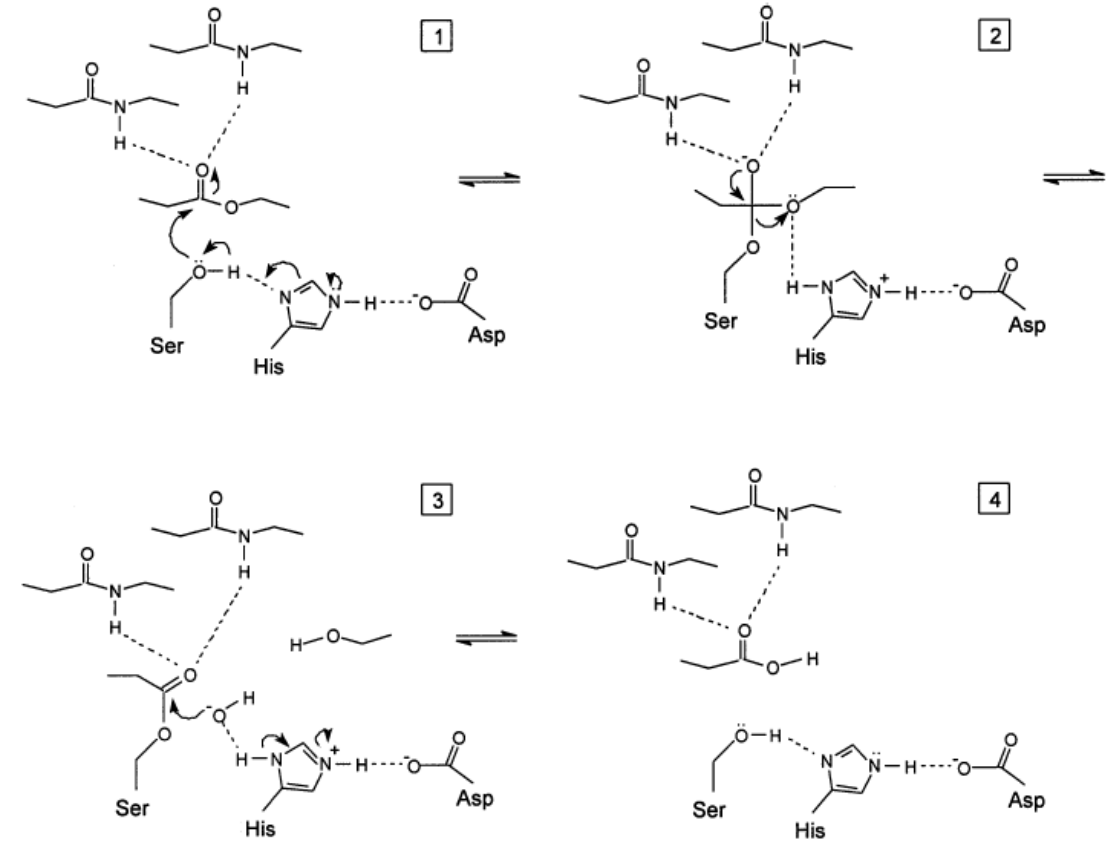


Figure 1.4. Reaction mechanism of lipases.
(Source: Jaeger, et al.1999).

1.3. Substrate Specificity of Lipases

The specificity of lipases is controlled by the structure of substrate, molecular properties of enzyme and factors affecting binding of the enzyme to the substrate (Saxena, et al. 1999). Lipases can be classified into three groups by their substrate specificity; positional specificity, fatty acid specificity and partial glyceride specificity (Rahman, et al. 2006). Lipase selectivity allows obtaining mostly one of the possible reaction products from the same substrate (Illanes 2008).

Positional specificity: Lipases hydrolyse triglycerides and release diglycerides, monoglycerides, glycerol and free fatty acids. Two group of lipases can be defined on the bases of their positional specificity. The first group consists of nonspecific lipases which releases fatty acids from all three positions of triglyceride randomly. However, the second group has 1,3 positional specificity which releases fatty acids only from the outer 1- and 3- positions of the triglyceride (Shimada, et al. 1994; Illanes, 2008). Many

extracellular microbial lipases, such as those from *A. niger* (Shu, et al. 2007), *R. japonicus*, *M. miehei*, *H. lanuginosa*, *C. viscosum*, and *P. fluorescens* show 1,3-(regio)-specificity (Saxena, et al.1999). *Rhizopus arrhizus, Rhizopus delemar* and *Candida cylindracea* have been reported to display partial stereospecificity in the hydrolysis of triacylglycerols. These lipases can be used to isolate optically pure esters and alcohols (Illanes 2008).

Fatty acid specificity: Monoacids and triglycerides are used to determine fatty acid specificity. Microbial lipases show different abilities to hydrolyse fatty esters. For example, *Streptomyces rimosus* lipase displayed highest hydrolysis rates with substrates *p*-NP-caprylate (C_8), *p*-NP-caprate (C_{10}) and *p*-NP-laurate (C_{12}) indicating the enzyme preference for medium acyl chain lengths. *p*-NP-palmitate and *p*-NP-myristate were also good substrates but *p*-NP-stearate was hydrolysed at considerably slower rate. The lipase activity on long chain of substrates was found between 70 to 100 % optimal for C_8 or C_{10} groups, and 30 and 50 % for C_{12} to C_{18}, whereas, this was less than 30 % with short chain substrates C_2-C_6. (Rahman, et al. 2006). The lipases from various *Rhizopus* species were reported to show maximum hydrolytic activity with the medium chain fatty acids C_8-C_{10}. (Saxena, et al. 1999).

Partial glyceride specificity: In addition to triglycerides, lipases can hydrolyse mono- and diglycerides into fatty acids and glycerol. Lipases that hydrolyses monoglycerides are called *monoglyceride lipases* (EC 3.1.1.23) (Shimada, et al. 1994).

1.4. Fungal Lipases

Lipases are produced by plants, animals and also by microorganisms (Rahman, et al. 2006) including fungi and bacteria (Haki and Rakshit 2003). In 1856, Claude Bernard discovered first lipase in pancreatic juice as an enzyme that hydrolysed insoluble oil droplets and converted them into soluble products. Lipases have traditionally been obtained from animal pancreas as a digestive aid for human consumption either in the crude mixture with other hydrolases (pancreatin) or as purified grade. Microbial lipases were first required because of a shortage of pancreas and difficulties of collecting available materials. These enzymes are mainly preferred over enzymes derived from plants and animals because of the high yields of enzyme possible, great variety of catalytic activities available, ease of genetic manipulations,

regular supply of enzyme due to the seasonal fluctuation and rapid growth of microorganisms on inexpensive media (Hasan, et al. 2006). In addition to these, microbial lipases have enormous biotechnological potential due to the facts that they are stable in organic solvents, do not require cofactors, possess a broad substrate specificity and exhibit a high enantioselectivity (Jaeger and Reetz 1998). In 1901, the presence of lipases has been observed for *Bacillus prodigiosus*, *B. pyocyaneus* and *B. fluorescens* which represent today's best studied lipase producing bacteria now named *Serratia marcescens*, *Pseudomonas aeruginosa* and *Pseudomonas fluorescens*, respectively. Among various microorganisms, moulds have been recognized as best lipase sources (Christakopoulos, et al. 1992). Since the lipases from the moulds of the *Penicillum* genus play an important role in developing characteristic flavours in ripened cheese, these enzymes mainly preferred in the dairy industry. The fungus *P. chrysogenum* can be used for the production of some antibiotics such as penicillin G or V. Molecular masses of most lipases from *Penicillum* strains have been reported to be in the range 30-60 kDa. (Ferrer, et al. 2000). The filamentous fungus *Aspergillus oryzae* has been reported to produce three extracellular lipases L1, L2, and L3; among these L1 has a high specificity towards dimercaptobutyrate, L2 catalyse the hydrolysis of mono- and diacylglycerols but not triacylglycerols and L3 catalyse the hydrolysis of mono-, di- and triacylglycerols. Lipases from *A.oryzae* is used in the production of koji for the manufacture of traditional Japanese fermented foods such as sake, miso and soy sauce. These lipases hydrolyze glycerides of soybeans and synthesize esters during miso fermentation, so that the flavors and tastes are formed (Toida, et al. 1998). The *Mucor miehei* lipase (Lipozyme^{TM}) and *Humicola* lipase expressed in *A.oryzae* (Lypolase^{TM}) are examples of lipases which show a high degree of specificity for the outer 1 and 3 positions of triglycerides. These lipases can be used in laundry detergents and for specific modifications of triglycerides (Zamost, et al. 2001).

1.4.1. Lipases from *Rhizopus* species

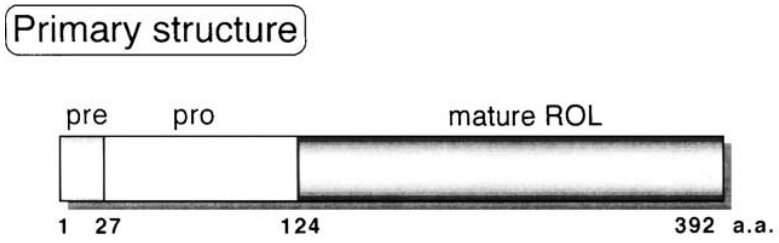
Rhizopus species was mainly divided into three groups which are *R. microsporus (R. chinensis), R. oryzae* and *R. stolonifer.* Among these, the more widely investigated species is *R. oryzae*. Lipase genes from *R. oryzae* and *R. stolonifer* have been reported to show % 84 amino acid sequence identitiy. The isolates named individually such as *R.*

oryzae, Rhizopus arrhizus, Rhizopus delemar and *Rhizopus javanicus* latterly recognized to be the same organism and were combined into *R. oryzae* despite some minor variations. *R. oryzae* lipase, *R. delemar* lipase and *R. javanicus* lipase have a substitution in the His134 and the Leu234 by an Asn and a Leu, respectively (Yu, et al. 2009).

Lipase from *R. chinensis* CCTCC M20102 was found to have very high ability of esterification of short chain fatty acids with ethanol. In other studies, two intracellular lipases, RCL-lip1 and RCL-lip2, were isolated from *R. chinensis.* RCL-lip2 is able to hydrolyze short chain esters and its N-terminal sequence shows a high homology with *Rhizopus oryzae* mature lipase. *Rhizopus chinensis* lipase gene structure has been reported to be similar to other *Rhizopus sp.* lipase genes which consist of signal sequence, prosequence and mature lipase sequence without intron (Yu, et al. 2009).

Eukaryotic lipases, *Rhizopus* lipases, are synthesized as pre-pro-proteins. Previous in vivo expression and in vitro refolding studies with *R.oryzae* lipase showed that the prosequence is necessary in the production of active lipase (Beer, et al. 1996). It has been concluded that the N-terminal sequence of prolipase can play important role on its specific activity, regioselectivity, stereoselectivity and its binding to a lipidic interface through a structural explanation of the enhanced penetration capacity as well as the catalytic activity of prolipase (Sayari, et al. 2005). Since prosequences help in overcoming kinetic block in the folding process, they seem to be necessary in the late stages of folding pathway. However, the residues of prosequence which mediate the folding are not strictly clear (Ueda, et al. 2002).

R. oryzae lipase comprises a signal sequence of 26 amino acids, a prosequence of 97 amino acids, and a mature lipase region of 269 amino acids (Takahashi, et al. 1998). This enzyme contains six cysteine residues which form three disulfide bridges (Derewenda, et al. 1994).



Presequence (26 a.a.) is for protein localization to the endoplasmic reticulum and secretion
Prosequence (97 a.a.)
The mature lipase (269 a.a.) is secreted as a 30 kDa protein.

Figure 1.5. *Rhizopus oryzae* lipase
(Source: Ueda, et al. 2002)

Since lipases from the genus *Rhizopus* are positionally selective, acting only at the sn 1 and sn 3 locations (1,3-regioselectivity) and are active only against esters of primary alcohols, they are attractive catalysts in lipid modification processes (Alberghina 2000). *R. delemar* lipase cDNA were previously expressed in *E.coli* for both the unprocessed lipase precursor and the mature product in insoluble form because *E.coli* lacks the necessary proteases to process fungal maturation signals. Attempts to produce this enzyme in active form in *E.coli* by site-directed mutagenesis revealed toxic effects to the host (Joerger and Haas 1992). However, the production of active *R. oryzae* lipase (ROL) and its precursor ProROL in soluble form was achieved in *E.coli* when Origami (DE3) and pET-11d were used as expression systems (Lorenzo, et al. 2005). The production of *Rhizopus* lipases in enzymatically active form has been also performed in *Pichia pastoris* (Minning, et al. 1998; Yu, et al. 2009) and *S. cerevisiae* in which these enzymes are converted to their mature forms by Kex-2 like proteases (Takahashi, et al. 1998).

1.5. Factors Affecting Lipase Production from Fungi

The type and concentration of carbon and nitrogen sources, the culture pH, growth temperature and dissolved oxygen concentration affects the production of lipase. Although a few authors have produced good yields of lipase in the absence of fats and oils, lipidic carbon sources seem to be necessary to obtain high yields of this enzyme (Sharma, et al. 2001).

1.5.1. Effect of Carbon Sources

One study explored 56 strains of molds for the ability to produce lipase. A strain identified as *Pe. wortmanii* was determined to be the best lipase producer. Maximum lipase production (12.5 U/mL) was obtained in a 7-day culture using olive oil (5% w/v) as the carbon source. The optimal pH and temperature for the crude lipase activity were 7.0 and 45 ^{0}C respectively (Costa and Peralta 1998). Similarly an alkaline lipase from *Penicillum expansum* exhibited maximum activity when biomass was grown in an oil-containing medium (0.1 % olive oil) at pH 8.3 (Sztajer, et al. 1993). A *Mucor hiemalis* strain has been reported to produce an inducable lipase and rape oil was found to be the best inducer. The highest lipase production was obtained with C:N about 0.1 (Hiol, et al. 1999). *Rhizopus oligosporus* has been determined to produce high yields of enzyme when Tweens were used as carbon source. The yield of lipase was maximum at 25 ^{0}C after 3 days at pH 6.5 (Nahas 1988). A fungus *Ophiostoma piceae* showed both high biomass and lipase activity when plant oils (olive, soybean, corn, sunflower seed, sesame, cotton seed or peanut) were used as carbon sources (Gao and Breuil 1995). For lipase production and cell growth of *Rhizopus oryzae*, rape seed and corn oil (3 %) were found to be the most suitable substrates (Essamri, et al. 1998). The efficient growth of *Candida rugosa* biomass and high lipase expression from this strain were supported with oleic acid as carbon source in the medium (Lotti, et al. 1998). Various types of oils were used to test their effects on lipase production and growth of *Fusarium solani.* The highest lipase activity of this strain was observed with sesame oil (0.5% (v/v)), followed by triolein contained in the medium. Corn oil and olive oil were also good carbon sources but they produced lower lipase activity (Maia, et al. 2001). A strain of *Aspergillus niger* was reported to produce lipase which was induced when olive oil, soybean, sunflower or rapeseed oil were used as carbon sources. In some previous studies, olive oil was found to be best inducer of lipase production. Sun flower groats and starch were also reported as best lipase inducers by some authors. Lipase activity was hardly detected when lipid materials were not added to the medium. However, a strain studied by Fukumoto et al. did not require an inducer and produced lipases in a medium with $CaCO_3$ (Pokorny, et al. 1997).

1.5.2. Effect of Nitrogen Sources

Maximum lipase production per unit of growth with *R . oligosporus* (about 47 U/mg) was obtained when the soybean meal extract was used as nitrogen source. Salts, amino acids, proteins and urea were used as nitrogen sources in media supplemented with 1 % glucose. *R. oligosporus* showed excellent growth with tryptone as nitrogen source but the lipase activity per mg dry weight was poor (Nahas 1988). *Penicillum citrinum* showed maximum lipase production in a medium including 5 % (w/v) peptone (pH: 7.2). Nitrogen sources such as corn steep liquor and soybean meal stimulated lipase production but to a lesser extent than peptone. Urea and ammonium sulfate were determined to inhibit lipase synthesis. Maximal lipase production from thermophilic fungus *Rhizopus oryzae* was also obtained when peptone was used as nitrogen source in the medium (Sharma, et al. 2001). Consistently high production of thermostable lipases from thermophilic fungi *Emericella rugulosa, Humicola sp., T. lanuginosus, Pe. purpurogenum,* and *Chrysosporium sulfureum*, was reported by use of yeast extract as the nitrogen source (Sharma, et al. 2001). High lipase production from *Aspergillus niger* was observed when the medium was supplemented with ammonium nitrate (Pokorny, et al. 1994). The fungus *Ophiostoma piceae* showed the best growth with casamino acids in the medium, whereas ammonium sulphate gave the best lipase production from this strain. The highest lipase productivity seen was obtained in a medium with olive oil as carbon source and a combination of ammonium sulphate and peptone as nitrogen source. The enzyme had optimal activity at 60 ^{0}C and pH 9.5 (Gao and Breuil 1995). For intracellular lipase production by the fungus *R. oryzae,* corn steep liquor (7%) was an optimal nitrogen source. At concentrations greater than 7%, corn steep liquor caused a rapid decline in cell growth and lipase production (Essamri, et al. 1998).

1.5.3. Effect of Metal Ions

Lipase activity of a *Aspergillus niger* strain was determined to decrease in the presence of $FeCl_3$ at 10mM concentration and it was completely inhibited at 100mM concentration (Pokorny, et al. 1997). Fe^{3+}, Hg^{2+}, and Cu^{2+} ions seemed to depress lipase activity of *R.oryzae* (Hiol, et al. 2000). The effects of various metal ions on *Humicola lanuginosa* lipase activity were investigated. Alkali metal ions (Li^+, K^+ or Ca^{2+})

enhanced but Co^{2+}, Ni^{2+}, Cu^{2+}, Sn^{2+} and Hg^{2+} inhibited the lipase activity (Omar, et al. 1987). *Mucor sp.* lipase activity has been reported to increase 3.5 times towards long chains triglycerides by the addition of $CaCl_2$ at 5mM concentration (Abbas, et al. 2002). Among the metal ions tested, only Mn^{2+} (27-39 %) enhanced the lipase activity of *Rhizopus homothallicus* whereas Mg^{2+}, Ca^{2+}, Cu^{2+} and Co^{2+} had no effect (Diaz, et al. 2006). Lipase activity of a *Penicillium camembertii* strain was inhibited significantly by both Fe^{2+} and Ca^{2+} (Tan, et al. 2004). $AgNO_3$, $NiCl_2$, $HgCl_2$ strongly inhibited the lipase activity of *Geotrichum candidum* whereas the presence of Ca^{2+} and Ba ions enhanced the activity of the enzyme (Gopinath, et al. 2003).

1.6. Thermostable/Alkalophilic Lipases

Thermostable lipases have growing importance for different applications. Mesophilic producers have been used in most of the studies so far. Many lipases from mesophiles are stable at elevated temperatures. Proteins from thermophilic organisms have been proved to be more useful than ones from mesophiles for biotechnological applications due to their stability (Imamura and Kitaura 2000). Thermostability of enzymes presents advantages allowing higher operation temperatures which provide higher reactivity (higher reaction and diffusional rates), higher process yield, lower viscosity and the reduced risk of microbial contamination. Thermostable enzymes can be obtained from mezophilic and thermophilic organisms; even psycrophiles have some thermostable enzymes. The obvious source of thermostable enzymes is thermophilic organisms because they will confer their proteins a high thermal stability (Illanes 1999).

In recent years, there has been a great demand for thermostable enzymes in industrial fields. They have been applied to the synthesis of biopolymers and biodiesel and used for the production of pharmaceuticals, agrochemicals, cosmetics, and flavours. Therefore, thermostable lipases from various sources have been purified and characterized (Kim, et al. 1998; Haki and Rakshit 2003).

Since they can be produced at low cost and exhibit improved stability, thermostable lipases from microorganisms are very advantageous. According to Razak et al. (1997) very few fungal lipases are active at temperatures above 40 ^{0}C but a lipase from a *Rhizopus* strain has been reported to be active at 50 ^{0}C and this lipase may have potential for applications requiring high temperatures (Koblitz and Pastore 2006). The

optimum temperature for a thermostable lipase from *Humicola lanuginosa* were reported as 45°C (Omar, et al. 1987). In another study, Arima et al. purified a lipase from *Humicola lanuginosa* strain Y-38 and found that the enzyme was optimally active at 60°C, at pH 8.0 and was stable in the pH range of 4 to 11. It showed appreciable activity at up to 65°C but was inactivated on heating at 80°C for 20 min (Maheshwari, et al. 2000). A lipase from a *Geotrichum*-like strain was also found to be thermostable and exhibited a very high activity after 1 hour incubation at 60 ^{0}C (Ginalska, et al. 2004).

Alkalophilic thermophiles are mainly preferred in detergent and leather industries. Lipases which are stable at pH ranges from pH 10 to 11, at temperatures from 30 to 60°C and in the presence of surfactants, are preferred as ingredients of laundry detergents (Maheshwari, et al. 2000). In various fields of industry, such as chiral molecule synthesis, biological wood pulping, and more production of sophisticated enzyme detergents, alkaline enzymes should find additional uses (Horikoshi 1999).

1.7. Applications of Lipases

Although lipases are primarily responsible for the hydrolysis of acylglycerides, a number of low and high-molecular weight esters, thiol esters, amides, polyol/polyacid esters etc. are also accepted as substrates by this unique group of enzyme. This broad substrate specificity of lipases is enlarged by the fact that lipases can catalyse the synthesis of esters efficiently and some lipases are known to be better in the synthesis reactions than hydrolysis reactions (Gandhi 1997).

Different reactions catalysed by lipases may be classified as follows;

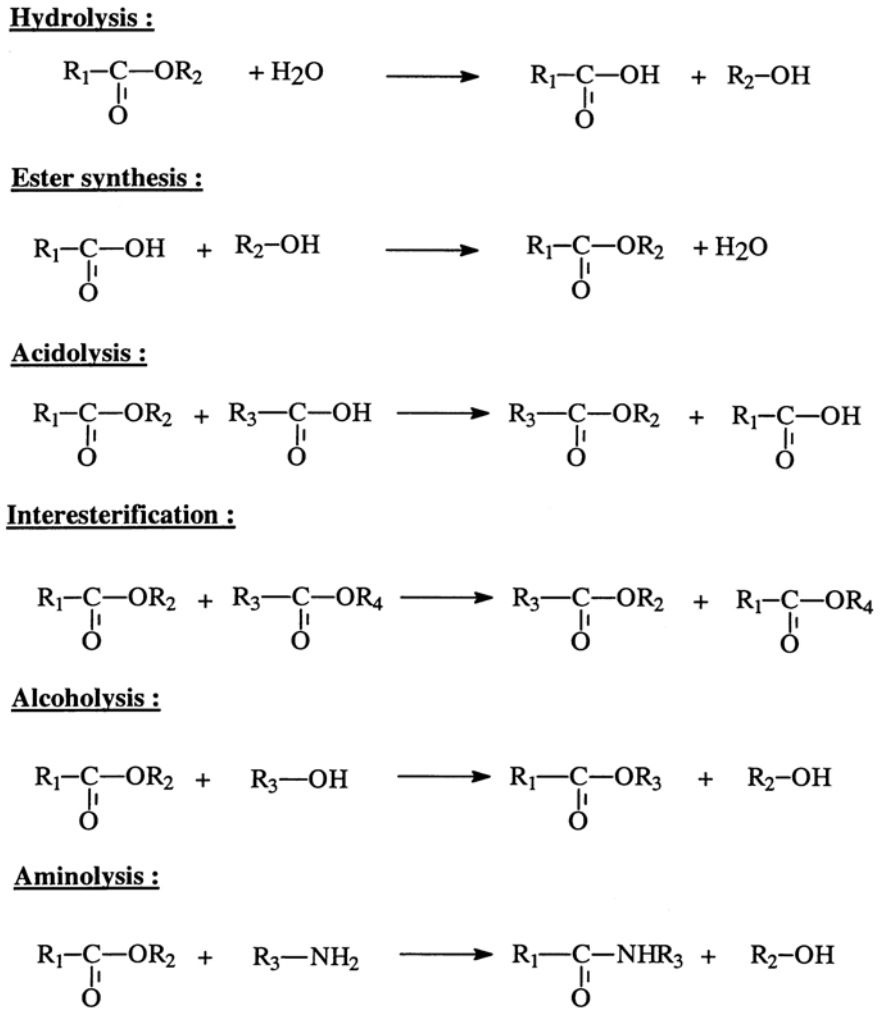


Figure 1.6. Different reactions catalyzed by lipases
(Source: Villeneuve, et al. 2000; Gandhi, 1997).

Since lipases show unique chemo- regio- and enantioselectivities, which provide the production of novel drugs, agrochemicals, and fine chemicals, they are important choice of biocatalysts (Saxena, et al. 2003). Reactions catalysed by lipases are often carried out in mixtures of the reactants to avoid the use of the solvents and for this, reaction mixture has to be heated from 50^0C to 80^0C for the fat to be liquid. Therefore, lipases have to be thermostable for optimal performance (Zamost, et al. 2001).

1.7.1. Fat and Oleochemical Industry

Lipases belongs to the family of serine hydrolases that act on carboxylic ester bonds and hydrolyse triglycerides into diglycerides, monoglycerides, free fatty acids and glycerol. In addition to hydrolysis function, lipases also catalyse esterification, interesterification and transesterification reactions in nonaqueous environment. This property makes lipases very important for various applications in the food, leather, detergent, textile, cosmetic, paper and pharmaceutical industries (Houde, et al. 2004). The physical properties and the nutritional and sensory value of lipids are influenced by some factors such as the chain length of the fatty acid, its degree of unsaturation and the position of the fatty acid in the glycrol backbone (Sharma, et al. 2001). Various chemical methods can be used to convert less valuable lipids into more valuable ones but these methods generally give random products. Lipases can modify the properties of lipids by changing the location of fatty acid chains in the glyceride and replacing one or more fatty acids with new ones. This way, higher value lipids can be produced from a relatively less desirable lipid. For example, lipases can be used in transesterification of cheaper oils and thus produces cocoa butter from palm mid-fraction, equivalent of cocoa butter, pharmaceutically important polyunsaturated fatty acids (PUFAs) rich/low calorie lipids and biodiesel from vegetable oils (Jaeger and Reetz 1998, Gupta 2000).

In oleochemical processing, use of lipases saves energy and minimize thermal degradation during alcoholysis, hydrolysis, and glycerolysis. Lipases, depending on the substrates, can catalyse acidolysis (where an acyl moiety is displaced between an acyl glycerol and a carboxylic acid), alcoholysis (where an acyl moiety is displaced between an acyl glycerol and an alcohol) and transesterification (where an acyl moieties are exchanged between two acylglycerols) (Sharma, et al. 2001).

1.7.2. Lipases in Detergent Industry

Because of the ability to hydrolyse fats, the most commercially important application field of lipases is their addition to detergents which are used in household dishwashers and industrial laundry. All detergents have similar ingredients and to improve detergency, one or more enzymes such as protease, amylase, cellulase and lipase are involved in modern types of heavy duty powder detergents and automatic

dishwasher detergents (Ito, et al. 1998). Environmental load of detergent products can be reduced by enzymes because they save energy by decreasing the washing temperature; allow less desirable chemicals to be reduced; are biodegradable; and do not leave harmful products to the environment. Lipases used in detergent industry are selected based on some requirements; (a) low substrate specificity to be able to hydrolyse various compositions of fats; (b) resistance to relatively harsh washing conditions (30-60 ^{0}C, pH 10-12) and (c) resistance to damaging surfactants and enzymes included in many detergent formulations. To remove fat-containing stains such as those resulting from frying fats, salad oils, butter, fat based sauces, soups, human sebum or certain cosmetics, lipase is used in detergent formulations. The enzyme hydrolyzes the original fats (triglycerides) into more soluble monoglycerides, diglycerides, free fatty acids and glycerol (Sharma, et al. 2001).

In the textile industry, removal of fatty stains is a problematic stage. In 1988, Novo Nordisk company developed a lipase capable of dissolving fatty stains. A fungal strain Humicola produces this enzyme naturally but the quantities of enzyme are not adequate for commercial applications. Hovewer, after cloning and insertion of this lipase encoding gene into *Aspergillus oryzae*, commercially relevant yields of enzyme was obtained and now this enzyme can be produced in high quantities and used in detergents allowing better washing performances and energy savings (http://www.efbweb.org/topics/genetic/menu4 3.htm)

The first commercial recombinant lipase 'Lipolase', introduced by Novo Nordisk in 1994, is originated from the fungus *Thermomyces lanuginosus* and expressed in *Aspergillus oryzae*. In 1995, two bacterial lipases were introduced—Lumafast from *Pseudomonas mendocina* and Lipomax from *Pseudomonas alcaligenes*, both produced by Genencor International, AU-KBC Research Center, Life Sciences, Anna University, Chennai, India (http://www.au-kbc.org/beta/bioproj2/uses.html). Lipases which are active at alkaline media are preferred for laundry detergents. For example, *Acinetobacter radioresistens* derived lipase has an optimum pH of 10 and is stable over a pH range of 6-10 and thus, this enzyme has a great potential as an additive of laundry detergents (Chen, et al. 1998). A great research studies have been performed to obtain more stable lipases at extreme temperature and pH conditions and many examples of microbial lipases included in the patent literature are said to be suitable for use in detergents. There are other common commercial applications for detergents such as decomposition of lipid contaminants in dry cleaning solvents, contact lens cleaning,

degradation of organic wastes on the surface of exhaust pipes, toilet bowls, etc., washing, degreasing and water reconditioning by using lipases along with oxidoreductases, which allows for smaller amounts of surfactants and operation at low temperatures (Hasan, et al. 2006).

1.7.3. Resolution of Racemic Mixtures

Lipases have the ability of retaining their activity in nonpolar organic solvents thus they can be used in the hydrolysis of water insoluble esters such as in the resolution of racemic mixtures through stereospecific hydrolysis (Kirchner, et al. 1985). In addition to this, lipases sythesize chiral building blocks for pharmaceuticals, agrochemicals and pesticides. Production of single enantiomers of drug intermediates has become increasingly important in pharmaceutical industry because chirality is key factor for the efficacy of many drugs (Patel 2002). Lipase from *C. antarctica* (Novozyme (R) 435) has been used for the kinetic resolution of racemic flurbiprofen by the method of enantioselective esterification with alcohols (Zhang, et al. 2005). Baclofen, chemically (*RS*)-beta-(aminomethyl)-4-chlorobenzenepropanoic acid, which is used as muscle relaxant and in the pain theraphy, produces two isomers and lipase from *C. cylindracea* has been used in the resolution of racemic mixture (Muralidhar, et al. 2001).

1.7.4. Diagnostic Tool

In the medical sector, lipases are important drug targets or marker enzymes. Their presence and high levels can indicate certain infection or disease and thus lipases can be used as diagnostic tools. In blood serum, the levels of lipases can be used as diagnostic tool in the detection of acute pancreatitis and pacreatic injury. Despite the most accurate laboratory indicators for pancreatitis, serum amylase and lipase levels are still used in the diagnosis of this disease (Lott and Lu 1991).

Propionibacterium acnes lipase in skin diseases and Unsei-in have been examined by Higaki and Morohashi (2003). Butyric acid is produced higher in axillary seborrheic dermatitis (ASD) than in other dermatitis, and that in acne vulgaris (AV) was significantly higher than in controls. *P. acnes* lipase is the pathogenic factor in AV and

fatty acids produced by lipase might be the pathogenic factor in ASD. In *Vibrio cholerae* infections, kanagawa haemolysin, slime, lipase, and colonial opacity have also been considered as virulence markers. Lipases derived from some pathogenic bacteria such as *P. acnes, Corynebacterium acnes* and *Staphylococcus aureus* have also been found to affect skin in acne patients (Hasan, et al. 2006).

1.7.5. Waste treatment

In aerobic waste processes, lipases are utilized to remove layers of fats continuously from the surfaces of aerated tanks to allow oxygen trasport (to maintain living conditions for biomass). For this purpose, *C. rugosa* lipase can be used. Clearing and prevention of fat blockage and effective breakdown of solids in waste systems are important in many industrial operations such as degradation of organic debris - for this purpose, commercial mixture of lipase, cellulase, amylase, inorganic nutrients is utilized, and cleaning of holding tanks, saptic tanks, grease traps. In food processing industry, leather industry and in the poultry waste processing, effective treatment is also necessary. In lipid-rich wastewater treatment, *P. aeruginosa* LP602 cells and lipase together were shown to be usable. Some environmental problems such as breakdown of fats in anaerobic digesters can be solved by bacterial lipases. During anaerobic digestion process, macromolecules are broken down into simpler compounds in the presence of extracellular enzymes. The increased sulphide concentration generated during the sulphate reduction process stimulates the enzymes (proteases, lipases and glucosidases) leading to enhanced solubilization of primary sewage sludge (Hasan, et al. 2006).

1.7.6. Pulp and Paper Industry

In pulp and paper manufacture, hydrophobic components of wood (or 'pitch') causes severe problems. Lipases are used to remove pitch from the pulp produced for paper making. Nippon Paper industries, Japan, have developed a pitch control method that uses the *Candida rugosa* fungal lipase to hydrolyze up to 90% of the wood triglycerides (Sharma, et al. 2001).

1.7.7. Medical Applications

The wax moth (*Galleria mellonella*) derived lipases and esterases were reported to be bacteriocidal on *Mycobacterium tuberculosis* (MBT) H37Rv. Since lipases are the activators of Tumor Necrosis Factor, they can be used in the treatment of malignant tumors (Annenkov, et al. 2004). Lipases may also be used as digestive aids and in the treatment of gastrointestinal disturbances, dyspepsias, cutaneous manifestations of digestive allergies, lipases have been used as therapeutics (Hasan, et al. 2006).

Lipase produced by *Candida rugosa* has been used in the synthesis of a drug, lovastatin, that lower serum cholesterol level. *S. marcescens* lipase has been reported to catalyze the asymmetric hydrolysis of 3-phenylglycidic acid ester which is a key intermediate in the synthesis of diltiazem hydrochloride, a widely used coronary vasodilator (Matsumae, et al. 1993)

1.7.8. Production of Biodiesel

The use of vegetable oils has been explored because of the limited resources of fossil fuels, increasing prices of crude oil, and environmental concerns (Shah and Gupta 2006). The biodiesel fuel produced from vegetable oils does not expose sulphur oxide and minimize the soot particulate one third times in comparison with the existing one from petroleum (Iso, et al. 2001).

Immobilized lipase of *P. cepacia* was used in the transesterification of soybean oil with ethanol and methanol (Noureddini and Philkana 2004). Two commercial lipases, Novozym 435 and Lipozyme IM, with n-hexane have been used in preparation of fatty acid ethyl esters from castor oil (Oliveira, et al. 2004). Immobilized lipases from *Thermomyces lanuginosa* and *C. antarctica* were used as biocatalysts in preparation of simple alkyl ester derivatives of restaurant grease (Hsu, et al. 2002).

Lipases are extremely versatile groups of microbial enzymes that are able to catalyse a variety of reactions and thus presents a fascinating field for future research. Extensive screening of new microorganisms and their lipases will open new routes to solve environmental problems (Jaeger, et al. 2006).

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

A list of commonly used growth media, buffers, solutions and their compositions is represented in Appendix B.

2.2. Methods

2.2.1. Sample Collection

In this study, soil samples were collected from Erciyes, Kula, Aliağa, Balçova and Tuz Gölü in Turkey.

2.2.2. Screening for Lipase Producing Fungi

Lipase producing fungi were screened by using Rhodamin B – LB agar media (with 1 % olive oil). Lipase activity was detected due to orange-red flourescent halo around colonies under UV light. Each soil sample was plated on Rhodamin B agar media and incubated at temperatures ranging from 4 to 65°C for 3 days. The fungal mycelium was picked up aceptically and transferred on fresh media. The plates were then incubated at temperature ranges 4 to 65°C for 3 days to determine optimum temperature for growth. The fungus displaying the maximal lipase activity was selected for further studies.

Stock culture of the fungus was prepared by pepton water and glycerol. For this aim, the fungus was inoculated on PDA and incubated at room temperature for 3 days to provide spore formation. Pepton water was used to prepare spore suspension and 20% glycerol was added for long-term storage of fungal spores at -80°C .

2.2.3. Identification of the Fungus by 28S rRNA Gene Sequence Analysis

Total RNA was isolated from fungus according to the procedure developed by Mukhtar, et al. (1998). This method was reported to enable rapid isolation of intact RNA from various filamentous fungi. cDNA sythesis was performed from total RNA. First strand of cDNA was synthesized using the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas) and second strand synthesis was carried out according to the protocol supplied by www.fermentas.com.tr. Blunt end cloning of cDNA was performed by CloneJET™ PCR Cloning Kit (Fermentas) and transformation was carried out into competent DH5-α cells via heat shock method. Transformation mixture was plated on LB agar media containing 100 µg/ml ampicillin and incubated overnight at 37 °C. Only recombinant clones containing insert can grow on culture plates as recircularized pJET1.2/blunt vector expresses a lethal restriction enzyme. The grown colonies were screened by colony PCR method. Reaction mixture (20 µl) contained 0.5 µl of 10 µM pJET1.2 forward and reverse primers, 2 µl 10X Taq polymerase buffer with KCl (Fermentas), 1.2 µl of 25 mM $MgCl_2$, 2 µl of 1mM dNTP mixture, 0.1 µl of 5u/µl Taq polymerase (Fermentas). PCR was performed in a thermocycler, iCycler (Bio-Rad Laboratories, Inc.) with the following program: initial denaturation step at 95°C for 3 min. ; 25 cycles of denaturation step at 94°C for 30 sec., annealing at 60°C for 30 sec., extension at 72°C for 2 min. ; and final extension step at 72°C for 5 min. PCR products were sequenced at the Biotechnology and Bioengineering Central Research Laboratories, Izmir Institute of Technology. BLAST search was performed to determine the identity of the fungus.

2.2.4. PCR Amplification and Sequencing of the Gene Encoding Fungal Lipase

Genomic DNA isolation from fungus was performed by using a manual fungal genomic DNA isolation procedure developed by Zhang, et al. (1996) with minor modifications. This method involves disruption of cells using dry ice (instead, liquid nitrogen was used), efficient removal of polysaccharides by cetyltrimethylammonium (CTAB), denaturation of DNases by the high concentration of SDS, extraction with phenol/chloroform and finally precipitation of DNA with isopropanol at room

temperature. Fungal lipase gene was directly amplified from genomic DNA using primers RsLipF (CCG**ATG**GTTTCATTCATTTCCATTTCTC) and RsLipR (GC**TTA**CAAACAGCTTCCTTCGTT) which were designed by Yu et al., (2009) according to the consensus sequence of lipases from other *Rhizopus* sp. (GenBank accession nos. AB013496, DQ139862, AF229435). PCR was carried out in a volume of 25 µl containing 2,5 µl of 10X Taq buffer with KCl (Fermentas), 2 mM $MgCl_2$, 5 µl of 1mM dNTP mixture, 0,5 U of Taq DNA polymerase (Fermentas) and 2 µl of template DNA (10 ng/µl). PCR was performed in a thermocycler, iCycler (Bio-Rad Laboratories, Inc.) with the following program: 95°C for 3 min., followed by 25 cycles of denaturation at 94°C for 30 sec., annealing at 55°C for 2 min., and elongation at 72°C for 2 min., and final extension step at 72°C for 4 min. The PCR fragment was purified with a commercial kit (Invitrogen) and sequenced. Amino acid sequence of obtained lipase gene was revealed and homology with lipases from other Rhizopus species was searched by using UniProt/EBI program (http://www.uniprot.org/).

2.2.5. The Effect of Incubation Time and Carbon Source on Lipase Production

The fungal spor suspension from stock culture was inoculated on PDA media and incubated at 25°C for 3 days to obtain growth and spor formation. After incubation period, fungal spores were collected with pepton water and counted on Thoma lam. 0,5 ml of spor suspension (5 x 10^5 spore/ml) were inoculated into 30 (250 ml) erlenmeyer flasks containing 50 ml minimal media and 1 % olive oil. The fungal cultures in liquid media were then incubated at 25°C for 10 days. 3 flasks were collected at each day of incubation and filtered by whatman paper. Lipase activity of these filtrates was calculated as mentioned in section 2.2.8. and optimum incubation time for lipase production was detected. Whatman papers containing fungal mycelium were washed with aceton and distilled water and dried at 80 ^{0}C for 15 hours to determine dry weight.

In order to determine the effect of carbon sources on lipase production, M.M, MM + 1 % olive oil (v/v), M.M + 20% glucose (w/v) and M.M + olive oil + glucose mixtures (200 ml) were added into 500 ml erlenmeyer flasks. 2 ml (5 x 10^5 spore/ml) spore suspension was inoculated into these media and these growth cultures were incubated for 7 days at 25^{0}C. Finally, each growth media was filtered to remove cells and subjected to lipase activity assay.

2.2.6. Partial Purification of Fungal Lipase

Fungal spore suspension (2 ml) was inoculated into three 500 ml erlenmeyer flasks containing 200 ml minimal media. Olive oil (1%) was used as carbon source and growth was performed at 25°C for 7 days.

After 7-day of growth, culture broth was filtered through whatman paper and centrifuged at 9000 rpm for 30 minutes to remove the cells completely. The cell-free broth was then precipitated by 20% concentration of chilled aceton for 1 hour at - 20 °C and centrifuged at 9000 rpm for 30 minutes to collect the precipitate (Rıfaat, et al. 2010). Additional aceton was added to the supernatant to bring the concentration to 30% (v/v). The mixture was then incubated and centrifuged to remove the precipitate. The remaining supernatant was further subjected to aceton precipitation (40, 50, 60, ... 90% v/v) as previously described. The obtained precipitates was dissolved in a small volume (5 ml) of 50 mM Tris HCl buffer (pH 8.0). The lipase activity of solutions was detected. The protein solution (5 ml) that exhibited maximum lipase activity was then applied to the Microcon YM-100 centrifugal filters which separate proteins according to their molecular weight. The solutions which contains proteins higher and lower than 100 kDa were collected and subjected to lipase activity assay.

2.2.7. Substrate Specificity

The enzyme activity was measured using various chain length of p-nitrophenyl fatty acid esters under standart assay conditions. The substrates (Sigma) were *p*-nitrophenyl caprylate (C8), *p*-nitrophenyl decanoate (C10), *p*-nitrophenyl laurate (C12), *p*-nitrophenyl myristate (C14), *p*-nitrophenyl palmitate (C16) and *p*-nitrophenyl stearate (C18).

2.2.8. Lipase Activity Assay

Lipase activity was detected by p-nitrophenol (pNP) method (Cai, et al. 2009). 2 ml (50mM) solution of *p*-NP-substrate was prepared with acetonitrile. 10 µl of substrate solution and 10 µl of enzyme solution were added into 980 µl of Reagent A (pH 7.2)

and incubated at room temperature for 1 hour. Lipase activity was determined using the spectrophotometric measurement of *p*-nitrophenyl release at 410 nm.

2.2.9. Characterization of Fungal Lipase

2.2.9.1. Effect of Temperature and pH on Lipase Activity and Stability

The optimal temperature for lipase activity was determined by incubating enzyme-substrate mixture at temperatures ranging from 4°C to 65°C for 1 hour. At each temperature, *p*-nitrophenyl release was measured spectrophotometrically at every 10 minutes for 1 hour. Thermal stability of lipase was also detected by incubating the enzyme at temperature ranges from 45°C to 85°C for 45 minutes. Residual activity was calculated.

The optimal pH for enzyme activity was measured by incubating the enzyme-substrate mixture at various pH values (4-12) in sodium acetate buffer (pH 4-5), potassium phosphate buffer (pH 6-7), Tris-HCl buffer (pH 8-9) and glycine-NaOH buffer (pH 10-12) for 1 hour. For pH stability study, the enzyme was incubated at desired pH (6-10) for 45 minutes and residual activity was assayed at pH 7.

2.2.9.2. Effect of Metal Ions and Organic Solvents on Lipase Activity

The effect of various compounds on lipase activity was detected. 10% (v/v) concentrations of organic solvents and different concentrations of metal ions were applied to reaction mixture and remaining activity was measured as described previously.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Isolation and Identification of the Lypolytic Fungus

The soil samples were plated on LB agar media (with Rhodamin B dye and 1 % olive oil) and incubated at different temperatures (4-65°C) for 3 days in order to isolate lipolytic fungi. Two lipolytic fungi were observed to grow on plates including soil sample from Kula at 25°C. Optimum growth temperature for these fungi was observed to be 25°C and they did not grow above 35°C. Fungal mycelium were separately transferred on fresh Rhodamin B agar media and incubated at 25°C for 3 days. After incubation period, the fungus which gave the larger orange fluorescent halo under UV light was selected for further studies.

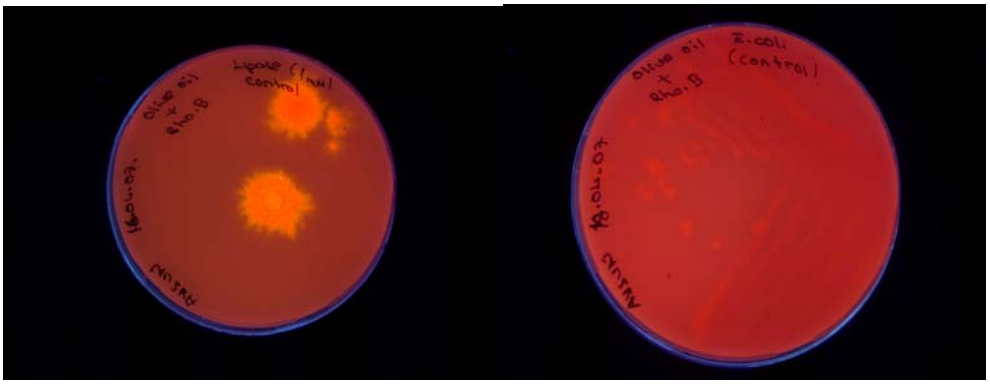


Figure 3.1. Positive and negative controls of lipase activity.

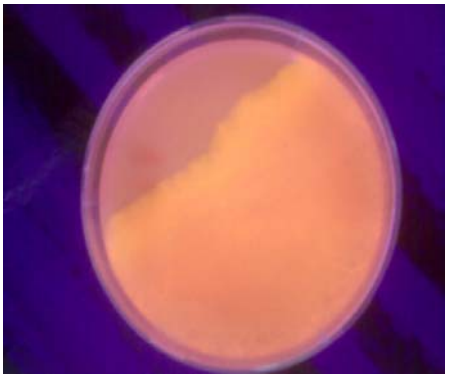


Figure 3.2. Fungal strain with higher lipase activity.

In order to identify this lipolytic fungus, firstly, genomic DNA isolation was performed and 28S and 18S rRNA genes were tried to be amplified by PCR using universal primers. However, these ribosomal genes were not amplified for any optimized PCR conditions. Since ribosomal RNA levels are very high in total RNA content of the cells, these total RNAs can be isolated and converted to the cDNA in order to obtain rRNA genes. For this aim, total RNA isolation from fungus was performed and cDNA synthesis was carried out with these RNAs. About 30 DNA fragments were cloned and sequenced to identify the fungus via rRNA gene sequence analysis. According to the sequencing results, 5 of these DNA fragments were determined to show highest (98-99%) sequence similarity to 28S rRNA partial gene of a *Rhizopus stolonifer* strain and 3 of the fragments exhibited about 98-99 % sequence similarity with 18S rRNA partial gene of the same species. Therefore, we concluded that our isolate is a strain of the *R. stolonifer* and named this fungus as *R. stolonifer K45*.

3.2. Sequence Analysis of the Lipase Gene

Fungal lipase gene was amplified by using genomic DNA and a pair of primers, RsLipF and RsLipR, which were designed in a previous study (Yu, et al. 2009) based on the consensus sequences of other *Rhizopus sp.* lipases. Agarose gel electrophoresis (1%) was carried out and about 1,2 kb fragment was observed on the gel (Figure 3.3).

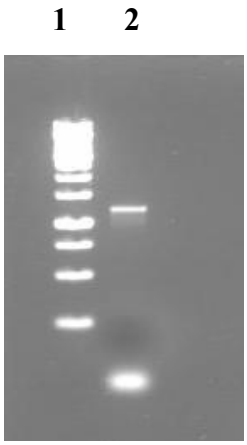


Figure 3.3. Agarose gel electrophoresis result of PCR amplified fungal lipase gene. Line 1 shows 1 kb DNA size marker (250, 500, 750, 1000, 1500bp,…) Line 2: ~ 1200 bp PCR product.

The lipase gene was sequenced and showed 96 % sequence identity with *Rhizopus oryzae* strain HU3005 lipase (ROL) mRNA.

Amino acid sequence of lipase gene was also detected and found homologous to *Rhizopus niveus* lipase (RNL, 98 % similarity, GenBank accession no. AB013496), *R. stolonifer* lipase (RSL, 83 % similarity, GenBank accession no. DQ139862), and *R. oryzae* lipase (ROL, 99 % similarity, GenBank accession no. AF229435) (Figure 3.4). According to the X-ray analysis of *R. niveus* lipase, S^{145}-H^{257}-D^{204} are the residues of the catalytic triad, and Ser^{145} is involved in the oxyanion hole stabilizing the tetrahedral intermediates (Kohno, et al. 1996). Since these residues are conserved in all homologous lipases, the catalytic triad of *R. stolonifer* mature lipase is composed of S^{145}-H^{257}-D^{204}, and that S^{145} involved in the oxyanion hole (Figure 3.4).

```
                Signal sequence M1          Prosequence V27
                    ↓                           ↓
RSK45L              ---------------------------------SGSSTTAVSASDNSALPPLISSRCAPPS 28
ROL                 MVSFISISQGVSLCLLVSSMMLGSSAVPVSGKSGSSTTAVSASDNSALPPLISSRCAPPS 60
RNL                 MVSFISISQGVSLCLLVSSMMLGSSVVPVSGKSGSSNTAVSASDNAALPPLISSRCAPPS 60
RSL                 MVSFISITQGVSLCLLVSSMMMGSSAVPVSGKTGASSDAVSTSGNFTLPPIISSRCAPPS 60
                                                :*:*. ***:*.* :***:*********
                                        Kexin-like protease recognition region
                                                  ↓
RSK45L              NKGSKSDLQAEPYYMQKNTEWYESHGGNLTSIGKRDDNLVGGMTLDLPSDAPPISLSGST 88
ROL                 NKGSKSDLQAEPYYMQKNTEWYESHGGNLTSIGKRDDNLVGGMTLDLPSDAPPISLSGST 120
RNL                 NKGSKSDLQAEPYNMQKNTEWYESHGGNLTSIGKRDDNLVGGMTLDLPSDAPPISLSSST 120
RSL                 GKGSSSDLQSEPYFVKKNTEWYTAHGGDLAAIGKRDDNQVGGMTLDLPENAPPIASSYSL 120
                    .***.****:*** ::****** :***:*::******* *********.:****: * *
                Mature sequence
                     ↓
RSK45L              NSASDGGKVVAATTAQIQEFTKYAGIAATAYCRSVVPGNKWDCVQCQKWVPDGKIITTFT 148
ROL                 NSASDGGKVVAATTAQIQEFTKYAGIAATAYCRSVVPGNKWDCVQCQKWVPDGKIITTFT 180
RNL                 NSASDGGKVVAATTAQIQEFTKYAGIAATAYCRSVVPGNKWDCVQCQKWVPDGKIITTFT 180
RSL                 DSVSDSS-VVAATAAQVQELTKYAGVAATAYCRSVVPGNKWDCKQCLKWVPDGKIITTFT 179
                    :*.**.. *****:**:**:*****:***************** ** *************

RSK45L              SLLSDTNGYVLRSDKQKTIYLVFRGTNSFRSAITDIVFNFSDYKPVKGAKVHAGFLSSYE 208
ROL                 SLLSDTNGYVLRSDKQKTIYLVFRGTNSFRSAITDIVFNFSDYKPVKGAKVHAGFLSSYE 240
RNL                 SLLSDTNGYVLRSDKQKTIYLVFRGTNSFRSAITDIVFNFSDYKPVKGAKVHAGFLSSYE 240
RSL                 SILSDTNGYVLRSDKQKTIYLVFRGTNSFRSAITDIVFNFSNYKPVSGAKVHTGFLSSYE 239
                    *:****************************************:****.*****:*******
                                          S145
                                           ↓
RSK45L              QVVNDYFPVIQEQLTANPTYKVIVTGHSLGGAQALLAGMDLYQREPRLSPKNLSIFTVGG 268
ROL                 QVVNDYFPVVQEQLTANPTYKVIVTGHSLGGAQALLAGMDLYQREPRLSPKNLSIFTVGG 300
RNL                 QVVNDYFPVVQEQLTAHPTYKVIVTGHSLGGAQALLAGMDLYQREPRLSPKNLSIFTVGG 300
RSL                 QVVNDYFPVIQAQLTANPSYQVIVTGHSLGGAQALLAGMDLYQREKRLSPKNLSIFTIGG 299
                    *********:* ****:*:*:********************** ***********:**

RSK45L              PRVGNPTFAYYVESTGIPFQRTVHKRDIVPHVPPQSFGFLHPGVESWIKS---------- 318
ROL                 PRVGNPTFAYYVESTGIPFQRTVHKRDIVPHVPPQSFGFLHPGVESWIKSGTSNVQICTS 360
RNL                 PRVGNPTFAYYVESTGIPFQRTVHKRDIVPHVPPQSFGFLHPGVESWIKSGTSNVQICTS 360
RSL                 PRVENPTFAYYVESTGIPFHRTVHKRDIVPHVPPQAMGFLHPGVESWIKSGDSNVQICNS 359
                    *** ***************:***************::*************

RSK45L              --------------------------------
ROL                 EIETKDCSNSIVPFTSLLDHLSYFDINEGSCL 392
RNL                 EIETKDCSNSIVPFTSILDHLSYFDINEGSCL 392
RSL                 QIETKACSNSIVPFTSIADHLSYFGINEGSCL 391
```

Figure 3.4. Alignment of amino acid sequence from *Rhizopus* sp. lipase

3.3. The Effect of Incubation Time and Carbon Source on Lipase Production

Fungal spore suspension (0,5 ml) was inoculated into 30 flasks including 50 ml minimal media with 1 % olive oil and these culture media were incubated at 25^{o}C for 10 days. Three of these media were collected each day and filtered by Whatman paper. The filtrates were subjected to lipase activity assay. The fungal mycelium remained on the filters was dried for 15 hours at 80 ^{o}C and dry weight of mycelium was detected for each day of incubation (Figure 3.6). According to the results indicated in the Figure 3.5, optimum incubation time for lipase production was determined to be 7 days. Lipase activity decreased significantly at 8th day of growth but an increase was observed in the enzyme activity at 10th day. The increase in the lipase activity might be due to the release of intracellular lipases because cell lysis was observed at 10th day of growth (Figure 3.6).

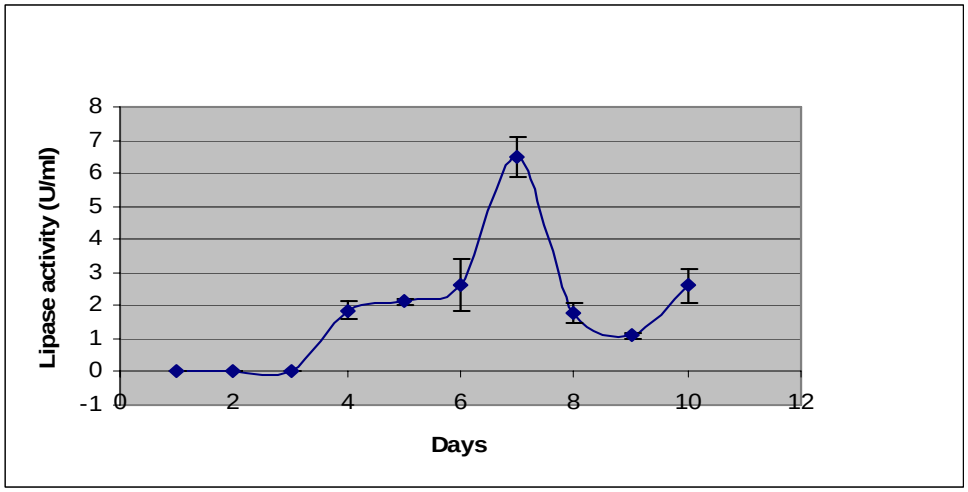


Figure 3.5. The effect of incubation time on lipase activity.

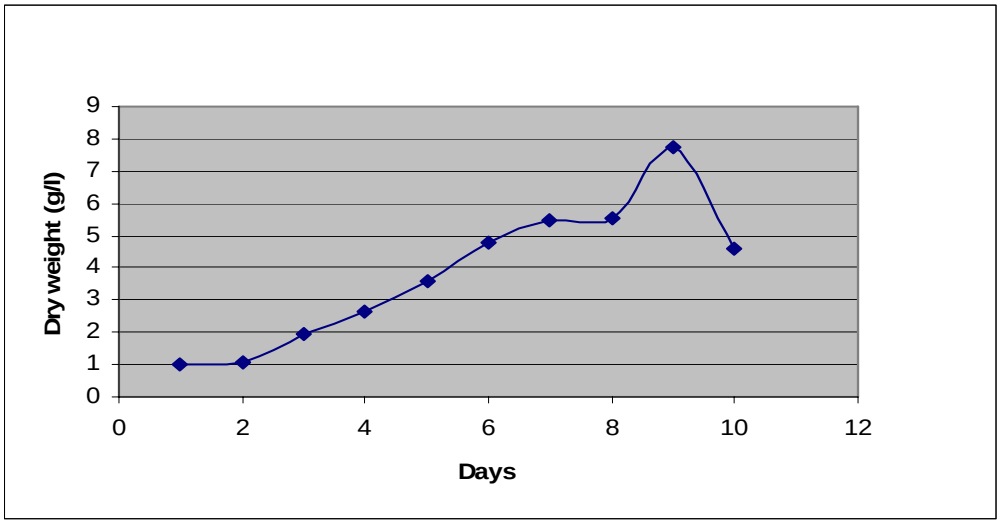


Figure 3.6. Dry weight of mycelium.

In order to understand the effect of olive oil and glucose on lipase activity, the growth media were supplemented with these carbon sources. Four different media (200 ml) were prepared which contains minimal media without carbon sources, minimal media and only olive oil (1%), m.m and only glucose (20%) and m.m with both carbon sources. These growth media were inoculated with 2 ml spore suspension and incubated 7 days at 25^{o}C. The fungal mycelium was filtered by whatman paper and cell free media were subjected to lipase activity assay. As shown in the Table 1. there was no activity in the media containing only minimal medium. The best enzyme activity was observed for the media containing only olive oil as carbon source. In the two media containing glucose, the amount of lipase produced was higher in the media containing olive oil than in media without olive oil which suggested that the addition of olive oil into the media enhances the production of lipase from fungus. However, in the media containing olive oil as only carbon source, lipase production was higher than in the media containing olive oil and glucose. In addition to this, maximum growth was observed in the media containing minimal media and both carbon sources. According to the results, we can conclude that olive oil is an inducer for lipase production but the addition of glucose into the media reduces lipase activity while enhancing the growth of the fungus. This behaviour was also observed by Nahas (1988) for *R. oligosporus* lipase and by Fadıloğlu and Erkmen (1999) for *R. oryzae* lipase. Annibale et al. (2006) and Brozzoli et al. (2009) also confirmed that lipase production by *Candida sp.* was found to be completely repressed by the presence of simple sugars and induced by using natural oils.

Table 3.1. The effect of carbon source on lipase production

Culture medium	Growth	Activity (U/ml)
M.M	+	**No activity**
M.M+Olive oil	+++	**3.1**
M.M+Glucose	+++++	**2.1**
M.M+Glu+O.O	++++++	**2.3**

3.4. Partial Purification of Fungal Lipase

Aceton precipitation and gel filtration medhods were used for the partial purification of lipase. The precipitation of lipase with the 30% concentration of chilled aceton was determined as best for total activity. Increased concentrations of aceton were observed to decrease total activity till 80 % at which lipase activity ended. 200 ml of culture broth was precipitated by 30% of chilled aceton and centrifuged. The precipitate was dissolved in 5 ml of TrisHCl buffer (pH 8.0). The mixture was then applied to ultrafilters which have a molecular weight limit of 100kDa. Solutions which passed through the membrane and remained on it were collected. According to the lipase activity assay, the solution remaining above the filter showed higher lipase activity and used for further experiments.

3.5. Substrate Specificity

The lipase activity was examined using different *p*-nitrophenyl esters (C8,C10,C12,C14,C16 and C18) as substrate. Lipase activity assay was performed with these substrates for 1 hour and relative activities were calculated. According to the results shown in Figure 3.7, fungal lipase exhibited maximal activity with *p*-nitrophenyl laurate (C12). Substrates with a carbon chain longer than 12 were observed to decrease enzyme activity greatly.

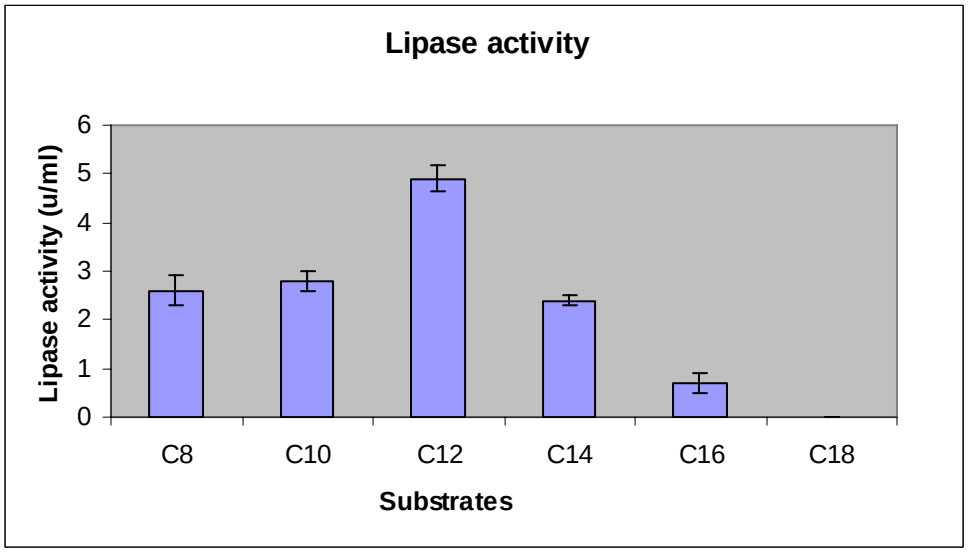


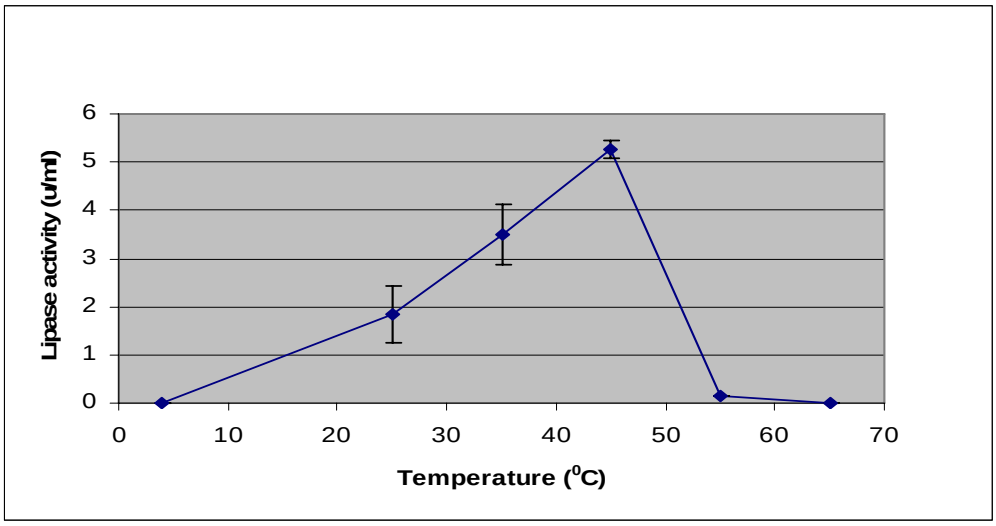
Figure 3.7. Substrate specificity of *Rhizopus stolonifer K45* lipase. C8, C10, C12, C14, C16, C18 were *p*-nitrophenyl octanoate, *p*-NP decanoate, *p*-NP laurate, *p*-NP myristate, *p*-NP palmitate and *p*-NP stearate, respectively.

3.6. Effect of Temperature and pH on Lipase Activity and Stability

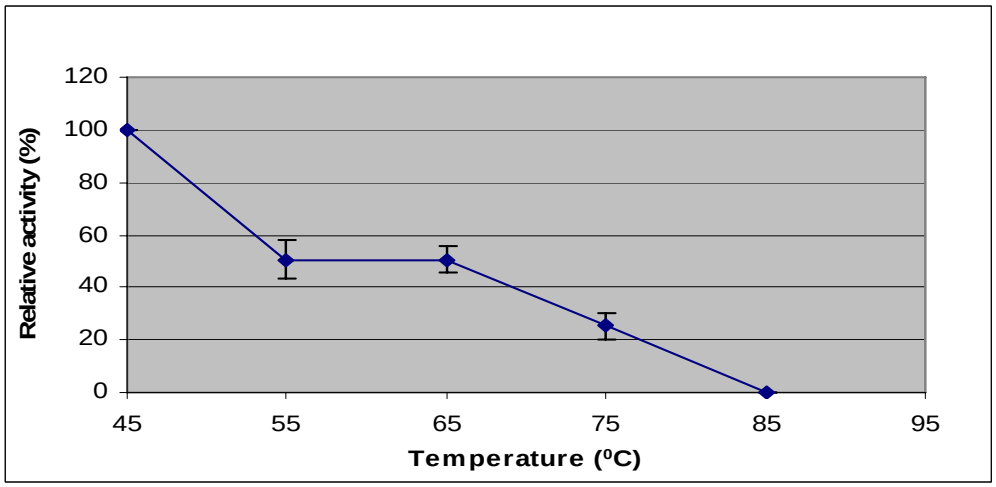
The effect of temperature on lipase activity and stability was examined at different temperatures (4°C - 85°C). As shown in Figure 3.8.a, lipase did not exhibited activity at 4°C and a linear increase was detected at temperatures between 25-45°C. Maximum enzyme activity was observed at 45°C. However, the enzyme lost its activity significantly at 55°C and no activity retained at 65°C. Thermophilic fungi which grow at high temperatures are known to produce thermophilic enzymes. The fungus *R. stolonifer* is a mesophilic fungus which grows best at 25°C but its lipase showed maximal activity at 45°C. This result showed that enzymes which show maximal activity at high temperatures can be produced from mesophilic organisms. According to Razak et al. (1997) most fungal lipases do not exhibit temperature optima above 40°C. The optimum lipase activity for *Aspergillus carneus* was 37°C (Saxena, et al., 2003), it was 40°C for *A. nidulans* lipase (Mayordono, et al., 2000), 35°C for *Mucor sp*. lipase (Abbas, et al., 2002) and between 25-45°C for many *Rhizopus sp*. lipases. It can be concluded that *R. stolonifer K45* lipase may have potential in high temperature reactions. Stability was also tested after incubation at temperatures between 45-85 for 45 minutes. The enzyme was detected to be stable at 45°C. At 55°C the lipase retained

50 % of its full activity while at 85°C the enzyme completely lost activity (Figure 3.8.b).

The enzyme activity and stability at different pH was detected using various buffers. Data of figure 3.8.c. indicate that the activity of lipase increased from pH 6.0 to 8.0 and optimal pH was observed to be 8.0 in TrisHCl buffer. Lipase activity was highly decreased above pH 8.0. The effect of pH on lipase stability was also determined and the enzyme was observed to be stable at pH range of 7-8 (Figure 3.8.d)



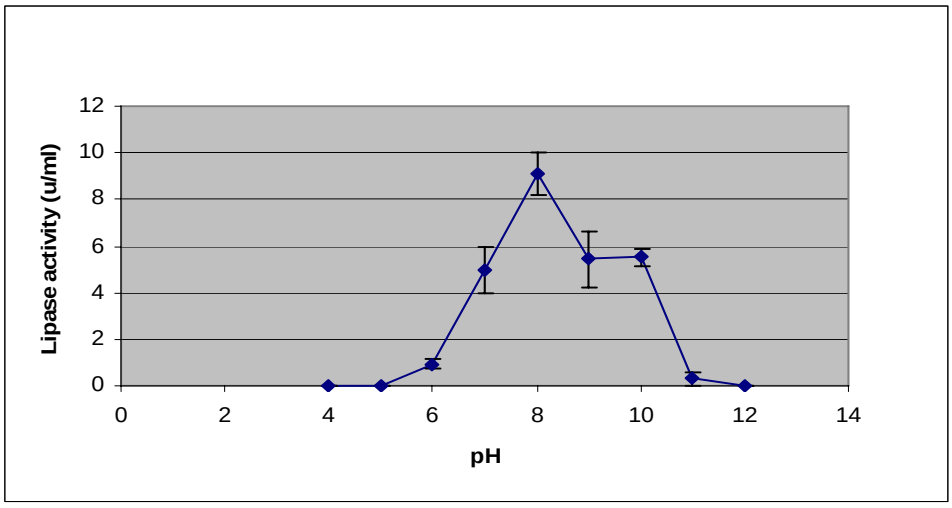
a) Effect of temperature on lipase activity



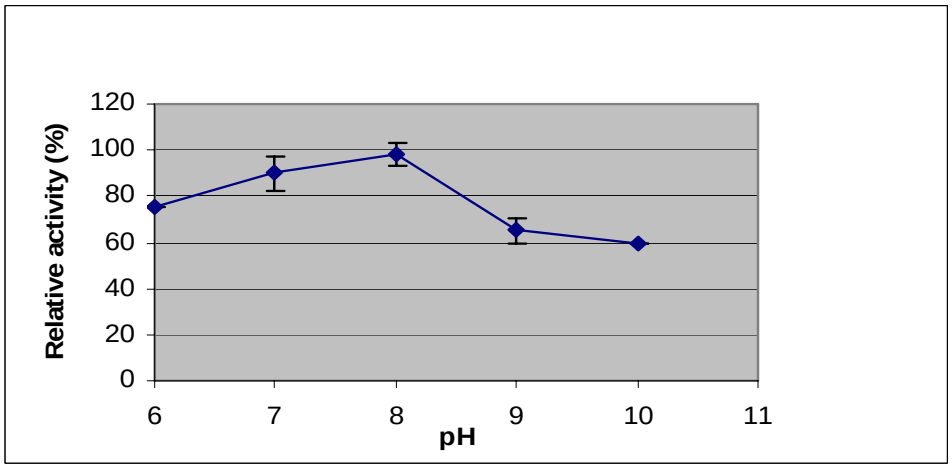
b) Effect of temperature on lipase stability.

Figure.3.8. Effect of temperature and pH on lipase activity and stability

(Cont. on next page)



c) Effect of pH on lipase activity.



d) Effect of pH on lipase stability

Figure.3.8. (cont.)

3.7. Effect of Metal Ions and Organic Solvents on Lipase Activity

The effect of various metal ions and organic solvents on *Rhizopus stolonifer K45* lipase was studied. According to the results shown in Table 3.2, the enzyme activity was decreased by ethanol, acetone, propanol, dimethyl formamide and strongly inhibited by n-hexane. However, methanol and DMSO were observed to increase the lipase activity. The stability of lipases in organic solvents offers advantages in many application fields.

Table 3.2. Activity of *R. stolonifer* K45 lipase in various organic solvents and compounds

Solvent	Relative activity during the assay (% ± SD)
None	100,00 ± 0,00
Ethanol	80,33 ± 22,03
Methanol	117,66 ± 14,36
Aceton	30,33 ± 4,04
Propanol	37,00 ± 5,00
n-Hexane	0,00 ± 0,00
Dimethyl formamide	39,00 ± 23,64
DMSO	123,33 ± 9,81

Among the metal ions tested, Mg^{+2} slightly enhanced the lipase activity. Zn^{+2} and Ni^{+2} had little effect on enzyme activity whereas Ca^{+2} reduced the activity significantly (Table 3.3). Furthermore, EDTA had little effect on lipase activity which suggested that *R. stolonifer K45* lipase is not a metalloenzyme and so it does not require any metal cofactors for activity. This is also the same for other fungal esterases/lipases (Akoh 2004).

Table.3.3. Effect of various metal ions on the activity of lipase

Metal ions	Concentration (Mm)	Relative activity during the assay (% ± SD)
Control		100,00 ± 0,00
NaCl	5	71,00 ± 3,60
$MgCl_2$	5	104,66 ± 5,03
KCl	5	77,00 ± 9,85
$CaCl_2$	1	47,33 ± 5,03
$ZnCl_2$	1	81,00 ± 6,08
$NiCl_2$	1	101,00 ± 4,58
EDTA	10	95,72 ± 0,11

CHAPTER 4

CONCLUSIONS

Lipases are serine hydrolyses which have the ability to catalyse both hydrolysis and synthesis of esters formed from glycerol and long-chain fatty acids. Since lipases have broad substrate specificity and many suitable properties, they find promising applications in organic chemical processing, detergent formulations, synthesis of biosurfactants, the oleochemical industry, the dairy industry, the agrochemical industry, paper manufacture, nutrition, cosmetics, and pharmaceutical processing. Lipases can be obtained from plants and animals but only microbial lipases are commercialy significant.

In this research, lypolytic fungus with very strong lipase activity was screened from soil samples. Rhodamin B plate assay was used to select lipase producing fungus and lipase activity was detected according to orange-red flourescent halos around colonies under UV light. The lypolytic fungus was identified by 28S rRNA gene sequence analysis and found to be a strain of *Rhizopus stolonifer* by BLAST (NCBI) analysis.

The lipase gene sequence of the fungus was revealed using a pair of primers which were designed in a previous study based on the consensus sequences of other Rhizopus lipases. The aminoacid sequence of the lypolytic fungus was also detected and homology with lipases from other Rhizopus species was searched using multiple alignment program (http://www.uniprot.org/). The lipase from our isolate was found homologous to *Rhizopus niveus* lipase (RNL, 82 % similarity, GenBank accession no. AB013496), *R. stolonifer* lipase (RSL, 69 % similarity, GenBank accession no. DQ139862), and *R. oryzae* lipase (ROL, 84 % similarity, GenBank accession no. AF229435). X-ray analysis of *R. niveus* lipase in a previous study indicated that its mature lipase is composed of S^{145}-H^{257}-D^{204}, and that S^{145} involved in the oxyanion hole. The lipase from *R. stolonifer*, our isolate, also composed of these residues since they are conserved in all homologous lipases.

As we corrected with Rhodamin B plate assay, our lypolytic fungus secreted its lipase to the extracellular environment like many other fungi. Therefore, isolation and

purification of the fungal lipase were performed without lysing the mycelium. Before purification steps, the effect of incubation time and carbon source on lipase production was studied. Optimum lipase production was obtained at 7^{th} day of growth and in the media containing minimal media and 1 % olive oil. It was also detected that addition of glucose into the growth media reduced the enzyme activity. In order to detect some properties of lipase, we purified the enzyme by (30%) acetone precipitation and ultrafiltration medhods. The chain length specificity of this lipase was analyzed using *p*NP esters. Middle (C8-C10) and long chain length substrates (C12-C14-C16-C18) were used and maximum lipase activity was obtained with *p*-NP laurate (C12).

Lipase activity was also tested at different temperature and pH values. The lypolytic fungus was observed to grow best at 25^0C but its lipase showed maximum activity at 45^0C. The enzyme activity was very low at 4^0C but a rapid increase was observed between 25-45^0C. Maximum activity of enzyme dropped significantly above 45^0C and no activity was remained at 65^0C. It can be concluded that the lipase from *R. stolonifer* has maximal activity at high temperature which means that enzymes working at elevated temperatures can be produced by mesophilic organisms. The effect of pH on enzyme activity was also studied and the results showed that the lipase from *R. stolonifer* is an alkaline lipase which is most active at pH 8.0. Lipase enzyme had no or little activity at acidic pH values.

In addition, partially purified lipase was exposed to some organic solvents and metal ions with different concentrations. Among them, Mg^{+2}, methanol and DMSO increased the lipase activity whereas Ca^{+2}, ethanol, acetone, propanol, dimethyl formamide and n-hexane reduced the activity significantly.

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APPENDIX A

PARTIAL SEQUENCES OF 28S rRNA AND LIPASE GENES OF IDENTIFIED FUNGAL STRAIN

Rhizopus stolonifer 28S ribosomal RNA gene

CGTTATCTTCGGATGGCTCGAGTTTTTCAGCAAGATCTTGGAATTGACCGAAGACAAACTAC
TGCGAAAGCATTTGACGATGGCCAGAAAGTGGTGTTGACGCAATGTGATTTCTGCCCAGTGC
TCTGAATGTCAAAGTGAAGAAATTCAACCAAGCGCGGGTAAACGGCGGGAGTAACTATGAC
TCTCTTAAGGTAGCCAAATGCCTCGTCATCTAATTAGTGACGCGCATGAATGGATTAACGAG
ATTCCCACTGTCCCTATCTACTATCTAGCGAAACCACAGCCAAGGGAACGGGCTTGGCAGAA
TCAGCGGGGAAAGAAGACCCTGTTGAGCTTGACTCTAGTTTGACATTGTGAAAAGACATAG
AGGGTGTAGCATAAGTGGGAGCTTCGGCGCCAGTGAAATACCACTACCTCTATTGTTTTTTT
ACTTAAATAATTAAGTGGGATTGAGTCGCAAGACTCACCTTCTAGCTTTAAGCATCCATTAG
GGTGCGACCCATGTTATTGACATTGTCAAGTGGGGAGTTTGGCTGGGGCGGATCTTTCTAGA
AGATCTCCTACAATATTCTCAGCTGCCATGGAAAATCGAATGTTCTTA

Partial sequence of lipase gene from *Rhizopus stolonifer* K45

AATCTGGATCTTCCACTACCGCCGTCTCTGCATCTGACAATTCTGCCCTCCCTCCTCTCATTTC
CAGCCGTTGTGCTCCTCCTTCTAACAAGGGAAGTAAAAGCGATCTTCAAGCTGAACCTTACT
ACATGCAAAAGAATACAGAATGGTATGAGTCCCATGGTGGCAACTTGACATCCATCGGAAA
GCGAGATGACAATTTGGTTGGTGGCATGACTTTGGATTTACCTAGCGATGCTCCTCCTATCA
GCCTCTCTGGATCTACCAACAGCGCCTCTGATGGTGGTAAGGTTGTTGCTGCTACTACTGCTC
AAATTCAAGAGTTCACCAAGTATGCTGGTATCGCTGCCACTGCCTACTGTCGTTCTGTTGTCC
CTGGTAACAAGTGGGACTGTGTCCAATGTCAAAAGTGGGTTCCTGATGGCAAGATCATCACT
ACCTTTACCTCCTTGCTTTCCGACACAAATGGTTACGTCTTGAGAAGTGATAAACAAAAGAC
CATTTATCTTGTTTTCCGTGGTACCAACTCCTTCAGAAGTGCCATCACTGATATCGTCTTCAA
CTTTTCCGACTACAAGCCTGTCAAGGGCGCCAAGGTTCATGCTGGTTTCCTTTCCTCTTATGA
GCAAGTTGTCAATGACTATTTCCCTGTCATCCAAGAACAACTGACCGCTAACCCTACTTACA
AGGTCATCGTTACCGGTCACTCACTCGGTGGTGCACAAGCTTTGCTTGCCGGTATGGATCTCT
ACCAACGTGAACCAAGATTGTCTCCCAAGAATTTGAGCATCTTCACTGTTGGTGGTCCTCGT
GTTGGTAACCCCACCTTTGCTTACTATGTTGAATCTACCGGTATTCCTTTCCAACGTACCGTT
CACAAGAGAGATATCGTTCCTCACGTTCCTCCTCAATCCTTCGGATTCCTTCATCCCGGTGTT
GAATCTTGGATCAAGTCCGG

APPENDIX B

BUFFERS AND STOCK SOLUTIONS

PDA (potatoe dextose agar), per liter

39 g PDA and dH_2O up to 1 L.

Luria Bertani (LB) agar, per liter

10 g tryptone, 5 g yeast extract, 5 g NaCl, 10g gellan gum instead of 15g agar and dH_2O up to 1 L.

Rhodamine B solution (0.1% w/v)

0.1 g rhodamine B in 100 ml dH2O.

5X minimal salt solution

64g $Na_2HPO_4.7H_2O$, 15g KH_2PO_4, 2,5g NaCl and 5g NH_4Cl (nitrogen source) dissolved in 1 liter water and autoclaved at 121^oC, 1 bar for 15 minutes.

Minimal Medium, per liter

200 ml of 5X minimal salt solution, dH_2O up to 1 L. After autoclave, 2ml 1M $MgSO_4$ and 0,1ml 1M $CaCl_2$ was added.

Sodium phosphate buffer (100 mM), pH 7,2

0,3g $NaH_2PO_4.H_2O$, 1,09g Na_2HPO_4 (anhydrous) dissolved in 100 ml water and then 150 mM NaCl and % 0.5 Triton-X was added.

Sodium acetate buffer (50 mM), pH 4 and 5

0,4 g sodium acetate dissolved in 100 ml water and then its pH was adjusted with acetic acid.

Potassium phosphate buffer (50 mM), pH 6 and 7

0,68 g potassium dihydrogen phosphate dissolved in 100 ml water and then pH was adjusted with phosphoric acid.

Tris-HCL biffer (50 mM), pH 8 and 9

0,6 g tris base dissolved in 100 ml water and then pH was adjusted with HCL

Glycine-NaOH buffer (50 mM), pH 10, 11 and 12

0,36 g gylcine dissolved in 100 ml water and then pH was adjusted with NaOH.

p-nitrophenyl laurate solution (50 mM)

1,6 g *p*-nitrophenyl laurate dissolved in 100 ml acetonitrile.