MOLECULAR CLONING, OVEREXPRESSION AND CHARACTERIZATION OF THERMOSTABLE ESTERASE AND LIPASE FROM *Thermophilic Bacillus sp.*

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ABSTRACT

MOLECULAR CLONING, OVEREXPRESSION AND CHARACTERIZATION OF THERMOSTABLE ESTERASE AND LIPASE FROM *Thermophilic Bacillus sp*.

The organisms that reside in hot places called thermophiles become very useful tool for biotechnology. The natural consequence of adapting to hot environments for thermophiles is encoding thermostable enzymes which make them a target for scientists.

We have aimed to use microorganisms that were previously isolated and characterized as a *Bacillus sp*. from Balçova Geotermal region in İzmir for their lipase and esterase activity. In order to measure esterase and lipase activity, the strains were incubated in the media that contain the detergent tween 20 and media containing rhodamin-B, respectively. Three strains out of almost 110 bacterial strains have displayed high lipase and esterase activity at the same time. Three different esterase (Est1, Est2, Est3) and two different lipase (Lip1, Lip2) from different environmental samples were cloned directly by PCR amplification using consensus degenerate primers from genomic DNA. The deduced amino acid sequence of the three types of esterase gene exhibited similar amino acid sequence identity with few amino acid differences. However sequenced lipase genes were complicated to explain so that characterization studies have been made for only esterases.

For over expression in *Escherichia coli,* the esterase genes and lipase genes were sub-cloned in pET28a vector with a strong T7 promoter. A one step purification of the recombinant esterases and lipases was achieved using His-Select HF nickel affinity gel. Enzyme assays using variety of *p*-nitrophenyl (*p*-NP) esters with different acyl chain lengths (C2-C16) as the substrate have confirmed the esterase activity.

All three esterase showed a very high specific activity toward all tested *p*-NP esters. Optimum pH and temperature, stability in terms of pH and temperature, the effect of several metal ions, inhibitors and detergents on activity were determined for purified Est1, Est2, Est3 separately and compared to each other.

ÖZET

TERMAL KARARLI ESTERAZ VE LİPAZ ENZİMLERİNİN *Thermophilic Bacillus sp.*` DEN MOLEKÜLER KLONLANMASI, İFADELENMESİ VE KARAKTERİZASYONU

Termofil olarak adlandırılan ve sıcak yerlerde yaşayan canlılar biyoteknoloji için çok önemli bir konu oldular. Sıcak alanlara adapte olmanın doğal bir sonucu olarak termofiller kendilerini biyoteknolojinin hedefi haline getirecek sıcaklığa dayanıklı enzimleri üretirler.

Çalışmamızda daha önceden Balçova (İzmir) Termal Bölgesinden lipaz ve esteraz aktivitelerine göre izole edilip karakterize edilen *Bacillus sp*. bakterilerini kullandık. Esteraz ve lipaz aktivitesini ölçmek için bakteriler sırasıyla tween 20 deterjanı ve rhodamin-B içeren besi ortamında inkübe edildi. Yaklaşık 110 bakteri gurubundan (strain) sadece 3 tanesi yüksek lipaz ve esteraz aktivitesi gösterdi. Farklı farklı çevrelerden toplanan farklı üç esteraz (Est1, Est2, Est3) ve iki lipaz (Lip1, Lip2) konsensüs dejenere primerler kullanılarak genomik DNA'dan direk olarak PCR çoğaltma metoduyla klonlandı. Bu üç farklı esterazdan elde ettiğimiz amino asit dizileri gösterdi ki aralarında sadece birkaç amino asit farkı var. Ancak dizilenen lipaz genlerinin karakterizasyonu o kadar karışıktı ki karakterizasyon çalışması sadece esterazlar için yapıldı.

Escherichia coli 'deki yüksek yazılımı sağlamak için esteraz ve lipaz genleri güçlü bir promotor olan T7 promotorunu içeren pET28a vektörüne klonlandı. Rekombinant lipaz ve esteraz proteinlerinin saflaştırılması His-Select HF nikel afinite jel metodu kullanılarak tek basamakta yapıldı. Farklı açil zincir uzunluklarına (C2-C16) sahip çeşitli *p*-nitrophenyl (*p*-NP) esterlerinin substrat olarak kullanıldığı Enzim Assayleri esteraz aktivitesini kanıtladı.

Bütün esterazlar test edilen *p*-NP esterlerinin hepsine karşı oldukça yüksek spesifik aktivite gösterdiler. Saflaştırılmış olan esterazlar; Est1, Est2 ve Est3'ün sağlamlığı ve çalışması için gereken optimum pH ve sıcaklık, çeşitli metal iyonlarının etkisi, inhibitörler ve deterjanları belirlendi ve bulduklarımız karşılaştırıldı.

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ABBREVIATIONS

bp	Base pair
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
kb	Kilo base
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel
PCR	Polymerase Chain Reaction
sp.	Species
kb	Kilobase pairs
l	Liter
M	Molar
mM	Millimolar
µM	Micromolar
ml	Milliliter
µl	Microliter
µg	Microgram
ng	Nanogram
nm	Nanometer
w/v	Weight per volume
rpm	Revolutions per minute
Taq	Thermus aquaticus (DNA polymerase)
UV	Ultraviolet
X-Gal	5-bromo-4-chloro-3 indolyl-B-D-galactoside
LB	Luria-Bertani
IPTG	Isopropyl-thio-β-D-galactopyranoside
DMSO	Dimethylsulphoxide
TAE	Tris/acetate/ethylenediamine tetra-acetic acid (buffer)
EDTA	Ethylenediamine tetra acetic acid
TEMED	Tetramethylethylenediamine
min	Minute

CHAPTER 1

INTRODUCTION

1.1. Extremophiles

A man with a naked eye can easily be mistaken about the complexity of the life. Without a careful investigation, the life seems to exist in just so called “moderate” conditions. However when the life is examined deeply it is appreciated that the term “moderate” is a relative term which is set by the evolutionary path of the organism. One moderate condition for a specific organism can be a severe condition for another organism. This is the case for especially microorganisms called extremophiles. Extremophiles are the organisms that have adapted to survive (sometimes obligate) in extreme conditions. Their resistance to extreme conditions make them important tool for industrial applications. Extreme conditions can be any factor that effects the growth of the organism and extremophiles are categorized according to these factors.

Acidophiles are the microorganisms that adapted to live in acidic (low pH) environments. Sulfur oxidation and chalcopyrite concentrate enzymes are the essential enzymes that have important applications in industry. While sulfur oxidation is used in desulfurization of coal, chalcopyrite concentrate is used for valuable metals recovery. On the other hand alkalophiles are the microorganisms that live in basic environments (high pH). Most important enzyme of alkalophiles is cellulase enzyme which is added to detergents in order to degrade polymers.

In addition to pH factor, there are also microorganisms reside in high salt concentration called halophiles. Halophiles are useful in getting rid of ion exchange resin regenerant, producing poly(γ-glutamic acid) (PGA) and poly(β-hydroxy butyric acid) (PHB). Piezophiles which can live in high pressure are useful in forming gels and starch granules. Besides industrial applications, some extremophiles like metalophiles and

radiophile are very important player of remediation trails. Metalophiles are resistant to high metal concentrations whereas radiophiles are resistant to high radiation levels and these two extremophiles are used in cleaning or stabilizing the contaminated areas with either metal or radioactive contaminants respectively. In the next section, the last class of extremophiles which is called thermophiles is explained in detail.

Table 1.1. Industrial applications of enzymes isolated from extremophiles (Source: Demirjian, et al. 2001)

Industrial applications of enzymes isolated from extremophiles.

Extremophile	Habitat	Enzymes	Representative applications
Thermophile	High temperature Moderate thermophiles (45–65°C) Thermophiles (65–85°C) Hyperthermophiles (<85°C)	Amylases Xylanases Proteases DNA polymerases	Glucose, fructose for sweeteners Paper bleaching Baking, brewing, detergents Genetic engineering
Psychrophile	Low temperature	Proteases Dehydrogenases Amylases	Cheese maturation, dairy production Biosensors Polymer degradation in detergents
Acidophile	Low pH	Sulfur oxidation Chalcopyrite concentrate	Desulfurization of coal Valuable metals recovery
Alkalophile Halophile	High pH High salt concentration	Cellulases	Polymer degradation in detergents Ion exchange resin regenerant disposal, producing poly(γ-glutamic acid) (PGA) and poly(β-hydroxy butyric acid) (PHB)
Piezophile	High pressure	Whole microorganism	Formation of gels and starch granules
Metalophile	High metal concentration	Whole microorganism	Ore-bioleaching, bioremediation, biomineralization
Radiophile	High radiation levels	Whole microorganism	Bioremediation of radionuclide contaminated sites
Microaerophile	Growth in <21% O_2		

1.1.1. Thermophiles

Organisms that are evolved to grow optimally at elevated temperatures are called thermophiles (Baker, et al. 2001). Thermophiles usually reside in thermal habitats where temperature is noticeably greater than usual habitats(Lebedinsky, et al. 2007). The reasons for thermal habitats being hot can be natural factors as well as artificially heating. Natural habitats include diverse hot springs or soils of volcanic, rift zones, subterranean hot hydrosphere and anthropogenic biocenoses whereas artificially maintained parts are include bioreactors and hot water supply systems (Ladenstein and Antranikian 1998, Niehaus, et al. 1999, Lebedinsky, et al. 2007).

Although there are some known thermophilic eukaryotes like *Cyanidium caldarium* (T_{max} = 56°C, pH 2-3) (Doemel and Brock 1970), the amoeba *Echinamoeba*

thermarum (T_{opt} = 50°C) (Baumgartner, et al. 2003) and microscopic fungi (T_{opt} = 45-50°C) (Baumgartner, et al. 2003) , the majority of characterized thermophiles are bacteria.

These organisms are classified into subgroups according to their optimal growth temperatures: psychrophiles (below 20°C), mesophiles (moderate temperatures), and thermophiles (high temperatures, above 55°C) (Turner, et al. 2007). Further characterization efforts resulted in advanced thermophilic groups: thermophiles, extreme thermophiles and hyperthermophiles (Baker, et al. 2001) . Overall classification based on the optimum temperature is summarized in the Figure 1.1. It should be noted that even though most of characterized thermophiles are bacteria.

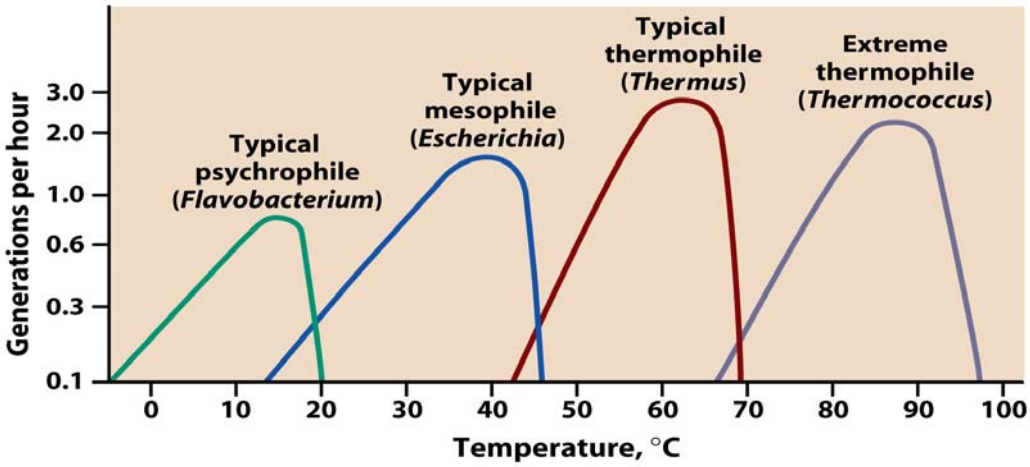


Figure1. 1. The overall classification of bacteria according to optimal growth

(Source: Microbiology, 6/e 2005 John Wiley & Sons)

1.1.1.1. Thermophilic Bacillus

The genus Bacillus contains large and varied collection of aerobic and facultatively anaerobic, Gram-positive, rod-shaped, endospore-forming bacteria(Claus and Berkeley 1872). Bacillus genus consists of wide range of extremophiles including thermophilic, psychrophilic, acidophilic, alkalophilic, freshwater and halophilic bacteria (Wisotzkey, et

al. 1992). The studies with 16S rRNA gene have showed high phylogenetic heterogeneity in the genus Bacillus (Ash, et al. 1991, Rainey, et al. 1994)

Bacillus, Alicyclobacillus, Brevibacillus, Aneurinibacillus, Sulfobacillus, Thermoactinomyces and Thermobacillus are the main genus that contains thermophilic aerobic spore-forming bacteria whose optimum growth temperature range is 45 to 70 °C (Claus and Berkeley 1872, Wisotzkey, et al. 1992, Dufresne, et al. 1996; Heyndrickx, et al. 1998; Touzel, et al. 2000). Among all the thermophiles *Bacillus smithii*, *Bacillus coagulans* and *Bacillus subtilis* are the important thermophilic bacillus (Ash, et al. 1991, Rainey, et al. 1994). One of the previous studies characterized *Bacillus stearothermophilus, Bacillus thermoglucosidasius, Bacillus kaustophilus* at Balçova (Agamemnon) Geothermal region.

1.1.2. Thermophilic Enzymes

It is known that endospore forming bacteria form spores when the environmental conditions become harsh in order to survive up to millions years (Jedrzejas and Huang 2003). While mesophiles forms spores at elevated temperatures thermophiles conserve their stability without transforming into spores. This situation suggests that thermophiles somehow evolved several mechanisms to maintain their structural unity. Despite these structural differences, similarity in key physiological events such as respiration, anatomy and metabolic processes suggests that there is an evolutionary relation in some way between mesophiles and thermophiles (Ljungdahl and Sherod 1960).

For many years, the factors that contribute to the durability of thermophiles attracted scientist which lead to an extensive researches nevertheless the recovered information is still restricted and insufficient (Jaenicke and Bohm 1998). Currently, there are some mechanisms proposed to explain thermophilic resistance including DNA gyrase, chaperons and cell membrane composition. DNA gyrase is a special DNA topoisomerase Type I which introduces to DNA positive supercoils. These supercoils provide extra resistance to by increasing the melting point of the host DNA. Moreover thermophiles have very effective chaperon system. Chaperons are universal repair mechanisms that refold the proteins which are misfolded or lost proper 3D structures. Most over effect of

heat to proteins is the loss of 3D structure which is reversed by chaperons. On top of that the cell membrane composition adds extra strength against high temperatures. Some of the thermophilic Bacillus species possess ω-alicylic fatty acid as the main membranous lipid component (Wisotzkey, et al. 1992). These unique saturated fatty acids provide extra rigidity to cells at increased temperatures (Haki and Rakshit 2003).

In addition to the compositional modifications, there is one more proposed mechanism which explains the stability with extra forces and amino acid change (Scandurra, et al. 1998). Extra electrostatic interactions, formation of hydrogen and disulfide bonds, enhancement of hydrophobic interactions or compaction of the structure are provided by change in amino acid sequence. Scandurra *et al.* reported substitution of Lys to Arg, Ser to Ala, Ser to Thr and Val to Ile (Scandurra, et al. 1998) which leads to increased hydrophobicity and decreased flexibility. These substitutions increase the frequency of helix forming residues while lowering the rate of helix breaking residues (Kumar, et al. 2000).

It is also known that some thermophilic enzymes set their stability by binding metal ions(Mozhaev and Martinek 1984). Lastly distinctions in the numbers of hydrogen bonds and salt bridges are also factors for stability. Mozhaev and Martinek showed 19 hydrogen bonds which do not exist in mesophilic enzymes and they suggested these two forces are the main constituent of thermostability (Mozhaev and Martinek 1984) along with oligomerisation of proteins by shortening of loops (Kumar, et al. 2000)

1.2. Thermostable Enzymes and Industry

1.2.1. Lipases

Lipases are lipolytic enzymes responsible for the catalysis of the both hydrolysis and synthesis of long chain fatty acid esters (Sarda and Desnuelle 1958, Ferrato, et al. 1997). Lipases are found in a wide range of organisms including bacteria, plants and animals but they haven't been identified in archaea so far. Although there are some

exceptions (Martinez, et al. 1992, Hjorth, et al. 1993, Lesuisse, et al. 1993), lipases are activated at the interface between the ester and water where substrates form an emulsion (Sarda and Desnuelle 1958; Verger and Dehaas 1976, Brzozowski, et al. 1991; Ferrato, et al. 1997), which is referred as the "interfacial activation". The reactions catalyzed by lipases involving a large spectrum of esters are stereospecific and regioselective (Jaeger and Reetz 1998, Schmidt-Dannert 1999).

Three dimensional structures of lipases are conserved (Arpigny and Jaeger 1999) having the similar α/β folding in hydrolase domain. Active site of the enzyme contains aspartic-glutamic acid, serine, histidine amino acids. In spite of the structural homology, primary sequences of different lipases are variable and their optimal reaction conditions such as temperature and pH changes considerably (Ma, et al. 2006).

Lipases attract increasingly more attention due to their unique features. Stereoselectivity and regioselectivity of lipases make them valuable tools for organic chemists. Microbial lipases are excreted which facilitates isolation in high amounts. Because of their nature, secreted enzymes are more stable in non-natural conditions and thus microbial lipases gain importance for usage in organic synthesis in various industrial sectors. In addition to the biotechnological applications, some lipases secreted from the pathogenic bacteria have virulent properties that worth a closer look to treat the bacterial infections (Rosenau and Jaeger 2000). Knowledge about the crystal structures of most of the lipases facilitates the engineering strategies for improving their usability in the industry. Generally, lipases don't catalyze some other side reactions and there is no need for cofactors for enzymatic activation most of the time (Jaeger and Eggert 2002). Those features of lipases make them important for research and biotechnology applications as will be discussed in the next section.

1.2.1.1. Biotechnological and Industrial Applications of Lipases

Lipases have numerous biotechnological applications in fine chemistry, cosmetics, laundry, food and pharmaceutical industries, biodiesel production and in sewage treatment(Almeida, et al. 2006). Advancement of the enzyme technology adds new applications to the lipases especially by producing more stable enzymes which is still

functional in unfavorable conditions such as temperatures exceeding 45 ^{o}C (Sharma, et al. 2002). Some of the fats undergo desirable physical changes at high temperatures which contribute to the importance of development of thermostable lipases which are currently being derived from thermophilic bacterial strains (Kambourova, et al. 2003). Besides isolating the thermostable enzymes, recombinant DNA technology provides an opportunity to engineer the enzymes to improve their performance in certain industrial applications (Boston, et al. 1997, Svendsen, et al. 1997, Schmid and Verger 1998).

Lipases are actively being used for the synthesis of new biopolymers. Combinatorial approach is used to synthesize novel polyesters by the high-throughput catalysis of lipase isolated from different sources. By this way, highly diverse polymer libraries are created by using diols and diesters as monomers via the enzymatic catalysis. In addition to this, transesterification or transacylation reactions involving macromolecules is also possible by the same approach(Kim and Dordick 2001).

Biodiesel is a newly emerging fuel derived from natural sources. It is produced chemically by converting oil originating from plants into short chain alcohol esters. Production of biodiesel from the vegetable oil is catalyzed by lipases in a single step reaction which is an example of transesterification reactions. Although this approach is not applicable in the industrial scale because of the cost of the biocatalyst, there are some strategies to overcome this problem such as immobilization of lipase to increase its stability and overexpression in yeast resulting in a solvent-free whole cell biocatalyst (Iso, et al. 2001, Matsumoto, et al. 2001).

One of the most important features of lipases is their enantioselectivity. Creating enantiopure products is essential in the synthesis of pharmaceuticals. Thus lipases become particularly important as biocatalysts for this purpose. Some examples of pharmaceuticals synthesized by the lipase catalysis are anticancer drug epithilone A4 (catalyzed by *Pseudomonas fluorescens* lipase) (Zhu and Panek 2001), antimicrobial compounds (S)- and (R)-elvirol and their derivatives (Ono, et al. 2001) and intermediates 9 and 11 of antimicrobial compound chuangxinmycin (Kato, et al. 1997). Besides these pharmaceuticals, lipases are also used in the production agriculturally important chemicals such as herbicide (S)-indanofan (Tanaka, et al. 2002). cis-4-hydroxy-D-proline or trans-4-hydroxy-D-proline are two diastereomers created from the lipase-catalyzed enantiospecific hydrolysis of racemic 4-oxo-1,2-pyrrolidinedicarboxylic acid dimethyl ester which is an important compound for the synthesis of agriculturally and pharmaceutically important chemicals.

Lipase catalyzed synthesis is well known for the production of flavor compounds and the fragrant chemicals. *Burkholderia cepacia* lipase is utilized to isolate (-)-menthol esters through a transesterification reaction yielding menthyl methacrylate 16 which would then be used for the production of a kind of perfume (Athawale, et al. 2001). (-)-methyl jasmonate is another example of fragrant compounds whose production involves lipase catalysis (Kiyota, et al. 2001).

As it is seen from the limited examples above, lipases have a wide range of biotechnological applications. Producing more stable enzymes which would work even in higher temperatures than the current ones might put lipases into usage in different industrial sectors.

1.2.2. Esterases

Esterases are enzymes catalyzing the reactions in which oxo- and thio-fatty acid esters are hydrolyzed. According to the type of the esterase, maximum enzymatic activity can be achieved towards the substrates with long acyl chains (containing 12-18 carbons) or towards the substrates with shorter chains (containing 2-6 carbons) (Kuznetsova, et al. 2005). Esterases are found in most of the organisms ranging from microbes to the plants and animals; bu in most of the cases, commercially valuable esterases are derived from microorganisms (Kuznetsova, et al. 2005). Enzymatic activity in both aqueous and nonaqueous solvents and stereospecific nature of the catalysis make esterases valuable tools in various aspects of biotechnology. Industrial usages of esterases require stable enzymes because of the similar reasons as seen in lipases. Studies showed that the resistance of the enzyme in organic solvents is proportional to its stability in high temperatures in an aqueous solution (Gao, et al. 2003). Thus production of thermostable enzymes becomes particularly important for industrial purposes.

Esterases have been studied extensively so far creating a collection of information regarding to the sequence data, x-ray crystallography and biochemical properties. This knowledge makes classification of esterases possible in at least 8 subfamilies (Jaeger, et al. 1999, Fojan, et al. 2000). Each of those subgroups have signature amino acid sequences which determine differential selectivity towards the side chains around the ester bond.

1.2.2.1. Biotechnological and Industrial Applications of Esterases

Esterases have a wide variety of applications like lipases. They are involved in oleochemical, food, detergent and pharmaceutical industries. Since esterases are useful for breakdown of the ester bonds, especially thermostable forms of this enzyme is important for laundry for cleaning of oil (DeFlaun, et al. 2007). Esterases can work in aqueous and nonaqueous systems which make them useful for reactions including stereospecific hydrolysis, transesterification and ester synthesis (Bornscheuer 2002, Gao, et al. 2003).

Stereo-selective nature of the enzyme makes it useful for production of pharmaceuticals through the reactions involving various amines and primary or secondary alcohols. This example shows the usage of esterase as a chiral biocatalyst in the area of fine chemical synthesis (Tirawongsaroj, et al. 2008). Esterases also provide opportunity to modify the physicochemical properties of triglycerides for organic synthesis reactions (Kademi, et al. 1999). Stereospecificity of the enzyme is also used for resolution of the racemic mixtures in numerous aspects of organic chemistry.

Despite its attractive properties, isolation of esterases in high amounts is not an easy task unlike the lipases (Kademi, et al. 2000). Perhaps more importantly, enzyme stability should be increased to resist harsh conditions such as high temperatures or the organic solvents in the reaction environment. Previous reports indicated that these types of resistance are related to each other; meaning thermostable enzymes are also resistant to the presence of organic solvents in the environment (Gao, et al. 2003). Recombinant DNA technology has a great potential in this case for the production of more desirable enzymes. By enhancing such properties, esterases might gain increasingly more attention for the industrial usage.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

2.1.1. Chemicals

Chemicals used in this study were shown in Appendix A.

2.1.2. Media

Media were listed in Appendix B.

2.1.3. Reagents and Solutions

Reagents and solutions were presented in Appendix C.

2.1.4. Strains

The strains used in our studies were from one of the previous studies in our Institute carried by Elif Yavuz et al. (2004). Three different thermal environmental samples out of 112 strains from Balçova (Agamemnon) geothermal site were selected for their high lipase and esterase activity. Est1, Est2, Est3 were named according to Esterases from mud, reinjection water and uncontrolled thermal leak, respectively. Lip1, Lip2, Lip3 were named according to Lipases from mud and reinjection water and uncontrolled thermal leak, respectively.

2.2. Methods

2.2.1. Bacteria Growth Conditions

All bacterial strains have been stored at -86 °C for almost three years before our studies. Not only the viability of each strain but also the optimum growth condition of them was tested by monitoring the bacterial growth curve at different temperature and using different media environment.

2.2.1.1. Growth at Different Temperatures

Characterized isolates were grown in nutrient agar plates and incubated for 1-3 days at 30^{o}C, 37^{o}C, 50^{o}C, 65^{o}C. Growth process was checked periodically.

2.2.1.2. Growth at Different Media

The isolates from the -86^{o}C stock were grown in 2xY , LB and Nutrient agar plates and incubated for 1day at 55^{o}C. Growth process was checked periodically.

2.2.2. Screening for Lipase and Esterase Activity

The isolates from the -86^{o}C stock were screened for esterase/lipase activity and media environment used for lipase and esterase screening were listed in Appendix B. One way of screening the precedence of lipase enzyme on agar plates is commonly carried out using Tween 80 as a substrate (Kouker and Jaeger 1987). Other way used in our studies is also commonly used with Rhodamin B +Olive Oil media for lipase activity screening. Tween20 as a substrate was used for esterase enzyme activity screening in our studies.

2.2.3. DNA Isolation and Manipulation

2.2.3.1. Genomic DNA Extraction

There is much different type of methods and commercial extraction kits are available for the Genomic DNA isolation. Selected isolates were activated in 5 ml LB media without any antibiotics, shaking approximately for 18 hours at 55 °C. Genomic DNA was extracted from each isolate by using the Genomic DNA Purification Kit which enables to isolate Gram-positive and Gram-negative bacteria (Fermentas). In the procedure, DNA isolation can be divided into four stages that includes disruption, lysis, removal of proteins and contaminants and finally recovery of the DNA. In our research,

procedure of genomic DNA kit`s was followed according to instructions of manufacturer. After using of the kit, isolated genomic DNA concentration was measured visually with 1% agarose gel electrophoresis.

2.2.3.2. Primer Design

Different bacilli thermostable esterases and lipases were aligned and then designed using Clustal X. program downloaded from internet page of the company with free of charge. The esterase encoding gene was chosen from the genome of B.*Subtilis* and B.*Stearothermophilus.* The gene amplified from genomic DNA by PCR using a pair of degenerate oligonucleotide primers. Forward primer was 5` -CAT ATG ATG AAA RTT GTT MCG CCG AAG-3` , reverse primer was 5'-AAG CTT TTA CCA ATC TAA CKW TTC AAG-3`. .NdeI and HindIII cutting sites are underlined, respectively were introduce to facilitate the cloning procedure. For lipase gene, encoding gene was chosen from the B.*stearothermophilus,* B.*thermoleovorans* and B.*thermocatenulatus*. The gene amplified from genomic DNA by PCR using a pair of degenerate primers. Forward primer was 5` -CAT ATG ATG AAA KGC TGY CGG GTK-3` and reverse primer was 5-AAG CTT TTA AGG CYG CAA RCT CGC-3` . Also, NdeI and HindIII cutting sites are underlined, respectively were introduce to facilitate the cloning procedure.

2.2.3.3. PCR Amplification

PCR was conducted for three selected genome DNA as a template and the following parameters for lipase: an initial denaturation step at 94 °C for 5 min, followed by 30 cycles at 94 °C for 1 min, annealing at 56 °C for 2 min, and extension at 72 °C for 2 min, at 72 °C for 10 min and preservation at 4 °C using Taq DNA polymerase. For esterase 94 °C for 5 min, followed by 30 cycles at 94 °C for 1 min, annealing at 58 °C for 2 min, and extension at 72 °C for 2 min, at 72 °C for 10 min and preservation at 4 °C

using Taq DNA polymerase Amplified products were run on a 1% agarose gel stained with ethidium bromide and visualized under UV light.

2.2.3.4. Agorose –Gel Electrophoresis

In order to visualize PCR products, agarose gel electrophoresis technique was used. Firstly, to prepare 1% (w/v) agarose gel 0.5 gram agarose was dissolved in 50 ml 1X TAE buffer and boiled in microwave until a clear solution was observed. After that solution was cooled and 0.5 μg/ ml EtBr from a 10 mg/ml was put into the solution. Then solution was transferred to horizontal gel apparatus and comb was positioned in it and it was cooled at room temperature in order to solidification. After the solidification, comb was removed gently and gel was transferred in to tank that included 1X TAE electrophoresis buffer. Next, DNA samples which were mixed before 6X loading dye were loaded into gel wells. Then, 100V electric field was applied for 30-45 minutes. Finally, gel was visualized under the UV in gel documentation system in order to observe the DNA bands.

2.2.3.5. PCR Product Purification

The desired PCR products were isolated from the gel by PCR purification Kit (DNA Gel Extraction Kit, FERMENTAS). The quantity of each samples were calculated by measuring the ratio of absorbance at 260 nm and 280 nm. Also qualities of samples were evaluated by calculating the ratio of A260/A280**.**

2.2.3.6. The Cloning of Lipase and Esterase Genes

2.2.3.6.1. Bacterial Transformation

2.2.3.6.1.1. Protocol for Ligation to pTZ57R/T vector

The amplified and purified selected PCR fragments were cloned by using TA cloning vector, pTZ57R/T PCR cloning Kit (Fermentas). All ligation reaction was set up in 10 µl and incubated 1 hour at 22 °C. The cocktail contains 1 µl T4 DNA ligase (3 Weiss units/µl), 1 µl pGEM-T Easy vector (50 ng), and 5 µl 2X Rapid ligation Buffer of T4 DNA ligase. The appropriate insert volume was adjusted according to Insert:Vector molar ratio which optimized as 3:1 and it was calculated for each reaction by using the following equation.

$$\frac{\text{ng of vector x kb size of insert}}{\text{kb size of vector}} \times \text{insert : vector molar ratio} = \text{ng of insert}$$

2.2.3.6.1.2. Protocol for Competent Cell Preparation

In order to activate stock of *E. coli Dh5α* strain that stored in – 80°C was spread on a LB agar plate and incubated overnight in incubator at 37^{o}C. A single colony was chosen from the incubated plate and was preincubated in 10 ml LB broth for 6 hours in the incubator-shaker at 200 rpm at 37°C. After the preincubation, cells was transferred into 200 ml SOB medium and incubate in orbital shaker (200 rpm) overnight at 10°C for to reach the mid-log phase. Following day, bacterial culture in SOB medium was incubated in ice for 10 minutes. At the same time, 5 pieces 50 ml falcon tubes were cooled in ice.

Next cultures were aliquoted in previously cooled 50 ml falcon tubes with the volume of 40 ml. After that cultures were centrifuged at 4000 rpm for 10 minutes at 4°C to pellet the cells and supernatant were removed gently and pellet was resuspended in 5 ml ice-cold TB. Subsequently tubes were centrifuged (4000 rpm) for 10 minutes at 4°C and supernatant was discarded again. In next step 2.5 ml ice-cold TB and 300 µl DMSO were added onto the pellet resuspended gently by pipetting. At last, final solution was aliquited with the volume of 100 µl to sterile eppendorf tubes which were already cooled on ice. The prepared cells were not only ready to use also can be stored at -80°C for long term storage without loose of competence ability.

2.2.3.6.1.3. Protocol for Transformation of Cloned pTZ57R/T easy Vector into Compotent Cells

Previously prepared frozen competent cells were removed from –80°C and waited on ice until thawed (about 5 minutes). 4µl of each ligated vectors was added on 100 µl competent *E.coli Dh5α* cells and tubes were gently flicked to mix. After that tubes were cooled on ice for 10 minutes and in order to heat-shock placed in water bath at exactly 42°C for 30 minutes. Next again tubes were cooled on ice for 2 minutes. Finally 250 µl SOC medium at room temperature was added to the tubes and allowed to incubation at 37°C for 1 hour on shaker (200 rpm).

2.2.3.6.1.3.1. Screening Transformants for Verification

20 µl transformated celles were spread on LB plates which include 80µg/ml X-Gal, 0.5 mM IPTG and 100 µg/ml ampicillin then incubated overnight at 37°C in incubator. In successful insertion, pGEMT-Easy vector interrupts the β-galactosidase enzyme synthesis so tranformated clones can be observed easily by color screening on indicator plates. At

this Stage of expreriment, white colonies were chosen for the following experiment, cloning to the expression vector.

2.2.3.6.1.4. Protocol for Ligation to pET-28a(+) Expression Vector

pET-28a(+) Expression Vector was used for the expression of the designed proteins. The features of the vector are shown in Figure 2.1 and Figure 2.2. The esterase and lipase genes were restricted from the pTZ57R/T vector using *NdeI* and *HindIII* restriction endonucleases and purified using 1% Agarose gel electrophoresis. The gel-extracted DNA fragments were ligated with *NdeI*/*HindIII* restricted pET-28a(+) expression vector (Novagen). After this final subcloning step, both genes were sequenced in the pET-28a(+) vector to confirm their sequence.

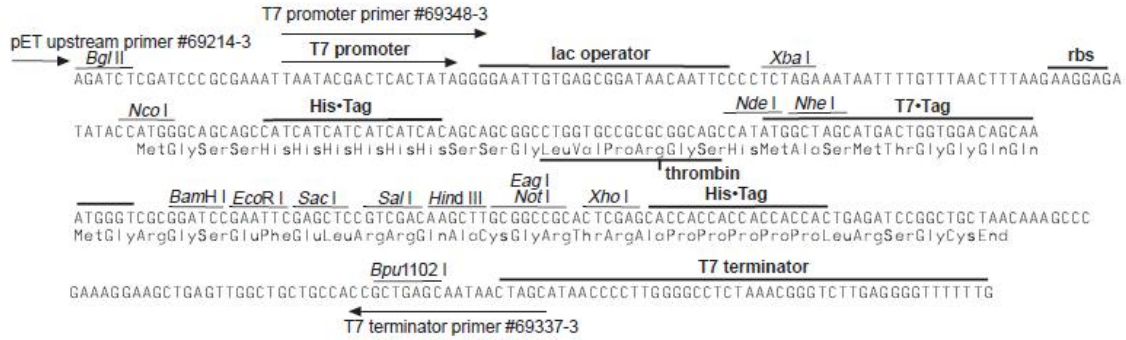


Figure 2. 1. pET28 a (+) expression region

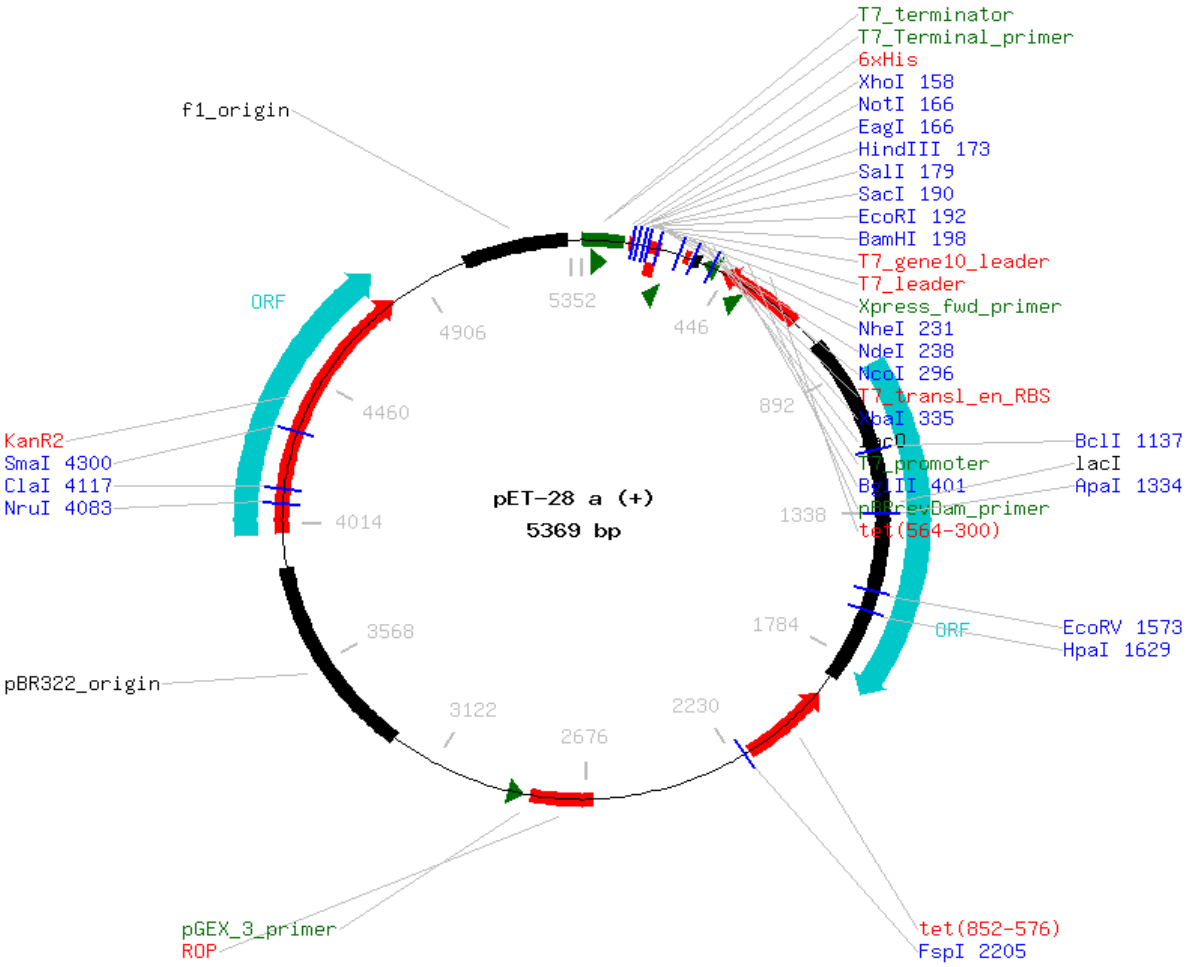


Figure 2. 2. pET28 a (+) expression vector and multiple cloning site

2.2.4. Protein Sample Preparation

2.2.4.1. Cultural Conditions

The transformant cell was grown in different media and different temperatures in order to optimize the expression level of the proteins. LB (Appendix B), Terrific Broth (Appendix B) and 2XYT as a media were used for the optimization of enzyme production.

Expression were carried out at 30°C, 40°C, 50°C in order to find out the better level of expression for the designed proteins.

2.2.4.2. Expression of the Transformated Genes

Esterase and lipase genes in the pET-28a(+) expression vector were transformed into *E. coli* strain BL21(DE3). This expression vector system is based on bacteriophage T7 RNA polymerase the BL21(λDE3) host contains a gene for the T7 RNA polymerase, under control of isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter and T7 transcriptional promoter (the target gene, esterases, is placed downstream from the strong phage T7 transcriptional promoter on the pET expression vector). The expression of the esterase and lipase genes is induced by addition of the lactose analog isopropyl-β-D-thiogalactopyranoside (IPTG) which derepresses the lac operator and allows the expression of T7 RNA polymerase , which in turn transcribes the target gene. Because the pET expression vector also contains an kanamycin resistant marker, 30 ug/ml kanamycin was included in all media to maintain selective pressure for the vector. In the expression experiment, fresh streaks of transformed *E.coli* were used to inoculate overnight cultures of 100 ml Lowry Broth (LB) media (Sambrook *et. al.,* 1989). The following day the cultures were diluted 1:10 into 1000 ml fresh LB media and grown at 37°C to an optical density of A_{600}=1.0 which is half-stationary phase. At that point expression of the esterase and lipase genes was induced by the addition of 1 mM IPTG. The cells were allowed to grow for an additional 4.0 h and were then harvested by centrifugation (8,000 X g for 10 min). The harvested cell pastes were stored frozen at –20 °C until ready for use. Expression of esterase proteins was evaluated using sodium dodecylsulfate (SDS) PAGE.

2.2.4.3. Total Protein Extraction

Recombinant lipases and esterase's cells were harvested by high-speed centrifugation at 10000 rpm, 10 min, +4 °C. Cell pellets were re-suspended in 20mM Tris-HCl buffer (pH 7.0) and disrupted by sonication for 10 min. Cell debris were removed by centrifugation at 5.000 g, 15min, +4 ºC. The supernatant was used for SDS-PAGE analysis and protein purification.

2.2.4.4. Affinity Chromatography

The purification procedure was carried out on ice using Low Pressure Liquid Chromatography system (Pro Team LC™ 320, Teledyne Isco). The frozen cell pastes were thawed and resuspended in 10x weight:volume of 50mM sodium phosphate, pH 7.0 (phosphate buffer). The cells were lysed by a sonicator (Sim-Aminco, Spectronic Instruments) for 5 minutes. The lysed sample was centrifuged at 10,000 x g for 30 minutes in a Beckman GSA rotor. Insoluble material was discarded and the soluble fraction was retained for further purification. The soluble fraction from the cell lysis step was loaded onto a 2.5 cm x 10 cm His-taq Nicel Affinity (Sigma) column previously equilibrated with phosphate buffer. The column was washed with phosphate buffer including 0.3 M NaCl, and the bound proteins eluted with a step elution of 250 mM immidazole in phosphate buffer including 0.1 M NaCl. The eluted proteins were fractioned as 20 drops in each collection tubes. The elution peak from the Nicel column "pool" was collected and dialysed against phosphate buffer.

2.2.4.5. Dialysis

In order to get rid of the imidazole that comes from elution step of the affinity chromatography, enzyme in 250 mM imidazole was dialyzed against 50 mM phosphate buffer at pH7.0. The final dialyzed sample was aliquoted and stored frozen at –70 C prior to use. Protein homogeneity was evaluated using Coomassie Blue visualized SDS PAGE.

2.2.4.6. SDS-PAGE

To determine the homogeneity and molecular weight of the lipase and esterase, SDS-PAGE was carried out following the Laemmli method (Laemmli 1970). Separation device was carried out in the Thermo Scientific electrophoresis. The gel contained 12% (w/v) polyacrylamide gels in the presence of 10% (w/v) SDS and 30% acrylamide mixture, 1.5 M Tris-Hcl buffer, Ph 8.8, 10% (w/v) ammonium per sulfate and TEMED. Stained with Coomassie briliant blue R-250. The molecular marker used in the analysis were myosin(200.0 kd), β-Galactosidase (116.0 kd), bovine serum albumin (68.0 kd), ovalbumin (43 kd), carbonic anhydrase (29.0 kd), trypsin inhibitor (20.0 kd), lysozyme (14.4 kd) and aprotinin (6.5 kd).

During the preparation of resolving gels; polymerization started instantly after adding TEMED to mixture. Resolving mixture poured into the thin gap between two plates to polymerization for 30 minutes. In order to avoid drying, the gel was covered by distilled water in each steps of SDS-PAGE. This step was followed with the preparation of stacking gel. In preparation of stacking gels; the mixture was poured into the polymerized resolving gel and then comb which has 10 holes placed on the gel before polymerization process. After the polymerization of stacking gel, the comb removed from the top of gel. Gel was placed into the buffer and reservoirs were filled until it reached the filling line level with 1X Tris-Glycine-SDS running buffer.

Sample buffer used for mixing with the sample and diluted with the ratio of 1:4 (v/v) .Then Samples were heated at 95°C in water bath for 4-5 minutes.

Colour of samples turned into blue after heating. 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 100 volts for 2 hours until the blue dye reached the bottom of the gel. After electrophoresis, the gel was taken out from two glass plates by the help of the distilled water and placed into the shallow staining tank. Staining solution which was prepared with the coomassie colloidal blue was applied on gel for min. 24 hours with 25 rpm shaking. The gel was washed with water after the staining process and then treated with neutralization buffer for three minutes. Another step is that destaining solution applied on gel less than one minute. Last step, gel placed on fixation solution for one day. After each process, gel washed with water very carefully and photo of gel was taken using the gel photo system.

2.2.4.7. Protein Determination

Quantitative protein determination was spectrophotometrically measured at 595 nm according to Bradford's method. Bovine serum albumin was used as a standard protein.

2.2.5. Esterase Enzyme Characterization

Because of better expression level and no problems with the purification process of esterase enzyme, only esterases from three different sources were characterized and compared to each other.

2.2.5.1. Assay of Esterase Activity

The esterase activity was assayed spectrophotometrically using *p*-nitrophenyl acetate as a substrate having one of the best hydrolytic activity among the variety of *p*-nitrophenyl (*p*-NP) esters with different acyl chain lengths (C2-C16) in our studies. The assay was based on hydrolytic cleavage of *p*-nitrophenyl ester by enzyme to release *p*-nitrophenol and acetic acid. The assay mixture (1 ml) contained 0.5 mM *p*-nitrophenyl acetate substrate dissolved in acetonitrile including Reagent A buffer (100 mM sodium phosphate buffer with 150 mM sodium chloride and 0.5 % (v/v) Triton X-100, pH 7.2). 10 mM purified enzyme was used and initial rates were estimated by measuring the increase in absorbance at 400 nm as a function of time. The micromolar extinction coefficient of p-nitrophenol at 400 nm was 0.0148 described by Quinn, D.M. et al. The assay was performed using a suitably thermostatted spectrophotometer (Perkin Elmer) at 55 °C observed to be optimum temperature for esterase enzyme activity in our studies. One unit of esterase activity was defined as the amount of enzyme releasing 1.0 nanomole of p-nitrophenol per minute at pH 7.2 at 55 °C using p-nitrophenyl acetate as a substrate.

2.2.5.2. Determination of Substrate Specificity

In order to determine substrate preference, enzyme activity of purified esterase enzymes towards p-nitrophenyl esters of various chain lengths was performed spectrophotometrically at 55 °C. The following compounds were used as substrates at a concentration of 50 mM; p-nitrophenyl acetate, p-nitrophenyl butyrate, p-nitrophenyl p-nitrophenyl p-nitrophenyl p-nitrophenyl p-nitrophenyl p-nitrophenyl.

2.2.5.3. Kinetic Studies

In order to determine the Michaelis-Menten kinetic parameters K_M and V_{max} values of esterase enzymes from different sources of Balcova geothermal region, Lineweaver-Burk plots were used assuming that the reactions followed a simple Michaelis-Menten kinetics. Lineweaver-Burk curves were obtained for p-NP acetate substrate at pH 7.0 and 55^0C using standard enzyme assay. The enzyme activity was measured at 6 different p-NP acetate substrate concentration (0.02, 0.04, 0.08, 0.1 and 0.2 mM).

2.2.5.4. Effect of Temperature on Enzyme Activity

Effects of temperature on esterase activities were evaluated by testing a wide range of temperature (30-90 ºC). During the experiment, buffer was heated to relevant temperature before the assay. The results were expressed as relative activities (%).

2.2.5.5. Effect of pH on Enzyme Activity

Effect of pH on enzyme activity was investigated using 0.1 M sodium phosphate buffer at different pH values. The optimum pH of the enzyme was determined under the standart assay conditions by measuring activity in the presence of buffers at different pH values ranging from 4.0 to 11.0. The results were expressed as relative activities (%).

2.2.5.6. Effect of Various Agents and Metal Ions on Enzyme Activity

In order to understand how different agents may effect the enyzme activity, esterase activity assay was carried out in the presence of different metal ions with %1 by volume. For this purpose, $CaCl_2$, $CuSO_4$, $MgSO_4$, KCl, $MgCl_2$, NaCl and $ZnCl_2$ were used.

The effect of some agents such as ethylenediaminetetraacetic acid (EDTA), sodium flouride, dimethyl sulfoxide (DMSO), dithiothreitol (DTT), sodium diethyldithiocarbamate, SDS, ethanol, isopropanol, b-mercaptoethanol and Triton X-100 on enzyme activity were also examined. For this purpose, the enzyme was incubated in the presence of different agents for 10 minutes at room temperature. The concentrations of the agents were; 5% (v/v) of Triton X-100 or 1mM of other agents in 1ml assay mixture.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Investigation of Lipase and Esterase Activity on Agar Plates

The bacterial strains used in our studies were isolated and characterized previously by Elif Yavuz et. al. The 112 isolates were tested according to lipase and esterase enzyme activity from Balçova Geothermal region in Izmir. This region could serve as a good source for new thermophilic microorganisms and enzymes with the industrially important properties. Usage of thermostable enzymes such as lipase and esterase from microorganism is being used in many industrial fields so in our research that we have aimed to discover advantage of thermophilic enzyme. Moreover, Recombinant DNA technology and effective purification techniques were used for the production of selected lipase and esterase enzyme and also characterization of esterase genes.

In this study, 112 bacterial strains were screened and three of them were selected due to their high lipase and esterase activity and were named Est1, Est2, Est3 and Lip1, Lip2, Lip3 respectively. Est1, Est2, Est3 were named according to Esterases from mud, reinjection water and uncontrolled thermal leak, respectively. Lip1, Lip2, Lip3 were named according to Lipases from mud and reinjection water and uncontrolled thermal leak, respectively.

Our research studies are based on the previous studies of Elif Yavuz graduated from Biotechnology program in our Institute. They have isolated strains showing extracellular enzyme activities were obtained from different sources. 45% of the isolates were from uncontrolled thermal leak, 31% of the isolates were from re-injection water samples and 24% of the isolates were obtained from the mud samples and all these isolates were collected from Balçova Physical Therapy and Rehabilitation Centre (Yavuz et al., 2003;2004). Schematic diagram of isolates are shown below (Table 4.1).

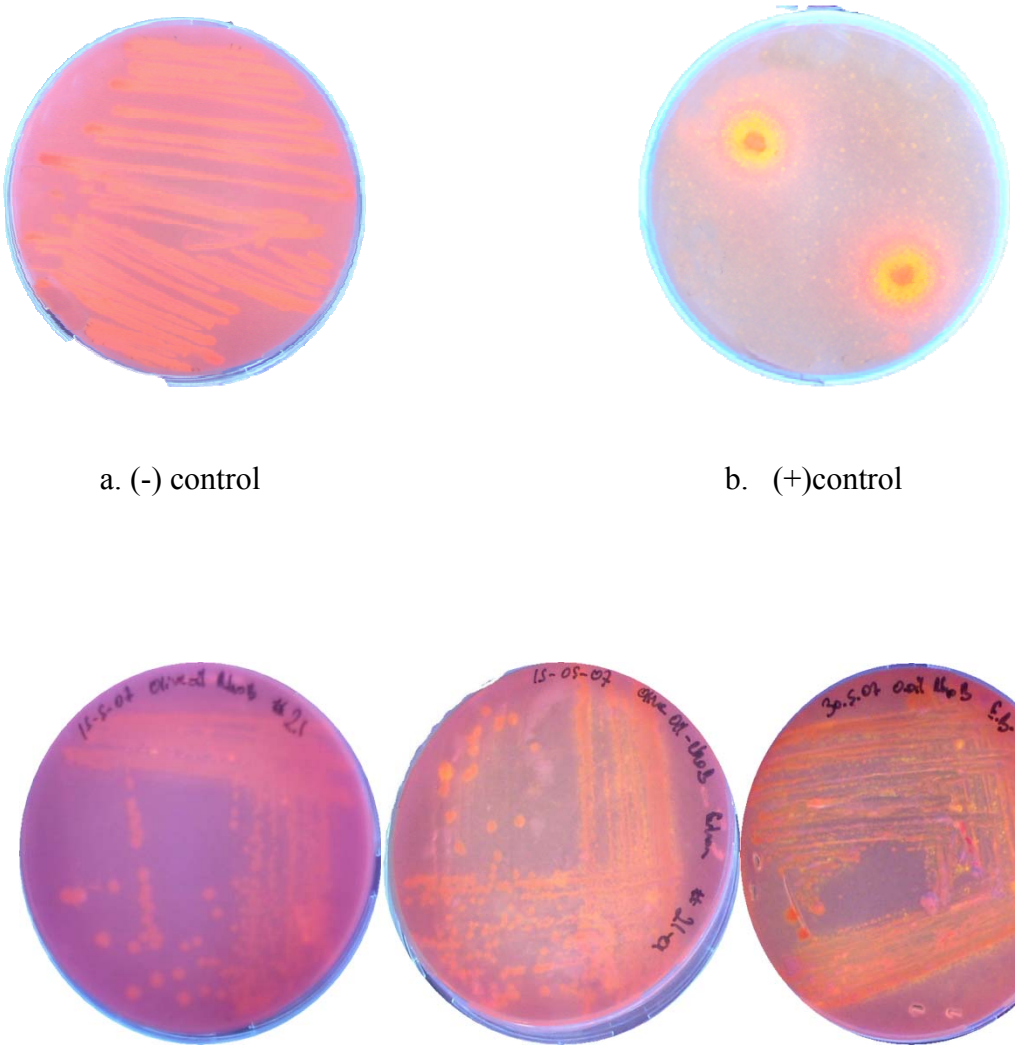
All bacterial strains have been stored at -86 °C for almost three years before our studies. Not only the viability of each strain but also the optimum growth condition of them was tested by monitoring the bacterial growth curve at different temperature and using different media environment. According to our results the viability of bacterial strains were good (data not shown) that resulted in typical bacterial growth curve includes lag, log and stationary phases.

The isolates from the -86^{o}C stock were then screened for esterase/lipase activity. Rhodamin B +Olive Oil media was used for lipase activity screening and Tween20 was used for esterase enzyme activity screening in our studies.

Table 3.1. Sample, names and numbers of isolates.

Sample	**Isolate Number**	**Isolate Name**
Uncontrolled thermal leak	50	1,2,3,4,5,6,8,9,10,11,28a,13,14,16,17,18,19,20,21a,23,24,64b,26,651a,28,36,37,42,43,44,45,46,47,651b,50,53,58,59,60,61,63,64,65,66,67,70,86,87,90,92
Reenjection Water	35	75A,38y,21,62B,74B,30,75B,32,33,34,35,38,39,40,41,49,52,54,55,56,57,62,71,72,73,74,75,76,77,78,79,80,84,85,94
Mud	27	Ç1A, Ç1,Ç-2, Ç-3, Ç-4, Ç-5, Ç-6, Ç-7, Ç-9, Ç-10, Ç-11, Ç-13, Ç-14, Ç-16, Ç-17, Ç-19, Ç-20, Ç-21, Ç-22, Ç-23, Ç-24,Ç-31,Ç-33, Ç-34,Ç-32,Ç-35,Ç-30

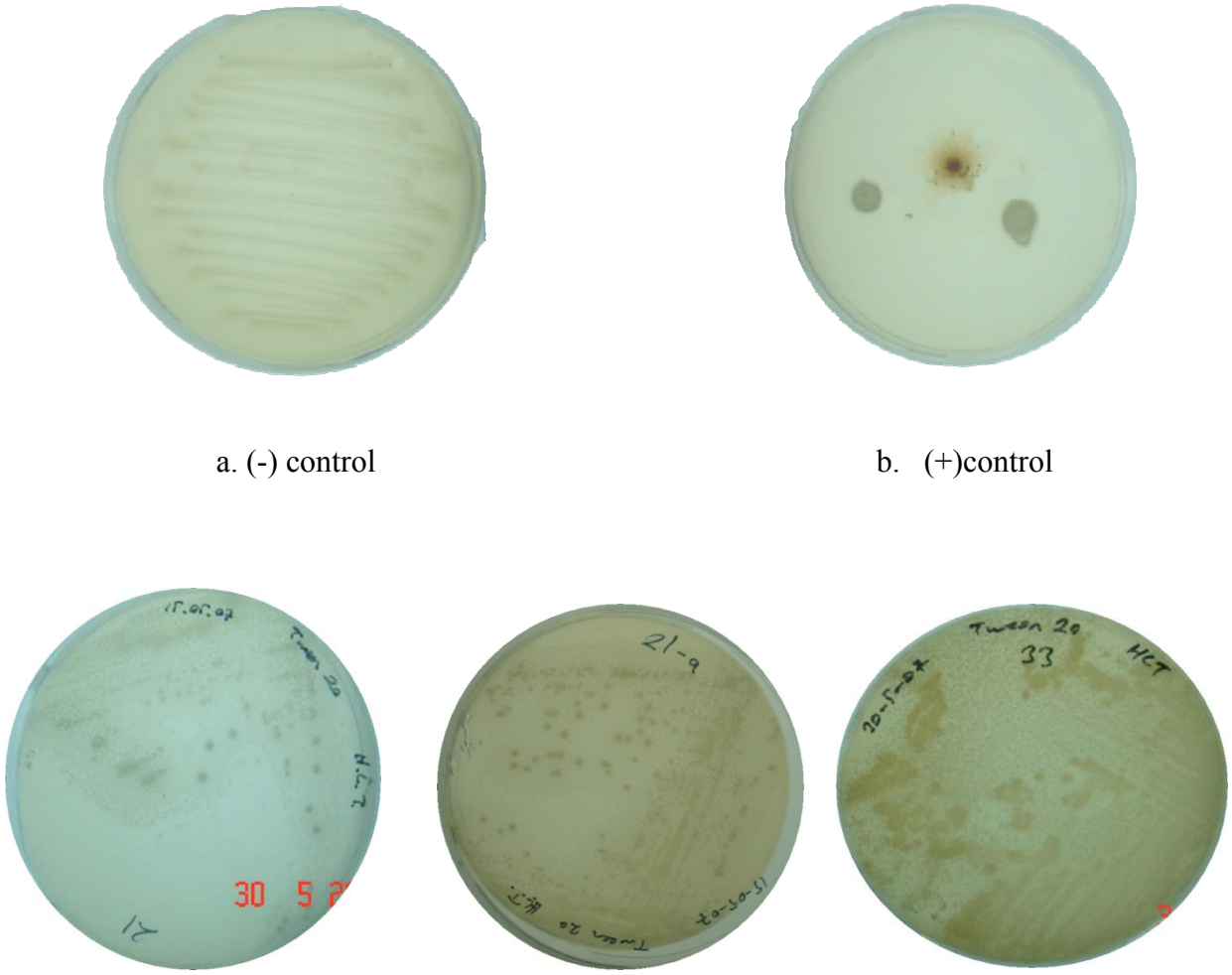
For lipase screening, inoculated bacterial isolates on specified agar plate after 24-36 h incubation at 55 ^{o}C have shown orange fluorescence around colonies under UV irradiation indicated true lipase activity. On the other hand strains without lipase activity have formed pink colonies (Figure 3.1). Esterase activity determination was detected by observing clear zones around the isolates which have esterase enzyme activity and no zones around colonies has indicated lack of esterase activity (Figure 3.2).



a. (-) control b. (+)control

c. Lip1 d.Lip2 e.Lip3

Figure 3. 1. Production of lipase activity from three selected isolates



a. (-) control b. (+)control

c. Est1 d .Est2 e. Est3

Figure 3. 2. Production of esterase activity from selected three isolates

3.2. Cloning of the Esterase and Lipase Gene from *Bacillus sp.*

The characterization of sequence similarity in distantly related proteins has proved useful for understanding the evolution of gene families. Nowadays, knowledge of bacterial lipolytic enzymes is increasing at a rapid and exciting rate. The thermostable esterase and lipase genes were amplified from genomic DNA of three different isolates

from different sources (mud, reinjection water and uncontrolled thermal leak) by designing a pair of degenerate primers through aligment of reported sequences of thermostable lipase and esterase genes from database. The amplified genes for lipase were ~1250 bp and ~750bp for esterase genes. Cloning of the amplified genes to Ptz57R/T vector was facilitated by single 3'-T overhang of the vector and a single 3'-A overhang of the PCR product. The Taq DNA polymerase conferred 3'-A overhang to the PCR product. All experimental procedure related to cloning studies were described by schematic for Esterase gene in Figure3.3. and Lipase gene in Figure 3.4.

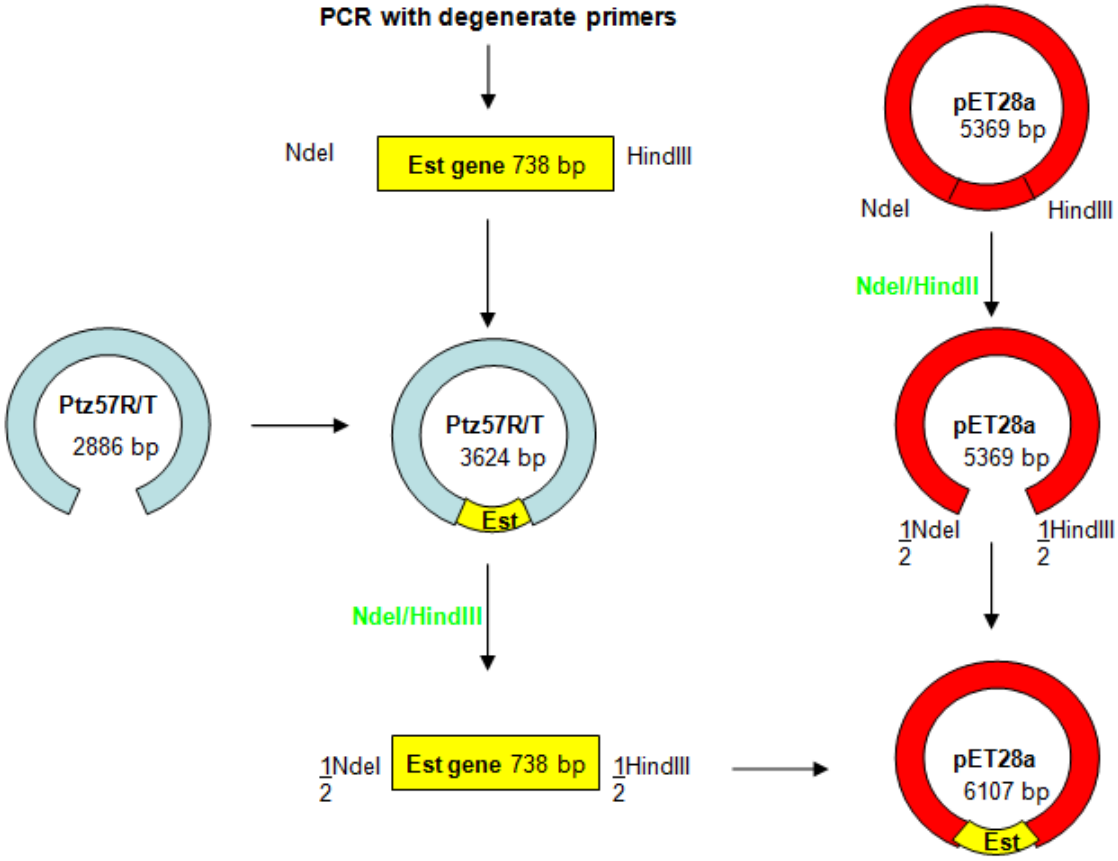


Figure 3. 3. Schematic representation of the esterase gene experiment flow chart

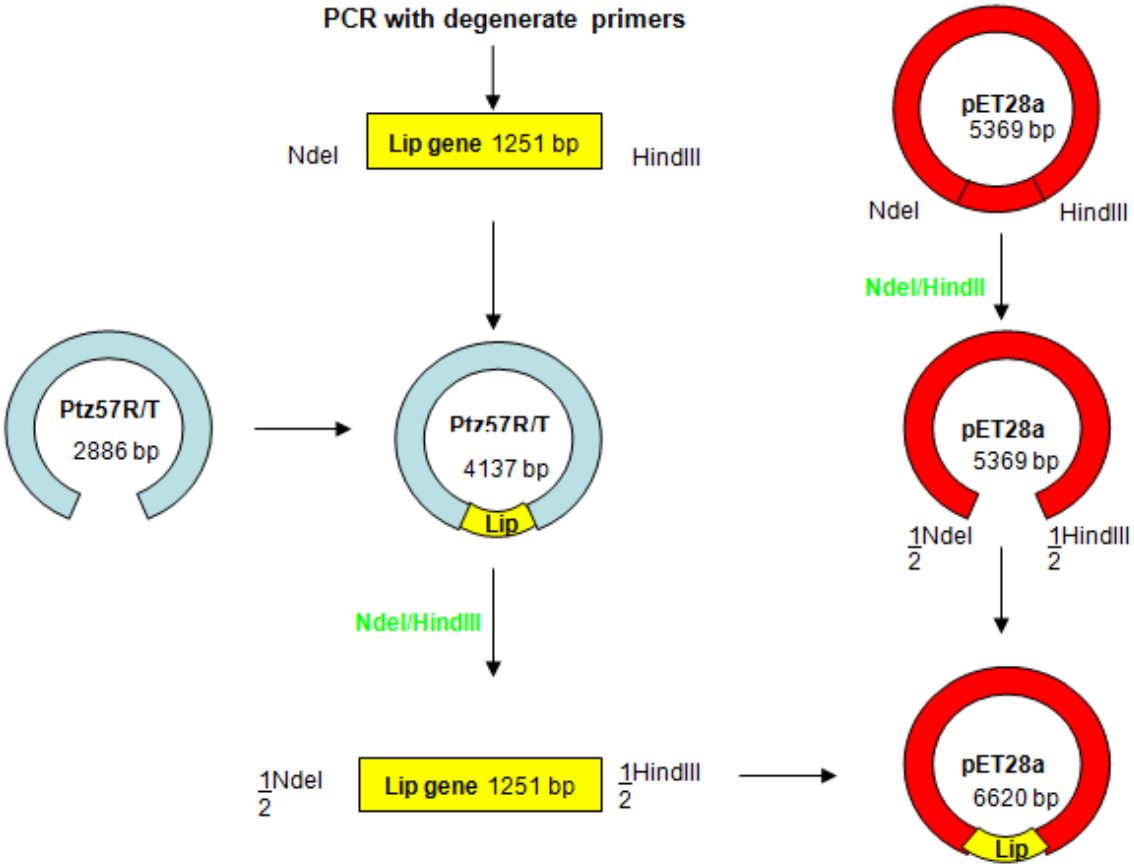


Figure 3. 4. Schematic representation of the lipase gene experiment flow chart

In our study we prefer InsTAclone™ PCR Cloning Kit as cloning system and pET 28 a (+) as expression vector. In the first step of cloning procedure the genes that encode these two proteins are amplified by using Polymerase Chain Reaction (PCR) technique. The integrity and quantity of amplified two gene were visualized in 1% agarose gel electrophoresis before ligation into the cloning vector (Figure 3.5.). During PCR reactions special primers are used to add the restriction sites of both HindIII and NdeI endonucleases to flanking regions of the enzymes. These restriction sites will be useful to remove our genes from cloning vector and to ligate into expression vector in the right orientation. Among the several endonucleases, we prefer HindIII and NdeI because the genes do not contain their restriction sites and they can run in the same buffer efficiently. During PCR amplification reaction Taq Polymerase who lacks proofreading mechanism (3'→5' exonuclease activity) is enzyme used as DNA polymerase enzyme. Because of the lack of an efficient proofread mechanism DNA Taq Polymerase adds extra dNTPs at the 3' end which is mostly characterized by last amplified nucleotide. The trick of TA cloning hides here because these amplified fragments are ligated into a special vector called

pTZ57R/T which contains additional dTTP at 3' ends. Due to complementarity issues the plasmid is not ligated without a proper insert which increases the efficiency of vector dramatically (InsTAclone™ PCR Cloning Kit)(Figure 3.6.).

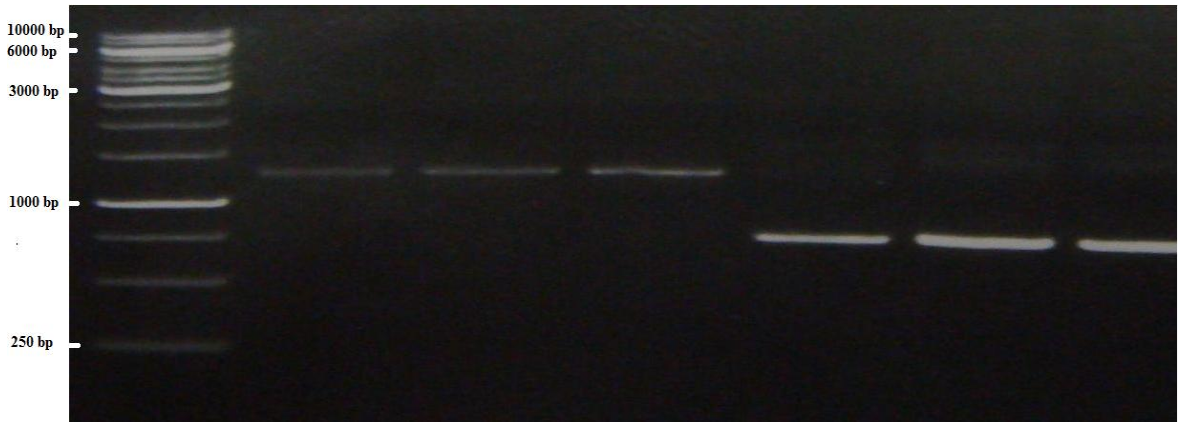


Figure 3. 5. Agarose gel picture of PCR amplified Lipase and Esterase

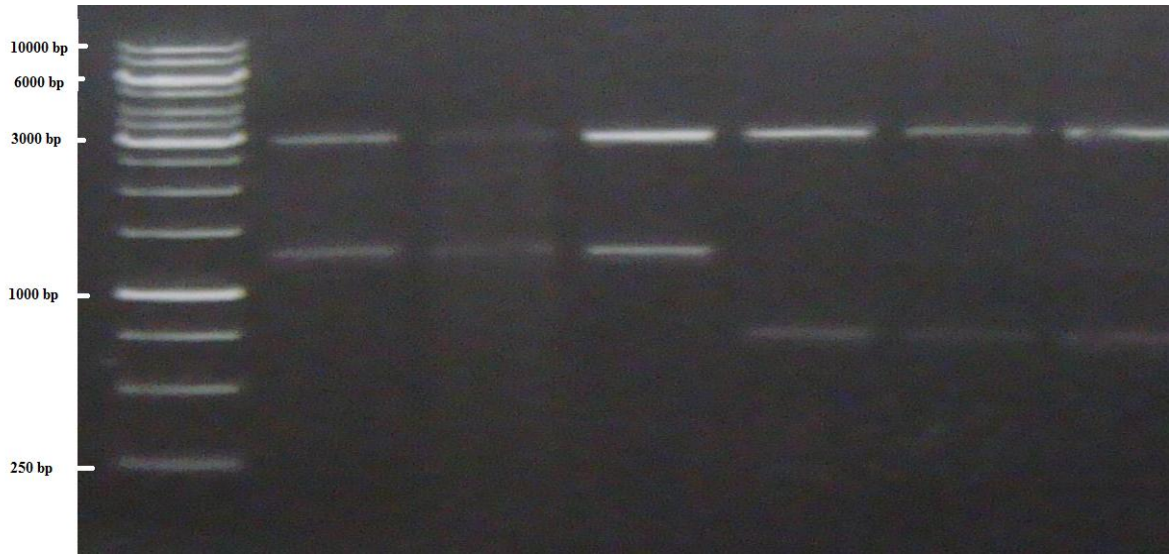


Figure 3. 6. The restriction analysis of lipase and esterase in pTZ57R/T(cloning vector)

3.3. Sequences Analysis

Sequence of esterase genes showed that Est1, Est2 and Est3 were 744 bp in length which codes for 247 amino acids and stop codon (Figure 3.7.). All three genes were

compared and total four nucleotide differences were observed. These differences also have resulted in total three amino acid differences among all three esterase genes (Figure 3.8.)

```
Est21a          ATGATGAAAATTGTTCCGCCGAAGCCGTTTTTCTTTGAAGCCGGGGAGCGGGCGGTGCTG 60
Est33           ATGATGAAAGTTGTTCCGCCGAAGCCGTTTTTCTTTGAAGCCGGGGAGCGGGCGGTGCTG 60
Est21           ATGATGAAAGTTGTTACGCCGAAGCCGTTTTTCTTTGAAGCCGGGGAGCGGGCGGTGCTG 60
                ********* ***** ******************************************

Est21a          CTTTTGCATGGGTTTACCGGCAATTCCGCCGACGTTCGGATGCTTGGGCGATTCTTGGAA 120
Est33           CTTTTGCATGGGTTTACCGGCAATTCCGCCGACGTTCGGATGCTTGGGCGATTCTTGGAA 120
Est21           CTTTTGCATGGGTTTACCGGCAATTCCGCCGACGTTCGGATGCTTGGGCGATTCTTGGAA 120
                ************************************************************

Est21a          TCGAAAGGGTATACGTGCCACGCTCCGATTTACAAAGGGCATGGCGTGCCGCCGGAAGAG 180
Est33           TCGAAAGGGTATACGTGCCACGCTCCGATTTACAAAGGGCATGGCGTGCCGCCGGAAGAG 180
Est21           TCGAAAGGGTATACGTGCCACGCTCCGATTTACAAAGGGCATGGCGTGCCGCCGGAAGAG 180
                ************************************************************

Est21a          CTCGTCCACACCGGACCGGATGATTGGTGGCAAGACGTCATGAACGGCTATCAGTTTTTG 240
Est33           CTCGTCCACACCGGACCGGATGATTGGTGGCAAGACGTCATGAACGGCTATCAGTTTTTG 240
Est21           CTCGTCCACACCGGACCGGATGATTGGTGGCAAGACGTCATGAACGGCTATCAGTTTCTG 240
                ************************************************************ **

Est21a          AAAAACAAAGGCTACGAAAAAATTGCCGTGGCTGGATTGTCGCTTGGAGGCGTATTTTCT 300
Est33           AAAAACAAAGGCTACGAAAAAATTGCCGTGGCTGGATTGTCGCTTGGAGGCGTATTTTCT 300
Est21           AAAAACAAAGGCTACGAAAAAATTGCCGTGGCTGGATTGTCGCTTGGAGGCGTATTTTCT 300
                ************************************************************

Est21a          CTCAAATTAGGCTACACTGTACCTATAGAAGGCATTGTGACGATGTGCGCGCCGATGTAC 360
Est33           CTCAAATTAGGCTACACTGTACCTATACAAGGCATTGTGACGATGTGCGCGCCGATGTAC 360
Est21           CTCAAATTAGGCTACACTGTACCTATAGAAGGCATTGTGACGATGTGCGCGCCGATGTAC 360
                *************************** ******************************

Est21a          ATCAAAAGCGAAGAAACGATGTACGAAGGTGTGCTCGAGTATGCGCGCGAGTATAAAAAG 420
Est33           ATCAAAAGCGAAGAAACGATGTACGAAGGTGTGCTCGAGTATGCGCGCGAGTATAAAAAG 420
Est21           ATCAAAAGCGAAGAAACGATGTACGAAGGTGTGCTCGAGTATGCGCGCGAGTATAAAAAG 420
                ************************************************************

Est21a          CGGGAAGGGAAATCAGAGGAACAAATCGAACAGGAAATGGAACGGTTCAAACAAACGCCG 480
Est33           CGGGAAGGGAAATCAGAGGAACAAATCGAACAGGAAATGGAACGGTTCAAACAAACGCCG 480
Est21           CGGGAAGGGAAATCAGAGGAACAAATCGAACAGGAAATGGAACGGTTCAAACAAACGCCG 480
                ************************************************************

Est21a          ATGAAGACGTTGAAAGCCTTGCAAGAACTCATTGCCGATGTGCGCGCCCACCTTGATTTG 540
Est33           ATGAAGACGTTGAAAGCCTTGCAAGAACTCATTGCCGATGTGCGCGCCCACCTTGATTTG 540
Est21           ATGAAGACGTTGAAAGCCTTGCAAGAACTCATTGCCGATGTGCGCGCCCACCTTGATTTG 540
                ************************************************************

Est21a          GTTTATGCGCCGACGTTCGTCGTCCAAGCGCGCCATGATGAGATGATCAATCCAGACAGC 600
Est33           GTTTATGCACCGACGTTCGTCGTCCAAGCGCGCCATGATGAGATGATCAATCCAGACAGC 600
Est21           GTTTATGCGCCGACGTTCGTCGTCCAAGCGCGCCATGATGAGATGATCAATCCAGACAGC 600
                ******** ********************************************************

Est21a          GCGAACATCATTTATAACGAAATTGAATCGCCGGTCAAACAAATCAAATGGTATGAGCAA 660
Est33           GCGAACATCATTTATAACGAAATTGAATCGCCGGTCAAACAAATCAAATGGTATGAGCAA 660
Est21           GCGAACATCATTTATAACGAAATTGAATCGCCGGTCAAACAAATCAAATGGTATGAGCAA 660
                ************************************************************

Est21a          TCAGGCCATGTGATTACGCTTGATCAAGAAAAAGATCAGCTGCATGAAGATATTTATGCA 720
Est33           TCAGGCCATGTGATTACGCTTGATCAAGAAAAAGATCAGCTGCATGAAGATATTTATGCA 720
Est21           TCAGGCCATGTGATTACGCTTGATCAAGAAAAAGATCAGCTGCATGAAGATATTTATGCA 720
                ************************************************************

Est21a          TTTCTTGAATCGTTAGATTGGTAA 744
Est33           TTTCTTGAATCGTTAGATTGGTAA 744
Est21           TTTCTTGAATAGTTAGATTGGTAA 744
                ********** ************
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Figure 3. 7. Nucleotide sequence of esterases genes

```
ESt21a          MMKIVPPKPFFFEAGERAVLLLHGFTGNSADVRMLGRFLESKGYTCHAPIYKGHGVPPEE 60
Est33           MMKVVPPKPFFFEAGERAVLLLHGFTGNSADVRMLGRFLESKGYTCHAPIYKGHGVPPEE 60
Est21           MMKVVTPKPFFFEAGERAVLLLHGFTGNSADVRMLGRFLESKGYTCHAPIYKGHGVPPEE 60
                ***:*.************************************************

ESt21a          LVHTGPDDWWQDVMNGYQFLKNKGYEKIAVAGLSLGGVFSLKLGYTVPIEGIVTMCAPMY 120
Est33           LVHTGPDDWWQDVMNGYQFLKNKGYEKIAVAGLSLGGVFSLKLGYTVPIQGIVTMCAPMY 120
Est21           LVHTGPDDWWQDVMNGYQFLKNKGYEKIAVAGLSLGGVFSLKLGYTVPIEGIVTMCAPMY 120
                ************************************************:**********

ESt21a          IKSEETMYEGVLEYAREYKKREGKSEEQIEQEMERFKQTPMKTLKALQELIADVRAHLDL 180
Est33           IKSEETMYEGVLEYAREYKKREGKSEEQIEQEMERFKQTPMKTLKALQELIADVRAHLDL 180
Est21           IKSEETMYEGVLEYAREYKKREGKSEEQIEQEMERFKQTPMKTLKALQELIADVRAHLDL 180
                ************************************************************

ESt21a          VYAPTFVVQARHDEMINPDSANIIYNEIESPVKQIKWYEQSGHVITLDQEKDQLHEDIYA 240
Est33           VYAPTFVVQARHDEMINPDSANIIYNEIESPVKQIKWYEQSGHVITLDQEKDQLHEDIYA 240
Est21           VYAPTFVVQARHDEMINPDSANIIYNEIESPVKQIKWYEQSGHVITLDQEKDQLHEDIYA 240
                ************************************************************

ESt21a          FLESLDW 247
Est33           FLESLDW 247
Est21           FLE-LDW 246
                *** ***
```

Figure 3. 8. Amino acid sequence of esterases genes

Sequence of lipase genes showed that Lip1 and Lip2 were 1263bp in length which codes for 420 amino acids and stop codon (Figure 3.9.). All three genes were compared and many nucleotide differences were observed (5% of total nucleotide) between them. These differences also have resulted in several amino acid differences among the two lipase genes (Figure 3.10.). Sequence alignment of lipases also has demonstrated that several inborn mutation have been occurred during our studies so that we could not able to continue the characterization studies for lipases.

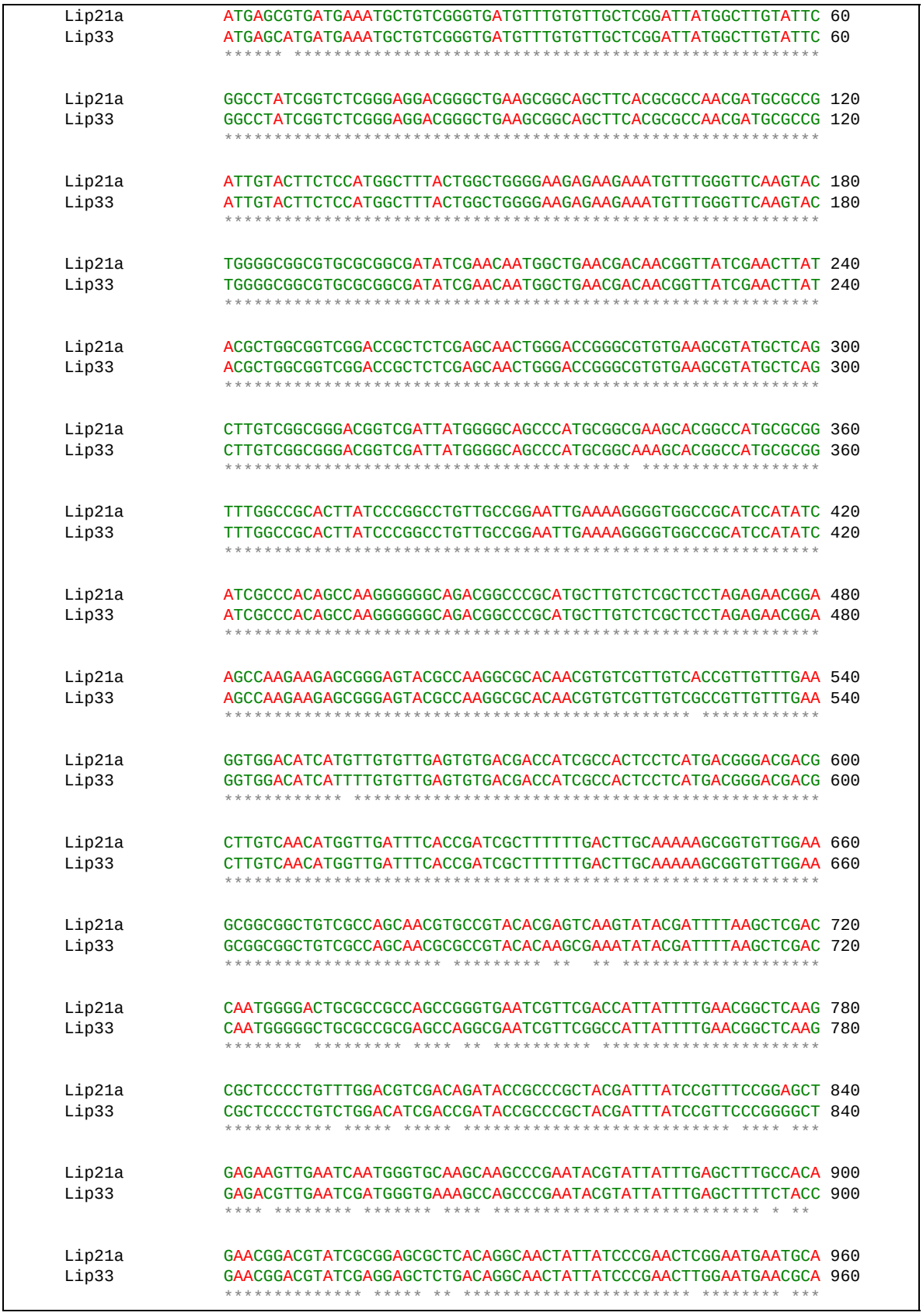

Figure 3. 9. Nucleotide sequence of lipases genes

(Cont. on next page)

```
Lip21a          TTCAGCGCGGTCGTATGCGCTCCGTTTCTCGGGTTCGTACCGCAATCCGACGCTCGGCAT 1020
Lip33           TTCAGCGCGATTGTCTGCGCCCCGTTTCTCGG-CTCGTACCGCAATGCGGCGCTTGGCAT 1019
                ********* * ** ***** ***********  ************ ** **** *****

Lip21a          TGACGACCGCTGGCTTGAAAACGATGGCATTGTCAATACGG--TTTCCATGAACGGTCCA 1078
Lip33           TGACAGCCATTGGCTTGAGAACGACGGCATTGTCAATACCAATTTTCGATGAACGGTCCG 1079
                ****  **  ******** ***** **************    **** ***********

Lip21a          AAGCGTGGATCAAGTGATCGGATCGTTACCGTATGACGGGGGCGTTGAAAAAAGGGGTTT 1138
Lip33           AAGCGTGGATCAAGCGATCGGATCGT-ACCGTATGACGGGGGCGTTGAAAAAAGGGGTTT 1138
                ************** *********** ********************************

Lip21a          GGAATGACATGGGAACGTACAATGTCGACCATTTGGAAATCATCGGCGTTGACCCGAATC 1198
Lip33           GGAATGACATGGGAACGTACAATGTCGACCATTTGGAAATCATCGGCGTTGACCCGAATC 1198
                ************************************************************

Lip21a          CGTCATTTGATATTCGCGCCTTTTATTTGCGACTTGCCGAGCAACTGGCGAGCTTGCGGC 1258
Lip33           CGTCATTTGATATTCGCGCCTTTTATTTGCGACTTGCCGAGCAGTTGGCGAGTTTGCGGC 1258
                ****************************************  ******* *******

Lip21a          CTTAA 1263
Lip33           CTTAA 1263
```

Figure 3. 9. (Cont.) Nucleotide sequence of lipases genes

```
Lip21a           MSVMKCCRVMFVLLGLWLVFGLSVSGGRAEAAASRANDAPIVLLHGFTGWGREEMFGFKY 60
Lip33            MSMMKCCRVMFVLLGLWLVFGLSVSGGRAEAAASRANDAPIVLLHGFTGWGREEMFGFKY 60
                 **:************************************************************

Lip21a          WGGVRGDIEQWLNDNGYRTYTLAVGPLSSNWDRACEAYAQLVGGTVDYGAAHAAKHGHAR 120
Lip33           WGGVRGDIEQWLNDNGYRTYTLAVGPLSSNWDRACEAYAQLVGGTVDYGAAHAAKHGHAR 120
                ************************************************************

Lip21a          FGRTYPGLLPELKRGGRIHIIAHSQGGQTARMLVSLLENGSQEEREYAKAHNVSLSPLFE 180
Lip33           FGRTYPGLLPELKRGGRIHIIAHSQGGQTARMLVSLLENGSQEEREYAKAHNVSLSPLFE 180
                ************************************************************

Lip21a          GGHHVVLSVTTIATPHDGTTLVNMVDFTDRFFDLQKAVLEAAAVASNVPYTSQVYDFKLD 240
Lip33           GGHHFVLSVTTIATPHDGTTLVNMVDFTDRFFDLQKAVLEAAAVASNAPYTSEIYDFKLD 240
                ****.*******************************************.****::******

Lip21a          QWGLRRQPGESFDHYFERLKRSPVWTSTDTARYDLSVSGAEKLNQWVQASPNTYYLSFAT 300
Lip33           QWGLRREPGESFGHYFERLKRSPVWTSTDTARYDLSVPGAETLNRWVKASPNTYYLSFST 300
                ******:*****.***********************.***.**:**:**********:*

Lip21a          ERTYRGALTGNYYPELGMNAFSAVVCAPFLGFVPQSDARHRP--LAKRWHCQYGFHERSK 358
Lip33           ERTYRGALTGNYYPELGMNAFSAIVCAPFLGSYRNAALGIDSHWLENDGIVNTNFRTVRS 360
                ***********************:*******   ::     .  * :    : .*:   .

Lip21a          AWIKSDRYRMTGALKKGVWNDMGTYNVDHLEIIGVDPNPSFDIRAFYLRLAEQLASLRP 417
Lip33           VDQAIGSYRMTGALKKGVWNDMGTYNVDHLEIIGVDPNPSFDIRAFYLRLAEQLASLRP 419
                .    . ************************************************
```

Figure 3. 10. Amino acid sequence of lipases genes

3.4. Expression and Purification of the Recombinant Lipases and Esterases in *E.coli*

3.4.1. Expression

The lipase and esterase expression vector, pET28a(+) were constructed by ligating 1263 bp NdeI/HindIII fragment of lipase from subcloning vector and 744 bp NdeI/HindIII fragment of esterase from subcloning vector. By using pET 28 expression vector only the esterase enzymes (Est1, Est2, Est3) were overexpressed (Figure 3.11.). The efficient expression of lipases has not been demonstrated very good which may be as a result of problems associated with sequence results. After subcloning of the esterase genes, we have demonstrated high level expression of the all three esterases in *E.coli* upon derepression of the *lac* operator by the lactose analog IPTG. Induction of expression by IPTG in the pET-28(+) expression vector in the BL21(λDE3) *E. coli* host resulted in the production of a ~28 kDa protein for each esterase gene (Figure 3.11.). The control cells with no added IPTG showed no such proteins. This level of expression indicates that esterases represent the major proteins in the induced cells. The expression reached maximum levels within 4 hours after induction by IPTG. Additional experiments with heterologous expression of each esterase gene indicate that approximately 30-50 mg of purified active protein can be isolated from 1.0 liter of bacterial culture in LB media.

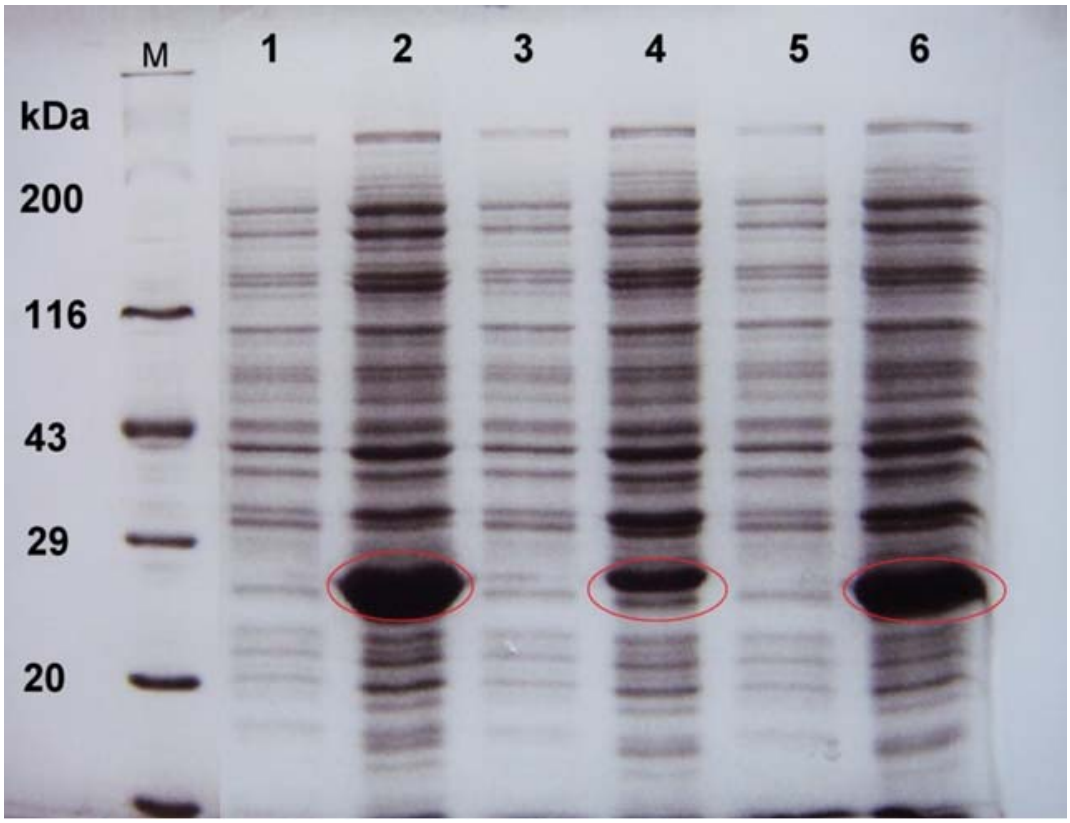

Figure 3. 11. IPTG induction of the esterases gene Gel images; Lane M: Marker; lane1: *E.coli* BL21 (DE3)/pET28 a(+)-Est1 before induction; lane2:*E.coli* BL21 (DE3)/pET28a(+)-Est1after induction lane3:*E.coli* BL21 (DE3)/pET28a(+)-Est2before induction lane 4:*E.coli* BL21 (DE3)/pET28a(+)-Est2after induction lane 5:*E.coli* BL21 (DE3)/pET28a(+)-Est3before induction lane6:*E.coli* BL21 (DE3)/pET28a(+)-Est3after induction

3.4.2. Purification & Affinity Chromatography

In order to ease the purification of heterologous esterases by an affinity chromatography, genes were cloned in the pET-28a(+) vector which carries the codons encoding both C-terminal and N-terminal His-taq region.

The purification of esterases using one step His-taq nicel affinity chromatography led to a nearly homogenous proteins. This type of purification has allowed a one-step purification of the esterase proteins using Ni-NTA affinity chromatography. Elution of the protein from the affinity column was done by elution buffer at 4ºC. Protein elution has

started to came out of column at fraction number 14 and continued until fraction number 31(Figure 3.12.) Selected fractions from peak were analyzed on 15% SDS-PAGE(Figure 3.13.). The purified recombinant esterases migrated as a single band, with a relative molecular mass of approximately 28 kDa on SDS-PAGE

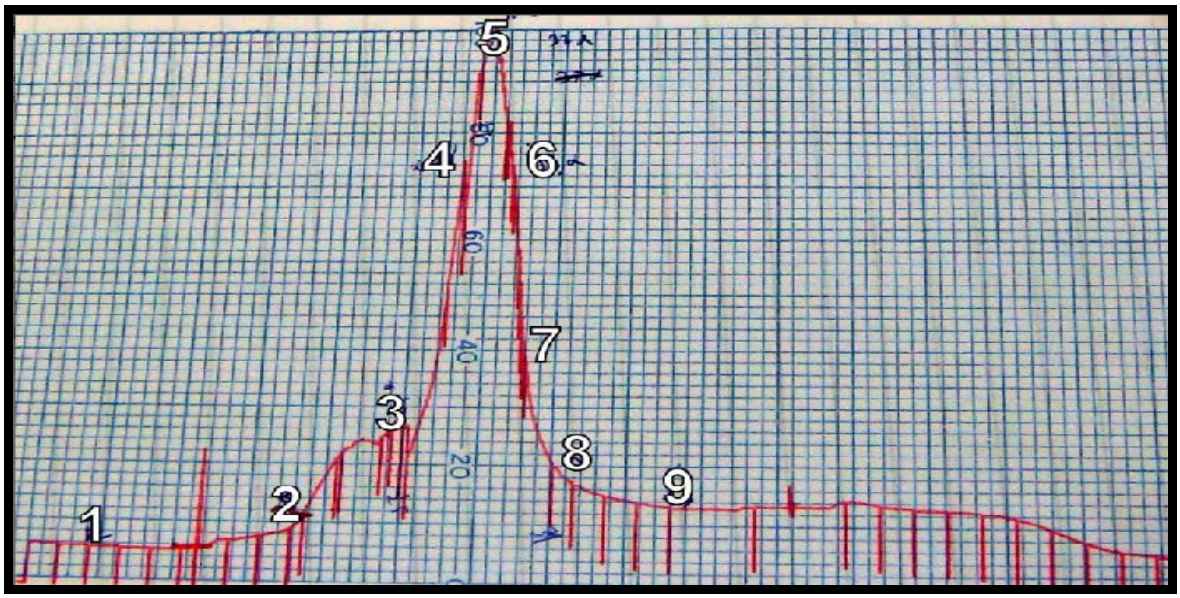

Figure 3.12. Elution profile of the His Select® HF Nickel Affinity Gel Column

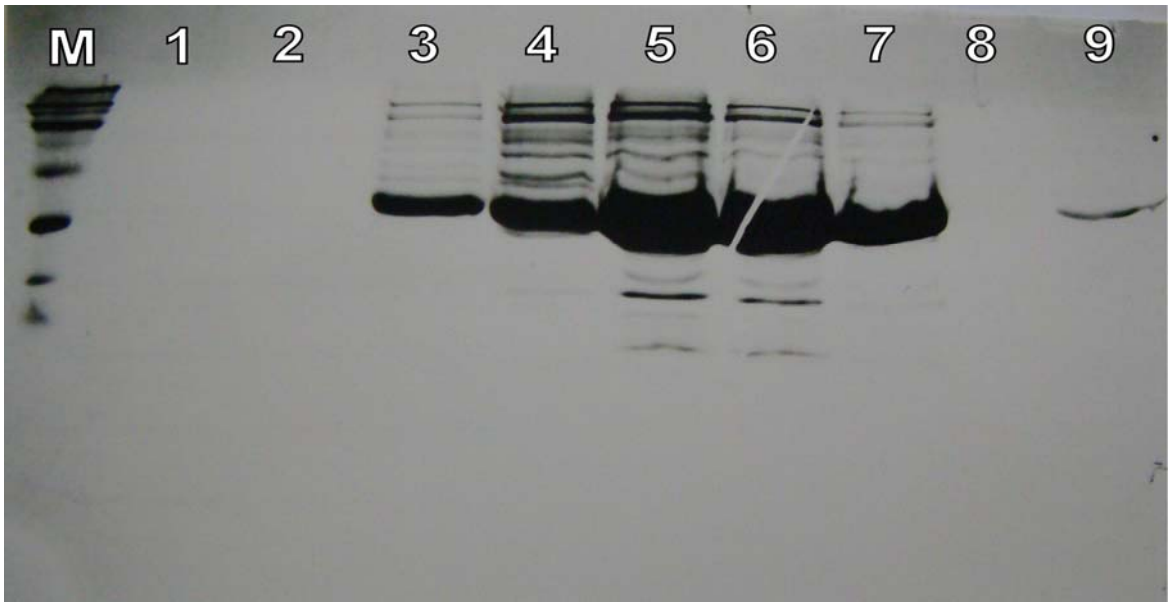

Figure 3.13. 15% SDS-PAGE analysis of selected fractions. M: molecular mass markers top the bottom 200, 116, 68, 43, 29, 14.4, and 6.5 kDa 1, 2, 3, 4, 5, 6, 7, 8, 9: selected fractions.

3.5. Enzyme Characterization

The purified recombinant esterases have been characterized in terms of substrate specificity, optimum pH, optimum temperature, Effect of Metal Ion, Organic Solvents and Detergent on Enzyme Activity.

3.5.1. Substrate Specificity

Substrate specifity was initially tested toward several *p*-nitrophenyl esters of different chain lenghts using a photometric assay. As it can be seen from the Figure 3. 14. , the highest activity of esterase was obtained toward *p*-nitrophenol acetate for Est1, Est2, Est3. When the acyl chain length of the substrate was increased starting with C2 , there was a decrease in the enzyme activity. Similar results have been demonstrated with the highest activity towards *p*-nitrophenyl by Soliman, et al. 2007. This results were not surprising because estearses use short chain fatty acids as a substrate for their catalytic activity.

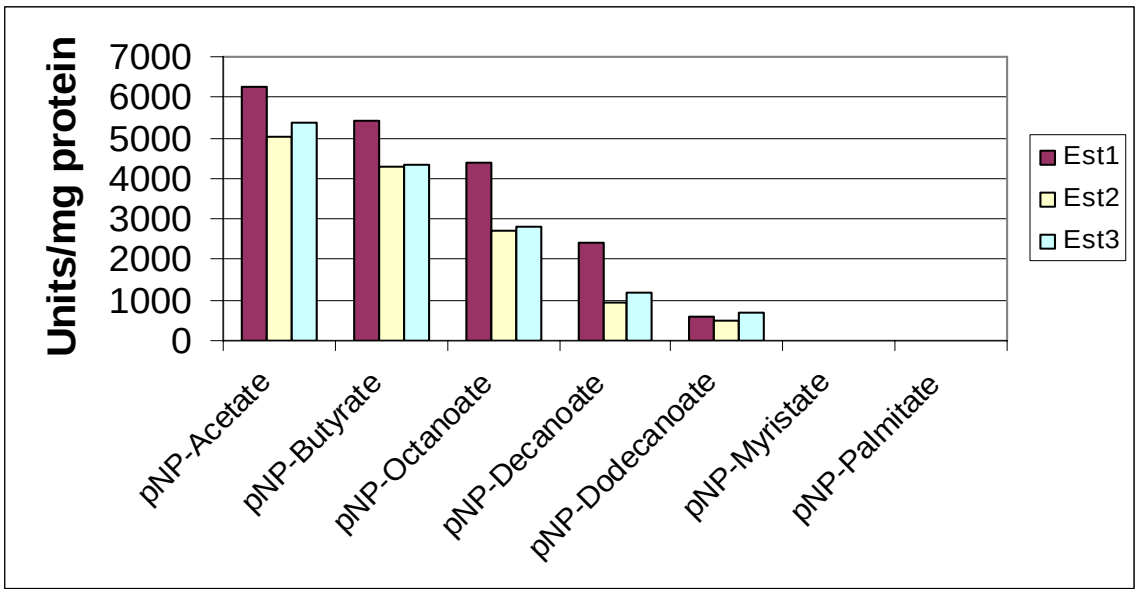

Figure 3. 14. Relative enzyme activity values aganist substrates containing different number of carbon chain leghts.

3.5.2. Optimum pH and Temperature

Effect of temperature and pH on the enzymes activities were measured using p-nitrophenyl acetate as substrate in the case of esterase. The lipase activity was measured at various pHs in buffers with the same ionic concentrations. Esterase showed high activity mainly in alkaline conditions in a pH range between 8 and 11. (Figure 3. 15.) *Bacillus licheniformis* lipase also showed maximum activity at pH 10-11.5 and was remarkably stable at alkaline pH values up to 12 (Nthangeni et al., 2001). This is in fact a desirable property for industrial applications. Similar to our results esterase enzyme from Geobacillus thermoleovorans have been reported to be more active at alkaline pH. (Soliman, et al. 2007). So it can be conclude that enzyme is resisted to alkaline pH changes. The recombinant esterases retained more than 80% of its activity following incubation for 2 hours at 55 ºC in various buffers over a ph ranging from 7 to 12, indicating that the recombinant enzymes are extremely alkali tolerant protein (data not shown).

In order to determine the effect of temperature on enzyme activity, esterase activities at different temperatures ranging from 30 to 90ºC were measured. The results of these measurements indicated that the enzyme showed highest activity around 65-70 ºC (Figure 3. 16.)All three enzyme have shown same trend in terms of temperature effect. Also, all three enzyme have retained 90% activity after incubation for 1hour at 55ºC. Similar results have been shown with esterase enzyme from *Geobacillus thermoleovorans* (Soliman, et al. 2007).

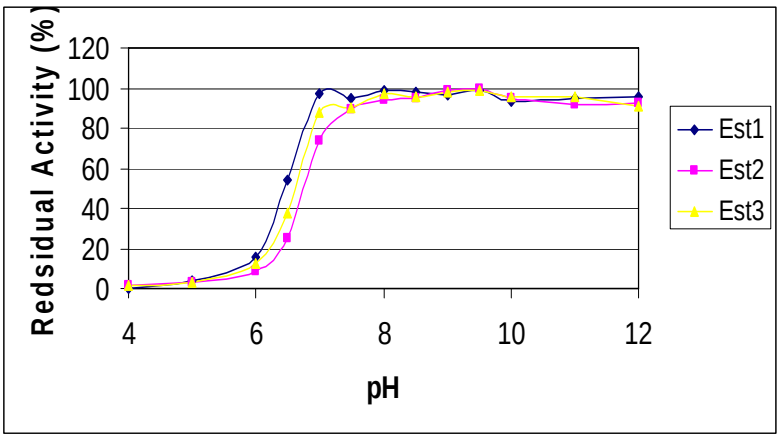

Figure 3. 15. Residual enzyme activity values at different pH

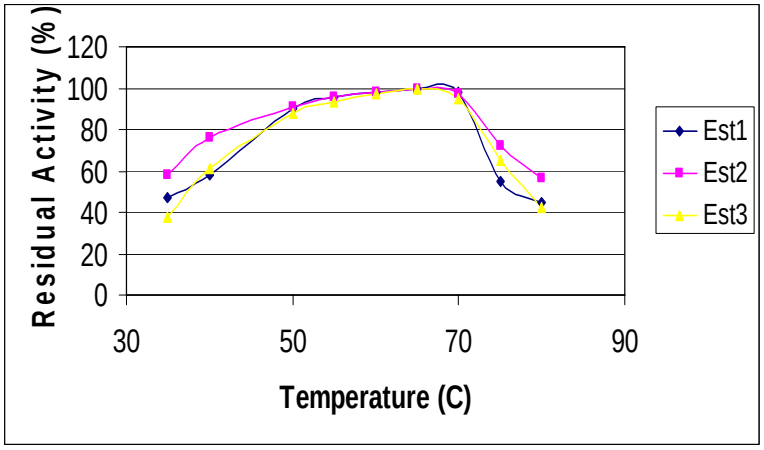

Figure 3. 16. Residual enzyme activity values at different temperatures

3.5.3. Effect of Metal Ion, Organic Solvents and Detergent on Enzyme Activity

The enzyme activity of esterases was studied in the presence of organic solvents, detergent and metal ions. The effects of various reagents, solvents and metals on estases are shown in Table 3.1. Stability against organic solvents is very important when using enzymes for synthesis of esters. The results are shown that, the activities of esterase were enhanced by $CaCl_{2+}$, NaCl , $ZnCl_{2+}$ but slightly inhibited by $MgCl_{2+}$. SDS and Isopropanol showed strong inhibitory effect on the esterases.

Table 3.2. Relative enzyme activity values in the presence of organic solvents, reagent, detergent

Treatment	Est1	Est2	Est3
None	100	100	100
MgCl2 (1 mM)	92	72	73
CaCl2 (1 mM)	110	94	100
NaCl (1 mM)	123	112	108
ZnCl2 (1 mM)	111	104	115
DMSO (1%)	101	113	110
DTT (1%)	111	112	109
SDS (1%)	81	57	50
EDTA (1%)	122	116	124
β-Mercaptoethanol(1%)	112	106	101
Isopropanol (1%)	41	72	69
Ethanol (1%)	89	95	87

CHAPTER 4

CONCLUSIONS

In this study, the main goal was characterization, purification and investigation of selected two extracellular enzymes due to potential use as biocatalysts in variety of biotechnological applications. Approximately 110 thermophilic strains were screened for the presence of 2 extracellular enzyme activities. These two enzyme were; lipases and esterases, for enzyme screening Tween 20 and Rhodamin-B with olive oil used as substrate.

As the second step the genes that encode these two enzymes are amplified by PCR technique and cloned into to *Escherichia coli* to express. By using pET 28 a(+) expression vector three esterases were entirely over expressed on the other hand three lipase were partially overexpressed. The overexpressed enzymes then purified via affinity chromatography techniques. The expression system pET 28 a(+) adds his-tag site to gene of interest which have affinity to bead of some metals like nickel. The total cell proteins were run through the nickel column and the content of the column fractionized by LPLC (Low Pressure Liquid Chromatography)

According to substrate specificity results, the highest enzyme activity was seen in p-nitrophenly acetate which has a relatively long, twelve, carbon chain. The optimum pH of esterase enyzme was investigated that relatively alkaline pH values. Bacterial esterase show resistance to temperature and pH change. So esterase can be used and applied on many fields by further characterizaiton methods and studies.

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APPENDIX A

CHEMICALS USED IN EXPERIMENTS

Agar- Agar

Bacteriological pepton

Yeast Extract

Glycerol

NaCl

K2HPO4

MgSO4.7H2O

Disodium hydrogen phosphate

Ammonium sulfate

KH2PO4

Calcium chloride

Nutrient broth

Tween80

Tween 20

Sodium carbonate

Tris Base Sigma

EDTA

Isopropanol

Ethidium bromide

Taq DNA polymerase

dNTP set

Nde I

Hind III

Sma I

Bromophenol blue

Standard agarose

APPENDIX B

MEDIAS

Luria Bertani (LB) broth, per liter

10 g tryptone, 5 g yeast extract, 5 g NaCl and dH2O up to 1 L.

Luria Bertani (LB) agar, per liter

10 g tryptone, 5 g yeast extract, 5 g NaCl, 15 g agar and dH2O up to 1 L.

2x YT

16 g tryptone 10 g yeast extract 5 g NaCl disolved in one liter dH2O and sterilized.

SOC Medium, per 100 ml

2 g Tryptone, 0.5 g Yeast Extract, 1 ml 1M NaCl, 0.25 ml 1M KCl, 1 ml 2M Mg2+ Stock, 1 ml 2M Glucose were dissolved in dH2O up to 100 ml.

SOB Medium, per 100 ml

g Tryptone, 0.5 g Yeast Extract, 1 ml 1M NaCl, 0.02 g KCl, 1 ml 1M MgCl,
1 ml 1M MgSO4 were dissolved in deionized dH2O up to 100 ml.

TB Medium, per 100 ml

0.3 g PIPES, 3 ml 1 M CaCl2 and 1.85 g KCl were dissolved in 100 ml deionized water and the solution pH was adjusted to 6.7 with KOH. Then 1.4 g MnCl2was added and the solution was filtered sterilized.

APPENDIX C

REAGENTS AND SOLUTIONS

3M Sodium Acetate (pH 5.2)

408.1 g sodium acetate.3H2O was dissolved in 800 ml of deionized water and the pH isadjusted to 5.2 with glacial acetic acid. The volume is adjusted to 1000 ml. Solution was sterilized by autoclaving.

95% Ethanol/Water, 70% Ethanol/Water

Ultra pure H2O was used to dilute the 100% (molecular biology grade) ethanol to required concentration as mentioned above and the solutions were stored at -20°C.

10% Ammonium Persulfate

0.1 g ammonium persulfate was dissolved in 1.0 ml dH2O.

2x Gel Loading Dye Reagents

0.25 ml 2% bromophenol blue, 0.25 ml 2% xylene cyanol, 7 ml 100% glycerol and 2.5 ml dH2O were mixed in total volume of 10 ml**.**

final volume.