T.C.
İSTANBUL UNIVERSITY
INSTITUTE OF GRADUATE STUDIES
IN SCIENCE AND ENGINEERING

M.Sc. THESIS

CLONING AND CHARACTERIZATION OF A LIPASE ENZYME ISOLATED FROM METAGENOMIC LIBRARIES

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June, 2017

İSTANBUL

This study was accepted on 16/6/2017 as a M. Sc. thesis in Department of Genetics and Bioengineering, Genetics and Bioengineering Programme by the following Committee.

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This thesis is supported by the project numbered 268201 of European Union FP7: Marie Curie IRG. under the supervision of Assoc. Prof. Dr. Gönül SCHARA.

FOREWORD

I've truly enjoyed all the exciting, productive times that I have experienced during my master period. I am very grateful to all the people who have supported and encouraged me on my master journey.

Firstly, I would like to present my special and heartfelt thanks to Assoc. Prof. Dr. Gönül SCHARA who I worked with during this work and my previous studies. I have to say that she is the most enthusiastic, enlightened, most committed person on science I've ever seen. She has always been a friend to me besides being a teacher, always supporting me in all matters with her patience and tolerance. I have succeeded in some tough areas thanks to her, and the knowledge that I have learned from her will be in the forefront in my scientific studies.

I thank the European Union FP7: Marie Curie IRG. for funding the project numbered 268201.

I would like to thank my research team for providing a wonderful work environment. Kübra Cansu YANIK YILDIRIM has always been there and helped whenever I get stuck and I thank for her support and friendship. I also want to thank my other group member, Cansu KILCI. I was so glad doing my master by her side and for her motivation, support and lovely friendship.

I would like to give my special thanks to Prof. Dr. Emine Şeküre Nazlı ARDA, my adviser at Istanbul University. I am very grateful that she always supported and helped me in my tough times and did not hesitate to share her knowledge.

Finally, a very warm and special thanks and gratitude to my dear family; my mother Zehra and my father Osman, my sister Şeyma for their unconditional love and also their support and belief in my decisions whole my life and my deep appreciation and gratitude goes to my lovely grandparents Şadiye and Bayram AYAN.

June 2017 Fatma Feyza ÖZGEN

TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

LIST OF TABLES

Page

LIST OF SYMBOLS AND ABBREVIATIONS

Symbol	Explanation
%	: Percentage
°C	: Centigrade degrees
h	: Hour
H_2O	: Water
L	: Liter
M	: Molar
mM	: Milli Molar
µL	: Microliter
µM	: Micromolar
mg	: Milligram
min	: Minute
ng	: Nanogram
nm	: Nanometer
p-	: *Para*
pmol	: picomole

Abbreviation	Explanation
bp	: Base pair
BSA	: Bovine serum albumin
ddH_2O	: Double distilled water
dH_2O	: Distilled water
dNTPs	: Deoxyribonucleotide triphosphate
E. coli	: *Escherichia coli*
IPTG	: Isopropyl β-D-1-thiogalactopyranoside
kDa	: Kilodalton
LB	: Luria-Bertani
NaCl	: Sodium chloride
NaOAc	: Sodium acetate

OD : Optical density

PCR : Polymerase chain reaction

***p*-NP** : *p*-Nitrophenol

rpm : Revolutions per minute

RT : Room Temperature

SDS-PAGE : Sodium dodecyl sulfate polyacrylamide gel electrophoresis

UV : Ultraviolet

ÖZET

YÜKSEK LİSANS TEZİ

METAGENOMİK KÜTÜPHANELERİNDEN İZOLE EDİLEN BİR LİPAZ ENZİMİNİN KLONLANMASI VE KARAKTERİZASYONU

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Lipaz enzimleri, farklı pH, sıcaklık ve organik ortamlardaki yüksek aktivite ve stabilitelerinden dolayı çeşitli kimyasal ve biyoproses endüstrilerinde biyokatalizörler olarak kullanılırlar. Lipaz enzimlerinin kullanımının milyar dolarlık bir ticaret olmasından dolayı, yeni lipaz enzimlerinin izole edilmesi için büyük bir baskı mevcuttur.

Yürütülen çalışma boyunca, metagenomik kütüphanelerinden keşfedilen lipaz geninin, klonlama ve ekspresyon çalışmaları yapıldıktan sonra enzim saflaştırma kademesine geçilmiştir. Karakterizasyon çalışmalarında iki farklı substrat kullanılmıştır. Farklı substrat konsantrasyonlarının yanı sıra pH ve sıcaklıklığın enzim aktivitesi ve stabilitesi üzerindeki etkisi araştırılmıştır. Çalışma sonucunda lipaz enzimin triaçilgliserol (true lipase, LipA) ailesinden olduğu anlaşılmıştır. Keşfedilen lipaz enzimi kısmi olarak saflaştırılmış, karakterize edilmiş ve enzimin geniş bir sıcaklık (25–80°C) ve pH (5-12) aralığında aktivite gösterdiği saptanmıştır. Enzimin ayrıca %70'lik metanol ortamında aktivitesinin yaklaşık %60'ını koruduğu tespit edilmiştir. Bu özellikler enzimin bilhassa deterjan ve biyodizel endüstrilerinde kullanılabilme potansiyelini göstermektedir.

Haziran 2017, 79 sayfa.

Anahtar kelimeler: lipaz, saflaştırma, hidrolitik aktivite, biyoproses, biyodizel

SUMMARY

M.Sc. THESIS

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Lipase enzymes are used as biocatalysts in various chemical and bioprocess industries because of their high activity and stability at different pH, temperature, and organic environments. Since the use of lipase enzymes is a billion-dollar trade, there is a great deal of pressure to isolate new lipases.

Throughout this study, the enzyme purification step was carried out after the cloning and expression studies of the lipase gene discovered from the metagenomics libraries. In the characterization studies, two different substrates were used. The effect of different substrate concentrations as well as pH and temperature on the enzyme activity and stability have been studied. The study concluded that the lipase enzyme is from a family of triacylglycerol (true lipase, LipA). This novel lipase enzyme was partially purified, characterized, and found to show activity in a wide range of temperature (25-80˚C) and pH (5-12). Furthermore, it was discovered that the enzyme retained about 60% of its activity in the presence of 70% methanol. These properties show its potential to be possibly used especially in detergent and biodiesel industries.

June 2017, 79 pages.

Keywords: Lipase, purification, hydrolytic activity, bioprocess, biodiesel

1. INTRODUCTION

Enzymes are powerful biocatalysts because of their ability to be very sufficient in industrial applications. Enzyme-based technology is one of the effective alternatives to conventional processing because of its beneficial features such as being highly specific, fast and biodegradable (Choi et al., 2015).

One of the most common enzyme groups used in industry are lipases (Dror et al., 2014). Lipases are widely found enzymes, catalyzing the hydrolysis and synthesis reactions of ester bonds of glycerol and long-chain fatty acids (Akbulut et al., 2013; Salameh and Wiegel, 2010). Biotechnological potentials of lipases are enormous due to their resistance to organic solvents, selectivity and stability, and broad substrate specificity. Furthermore, lipases are resistant to high temperatures and stable in alkaline environments which endow many advantages on bioprocessing. For industrial applications, the lipase enzymes are commercially proficient in several industries, such as laundry, food, oil chemistry, pharmaceutical, and paper, as well as used in biodiesel production and waste treatment (Hasan et al., 2006; Ru et al., 1997). In particular, small-scale aspects of bacterial lipases provide ease of use in enzyme studies and substrate specificities make these enzymes valuable in biotechnological fields. These properties demonstrate the importance of the lipase enzymes. As a result, despite the existence of lipases which are very broad in nature, there is still a need for ideal lipases with novel characteristics and advantageous properties.

This work was supported by the European Union FP7: Marie Curie IRG # 268201 Project and completed under the supervision of Assoc. Prof. Dr. Gönül Schara. In the first phase of the Marie Curie Project, a novel lipase enzyme was discovered from metagenomics libraries. The results show that the lipase enzyme is similar to triacylglycerol lipase (true lipase, LipA) which is very significant in industrial areas (Arpigny and Jaeger, 1999).

The aim of this work is to continue with the second phase of the Marie Curie Project and carry out the cloning and characterization of the lipase enzyme discovered from the Turkey soil metagenome for use in the biofuels and biotechnology industry. Once the

optimal purification protocol was established, the application potentials in the industry were examined by testing the enzyme activity, stability, kinetic parameters, optimum pH and temperature values, and substrate specificity of the pure lipase enzyme. This work may provide tremendous economic and strategic benefits to our country and the novel enzyme may eliminate our foreign- dependence on this matter. The research results have a high potential to be patented. Other expected outcomes are publications in the peer-reviewed journals, which will enrich our country's background in science and technology.

1.1. OVERVIEW ENZYMES

Energy is an indicator of the level of development of the countries and indispensable for people living comfortably. Nowadays, energy is a problem all over the world because of the growing global population and the rises in energy consumption. The primary sources of energy production in the world are non-renewable energy sources such as oil, natural gas, and coal. However, the limited capacity of raw materials and energy sources and also the constant and rapid increase in energy requirement has led that situation to alternative sustainable sources. Enzymes are now one of the most important alternative sources for their use in industrial areas, as they are edible and sustainable (Choi et al., 2015; Zhao et al., 2015; Kumar et al., 2014).

Enzymes are proteins that are formed by living cells and have ability to specifically catalyze chemical reactions (Jegannathan and Nielsen, 2013). Enzymes are highly effective and can increase reaction rates more than normal chemical reactions. They are able to show intracellular or extracellular functions and protect their structures during the processes. These are commonly known as "Biocatalysts" (Gurung et al., 2013).

1.1.1. Historical Outline

Although any knowledge of the enzymes is unknown, they have already been observed and used more than five thousand years ago. Enzymatic reactions such as milk souring, alcohol fermentation of glucose by yeast, producing cheese, yogurt, bread, wine have been in known for a long time (Schäfer et al., 2005) and it has been assumed that enzymatic reactions may be possible by living microorganisms. In 1833, the enzyme that digested starch with alcohol was precipitated by the French chemist Payen and was named as diastase (Gurung et al., 2013). In the 1850s, Louis Pasteur reached the conclusion that

sugar was catalyzed by the fermentation of alcohol through yeast via 'fermenters', and as a result of the experiments, fermentation can only take place in living cells. In 1878, Frederick W. Kühne gave these ferments the scientific term *enzyme* which literally means “in yeast” (Binod et al., 2013). In 1897, Eduard Buchner proved that fermentation enzymes can also function when they are removed from living cell structure. Nobel Prize winner James B. Sumner has isolated and crystallized urease in 1926 and found that it is fully protein, and this work has opened the way for preliminary studies of specific enzymes. With these studies, the workings of the enzymes have been understood, and thousands of enzymes have been purified and used. Today around 5500 enzymes are known and 200 are commercially available. The first commercial of these is the *Bacillus* protease. It was produced by Novozymes and used by detergent companies in 1965 (Jegannathan and Nielsen, 2013; Gurung et al., 2013; Binod et al., 2013).

1.1.2. Enzyme Classification

Enzymes are classified based on the type of chemical reaction that they catalyze, the type of bond that is broken down or formed, and the type of chemical group that they separate or transfer. As a result of mixed naming, sometimes two or more names were used for the same enzyme; sometimes the same name was used for two different enzymes. Enzymes have been named and categorized by the systematic nomenclature proposed by the International Union of Biochemistry and Molecular Biology (IUBMB) to get rid of this problem. The Enzyme Commission number (EC number) is a numerical classification that the enzymes are divided into six main classes according to the catalyst reactions shown in Table 1.1 (Gurung et al., 2013; Webb, 1992). Each class has subclasses based on the catalyzed reaction type (Singh et al., 2016). Accordingly, the enzyme classes are:

Enzyme Class 1: Oxidoreductases

An enzyme class that transfer of hydrogen or electrons to another substrate and play a very important role in metabolism. Subgroup enzymes are dehydrogenases, oxidases, oxygenases, and peroxidases (Schäfer et al., 2005; Karigar and Rao, 2011; Singh et al., 2016). For example, alcohol dehydrogenase catalyzes reactions;

$R\text{-}CH_2OH + A \rightarrow R\text{-}CHO + AH_2$

Enzyme Class 2: Transferases

In biochemistry, transferase is an enzyme that transfers (transfers) a functional group (e.g., a methyl or phosphate group) to a donor molecule (Chaloupkova and Damborsky, 2013). Subgroup enzymes are fructosyltransferases, transketolases, acyltransferases, transaminases (Karigar and Rao, 2011; Singh et al., 2016).

Enzyme Class 3: Hydrolases

The enzymes that catalyze the hydrolysis reaction that is the disruption of the substrate by water addition. These enzymes provide the hydrolysis of the substrate by adding water to the bond of the substrate to be cleaved. The classification of hydrolysis is based on the type of linkage. For example, amylases, cellulases, lipases, mannanases pectinases, phytases, proteases, pullulanases, xylanases are used in industrial processes (Monsef Shokri et al., 2014; Anobom et al., 2014; Singh et al., 2016).

$R_1CO{-}OR_2 + H_2O \rightarrow R_1CO{-}OH + R_2{-}OH$

Enzyme Class 4: Lyases

In biochemistry, lyase is an enzyme that breaks down various chemical bonds rather than hydrolysis and oxidation, often by forming a new double bond or ring structure. Subgroup lyases are Pectate lyases, hydratases, dehydratases, decarboxylases, fumarase, argino succinase (Reetz, 2013; Singh et al., 2016).

Enzyme Class 5: Isomerases

Isomerases are enzymes that catalyze new atomic organization within the molecule and alter its spatial organization. Epimerase can be given as an example (Jegannathan and Nielsen, 2013; Singh et al., 2016).

Enzyme Class 6: Ligases

The class of ligase enzymes is an enzyme that combines them by forming a chemical bond between two large molecules; Usually, a small chemical group of one of these large molecules is hydrolyzed during the reaction (Reetz, 2013).

Table 1.1: Enzyme classes and types of reactions (Singh et al., 2016).

EC number	Class	Reactions
EC 1	Oxidoreductases	catalyze reduction-oxidation reactions.
EC 2	Transferases	catalyze the transfer of functional groups from one molecule to another.
EC 3	Hydrolases	catalyze hydrolysis reactions in which the bonds are cleaved by the addition of water.
EC 4	Lyases	cleave the bonds in other ways than oxidation or hydrolysis.
EC 5	Isomerases	catalyze changes in a molecule.
EC 6	Ligases	catalyze the linking of two molecules by the hydrolysis of an enriched bond.

Presently the role of enzymes as a part of industrial biotechnology is increasing significantly in the worldwide situation (Angajala et al., 2016). Within the various enzyme classes, *lipases* are one of the most important enzyme group in the industrial fields (Messaoudi et al., 2010).

1.1.3. Enzyme Production in Turkey

The use of enzyme-assisted methods has been well documented throughout past civilizations and the emergence of enzymology indicates one of the most important breakthroughs in the biotechnological processes (Angajala et al., 2016). Enzymes have a broad range of usage within various industries due to their high specificity and efficiency (Bidmanova et al., 2014).

Denmark and the United States are the top countries in the world on the production of industrial enzymes. Especially Novozymes (Denmark) is a leader among all the companies in the world with more than 700 products capacity (Binod et al., 2013; Adrio and Demain, 2014).

Although the importance of enzymes is known, the enzyme production in Turkey has not improved on industrial scale, especially lipases are used in many sectors in the country. Importing enzyme is a disadvantage for Turkey. The amount of enzymes that Turkey imported from strong competitors abroad continues to increase with each passing year. When the data of the Turkish Statistical Institute (TÜİK) is examined, the total amount of enzyme taken from abroad in 1996 was 20 million (US $ basis), 26 million in 2000, 59 million in 2005, 92 million in 2008, 123 million in 2012 and it has reached 138 million in 2014.

This result shows that the consumption of enzymes will continue to increase and it will bring harm to the country's economy in the future. The reduction of economic dependence on external sources and the development of Turkey's own processes for obtaining lipase enzymes depend on the success of our research and development work in this area. This issue has a strategic importance. Since the enzymes used in the industrial enzyme production process are patented products, one of the purposes of this study is to study the R&D of the lipase enzymes that may be patented.

1.2. LIPASES

The interest in commercial enzymes is increasing day by day due to the wide range of applications. One of the most popular of enzyme group is lipases. Lipases are biocatalysts that are widely used in many industries such as food and beverage, pharmaceuticals, detergent industry, textile and cosmetics (Hasan et al., 2006; Bhosale et al., 2016; Sharma and Kanwar, 2014; Eggert et al., 2004).

First lipase enzyme was isolated by Eijkman (1901) from *Bacillus prodigious, Bacillus pyocyaneus,* and *Bacillus fluorescens* and nowadays there are so many studies on lipases isolated by various organisms, including animals, plants, fungi, yeast and microorganisms (Nthangeni et al., 2001; Bancerz et al., 2005; Ejedegba et al., 2007; Bussamara et al., 2010; Kumar et al., 2012; Jiewei et al., 2014; Saranya et al., 2014; Maharana and Ray, 2015).

Lipases from the hydrolases class are a group of enzymes that catalyze the hydrolysis of ester bonds of lipids (Glogauer et al., 2011). Due to the low solubility of lipases in

aqueous media, triacylglycerol is the main substrates (Angajala et al., 2016). Lipases with different chemical properties are divided into two main groups: Carboxylesterases (EC 3.1.1.1) and “true” lipases (EC 3.1.1.3) and they have several usages in biotechnological and biomedical fields (Messaoudi et al., 2010).

Microbial lipases are commonly used in industrial applications due to their selectivity, stability and broad substrate specificity. They can be also used as biocatalyst in producing biodiesel in transesterification process (Korman et al., 2013). They are generally active in wide range of temperature from 20-60 °C and the optimum pH of most lipase is in between 7 and 9 (Cheng et al., 2011; Sifour et al., 2010). There is still a considerable interest in advancing new or developed lipases for using in different scale of industries (Panizza et al., 2014).

The famous biochemist J. B. S. Haldane mentioned in 1930 in his article named as "Enzymes" that there could be millions of potential substrates for the lipase enzyme, and he referred to the wonderful potential of lipase enzyme in the industry (Haldane, 1930). Approximately 85 years later, although there have been many studies and improved products on lipases, it has been suggested that lipases in the industrial sector are still active biocatalysts. This view supports a number of patents taken on lipase enzyme and continuing to be taken. The study in 2015, when scientific publications and patents were examined and summarized, emphasized the need new lipases to reach the full potential of the industry (Daiha et al., 2015).

When we examined the national and international patent databases, we found that there are many patents on lipase enzymes isolated from natural sources. For example, patented lipase products used in the detergent industry are resistant to different temperature and pH values and have increased substrate specificities. The patents of lipase enzymes which are very similar primer structure our lipases studied in this project are briefly summarized below.

According to the Espacenet patent search engine:

• The patent coded TR 9800872 (T2) is entitled “Laundry detergent compositions comprising a lipolytic enzyme and selected quaternary ammonium compounds” is about the addition of lipolytic enzyme (lipase) to dirt/oil/stain remover solutions.

• The patent coded TR 9901337 (T2) is entitled “The immobilized enzyme and its processing of triglyceride oils” is about immobilized enzyme processes and preparatory steps and preparation of the selected lipase enzyme by fermentation and regulation of the renewable triglyceride fatty esters and removal of the acid from the medium.

• The patent coded EP 0305216 B1 was taken by Novo Nordisk Company and is about Lipolase™ the first commercial lipase of Novo Nordisk A/S in the detergent industry.

1.2.1. Classification of Lipases

In the classification of lipases, the structure of the substrate, substrate affinity and molecular properties of the enzyme are influential. The specificities of lipases are examined in three main groups:

(I) Non-specific lipases: This group has the ability to break acyl groups at all positions of triglycerides. The reaction results in diacyl and monoacylglycerols as intermediates. Triglycerides are broken down into glycerin and fatty acids. Non-specific lipases produced by organisms such as *Candida rugosa, Staphylococcus aureus* and *Pseudomonas sp.* (Ribeiro et al., 2011).

(II) 1,3-specific lipases: This type of enzymes release the fatty acids from glycerol position 1 and 3 and then, products with specific construction may be formed. *Aspergillus niger, some Rhizopus strains,* and *Penicillium roquefortii* are examples of organisms in which these lipases are produced (Ribeiro et al., 2011).

(III) Fatty acid-specific lipases: These lipases cause the degradation of esters having long chain fatty acids and sensitive to cis-9 position. An example is *Geotrichum candidum* lipase (Ribeiro et al., 2011).

Lipases belong the structural superfamily of α/β-hydrolases and have generally catalytic triad residues; Ser, His and Asp. The usage of true lipase group from α/β-hydrolases in industrial applications makes them attractive due to their unique catalytic features (Arpigny and Jaeger, 1999; Widmann et al., 2010).

In the Triacylglycerol Lipase (3.1.1.3) Database, 74 subfamilies were obtained, in particular, according to the classification procedure for triacylglycerol lipases (Indian Institute of Technology, 2016). Some of the sub-family groups are below;

• Sub-family 2: Pancreatic lipases related proteins (PLRP)

• Sub-family 3: Cold-active lipases.

• Sub-family 4: *Bacillus* source lipases.

• Sub-family 5 to 11: Microbial lipases with industrial importance.

• Sub-family 58: Lipases to single biological process.

1.2.2. Lipase Reactions

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) catalyze the hydrolysis and the synthesis of ester bonds from triacylglycerol substrate (Figure 1.1). Since lipases are resistant to non-polar organic solvents and have specificity a large number of different substrates and their flexible protein structures allow them to catalyze many reactions such as hydrolysis, interesterification, aminolysis, oximolysis and thiotransesterification (Xiao et al., 2009; Stergiou et al., 2013).

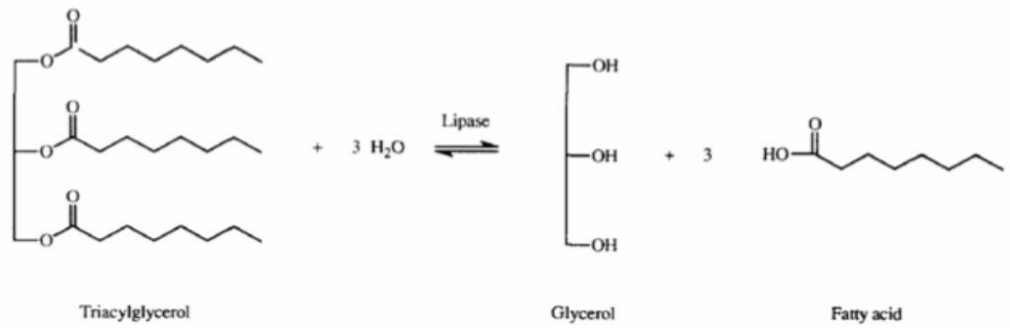


Figure 1.1: Enzymatic reaction of lipase (Messaoudi et al., 2010).

Esterification occurs in the low-water-content environment and is the opposite of hydrolysis. Transesterification can be defined as the exchange of acyl radicals between an ester and an acid (acidolysis), an ester and another ester (interesterification) or an ester

and an alcohol (Sharma et al., 2001; Guncheva and Zhiryakova, 2011). The reactions are as follows.

A. Ester hydrolysis reaction: $R_1COOR_2 + H_2O \rightarrow R_1COOH + R_2COH$

B. Ester synthesis reaction: $R_1COOH + R_2COH \rightarrow R_1COOR_2 + H_2O$

C. Transesterification reaction: $R_1COOR_2 + R_3COOR_4 \rightarrow R_3COOR_2 + R_1COOR_4$

1.2.3. 3D Structure and Catalytic Mechanism of True Lipases

The three-dimensional structures of lipases obtained from all sources are almost identical. In the 1990s, different lipase species was resolved. It has been observed that the majority, size, ranking similarity, substrates, and activators, have a similar structure. When the internal structure of α/β hydrolase proteins is examined by a central β-band, which characteristically contains catalytic groups in all the lipase studies, it is seen that the parallel β-folded bands are separated by a helical α structure and superheterically obliquely striped (Figure 1.2 and Figure 1.3). Peptide fragments of the helical structure are located on the outside of this lane. Lipases generally consist of a polypeptide chain separated into two parts, C and N. The N-part contains the active center with a hydrophobic tunnel carrying a long fatty acid chain up to the surface of the catalytic column (Öztürk, 2002; Eggert et al., 2004; Akoh and Min, 1998).

In addition to catalytic serine amino acid, the active center of most lipases contains histidine and another amino acid (Asp or Glu). The nucleophilic serine is located on the α-helix and the histidine, aspartic acid, and glutamic acid are located on the other side of the α-helix. Most amino acids containing the catalytic domain are conserved in the lipase structure (Figure 1.4) (Öztürk, 2002; Pleiss et al., 2000).

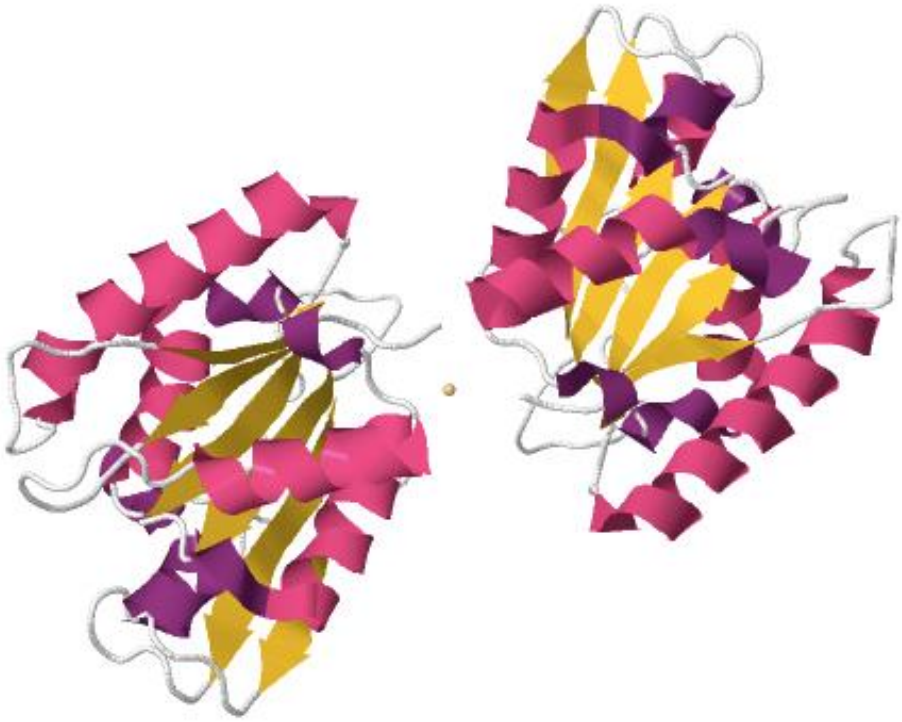


Figure 1.2: Displaying of *Bacillus subtilis* lipase: alpha/beta hydrolase fold enzyme with cadmium ion (yellow sphere) (PDB Code: 1I6W) (van Pouderoyen et al., 2001).

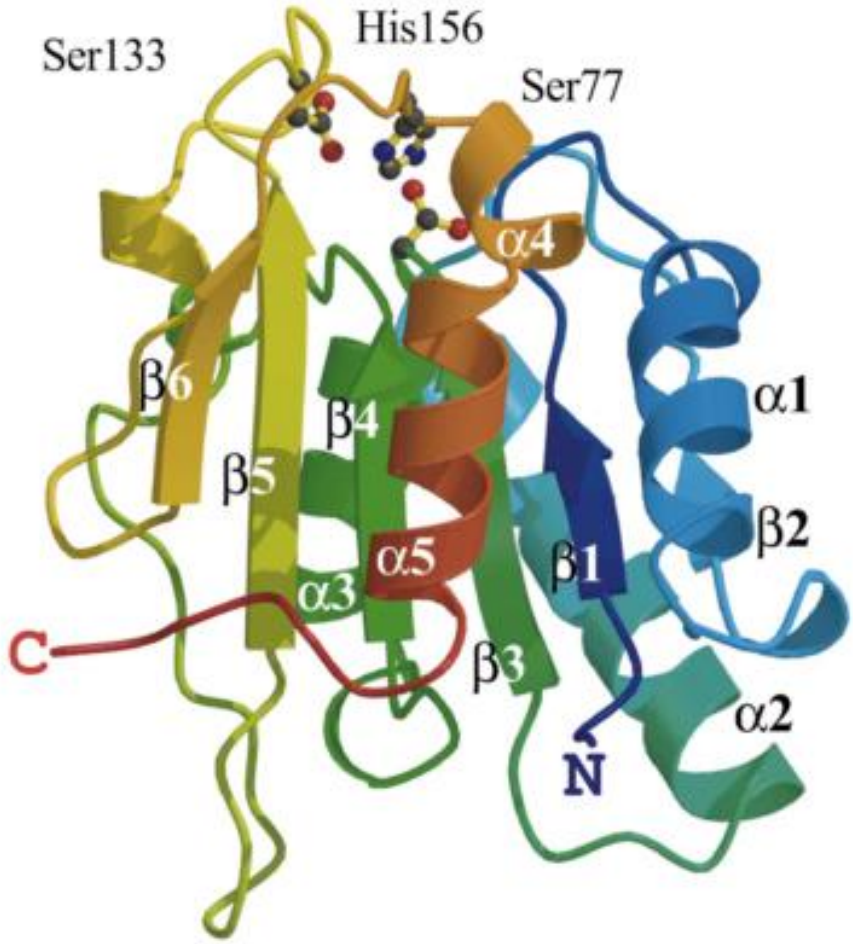


Figure 1.3: Crystal structure of *Bacillus subtilis* lipase (PDB Code: 1ISP) (Kawasaki et al., 2002).

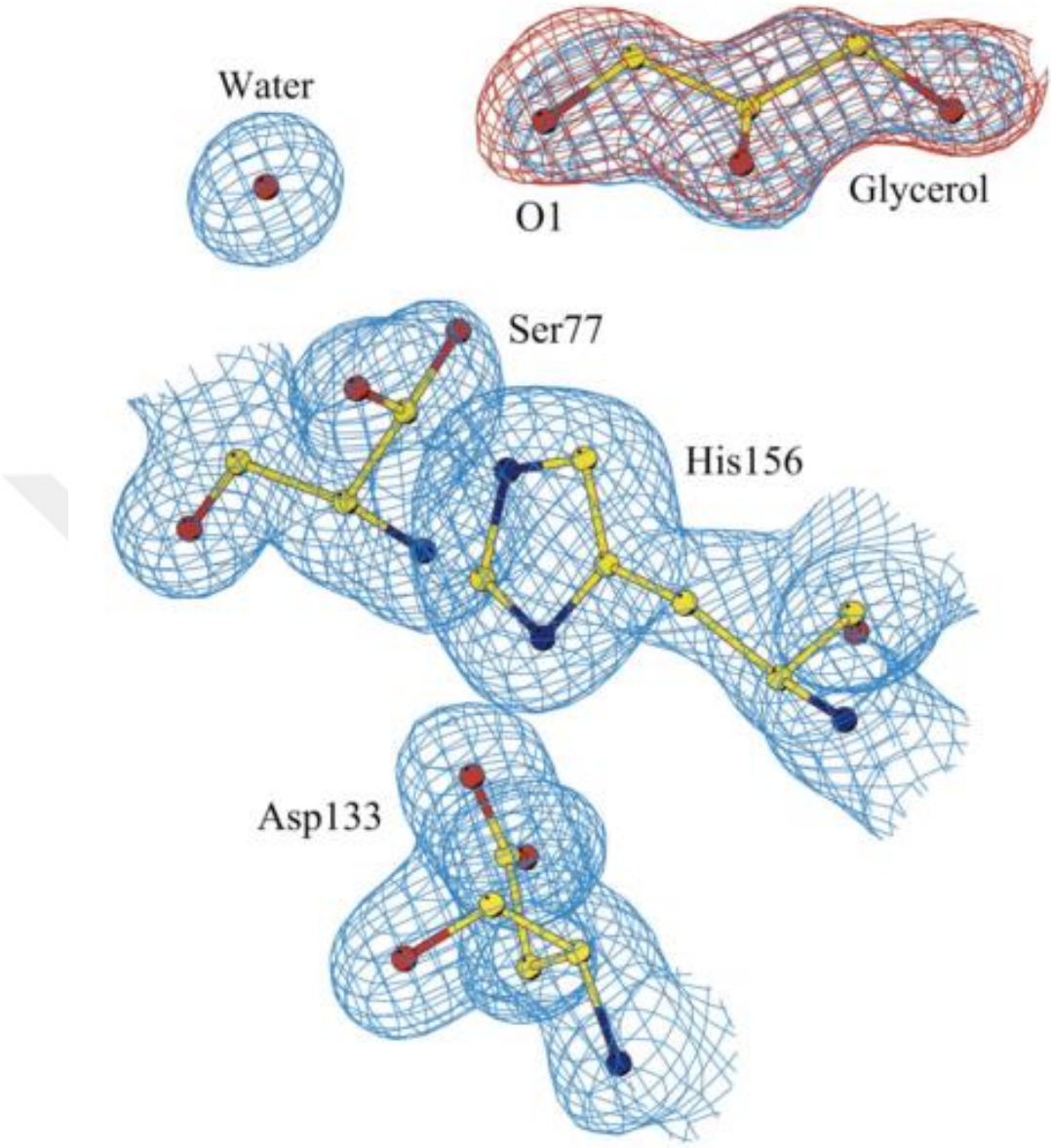


Figure 1.4: Crystal structure of *Bacillus subtilis* lipase (PDB Code: 1ISP), glycerol molecule at the active site (Kawasaki et al., 2002).

1.2.4. Applications of Lipases

Microbial lipases are mainly popular in industrial applications due to their high availability in nature, their specificity and their ease of production due to being small enzymes. They act under mild conditions, are highly stable in organic solvent environments, and demonstrate widely substrate specificity and generally show high regio - and/or stereoselectivity in the catalyst (Hasan et al., 2006). Lipases constitute the main product of industrial studies ranging from milk, pharmaceuticals, pesticides, and detergents to chemicals, tea industries, cosmetic products, leather products, biodiesel production and various bioremediation processes (Table 1.2) (Sharma and Kanwar, 2014).

Lipases in the Detergent Industry: Lipases are used in the composition of laundry detergents for the purpose of removing oil stains. Other common commercial applications of lipases in detergents; bleaching, dry cleaning solvents, liquid skin and contact lens cleaners, the disintegration of organic wastes on the surfaces of toilets and outlet pipes. They are also used in the synthesis of surfactants for daily products such as soaps and shampoos (Hasan et al., 2006; Tan et al., 2015).

Lipases in Food Industry: Lipases are used in food industry to improve the shelf life, rheological properties or aroma production of bread, cheese and other foods with other enzymes in situ. These enzymes are used for modification of the composition and structure of acylglycerols obtained by interesterification or transesterification to enhance taste production and nutritional value (Sharma et al., 2001; Yu et al., 2014).

Lipases in Textile Industry: The processing of leather and post processing is based on ancient dates. Leather processing involves the removal of hair, removal of subcutaneous fat, filling (Sharma and Kanwar, 2014). Using lipase in leather processing is an alternative to other processes due to being nontoxic. In addition, lipases are generally used to develop the qualification of a polyester fabric.

Cosmetics and Personal Care Products: Lipases are used in the production of industrial cosmetic and fragrant compounds. In the cosmetic industry, lipases are used as emollients and fragrances and are found in skin and sun creams and oils and body moisturizers. (Sharma and Kanwar, 2014).

Lipases in Paper Making Industry: Lipase is used to remove tar from the paper-made paper pulp. Tar is used to define the triglycerides and the hydrophobic components of the wood called wax, which causes serious problems in paper clay and paper production (Arpigny and Jaeger, 1999).

Biodiesel Production: Biodiesel is an alternative, non-toxic, biodegradable and renewable diesel fuel resulting from the transesterification of different kind of oils. Biodiesel does not contain petroleum; But it can be used purely or as petrol-based diesel fuel at any time. Pure biodiesel and diesel-biodiesel blends can be used for any diesel engine, with little or no modification to the engine. (Kimtun et al., 2015; Schneider et al., 2012; Hwang et al., 2014; Tripathi et al. 2014; Li et al., 2014).

Biodiesel production is usually carried out in anhydrous environments by transesterification (Aarthy et al., 2014). The transesterification reaction during biodiesel synthesis is shown in Figure 1.5.

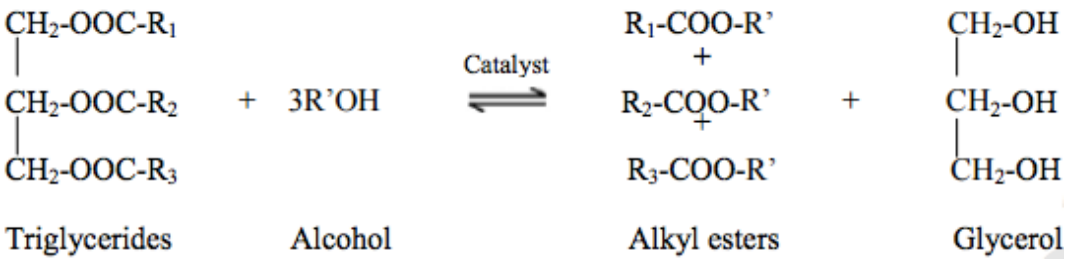


Figure 1.5: Transesterification reaction of the triglyceride molecule (Aarthy et al., 2014).

Table 1.2: Industrial applications of microbial lipases (Sharma et al., 2001).

Industrial Area	Function	Product
Detergent	Hydrolysis of Oils	Removal of oil stains from fabrics
Dairy products	Milk oil hydrolysis, faster cheese ripening	Development of sweeteners in cheese, milk, and butter.
Cosmetic	Synthesis	Humidifiers, skin care
Chemistry	Enantioselectivity	Chiral chemistry
Cleaning	Synthesis, Hydrolysis	Cleaning chemicals like surfactants, removal of agents
Drug	Transesterification	Digestive regulators
Paper	Hydrolysis	Paper products, pitch control
Leather	Hydrolysis	Leather products, degreasing
Bakery products	Flavor enhancer, shelf life extension	Bakery products, dough stability, and conditioning
Drinks	Aroma developer	Drinks

2. MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. Chemicals List of chemicals are in Appendix 1.

2.1.2. Enzymes List of enzymes are in Appendix 2.

2.1.3. Equipments List of equipment is in Appendix 3.

2.1.4. Molecular Biology Kits List of buffers and solutions are in Appendix 4.

2.1.5. Buffers and Solutions List of molecular biology kits are in Appendix 5.

2.2. METHODS

This work is a part of the European Union FP7: Marie Curie IRG # 268201 project, in which additional cloning experiment was performed to express and characterize the lipase enzyme (LIPA-TR) discovered from the Turkish metagenomics libraries. The enzyme purification protocol was optimized and to study the application potential of the new lipase enzyme (LIPA-TR) characterization experiments were performed by testing the enzyme activity, stability, kinetic parameters, optimum pH and temperature values, and substrate specificity.

All of the work was performed under the supervision of Associate Prof. Dr. Gönül Schara.

2.2.1. Maintenance and Cultivation

The culture growth condition of *Escherichia coli* BL21(DE3) pLysS is in Luria-Bertani (LB) medium; 5 g Yeast-Extract, 10 g Tryptone, 10 g NaCI at 37 °C. The pH was adjusted to 7.4 prior to autoclaving.

pET-21a (+) plasmid (host DH5α/*E. coli*) was prepared in LB medium with 100 μg/mL Ampicillin. Glycerol stock was prepared as 25%.

Escherichia coli strain TG1 was utilized as the host for the molecular cloning and expression of the gene in LB medium. 25% glycerol stocks were prepared, and storage was at -80 °C.

2.2.2. Genomic DNA Isolation

To isolate the genomic DNA containing the positive lipase gene (LIPA-TR), the host was streaked on LB agar plate and incubated overnight at 37 °C. The following day, a colony was selected from the plate, transferred to a liquid of 5 mL of LB broth and incubated at 37 °C for about 17 hours. 1 mL was taken from the liquid samples and transferred to microcentrifuge eppendorf tubes. After centrifugation at 11000 x g for 1 minute, the supernatant was discarded and the same procedure was applied to the remaining 4 mL. For isolation procedures, GeneJet Genomic DNA Purification Kit with Gram Negative Bacteria Genomic DNA Purification Protocol-Thermo Scientific was used. The pellet was resuspended in 180 µL of digestion solution and 20 µL of Proteinase K Solution were added to the cell suspension and mixed by pipetting and incubated for approximately 30 minutes at 56 °C. Then 20 µL of RNase solution was added and incubated for 10 minutes at room temperature. 200 µL of Lysis Solution was added to the sample and vortexed for 15 seconds and mixed. After addition of 400 µL of 50% ethanol, the cell lysate was transferred to the column. After centrifugation at 6000 x g for 1 min, the liquid was discarded and 500 µL of Wash Buffer was added and centrifuged for 1 min at 8000 x g. The procedure was repeated 2 times and the ethanol was allowed to flow for 30 minutes in a sterile environment. 100 µL of the Elution Buffer was added to the center of the Genomic DNA Purification Column membrane to elute the genomic DNA. After 2 minutes of incubation at room temperature, the genomic DNA was centrifuged at 8000 x g for 1 min and stored at -20 °C.

2.2.3. Nucleic Acid Isolation Techniques

Firstly, pET-21a (+) plasmid was prepared on an LB agar plate with 100 µg/mL Ampicillin and incubated overnight at 37 °C. After overnight, two colonies were taken from the plate, transferred into a liquid of 5 mL of LB broth with 100 µg/mL Ampicillin and incubated at 37 °C. 1 mL was taken from the liquid samples and transferred to microcentrifuge eppendorf tubes to centrifuge at 11000 x g for 1 minute. The step was repeated for all 5 mL solution one by one, the supernatant was discarded and the liquid

was removed as much as possible. Then pET-21a (+) plasmid was isolated by using Plasmid DNA Purification method of Nucleospin Plasmid Kit (Macherey-Nagel, Düren, Germany).

Quantification of plasmid DNA was measured by absorbance at 260 nm and 280 nm and the purity of plasmid DNA was signified with the ratio of absorbance values of 260 and 280 nm using by using NanoDrop (Thermo-Scientific, USA).

2.2.4. The Polymerase Chain Reaction

After isolation of genomic DNA showing lipolytic activity obtained from metagenomics libraries, lipase gene (640 bp) was amplified using polymerase chain reaction (PCR) method.

Two oligonucleotide primers, STRFI_BamHI front (5'- CAG CAG GGA TCC ATG AAA TTT GTA AAA AGA AGG-3') and LIP_NotI rear (5'- CAG CAG CGG CCG CTT AGT TTG TAT TTT GGCC-3'), were designed by using Vector NTI Advance™ 11 (Invitrogen, USA) program (Lu and Moriyama, 2004).

30 pmol of each primer was used to amplify the target site and the Polymerase Chain Reaction was performed by using Intron Biotechnology-I-Taq™ DNA Polymerase Kit.

PCR conditions to amplify target 640 bp lipase gene region from genomic DNA were, 2 minutes at 94 °C (first denaturing), 30 cycles included 30 seconds at 94 °C (denaturing), 30 seconds at 55 °C (annealing), 2 minutes at 72 °C (extension) and 5 minutes at 72 °C (final extension) (Table 2.1).

Table 2.1: Components are listed in the order of adding in a PCR tube.

PCR Mixture	
Components	***Amount***
10X Buffer	5 µL
Primer mix	30 pmol each
dH_2O	up to 50 µL
dNTP mixture (2.5 mM)	5 µL
DNA template	25 ng
i-Taq DNA polymerase (5 U/µL)	1 µL

After running the PCR program, Taq polymerase was added before denaturing step. The samples were first held at 4 °C then stored at -20 °C.

2.2.5. Restriction Enzyme Digestion of DNA

In this step, two single digestions were completed to prepare the vector and the insert for cloning. Approximately 5400 bp of pET-21a (+) isolated as an expression the vector and the lipase PCR sample, firstly followed by digestion with the NotI enzyme. 50 µL reaction was established using 39 µL DNA, 10 × BSA, 10 × Cut Smart Buffer, 1 µL NotI enzyme and cut at 37 °C for 3 hours. Then, for enzyme inactivation, the reaction was left at 65 °C for 20 minutes. In the second single digestion step, 39 µL DNA sample, 10 × BSA, 10 × Buffer 3, 1 µL BamHI enzyme was incubated at 37 ° C for 4 hours for 50 µL reaction. Agarose gel with 1x TAE buffer and Ethidium bromide (5 mg/mL) was prepared for digested products and samples were loaded on 0.7% gel and run at 70V for 40 minutes. The gel was cut at 640 bp (lipase PCR sample) and 5400 bp (pET-21a (+) plasmid) under UV light and the PCR sample and plasmid were extracted by using Nucleospin Extract II Kit.

2.2.6. Ligation

In order to clone the newly prepared lipase gene into pET-21a (+) vector, 20 µL of reaction was established with 79 ng cut vector, 28 ng cut insert (lipase gene), 2 µL 10X

Ligase Buffer, 1 µL T4 DNA Ligase, 0.3 µL distilled water, the reaction incubated at 16 °C for overnight (Table 2.2). The next day, incubation was continued for 1 hour at 22 °C and then for 10 minutes at 65 °C for ligase enzyme inactivation. The purity of ligation reaction was measured with dividing absorbance values of 260/230.

Ligation was set up according to following equation:

$$[\text{vector}] = (100ng\_\text{insert})\text{x}\ \frac{1}{3}\text{x}\frac{\text{vector kb}}{\text{Insert kb}} \quad (2.1)$$

Table 2.2: Ligation reaction components are listed.

Ligation Mixture (20 µL)	
Components	***Amount***
10X Ligation Buffer	2 µL
pET-21a (+) vector	14 µL (79 ng)
Lipase PCR	2.7 µL (28 ng)
dH_2O	0.3 µL
T4 DNA Ligase	1 µL

2.2.7. Transformation

The transformation process was performed in two stages. First, cloned lipase enzyme was transferred to *E. coli* TG1 cells by electroporation using GenePulser pulse controller (Bio-Rad, Hercules, USA).

In order to prepare the electrocomponent cells, the *E. coli* TG1 cells were streaked from its glycerol stock one day before the transformation experiment. The next day, one fresh colony was inoculated into 20 mL of LB broth. The cells were grown until OD_{600} reach 0.5-0.6 (~2 hours) and spinned for 10 min at 6000 rpm, 4 °C. After discarding the supernatant, the cells were washed with 1 volume of 300 mM Sucrose (20 mL) and spinned for 10 min at 6000 rpm, 4 °C. This step was repeated with 0.5 volume of 300 mM sucrose (10 mL) and spinned as described before. The supernatant was thrown and cells were resuspended with 120 mL Sucrose Solution. 40 µL electrocomponent cells with 10

μL ligated mix was electroporated at 15 kV/cm, 25 μF, and 200 Ω in a 0.1 cm gap electroporation cuvette.

After electroporating the cells and ligated DNA, 1 mL LB broth medium was added immediately to the mix and incubated at 37 °C for 1 hour in a shaker. Then the mix was plated onto LB (100 μg/mL Amp) agar at 37 °C for overnight. Glycerol stock was prepared as 25% for *E. coli* TG1/pET-21a (+)_LIPA-TR cells and stored at -80 °C.

A randomly chosen single colony from *E. coli* TG1/pET-21a (+)_LIPA-TR transformation plate was isolated and 1 μL of the supercoiled DNA was transferred to 45 μL of *E. coli* BL21(DE3) pLysS cells using electroporation. Glycerol stock was prepared as 25% for *E. coli* BL21(DE3) pLysS/pET-21a (+)_LIPA-TR cells and stored at -80 °C. To check the lipolytic activity of *E. coli* BL21(DE3) pLysS/pET-21a (+)_LIPA-TR, 1% tributyrin plates with 100 μg/mL Ampicillin were prepared and cells were streaked on it. Negative control experiments were also performed. In addition, *E. coli* BL21(DE3) pLysS /pET-21a (+)_LIPA-TR plate containing 1% olive oil (100 μg/mL Amp + 0.2 mM IPTG) was also prepared to see the activity of lipase towards olive oil. At 37 °C overnight, the screening plate was later left for 3 days at room temperature and then it was observed at 4 °C 2 days later.

Isopropyl-β-D-thiogalactopyranoside (IPTG) test was performed for *E. coli* BL21(DE3) pLysS culture incubated in LB (100 μg/mL Amp) broth by adding 0.2 mM IPTG into the laboratory tubes after two hours of incubation at 37 °C in a shaker.

2.2.8. DNA Sequencing and Data Analysis

A dideoxy chain termination technique was applied to pET-21a (+)_LIPA-TR product with 10 mM T7 promoter front primer (5'-TAATACGACTCACTATAGGG-3') by BIO BASIC Inc, Shanghai, China. The sequence results were analyzed using the Vector NTI Advance^{TM} 11 (Invitrogen, USA) program and the three-dimensional structure was analyzed with the Swiss Model using the amino acid sequences.

The obtained 3-dimensional model was studied in detail with PyMOL (DeLano, 2002).

2.2.9. Purification and SDS-PAGE Analysis

Affinity chromatography, ammonium sulphate precipitation, and dialysis membrane methods were used for partial purification of LIPA-TR enzyme. Extracellular analysis of the enzyme activity was also performed.

2.2.9.1. Determination of the Molecular Weight of the Enzyme

a-Preparation of Cells

The cloned lipase gene is incubated overnight at 37 °C and then 450 µL is inoculated into 150 mL LB (100 µg/mL Amp) broth. The absorbance value was reached 0.2 at 37 °C about 2 hours later, then 0.2 mM IPTG was added and incubated overnight at 22 °C. The following day, the cells were centrifuged for 5 minutes at 4 °C at 6000 rpm and 0.25 mM AEBSF solution was added as a protease enzyme inhibitor to 50 mM sodium phosphate 200 mM sodium acetate buffer (pH 7.4) and sonicated for 30 sec at 10 watts for 4-5 times. After centrifugation at 9500 rpm for 20 minutes, the pellet and cell lysate were separated.

b-Jel Installation and Running

Cell lysis was performed in 20 µL of SDS gel wells and separated by molecular weight by SDS-PAGE electrophoresis. The voltage is set to 100 V during operation. The gels were then visualized for 1 day with a solution of Coomassie blue and 1% Acetic Acid in 90% Acetic Acid to make protein bands visible.

2.2.9.2. Affinity Chromatography

Purification was carried out at pH 7.4 at room temperature. Cell lysates were prepared as in SDS-PAGE studies and 10 mL of it was transferred to a HIS-Select ™ HF nickel affinity gel column. Then, 50 mM sodium phosphate prepared using 0.22 µm filter was loaded in 200 mM sodium acetate buffer solution. The column was equilibrated by washing with 50 mM sodium phosphate 500 mM imidazole buffer solution and finally, elution was performed.

2.2.9.3. Ammonium Sulfate Precipitation

In this section, the cells were slightly different. LIPA-TR was incubated overnight at 37 °C and then 450 µL is inoculated into 150 mL LB (100 µg/mL Amp) broth. The absorbance value was reached 0.2 at 37 °C about 2 hours later, then 0.2 mM IPTG was added and incubated overnight at 22 °C. The following day, the cells were centrifuged

for 5 minutes at 4 °C at 6000 rpm and resuspended in 20 mM sodium phosphate buffer (pH 7) and sonicated for 30 sec at 10 watts for 4-5 times. After centrifugation at 9500 rpm for 30 minutes at 4 °C, the pellet and as wells as the supernatant was separated.

The supernatant obtained by centrifugation was treated with ammonium sulphate to 25 % saturation and incubated on ice for 30 min and then centrifuged at 9500 rpm for 30 minutes at 4 °C. The supernatant after second centrifugation was filtered (0.2 μM size filter) and applied to a HiTrap® Phenyl High-Performance column (1mL). The column was washed with a linear gradient of Buffer B (20 mM sodium phosphate + 1 M ammonium sulfate buffer) at a flow rate of 0.5 mL min^{-1}. Six gradients were prepared with different percentages at which the lipase can be obtained purely. These are;

Gradient 1:100% Buffer B

Gradient 2: 20% Buffer A + 80% Buffer B

Gradient 3: 40% Buffer A + 60% Buffer B

Gradient 4: 60% Buffer A + 40% Buffer B

Gradient 5: 80% Buffer A + 20% Buffer B

Gradient 6:100% Buffer A

All gradients were observed by SDS-PAGE analysis.

2.2.9.4. Concentration of Extracellular Lipase

LIPA-TR cells were incubated overnight at 37 °C and then 450 μL is inoculated into 150 mL LB (100 μg/mL Amp). The absorbance value was reached 0.2 at 37 °C about 2 hours later, then 0.2 mM IPTG was added and incubated overnight at 22 °C. The following day, the cells were centrifuged for 5 minutes at 4 °C at 6000 rpm. The supernatant was placed into a dialysis membrane (12 kDa). The dialysis membrane was cut to a sufficient length and the surfaces of the membrane were washed with distilled water. The ends of the membrane were closed using dialysis clips. Dialysis process was performed 3 to 4 times in 20 mM sodium phosphate buffer environment to remove the salt. Then, the environment around dialysis membrane which included extracellular lipase was changed to 20 mM sodium phosphate + 55% glycerol mix for overnight. Next day, the sample inside dialysis membrane was concentrated with Protein Centrifugal Filter (Amicon® Ultra-0.5 mL, 10 kDa).

The extracellular lipase sample was used for characterization studies and SDS- PAGE analysis.

2.2.10. Characterization of Lipase (LIPA-TR)

The cloned lipase characterization studies were first included the modelling of a growth curve of *E. coli* BL21(DE3) pLysS/pET-21a (+)_LIPA-TR, then *para*-nitrophenol (*p*-NP) calibration curve and several characterization studies was carried out to determine lipase activity using 96 well-plate readers (Fisher Scientific™ UV/Vis Microplate Spectrophotometer) at the last stage.

2.2.10.1. Growth Curve of E. coli BL21(DE3) pLysS/pET-21a (+)_LIPA-TR

Optical density measurements were done with fresh exponential grown *E. coli* BL21(DE3) pLysS/pET-21a (+)_LIPA-TR in 150 mL of LB (100 µg/mL Amp) broth and 375 µL of its overnight culture, at 600 nm, via Spectrophotometer (6300 Jenway, UK) at 37°C. OD_{600} was quantified by 1:1 dilution in cuvette, and about two hours later when OD_{600} was reached 0.23 absorbance value, 0.2 mM IPTG was added to the culture.

2.2.10.2. Calibration of para-Nitrophenol

p-NP samples of 0.01 mM, 0.02 mM, 0.05 mM, 0.1 mM, 0.25 mM, 0.5 mM, 1 mM were prepared in a 50 mM sodium phosphate buffer solution at pH 7 in order to generate molar absorbance coefficients at different concentrations of *p*-NP. And the absorbance at 405 nm was measured and the concentration plots were drawn.

2.2.10.3. Effect of Temperature on Lipase Activity

The cell lysate prepared using 50 mM sodium phosphate buffer was incubated at 30, 40, 50, 60, 70, 80 °C for 30 minutes to observe the effect of temperature changes on the lipase enzyme. 1 mM *p*-nitrophenyl acetate and *p*-nitrophenyl butyrate substrates were used, followed by spectrophotometric measurement at 405 nm. As a negative control, cell lysates were tested without the substrates.

2.2.10.4. Effect of Different pH Values on Lipase Activity

To measure the lipase activity of the different pH values, the pH was varied between 3 and 13, prepared at room temperature. pH values were prepared using NaOH and Acetic Acid. The spectrophotometric method was used for measurement at 405 nm for 1 hour.

2.2.10.5. Effect of Different Substrate Concentrations on Lipase Activity

p-Nitrophenyl acetate and *p*-nitrophenyl butyrate substrates were prepared for activity studies at different concentrations of 0.01 mM, 0.02 mM, 0.05 mM, 0.1 mM, 0.25 mM, 0.5 mM and 1 mM at room temperature and pH 7.4. Absorbance values at 405 nm were measured intermittently for about 1 hour from the first minute. Thus, one unit of enzyme value was calculated based on the 1 µmol *p*-NP value that was reached at the minute. Also, the values of Michaelis-Menten constant (K_m) and maximum velocity (V_{max}) were calculated using Lineweaver-Burk graph.

2.2.10.6. Effect of Methanol/Ethanol on Enzyme Stability

The concentrations of *p*-nitrophenyl acetate and *p*-nitrophenyl butyrate were used to measure enzyme saturation at high methanol and ethanol concentrations. 50%, 60%, 70%, 80% and to prepare negative control without methanol or ethanol; 140 µL of 50 mM sodium phosphate (pH 7) buffer solution, 50 µL of cell lysate mixture and 10 µL of 1 mM of substrate solution.

2.2.10.7. Determination of Extracellular Activity

Experiments were carried out using 1 mM *p*-nitrophenyl acetate and 1 mM *p*-nitrophenyl butyrate to determine the extracellular activity of the lipase enzyme (LIPA-TR) cloned.

3. RESULTS

3.1. MOLECULAR CLONING OF LIPASE ENZYME

3.1.1. Molecular Cloning

The lipase gene from the metagenomic DNA was isolated using the forward and rear primers by applying the PCR conditions mentioned in the method section. PCR was needed to amplify 640 bp region which corresponds to the putative lipase gene from the metagenomics library- positive clone.

After PCR reaction, 640 bp lipase gene band was clearly visible on 0.7% Agarose Gel (Figure 3.1).

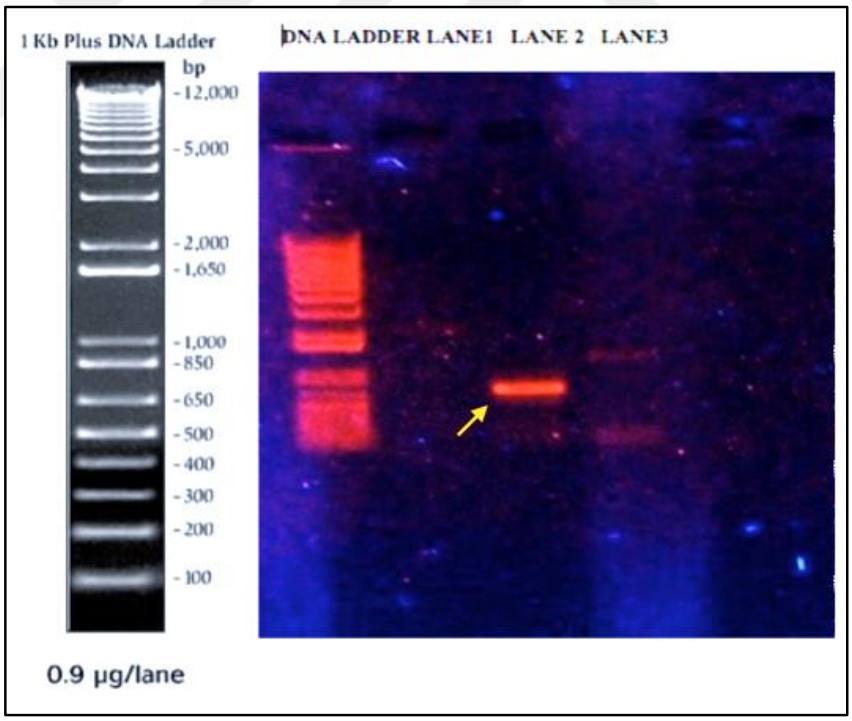


Figure 3.1: Gel electrophoresis result of PCR product of the lipase gene from metagenomic DNA. On Lane 2, yellow arrow indicates the lipase gene.

pET-21a (+) plasmid vector was prepared for cloning procedures. Digestions for both PCR products and vector plasmid were done in two steps since NotI and BamHI requires

different reaction buffers. To prevent the loss of products more than one digestion reaction mixtures were prepared for each PCR product and pET-21a (+) plasmid. Finally, ligation and electroporation were performed for expression and transfer to firstly *E. coli* TG1 cells. Then one supercoiled DNA colony from *E. coli* TG1/pET-21a (+)_LIPA-TR plate was transferred to *E. coli* BL21(DE3) pLysS/pET-21a (+)_LIPA-TR by using electroporation (Figure 3.2).

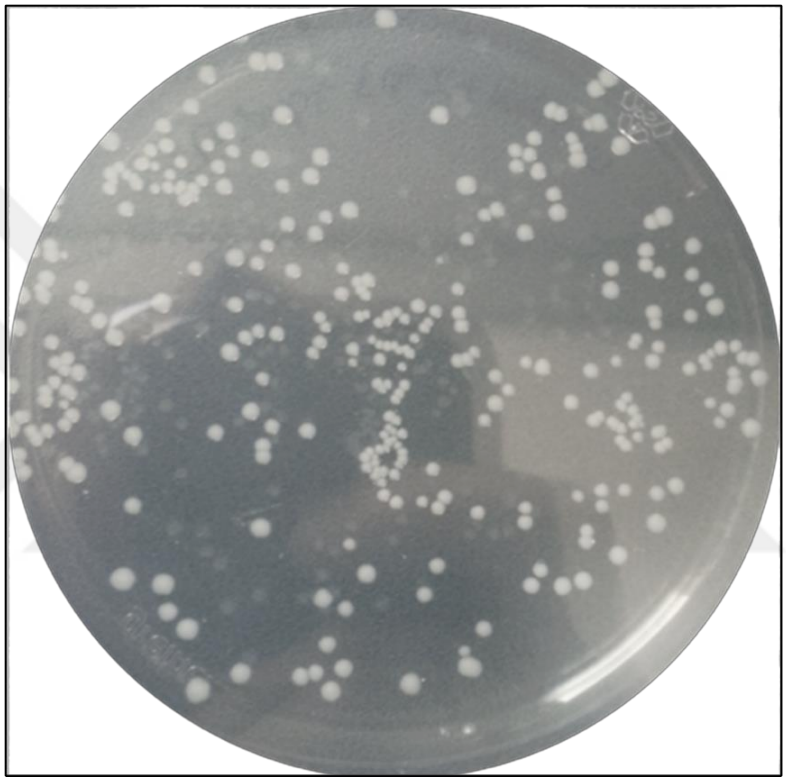


Figure 3.2: Plate containing colonies with possible lipase gene after expression.

3.1.1.1. Screening for Olive Oil Activity

E. coli BL21(DE3) pLysS/pET-21a (+)_LIPA-TR was streak on 1% olive oil +100 µg/mL Amp + 0.2 mM IPTG LB Agar Plate for overnight at 37 °C. Next day, there was no clearance around colonies. After 3 days at room temperature, around 1.5 mm clearance around those colonies was occurred and then 2 days later at 4 °C there was still clearance around colonies 2 days later at 4 °C (Figure 3.3).

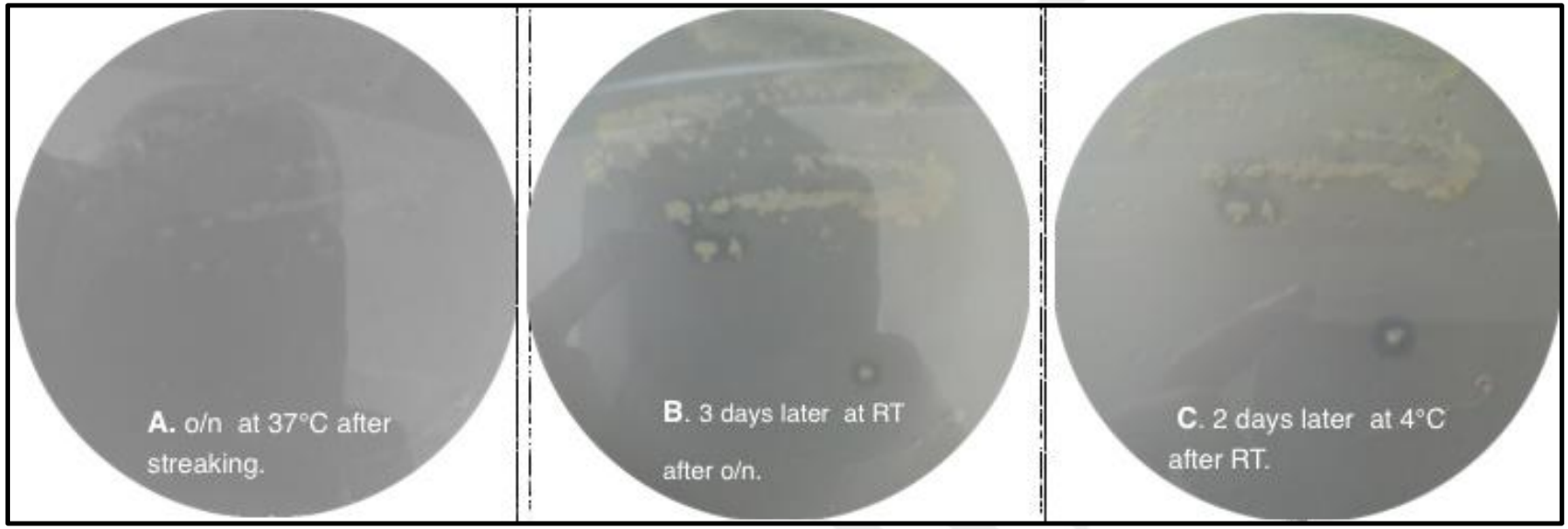


Figure 3.3: *E. coli* BL21(DE3) pLysS/pET-21a (+)_LIPA-TR was streak on 1% olive oil +100 µg/mL Amp + 0.2 mM IPTG LB Agar Plate and checked the appearances at different time.

3.1.1.2. Screening for Tributyrin Activity

E. coli TG1 and *E. coli* BL21(DE3) pLysS hosts without plasmids were streak as a negative control. *E. coli* TG1/pET-21a (+)_LIPA-TR and *E. coli* BL21(DE3) pLysS/ pET-21a (+)_LIPA-TR were streak on 1% tributyrin +100 µg/mL Amp + 0.2 mM IPTG LB Agar Plate for o/n at 37 °C. Next day, there was clearance around *E. coli* BL21(DE3) pLysS/pET-21a (+)_LIPA colonies. No clearance was observed with the negative control (host strain) (Figure 3.4 and 3.5).

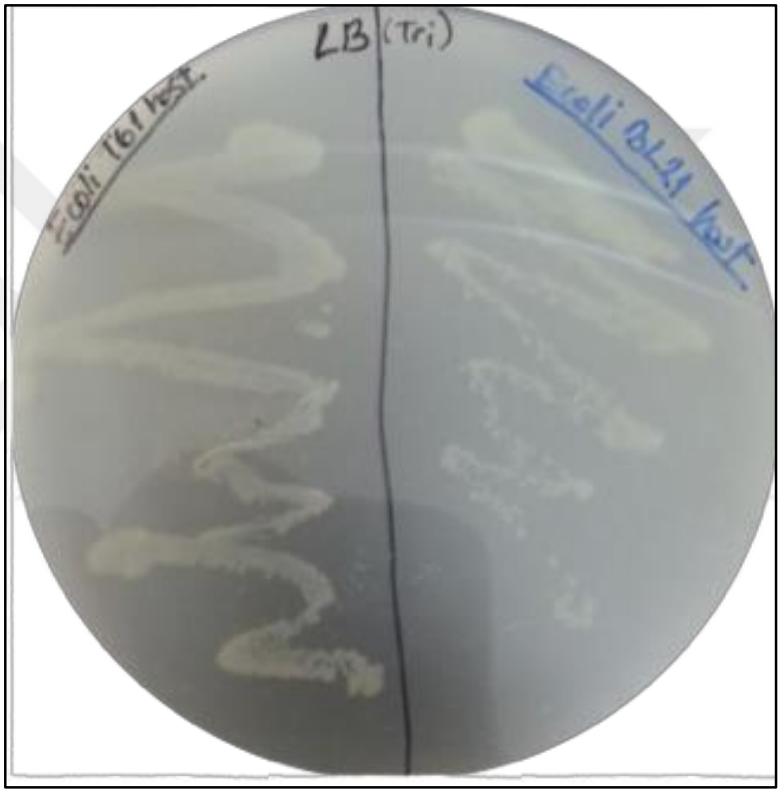


Figure 3.4: *E. coli* TG1 (left hand side) and *E. coli* BL21(DE3) pLysS (right hand side) hosts without plasmids were streak on LB Agar Plate.

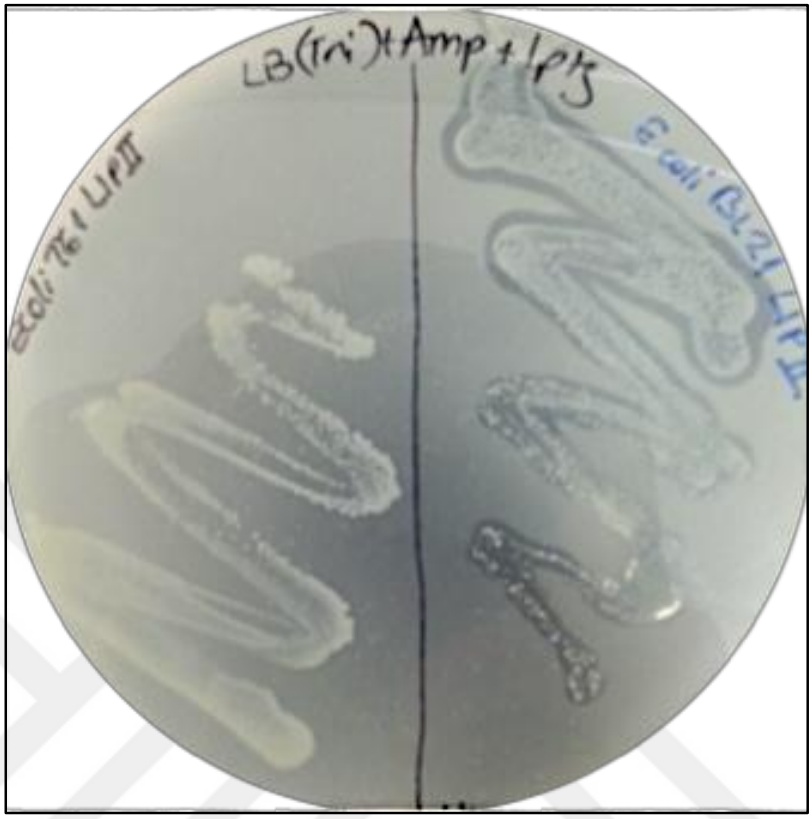


Figure 3.5: *E. coli* TG1/pET-21a (+)_LIPA-TR (left hand side) and *E. coli* BL21(DE3) pLysS/pET-21a (+)_LIPA-TR (right hand side) were streak on 1% tributyrin +100 µg/mL Amp + 0.2 mM IPTG LB Agar Plate.

3.1.2. Restriction Enzyme Digestion

NotI and BamHI restriction enzymes were used for the digestion of purified constructed plasmid and chosen according to their location on the plasmid map (Figure 3.6).

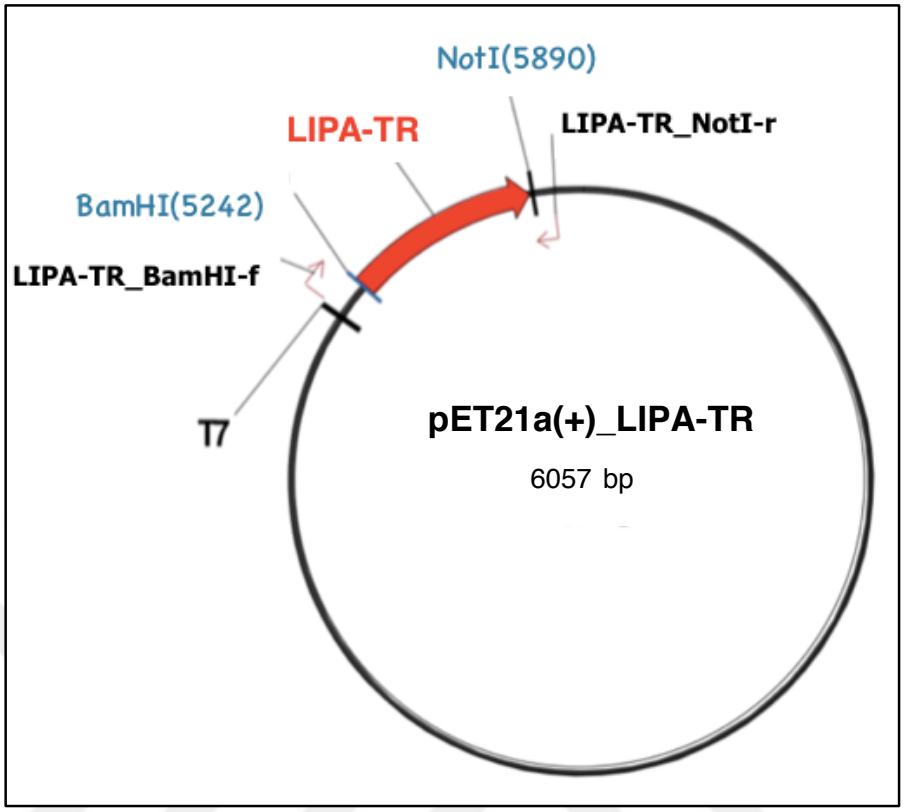


Figure 3.6: pET-21a (+)_LIPA-TR plasmid map. The T7 promoter region, cleavage enzymes (BamHI and NotI) and primer regions (LipA-BamHI-forward and LipA-NotI-rear) required for cloning are shown.

Plasmid isolation was performed using a couple of colonies after the transformation and NotI and BamHI cleavage enzymes were used to determine if the correct plasmid and lipase gene was presented. As seen in Figure 3.7, expected plasmid (5400 bp) and insert (640 bp) was observed which can be seen in the last two lanes on the right. Other 4 samples were found to contain the empty vector.

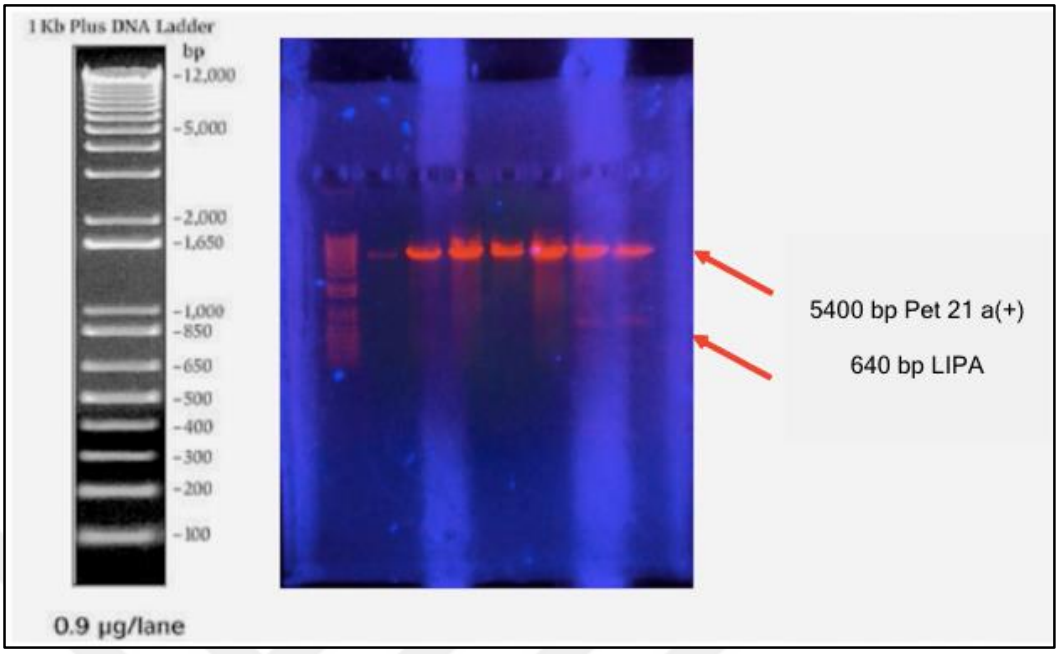


Figure 3.7: Gel electrophoresis result of restriction enzyme digestions for constructed plasmid.

3.1.3. DNA Sequencing

A dideoxy chain termination technique was used to determine the nucleotide sequence of pET-21a (+)_LIPA-TR in *E. coli* TG1 host. T7 front primer was used for sequencing studies. According to the results of the chromatogram data, the sequence reading was clear (Figure 3.8).

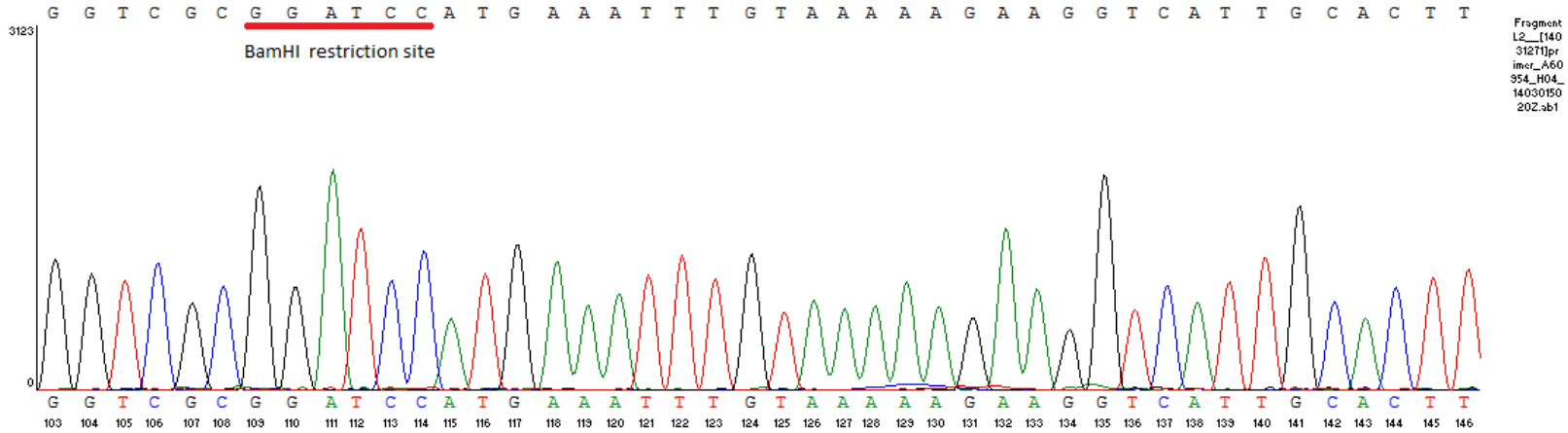


Figure 3.8: DNA sequencing results using T7 front primer and pET-21a (+)_LIPA-TR plasmid DNA. Chromatogram data includes only 44 bases, showing BamHI restriction site. Read length: 1246 bp (Vector NTI Advance™ 11).

3.2. CHARACTERIZATION OF LIPASE ENZYME

3.2.1. Growth Curve of *E. coli* BL21(DE3) pLysS/pET-21a (+)_LIPA-TR

Optical density at 600 nm (OD_{600}) measurements was done with *E. coli* BL21(DE3) pLysS/pET-21a (+)_LIPA-TR, via Spectrophotometer (6300 Jenway, UK) at 37 °C. 0.2 mM IPTG was added when OD_{600} reaches to 0.23. The result of growth curve at 600 nm is shown in (Figure 3.9, Table 3.1).

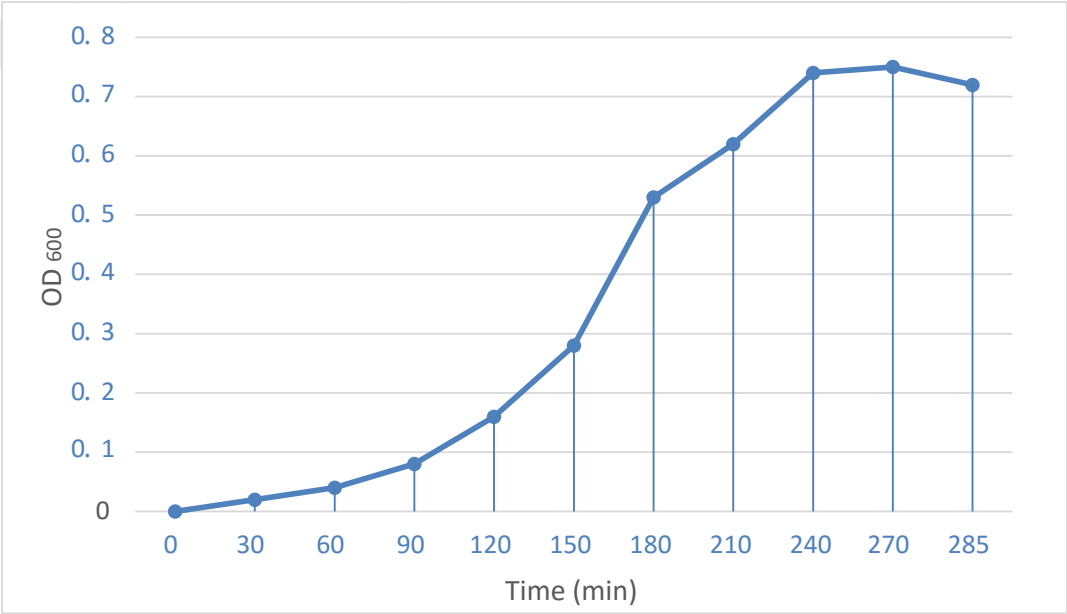


Figure 3.9: Growth curve of *E. coli* BL21(DE3) pLysS/pET-21a (+)_LIPA-TR. Spectrophotometer results were taken at 600 nm by 6300 Jenway, UK.

Lag phase of *E. coli* BL21(DE3) pLysS/pET-21a (+)_LIPA-TR which is the time for an adaptation of bacteria to new growth conditions lasted about one and a half hours. Cell doubling is happening in the exponential (log) phase for approximately 2 hours. The mature bacterial cells were provided at the stationary phase where growth slowed and OD_{600} values became constant.

Table 3.1: OD_{600} measurements of *E. coli* BL21(DE3) pLysS/pET-21a (+)_LIPA-TR over time.

TIME (MINUTE)	OD_{600}
0	0.00
30	0.02
60	0.04
90	0.08
120	0.16
150	0.28
180	0.53
210	0.62
240	0.74
270	0.75
285	0.72

3.2.2. SDS – PAGE Analysis

The SDS-PAGE method was used to determine the molecular weight of the lipase enzyme, to observe whether the enzyme was expressed or not and to observe the purity level of the enzyme after purification studies. After DNA sequence analysis, the calculated enzyme size was determined to be about 21 kDa and the same value was observed after SDS-PAGE. When negative control and positive control were examined together, it was clearly observed that the lipase region was expressed (Figure 3.10). Affinity chromatography was performed to purify the 21 kDa lipase enzyme. At this stage, however, the problem of aggregation was encountered. Since 100% pure enzyme could not be obtained for the characterization studies, experiment was carried on using protein cell lysis.

Second purification method was hydrophobic interaction method. The lipase was partially purified (approximately 80%) according to the hydrophobic purification column by ammonium sulfate precipitation (Figure 3.11).

Intracellular and extracellular enzyme activity experiments were also performed (Figure 3.12 and 3.13). Accordingly, approximately 20% of the enzyme activity was observed

extracellularly, and 80% of the enzyme activity was observed in the intracellular structure.

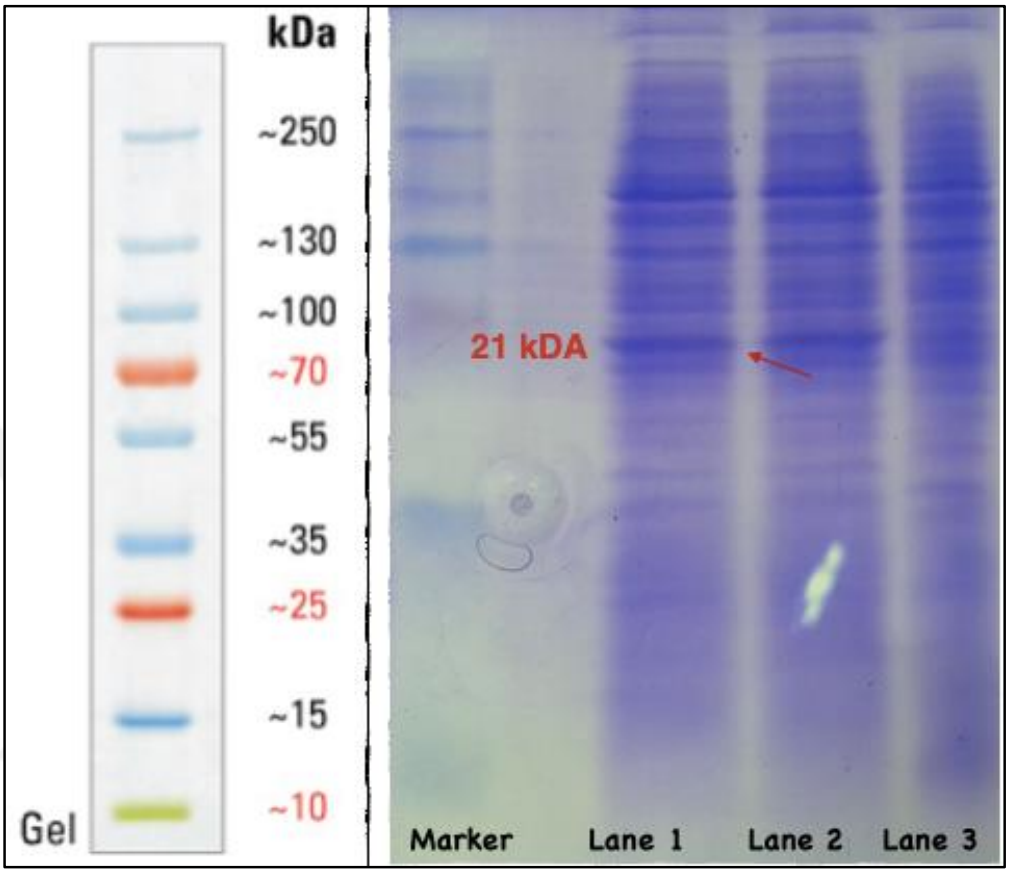


Figure 3.10: Determination of the molecular weight of lipase by SDS-PAGE. Lane 3 is negative control (*E. coli* BL21 (DE3) pLysS), Lane 2 and 3 are a positive control-lipase gene (*E. coli* BL21 (DE3) pLysS/pET-21a (+)_LIPA-TR). The arrow shows the lipase express band at about 21 kDa.

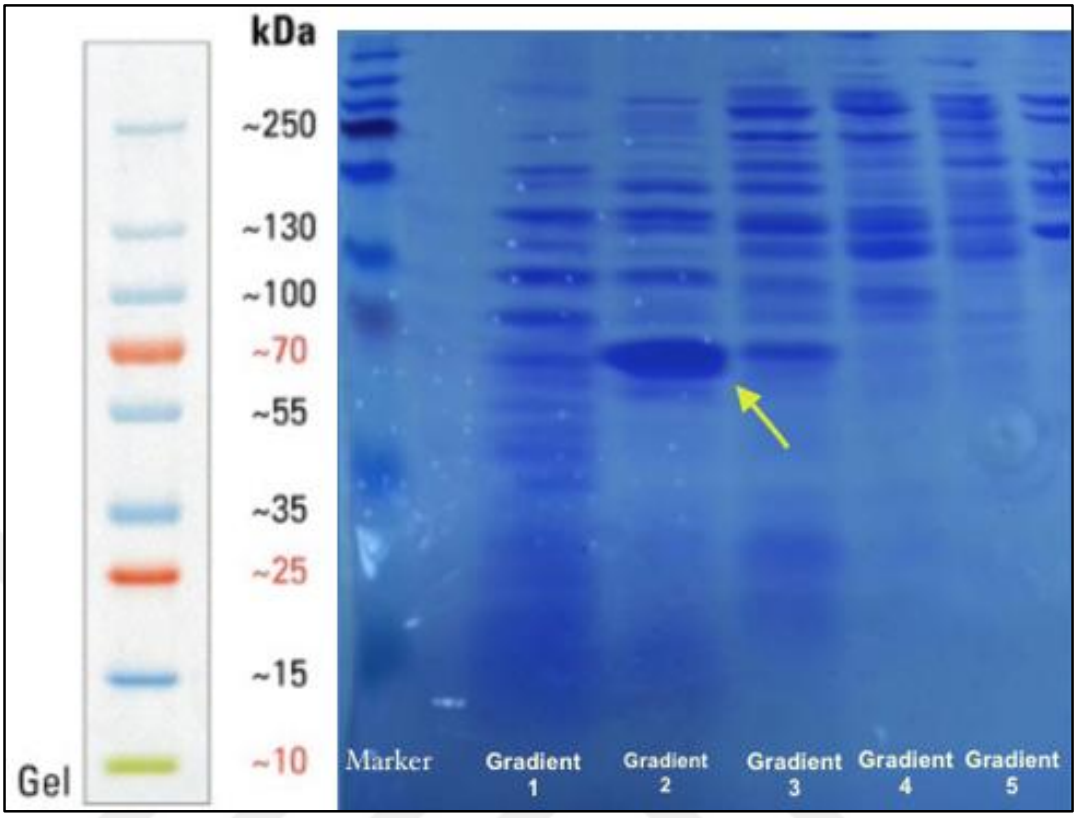


Figure 3.11: SDS-PAGE analysis of partially purified LIPA-TR using hydrophobic interaction column with ammonium sulfate participation.

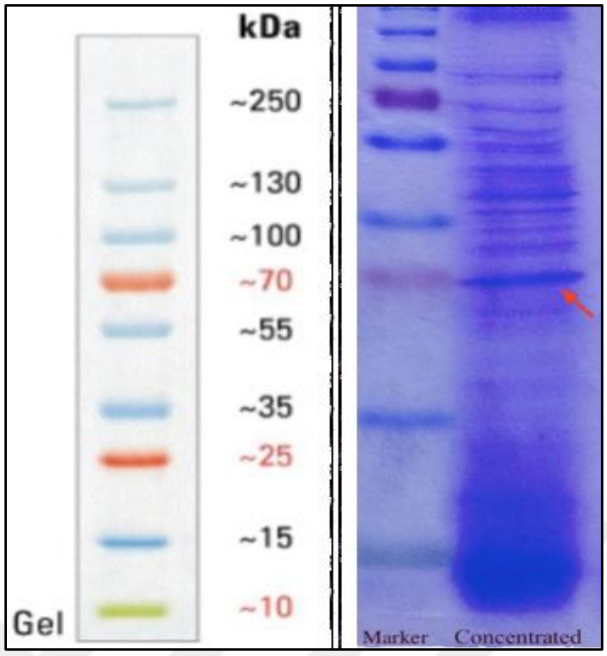


Figure 3.12: SDS-PAGE analysis of the extracellular concentrated LIPA-TR (red arrow).

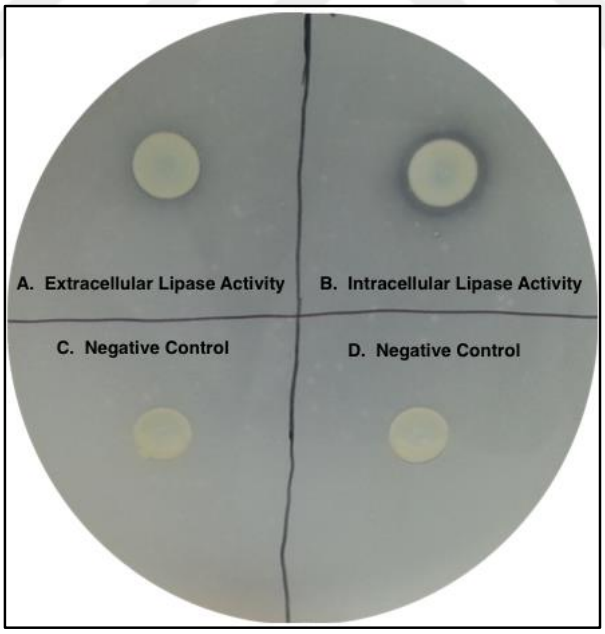


Figure 3.13: Activity study of cloned lipase (*E. coli* BL21 (DE3) pLysS/pET-21a (+)_LIPA-TR) on 1% tributyrin plate. Extracellular activity in the upper left compartment, intracellular activity in the upper right compartment. Left-right lower compartments are negative controls (*E. coli* BL21 (DE3) pLysS).

3.2.3. Characterization Studies of Lipase

p-NP samples of 0.01 mM, 0.02 mM, 0.05 mM, 0.1 mM, 0.25 mM, 0.5 mM, 1 mM were prepared in a 50 mM sodium phosphate buffer solution pH 7 to produce molar absorbance coefficients at different concentrations of *p*-nitrophenol and 405 nm. The absorbance was measured and the concentration graphs were plotted using 96 well-plate readers (Fisher Scientific™ UV/Vis Microplate Spectrophotometer) (Table 3.2).

Table 3.2: Absorbance values at different concentrations of *p*-nitrophenol, at OD_{405} nm.

***p*-NP Concentration (mM)**	**Absorbance (OD_{405})**
0	0
0.01	0.1018
0.02	0.1525
0.05	0.3054
0.1	0.643
0.25	1.3216
0.5	2.5397
1	3.4441

3.2.3.1. Effect of pH and Temperature Changes on Lipase Activity

The *p*-NP acetate and *p*-NP butyrate substrates were used to determine the effect of temperature and pH conditions on the cloned lipase enzyme. Lipase activity was measured at pH 7 after incubation for 30 min at different temperatures using 1 mM *p*-NP acetate substrate and 1 mM *p*-NP butyrate separately and activities were measured 75 min later. The highest activity for *p*-NP acetate was shown at 30 °C and a steady decrease was observed at 80 °C (Table 3.3, Figure 3.14). The highest activity for *p*-NP butyrate was found at 50 °C and the lowest activity at 60 °C, while the lipase enzyme maintained 80% activity at 80 °C (Table 3.4, Figure 3.15).

The effect of pH changes on activity was investigated this time using the same subsets of lipase enzyme. Lipase activity was measured by measuring the different pH (3- 13) values for the 1 mM acetate and 1 mM *p*-NP butyrate separately and activities were measured after 60 min. The lipase enzyme cloned in the characterization assay prepared using

acetate substrate has very low activity at acidic pH values. However, the pH value remained around 95-100% when in alkaline conditions (Table 3.5). In the butyrate supplement, the lipase gave a different result. At pH 6, it reached maximum activity and maintained 100% activity. However, at pH 8-13, approximately 50% of the loss is the equilibrium state of lipolytic (Table 3.6).

Table 3.3: Effect of temperature on lipase activity for *p*-NP acetate (1 mM).

Temperature (°C)	Concentration of *p*-NP (mM)	Conservative Activity %
30	0.36500 ± 0.00199	100
40	0.35714 ± 0.01481	98
50	0.31684 ± 0.00584	87
60	0.19624 ± 0.00372	54
70	0.17346 ± 0.00099	48
80	0.16072 ± 0.00039	44

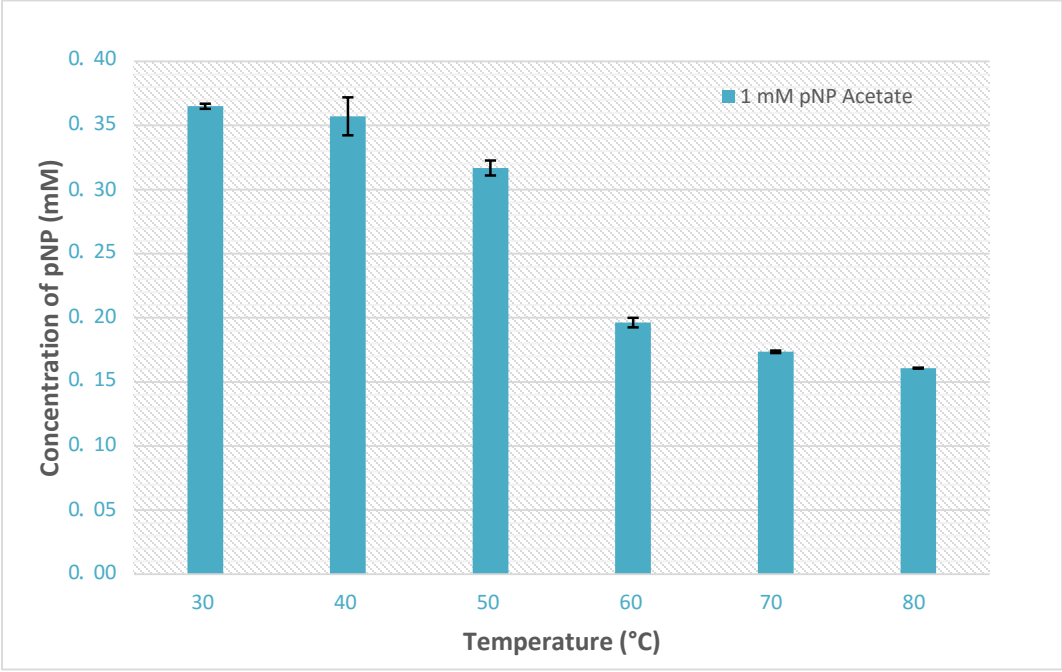


Figure 3.14: The change in temperature versus the concentration of *p*-NP for *p*-NP acetate (1 mM).

Table 3.4: Effect of temperature on lipase activity for *p*-NP butyrate (1mM).

Temperature (°C)	Concentration of pNP (mM)	Conservative Activity %
30	0.02978 ± 0.00229	77
40	0.02807 ± 0.00251	72
50	0.03890 ± 0.00031	100
60	0.02444 ± 0.00132	63
70	0.02587 ± 0.00190	67
80	0.03244 ± 0.00279	84

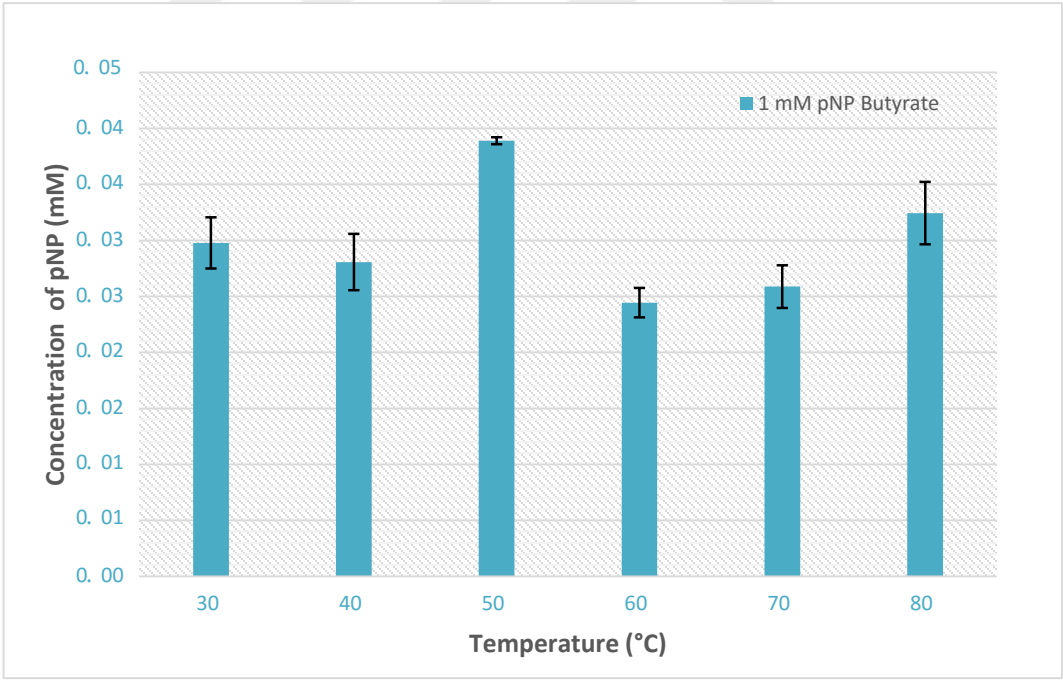


Figure 3.15: The change in temperature versus the concentration of *p*-NP for *p*-NP butyrate (1 mM).

Table 3.5: Effect of pH on lipase activity for *p*-NP acetate (1mM).

pH	Concentration of pNP (mM)	Conservative Activity %
3.1	0.14365 ± 0.06788	15
3.9	-0.06068 ± 0.04536	-7
4.5	0.03304 ± 0.00147	4
5.5	0.00190 ± 0.00539	0
6.3	0.08723 ± 0.00209	9
7.4	0.80233 ± 0.00517	86
8.3	0.81774 ± 0.01736	88
9	0.83004 ± 0.03475	89
10	0.90766 ± 0.00597	98
10.6	0.92233 ± 0.00273	99
11.3	0.92253 ± 0.00171	99
12.2	0.92181 ± 0.00194	99
12.7	0.92983 ± 0.00226	100

Table 3.6: Effect of pH on lipase activity for *p*-NP butyrate (1mM).

pH	Concentration of pNP (mM)	Conservative Activity %
3.1	0.02745 ± 0.05572	37
3.9	-0.16143 ± 0.06966	-72
4.5	-0.00625 ± 0.00319	-3
5.5	0.00025 ± 0.00419	0
6.3	0.22401 ± 0.05146	100
7.4	0.04625 ± 0.00971	26
8.3	0.08320 ± 0.00079	37
9	0.08984 ± 0.00211	40
10	0.12161 ± 0.01138	54
10.6	0.11001 ± 0.00291	49
11.3	0.11592 ± 0.01285	52
12.2	0.10541 ± 0.00278	47
12.7	0.11285 ± 0.00110	50

3.2.3.2. Stability of Lipase Enzyme in Methanol/Ethanol Environment

Intracellular activity studies were performed with recombinant lipase. To determine enzyme stability, measurement was done at specific minutes using 1 mM *p*-NP acetate and *p*-NP butyrate, after 30 minutes in methanol/ethanol solutions with different concentrations. Accordingly, in the acetate assay at 6 min, the lipolytic activity decreased as the methanol concentration increased. In addition, when ethanol was used, the activity loss was greater than that of methanol. The same experiment was repeated using *p*-NP butyrate substrate (Figure 3.16 and 3.17). In general, the most destructive effect on enzyme stability appears with 80% methanol and ethanol.

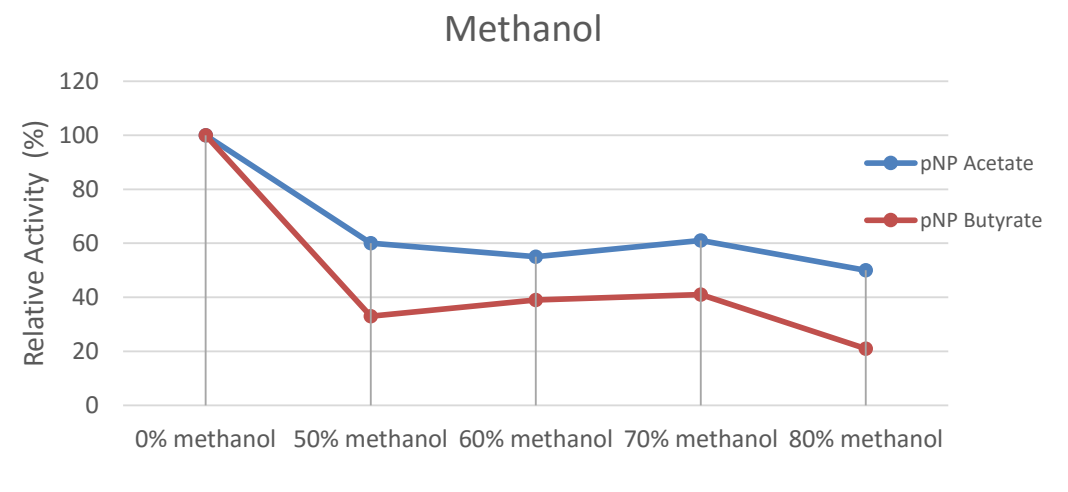


Figure 3.16: Effect of methanol solvent with different percentages on lipase enzyme stability. Stability chart showing *p*-NP acetate and *p*-NP butyrate prepared at 1 mM for 30 minutes at room temperature in the presence of methanol.

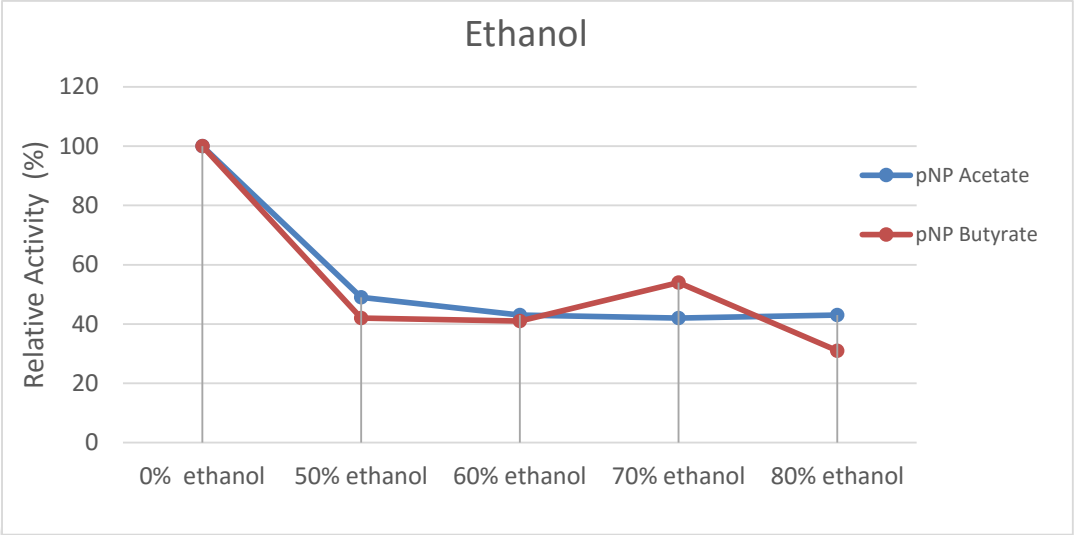
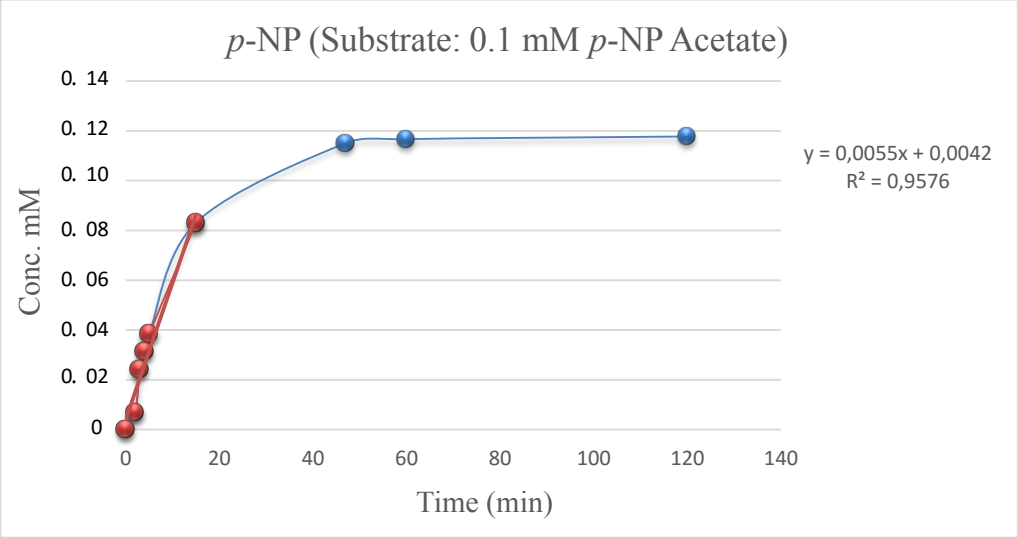


Figure 3.17: Effect of ethanol solvent with different percentages on lipase enzyme stability. Stability chart showing *p*-NP acetate and *p*-NP butyrate prepared at 1 mM for 30 minutes at room temperature in the presence of ethanol.

3.2.3.3. Effect of Different Substrate Concentrations on Enzyme Activity

The lipase enzyme kinetic values (V_{max} and K_m) was determined by using the Michaelis-Menten equation and the Lineweaver Burk-Chart. As shown in Figure 3.18, the initial velocity was calculated and other values were applied in a similar manner to create kinetic constants in Table 3.7 and Table 3.8. The V_{max} and K_m values for the *p*-NP acetate substrate is 0.48 U/mg protein and 0.69 mM, respectively (Table 3.9). The maximum rate for *p*-NP butyrate was lower and the time to reach saturation was higher.

A



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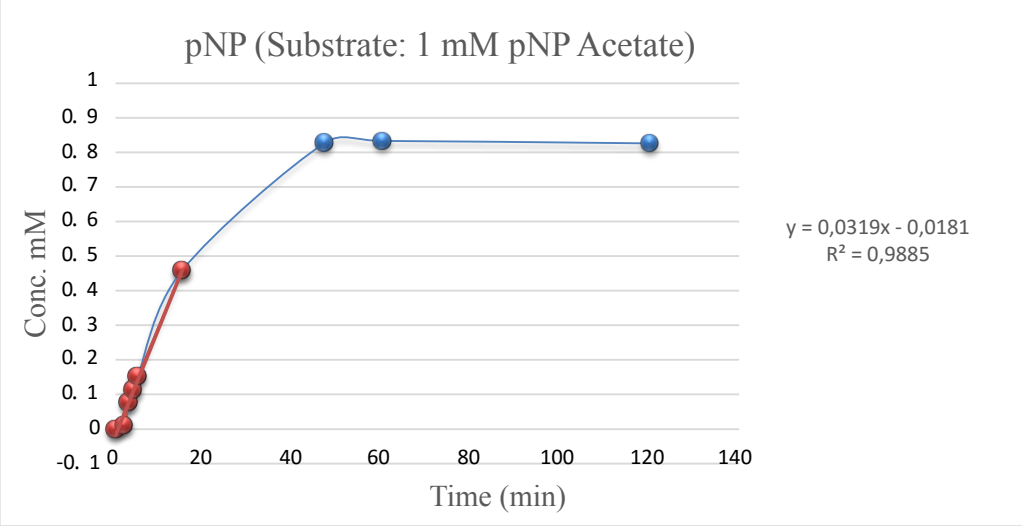

Figure 3.18: (A-B) Graphical examples of the time-*p*-nitrophenol constants for *p*-nitrophenyl acetate. To calculate the initial velocity in the first 15 minutes, the slope of the linear sections are shown in the figures.

Table 3.7: *p*-NP acetate substrate kinetic values.

Substrate: *p*-NP Acetate, mM	**Specific Activity, U/mg protein**
0.01	0.011
0.02	0.017
0.05	0.028
0.1	0.046
0.25	0.108
0.5	0.179
1	0.266

Table 3.8: *p*-NP butyrate substrate kinetic values.

Substrate: *p*-NP Butyrate, mM	**Specific Activity, U/mg protein**
0.05	0.003
0.1	0.005
0.25	0.015
0.5	0.026
1	0.053

Table 3.9: Kinetic parameters resulting from the hydrolysis of *p*-nitrophenyl esters.

***p*-NP Acetate**		***p*-NP Butyrate**	
$\mathbf{V_{max}}$	0.48 U/mg protein	$\mathbf{V_{max}}$	0.19 U/mg protein
$\mathbf{K_m}$	0.69 mM	$\mathbf{K_m}$	3 mM

4. DISCUSSION

Metagenomes coming from soil samples are essential sources for the discovery of novel enzymes. My research team under the supervision of Assoc.Prof. Dr. Gönül SCHARA previously identified a novel lipase gene from a fosmid metagenomic library isolated from Turkey alkali soil samples and the source was unknown.That's why the study could have unique properties.

In the literature, there are different purification studies related to other *Bacillus* type lipases. For example; as a result of studies performed by Ramachandran and Aran (2016), lipase enzyme was purified by ammonium sulphate precipitation and ion-exchange chromatography and 5.1-fold purification and 10.5% yield have been identified. For the purification of *Bacillus* sp. RSJ-1 lipase, a gel filtration technique was used and the specific activity of the enzyme was determined as 428.08 U/mg (Sharma et al., 2002). On the extracellular lipase studies, Sifour et al. (2010) purified the lipase from a thermophilic *Geobacillus stearothermophilus* strain-5 and used ultrafiltration followed by three column chromatographies: Q-Sepharose ion exchange chromatography, Sephadex G-100 gel filtration and adsorption on hydroxyl apatite and identified the result as 22.6-fold with 8.8% recovery.

The optimum temperatures for bacterial lipases generally are 30-60 °C (Sharma et al., 2011). Hiol et. al. (2000) used extracellular lipase from the thermophilic Rhizopus Oryza strain and identified that the optimum pH of the lipase was 7.5 and the optimum temperature was 35 °C. Ramachandran and Aran (2016) also found the optimum pH and temperature for lipase activity were 6.5 and 37 °C respectively. For the kinetic parameters, Asoodeh and Ghanbari (2013) found that the lipase enzyme has K_m of 4.2 mM and V_{max} of 151 μmol min^{-1} mg^{-1} for *p*-nitrophenyl acetate. According to Shi et. al. (2010), the lipase activity was the highest at 50 °C and at pH 8.0. The enzyme retained over 70% of the maximal activity at temperatures of 40–60°C and pH 6.0–11.0 and tributyrin was the substrate.

Considering the studies done, it has been found that the optimum pH of the lipases is between pH 6.0-11.0 and the temperature is in a very wide range (30-60 °C).

Yoo et al. (2011) found that methanol and ethanol environment have negative effects on the enzyme activity and stability. The enzyme was lost its activity at a concentration of 25% and 50% methanol concentrations. At concentrations of 2.5%-25% of ethanol, it was determined that the enzyme was active and stable, but it then inhibited at 50% concentration of ethanol.

In the current work, a second cloning experiment was performed to isolate and express the lipase enzyme contained in the fosmid clone obtained at the end of the screening. The lipase enzyme discovered in these cloning experiments was constructed by pET-21a (+)_LipA-TR plasmid by binding to the pET-21a (+) vector and transferred to *E. coli* BL21 (DE3) pLysS cells for expression of the enzyme. Partial purification of the expressing lipase enzyme was completed and some of the characterization experiments were completed. In this study, the purification protocol was optimized up to 80% purity level and some of the lipase enzyme characterization data were obtained by working with cell lysis. It was observed that the lipase enzyme was effective both intracellular and extracellular environment (Figure 3.13).

Accordingly, the amount of *p*-nitrophenol resulting from the cleavage of *p*-nitrophenyl acetate and *p*-nitrophenyl butyrate substrates from *p*-nitrophenyl esters was measured spectrophotometrically at 405 nm and examined at different substrate concentration ranges. pH studies showed that lipase enzyme was stable in alkaline medium and it was also found to be highly active between 30 °C and 50 °C.

In addition, it was determined that the same substrates were used to measure the enzyme stability in the methanol/ethanol medium, which is the front of the biodiesel studies, and the enzyme activity preservation can be seen in approximately 80% methanol and ethanol by standing in methanol/ethanol solutions for 30 minutes.

When the results were analyzed, it is concluded that the lipase enzyme we have discovered has the potential to be used especially in the detergent and biodiesel industries. We are also excited about the fact that the novel enzyme isolated from an unknown source can further be engineered with protein engineering methods which are frequently used for

the development of industrial enzymes. There are many successful studies on lipase protein engineering (Rathi et al., 2016; Tian et al., 2014; Boersma et al., 2008; Reetz and Carballeira, 2007; Ni et al., 2011). Protein engineering is an efficient technique to increase the catalytic function as well as improve the substrate specificity and stability of an enzyme (Dror et al., 2014; Hwang et al., 2014). There are three main protein engineering approaches;

Rational Design;

– requires knowledge of the structure-function relation of an enzyme.
– provides knowledge to select potential modifications sites.
– the structure of a homologous enzyme utilized.

Directed Evolution;

– not required structural features of lipases.
– rapid and efficient.

The semi-rational design is formed by the combination of rational design and directed evolution techniques.

– In this method, the mutation of predetermined amino acid position is replaced with the other 19 possible amino acids.

Newly discovered lipase can be further developed in future projects, using protein engineering techniques, further enhancing its particular hydrolytic. In addition, for biodiesel production potential studies, methanol and ethanol tolerances of the lipase enzyme and its variants could be measured using different substrates. Particularly biodiesel studies may be performed using lipase enzymes and/or variants using olive oil substrate which was shown the highest stability to methanol/ethanol and exhibit the most hydrolytic activity. It is thought that olive oil and palm oil, as well as waste cooking oils, could be tested as a substrate (Kimtun et al., 2015; Xiao et al., 2009; Sanchez and Vasudevan, 2006; Yücel et al., 2012; Whangsuk, 2013).

5. CONCLUSION AND RECOMMENDATIONS

In this project, a new lipase enzyme discovered from Turkish alkaline soils was cloned, expressed, partially purified and characterized. This new enzyme named as LIPA-TR due to its similarity with true lipases. The lipase enzyme was found, characterized and the enzyme was found to have activity at a broad temperature (25-80 ° C) and pH (5-12). The enzyme has also been found to retain about 60% of its activity in a 70% methanol environment.

These properties demonstrate the potential of the enzyme to be used in detergent and biodiesel industries. However, there is still a need for protein engineering studies since lipases are becoming increasingly important for industrial biotechnology and their activities can be enhanced using protein engineering techniques. The lipase enzyme (s) to be improved by the protein engineering method are expected to be patented.

Rational design, a protein engineering process can be performed to analyze enzymes better. However, this method needs information about similar enzyme structure. For this reason, modeling of LIPA-TR was performed using Swiss-Model (Figure 5.1) and the results were analyzed on PyMOL Molecular Graphics System, Version 1.3, Schrodinger LLC. This method will be useful to find out the significant amino acid positions in the enzyme for my future work.

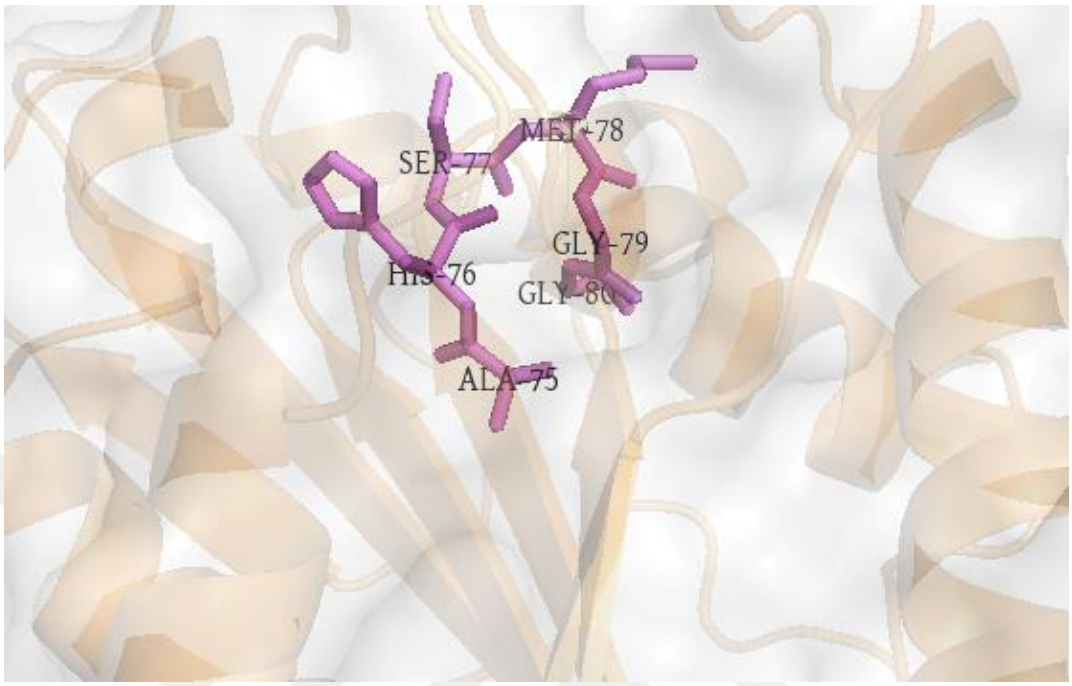

Figure 5.1: Modelling results of α-subunit of cloned LIPA-TR using Swiss-Model and PyMOL, Schrodinger LCC programs. The 3D structure includes the active site residues shown as pink.

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APPENDICES

APPENDIX 1: List of Chemicals.

4-Nitrophenol	Carlo ERBA	Italy
4-Nitrophenyl Acetate	Sigma-ALDRICH	Germany
4-Nitrophenyl Butyrate	Sigma-ALDRICH	Germany
Acetic acid (glacial) 100%	MERCK	Germany
Acrylamide/Bis solution (30%)	MERCK	Germany
Agarose	Bio-Rad	USA
Ammonium Sulfate	MERCK	Germany
Ampicillin	AppliChem	Germany
Coomassie Brilliant Blue R-250	Bio-Rad	USA
Ethanol 96%	MERCK	Germany
Ethanol absolute	MERCK	Germany
Ethidium Bromide	Sigma-ALDRICH	Germany
Gene Ruler 1 kb Plus DNA Ladder	Thermo Scientific	USA
Glycerol anhydrous	AppliChem	Germany
Glycine	Sigma-ALDRICH	Germany
Hydrochloric acid (HCl)	MERCK	Germany
Imidazole	Sigma-ALDRICH	Germany
Isopropyl-β-D-thiogalactopyranoside (IPTG)	Fisher	USA
LB-Agar (MILLER)	MERCK	Germany
LB-Broth (MILLER)	MERCK	Germany
Methanol for liquid chromatography	MERCK	Germany
PageRuler Plus Prestained Protein Ladder (#26619)	Thermo Scientific	USA
SDS	MERCK	Germany
SDS loading buffer pack	Fermentas	USA
Sodium acetate anhydrous	AppliChem	Germany

Sodium Chloride	MERCK	Germany
TEMED	Sigma-ALDRICH	Germany
4-(2-Aminoethyl)benzene sulfonyl fluoride hydrochloride	Sigma-ALDRICH	Germany

APPENDIX 2: List of Enzymes.

Restriction Endonucleases

BamHI	Buffer 3	NEB, UK
NotI-HF	CutSmart® Buffer	NEB, UK

DNA Polymerases

i-Taq™ DNA Polymerase	10x TaqPol Reaction Buffer (w/ 20mM $MgCl_2$)	Intron Biotechnology

DNA Ligase

T4 DNA Ligase	10X Ligation Buffer	Fermentas, USA

APPENDIX 3: List of Equipment.

Analytical Balance	Explorer E12140, OHAUS, Switzerland
Autoclave	3870ELC, Tuttnauer, Netherlands
Centrifuge	Microfuge 16, Beckman Coulter, Germany
Centrifuge	Allegra X-22R, Beckman Coulter, Germany
Dry Bath	MK-10, BioJinn, China
Drying Oven	Ecocell, MMM Group, Germany
Electrophoresis Equipment	Bio-Rad, USA
Electrophoresis Power Supply	Power Source 300V, VWR, Singapore
Fume Hood	Labortex, Turkiye
Ice Maker	AF80 Frimont, Scotsman, Italy
Incubator	EN500, Nüve, Turkey

Incubator Shaker	Innova 40R, New Brunswick Scientific, USA
Magnetic Stirrer	Labworld, Germany
Micro Syringe Pipette	Hamilton, Switzerland
Micropipettes	Research Plus, Eppendorf, USA
Microwave Oven	Beko, Turkey
NanoDrop	Thermo Scientific, USA
Orbital Shaker, Wisemix	RK-1D, Wisd Laboratory Instruments, Korea
pH meter	Hanna Instruments, Clarkson Laboratory, USA
Refrigerated Vapor Trap	Speed Vac Plus, USA
Refrigerator (-20°C/ +4°C)	Arcelik, Turkey
Spectrophotometer	6300, Jenway, UK
Thermal Cycler	VWR, Gene Technologies, UK
ThermoSavant	Speed Vac Plus, USA
Transilluminator	Bio-Rad GelDoc 2000, USA
Ultra Low Temperature Freezer (-80°C)	Elcold, Denmark
Ultrasonic Homogenizer	Sonopuls HD 2070, Bandelin, Germany
UV-Vis Spectrophotometer	Schimadzu, Japan
Vertical Electrophoresis System	MiniVE, PS300-B Hoefer, USA
Vertical Laminar Air Flow	SafeFast Classic, Italy
Vortex	ReaxTop, Heildolph, Germany
Water Purification System	Millipore, Milli-Q Direct-16, France

APPENDIX 4: List of Molecular Biology Kits.

Genomic DNA Purification Kit	Thermo Scientific, USA
PCR Clean-Up, Gel Extraction Kit	Nucleospin Plasmid, MACHEREY- NAGEL, Germany
Plasmid DNA Purification Kit	Nucleospin Plasmid, MACHEREY- NAGEL, Germany

APPENDIX 5: List of Buffers and Solutions.

10X Running Buffer

In a glass of the bottle, 288 g glycine, 60.4 g Tris base, 20 g SDS was added and the mixture is completed the solution to 2 L of ddH_2O.

50X TAE buffer (pH 8.5)

In a glass of the bottle, 242 g Tris base, 57.1 mL glacial acetic acid and 100 ml 0.5 M EDTA are added and the mixture is completed the solution to 1 L of ddH_2O.

Coomassie Blue Staining Solution

In a glass of the bottle, 53% ddH_2O, 40% methanol, 7% acetic acid and 0.25% Coomassie Brilliant Blue R-250 are added.

Destaining Solution

In a glass of the bottle, 90 % dH_2O, 10% acetic acid are mixtured.

Glycerol Stock Solution 50%

5 mL ddH2O are added into 5 mL glycerol and the solution is autoclaved.

IPTG Stock Solution (0.8 M)

0.2 g IPTG into 10 ml ddH2O are mixtured and the solution is filtered with 0.2 mm filter and syringe, load to microcentrifuge tubes.

Sodium Acetate (NaOAc) (3 M, pH 5.2)

6.15 g NaoAc is added into 15 mL dH_2O and then 5 mL of acetic acid is added until pH reach to 5.2. The solution is completed to 25 mL dH_2O and then autoclaved.

Tris (1 M, final volume 100 mL)

10 mM Tris.Cl and 1 mM EDTA are mixtured into 98.8 ml dH_2O (autoclaved).

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