CLONING AND EXPRESSION OF THE *Pseudomonas* KE38 EXTRA-CELLULAR LIPASE GENE IN *E. coli*

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ABSTRACT

CLONING AND EXPRESSION OF THE *Pseudomonas* KE38 EXTRA-CELLULAR LIPASE GENE IN *E.coli*

Lipases are serine hydrolases that catalyze both the hydrolysis and synthesis of insoluble or poorly soluble long-chain triacylglycerols with an acyl chain length ≥ 10 carbon atoms based on the presence or absence of water. Lipases are produced and secreted by all kingdoms of life that are eukaryotes including plants, animals, fungi and prokaryotes including bacteria and archaea. However, microbial lipases, especially from bacteria, more useful than their plant and animal derivatives because of several important properties. Because of their acitivities in both aqueous and nonaqueous environments, lipases have specific applications in industry and medicine.

The primary goals of this thesis were to clone and express the extra-cellular lipase gene from *Pseudomonas sp.* KE38, isolated from soil samples of Erciyes mountain in Kayseri, in *E. coli* and partial purification of the gene product. To achieve this aim, genome walking technique was used to obtain lipase gene from *Pseudomonas sp.* KE38, that gene was then cloned into pET28a expression vector and expressed in *E. coli*. The lipase expression of *E. coli* BL21 and its activity was screened with olive oil-Rhodamin B plate assay. After expression recombinant lipase was partially purified via inclusion body isolation. Moreover the optimum lipase production time of *E. coli* BL21 cells were determined and analyzed with SDS-PAGE. According to SDS-PAGE analysis the recombinant lipase was approximately 64 kDa and lipase production reached to the highest level after two hours of IPTG induction.

As conclusion, recombinant lipase from *Pseudomonas sp.* KE38 was cloned into *E. coli*, expressed and partially purified.

ÖZET

Pseudomonas KE38 HÜCRE DIŞI LİPAZ GENİNİN KLONLANMASI VE *E. coli* DE İFADELENMESİ

Lipazlar uzun zincirli triaçilgliserollerin hem hidroliz hemde sentezlerini katalizleyen serin hidrolazlardır ve endüstrideki yaygın kullanımları nedeniyle büyük öneme sahiptirler. Lipazlar yaşayan tüm organizmalar, yani mikrooraganizmalar (bakteri ve mantarlar), bitkiler ve hayvanlar tarafından üretilmektedirler. Ancak, mikrobiyal lipazlar özellikle bakteri kaynaklı olanlar, birçok önemli ve ayırt edici özelliklerinden dolayı bitki ve hayvan kaynaklı olanlara nazaran daha kullanışlıdırlar. Lipazlar su bulunan ve bulunmayan ortamlarda aktivite gösterebilirler ve bu sebeple biyoteknolojideki kullanım alanları oldukça geniştir.

Bu çalışmanın amacı Kayseri Erciyes dağının toprak örneğinden izole edilerek *Pseudomonas sp.* KE38 olarak adlandırılan bakteriden lipaz geninin elde edilmesi, *E. coli* bakterisine klonlanması ve yine aynı bakteride rekombinant olarak üretildikten sonra *E. coli* BL21 bakterisinin en uygun lipaz üretim zamanı belirlenerek recombinant lipazın kısmi saflaştırılmasının yapılmasıdır. SDS-PAGE sonucuna göre rekombinant lipazın molekül ağırlığı yaklaşık olarak 64 kDa olarak belirlenmiş ayrıca *E. coli* BL21 IPTG ile indüklendikten iki saat sonra lipaz üretimi en üst seviyeye ulaşarak devam eden sekiz saat boyunca herhangi bir değişim gözlemlenmemiştir. Sonuç olarak çalışmada amaç olan *Pseudomonas sp.* KE38 bakterisinden izole edilen lipaz geninin klonlanması, *E. coli'*de ifadelenmesi ve kısmi saflaştırılması gerçekleştirilmiştir.

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ABBREVIATIONS

LB	Luria-Bertani
UV	Ultraviolet
rpm	Revolutions per minute
PCR	Polymerase chain reaction
OD	Optic density
DNA	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 Source of Enzymes

The diversity of microorganisms spread all over the biosphere and they have important functions like global primary energy or element cycling. The most common source of genes that are used in several industrial and research applications are based on this diversity (Beloqui, et al. 2008).

Microorganisms can be found at high atmospheric pressure, at unusually high and cold temperature, in the deepest ocean sediments, even in highly polluted environments. Microbes are able to break down a variety of usual and unusual carbon and energy sources and convert them into amino acids, nucleotides, vitamins, carbohydrates and fatty acids by producing specific enzymes. Enzymes that carry out metabolic processes in microbes also have several practical and industrial usages 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).

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 wide substrate specificity. Carbohydrases, proteases, pectinases and lipases are classified into hydrolases. They catalyze the hydrolysis of natural organic compounds (Rajan 2001, Underkofler, et al. 1957).

1.2 . Lipases

Lipids are essential to all living systems because they play structural roles in membranes, are involved in signaling events and the most important role that they have is being the most important source of energy. During lipid metabolism, lipolytic enzymes are required for lipids to carry out these functions. The turnover of these

water-insoluble compounds are catalyzed by lipolytic enzymes which either breakdown lipids and make them mobile within the cells of individual organisms (Beisson, et al. 2000, Gilham and Lehner 2005). Lipolytic enzymes are classified into 3 main categories which are **esterases** catalyze hydrolysis of glycerol esters with an acyl chain length of <10 carbon atoms, **phospholipases** hydrolyze phospholipids into fatty acids and other lipophilic substances and **lipases** catalyze hydrolysis of triacylglycerols with an acyl chain length ≥ 10 carbon atoms to glycerol and free fatty acids at the lipid-water interface (Arpigny and Jaeger 1999, Jensen 1983, Sharma et al.,2011).

Claude Bernard first discovered lipases in 1856 while he was studying the role of the pancreas in fat digestion (Peterson and Drabløs 1994). Several researchers reported that a variety of bacteria, fungi and actinomycetes produce and secrete many different lipases since then (Sztajer et al.,1988, Rapp and Backhaus 1992, Kulkarni and Gadre 2002). 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).

Sarda and Desnuelle first defined lipases in 1958 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).

Because lipases catalyze the hydrolysis of long-chain triacylglycerols to form glycerol and fatty acid in the presence of excess water (Figure 1.1), they are basically defined as fat-splitting enzymes. As well, the reverse reaction which is synthesis of triacylglycerols can be catalyzed by lipases at the absence of water (Jaeger, et al. 1999, Gupta, et al. 2004, Pascale, et al. 2008). At the absence of water, they play role in the esterification, transesterification, acidolysis and aminolysis reactions. Because of their acitivities in both aqueous and nonaqueous environments, lipases have specific applications in industry and medicine (Jaeger, 1994). In addition, glycerolesters with an acyl chain length ≥ 10 carbon atoms are known as lipase substrates (Jensen 1983). Besides, lipases and esterases were confused for a long time due to their hydrolase activity. But now, they are known as different enzymes because they have different substrate specificity (Zhang and Zeng 2008). Esterases break ester bonds of glycerol esters with an acyl chain length of <10 carbon atoms, whereas lipases catalyze the

hydrolysis of long chain fatty acids that are insoluble or poorly soluble. Thus, while esterase activity is found to be highest towards more water soluble substrate, lipase is capable of identifying an insoluble or aggregated substrate (Fojan, et al. 2000).

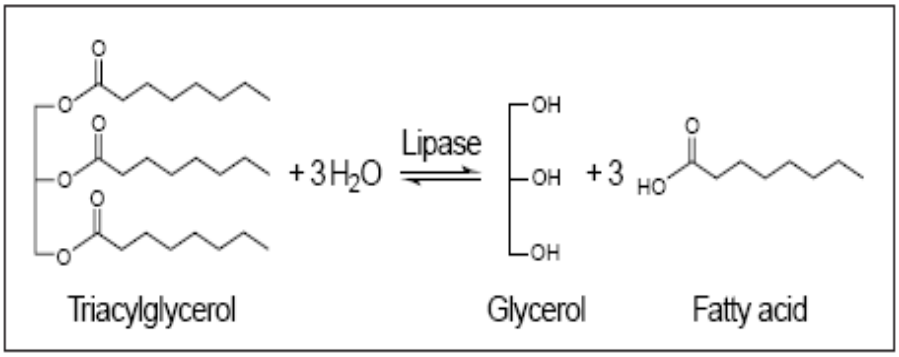


Figure 1.1. Hydrolytic and Synthetic Actions of Lipase
(Source: Jaeger and Reetz 1998)

1.3 . Lipase Producing Microorganisms

Due to the great variety of biochemical activities of microbial lipases and easiness of their isolation and production more attention has been paid for them recently (Wang, 2009). Also, microbial lipases are more stable in comparison with their plant and animal derivatives and for industrial and research applications their production is safer (Schmidt-Dannert, 1999). On the other hand, bacterial enzymes have higher activities at neutral or alkaline pH optima and they are more stable, so that among all the microbial lipases they are more preferred over fungal enzymes. Furthermore, bacterial cells have simple nutrional needs, short generation times and easy screening procedures for desired properties, so that genetic and enviromental manipulations are easier to perform on them (Hasan, 2006). Because of these properties, bacterial lipases have found immerse applications in food, detergent, pulp and paper and leather industries, envorimental management and pharmacy (Hasan, 2006; Jaeger and Reetz 1998).

1.4. Bacterial Lipases

Both Gram-positive and Gram-negative bacteria produce lipases but, Gram-negative bacteria genetare the greater part of that enzymes and the most important Gram-negative genus is *Pseudomonas* which contains at least seven lipase producing

species, which are *P. aeruginosa, P. alcaligenes, P.fragi, P. glumae, P. cepacia, P. fluorescens and P. putida* (Jaeger, 1994; Kojima, 2003). Along with *Pseudomonas* species, the most common lipase producing gram-negatives are *Achromobacter*, *Alcaligenes, Burkholderia* and *Chromobacterium* strains (Gupta and Rathi 2004). On the other hand, although *Bacillus* is the most useful lipase producer genus used in industry among gram-positive bacteria, *Staphylococcus* (especially, *S.aureus and S.hyicus)* and *Streptomyces* species are lipase producers too (Jaeger, et al. 1994).

Pseudomonas lipases are classified into three groups based on their amino acid homologies and some biological properties (Zhang, 2008). *P.aeruginosa, P.alcaligenes* and *P.fragi* are classified in Group I lipases, while *P.glumae* and *P.cepacia* are prototypes of group II and *P.fluorescens* lipases are prototypes of group III (Arpigny and Jaeger 1999). Group I lipases consist of approximately 285 amino acids with a molecular weight of 30 kDa and a specific sequence at their N-terminal for their effective secretion. Another protein called lipase-specific foldase is needed for the correct folding and secretion of group I lipases. Generally, Group II lipases consist of 320 amino acids with a molecular weight of 33 kDa, one disulfide bond and like group I lipases they have an N-terminal signal sequence. It is important that group I and group II lipases share % 60 amino acid homology and in order to be active and secreted into extracellular environment they also require lipase specific foldases. The larger lipases are represented as Group III and they are composed of about 475 amino acids with a molecular weight of around 50 kDa. There are no cysteines, nor an N-terminal signal sequence in group III lipases They also do not require a lipase specific foldase for their folding and secretion. While group I and II lipases are secreted via type II secretion pathway (also called secreton-mediated secretion), group III lipases are secreted via type I secretion system (also named ABC exporters) (Rosenau and Jaeger 2000). Due to these differences they are separated from group I and II lipases. Although Group III lipases do not contain a typical N-terminal signal sequence, they have a C-terminal targeting signal sequence responsible for the secretion of lipase by an ABC exporter, (Amada, et al. 2000, Duong, et al. 1994).

1.5. Industrial Applications of Microbial Lipases

Nowadays lipases are used in a wide variety of biotechnological applications such as dairy, paper, agrochemical, detergent and food industry, chemical processing, cosmetics, synthesis of surfactants, polymer synthesis and personal care products as biocatalysts due to their favorable properties compared with chemical catalysts. Commercial use of lipases is a billion dollar business (Sharma et al., 2011).

1.5.1. Lipases in Food Industry

Recently, rather than traditional chemical processes, lipases have become essential part of applications in the modern food industry (Theil 1995, Sharma et al. 2011). Nowadays, commonly preferred enzymes are lipases for the production of a variety of products like butter, cheese, baked foods, dressings, fermented vegetables, fruit juices, soups and sauces.

Lipases are used to form modified lipids (oils and fats) in food industry by adding or deleting some parts of triacylglycerols through esterification and transesterification reactions. Especially region-specific 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).

Flavor and fragrance compounds are made by lipases that give special flavour and taste to food by synthesis of fatty acids and alcohols (Gandhi 1997). In this case, lipases are commonly employed in dairy industry for the hydrolysis of milk fat. Also, lipases are used for accelerating cheese ripening, improvement of flavor in cheeses, the lipolysis of butterfat and cream and the production of cheese like products (Saxena, et al. 1999, Sharma et al.,2011).

1.5.2. Lipases in Detergents

Ever since the discovery of lipases, they became part of the detergent industry along with proteases, amylases and cellulases (Pandey, et al. 1999, Sharma et al.,2011).

Lipases are mainly used as additives to detergents that are used commonly in household and industrial laundry due to their fat splitting activity. Lipases catalyze the hydrolysis of fatty stains into more hydrophilic parts that are easily removed than similar non-hydrolyzed stains (Joseph, et al. 2007).

Novo Nordisk brought in the first commercial recombinant lipase to the detergents in 1994 which was named as Lipolase produced from fungus *T. Lanuginosus* and expressed in *Aspergillus oryzae* (Jaeger and Reetz 1998). Later in 1995, two bacterial lipases named as 'Lumafast' from *Pseudomonas mendocina* and 'Lipomax' from *Pseudomonas alcaligenes* were produced by Genencor International (Jaeger and Reetz 1998).

Lipases should be capable of functioning in the presence of the various components of washing powder formulations like surfactants and proteases, also should be both alkalophilic (pH 10-11) and thermophilic (30-60 °C). Besides, they should have wide substrate specifity to be able to hydrolyze fats of various compositions (Sharma, et al. 2001, Jaeger and Reetz 1998).

Lipases are also used for contact lens cleaning, dish washing and degradation of organic wastes on the surface of exhaust pipes and toilet bowls, and etc (Hasan, et al. 2006).

1.5.3. Lipases as Biosensors

Lipases can be manipulated as biosensors because of their ability to sense lipids and lipid-binding proteins. The most common application of lipases as biosensors is especially in food industry and in clinical diagnosis by quantitative determination of triacylglycerols. The released glycerol in the analytical sample which was formed by lipase activity from triacylglycerol is quantified by a chemical or enzymatic method in clinical diagnosis. It was shown that the patients with cardiovascular complaints have been diagnosed very precisely by this principle (Pandey, et al. 1997, Sharma et al. 2011). Also a biosensor which is a lipid sensing device is rather cheaper and less time consuming in comparison with chemical methods (Sharma et al. 2011).

1.5.4. Lipases in Bioremediation

It is a new approach using lipases for environmental management in biotechnology. Lipases from different derivations could be used for treating wastes of lipid processing factories, cleaning restaurants and handling oil spills in refinery (Nakamura et al., 1994). Also, degradation of the wastewater contaminants like olive oil from oil mills and removal of biofilm deposits from cooling water systems could be achieved by lipases (Anonymous, 1995; Sharma et al. 2011).

1.5.5. Lipases in Pharmacy

Lipases have region-selective property which cause them commonly used in pharmaceutical industry. The usage of lipases in pharmaceutical industry is such as catalysis of synthetic reactions, production of enantiomeric compounds, racemic esters and kinetic resolution process for the preparation of optically active chiral compounds. Particularly, in that case the vital applications of lipases are like synthesis of anti-tumor agents, alkaloids, antibiotics and vitamins and preparation of homochiral compounds (Jaeger and Eggert 2002).

1.5.6. Lipases in Pulp and Paper Industry

Wood is the main source of paper and pulp industry. 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).

1.5.7. Lipases in Leather Industry

The main processes in leather industry are the removal of subcutaneous fat and hair. 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).

1.6. The Aim of this Work

The primary goals of this thesis were to clone and express the extra-cellular lipase gene from *Pseudomonas sp.* KE38, isolated from soil samples of Erciyes mountain in Kayseri, in *E. coli* and partial purification of the gene product. To achieve this aim, genome walking technique was used to obtain lipase gene from *Pseudomonas sp.* KE38, that gene was then cloned into the cloning vector pJET1.2 and then the expression vector pET28a, expressed in *E. coli* and partial purification of the recombinant protein via inclusion body isolation method was carried out.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

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

2.2. Methods

2.2.1. Genomic DNA Purification of *Pseudomonas* sp. KE38

A single colony of *Pseudomonas* sp. KE38 was inoculated to 10 ml Luria-Bertani (LB) broth and incubated at room temperature (25 °C) with overnight shaking at 160 rpm. After overnight incubation, 3ml of that culture was used to purify genomic DNA. Genomic DNA was purified using Fermentas Genejet Genomic DNA Purification Kit according to the manufacturer's instructions. Isolated genomic DNA concentration was measured with a nanodrop specktrophotometer (Thermo) and visualised by electrophoresis on 1% agarose gel.

2.2.2. Genomic DNA amplification by Genome Walking PCR Technique

Genome walking technique was used in order to obtain the whole lipase gene by using isolated genomic DNA in PCR reaction. In this technique forward nested specific primers which are LIP7 (5' ACGTGATCAACGACCTGCTGG 3'), LIP5 (5' CCAATGGCTTGTCGGGAAAAG 3') and LIP4 (5' CCAAAGAGTCGGCCACCG 3') and a universal walker primer which is Semi-2 (5' GCCTTAAGGCCTANGARMSNCCNAG 3') as reverse primer were used as primer pairs. First round PCR was performed as follows: 1 µl of genomic DNA (41,3 ng/ µl),

10 µl each of LIP7-Semi2 primers (2 µM), 10 µl of dNTPmix (2 µM), 10 µl of 10X Fermentas Taq DNA Polymerase Buffer, 12 µl of $MgCl_2$ (25 µM), 0,5 µl of Fermentas Taq DNA Polymerase (5U/ µl) and finally 46,5 µl of dH_2O were mixed in a total volume of 100 µl. The conditions for PCR amplification were as follows: an initial denaturation step at 94 °C for 2 minutes; followed by 30 cycles including denaturation at 94°C for 30 seconds, gradient primer annealing at 45-65 °C for 1 minute and elongation at 72°C for 2 minutes and also final elongation at 72°C for 10 minutes. Then, 1 µl each gradient PCR product was used as template for the second round PCR amplification. The reaction mixture and PCR conditions were the same as first round PCR, except LIP5-Semi2 primer pair was used instead of LIP7-Semi2. These second round PCR products were used as templates for the third round PCR in which LIP4-Semi2 primer pair and 2 µl of template were used. Any bands that were visualized by 1% agarose gel electrophoresis after the 2^{nd} or 3^{rd} round of PCR were cut from agarose gel, purified with QIAquick Gel Extraction Kit (Qiagene), and directly sequenced as a PCR product with LIP4-Semi2 primers at the Biotechnology and Bioengineering Central Research Laboratories, Izmir Institute of Technology. Homology analysis using BLAST searches (National Center for Biotechnology Information, NCBI) were performed on the DNA sequence to determine the most closely related gene sequence.

2.2.3. PCR Amplification, Cloning, and Expression of PfKE38 Lip

According to the sequence obtained with genome walking, new primers, LIP_pet_start (5'CATATGGGTGTGTATGACTACAAGAAC3'), and LIP_stop (5'TCAGGCAATCACAATCCCTGTACC3') which contained the recognition sequences for the restriction enzymes *Bgl* II and *Bam*HI respectively were designed and PCR reaction was performed with these primers. PCR was performed as follows: 3 µl of genomic DNA (41,3 ng/ µl), 5 µl each of LIP_pet_start- LIP_stop primers (2 µM), 5 µl of dNTPmix (2 µM), 5 µl of 10X Fermentas Pfu DNA Polymerase Buffer, 1 µl of Fermentas Pfu DNA Polymerase (5U/ µl) and finally 26 µl of 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 30 cycles including denaturation at 94°C for 30 seconds , primer annealing at 55 °C for 1 minute and elongation at 72°C for 5 minutes and also final elongation at 72°C for 15 minutes. PCR products were

visualized by 0,8 % agarose gel electrophoresis and the band at 1800 bp corresponding to lipase gene was cut and purified with QIAquick Gel Extraction Kit (Qiagen). The purified fragment was cloned into pJET1.2/blunt cloning vector by using Fermentas CloneJET PCR Cloning Kit according to the manufacturer's instructions. *E. coli* DH5α competent cells were transformed with the resulting mixture. Transformation mixture was inoculated on LB plates with ampicillin. Colonies were checked by colony PCR using LIP_pet_start and LIP_stop primers under the same conditions as described above. PCR product size was checked by 1% agarose gel. A colonoy containing the correct insert was cultured overnight and its plasmid bearing the lipase insert was purified using Roche High Pure Plasmid Isolation Kit. Then, lipase gene was removed from the construct via *Nde* I/*Bgl* II double digestion and then ligated into the pET28a expression vector which was cut via *Bam*HI/*Nde* I double digestion. Digested products were visualized on 0,8 % agarose gel and lipase fragment and pET28a expression vector was purified with QIAquick Gel Extraction Kit. The purified fragments was ligated with Fermentas T4 DNA ligase enzyme and transformed into *E. coli* DH5α. The transformation product was plated on LB agar containing (35 μg/ml) kanamycin. Plates were evaluated after overnight incubation at 37°C. Colony PCR was also performed to control the presence of lipase gene using LIP_pet_start and LIP_stop primers with the same PCR conditions as mentioned above. Colony PCR products was checked on 1 % agarose gel. Plasmid from one of the positive clones, based on gel result, was isolated from overnight *E. coli* DH5α culture using Roche High Pure Plasmid Isolation Kit and then used for the transformation of *E. coli* BL21 cells in order to express the lipase gene.

2.2.4. Partial Purification of Recombinant Lipase with Inclusion Body Isolation

The expressed recombinant lipase was purified partially by inclusion body isolation procedure according to Akbari et all., 2009 with slight changes. First of all, 2 ml overnight culture of the *E. coli* BL21 pET28a Lip was used to seed 200 ml culture which includes LB medium supplemented with kanamycin (35 μg/ml) and incubated overnigth at 37°C. When the optical density (OD) at 600 nm reached about 0.6, IPTG was added to a final concentration of 1 mM. After incubation for overnigth, cells were

harvested by centrifugation (10,000g, 10 min, 4°C). The cell pellets from 200 ml culture (<1 g wet weight cells) were suspended in 2 ml Tris–HCl buffer (100 mM, pH 8) containing 1 mM EDTA. After seven cycles of 30 s sonication, the cell lysate was centrifuged at 12,000g for 20 min. The supernatant and the precipitate were collected as soluble and insoluble fractions, respectively. The cell pellets containing the insoluble protein were washed several times with Tris–HCl buffer (100mM, pH 8.0) to remove impurities. Inclusion bodies were then resuspended in 10 ml buffer A (50 mM Tris–HCl pH 8, 8 M urea, 10 mM DDT) and incubated at 37 °C for 60 min with mild stirring. Following centrifugation (12,000g, 20 min, 4 °C), the supernatant was dialyzed against Tris–HCl buffer (50mM, pH 8.0). After dialyses, the 10 ml sample was concentrated with Pierce Protein Concentrators 20K MWCO and final protein sample was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

2.2.5. SDS-PAGE

The molecular mass of partially purified recombinant lipase was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 10 % resolving gel used together with 5 % stacking gel and stained with coomassie brilliant blue, as described by Laemmli (1970). Lipase containing fractions were loaded on SDS gel. Electrophoresis was performed at 60 volts for 60 minutes and then 100 volts for 2,5 hours. Gels were stained with Coomassie Brilliant Blue overnight. PageRuler Plus Prestained Protein Ladder (Fermentas) was used as molecular mass marker.

2.2.6. Determination of Optimum Recombinant Lipase Production Time

A 150 µl overnight culture of the *E. coli* BL21 pET28a Lip and *E. coli* BL21 pET28a as negative control were used to seed 15 ml LB culture supplemented with kanamycin (35 µg/ml) and incubated at 37°C for 8 hours. 1,5 ml aliquots from each culture were withdrawn and then IPTG was added to a final concentration of 1 mM. After that, 1,5 ml aliquots from each culture broth were withdrawn at every 2 hours and harvested (10,000g, at 4°C for 30 min) to determine the optimum time for recombinant lipase production. The cell pellets from these samples were suspended in 0,5 ml Tris–

HCl buffer (100 mM, pH 8) containing 1 mM EDTA. After seven cycles of 30 s sonication, the cell lysate was centrifuged at 12,000g for 20 min. The supernatant was disposed and the cell pellets containing the insoluble protein were washed several times with Tris–HCl buffer (100mM, pH 8.0) to remove impurities. After washing, the cell pellets were resolved in 50 µl Tris–HCl buffer (50mM, pH 8.0) and analyzed with SDS-PAGE.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Amplification of the Lipase Gene of *Pseudomonas* sp. KE38 by Degenerate PCR and Genome Walking Technique

In a previous study performed in our lab, an extracellular lipase producing bacterium, *Pseudomonas* sp. KE38, which was identified as a strain of *Pseudomonas fluorescens* by 16s rRNA gene analysis, was isolated from soil samples taken from Erciyes mountain in Kayseri (Adan Gökbulut A. and Arslanoğlu A. 2013).

In order to amplify the extracellular lipase gene of *Pseudomonas* sp. KE38 (PfKE38 Lip), two degenerate primers (KE38Lip_F and KE38Lip_R) were designed by careful analysis of the aligned sequences of already sequenced lipase genes from *Pseudomonas* species that are phylogenetically closely related to *Pseudomonas fluorescens* (Figure 3.1). The sequences were obtained from NCBI and alligned by the Clustal Omega software (www.clustal.org). Degenerate primers were designed to regions that showed high level of sequence identities. Although the 5' end of the alligned lipase sequenced contained high level of identitiy, the 3' end was poor in identity forcing us to designed the 3' primer to a region in the middle of the sequence.

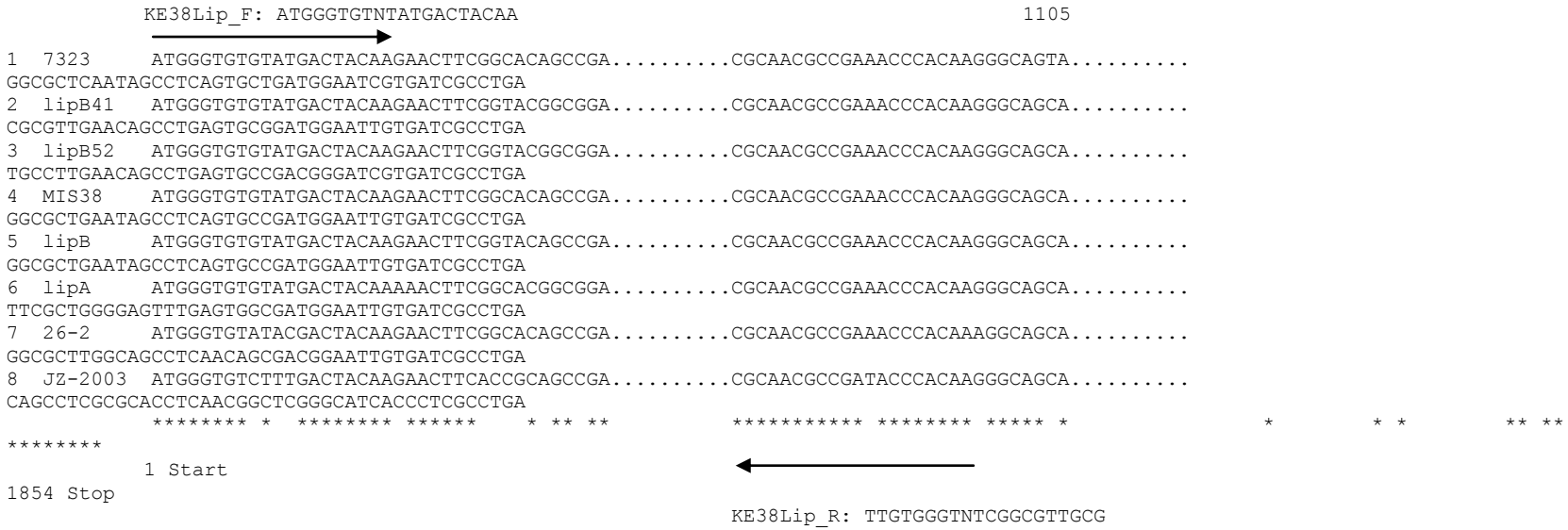
Partial amplification of the PfKE38 Lip was succesfully achieved by using KE38Lip_F and KE38Lip_R primers. The amplified 1105 bp fragment was then cloned into pTZ57RT/A cloning vector and sequenced by using vector specific primers M13F and M13R.

In order to amplify the 3' end of the PfKE38 Lip, genome walking technique according to Guo and Xiong, was employed by using isolated genomic DNA in PCR reaction as described in materials and methods (Guo and Xiong, 2006). This technique is based on nested PCR-based strategy for genome walking in order to extend a known sequence region to its uncharacterized flanking regions. A walker primer which is partially degenerate (Semi2) and a set of nested gene specific primers (LIP7, LIP5, LIP4) are used in a, two to three successive rounds of nested PCR reaction (Figure 3.2). The walker primer is expected to bind randomly to several sites on the bacterial

chromosome, potentially generating many nonspecific as well as specific PCR products. Specific products are then further amplified in the second and/or third round of PCR using second and third nested gene specific primers, respectively eliminating nonspecific artifacts (Guo and Xiong, 2006).

Figure 3.1. Multiple aligment of 8 different *Pseudomonas fluorescens* lipase genes.

1. *Pseudomonas* sp. 7323 lipase gene, **2.** *P.fluorescens* lipase (lipB41) gene, **3.** *P. fluorescens* lipase (lipB52) gene, **4.** *Pseudomonas* sp. MIS38 gene for lipase, **5.** *P.fluorescens* lipase (lipB) gene, **6.** *P.fluorescens* lipA gene for lipase, **7.** *P.fluorescens* strain 26-2 lipase class 3 gene, **8**. *Pseudomonas* sp. JZ-2003 lipase gene



The three round of PCR products were visualized by 1% agarose gel electrophoresis (Figure 3.3). There were no band obtained at the first and second round PCR while the third round PCR product which was approximately 1500 bp band was cut from agarose gel and sequenced from both ends using LIP4 and Semi2 primers. Homology analysis using BLAST revealed that the sequenced fragment had high homology to the 3' end of extracellular lipase genes from various *Pseudomonas* species confirming the specific amplification of the 3' end region of the PfKE38 lipase gene (figure 3.4). This sequence was then used for designing new primers, LIP_pet_start and LIP_stop, for the amplification of whole PfKE38 Lip open reading frame to be cloned into an expression vector.

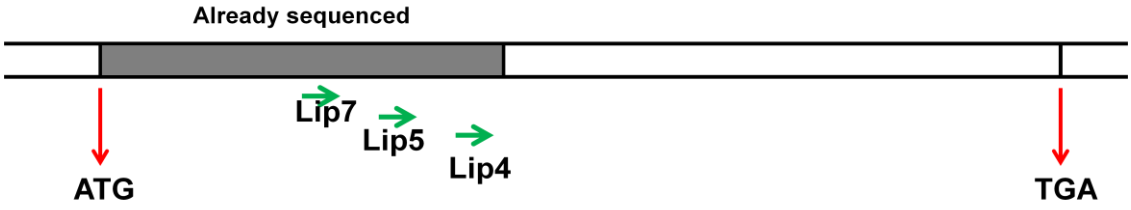


Figure 3.2. Shematic illustration of lipase gene with already sequenced part, start and stop codons and nested PCR primers.

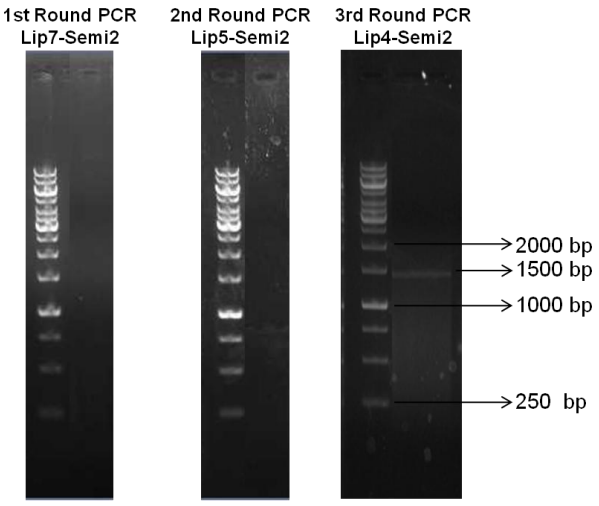


Figure 3.3. Agarose gel analysis of genome walking PCR amplified lipase gene. Fermentas 1 kb DNA size marker (from bottom to top: 250, 500, 750, 1000, 1500 bp, 2000bp….). 3rd round PCR ~1500 bp PCR product.

3.2. Expression of PfKE38 Lip Gene

PfKE38 Lip open reading frame of about 1.8 kbp in length was amplified by PCR using Pfu DNA Polymerase due to its high proof reading activity using primers Lip_pet_start and Lip_stop (figure 3.5).

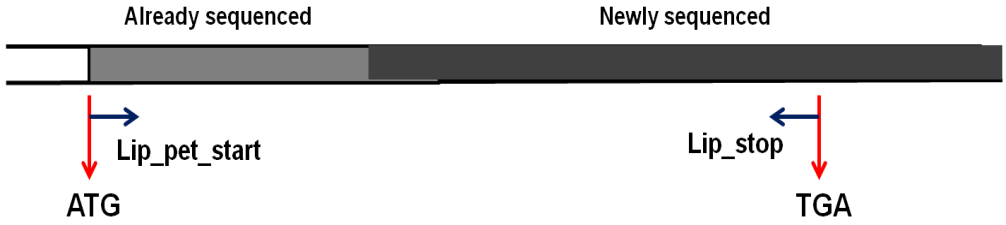


Figure 3.4. Shematic illustration of lipase gene with already and newly sequenced parts, start and stop codons.

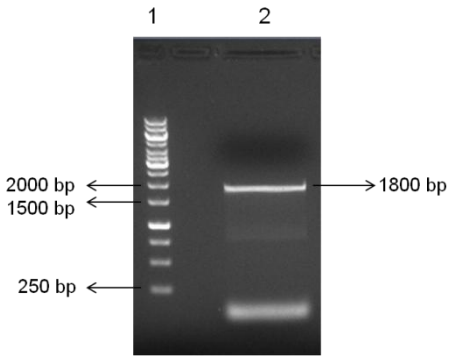


Figure 3.5. Agarose gel analysis of PCR amplified PfKE38 Lip. Line 1: Fermentas 1 kb DNA size marker (from bottom to top: 250, 500, 750, 1000, 1500 bp, 2000bp….). Line 2: 1800 bp PCR product.

After obtaining the purified lipase fragment, it was cloned into pJET1.2/blunt PCR cloning vector and the transformants were confirmed by colony PCR (figure 3.6).

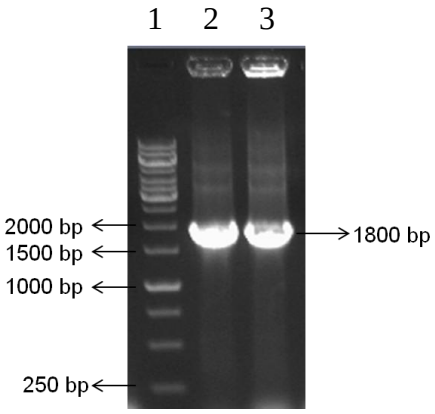


Figure 3.6. Agarose gel analysis of colony PCR of two transformants for the presence of lipase gene. Line 1: Fermentas 1 kb DNA size marker (from bottom to top: 250, 500, 750, 1000, 1500 bp, 2000bp….). Line 2-3: 1800 bp colony PCR product from two different transformants.

The plasmid isolated from one of these positive colonies digested via *Nde* I/*Bgl* II, lipase gene was removed from the construct and ligated into the pET28a expression vector which was cut via *Bam*HI/*Nde* I double digestion (figure 3.7).

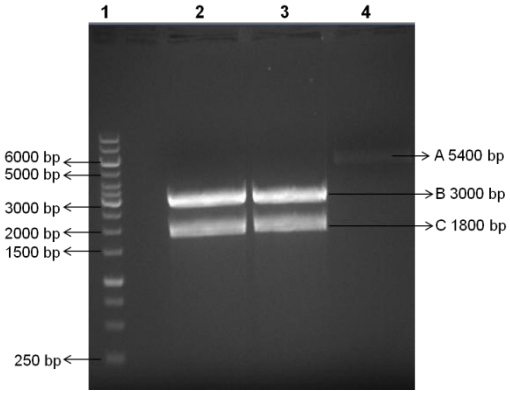


Figure 3.7. Agarose gel analysis of double digestion. Line 1: Fermentas 1 kb DNA size marker (from bottom to top: 250, 500, 750, 1000, 1500 bp, 2000bp….). Line 2-3: B: pJET1.2 vector, C: Lipase gene 1800 bp. Line 4-A: pET28a vector.

The purified fragment of lipase gene was then cloned into pET28a expression vector and the transformants were confirmed by colony PCR (figure 3.8) The plasmid isolated from one of the positive clones transformed into *E. coli* BL21 cells to express lipase gene (Figure 3.9).

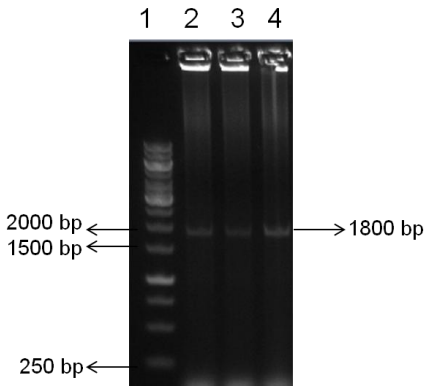


Figure 3.8. Agarose gel analysis of colony PCR of pET28a Lip. Line 1: Fermentas 1 kb DNA size marker (from bottom to top: 250, 500, 750, 1000, 1500 bp, 2000bp….). Line 2-3-4: 1800 bp colony PCR product.

Lipase production can be identified by the formation of orange fluorescent halos around the colonies when olive oil-rhodamine B spread plates incubated at appropriate temperature and 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 rhodamine B-long chain fatty acid conjugate in this method (Jaeger, et al. 1994).

As seen in figure 3.9 the *E. coli* BL21 harboring pET28a Lip, *Pseudomonas sp.* KE38 as a positive control, *E. coli* BL21 and *E. coli* BL21 harboring pET28a as negative controls grow on LB agar including olive oil and rhodamine B plates. It is shown in figure 3.9 A the positive control *Pseudomonas sp.* KE38 and 3.9B *E. coli* BL21 pET28a Lip secreted lipase, these lipases splitted the substrate olive oil and the formation of fluorescence orange halos around colonies proved that activity. However there was no fluorescence on the negative controls, *E. coli* BL21 and *E. coli* BL21 pET28a, plates shown in figure 3.9C and 3.9D respectively which proves that these cells did not express lipase. However, the *E. coli* BL21 pET28a Lip did not cover the whole plate because of unefficient inoculation (Figure 3.9B).

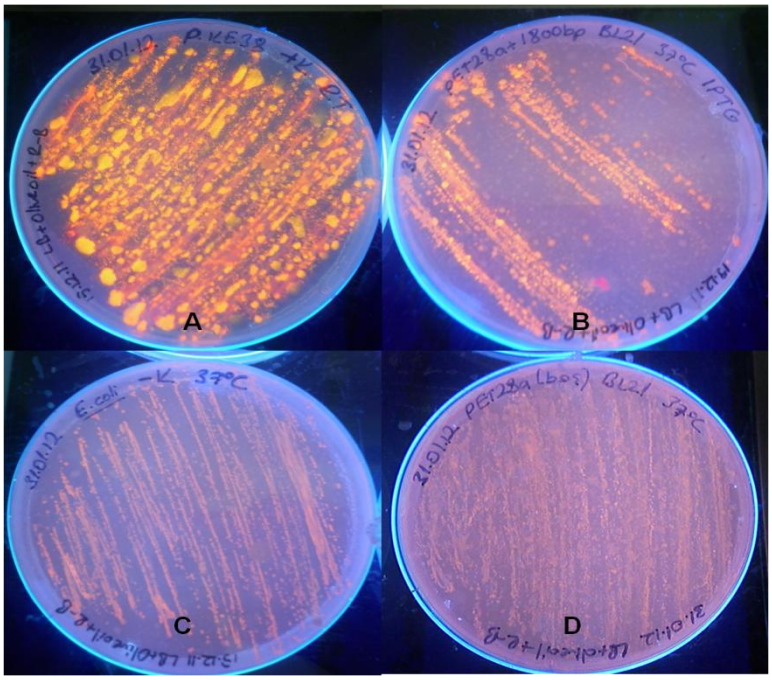


Figure 3.9. Photos of lipase expression on LB agar plus olive oil plates. A: *Pseudomonas sp.* KE38 as positive control. B: *E. coli* BL21 harboring pET28a Lip. C: *E. coli* BL21 and D: *E. coli* BL21 harboring pET28a vector, respectively.

Minimal medium (see appendix A) with %1 olive oil was used to check whether lipase was produced or not. Only lipase producing bacteria can use olive oil as carbon source and grow in this medium. As seen in figure 3.10 *Pseudomonas sp.* KE38 as positive control and *E. coli* BL21 pET28a Lip could grow on this medium. *E. coli* BL21 and *E. coli* BL21 pET28a were not able to grow. These results proved that the *E. coli* BL21 pET28a Lip expressed the lipase gene. However, *E.coli* BL21 pET28 Lip growth was much less compared to the positive control. This might be due to the consequence of the formation of lipase inclusion bodies. Inclusion bodies are composed of densely packed denatured protein molecules in the form of particules, and may cause the decrease in lipase activity.

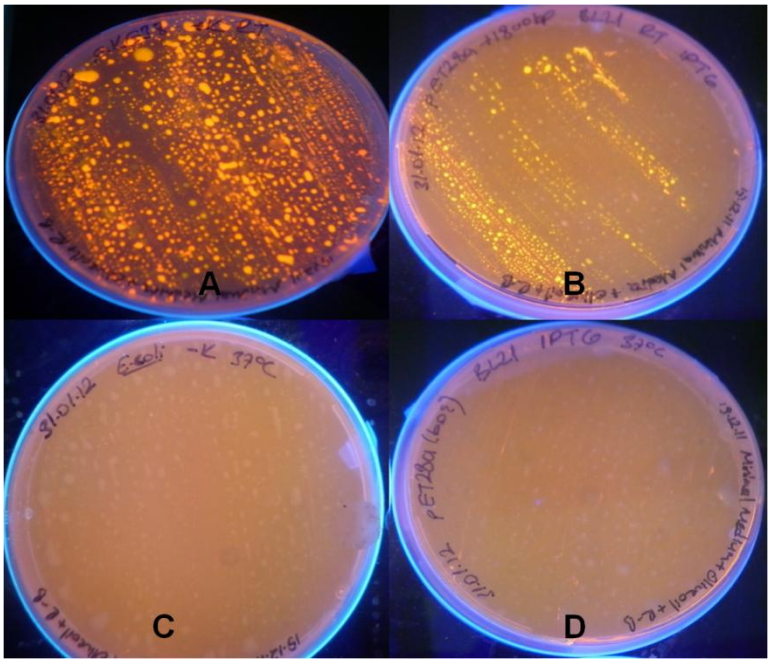


Figure 3.10. Photos of lipase expression on minimal media plus olive oil plates. A: *Pseudomonas sp.* KE38 as positive control. B: *E.coli* BL21 pET28a Lip C: *E. coli* BL21 and D: *E. coli* BL21 pET28a vector, respectively.

3.3. Partial Purification of Recombinant Lipase with Inclusion Body Isolation and SDS-PAGE Analysis

Inclusion bodies are derived from high-level expression of recombinant proteins in bacterial hosts and they are insoluble and inactive form (Akbari et all., 2009). Also they are composed of densely packed denatured protein molecules in the form of particules (Sing and Panda, 2005). Solubilization of inclusion bodies can be accomplished by using of a high concentration of denaturants such as urea or guanidine hydrochloride, along with a reducing agent such as β-mercaptoethanol (Rudolph et all., 1996, Clark, E.D.,1998). Refolding of solubilized proteins can be done by slow removal of the denaturant (Rudolph et all., 1997). Isolation of inclusion bodies from cell homogenate is a suitable and effective way of purifying the interested protein (Sing and Panda, 2005). There are some adventages of formation of inclusion bodies such as; they have different size and density as compared with cellular contaminants so that they can be easily isolated from cells, the expressed protein is exposed of lower degradation, the interested protein is higly expressed compared to cellular protein and the expressed protein resists to proteolytic attack by celular proteases (Sing and Panda, 2005).

The recombinant lipase had higly hydrophobic nature and high level expression of that protein in *E. coli* made lipase accumulate as inclusion bodies. Therefore the expressed lipase was partially purified with inclusion body isolation procedure according to Akbari et all., 2009. Isolated lipase was checked on the SDS-PAGE. Figure 3.11 line 2 and 4 show the results of inclusion body isolation, line 3 and 5 indicate the supernatant taken after ultrasonication. As seen in figure 3.11 line 2 the recombinant lipase was partially purified and this result was consistent with Akbari et all., 2009 and the knowledge. In fact, recombinant lipase in the form of inclusion bodies had some adventages such as high expression, resistance against proteolytic attack and easy isolation. Also the partially purified recombinant lipase from the strain KE38 had approximately 64 kDa molecular weight based on the position of the protein marker. This result was consistent with the knowledge that Group 3 *Pseudomonas* lipases are the largest ones compared with other two groups and the recombinant lipase verified that knowledge. On the other hand there was no expressed protein in line 3, 4 and 5 as expected. Because line 3 was the supernatant taken after ultrasonication and line 4 and 5 were belong to negative control which did not contain lipase.

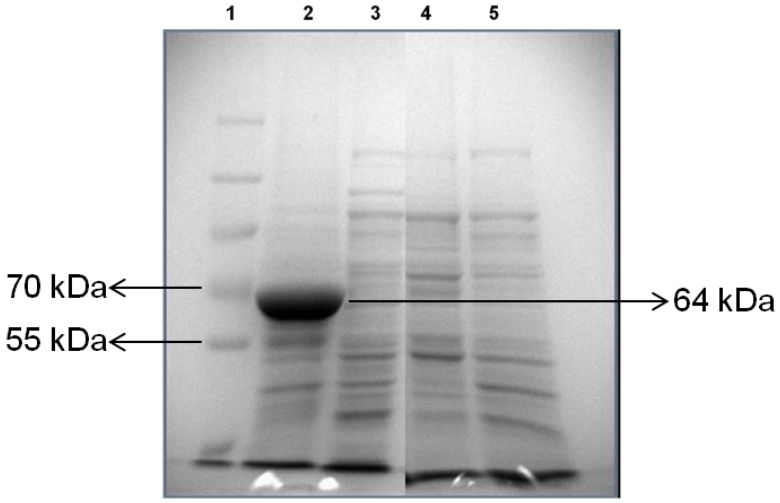


Figure 3.11. SDS-PAGE of partially purified recombinant lipase enzyme. Line 1: Fermentas PageRuler Plus Prestained Protein ladder (from top to bottom: 250, 130, 100, 70, 55, 35, 25, 15, 10 kDa) Line 2: pET28a Lip after inclusion body isolation Line 3: pET28a Lip supernatant after ultrasonication Line 4: Negative control pET28a after inclusion body isolation Line 5: Negative control pET28a supernatant after ultrasonication.

3.4. Optimum Recombinant Lipase Production Time

The *E.coli* BL21 pET28a Lip and *E.coli* BL21 pET28a as negative control were grown at 37 °C for 10 hours for determination of optimum recombinant lipase production time after IPTG induction. Figure 3.12 shows optimum lipase production time on SDS-PAGE, as seen in line 2 there was not any expression of lipase before IPTG induction. Because, the *lacI* gene binds to the *lac* operator and represses T7 promoter of pET28a vector. However, when IPTG is added, IPTG binds to the *lac* repressor and releases it, thereby allows the T7 promoter work and transcription initiates. Consistent with that knowledge, as seen in figure 3.12 line 3, 4, 5, 6 and 7, lipase was expressed with 64 kDa molecular weight after IPTG induction. Yet there is no change of the expression during ten hours supporting that lipase expression reached maximum level after two hours of IPTG induction. Negative control of expression is shown in figure 3.13 and there is no change before and after IPTG induction during ten hours as expected since no lipase gene is present in the control.

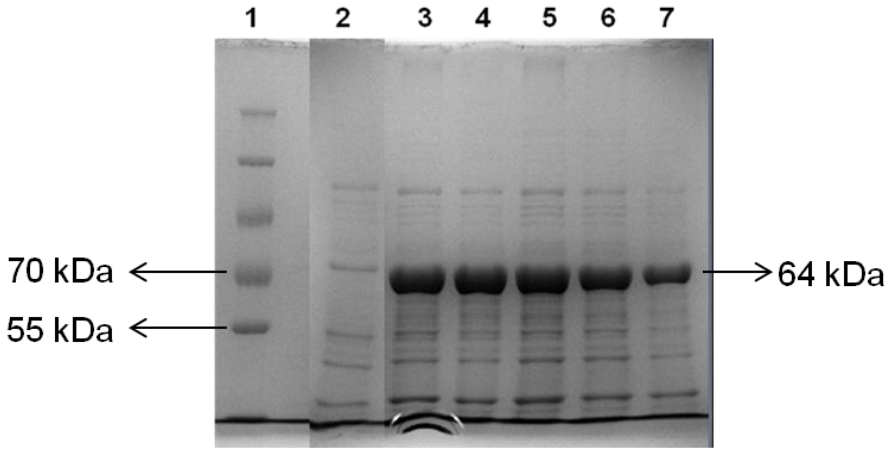


Figure 3.12. SDS-PAGE of optimum recombinant lipase production time Line 1: Fermentas PageRuler Plus Prestained Protein ladder (from top to bottom: 250, 130, 100, 70, 55, 35, 25, 15, 10 kDa) Line 2: Lipase before IPTG induction Line 3-4-5-6-7: Lipase after IPTG indution, 2, 4, 6, 8 and 10th hours (respectively).

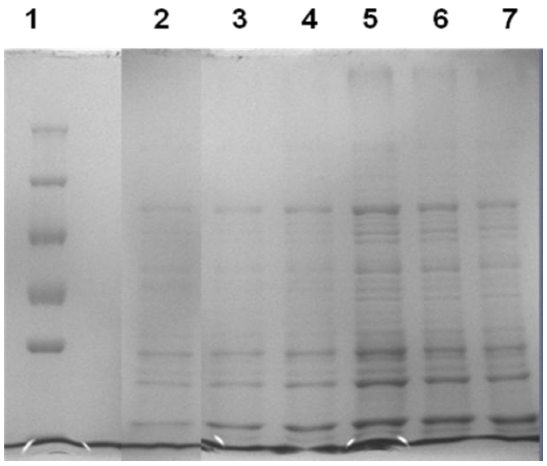


Figure 3.13. SDS-PAGE of negative control for optimum recombinant lipase production time Line 1: Fermentas PageRuler Plus Prestained Protein ladder (fom top to bottom: 250, 130, 100, 70, 55, 35, 25, 15, 10 kDa) Line 2: pET28a before IPTG induction Line 3-4-5-6-7: pET28a after IPTG indution, 2, 4, 6, 8 and 10^{th} hours (respectively).

CHAPTER 4

CONCLUSIONS

Microbial lipases have specific applications in biotechnology because of their several important properties especially their acitivities in both aqueous and nonaqueous environments. Among microbial lipases *Pseudomonas* lipases have been paid much attention due to their significant potential in detergent, food and leather industries and so on.

In this study, we succesfully cloned a novel lipase enzyme from *Pseudomonas sp.* KE38 which was isolated from soil samples of Erciyes mountain in Kayseri. To achieve that goal, we used degenerate PCR and genome walking technique. After extending the 3' end of the PfKE38 Lip, we cloned and expressed lipase in *E. coli* and partially purifed the recombinant enzyme succesfully. According to the results of gene and aminoacid homology search, PfKE38 Lip gene had high similarity with lipases classified in group 3 lipases (it was % 87 identical to *Pseudomonas* sp. 7323 lipase, % 83 identical to *Pseudomonas sp.* MIS38 lipase, *Pseudomonas sp.* CR-611 lipase and *P. fluorescens* PfO-1 lipase, and % 80 identical to *P. fluorescens* strain26-2 lipase class 3 gene). The deduced aminoacid sequence however contained a lipase consensus sequence, GHSLGG, which is most conserved in *Pseudomonas* group 3 lipases, did not contain cysteine residues to form disulfide bonds, another important feature of group 3 lipases (Angkawidjaja and Kanaya 2006) and had high aminoacid similarity with lipases classified in group 3 lipases (it was % 91 identical to *Pseudomonas* sp. 7323 lipase, % 87 identical to both *P. fluorescens* PfO-1 lipase and *Pseudomonas sp.* MIS38 lipase).

Because PfKE38 Lip is a novel enzyme and it has different biochemical characteristics from the other defined lipases, it can be properly used for biotechnological purposes especially in detergent industry.

For further studies, lipase will be purified completely, detailed biochemical characterization such as the effects of temperature and pH on activity, organic solvent tolerance, effects of several metal ions etc. of the purified lipase are planed and lipase gene will be knocked out by preparation of mutant gene to screen if there are more than one lipase in the genome of *Pseudomonas sp.* KE38 or not.

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APPENDIX A

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

Kanamycin (100 mg/ml)

0.1 g kanamycin in 1 ml dH_2O.

5X minimal salt solution

64g $Na_2HPO_4.7H_2O$, 15g KH_2PO_4, 2,5g NaCl and 5g NH_4Cl 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 buffer (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.