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MOLECULAR CLONING, EXPRESSION AND PURIFICATION OF α, β-HYDROLASE GENE FOR LIPASE ACTIVITY FROM *SHEWANELLA PUTREFACIENS*

by

Mohammad Kaifee ARMAN

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Mohammad Kaifee ARMAN

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APPROVAL PAGE

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M.S. Thesis – Genetics and Bioengineering
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ABSTRACT

Shewanella putrefaciens is a gram negative bacterium. *S.putrefaciens* is also a facultative anaerobe with the ability to reduce iron and manganese as the terminal electron acceptor in the electron transport chain in both solid and liquid media. *S.putrefaciens* is also recognized by its bright pink colour. They are resistant to changeable environmental conditions. Lipases are also found to be widespread among bacteria, based on homology searches in protein databases and bacterial genomes. Lipase genes have been cloned from fungi and bacteria and then have been searched enzyme activity. In this study, we preferred *S. putrefaciens* ATCC 8071 because its lipase enzyme was not characterized before. Lipase genes in *Shewanella putrefaciens* were transferred into *E. coli* with molecular cloning techniques. Also, lipase enzyme was purified and characterized.

Keywords: *Shewanella putrefaciens*, lipase, cloning, gene expression.

SHEWANELLA PUTREFACIENS BAKTERISINDEN α, β-HYDROLASE GENININ KLONLANMASI VE LIPAZ AKTIVITESININ ARAŞTIRILMASI

Mohammad Kaifee ARMAN

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ÖZ

Shewanella putrefaciens topraktan ve denizden izole edilebilen, lipaz aktivitesi içeren gram negatif bir bakteridir. Metabolik olarak manganez kullanırlar ve demiri indirgeyebilen fakültatif anerobtur. Hem katı hem de sıvı ortamda parlak pembe renkleriyle ayrılırlar. Ortam koşullarının değişmesine çok dayanıklı bir mikroorganizmadır. Lipazların mantar ve gelişmiş bitkilerin yani sıra çoğu bakteri türünde de yaygın olarak bulunduğu, homolojik olarak yapılan protein bilgi bankası ve bakteri genom araştırmaları ile tespit edilmiştir. Lipaz geni hem fungal sistemlerden hem de çeşitli bakterilerden klonlanıp fonksiyonel analizleri yapılmıştır. Bu çalışmada, daha önce lipaz geninin varlığı bilinen ancak karakterizasyonu yapılmamış olan *S. putrefaciens* ATCC 8071 seçildi. *S. putrefaciens'*teki lipaz geni indüklenebilir bir ekspresyon vektörüne klonlandı ve *E.coli* bakterisine aktarıldı. Aynı zamanda, lipaz enzimi saflaştırılarak karakterizasyonu yapıldı.

Anahtar Kelimeler: *Shewanella putrefaciens,* lipaz, klonlama, genin ifade edilmesi.

.

This thesis is dedicated to my father Abul Kalam, my mother Aftabun Nisha, my sisters Shabnam, Gulshan, Sanubar and Nishat for their endless love and support.

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LIST OF SYMBOLS AND ABBREVIATIONS

SYMBOL/ABBREVIATION

APS	Ammonium persulphate
Asp	Aspartate
ATCC	American Type Culture Collection
Blast	Basis Local Alignment Search Tool
BSA	Bovine serum albumin
CDC	Centre for Disease Control and Prevention
CIAP	Calf intestinal alkaline phosphatase
dNTP	Deoxydiribonucleotide triphosphate
DOE	Department of Energy
DMSO	Dimethyl sulfoxide
DNA	Deoxy ribonucleic acid
E. Coli	Escherichia
EDTA	Ethylene diamine tetra acetic acid
GC	Guanine plus Cytosine
Gly	Glycine
IPTG	Isopropyl-β-D-1-thiogalactopyranoside
LB	Luria- Bertani
Leu	Leucine
Lif	Lipase-specific fold
MFCs	Microbial fuel cells
PCR	Polymerase Chain Reaction
rRNA	ribosomal ribonucleic acid
S. putrefaciens	Shewanella putrefaciens
SDS-PAGE	Sodium Dodecyl Suphate-Polyacrylamide Gel-Electrophoresis
Ser	Serinine
Seq	Sequence

TAE	Trisacetic acid-Ethylenediammineacetic acid
TEMED	Tetramethylethylenediammine
UV	Ultraviolet

CHAPTER 1

INTRODUCTION

Genus *Shewanella* are a type of gram-negative, facultative anaerobic bacteria mostly found in aquatic and marine environments and can often be isolated from spoiling fish. Usually, *Shewanella* are known to be the members of the γ-subclass of the Proteobacteria that are gram-negative rods, 0.4 – 0.7 µm in diameter, 2–3 µm in length, and motile by a single polar flagellum [1]. Many types of Shewanella are grown in the laboratory with general growth media following enhancement from environmental samples [2] with a range of salt concentrations, temperatures, and barometric pressures and have distinct roles from food spoilage organisms to symbionts, epibionts, and opportunistic pathogens [1]. In the absence of oxygen, *Shewanella* is able to carry out anaerobic respiration by using a broad range of final electron acceptors. This feature not only enables the members of the genus *Shewanella* to survive in various environments with the capacity of growing naturally almost anywhere, but also to be utilized for bioremediation of contaminated environments by reducing some certain metals and compounds in an altered state [3]. For example, *Shewanella* can convert uranium dissolved in contaminated groundwater to a form unable to dissolve in water, so that the uranium will not spread as the groundwater flows. Hence, areas such as DOE sites contaminated during the manufacture of nuclear weapons can be cleaned and confined. *Shewanella* has benefits that make it easier for researchers to study. *Escherichia* and *Shewanella* are well known to be related. Tools and techniques developed since 1970s for *Escherichia* works with *Shewanella,* which can tolerate oxygen a useful ability that makes it easier to work with in the laboratory and is missing in other groups of metal metabolizing bacteria.

S. putrefaciens is also a facultative anaerobe with the ability to reduce iron and manganese metabolically; that is, it can use iron and manganese as the terminal electron acceptor in the electron transport chain in both solid and liquid media, *S. putrefaciens* is often recognizable by its bright pink colour. They are resistant to changeable environmental conditions. Lipase in *S. putrefaciens* is likely to be resistant to hard conditions.

Lipases are versatile group of enzymes that not only hydrolyse the esters of long chain aliphatic acids from glycerol at oil/water interface but also involved in the transesterification reaction. Lipases are part of the family of hydrolases that act on carboxylic ester bonds. The physiologic role of lipases is to hydrolyse triglycerides into diglycerides, monoglycerides, fatty acids, and glycerol. In addition to their natural function of hydrolyzing carboxylic ester bonds, lipases can catalyse esterification, interesterification, and transesterification reactions in non aqueous media. This versatility makes lipases the enzymes of choice for potential applications in the food, detergent, pharmaceutical, leather, textile, cosmetic, and paper industries [4]. Lipases are valued biocatalysts because they act under mild conditions, are highly stable in organic solvents, show broad substrate specificity, and usually show high regio- and/or stereo selectivity in catalysis [5].

Lipases are produced by a variety of microorganisms, including fungi [6] and bacteria [7-11]. Many microbial lipase genes have been cloned during past few decades. Several of these genes are from fungi, including *Aspergillus oryzae* [12-14], *Rhizopus delemar* [15], *Rhizomucor miehei* [16], *Penicillium camembertii* [17], *Penicillium cyclopium* [18], and *Geotrichum candidum* [19]. Most of these lipases have been expressed heterogeneously and their properties characterized [20-23]. Also bacterial lipases have been a source of attraction because of their valuable applications in various biotechnological aspects [24-26].

In this study, we preferred *S. putrefaciens* ATCC 8071 because its lipase enzyme has not been characterized ever before. Lipase genes in *Shewanella putrefaciens* were transferred into *E. coli* with molecular cloning techniques. Also, lipase enzyme was purified and characterized.

CHAPTER 2

LITERATURE REVIEW

2.1 SHEWANELLA AS A VERSATILE ORGANISM

2.1.1 A Brief History of the Isolation of *Shewanella*

The isolation of the first *Shewanella* was performed in 1931 as one of various contaminating microorganisms in charge of butter putrefaction [27]. Derby and Hammer were hesitant to identify the microorganism as *Achromobacter putrefaciens* [27], although the taxon was renamed to Pseudomonas (*Pseudomonas putrefaciens*) following further growth and biochemical characterizations in 1941 [28]. In 1960, Shewan *et al.* suggested a classification scheme [29] based on the oxidase positive reaction and motility by means of polar flagella reinforced reclassification into the genus *Pseudomonas*. On the other hand, Baumann and coworkers introduced a new scheme in 1972 primarily based on moles percent guanine plus cytosine (mol % GC) content of DNA. A numerous strains of non fermentative marine bacteria previously classified as *Pseudomonas* was placed into a newly created genus, *Alteromonas* [30].

The type species isolated in 1931 [27] was changed from *Pseudomonas putrefaciens* to *Alteromonas putrefaciens* [31] in 1977. Finally, based on 5S rRNA sequence data, MacDonell and Colwell [32] proposed reclassification into a new genus, *Shewanella*, in honor of the late Dr. James M. Shewan and in recognition of his contributions to fisheries microbiology. In 1985, *Shewanella putrefaciens* was born, and no more reclassifications at the genus level have been made up to date. Approximately 40 species are assigned to the genus *Shewanella* based primarily on DNA at present: DNA hybridization and 16S rRNA sequences.

2.1.2 Species Characterization

Although the first *Shewanella* were characterized based on phenotypic characteristics like morphology, standard biochemical reactions, gram stain, and growth at different physiological conditions, molecular analysis have enabled evaluation, comparison, and classification of phenotypically distinct bacteria into the genus Shewanella based primarily on DNA hybridization and 16S rRNA gene sequences. Thus, several phenotypically distinct bacteria have been reclassified under the genus Shewanella, and existing Shewanella species have been assigned new species names or better characterized. Phylogenetic associations based on 16S rRNA gene sequences are presented in Figure 2.1 for well-characterized type strains of Shewanella. Most new *Shewanella* strains were then classified as *S. putrefaciens* despite indications that the group was more diverse [33]. The study of Owen and coworkers [33] showed that the species *S. putrefaciens* included at least four clearly different DNA homology groups (IIV) based on DNA-DNA hybridization experiments that included several *P. putrefaciens* strains. While the number of species increases, the phenotypic characteristics of the Shewanella are as diverse as the environments in which they live [1,2]. The distribution of *Shewanella* on the earth appears to be rooted in two basic physiological observations: (*a*) their incomparable ability to respire compounds found in the environment and (*b*) their ability to survive at low temperatures.

Some groups of *Shewanella* are grouped together (different colours) that were isolated from similar environments. For instance, several fish intestinal isolates cluster together (*S. pneumatophori, S. schlegeliana, S. marinintestina,* and *S. sairae*). Nevertheless, other examples such as the clustering of the butter surface taint isolate *S. putrefaciens* with the deep-ocean sediment isolate *S. profunda* indicate that 16S rRNA gene comparisons have restricted predictive power in terms of ecology. *S. abyssi* was not taken into account in this analysis because of some difficulties determining the appropriate 16S rRNA sequence for this type strain [34]. Sequences were trimmed to ~1200 bp to smooth the progress of making comparisons between species with incomplete 16S rRNA gene sequences.

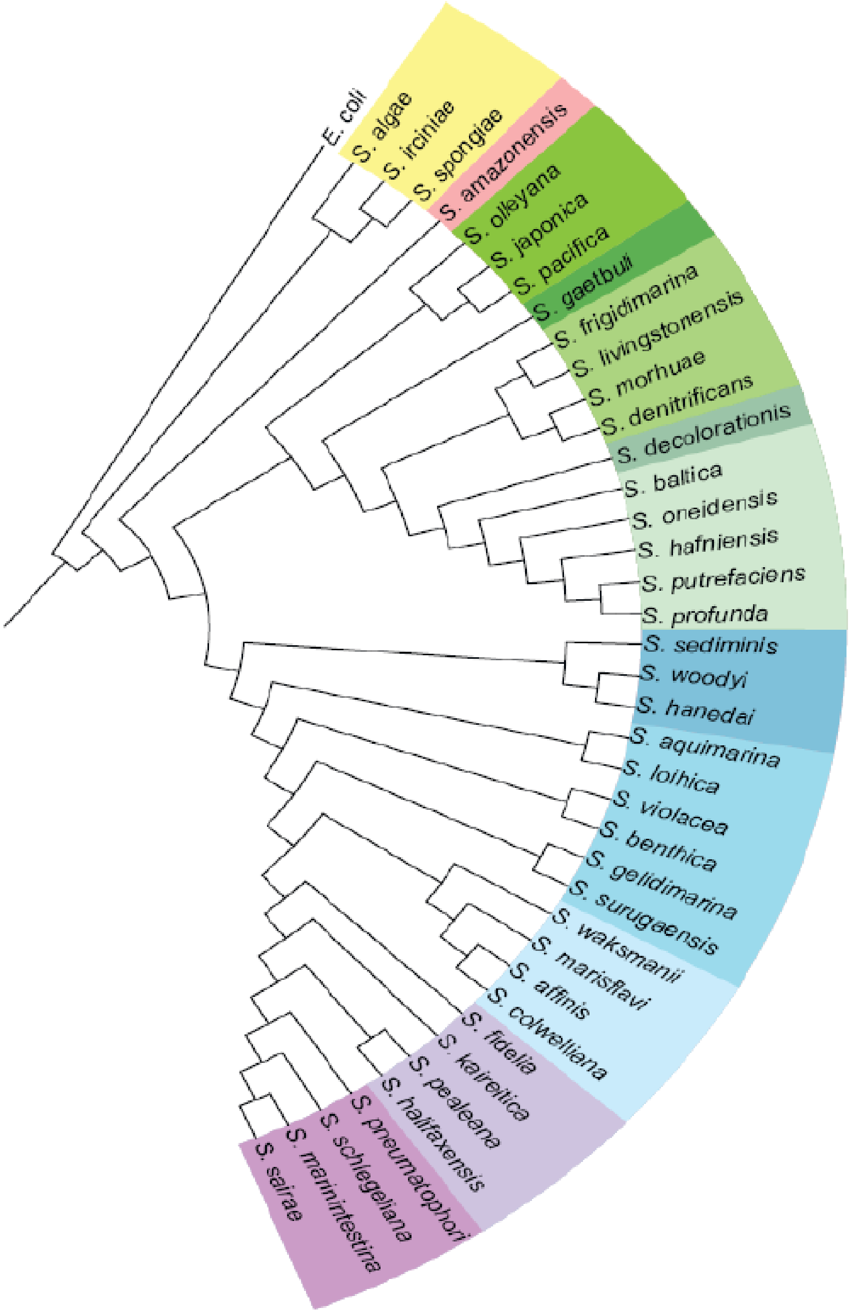


Figure 2.1 Phylogenetic analysis of *Shewanella* type strains.

2.1.2.1 Respiratory Diversity

As a genus, *Shewanella* are the most diverse respiratory organisms described so far. There are roughly twenty inorganic and organic compounds that can be respired by *Shewanella* and these have several insoluble metals and toxic elements. A partial list is as follows:

Trimethylamine-*N*-oxide (TMAO) → Trimethylamine (Me_3N),
Fe (III) Chelate and Fe (III) Oxide → Soluble Fe (II),
Mn (III and IV) Chelates and Mn (III and IV) oxides → Soluble Mn (II),
Sulfur/polysulfide → H_2S,
Sulfite → H_2S,
Thiosulfate→ H_2S,
Dimethyl sulfoxide (DMSO) → Dimethylsulfide,
Arsenate → Arsenite, Fumarate, and Succinate.

A complete list and vivid illustrations can be found in the literature [2]. For some compounds reduction has been exhibited but growth has not (e.g., selenite). The respiratory diversity of Shewanella is one of their greatest benefits in terms of survival in the environment. The fact that all isolates seem to be facultative anaerobes and the anaerobic electron acceptors are various, suggests these organisms are normally localized in both oxic and anoxic environments. Oxygen can be limiting in sediments, in intestinal tracts of higher organisms, and in organic-rich flocculates such as marine snow and faecal pellets. Some aquatic systems are permanently or temporarily stratified, allowing the formation of large anoxic zones. The respiratory diversity of Shewanella allows them to breath almost anywhere. In anoxic environments, Shewanella are likely to respire one if not several compounds. In organic-rich flocculates the electron acceptor may be DMSO. In sedimentary environments, the electron acceptors may be insoluble iron or manganese oxide minerals. The mechanism of anaerobic respiration in these organisms is implicit at the genetic level for some compounds (Fumarate, DMSO, As, V, Fe, and Mn). Many of these compounds are reduced by terminal reductases located outside of the cell, and reviews have recently concentrated on the molecular details of this process [35,36].

2.1.2.2 Low- Temperature Growth

Growth at low temperatures (4°C) appears to be a hallmark of the *Shewanella* genus. Most *Shewanella* strains are psychrotolerant, meaning that they have the capacity to grow at low temperatures (<5°C), but their optimum temperature is above 16°C. Though some isolates do not demonstrate robust growth at low temperatures, many behave as true psychrophiles with temperature optima below 16°C [1,37,38]. The capacity to thrive at low temperatures gives these organisms an advantage in permanently cold environments such as the ocean and the polar regions. This ability is also useful in environments with large temperature fluctuations. For example, the temperature of Oneida Lake drops dramatically in the winter months, with the lake freezing over completely for several months [39]. The physiological changes that take place to allow low-temperature growth in the Shewanella are unknown.

2.1.3 Ecology

2.1.3.1 Syntrophy

The diverse distribution of *Shewanella* is explained by their potential to build syntrophic affiliations with fermentative microbes or those microbes that can use Shewanella's by-products. Most Shewanella strains are non fermenters, but colonization with fermenters would allow them to employ products of fermentation (lactate, formate, hydrogen, and some amino acids) for anaerobic respiration. By-products produced during anaerobic respiration such as acetate, ammonia, and alanine could be further consumed by acetogens, methanogens, organotrophs, or lithotrophs, depending on the environment [2].

2.1.3.2 Pathogenicity

Shewanella most commonly known as secondary or opportunistic pathogens, and the possibility of infections caused by them are very rare; nevertheless, infections are being reported with higher frequency because of better diagnostics in the clinical microbiology laboratory [2,40]. *S. putrefaciens* and *S. algae* are most commonly isolated though human clinical specimens examined thus far are very narrow. The reason for this is a short of criteria for good speciation as an example molecular characterizations to differentiate between *S. putrefaciens*, *S. algae*, and other

Shewanella species. Additionaly, during isolation of the *Shewanella*, other bacterial pathogens are also isolated beside the *Shewanella*. Because of this fact that, the function of the *Shewanella* in pathogenesis and their clinical importance have not been understood yet.

On the other side, there are reports of monomicrobial *Shewanella* infections; patients present clinically with cellulitis in the context of other skin and soft tissue manifestations, bacteremia/septicemia, otitis media or otitis externa, respiratory distress, intra-abdominal infection, pneumonia, and empyema [40,41]. Beside there are some exceptions, *S. putrefaciens* and *S. algae* are vulnerable to common antibiotics used to treat bacterial infections [40,41] though drug-resistant strains have been documented to emerge during the course of patient treatment [42].

Since earlier isolates have been classified under another genus, in 1963 the first *S. putrefaciens* was isolated from human clinical specimen. From 1963 to 1997, about 75 case of human infection caused by *S. putrefaciens* had been reported [43]. There is a strong relation between *S. putrefaciens* infection and an immunocompromised state, and liver disease is a strong risk factor [43]. In 1985, three different biovars were before construction of the species *S. algae* in 1990 commonly, but incorrectly, there were reports which identified many human isolates as *S. putrefaciens* rather than *S. Algae* [40]. After understanding of the fact that *S. algae was* different from *S. putrefaciens*, Gilardi biovar 2 was used to illustrate *S. algae* [45,46].

Because of the fact that the possibility of infections caused by *Shewanella* is very low, *S. algae* become the most common human pathogen. In addition to this, *S. putrefaciens* are responsible for nonhuman pathogenicity [40,44]. CDC biotype 1 (*S. putrefaciens*) and CDC biotype 2 (*S. algae*) were recognized as two types of clinical isolates in 1995 by Centers for Disease Control and Prevention [44]. But there was need for more detailed molecular characterizations to differentiate into subgroups other than *putrefaciens* and *algae*. A review by Holt *et al.* that was recently published shows important points in differentiating to the clinical microbiologist. On the other hand, DNA:DNA hybridization or 16S rRNA sequences that are examples of molecular characterizations can only distinguish *S. putrefaciens* and *S. algae* from other members of the genus *Shewanella*. There has not been information that describe the virulence factors for *Shewanella* clinical isolates yet but according to one study, after comparison many human and nonhuman *Shewanella* clinical isolates, it was found that *S. algae* was more pathogenic than *S. putrefaciens* on the strength of resistance to antimicrobials, production of haemolysin, and pathogenicity in mice [44]. According to the study that examined pathogenicity of various *S. putrefaciens* isolates in mice, it was found that doses ≥1 × 109 organisms per mouse were only lethal [47]. Extracellular virulence factors for instance siderophores, exoenzymes, and tetrodotoxin, a potent marine neurotoxin produced by *S. algae* have been determined and by some investigators and may be a part of in pathogenesis [40].

2.1.4 Applications in Biotechnology

With a diverse group of electron acceptors, genus *Shewanella* have got ability to respire and have shown an adaption to the life in extreme and different environments. *Shewanella* can easily grow in the lab conditions and are open to genetic manipulation. So, *Shewanella* have potential to remediate environmental pollutants and in microbial fuel cells (MFCs), where their metabolism have the capacity to produce electricity [48-51].

2.1.4.1 Bioremediation of Radionuclides and Toxic Elemental Waste

Contaminated environments can be effectively and inexpensively cleaned up by the help of microorganisms which prevent difficulties in efficient bioremediation strategies [52]. *Shewanella* are superior candidates for potential use in pollutant bioremediation among dissimilatory metal-reducing bacteria due to their intrinsic ability to respire using a wide range of electron acceptors [2]. The solubility and mobility of elements in soils, sediments, and water can be influenced with their oxidation states. Microorganisms display one means by which changes in oxidation states are catalyzed so that transport into rivers and groundwater can be blocked and cleanup facilitated [2,53]. Applications might contain ex situ remediation strategies and in situ bioremediation in storage tanks or areas of environmental contamination [2]. For instance, in manufacturing of nuclear weapons and as a main fuel for nuclear reactors, uranium (U) is used. Some Shewanella can able to play a role in the reduction of U (VI) to the insoluble U (IV) form [2] and this would cause to precipitation and prevent further spread in groundwater at the contaminated site. There is another radionuclide, Technetium (^{99}Tc VII), which is obtain as an end product of nuclear reactor operations and fallout from nuclear weapons testing. Many oxidations states of Technetium (^{99}Tc VII) can be observed but the most reduced form is largely immobile [54]. ^{99}Tc can be reduced by *S. putrefaciens* [2,54], *S. oneidensis* MR-1 [55], and *S. algae* [54] and they can be used in remediation of Tc contaminated environments and waste streams. Similar to Technetium (^{99}Tc VII), Cobalt (^{60}Co) is a radionuclide and formed after weapons operations. Co(III)EDTA− is the mostly found form of cobalt at contaminated sites and in groundwater, and some Shewanella can use this form of cobalt as an electron acceptor [56,57].

^{60}Co(III)EDTA is reduced to ^{60}Co(II)EDTA$_{2-}$ which causes reduction of ^{60}Co(III)EDTA$_{-}$ to ^{60}Co(II)EDTA$_{2-}$ leads to immobilization and therefore restricted transport in subsurface environments.^{60}Co(II)EDTA$_{2-}$ that is the reduced form is not stable sorbs to mineral surfaces facilitating elimination [56,57]. Chromium (Cr), mercury (Hg), and arsenic (As) can be reduced by some type of Shewanella but there can be advantages and disadvantages of these reductions. Cleanup efforts can be made possible by the formation of solid oxides as a result of reduction of soluble Cr(VI) to Cr(III) by some *Shewanella* [2,58]. But *S. oneidensis* MR-1 reduce ionic mercury [Hg(II)] to elemental mercury [Hg(0)] and this cause an increase in mobility and an oxidation state readily available to form methylmercury, a bioaccumulative environmental toxin. In addition to these examples, reduction of arsenate [As(V)] to arsenite by *Shewanella* cause big problems. Drinking waters are contaminated and poisoned by As (III) since this form of arsenate is mobile.

As a result, understanding the role of *Shewanella*, as well as other microorganisms in the oxidation of target compounds and the results in varying these oxidation reactions are precursor factors to optimize cleanup strategies. Bioremediation strategies consisting *Shewanella* can be only applied in the laboratory and have not been used outside the lab yet. The future applications of *Shewanella* in bioremediation strategies consist of cleanup of contaminated global environments and groundwater. Since *Shewanella* mainly lives in aqua, further studies will be focused on how *Shewanella* behave in soil and contaminated groundwater environments [59].

2.2 LIPASE

Lipases are members of the α/β hydrolase super family, which is characterized by having a semi-conserved pentapeptide domain GXSXG (X represents any amino acid) and the Ser, Asp (or Glu), His catalytic triad [60,61]. The enzymes catalyze the hydrolysis and synthesis of fatty acid esters [62,63]. Despite sharing a common catalytic mechanism and a similar structure, the amino acid sequences of different lipases/esterases are greatly variable [64], which endow them potential specific catalytic activities on different ester substrates or functions under various tolerable conditions. Lipases are versatile group of enzymes that hydrolyze the esters of long chain aliphatic acids from glycerol at oil/water interface and are involved in the transesterification reaction. Lipases hydrolyze complex forms like triacylglycerol, diacylglycerol and monoacylglycerol to simpler forms like glycerol and free fatty acids. According to Vajanti Mala Pahoja and Mumtaz Ali Sethar the pH ranges of plant lipase is between 4.0 to 9.0 and of those animals between 5.5 to 8.5 and of microorganisms it ranges somewhat higher than other two groups i.e, 6.0 to 10.0. The temperature ranges at which plant lipase remain active is between 20 to 38°C and of animals it lies between 37 to 60°C. The microbial lipase temperature is between 37 to 55°C. The plant and animal lipase activate in the presence of calcium and zinc, while they in inhibit in the presence of EDTA, Triton X-100 and Tween 80. However, the microbial enzymes inhibit in the presence of FeCl3, ZnCl2 and HgCl2. The molecular weight of lipase in plants ranges between 19000 to 270000. While animal lipase ranges 43000 to 300000. The microbial lipase molecular weight lies between 32000 to 97000 [65].

2.2.1 Classification of Lipases

2.2.1.1 Plant Lipases

According to Vulfson lipases are widely distributed in the plant kingdom, yet the knowledge of lipases from plants is still limited compared with those on mammalian and microorganism lipases [66]. Many of the lipases so far studied in plants have been found to be membrane-bound and have been examined whilst still attached to the membranes. Lipases from maize, rape and castor bean have been solubilised and either purified to homogeneity or at least substantially purified [66].

2.2.1.2 Mammalian Lipases

Gargouri states that three groups of lipolytic enzymes may be distinguished in mammals : the lipases discharged into the digestive tract by the specialized organs, the tissue lipases and the milk lipases [67]. Several tissues and organs of mammals, such as the heart, brain, muscle, arteries, kidney, spleen, lung, liver, adipose tissue and serum, contain lipases [66]. In 1856, Claude Bernard first discovered a lipase in pancreatic juice as an enzyme that hydrolysed insoluble oil droplets and converted them to soluble products.

2.2.1.3 Fungal Lipases

Many lipase genes have been cloned during past few decades. Several of these genes are from fungi, including *Aspergillus oryzae* [12-14], *Rhizopus delemar* [15], *Rhizomucor miehei* [16], *Penicillium camembertii* [17], *Penicillium cyclopium* [18], and *Geotrichum candidum* [19]. Lipases isolated from fungi are best studied among all microbial lipases. Most of these lipases have been expressed heterogeneously and their properties characterized [20-23].

2.2.1.4 Bacterial Lipases

Microbial sources are considered to be better to for enzyme production as compared to plants and animals because of the ease with which they can be mass cultured and genetically manipulated [68]. Bacterial strains are being constantly screened and improved for lipase production using different technologies. The presence of lipases has been observed as early as in 1901 for *Bacillus prodigiosus, B. pyocyaneus* and *B. fluorescens* which represent today’s best studied lipase producing bacteria now named *Serratia marcescens*, *Pseudomonas aeruginosa* and *Pseudomonas fluorescens,* respectively [68]. Production of lipase by many different bacterial species has been extensively studied so far. There are various research works targeting the production of bacterial lipase enzymes particularly from the Pseudomonas and Bacillus species, for example *P.aeruginosa* [69]. *Pseudomonas fluorescens* [70], *Bacillus pumilus* [71], *B. thermocatenulatus* [72], *B. subtilis* [73], *B. licheniformis* [74], *B.coagulans* [75], *B. cereus* [76], and *B.halodurans* [77]. other genera like *Acinetobacter* [78], *Staphylococcus* [79], *Streptococcus* [80], *Burkholderia* [81], *S.marescens* [82], *Achromobacter, Arthrobacter, Alcaligenes and Chromobacterium* [83], have also been studied extensively.

2.2.2 Classification of Bacterial Lipases

Lipases are enzymes which belong to the family of serine hydrolases and their activity relies on a catalytic triad comprising of serine, histidine and aspartate and an α/β hydrolase fold [84]. Bacterial lipolytic enzymes were classified into 8 families and the largest family was subdivided into 6 sub-families by [85]. The basis of this classification was the conserved sequence motifs and biological properties of the enzymes. True lipases belong to family I which comprises most of the *Pseudomonas, Bacillus* and *Staphylococcus* lipases. These lipases possess the conventional catalytic pentapeptide Gly-Xaa-Ser-Xaa-Gly. Family II lipases exhibit Gly-Asp-Ser-Leu motif at the active site and esterases of *Streptomyces, Aeromona* and *Salmonella* belong to this family. Family III comprises of lipases of *Streptomyces* sp but unlike family II esterases these are extracellular lipases. Lipases having similarity with mammalian hormone sensitive lipases are grouped under family IV while lipases of mesophilic bacteria like *P. oleovorans* and *Haemophilus influenza* belong to family V. Family VI lipases are the smallest esterases and the active enzymes are dimeric. Family VII lipases are large esterases and their amino acid sequence is homologous to that of eukaryotic acetyl choline esterases. Family VIII lipases are similar to β-lactamases. The sequences of few other enzymes could not be grouped into any of the eight super families described by Arpigny and Jaeger and have been arbitrarily classified as new family 9 and 10. A cold active lipase reported by De Pascale *et al.* (2008) [84] could not fit into the traditional classification and hence reported as a lipase belonging to a novel lipolytic family.

2.2.3 Cellular Location of Lipases

Bacterial lipases exist as intracellular, membrane-bound or extracellular. *B. clausii* strain which produces only intracellular lipase has been reported [86]. Strains which produce only intracellular lipase strictly grow on long chain triglycerides [87]. Ertugrul *et al*. have observed the production of both intracellular and extracellular lipase in *Bacillus sp*. Boekema *et al*. have reported the production of extracellular lipase as consequence of secretion of accumulated intracellular lipase by membrane-bound chaperones. According to Sangeetha *et al*. bacteria secrete lipase to the external medium through different types of secretory systems. The type I Secretory System (T1SS) comprises of an energy driven exporter complex made up of three protein subunits. The type II Secretory System (T2SS) has two components-the (general protein secretion) Sec-dependent pathway and the (twin-arginine translocation) Tat-dependent pathway. Bacterial lipases are secreted in the unfolded state via the Sec-dependent pathway into the periplasmic space where folding takes place with the assistance of chaperone called lipase-specific foldase (Lif). The folded lipases are then transported out into the external medium by a transporter complex [88,89].

2.2.4 Biochemical Characteristics of Bacterial Lipases

The biochemical characterization of lipase is indispensable to understand its requirements by which they exhibit maximal catalytic performance in order to exploit that enzyme to its maximum. The characteristics that are usually studied are the optimal pH and temperature of enzymes, influence of the presence of cofactors, inhibitors and enhancers on catalytic activity, tolerance of the enzyme to organic solvents and proteases.

2.2.4.1 Acidic and Alkaline Lipases

Most of the known bacterial enzymes are alkaline in nature and alkaline lipases are suitable catalysts for many industrial processes [90,91]. The documents on acidic lipases are very few despite several reports on lipase and lipase producing bacteria. The few reports on acidic lipases are from fungi, especially *Aspergillus nijer*. The other report was cited by Ramani *et al.* who investigated the production of acidic lipase by *P. gessardii* [92].

2.2.4.2 Thermophilic and Psychrophilic Lipases

One of the most demanding characteristics of an industrial enzyme is its thermal stability that requires temperature greater than equal to 60°C. Thermostability is the most preferable feature of lipase because of the high temperature employed in lipolytic reactions, mainly because of the high melting point of the lipidic substrates participating in the process. Thermostable lipases from many *Pseudomonas* and *Bacillus sp.* have been isolated and studied [93,94]. The broad range of the industrial potential of cold adapted lipases includes a wide range of biotechnological applications. These enzymes show maximum catalytic activity at temperatures between 0 and 30°C [95] and are generally produced by psychrophilic microorganisms which survive at temperatures around 5°C [96]. Cai *et al*. (2009) have also reported cold-adapted lipases from a mesophilic *Geotrichum sp*.

2.2.4.3 Effectiveness of Detergents and Surfactants

Surfactants enhance the rate of lipolysis by increasing the lipid-water interface. But all surfactants do not follow this rule. Apart from this, the effect of surfactants is concentration dependent. As for example, high concentrations of Tween 80 (1%) inhibited lipase production by *B. pumilus* while at 0.5% concentration Tween 80 exhibited maximum lipase production [97,98]. The effect of SDS was found to be inhibitory on lipases while Triton X-100 and Tween increased reaction rates [72,99]. The reversal of this is also likely to be true; it has been observed that SDS exhibited stimulatory effect while Triton and Tween inhibited lipase activity [76].

2.2.4.4 Effect of Metal Ions

Metal ions are known to enhance the catalytic activity of enzymes and also provide thermostability [100]. Enzymes also require the presence of metal ions in order to maintain their active structures [101]. Different lipases show different response to metal ions. The ions which function as activators for certain lipases inhibit the activity of few others. The commonly studied metal ions are Mg2+, Mn2+, Co2+, Hg2+, Cu2+, Fe2, Ca2+, Zn2+ etc. Many metal-dependent lipases have been studied and Ca2+ has been found to show a stimulatory effect in all those enzymes [97,98,100,102]. This special effect could be attributed to the structural modifications imposed by the binding of Ca2+ to the enzyme. According to R. Sangeetha the folded enzyme includes a region of negatively charged amino acid residues which try to move apart to reduce the electrostatic repulsions and in turn proves detrimental to the stability of the enzyme. The metal ion, however, binds to the enzyme and forms a bridge that cross-links the polypeptide chain and the enzyme-metal ion complex becomes stable. Calcium-independent lipases are known to function effectively in the presence of chelating agents like EDTA which are usually found in detergents

2.2.4.5 Effect of Organic Solvents

Organic solvents are advantageous in reaction systems involving biocatalysts than their inorganic counterparts. They increase the solubility of substrates, help in easy recovery of products and assist in shifting the equilibrium in the forward direction in synthetic reactions [97,98]. Organic solvent tolerant-lipases are effectively being used as catalysts in the synthesis of biopolymers, transesterification and production of biodiesel [103,104]. The stability and activity of lipase is usually tested in the presence of organic solvents like isopropanol, methanol, ethanol, acetone, glycerol, n-hexane, n-heptane, n-octane, n-decane, benzene, toluene, xylene, styrene, benzene, ethylbenzene, cyclohexane, dimethylsulfoxide, tetrahydrofuran, chloroform and acetic acid [97,98,105]. The sensitivity of lipases to solvents varies depending on their polarity with polar solvents being more destabilizing than non-polar solvents [73]. Commercial exploitation of lipase demands a thorough analysis of tolerance against these solvents as they exhibit different extents of tolerance to organic solvents.

2.2.4.6 Effect of Protease

Sometimes lipases are employed have a wide range of applications of which few employ lipase in combination with other enzymes like amylase and protease. Proteases are known to be hydrolytic enzymes which are capable of auto-digestion and also digest other enzymes produced simultaneously [106]. A detailed study of literature states that the production of lipase and protease are inter related [107]. These studies have shown that when the production of protease is affected either due to the influence of production parameters or due to genetic alterations, the production of lipase is enhanced. For instance, it has been proved that the increased air pressure during fermentation decreased protease production and thereby increased lipase production [108]. The susceptibility of a lipase produced by *B. subtilis* to degradation by extra cytoplasmic proteases located in the cell wall or in the growth medium has been also investigated [109]. But there are few documents on proteolysis-resistant lipases. For example Zhang and Dutta have produced a lipase from *Streptomyces fradiae* and *B. cereus* respectively which was known to be resistant to commercial neutral and alkaline proteases [76,110]. Lipases produced by *P. aeruginosa, B. pumilus* and *B. licheniformis* were found to be resistant to co-produced native proteases [71,74,111].

2.3 RECOMBINANT BACTERIAL LIPASES

Recombinant DNA technology acquaints us about the binding and catalytic site of lipases, overexpress lipases in suitable hosts to meet required demands and to manipulate the enzyme according to need. Lipases which are obtained from culture supernatants have various disadvantages like non-reproducibility of results, undesirable side-effects and requires frequent purification processes [87]. On the other hand recombinant lipases overcome all these constraints and as a result help in mass production of pure lipases which may or may not be tailor-made [112]. The choice of the expression system employed for over expression and the thorough knowledge on the genetic modifications to be made are the backbones of success of this technology. Cloning and sequencing of lipase genes has been an area of study attracting attention from researchers worldwide. Many bacterial lipases have been cloned, sequenced and expressed in homologous or heterologous hosts. The popular expression host is *Escherichia coli* [113], while other suitable hosts include *Pichia pastoris* and *Saccharomyces cerevisiae* [77,114]. The high-level expression of lipase in *E. coli* often results in formation of insoluble and inactive inclusion bodies [115]. The overexpressed proteins may not be efficiently processed by the post-translational machinery of the host and this result in protein mis-folding. Dictated by the cell’s degradation capacity, the misfolded proteins may be degraded or form insoluble aggregates called inclusion bodies in the cytoplasm or periplasm [116]. Akbari *et al.* have optimized the refolding conditions of a recombinant lipase from *Pseudomonas* sp. Using response surface methodology. Conventional methods make use of only chemical additives to assist enzyme refolding. The role of lipase-specific foldase (Lif), the chaperones which assist in lipase secretion has been well explored [87]. These proteins can assist in lipase folding both in vitro and in vivo. The gene encoding Lif was cloned with the lipase coding gene in the same plasmid and evaluated for enhanced expression of lipase in *E. coli*. The yield of lipase was low and this result was attributed to complex gene regulatory and secretion mechanisms. Alternatively, the genes coding lipase and Lif were cloned in separate expression vectors and expressed in different hosts. Lipase expression was found to increase but as inclusion bodies [113]. In a different attempt, the foldase can be isolated and used along with chemical aids to enhance the protein refolding yield. This strategy was adopted to refold a *Pseudomonas* lipase expressed as inclusion bodies in *E. coli* and was observed several fold increase in the refolding yield over the conventional method [115]. Surprisingly, lipases from *P. fragi* IFO 34584, *P. fluorescens* C9 and *P. fluorescens* JCM 5963 have been expressed in enzymatically active forms in *E. coli* [97,98].

2.4 APPLICATIONS OF LIPASES

Microbial lipases are an eminent group of biotechnologically valuable enzymes. This is because of the diversity of their applied properties and ease of mass production. Microbial lipases are known for their diverse enzymatic properties and substrate specificity, which are the attractive features for industrial applications. In the industrial segment, lipases and cellulases are supposed to post the best gains. Lipases are valuable biocatalysts with diverse applications. Though lipases share only 5% of the industrial enzyme market, they have gained focus as biotechnologically valuable enzymes [87]. They play vital roles in food, detergent and pharmaceutical industries.

2.4.1 In Oleochemical Industry

The scope for the application of lipases in the oleochemical industry is enormous. Lipases are part of the family of hydrolases that act on carboxylic ester bonds. The physiologic role of lipases is to hydrolyse complex forms like triglycerides into diglycerides, monoglycerides, fatty acids, and glycerol. Apart from this, lipases have got role in catalyzing esterification, inter esterification, and transesterification reactions. This special versatility makes lipases the enzymes of choice for potential applications in the food, detergent, pharmaceutical, leather, textile, cosmetic, and paper industries [117]. Because of the special structures, some fats are much more valuable than others. Chemical methods tend to give quite random products in conversion of less valuable fats into more useful species [68]. As such lipase catalysed transesterification of cheaper oils can be implemented, for example to produce cocoa butter from palm mid-fraction [118]. The lipase catalysed transesterification in organic solvents is an emerging industrial application such as production of cocoa butter equivalent, human milk fat substitute “Betapol”, pharmaceutically important polyunsaturated fatty acids (PUFA) rich/low calorie lipids, “designers fats or structured lipid” and production of biodiesel from vegetable oils [119,120]. *Mucor miehei* (IM 20) and *Candida antarctica* (SP 382) lipases were used for esterification of free fatty acids in the absence of organic solvent or transesterification of fatty acid methyl esters in hexane with isopropylidene glycerols [121]. Lipolysis is the “constructive” consequences of the ability of lipase to hydrolyse lipids so as to obtain fatty acids and glycerol, both of which have important industrial applications. For example, fatty acids are used in soap production [122].

2.4.2 Synthesis of Biodegradable Polymers

Lipases have got an indispensable importance for its applications in organic syntheses. Lipases are frequently used as biocatalyst in the production of useful biodegradable compounds. 1-Butyl oleate was produced by direct esterification of butanol and oleic acid to decrease the viscosity of biodiesel in winter use [68]. Trimethylolpropane esters were also similarly synthesized as lubricants. Lipases act as catalyzing agent in ester syntheses. They are also known to catalyse transesterification reactions in organic solvent systems which has created the possibility of enzyme catalysed production of biodegradable polyesters. Aromatic polyesters can also be synthesized by lipase biocatalysis [123].

2.4.3 In Textile Industry

Desizing is a process which is required in order to remove the size material which has been impregnated from the fabric prior to weaving. Traditional desizing uses acid or oxidizing agents which damages the cellulose material in the fabric. As compared to traditional desizing methods, enzymatic desizing has several advantages over the traditional process and uses enzymes like cellulase, amylase, protease and lipase depending on the sizing agent. The use of lipases in textile industry assists in the removal of size lubricants which provides a fabric with greater absorbency for improved levelness in dyeing. Its use also reduces the frequency of streaks and cracks in the denim abrasion systems [87]. The commercial preparations which are used for the desizing of denim and other cotton fabrics, contains both alpha amylase and lipase enzymes [124]. Synthetic fibers have been modified enzymatically for the use in the production of yarns, fabrics, textiles, rugs and other consumer items. It modifies the characteristics of a polyester fiber so that such polyesters are more susceptible to post-modification treatments. The use of polyesterase which is closely related to lipase to improve the ability of a polyester fabric to uptake chemical compounds, such as cationic compounds, fabric finishing compositions, dyes, antistatic compounds, anti-staining compounds, antimicrobial compounds, antiperspirant compounds or deodorant compounds [125].

2.4.4 In Detergent Industry

The most important application of hydrolytic lipases is their use in house-hold and laundry detergents. After the successful introduction of proteases in powder and liquid detergents, lipases were developed as detergent enzymes. The efficiency of cleaning power of detergents seems to have increased. Almost all detergents contain similar ingredients and follow similar detergency mechanisms [87]. In order to improve detergency, new launched heavy duty powder detergents and automatic dishwasher detergents usually contain one or more enzymes, such as protease, amylase, cellulase and lipase [126]. The use of enzymes reduce the environmental load of detergent products as they save energy by enabling a lower wash temperature to be used; allow the content of other, often less desirable, chemicals in detergents to be reduced; are biodegradable, leaving no harmful residues; have no negative impact on sewage treatment processes; and do not present a risk to aquatic life [127]. Though fungal lipase was the first introduced detergent enzyme, bacterial lipases captivated the detergent market. This was due to the acidophilic nature of fungal enzymes that make them incompatible with the alkaline wash conditions [128]. During laundering, the lipase adsorbs on to the fabric surface to form a stable fabric-lipase complex which then acts on the oil stains and hydrolyses them. The complex is resistant to the harsh wash conditions and is retained on the fabric during laundering [68]. Lipase retards the redeposition of oil and oil hydrolysis by-products during laundering of fabric. Oil hydrolysis by-products are removable during laundering of fabric at a basic pH or in the presence of a surfactant [129]. The first commercial lipase, Lipolase, which originated from the fungus *T. lanuginosus* and was expressed in *A. oryzae* was introduced by Novo Nordisk in 1994. In 1995, two bacterial lipases were further introduced—Lumafast from *Pseudomonas mendocina* and Lipomax from *Pseudomonas alcaligenes,* both produced by Genencor International, AU-KBC Research Center, Life Sciences, Anna University, Chennai, India [130]. Lipases used as detergents also include those from *Candida* [131]. As laundering is generally carried out in alkaline media, lipases active under alkaline conditions are preferred [132,133], for example, the *A. oryzae* derived lipase. Lipase produced by *Acinetobacter radioresistens* is also alkaline in nature with optimum pH of 10 and having stability over a pH range of 6–10, is proving to have a great potential for application in the detergent industry [134].

2.4.5 Resolution of Racemic Mixtures

Lipases are also used to resolve the racemic mixtures. They are being used for the synthesis of the chiral building blocks for pharmaceuticals, and pesticides agrochemicals. Some lipases retain their activity in nonpolar organic solvents, thus they can be used in the hydrolysis of water insoluble esters, for example in the resolution of racemic mixtures through stereospecific hydrolysis. The resolution of stereoisomers by enantio selective hydrolysis or esterification, have been developed [135]. Lipase from *C. antarctica* (Novozyme (R) 435) has been used for the kinetic resolution of racemic flurbiprofen by the method of enantioselective esterification with alcohols [136]. Chirality is known to be an indispensable factor in the efficacy of several drugs; therefore, it is of utmost importance for the production of single enantiomers of drug intermediates in the pharmaceutical industry. As a result chiral intermediates and fine chemicals are in increasing demand from the pharmaceutical and agrochemical industries which are used for the preparation of several drug substances and agricultural products. An increasing awareness of the enormous potential of microorganisms and enzymes for the transformation of synthetic chemicals with high chemo-, regio- and enantioselectivity has been noticed [137]. Baclofen, an analgesic and muscle relaxant is chemically (RS)-beta-(aminomethyl)-4 chlorobenzene propanoic acid. It synthesizes two isomers. The lipase obtained from *C. cylindracea* has been used as a catalyst for resolving racemic mixture [138]. Lipases are currently being consumed in large amount by many pharmaceutical companies world-wide for the preparation of optically active intermediates and their demand is increasing day by day. Biotechnological companies, such as Enzymatix in the UK, deals in biotransformations and offer a whole variety of intermediates prepared via lipase mediated resolution [68]. Regioselective modification of castanospermine. a promising drug for the treatment of AIDS have also been successfully achieved by using lipases [139].

2.4.6 In Food Processing, Increasing Flavour and Quality Improvement

Fat and oil modification is one of the important areas in food processing industry that demands novel economic and green technologies. Modified vegetable oils with nutritionally important structured triacylglycerols together with altered physicochemical properties have an increasing demand. Modification of vegetable oil is being done by using microbial lipases which are regiospecific and fatty acid specific in nature. By upgrading cheap oils, nutritionally important structured triacylglycerols like cocoa butter substitutes, and oleic acid enriched oils, low calories triacylglycerols could be synthesized [68]. With increasing useful modifications, lipases are going to occupy a prominent place in oil industry for tailoring structured lipids since enzymation modifications are specific and can be carried out at moderate reaction conditions [140]. Lipases have also been used for addition to food to modify flavour by synthesis of esters of short chain fatty acids and alcohols, which are known flavour and fragrance compounds [141]. Psychrotrophic gram-negative bacteria, such as *Pseudomonas* species, are known for causing spoilage problem in refrigerated meat and dairy products due to secretion of hydrolytic enzymes like lipases and proteases. This study characterized the enzymes produced by strains of *P. fluorescens* isolated from pasteurized milk [142]. Lipases have been used in production of leaner meat such as in fish by procedure called bio lipolysis in which fat is removed during the processing of the fish meat by adding lipases [68]. Another important role of lipases is in the fermentative steps of sausage manufacture and to further determine changes in long chain fatty acid liberated during ripening. In recent past lipases of different microbial origin have been used for refining rice flavour, modifying soybean milk and for improving the aroma and accelerating the fermentation of apple wine [143].

2.4.7 As Diagnostic Tool

Lipases have got much importance as they are used as drug targets or marker enzymes in the medical fields. They are also being used as diagnostic tools and their increasing or decreasing levels can indicate certain infection or disease. Lipases are also being used in the enzymatic determination of serum triglycerides by generating glycerol as product which is further determined by enzyme linked colorimetric reactions [68]. Acute pancreatitis and pancreatic injury are also diagnosed by measuring the level of lipase in blood serum [144]. Higaki and Morohashi (2003) [145] have examined

Propioni bacterium acnes lipase in skin diseases and Unsei-in. The production of butyric acid in axillary seborrheic dermatitis (ASD) was higher than in other dermatitis, and that in *Acne vulgaris* (AV) was significantly higher than in controls [68]. *P. acnes* lipase is the pathogenic factor in AV and fatty acids produced by lipase might be the pathogenic factor in ASD. Kanagawa haemolysin, slime, lipase, and colonial opacity have also been considered as virulence markers in infections by *Vibrio cholerae* [146].

2.4.8 Bread Making and Cheese Flavouring

The lipases which are being obtained from lactobacillus play a prominent role in manufacture of bacterial ripened cheese like Parmesan and Grana Padano cheese [87]. The role of lipase/esterase from *Alicyclobacillus acidocaldarius* in milk and cheese models have also been investigated [147]. According to their investigations it was being observed that the recombinant enzyme was more efficient than the native enzyme and therefore could be used in dairy industry to impart flavor or enhance cheese ripening. Enzyme Modified Cheese (EMC) is a concentrated cheese flavor food ingredient produced by treating cheese curd with enzymes. It is used as cheese powders, in soups, salads, sauces and coatings [148]. The flavor of EMC has more intensity than naturally ripened cheese and this flavor is mainly due to small chain fatty acids (C2-C6) (www.amano-enzyme.co.jp). Other special characteristic flavors are as a result of esterification which produces new esters like ethyl butanoate, ethyl hexanoate [149]. Emulsifiers which are additives are being used as a bread improver. They improve bread volume and texture and dough stability. Lipase from *B. subtilis* has been proved to play a role in bread making [150]. These enzymes can completely or partially replace the traditional volume improving agents [151].

2.4.9 In Cosmetics and Personal Care Products

The flavors being produced by transesterification and resolution of racemic intermediates by lipases are used in the cosmetic and perfume industry. Lipases produced by *P. cepacia* have been used to resolve the racemic rose oxides produced by the bromomethoxylation of citronellol [152]. Esters of aliphatic and aromatic acids, alcohols including terpene alcohols, aldehydes, phenols are commonly present in the flavor materials used in perfumes and other personal care products [153]. The synthesis of hydrocinnamic acid esters by *P. cepacia* has also been studied [154]. Mouth washes and shaving creams contain menthol to provide a peppermint flavor and a cooling sensation. Menthol can be artificially produced by the esterification process when there is a dearth for the natural menthol [87]. A process to produce menthol esters and similar compounds has been patented and this process utilizes lipases of *P. fluorescens* and *P. cepacia* [155].

2.4.10 Biosensors

Lipases are widely being used as biosensors. Lipases are used in the qualitative and quantitative determination of lipids and lipid-binding proteins which is being used as biosensors. As biosensors immobilized lipases are used. Such biosensors are used to detect triglycerides in food and clinical samples, pollution analysis like pesticide contamination and pharmaceutical industry [156]. Huang immobilized lipase on a micro-emulsion based gel to fabricate a glass-electrode-based lipase biosensor [157]. A potentiometric biosensor using a lipase immobilized on a mesoporous silica matrix has also been fabricated [158].

2.4.11 Bioremediation

Lipases have got an eminent role in bioremediation. Lipases are actively being used in sludge and other aerobic waste processes. Oil spills in the soil and water during rigging and refining can be handled using lipases [156]. Cultivation of pure culture is an important method of treating waste water which produces lipase and other enzymes which degrades lipids and other pollutants. Effluents emanating from food processing, tannery, automobile industries and restaurant and fast-food outlets can be treated with by cultivating lipase producing bacteria [156,159]. The commonly used bacterial genera are *Pseudomonas, Bacillus* and *Acinetobacter* [160].

2.5 PROPERTIES OF LIPASES

There has been a rapid increase in the production and number of lipases in recent past. These are being used as industrial biocatalysts as they have several attractive properties like high specificity, bio-degradability, and high catalytic efficiency. They have also several other unique properties like their specificity, temperature, pH dependency, activity in organic solvents and nontoxic nature which are frequently being exploited in the food processing industries. There has been deep investigation of lipases from different sources targeting their hydrolytic and synthetic properties. Among different properties of lipases, the most desired features of the lipase are their low product inhibition, ability to utilize all mono-, di-, and tri-glycerides as well as the free fatty acids in transesterification, high activity/yield in non-aqueous media, low reaction time, resistance to altered temperature, pH, alcohol and reusability of immobilized enzyme [161].

2.5.1 Stability in Organic Solvents

Stability in organic solvents is the prime requisite in synthesis reactions. In organic solvents lipases are generally stable except few exceptions of stimulatory or inhibitory effect [162]. The lipase activity of *B. thermocatenulatus* and AG-8 lipase was remarkably increased by ethanol and methanol [163].

2.5.2 Effect of Lipase Inhibitors

Mechanistic and structural properties of lipase have been studied by using lipase inhibitors. Study for lipase inhibitors is also targeted for pharmacological values. Designing drugs for the treatment of many diseases like obesity and the problem of acne are being done by lipase inhibitors. Generally two types of inhibitors are known - reversible or irreversible [162].

2.5.3 Effect of Metal Ions

Metal ions can have a dual effect on microbial enzyme production i.e they can either stimulate or inhibit enzyme production. Among different metal cations, Ca2+ has got roles in the structure and function of enzymes, and some of the known lipases strictly depend on calcium ions [164]. Activation of enzyme is promoted by Ca2+ ions, while Zn2+, Fe2+, Fe3+ strongly inhibited its activity. Salt ions like Ca2+, Cd2+, and Fe2+ promoted the activity of immobilized biocatalyst while a few ions like Co2+, Zn2+, Mg2+, Mn2+, Al3+, and Na+ had mild inhibitory effect [165].

2.5.4 pH and Temperature Kinetics

Most of the bacterial lipases have a neutral or alkaline optimum pH with the exception of lipase from *P. fluorescens* SIK W1 that had an acidic optimum pH 4.8. However, lipases from *Bacillus stearothermophilus* SB-1, *B. atrophaeus* SB-2 and *B. licheniformis* SB-3 are active over a broad pH range 3-12 [166]. Bacterial lipases generally have optimum activity in the temperature range the 30-60°C [167]. Maximum lipase production in *B. licheniformis* MTCC-10498 was observed at pH 7.5 (~0.4 U/ml) [168]. However, bacterial lipases with optima in both lower and higher ranges have been reported. The thermostability of the enzyme from *Bacillus sp.* was enhanced by the addition of stabilizers such as ethylene glycol, sorbitol, glycerol, with the enzyme retaining activity at even after 150 min of incubation at 70°C [169].

2.6 MOLECULAR MODIFICATIONS

Genetic engineering techniques together with protein engineering are proving to be a great asset for maximum exploitation of enzymes for different needs. Substrate binding sites of lipases plays an important role in deciding their specific functions. Therefore knowledge on structure function relationship is required [170]. Designing and engineering of lipases for different important functions is based on the knowledge of the three dimensional structure of lipase. Several lipases from various sources have been crystallized and extensive information on lipase engineering has been mentioned [162]. Schmidt Dannert states a set of information to produce lipase for special applications [112]. The most appropriate enzyme for particular reaction can be modified via mutagenesis. Studies based on the sequence information of protein engineering of lipases has been done since the mid of 1980s. The first lipase enzyme to be engineered was from *P. mendocina* [171]. Thermostability of lipases can be tailored by changing the amino acid sequence when modifying the enzyme for food application. A patent was also being registered on *H. lanuginosa* lipase with increased temperature stability. Several lipases have been tailored for protease stability, thermostability and for oxidative stability [171]. A bacterial lipase gene (from *B. subtilis*) was successfully expressed in in baker’s yeast, *S. cerevisiae* for the first time by Sanchez et al [150]. Another lipase B that showed unique substrate specificity for long-chain cis-9 unsaturated triacylglycerols were overexpressed in *Pichia pastoris* [172]. A nongenetically modified mutant strain *Yarrowia lipolytica* CBS6303 with high productivity was developed by Fickers *et al* by chemical mutagenesis [173]. The detailed explanation about the use of *A. oryzae* as a host for the production of recombinant lipases was explained by Huge-Jensen et al [174]. The different methods for the production of recombinant lipase in different expression systems have been described by Schmidt-Dannert [112]. Holmguist et al reported accumulation of active recombinant *G. candidum* lipase in *P. pastoris* without any contaminating protein [175].

CHAPTER 3

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Chemicals

All laboratory chemicals were analytical grade from Sigma –Aldrich (Germany), Merck (Germany), Riedel-de Haen (Germany), Fluka (Switzerland), Bio-Rad (USA), Fermentas (USA).

Table 3.1 List of chemicals.

Acetic acid	Merck (Germany)
Acrylamide	Sigma –Aldrich (Germany)
Agarose	Sigma –Aldrich (Germany)
Ammonium persulphate (APS)	Merck (Germany)
Ammonium sulphate	Riedel-de Haen (Germany)
B-Mercaptoethanol	Merck (Germany)
Bovine serum albumine (BSA)	Sigma-Aldrich (Germany)
Bromophenol Blue	Sigma-Aldrich (Germany)
Bisacrylamide	Sigma-Aldrich (Germany)
Calcium chloride dihydrate	Merck (Germany)
Cobalt chloride hexahydrate	Merck (Germany)
Coomassie Brilliant Blue	Riedel-de Haen (Germany)
Copper sulfate pentahydrate	Bio-Rad (USA)
Glucose	Merck (Germany)
Glycine	Sigma –Aldrich (Germany)
Glycerol	Merck (Germany)
Hydrochloric acid	Fluka (Switzerland)
Manganese chloride tetrahydrate	Merck (Germany)
Manganese sulfate monohydrate	Merck (Germany)
Methanol	Merck (Germany)
Protein molecular weight marker	Merck (Germany)

Table 3.1 (cont.)

Sodium bicarbonate	Merck (Germany)
Sodium carbonate	Riedel-de Haen (Germany)
Sodium dodecyl sulphate	Merck (Germany)
Sodium hydroxide	Sigma-Aldrich (Germany)
Sodium dihydrogen phosphate	Merck (Germany)
Sodium tartarate dihydrate	Riedel-de Haen (Germany)
TEMED	Riedel-de Haen (Germany)
Tris-HCl	Merck (Germany)
Yeast extract	Sigma-Aldrich (Germany)
Sodium fluoride	Merck (Germany)
Tween 80	Merck (Germany)

3.1.2 Laboratory Equipments

Table 3.2 List of equipments.

Autoclaves	Nüve OT 4060 Steam Sterilizer (Turkey)
Balances	Precisa XB620C (Germany)
Cellulose nitrate filter 0.20 µm cellulose nitrate membrane filter	Sartorius (USA)
Centrifuges	Beckman Coulter (Germany)
Deep Freezers -20°C	Arçelik (Turkey)
Deep Freezers -80°C	New Brunswick Scientific U410 Premium (England)
Electrophoresis equipment	Bio-Rad (USA)
Ice machine AF 10	Scotsman (UK)
Incubators	Nüve EN400 (Turkey)
Magnetic stirrers	Labworld (Germany)
Microplate reader	Model 3559 UV Microplate, Bio-Rad
Micropipettes	Thermo (USA), 1000µl, 200µl, 100µl, 10µl
Microwave Oven	Beko (Turkey)
Orbital Shakers	Thermo (USA)
pH-metre	Mettler Toledo MP220 (Switzerland)
Power Supplies	Bio-Rad Power PAC (USA)
Thermocycler	Techne(UK)
Transilluminator	Bio-Rad GelDoc 2000 (USA)
UV-Visible Spectrophotometers	NanoDrop, Thermo (USA)
Vortex apparatus	Heidolph Reax top (Germany)
Water Purification System	Millipore(Germany)
Waterbath	Nüve (Turkey)

3.1.3 Preparation of Media and Reagents

3.1.3.1 Shewanella Culture Medium

Shewanella putrefaciens was provided from ATCC and number was 8071. Luria-Bertani (LB) agar and broth (Merck) were used for the cultivation of *Shewanella putrefaciens*. The composition of the growth media for *S. putrefaciens* was as follows:

Table 3.3 Luria-Bertani (LB) broth medium: (per litre).

Tryptone (pancreatic digest of casein)	10 g
Yeast extract	5 g
NaCl	5 g

For the preparation of 500 mL LB broth medium, 10g powder was suspended in 500 mL of distilled water. The media were autoclaved at 121°C for 15 minutes, then cooled to 50°C, and stored at 4°C until use.

Table 3.4 Luria-Bertani (LB) agar medium: (per litre).

Tryptone (pancreatic digest of casein)	10 g
Yeast extract	5 g
NaCl	5 g
Agar	15 g

For the preparation of 500 mL LB agar medium, 17.5 g powder was suspended in 500 mL of distilled water. The suspension was heated to boiling while stirring to dissolve all ingredients completely. The media were autoclaved at 121°C for 15 minutes, then cooled to 5°C. Afterwards, the sterile media were poured into sterile petri dishes. Petri dishes were cooled in order to solidify the media under aseptic conditions, and then sealed with stretch film and stored at 4°C until use.

3.1.3.2 Media for the Growth of E. Coli

LB is the most commonly used medium for the growth and expression of *E. coli*. After sterilization of LB broth, the medium was allowed to cool to ~55°C before adding antibiotic (kanamycin, 100 mg/mL final concentration).

For preparation of LB plates plus antibiotic; after autoclaving, the medium was allowed to cool to 50°C before adding kanamycin to a final concentration of 100 mg/mL and poured the plates. 30-35 mL of medium was poured into 100 mm petri dishes. The agar was let to harden. All plates were stored at 4°C for up to 1 month or at room temperature for up to 1 week.

3.1.4 Molecular Biology Kits

All of the molecular biology kits used for DNA isolation, plasmid isolation, and PCR purification are listed below:

Table 3.5 List of molecular biology kits.

High Pure PCR Template Preparation kit	Roche (Germany)
High Pure PCR Template Purification Kit	Roche (Germany)
High Pure Plasmid Isolation Kit	Roche (Germany)
Rapid DNA Ligation kit	Fermentas (USA)
Transform Aid Bacterial Transformation Kit	Fermentas (USA)

3.1.5 Buffers and Solutions

3.1.5.1 Kanamycin Stock Solution

100 mg/mL of kanamycin was dissolved in water, filter sterilized, stored in aliquots at -20°C.

3.1.5.2 IPTG Stock Solution

1.2 g of IPTG was added in deionized water to a final volume of 50 mL. Filtered sterilized and stored at 4°C.

3.1.5.3 Tween 80 Stock Solution

3 ml of Tween 80 was dissolved in 250 mL of distilled water to make 1% solution of Tween 80.

3.1.5.4 $CaCl_2$ Stock Solution

Dissolved 2.31g of $CaCl_2$ in 250 mL of distilled water to make 80 mM solution of $CaCl_2$.

3.1.5.5 SDS-PAGE Solutions and Buffers

Monomer solution for SDS-PAGE: 58.4 g acrylamide, 1.6 g bisacrylamide was dissolved in 200 mL of distilled water and stored at 4°C in the dark.

4X Running (Separating) gel buffer for SDS-PAGE: 1.5M Tris-HCl (pH 8.8) was dissolved in 200 mL of distilled water.

4X stacking gel buffer for SDS-PAGE: 0.5M Tris-HCl (pH 6.8) was dissolved in 50 mL of distilled water.

2X sample buffer for SDS-PAGE: 2.5 mL 4X Stacking buffer, 4 mL SDS solution (10%), 2ml Glycerol, 1mL β-Mercaptoethanol and 0.05 % (w/v) Bromophenol blue were dissolved in 10 mL of distilled water.

Running buffer for SDS-PAGE: 3g Tris, 14.4g Glycine, 10 mL SDS (0.1%) were dissolved in 1L of distilled water.

Coomassie Blue Staining Solution for SDS-PAGE: 0.1g Coomassie brillant blue, 50 mL Methanol and 10 mL Acetic acid were dissolved in 100 mL of distilled water.

Gel Destain Solution for SDS-PAGE: 5 ml Methanol, 10 mL Acetic acid were dissolved in 100 mL of distilled water.

Ammonium persulphate (APS) concentration 10% (w/v)

Sodium dodecyl sulphate (SDS) concentration 10% (w/v)

3.1.6 Bacterial Strains

Shewanella putrefaciens were purchased from ATCC (USA) number of 8071. For sub cloning purposes *Escherichia coli* XL1 Blue cells (Invitrogen, USA) are used. During expression studies, *Escherichia coli* BL21(DE3)pLysS cells (Invitrogen, USA) , are used.

3.1.7 Plasmids and Vectors

pET-28a vectors are purchased from Novagen (Germany).

3.1.8 Primers

All primers were designed by primer3 software and purchased from IDT (Integrated DNA Technology, USA). The primers were diluted using PCR grade water according to the manufacturer’s instructions.

3.1.9 Enzymes

Table 3.6 List of enzymes.

Restriction enzymes	EcoRI, HindIII (NEB enzyme, UK)
PCR amplification	Primestar HS DNA polymerase (Takara,Japan) Taq DNA Polymerase(Takara, Japan)
Ligation	T4 DNA ligase (Fermentas,USA)
Dephosphorylation	Calf Intestinal Alkaline Phosphatase (CIAP) (Fermentas,USA)

3.2 METHODS

3.2.1 Cultivation of Microorganisms

3.2.1.1 Aerobic Growth of Shewanella Putrefaciens ATCC 8071

S. putrefaciens was grown on LB agar at 25°C. For aerobic growth, fresh cultures of 200 mL in 1000 mL flasks were shaken continuously on a rotary shaker (Sartorious Certomat IS) at 160 rpm and 25°C. After 36 h of growth, the suspension were pipette into a 50 mL sterile falcon tubes and centrifuged at 13,000 X g, for 15 min at 4°C. After centrifugation, the supernatant was discarded and the pellet was washed three times with 10 mL of 50 mM Na-phosphate buffer, pH 7.0, containing 0.1 M NaCl. After sub-culturing of *Shewanella putrefaciens*, 0.5 ml of fresh culture was aliquoted into 5 sterile 1.5 mL ependorf tube and stored, following the addition of sterile 50% glycerol in each tube, at -70°C for up to 1 year.

3.2.1.2 Growth of E. coli

Escherichia coli XL-1 Blue and BL21 (DE3) pLysS served as host for DNA manipulation. *E. coli* transformants were grown at 37°C in the Luria- Bertani (LB) broth and on LB agar plates containing 100 mg of kanamycin per mL with shaking.

3.2.2 Cloning of Lipase Gene into pET28a Vector

3.2.2.1 Isolation of Genomic DNA from Shewanella putrefaciens ATCC 8071

Genomic DNA isolation was performed using High Pure PCR Template preparation kit (Roche, Germany) according to the manufacturer's instructions. Quantification of total DNA was carried out by absorbance at 260 and 280 nm using a NanoDrop. The absorbance at 260 and 280 nm ratio (260/280) was used to assess the purity of the DNA.

3.2.2.2 Amplification of Lipase Gene

One of the most important cloning strategy is the addition of restriction endonuclease sites into PCR product if necessary. For that reason, the oligonucleotide primers were designed to include restriction enzyme recognition sites near their 5' termini.

Suitable forward and reverse primers including the appropriate restriction-enzyme site at the 5' end were designed using a websites that were useful in aiding primer design. A particularly good one used in this study was Primer 3 (www.genome. wi.mit.edu/cgi bin/primer/ primer3_ www.cgi). In order to amplify the lipase gene of *Shewanella putrefaciens* nested PCR strategy was used. Both first and second PCR primers listed on Table 3.7. Primers, F1 and R1 were used to first amplify the lipase gene. At the end of the first PCR, amplicon expected size was ~1150 bp. A second set of primers, F2 and R2 used for the second PCR was designed in this study. The second protocol (nested PCR) was predicted to amplify a 896 bp DNA fragment.

Primer F2 (5'- AGAATTCATGAATTATGTTTCCTCAG -3'), containing a EcoRI restriction site at 3’ end was used as the forward primer for the second PCR. The reverse primer, R2 (5'- TTAAGCTTTTGGCACATTACCTTGC -3'), contained a HindIII restriction site. Both primers were obtained from IDT (Integrated DNA Technology, USA).

3.2.2.3 Nested PCR Conditions

PCR was carried out in a Thermocycler Techne TC512 Gradient Thermal Cycler in thin walled 0.2 mL PCR tubes. The first PCR protocol was as follows: 1 µl (~100-150 ng) of genomic DNA was added to a 25 µl reaction containing 12.5 µl of Emerald Master Mix, 0.7 µl (10µM) forward (F1) and reverse primers (R1), IDT (Integrated DNA Technology, USA) and 10.8 µl PCR graded H2O. The reaction mixture was spin down for 10 s and performed for the first PCR reaction. The PCR conditions were as follows; initial denaturation at 94ºC for 1 minute followed by 30 cycles of denaturation step (at 98ºC for 10 seconds), annealing step (at 60ºC for 30 seconds) and an extension step (at 72ºC for 30 seconds). These cycles were then followed by a final extesion step at 72ºC for 5 minutes.

Table 3.7 Oligonucleotide primers used for PCR of lipase gene of Shewanella Putrefaciens.

Primer	TM(°C)	Length of primers(bp)	Oligonucleotide Sequences
F1	64	20	5'- TATCAGAATCGCGGCAATCG
R1	63	20	5'- GCGCAGCCGAGAAATAACAC
F2	59	26	5'- AGAATTCATGAATTATGTTTCCTCAG
R2	60	25	5'- TTAAGCTTTTGGCACATTACCTTGC

The expected PCR product size was 1150 bp. First PCR product was run on a 1.5% TAE gel stained with ethidium bromide (5 mg/mL) to confirm successful amplification. The second PCR was done under same conditions. 1 µl (~100-150 ng) of genomic DNA was added to a 25 µl reaction containing 12.5 µl of Emerald Master Mix, 0.7 µl (10µM) forward (F2) and reverse primers (R2), IDT (Integrated DNA Technology, USA) and 10.8 µl PCR graded H2O. The reaction mixture was spin down for 10 s and performed for the second PCR reaction. All conditions were same as first round PCR. The reaction was ended and cooled to 4ºC for storage. Amplification products were fractionated by electrophoresis in 1% agarose / TAE gels and appropriate bands (about 896 bp) were seen.

3.2.2.4 Gel Electrophoresis

The PCR products were run at 85V for 45 minutes using 1% agarose gels. Agarose gels were prepared using 1X TAE, that had been prepared according to the protocol in Molecular Cloning: A Laboratory Manual. The gel was examined under UV light in order to determine whether DNA band of interest was present on the appropriate place on the gel according to molecular size marker or not. Only a band was detected about 896 bp. This band was purified using the High Pure PCR Purification Kit (Roche, Germany) as per the manufacturer's instructions. Concentration of DNA in the samples was measured by spectrophotometry by taking the OD260 reading and using the Nano Drop.

3.2.2.5 Restriction Enzyme Digestion of PCR Product and Vector

Restriction of the PCR product and vector were done according to the protocols provided by NEB. We used EcoR1 and HindIII restriction endonucleases.

PCR product was digested with EcoR1 and HindIII. The reaction mixture was pipette and spinned to ensure that the contents was at the bottom of the microcentrifuge tube. The tube was incubated for 30 minutes at 37°C. Following the double digestion of insert, mixture was purified to remove the enzyme with High Pure PCR Purification Kit (Roche, Germany).

Table 3.8 Amount of enzymes and buffer for digestion of insert DNA.

Restriction Enzyme Buffer	10 µl
Insert DNA	10 µl
HindIII	2.5 µl
EcoR1	2.5 µl
Sterile dd H2O	75 µl
Final Volume	**100 µl**

Table 3.9 Amount of enzymes and buffer for digestion of pET28a vector.

10X Restriction Buffer	10 µl
Vector DNA	7 µl
HindIII	2.5 µl
EcoR1	2.5 µl
Sterile ddH2O	78 µl
Final Volume	**100 µl**

The suspension was mixed by pipetting and spinned to ensure that the contents was at the bottom of the microcentrifuge tube. The tube was incubated at 37°C for 30 minutes. Dephosphorylation of digested vector was performed with CIAP (calf intestinal alkaline phosphatase) enzyme immediately. For that purpose, 4 µl of CIAP (1u/ µl) was added directly to the digested vector DNA sample to prevent the self ligation of the cut plasmid. The reaction mixture was incubated at 37°C for 35 minutes. Afterwards, CIAP was inactivated by heating at 75 °C for 5 minutes.

3.2.2.6 Gel Extraction of the Vectors

The cut vectors were run at 85V for 45 minutes using 1% agarose gels. Agarose gels were prepared using 1X TAE that had been prepared according to the protocol in Molecular Cloning: A Laboratory Manual, Sambrook et al., 2001. High Pure PCR Template Purification Kit (Roche, Germany), then used to extract the vectors.

3.2.2.7 Ligation

Plasmid DNA and purified PCR fragment were digested with the appropriate restriction enzyme for ligation. Then, plasmid DNA was dephosphorylated with calf intestine alkaline phosphatase (Fermentas). Dephosphorylation reaction was done according to the protocols provided by Fermentas. Digested and dephosphorylated plasmid DNA and digested PCR fragment were then purified using the High Pure PCR Purification Kit (Roche, Germany). Ligation reaction was performed with a vector insert molar ratio of 1:3 using vector and insert using Rapid DNA Ligation kit from Fermentas. The reaction was done according to the protocols provided by Fermentas. Then 5 µl aliquots of the ligation reaction were used to transform *E. coli* XL1 Blue.

3.2.2.8 Transformation into Competent XL-1 Blue Cells

Competent cells were prepared using Transform Aid Bacterial Transformation Kit (Fermentas) according to the manufacturer’s instructions.The transformation was carried out as explained below:

One tube containing 100 µL of *E. coli* XL-1 Blue competent cells was removed from -80ºC and thawed on ice. For each transformation, 75 µL of bacterial suspension was aliquoted into a sterile Ependorf tube. Approximately 1 ng of plasmid DNA (control) was added into the tube containing competent cell for the control of transformation, and 5 µL ligation products were added into the other tube and mixed very gently. Pipetting up and down or vortexing the tube was not being done at all. For each set of transformations, a negative control that consists of competent cells without DNA, and a positive control using a standard plasmid were prepared. The transformation mix was placed on ice for 20 min. The transformation mix was transferred to a 42°C water bath and incubated for exactly 45 s. The temperature and time was crucial in this step. The tubes were not shaken and were handled very carefully. The transformation mix was immediately placed on ice for 2 min. 900 µL of LB broth at room temperature was added. The tubes were incubated at 37°C for 30-45 minutes, shaking vigorously (approx 250 rpm) to ensure good aeration. A range of serial dilutions of the bacteria was prepared from the transformation mix (50 µL and 100 µL from each transformation) and spread on to the pre warmed LB agar plates containing 50 µg of kanamycin per mL. The agar plates were incubated overnight at 37°C.

After incubation, at least ten colonies were selected and each colony was inoculated into 5 mL of LB broth medium containing kanamycin (50 µg /mL). Then each tube was incubated overnight at 37°C. Afterwards, plasmid DNA was extracted from each transformants using High Pure Plasmid Isolation kit (Roche) and analyzed by restriction digestion.

3.2.2.9 Screening of Clones by PCR, Restriction Enzyme Digestion and Sequence Analysis

This technique was used to screen for containing of a lipase gene insert into the pET28a vector. Colony PCR was performed with the insert-specific forward and reverse PCR primer. Eighteen colonies were chosen from the cloning experiment for analysis. Colony preparation for PCR was as the following: The transformation plates were examined and eighteen well-isolated colonies were picked, then transferred to 5 ml sterile LB broth containing appropriate kanamycin for overnight culture. Each colony to be screened was labelled. 10 µl of culture from each tube was taken for performing colony PCR. 10 µl of grown culture from each colony was diluted in 90 µl sterile water, respectively. Each suspension was boiled for 5 min at 95°C in a thermal cycler. 1 µl of the boiled culture was used as a template for each amplification. Positive clones with a suitable size DNA fragment insert were screened by PCR. A master PCR reaction protocol was; 1 µl of bacterial cell suspension was added to a 15 µl reaction containing 7.5 µl Sapphire Master Mix(Takara)), 0.4 µl (10 µM) forward (F2) and reverse primers (R2), 6.1µl PCR graded H_2O. PCR conditions were as follows: 1 cycle of 94ºC for 2 min for initial denaturation; 35 cycles of 95ºC for 10 sec, 60ºC for 30 sec and 72ºC for 1 min; then a final extension at 72ºC for 5 min before storage at 4ºC. Amplification products (5 µl) were analyzed on a 1% agarose gel. Positive clones with insert lipase gene were stored at -80ºC in 50 % glycerol. As an important note here, the amount of bacteria needed for PCR screening is usually very small. Too many bacteria in the mix will inhibit the PCR reaction.

In addition to screen transformants by PCR, a plasmid miniprep followed by restriction digestion was performed. Well-isolated colonies were picked from a plate and transferred to LB broth culture medium containing the appropriate antibiotic for selection. All cultures were incubated over night with shaking (~250 rpm). The plasmid isolation experiment was performed using a High Pure Plasmid Isolation (miniprep) kit (Roche).

Once the DNA was purified, a portion of plasmid was screened by restriction digestion. 1 µg of plasmid was used in our digest. HindIII and EcoR1 were used for screening the orientation of the insert.

Table 3.10 Amount of enzymes and buffer for digestion of pET28a vector for screening the orientation of the insert.

:

10X Restriction Buffer	2.5 µl
Vector DNA	8 µl
HindIII	1 µl
EcoR1	1 µl
Sterile ddH2O	12.5 µl
Final Volume	**25 µl**

Reaction mixture was incubated for 30 min at 37ºC. Then, all tubes were analysed on 1% agarose gel and positive clones were sequenced by RefGen, Ankara, Turkey.

3.2.3 Heterologous Expression of Lipase Gene form pET28a /lipase plasmid in *E. coli* BL21 (DE3) pLysS

The PCR product was digested with HindIII and EcoR1 and then ligated into HindIII and EcoR1–treated expression vector pET-28a and transformed into *E.coli* BL21(DE3) plysS. The *E.coli* cells transformed with this plasmid were plated on LB agar containing 100 mg/mL kanamycin. The transformant was grown in a 15 mL falcon containing 5 mL LB medium supplemented with 100 µg/mL kanamycin at 37°C shaking 250 rpm overnight. Next day we used 1:100 dilution overnight culture into 100 mL flask containing 50 mL LB with no antibiotic. The optical density at 600 nm reached to 0.6–1.0, and then 50µl 1 mM IPTG was added to induce target protein expression. We collected expressed protein each hour by centrifugation and were stored at -20°C until further use.

3.2.4 Characterization of Lipase

3.2.4.1 SDS-PAGE

SDS-PAGE was carried on a 10% separating gel and a 10% stacking gel. When all the samples had been collected from protein induction step, each cell pellet was resuspended in 100 µl of 2X SDS-PAGE sample buffer and was being vortexed. The cell suspention was boiled for 5 minutes and incubated on ice for 2 minutes. Then, all tubes were centrifuged at 13,000 rpm for 1 minute. The gel apparatus was assembled and filled with 1X SDS-PAGE running buffer. 15µl of each sample was loaded on SDS PAGE gel and electrophoresis was being done. The samples were saved by storing them at -20°C. When the dye front of the sample buffer reached the bottom of the plate, the gel was removed. In order to analyze the samples, the polyacrylamide gel was stained with Coomassie blue for 1h with slow shaking. The gel was incubated in destaining solution with slow shaking, until the protein bands were clearly visible. The gel was being looked for a band of increasing intensity in the expected size range for the recombinant protein. The non induced culture was used as a negative control. Molecular Weight Standard (Fermentas), was loaded in the same gel for control identification. Samples were separated by electrophoresis for 2-3 hours at 120 volts.

3.2.4.2 Lipase Enzyme Assay

The activity with Tween 80 as substrate was measured spectrophotometrically at 450 nm using 1% (w/v) Tween 80 in 20 mM Tris-HCl (pH 7), containing 80 mM $CaCl_2$. After the addition of the enzyme, the increase in absorbance was monitored as a function of time. The increase in absorbance was the result of increasing cloudiness of the in situ formed calcium oleate. Lipase activity were monitored UV-Vis Spectrophotometer. The reaction mixture which contained 500 µl Tris-HCl buffer, 200 µl Tween 80 solution (1% w/v), 200 µl $CaCl_2$ solution (80 mM) and 100 µl enzyme, loaded into spectrophotometer and measured at 450 nm for 20 minutes of reaction time at room temperature.

3.2.4.3 Effect of Temperature on the Enzyme Activity

The influence of temperature on lipase activity was studied spectrophotometrically. The reaction buffer contained 500 µl Tris-HCl buffer, 200 µl Tween 80 solution (1% w/v), 200 µl $CaCl_2$ solution (80 mM) and 100 µl enzyme. The effect of temperature on lipase activity was determined by incubating reaction buffer at various temperatures ranging 30ºC to 100°C.

3.2.4.4 Effect of pH on the Enzyme Activity

The influence of pH on lipase activity was studied spectrophotometrically. The pH dependence was determined with Tween 80 solution (1% w/v) as the substrate. The reaction buffer contained 500 µl Tris-HCl buffer, 200 µl Tween 80 solution (1% w/v), 200 µl $CaCl_2$ solution (80 mM) and 100 µl enzyme. pH of the reaction buffer was adjusted from 2 to 10 using 50 mM glycine HCl buffers (pH 2.0 to 3.0), 50 mM sodium acetate buffers (pH 4.0 to 5.0), 50 mM sodium phosphate buffers (pH 6.0 to 7.0) and 2M NaOH (pH 8.0 to 10.0).

CHAPTER 4

RESULTS AND DISCUSSION

4.1 MOLECULAR CLONING OF LIPASE GENE INTO pET-28a VECTOR AND HETEROLOGOUS EXPRESSION OF LIPASE GENE

In order to express lipase gene in *Shewanella putrefaciens*, pET28a (as depicted in Figure 4.1) vectors were used to construct and transcript a recombinant lipase enzyme in prokaryotic host cells. After performing of the plasmid construction, lipase gene was tried to express into *E. coli* host cells.

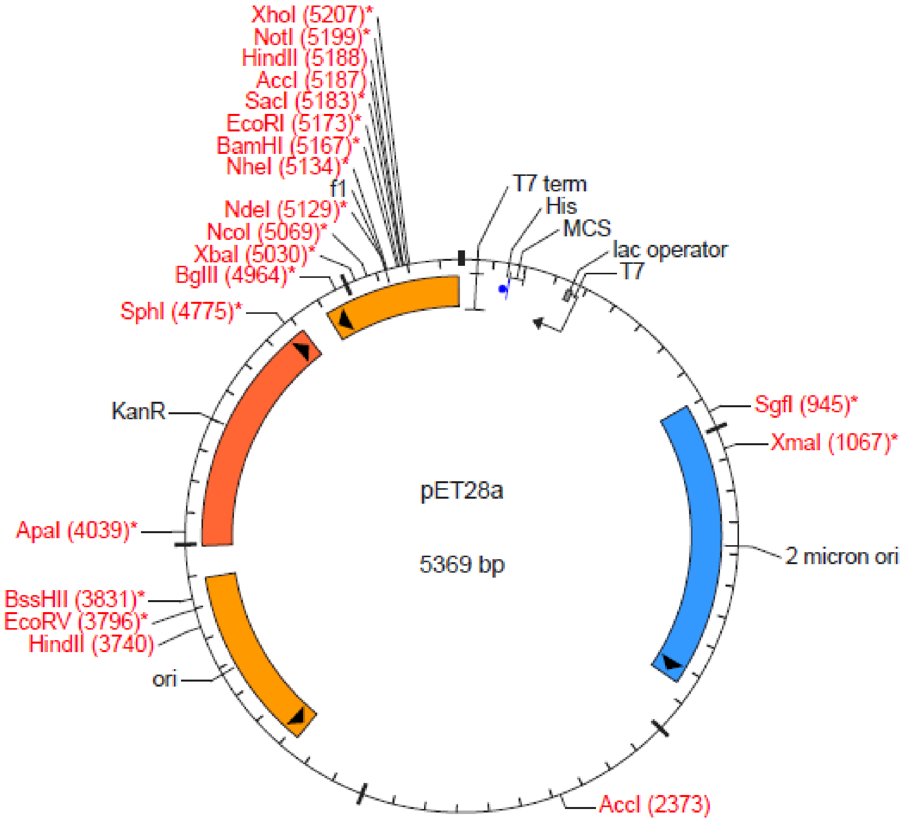


Figure 4.1 Schematic representation pET28a expression vector map.

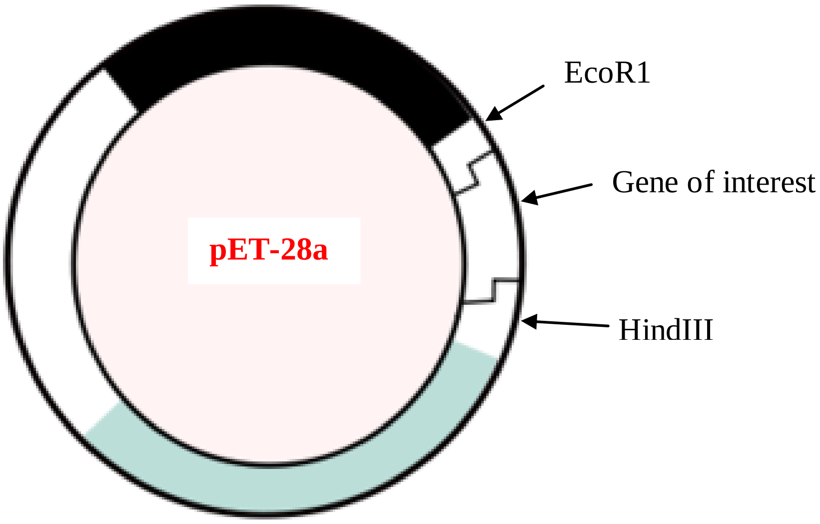


Figure 4.2 Schematic representation of pET28a and lipase gene ligation.

The general outline of the cloning strategy in order to construct pET28a/lipase is presented above in Figure 4.2. The lipase insert DNA (896 bp) lies between the EcoR1 and HindIII restriction site. Lipase was cloned into the pET-28a vector using standard cloning techniques, incubating the digested PCR product with the pET-28a expression vector in a ligation reaction for and transforming the ligation mixture into competent cells of *E. coli*. The genomic DNA isolated from *Shewanella putrefaciens* was quantified spectrophotometrically by absorbance at 260 nm and 280 nm. The concentration of genomic DNA of *Shewanella putrefaciens* was found according to the value of 260 nm absorbance. This total DNA solution had a concentration of 2 µg/ µL and OD 260/ 280 =1.82. This demonstrates that the total DNA extracted was high quality to perform the subsequent analysis. This genomic DNA from *Shewanella putrefaciens* was used as template to amplify the lipase gene by PCR with Emerald Master Mix. The lipase gene fragment synthesized by the nested polymerase chain reaction (PCR) was analyzed by agarose gel electrophoresis. Figure 4.3 shows only one band that belongs to lipase gene and was obtained from total DNA isolated from *Shewanella putrefaciens*. The expected size of the first PCR product was 1150 bp, as demonstrated by its migration on the agarose gel. After performing the nested PCR, 896 bp was obtained.

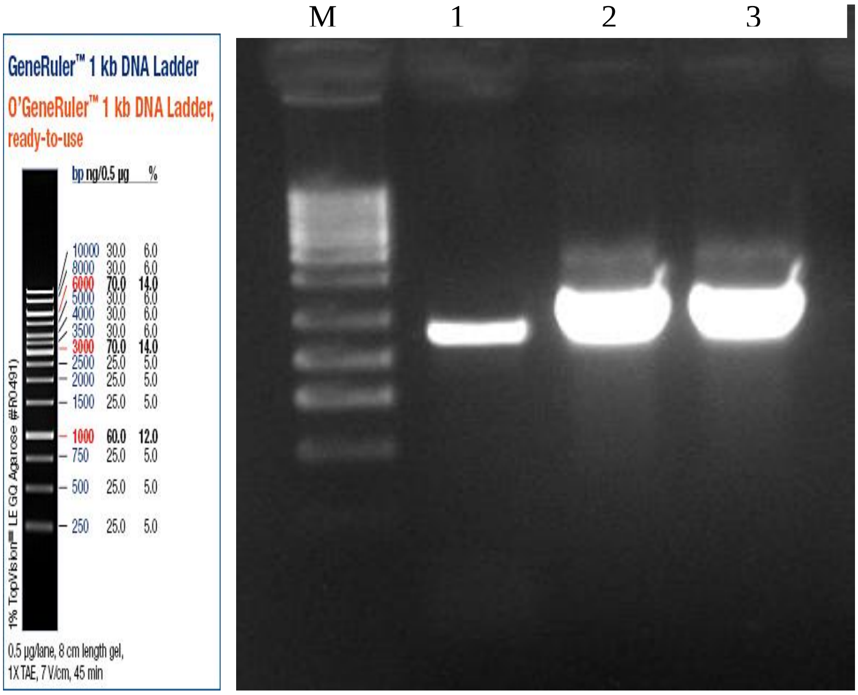


Figure 4.3 First PCR and second PCR.M-1 kb ladder; 1- second PCR; 2 and 3-first PCR.

After obtaining PCR product in correct size, PCR product of lipase gene and expression vector pET-28a were digested by *HindIII and EcoR1* restriction enzymes and both digested products were analysed on a 1% agarose gel and were also purified. The concentrations of the both digested DNA insert and plasmid vector were calculated after measurement optical density at 260 nm. This PCR product had a concentration of 530 ng/ µL and vector concentration had 295 ng/ µL.

It is necessary to estimate the concentration of both vector and insert DNA before ligation. This may be estimated by agarose gel electrophoresis when run against molecular-weight markers of known concentration or by using a spectrophotometer. After performing ligation step with Rapid DNA Ligation Kit, all ligation products were transformed into *E. coli* host cell, and then incubated at 37ºC for overnight period. The next day, eighteen transformants were chosen and mini-preparations of plasmids encoding the lipase were done for screening positive clones that contain the proper orientation of insert DNA by PCR using the F2 and R2 primers as depicted in Figure 4.4.

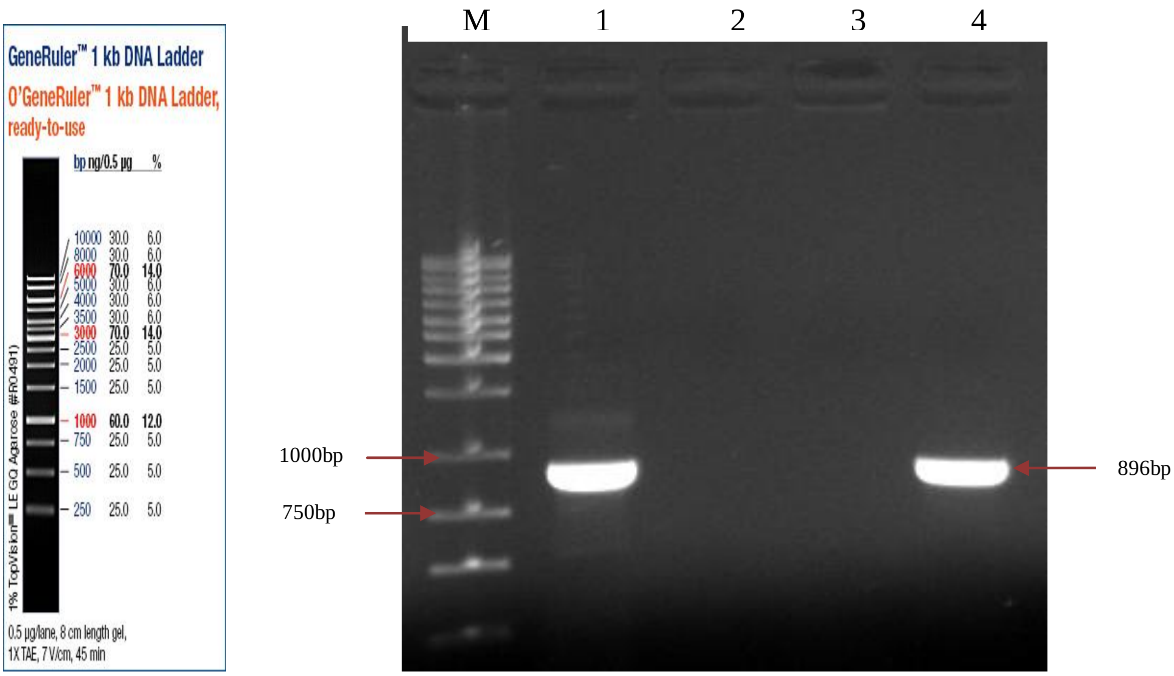


Figure 4.4 Schematic representation of colony PCR and it shows positive clone. M-1 kb ladder; 1- Positive control; 4- Positive colony.

As shown in Figure 4.4, only one construct contains 896 bp lipase gene insert. No insert of interest was seen within the other clones. The positive clone containing the insert lipase gene was sequenced by cycle sequencing.

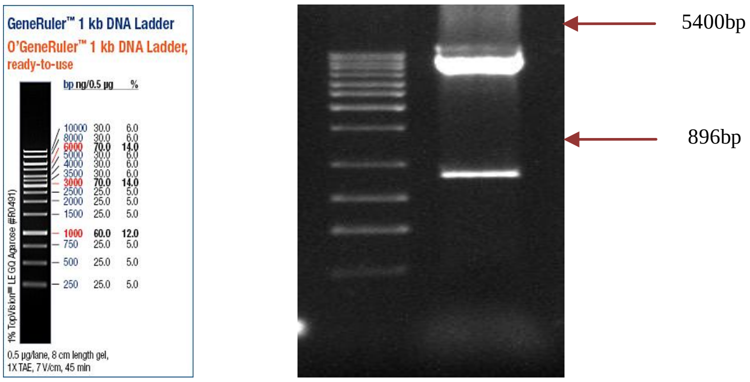


Figure 4.5 Positive clone after digestion with HindIII and EcoR1.

After performing cycle sequencing, only one clone was selected for the expression studies. Once sequenced, plasmid selected for the protein expression was denominated pET-28a/lipase. After this, clone was transformed into strain BL21 (DE3) pLysS hostcell. Sequence analysis was performed using the computer program pDRAW32 and the sequence of lipase gene was compared with GenBank database. The most effective pET-28a/lipase expression transformant was selected by protein expression studies. Recombinant lipase expression was confirmed by SDS-PAGE analysis. Additionally, for the control of the IPTG expression system, pET-28a original vector was induced. Recombinant lipase was secreted into the bacterial cell almost as a single band on SDS-PAGE gel. The expression vector pET-28a /lipase was transformed into *E. coli* BL21 (DE3) pLysS host cell. Eight colonies were isolated and the expression of the recombinant lipase was determined.

Heterologous expression of lipase from pET-28a /lipase was achieved within 4h of induction with 1mM IPTG at 37ºC in *E. coli* BL21 (DE3) pLysS. The choice of such a bacterial host strain for protein expression was dictated by the observation that, due to its high GC content. Expression of recombinant lipase was observed by SDS-PAGE analysis.

SDS-PAGE of bacterial cell *E. coli* strain BL21 (DE3) pLysS containing recombinant pET-28a/lipase vector in Figure 4.6. Lane 1 shows bacterial cell containing pET-28a/lipase plasmid before IPTG induction. Lane 2 shows bacterial cell containing pET-28a/lipase plasmid after 1 h of 1mM IPTG induction. Lane 3 contains bacterial cell containing pET-28a/lipase plasmid after 2 h of 1mM IPTG induction. Lane 4 contains bacterial cell containing pET-28a/lipase plasmid after 3 h of 1mM IPTG induction. It was used as negative control sample (zero time point). Lane Marker: Prestained Protein Molecular Weight Marker (Fermentas). The black arrows show the lipase gene (29 kDa) expression.

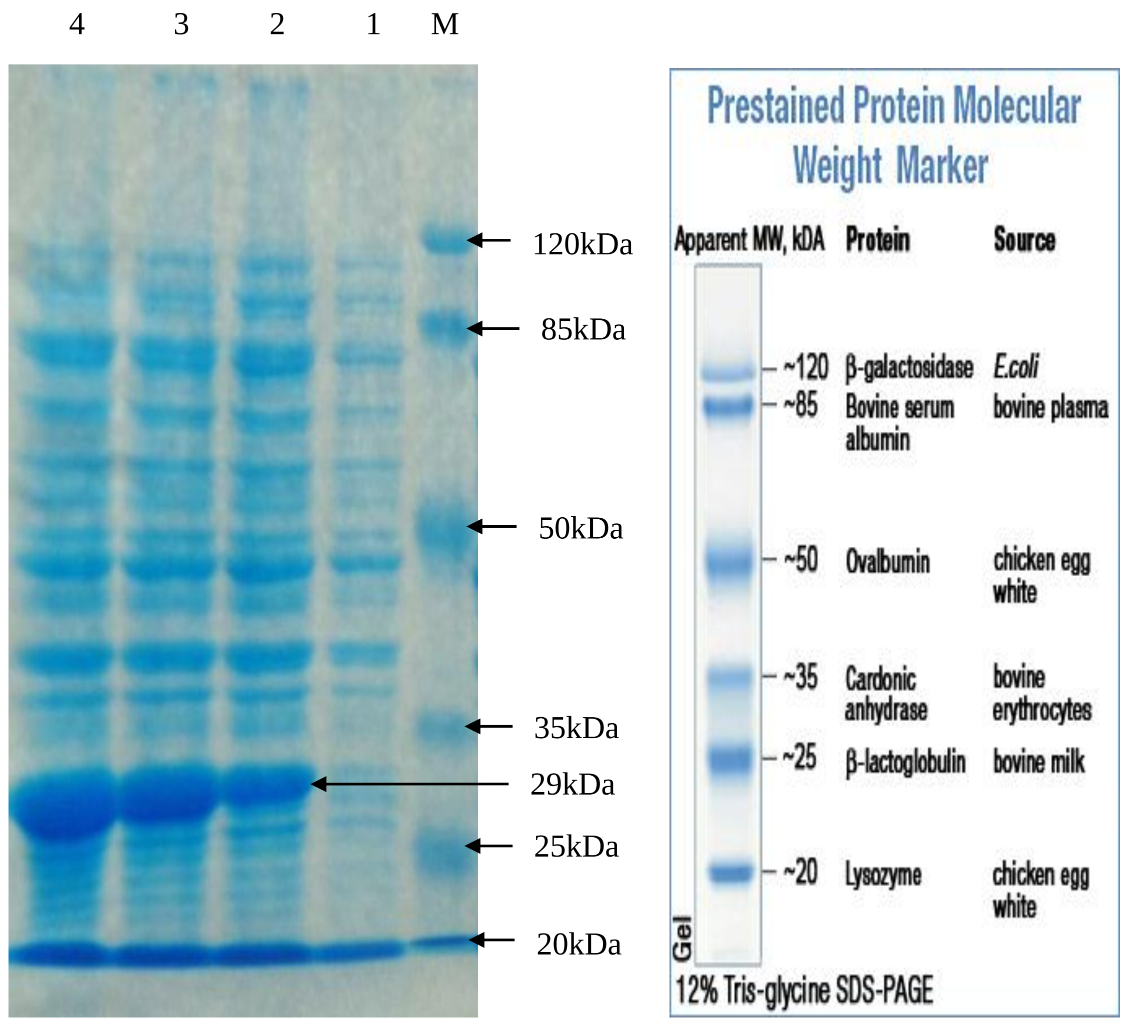


Figure 4.6 SDS- PAGE analysis: Marker is prestained protein molecular weight marker (Fermentas), First lane shows before IPTG induction. Second line shows after 1 hour IPTG induction, third line shows after 2 hours IPTG induction and fourth lane shows after 3 hours IPTG induction.

4.2 CHARACTERIZATION OF LIPASE ENZYME

4.2.1 Enzyme Assay Analysis

For the enzyme assay, Tween 80 was used. The activity with Tween 80 as substrate was measured spectrophotometrically at 450 nm using 1% (w/v) Tween 80 in 20 mM Tris-HCl (Ph 7) buffer, containing 80 mM $CaCl_2$. After the addition of the enzyme, the increase in absorbance was monitored as a function of time. The increase in absorbance was the result of increasing cloudiness of the *in situ* formed calcium oleate. Lipase activity were monitored UV-Vis Spectrophotometer. The reaction mixture which contained 500 µl Tris-HCl buffer, 200 µl Tween 80 solution (1% w/v), 200 µl $CaCl_2$ solution (80 mM) and 100 µl enzyme, loaded into spectrophotometer and measured at 450 nm for 20 minutes of reaction time at room temperature.

Then we searched effect of temperature and pH on the enzyme activity. It was determined by spectrophotometer. The lipase enzyme was tested for its characteristics under different pH conditions such as 2 to 10 and under different temperatures such as 30 to 100°C. The results demonstrated that the enzyme had an optimal pH value at pH 5.0 with Tween 80 as the substrate. It almost did not show any activity after pH 8.0.

Table 4.1 Absorbance at respective pH.

pH	**Absorbance(U/ml)**
2	0.135
3	0.27
4	0.36
5	0.5
6	0.37
7	0.16
8	0.01
9	0.01
10	0

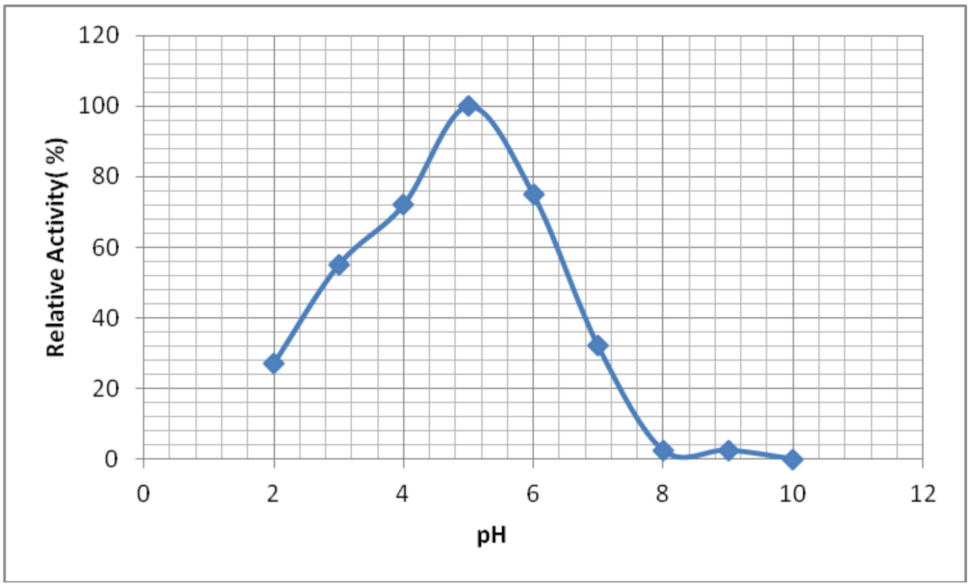


Figure 4.7 Effect of pH on the enzyme activity.

The results showed that the optimum temperature for lipase was 80°C. The enzyme started to lose its activity after 80°C. But it even showed some activity at 90°C and 100°C and was found to be thermostable. Three repeats were done for enzyme analysis with respect to pH and temperature and their average is mentioned in Table 4.1 and 4.2 respectively.

Table 4.2 Absorbance at respective Temperature.

Temperature	**Absorbance(U/ml)**
30	0.04
40	0.1
50	0.11
60	0.17
70	0.31
80	0.43
90	0.22
100	0.1

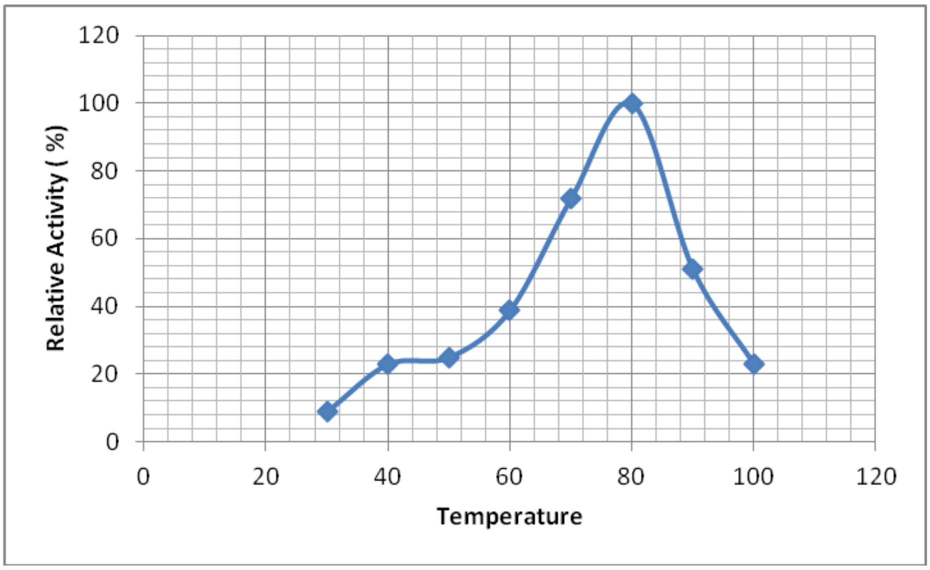


Figure 4.8 Effect of Temperature on the enzyme activity.

4.3 BIOINFORMATIC ANALYSIS

Sequence analysis was performed using the computer program pDRAW32 and the sequences of lipase gene were compared to GenBank database. We converted to recombinant lipase gene to protein sequence. After BLASTp program was used from NCBI, result of BLASTp was compared with various organisms and was found complementary domain and super family.

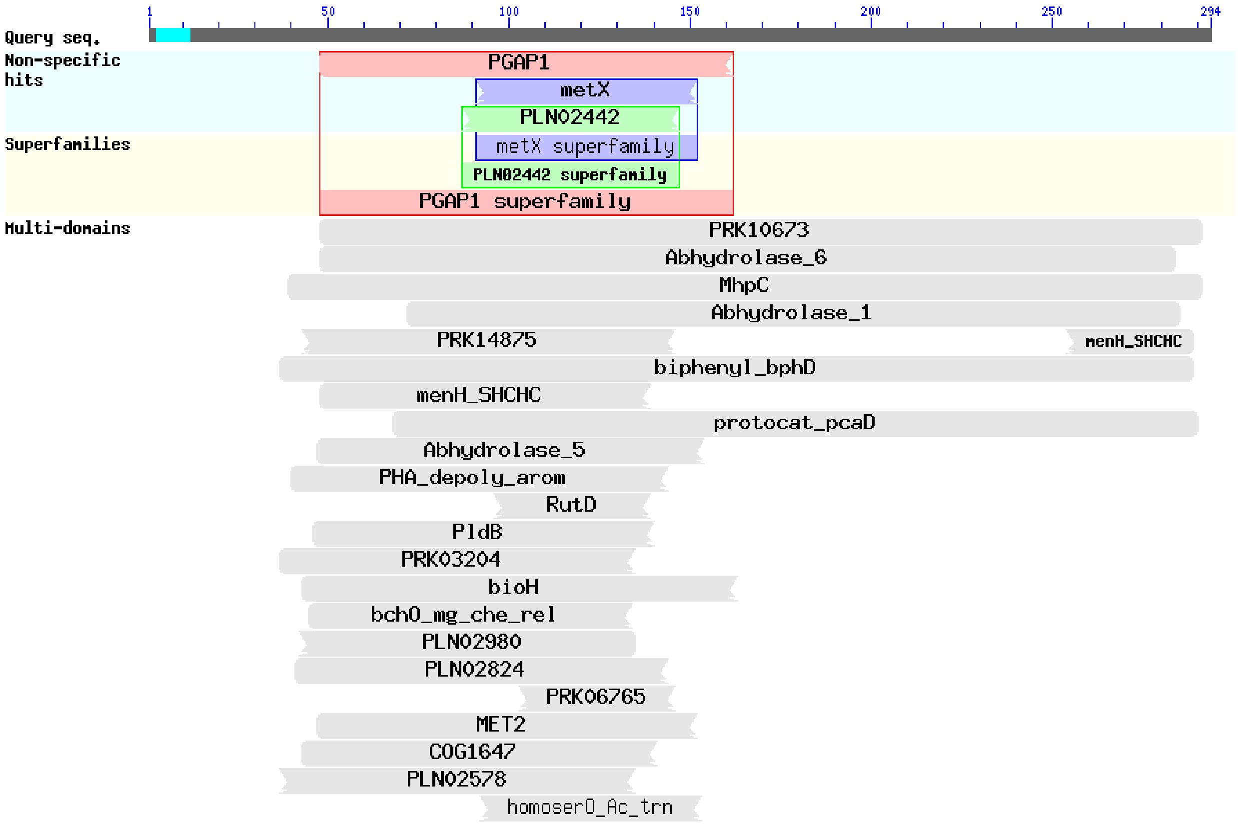


Figure 4.9 BlastP results and its domain study.

We compared Wild type *S.putrefaciens* lipase gene and protein and recombinant lipase gene and protein with CLC-BIO main workbench software and alignment study was being done.

```
ATGAATTATGTTTCCTCAGGTCAAGGACATGCGGTGCTAATAATCCATGGCCTGTTTGGTAATTTAGATAATCTCAAA
GGCTTAGGCCAAGTGTTAGAAAGCCAGCACCAAGTGATCCGCGTTGATGTGCCCAACCATGGGTTAAGTGAGCATTGG
GATCATATGGATTATCCTCGCCTTGCCCATGCCATGATAGATTTGCTCGATAACCTTGACATCGCTCACGCGCACATC
ATCGGCCACTCTATGGGCGGTAAAATCGCCATGGCCACGGCATTAGCCTTTCCAGAGCGCATTATCAGCATGGTCGCT
GCCGATATTGCACCTGTCGCATATGAGCCTAGACACGATATTGTATTTGCTGCATTAGAAAGCCTGCCCTTAGAAGGC
CATACCGACAGACGCTTTGCCCTAAACCACTTAATCAACCATGGTATTGATGAAGCCACTGCCCAATTTTTGCTGAAA
AACTTGCAACGTACCGATACTGGATTTCGTTGGAAAATGAACTTAAGTGGTTTAAAAACCTGTTATCCAAACATTATT
GGCTGGCATAATCAGCCACCGAACCCCGTGCTGAGCTATTCTGGCCCAAGTCTATTTATTCGTGGCGGTGATTCAAAT
TATGTCAATAGCGAACATAGGGACGCGATTATGGCGCAATTCCCTACTGCACAAGCAAAAACACTTGAAGGTTGTGGA
CATTGGTTGCATGCCCAAAAACCTGCGATTTTTAATCGAATTGTGTCTGAATTTATTGACAAACAGGCAATG
```

Figure 4.10 Wild type *S.putrefaciens* lipase gene.

```
ATGGGCAGCAGCCATCATCATCATCATCACAGCAGCGGCCTGGTGCCGCGCGGCAGCCATATGGCTAGCATGACTGGT
GGACAGCAAATGGGTCGCGGATCCGAATTCATGAATTATGTTTCCTCAGGTCAAGGACATGCGGTGCTAATAATCCAT
GGCCTGTTTGGTAATTTAGATAATCTCAAAGGCTTAGGCCAAGTGTTAGAAAGCCAGCACCAAGTGATCCGCGTTGAT
GTGCCCAACCATGGGTTAAGTGAGCATTGGGATCATATGGATTACCCTCGCCTTGCCCATGCCATGATAGATTTGCTC
GATAACCTTGACATCGCTCACGCGCACGTCATCGGCCACTCTATGGGCGGTAAAATCGCCATGGCCACGGCATTAGCC
TTTCCAGAGCGCATTATCAGCATGATCGCTGCCGATATCGCGCCCGTTGCTTATGAGCCTAGACACGATATTGTATTT
GCAGCATTAGAAAGTCTGCCCTTAGAAGGCCATACCGACAGACGCTTTGCCCTAAACCACTTAATCAACCATGGTATT
GATGAAGCCACTGCCCAATTTTTGCTGAAAAACTTGCAACGTACCGATACCGGATTTCGTTGGAAAATGAACTTAAGT
GGTTTAAAAACCTGTTATCCAAACATTATTGGCTGGCATAATCAGCCACCGAACCCCGTGCTGAGCTATTCTGGCCCA
AGTCTATTTATTCGTGGCGGTGATTCAAATTATGTCAATAGCGAACATAGGGACGCGATTATGGCGCAATTCCCTACT
GCACAAGCAAAAACACTTGAAGGTTGTGGACATTGGTTGCATGCCCAAAAACCTGCGATTTTTAATCGAATTGTGTCT
GAATTTATTGACAAACAGGCAATGTAA
```

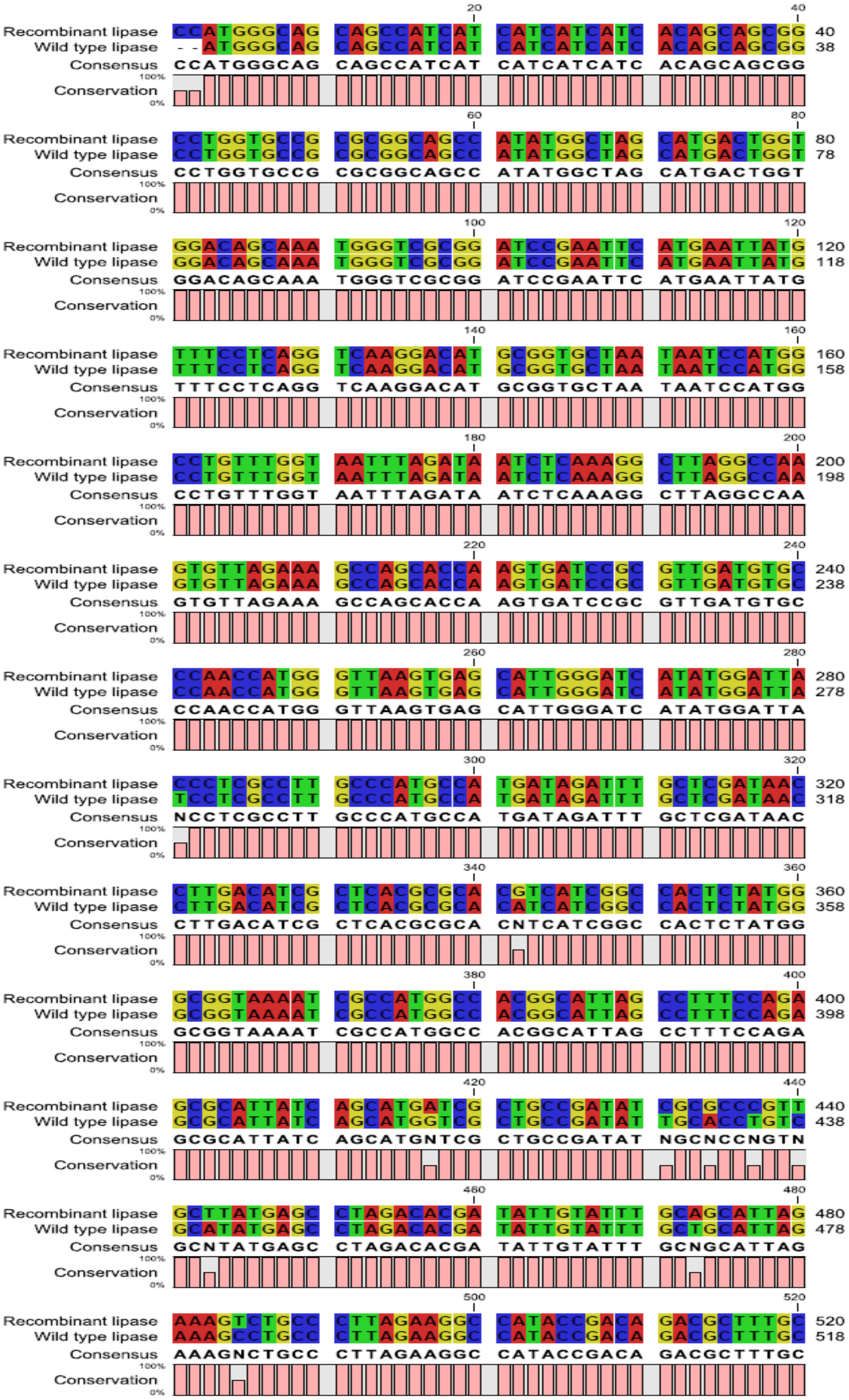
Figure 4.11 Recombinant *S.putrefaciens* lipase gene.

```
MNYVSSGQGHAVLIIHGLFGNLDNLKGLGQVLESQHQVIRVDVPNHGLSEHWDHMDYPR
LAHAMIDLLDNLDIAHAHIIGHSMGGKIAMATALAFPERIISMVAADIAPVAYEPRHDI
VFAALESLPLEGHTDRRFALNHLINHGIDEATAQFLLKNLQRTDTGFRWKMNLSGLKTC
YPNIIGWHNQPPNPVLSYSGPSLFIRGGDSNYVNSEHRDAIMAQFPTAQAKTLEGCGHW
LHAQKPAIFNRIVSEFIDKQAM
```

Figure 4.12 Wild type *S.putrefaciens* lipase gene protein.

```
MGSSHHHHHHSSGLVPRGSHMASMTGGQQMGRGSEFMNYVSSGQGHAVLIIHGLFGNLD
NLKGLGQVLESQHQVIRVDVPNHGLSEHWDHMDYPRLAHAMIDLLDNLDIAHAHVIGHS
MGGKIAMATALAFPERIISMIAADIAPVAYEPRHDIVFAALESLPLEGHTDRRFALNHL
INHGIDEATAQFLLKNLQRTDTGFRWKMNLSGLKTCYPNIIGWHNQPPNPVLSYSGPSL
FIRGGDSNYVNSEHRDAIMAQFPTAQAKTLEGCGHWLHAQKPAIFNRIVSEFIDKQAM
```

Figure 4.13 Recombinant type *S.putrefaciens* lipase gene protein.



Recombinant lipase CCATGGGCAG CAGCCATCAT CATCATCATC ACAGCAGCGG 40
Wild type lipase - - ATGGGCAG CAGCCATCAT CATCATCATC ACAGCAGCGG 38
Consensus CCATGGGCAG CAGCCATCAT CATCATCATC ACAGCAGCGG

Recombinant lipase CCTGGTGCCG CGCGGCAGCC ATATGGCTAG CATGACTGGT 80
Wild type lipase CCTGGTGCCG CGCGGCAGCC ATATGGCTAG CATGACTGGT 78
Consensus CCTGGTGCCG CGCGGCAGCC ATATGGCTAG CATGACTGGT

Recombinant lipase GGACAGCAAA TGGGTCGCGG ATCCGAATTC ATGAATTATG 120
Wild type lipase GGACAGCAAA TGGGTCGCGG ATCCGAATTC ATGAATTATG 118
Consensus GGACAGCAAA TGGGTCGCGG ATCCGAATTC ATGAATTATG

Recombinant lipase TTTCCTCAGG TCAAGGACAT GCGGTGCTAA TAATCCATGG 160
Wild type lipase TTTCCTCAGG TCAAGGACAT GCGGTGCTAA TAATCCATGG 158
Consensus TTTCCTCAGG TCAAGGACAT GCGGTGCTAA TAATCCATGG

Recombinant lipase CCTGTTTGGT AATTTAGATA ATCTCAAAGG CTTAGGCCAA 200
Wild type lipase CCTGTTTGGT AATTTAGATA ATCTCAAAGG CTTAGGCCAA 198
Consensus CCTGTTTGGT AATTTAGATA ATCTCAAAGG CTTAGGCCAA

Recombinant lipase GTGTTAGAAA GCCAGCACCA AGTGATCCGC GTTGATGTGC 240
Wild type lipase GTGTTAGAAA GCCAGCACCA AGTGATCCGC GTTGATGTGC 238
Consensus GTGTTAGAAA GCCAGCACCA AGTGATCCGC GTTGATGTGC

Recombinant lipase CCAACCATGG GTTAAGTGAG CATTGGGATC ATATGGATTA 280
Wild type lipase CCAACCATGG GTTAAGTGAG CATTGGGATC ATATGGATTA 278
Consensus CCAACCATGG GTTAAGTGAG CATTGGGATC ATATGGATTA

Recombinant lipase CCCTCGCCTT GCCCATGCCA TGATAGATTT GCTCGATAAC 320
Wild type lipase TCCTCGCCTT GCCCATGCCA TGATAGATTT GCTCGATAAC 318
Consensus NCCTCGCCTT GCCCATGCCA TGATAGATTT GCTCGATAAC

Recombinant lipase CTTGACATCG CTCACGCGCA CGTCATCGGC CACTCTATGG 360
Wild type lipase CTTGACATCG CTCACGCGCA CATCATCGGC CACTCTATGG 358
Consensus CTTGACATCG CTCACGCGCA CNTCATCGGC CACTCTATGG

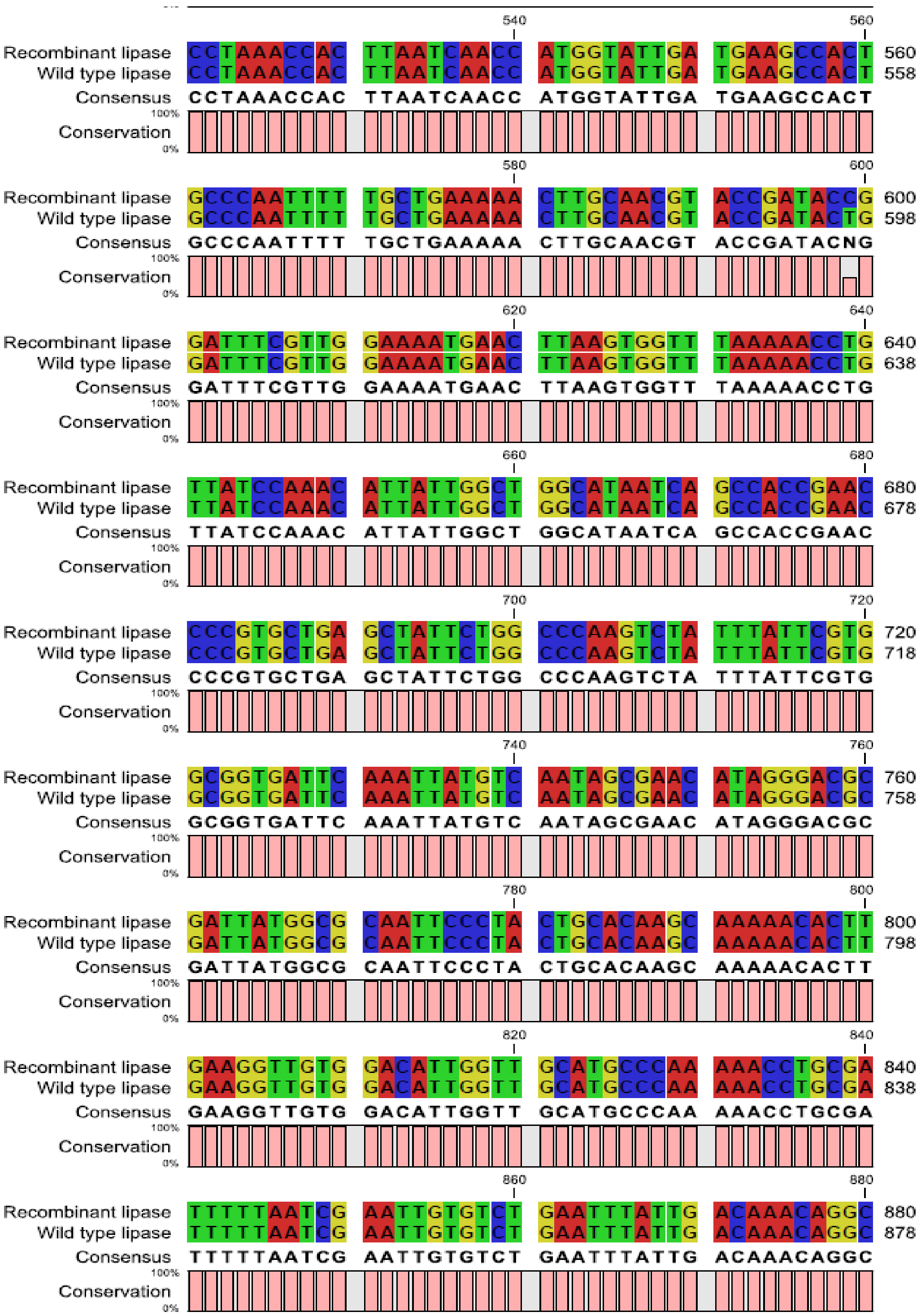
Recombinant lipase GCGGTAAAAT CGCCATGGCC ACGGCATTAG CCTTTCCAGA 400
Wild type lipase GCGGTAAAAT CGCCATGGCC ACGGCATTAG CCTTTCCAGA 398
Consensus GCGGTAAAAT CGCCATGGCC ACGGCATTAG CCTTTCCAGA

Recombinant lipase GCGCATTATC AGCATGATCG CTGCCGATAT CGCGCCCGTT 440
Wild type lipase GCGCATTATC AGCATGGTCG CTGCCGATAT TGCACCTGTC 438
Consensus GCGCATTATC AGCATGNTCG CTGCCGATAT NGCNCCNGTN

Recombinant lipase GCTTATGAGC CTAGACACGA TATTGTATTT GCAGCATTAG 480
Wild type lipase GCATATGAGC CTAGACACGA TATTGTATTT GCTGCATTAG 478
Consensus GCNTATGAGC CTAGACACGA TATTGTATTT GCNGCATTAG

Recombinant lipase AAAGTCTGCC CTTAGAAGGC CATACCGACA GACGCTTTGC 520
Wild type lipase AAAGCCTGCC CTTAGAAGGC CATACCGACA GACGCTTTGC 518
Consensus AAAGNCTGCC CTTAGAAGGC CATACCGACA GACGCTTTGC

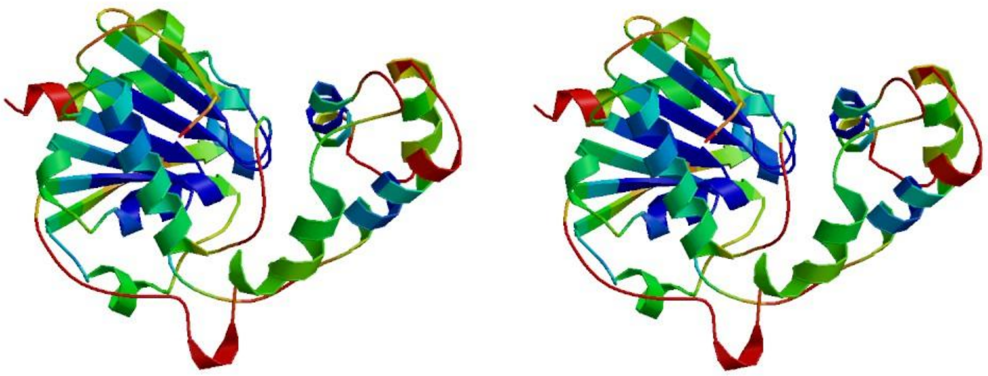
Figure 4.14 Alignment of wild type *S.putrefaciens* lipase gene and recombinant *S.putrefaciens* lipase gene.



```
                                               540                                 560
Recombinant lipase  CCTAAACCAC  TTAATCAACC  ATGGTATTGA  TGAAGCCACT  560
  Wild type lipase  CCTAAACCAC  TTAATCAACC  ATGGTATTGA  TGAAGCCACT  558
         Consensus  CCTAAACCAC  TTAATCAACC  ATGGTATTGA  TGAAGCCACT
              100%
      Conservation
                0%
                                               580                                 600
Recombinant lipase  GCCCAATTTT  TGCTGAAAAA  CTTGCAACGT  ACCGATACCG  600
  Wild type lipase  GCCCAATTTT  TGCTGAAAAA  CTTGCAACGT  ACCGATACTG  598
         Consensus  GCCCAATTTT  TGCTGAAAAA  CTTGCAACGT  ACCGATACNG
              100%
      Conservation
                0%
                                               620                                 640
Recombinant lipase  GATTTCGTTG  GAAAATGAAC  TTAAGTGGTT  TAAAAACCTG  640
  Wild type lipase  GATTTCGTTG  GAAAATGAAC  TTAAGTGGTT  TAAAAACCTG  638
         Consensus  GATTTCGTTG  GAAAATGAAC  TTAAGTGGTT  TAAAAACCTG
              100%
      Conservation
                0%
                                               660                                 680
Recombinant lipase  TTATCCAAAC  ATTATTGGCT  GGCATAATCA  GCCACCGAAC  680
  Wild type lipase  TTATCCAAAC  ATTATTGGCT  GGCATAATCA  GCCACCGAAC  678
         Consensus  TTATCCAAAC  ATTATTGGCT  GGCATAATCA  GCCACCGAAC
              100%
      Conservation
                0%
                                               700                                 720
Recombinant lipase  CCCGTGCTGA  GCTATTCTGG  CCCAAGTCTA  TTTATTCGTG  720
  Wild type lipase  CCCGTGCTGA  GCTATTCTGG  CCCAAGTCTA  TTTATTCGTG  718
         Consensus  CCCGTGCTGA  GCTATTCTGG  CCCAAGTCTA  TTTATTCGTG
              100%
      Conservation
                0%
                                               740                                 760
Recombinant lipase  GCGGTGATTC  AAATTATGTC  AATAGCGAAC  ATAGGGACGC  760
  Wild type lipase  GCGGTGATTC  AAATTATGTC  AATAGCGAAC  ATAGGGACGC  758
         Consensus  GCGGTGATTC  AAATTATGTC  AATAGCGAAC  ATAGGGACGC
              100%
      Conservation
                0%
                                               780                                 800
Recombinant lipase  GATTATGGCG  CAATTCCCTA  CTGCACAAGC  AAAAACACTT  800
  Wild type lipase  GATTATGGCG  CAATTCCCTA  CTGCACAAGC  AAAAACACTT  798
         Consensus  GATTATGGCG  CAATTCCCTA  CTGCACAAGC  AAAAACACTT
              100%
      Conservation
                0%
                                               820                                 840
Recombinant lipase  GAAGGTTGTG  GACATTGGTT  GCATGCCCAA  AAACCTGCGA  840
  Wild type lipase  GAAGGTTGTG  GACATTGGTT  GCATGCCCAA  AAACCTGCGA  838
         Consensus  GAAGGTTGTG  GACATTGGTT  GCATGCCCAA  AAACCTGCGA
              100%
      Conservation
                0%
                                               860                                 880
Recombinant lipase  TTTTTAATCG  AATTGTGTCT  GAATTTATTG  ACAAACAGGC  880
  Wild type lipase  TTTTTAATCG  AATTGTGTCT  GAATTTATTG  ACAAACAGGC  878
         Consensus  TTTTTAATCG  AATTGTGTCT  GAATTTATTG  ACAAACAGGC
              100%
      Conservation
                0%
```

Figure 4.14 (cont.)

Protein alignment has been performed and protein modeling has been performed with swiss-model programming and 3D structure of protein was compared with each other.



Wild type *S.putrefaciens* lipase Recombinant *S.putrefaciens* lipase

Figure 4.15 3D modelling of wild type *S.putrefaciens* lipase and recombinant *S.putrefaciens* lipase.

DISCUSSION

The lipase enzyme obtained from *Shewanella putrefaciens* ATCC 8071 showed many useful properties as compared to other lipases. For example our lipase enzyme was thermostable which showed maximum activity at 80 °C and at pH 5. It continued to show its activity at 80 °C for 20 minutes. It also showed some activity even at 90 °C and 100 °C.

Lipases are active and stable under alkaline conditions and over a broad temperature range, which makes them ideal for incorporation in various applications. Lipases are also stable in detergents containing protease and activated bleach system [176]. The purified *Aspergillus terreus* lipase showed excellent temperature tolerance (15-90°C) and was highly thermostable retaining 100% activity at 60°C for 24 hours. It showed good pH tolerance (3-12) and was stable over a pH range of 4.0-10.0 for 24 hours [177]. Activity of lipase II from *Penicillium cyclopium* [178] and *Penicillium wortmani* [179] is stable at pH 7.0 and 40–45 °C. Activity is lost at temperatures above 50 °C. The lipases from *Penicillium roqueforti* 141 were stable to heating (up to 55 °C) and within a wide pH range. *Penicillium cyclopium* lipase was also found to be stable at 35 °C for 60 min and has maximal activity in a pH range of 8–10 [180]. The optimum temperature for enzyme activity of lipases from a Brazilian strain of *Penicillium citrinum* was also found in the range of 34–37 °C. However, after 30 min at 60 °C, the enzyme was completely inactivated. The enzyme also showed optimum at pH 8.0 [181].

The Brazilian strain of *Fusarium solani* FSI lipase activity was stable below 35 °C but above this temperature activity losses were observed [182]. The optimum pH of the solvent-tolerant lipase from *Fusarium heterosporum* at 40 °C and optimum temperature at pH 5.6 were 5.5–6.0 and 45–50 °C, respectively, when olive oil was used as the substrate. The lipase was stable over a pH range of 4–10 at 30 °C for 4 h, and up to 40 °C at pH 5.6 for 30 min [183]. At 55 °C, the *Pseudomonas aeruginosa* LP602 lipase had the highest activity but not stability [184]. An alkaline lipase from *Pseudomonas fluorescens* AK 102 [183] and *Pseudomonas putida* 3SK [185] were found to have pH stability in the range 4–10 and optimum pH 8–10.

The purified extracellular lipase of *Staphylococcus warneri* was found to have an optimum pH of 9.0 and an optimum temperature of 25 °C. The enzyme was stable within the range pH 5.0–9.0 [186]. The optimum temperature of the purified lipase *Staphylococcus aureus* 226 enzyme was 60 °C with triolein [187]. Optimum temperature and pH for the hydrolysis of olive oil by *Staphylococcus haemolyticus* L62 was found to be 28 °C and pH 8.5, respectively. The enzyme was stable up to 50 °C in the presence of Ca2+ and over the pH range 5–11 [188]. The *Staphylococcal* lipase preparation was found to be stable within pH 5.0 to 9.0 and exhibits maximal activity at pH 8.0. The optimal temperature for the enzymatic reaction was established at 55 °C [189].

The lipase from psychrotrophic *Pseudomonas sp*. [190] and *Pseudomonas fluorescens* strain AFT 36 [191] were found to be stable between pH 6 and 9. *Pseudomonas sp*. Enzyme was found to be stable between pH values 6 and 12 and below 60 °C [192]. Whereas, *P. fluorescens* strain AFT 36 was relatively heat-labile in phosphate buffer, in the temperature range 60–80 °C. Maximum activity of lipase from *Bacillus thermocatenulatus* was found at pH 7.5–8.0 and 60–70 °C with p-nitrophenyl palmitate and olive oil as substrates [193]. The lipase purified from *Pseudomonas aeruginosa* EF2 was very stable to freezing and thawing [194]. *Pseudomonas* species, KWI-56, extracellular thermostable lipase resisted more than 90% of the enzyme activity after 24 h of heat treatment at 60 °C [195]. The lipase produced by *Bacillus sp.* LBN4 had temperature optima of 65 °C. Over 80% of its peak activity was at the pH range of 7–8, with an optimum of 8.0. The enzyme was found to be most stable at 80 °C for 10 min. Almost 80% of the residual activity was retained at 80 °C [196].

According to Vajanti Mala Pahoja and Mumtaz Ali Sethar, the pH ranges of lipases obtained from microorganisms ranges between 6.0 to 10.0. The temperature at which the microbial lipase is active ranges between 37 to 55°C [65]. But our lipase enzyme showed maximum activity at pH 5 and at temperature 80 °C which showed it to be thermostable in nature.

Lipases which are obtained from culture supernatants have various disadvantages like non-reproducibility of results, undesirable side-effects and requires frequent purification processes [87]. The choice of the expression system employed for over expression and the thorough knowledge on the genetic modifications to be made are the backbones of success of this Recombinant DNA Technology. Microbial lipases are an eminent group of biotechnologically valuable enzymes. This is because of the diversity of their applied properties and ease of mass production. Microbial lipases are known for their diverse enzymatic properties and substrate specificity, which are the attractive features for industrial applications. Genetic engineering techniques together with protein engineering are proving to be a great asset for maximum exploitation of enzymes for different needs. Substrate binding sites of lipases plays an important role in deciding their specific functions. Therefore knowledge on structure function relationship is required [170]. We will try to improve our enzyme through modification of its structure and using different genetic engineering tools.

For the characterization of lipase activity, tween 80 was being used as substrate in my thesis [197]. As compared to this, many researchers used different other chemicals as substrates in lipase characterization. Schmidt-Danert et al. used p-NPP and olive oil as substrates [198]. The purified *Pseudomonas fluorescens* strain AFT 36 lipase showed maximum activity on tributyrin [199]. *Pseudomonas aeruginosa* strain MB 5001 lipase was found to be more active on short-chain versus long-chain triglycerides, and hydrolyzes C18-unsaturated fatty acid esters more efficiently than it hydrolyzes C18-saturated fatty acid esters [200].

The cloned lipase enzyme from *Shewanella putrefaciens* ATCC 8071 belongs to alpha-beta hydrolase group enzyme in superfamily PGAP1 possessing many useful and unique properties.

CHAPTER 5

CONCLUSION

In this study, we preferred *S. putrefaciens* ATCC 8071 because its lipase enzyme was not characterized ever before. Lipase genes in *Shewanella putrefaciens* ATCC 8071 was amplified and transferred into pET28a expression vector and *E.coli* (DE3)pLysS competent cell. Our lipase gene was expressed, then was analysed by SDS- PAGE and bioinformatic studies.

According to the bioinformatic studies our recombinant lipase has got no transformation in its active site. This can be confirmed by looking at the 3D modelling of recombinant lipase as it is almost similar to the 3D modelling of wild type lipase. There are only few nucleotide changes in our recombinant lipase as it is clear from alignment studies. These changes may be due to polymorphism. No mutation was being seen in our recombinant lipase. These results show that our enzyme is free from any adverse mutations and shows that our recombinant protein may be more active than others. SDS-PAGE results are the same as bioinformatic analysis. Also, this recombinant lipase gene has more thermostability as compared to other lipases which were being studied previously. This shows that our recombinant protein may be more active at high temperatures than others.

This is the first record in the literature in which lipase enzyme from *S. Putrefaciens* ATCC 8071 was isolated, cloned, purified and characterized. We think that our recombinant lipase has different characteristics from another lipase enzyme. In future we will continue to characterize our enzyme and will develop our enzyme with protein engineering techniques. Enzyme kinetics will also be studied in future. Also we will use this enzyme in order to develop some biosensors and also use it in pharmaceuticalfieldsetc.

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