# ISOLATION AND IDENTIFICATION OF A LIPASE PRODUCING PSYCHROTROPHIC BACTERIA FROM SOIL: CLONING AND PARTIAL CHARACTERIZATION OF ITS LIPASE

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# ABSTRACT


## ISOLATION AND IDENTIFICATION OF A LIPASE PRODUCING PSYCHROTROPHIC BACTERIA FROM SOIL: CLONING AND PARTIAL CHARACTERIZATION OF ITS LIPASE

Lipases are serine hydrolases and catalyze both the hydrolysis and synthesis of long-chain triacylglycerols. Lipases have great importance since their wide usage in industry. Lipases are produced by microorganisms (bacteria and fungi), plants and animals. However, microbial lipases, especially from bacteria, more useful than their plant and animal derivatives because of several important properties.

The primary goals of this thesis were to isolate and identify a lipase producing bacterium from soil sample from Erciyes mountain in Kayseri by 16S rRNA sequence analysis, find the sequence of lipase gene by degenerate PCR and inverse PCR and analyze of lipase gene for some important features like active site residues. The other purposes of this study were determination of lipase production conditions like optimum production time, partial purification and characterization of native lipase enzyme. Purification was performed by ammonium sulfate precipitation and gel filtration. Spectrophotometric lipase assay was used for enzyme characterization.

As conclusion, a lipase producer bacterium was isolated from soil using rhodamine B-olive oil plate assay and identified as a strain of *Pseudomonas fluorescens* based on 16S rRNA sequence homology. Its partial lipase gene was obtained and it was suggested that the lipase belong to group 3 *Pseudomonas* lipases according to gene and amino acid homology search. Moreover, native lipase partially purified and characterized. The molecular mass of purified lipase was estimated to be approximately 43 kDa by SDS-PAGE. The optimum temperature and pH for the lipase were found to 45°C and pH 8, respectively.

# ÖZET

## LİPAZ ÜRETEN PSİKROTROF BİR BAKTERİNİN TOPRAKDAN İZOLASYONU VE TANIMLANMASI: LİPAZIN KLONLANMASI VE KISMİ KARAKTERİZASYONU

Lipazlar serin hidrolazlardır ve uzun zincirli triaçilgliserollerin hem hidroliz hemde sentezlerini katalizlerler. Lipazlar endüstrideki yaygın kullanımları nedeniyle büyük öneme sahiptirler. Lipazlar mikrooraganizmalar (bakteri ve mantarlar), bitkiler ve hayvanlar tarafından üretilirler. Fakat, mikrobiyal lipazlar, özellikle bakteri kaynaklı olanlar, birkaç önemli özellikleri nedeniyle bitki ve hayvan türevlerinden daha kullanışlıdırlar.

Bu çalışmanın öncelikli hedefleri; Kayseri Erciyes'den gelen toprak örneğinden lipaz üreten bir bakterinin izole edilmesi ve 16S rRNA sekans analizi ile tanımlanması, lipaz geninin sekansının dejenere PCR ve invers PCR ile bulunması ve lipaz geninin aktif bölge rezidüleri gibi bazı önemli özellikler bakımından incelenmesidir. Bu çalışmanın diğer amaçları ise optimum lipaz üretim zamanı gibi lipaz üretim koşullarının belirlenmesi, doğal lipaz enziminin kısmi olarak saflaştırılması ve karakterizasyonudur. Saflaştırma amonyum sülfat çöktürmesi ve jel filtrasyonu ile gerçeklestirilmiştir. Enzim karakterizasyonu için spektrofotometrik lipaz methodu kullanılmıştır.

Sonuç olarak, lipaz üreten bir bakteri rodamine B-zeytinyağı petri yöntemi kullanılarak toprakdan izole edilmiş ve 16S rRNA sekans homolojisine dayanarak *Pseudomonas fluorescens* in bir suşu olarak tanımlanmıştır. Lipaz geninin kısmi sekansı elde edilmiş ve aminoasit ve gen homoloji analizine göre *Pseudomonas* grup 3 lipaz grubuna ait olduğu önerilmiştir. Ayrıca, doğal lipaz enzimi kısmi olarak saflaştırılıp karakterize edilmiştir. Molekül ağırlığı sodyum dodesilsülfat poliakrilamit jel elektroforezi ile yaklaşık 43 kilodalton olarak hesaplanmıştır. Lipaz için optimum sıcaklık ve pH sırasıyla 45°C ve pH 8 olarak bulunmuştur.

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# ABBREVIATIONS

| | |
|---|---|
| LB | Luria-Bertani |
| UV | Ultraviolet |
| rpm | Revolutions per minute |
| CTAB | Cetyl trimethylammonium bromide |
| PCR | Polymerase chain reaction |
| OD | Optic density |
| rRNA | Ribosomal ribonucleic acid |
| rDNA | ribosomal deoxyribonucleic acid |
| ng | Nanogram |
| µg | Microgram |
| Taq | Thermus aquaticus (DNA polymerase) |
| µl | Microliter |
| ml | Milliliter |
| µM | Micromolar |
| mM | Millimolar |
| IPTG | Isopropyl-thio-β-D-galactopyranoside |
| X-Gal | 5-bromo-4-chloro-3 indolyl-B-D-galactoside |
| 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 |

# CHAPTER 1

# INTRODUCTION

## 1.1. Microorganisms as Bio-Factories

The biosphere is occupied by a wide variety of microorganisms that carry out important functions like global primary energy and element cycling and they form the largest part of living organisms in the sense of total biomass cell numbers (6 $x10^{30}$ bacteria, 1.3 $x10^{28}$ archaea, 3.1 x $10^{29}$ eukarya), cell biomass (6 x $10^{18}$ kg bacteria, 1.3 x $10^{16}$ kg archaea and 3.1 x $10^{17}$ kg eukarya) and species diversity. This diversity of microorganisms is the most common source of genes which can be used in several industrial and research applications (Beloqui, et al. 2008).

Microorganisms are found everywhere. It means that they can be observed in the deepest ocean sediments, at high atmospheric pressure, at unusually high and cold temperatures and also in highly polluted environments. Microbes are able to break down a variety of usual and unusual carbon and energy sources and convert them into aminoacids, nucleotides, vitamins, carbohydrates and fatty acids by producing specific enzymes. Enzymes that carry out metabolic processes in microbes also have several practical and industrial usage to perform certain reactions apart from the cell. So, due to their ability to adapt a wide range of conditions and produce specific enzymes, microorganisms have been paid attention as little bio-factories (Sanchez 2005).

In the beginning of enzyme technology, crude preparations from certain animal tissues like pancreas and stomach mucosa, or plant tissues found applications in textile, leather and other industries. However, such preparations had some disadvantages such as high cost and shortage of tissues from animals and plants. It was realized that some microorganisms produce enzymes similar to that of plants and animals in terms of actions. Dr. Jokichi Takamine (1894, 1914) was the first person to introduce microbial enzymes to industry. Although he was mainly interested in fungal enzymes, Boidin and Effront (1917) were pioneers in the production of bacterial enzymes. Since that time microbial enzymes have taken the place of enzymes from plants and animals (Underkofler, et al. 1957).

Microbial enzymes have a great number of usage in food, pharmaceutical, textile, paper, leather and other industries (Hasan, et al. 2006). Their applications have been increasing rapidly. Among industrially important enzymes, hydrolases come in the first place and include enzymes with a wide substrate specifity. Carbohydrases, proteases, pectinases and lipases are classified into hydrolases. They catalyze the hydrolysis of natural organic compounds (Rajan 2001, Underkofler, et al. 1957). We will focus on lipase from a cold-adapted bacterium in this particular study.

## 1.2. Definition of a Lipase

The large part of the earth's biomass is represented by lipids. Lipids are essential to all living systems. They are the most important source of energy, play structural roles in membranes and are involved in signaling events. To be able to carry out these functions, lipids require lipolytic enzymes during their metabolism. Lipolytic enzymes catalyze the turnover of these water-insoluble compounds (Gilham and Lehner 2005). They also breakdown lipids and make them mobile within the cells of individual organisms (Beisson, et al. 2000). Lipolytic enzymes are grouped into 3 main categories, which are esterases, phospholipases and lipases (Arpigny and Jaeger 1999).

Lipases were first discovered in 1856 by Claude Bernard when he studied the role of the pancreas in fat digestion (Peterson and Drabløs 1994). Since then, many different lipases have been identified in and isolated from bacteria, fungi, plants, and animals. The presence of lipases has been noticed as early as in 1901 for *Bacillus prodigiosus*, *B. pyocyaneus* and *B. fluorescens* which are now called *Serratia marcescens*, *Pseudomonas aeruginosa* and *Pseudomonas fluorescens*, respectively (Jaeger, et al. 1999, Hasan, et al. 2006).

In 1958, lipases were first defined by Sarda and Desnuelle based on the phenomenon of interfacial activation, which was first observed in 1936 by Holwerda et al and then in 1945 by Schonheyder and Volqwarts. After the discovery of this phenomenon, Sarda and Desnuelle concluded that the activity of lipases were related to the formation of interface between the water-insoluble substrate and water (Verger 1997).

Human pancreatic lipase and *Rhizomucor miehei* lipase were the first ones whose 3D structures elucidated in 1990. These two lipases were found to have a lid like

structure covering the active site of the enzyme and the presence of such a lid might be a good explanation for interfacial activation of the enzyme. It was postulated that this structure had to show a change in conformation in the presence of lipid-water interface and this might be related to the activation of enzymes (Frenken, et al. 1992). Finally, it was clearly identified that both lipases were active via the movement of lid that closes the active site. Since, the active site of the enzyme became accessible to the substrate. On the other hand, The European BRIDGE-T-LIPASE project, carried out from 1990 to 1994, found some lipases with exceptional 3D structures from *P.glumae*, *P.aeruginosa* and *Candida antartica* B. All of them have a lid structure, but do not show interfacial activation to be active (Schmid and Verger 1998, Verger 1997).

In conclusion, based on exceptions, interfacial activation as well as presence of a lid domain are not a suitable criteria to determine true lipases. Therefore, lipases are defined basically as fat-splitting enzymes that catalyze the hydrolysis of long-chain triacylglycerols to form glycerol and fatty acid in the presence of excess water (Figure 1.1). Also, they can catalyze the reverse reaction, synthesis of triacylglycerols, under non-aqueous conditions (Jaeger, et al. 1999, Gupta, et al. 2004, Pascale, et al. 2008). Glycerolesters with an acyl chain length $\geq$ 10 carbon atoms can be said as lipase substrates (Jensen 1983). In addition to lipases, esterases are also grouped into hydrolases and these two enzymes were confused for a long time. Now, it is realized that they are really different from each other in terms of substrate specificity (Zhang and Zeng 2008). Esterases break ester bonds of short chain fatty acids whereas lipases catalyze the hydrolysis of long chain fatty acids that are insoluble or poorly soluble. Therefore, the lipase must be capable of identifying an insoluble or aggregated substrate. Esterase activity is found to be highest towards more water soluble substrate (Fojan, et al. 2000).

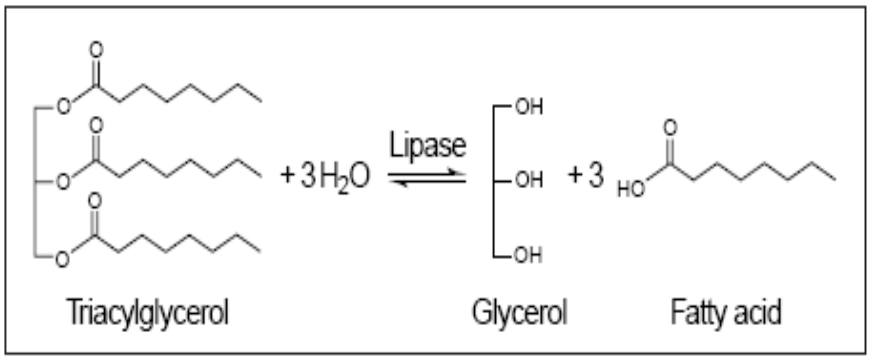


Figure 1.1. Hydrolytic and Synthetic Actions of Lipase
(Source: Jaeger and Reetz 1998)

## 1.3. General Lipase Structure

It is very important to know three-dimensional structures of lipases in order to make them fit for specific applications. Human pancreatic lipase and the lipase from the fungus *Rhizomucor miehei* were the first ones whose 3D structures elucidated. Various other fungal lipase structures, from *Geotrichum candidum*, *Fusarium solani*, *Candida rugosa*, *Candida antarctica*, *Humicola lanuginose* and *Rhizopus delemar,* followed them (Jaeger, et al. 1999, Jaeger, et al. 1994). In contrast, 3D structues of bacterial lipases were determined slowly. The first bacterial lipase structure, from *Pseudomonas glumae,* was clarified in 1993 (Noble, et al. 1993). Many bacterial lipases have been studied structurally since 1993.

After the determination of first lipase structures, it was realized that they shared a common folding pattern in spite of not showing sequence similarity (Fan, et al. 2008). This folding pattern was identified in 1992 by Ollis et al. Ollis and his friends compared five hydrolytic enzymes which were dienelactone hydrolase, haloalkane dehalogenase, wheat serine carboxypeptidase II, acetylcholinesterase and the lipase from *Geotrichum candidum*. They concluded that they share a same folding pattern called α/β hydrolase fold. Because they all catalyze a hydrolysis reaction (Arpigny and Jaeger 1999, Nardini and Dijkstra 1999). So, the alpha/beta-hydrolase fold family consists of structurally related enzymes with diverse catalytic functions.

The α/β hydrolase fold includes a central, mostly parallel eight- stranded β sheet (only the second β strand is antiparallel ) surrounded on both sides by α helices (Figure 1.2). The β sheet displays a left-handed superhelical twist and the first and the last

strands cross each other at an angle of approximately 90° (Jaeger, et al. 1999). The bacterial lipase structures known so far possess the α/β hydrolase fold with some variations. The number of β strands in β sheet can be changeable for lipases from different bacterial species. For instance, the lipases from *B.glumae* and *B.cepacia* have six parallel β strands whereas that from *S.exfoliatus* contains an extra antiparallel β strand in the central β sheet of the α/β hydrolase fold (Nardini and Dijkstra 1999).

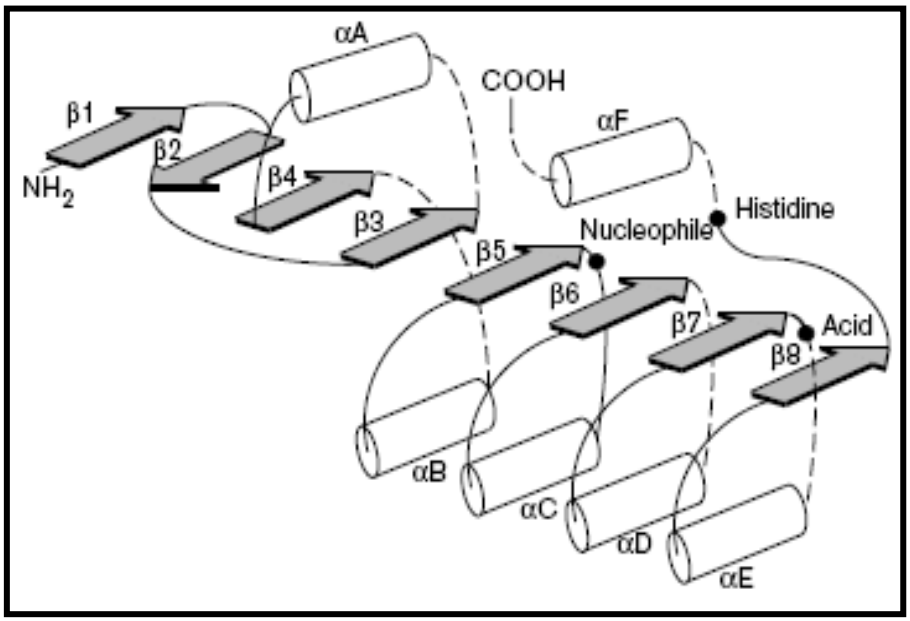


Figure 1.2. The α/β hydrolase fold
(Source: Jaeger, et al. 1999)

The active site of the α/β hydrolase fold enzymes contains three catalytic residues which are nucleophilic residues (serine, cysteine or aspartate), a catalytic acid residue (aspartate or glutamate) and a histidine residue. In lipases the nucleophile residue has been determined to be a serine residue, but the catalytic acid can be either an aspartate or a glutamate residue. The active site serine residue is located in a higly conserved Gly-X-Ser-X-Ser pentapeptide (Pascale, et al. 2008, Joseph, et al. 2008). In contrast, this well conserved pentapeptide differs in lipases from *Bacillus* strains where the first Gly residue is replaced by an Ala (Jaeger, et al. 1994).

Another unusual and interesting feature of the structure of most lipases is the presence of a lid-like structure that consists of one or two α-helices. This property results in a conformational change in lipase if there is an interface between oil and water (interfacial activation phenomenon). The lid moves away from the active site, thereby

allowing it to become accessible for the substrate (Angkawidjaja and Kanaya 2006). However, this phenomenon does not include all lipases. Remarkable exceptions are the lipases from *P.glumae* and *C.antartica B*. In spite of having a lid, these lipases do not show interfacial activation (Schmid and Verger 1998).

Finally, some *Pseudomonas* lipases are shown to have a calcium binding site (Figure 1.3). This calcium binding site is located near the active site, but it is not related to catalytic activity. It is thought to play a role in stabilization of the general structure of the enzyme.

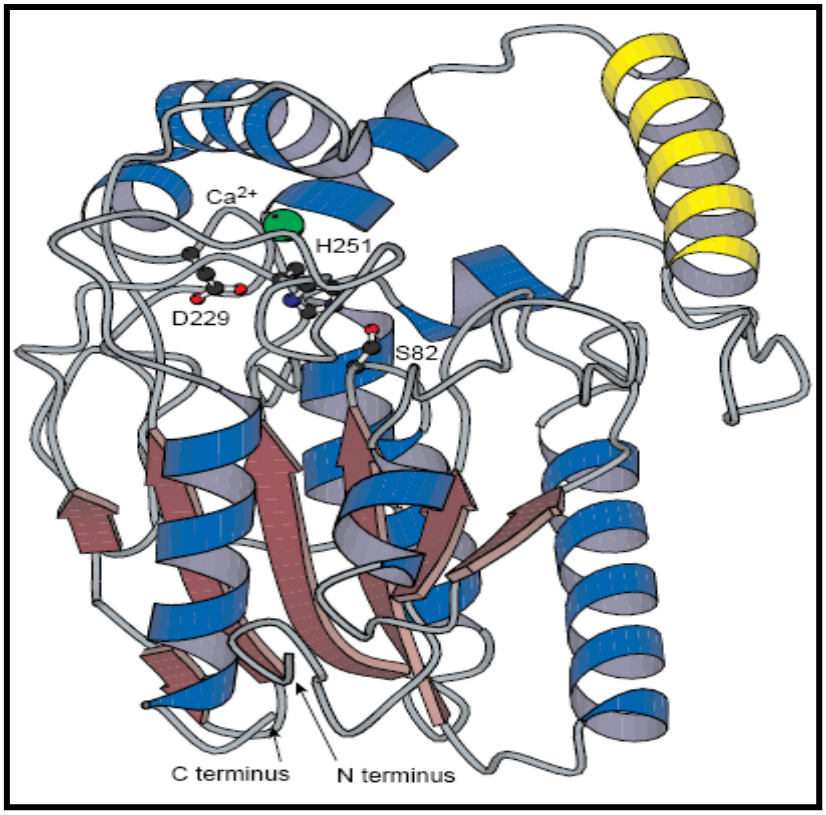


Figure 1.3. The 3D structure of the lipase from *Pseudomonas aeruginosa* (Source: Jaeger and Reetz 1998)

## 1.4. Sources of Lipases

Lipases are found throughout all kingdoms of life, which are prokaryotes including bacteria and archaea and eukaryotes including plants, animals and fungi (Cai-hong, et al. 2008). Microbial lipases are more useful than enzymes derived from plants and animals, since they have great variety of catalytic activities and microorganisms are

easy to manipulate genetically and cabaple of rapid growth on inexpensive media. Furthermore, microorganisms are not affected by seasonal fluctuations so they can be supplied regularly and high amounts of lipases may be obtained from microbial cells. Microbial lipases are also more stable than their plant and animal derivatives and their production is easier and safer for industrial and research applications (Schmidt-Dannert 1999).

Bacterial enzymes are more preferred over fungal enzymes because of their higher activities and neutral or alkaline pH optima. In order to increase the cell yields and the enzymatic activities of the cells or to produce altered enzymes, genetic and enviromental manipulations can be performed more readily on bacterial cells due to their short generation times, their simple nutrional needs and easy screening procedures for desired properties (Hasan, et al. 2006).

## 1.5. Bacterial Lipases

A variety of lipases are produced from both Gram-positive and Gram-negative bacteria. Greater part of bacterial lipases comes from Gram-negative bacteria and the most important Gram-negative genus is *Pseudomonas* which contains at least seven lipase producing species, that are *P. aeruginosa, P. alcaligenes, P.fragi, P. glumae, P. cepacia, P. fluorescens and P. putida* (Jaeger, et al. 1994, Kojima, et al. 2003).

*Pseudomonas* lipases are classified into three groups based on their amino acid homologies and some biological properties (Zhang, et al. 2008). Group I contains lipases from *P.aeruginosa, P.alcaligenes and P.fragi*. Lipases in this group are composed of approximately 285 amino acids with a molecular weight of 30 000 dalton. Also, they include two cysteine residues to form a disulfide bond and need another protein called lipase-spesific foldase for correct folding and secretion. Moreover, they include a specific sequence at their N-terminal. Group II lipases contain 320 amino acids correspondig to a molecular weight of 33 000 dalton, one disulfide bond and an N-terminal signal sequence like group I lipases. Additionally, There is % 60 amino acid homology between group I and group II lipases and they also require lipase specific foldases in order to be active and secreted into extracellular environment. Lipases from *P.glumae* and *P.cepacia* are prototypes for group II. Group III *Pseudomonas* lipases are larger containing about 475 amino acids and a molecular weight of 50 000 dalton.

*P.fluorescens* lipases are prototypes of this group (Arpigny and Jaeger 1999). Group III lipases are separeted from group I and group II lipases in several ways a) they do not contain cysteine residues, b) they do not require any lipase specific foldases and c) they do not contain an N-terminal signal sequence. According to these differences, it is said that group III lipases use a different secretion pathway. Group I and II lipases use type II secretion pathway (also called secreton-mediated secretion) while group III lipases are secreted via type I secretion system (also named ABC exporters) (Rosenau and Jaeger 2000).

As mentioned above, although group III lipases do not have a typical N-terminal sequence, they contain a C-terminal targeting signal sequence responsible for the secretion of lipase by an ABC exporter (Figure 1.4) (Amada, et al. 2000, Duong, et al. 1994). The type I secretion pathway includes three different proteins a) an inner membrane ATPase confers the substarete specificity to the system b) membrane fusion protein (MFP) functions like a bridge between the inner and outer membrane because it is connected with both the inner and the outer membrane c) the last protein is an outer membrane protein (Rosenau and Jaeger 2000, Jaeger, et al. 1999). Lipases are secreted directly into the extracellular area by this system in which there is no need for an extra step like the formation of enzymatically active periplasmic intermediates appeared in type II secretion pathway.

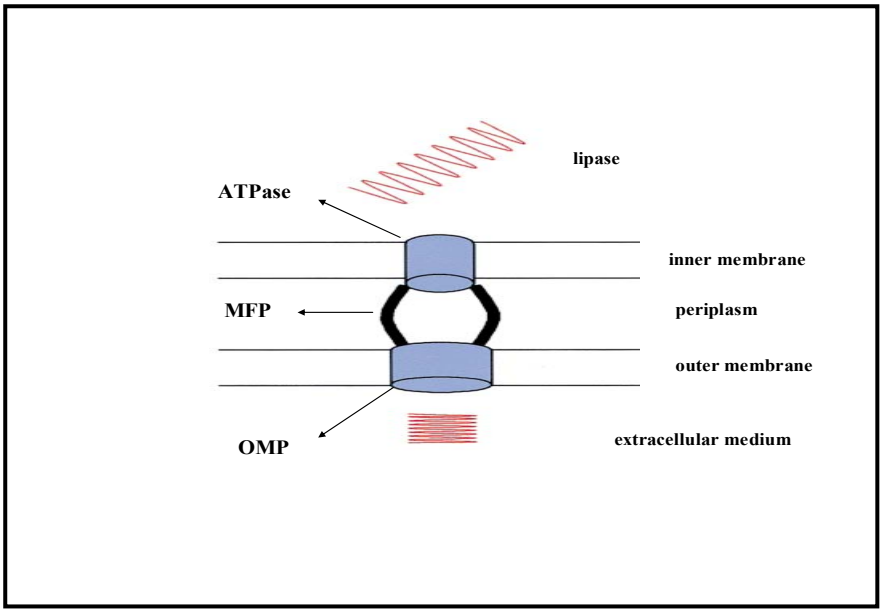


Figure 1.4. Type I secretion pathway
(Source: Rosenau and Jaeger 2000)

On the other hand, group I and group II lipases have an N-terminal signal sequence which is used for efficient secretion through the inner membrane by a Sec-dependent mechanism. This mechanism contains a multisubunit protein complex called Sec translocase that recognizes the N-terminal sequences of lipases. While lipase is secreted through the inner membrane, signal sequence is removed and lipase interact with its specific foldase as well (Rosenau, et al. 2004). As a result, lipases fold into an enzymatically active conformation in the periplasm. Moreover, this process is assisted by the Dsb (disulfide bond formation)-proteins which catalyzes the formation of disulfide bonds. Alternatively, misfolded lipases may be degraded by periplasmic proteases. Finally, lipases are transported through the outer membrane by the help of a complex machinery called secreton which contains different proteins forming type II secretion pathway or secreton mediated- secretion (Figure 1.5).

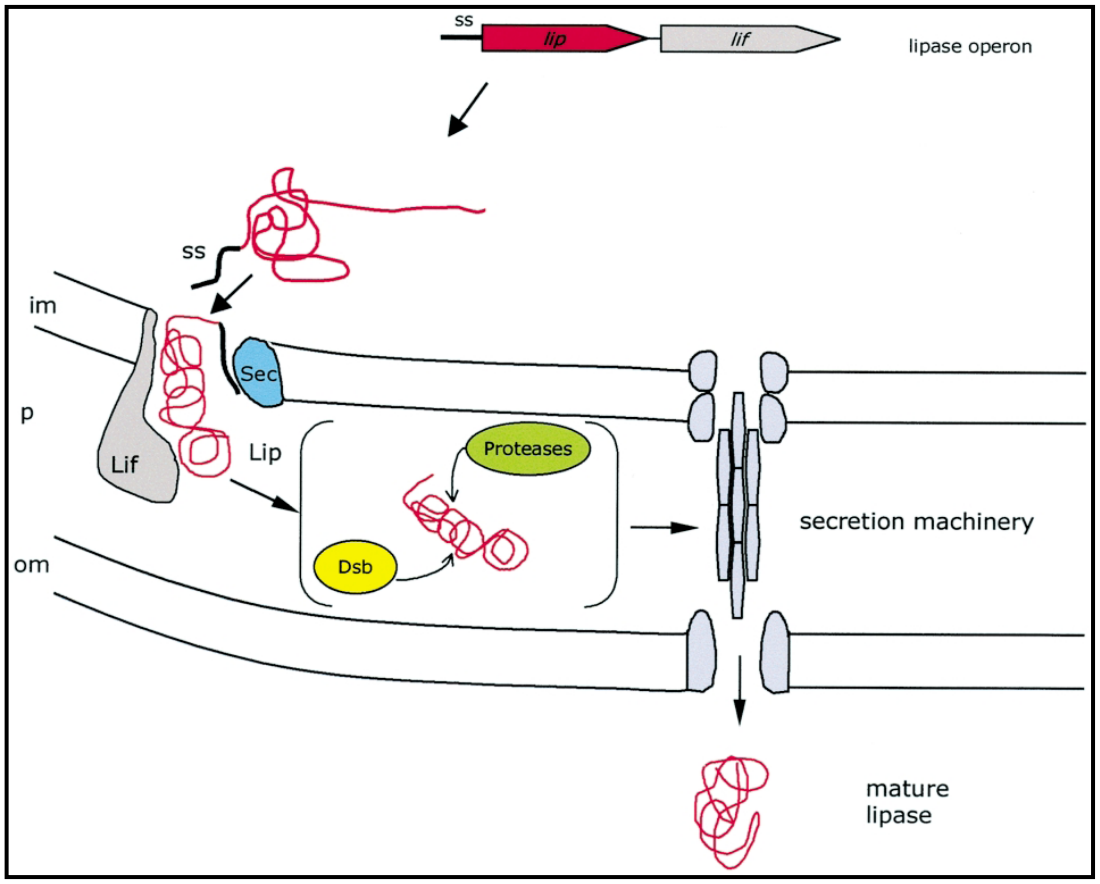


Figure 1.5. Model for *Pseudomonas aeruginosa* lipase secretion pathway
(Source: Rosenau and Jaeger 2000)

Besides *Pseudomonas* species *Achromobacter*, *Alcaligenes*, *Burkholderia* and *Chromobacterium* strains are the most common lipase producing gram-negatives (Gupta and Rathi 2004).

Gram-positive lipase producers are *Staphylococcus* (especially, *S.aureus and S.hyicus*), *Streptomyces* and *Bacillus* species. The most useful lipase producer genus used in industry is *Bacillus* among gram-positive bacteria (Jaeger, et al. 1994).

Most bacterial species studied for lipase production are non-pathogenic, mainly because these lipases were aimed to be used for biotechnological applications. It is a fact that extracellular lipases are important microbial virulence factors in addition to their industrial usage. In connection with this aspect, human pathogenic bacteria has been examined recently. One of them is *Staphylococcus epidermidis*, which is an opportunistic pathogen. During infection, it secretes two lipases that are considered to play a role in the colonization on the skin by breaking sebum-derived triacylglycerols. As well as *Staphylococcus,* other bacterial species seem to produce lipases for pathogenicity. *Propionibacterium acnes*, acting as the cause of acne vulgaris, produces an extracellular lipase, which functions in the microbial colonization of human skin (Stehr, et al. 2003).

## 1.6. Detection of Lipolytic Microorganisms

There must be three factors to detect a lipase-positive bacterium by culturing it. These factors include (i) growth of the organism, (ii) production of lipase by that organism under suitable growth conditions and (iii) the presence of a sensitive method to detect lipase acitivity (Shelley, et al. 1987).

Growth conditions affect the synthesis of lipase by microorganisms. Carbon and nitrogen sources, the presence of activators and inhibitors, incubation temperature, pH, inoculum amount and oxygen tension can influence lipase production (Gupta, et al. 2004). The carbon source has been reported as the major factor that affect lipase expression, since lipases are inducible enzymes. The production of these enzymes depends on the presence of a lipid, such as olive oil or any other inducer, such as triacylglycerols, fatty acids and tweens (Treichel, et al. 2009).

Lipase activity is identified by using triacylglycerols composed of long-chain fatty acids. Triolein is the most ideal substrate due to its liquid form at common assay

temperatures. This feature facilitates emulsification of it into growth media. Alternatively, olive oil can be used instead of triolein. Olive oil has the advantage of including high concentration of oleic acid and being more economical (Jensen 1983). Substrates like tweens and tributyrin can also be used for the detection of lipases. However, since they can also be hydrolyzed by esterases, they don't give lipase specific results (Jensen 1983, Shelley, et al. 1987). In conclusion, tweens and tributyrin can be used for only primary screening procedures.

Microorganisms are often tested for lipase production on solid media. All methods containing agar can be grouped into two categories; (1) Methods based on substrate changes in appearance as a result of lipolysis and (2) methods including the usage of an indicator dye to detect lipolysis (Thomson, et al. 1999). In all methods, it is important that there must be a contact between substrate and enzyme. Thus the agar content in screening media can be reduced for increased diffusibility of extracellular lipase (Hou and Johnston 1992).

### 1.6.1. Methods Based on Changes in Substrate Appearance

It is possible to visualize lipase-producing microbial colonies on solid media depending on the used lipase substrate. This type of detection have several advantages like the elimination of the requirement for specific dyes, that can reduce the cost of analysis and prevent the inhibiton of microbial growth because microorganisms may be sensitive to dyes (Thomson, et al. 1999).

A diffusion assay can be used to identify lipase-producing microorganisms. In this method, a growth medium with tributyrin or triolein is prepared and microbial culture is put into wells cut in the agar. Lipase activity is defined by a clear zone formation around colonies after incubation (Thomson, et al. 1999).

In direct observation methods, the formation of clear or turbid zones around colonies, or the production of crystals on the agar surface displays the presence of lipolytic activity (Shelley, et al. 1987). Triolein is used as the substrate in lipase detection methods; however, it is difficult to visualize zones of hydrolysis. When Tweens are used as lipase substrates, clear zones are easy to observe (Shelley et al. 1987). Shelley et al. (1987) concluded that agar media can be useful for only screening of lipase production, but not to measure lipase acitivity.

### 1.6.2. Dye-Based Methods

Lipolytic activity of microorganisms can also be detected by using specific dyes like Victoria blue B, Spirit blue, Nile blue sulfate, and night blue. These dyes can be used by either directly putting into the growth medium or applying to the plate after incubation. In dye-based methods, indicator dyes must show a clear and distinguishable color changes as a result of pH changes that occur due to the release of free fatty acids from triacylglycerols during lipolysis. Various combinations of substrates like tributyrin and tweens and dyes such as Victoria blue B and night blue can be used in these methods (Thomson, et al. 1999). However, These substrates are not suitable to detect true lipases because they are hydrolyzed by esterases, too. The formation of clear zones around colonies against an opaque background on tributyrin agar lacking Victoria blue B indicates lipolytic microbes, but in the presence of Victoria blue B lipolytic colonies are surrounded by dark zones against an opaque, light blue background (Jones and Richards 1952).

Microbial lipase activity can also be identified by using fluorogenic dye Rhodamine B. The method containing Rhodamine B as an indicator of the presence of lipase and olive oil as lipid substrate was firstly found by Kouker and Jaeger (1987). Agar plates containing olive oil and rhodamine B are opaque and pink colored. Lipase producing bacteria forms orange fluorescent halos around their colonies under UV light, but lipase negative bacteria do not show orange fluorescence upon UV irradiation (Kouker and Jaeger 1987). The fluorescence is related to the formation of a rhodamine B-long chain fatty acid conjugate (Jaeger, et al. 1994). Methods used to find lipolytic microbes with triacylglycerols and pH indicator dyes such as Victoria blue B are sensitive to any pH changes and can inhibit the growth of some bacteria. The rhodamine B plate method is not affected by pH changes and does not inhibit the growth of test microorganism or change its physiological properties (Kouker and Jaeger 1987, Thomson, et al. 1999).

## 1.7. Industrial Applications of Microbial Lipases

The global industrial enzyme market was about $ 2 billion in 2004 and is estimated to reach nearly $ 2.4 billion in 2009. Lipases represent about % 4 of this

market and has been paid an increasing attention due to their biotechnological potential (Hasan, et al. 2006).

Lipases as biocatalysts have many favourable properties that make them suitable for specific applications compared with chemical catalysts. Lipase-catalyzed reactions are higly specific because of their broad substrate specificity and high regio and/or stereoselective features while chemical processes are typically more non-specific. Due to the specificity of lipases, the production of unwanted products in the waste stream are decreased or eliminated. Moreover, the use of enzymes decreases the side reactions and make post-reaction separation problems simpler (Pandey, et al. 1999, Hasan, et al. 2006). Thus, it can be said that lipases are environmental friendly.

Additionally, lipases can carry out reactions under mild conditions of pH and temperature and this reduces energy needs to direct reactions at unusual temperatures and pressures. As a result, unstable reactants and products are protected from destruction. Other reasons for biotechnological potential of microbial lipases are their stability in organic solvents and being active without the aid of cofactors (Jaeger and Reetz 1998).

The lipases can be employed in industry by cultivating lipase producing microorganism in the medium containing a suitable substrate (especially in the food industry), which is named *in situ* application or by using immobilized pure enzymes (especially in the production of fine chemicals) called *ex situ* application (Pandey, et al. 1999).

### 1.7.1. Lipases in the Food Industry

Lipases have found broad applications in the modern food industry instead of traditional chemical processes. Nowadays, lipases are commonly used in the production of a variety of products like fruit juices, baked foods, fermented vegetables, cheese, butter, dressings, soups and sauces.

Modification of lipids (oils and fats) is one of the important processes in food industry. Some moities can be added to or deleted from triacylglycerols by lipase-catalyzed specific reactions. Also, several esterification and transesterification reactions can be carried out by lipases to form modified lipids. Especially regiospecific and fatty acid specific microbial lipases have been used to convert oils into more nutritionally

valuable ones. Cheap oils could be improved to synthesize nutritionally important modified triacylglycerols such as cocoa butter substitutes, triacylglycerols with low calorie and oils containing high amounts of oleic acid (Hasan, et al. 2006). For instance cocoa butter includes palmitic and stearic acids and has a melting point about 37°C that makes it melt in the mouth. In 1976, Unilever found a method with a mixed hydrolysis and synthesis reaction and produced a cocoa-butter substitute using an immobilized lipase. This method based on an immobilized lipase from *R. miehei* is now used commercially by Quest-Loders Croklaan. This lipase is responsible for a transesterification reaction replacing palmitic acid in palm oil with stearic acid to form the stearic- oleic- stearic triglyceride with the desired melting point for use in cholotae (Sharma, et al. 2001, Jaeger and Reetz 1998).

Lipases are also used to give special flavour and taste to food by synthesis of fatty acids and alcohols, which are accepted as flavour and fragrance compounds (Gandhi 1997). In this case, lipases are commonly employed in dairy industry for the hydrolysis of milk fat. Improvement of flavour in cheeses, the acceleration of cheese ripening, the production of cheese like products and the lipolysis of butterfat and cream is achieved by the help of lipase enzymes. Addition of lipases to such products primarily releases short- chain ($C_4$ and $C_6$) fatty acids that form sharp flavour but the release of medium- chain ($C_{12}$ and $C_{14}$) fatty acids leads to formation of a smooth taste (Saxena, et al. 1999).

Lipases have been used to produce meat like fish meat without excess amount of fat. The fat is removed during the processing of the fish meat by adding lipases and this procedure is called biolipolysis (Sharma, et al. 2001, Seitz 1974).

### 1.7.2. Lipases in the Detergents

Lipases are largely employed as additives to detergents, which are used commonly in household and industrial laundry and in household dishwashers. Approximately 1000 tons of lipases are sold every year in this area. Lipases are generally added to the detergents primarily in association with proteases and cellulases (Pandey, et al. 1999). However, other enzymes such as amylases, peroxidases and oxidases should be also included**.** Lipases catalyze the hydrolysis of fatty stains into

more hydrophilic parts that are easily removed than similar non-hydrolyzed stains (Joseph, et al. 2007).

In 1994, the first commercial lipase was introduced by Novo Nordisk. This lipase ,named Lipolase™,was produced by the fungus *T. Lanuginosus* and expressed in *Aspergillus oryzae*. In 1995, Genencor International produced two bacterial lipases, which are Lumafast™ from *Pseudomonas mendocina* and Lipomax™ from *Pseudomonas alcaligenes* (Jaeger and Reetz 1998).

To be a suitable additive in detergents, lipases should be both thermophilic (30-60 °C) and alkalophilic (pH 10-11) and capable of functioning in the presence of the various components of washing powder formulations like surfactants and proteases. In addition, they should have wide substrate specifity to be able to hydrolyze fats of various compositions (Sharma, et al. 2001, Jaeger and Reetz 1998). Lipases with these properties can be identified by the aid of a combination of genetic and protein engineering.

In addition to their application in laundry, lipases are also included in dish washing, contact lens cleaning, degradation of organic wastes on the surface of exhaust pipes and toilet bowls, and etc (Hasan, et al. 2006).

### 1.7.3. Lipases in Pulp and Paper Industry

Wood is the main source of paper and pulp industry and the presence of the hydrophobic components (mainly triglycerides and waxes), also named pitch, in wood causes serious problems in the production of paper and pulp. Lipases are used to remove the pitch from pulp produced for paper making. In Japan, Nippon Paper Industries have found a pitch control system to remove most of the wood triglycerides. This system is based on the usage of a lipase from *Candida rugosa* (Jaeger and Reetz 1998).

### 1.7.4. Lipases in Leather Industry

The main processes in leather industry are the removal of subcutaneous fat and dehairing. Conventional methods including organic solvents and surfactants to remove fat from animal skins can be harmful to environment due to the production of dangerous final products like volatile organic compound (VOC) emissions (Hasan, et al. 2006).

Usage of lipases in association with other hydrolytic enzymes like proteases is a new approach in leather processing. Since the process is carried out at alkaline pH, alkalophilic lipases are used in combination with alkaline or neutral proteases and other necessary hydrolytic enzymes (Pandey, et al. 1999).

### 1.7.5. Lipases in Environmental Management

Employment of lipases in bioremediation processes is a new aspect in lipase biotechnology. The wastes of lipid- processing factories and restaurants can be cleaned by the help of lipases from different origins. In this sector, lipases could be used by either *ex situ or in situ* (Pandey, et al. 1999).

### 1.7.6. Lipases as biosensors

Lipases have an ability to sense lipids and lipid-binding proteins. Therefore, lipases can be manipulated as biosensors. The quantitative determination of triacylglycerols especially in food industry and in clinical diagnosis is the most common application of lipases as biosensors. In clinical diagnosis, lipases are used to form glycerol from the triacylglycerol in the analytical sample and the released glycerol is quantified by a chemical or enzymic method. It was shown that the patients with cardiovascular complaints have been diagnosed very precisely by this principle. Shoemaker et al. discovered a method including the use of microbial lipase. In this method, the glycerol produced as a result of lipid hydrolysis was oxidized by glycerol dehydrogenase. NADH formed during the reaction was measured by fluorescence spectroscopy. Non- specific lipases are generally employed as biosensors (Pandey, et al. 1997).

### 1.7.7. Lipases in Pharmacy

Lipases are very commonly used in pharmaceutical industry because of its regioselective property. Production of enantiomeric compounds, racemic esters, catalysis of synthetic reactions, kinetic resolution process for the preparation of

optically active chiral compounds can be examples to the usage of lipases in pharmaceutical industry. Especially, preparation of homochiral compounds that are used against HIV and synthesis of anti-tumour agents, alkaloids, antibiotics and vitamins can be said to be the vital applications of lipases (Jaeger and Eggert 2002).

## 1.8. The Aim of this Work

The primary goals of this thesis were to isolate and identify a lipase producing bacterial strain from soil samples from Erciyes mountain in Kayseri, find and then analyze the sequence of its lipase gene. Rhodamine B-olive oil screening system was used to find lipase producers from soil samples. To identify the selected lipase producer strain, 16S rRNA gene sequence analysis was applied as well as the some taxonomical studies. Degenerate PCR and inverse PCR were performed to find the sequence of the lipase gene. The other purposes of this study were determination of lipase production conditions like optimum production time, partial purification and characterization of native lipase enzyme. Purification was performed by ammonium sulfate precipitation and gel filtration. Spectrophotometric extracellular lipase assay was used for enzyme characterization.

# CHAPTER 2

# MATERIALS AND METHODS

## 2.1. Materials

A detailed list of commonly used chemicals, buffers, solutions and their compositions are presented in Appendix C.

## 2.2. Methods

### 2.2.1. Selection of a Lipase Producer Bacterial Strain From a Soil Sample

A soil sample was collected from Erciyes Mountain in Kayseri in september 2006. This soil sample was diluted several times with Luria-Bertani (LB) broth and diluted soil mixtures were plated on agar plates of Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 1% gellan gum, pH 7.2) supplemented with 1% v/v olive oil (Applichem) as substrate and 1% rhodamine B solution (0.1% w/v) (Applichem). Lipase producing strains were identified on spread plates after several days incubation at 25 °C by the formation of orange fluorescent halos around the colonies monitored by fluorescence with UV light at 350 nm. A strain showing the highest lipolytic activity was picked out for further study. This selected strain was plated on fresh LB agar to obtain pure cultures and grown at several temperatures as well as 25°C to identify its growth and lipase production temperature range using LB agar alone and supplemented with olive oil and rhodamine B. At the same time the positive control with commercial lipase from porcine pancreas (Applichem) and the negative control with *E.coli* were made using rhodamine B-olive oil LB plates. These two control groups were incubated overnight at 37 °C.

### 2.2.2. Genetic Identification of Isolated Strain by PCR Amplification of Partial 16S rRNA Gene

Total genomic DNA of the strain was isolated by CTAB/NACI method (Jaufeerally-Fakim and Dookun 2000) which consists of chemical lysis with detergent, incubation with a nonspecific protease, followed by a CTAB extraction in which CTAB complexes both with polysaccharides and with residual protein. This procedure is very effective in producing high quality chromosomal DNA from a variety of gram-negative bacteria, all of which normally produce large amounts of polysaccharides (Wilson 2001). 30 ml of 24 hour bacterial culture ($OD_{600}$ :1) grown at 25 °C with shaking (200 rpm) was used for the genomic DNA isolation. Isolated genomic DNA concentration measured visually after electrophoresis in 1% agarose gel.

16S rRNA gene of isolated strain was amplified from its genomic DNA using a pair of universal bacterial primer, E334F (5' CCAGACTCCTACGGGAGGCAG 3') as forward primer and E1115R (5'CAACGAGCGCAACCCT3') as reverse primer. PCR was performed as follows: genomic DNA ( 100 ng/ µl ) 1 µl, E334F- E1115R primers (10 µM) 1 µl each, 25 µl 2X PCR master mix (Fermentas) and 22 µl $dH_2O$ were mixed in a total volume of 50 µl. The conditions for PCR amplification were as follows : an initial denaturation step at 94 °C for 2 minutes; followed by 25 cycles including denaturation at 94°C for 30 seconds , primer annealing at 62°C for 30 seconds and elongation at 72°C for 1 minute and also final elongation at 72°C for 5 minutes. PCR products were visualized by agarose gel electrophoresis and purified by PCR purification Kit (Qiagen). The amplified and purified 16S rDNA fragment was cloned into the pTZ57R/T cloning vector (Fermentas), used as TA cloning vector for PCR product amplified with taq polymerase, according to the manufacturer's instructions. *E.coli* Dh5α competent cells were transformed with pTZ57R/T- amplified 16S rDNA fragment via heat-shock method. Transformation mixtures were plated on LB plates containing 100 µg/ml ampicillin, 0.5 mM IPTG and 80 µg/ml X-Gal and incubated overnight at 37°C. Recombinant clones were identified by blue-white screening. Clones that contain PCR product produce white colonies in most cases. White colonies were chosen for colony PCR to verify the insert presence in the vector. Plasmid specific primers M13F (5'gtaaaacgacggccagt 3') and M13R (5'caggaaacagctatgacc 3') were used in colony PCR. The PCR reaction mixture included 12.5 µl 2X PCR Master Mix (Fermentas), 1 µl of M13F (10µM), 1 µl of M13R Primer (10µM), 10.5 µl $dH_2O$ and

white colonies that picked up from plates via pipette tip and suspended into 25 µl PCR reaction mixture. The conditions for PCR amplification were as follows: 94°C /2 min; 94°C /1 min, 55°C /1 min, 72°C /1 min for 25 cycles; 72°C /5 min; hold at 4°C. The positive clones based on the result of colony PCR were selected and pTZ57R/T-16S rDNA plasmids were isolated from overnight cultures of positive clones using GeneJet Plasmid Miniprep Kit (Fermentas). All steps in plasmid isolation were carried out according to the manufacturer's instructions. Finally purified plasmids were sequenced with vector (pTZ57R/T) specific primers (M13F and M13R) to determine the sequence of the cloned DNA fragments. Blast searches (National Center for Biotechnology Information, NCBI) were performed on the DNA sequence to determine the most closely related 16S rRNA species and to further testify the subfamily of the lipase from the experimental strain. A phylogenetic tree was also made based on the homology of known 16S rRNA sequences deposited in GenBank.

### 2.2.3. Design of an *E.coli* Expression Vector

PUC19 cloning vector was used as a starting template to design of an inducible *E.coli* expression vector by PCR-based cloning. For this purpose, a pair of specific primer was designed to remove lacZ structural gene except the inducible lac promoter from PUC19 vector. These primers were named PUC5' (5' CTG ATA TC**G GAT CC**T AGC TGT TTC CTG TGT G 3') (The underlined and the thick sequences are ECORV and BamHI restriction sites, respectively) and PUC3' (5' CTG ATA TC**G AAT TC**G GTG GCA CTT TTC GGG 3') (The underlined and the thick sequences are ECORV and ECORI restriction sites, respectively). PCR was performed in a total reaction volume of 50 µl containing 25 µl 2X PCR master mix (Fermentas), 1 µl PUC5' (20 µM), 1µl PUC3' (20 µM) and 1 µl PUC19 (100ng). PCR cycles were as follows: 94°C /2 min; 94°C /30 s min, 55°C /30 s, 72°C /2.5 min for 30 cycles; 72°C /5 min; hold at 4°C. Amplified products were run on a 1% agarose gel to control its size. PCR product was cleaned using High Pure PCR Purification Kit (Roche) and blunted with a thermostable DNA blunting enzyme (Genejet PCR cloning Kit, Fermentas). These two reactions were carried out according to the manufacturer's instructions. Self circularization of blunt ended linear PCR fragment was performed using T4 DNA ligase and *E.coli Dh5α* competent cells were transformed with the resulting mixture.

Transformation mixture was inoculated on LB plates with ampicillin. Colonies were controlled by colony PCR after overnight incubation at 37°C. Colony PCR was performed using M13R and PUC5' primers. PCR cycles were 94°C /2 min; 94°C /30 s, 55°C /30 s, 72°C /2.5 min for 30 cycles; 72°C/5 min; hold at 4°C. PCR product size was checked by 1% agarose gel. The expected plasmid based on gel result was isolated from overnight *E.coli* cultures using plasmid isolation kit (Roche). Furthermore, the isolated plasmid was digested with TaqI, ECORV and Cfr113I enzymes to control whether it was correct one or not. PUC19 was also digested with the same enzymes to compare two plasmid restriction results.

### 2.2.3.1 Control the Expression Capacity of the Designed Plasmid

In order to check whether the designed plasmid has the ability to express any gene inserted in it, the tetracycline resistance gene open reading frame (orf), 1210 bp, from pBR322 cloning vector was amplified by a PCR with the primer set, tet5' ( 5' tcg *aga tct* atg aaa tct aac aat gcg c 3') and tet3' (tcg *gaa ttc* tca ggt cga ggt ggc c 5'), containing restriction enzyme sites of the italic letters with Bgl II/EcoRI, respectively. PCR was run with the following conditions: an initial denaturation at 94°C for 2 min, denaturation at 94°C 30 s, annealing at 65°C for 30 s and an extension step at 72°C for 2 min. The sequence was repeated 25 times followed by a 5-min final extension step at 72°C. PCR result was evaluated by 1% agarose gel and PCR product was purified with commercial kit (Invitrogen). The pure tetracyline gene fragment was cloned into TA cloning vector (pTZ57R/T, Fermentas) following the protocol in kit and *E.coli* Dh5α competent cells were used as host cells. Following transformation, blue-white screening was performed and white colonies were picked up for colony PCR to control insert presence. In colony PCR tet5' and tet3' primers were used so the colony PCR cycles were same as described above. Positive plasmid construct (pTZ57R/T- tet gene) was isolated using GeneJET Plasmid Miniprep Kit (Fermentas) and tetracyline gene was removed from the construct via EcoRI/BglII double digestion and then ligated into EcoRI/BamHI digested newly designed expression vector and transformed into *E.coli* Dh5α. The *E. coli* transformed with this plasmid was plated on both LB agar containing (12.5 μg/ml) tetracycline plus 0.5 mM IPTG and only tetracyline. Plates were evaluated after overnight incubation at 37°C. Colony PCR was also performed to control the

presence of tetracyline gene using tet5' and tet3' primers with the same PCR conditions as mentioned above.

### 2.2.4. PCR Amplification, Cloning, and Sequence Analysis of Lipase Gene from *Pseudomonas* sp. KE38

For initial cloning of part of the lipase gene from the experimental strain, complete lipase gene sequences from 53 different *Pseudomonas* strains were downloaded from the Entrez Search and Retrieval System at the National Center for Biotechnology Information (NCBI), they were aligned using ClustalW and a phylogenetic tree was constructed. 23 different *Pseudomonas fluorescens* lipases were selected from this tree and they were aligned using ClustalW program. According to the aligment result, a set of degenerate primers LIPPFF, 5'-ATG GGT ITN TIY GAC TAY AAI AAC-3' and LIPPRR, 5'-GCG TAR TGR TCG TTG AAG ITG ACG ATR-3' was constructed at higly conserved regions in the lipases. PCR amplification was performed in a 25 µl volume containing 10 ng of DNA template, 5 µM of each primer and 12.5 µl 2X PCR master mix. The reaction mixture was subjected to 30 cycles of amplification with the following conditions: 94°C/ 2 min for denaturing, 45°C/55°C (temperature gradient) for 1 min for annealing, 72°C/1 min for extension and 72°C/5 min for final extension. Purified PCR product was cloned into pTZ57R/T prior to sequencing. Ligation mixture was transformed into *E.coli* Dh5α competent cells. After colony PCR, the recombinant plasmids were purified with the GeneJET Plasmid Miniprep Kit and sequenced at the Biotechnology and Bioengineering Central Research Laboratories, Izmir Institute of Technology. Homology analysis using BLAST was performed on the obtained partial sequence. Also, partial amino acid sequence was analyzed using UniProt/EBI.

The rest of the lipase gene was tried to be obtained by performing a kind of PCR method called inverse PCR. Inverse PCR resembles the PCR, but it uses the primers oriented in the reverse direction. These primers must be synthesized from known sequences. The template for the inverse PCR is the self-circulated genomic DNA fragments digested with suitable restriction enzymes that have no restriction sites within the known sequence.. In this method, the DNA regions that flank a known DNA sequence can be amplified (Ochman, et al. 1988).

Based on the sequence of the DNA fragment, three pairs of primers, lip3/lip4, lip5/lip6 and lip7/lip8, for inverse PCR were constructed in the opposite direction. The sequences and the positions of these primers were given in Appendix B. Partial lipase fragment was analyzed to find appropriate restriction enzymes using Clone Manager 5 software. Through this analysis, PstI, HindIII and SmaI enzymes were chosen. The genomic DNA (20 μg) of the strain was subjected to restriction enzyme digestion by these enzymes (10 units each). Digested DNA was purified and self-circulated by T4 DNA ligase. Three Inverse PCR reactions were performed with lip3/lip4, lip5/lip6 and lip7/lip8 primer pairs using the circularized, digested genomic DNA as template. These three reactions were subjected to predenature for 5 min at 94°C, and then the following 30 cycles were carried out: 94°C for 30 s, 55°C for 1 min, 72°C for 2 min, and finally 72°C for 5 min. The amplified DNA was sequenced and analyzed.

### 2.2.5. Purification and Characterization of Lipase from *Pseudomonas* sp. KE38

#### 2.2.5.1. Determination of Optimum Lipase Production Time and Carbon Source

To increase the inoculum size for enzyme production, *Pseudomonas* sp. KE38 was grown at 25°C under shaking (200 rpm) for 24 hour in 10 ml minimal medium supplemented with %1 (v/v) olive oil as lipase substrate and sole carbon source. 2 ml of the seed culture was transferred into a 500 ml flask containing 200 ml minimal medium with %1 (v/v) olive oil and grown at 25°C with shaking (200 rpm) for 1 to 5 days. Aliquots of the culture broth were withdrawn at each day and harvested (10,000g, at 4°C for 30 min) to determine the optimum time for lipase production. The supernatants were used for measurement of lipase activity.

To monitor the effect of glucose, olive oil and their combination on the production of lipase, 4 ml of %20 (w/v) glucose solution, %1 (v/v) olive oil and both glucose and olive oil were added to 200 ml minimal medium seperately. *Pseudomonas* sp. KE38 was grown on these three media at 25°C with shaking (200 rpm) for 4 days. After incubation, all cultures were harvested at 10,000 g for 30 min. The cell free supernatants were subjected to lipase assay described below.

### 2.2.5.2. Partial Purification of KE38 Lipase

After the determination of optimum lipase production time and suitable carbon source, *Pseudomonas* sp. KE38 was cultured at 25°C with shaking (200 rpm) for 4 days in 200 ml minimal medium plus %1 (v/v) olive oil. After incubation, the culture broth was centrifuged (10,000g, at 4°C for 30 min) and the cell-free culture supernatant was collected. The supernatant was passed through a 0.22 μm filter to remove any remaining cells. This filtrate was called as crude lipase. The obtained crude lipase was partially purified by ammonium sulfate precipitation and Sephadex G-100 size-exclusion chromatography.

In ammonium sulfate precipitation, sufficient amount of solid ammonium sulfate was added slowly to crude enzyme mixture (200 ml) until % 30 saturation (w/v) with stirring at 4°C. The mixture was allowed to stand overnight at 4°C with gentle stirring and then centrifuged at 4°C and 10.000g for 30 min. The precipitate was dissolved in 3 ml of 50 mM Tris-HCl buffer, pH 8.0. This solution was loaded on Sephadex G-100 column equilibrated with 50 mM Tris-HCl buffer, pH 8.0 and fractions were collected. Spectrophotometric lipase assay described below (at 25°C) was applied to all fractions to find lipase-containing ones. Fractions contained lipase were combined and used for enzyme characterization.

### 2.2.5.3. Characterization of Partially Purified Lipase Enzyme

Lipase assay: Lipase activity was measured by spectrophotometer using *p*-nitrophenyl laurate as substrate. In this assay, amount of released *p*-nitrophenyl was measured at 400 nm. The reaction mixture contained 980 μl 100 mM sodium phosphate buffer including 150 mM sodium chloride and % 0,5 triton X-100, pH 7,2, 10 μl 50 mM *p*-nitrophenyl laurate and finally 10 μl enzyme solution. Blank contained the same components except enzyme solution.

### 2.2.5.3.1. Effect of Temperature and pH on Lipase Activity

The optimum temperature for lipase activity was determined by incubating the assay mixture described above at different temperatures between 4°C and 65°C for 2 hours at 10°C intervals and the resulting enzyme activity was measured spectrophotometrically at 400 nm based on the release of *p*-nitrophenyl.

In order to find the pH profiling of lipase activity, sodium acetate buffer (pH 3-5), potassium phosphate buffer (pH 5-7), tris-HCL buffer (pH 7-9) and glycine-NaOH buffer (pH 9-12) were selected. All buffers were at 50 mM concentration. The assay mixture (980 µl each buffer, 10 µl 50 mM *p*-nitrophenyl laurate and 10 µl enzyme solution) was incubated at 45°C for 2 hours in different pH values of above buffers and the enzyme activity was determined by spectrophotometric lipase assay.

### 2.2.5.3.2. SDS-PAGE and Native PAGE

The molecular mass of partially purified KE38 lipase was determined by SDS-PAGE according to the protocol of Laemmli (1970). %12 polyacrylamide gel (resolving gel) was used together with % 4 stacking gel. Lipase containing (7) fractions were loaded on SDS gel. Electrophoresis was performed at 65 volts for 30 minutes and then 105 volts for 2 hours. Gels were stained with Bromophenol Blue overnight. Spectra™ Multicolor Broad Range Protein Ladder (Fermentas) was used as molecular mass marker.

Native PAGE (% 7.5 resolving gel together with % 4 stacking gel) was also performed to observe native lipase enzyme via activity staining. Plates containing rhodamine-B solution, %1 (v/v) olive oil and % 1 (w/v) gellan gum were prepared for activity staining. The gel was placed on the plates after running the native PAGE. In native PAGE, same SDS-PAGE procedure was followed without using SDS in required steps. Also, the protein samples were not boiled before loading them on the gel and the sample buffer solution did not contain 2-mercaptoethanol. The running conditions for native gel was same with the SDS-PAGE.

# CHAPTER 3

# RESULTS AND DISCUSSION

## 3.1. Isolation and Identification of a Lipase Positive Bacterium from Erciyes Soil Sample

A soil sample taken from Erciyes Mountain in Kayseri was examined for the presence of lipase positive strains using a screening method suitable for detection of lipase producers as described in materials and methods. Lipase producer strains in the soil were identified by the formation of orange fluorescent halos around the colonies when olive oil-rhodamine B spread plates incubated at 25°C were exposed to UV light at 350 nm. Olive oil is used as lipase substrate and rhodamine B is the indicator of lipase activitiy. The fluorescence is related to the formation of a rhodamine B-long chain fatty acid conjugate in this method, but the mechanism underlined this situation is not known (Jaeger, et al. 1994). Several bacteria were screened and a strain was selected as the most effective producer of lipase because it formed the largest halo. Selected strain was plated on LB agar alone and LB agar including olive oil and rhodamine B and incubated at temperatures other than 25°C to identify its growth and lipase production temperature range. As a result, it was found that the optimal and highest temperatures for growth of strain were 25°C and 30°C and also it exhibited sigmoidal growth even at +4°C. It was not able to grow at temperatures higher than 30°C. So, it can be concluded that the experimental strain is a psychrotrophic bacterium which is not fully adapted to the cold and have an upper growth limit at temperatures >20°C (Gerday, et al. 2000). Furthermore, the lipase activity was detected only when the strain was grown at temperatures between +4°C and 25 °C (Figure 3.1). No activity could be detected when cells were grown at 30°C. However, its growth and lipase production took longer time with decreasing temperatures.

On solid media, it formed yellowish-white, smooth, mucoid and large colonies. Taxonomical studies on lipase-producing strain showed that the strain is gram-negative, rod-shaped, aerobic, catalase and oxidase-positive. From these results, this strain was classified into the genus of *Pseudomonas* according to Bergey's manual.

To be able to control whether rhodamine B plates works or not, positive control with commercial lipase and negative control with *E.coli* known as lipase-negative (Jaeger, et al. 1999) were also made and their results showed that this lipase screening system works efficiently (Figure 3.2).

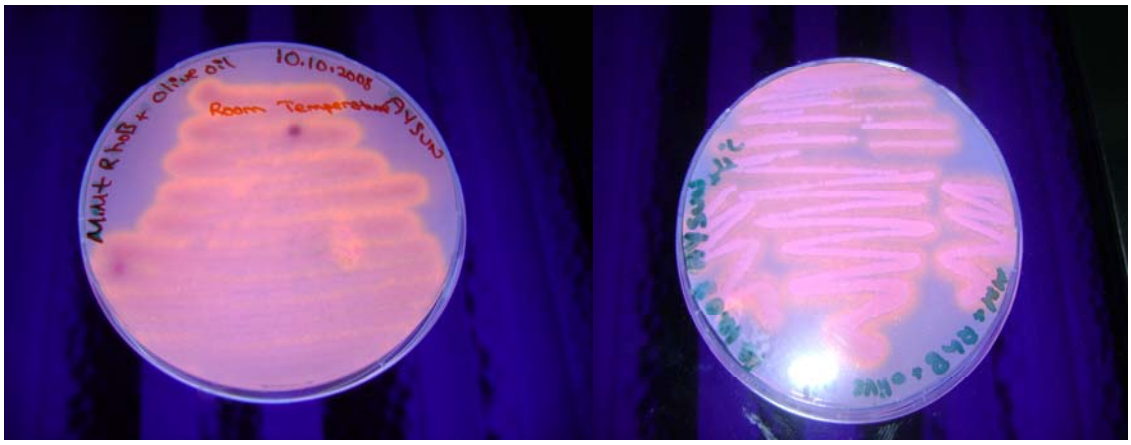

Figure 3.1. Photos of selected lipase positive strain under UV light, grown at 25 °C and 4 °C, respectively

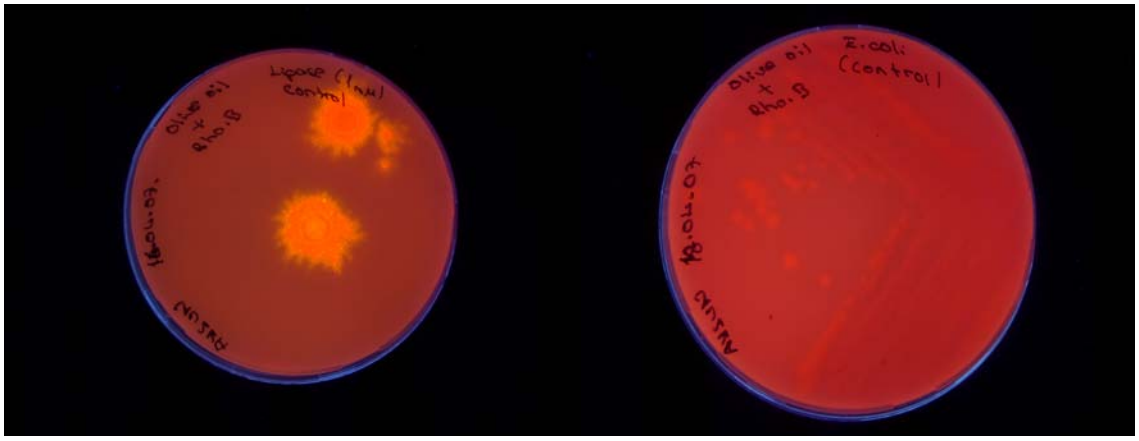

Figure 3.2. Positive and negative controls under UV light

To identify the experimental strain exactly according to 16S rRNA sequence analysis as well as taxonomical studies, genomic DNA of the strain was used as template to amplify partial 16S rRNA using universal bacterial primer pairs, E334 and E1115 (Figure 3.3) and amplified fragments were cloned into pTZ57R/T cloning vector. False-positive clones were eliminated by colony PCR using vector specific primers and the expected length of fragment (~780 bp) was observed in 1% gel electrophoresis

(Figure 3.4). The chosen positive clones were sequenced. Finally, the obtained partial 16S rRNA sequence of this strain was analyzed with BLASTn. It was found to have 95-98% identity with different strains of *Pseudomonas fluorescens.* Among them, it showed high similarity (%98) with *Pseudomonas fluorescens* WR3-08 and *Pseudomonas fluorescens* CTS22. However, no strain identical to isolated strain could be found. So the strain was named as *Pseudomonas* sp. KE38. The sequence of amplified partial region of 16S rRNA gene of identified bacterial strain was given in Appendix A. A phylogenetic tree was also constructed based on the homology of known 16S rRNA sequences (Figure 3.5).

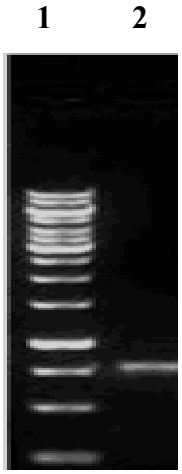


Figure 3.3. Agarose gel analysis of PCR amplified partial 16S rRNA gene region of the experimental strain. Line 1: 1 kb DNA size marker (Fermentas) ( 250, 500, 750, 1000, 1500 bp….). Line 2: ~780 bp PCR product

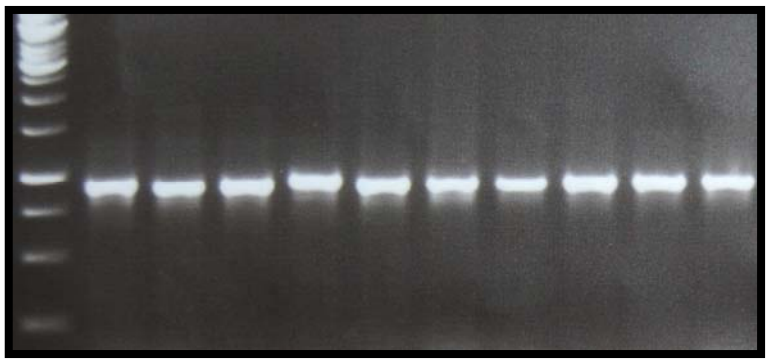

Figure 3.4. Colony PCR result. Line 1: 1 kb DNA size marker (Fermentas). Line 2: ~780 bp insert (16S rRNA gene)

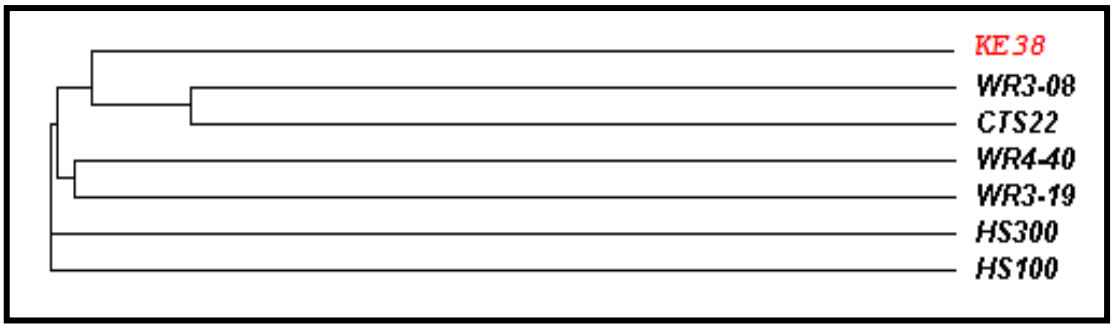


Figure 3.5. The construction of phylogenetic tree based on 16S rRNA analysis (Source: European Bioinformatics Institute Server 2009)

*Pseudomonas* species are commonly found both in soil and water and they have great scientific and technological importance. *Pseudomonas* species have the ability to use various simple and complex organic compounds so they are involved in biodegradation of natural or man-made chemical compounds. Morover; the bacterial genus *Pseudomonas* is the most important producer of extracellular enzymes like lipases (Hasanuzzaman, et al. 2004). As a conclusion, a lipase producing bacterial strain was isolated from Erciyes soil sample and identified as a strain of *Pseudomonas fluorescens*.

## 3.2. Designing an Inducible *E.coli* Expression Vector by PCR-Based Cloning

Strong expression of lipase gene can lead to accumulation of large amounts of lipase in the cytoplasm due to the hydrophobic nature of the enzyme. This situation results in death of host cells since lipase produces toxicity to host cells (Zhang, et al. 2008). So it is so important to control the expression level of lipase in the host cell. In this study, an expression vector, called pUCex, was designed to overcome such difficulties explained above. pUC19 cloning vector was used as starting plasmid to design pUCex by PCR-based cloning (Figure 3.6) as mentioned in material and methods. A specific primer pair, PUC5' and PUC3', was designed to remove 540 bp lacZ structural gene except lac promoter from pUC19 (2686 bp in length). The expected size of the plasmid was, 2175 bp, verified by agarose gel electrophoresis after PCR reaction. ECORV, ECORI and BamHI enzyme sites were also added to ends of resulting plasmid pUCex with this primer pair.

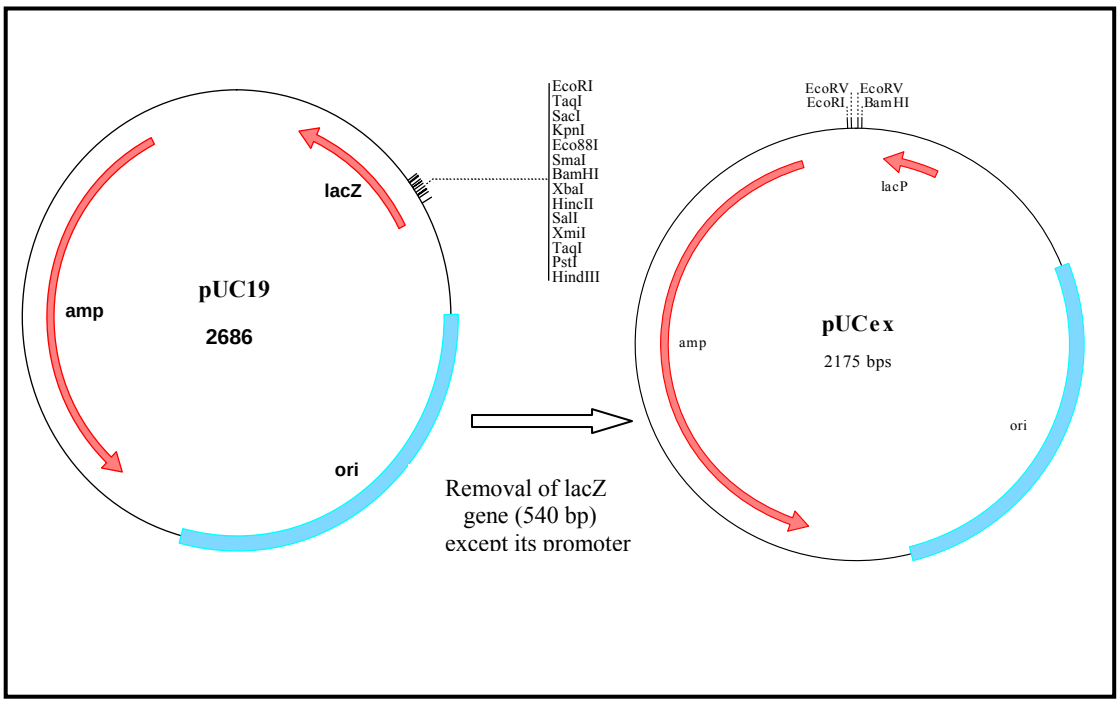


Figure 3.6. Construction of pUCex by PCR-based cloning

After making the amplified fragment blunt-ended and self-circulated, *E.coli* Dh5α cells transformed with the ligation mixture. Colonies grown on LB agar with ampicillin were checked by colony PCR using M13R and PUC5' primers. This primer pair was used to understand whether obtained colonies were really the new plasmid or not because PCR with this primer pair amplifies both pUCex and pUC19. However, PCRs including these two plasmids as template gave bands with different sizes, 2175 bp for the new plasmid and 2686 bp for pUC19. According to the colony PCR result, only one colony was positive. The plasmid isolated from this colony was also verified by digesting it with TaqI, ECORV and Cfr113I enzymes. The restriction profile of pUCex was compared with pUC19 digested with the same enzymes and the newly designed plasmid was the expected one according to the restriction profile.

This plasmid is an IPTG inducible plasmid due to the presence of lac promoter so expression level of a gene cloned into it can be controlled via increasing or decreasing amount of IPTG. To contol whether pUCex express any gene or not, tetracyline gene from pBR322 was cloned into ECORI/BamHI site of it as mentioned in material and methods and expression was observed on LB plates with IPTG and tetracyline since the transformants were cabaple of growing in the presence of tetracyline. It means that the pUCex expressed tetracyline gene successfully. Growing

colonies on tetracyline plates were also checked by colony PCR in the means of insert presence (Figure 3.7) and all selected colonies contained the tetracyline gene. Also, there was little or no expression in the absence of IPTG on tetracyline plates. It also showed that pUCex was an IPTG inducible expression vector.

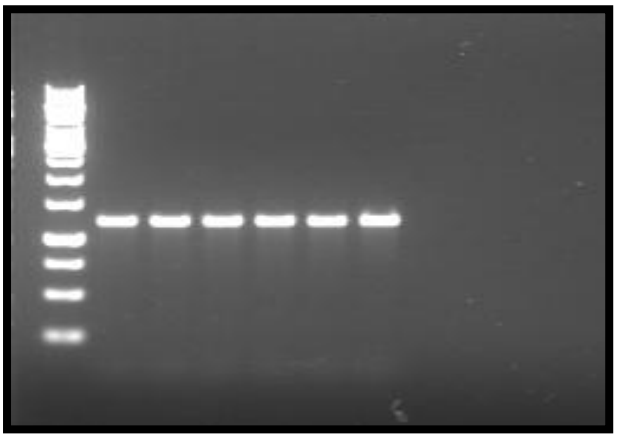

Figure 3.7. Control the presence of tetracyline gene cloned into pUCex by colony PCR. Line 1: 1 kb DNA size marker (Fermentas) Line 2: The tetracyline gene

## 3.3. Cloning and Sequence Analysis of a Partial Lipase Gene from a Psychrotrophic *Pseudomonas* sp. KE38

A pair of degenerated oligonucleotide primers (LIPPFF and LIPPRR) was carefully designed based on the higly conserved regions in the 23 different *Pseudomonas fluorescens* lipases which were chosen according to the tree (B part) constructed using lipases from 53 different *Pseudomonas* strains (Figure 3.8). This tree was constructed to be able to group and differentiate these lipases. Especially, *P. fluorescens* lipases were selected because 16S rRNA sequence of the strain showed high homology (%98) with *Pseudomonas fluorescens* WR3-08 and *Pseudomonas fluorescens* CTS22. Most of the lipases from *P. fluorescens* strains belong to group 3 lipases. Therefore, the strain has been suggested as a member of *Pseudomonas* group 3 lipase. Detailed information about group 3 lipases was given in section 1.5. The conserved regions were identified as a result of aligment procedure using ClustalW (Figure 3.9).

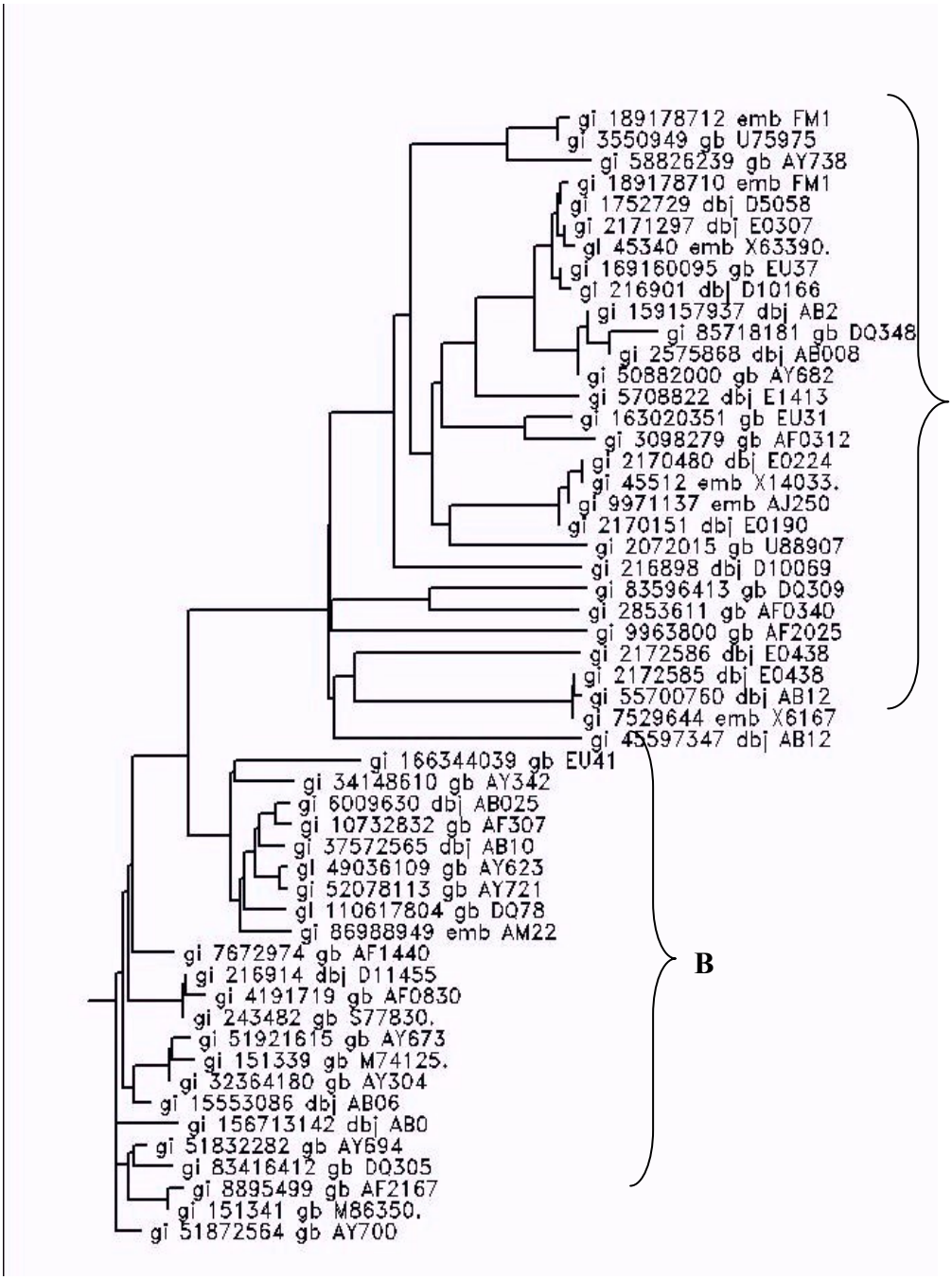


Figure 3.8. Phylogenetic tree predicted from sequence aligment of lipases from 53 different *Pseudomonas* strains.**A**: Lipases from *P. aeruginosa, P. alcaligenes P. fragi* and *P. wisconsinensis* **B**: Lipases from *P.fluorescens* strains. Lipases were represented with their accession numbers

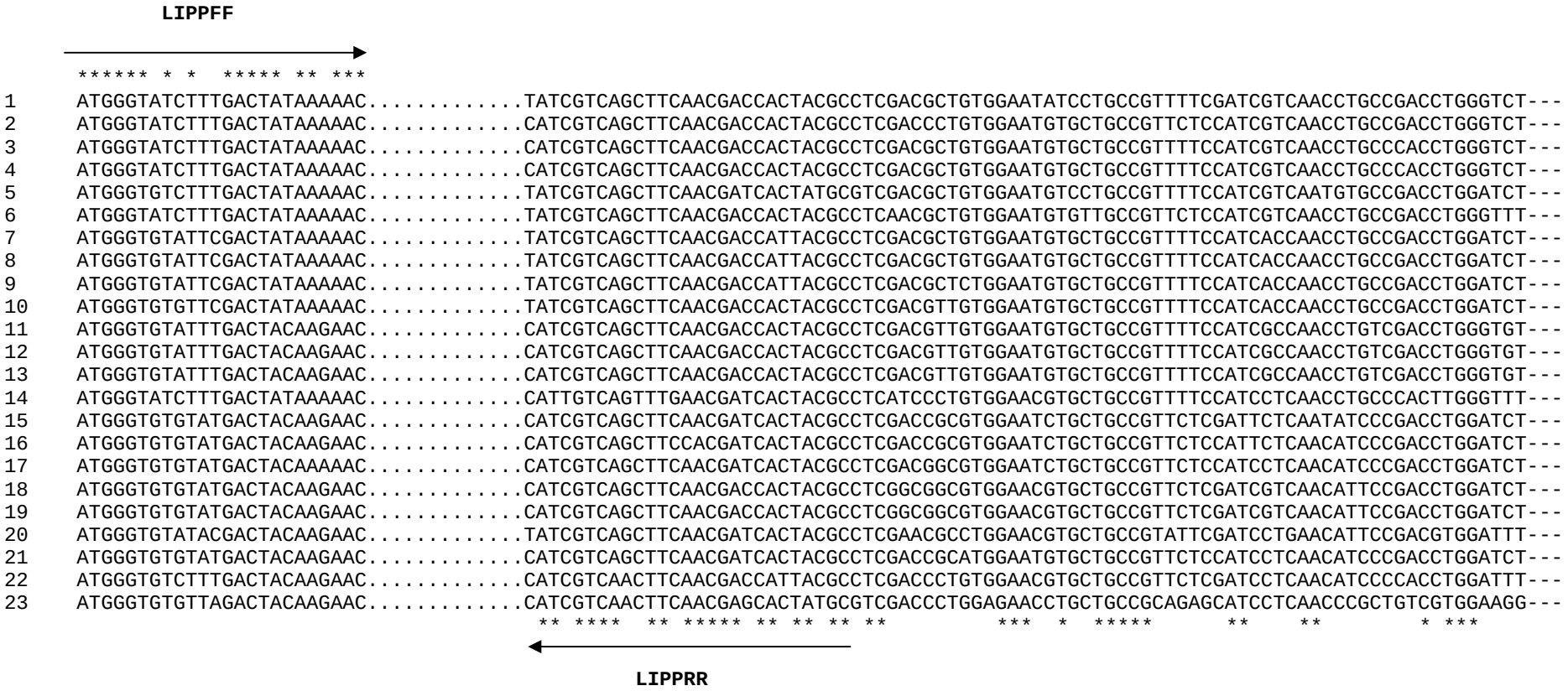


Figure 3.9. Design the degenerate primers LIPPFF and LIPPRR by multiple aligment of 23 different *Pseudomonas fluorescens* lipases.

**1.** *P.fluorescens* lipase (lipB68), **2**. *P.fluorescens* lipase (lip), **3**. *P.fluorescens* PrtB (prtB) and lipase (lipA), **4**. *P.fluorescens* lipase (M86350), **5**. *P.fluorescens* gene for lipase (AB009011), **6**. Uncultured *Pseudomonas* sp. lipase (lipJ03), **7.** Uncultured *Pseudomonas* sp. Lipase (lipJ02), **8**. *Pseudomonas* sp. Lipase (M74125), **9**. *P.fluorescens* lipase gene (AY304500), **10**. *Pseudomonas* sp. KB700A KB-lip gene for lipase, **11**. *P.fluorescens* gene for triacylglycerol lipase, **12**. *P.fluorescens*,W1, lipase, **13**. *P.fluorescens* PrtA, protease inhibitor Inh, ABC transporter TliD (tliD), ABC transporter TliE (tliE), ABC transporter TliF (tliF), and thermostable lipase TliA (tliA) genes, **14**. *P.fluorescens* polyurethanase lipase A, **15.** *Pseudomonas* sp. MIS38 gene for lipase, **16.** *P.fluorescens* lipase (lipB) gene **17**. *P.fluorescens* lipA gene for lipase, **18**. *P. fluorescens* lipase (lipB52) gene, **19**. *P.fluorescens* lipase (lipB41) gene, **20**. *P.fluorescens* strain 26-2 lipase class 3 gene, **21**. *Pseudomonas* sp. 7323 lipase gene, **22**. *Pseudomonas* sp. JZ-2003 lipase gene, **23**. *Pseudomonas* sp. lip35 lipase

Forward primer including initiation codon ATG was designed according to the relative high similarity at N-termination. Using these primers, a band of about 879 bp length was amplified from the chromosomal DNA of *Pseudomonas sp*. KE38 (Figure 3.10). Temperature gradient (45°C/55°C) PCR was performed due to the degeneracy of the primer pair. As is seen in Figure 3.9, the primer pair amplified the target sequence perfectly (there was no non-specific binding) at all temperatures between 45°C and 55°C. The amplified fragment was cloned into pTZ57R/T prior to sequencing. After sequencing with plasmid-specific M13F and M13R primers, obtained sequence was examined and sequence aligment by BLAST confirmed that the cloned 879 nucleotide fragment belonged to a lipase gene. The partial sequence of cloned fragment was given in Appendix A.

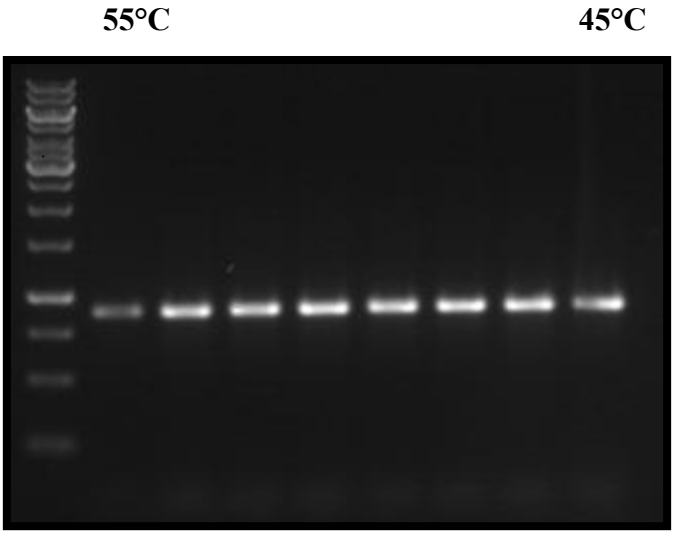


Figure 3.10. The partial lipase fragment amplified by temperature gradient PCR using degenarate primers, LIPPFF and LIPPRR.

A search for homology in protein and gene banks (UniProtKB for proteins and GenBank/NCBI for genes) indicated that the partial nucleotide and aminoacid sequence of lipase had high similarity with lipases classified in group 3 lipases as suggested before. This partial lipase was % 92 identical to *Pseudomonas* sp. 7323 lipase, % 90 identical to both *Pseudomonas* sp. MIS38 lipase and *Pseudomonas* sp. UB48 lipase, % 88 identical to *P. fluorescens* PfO-1 lipase, and % 79 identical to *Pseudomonas* sp. KB700A lipase (Figure 3.11 and Figure 3.12). The deduced aminoacid sequence contained a lipase consensus sequence, GHSLGG, which is most conserved in

*Pseudomonas* group 3 lipases (Arpigny and Jaeger 1999). The GXSXG sequence with the active site serine residue was found from residues 205 to 209. The localization of the active-site residues of group 3 lipases are $Ser^{207}$, $Asp^{255}$ and $His^{313}$. These residues are fully conserved and they form a catalytic triad (Angkawidjaja and Kanaya 2006). It was found that the partial lipase contained $Ser^{207}$ and $Asp^{255}$. Furthermore, cysteine residues were not found in this partial sequence so disulfide bond formation does not occur. This is the other most important property of group 3 lipases.

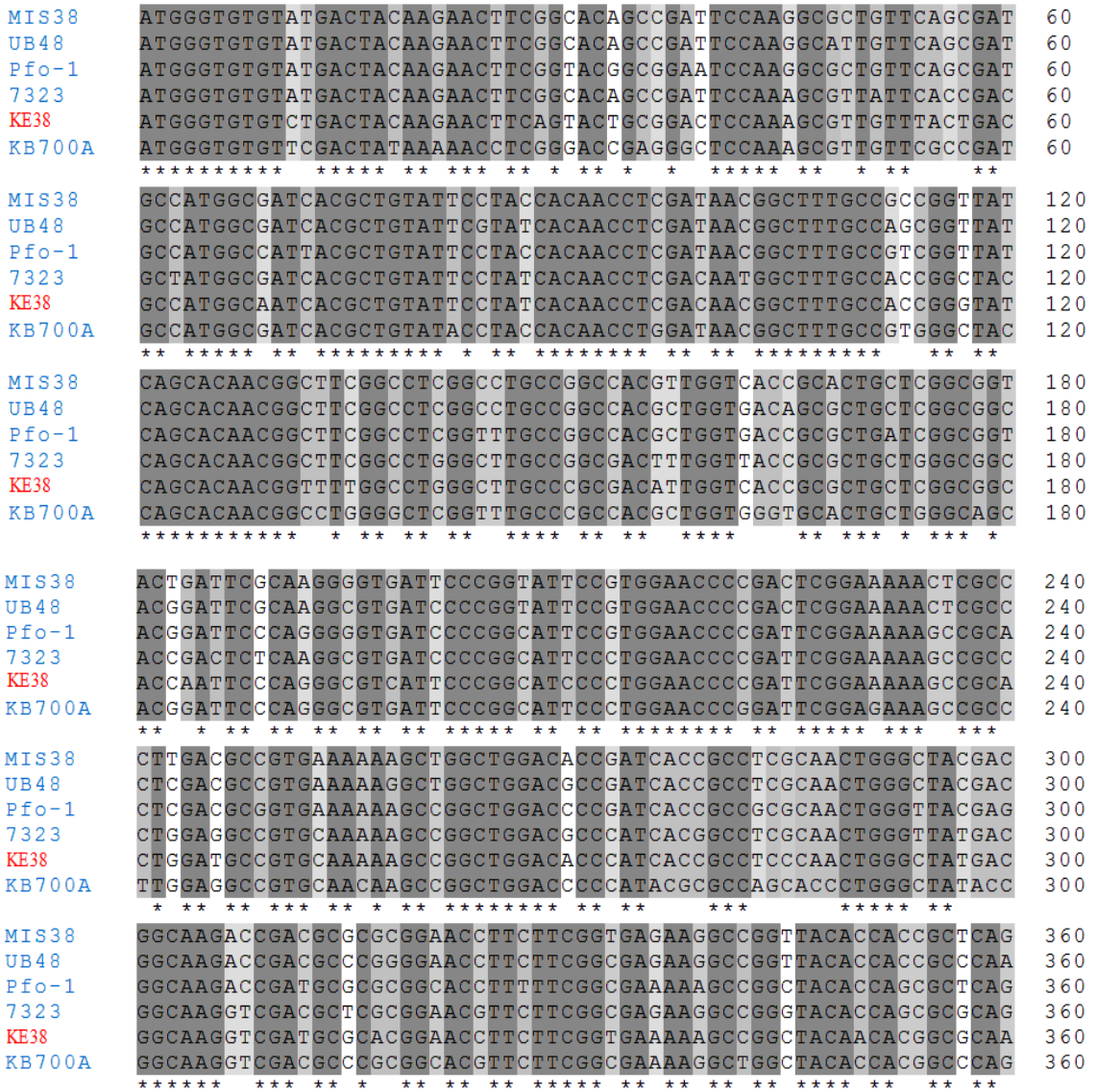


Figure 3.11. Multiple alignment of *Pseudomonas* sp. KE38 partial lipase gene sequence with other lipase sequences of *Pseudomonas fluorescens* strains.

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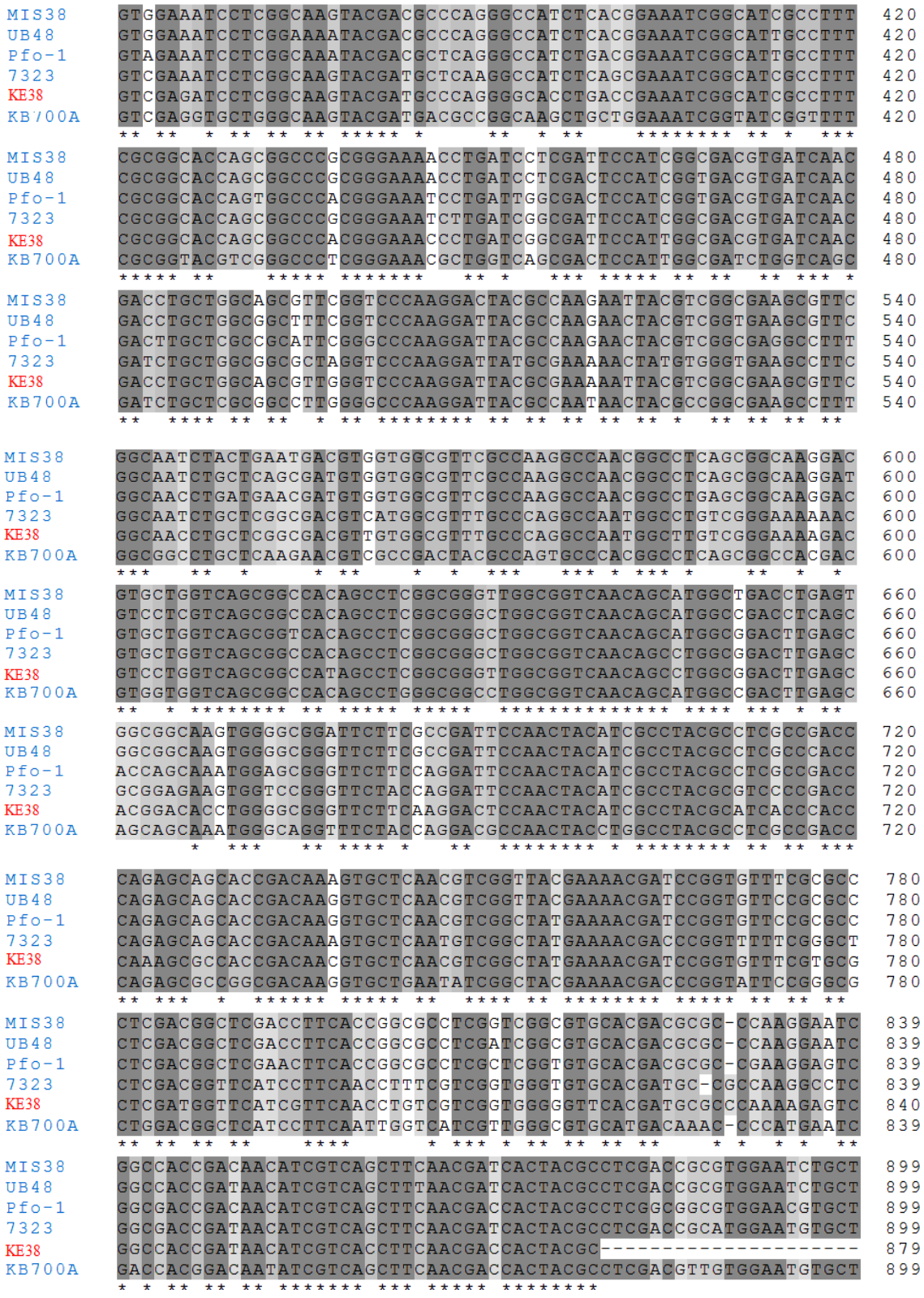



Figure 3.11. (cont.)

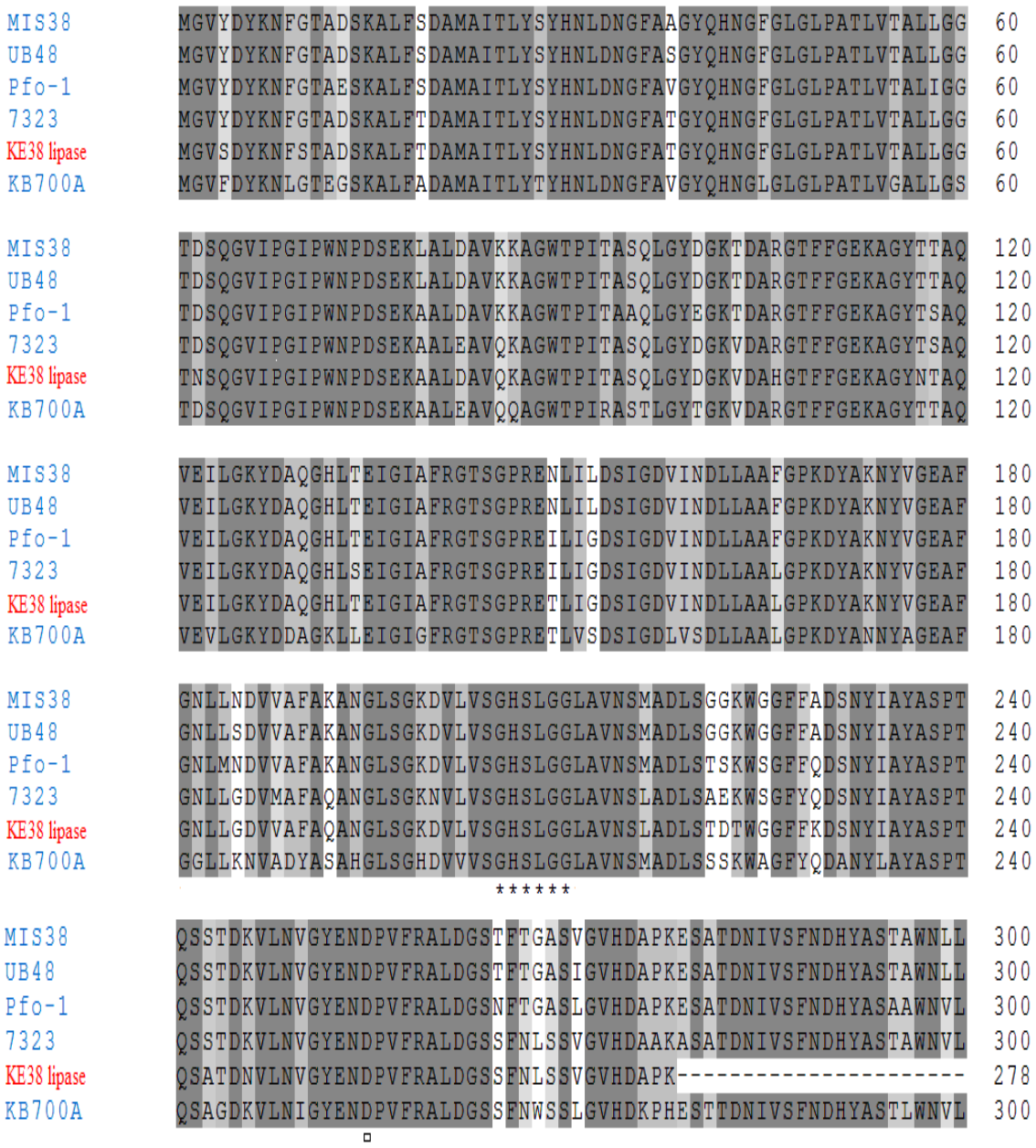



Figure 3.12. Multiple alignment of *Pseudomonas* sp. KE38 lipase with other lipases of *Pseudomonas fluorescens* strains. (Asterisks indicates lipase consenseus sequence, G-X-S-X-G. ▫ indicates $Asp^{255}$, catalytic acid residue)

Lipases show low homology at their C- termination so it is very difficult to obtain the full sequence of a lipase gene by performing PCR (Chai-hong, et al. 2008). Inverse PCR was selected to amplifiy C-termination in this study. Through the restriction analysis of partial lipase fragment, PstI, HindIII and SmaI enzymes were chosen to digest genomic DNA, but only PstI could amplify a fragment with desired size using only lip7/lip8 inverse PCR primer. This fragment was cloned and sequenced. The sequencing result showed that the fragment did not contain any sequence related to

lipase gene. Therefore, the rest of the lipase gene was not obtained. Native lipase enzyme purification was performed to find the rest of the protein by mass spectrometric approaches like MALDI analysis.

## 3.4. Partial Purification and Characterization of *Pseudomonas* sp. KE38 lipase

Minimal medium with %1 olive oil was selected as lipase production media. This medium contained only some minerals and inorganic nitrogen source. The strain was enforced to produce lipase by using only olive oil as sole carbon source. To find the optimum time for lipase production, *Pseudomonas* sp. KE38 was grown at 25°C for 1 to 5 days and supernatant for each day was evaluated in the means of lipase activity using spectrophotometric lipase assay as described in materials and methods. The results showed that higher lipase activity (1.64 units/ml) exhibited at incubation times of 4 days (Figure 3.13) and decreased after 5 days. Also, the lipase activity was found to increase along with the cell growth (Table 3.1)

Table 3.1. Optimization of lipase production time

| Incubation Days | Growth ($OD_{600}$) (1:100 dilution) | Activity (units/ml) |
|---|---|---|
| 1 | 0.0785 | 0.4 |
| 2 | 0.1301 | 0.78 |
| 3 | 0.2130 | 1.32 |
| 4 | 0.2405 | 1.64 |
| 5 | 0.2701 | 1.4 |

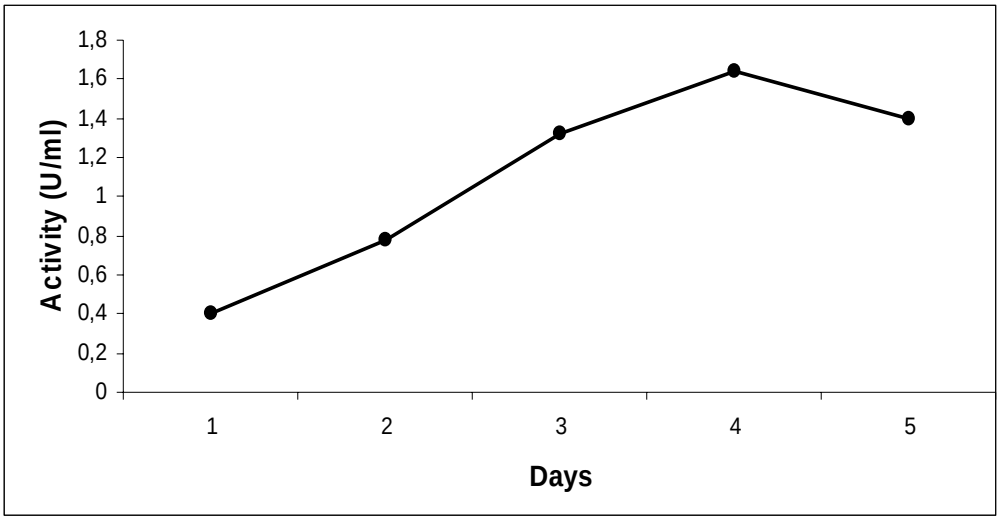



Figure 3.13. Time course of lipase production of *Pseudomonas* sp. KE38 in an olive oil containing medium.

After the determination of optimum incubation time, the effects of glucose and olive oil on both growth and lipase production were examined. It has been shown that the presence of lipids or fatty acids in the growth medium induce extracellular lipase production strongly (Kiran, et al. 2008). In this study, olive oil was found to induce lipase production significantly and to support good growth (Table 3.2). In the presence of other carbon sources like glucose, there was a reduction in lipase production. This could be due to catabolite repression by easily usable carbon sources in the medium (Kiran, et al. 2008). As is seen in table 3.12, the presence of glucose in the medium caused the great repression although it supported growth. Furthermore, to investigate the induction effect of the lipid-related substrates on lipase production, mixed carbon source (glucose+olive oil) medium was used. This kind of medium gives the bacterium the possibility to use both substrates in a sequential or simultaneous way based on its metabolism. Table 3.2 showed that the use of glucose and olive oil together did not improve the result obtained by using only olive oil as lipid and carbon source. Although the cells was grown perfectly, there was no increase in activity. This situation could be due to the preferential usage of glucose to olive oil or a relation between extracellular lipase production and lipids utilization. It was suggested that olive oil was not consumed completely during 4 days incubation period.

Table 3.2. Effect of glucose and olive oil on growth and enzyme production

| Medium type | Cell growth ($OD_{600}$) 1:100 dilution | Activity (units/ml) |
|---|---|---|
| M.M+glucose | 0.0810 | 0.473 |
| M.M+olive oil | 0.2405 | 6.2 |
| M.M+glucose+olive oil | 0.1713 | 1.01 |
| M.M as negative control | No growth | No activity |

*Ammonium sulfate precipitation was applied to cell-free extract to measure lipase activity.

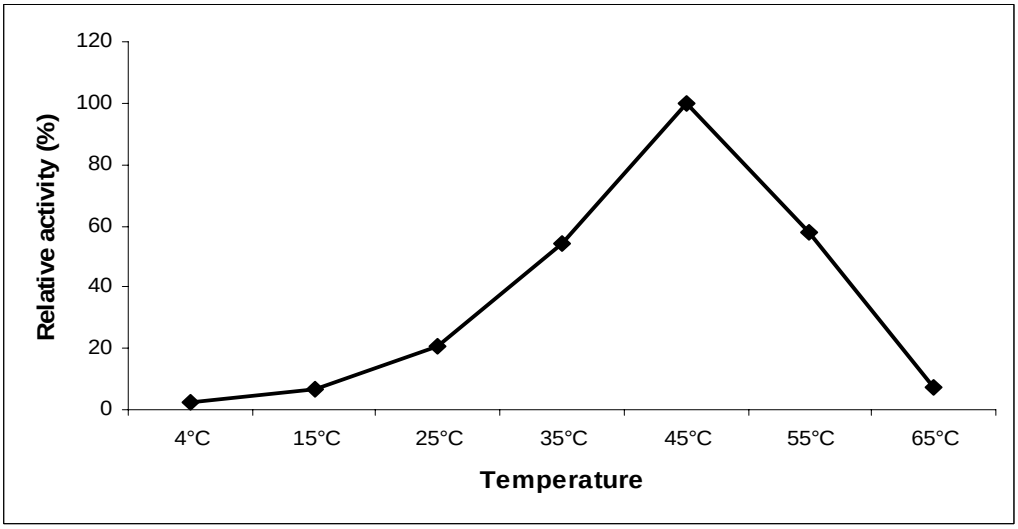
Lipase from *Pseudomonas* sp. KE38 grown in minimal medium with %1 olive oil for 4 days was partially purified by ammonium sulfate precipitation and then Sephadex G-100 size-exclusion chromatography. Lipase assay was applied to 84 fractions and only 7 fractions was found to contain lipase enzyme. These 7 fractions were combined and used for enzyme characterization.

### 3.4.1. Effect of Temperature and pH on Lipase Activity

The effect of temperature (a) and pH (b) on enzyme activity was indicated in Figure 3.13. The lipase showed activity in the temperature range of 25-45°C and the maximum activity was observed at 45°C. At higher temperature (55°C) the enzyme lost % 41.8 of the maximum activity. The lipase lost its activity rapidly above 55°C with only % 7.4 of the remaining activity at 65°C. *Pseudomonas* sp. KE38 used in this study grows optimum at 25°C, but its lipase has maximum activity at 45°C. So, it was the another evidence to classify KE38 lipase into group 3 lipases. Although group 3 lipases are generally produced from mesophilic or psychrotrophic bacteria, they have optimum temperature for activity in the mesophilic or thermophilic range (Angkawidjaja and Kanaya 2006). The effect of pH on enzyme activity was examined at different pH values ( pH 4 to 12) at 45°C. The lipase exhibited activity in the pH range of 7.0-9.0, and the optimum activity was observed at pH 8. The lipase retained % 65.11 of its maximum activity at pH 9.0 and pH 10.0 the enzyme lost % 66.86 of its maxiumum activity. In the acidic range, significant reduction in enzyme activity was observed. For instance, at pH 6.0 the lipase retained only % 6.48 of its maximum activity. No activity was observed at pH 4 and pH 5.

Also, lipase activity showed a linear increase only within 40 minutes at higher alkaline pH values (pH 11 and pH 12) and then activity decreased rapidly. So, activity was calculated according to the increase in 40 minutes. It was speculated that the active site of the lipase enzyme could be ionized irreversibly at too alkaline and acidic environment.

**a)**




**b)**

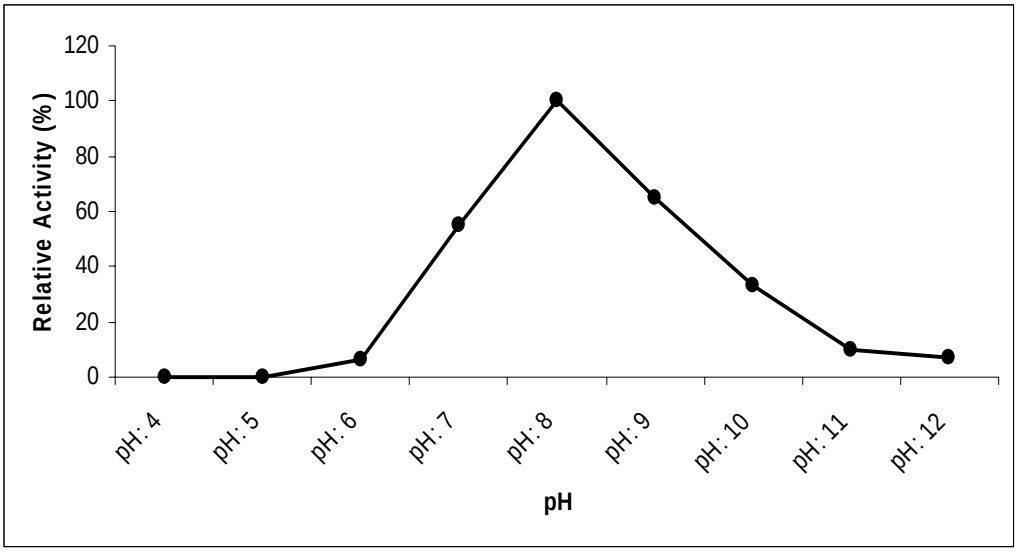



Figure 3.14. Effect of temperature a) and pH b) on enzyme activity based on relative enzyme activity values. The maximum activity of the enzyme was taken as %100

### 3.4.2. Determination of Molecular Mass

As shown in figure 3.14, the purified lipase from the strain KE38 was seemed to be homogeneous on SDS-PAGE gel and its molecular weight was estimated to be approximately 43 kDa based on the position of the protein marker. Group 3 *Pseudomonas* lipases are the largest ones compared with other two groups. So, KE38 lipase with 43 kDa molecular weight agreed with this knowledge.

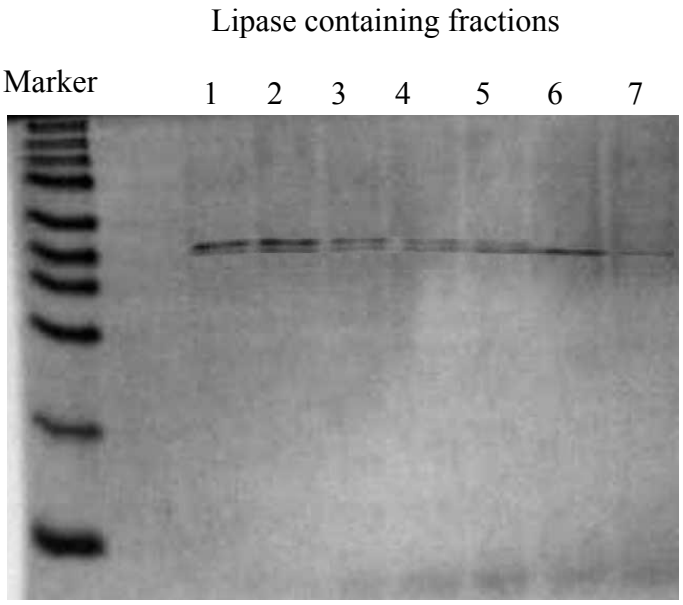



Figure 3.15. SDS-PAGE of partially purified lipase enzyme from *Pseudomonas* sp. KE38. Line 1: Protein size marker (Fermentas) ( 260, 140, 100, 70, 50, 40, 35, 25, 15, 10 kDa)

Native-PAGE was performed as well as SDS-PAGE. In native-PAGE, it was observed that a major part of the partially purified lipase failed to penetrate into the resolving gel. So, it was speculated that the lipase could have a strong tendency to aggregate due to its hydrophobic nature.

# CHAPTER 4

# CONCLUSIONS

Lipases are classified into serine hydrolases and they catalyze both the hydrolysis and synthesis of long-chain triacylglycerols. Extracellular lipases are produced by microorganisms, fungi and bacteria. Lipases from bacteria have been paid much attention due to their common usage in a variety of biotechnological applications. *Pseudomonas* lipases are the most important ones which have a significant potential in detergent industry, organic chemistry and so on.

In this study, we screened lipase positive bacterial strains from soil samples from Erciyes mountain in Kayseri using a screening system (rhodamine B-olive oil plates) suitable for the detection of lipase producers. We selected a bacterial strain among others based on the formation of orange-fluorescent halos with highest intensity around its colonies. In order to identify this bacterium, some taxonomical studies and 16S rRNA gene analysis were performed. As a result, it was identified as a strain of *Pseudomonas fluorescens* and no strain identical to our strain was found. So, we called it as *Pseudomonas* sp. KE38.

To be able to find the nucleotide sequence of the lipase gene from KE38, we carefully designed a pair of degenarate primer based on the higly conserved regions in the selected 23 different *Pseudomonas fluorescens* lipases. A fragment with 879 bp lenght was obtained by using this primers and this fragment was found to belong to a lipase gene based on BLAST analysis. According to the results of gene and aminoacid homology search, the lipase from KE38 has been found as a member of group 3 *Pseudomonas* lipases because of several facts: a) it had high similarity with lipases classified in group 3 lipases (it was % 92 identical to *Pseudomonas* sp. 7323 lipase, % 90 identical to both *Pseudomonas* sp. MIS38 lipase and *Pseudomonas* sp. UB48 lipase, % 88 identical to *P. fluorescens* PfO-1 lipase, and % 79 identical to *Pseudomonas* sp. KB700A lipase), b) the deduced partial aminoacid sequence contained a lipase consensus sequence, GHSLGG, which is most conserved in *Pseudomonas* group 3 lipases, c) the partial lipase contained $Ser^{207}$ and $Asp^{255}$, which are found to be conserved in *Pseudomonas* group 3 lipases, d) the partial lipase did not contain cysteine

residues to form disulfide bonds, another important feature of group 3 lipases (Angkawidjaja and Kanaya 2006). Several inverse PCR attempts were performed to find the complete sequence of the KE38 lipase, but the rest of the gene could not be obtained.

Furthermore, we determined optimum lipase production time and monitored the effect of glucose, olive oil and their combination on growth and lipase production. The maximum lipase production was observed at incubation times of 4 days and lipase activity increased along with the cell growth. Olive oil supported good growth and increased lipase activity significantly. So, olive oil was found to act as an inducer of lipase production for our strain. This result agreed with the knowledge in the literature in which lipase production has been shown to be induced remarkably in the presence of lipidic carbon sources like oils, fatty acids etc. (Kiran, et al. 2008). On the other hand, glucose was found to act as a repressor of lipase production because it caused a great repression although it supported growth. This was also an expected result based on the literature. In the precence of other carbon sources like carbohydrates, lipase production could decrease due to the catabolite repression (Dalmau, et al. 2000). We also investigated the inducer effect of olive using both glucose and olive oil in the same medium. The presence of two carbon sources supported good growth, but did not improve lipase production compared with olive oil containing media. We speculated that our strain used glucose as primary carbon source and started to use olive oil after the complete consumption of glucose. So, the lipase production was directly related to the lipid utilization. After determining optimum lipase production time and carbon source, native lipase enyzme was partially purified by ammonium sulfate precipitation and then Sephadex G-100 size-exclusion chromatography. Lipase containing 7 fractions were used for enzyme characterization.

The optimum temperature and pH for lipase activity were 45°C and pH 8. At temperatures above 45°C, enzyme losts its activity rapidly. At acidic pH values, no or little activity was observed. From these results, it is said that the lipase from strain KE38 was an alkaline enzyme which can be used in several industrial areas after more purification steps. Its molecular weight was estimated about 43 kDa by SDS-PAGE.

Mass spectrometry can be used to identify the rest of the amino acid sequence of enzyme. For this purpose, the bands were cut from SDS gel and digested with tripsin. The resulting peptides will be analyzed via MALDI analysis as a further study. At present, it is not known that the purified enzyme is the same one with the lipase whose

partial gene sequence has been found. After MALDI analysis, we will make a comment about this situation.

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# APPENDIX A

# PARTIAL SEQUENCES OF 16S rRNA AND LIPASE GENES OF IDENTIFIED BACTERIAL STRAIN

## *Pseudomonas sp.* KE38 16S ribosomal RNA gene

CCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAG
CCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGT
TGTAACCTAATACGTTGCAATCTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGC
CAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCG
CGTAGGTGGTTCGTTAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCAAA
ACTGACGAGCTAGAGTATGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTA
GATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTG
CGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCA
ACTAGCCGTTGGGAGCCTTGAGCTCTTAGTGGCGCAGCTAACGCATTAAGTTGACCGCCTGG
GGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAG
CATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGCCTTGACATCCAATGAACTTT
CCAGAGATGGATTGGTGCCTTCGGGAACATTGAGACAGGTGCTGCATGGCTGTCGTCAGCTC
GTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCT

## Partial sequence of lipase gene from *Pseudomonas* sp. KE38

ATGGGTGTGTCTGACTACAAGAACTTCAGTACTGCGGACTCCAAAGCGTTGTTTACTGACGC
CATGGCAATCACGCTGTATTCCTATCACAACCTCGACAACGGCTTTGCCACCGGGTATCAGC
ACAACGGTTTTGGCCTGGGCTTGCCCGCGACATTGGTCACCGCGCTGCTCGGCGGCACCAAT
TCCCAGGGCGTCATTCCCGGCATCCCCTGGAACCCCGATTCGGAAAAAGCCGCACTGGATGC
CGTGCAAAAAGCCGGCTGGACACCCATCACCGCCTCCCAACTGGGCTATGACGGCAAGGTC
GATGCGCACGGAACCTTCTTCGGTGAAAAAGCCGGCTACAACACGGCGCAAGTCGAGATCC
TCGGCAAGTACGATGCCCAGGGGCACCTGACCGAAATCGGCATCGCCTTTCGCGGCACCAG
CGGCCCACGGGAAACCCTGATCGGCGATTCCATTGGCGACGTGATCAACGACCTGCTGGCA
GCGTTGGGTCCCAAGGATTACGCGAAAAATTACGTCGGCGAAGCGTTCGGCAACCTGCTCG
GCGACGTTGTGGCGTTTGCCCAGGCCAATGGCTTGTCGGGAAAAGACGTCCTGGTCAGCGGC
CATAGCCTCGGCGGGTTGGCGGTCAACAGCCTGGCGGACTTGAGCACGGACACCTGGGGCG
GGTTCTTCAAGGACTCCAACTACATCGCCTACGCATCACCCACCCAAAGCGCCACCGACAAC
GTGCTCAACGTCGGCTATGAAAACGATCCGGTGTTTCGTGCGCTCGATGGTTCATCGTTCAA
CCTGTCGTCGGTGGGGGTTCACGATGCGCCCAAAAGAGTCGGCCACCGATAACATCGTCACC
TTCAACGACCACTACGC

# APPENDIX B

# THE SEQUENCES AND POSITIONS OF INVERSE PCR PRIMERS ON PARTIAL LIPASE FRAGMENT

Table B.1. The sequences of inverse PCR primers

| Primers | Sequences (5' → 3') |
|---|---|
| lip3 | CAACGCTTTGGAGTCCGC |
| lip4 | CCAAAAGAGTCGGCCACCG |
| lip5 | CCAATGGCTTGTCGGGAAAAG |
| lip6 | CAGTTGGGAGGCGGTGATGGG |
| lip7 | ACGTGATCAACGACCTGCTGG |
| lip8 | CTTGCGCCGTGTTGTAGCCGG |

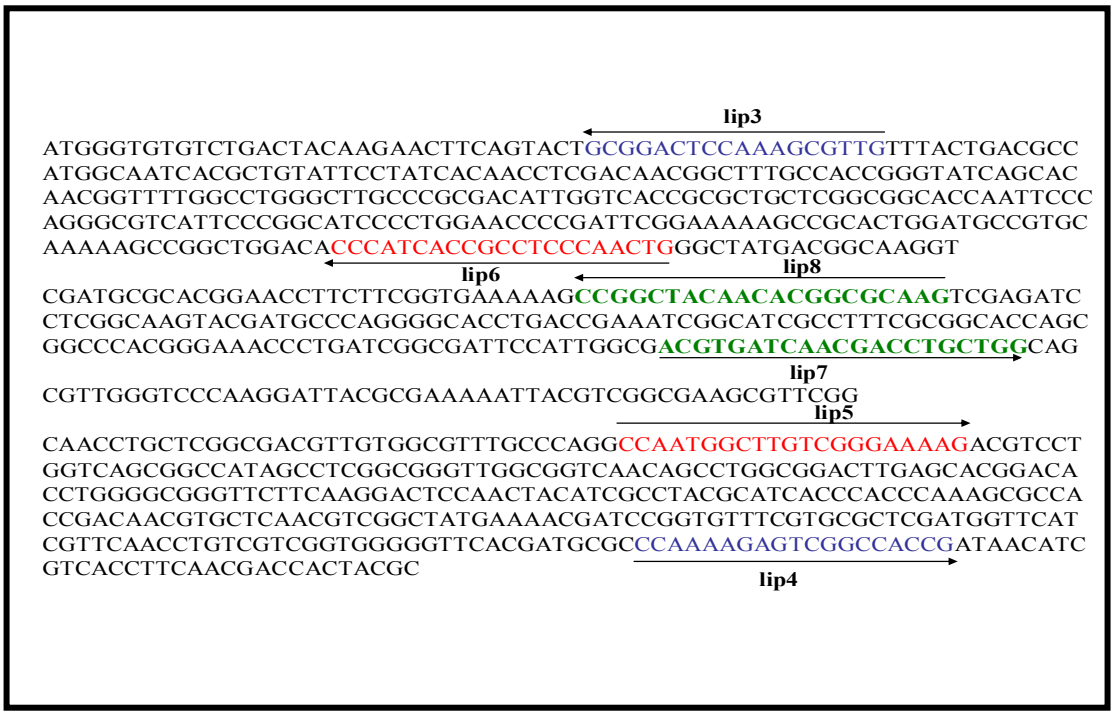


Figure B.1. Positions of inverse PCR primers on partial lipase fragment

# APPENDIX C

# BUFFERS AND STOCK SOLUTIONS

## Luria Bertani (LB) broth, per liter

10 g tryptone, 5 g yeast extract, 5 g NaCl and $dH_2O$ up to 1 L.

## Modified 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.

## Amphicilin (100 mg/ml)

0.1 g amphicilin in 1 ml dH2O

## X-GAL Stock Solution (50 mg/ml)

0.1 g x-gal in 2 ml N-N di-methyl formimide.

## IPTG Stock Solution (0.1 M)

1.2 g IPTG was dissolved in 50 ml of deionized water

## Tetracyline (12.5 mg/ml)

0.0125 g tetracyline in 1 ml $dH_2O$ and keep away from light.

## CTAB/NACI

Dissolve 4.1 g NaCl in 80 ml of water and slowly add 10 g CTAB while heating (≈65°C) and stirring. This takes more than 3 hrs to dissolve CTAB. Adjust final volume to 100 ml and sterilize by filter or autoclave.

## 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 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 to 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 to7

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

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

## Glycine-NaOH buffer (50 mM), pH 9 to 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.