PARTIAL PURIFICATION AND CHARACTERIZATION OF LIPASE ENZYME FROM A *Pseudomonas* STRAIN

A Thesis Submitted to
the Graduate School of Engineering and Sciences of
İzmir Institute of Technology
in Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

in Chemistry

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December 2008

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ACKNOWLEDGEMENTS

First of all, I would like to thank my supervisor Assoc. Prof. Dr. Talat YALÇIN, for his excellent guidance, understanding, encouragement and help throughout this study.

Also I would like to express my grateful thanks to my co-supervisor Assist. Prof. Dr. Alper ARSLANOĞLU, for his help and valuable comments during thesis study.

Special thanks are extended to Assist. Prof. Dr. Gülşah ŞANLI, for her valuable suggestions, comments and kind helps. I want to thank to Molecular Microbiology, Molecular Genetics and Biological Chemistry Laboratory members.

I express my thanks to my searching team; Pınar BAYDARA, Çağdaş TAŞOĞLU and Ahmet Emin ATİK; for their advise and helps.

I am also grateful to my laboratory friends Erhan BAL, Melda GÜRAY, Burcu ÜNSAL, Burcu ŞENGEZ and Aysun ADAN for their sincere help and kindness during studies.

Finally, I want to express my gratitude to my family. Thanks for all their motivation, encouragement and their support throughout not only this study and also all through my life. Lastly, I express my special thanks to my fiancée for his patience, supports and technological helps during thesis study.

ABSTRACT

PARTIAL PURIFICATION AND CHARACTERIZATION OF LIPASE ENZYME FROM A *Pseudomonas* STRAIN

Lipase is a triacylglycerol-hydrolyzing enzyme which is catalyzed the hydrolysis of water insoluble free fatty acid and glycerols and also a wide range of chemical reactions. Beside, microbial lipases show regiospecificity and enantioselectivity properties. Therefore, microbial lipases gain the great importance for industrial applications and organic synthesis. In this study, investigation, partial purification and characterization of lipase enzyme from a *Pseudomonas* strain was studied by using different analytical approach.

Purification step was done by size-exclusion chromatography. The molecular weight of partial purified lipase was determined by SDS-PAGE. Spectrophotometric lipase assay applied to find out the enzyme characterization. Kinetic study of enzyme was also investigated varying the substrates concentrations. Specific activity staining on gel procedures applied after native gel process. After electrophoresis, lipase activity responsive protein bands were appeared on gel.

After screening for the presence of lipase activity in *Pseudonomas* strain which was isolated from soil, it was decided to choose intracellular enzyme sample for characterization and purification studies. The enzyme gave the highest lipase activity when p-nitrophenyl laurate used as a substrate. The optimum pH range for activity of lipase was alkaline pH ranges, about pH 8.0 and 9.0. The optimum temperature was dedicated as 25^{o}C. In the presence of metal salts and organic solvents; while some additives sharply decreased enzyme activity, some additives were not effect the enzyme activity. Approximate molecular mass of partially purified enzyme was between 29 kDa and 43 kDa.

ÖZET

Pseudomonas SUŞUNDAN LİPAZ ENZİMİNİN KISMİ SAFLAŞTIRILMASI VE KARAKTERİZASYONU

Lipazlar enzimi, triaçilgliserol hidrolaz bir enzim olup suda çözünmeyen serbest yağ asitlerin ve gliserollerin hidrolizini ve birçok kimyasal reaksiyonu katalizlerler. Dahası mikrobial olarak üretilen bir lipase enzimi organik sentezlerde enantiomerik seçiciliği ve özel bölge bağlanmaları ya da kırılmaları seçiciliği gösterir. Bu yüzden mikrobial olarak üretilen lipaz enzimi endüstriyel uygulamalarda ve organik sentez tasarımlarında önem kazanmıştır. Bu çalışmada *Pseudonomas* suşundan elde edilen lipaz farklı analitik yöntemler kullanılarak varlığı kanıtlamış, kısmen saflaştırılmış ve karakterizasyonu yapılmıştır.

Saflaştırma işlemi boyut dışlamalı kromatografi kullanılarak yapılmıştır. Kısmı olarak saflaştırılan enzimin moleküler ağırlığı poli akrilamit jel elektroforezi kullanılarak tespit edilmiştir. Spektrofotometrik çalışma ile enzim karakterizasyonu yapılmıştır. Ayrıca bunun yanında enzimin kinetik çalışması da yapılmıştır. Elektroforez işleminden sonra, potansiyel lipase activitesi gösteren protein bantları gel üzerinde görüntülenmiştir.

Topraktan izole edilmiş *Pseudonomas* suşunun farklı denemelerde ortaya çıkan lipaz aktivite sonuçları gözlendikten sonra, enzimin hücre içinde çalışan bir enzim olduğuna ve yapılacak saflaştırma ve karakterizasyon çalışmalarının bu bilgi göz önüne alınarak yapılmasına karar verilmiştir. Enzim, p-nitrofenil laurate'in substrat olarak kullanıldığı çalışmalarda en yüksek enzim aktivitesini göstermiştir. Lipaz enziminin çalışması için en uygun pH aralığının pH 8.0-9.0 civarında, alkali pH aralığında olduğu tespit edilmiştir. Uygun sıcaklık değeri ise 25^{o}C olarak belirlenmiştir. Metal tuzları ve organik çözücüler varlığında, enzim aktivitesi kimi katkı bileşikleri varlığında hızlı bir şekilde düşerken, kimi katkı bileşikleri varlığında etkilenmeden sabit kalmıştır. Kısmı olarak saflaştırılmış lipaz enziminin yaklaşık molekül ağırlığı 23 kDa ve 49 kDa aralığında bulunmuştur.

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ABBREVIATIONS

BSA	Bovine Serum Albumin
CBB	Commasie brillant blue
CE	Capillary electrophoresis
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediamine tetra acetic acid
HPLC	High performance liquid chromatography
IPG	Immobilized pH gradient
IEF	Isoelectric focussing
LB	Luria Bertani
MM	Minimal medium
Mr	Molecular weight
PAGE	Polyacrylamide gel electrophoresis
RNA	Ribonucleic acid
SEC	Size exclusion chromatography
SDS	Sodium dodecyl sulfate
TEMED	Tetramethylethylenediamine
UV	Ultra violet
1-D	One-dimension
2-D	Two-dimension
3-D	Three-dimension

CHAPTER 1

INTRODUCTION

1.1. Amino Acids, Peptides and Proteins

1.1.1. Physical and Chemical Properties

A major role for many sequences of DNA is to encode the sequences of proteins. Proteins are the most abundant biological macromolecules. They are produced in all cells. Proteins also occur in great variety; different kinds, ranging in size from relatively small peptides to polymers. Moreover, proteins exhibit variety of biological function. All proteins are constructed from the same ubiquitous set of 20 amino acids. These amino acids covalently linked each other in a characteristic linear sequences. Cells can produce proteins with different properties and activities by joining the same 20 amino acids in many different combinations and sequences. Amoung these protein products, the enzymes are the most varied and specialized. All cellular reactions are catalyzed by enzymes. All 20 of amino acids are common in structure, carboxyl group and an amino group bonded to the same carbon atom (the α-carbon).They differ from each other in their side chains, or R groups. R groups define structure, size, electric charge, polaritiy and the solubility of the amino acids.

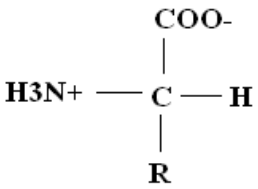


Figure 1.1. General structure of amino acid.

The peptides and proteins are the polymers of amino acids. Two or more amino acid molecules can be covalently joined through a peptide bond, to yield a dipeptide, oligopeptide and polypeptide. Four levels of protein structure are commonly defined. A description of all covalent bonds (peptide bonds and disulfide bonds) linking amino acid residues in a polypeptide chain is its primary structure. Secondary structure is

particularly stable arrangements of amino acid residues giving rise to recurring structural patterns. Tertiary structure describes the three-dimensional folding of a polypeptide. When a protein has two or more polypeptide subunits, their arrangement in space is called as quaternary structure (Nelson and Cox 2008).

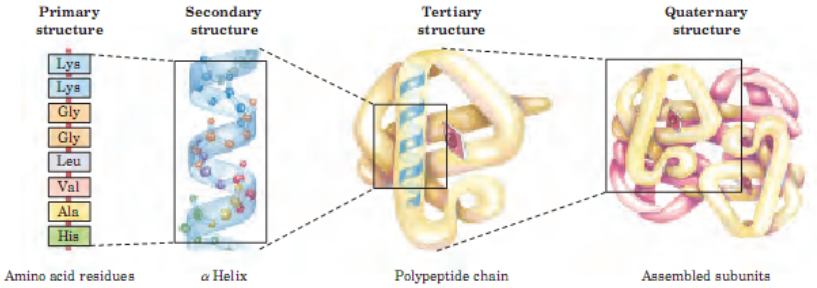


Figure 1.2. Levels of structure in proteins
(Source: Nelson and Cox 2008)

1.1.2. Protein Purification

A pure protein sample is important before determining protein's properties and activities. Different kinds of methods are used for purification process according to protein's properties, such as size, charge and binding properties (Nelson and Cox 2008). Biomolecules are purified using chromatography techniquies that seperate them according to differences in their specific properties. The application of chromatography to the study of both large and small biological molecules gain great importance in recent years. Chromatography allows the isolation of substance in very small amounts and in non-denaturating conditions.

Table 1.1. Chromatography techniques which used for protein purification according to their properties.

Property	Technique
Size	Gel filtration(size exclusion) chromatography
Charge	Ion exchance chromatography
Hydrophobicity	Hydrophobic interaction chromatography
	Reversed phase chromatography
Ligand specificity	Affinity chromatography

A porous solid material with convenient chemical properties (the stationary phase) is packed in a column, and a buffered solution (the mobile phase) elute through it. Individual proteins migrate faster or more slowly through the column depending on their properties.

Ion exchance chromatography separates proteins with differance in their charge (Figure 1.3.b). The separation is based on the interaction between a charged protein and an oppositely charged chromatographic medium (Nelson and Cox 2008). The elution is done with gradiently in order the change elution conditions. This elution is usually performed by increases in salt concentration or changes in pH (Stryer 1995).

Affinity chromatography seperates proteins is based on the binding affinity. The beads in the column have a covalently attached chemical group (specific ligand). A protein with affinity for this particular chemical group bind to the beads in the column. The technique can be used whenever a suitable ligand is available for the protein of interest. Affinity chromatography has high selectivity and resolution, also give specific results for protein interests.

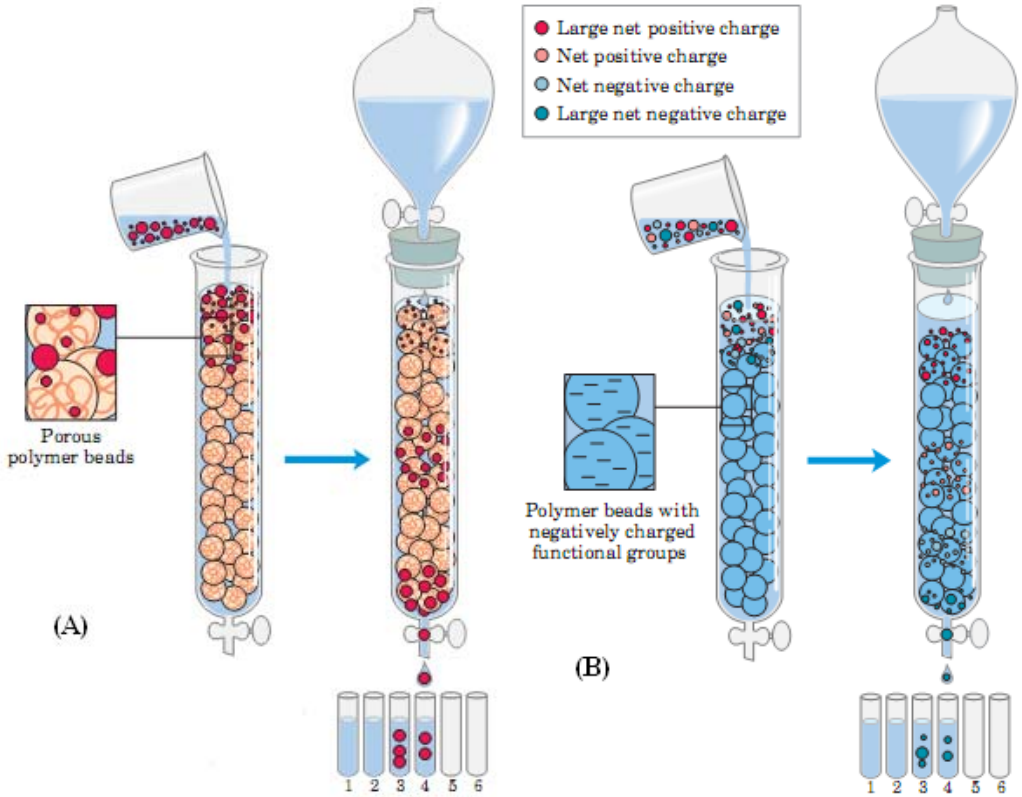


Figure 1.3. (a) shematic representation of size exclusion chromatography, (b) shematic representation of ion exchange chromatography (Source: Nelson and Cox 2008)

Size-exclusion chromatography separates proteins according to their size (Figure 1.3.a). In this method, large proteins elute through the column sooner than small ones. The solid phase consists of beads with pores of a particular size. Large proteins cannot enter the pores, and so take a short path through the column, whereas small ones enter the pores, and migrate through the column more slowly.

Another important technique for the separation in electrophoresis. Electrophoretic methods often adversely affect the structure and the function of proteins. So it is generally used as an analytical method. Electrophoresis allow to obtain number of seperated different proteins in a mixture, degree of purity of a particular proteins and also approximate molecular weight.

Dialysis method seperates proteins from small molecules such as salts through a semipermeable membrane. Proteins having huge dimensions can not pass though the membrane. But smaller molecules and ions can migrate the dialysis solution easily. This technique is useful for removing a salt or other small molecule (Figure 1.4).

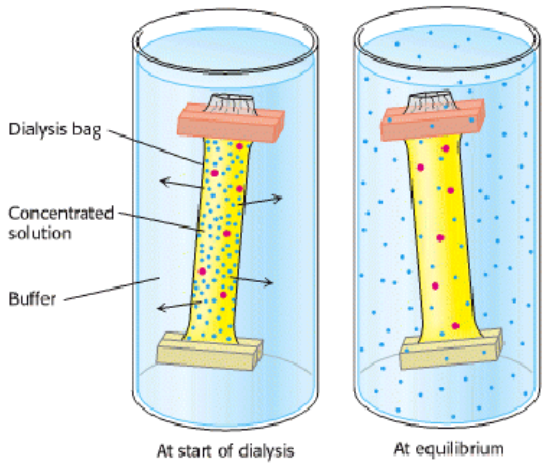


Figure 1.4. Shematic representation of dialysis process
(Source: Stryer 1995)

1.1.3. Size-exclusion Chromatography

Size-exclusion chromatography (gel filtration chromatography) is a technique for separating proteins and other biological macromolecules on the basis of molecular size. Size-exclusion chromatography (SEC) is a commonly used technique, because of the diversity of the molecular weights of proteins in biological tissues and extracts.

The technique is developed using crosslinked dextran. The solid-phase matrix consists of porous beads that are packed into a column with a mobile liquid phase which allow the elution through the column. The mobile phase has access to both the volume inside the pores and the volume external to the beads. Sephadex is the commercial name for a series of cross-linked, hydrated, dextrans which allow seperation of molecules on the basis of size. They manufactured in the form of small beads, 10-15 μm in diameter, which have an internal volume to small enough to penetrate the cross-linked lattice structure. Due to manufacturing in different size of pores, appropriate exclusion characteistics can be chosen for the system of interest.

Unlike many other chromatographic methods, size exclusion is not an adsorption technique. Seperation is depend on the ability of molecules to entrance the pores of bead volumes. Large molecules remain in the external volume of the beads, as they are unable to enter the pores. They prefer the shorter flow path so, they pass through the column relatively early.

Size exclusion is suited to commercially available standard low-pressure chromatography systems. Systems require a column packed with a matrix offering a suitable fractionation range, a detector to monitor the eluting proteins, a chart recorder for viewing the detector response, and a fraction collector for recovery of eluted proteins. The most commonly peristaltic pumps are used, which are relatively effective at low flow rates and also inexpensive. In SEC, resolution is dependent on column length. Column length is a balance between resolution and run time. Protein elution is most often monitored by absorbance in the ultraviolet range, either at 280 nm, which is suitable for proteins with aromatic amino acids, or at 206 nm, which detects the peptide bond (Cutler 2003).

1.2. Enzymes

Enzymes are biological catalysts that make easy the conversion of substrates into products by lowering the activation energy of the reaction. Nearly all known enzymes are proteins. The regions of the enzyme which are directly involved in the catalytic process is called active sites. Some enzymes require no chemical groups for activity other than their amino acid residues. Others require an additional chemical component which is called cofactor. Cofactors can be either one or more inorganic ions and a complex organic or metalloorganic molecule.

Substrate(s) ⟶ Product(s)

Enzyme nomenclature are related to the function of the enzyme, in other words, to the type of reaction catalyzed. All known enzymes fall into six categories. The six main divisions are the oxidoreductases, the transferases, the hydrolases, the lyases, the isomerases, and the ligases (synthetases). Oxidoreductases catalyze the transfer electrons and protons from a donor to an acceptor. Transferases catalyze the transfer of a functional group from a donor to an acceptor. Hydrolases promote the cleavage of C-C, C-O, C-N and other bonds by water. Lyases catalyze the cleavage of bonds by elimination, leaving double bonds (or, in the invert process, catalyze the addition of groups across double bonds). Isomerases promote geometric or structural rearrangements or isomerizations. Lastly, ligases catalyze the joining of two molecules, and often require the hydrolysis of a pyrophosphate bond in the cofactor adenosine triphosphate to provide the energy required for the synthetic step (Mikkelsen and Corton 2004).

1.3. Lipases

1.3.1. General Description

Lipases are acyl hydrolases and water-soluble enzymes that play a key role in fat digestion by cleaving long-chain triglycerides into polar lipids. Because of an opposite polarity between the enzyme (hydrophilic) and their substrates (lipophilic), lipase reaction occurs at the interface between the aqueous and the oil phases (Reis, et al. 2008).

1.3.2. Lipase as Biocatalysts

Lipases (EC.3.1.1.3,triacylglycerol acylhydrolases) are a group of enzymes, which have the ability to hydrolyze triacylglycerols at an oil-water interface to release free fatty acids and glycerol. Lipases are present in microorganisms, plants and animals (Jisheng, et al. 2005). Lipases catalyze a wide range of reactions, including hydrolysis, inter-esterification, alcoholysis, acidolysis, esterification and aminolysis(Joseph, et al. 2008). This hydrolytic reaction is reversible. In the presence of organic solvents, the enzymes are effective catalysts for various inter-esterification and trans-esterification

reactions. Furthermore, microbial lipases show regiospecificity and chiral selectivity (Gupta, et al 2003). Especially microbial lipases have different enzymological properties and substrate specificities. Many species of bacteria, yeast and molds are found to produce lipases (Liu, et al. 2008).

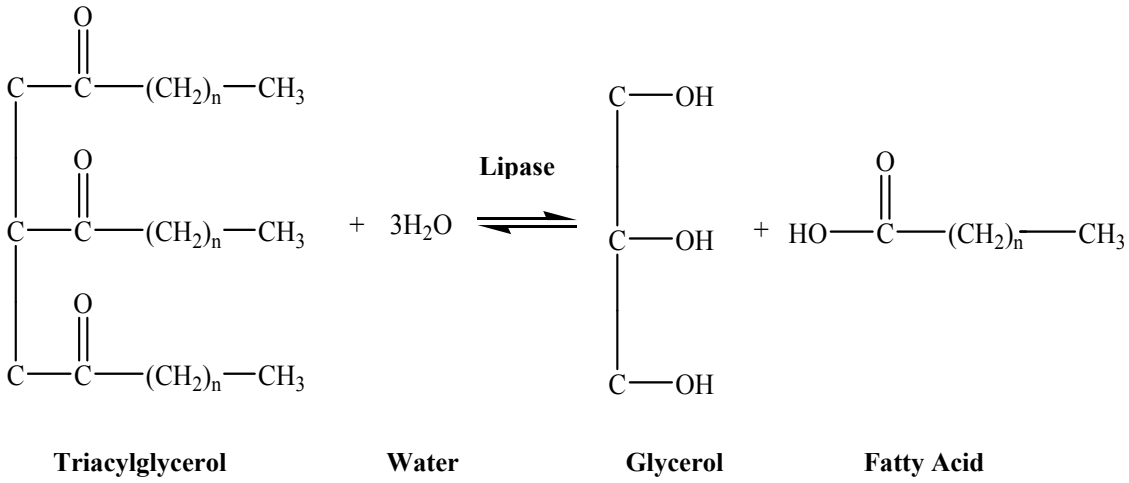


Figure 1.5. Hydrolysis or synthesis of a triacylglycerol substrate catalysed by a lipase enzyme (Source: Thomson, et al. 1999)

Their biotechnological potential is relying on their ability to catalyze not only the hydrolysis of a given triglyceride, but also its synthesis from glycerol and fatty acids. Therefore, microbial lipases have many industrial applications(Jaeger, et al. 1999). The temperature stability of lipases are the most important characteristic for industiral use (Choo, et al. 1997). The extracellular bacterial lipases are commercially valuable, because their bulk production is much aesier (Gupta, et al. 2004). Lipase catalized reactions are widely used in the manufacturing fats and oils, detergents and degreasing formulations, food processing, the synthesiss of fine chemicals and pharmaceuticals, paper manufacture, and production of cosmetics. Lipases are also used for accelerate the degradation of fatty wastes and polyurethane (Jisheng, et al. 2005).

1.3.3. Bacterial Lipases

Bacteria produce different classes of lipolytic enzymes including carboxylesterases (EC 3.1.1.1) and hydrolyzing water-soluble esters and lipases (EC 3.1.1.3) which hydrolyze long-chain triacylglycerol substrates (Rosenau and Jaeger 2000). Many bacterial species produce lipases which hydrolyze esters of glycerol with

long-chain fatty acids. They act at the interface generated by a hydrophobic lipid substrate in a hydrophilic aqueous medium. Lipases shows interfacial activation, a sharp increase in lipase activity observed when the substrate starts to form an emulsion. As a consequence, the kinetics of a lipase reaction does not follow the classical Michaelis-Menten model. With only a few exceptions, bacterial lipases are able to completely hydrolyze a triacylglycerol substrate although an ester bonds are more favourable (Jaeger, et al. 1994). Some important lipase-producing bacterial species are *Bacillus, Pseudomonas* and *Burkholderia* (Svendsen 2000). Numerous lipase assay methods are available using coloured or fluorescent substrates which allow spectroscopic and fluorimetric detection of lipase activity. Another important assay is based on titration of fatty acids released from the substrate.

Usually enzymes are not stable in organic solvents and they are tend to denature and lose their activities. But lipases remain stable and active in organic solvents without any stabilizer. Substrates of lipase are often insoluble or partially soluble in water and thus the use of organic solvents or organic–aqueous solutions is in favor of some reactions (Zhao, et al. 2008) Because of its ability to catalyze in organic solvents, many new biotechnological applications of lipases have been identified. One of the applications is the synthesis of chirally important drugs and drug intermediates (Singh and Benarjee 2007). Substrates and products of lipase-catalyzed mechanisms are often insoluble in aqueous solution, and the enzyme is usually insoluble in organic solvents. Reactions catalyzed by lipases are carried out in organic-aqueous interface. That process is desirable because the separation of enzyme from substrates or products is easy (Rahman, et al. 2005).

The synthesis and secretion of lipases by bacteria is influenced by a variety of environmental factors like ions, carbon sources, or presence of non-metabolizable polysaccharides.The secretion pathway is known for Pseudomonas lipases with *P.aeruginosa* lipase using a two-step mechanism and *P.fluorescens* lipase using a one-step mechanism. Additionally, some *Pseudomonas* lipases need specific chaperone-like proteins assisting their correct folding in the periplasm.These lipase-specific foldases which show a high degree of amino acid sequence homology among different Pseudomonas species are coded the lipase structural genes.A comparison of different bacterial lipases on the basis of primary structure revealed only very limited sequence homology. However, some of the bacterial lipases reveal a conserved folding pattern called the alpha/beta-hydrolase fold. The catalytic site of lipases is buried inside the

protein and contains a serine-protease-like catalytic triad consisting of the amino acids serine, histidine, and aspartate (or glutamate). The active site is covered by a lid-like alpha-helical structure which moves away upon contact of the lipase with its substrate, thereby exposing hydrophobic residues at the protein's surface mediating the contact between protein and substrate (Svendsen 2000).

1.3.4. Fermentation Conditions

Bacterial lipases are mostly released outside of the cell that is called extracellular enzyme. Bacterial lipases influenced by nutritional and physico-chemical factors; such as temperature, pH, nitrogen and carbon sources, presence of lipids, inorganic salts, stirring conditions, dissolved oxygen concentration (Rosenau and Jaeger 2000).

The major factor for the expression of lipase enzyme is carbon source. Lipases generally produced in the presence of lipid source such as oil, triacylglycerols, fatty acids, hydrolyzable esters, tweens and glycerols addition to carbon source, the type of nitrogen source is also influence the production of lipases. Generally, organic nitrogen source is prefered by bacteria, such as peptone and yeast extract (Gupta, et al. 2004).

The initial pH of the growht medium is improtant for lipase production. Most bacteria prefer pH around 7.0 for their best growth and lipase production. The optimum temperature for lipase production is parallel with the growth temperature of the respective microorganism. It has been reported that lipases are produced in the temperature range from 20 to 45 ^{o}C (jaeger, at al. 1999). Incubation periods change from few hours to many days until the maximum lipase production from bacteria is recorded.

1.3.5. Purification of Bacterial Lipases

Purification methods need to be applied in order to understand enyzme functions, it’s relations between environment and it’s 3-D structures (Reis, et al. 2008). In addition, purification of enzymes allows determination of their primary amino acid sequence and the three-dimensiona structure. The X-ray studies of pure lipases enable the establishment of the structure–function relationships. That knowledge provides a

better understanding of the kinetic mechanisms of lipase action on hydrolysis, synthesis and group exchange of esters (Saxena, et al. 2002). Knowledge of the three-dimensional structure of lipases plays an important role in designing and engineering lipases for specific purposes.

Pre-purification steps involve concentartion of culture by ultrafiltration, by ammonium sulfate or cold aceton precipitation and by extraction with organic solvents. In further process, affinity chromatography (especially hydrophbic interaction chromatography) is the best method since lipases are hydrophobic and have large hydrophobic surfaces in their active sites (Gupta, et al. 2004). Alternatively ion-exchange and size-exclusion chromatography are prefered after precipitation step because of high cost of hydrophobic resins.

1.3.6. Properties of Lipases

Lipases from several microorganisms have been stuied. According to their proved properties, lipase used variety of industies. Generally, bacterial lipases have neutral or alkaline pH value and show activity in a broad pH range (pH 4 to pH 11). The thermal stability of lipases ranging from 20^{o}C to 60^{o}C. Stability of organic solvents is desiable in synthesis reaction. Most of bacterial lipases are stable in organic solvents (Gupta, et al. 2004).

According to substrate specificity microbial lipases divided into three categories; nonspecific, regiospecific and fatty acid-specific. Nonspecific lipases behave randomly on the triacyglyceride molecule and produce complete breakdown of triacyglyceride to fatty acid and glycerol. Conversely, regiospecific lipases are 1,3-specific lipases which hydrolyze only primary ester bonds (ester bonds at atoms C1 and C3 of glycerol) and thus hydrolyze triacylglyceride to give fatty acids. Fatty acid-specific lipases display activity in fatty acid presence (Thomson, et al. 1999, Gupta, et al. 2004).

1.4. Lipase Assay

Lipases have lipolytic and esterolytic activity. So they have a wide range of substrate. Lipase activity can be obtained using an assay protocols which are differ in the terms of their basic principle, substrate selectivity and sensitivity. The activity of

lipases can be assayed by monitoring fatty acids or glycerols which is release from triacylglycerols. Since lipases act at the oil/water interface, changing the properties of the interface is an important gauge for measuring lipolytic activity (Gupta, et al. 2003). These tests are very suitable for fast screening on agar plates.

Agar plate assay is the most frequently used procedures for screening of lipase existant on petri dishes. In this assay coloured holes appear around the responsible colonies. The three substrate which are mostly used for plate assay is Tributylglycerol (tributyrylglycerol), Tween 80 and Tween 20. If Tweens are used as substrates for lipase, no dyes are required for visualization (Thomson, et al. 1999) They give the white clear zones around the colonies when hydrolysis is pointing out either esterase or lipase activity (Gupta, et al. 2003). These substrates are easily hydrolysed by esterase and may give wrong results for lipase presence. The fluorescence dye Rhodamine B agar plate assay is describe as a true lipase assay which indicate the zone of lipolysis as an orange fluorescence under UV light at 350nm (Figure 1.6). Regardless of substrate, the indicator dye used must undergo a definite color change with a change in pH. No fluorescent halos are formed when esterase is tested in that assay. Rhodamine B in the presence of olive oil forms a fluorescence complex with free fatty acids (Thomson, et al. 1999).

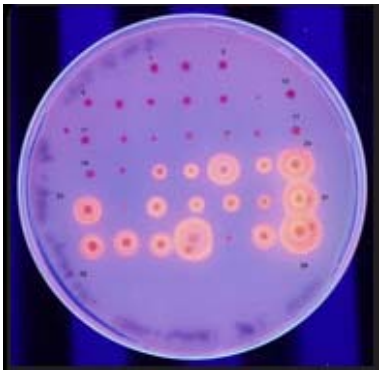


Figure 1.6. Lipase positive colonies on agar containing Rhodamine B dye and olive oil (Source: Applimexsystems 2008)

Titrimetric methods for assaying lipase are popular, the oldest and sensitivity in many applications. A successful measurement of lipase activity by titration requires stable emulsions and absolute control of pH during hydrolysis. Titration methods for the determination of the lipase activity are wide spread by using its tributyrin or olive oil as

a substrate (Starodub 2006). Reaction starts by addition of a lipase solution.Libetrated fatty acids are continuously titrated with suitable bases (Kordel, et. al 1991). Method performance is improved by special electrodes which is called pH-stat method. Triolein is generally used as a substrates rather than tributyrin, triacetin (triacetylglycerol) and tripropionin for estimation of enyzme activity (Gupta, et al. 2003, Thomson, et al. 1999). Titrimetric methods are time consuming process rather than others.

Spectrophotometric lipase assay is other important assay. P-nitrophenyl esters with aliphatic acyl chains of various lengths commonly used for investigating esterase and lipase activity. Hydrolysis of the substrate by lipase releases p-nitrophenol, which can be measured spectrophotometrically in 410 nm. Enzyme activity is expressed as μmoles of p-nitrophenol released per minute (Thomson, et al. 1999, Ruchi, et al. 2007). Short-chain esters, like acetate or butyrate, are used to measure esterase activity, while longer chains such as laureate, palmitate or oleate are used to investigate lipase activity. This method also has limitations. These esters, particularly with short acyl chains, can be hydrolyzed by non-specific esterases, non-enzymatic proteins or proteases. For that reason, these assays are best suited for purified lipases (Gildam and Lehner 2004).

1.4.1. Activity Staining for Lipases

Activity stains gain a great importance during the isolation, purification, and characterization of enzymes when a particular catalytic reaction is involved. The detection of this activity gives the unclear identification of the zone of interest on the electrophoresis gel. After separation, the gel is removed from the electrophoresis apparatus and is immersed in a substrate solution. Detection relies on the formation of a colored product, if the product of the reaction that enzyme catalyzes, by enzyme in the zones containing the enzyme. This type of staining cannot be done after SDS-PAGE, because the enzyme often loses its catalytic activity (Kralova 1999).The detection of enzymes by activity staining is important not only for the direct detection of separated enzymes, but also allows the identification of a particular analyte species in very complex sample mixtures (Mikkelsen and Corton 2004).

A common analytical technique for protein identification in a mixture is their electrophoretic separation and following detection on polyacrylamide gels. Enzyme activity staining after native PAGE is a widely used technique for enzyme analysis. It

allows the detection of a specific enzyme present in a small amount of extract (Rivoal, et al. 2001). The technique, based on SDS-PAGE, and substrate interaction with polyacrylamide gel in order to the detection of enzyme activity is called Zymography (Saminathan, et al. 2008). Samples are prepared in the standard SDS-PAGE treatment buffer but without boiling, and without a reducing agent (non-denaturating native page). Native PAGE protocols prevent the sulfide bonds so the proteins folding structure. By this way proteins do not loose their activity.

Methods for activity staining of lipases include fluorescent substrates, substrate analogs, or various esters which can be hydrolyzed by both lipases and esterases. Although highly specific esters have been used for that process, substrates can not differentiate between lipases and esterases. Use of pH indicators is the better way to detect hydrolases that cause a change in pH during catalysis (Ong and Chang 1997).

Lipase-specific oil-based dye detection methods have also been developed, which involve either fluorescent dye rhodamine or pH indicator dyes such as Victoria blue. Rhodamine-olive oil method is very commonly used, but it is less sensitive and requires prolonged incubation time for detectable fluorescence (Singh, et. al 2005).

1.5. *Pseudomonas* Lipases

Pseudomonas lipases play an important role in biotechnology both as hydrolases for detergent additives and synthases catalyzing the kinetic resolution of racemic compounds. Large-scale production of *Pseudomonas* lipases needs correct folding and secretion through the bacterial membranes. Lipase from Pseudomonas are excellent catalysts in a variety of synthetic organic transformations, also provide the enantioselectivity of the hydrolysis of a chiral ester (Manfred and Jaeger 1998).

Although a wide variety of Gram-positive and Gram-negative bacterial species produce lipases, the most widely used enzymes originate from the genus *Pseudomonas*. These lipases have been grouped into three categories according to amino acid sequence homology (Jaeger, et al. 1994). Group I include lipases from *P.aeruginosa, P.alcaligenes*, and *P.fragi* with a Mr of 30000; group II cover lipases from *P.cepacia* and *P.glumae* which have larger Mr of 33000; group III involve lipases from *P. fluorescens* has a Mr of 50000.

Lipases belonging to group I and II are similar and both contain two cysteine residues forming one disulfide bond, and they need a chaperone-like protein named

lipase-specific foldase (Lif) for correct folding and secretion. Lipases which belong to group III is different from group I and II. It is secreted by a different mechanism involving a transporter protein (Manfred and Jaeger 1998).

A number of investigations have concentrated on purification and characterization of lipase from Pseudomonas spp. *Pseudomonas fragi* produced extracellular and intracellular lipases when grown in whey media. They obtained partially purified lipase extracts by precipitation with ammonium sulphate. Native PAGE of the extracellular lipase showed the presence of a major band with a molecular weight of 25.5 kDa, whereas the intracellular lipase showed the presence of three fractions with molecular weights of 35.5, 49 and 70 kDa.

Extracellular *Pseudomonas* lipase is able to interact with alginate. Based on this observation, procedures were designed which allow rapid, simple and inexpensive purification of an extracellular lipase from a strain of *Pseudomonas aeruginosa.*

An extracellular alkaline lipase of alkalophilic *Pseudomonas pseudoalcaligenes* was purified to homogeneity using acetone precipitation and chromatography on Sephadex G-100. SDS-PAGE revealed the molecular weight of the lipase as 32 kDa and its pI was 7.3 (Saxena, et al. 2002)

Table 1.2. Pseudomonas lipases for biotechnological applications
(Source: Manfred and Jaeger 1998).

Lipase producing *Pseudonomas* strain	**Mr (kDa)**	**3-D structure (Ref.)**	**Biotecnological applications**	**Commercialized by**
P. aeruginosa	30	Jaeger, et al. 1993	Organic synthesis	Merck (USA)
P. alcaligenes	30		Detergent additive	Genencor international (USA)
P. mendocina	30	Boston, et al. 1997	Detergent additive	Genencor international (USA)
P. cepacia	33	Schrag, et al. 1997	Organic synthesis	Amano (Japan)
P. glumae	33	Noble, et al. 1993	Organic synthesis	Unilever (The Netherlands)
P. fluorescens	50		Organic synthesis	biocatalysts (UK)

CHAPTER 2

PROTEIN SEPERATION

2.1. Electrophoresis

Electrophoresis is a bioanalytical tool used in fundamental research for the isolation and identification of high molecular weight biomolecules. The separation is based upon the mobility of charged macromolecules under the influence of an electric field. Mobility of a macromolecule depends on the magnitude of its charge, its molecular weight, and its shape (its tertiary or quaternary structure). Most biopolymers, such as proteins and nucleic acids, are charged, so they can be separated and by electrophoretic methods (Mikkelsen and Corton 2004).

Two electrodes seperate macromolecules though the selected medium in electrophoretic seperation. One end of the medium is the positively charged anode, and the other is the negatively charged cathode. Along the medium, positively charged species migrate toward the cathode and negatively charged species move toward the anode. When a voltage is applied across the electrodes, a current is generated from the movement of ions in the electric field. Suitable running buffer is used in order to provide continous current between electrodes.

2.1.1. Electrophoretic Support Media: Polyacrylamide Gels

Polyacrylamide gels are prepared by the reaction of acrylamide (monomer) with N,N'-methylenebis(acrylamide) (cross-linker) in the presence of a catalyst and initiator. Initiators include ammonium persulfate and potassium persulfate, where the $S_2O_8^{-2}$ dianion decomposes into two $SO_4^{\cdot-}$ radicals, while the commonly used catalyst is tetramethylethylenediamine [TEMED, $(CH_3)_2N(CH_2)_2N(CH_3)_2$], which reacts with the sulfate radical anion to produce a longer lived radical species.

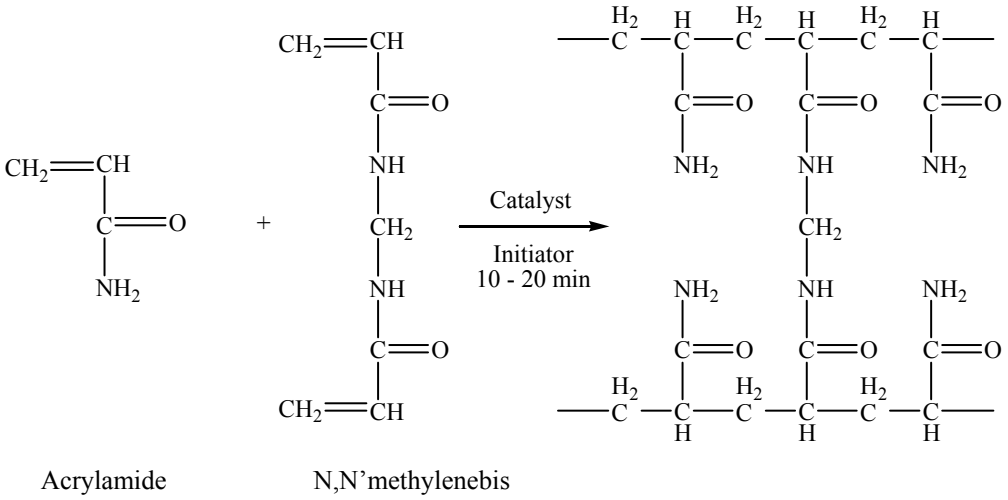


Figure 2.1. Reaction of polyacrylamide gel formation

The concentration of TEMED used for gel preparing determines the length of the polyacrylamide chains formed. The polymerization on polyacrylamide gel is a random process, diverse a distribution of pore sizes ocur (Figure 2.2). The average pore size depends on the total amount of acrylamide used and the degree of crosslinking. The total monomer concentration is generally between 5 and 20% by weight (Mikkelsen and Corton 2004)

2.2. Protein Seperation

There are three seperation approaches before the proteins are digest; 1D-SDS-PAGE, 2D-SDS-PAGE and isoelectric focussing. Beside these, chromatographic methods are also used; but, three of them are very popular and widely used in many studies. With electrophoresis methods, proteins seperate according to their physical properties such as molecular weight and isoelectric point.

An electrophoretic method commonly use the detergent sodium dodecyl sulfate (SDS) which equilibrate the charge of proteins. SDS binds to proteins in amounts with the proportional molecular weight of the protein, about one molecule of SDS for every two amino acid residues. The bound SDS contributes a large net negative charge, makes each protein a similar charge-to-mass ratio. In addition, the native conformation of a protein is change when SDS is bound, and most proteins assume in a similar shape. Electrophoresis in the presence of SDS therefore separates proteins almost on the basis of molecular weight (Nelson and Cox 2008).

2.2.1. Two-dimensional SDS-PAGE

Two-dimentional SDS-PAGE is the best method to resolve highly complex protein mixtures. Two-dimentional SDS-PAGE seperate proteins according to their two different phycical properties; isoelectric point (first dimension) and molecular mass (second dimension). In first dimension, proteins resolve on the basis of isoelectric point by the help of isoelectric focusing systems. In second dimension, focused proteins resolve by electrophoresis on a polyacrylamide gel. Seperated proteins seen on gel as a spot (Figure 2.3). In IEF process, immobilized pH gradient (IPG) strips, which have different pH ranges, are used for focussing. By changing PH range of IPG, different gel images obtain with different resolution properties. Narrow range of pH give beter resolution, so proteins with higly similar isoelectric point can be seperate.

On the other hand, two dimensional SDS-PAGE has very long and difficult procedure, also has low reproducibility; it is difficult to catch the same gel image in second trial. Other limitations are poor solubility of membrane and hydrophobic proteins and difficulties in resolving and identifying very acidic or basic proteins.

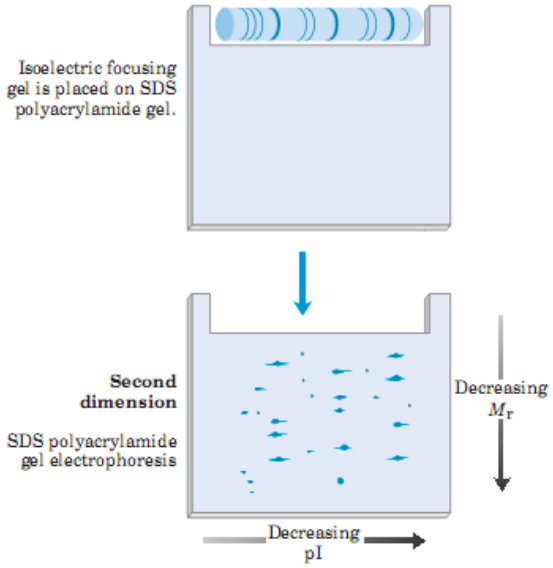


Figure 2.2. Two dimensional electrophoresis
(Source: Nelson and Cox 2008)

While two-dimensional SDS-PAGE use two physical properties of proteins, one-dimensional SDS-PAGE use only one physical property, molecular masses.

2.2.2. One-dimensional SDS-PAGE

One-dimensional SDS-PAGE is the most widely used protein seperation method in proteomic studies. The seperation method is based on the binding of SDS to the protein, which makes protein to negative charge; by this way, molecular weight of protein become the only one parameter for seperation. When the gel expose to high voltage, the protein-SDS complex begin to migrate on polyacrylamide gel based on their ability to penetrate the pores of gel (Figure 2.4). The seperated proteins seen on gel as a band.

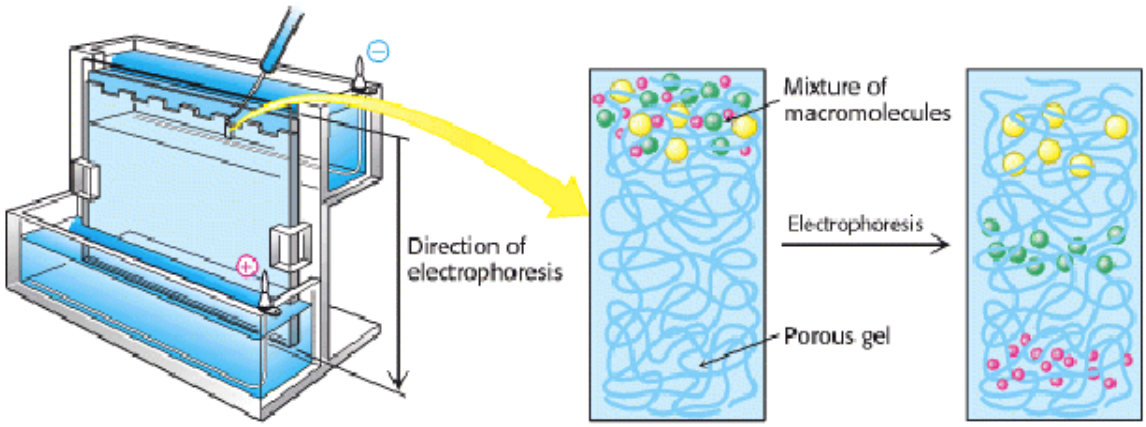


Figure 2.3. Polyacrylamide gel electrophoresis
(Source: Stryer 1995)

One-dimension-SDS-PGE is done on gels that have different degree of cross-linking. The lower degrees of cross-linking gel allow to pass larger protein transition. Degree of cross-linking should be choosen based on the characteristics of the protein. Sample containing relatively low molecular weight proteins resolve in highly cross-link gel, sample containing relatively high molecular weight proteins revolse in lower cross-link gel. Resolution on gel change by controling the molecular sieving effect. If the path leght of protein is getting longer, the beter seperation observed with higher resolution.

2.4. Stains and Dyes

The most common protein visualization method on gel is staining a gel with a special dyes that interacts in a nonselective way with proteins. Gels are removed from

the electrophoresis apparatus and treated with staining solutions for a sufficient time. There are lost of avaliable electrophoretic staining dyes and procedures which have different molecular mass dedection limits. Coomassie blue staining, silver staining and fluorescence staining are the most preferred detection methods for proteomic studies. By applying different staining techniques to same gel sample, different analytical data can be obtained.

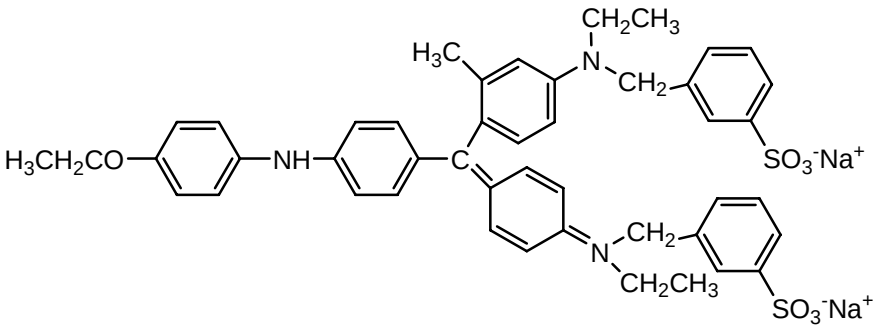


Figure 2.4. Coomassie brilliant blue G-250
(Source: Mikkelsen and Corton 2004)

Coomassie brilliant blue (CBB) staining is widely used as a protein detection technique (Figure 2.5). Coomassie brilliant blue staining procedure is easy to apply, dye can be removed from gel and then stain again, it is convenient for MS analysis, and also it is cheap method rather than other staining techniques. CBB colors the proteins on a gel with dark blue. Coomassie brilliant blue G-250 is the one of CBB dyes which is used for general protein staining. It has a medium range of detection limit (Kinter and Sherman 2000).

After staining, gels may be photographed under UV, visible light, or white light to yield qualitative information about the number and positions of the bands.

2.5. The Aim of the Study

In this study, investigation, characterization, purification and identification of lipase enzyme from a *Pseudomonas* strain was studied by using different analytical approach. The main purpose of this study to understand how lipase enzyme behave under condition of different substrates, pH values, organic solvents, metal ions and temperatures and also to find out enzyme's amino acid sequence. Colourimetric

methods on agar plates, activity staining on PAGE methods and spectrophotometric methods were used for investigation process. Chromatography was used for purification process.

CHAPTER 3

EXPERIMENTAL

3.1. Bacteria Growth Conditions

Responsible bacteria, a *Pseudonomas* strain, isolated from soil that is taken from Erciyes Mountain in Central Anatolian Region in Kayseri. Bacteria was grown at 25^{o}C for two days and at 4^{o}C for four days. Two different growth media, both agar and broth, used for a *Pseudonomas* strain. One is Luria Bertani (LB) media and the second is minimal medium (MM) media which contains only sufficient nutritions for bacterial growth, generally contain no proteins. According to desired procedure %1(v/v) higly refined olive oil, tween80 and tween20 added in both medium.

- LB medium: 10g tryptone, 5g yeast extract, 5g NaCl were weighted. After this step 800ml of water added and pH adjusted to 7.5 with NaOH. 15g of agar added if petri dishes used. The volume completed to 1 liter. The mixture was autoclaved for sterilization for 15 minutes at 121^{o}C under high pressure.
- Minimal medium: For 1 liter of minimal medium, 200ml of 5X minimal salt solution, 800ml of water and 15g of agar (if nedded) added. After autoclave process, 2ml 1M $MgSO_4$ (steril filtrated), 0,1ml 1M $CaCl_2$ (steril filtrated) and 20ml of %20 glucose (steril filtrated) (carbon source) solution added to media.
- 5X minimal salt solution: 64g Na_2HPO_4-$7H_2O$, 15g KH_2PO_4, 2,5g NaCl and 5g NH_4Cl (nitrogen source) weighted and dissolve in 1 liter water and autoclaved at 121^{o}C, 1 bar for 15 minutes.

3.2. Monitorize Lipase Activity on Agar Plates

The production of lipase activity by Pseudonomas strain was verified on both LB and MM agar containing %1 high refined olive oil(v/v) and fluorescent dye Rhodamine-B (%0.005)(dissolve in distilled water and steril filtrated). Orange-coloured fluorescent halos around lipase producing colonies could be seen when these agar plates were expose to UV light at 350nm. While plates were prepared gellan gum was used instead of agar.

Another lipase activity monitoring methods on agar plates is Tween20 and Tween80 methods. Agar plates prepared following LB agar procedure and added %1(v/v) Tween20 or Tween80 after autoclave process. White clear crystal occurs around the colonies which give pozitive results to lipase activity.

3.3. Protein Sample Preparation

Bacteria grown in both minimal medium broth and minimal medium broth containing %1(v/v) highly refined olive oil for three days at 25^{o}C, 200 rpm in incubator. Bacteria grown at 25^{o}C during three days in order to allow the strain releasing lipase enzyme. Bacterial culture was centrifuged at 7000rpm, 4^{o}C for 25 minutes at falcon tubes. Supernatants and pellets were seperated. A sufficient amount of supernatant taken and keep in ice for extracellular enzyme activity testing. All supernatant removed from pellets. Cells dissolved in 20mM tris-HCl buffer and vortex gently. In order to expose intracellular proteins, sonicator used for dissolved cells to decompose cell walls. Sonicator applied to solution for 5 minutes in 7X cycles in ice. Then sonicated mixture was centrifuged at 15000rpm, 4^{o}C, for 25 minutes. Supernatant used to determine the activity of intracellular enzyme activity. By this way, four types of crude enzyme extract obtained.

Also cold aceton precipitation method, that help to precipitate whole protein content in solution, was applied on these crude enzyme extracts. In aceton precipitation process, sufficient amount of sample was taken and two-fold volume of cold (-20^{o}C) aceton added into. After gentle vortex, store at -20^{o}C for 20 minutes. Then it was centrifuged at 15000rpm, 4^{o}C, 25 minutes in corex tubes. Supernatant removed and protein pellets dissolved in proper buffers (20mM tris-HCl buffer, pH 7.2). Prepared protein samples stored at 4^{o}C degree.

3.4. Enzyme Characterization

3.4.1. Substrate Specificity of the Bacterial Lipase

The substrate specificity of the bacterial lipase was studied with p-nitrophenly fatty acyl esters at various chain leghts (Table 3.1) . Lipase activity assayed by

measuring the amount of liberated p-nitrophenyl from its esters. Absorbance was continuously measured at 400nm in 1cm path lenght cell with double-beam ultraviolet-visible spectrophotometer. The substrates were dissolved in acetonitrile at a concentration of 50mM. Measurements done at 25^{o}C. In this assay, 100mM sodium phosphate buffer containing 150mM sodium chloride and 0,5% triton X-100, pH 7,2 was used for the purpose of keeping pH balance of the reaction media and helping the cleave of bonds. Substrates added in both blank and test cells. The reaction started by addition of 20µL of the enzymatic solution, so it is the last component of test cell.

Table 3.1. List of used p-nitrophenly fatty acyl esters at various chain leghts

Substrates	Abbreviations	Carbon Chains	Molecular Formula	Source
4-Nitrophenyl Acetate	pNPA	C2	$C_8H_7NO_4$	Sigma Ald.
4-Nitrophenyl Butyrate	pNPB	C4	$C_{10}H_{11}NO_4$	Sigma Ald.
4-Nitrophenyl Caprylate	pNPO	C8	$C_{14}H_{19}NO_4$	Fluka
4-Nitrophenyl Decanoate	pNPD	C10	$C_{16}H_{23}NO_4$	Sigma Ald.
4-Nitrophenyl Laurate	pNPDD	C12	$C_{18}H_{27}NO_4$	Sigma Ald.
4-Nitrophenyl Myristate	pNPM	C14	$C_{20}H_{31}NO_4$	Fluka
4-Nitrophenyl Palmitate	pNPP	C16	$C_{22}H_{35}NO_4$	Sigma Ald.

3.4.2. Effect of Temperature and pH on Activity

The substrate which gave the highest lipase activity selected and optimum pH and temperature tests done with it. In both studies, 100mM sodium phosphate buffer containing 150mM sodium chloride and 0.5% Triton X-100, pH 7.2 used as a buffer system. pH adjustment done with 3M HCl and 3M NaOH between pH4.0 to pH11.0.

After the investigation of optimum pH values, enyzme activities calculated at different temperatures by using desired pH and substrate. Enyzme activities studied at 4^{o}C, 15^{o}C, 25^{o}C and 35^{o}C.

3.4.3. Organic Solvent and Metal Ion Effect

In order to understand how organic solvent effect the enyzme activity, lipase assay was carried out in the presence of different organic solvents with %1 by volume.

One of the lipase assay test cuvette contained no organic solvent and that is used as a control sample.

The enyzme activity of lipase enyzme were also studied in the presence of metal salts. A certain amount of metal salt solution was added to lipase assay volume which makes the final concentration 1mM. Beside EDTA, chloride salts, sulfate salts and nitrate salts were used. $CoSO_4$, $Pb(NO_3)$, KNO_3, $MgCl_2$, $CaCl_2$, $ZnCl_2$, NaCl were the used compound in that study.

3.5. Kinetic Study

The main purpose of enzyme kinetic studies were the investigation of enyzme affinity to the proper substrates. For that purpose different parameters can be change whereas the others keep constant. Substrate concentration was the most preferable parameter for kinetic studies. The effect of substrate concentration on the hydrolysis rate was measured at different substrate concentrations varying 0.05mM to 0.7mM.

3.6. Molecular Weight Determination

In order to find out the approximate molecular weight of lipase enyzme, SDS-PAGE was run with the protein marker. The crude extract (the intracellular protein sample), selected fraction after size-exclusion chromatography and it's neigbours were used as a sample. Seperation was carried out in Thermo Scientific electrophoroses device. 12% polyacrylamide gel prepared according to protocol table.

3.7. Enzyme Activity Calculation

Lipase assay was studied with p-nitrophenly fatty acyl esters at various chain leghts. Absorbance reading was taken with the certain time interval at 400nm. According to absorbance versus minutes data, the curve, which has a raising behaviour, was drawn. Enzyme activity (units/ml enzyme) calculated acccording to equation below. The highest enzyme activity data assumed 100% and relative enzyme activity estimated for each data. To calculate specific enzyme activity (units/mg protein), bradford assay should be applied. Specific enzyme activity obtained by division of

enzyme activity value to protein content in mililiter. One unit release 1 nanomole (10^{-9} mole) of p-nitrophenol per minute at desired pH and temperature using p-nitrophenyl fatty acyl esters at various chain leghts.

$$\text{Units/ml enzyme} = \frac{(\Delta A\ 400nm/min\ Test - \Delta A\ 400nm/min\ Blank)(volume\ of\ assay,\ ml)}{(0{,}0148)(volume\ of\ enzyme\ used,\ ml)}$$

$$\text{Units/mg protein} = \frac{\text{Units/ml enzyme}}{\text{mg protein/ml enzyme}}$$

df: dilution factor

0,0148: micromolar extinction coefficient of p-nitrophenol at 400nm

Figure 3.1. Enzyme activity calculation equation (Source: Ateslier and Metin 2005)

3.8. Bradford Total Protein Assay

The Bradford total protein assay is the spectroscopic analytical methods which is used to determine the total protein concentration of responsible sample. In this method, Coomassie brilliant blue G-250 dye binds to proteins and change their colour from green to blue. That colour change is monitored at 595nm in UV-visible spectrophotometer. As the concentration of protein content is increase, the colour is getting darker and darker. Coomassie brilliant blue G-250 binds to arginine, lysine, and histidine residues in protein samples. Bradford assay applied samples before loading on SDS-PAGE gel and after every purification steps in order to calculate lost and gain of protein amount.

A series of Bovine serum albumin (BSA) Standard in different concentrations were prepared. According to this BSA standars, standard calibration curve was drawn with response to their absorbance values. Total protein content was calculated from standard calibration curve equation.

Table 3.2. Preparation of BSA standars

Test Sample	0.2mg/ml BSA volume, µl	Water Volume, µl	Coomassie Reagent Volume, µl
Blank	0	800	200
BSA Standard – 1 µg/ml	5	795	200
BSA Standard – 2 µg/ml	10	790	200
BSA Standard – 4 µg/ml	20	780	200
BSA Standard – 6 µg/ml	30	770	200
BSA Standard – 8 µg/ml	40	760	200
Protein Sample	50	750	200

Blank, BSA standards, and protein samples were prepared according to Table in 1cm disposable cuvettes and read at 595nm in spectrophotometer. The absorbance values of sample should be in the dynamic range of BSA standard calibration curve. For over concentrations, sample dilution could be done.

- Preparation of Coomassie Reagent: 10.0 mg of CBB G-250 (AppliChem) was dissolved in 5 ml of 95 % ethanol and then 10.0 ml of 85 % phosphoric acid (AppliChem) was added. The mixture was adjust to 100 ml with ultra pure water. The final solution was filtered through filter paper (Whatman No. 1) and was stored in a dark bottle at 4 ºC.

3.9. SDS-PAGE

SDS-PAGE applied on samples which are prepared in former steps and their proteins resolved according to their molecular weight differences. Seperation was carried out in Thermo Scientific electrophoroses device. 12% polyacrylamide gel prepared according to protocol table which consist of 30% acrylamide mixture, 1.5 M Tris-HCl buffer, pH 8.8, 10% SDS (w/v), 10% (w/v) ammonium per sulfate and TEMED. Glass plates sizes are 10x10cm and have ten holes for sample loading.

For resolving gels, 12% polyacrylamide gel was prepared by mixing 1.68 ml of water, 2 ml of 30 % acrylamide mixture, 1.25 ml of 1.5 M Tris-HCl (pH 8.8), 50 µl of 10 % SDS, 25 µl of 10 % ammonium per sulfate and lastly 2.5 µl of TEMED to final volume of 5ml. Polymerization started immediately after adding TEMED to mixture. The mixture was poured into the thin gap between two glass plates and allowed to

polymerization for 30 minutes. The gel covered by distilled water in each steps of SDS-PAGE to avoid drying.

For stacking gels, 4% gel was prepared by mixing 3.05 ml of water, 665µ of 30 % acrylamide mixture, 1.25 µl of 0.5 M Tris-HCl (pH 6.8), 50 µl of 10 % SDS, 25 µl of 10 % ammonium per sulfate and lastly 5 µl of TEMED to final volume of 5ml. The mixture was poured into the polymerased resolving gel. The comb containing 10 holes placed on the gel before polimerization.

After polymerization of stacking gel, the drag removed from the top of gel. The gel was placed in a buffer tank and the reservoirs were filled until it is reach the filling line level with 1X Tris-Glycine-SDS (TGS) running buffer.

The samples were mixed with sample buffer (SDS reducing buffer). The samples diluted with sample buffer with the ratio of 1:4(v/v). Then samples were heated at 95^{o}C in water bath for 4 minutes. Sample buffer make the samples's colour's blue. The samples loaded the gel in buffer tank in the presence of running buffer. Electrophoresis run at constant voltage at 65 volts for 30 minutes and followed by constant voltage at 105 volts for 2 hours until the blue dye reached the bottom of the gel.

After electrophoresis was finished, the gel was taken out from two glass plates by the help of the distillled water and place into the shallow staining tank. Coomassie colloidal blue staining solution was applied on gel for 24 hours with slow shaking. After staining process, the gel was washing with water and then treated with neutralization buffer (0.1 M of Tris-phosphate, pH 6.5) for three minutes. Then destaining solution (25 %, v/v, methanol solution) applied on gel less than one minutes. Lasty, gel place on fixation solution (20 %, w/v, of ammonium sulfate) for one day. After each process, gel was washed with water. After staining and destaining steps, the photograph of the gel under U.V. light was taken by the camera. The gel can be stored at 4^{o}C in the presence of %5 acetic acid solution(v/v) for years.

- Preparation of 5X Tris-Glycine-SDS (TGS) running buffer: 15.1 g of Tris-base (AppliChem) and 94 g of glycine (AppliChem) were weighed and dissolved in 900 ml of water. Then, 50 ml of a 10 % (w/v) SDS solution (not for native PAGE) was added and the final volume was adjusted to 1 liter with water. It can be stored at 4 ºC. For prepering 1X TGS, 5X TGS was diluted by ultrapure water.

- Preparation of 30 % acrylamide mixture: 29.0 g of acrylamide (AppliChem) and 1.0 g of N, N´-methylenebisacrylamide (AppliChem) were dissolved in a total volume of 60 ml of water. The total volume was adjusted to 100 ml with water. This solution can be stored at 4 ºC in dark bottles for one month.
- Preparation of 0.5 M, 1.5 M Tris-HCl, pH 8.8, pH 6.8: The desired amount of Tris-base was weighted and dissolved in 60 ml of water. pH was adjusted to 8.8 (for resolving gel) and pH 6.8 (for stacking gel) with 6 M HCl. Then the volume was adjusted to 100 ml with water. It can be stored at 4 ºC.
- Preparation of sample buffer: 3.8 ml of water, 1 ml of 0.5 M tris-HCl buffer, 0.8 ml of glycerol, 1.6 ml of 10% (w/v) SDS (not for native PAGE), 0.4 ml of 2-mercaptoethanol (not for native PAGE) and 0.4 ml of 1% (w/v) bromophenol blue were mixed to final volume of 8 ml. It can be store at room temperature.
- Preparation of Staining Solution: 40 g of ammonium sulfate (AppliChem) was dissolved in 300 ml of ultrapure water and mixed with 8 ml of 85 % phosphoric acid (AppliChem). Then 100 ml of methanol (Merck) was added and the final volume is adjusted to 500 ml with ultra pure water. Finally, 0.5 g of CBB G-250 was added to mixture. It can be stored at 4 °C.

While studing on native PAGE, same SDS-PAGE procedure followed without using SDS solution in each step and skipped protein sample-sample buffer heating process before loading samples on gel. Also 2-mercaptoethanol was not use when sample buffer solution prepared.

3.9.1. Activity Staining on Gel

Two native gels carring the same samples were run. One was stained with coomassie colloidal blue staining solution and the other is placed on the square petri dishes containing 15% (w/v) gellan gum, Rhodomine B reagent and 1% olive oil. It was incubated at 25^{o}C for 3 days. The protein bands which were responsible to lipase activity gave orange colour under U.V. light at 350nm. By comparing two gel images, the responsible protein bands were cut from the gel, which was stained with commassie colloidal blue staining solution, by the help of sharpe scalpel. Cutting bands were place

into eppendorf tubes with the presence of 5% acetic acid solution. Eight potential protein bands were delivered to proteome factory for their mass analyses.

3.10. Size-exclusion Chromatography

In size-exclusion chromatography, Sephadex G-100 column resin was used. 1g of Sephadex G-100 approximately reach volume to 15-20ml when swelled in water. According to calculated column volume(diameter: 1.25cm, height: 50cm), sufficient amount of sephadex G-100 weighted and dissolve in ultrapure water. It was incubated at 90^{o}C for 5 hours. Then column fixed with sephadex slurry and it was allowed to settle down completely. Column adapted to low pressure chromatographic systems and column condition done with approximately 300 ml of 50mM tris-HCL buffer, pH 8.2.

10 ml of sample loaded to column. The sample preparation process follow these steps. Bacteria grown in minimal medium containing %1 olive oil at 25^{o}C for three days. Broth culture centrifuged and supernatant removed. Pellets dissolve in 20mM tris-HCl buffer and sonicated for 5 minutes. Then mixtures was centrifuged and supernatant used as a sample which eluted in column. Elution continued with buffer until approximately 300 ml of buffer passing through the column.

Automatic fraction collector collected fifty drops in each glass tubes. Each fraction measured by UV-visible dedector, at 280 nm, and peaks were monitored by computer screen (Figure 3.1).

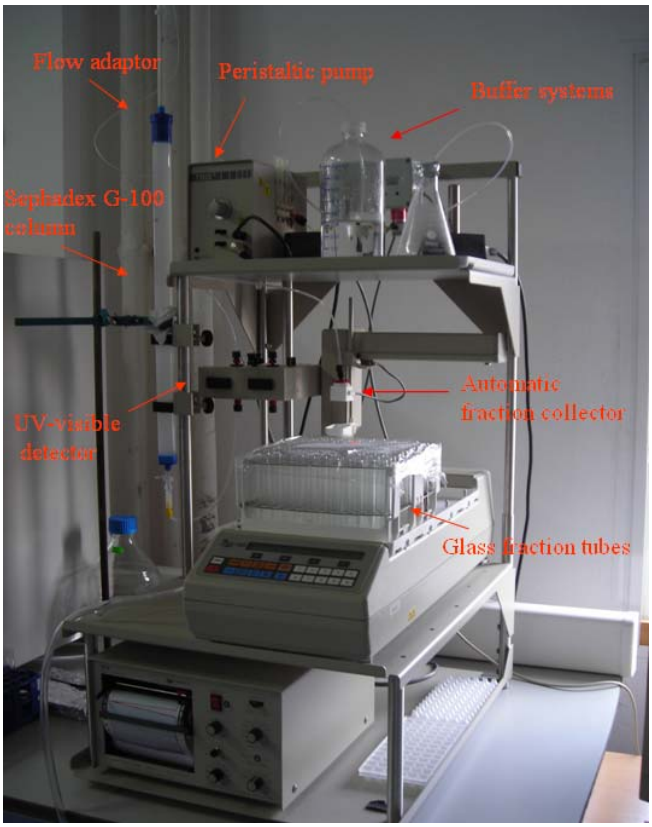


Figure 3.1. Size-exclusion chromatography system.

CHAPTER 4

RESULT AND DISCUSSION

In this study, Pseudonomas strain, which was isolated from Erciyes Mountain in Central Anatolian Region, was selected to investigate lipase activity. The study was deal with two subjects, one was characterization and the other was purification of bacterial lipase enzymes. In characterization part, the best substrate was found out and pH and temperature studies applied. Also, amino acid sequence obtained by mass analysis after activity staining process. In partially purification part, size exclusion chromatography used to seperate proteins according to molecular mass.

4.1. Investigation of Lipase Activity on Agar Plates

The production of lipase activity by Pseudonomas strain was verified on both LB and MM agar containing high refined olive oil and fluorescent dye Rhodamine-B at both 25^{o}C and 4^{o}C. Orange-coloured fluorescent halos around lipase producing colonies were seen when these agar plates (MM agar in figure) were expose to UV light at 350nm (Figure 4.1). While bacteria gave pozitive result to lipase activity after 3 days at 25^{o}C, orange colour observed after a week at 4^{o}C. Rhodomine-B dye test gave more convenient results than others. Because, the test show pozitive results to only lipase existance and not affected by bacterial metabolite wastes.

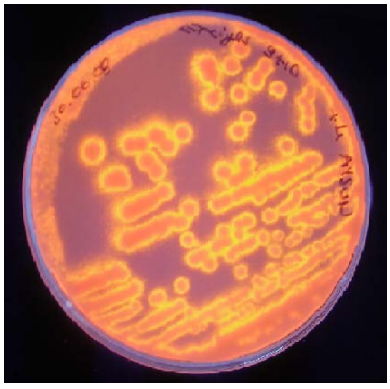


Figure 4.1. Photos of colonies under UV light, grown at 4^{o}.

Another lipase activity monitoring methods applied on Pseudonomas strain with Tween20 and Tween80 reagent. The use of Tweens as lipase substrates in screening lipase activity has been criticized. Because Tweens may be hydrolyzed by esterases, giving false-positive results for lipase test. White clear crystal occured around the colonies, seen in Figure 4.2, which give pozitive results to Tween test. Two different temperature, 25^{o}C and 4^{o}C, again was used for that test with different incubation times.

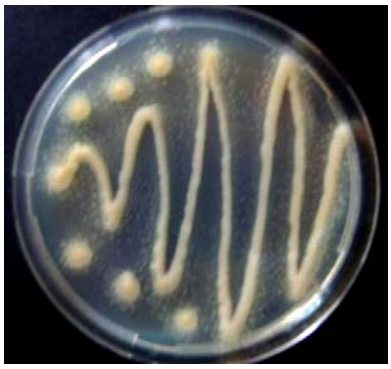


Figure 4.2. Photos of a *Pseudomonas* strain coloniesin the presence of Tween 80.

4.2. Activity Staining on Gel

Double native PAGE system was run which were carring the same samples. One gel staining with CCB dye, and the other is incubated with agar containing rhodomine-B reagent and olive oil. Bacteria grown both in only minimal medium and minimal medium with olive oil additives. After centrifuged, supernatant used as a crude sample. Accumulated cells dissolved in proper buffer and sonicated, then supernatant again used as a crude sample after centrifuge. By that way, eight of crude sample loaded to gel.

After incubation with agar, the only one sample's protein band give orange light under U.V. light. The sample is which grown in minimal medium with olive oil additives and sonicated after centrifuge step. This is show that lipase enzyme production induced by olive oil. Also it can be said that bacteria produce lipase enzyme but it can not released the media that live in it. Whereas many bacterial lipase enzymes are reported that they are extracellular enzymes, the used Pseudonomas strain show intracellular properties of lipase enzyme. Commercial lipase enzyme also studied and it gave pozitive result in activity staining.

The same procedure repeated by that samples. The responsible protein bands were detected in control gel which staining with CCB. Potential protein bands were cut and delivered to mass analysis.

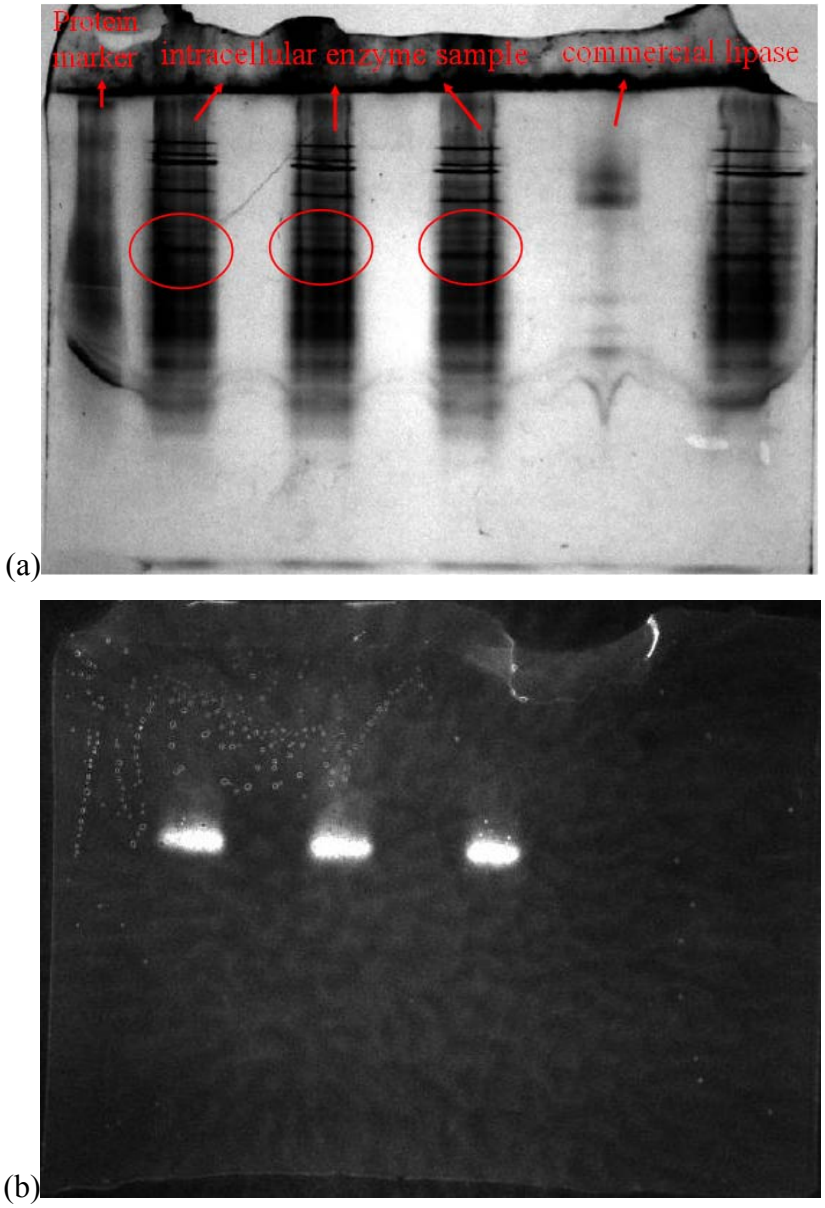


Figure 4.3. Gel images; (a)the native gel staining with CCB dye, (b) the native gel image under UV light which incubated with agar containing Rhodomine B reagent and olive oil.

4.3. Size-exclusion Chromatography

The size-exclusion chromatography was applied on intracellular protein sample which grown in minimal medium in the presence of olive oil. All drops collected in glass tubes. Spectrophotometric lipase assay was applied for each tubes, and p-nitrophenly deconate used as a substrate. Fraction with thirty three number give higher absorbance values so enzyme activities. After that knowledge, substrate specificity, pH, temperature and other characterization studies were done with fraction-33.

4.4. Visualization of Lipase Activity

Lipase activity assays were studied on U.V.-visible spectrophotometer at 400nm. Lipase assay results which done with both extracellular and intracellular crude protein samples showed that olive oil presence induced lipase activity. Also special activity staining on native gel showed that lipase enzyme produce in the cells, means that intracellular property.

4.5. Enzyme Characterization

4.5.1. Substrate Specificity

After size-exclusion chromatography, lipase assay applied on fraction-33 sample. Substrate specificity of a fraction-33 was examined with p-nitrophenyl esters of various fatty acids. The rates of hydrolysis for the fatty acids were as indicated in units/ml. In substrate specificity test, p-nitrophenly laurate which contain twelve carbon chains (C12), gave the highest enzyme activity. That is the another evidence that the responsible *Pseudomonas* strain produce lipase enyzme. Since lipases hydrolyze esters in emulsion and usually water-insoluble substrates, typically triglicerides composed of long-chain fatty acids, whereas esterases preferentially hydrolyze “simple” esters and usually only triglicerides bearing fatty acids shorter than six carbon chains (C6), these results strongly suggest that the enzyme used in this study showed an lipase activity. The better substrate with response to enzyme affinity was found out and futher characterization studies were done by using p-nitrophenly laurate as a substrate.

The graph (Figure 4.4) show the absorbance reading of sample during 24 hours with different substrates. For enzyme activity calculations, the sharp increase of absorbance values region was selected for each trial. Then according to equation (Figure 3.1) the enzyme activity of each trail was calculated (Figure 4.5.a). In relative activity calculation, the highest enzyme activity value assumed hundred percent, and other values relatively calculated (Figure 4.5.b). In Figure 4.4, p-nitrophenly laurate showed sharper increase than p-nitrophenly deconate between 20-25 hours, so it was show the higher enzyme activity comparing with other substrates.

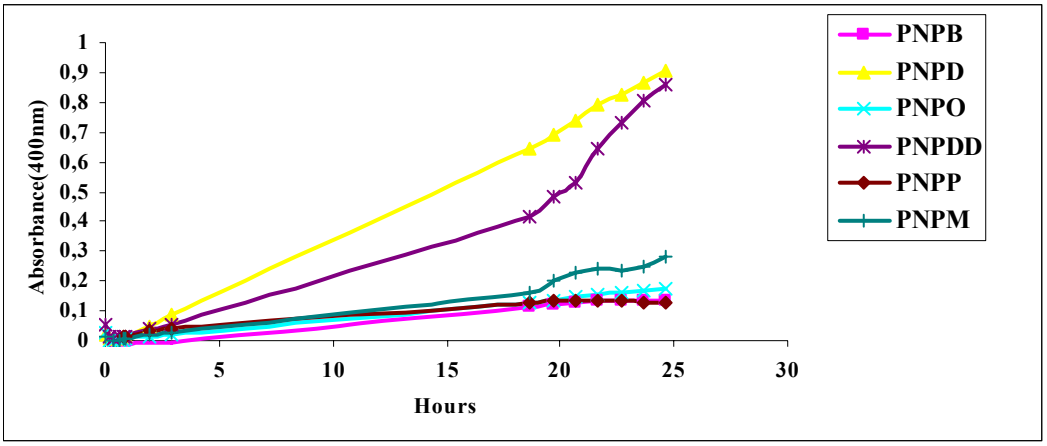


Figure 4.4. Absorbance values of substrate specificity test

(a)

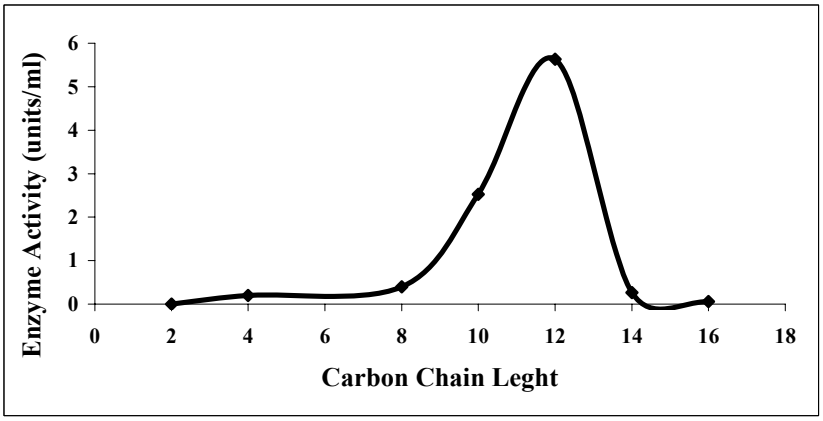


Figure.4.5. (Cont.) (a) Enyzme activity and (b)relative enzyme activity values aganist substrates containing different number of carbon chain leghts.

(Cont. on next page)

(b)

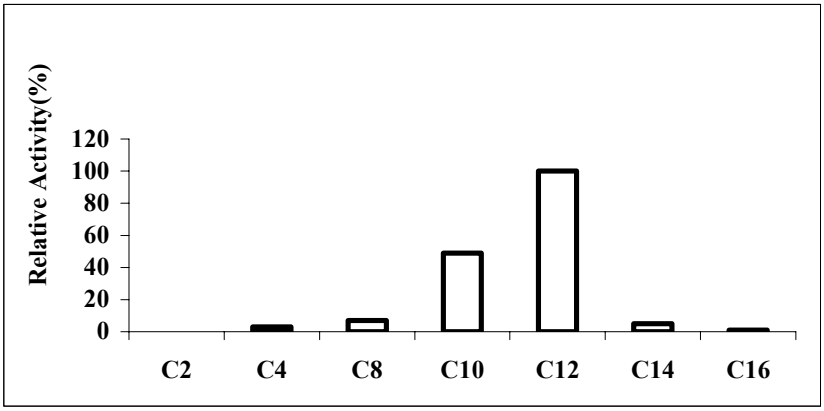
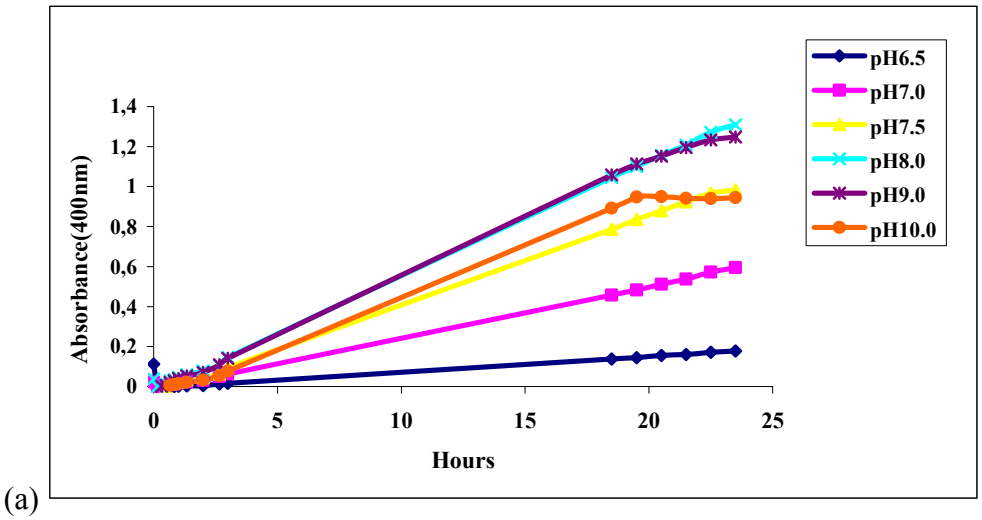


Figure.4.5. (Cont.) (a) Enyzme activity and (b)relative enzyme activity values aganist substrates containing different number of carbon chain leghts.

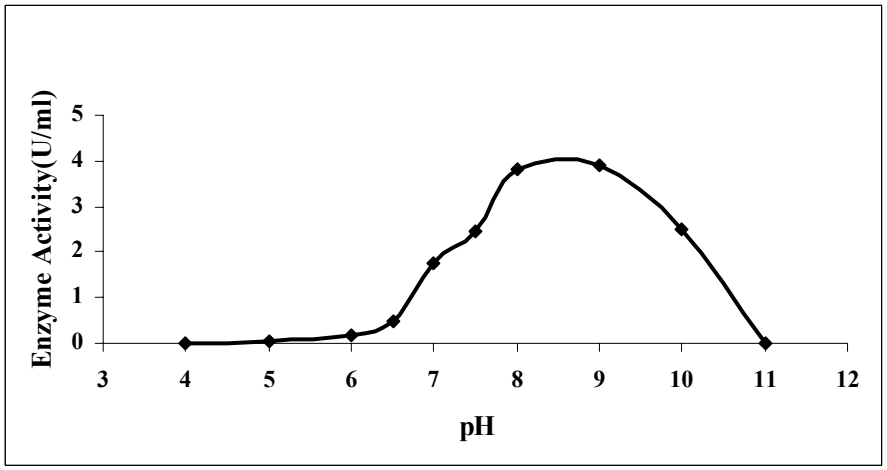
4.5.2. Optimum pH and Temperature

Optimum pH stability tests were continued with p-nitrophenly laurate as a substrate. The lipase activity was measured at various pHs in buffers with the same ionic concentrations. Enzyme gave better results at alkaline pH values such as pH 8.0 and pH 9.0 (Figure 4.6). It is desireable properties for industial applications. Generally *Pseudomonas* strain of lipase show their enzyme activity at alkaline pH. Furthermore, enzyme take place in a wide range of pH values. So it can be conclude that enzyme is resisted to pH changes.

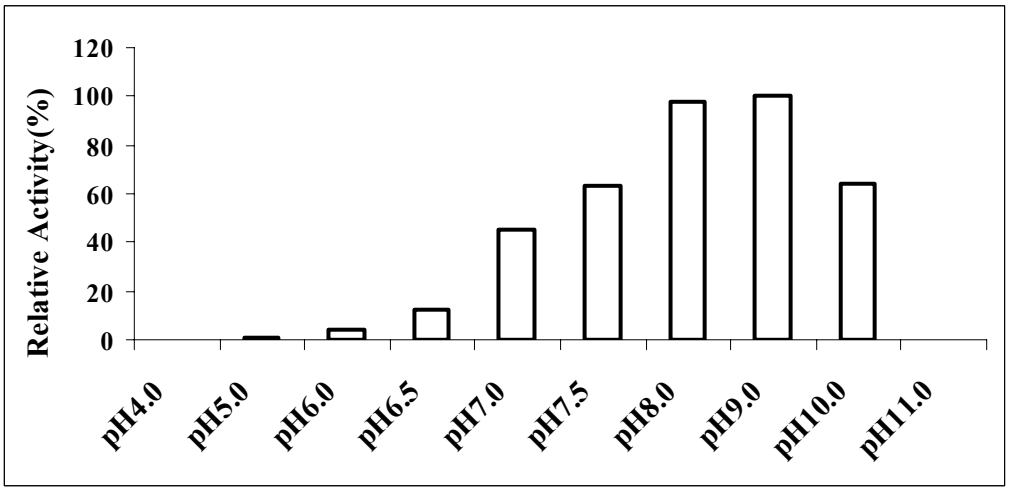
Temperature studies were done at pH 8.0 with p-nitrophenly laurate. The optimum temperature value was found as 25^{o}C. The optimum bacterial growth temperature was also 25^{o}C. Whereas bacteria can not be populate over 30^{o}C, enyzme works over that temperature values. Bacteria resisted a low range of temperature because, it was isolated from soil where cold climate use to prevail. Over 35^{o}C, the absorbance values of lipase assay were fluctuated and enzyme lost its activity. Temperature changes give rise to cleave of hydrogen bonds between substrates and enzyme active sides. Optimum temperature value promote binding potential of enzyme and substrates.



(a)



(b)



(c)

Figure 4.6. (a)Absorbance, (b)enzyme activity and (c) relative enzyme activity values at different pH.

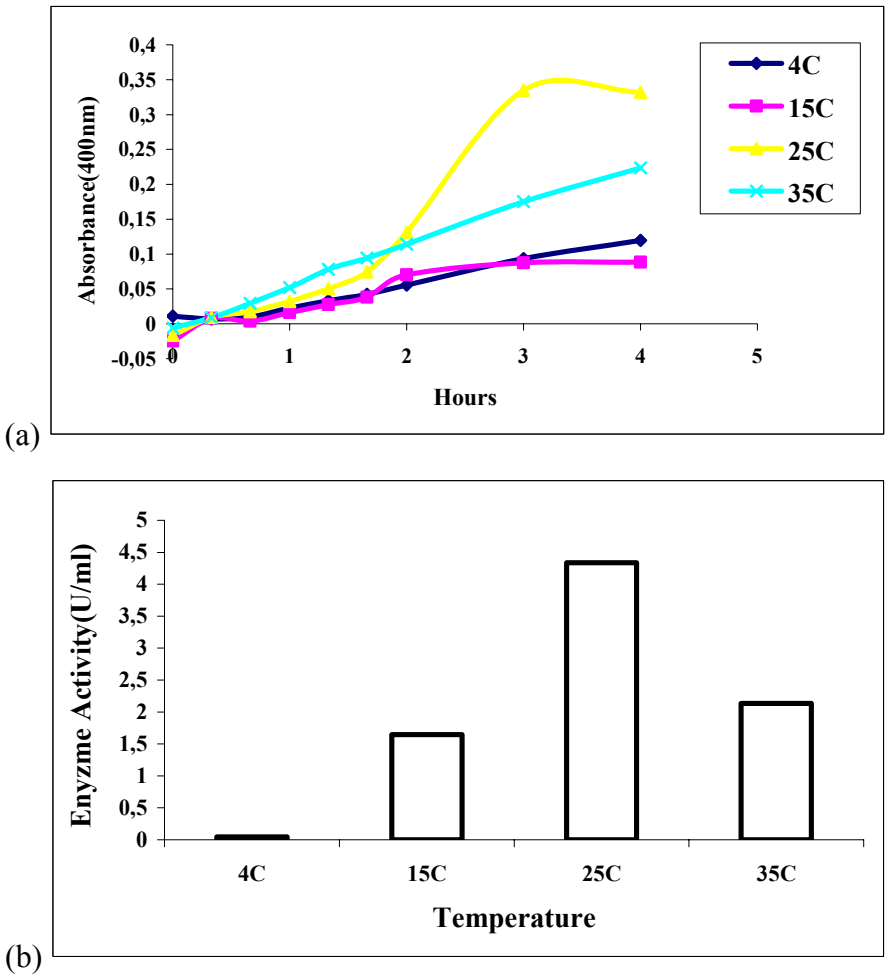


Figure 4.7. (a)Absorbance and (b)enzyme activity values at diffferent temperatures

4.5.3. Effect of Metal Ion on Enzyme Activity

The spectrophotometric enzyme activity of lipase were studied in the presense of metal salts. Relative enzyme activities were calculated from the absorbance values of sample with respect to the time. In that study control sample which did not contain any additives was used and it is assumed hundred percent while calculating relative enzyme activities. Other trials were calculated according to control sample.

If the results are compared, the enzyme activity decreased in the presence of zinc, magnesium, lead and EDTA. In other way, cobalt cause %60 increase in the activity of enzyme. Also, in the existance of calcium and potassium, the enzyme show higher activity than bare one. These metal ions may increase the binding affinity of enzyme to the substrate. Most of metal ions had a negative effect on the lipase activity,

except Ca^{2+}, Mg^{2+} and monovalent ions at a concentration of 10 mM. The chelating agent EDTA significantly reduced the lipase activity, it can be suggesting that the partial purified lipase may be a metaloenzyme.

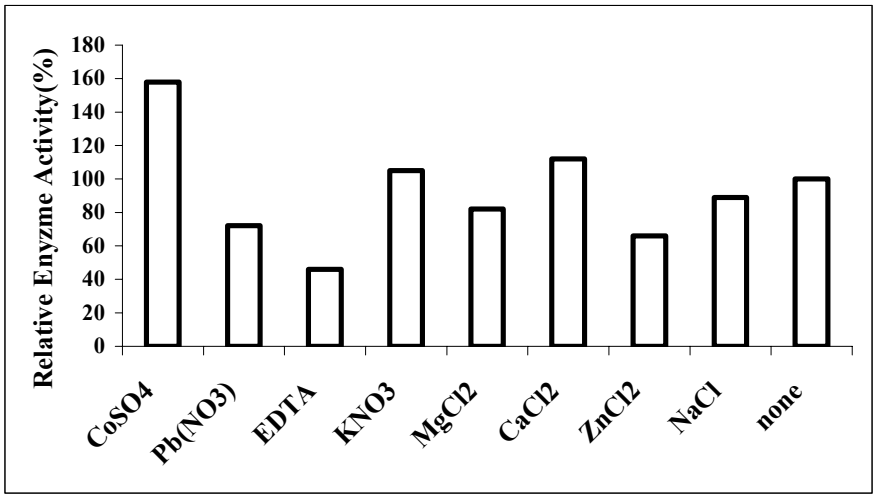


Figure 4.8. Relative enzyme activity values in the presence of metal salts

4.6.4. Effect of Organic Solvents on Enzyme Activity

The enzyme activity of lipase was studied in the presence of organic solvents. Stability against organic solvents is very important when using enzymes for synthesis of esters. The purified lipase exhibited good tolerance to organic solvents, such as acetonitrile and ethanol. Lipases from *Pseudomonas* and *Bacillus* strains were activated in the presence of several water-miscible organic solvents.

The results are shown that, iso-propanol, hexane and ethly acetate existance cause lost of enzyme activity (Figure 4.9). Although the three-dimensional structure of enzyme has not been known, the solvents probably convert the closed form of the enzyme to the open form folding structure which responsiblethe activate the of enzyme. Whereas the enzyme activity increase in the existance of acetonitrile and ethanol with response to control sample. As it was stated before, bacterial lipases were used in organic synthesis especially esterification reactions because of their regioselectivity properties. It is useful knowledge while choosing the organic solvent to lipase catalized synthesis reactions.

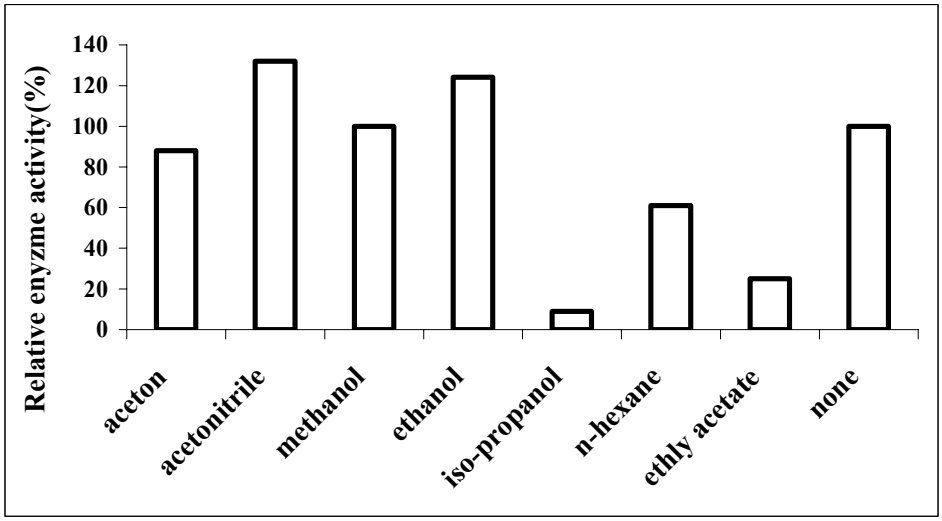


Figure 4.9. Relative enzyme activity values in the presence of organic solvents

4.7. Kinetic Study of Lipase Enzyme

The effect of p-nitrophenyl laurate concentration on the hydrolysis rate was measured at different substrate concentrations. The lipase showed a variable specificity towards various concentration of triacylglycerols. The spectrophotometric lipase assay applied for each substrate concentrations with respect to time (Figure 4.10). According to absorbance values, enzyme activity calculations were done for each concentration. By combining the data, Lineweaver-Burk plot which was 1/enzyme activity(V) versus 1/substrate concentration(S), was drawn (Figure 4.11).

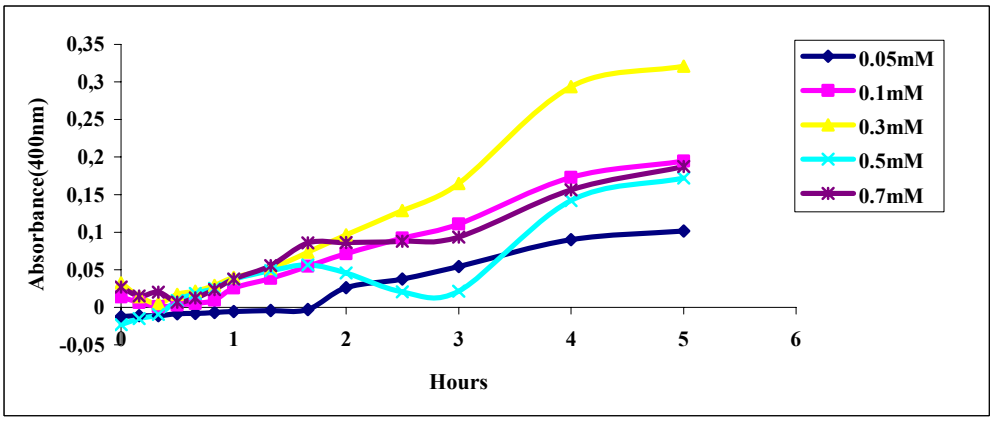


Figure 4.10. Absorbance values of kinetic study.

The Lineweaver–Burk plots were linear and indicated that hydrolysis of various triglyceride esters by the lipase followed Michaelis–Menten kinetics. That graph gave two kinetic parameters, K_m and V_{max}, which show the substrate affinity of enzyme. The smaller K_m value indicate the higher enzyme affinity to substrates. It was reported that the Km values of most industrial enzymes are varied in the range of 10^{-1} to 10^{-5} M when acting on biotechnologically important substrates. In that study the K_m and V_{max} values were determined, as a function of p-nitrophenly laurate concentration, K_m and V_{max} values were calculated as 0,2 mM and 0.089mM/min. These results reveal that the lipase from the bacteria displayed high affinity for p-nitrophenyl laurate. It is relatively small value but enzyme need further prufication process for better K_m and V_{max} values.

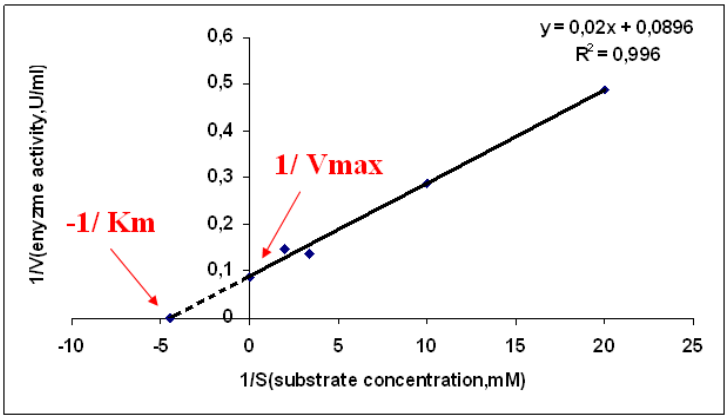


Figure 4.11. Graph of kinetic study

4.8. Molecular Weight Determination

In order to find out approximate molecular weight of partially purified lipase enzyme, SDS-PAGE was run with protein marker. The responsible protein fraction, fraction-33, its neigbours and crude protein extract were run on the gel. All fractions marked intense band in the same region. Fraction-33 marked darker band because it had more concentrated protein amount than others. On gel images, it was seen that the approximate molecular weight (Mr) of the partially purified lipase enzyme is between 43 kDa and 29 kDa (Figure 4.12).

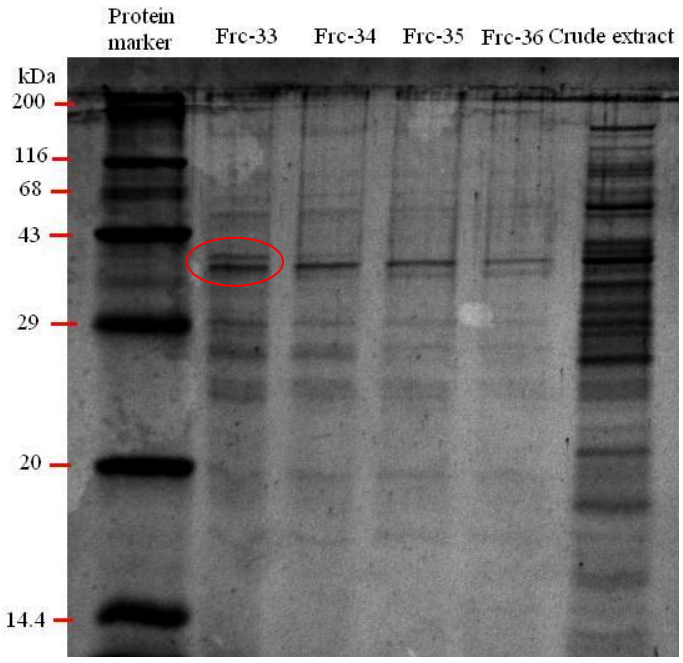


Figure 4.12. Gel image under white light

CHAPTER 5

CONCLUSION

The main purpose of this studies was investigation, purification and characterization of lipase enzymes which was isolated by soil. *Pseudonomas* strain gave positive results to lipase activity in different lipase assay protocols. Specific activity staining procedure applied on native gel carring extracellular and intracelluler bacterial crude extracts. The orange light under UV light was seen at intracellular sample. So it can be concluded that the enzyme is not release from the cell into grown media.

According to substrate specificity results, the highest enzyme activity was seen in p-nitrophenly laurate which has a relatively long, twelve, carbon chain. Only lipase enzymes clavege long carbon chains, whereas esterase is not. The optimum pH of that enyzme was investigated that relatively alkaline pH values. Although the bacterial optimum grow temperature is 25^{o}C, lipase enzyme showed activity in above and below that temperatures. The enzyme is not so much effected by organic solvents but relatively effected by metal ions.

In the cause of isolating from soil, bacterial lipase show resistance to temperature and pH change. So it can be applied on many fields by further characterization studies. Further purification procees can be allowed to get better enzyme activity. Also mass spectrometry can be used to identified amino acid sequence of enzyme after further purification. Beside this, the biological nomenclature of responsible *Pseudonomas* strain can be identified.

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APPENDIX A

BRADFORD ASSAY CALIBRATION GRAPH

Table A.1. BSA standarts

	Concentration	BSA stok(200ug/ml)	Water(uL)	Bradford Reagent (uL)
Blank	**0**	0	800	200
BSA std.	**1ug/ml**	5	795	200
BSA std.	**2ug/ml**	10	790	200
BSA std.	**4ug/ml**	20	780	200
BSA std.	**6ug/ml**	30	770	200
BSA std.	**8ug/ml**	40	760	200

Table A.2. Absorbance values of standarts

Standards	Absorbance(595nm)
1ug/ml	0,0782
2ug/ml	0,1427
4ug/ml	0,2073
6ug/ml	0,2806
8ug/ml	0,3282

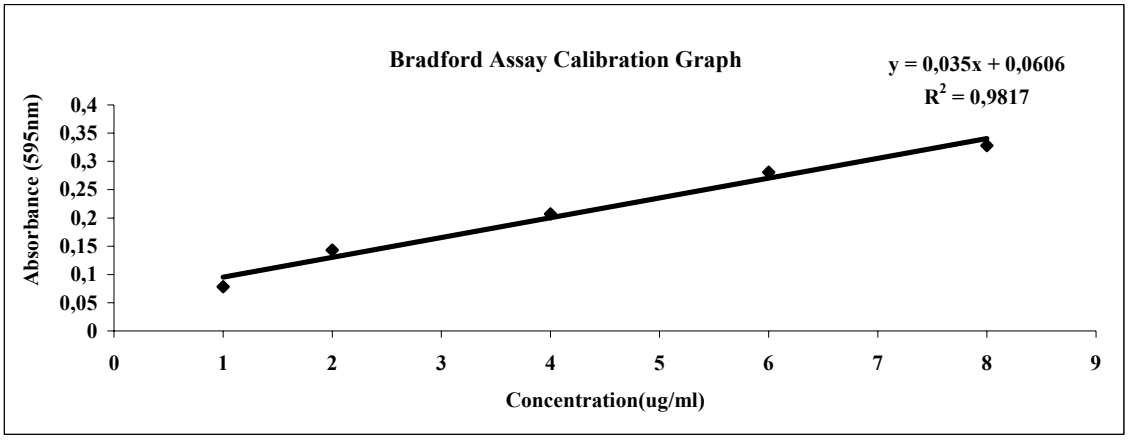

Figure A.1. BSA Standard calibration curve for Bradford assay