Biochemical and biophysical studies on thermostable and thermoactive enzymes and understanding the molecular determinants of protein thermostability

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By

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Nirma University

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Certificate

This is to certify that the thesis entitled **"Biochemical and biophysical studies on thermostable and thermoactive enzymes and understanding the molecular determinants of protein thermostability"** has been prepared by **Miss. Krupali Parmar** under my supervision and guidance. The thesis is his own original work completed after careful research and investigation. The work of the thesis is of the standard expected of a candidate for Ph.D. Programme in **Science** and I recommend that it be sent for evaluation.

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Abstract

This research work compiles biophysical and biochemical studies on two extracellular enzymes, a lipase from *Halomonas shengliensis* and an amylase from *Bacillus atrophaeus*. Both these enzymes belong to the group of candidate enzymes which possess promising industrial applications. The initial study is focused on purification, characterization, cloning and stability studies of lipase from a moderate haloalkalophile. The production of thermo-active lipase was carried out in optimized production medium containing; olive oil 2.0% (w/v), yeast extract 0.5% (w/v) and NaCl 6% (w/v), pH 8.5 at 35°C with agitation for 4 days. The protein exhibited high affinity towards anion exchanger resins. For purification, it was bound to Q-Sepharose Fast Flow column and eluted in 0.05 M-1M NaCl gradient. The crude lipase was purified with around 70% final yield and 10 fold purification. The molecular mass of lipase determined from SDS-PAGE was 41.35 kDa, while mass spectroscopy analysis estimated it to be 35.19 kDa, an anomaly which leads us to presume that the anomalous migration of lipase on SDS-PAGE could be due to the presence of glycan moiety on it. Primary glycan screens such as phenol-H_2SO_4 and stains-all staining of native protein on SDS-PAGE, supported our assumption. The purified lipase was found to be relatively thermostable, retaining its activity even at temperatures of up to 80°C, with optimal activity at 70°C. It was observed to be active at pH 6.0-8.5, with optimum activity at pH 7.5. This lipase could actively hydrolyse most of the p-nitrophenylester substrates, with slightly greater preference for short to medium chain fatty acid substrates (C2, C4, C6). Strong inhibition of lipase activity by phenylmethylsulfonidefluoride (PMSF), indicated the active role of serine residue at its catalytic site. The enzyme retained around 70% of its initial activity after exposure to 15% organic solvents (methanol, ethanol, acetone, benzene, acetonitrile, dimethylformmamide and n-hexane). Lipase showed slightly enhanced activity in presence of non-ionic detergents (0.25% of Tween-20, Tween-80 or Triton X-100) but completely lost activity in presence of laundry detergents. No significant effect on activity was observed when the enzyme was incubated with 1 mM concentration of metal ions (Na+, K+, Ca^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+}) but significant reduction

in activity was observed when the concentration was raised to 10 mM for divalent cations such as Ca^{2+}, Mg^{2+}, Zn^{2+} and Cu^{2+}. Michaelis-Menten constants, K_m and V_{max}, were determined for the hydrolysis of MUF-B and pNPA substrates by lipase. Crystals for the native lipase were obtained by sitting drop crystallization. Stability studies on the purified enzyme were carried out using chemical denaturation and thermal denaturation. Proteolysis and DSC (differential scanning calorimetry) analysis indicated presence of two structural domains in the lipase. Activity of the lipase enzyme at high temperatures, in presence of surfactants and its substantial tolerance towards various organic solvents makes it a satisfactory and promising candidate for industrial applications

Six sequences coding for lipase genes were retrieved from the whole genome sequence of the microorganism and five of these sequences were selected for structural studies including Homology modelling, structure prediction and validation. Multiple sequence analysis of all lipases among *Halomonas* species to explore conserved regions demonstrated very low sequential identity, but high structural homology. The structural and sequential studies for all available lipases from PDB (protein data bank), propounded the imperative role of electrostatic interactions (salt bridges) in the thermostability of lipases. The collective information gathered from this study could throw some light into understanding the molecular determinants of thermostability of proteins.

In the second study, two amylases screened from *Bacillus atrophaeus*, (of Molecular weights 64.3 kDa and 73.4 kDa) were purified partially by ion exchange chromatography. Ammonium sulphate fractionation (70%-80%) could segregate one amylase. The SDS-PAGE and zymography analysis showed that the purified amylase has molecular weight of 63.4 kDa. The optimum temperature and optimum pH for activity of the amylase were found to be 40°C and 5.0, respectively. Efforts were made to clone the genes of both amylases into *E.coli* DH5α strain. Amylase (64.3 kDa) was successfully cloned and expressed into *E.coli* BL21 strain, via ligation independent cloning. The recombinant protein showed activity at optimum temperature of 40°C and optimum pH 6.0. The recombinant protein could further be targeted to enhance the thermostability by protein engineering methods such as directed evolution.

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Declaration

I, **Miss. Krupali Parmar** registered as Research Scholar, bearing Registration No. **13FTPHDS28** for Doctoral Programme under the Faculty of **Science** of Nirma University do hereby declare that I have completed the course work, pre-synopsis seminar and my research work as prescribed under R. Ph.D. 3.5.

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I do hereby further declare that the text, diagrams or any other material taken from other sources (including but not limited to books, journals and web) have been acknowledged, referred and cited to the best of my knowledge and understanding.

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Abbreviations

CD: Circular dichroism

PDB: Protein Data Bank

SDS: Sodium Dodecyl Sulfate

4-MUFB: 4-Methylumbelliferyl butyrate

MU: Methylumbelipheryl

CBB: Coomassie brilliant blue

PAGE: Polyacrylamide gel electrophoresis

CTAB: Cetyl trimethylammonium bromide

4-NPA: 4-nitrophenyl acetate

pNPA: p-nitrophenyl acetate

CD: Circular dichroism

DCS: Differential Scanning calorimetry

GdnHCI: Guanidine hydrochloride

ATCC: American Type Culture Collection

DNSA: 3,5-dinitrosalicylic acid

PCR: Polymerase chain reaction

AS: Ammonium sulphate

EtOH: Ethyl alcohol

HPLC: High pressure liquid chromatography

Chapter 1

Introduction

Introduction

At present, only a minor fraction of the microorganisms on earth have been exploited for industrial applications and scientific research. The chemically catalysed synthesis of polymer intermediates, pharmaceuticals, specialty chemicals and agrochemicals is many a times done by expensive processes that have the disadvantages of low selectivity and formation of undesired by-products. A large proportion of enzymes that have been used to date have been isolated from mesophilic organisms. Although such organisms are advantageous by virtue of their easy cultivation and fast growth, the application of enzymes derived from such organisms is restricted in industries because they lack stability. As a result, the use of biocatalysts in organic reactions constituted only a small fraction of the industrial market in the past.

Extremophiles are the microorganisms that can grow and thrive in extreme environments, which were earlier considered to be too hostile to support life. Such extreme environments include those with either high (55 to 121 °C) or low (–2 to 20 °C) temperatures, high salinity (2–5 M NaCl) and high alkalinity (pH>8) or high acidity (pH<4). Several extremophiles can tolerate other extreme conditions including low oxygen tension, high pressure, high levels of radiation, high metal concentrations, toxic compounds, or some unusual conditions like, living in rocks deep below the surface of the earth or living in extremely dry areas with very low water and nutrient supply. Extremophiles can be divided into number of different classes that includes; thermophiles, acidophiles, alkalophiles, psychrophiles, piezophiles, halophiles, metalophiles, radiophiles and microaerophiles. Some extremophiles are adapted to multiple stress conditions. Extremophiles are vivid examples of adaptation and may answer important questions regarding the molecular basis of protein stability.

Enzymes produced by extremophiles, function best under the corresponding extreme conditions. Discovery of new extremophilic species and determination of their genome sequences may result in identification of new enzymes with potential novel applications (Sorokin et al., 2014). Extremophilic proteases, lipases, esterases, pullulanases, isomerases, dehydrogenases, acylases, oxidases, phytases and polymer-degrading enzymes, such as cellulases, chitinases, pectinases, xylanases, glucanase, glycosidases, mannanase and amylases have been shown to have huge industrial applications (Dumorné et al., 2017). Enzymes from thermophilic and hyperthermophilic microorganisms possess overall inherent stability and thus have been shown to be more resistant to a variety of enzyme denaturants. Such stable enzymes represent promising alternatives for the development of industrial biotransformations and biocatalytic processes (Hough and Danson, 1999; Niehaus et al., 1999; Demirjian et al., 2001; D'amico et al., 2003a; Egorova and Antranikian, 2005; Razvi and Scholtz, 2006) (Major approaches to extending the range of applications of extremozymes have emerged from recent works (Frock and Kelly, 2012; Dumorné et al., 2017).

In addition to the increasing potential applications in biotechnology, enzymes from extremophiles are of great interest to investigate the structural features and characteristics that render them their superior thermodynamic stability (Vieille and Zeikus, 1996; Zeikus et al., 1998). An emerging area of interest is the metagenomic approach to tap the microbial sources of novel enzymes from the huge, undiscovered wealth of molecular diversity (Henne et al., 2000; Ferrer et al., 2007; Dumorné et al., 2017; Khan and Sathya, 2018).

Lipases are triacylglycerol acylhydrolases (E.C.3.1.1.3) that catalyze the release of glycerol and free fatty acids from fats and oils at the oil-water interface (Zaks and Klibanov, 1985; Sadaf et al., 2018). The natural substrates of lipase are triacylglycerols. However, a wide range of structurally diverse esters, alcohols and carboxylic acids may also serve as substrates for this enzyme (Sharma et al., 2001; Sahonero-Canavesi et al., 2016; Agustian et al., 2017). Although lipases belong to many different protein families without sequence similarity, there is a much greater conservation in the secondary and tertiary structures of lipases (Taylor and Vaisman, 2010). It has been widely studied as the key enzyme in biotechnological applications, such as food processing, detergent, biomedical sciences, in industrial enzymology for production of flavonoid esters, oleo chemicals, pharmaceuticals, biopolymers, and bio-lubricants (Verma et al., 2012; Hwang et al., 2014; Sadaf et al., 2018). Moreover, lipase immobilized on a solid support has been proved to be an attractive bio interface for biodiesel production or biosensor to

detect serum triacylglycerol levels (Dror et al., 2014; Dutta and Saha, 2018; Rios et al., 2019). Lipases are extensively produced by various plants, animals and microorganisms. Depending on the source of the enzyme, purified lipases have variable properties. Hence, efforts have been put in to find lipases that meet the requirements for a given application. However, relatively fewer lipases have been identified or purified from haloalkalophillic microorganisms. Lipases are amongst the most promising and important biocatalysts for carrying out reactions in both aqueous and non-aqueous media (Kourist et al., 2010). This is primarily due to their ability to utilize a broad spectrum of substrates having high chemo-, regio- and enantio-selectivities in the synthesis or hydrolysis of lipid compounds, as well as their stability under a wide range of conditions(De Miranda et al., 2015; Li et al., 2018; Urrutia et al., 2018).

Most thermostable lipases have other properties which make them unique in the reactions they catalyse. When a new lipase is identified, its properties such as organic solvent tolerance and substrate specificity should be assessed to know whether these novel biocatalysts have an adequate fit to various applications (Padilha and Osorio, 2019). Any purification process that leads to substantial yield and high specific activity is considered appropriate. The scope of thermostable lipase studies is challenging and needs careful consideration of the enzyme production, purification and characterization (Haki and Rakshit, 2003; Dumorné et al., 2017). Thermostable lipases find applications in several biocatalytic reactions and some of the advantages of catalysis at higher temperatures include high rate of product formation with minimal diffusional restriction, high dissolution of hydrophobic substrates, high conversion efficiency, increased kinetic energy of reactants and limited chance of contamination (Chopra et al., 2018). Noncovalent interactions such as salt-bridges, contribute to the stabilization of enzymes at elevated temperatures by entropically restricting the flexibility of the enzyme active site. As a result, activity and specificity tend to be temperature dependent as seen in most lipases (D'amico et al., 2003b). The demand for thermostable lipases has led researchers to explore different samples for isolation and to develop medium for production of lipases with desirable properties. This is based on the fact that most industrial applications require lipases that are thermostable in addition to other characteristics for catalyzing the reactions of interest (Parapouli et al., 2018).

In chapter 3, we have described the purification and biochemical and biophysical characterization of a Lipase.

A moderate haloalkalophile, *Halomonas shengliensis*, extracellularly secretes lipase. The media was optimized for the optimum production of the enzyme in minimum time. The cell free supernatant was screened for extracellular enzyme activity. The proteins in the cell free supernatant were precipitated out with suitable protein precipitation method. The enzyme was purified from the crude preparation by general chromatography techniques i.e. ion-exchange chromatography and gel filtration chromatography. SDS-PAGE was performed at each step to check the progress of purification of the enzyme. Enzymatic characterization was carried out for the purified enzyme which included, determination of relative molecular mass, size, optimum pH, optimum temperature, optimum salt concentration, Michaelis-Menton constant (K_m, V_{max}), effect of inhibitors, effect of metal ions, effect of carbon chain length of substrate, tolerance towards organic solvents and detergents, etc. Crystals for the native lipase were obtained by sitting drop crystallography.

In chapter 4, we have carried out stability studies of the wild type lipase using chemical denaturation and thermal denaturation. The enzyme was also checked for the presence of glycosylation.

The whole genome sequence of the microorganism was analysed for presence of possible genes of lipases. Structural studies were undertaken with the retrieved lipases, including homology modelling, structure prediction and validation. Multiple sequence analysis of all lipases among Halomonas species was done to check conserved regions and sequence similarities. The structural and sequential studies were done for all available lipases from PDB (protein data bank), to get some insight into the molecular determinants of the tolerance and stability of extremozymes towards various extreme conditions.

Structure and stability studies can reveal some facts about molecular determinants for tolerance of extreme conditions like halotolerance and thermotolerance (Kovacic et al., 2015). Factors that contribute to the remarkable stability of extremozymes include an increased number of ion pairs, reduction in the size of loops and in the number of cavities, reduced ratio of surface area to volume, changes in specific amino acid residues, increased hydrophobic interaction at subunit interfaces, changes in solvent-exposed surface areas, increase in the extent of secondary structure formation and truncated amino and carboxyl termini (Demirjian et al., 2001; Stepankova et al., 2013; Zhou and Pang, 2018).

Studies that compare proteins from extrermophilic and mesophilic organisms can provide insights into ability of extremophiles to adapt in adverse conditions and may provide clues that enable us to better define the molecular forces that stabilize all proteins (Szilágyi and Závodszky, 2000; Sarmiento et al., 2015). This approach requires high-resolution structural data for homologous enzymes from mesophiles and extremophiles both, so that differences, which might result in enhanced stability of the extremozyme, can be identified by comparison of the structures (Meruelo et al., 2012; Fulton et al., 2015). Comparative studies have mainly focused on thermal stability and as expected showed that thermophilic proteins have higher T_m values than their mesophilic counterparts. Although these comparisons are helpful, more detailed thermodynamic studies are essential to understand the mechanisms that extremozymes employ to remain folded over a wider range of diverse conditions (Vijayabaskar and Vishveshwara, 2010). It has also been proposed that the structural alterations and changes to the amino acid sequences might give rise to varied ways of gaining thermostability (Chakravarty and Varadarajan, 2002; Yang et al., 2017).

In chapter 5, *Bacillus atrophaeus* was screened for extracellular amylase, two amylases were purified and partially characterized. Efforts were made to clone genes of these two amylases into *E.coli* DH5α strain. One of the amylase (63.4 kDa) was successfully cloned and expressed into *E.coli* BL21 strain. The recombinant amylase was checked for the activity and was partially characterized.

Bacillus atrophaeus is a spore-forming bacterium which has been studied by researchers as well as industries for its vast applications. Among other applications, *Bacillus atrophaeus* has been established in industries as bio-indicator for sterilization, as potential adjuvants or vehicles for vaccines, in studies of biodefense and astrobiology methods as well as disinfection agents in treatment evaluation (Sella et al., 2015). Recently, a mutant thermoalkalophilic lipase production was reported from the bacteria (Ameri et al., 2019). This work aimed to purify and characterize an extracellular amylase which can meet the needs required to maximize its applications in industries, research and academics, in the future.

Chapter 2

Review of Literature

Review of literature

2.1 Extremozymes from Extremophiles

The synthesis of polymer intermediates, pharmaceuticals, specialty chemicals and agrochemicals which are catalysed by chemical catalyst, is often done by expensive processes that suffer from low selectivity and undesired by-products. Mesophilic enzymes are frequently not well suited for the harsh reaction conditions required (extremes of temperature, pH, ionic strength, pressure and salinity etc.) in industrial processes because of the lack of enzyme stability (Hough and Danson, 1999). Because of this reason, the use of biocatalysts in organic reactions offered only a small fraction of the industrial market in the past. Therefore, the discovery of new extremophilic species and genome sequencing provide a way to unique robust enzymes with novel metabolic pathways, which may lead to novel activities and applications. Extremophiles provide vivid examples of adaptation and raise important questions about prerequisites for stability of essential biomolecules. Thus, microorganisms which can produce extremonzymes that can withstand the process conditions has had a great impact on the field of biocatalysis and biotransformation (Raddadi et al., 2015). Extreme environmental conditions require optimized interactions within the protein, at the protein-solvent boundary, or with the influence of extrinsic factors such as metabolites, cofactors and compatible solutes (Ladenstein and Antranikian, 1998; Demirjian et al., 2001).

Industrial applications of microbial enzymes are well established and growing day by day (Chand and Mishra, 2003). Enzymes are ideal candidates for catalyzing the new synthetic routes in industrial processes due to their specificity, mild reaction condition and both eco and consumer friendly (Schmid et al., 2001). The uses of enzyme at industrial scale also reduce side product

formation and in turn, downstream processing cost. The enzymes are only alternative for bio-catalytic use in food industry.

The use of enzymes as biotransformation catalysts is well established and has been the subject of numerous texts, documents, and reviews. Novel developments related to the cultivation and production of extremophile, cloning and expression of their genes successfully in a mesophilic host such as *E. coli*, has been done, although difficulties might be faced when the expressed enzyme requires specific cofactors or metal ions that the host does not digest. Expression normally yields a soluble molecule that has similar activity to the native proteins when assayed at an appropriate temperature (Connaris et al., 1998; Hough and Danson, 1999). This process can increase the number of enzyme-driven transformations in chemical, food, biopharmaceuticals, biotechnological, detergent industries, food industries, etc. However, to fully realize the efficiency of designer enzymes in industrial applications, it is necessary to monitor catalyst properties so that they are optimal not only for a given reaction but also in the context of the industrial process in which the enzyme is applied.

An emerging area of interest is the metagenome approach to tap the microbial sources of novel enzymes from the hitherto undiscovered wealth of molecular diversity (Henne et al., 2000). Enzymes from thermophilic and hyperthermophilic microorganisms have found a number of commercial applications because of their overall inherent stability and resistance to a variety of enzyme denaturants and, thus, represent promising alternatives for the development of industrial biocatalytic processes (Niehaus et al., 1999b; Demirjian et al., 2001). In addition to the increasing potential applications in biotechnology, enzymes from extremophiles are of great interest to investigate the structural requirements that are beyond their superior thermodynamic stability (Zeikus et al., 1998; Pantazaki et al., 2002).

Factors that contribute to the remarkable stability of extremozymes include an increased number of ion pairs, reduction in the size of loops and in the number of cavities, reduced ratio of surface area to volume, changes in specific amino acid residues, increased hydrophobic interaction at subunit interfaces, changes in solvent-exposed surface areas, increase in the extent of secondary structure formation and truncated amino and carboxyl termini (Demirjian et al., 2001).

The studies on enzymes as regards to their conformational stability, irreversible unfolding, heat inactivation, activation parameters of the reaction, properties in complex with a transition state analogue, and structural permeability can allow us to propose an energy landscape for a family of

extremophilic enzymes based on the folding funnel model, combining the main differences in conformational energy, temperature dependence of the activity and cooperativity of protein unfolding. Particularly, the shape of the funnel bottom, which illustrates the stability of the native state at once, also narrates for the thermodynamic parameters of activation that characterize these extremophilic enzymes, therefore provide a rational basis for stability-activity relationships of protein resistance to extreme temperatures (D'amico et al., 2003). Understanding the structure and stability studies can reveal some facts about the molecular determinants of the tolerance of extremozymes towards various extreme conditions.

Cited Literature

Isolation and characterization of extremozymes opens doors to understand the stability of extremozymes at a molecular level. Methods like protein engineering and directed evolution can create novel biocatalysts with greater stability and modified specificity which may not exist in the natural world.

Studies on Phosphoglucomutase and a novel glycolipid from an Extreme Thermophile, *Flavobacterium thermophilum* HB8 has been done (Yoshizaki et al., 1971; Oshima and Imahori, 1974). There has been report of extracellular secretion of gelatinase and amylase from *Flavobacterium thermophilum* but their characterization is yet not done (Yoshizaki et al., 1971; Degryse et al., 1978). The *Virgibacillus marismortui* has been screened for the secretion of an extracellular gelatinase (Essghaier et al., 2012) but there is no report on the purification and characterization of the extracellular proteases. Despite the extensive research on several aspects of proteases, knowledge about the roles that govern the diverse specificity of these enzymes is lacking. Uncovering these secrets would make us able to exploit proteases for their applications in biotechnology (Hiraga et al., 2005; Sriket et al., 2011). Lot of work have been done on the *Alicyclobacillus acidocaldarius* and its enzymes. From *Alicyclobacillus acidocaldarius*, thermostable esterase, endoglucanase, carboxylesterase, mannanase, thermoacidophilic cellulose, beta-galactosidase, thermophilic L-arabinose isomerase, acidophilic amylase, neopullulanase have been purified and characterized (Matzke et al., 2000; Manco et al., 2001) (Eckert et al., 2002; Lee et al., 2005; Di Lauro et al., 2008; Morana et al., 2008; Zhang et al., 2008). The genome sequencing of the organism has also been done (Mavromatis et al., 2010). There was a report on extracellular secretion of gelatinase from *Alicyclobacillus acidocaldarius* but no purification and characterization is reported for gelatinase yet.

The characterization of extremozymes and increased access to genomic sequence data can allow the analysis of the protein families related to stress responses. Heat shock proteins appear to be ubiquitous in extremophiles. Biochemical studies on sHSPs are limited to thermophilic and hyperthermophilic organisms, and the only two available crystal structures of sHSPs from *Methanocaldococcus jannaschii*, a hyperthermophilic archaeon and a mesophilic eukaryote, have contributed significantly to an understanding of the mechanisms of action of sHSPs, although many aspects remain unclear (Laksanalamai and Robb, 2004).

2.2. Thermophiles and thermozymes

Microorganisms that can tolerate high temperatures are known as thermophiles, are found in hot springs, or deep-sea thermal vents, and are typically part of the group of Archaea. Thermophilic extremophiles have attracted most attention (Gomes and Steiner, 2004). In terms of microorganisms or enzymes, a thermophile is generally considered as the one that maintains life or activity at temperatures above 40ºC. Thermophiles can be generally classified into moderate thermophiles (growth optimum 50–60°C), extreme thermophiles (growth optimum 60–80°C) and hyperthermophiles (growth optimum 80–110°C) (Madigan and Marrs, 1997; Van Den Burg, 2003; Gomes and Steiner, 2004; De Champdore et al., 2007).

Thermophilic microorganisms are able to survive because of their ability to produce thermophilic enzymes. Most normal enzymes, being proteins, begin to denature around 40ºC, and are generally completely inactive beyond 50-60ºC. Some thermophilic enzymes have been discovered that can maintain at least half of their specific activity at temperatures as high as 80ºC, or in rare cases even higher. In other cases, enzymes might partially denature at high temperatures but have adaptive systems that allow them to renature once removed from such extreme conditions (Beg et al., 2001). The reasons to exploit enzymes that are stable and active at elevated temperatures are obvious. At elevated temperatures the solubility of many reaction components, in particular polymeric substrates, is significantly improved (Van Den Burg, 2003; De Champdore et al., 2007). Enzymes are the catalytic cornerstone of metabolism, and as such are the focus of intense worldwide research, not only in the biological community, but also with process designers/engineers, chemical engineers, and researchers working in other scientific fields (Beg et al., 2001).

It is difficult to isolate extreme thermophiles because of the harsh and often inaccessible environments from which they come, and subsequently cultivating these microorganisms in

laboratory settings, had presented significant challenges to their study and, consequently, to associated biotechnological applications. Successful attempts to clone and express genes from hyperthermophiles in mesophilic recombinant hosts (e.g. *Escherichia coli*) had been done which has facilitated efforts to produce specific enzymes for characterization and application. Once the gene for a potentially useful thermophilic enzyme has been identified, cloning techniques can be applied to express the enzyme under the control of a strong promoter, in a fast-growing organism that has been proven to show robust growth in large-scale fermentation systems (Phillips et al., 2013).

Advances in this area have been possible with the isolation of a large number of beneficial thermophilic microorganisms from different exotic ecological zones of the earth and the subsequent extraction of useful enzymes from them (Antranikian et al., 1987; Groboillot et al., 1994; Bhushan and Hoondal, 1998; Bauer et al., 1999; Bruins et al., 2001). While the most widely used thermostable enzymes are the amylases in the starch industry (Nigam and Singh, 1995; Crabb and Mitchinson, 1997; Lévêque et al., 2000; Sarikaya et al., 2000), a number of other applications are in various stages of development. In the food related industry, they have been used in the synthesis of amino acids (Hanzawa et al., 2001). In the petroleum, chemical and pulp and paper industries, for example, thermostable enzymes have been used for the elimination of sulphur containing pollutants through the biodegradation of compounds like dibenzothiophene (Bahrami et al., 2001), in the production of 1,3 propanediol from glycerol and in replacing polluting chemical reagents causing toxic products (Wittlich et al., 2001). Currently, a number of publications have extensively discussed developments in this area. Adaptation of extremophiles to hot environments (Danson et al., 1992; Stetter, 1999), production of heat-stable enzymes from thermophiles and hyperthermophiles (Niehaus et al., 1999a; Pantazaki et al., 2002), structure and function relationships of thermozymes (heat-tolerant enzymes) (Zeikus et al., 1998) and biotechnological and industrial applications of thermostable enzymes (Franks, 1993; Lasa and Berenguer, 1993; Leuschner and Antranikian, 1995; Cowan, 1996; Holst et al., 1997; Hough and Danson, 1999; Eichler, 2001) are among the topics that have been studied.

2.3 Haloalkalophile

Microbial diversity studies have been performed in a great variety of environments. These include saltern ponds worldwide, Great Salt Lake, the Dead Sea (Bodaker et al., 2010), saline lakes in Inner Mongolia (Pagaling et al., 2009), African soda lakes, deep-sea brines (Van Der Wielen et

al., 2005) and many others. In many saline environments, life at the extremes of high salt is combined with the need to thrive at alkaline pH and elevated temperatures, and organisms growing there do so at the physico-chemical boundary for life (Bowers and Wiegel, 2011). The question of the longevity of different types of halophiles within salt crystals has become a popular topic, relevant to disciplines including geology, biogeography, evolution, and even space exploration (Mcgenity et al., 2000). In comparison to other groups of extremophilic microorganisms such as the thermophiles and the alkaliphiles, the halophiles of all three domains have been relatively little exploited in biotechnological processes, with notable exceptions of carotene from *Dunaliella*, bacteriorhodopsin from *Halobacterium*, and ectoine from *Halomonas* (Oren, 2010). In recent years, there has been more interest in studying the microbiological flora of soda lakes because these naturally occurring alkaline hypersaline environments are the potential source of assorted microorganisms (Jones et al., 1998; Horikoshi, 1999). Hypersaline environment are extreme habitats were the salinity is high (much higher than that of sea water) and can be divided into two main types i.e. thalassohaline and athalassohaline environments, depending on whether they originated from sea water or not, respectively. Examples of thalassohaline environments that have been studied in detail are the solar salterns (used for the production of salt by evaporation of seawater), some lakes such as the Great Salt Lake in Utah, or saline soils. The athalassohaline environments that have been more extensively studied are the Dead Sea and some alkaline lakes (soda lakes) that are poor in magnesium and calcium ions and abundant in carbonates, resulting in a very alkaline pH (9-11.5). Most ecological studies in hypersaline environments have been based on the conventional methods of isolation and identification of pure cultures. However, recent molecular techniques have improved our knowledge of the microbial diversity of these habitats. The biodiversity is higher than was reported, but obviously lower than that of other nonsaline environments. Besides some eukaryotic representatives (e.g., the brine shrimp *Artemia salina* or the unicellular algae *Dunaliella*), most microbial inhabitants of hypersaline environments are phototrophic and heterotrophic prokaryotes. In the most hypersaline environments, aerobic archaea, member of the Halobacteriales are very abundant (e.g., species of the genera *Haloarcula*, *Haloferax*, *Halobacterium*, *Halorubrum*, as well as *Natronobacterium* or *Natronococcus* in the alkaline lakes). However, recent studies have also determined that bacteria represent high proportion of the inhabitants of these habitats (the bacterial species *Salinibacter ruber*). Saline environments with intermediate salinity are more diverse, in which, both phototrophic and heterotrophic bacteria are observed. These are represented by a variety of gram-negative and gram-positive aerobic and strictly anaerobic microorganisms that are moderately or extremely halophilic,

as well as some halotolerant bacteria. They play an important ecological role in these extreme environments (Arvanitis et al., 1995).

The groups of bacteria able to grow under alkaline conditions in the presence of salt are referred as haloalkaliphiles. They possess special adaptation mechanisms to survive and grow under salinity and alkaline pH. These properties of dual extremity of halophiles and alkaliphiles make them interesting from both, fundamental research and biotechnological points of view (Feng et al., 2005; Joshi, 2006). The world of halophilic microorganisms is highly diverse. Microbes adapted to life at high salt concentrations are found in all three domains of life: Archaea, Bacteria, and Eucarya. The brines of saltern crystallizer ponds worldwide are coloured pink-red by Archaea (*Haloquadratum* and other representatives of the *Halobacteriales*), Bacteria (*Salinibacter*), and Eucarya (*Dunaliella salina*) (Yanhe Ma et al., 2010). Most known halophiles are relatively easy to grow, and genera such as *Halobacterium, Haloferax,* and *Haloarcula* have become popular models for studies of the archaeal domain as they are much simpler to handle than methanogenic and hyperthermophilic Archaea. Some halophilic and halotolerant microorganisms have found interesting biotechnological applications.

Haloalkaliphilic bacteria have largely been explored and studied from the concentrated hyper saline environments; Soda Lake, Solar Saltern, Salt brines, Carbonate springs and Dead Sea (Singh, 2006), the exploration of the natural saline and alkaline environments beyond the above boundaries is just the beginning (Dodia et al., 2008; Manikandan et al., 2009; Purohit and Singh, 2009; Thumar and Singh, 2009).

Halomoas sp.

The genus *Halomonas,* belonging to the family Halomonadaceae (Franzmann et al., 1988; Dobson et al., 1993; Ben Ali Gam et al., 2007; Ntougias et al., 2007), was first proposed by Vreeland et al., 1980 with the description of *Halomonas elongate* (Chen et al., 2009). The bacterial genus *Halomonas* contains numerous moderately halophilic species with different biochemical functions including: denitrification by *Halomonas desiderata* (Lasa and Berenguer, 1993) *H. campisalis* (Mormile et al., 1999), production of exopolysaccharides *Halomonas eurihalina* (Mellado et al., 1995), *H. maura* (Bouchotroch et al., 2001), *H. ventosae* (Martinez-Canovas et al., 2004) and degradation of aromatic compounds *Halomonas organivorans* (Garcia et al., 2004).

A moderately halophilic bacterium, designated strain SL014B-85T, was isolated from a crude-oil-contaminated saline soil from Shengli oil field, Shandong Province, China. Cells are

moderately halophilic, Gram-negative, aerobic, crude-oil utilizing short rods (0.6–0.86, 1.0–1.6 mm) with several lateral flagella. Colonies on ASW agar are creamy and circular. Grows at 0–15% (w/v) NaCl, pH 8.0–9.0 and 10–42ºC; optimum growth occurs at 5–15% (w/v) NaCl, pH 8.5 and 30℃. Utilizes glucose, sucrose, lactose, mannose, galactose, dextrin, gluconate, malate, malonate, succinate and sorbitol as sole carbon sources. Oxidase, urease and catalase-positive but negative for hydrolysis of starch, Tween 80 and gelatin and production of H_2S. It is able to reduce nitrate anaerobically as well as nitrate and nitrite aerobically (Wang et al., 2007).

2.4 Lipases

Lipases (triacylglycerol lipase, EC 3.1.1.3.) are serine hydrolases, which are able to catalyze the hydrolysis of fatty acyl esters. The natural substrates of lipase are triacylglycerols. However, a wide range of structurally diverse esters, alcohols and carboxylic acids may also serve as substrates of this enzyme (Sharma and Kanwar, 2014). Extremophilic lipases can be used as a bio-detergent as they have the relevant activity and is stable at high temperature and in an alkaline environment. The increasing interest in lipase is mainly due to the wide range of industrial applications of the enzyme, including detergent formulation, oil/fat degradation, pharmaceutical synthesis, and cosmetics production (Chen et al., 2009). Industrial lipases used were mainly produced by microorganisms because of their wide variety of sources, good stability, diversified enzymatic properties, and various applications compared with those enzymes from animals and plants (Kumar and Gupta, 2008). Potential applications include modification of sugars, synthesis of flavour esters for the food industry, and the resolution of racemic mixtures to produce optically active compounds (Kazlauskas and Bornscheuer, 1998; Schmid and Verger, 1998; Bornscheuer, 2002).

Lipases are the most widely used in enzyme technology. They are able to recognize wide variety of substrates and can catalyse many different reactions like hydrolysis or synthesis of ester bonds, alcoholysis, aminolysis, peroxidations, epoxydations, transesterification, inter esterifications and acidolysis etc. (Reis et al., 2009; Rodrigues and Fernandez-Lafuente, 2010). However, lipase in free form is often unstable and possesses low activity in organic solvents or in harsh environment such as high temperature and extreme pH. Several methods like physical, chemical or genetic modification of enzymes are desirable to improve their catalytic activity and stability so that they can be successfully applied in the industrial applications (Alloue et al., 2007). Rising demand for fossil fuels has led to increases in the price of crude oil and thus it is important to find alternative fuels, such as biodiesel, which can be made from vegetable oils by lipase via

transesterification (Modi et al., 2006). Lipases show extreme versatility regarding fatty-acyl-chain-length specificity, regiospecificity and chiral selectivity (Beisson et al., 2000).

Lipases can be obtained from various sources, such as plants, microorganisms and animals (Chen et al., 1998). Recent interest in the potential use of microbial lipases in biotechnology has stimulated work on the purification and characterization of several of these enzymes from bacteria (Macrae, 1983; Macrae and Hammond, 1985; Harwood, 1989). The number of bacterial lipases that have been purified and characterized and whose genes have been sequenced is increasing (Brune and Götz, 1992). To date most lipases has come from microorganisms due to the low production cost. Several microorganisms, such as *Candida rugosa*, *Candida antarctica*, *Burkhoderia cepacia*, and *Pseudomonas alcaligenes*, produce lipase efficiently, and this is now are commercially available (Jaeger and Reetz, 1998). Lipases are responsible for hydrolysis reactions, they also possess the ability to catalyse several other types of biotransformations (e.g., esterification and transesterification) in environments with low water content. This distinctive feature has increased the biotechnological interest on these enzymes for a number of industrially significant biotransformations, either as hydrolytic or as synthetic catalysts (Hari Krishna and Karanth, 2002). Lipases have become an excellent bio-catalyser in a broad range of industrial application, including the food industry, pharmaceuticals, production of biodiesel, biological detergents, medicinal applications, and enzymatic production of lipophilic fine chemicals (Zaks and Klibanov, 1985; Rubin and Dennis, 1997; Jaeger and Reetz, 1998; Kazlauskas and Bornscheuer, 1998; Gupta et al., 2004).

Over the last few years, there has been a progressive increase in the number of publications related to properties and industrial applications of lipase catalysed reactions (Verma et al., 2012). In spite of the increasing interest in microbial lipases, very few studies have been done on fungal lipases (Tomizuka et al., 1966; Semeriva et al., 1967; Tsujisaka et al., 1973). Owing to their immense importance, these multi-faceted enzymes from microorganisms have tremendous potential in areas such as oil/fat degradation, the chemical industry and biomedical sciences such as, pharmaceutical synthesis, and cosmetics production. Hence, reviews on lipases for several years have focused on their biotechnological impetus (Pandey et al., 1999). Several alkaline lipases with high levels of activity at alkaline pHs were obtained from various microorganisms, such as Achromobacter spp., Alcaligenes spp. (Kokusho et al., 1982), Bacillus spp. (Lesuisse et al., 1993), Humicola spp. (Huge-Jensen and Gormsen, 1989), and Pseudomonas fragi (Nishio et al., 1987).

Due to the increase in demand for lipase, increasing attention has been paid to how to produce it efficiently and economically.

A lot of work has been done for the purification of lipases at international level. Many bacteria are known to secrete extracellular lipase which has been purified. For example, from *Pseudomonas aeruginosa* an alkaline lipase was isolated from putrid mineral oil, purified and characterized and preliminary X-ray diffraction data has been generated (Stuer et al., 1986; Gilbert et al., 1991; Kordel et al., 1991). Acinetobacter is known to produce novel extracellular lipase which is capable of catalyzing hydrolysis of oleyl benzoate (Yeo et al., 1998; Choi et al., 2008). *Bacillus stearothermophilus* is known to secrete a thermostable lipase which was produced at high level and purified (Kim et al., 2000). A gene for production of extracellular lipase was cloned into *E.coli* from *Serratia marcescense* (Li et al., 1995), purification and preliminary characterization was carried out for an extremely basic pH-tolerant extracellular lipase produced by *Bacillus subtilis* (Lesuisse et al., 1993). Purification and characterization of extracellular lipase has been reported for *Pseudomonas fluorescens* (Kojima and Shimizu, 2003). Purification and partial characterization of an alkaline lipase has been done for *Pseudomonas pseudoalkaligenes* (Lin et al., 1996). Bacillus sp. RN2 is known to secrete lipase which is tolerant to organic solvents and detergents, it has been purified and characterized (Kanjanavas et al., 2010). Purification and characterization of lipase has been reported from animals too i.e. *Todarodes pacificus* (Park et al., 2008). Production of extracellular lipases has been reported for fungi like *Penicillium cyclopium*, the purification and characterization was done for partial Acylglycerol lipase from it (Chahinian et al., 2000). *Halomonas shengliensis* has been reported as novel sp. a moderately halophilic, denitrifying, crude-oil-utilizing bacterium but no work has been reported for production of extracellular enzymes from the bacterium yet (Wang et al., 2007). Despite, lipases belong to many different protein families having no sequence analogy, there is a much greater conservation in the secondary and tertiary structures of lipases.

2.5 Amylases

Amylases have potential application in a wide number of industrial processes such as food, fermentation and pharmaceutical industries. α-Amylases can be obtained from plants, animals and microorganisms. However, enzymes from fungal and bacterial sources have dominated applications in industrial sectors (Hyun and Zeikus, 1985). Amylases are the enzymes, which hydrolyses starch to different extents. Enzymes hydrolysing starch are produced by a wide variety

of living beings, including humans. Enzymes used in starch- saccharification process are α-amylase (EC 3.2.1.1), β- amylase (EC 3.2.1.2), amyloglucoside or glucoamyalse or γ-amylase (EC 3.2.1.3), pullulanase (EC 3.2.1.41) and isoamylase (EC 3.2.1.68). In the hydrolysis of starch, enzymes should specifically hydrolyze both α- 1, 4 and α- 1, 6 linked glucose molecules in starch. The α-amylases are among the most important commercial enzymes, having wide applications in starch-processing, brewing, alcohol production, textile, and other industries. Numerous α-amylases from eubacteria, fungi, plants, and animals have been characterized, and their genes have been cloned. The most thermostable α-amylase used in industry is produced from *Bacillus licheniformis* (Dong et al., 1997).

Several amylolytic enzymes, such as α-amylase (EC 3.2.1.1; glycosyl hydrolase family 13), α -amylase (EC3.2.1.2; glycosyl hydrolase family 14), and glucoamylase (EC 3.2.1.3; glycosyl hydrolase family 15), with different specificities can contribute to starch degradation. Amylases [α -amylase, β-amylase and glucoamylase (GA)] are among the most important enzymes in present-day biotechnology. The enzymes of amylase family have great significance due to its wide area of potential application. The spectrum of amylase application has widened in many other fields, such as clinical, medical analytical chemistry, and pharmaceutical aid for the treatment of digestive disorders. Thermostable amylases have wide application in a number of industrial productions, most prominently glucose and beer production. The high cost of malting has demonstrated the need for replacement of indigenous barley enzymes with microbial amylases and proteases (Obi and Odibo, 1984). The production of sweetener from corn starch by microbial saccharidases is an important application of enzyme technology in the food industry. The current process for high-fructose corn syrup production involves several separate enzymatic steps, including liquefaction by α-amylase, saccharification by glucoamylase, and isomerization by glucose isomerase (Lee et al., 1990).

Chapter 3

Isolation, Purification and Characterization of Lipase

Isolation, Purification and Characterization of Lipase

3.1 Preamble

Lipases (triacylglycerol ester hydrolases (EC 3.1.1.3) have the ability to catalyze several reactions of biochemical importance including hydrolysis of triglycerides, esterification, transesterification, interesterification, acidolysis, aminolysis, alcoholysis, acylation and resolution of racemates (Romdhane et al., 2010; Roy et al., 2012; Yang et al., 2016). It is thus an enzyme of great industrial importance. Characterization of these enzymes with respect to enzyme activity, specificity, thermostability, enantioselectivity and tolerance to various solvent systems are the prerequisites for selection of thermostable lipases, which make them highly demanding in lipase catalyzed reactions. The study presented here reports the isolation of a novel lipase from a haloalkalophilic microorganism, *Halomonas shengliensis*, and its enzymatic characterization to obtain all information about the properties of the new lipase.

3.2 Materials and Methods

All organic solvents, Sodium Dodecyl Sulfate (SDS), Glycine, Sodium chloride, Ammonium sulphate, Sodium phosphate monobasic and dibasic were purchased from Merck. β-mercaptoethanol, Ammonium persulfate, 8-Anilinonaphthalene-1-sulfonic acid (ANS), Dithiothreitol, p-Nitrophenyl esters, 4-Methylumbelliferyl butyrate (4-MUFB), Coomassie brilliant blue-R250, Q-Sepharose Fast Flow slury and dialysis membranes were purchased from Sigma–Aldrich (St. Louis, MO, USA). Ammonium sulfate, Olive oil, Tributyrin, Triton X 100, Victoria Blue B, Bis-Acrylamide and all microbial media supplements were purchased from

Himedia Laboratories Private Limited (Mumbai, India). Acids were purchased from S D Fine-Chem Limited (Mumbai, India). Rhodamine B was purchase from MP Biomedicals. PCR reagents were purchased from Thermofisher. All chemicals and reagents used for electrophoresis were of Analytical grade. All buffer solutions were filtered through a 0.22 µm Micro Syringe filter (Millipore, USA) and pH was checked with pHTestr® 3 pH-meter (Oakton).

3.2.1 Microorganism growth and maintenance

Halomonas shengliensis is a soil isolate, isolated by Dr. Vijay Kothari (Institute of Science, Nirma University) and his students from saline soil of Khambhat. The seed culture was grown at 30ºC for 8 hours in a liquid medium containing: 1% (w/v) Casein peptone, 0.5% (w/v) Yeast extract, 0.5% (w/v) Glucose and 6% (w/v) NaCl, pH 8.5 with vigorous shaking. The bacteria grow well at temperatures between 30ºC and 37ºC. The cells were maintained as agar stabs in the same medium (2.0% w/v agar) at 4°C. 1% of an actively growing culture was dispensed into the respective growth medium for further experiments.

3.2.2 Screening of Lipase from *Halomonas shengliensis*

3.2.2.1 Rhodamine agar plate assay

The production of extracellular lipase by *Halomonas shengliensis* was screened on 2% (w/v) agar media plates containing 0.5% (w/v) Yeast extract, 6% (w/v) NaCl and 2% (w/v) Tributyin, pH 8.5. Sterile fluorescent dye 0.005% (w/v) Rhodamine-B was added to autoclaved media before pouring plates. The agar plates were inoculated with the microorganism and incubated at 35ºC for 8 days. The colonies were examined every day for lipase secretion by exposing them to UV light of 354 nm (Niño De Guzmán et al., 2008).

3.2.2.2 Victoria blue B agar plate assay

To monitor the extracellular lipase activity from colonies of *H.shengliensis*, 2% (w/v) agar media plates were prepared using 0.5% (w/v) Yeast extract, 6% (w/v) NaCl and 2% (w/v) Tributyrin, pH 8.5. Sterile Victoria Blue B dye 0.01% (w/v) was added to the autoclaved media before pouring plates. The plates were inoculated by the bacteria and incubated at 35ºC. They were repeatedly monitored for lipase secretion for 5 days (Sharma et al., 2001).

3.2.3 Optimization of media and culture conditions for lipase production

Attempts were made to increase the enzyme production by optimization of medium components at 35°C and pH 8.5 by substituting the respective carbon or nitrogen source in the production medium. Different substrates like Tween-20, Glycerol, Olive oil and Tributyrin were tried out as carbon source for production of lipase. Different amounts of yeast extract (0.5%, 1% and 1.5%) as the nitrogen source and different concentration of salt (NaCl) i.e., 6%, 10% and 15% were used to formulate optimum medium conditions for maximum production of lipase (Cardenas et al., 2001).

1% inoculum from an actively growing seed culture was dispensed into medium containing the above-mentioned components for lipase production. The culture was allowed to grow at 35ºC under shaking conditions and lipase production was monitored till a maximum production level was achieved. 5 ml of the growing culture was removed at regular intervals of 3 h. The cell density from its optical scatter at 600nm and the level of lipase production were measured simultaneously. Lipase activity was estimated from the cell free supernatant or crude enzyme preparation by the standard lipase activity assay, as described in the following section. The cell free supernatants collected at each time point was also loaded into wells bored in tributyrin agar plates to check for the amount of lipase. The best formulated production medium for optimum production of lipase and the optimum time for maximum enzyme secretion were selected to carry out larger scale production of enzyme for purification and follow up experiments.

3.2.4 Lipase assay

Lipase activity was initially determined spectrophotometrically as described by Winkler and Stuckmann, 1979, with slight modifications. Solution A was made by dissolving pNPA (p-nitrophenyl acetate) in 10 ml of 2-propanol to a concentration of 20 mM. Solution B consisted of 50 mM Tris.HCl buffer (pH 7.5) containing 50 mM NaCl. The final reaction mixture was prepared fresh every time before the assay by mixing 1 part of solution A to 9 parts of solution B. A 100µl volume of an appropriate dilution of the enzyme solution (~50 µg) was added to 900µl of the reaction mixture. The reaction mixture was then incubated at 37°C for 15 minutes after which the reaction was stopped by addition of 1:1 chilled acetone-ethanol mixture. Lipase activity was measured by monitoring the change in absorbance at 410 nm that represents the amount of released pNP (p-nitrophenol). All measurements were carried out under first-order reaction conditions. All the assays, unless stated otherwise, were executed in triplicate. One unit of lipase activity was defined as the amount of enzyme that released 1 µmol p-nitrophenol from pNPA per min under the

assay conditions. The activity of enzyme was expressed in U/L. Due to autohydrolysis of pNPA at higher pH and higher temperature, the spectrophotometric measurement of activity was recorded at 348nm, which is the isosbestic point of pNP (Woods and Walker, 2013; Peng et al., 2016).

The enzyme activity was also measured by a rapid, sensitive and convenient fluorescent assay which was developed to screen for lipase activity (Roberts, 1985). The non-fluorescent substrate, 4-methylumbelliferyl butyrate (4-MUB), is hydrolyzed to butyric acid and the highly fluorescent compound, 4-methylumbelliferone (4-MU) by lipase/esterase. 4-MUB was dissolved in DMSO to a final concentration of 25 mM. The reaction mixture was prepared using ~20 µg enzyme in 25 mM phosphate buffer containing 25 mM NaCl (pH 7.5) and 0.1 mM 4-MUB. Assay was carried out at 37°C for 10 min and reaction was terminated by addition of cold (4°C) 1M phosphate buffer (pH 7.5). The changes in fluorescence were measured using a Cary eclipse Fluorescence spectrophotometer (Agilent Technologies). The emission at 448 nm upon excitation at 323 nm was recorded.

3.2.5 Preparation of crude enzyme extract

The seed culture of *H.shengliensis* was grown in growth medium for 8 h from which actively growing cells were used to inoculate production medium in Erlenmeyer flasks. The culture was allowed to grow for four days at 35˚C, 180 rpm in a shaker incubator, in order to allow the strain to release extracellular lipase. Cells were harvested by centrifugation at 7500 rpm for 20 minutes at 4°C. The cell free supernatant was collected and filtered using Whatman filter paper grade 1. The cell free filtrate was used as the extracellular crude enzyme. Sufficient amount of cell free supernatant was taken and kept on ice to check extracellular enzyme activity and to load into SDS-PAGE later.

3.2.6 Purification of Lipase

3.2.6.1 Ammonium sulphate precipitation

Ammonium sulphate (AS) precipitation and/or fractionation was performed as described by Simpson et al., 2006. The commonly used AS precipitation procedure is to add solid AS to a protein extract to give a certain percent saturation which is convenient, reproducible, and practical (Deutscher, 2009). Solid AS was added slowly into the crude enzyme preparation with gentle stirring to 100% saturation at 4ºC (on an ice bed). The proteins were allowed to precipitate out for

about 2 h. The suspension was centrifuged at 7500 rpm for 30 minutes at 4ºC to collect the precipitated protein pellet. The pellet was dissolved in a minimum volume of 25 mM phosphate buffer (pH 7.5) containing 25 mM NaCl. The resuspended sample was then extensively dialyzed against the same buffer at 4ºC using dialysis tubing of 11 kDa molecular weight cut off (Sigma Aldrich). The dialyzed protein sample was filtered through a 0.45 µm filter (Merck Millipore, Germany) and stored at −20ºC until further analysis (Englard and Seifter, 1990). Lipase activity assay was carried out to monitor the functional lipase.

3.2.6.2 Ion-Exchange chromatography

Purification of the enzyme from a crude preparation was done by anion-exchange chromatography. Q- Sepharose Fast Flow (Sigma Aldrich) preswollen in 20% EtOH (ethanol) was used to pack a glass column with 45 ml resin at room temperature. All buffers used in the chromatography process were previously filtered using a 0.22 µm filter (Merck Millipore). A constant flow rate of 1 ml/min was maintained using a peristaltic pump. The column was equilibrated with 4-5 column volumes of 25 mM phosphate buffer (pH 7.5) containing 25 mM NaCl. The previously concentrated filtrate containing the extracellular protein fraction was loaded into the column. The flow through was collected and the unbound proteins were washed out with two column volumes of the same buffer. The bound proteins were eluted from the column by a step gradient of increasing NaCl concentration from 50 mM to 1 M (at steps of 50 mM) and 50 ml fractions for each salt concentration were collected. After elution, the column was washed extensively with 4 M NaCl to remove the residual proteins, followed by water wash and then stored in 20% ethanol.

The collected fractions were checked for the presence of the protein by the spot test, whereby, 20µl of each fraction was spotted onto Whatman filter paper No.1. The paper was stained with Coomassie brilliant blue (CBB) after the spots were dry, and then destained with destaining solution till blue spots of protein were visible.

The fractions having protein were then analyzed for the presence of the lipase enzyme by SDS-PAGE. 15 µl from each fraction was mixed with SDS PAGE loading Buffer and loaded to 12% SDS-PAGE and subsequently stained with CBB to assess the presence and purity of the target protein. The different fractions containing the pure lipase were pooled together and concentrated by centrifugation at 4,500g and 4°C in a 10kDa cut off Amicon Ultra-15 centrifuge tube (Millipore) until a final volume of 5ml was attained. The sample was then assayed for lipolytic activity. The

resulting concentrated and desalted sample was mixed with glycerol at 50:50 (v/v) ratio and stored at -20°C. Protein quantification was carried out by the method of Bradford (1976), using bovine serum albumin as a standard.

3.2.6.3 Gel filtration chromatography

The active fraction concentrate obtained from Ion exchange chromatography was loaded onto a size exclusion chromatography column (Zorbax GF-250, 9.4 x 250mm, 4 micron, analytical) connected to a semi-preparative HPLC (1200 infinity series, Agilent technologies). Isocratic elution of the enzyme was carried out at a flow rate of 0.1 ml/min using double filtered 25 mM phosphate buffer (pH 7.5) containing 25 mM NaCl. The samples were examined for purity and the protein concentration was determined from its absorbance at 280nm.

3.2.7 Biochemical characterization of *Halomonas shengliensis* lipase

3.2.7.1 Molecular weight determination by SDS-PAGE

Molecular weight of the purified protein sample was determined by carrying out Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of (Laemmli, 1970). The purified protein was loaded onto a 12% cross linked polyacrylamide gel along with crude enzyme preparation, standard proteins like bovine serum albumin, ovalbumin, lysozyme, β-lactoglobulin, and a broad range protein molecular weight marker (Genei). Electrophoresis was carried out at constant voltage of 150V and 50mA for 140 minutes at room temperature. Coomassie brilliant blue as well as silver staining were used to visualize protein bands on the gels (Morrissey, 1981). The Rf value for each band of protein molecular weight marker and each standard protein was calculated and the graph of log of molecular weight (daltons) vs the respective Rf value was plotted to obtain the molecular weight of the new unknown lipase.

3.2.7.2 Determination of temperature optima and thermal stability of enzyme

The effect of temperature on the activity of the purified lipase enzyme was studied by assaying the enzyme activity at different temperatures, 20°C, 30°C, 40°C, 50°C, 60°C, 70°C, 80°C, and 90°C in 25 mM phosphate buffer, pH 7.5, containing 25 mM NaCl and 4-MUFB as the substrate for 15 minutes. The optimum temperature for lipase activity was determined from a graph of activity vs temperature. To determine the thermostability of the lipase, the purified enzyme was incubated at each of the above mentioned temperatures (30-90°C) in the reaction buffer. Aliquots

were withdrawn from the incubated enzyme at regular time intervals and their residual lipolytic activity was measured.

3.2.7.3 Determination of pH optima of enzyme

The optimum pH of the lipase enzyme was determined by assaying the enzyme at different pH buffers. The standard lipase assay method was followed using 5% w/v gum acacia emulsified olive oil as the substrate in different buffer systems such as citrate buffer (pH 3.5-6.5), phosphate buffer (pH 7.0-8.5) and glycine-NaOH buffer (pH 9.0- 9.5). The activity of the enzyme was plotted as a function of pH to obtain the pH optimum value.

3.2.7.4 Effect of organic solvents on enzyme activity

To monitor the effect of organic solvents on lipase, the enzyme solution was treated with 15% or 30% (v/v) concentrations of different organic and the mixture was incubated at 37°C for 1 h. The solvents used were acetone, methanol, ethanol, isopropanol, butanol, DMSO, chloroform, toluene, DMF, glycerol, benzene, n-Hexane and acetonitrile. The residual enzyme activity after the incubation was measured using standard assay conditions and compared with that of the control, which was the enzyme not treated with any organic solvent.

Far-UV CD spectra of native lipase and lipase incubated with 15% organic solvents at 37˚C for 1 h was recorded from 199 nm to 260 nm on a Jasco J815 spectropolarimeter using a 0.2 cm path length cuvette. A bandwidth of 1nm and a scan rate of 100 nm/min was used to record the spectra. Each far-UV spectrum was an average of three scans and corrected for buffer contributions. The organic solvents used were methanol, ethanol, 2-propanol and acetone. The spectra were recorded only for lipase in 15% organic solvents and not in 30% organic solvents due to high HT at higher solvent concentration.

3.2.7.5 Effect of detergents and surfactants on enzyme activity

To examine the effect of various detergents on the lipolytic activity of the purified lipase, the buffered enzyme solution was treated with various detergents. Solid detergents (diluted in water) and liquid detergents were diluted to give a final concentration of 0.25% or 0.5%. The enzyme-detergent mixture was incubated at 37°C for 1 h after which the residual enzyme activity was measured using the standard activity assay. The detergents that were studied are Nirma, Ariel, Henko, Surf Excel, Tide (commercially available laundry detergents in India), SDS (sodium

dodecyl sulphate), CTAB (cetyl trimethylammonium bromide), Tween 80, Tween 20 and Triton X-100. The activity of the untreated enzyme was used as a reference to calculate the relative activity.

3.2.7.6 Effect of inhibitors on enzyme activity

To determine the effect of inhibitors on lipase activity, the purified enzyme was preincubated with 1 mM or 10 mM inhibitor for 1 h at 37ºC before assaying its activity. EDTA, PMSF, DTT and β-ME were used for this study. The residual enzyme activity was determined under standard assay conditions with an untreated control.

3.2.7.7 Effect of metal ions on enzyme activity

To study the effect of metal ions on the enzyme, the purified lipase was preincubated with each of the selected metal ions at a final concentration of 1 mM or 10 mM at 37°C for 1 h and then its activity was measured by the standard assay. The lipase activity of the purified enzyme in the absence of any metal ion was taken as control (100%). The metal ions selected for the study were Manganese, Sodium, Magnesium, Copper, Zinc, Barium, Aluminum, Calcium, Potassium, Mercury and Ferrous.

3.2.7.8 Determination of substrate specificity (carbon chain length) and enzyme kinetics

The hydrolytic activity and substrate specificity for fatty acid chain length of *H.shengliensis* lipase was investigated under standard assay conditions using different p-nitrophenylesters as substrates such as 4-nitrophenyl acetate (4-NPA, C2), 4-nitrophenyl butyrate (4-NPB, C4), 4-nitrophenyl oleate (4-NPB, C8), 4-nitrophenyl dodecanoate (4-NPD, C12) and 4-nitrophenyl palmitate(4-NPP, C16), at various substrate concentrations (0.2–2 mM). The purified lipase was incubated with various concentration of the substrates and the influence of substrate concentration on the reaction velocities of the purified lipase was studied. The Michaelis–Menten enzyme kinetic constants (K_m) and maximum velocity (V_{max}) were determined from the Lineweaver–Burk and Woolf–Hanes plots for pNPA and MUF-B. In all cases, the enzymatic activity was assayed under optimum assay conditions. Each value in all the above experiments is the mean of triplicate experiments.

3.2.7.9 X-Ray crystallography

Attempts were made to crystallize the purified lipase protein by the sitting drop method. Commercially available crystallization screening kits from Hampton Research (Index, Crystal Screen 1 and Crystal Screen 2) were used to do an extensive screening of crystallization conditions. The protein solution, that was concentrated to 6 mg/ml using Amicon Ultra-15, was mixed with the screening solution in a 1:1 ratio and placed onto the wells of 96 well plates. The drops were then over-layed with a 1:1 paraffin:silicone oil mixture and the plates were incubated at 20°C in a vibration free chamber. A total of 194 screening conditions were set up and the protein- precipitant drops were regularly observed every three to four days under a microscope to see if there were any protein crystals.

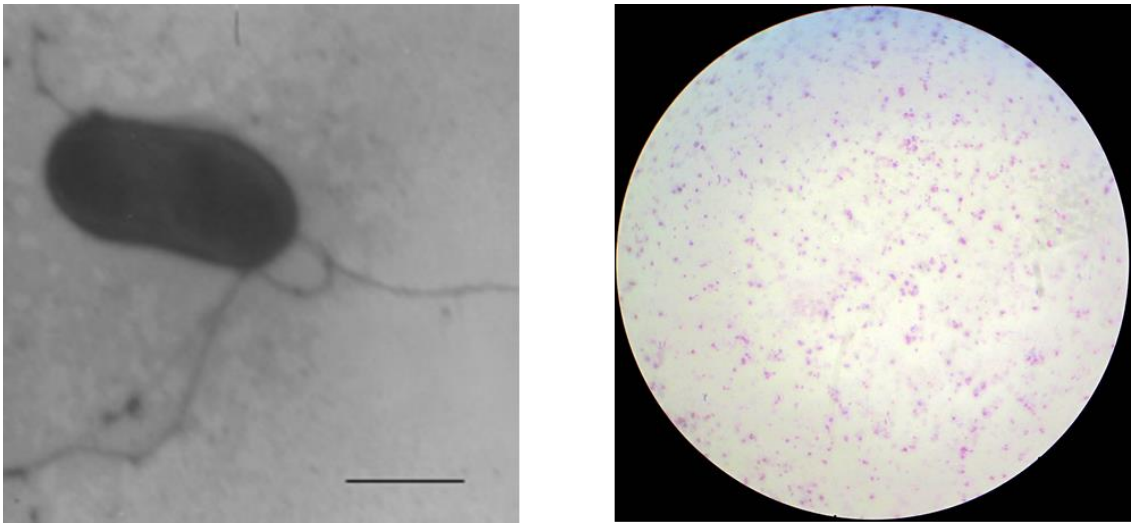
3.2.7.10 Cloning of *H.shengliensis* lipases

Genomic DNA from *H.shengliensis* was extracted and purified as described in Sambrook and Russell (2001) and its purity was spectrophotometrically evaluated by the A260/A280 ratio. The universal 16S rDNA primers were used for amplification of the 16S rDNA gene (Chen et al., 2015). Amplification of 16S rDNA gene was done with a PCR machine (Life Technologies, US) under the following conditions: initial denaturation at 95ºC for 5 min; 30 cycles of denaturation at 94ºC for 30 sec, annealing at 52ºC for 1 minute, extension at 72ºC for 90 sec, and a final extension at 72ºC for 10 min. The amplified PCR product was analyzed on an agarose gel, purified and sent for sequencing. The primers were designed for five genes of lipases (confirmed from genome sequence) to amplify the respective genes for cloning purposes.

3.3 Results and Discussion

3.3.1 Microorganism growth and maintenance

Halomonas shengliensis, a moderate holoalkalkalophile, is a gram negative, aerobic, short rod bacilli (**Figure 3.1**) with several lateral flagella. Colonies on agar plates are creamy and circular. 16S rRNA sequencing done by Gujarat State Biotechnology Mission (GSBTM) confirmed that the organism is *Halomonas shengliensis*.



A **B**

Figure 3.1 (A): Transmission electron micrograph of negatively stained cells of strain SL014B-85T (Wang et al., 2007). **(B)** Gram staining of *H. shengliensis*.

The designated strain SL014B-85T, is considered to represent a novel species of the genus *Halomonas* and was first isolated from a crude oil contaminated saline soil from Shengli oilfield of China, for which the name *Halomonas shengliensis* sp. nov. is proposed. The bacteria grows at 0–15 % (w/v) NaCl (optimum growth at 5–15 % (w/v) NaCl), pH 8.0–9.0 (optimum pH 8.5) and 10–42°C (optimum growth temperature 30°C). It is able to utilize glucose, sucrose, lactose, mannose, galactose, sorbitol, malate, malonate, succinate, gluconate, and dextrin as the sole carbon source. The bacteria is urease (to hydrolyze urea), oxidase (for oxidation-reduction reaction) and catalase (decomposition of hydrogen peroxide) positive, but negative for hydrolysis of starch, Tween 80, gelatin and for production of H_2S. It is able to reduce nitrate and nitrite aerobically and nitrate anaerobically (Wang et al., 2007). The growth medium used to optimally grow *H.shengliensis* was 1% (w/v) casein peptone, 0.5% (w/v) yeast extract, 0.5% (w/v) glucose, 6% (w/v) NaCl and pH 8.5. Optimum growth occurred at 35°C within 8 h in the above mentioned growth medium. Same medium was used to maintain the bacterial culture on 2% (w/v) agar plates and bacteria was transferred to fresh plate after every 20 days.

3.3.2 Screening of Lipase from *Halomonas shengliensis*

3.3.2.1 Rhodamine agar plate assay

We screened *Halomonas shengliensis* for secreted extracellular enzymes and it was found to be positive for extracellular lipase just as we had anticipated, given the fact that it was first isolated from oil spills. Formation of yellow orange fluorescent halos were observed around the bacterial colonies growing on Rhodamine B agar plates upon exposure to UV light of 354 nm wavelength. A gradual increase in fluorescence intensity around the bacterial colonies was observed from day 2 through day 5. We continued the incubation of bacteria for 8 days. **Figure 3.2** shows fluorescence halos around colonies of *H. shengliensis* after 8 days of incubation, confirming presence of strong lipase activity. Free fatty acids are produced as a result of hydrolysis of Tributyrin by lipase, when Tributyrin was used as the sole carbon source in agar plates. The fluorescent halos are formed as the result of reaction between free fatty acids and Rhodamine B

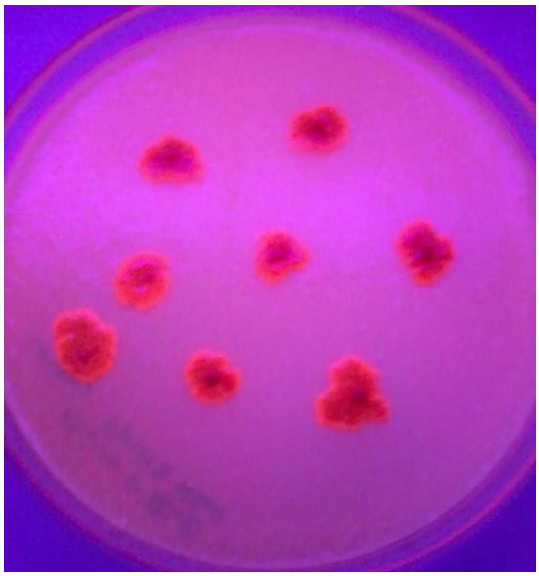


Figure 3.2: Plate showing yellow orange fluorescent halos around colonies on modified HM media containing Olive oil as a substrate and Rhodamine B as an indicator, indicating positive Lipase activity. Fluorescence was observed with UV light of 354 nm wavelength.

3.3.2.2 Victoria blue B agar plate assay

Secretory lipase activity of the *H. shengliensis* was also confirmed by the intensification of blue color around the lipase producing bacterial colonies grown on the agar plates containing Victoria Blue B. There was a gradual intensification of blue color around the growing colonies as shown in **Figure 3.3**, indicating presence of lipolytic activity. The functional group of Victoria Blue B dye reacts with the product i.e. free fatty acid, and intensifies the blue color.

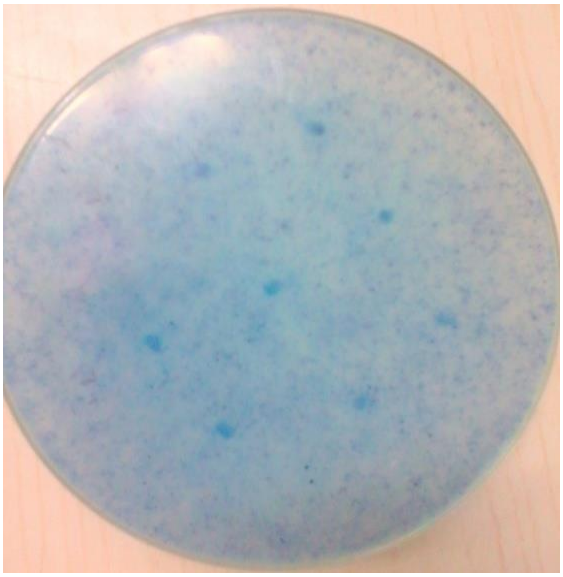


Figure 3.3: Plate showing intense blue colour formation around the colonies of *Halomonas shengliensis* on media containing Victoria Blue B as an indicator dye and tributyrin as sole source of carbon. The formation of blue colour around the colonies indicates strong lipase activity.

3.3.3 Optimization of media and culture conditions for lipase production

Once it was confirmed that *H.shengliensis* secretes lipase enzyme, the next step was to identify the most efficient media conditions that would result in the organism secreting maximum amount of enzyme. We obtained a range of lipase activity from ~30U/ml to ~220 U/ml, indicating that the optimization of medium was necessary to ensure higher levels of enzyme production. Extracellular lipase production by *H.shengliensis* was found to be influenced by physical factors such as type of carbon source and incubation period. Some lipases have been reported to be optimally produced in other organisms in presence of olive oil as well as tributyrin (Pandhi and Rana, 2012). The lipase from *H.shengliensis* was also shown to be produced at similar levels when the organism was grown in the presence of either 2% (w/v) olive oil or 2% (w/v) Tributyrin as the carbon source. After a few rounds of optimization, the media composition at which we obtained maximum lipase production from *H.shengliensis* was: Olive oil (2% w/v) or Tributyrin (2% w/v), peptone (1% w/v), yeast extract (0.5% w/v), salt (NaCl 6% w/v), and pH 8.5. Since both, olive oil and tributyrin, showed approximately equal lipase production (**Figure 3.4)**, olive oil was chosen as a sole carbon source for further experiments for lipase production, as the economical choice. The results shown in F**igure 3.4** indicate an approximately 54% increase in relative lipase activity after optimization of media parameters.

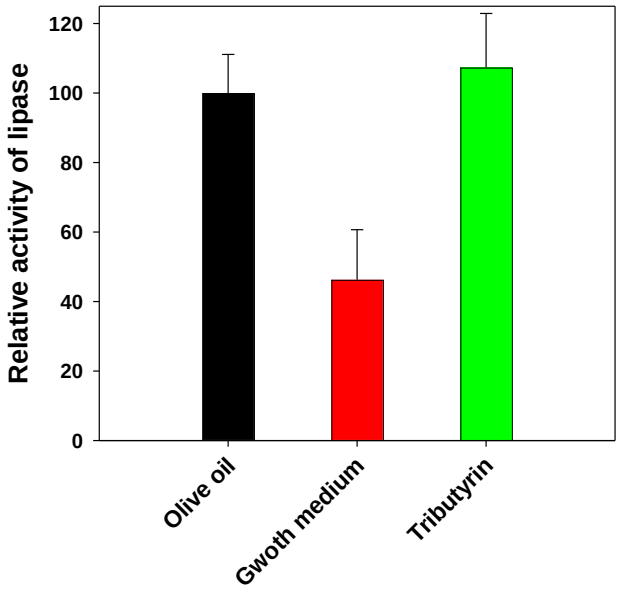


Figure 3.4: Relative activity of lipase estimated from growth media, from medium supplemented with olive oil (2%) as sole source of carbon and medium supplemented with tributyrin (2%) as sole source of carbon.

Maximum lipase production was obtained after 96 hours of growth at 35ºC under shaking at 200 rpm. As shown in **Figure 3.5**, the crude enzyme that was withdrawn every day from the optimized medium and loaded into the wells bored in tributyrin agar plates, displayed gradual intensification of blue color around the well till the 4th day. The crude lipase activity measured along with the growth curve also gave the same results (**Figure 3.6**).

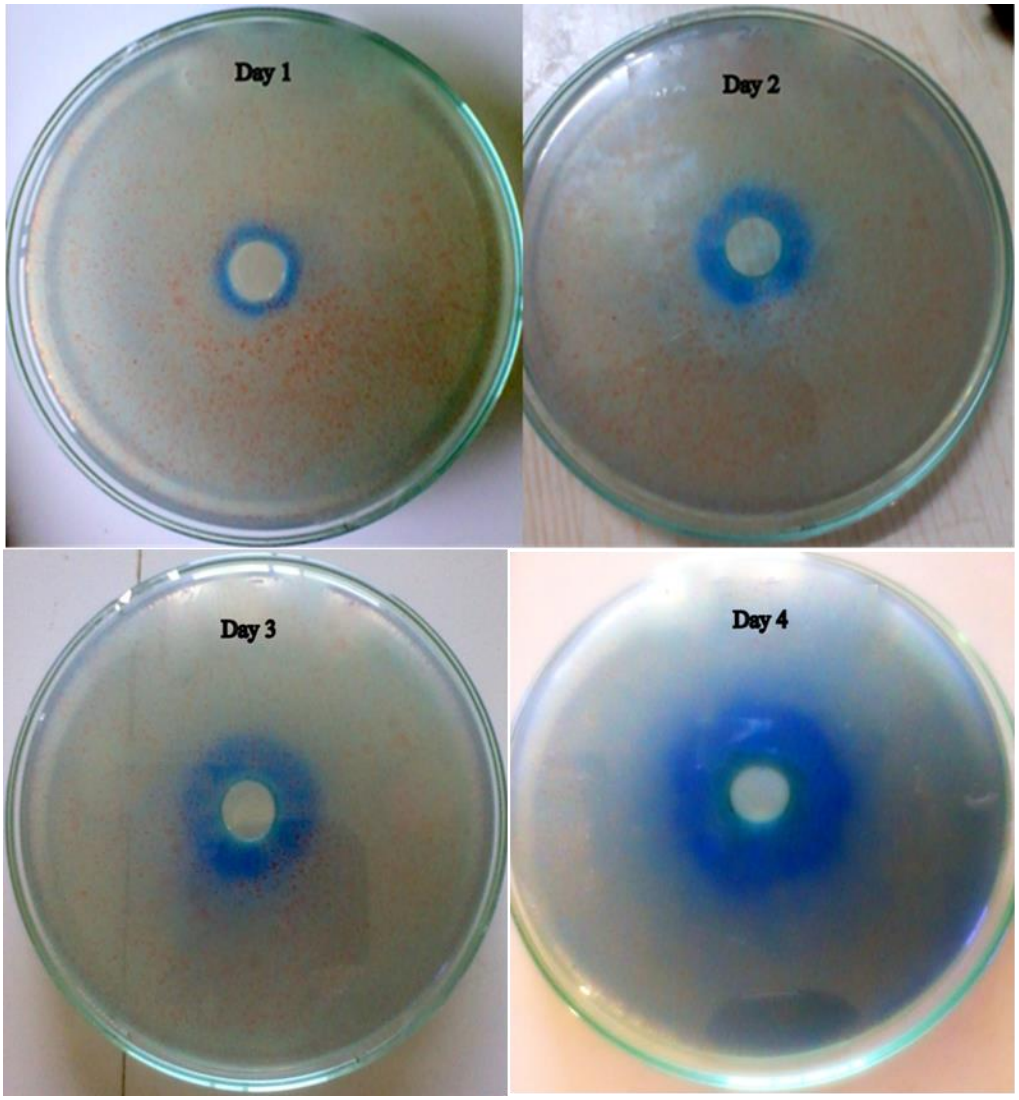


Figure 3.5: The crude enzyme preparation was removed each day and put in the wells bored on agar plate with Victoria Blue indicator. The maximum lipase production was obtained after 4 days of inoculation of seed culture into production media as seen from the maximum diameter of the dark blue zone around the bore indicating lipase enzyme activity.

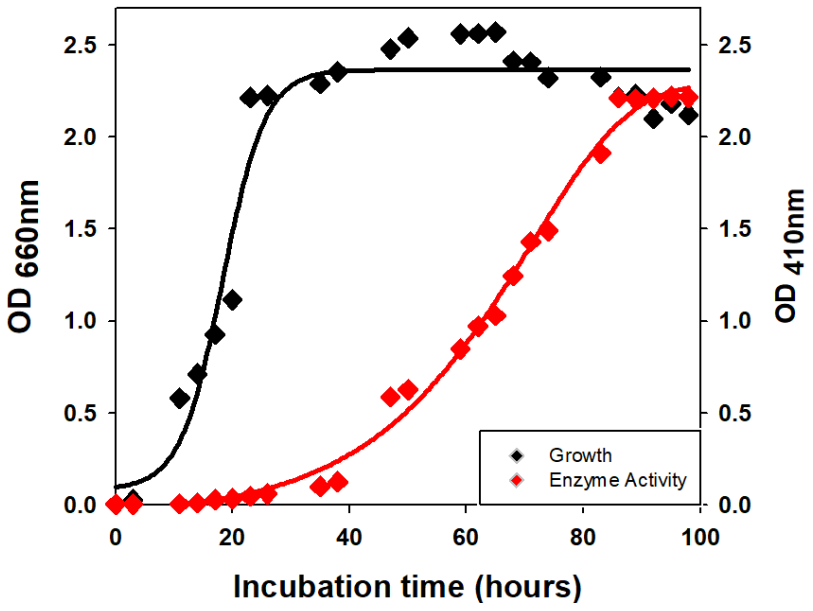


Figure 3.6: Monitoring growth and extracellular lipase production by *Halomonas shengliensis* in optimized production media. The increase in the cell mass of the organism is measured from the optical scatter by the bacterial cells at 600 nm using a spectrophotometer. The degree of turbidity in the broth culture is directly related to the number of microorganism present. The enzyme activity from the cell free supernatant was simultaneously measured to determine the time of optimum enzyme production.

3.3.4 Lipase assay

Activity assay of lipase using p-NPA as the substrate is extensively reported and widely used by many scientific groups from all across the globe (Mathias et al., 1981; Kordel et al., 1991; Schmid et al., 2001) and was our first choice to determine the enzymatic activity of our lipase samples. Unfortunately, we noticed erroneous results in the measurement of enzyme activity by this method under slightly alkaline pH of 7.5. Upon conducting substantial literature search on lipase activity assay, we got to know that the errors in activity measurement were due to the fact that p-nitrophenyl acetate (pNPA) readily undergoes autohydrolysis to p-nitrophenol (pNP), at higher pH and higher temperatures. pNPA has an absorbance maximum at 270 nm and PNP an absorbance maximum between 405 and 410 nm, depending on the temperature. To accurately measure the activity of the enzyme using this substrate, we took the absorbance readings at 348 nm, the isosbestic point of pNP, and at 410 nm. Activity measurements were found to be consistent after incorporation of this correction in the assay procedure

Later on, we shifted to a fluorescence-based enzyme activity assay using the substrate 4-methylumbelliferyl butyrate (4MUF), which is more convenient, rapid (requires very brief incubation time) and highly sensitive to lower concentrations of enzyme. In this method the amount of highly fluorescent 4-methylumbelliferone released upon hydrolysis of (MUF)-derivative substrate by lipase is measured (Wilton, 1990; Wilton, 1991; Prim et al., 2003)**.** This method has been used for the detection of lipase activity from microbial colonies, cell culture suspensions, or from proteins separated on SDS-polyacrylamide or isoelectric focusing gels. The crude enzyme under standard assay conditions showed positive lipolytic activity by this method

3.3.5 Preparation of crude enzyme extract

As shown in **Figure 3.7**, the crude lipase sample obtained from *H.shengliensis* showed a very prominent band of native lipase amongst other extracellular proteins, on SDS-PAGE. Upon loading varying concentrations of the protein sample, the intensity and thickness of the band also varied as expected. The crude enzyme extract was stored at 4°C until further treatments.

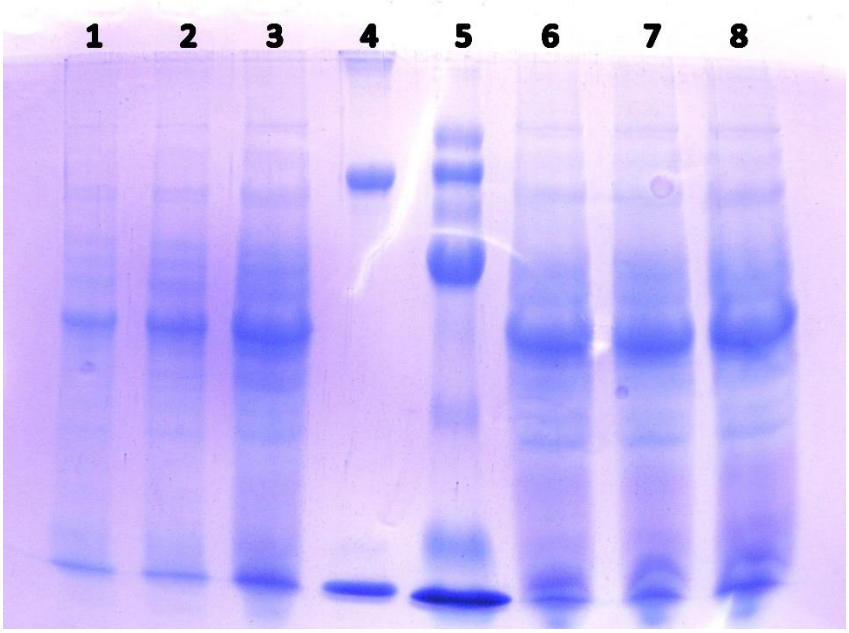


Figure 3.7: 10% SDS-PAGE of various amounts of the crude lipase. Lane 1: 2 µl, Lane 2: 5 µl, Lane 3: 10 µl, Lane 4: BSA 2 µg, Lysozyme 2 µg, Lane 5: Ovalbumin 5 µg, β-lactoglobulin 2 µg, Lane 6: 15 µl, Lane 7: 15 µl, Lane 8: 20 µl of the crude enzyme preparation.

3.3.6 Purification of Lipase

3.3.6.1 Ammonium sulphate precipitation

Ammonium sulphate precipitates out proteins by the salting out process and the amount of the ammonium sulphate required depends upon the percent saturation required. Ammonium sulphate was added in small amounts to the cell-free supernatant with constant stirring on ice till no more of the salt dissolved (100% saturation). Complete precipitation was observed after the suspension was left under stirring condition on ice for 2 h. The precipitates formed a tight pellet after centrifugation at low temperature. The pellet was resuspended in 25 mM phosphate buffer (pH 7.5) containing 25 mM NaCl and dialyzed against the same buffer. Lipase activity was found to be higher in the dialyzed sample compared to cell free supernatant indicating that the protein has been concentrated in this process.

3.3.6.2 Ion-Exchange chromatography

In ion exchange separations, the distribution and net charge on the protein's surface determines the interaction of protein with the charged groups on the surface of the packing material (Berg et al., 2001). The lipase was purified from the concentrated crude extract by passing it through anion exchange chromatography column followed by elution with a step gradient of NaCl. Spot test was done with the collected fractions to get a quick idea about fractions containing maximum amount of proteins, which we would further check for purity in SDS-PAGE. As shown in **Figure 3.8**, fractions eluted with 250 mM NaCl, 300 mM NaCl, 350 mM NaCl, 400 mM NaCl, 450 mM NaCl, & 500 mM NaCl showed visible dark spots after staining and destaining in the spot test.

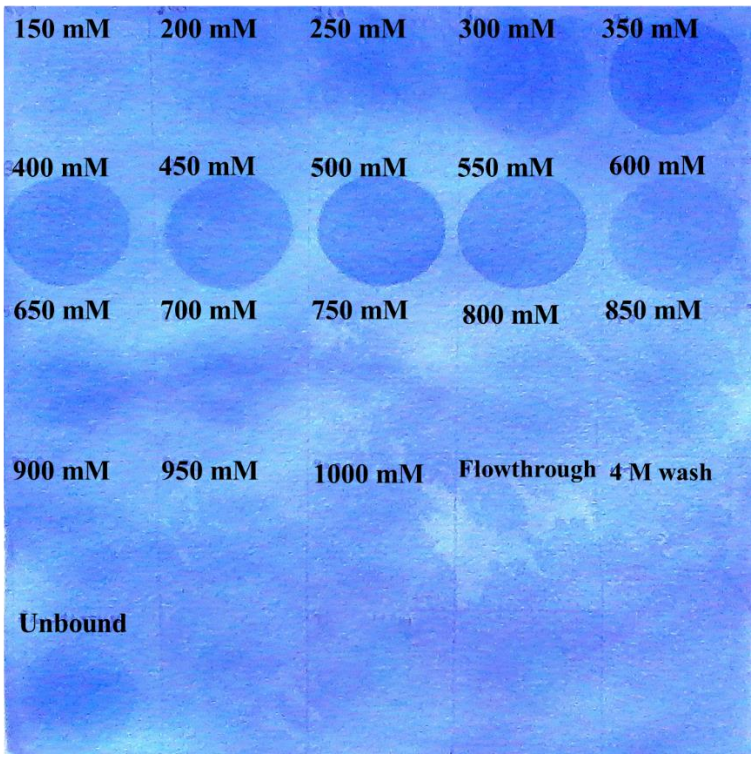
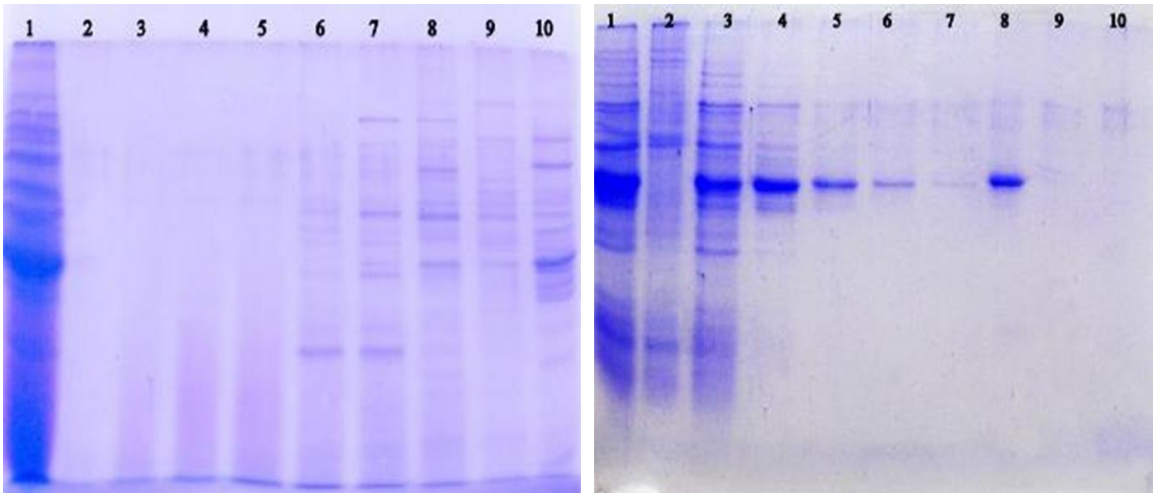


Figure 3.8: Spot test of fractions of protein collected from Ion-Exchange Chromatography at different eluting salt concentrations.

SDS-PAGE analysis of fractions from ion-exchange chromatography, as shown in **Figures 3.9 (A)** and **(B)**, showed that the fractions eluted with 450 mM NaCl, 500 mM NaCl, 550 mM NaCl. 600 mM NaCl and 650 mM NaCl showed the lipase band. The fraction eluted with 500 mM NaCl had the maximum amount of lipase, but relatively less pure. The fraction eluted with 600 mM NaCl showed highest purity but lesser amount of protein. All fractions containing pure enzyme were pooled together and concentrated for further experiments. The pooled pure enzyme band is shown in lane 8 of **Figure 3.9 (B).**



A **B**

Figure 3.9: 12% SDS-APGE analysis of fractions eluted with different concentrations of NaCl from ion exchange chromatography through Q-Sepharose. (A) Lane 1:15µl crude lipase, Lane 2:15µl 50mM NaCl fraction, Lane 3:15µl 100mM NaCl fraction, Lane 4:15µl 150mM fraction, Lane 5:15µl 200mM NaCl fraction, Lane 6:15µl 250mM NaCl fraction, Lane 7:15µl 300mM NaCl fraction, Lane 8:15µl 350mM NaCl fraction, Lane 9:15µl 400mM NaCl fraction, Lane 10:15µl 450mM NaCl fraction **(B)** Lane 1:15µl crude lipase, Lane 2: Flow through, Lane 3:15µl 500mM NaCl fraction, Lane 4:15µl 550mM NaCl fraction, Lane 5:15µl 600mM NaCl fraction, Lane 6:15µl 650mM NaCl fraction, Lane 7:15µl 700mM NaCl fraction, Lane 8:15µl Pooled pure fractions, Lane 9:15µl 800mM NaCl fraction, Lane 10:15µl 850mM NaCl fraction.

The purification table **(Table 3.1)** showed that a10-fold purification and 70% yield of lipase was achieved using ion exchange chromatography and the maximum specific enzyme activity (SEA) for the enzyme was 294.74 U/mg protein.

Table 3.1: Purification table for lipase

Purification step	Total Protein (mg)	Activity (Units)	Specific activity U/mg protein	Purification fold	% Yield
Ammonium sulphate precipitated cell free supernatant	29.6	794	26.82	1	100
Q-sepharose purification	1.9	560	294.74	10.89	70.53

The **Figure 3.10**, shows purified lipase with crude enzyme preparation to compare the fold purification and yield.

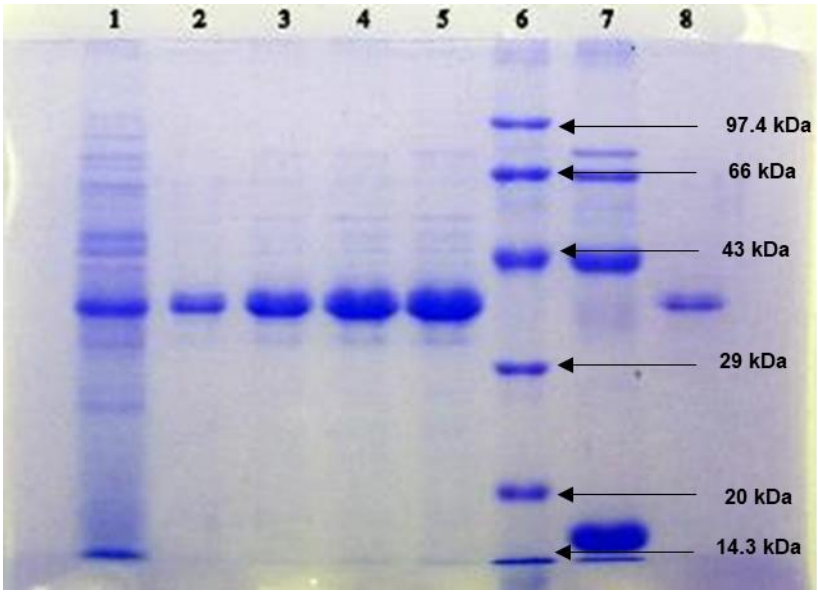


Figure 3.10: 12% reducing SDS-PAGE of lipase. (Lane1- 5µl Crude lipase, Lane 2- 2µg Pure lipase, Lane 3- 5µg Pure lipase, Lane 4- 10µg Pure lipase, Lane 5- 15µg Pure lipase, Lane 6- Protein molecular weight marker (Genei), Lane 7- 5µg BSA, OVA, lysozyme, Lane 8-1µg lipase).

3.3.6.3 Gel filtration chromatography

Size exclusion chromatography of the lipase was carried out by semi-preparative HPLC. The elution profile is shown in **Figure 3.11.** Since, lipase preparation was relatively pure with a purity of about 90% after ion-exchange chromatography, it was used as method of protein purification for further experiments.

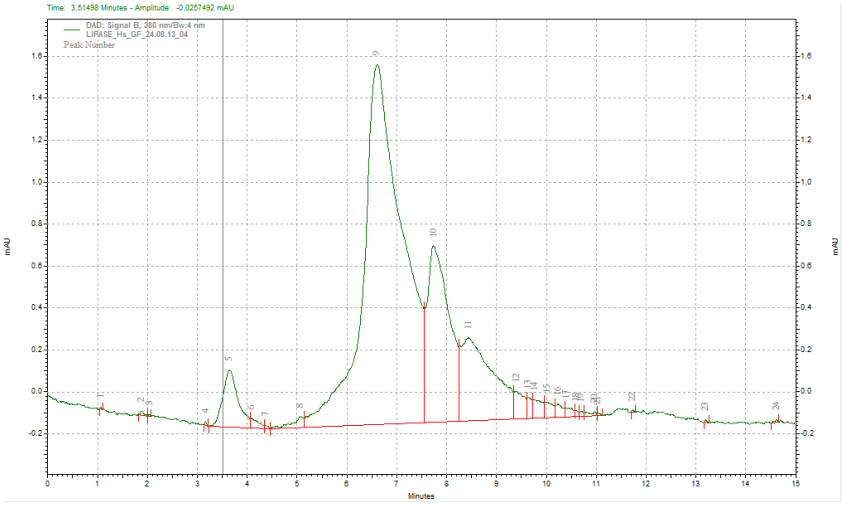
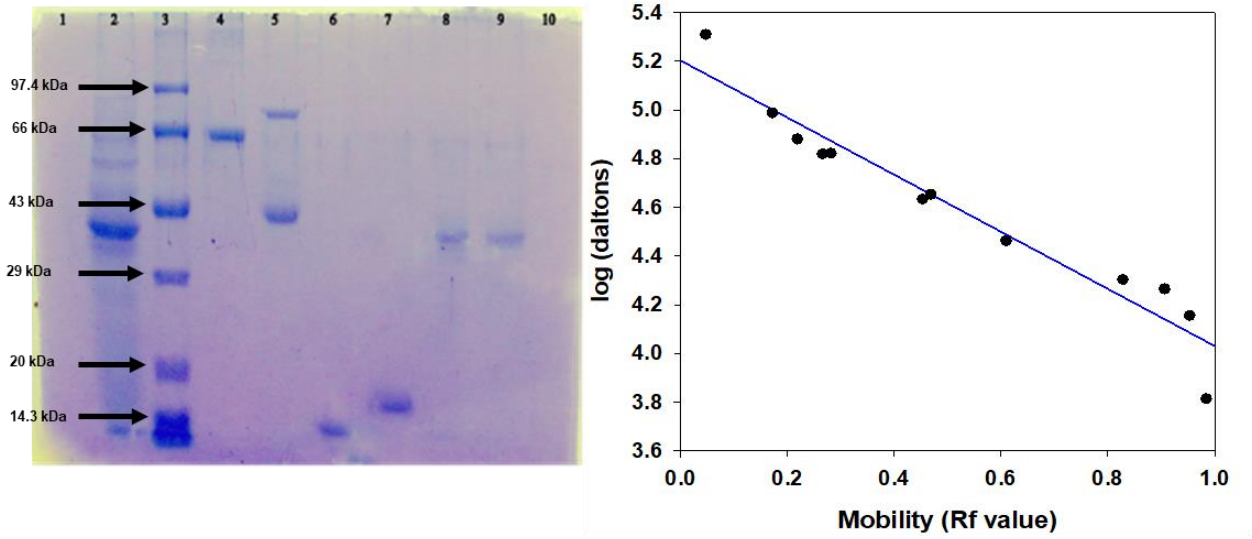


Figure 3.11: Size exclusion chromatography of lipase after ion exchange chromatography. Gel filtration column (Zorbax GF-250, 9.4 x 250mm, 4 micron, analytical) was used with HPLC (Agilent technologies, 1200 infinity series, semi-preparative) system. Flow rate was kept at 0.1 ml/min, 5 µg protein in 20 µl buffer was loaded into column.

3.3.7 Biochemical characterization of *Halomonas shengliensis* lipase

3.3.7.1 Molecular weight determination by SDS-PAGE

The molecular weight of the purified lipase was estimated from its mobility on SDS-PAGE. Standard proteins were simultaneously run on the 12% SDS-PAGE (**Figure 3.12A**) and their Rf values were calculated. A standard plot of log molecular weight versus Rf value of the standard proteins was plotted (**Figure 3.12B**) and the molecular weight of the lipase was read as 41.35 kDa from the standard plot from its Rf value.



A **B**

Figure 3.12 (A) SDS-PAGE of purified lipase and standard proteins for determination of molecular weight. Lane 1: empty; Lane 2: Crude lipase; Lane 3: Genei Protein Molecular Weight Marker (15µl); Lane 4: Bovine Serum Albumin (2µg); Lane 5: Ovalbumin (2µg); Lane 6: Lysozyme (2 µg); Lane 7: β- Lactoglobulin (2µg); Lanes 8 & 9: Lipase purified after Ion-exchange chromatography (1µg); Lane 10: empty. **(B)** Standard plot showing the relative mobility (Rf) of standard proteins as a function of their log molecular weights**.**

The pure lipase sample was also sent for mass spectrometry analysis to determine the exact molecular weight. The molecular weight of lipase obtained from mass spectrometry was 35.19 kDa (**Figure 3.13**).

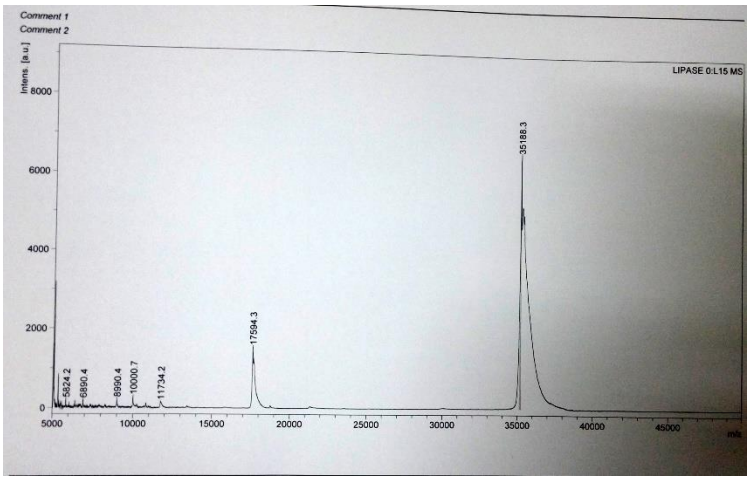


Figure 3.13: Mass spectrometry of pure lipase to determine exact molecular weight.

3.3.7.2 Determination of temperature optima and thermal stability of Lipase

A plot of lipase activity as a function of assay temperature over a range of 20 - 100ºC showed the classic inverted bell shape that is obtained for most enzymes (**Figure 3.14)**. The optimum temperature obtained for the purified lipase was 70°C. There was a gradual increase in the enzyme's activity from 20°C to 70°C. There was a sharp fall in enzyme activity when the temperature was increased beyond 70°C. The enzyme was highly active between the temperatures ranging from 60℃ to 70℃, which is quite a high temperature considering that it is isolated from a non-thermophile, but this implies that it is a thermoactive enzyme and hence would be more important for practical industrial use.

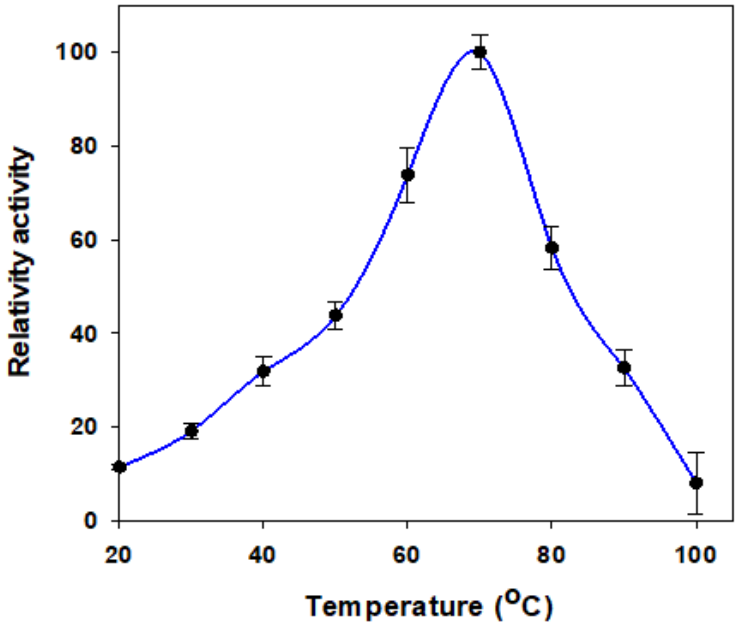


Figure 3.14: Relative activity of purified lipase as a function of temperature. The maximum activity obtained was taken as 100% and the remaining were calculated as a relative percentage of this value. All assay parameters were kept constant except for the temperature which was varied from 20°C to 100°C. Each point is an average of three individual experiments. The optimum temperature was found to be 70°C.

The thermostability of the purified lipase was then examined by incubating the enzyme at a specific temperature for six hours and measuring its activity at regular time intervals. Data represented in **Figure 3.15** show that the lipase retained its full activity for more than six hours when it was incubated at temperatures ranging from 30°C to 50°C. When the lipase was incubated at 60°C, it exhibited 100% activity for up to 4.5 hours, after which the activity decreased, but was still partially active (40% activity) after 6 hours of incubation. At 70°C, the lipase was fully active for 3 hours, and then lost about 85% of its activity after 6 hours of incubation. When incubated at 80°C, the enzyme started showing a reduction in activity within a few minutes of incubation and completely lost its activity before 3 h incubation. A sharp fall in lipase activity was observed when it was incubated at 90°C with complete loss of activity by 30 minutes of incubation. The thermostability profile of lipase intrigued us into believing that studying this enzyme could get us some insight about mechanisms of thermostability as well as about the molecular determinants of protein thermostability.

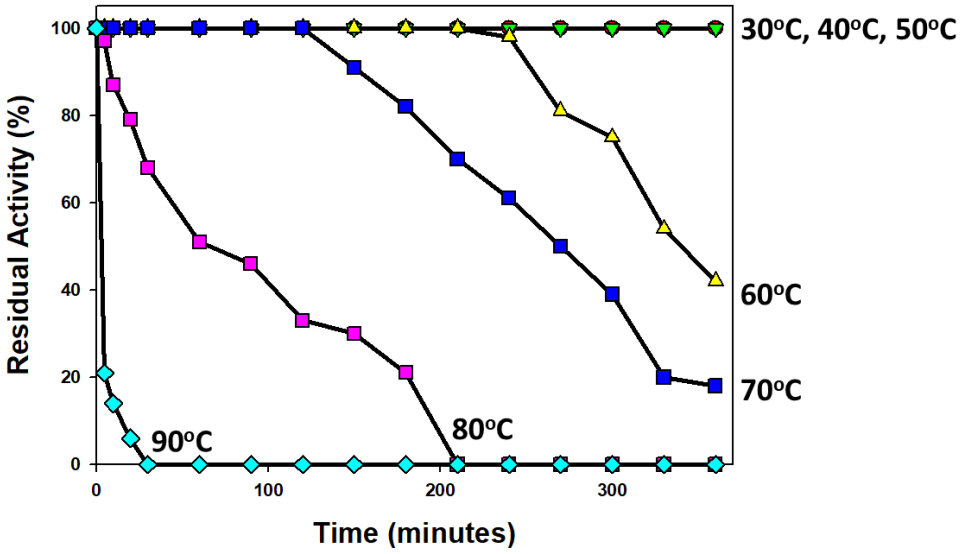


Figure 3.15: Thermostability of lipase at different temperatures. The enzyme was incubated at the specified temperature for six hours and aliquots were removed at regular time intervals and assayed for lipolytic activity. The activity of the native enzyme was taken as 100% and all other activities were calculated relative to this value.

3.3.7.3 Determination of pH optima of enzyme

When the lipolytic activity of the purified enzyme was measured as a function of pH, the lipase was found to have a pH optimum of 7.5 **(Figure 3.16)**. The enzyme was highly active at pH values between 7.0 and 8.5, which indicated that the enzyme requires moderately alkalophilic conditions for optimum activity.

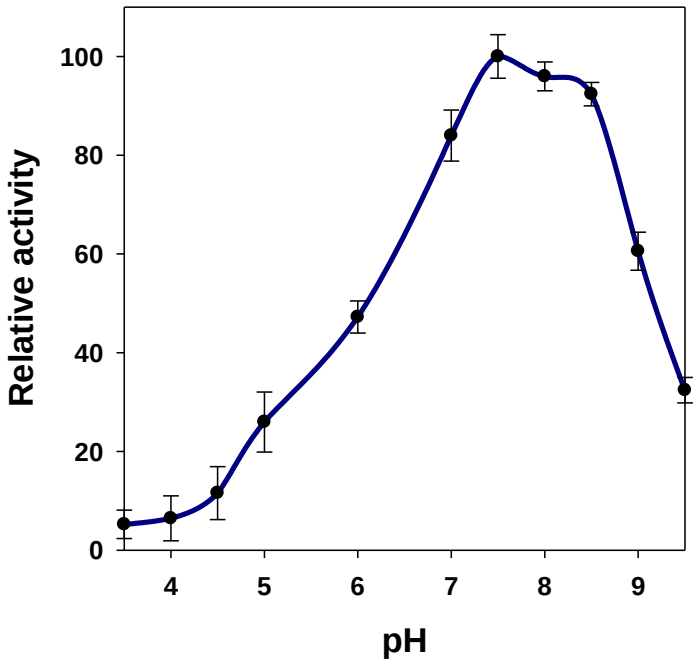
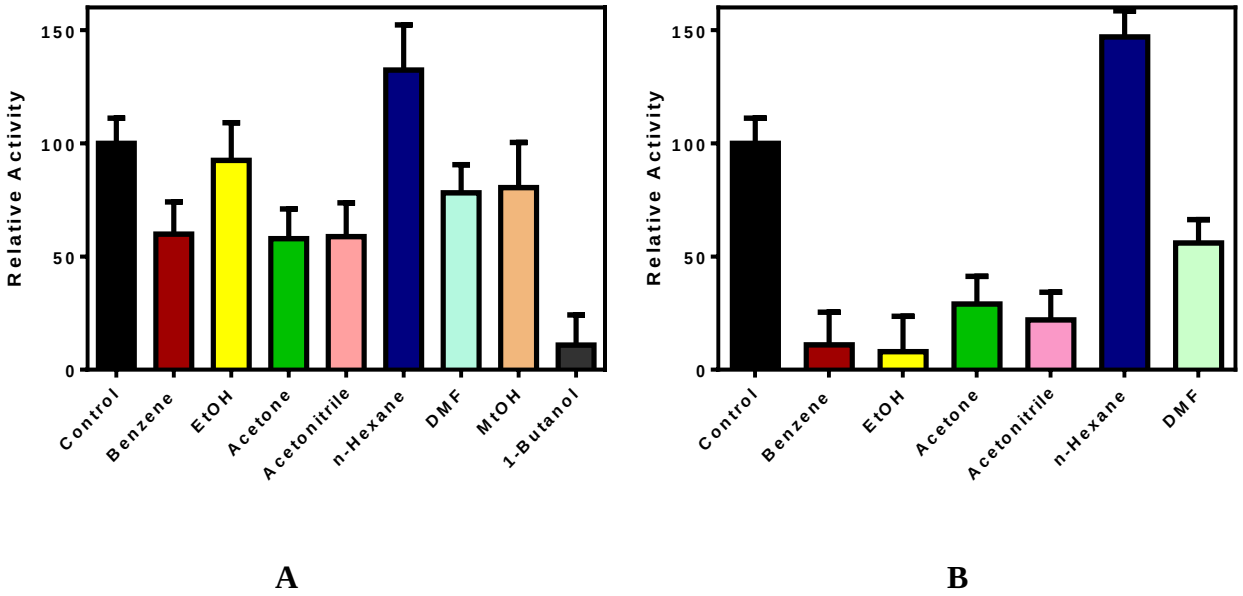


Figure 3.16: Relative Lipase activity as a function of pH (optimum pH 7.5). All the assay parameters except the pH were kept constant for all the assays and the curve obtained is an average of three independent experiments. The maximum activity obtained was taken as 100% and all other values were calculated relative to this value.

3.3.7.4 Effect of organic solvents on enzyme activity

There has been much interest in the development of rules to predict the effects of various solvents on a biocatalyst (Valivety et al., 1994). Presence of organic solvents which can penetrate into the active site of enzyme can cause the unfolding of proteins by disruption of the stabilizing forces. Substantial efforts have been made in the past to rationalize the correlation between enzyme activity and the nature of organic solvents. In an aqueous-organic solvent two phase system, the interaction of enzyme with the bulk organic medium results in inactivation of the enzyme (Ghatorae et al., 1994). The interfaces destabilize the electrostatic, hydrophobic and hydrogen bonding interactions of the protein, causing irreversible denaturation of enzyme during biocatalysis in interfacial system (Wu et al., 1993). Quantity of water is also thought to be an important factor required for lipase action. When the water is present around the enzyme molecules, the rest of water in solution can be replaced with an organic solvent without adversely affecting the performance of the enzyme. Stability of lipases in organic solvents makes their uses commercially feasible in the enzymatic esterification reactions (Hari Krishna and Karanth, 2001; Kiran and Divakar, 2001).

We checked the tolerability of pure lipase towards various organic solvents as it is known that the thermostability of an enzyme is positively associated with its stability in organic solvents. Also, the substrates of this enzyme should be more soluble in organic solvents and hence organic solvent tolerant lipase would be more useful industrially. As shown in **Figure 3.17**, the purified lipase showed highest activity in the presence of 15% and 30% n-hexane. In the presence of 15% methanol, ethanol or dimethylformamide, it retained about 80% of its activity, while it retained only about 60% activity in the presence of 15% benzene, acetone or acetonitrile. The enzyme exhibited negligible activity in presence of 15% butanol. In the presence of 30% acetone, acetonitrile, benzene or ethanol, the enzyme showed more than 50% to complete loss of activity.



A **B**

Figure 3.17: (A) Relative lipase activity in 15% organic solvents and **(B)** Relative lipase activity in 30% organic solvents. All the assay parameters were kept constant except for different organic solvents and each activity value is an average of three independent experiments. The activity of lipase in the absence of any organic solvent was taken as 100%, and the activity in presence of the organic solvents were calculated relative to this value.

We also examined whether 15% organic solvent caused any structural perturbations in the lipase by recording the CD spectra of the protein in the presence of these solvents (**Figure 3.18**). A shift in the peak at 208nm was observed in the CD spectra when 5µg lipase was incubated in presence of methanol, ethanol, isopropanol and acetone. This suggests that there is some minor changes in the lipase structure in the presence of organic solvents. The peak shift at 208 nm is

associated with change in α helical content of protein. The decrease in α helical content is often correlated with decreased stability of protein. As shown in **Table 3.2**, the decrease in α helical content was observed in lipase in presence of organic solvents in order, Native>Methanol>Ethanol>Isopropanol>Acetone. The increase in amount of coils was also observed with decrease in α helical content, indicating structural unfolding of protein. It has been reported that disulfide bonds and amino acid residue(s) present on the surface of the enzyme play a vital role in the stability towards organic solvents and it is closely related to the secondary structure of the molecule. Several factors, including conformational changes, are responsible for decrease or complete loss of catalytic activity when lipases are subjected to act in an organic medium (Kumar et al., 2016).

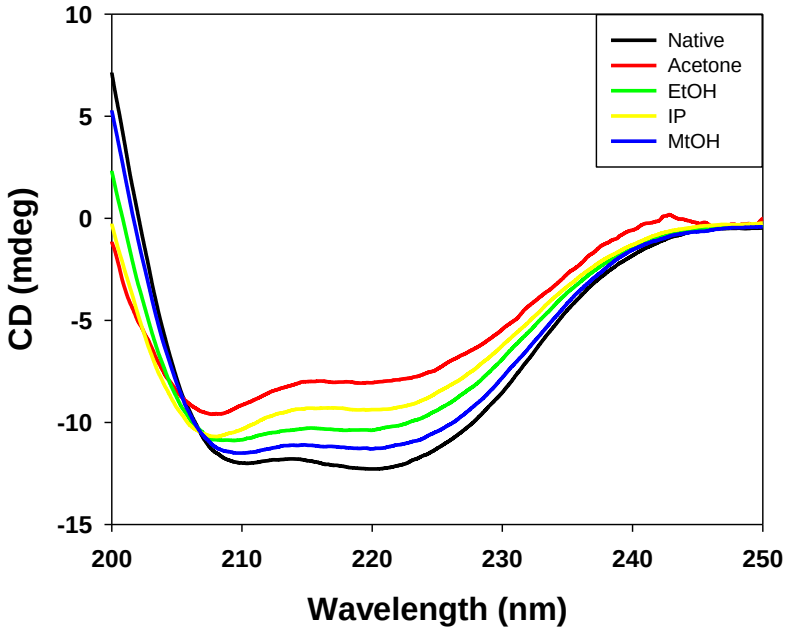


Figure 3.18: Far UV-CD spectra of lipase in presence of 15% organic solvent.

Table 3.2: Secondary structure content of lipase and lipase in presence of organic solvents

Sample	% α Helix	% β Sheets	% Turns	% others
Native	74.2	10.3	1.7	13.7
Methanol	75.7	3.9	3.0	17.5
Ethanol	73.6	0	0.2	26.2
Isopropanol	66.9	6.1	1.0	26.2
Acetone	60.6	11.6	4.5	23.3

3.3.7.5 Effect of detergents and surfactants on lipase activity

Many laundry detergent products nowadays are known to contain cocktails of enzymes including proteases, amylases, cellulases, and lipases (Jeon et al., 2009). Lipases are the prime choice for detergents because of their generally low substrate specificity, stability under alkaline pH conditions and in the presence of surfactants and enzymes, which are the typical properties of most detergent formulations (Hasan et al., 2010). Most of the chemical detergent ingredients are toxic for humans and the environment; hence the use of alkaline lipases in detergent formulation has gained attention to overcome this problem (Niyonzima and More, 2015). Different detergents influence lipase activity differently. Substrate and detergent interactions with lipases can be uncertain and complicated to understand (Misiorowski and Wells, 1974; Hermoso et al., 1996; Mogensen et al., 2005; Moh'd A and Wiegel, 2010).

We examined the activity of the purified lipase in the presence of laundry detergents and surfactants. We used a concentration of 0.25% for the selected detergents because the recommended concentration of commercial detergents for routine wash is 0.20-0.24%. Activity of the lipase was adversely affected by various routinely used commercial detergents. The complete loss of enzyme activity was observed, which could be explained by fact that we directly exposed native lipase to detergents without any treatments or protective coating around it or the presence of detergents could induce conformational changes in the enzyme thereby leading to enzyme inactivation.

The nonionic detergents (surfactants), Triton X-100, Tween 20, and Tween 80, slightly enhanced the activity of the lipase at a concentration of 0.25%. Particularly, in presence of Tween 80 there is an increase in around 25% lipase activity, this may be a consequence of formation of a micellar system by a non-ionic detergent in presence of fatty acid. The micelle packing density of Tween 80 is greater than other mentioned nonionic surfactants, which is known to affect the lipase penetration into the micelle surface and enzyme activation (Plou et al., 1998). Lipase retained 100% of its original activity upon incubation with non-ionic surfactants, but lost around 70% activity in presence of 0.25% SDS (**Figure 3.19**). Normally, nonionic detergents are considered mild compared to chemically formulated laundry detergents. They do not interact extensively with the protein surface, whereas SDS in particular, being an ionic detergent, normally bind non-specifically to the protein surface, thereby leading to protein unfolding.

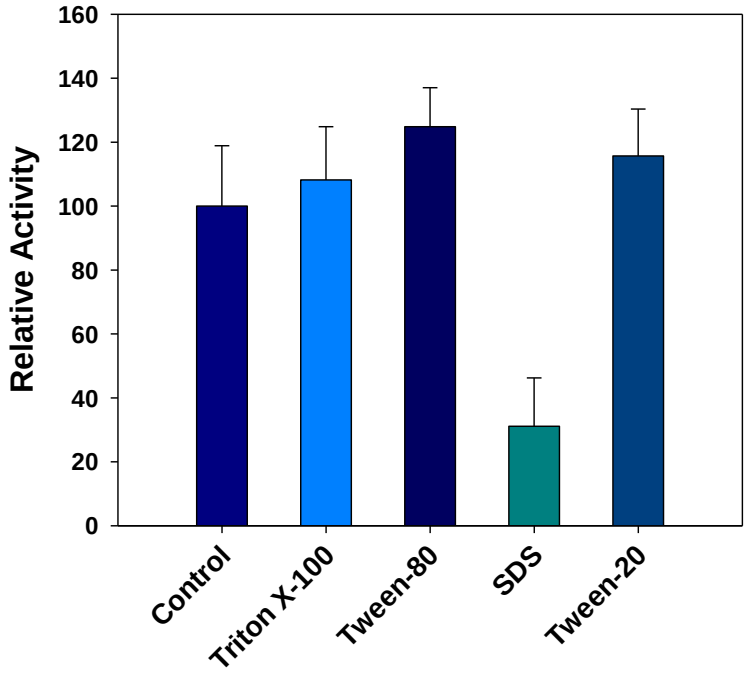
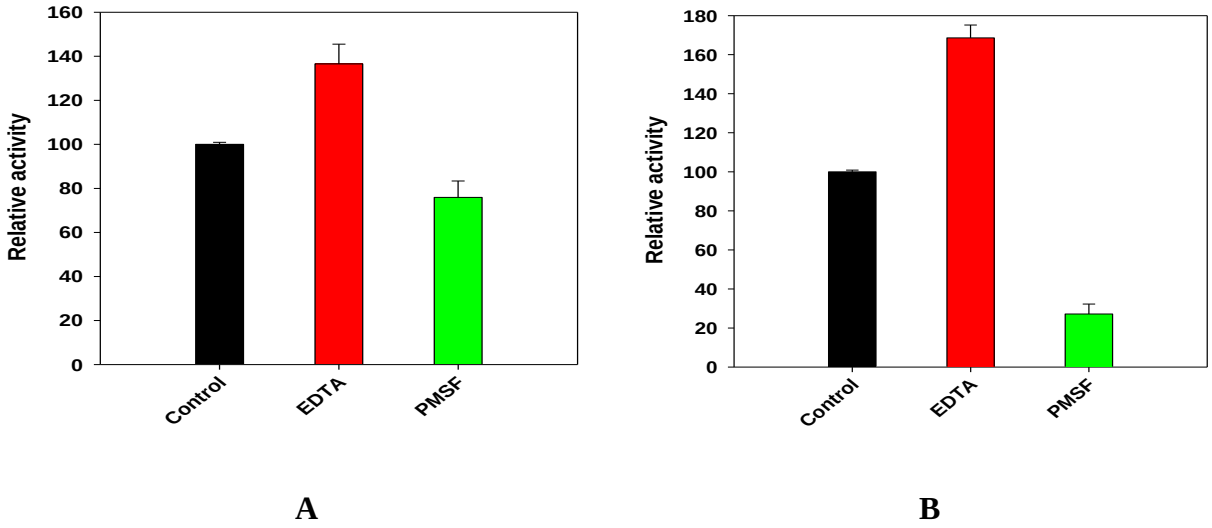


Figure 3.19: Relative lipase activity in presence of 0.25% detergents. All the assay parameters were constant except different detergents and each activity value is an average of three independent experiments.

3.3.7.6 Effect of inhibitors on enzyme activity

Various inhibitors are generally used to find catalytic site residues of an enzyme. We studied the effect of commonly known enzyme inhibitors on the activity of the purified lipase enzyme. Lipase was strongly inhibited by PMSF at 1 mM and 10 mM concentrations, indicating

that a serine residue might be present at the active site. The enzyme completely lost its activity when incubated in 1 mM & 10 mM reducing agents, βME and DTT. As shown in **Figure 3.20,** the enzyme activity was considerably enhanced upon incubation with 1 mM and 10 mM EDTA. The stability of lipase in EDTA is essential for it to be used in combination with detergent, as the chelating agent contributes to removing ions accountable for hardness of water, helping in proper stain removal.



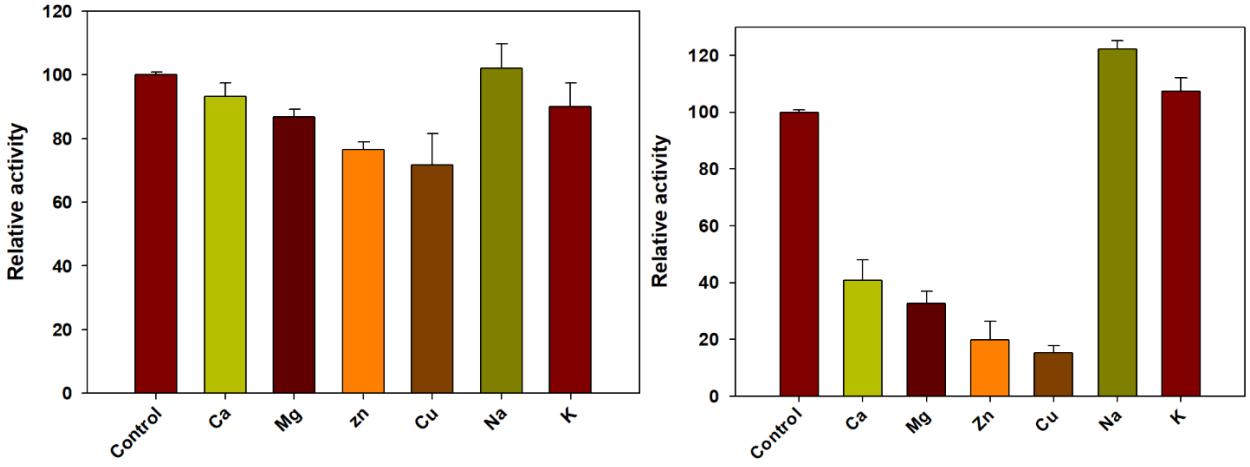
A **B**

Figure 3.20: Relative activity of lipase in presence of 1 mM **(A)** and 10 mM **(B)** concentrations of inhibitors**.** All the assay parameters were constant except different detergents and the results are average of three independent experiments. The activity of lipase in the absence of any inhibitor was taken as 100% and others were calculated relative to that value.

3.3.7.7 Effect of metal ions on enzyme activity

We examined the effect of various monovalent and divalent metal ions on the activity of the lipase. As can be seen from **Figure 3.21**, lipase retained most of its activity even in the presence of 10mM concentration of monovalent cations, Na^{+} and K^{+}. Among the divalent cations at a concentration of 1 mM, Ca^{+2} and Mg^{+2} had no significant effect on enzyme activity, whereas Zn^{+2} and Cu^{+2} reduced the activity by up to 25%. At concentrations of 10 mM, Ca^{+2}, Mg^{+2}, Zn^{+2} and Cu^{+2} greatly inhibited the lipase activity. On the other hand, even 1 mM Hg^{+2}, Mn^{+2}, Ba^{+2}, Al^{+3} and Fe^{+2} led to complete loss of lipase activity (data not shown), indicating inhibitory effect of divalent metal ions on lipase activity. EDTA is known to chelate most of the divalent metal ions, considerable increase in lipase activity was observed in presence of EDTA (**Figure 3.19**)

suggesting no divalent metal ions are needed for the reaction and that EDTA probably chelates the inhibitory metal ions (Ghori et al., 2011).



A **B**

Figure 3.21: Relative activity of lipase in presence of 1 mM metal ions **(A)** and 10 mM metal ions **(B).** All the assay parameters were constant except for different metal ions and each activity is an average of three independent experiments. The activity of lipase in the absence of any metal ion was taken as 100% and others were calculated relative to that value.

3.3.7.8 Determination of substrate specificity (chain length) and enzyme kinetics

Lipases are known to digest a wide variety of substrates and exhibit broad substrate specificity. The lipase from *H.shengliensis* was able to readily hydrolyze all tested p-nitrophenyl substrates of varying carbon chain lengths. With respect to the fatty acid specificity, the enzyme was more active against p-nitrophenyl butyrate, and showed slight preference towards substrates containing shorter fatty acid chain length. Lipases generally exhibit different kinetic behaviors depending on the properties and concentration of hydrolyzed substrate. The influence of substrate concentration on the reaction velocities of the purified lipase was studied with pNPA and 4-MUFB. From the plots shown in **Figure 3.22**, the K_m values for lipase obtained using 4-MUFB and pNPA as substrates were 90 µM and 420 µM respectively. A lower K_m value for 4-MUF indicates that the purified lipase has higher affinity for 4-MUF. V_{max} values for lipase obtained using 4-MUFB and pNPA were recorded 0.616 µM /ml/min and 0.423 µM /ml/min.

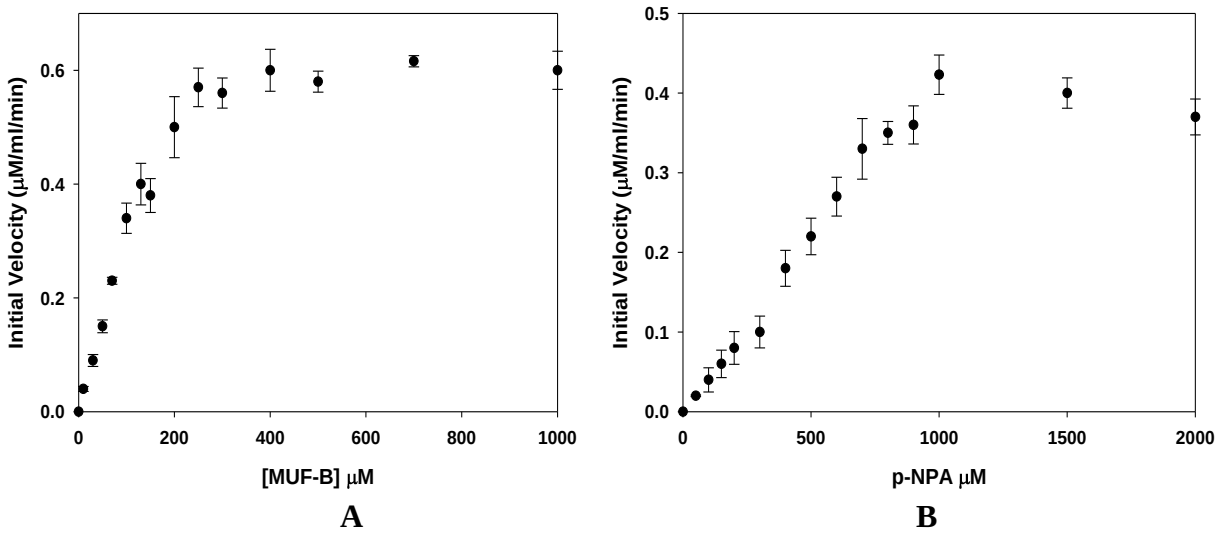
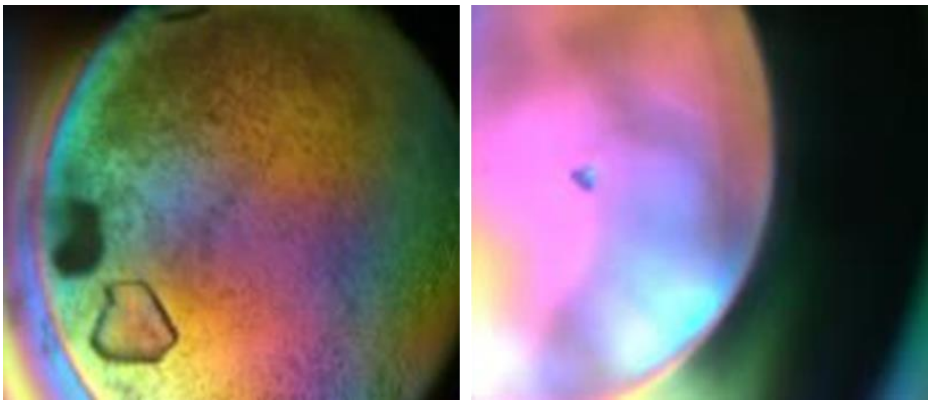


Figure 3.22: Michaelis-Menten plot of lipase to determine K_m & V_{max} **(A)** with 4-MUFB and **(B)** with pNPA**.**

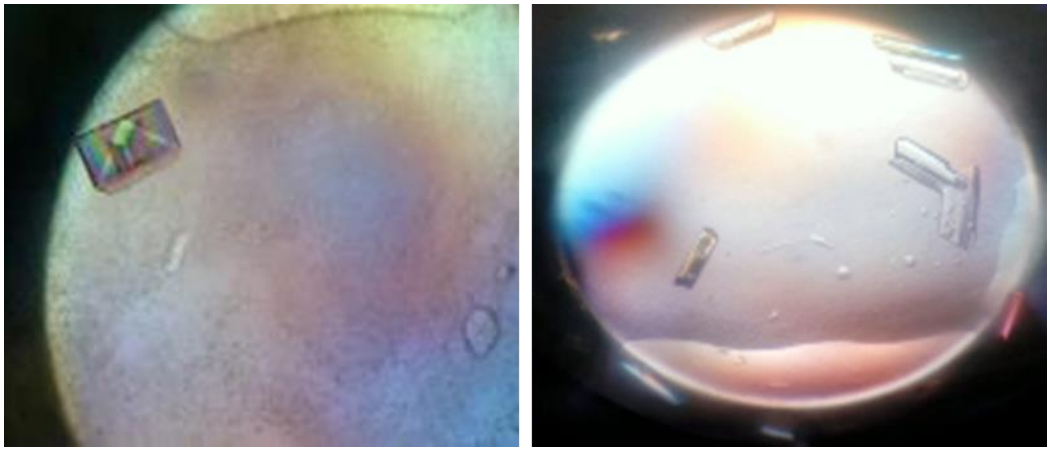
3.3.7.9 X-Ray crystallography

Since the isolated protein is a new one whose structure is not known, we attempted to crystallize the enzyme so that its molecular structure can be determined. 194 conditions of crystal screens were set up and among them, several conditions gave crystals as shown in **Figures 3.23 (A-J).** The first signs of crystal formation could be seen 25 days after setting up crystallization. The crystals were monitored for growth for the next 20 days. Full grown crystals were obtained after 40 days of crystallization. Data collection and structure solution are not yet done as it has to be outsourced elsewhere as we do not have the facility.



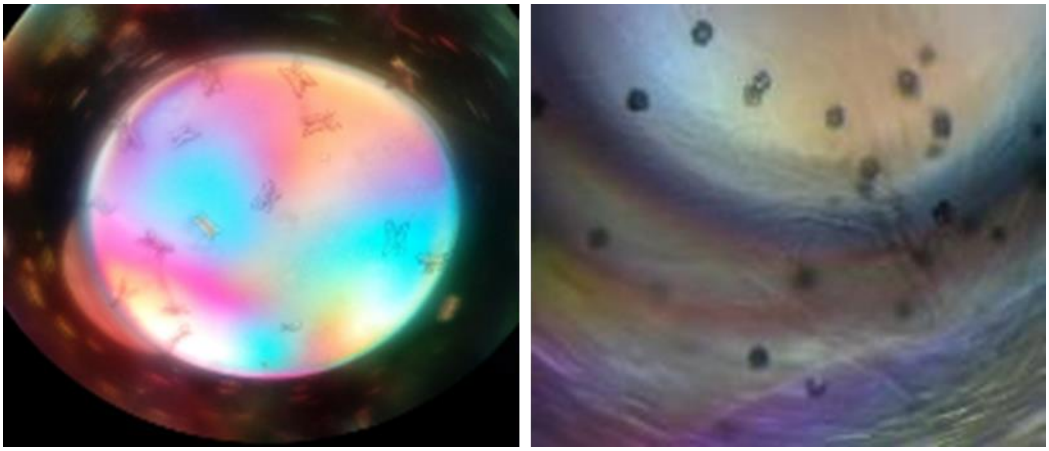
A **B**

Figure 3.23: (A) Crystals obtained in Index 1 (0.1 M BIS-Tris pH 6.5, 0.5 M Magnesium formate dehydrate) **(B)** Index 1 (1.8 M Ammonium citrate tribasic pH 7.0).



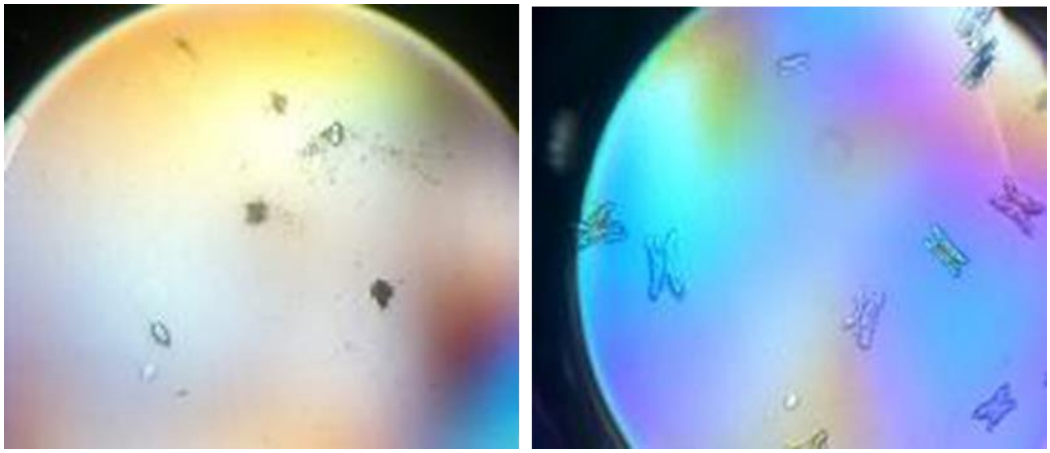
C **D**

Figure 3.23: (C) Crystals obtained in Index 2: 5 mM Cobalt(II) chloride hexahydrate, 5 mM Nickel(II) chloride hexahydrate, 5 mM Cadmium chloride hydrate, 5 mM Magnesium chloride hexahydrate dehydrate) **(D)** Index 2: 0.05M Magnesium chloride hexahydrate, 0.1M HEPES, pH 7.5, 30% v/v Polyethylene glycol monomethyl ether 550.



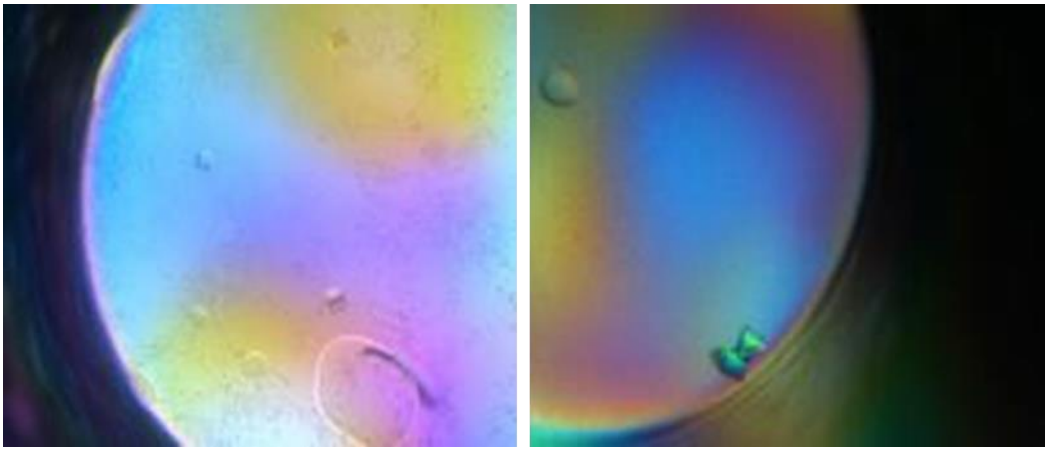
E **F**

Figure 3.23: (E) Crystals obtained in Crystal screen 1 (0.2M Magnesium chloride hexahydrate, 0.1M HEPES Sodium, pH 7.5, 30% v/v 2-Propanol) **(F)** Crystal screen 1 (0.2M Zinc acetate dehydrate, 0.1M Sodium cacodylate trihydrate pH 6.5, 18% (w/v) PEG 8000).



G **H**

Figure 3.23: (G) Crystal obtained in Crystal screen 1 (0.1 M Sodium cacodylate trihydrate pH 6.5, 1.4 M Sodium acetate trihydrate) **(H)** Crystal screen 2 (0.2M Magnesium chloride hexahydrate, 0.1M Tris, pH 8.5, 3,4 M 1,6-Hexanediol).



I **J**

Figure 3.23: (I) Crystal obtained in Crystal screen 2 (0.2 M Potassium sodium tartrate tetrahydrate, 0.1 M Sodium citrate tribasic dihydrate pH 5.6, 2.0 M Ammonium sulfate) **(J)** Crystal Screen 2 (0.1M HEPES Sodium pH 7.5, 2% v/v polyethylene glycol 400, 2.0 M Ammonium sulphate).

3.3.7.10 Cloning of *H.shengliensis* lipase

The amplification of 16S rDNA gene was achieved by PCR amplification of genomic DNA with universal 16S rDNA bacterial primers; forward 27F (5´-AGTTTGATCCTGGCTCAG-3´) and reverse 1492R (5´-GGTTACCTTGTTACGACTT-3´), under conditions described under

methods (**Figure 3.24**). The obtained PCR product of the 16S DNA was sequenced and the sequence was aligned against bacterial DNA data base and the bacterium was identified to species and genus level as *Halomonas shengliensis*.

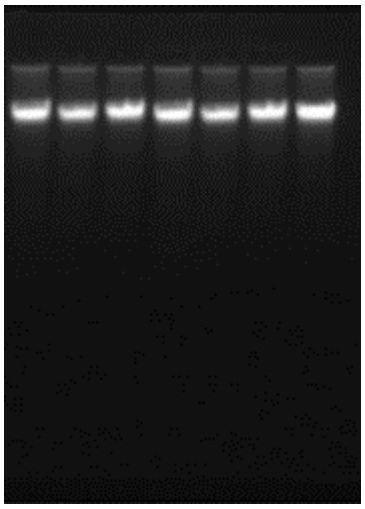


Figure 3.24: Genomic DNA isolated from *H.shengliensis.* (Lane 1 to Lane 7).

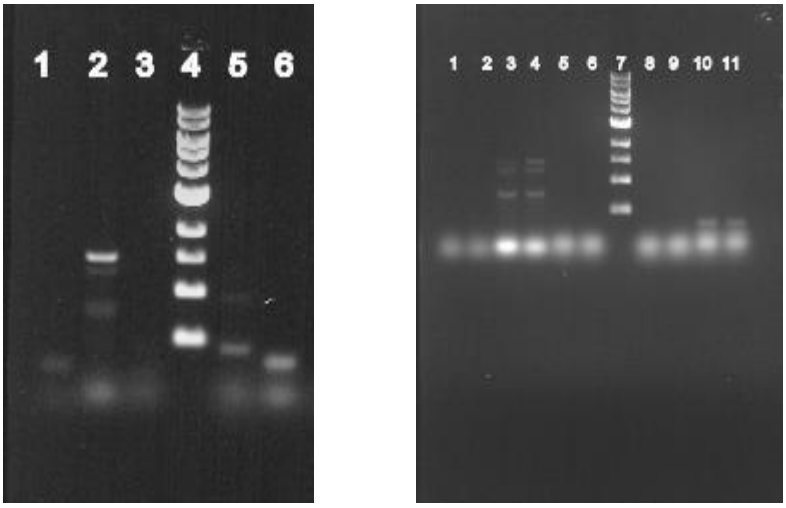
The primers designed for genes of lipases, are mentioned in **Table 3.2** (to clone into pLATE31 vector).

Table 3.2: Sequence of the primers designed for *H.shengliensis* lipases

1	Lysophospholipase 1	Forward primer	5’-AGAAGGAGATATAACTATGCAGCGACTTGATGGGTTCAGCCTAGACGATCTCGAGGTGGC-3’
1	Lysophospholipase 1	Reverse primer	5'-GTGGTGGTGATGGTGATGGCCGGCCACTCCCGCAGCGAGGAAGCGGC-3'
2	Lysophospholipase 2	Forward primer	5'-AGAAGGAGATATAACTATGAGCGACTTCACCCCCCTACAGGCCCT-3'
2	Lysophospholipase 2	Reverse primer	5'-GTGGTGGTGATGGTGATGGCCTGGTGAGGTCTTCTCCACGGCGGCGC-3'
3	Acetyl esteraselipase	Forward primer	5'-AGAAGGAGATATAACTATGCCGGCAGCGGCCTGGTGGCGAGGCGCCCTGCTGCTG-3'

3	Acetyl esteraselipase	Reverse primer	5'-GTGGTGGTGATGGTGATGGCCGCGGTCCCTCCTTTGCCCAGAGCGGCCCAC-3'
4	Phospolipase carboxylesterase	Forward primer	5'-AGAAGGAGATATAACTATGAGCGAACCCGGTGAGCTGATCATCGATCCCCAGGGCGGCAC -3'
4	Phospolipase carboxylesterase	Reverse primer	5'-GTGGTGGTGATGGTGATGGCCGCGCGCCAGGCGCTGGCCCAGCCAGT-3'
5	Phospholipase A1	Forward primer	5'-AGAAGGAGATATAACTATGCGTGCCCCGCTTCGCCCCTCCGTCGCCCTCATCGCC-3'
5	Phospholipase A1	Reverse primer	5'-GTGGTGGTGATGGTGATGGCCGCGCAGCATGCCCGCTGGTCGGGG-3'

There was no amplification of any of the expected gene products (**Figures 3.25 A** & **B**) even after alterations of various parameters in the PCR cycles. This indicates that the isolated organism is a different strain from that for which the whole genome has been sequenced (NCBI Reference Sequence: NZ_FNIV01000001.1) (*Halomonas shengliensis* SL014B-85, strain CGMCC 1.6444). This sequence was used to design primers for gene amplification and hence it has not worked.



A **B**

Figure 3.25: 1% agarose gel electrophoresis. (**A**) Lane 1: PCR reaction for gene 1- 6µl, Lane 2: PCR reaction for gene 2- 6µl, Lane 3: PCR reaction for gene 3- 6µl, Lane 4: 1kB DNA ladder, Lane 5: PCR reaction for gene 4- 6µl, Lane 6: PCR reaction for gene 5- 6µl (**B)** Lane 1: PCR

reaction for gene 1- 6µl, Lane 2: PCR reaction for gene 1- 6µl, Lane 3: PCR reaction for gene 2- 6µl, Lane 4: PCR reaction for gene 2- 6µl, Lane 5: PCR reaction for gene 3- 6µl, Lane 6: PCR reaction for gene 3- 6µl, Lane 7: 1kB DNA ladder, Lane 8: PCR reaction for gene 4- 6µl, Lane 9: PCR reaction for gene 4- 6µl, Lane 10: PCR reaction for gene 5- 6µl, Lane 11: PCR reaction for gene 5- 6µl.

3.4 Conclusions

The data presented in this chapter shows that we have achieved the isolation, purification and enzymatic characterization of a novel thermoactive lipase from *H. shengliensis*. This enzyme can be economically produced at high level in the presence of olive oil as a carbon source. Its activity at high temperature, detergents and tolerance towards various organic solvents makes it a satisfactory and promising candidate for industrial applications. Stability studies shows that the lipase is stable at higher temperatures. The X-ray crystals obtained for lipase can be further sent for the collection of diffraction pattern to solve the structure. Method of cloning of the genes of lipases needs to be carefully optimized for further protein engineering and relevant experiments.

Chapter 4

Stability Study of Lipase

Stability studies of Lipase

4.1 Preamble

The determinants of native state stability in aqueous solutions are the amino acid sequence of the protein as well as the variable conditions of pH, temperature, and the concentration of salts and ligands. Although the native conformation is essential for activity, the conformational stability is remarkably low. The native state of most naturally occurring proteins is only about 5-15 kcal/mol more stable than its unfolded conformations. In this chapter, we carried out various experiments to study the stability of lipase. Chemical denaturation was carried out using chaotropic reagents i.e. GdnHCl and Urea, which denature proteins by forming hydrogen bonds. The process of denaturation was monitored using CD and fluoresence spectroscopy. Thermal denaturation, which disrupts the attractive forces, was monitored using CD spectroscopy and DSC. The change in structural conformation was measured and the process of denaturation of protein was thoroughly analysed. Acrylamide quenching was measured for native and denatured lipase to obtain data concerning the proximity of the Trp, Tyr & Phe to (+) or (-) charged groups. Comparative study of structural parameters was carried out to get an idea about the factors contributing vastly towards the thermostability of lipases.

4.2 Materials and Methods

GdnHCl, Urea, Acrylamide and Stains-all were purchased from Sigma–Aldrich (St. Louis, MO, USA). Phenol and Trypsin were purchased from HiMedia. All other chemicals and reagents

used were of analytical grade. All buffer solutions were filtered through a 0.22 µm Micro Syringe filter (Millipore, USA). The pH was checked with pHTestr® 3 pH-meter (Oakton).

4.2.1 Chemical denaturation of lipase

4.2.1.1 GdnHCl and Urea denaturation monitored by CD

8 M GdnHCl and 10 M Urea stock solutions were prepared in water and their molar concentrations were accurately determined by measuring the refractive index of the solutions. The urea stock solution was prepared fresh every time just before the experiment and used within 24 hrs. 0.5mg/ml concentration of lipase in 25 mM phosphate buffer (pH 7.5) was incubated in presence of different concentration of guanidine hydrochloride (GdnHCl) or Urea at 37°C for 12 h. Circular dichroism (CD) spectral change of the protein after GdnHCl/Urea treatment was measured in the far UV region (200 nm to 250 nm) in a Jasco J-715 spectropolarimeter, equipped with a constant temperature cell holder. Temperature was controlled and monitored with a water bath. The spectra were recorded in a 0.2 cm path length quartz cuvette, using a bandwidth of 1nm and a scan rate of 50 nm/min. Each spectrum was an average of three scans and corrected for buffer contributions.

For determination of C_m, equilibrium unfolding was monitored from the change in CD signal at 222 nm as a function of denaturant concentration. Both, urea and GdnHCl were used as chemical denaturants for unfolding the lipase.

4.2.1.2 GdnHCl and Urea denaturation monitored by Fluorescence

20 µg lipase in 25 mM phosphate buffer (pH 7.5) was incubated separately with different concentrations of GdnHCl (pH 7.5) or urea for 12 h at 37°C to examine the effect of the denaturants on the intrinsic fluorescence of lipase. Fluorescence emission spectra of the denaturant treated samples were recorded from 310 nm to 450 nm with excitation at 280 nm in a Cary Eclipse fluorescence spectrophotometer. The excitation and emission slit widths were both set at 5 nm and an averaging time of 0.1 s was used. Fluorescence spectra were corrected for the corresponding buffer and denaturant concentration.

4.2.1.3 Urea gradient polyacrylamide gel electrophoresis

Urea PAGE gel preparation, gel sandwich assembly set up, protein sample preparation, Loading and running the gel and processing of the gel, were done according to the protocol

described by (Goldenberg, 1996). Transverse urea-gradient gels were prepared with a gradient of urea (0 M-10 M) concentration perpendicular to the direction of electrophoresis (Creighton, 1979). A single sample was applied to the top of the gel and the protein was electrophoresed. The gel was prepared with the glass plates rotated 90° from the orientation used for electrophoresis, which requires minor modifications of most standard electrophoresis equipment. The major requirements are spacers to fit the sides of the glass plates as oriented for casting and some arrangement for holding the glass plates in this orientation. After the run gel was rinsed with water and stained with CBB R-250 staining solution and destained. The pattern of protein denaturation by increasing concentrations of urea was monitored from the gel.

4.2.2 Thermal denaturation of lipase

4.2.2.1 Thermal denaturation of lipase monitored by CD

0.5mg/ml lipase in 25 mM Na-Phosphate buffer (pH 7.5) was heated from 20°C to 95°C and then cooled down back to 20°C in a 0.2 cm path length cuvette placed in the Peltier controlled cuvette holder of the Jasco J-715 spectropolarimeter. The CD signal at 222 nm was monitored as a function of temperature during the heating as well as the cooling. A bandwidth of 1 nm was set for this experiment. The melt profile thus obtained was used to determine the Tm value. The far UV CD spectra of the native and denatured lipase were also recorded from 200nm to 250 nm at a scan rate of 50 nm/min. each spectrum was an average of three scans and corrected for buffer contribution. CD spectrum of the native lipase was recorded in far (200 nm to 250 nm) UV region. CD spectral change of the protein was measured in far (200 nm to 250 nm) UV region in a spectropolarimeter. The spectra were recorded in 0.2 cm (far UV) and 1 cm (near UV) path length cuvettes, respectively.

4.2.2.2 Thermal denaturation of lipase monitored by Differential Scanning calorimetry (DSC)

Thermodynamic parameters for the thermal denaturation of lipase was measured by DSC in a VP-DSC calorimeter from MicroCal Inc. (Northampton, MA) as described in (Das et al., 2007). 1mg/ml lipase in 25 mM Na-Phosphate buffer (pH 7.5) was subjected to heating from 20°C to 100°C at a scan rate of 60°C/h and the excess heat capacity was measured as a function of temperature. Data were fit using the ORIGIN software (MicroCal) supplied with the instrument, and the melting temperature (T_m), at which the excess heat capacity is maximum, was determined.

4.2.3 Proteolytic Cleavage of lipase by Trypsin

The purified lipase sample was subjected to proteolytic cleavage by trypsin at varying Trypsin:Protein ratios, namely, 1:500, 1:1 and 4:1. The lipase protein in 25 mM phosphate buffer, pH 7.5, was incubated with the specified concentrations of trypsin at 37°C. Aliquots from the digestion mixture were withdrawn at different time points and the reaction was stopped by adding PMSF. The digested samples were then subjected to SDS-PAGE to check the pattern of proteolysis.

4.2.4 Quenching of lipase fluorescence by Acrylamide

Quenching of protein fluorescence by Acrylamide was performed as described in (Möller and Denicola, 2002), for the purified lipase. A 1M stock solution of Acrylamide was prepared fresh just before the experiment. 20µg of lipase taken in a 1.0 cm path length quartz cuvette was titrated with increasing concentrations of Acrylamide in 25 mM Na-Phosphate buffer, pH 7.5 at 25°C. The fluorescence emission spectrum of lipase after each addition of acrylamide was recorded between 310 nm and 450 nm at an excitation wavelength of 280 nm using a Cary Eclipse fluorescence spectrophotometer (Agilent Technologies, USA). Excitation and emission slit widths were both set at 5 nm and an averaging time of 0.1 s was used. The background fluorescence that might arise due to the quencher was corrected by subtracting the corresponding blanks.

4.2.5 Detection of Glycosylation on *Halomonas shengliensis* lipase

4.2.5.1 By Stains-all staining on SDS-PAGE gel

The protocol for Stains-all was adapted from that described previously (Goldberg and Warner, 1997). After the run of SDS-PAGE, the gel was rinsed three times with 25% (v/v) isopropanol followed by washing in 30–50 ml of the same solution on a gel rocker for 10 min. The cycle of rinsing (three times) and washing were carried out for removal of all SDS, which, if present, would cause the precipitation of the Stains-all dye. Isopropanol was then replaced by 30 ml of Stains-all solution (30 mM Tris, 7.5% (v/v) formamide, 25% (v/v) isopropanol, adjusted to pH 8.8 with HCl, followed by addition of 0.025% (w/v) Stains: Staining with Stains-all and Silver all). Due to the photosensitivity of Stains-all, gels were analyzed immediately and kept in dark all the time.

4.2.5.2 Phenol-H_2SO_4 assay

The phenol sulphuric acid method (PHS) is was used to confirm the presence of glycan moiety on lipase. To 1 ml of 25 mM phosphate buffer containing 25 mM NaCl, pH 7.5 and 20 µg lipase, 1ml of 5 % (w/v) phenol was added followed by the addition of 5 ml concentrated sulphuric acid. The sample tubes were kept on ice while adding sulphuric acid. The mixture was incubated at room temperature for 20 min and then its absorbance was read at 480 nm and 490 nm for the detection of pentoses and hexoses, respectively.

4.2.6 Computational studies for the determination of factors involved in protein thermostability

4.2.6.1 Lipase structure prediction and evaluation

The amino acid sequence of *Halomonas shengliensis* lipase was retrieved from NCBI (Reference Sequence: WP_089677621.1). Multiple PDB structures were used as template for modeling of lipase structure by I-TASSER, which models the protein structure based on remote homology/fold recognition and also predicts protein function (Roy et al., 2010; Yang et al., 2015; Yang and Zhang, 2015). The obtained models had their quality analyzed using PROCHECK (assesses the stereochemical quality) (Laskowski et al., 2006). The best selected model was analyzed for polar contacts, salt bridges and electrostatic interactions and compared with the template structre (PDB ID: 4N5I).

4.2.6.2 Comparison of modelled lipase with other thermophilic lipases

The structure predicted by I-TASSER was compared with three structures of thermophilic lipases (1JJI, 2DSN, 3RLI) available in PDB. Comparisons of the number of salt bridges, electrostatic charges, H-bonds, solvent accessibility and π-cation interactions were considered.

4.2.6.3 Comparison of all thermophilic and mesophilic lipases (available in PDB)

A comparative study between structures of thermophilic and mesophilic lipases available in PDB was carried out. All structures of lipases were obtained from RCSB PDB (https://www.rcsb.org/) (categorized based on its origin i.e. from thermophilic or mesophilic microorganisms, **Table 4.2**). We explored the parameters like, solvent accessibility of each residues, presence of salt bridges, electrostatic interactions, number of H-bonds, hydrophobic interactions, % of secondary structure and number of different amino acid residues present in all the selected lipases. The intramolecular bonds in structures were calculated with the help of Discovery studio visualizer and Solvent accessibility of each residue was obtained from PDBePISA server

(https://www.ebi.ac.uk/pdbe/prot_int/pistart.html) (Krissinel and Henrick, 2007). It distinguishes buried residues from solvent exposed ones in 3D protein structure.

4.3 Results and Discussion

4.3.1 Chemical denaturation of lipase

Denaturation curves using urea and guanidine hydrochloride are a convenient method for estimating the conformational stability of a protein. Under physiological conditions, the equilibrium of most proteins hugely favors the native state, as a result of which, measuring the equilibrium constant for the folding/unfolding reaction is difficult. However, in the presence of chaotropic agents such as urea or guanidine hydrochloride, the folding/unfolding equilibrium is shifted towards the unfolded state and hence the equilibrium constant can be accurately measured, and an extrapolation can be made to physiological conditions.

4.3.1.1 Guanidine hydrochloride (GdnHCl) and Urea denaturation of lipase monitored by CD

Proteins exhibit distinct near and far UV CD spectra. Far UV CD can be used to assess quantitatively the overall secondary structure content of a protein and the different forms of regular secondary structure found in peptides. The near UV CD of proteins arises from the environments of each aromatic amino acid side chain as well as possible contributions from disulfide bonds, or non-protein cofactors which might absorb in this spectral region. Structural conformation of the protein determines the wavelengths and magnitudes of the ellipticity bands and hence these parameters serve as a useful index for protein unfolding. A well folded protein with a high degree of conformation show distinctive CD spectra which gradually disappear during its unfolding.

A number of factors can influence the near UV CD spectra of aromatic amino acids. A very important one is the rigidity of the protein, with the more highly mobile side chains giving lower intensities in the spectrum. Other factors include the environment of the aromatic residue in terms of hydrogen bonding, polar groups and polarizability. Each of the aromatic amino acids tends to have a characteristic wavelength profile: tryptophan shows a peak close to 290 nm with fine structure between 290 nm and 305 nm; tyrosine exhibits a peak between 275 and 282 nm (the fine structure at longer wavelengths may be obscured by that from tryptophan); phenylalanine gives sharp fine structure between 255 and 270 nm.

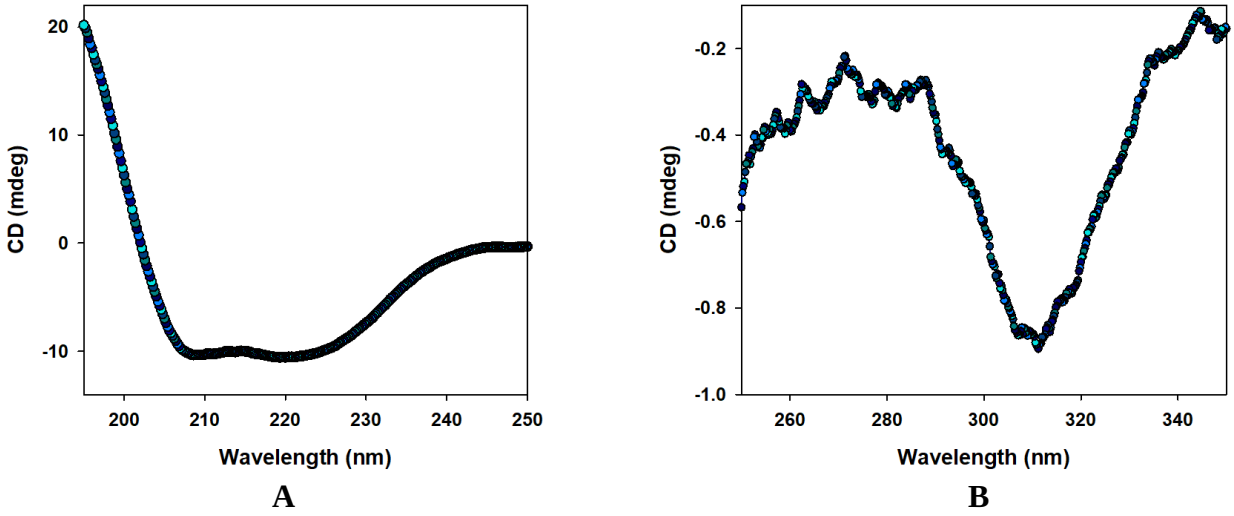


Figure 4.1: (A) Shows the far UV CD spectra of native lipase that was purified from *Halomonas shengliensis*. The far UV CD spectrum indicates that the protein has a well folded structure that is predominantly alpha helical. **(B)** shows distinctive near UV CD spectrum of native lipase.

CD was used for studying the secondary structure and conformational changes that occurred during urea or GdnHCl induced unfolding of the purified lipase. **Figure 4.2** shows the unfolding profile of lipase as the ellipticity at 222 nm at increasing concentrations of GdnHCl. The profile clearly shows two phases of unfolding of the protein. The C_m values for the two phases were 0.7 M GdnHCl and 3.0 M GdnHCl, respectively. This may indicate the presence of two domains in the lipase protein, which are independently folded. A small increase in negative ellipticity at 222 nm was observed when the lipase was treated with low concentrations of GdnHCl, viz. 0.1 – 0.2 M. This may indicate an intermediate more structured and compact intermediate induced by low concentrations of the denaturant. There are reports of such intermediates induced by low concentrations of GdnHCl (Shirley, 1995; Das and Dasgupta, 1998; Del Vecchio et al., 2002).

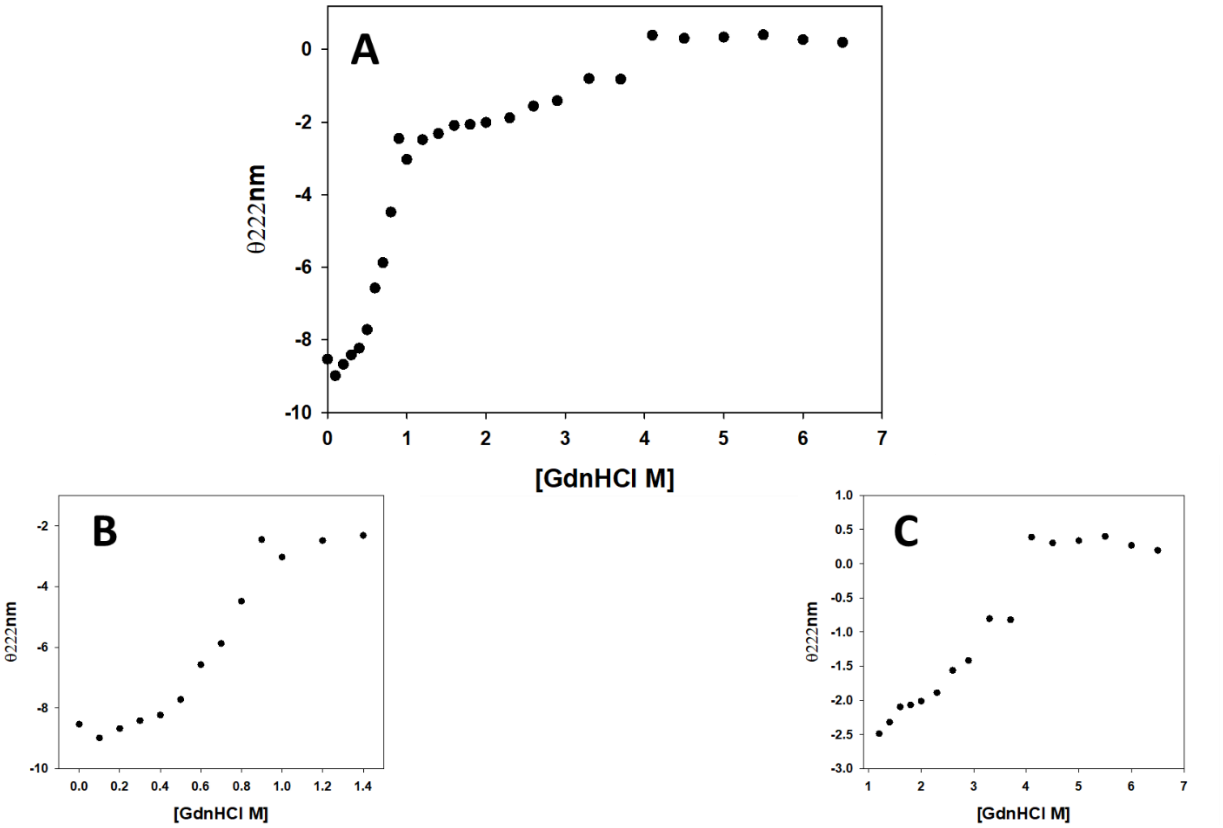


Figure 4.2: (A) Unfolding profile of lipase in the presence of varying concentrations of GdnHCl as monitored by Far UV CD. The ellipticity values of the lipase at 222 nm is plotted as a function of increasing concentrations of GdnHCl. The two phases of unfolding seen in the profile are separately depicted in the lower panels **(B)** and **(C)**.

In contrast to the unfolding profile by GdnHCl, the urea unfolding shown in **Figure 4.3 (A)** showed a single phase of unfolding with a C_m value of 4.0 M. The unfolding profile though showed an initial dip at low concentrations of urea, as seen in case of GdnHCl too. The protein exhibited a greater negative ellipticity at 222 nm in presence of low concentrations of urea up to 1.4 M, indicating an increase in helicity of the lipase under those conditions. It could be an intermediate compact state induced by lower concentrations of urea. The CD spectrum of the lipase in the presence of 1.4 M urea, shown in **Figure 4.3 (B)** also shows an increase in helicity. The spectrum of lipase in the presence of 8.5 M Urea shows a completely denatured protein, as expected.

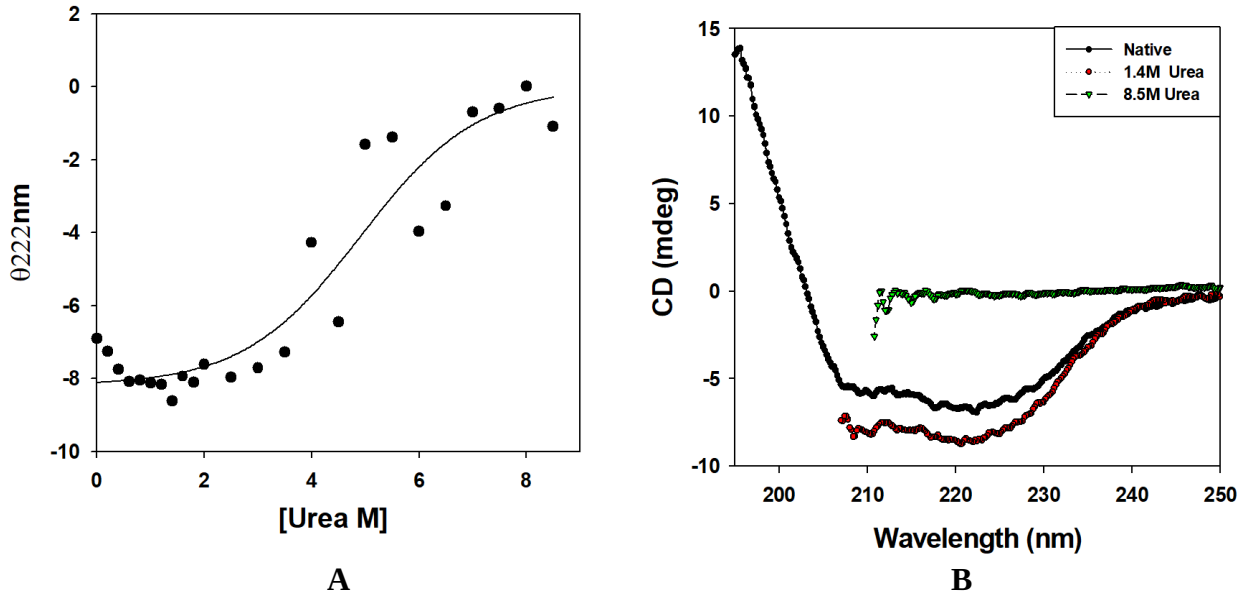


Figure 4.3: **(A)** Urea denaturation profile of lipase as monitored by far UV CD. The ellipticity values at 222 nm is plotted as a function of urea concentration. **(B)** Far UV CD spectrum of native lipase and lipase in the presence of 1.4 M and 8.5 M urea, respectively, after 12 hours of incubation.

4.3.1.2 Guanidine hydrochloride (GdnHCl) and Urea denaturation of lipase monitored by Fluorescence

The purified lipase has 7 tryptophan residues, 15 phenylalanine residues and 3 tyrosine residues and hence should have a good fluorescence quantum yield. The fluorescence emission spectra of the purified lipase upon excitation at 280 nm was recorded after treating the enzyme with varying concentrations of GdnHCl for 12 hours. The native enzyme has an emission maximum at 342 nm. As shown from the spectra of the protein at different concentrations of the denaturant in **Figure 4.4 (A)**, there was no change in emission intensity up to 3.0 M GdnHCl. The interesting feature that can be observed is that there is initially a blue shift of the emission maxima at low concentration of GdnHCl upto 0.5 M (spectrum shown only for 0.5 M GdnHCl in the figure for clarity) after which there was a gradual red shift with increasing concentrations of the denaturant, with the completely denatured protein in the presence of 6.5 M GdnHCl having a maximum at 357 nm, indicating the exposure of the fluorophores to highly polar aqueous environment. The constant emission intensity till 3.0 M GdnHCl was followed by a large increase in intrinsic fluorescence of the lipase with increasing concentrations of the chaotropic agent. The unfolding profile shown in **Figure 4.4 (B)** gives a C_m value of 3.7 M GdnHCl.

The initial blue shift in emission maximum is consistent with our observation of an increase in negative ellipticity of the protein when treated with low concentrations of GdnHCl, indicating a probably more compact structure of the lipase under those conditions. The increase in fluorescence upon denaturation of the enzyme indicates that the 7 tryptophans and the 3 tyrosines in the protein are highly quenched in the native state due to their environment. Hence the resultant emission intensity of the folded lipase is low. Upon denaturation and opening up of the structure at higher GdnHCl concentrations, these residues are dequenched and hence the quantum yield highly increases, as reflected by the huge increase in fluorescence intensity. Of course, the exposure of the fluorophores to highly polar aqueous environment upon unfolding causes quenching of their fluorescence, but the final emission intensity depends upon the relative balance of the two factors. The data indicate that the fluorophores are highly quenched in the native protein.

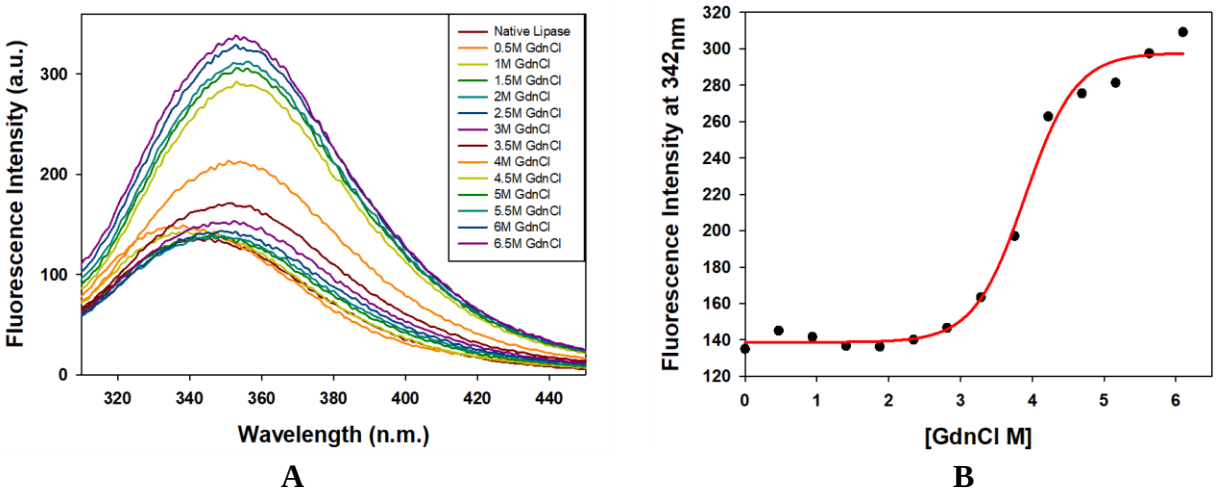
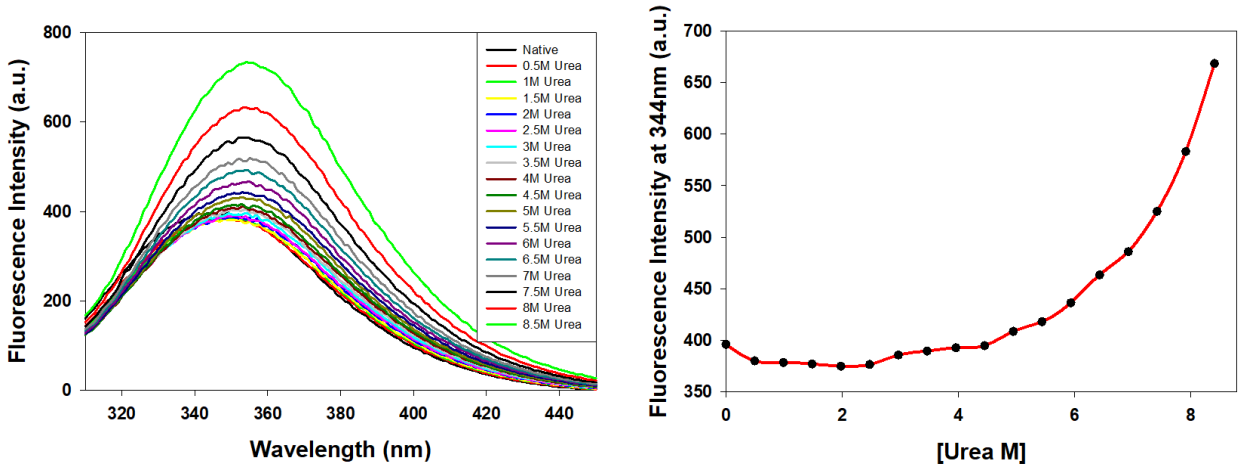


Figure 4.4: GdnHCl denaturation profile of purified lipase enzyme monitored by its intrinsic fluorescence. Excitation was at 280 nm and each spectrum is an average of three scans. **(A)** The intrinsic fluorescence spectra of lipase after treatment with varying concentrations of GdnHCl. **(B)** Fluorescence intensity at 342 nm as a function of GdnCl concentration.

Similar increase in fluorescence emission intensity was observed when lipase was treated with increasing concentrations of urea. The emission intensity was more or less constant up to 4.0 M urea, after which there is a huge increase in emission intensities **(Figure 4.5A)**. The initial blue shift in emission maxima observed with GdnHCl was not very prominent in case of treatment with urea. The red shift upon unfolding was observed in this case also, but the emission intensity did

not plateau out even upon treatment of the enzyme with 8.5 M Urea. Hence a C_m value could not be determined for urea denaturation of lipase.



A **B**

Figure 4.5: Urea denaturation profile of purified lipase enzyme monitored by its intrinsic fluorescence. Excitation was at 280 nm and each spectrum is an average of three scans. **(A)** The intrinsic fluorescence spectra of lipase after treatment with varying concentrations of urea. **(B)** Fluorescence intensity at 342 nm as a function of urea concentration.

4.3.1.3 Urea gradient gel electrophoresis

Urea-gradient gel electrophoresis was developed by Creighton (1979), as a simple alternative to spectroscopic methods for studying protein folding and unfolding. As the sample travels through the gel during electrophoresis, protein molecules at different positions across the gel are exposed to different urea concentrations as the polyacrylamide gel has urea gradient. Molecules that are unfolded have a larger hydrodynamic volume than the folded protein molecules and therefore migrate more slowly. After electrophoresis, the stained gel produces a graphic representation of the unfolding transition. The generated pattern provides information about the protein undergoing a urea-induced unfolding, information about the presence of significantly populated intermediate states in this transition, and semi-quantitative information about the net stability of the folded protein and the rates of interconversion between the native and unfolded states. The urea induced unfolding transition of the purified lipase obtained from the urea gradient gel electrophoresis is shown in **Figure 4.6.** As observed with the urea denaturation profile monitored by CD, there are indications of the formation of a more compact intermediate state in

the presence of low concentrations of urea up to 1.5 M. This more compact state has a lower hydrodynamic radius and thus greater mobility through the gel, as indicated by the initial dip in the profile.

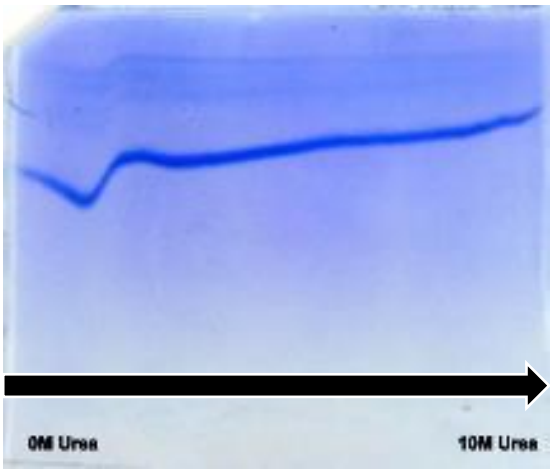
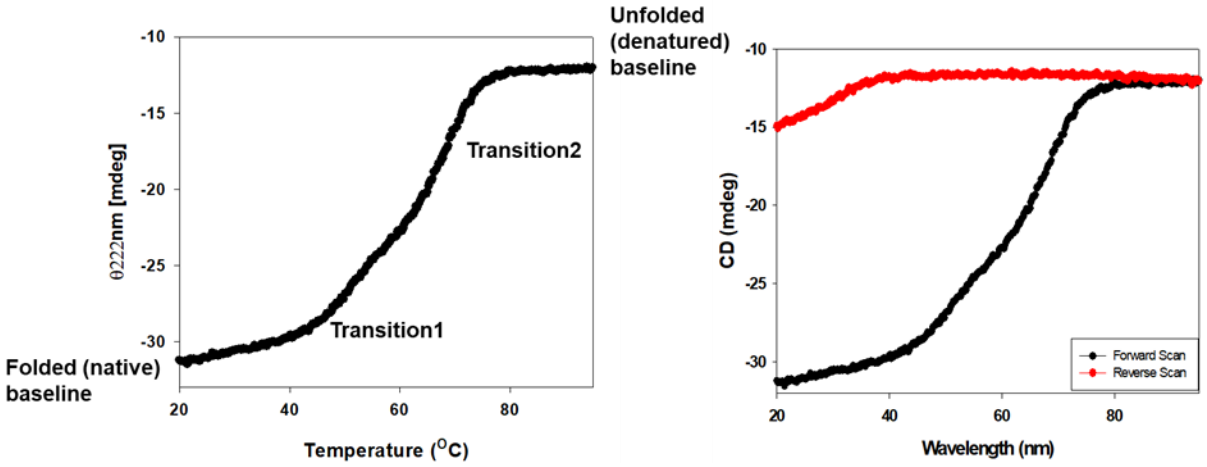


Figure 4.6: Urea induced unfolding profile of purified lipase monitored by urea gradient polyacrylamide gel electrophoresis. The urea concentration increases from 0M to 10 M from left to right. The gel was stained with Coomassie Brilliant Blue.

4.3.2 Thermal denaturation of lipase

5.2.2.1 Thermal denaturation of lipase monitored by CD

Figure 4.7 (A) shows the thermal denaturation profile of lipase as monitored by the change in ellipticity of the protein at 222 nm. As observed with the GdnHCl denaturation profile, the thermal melt profile also shows two phases of transition during unfolding. The Tm values for the two transitions were 50°C and 70°C, respectively. This also indicates the presence of two structural domains in the lipase enzyme. The reverse thermal melt shown in **Figure 4.7 (B)** indicates that the process of thermal denaturation was irreversible.

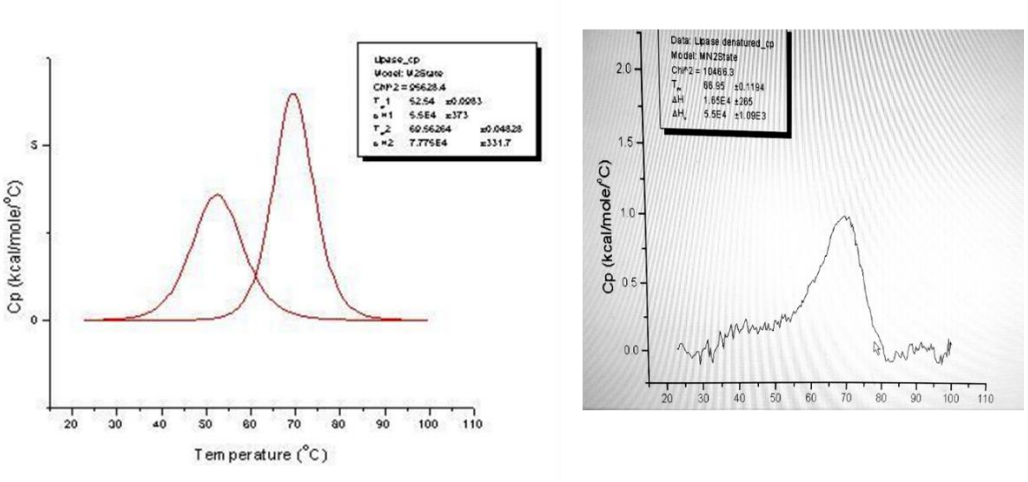


A **B**

Figure 4.7: Thermal melt of lipase monitored by far UV-CD. **(A)** The ellipticity at 222 nm is plotted as a function of temperature**. (B)** The reverse scan when cooled back to 20°C shows that thermal melt of lipase is irreversible.

4.3.2.2 Thermal denaturation of lipase monitored by Differential Scanning calorimetry (DSC)

Differential Scanning Calorimetry (DSC) is an analytical technique used for measuring the stability of proteins or other biomolecules directly in their native form. In this technique, the heat change associated with the thermal denaturation of the biomolecule when heated at a constant rate is measured. The DSC scan obtained for the purified lipase showed two different thermal transitions upon deconvolution **(Figure 4.8)**. The T_m values of the two transitions were found to be 52.54°C and 69.56°C, respectively. This clearly indicates the presence of two independently folding domains in the lipase, just as indicated from thermal denaturation profile of lipase monitored by far UV CD. After the first DSC scan, the sample was allowed to cool in the sample cell itself and then another scan was performed on it to check for the reversibility of thermal denaturation of lipase. The sample now showed only a single transition with a T_m value of 66.95°C. This may indicate that the first domain is irreversibly denatured by heat, while the second domain is thermally more resistant and the active site may be located in this domain as the enzyme shows high activity at high temperatures, with a temperature optimum of 70°C.

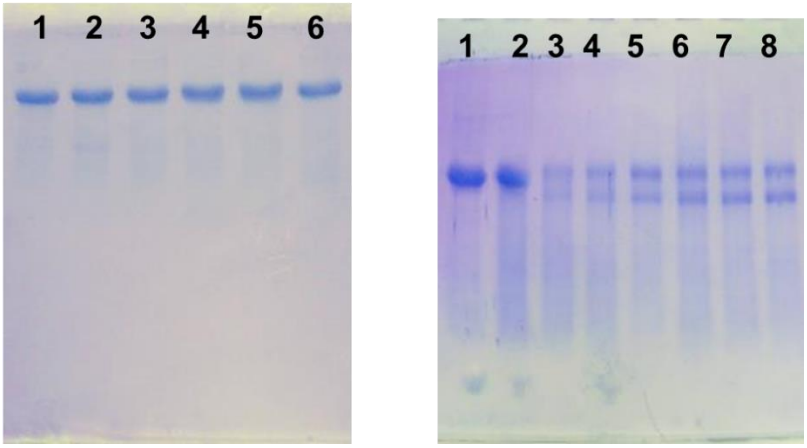


A **B**

Figure 4.8 (A): Differential Scanning Calorimetry (DSC) scan of lipase enzyme after deconvolution. **(B)** DSC scan of reheating of the once heated and cooled sample after the first scan.

4.3.3 Trypsin digestion of lipase

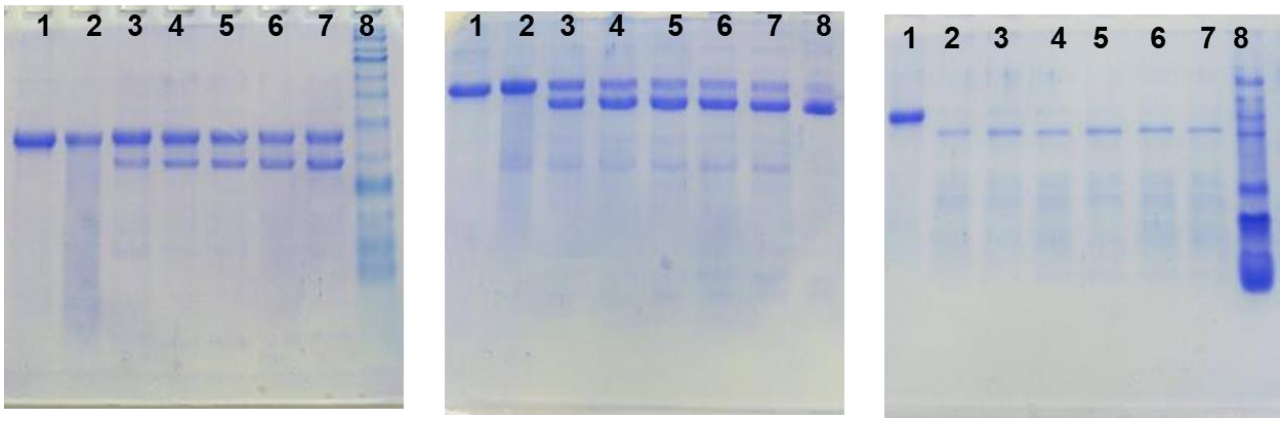
We carried out a limited proteolysis experiment on the lipase using a trypsin:lipase ratio 1:500. We observed that there was no cleavage of the enzyme even after 6 hours of incubation with the proteolytic enzyme as can be seen in **Figure 4.9 (A)**. Hence the concentration of trypsin was raised significantly to investigate the susceptibility or resistance of lipase to trypsin digestion. The experiment was done using trypsin:lipase ratios of 1:1 and even 4:1. At 1:1 ratio of trypsin to protein, the lipase started to show cleavage after 3 hours of incubation. Two bands were visible on SDS-PAGE: one of undigested protein and another of digested lipase of lower molecular weight. One of the two domains of the enzyme must be susceptible to cleavage while the other must be relatively resistant. As shown in **Figure 4.9 (B),** the two bands were visible even after three days of incubation, indicating that the lipase is considerably stable to trypsin cleavage.



A **B**

Figure 4.9 (A): SDS-PAGE of samples at different time points during limited trypsin digestion of lipase at **1:500** ratio of trypsin to protein. Lane1- 10µg native lipase, Lane 2- 0 min, Lane 3-1hour, Lane 4- 2 hours, Lane 5- 3 hours, Lane 6- 6 hours of digestion. **(B)** SDS-PAGE of samples at different time points during limited trypsin digestion of lipase at **1:1** ratio of trypsin to protein. Lane1- 10µg native lipase, Lane 2- 0 min, Lane 3- 3 days, Lane 4- 24 hours, Lane 5- 12 hours, Lane 6- 9 hours, Lane 7- 6 hours, Lane 8- 3 hours of digestion.

Since 1:1 ration of trypsin to lipase failed to completely digest the enzyme even in three days, a higher concentration of the protease was used at a trypsin to protein ratio of **4:1**. The digested band of lipase was highly resistant to proteolysis by trypsin, with the band being visible even after 7 days of digestion as shown in **Figure 4.10**. These results indicate that the two structural domains of the lipase are separated by trypsin at a relatively higher concentration. The smaller domain is unstable and further digested, but the larger domain is considerably stable. Such an indication was also observed in the DSC scans of the lipase, whereby one of the domains were stable to denaturation.



A **B** **C**

Figure 4.10: SDS-PAGE of samples at different time points during limited trypsin digestion of lipase at **4:1** ratio of trypsin to protein. **(A)** Lane1- 10µg native lipase, Lane 2- 0 min, Lane 3-1 hour, Lane 4- 2 hours, Lane 5- 3 hours, Lane 6- 5 hours, Lane 7- 6 hours of digestion, Lane 8- Protein molecular weight marker (NEB). **(B)** Lane1- 10µg native lipase, Lane 2- 0 min, Lane 3- 7 hours, Lane 4- 8 hours, Lane 5- 9 hours, Lane 6- 10 hours, Lane 7- 12 hours, Lane 8- 24 hours of digestion **(C)** Lane1- 10µg native lipase, Lane 2- 2 days, Lane 3- 3 days, Lane 4- 4 days, Lane 5- 5 days, Lane 6- 6 days, Lane 7- 7 days of digestion, Lane 8- Protein molecular weight marker (NEB).

4.3.4 Quenching of lipase fluorescence by Acrylamide

Intrinsic fluorescence of proteins can be used not only to study ligand binding, but also to examine structural, physicochemical, and functional properties of polypeptides (Permyakov et al., 1982). When the change in intrinsic fluorescence of lipase when treated with different concentrations of GdnHCl was monitored, it was observed that there may be an intermediate, more compact conformational state in the presence of lower concentrations of the denaturant (**Figure 4.4 A**). This was indicated by an initial blue shift in the emission maximum. So further investigate whether such a compact intermediate is formed, we carried out acrylamide quenching of lipase fluorescence upon treatment with varying concentratios of GdnHCl. The Stern-Volmer plots are shown in **Figure 4.11.** The slope of the Stern-Volmer plot was found to be reduced at lower concentrations of GdnHCl. Indicating a more compact state where the fluorophores are less accessible to the quencher. At higher concentrations of GdnHCl, the slope increases suggesting an opening up of the lipase structure.

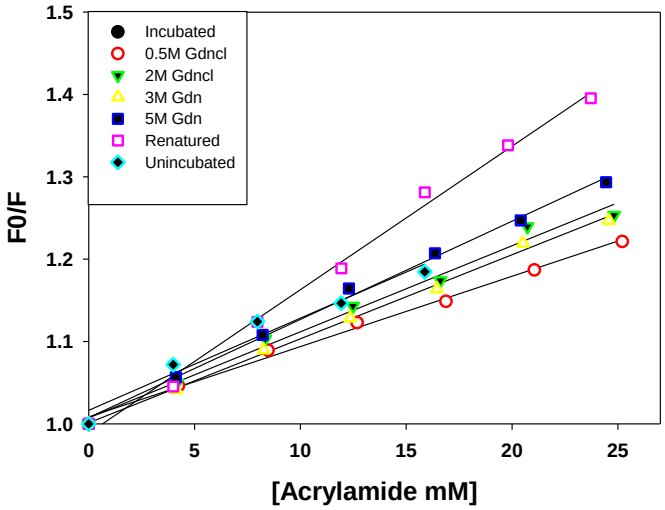


Figure 4.11: Stern-Volmer plot for quenching of lipase by acrylamide in the presence of different concentrations of GdnCl.

4.3.5 Detection of glycosylation in *Halomonas shengliensis* lipase

4.3.5.1 By Stains-all staining on SDS-PAGE gel

The lipase from *Halomonas shengliensis* showed many characteristics that led us to speculate the presence of glycan moieties on the protein. The anomalous migration of lipase on SDS-PAGE and higher thermostability might be due to glycosylation. Stains-all is a dye which can detect the presence of nucleic acids, anionic proteins, anionic polysaccharides, etc. It is used in SDS-PAGE, agarose gel electrophoresis and histologic staining. **Figure 4.12** shows the SDS-PAGE of lipase stained with stains-all. The lipase bands stained as blue bands. Stains-all stains glycoproteins on SDS-PAGE as blue bands when they have 60% or more of sialic acids and contain at least 10-100 ng sialic acid (King Jr and Morrison, 1976). The results clearly prove the presence of glycosylation in the lipase protein, although it is isolated from a bacterium. Glycosylation may thus explain the thermostability of the enzyme also.

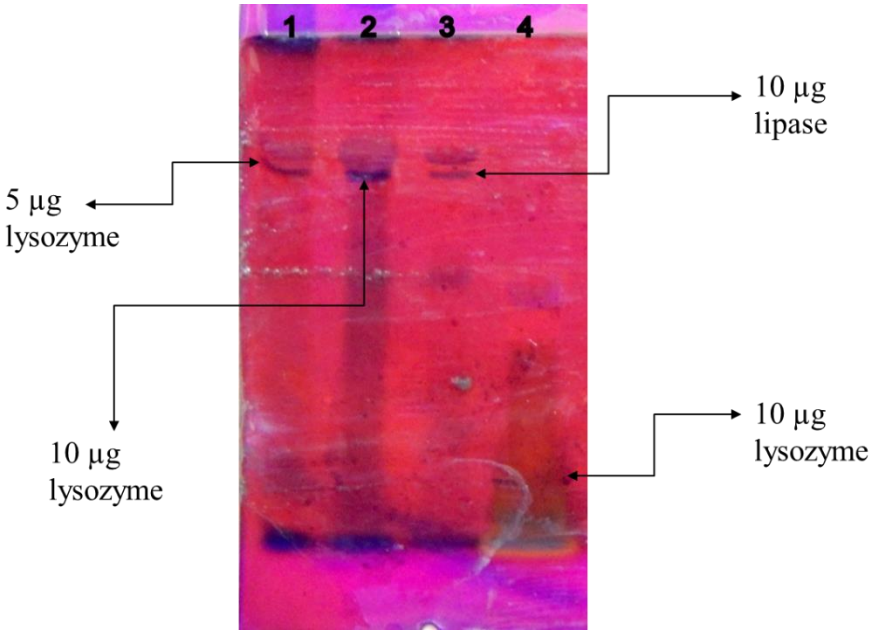
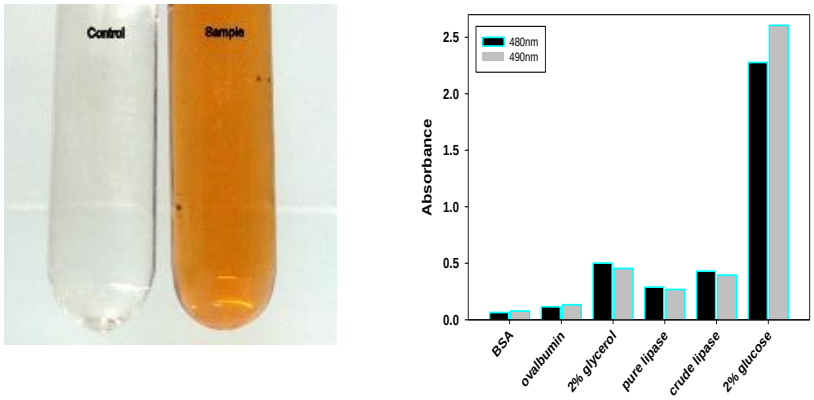


Figure 4.12: Stains-all staining of 12% SDS-PAGE for the in-gel detection of glycoproteins. (Lane 1: 5 µg lipase, Lane 2: 15 µg lipase, Lane 3: 10 µg lipase, Lane 4: 10 µg Lysozyme).

4.3.5.2 By phenol-H_2SO_4 assay

The phenol sulphuric acid method (PhS) is a great way to show the presence of glycan moieties. This method is based on a colorimetric product formed when phenol, sulphuric acid, and sugar react together and was first described by Dubois et al. in 1951. This assay is broadly applicable and measures hexoses and pentoses in a variety of oligosaccharides, making it useful for quantifying neutral sugars. It has been used for measuring neutral sugars in oligosaccharides, proteoglycans, glycoproteins, and glycolipids. This method is used widely because of its sensitivity and simplicity (Masuko et al., 2005). Exopolysaccharides were estimated as total carbohydrates by phenol-sulphuric acid method (Dubois et al., 1956). Concentrated sulphuric acid causes hydrolysis of glycosidic linkages, these hydrolysed neutral sugars are then partially dehydrated with the elimination of three molecules of water to form furfural or furfural derivatives. The colored compounds developed by the condensation of furfural or furfural derivatives with phenol are measured at 490 nm. As, shown in **Figure 4.13 (A)**, the sample of lipase when treated with PhS, developed orange color, as compared to no color developed in case of bovine serum albumin, a non-glycosylated protein. **Figure 4.13 (B)** shows the intensities of color developed by BSA, ovalbumin and lipase as compared to that developed by free glucose.



A **B**

Figure 4.13: (A) Qualitative determination of glycosylation by Phenol-H_2SO_4 test **(B)** Absorption of Phenol-H_2SO_4 treated samples at 480 & 490nm.

4.3.6 Computational studies for the determination of factors involved in protein thermostability

The *Halomonas shengliensis* genome consists five esterases/lipases. Here, we chose only one lipase as its predicted parameters are significantly similar with the lipase which we have studied experimentally. The protein sequence of the selected lipase is as follows:

>WP_089677621.1 Acetyl esterase [*Halomonas shengliensis*]
MPAAAWWRGALLLASLGLAGCASTHLDSDGREVVAHDIDMPNGEPETVRDLGELRYTP
DDWPEPLNARVLLPEGDDGSLRPAALLVHGGGWRNRTPDDMEAIAESLSRRGFVTLNIE
HRFAPEHRFPAQLHDLQQAMAWLHANATAWRVDTERVVGVGFSSGAHLVSLLALAGS
DGPLSSPHGGEHARLAAVVAGGTPTDLFKFDDGRLVVQFLGGTRAEVPEQYRLASPARQ
IPEAPPPFFLFHGTWDTLVPVDHATDFYQALRDQGGEAELFLQHGRGHFLSFLTRGSAID
AGLDFLERQVGRSGQRRDR

4.3.6.1 Lipase structure prediction and evaluation

The structure of this lipase protein was predicted by homology modelling. The predicted lipase structure **(Figure 4.14 A**) has an ordered, helical conformation that is in excellent agreement with template structures. Although *Halomonas shengliensis* lipase shares significantly low sequence similarity with its templates, it has an almost identical fold (**Figures 4.14 C and D**).

PROCHECK examines the overall stereochemical quality of a predicted protein structure. Ramachandran plots show the phi (ϕ) - psi (ψ) torsion angles for every residue of a polypeptide. Good quality models are required to have over 90% residues in the favored and allowed regions of the plot. The predicted lipase model can be considered to be of good quality as 80.4 % of the amino acid residues were found to be in the favored regions, 14.8 % residues were found in the allowed regions while only 4.8 % residues were found in outlier regions (**Figure 4.14 B**). We observed that predicted lipase has significantly higher proportion of salt bridges compared to one mesophilic template 4N5I **(Figure 4.15 A** and **B)**.

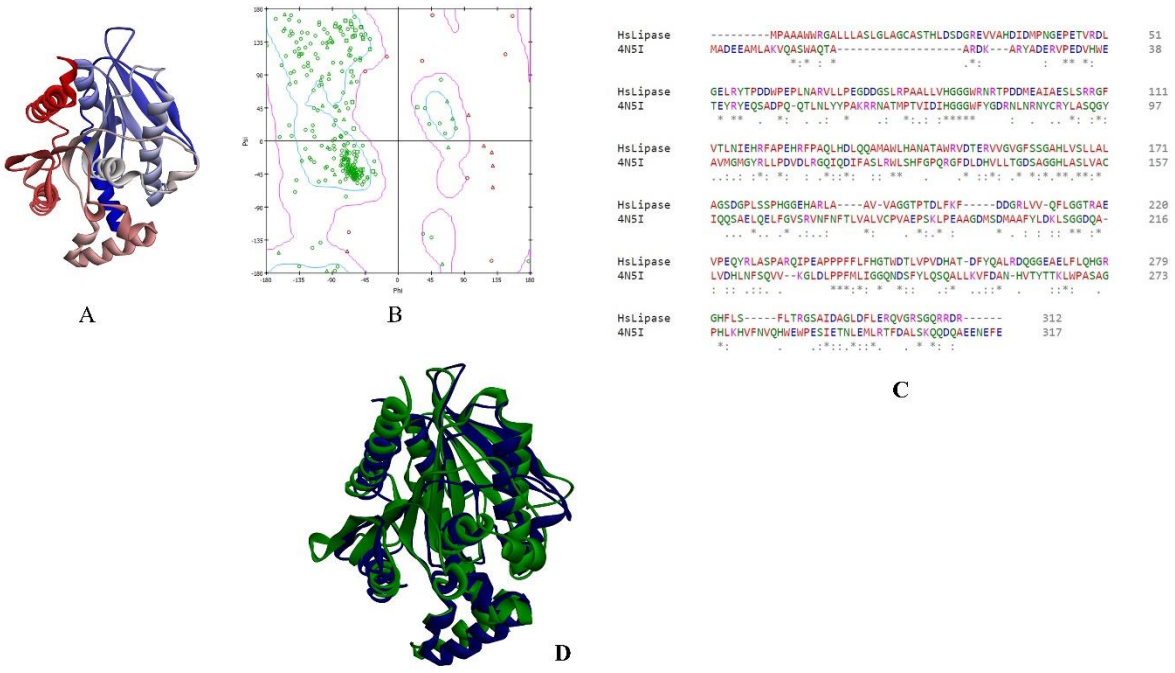


Figure 4.14: (A) Predicted structure of *Halomonas shengliensis* lipase using I-TASSER server. **(B)** Validation of the predicted lipase structure by Ramachandran plot. **(C)** Sequence alignment between predicted lipase and one of the templates 4N5I using Clustal Omega (Sievers et al., 2011). The obtained sequence similarity between predicted lipase and 4N5I is 23 %. **(D)** Superimposition of predicted lipase (Blue) with 4N5I (Green). The observed RMSD is 2.62 Å.

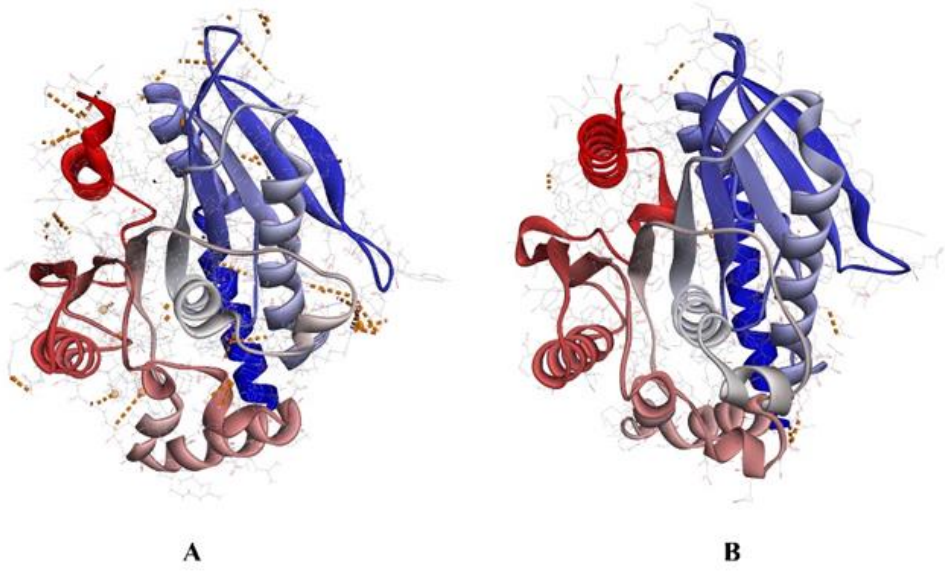


Figure 4.15: Representation of salt bridges in **(A)** predicted *Halomonas shengliensis* lipase (22.43 salt bridges per 100 residues) and **(B)** 4N5I (8.03 salt bridges per 100 residues). Salt bridges are shown as orange dashed line.

4.3.6.2 Comparison of modeled lipase with other thermophilic lipases

The modeled lipase structure was compared with other lipases. Structural parameters like the number of H-bonds, electrostatic charges, salt bridges and cation- π interactions were selected for the structural comparison, which are considered to be determinants of thermostability of a protein. **Table 4.1** lists the differences or similarities of the lipase from other thermophilic lipases with respect to these structural parameters. All the lipases compared have similar parameters. We noted that the predicted lipase has significantly higher proportion of salt bridges relative to one mesophilic template 4N5I.

Table 4.1: Comparison of structural parameters of modeled lipase with other thermophilic lipases.

Per 100 residues	**Lipase**	**1JJI**	**2DSN**	**3RLI**
Electrostatic Interactions	21.21	17.36	12.66	9.6

Salt bridges	11.2	7.7	4.9	1.8
No. of H-bonds	123	125	116	102
Cation-π interactions	1	0.3	1	1

1JJI: Hyper-thermophilic Carboxylesterase from the Archaeon *Archaeoglobus fulgidus* (De Simone et al., 2001).

2DSN: Thermoalkalophilic lipase from the *Geobacillus zalihae* (Matsumura et al., 2008).

3RLI: Monoacylglycerol lipase from *Bacillus* sp. H257 (Rengachari et al., 2012).

4.3.6.3 Comparison of all thermophilic and mesophilic lipases (available in PDB)

The structures of all thermophilic and mesophilic lipases available in the PDB were compared to each other with respect to various parameters (the list of selected lipases is given in **Table 4.2**). This was done to enumerate the common features that determine the thermostability of a protein. In this comparative study, we observed that proportions of charged residues such as Arg and Lys at exposed sites are relatively higher for thermophilic proteins as compared to their mesophilic counterparts **(Figures 4.16 & 4.17)**. Polar residues i.e. Glu, Gln and Thr contents were significantly lower in thermophilic proteins. The gain in Pro residues in thermophiles was significantly higher than their losses. At exposed sites, the large increase in charged residues is accompanied by a decrease in polar residues (Ser, Thr, His, Asn, Gln, Tyr). The overall high proportion of residues Met, Ala, Leu, Glu and Lys in thermophilic proteins suggests the high helix-forming propensities for thermophilic proteins. The results obtained showed similarity with a previous report that suggested that the relative percentages of Arg, Pro and Glu increase in going from mesophile> thermophile> hyperthermophile (Chakravarty and Varadarajan, 2002).

Table 4.2: List of experimental coordinates of thermophilic and mesophilic proteins obtained from PDB for comparative study.

Sr. No.	PDB ID	Title	Reference
Thermophilic			
1	1JJI	The Crystal Structure of a Hyper-thermophilic Carboxylesterase from the Archaeon *Archaeoglobus fulgidus*	(De Simone et al., 2001)
2	2DSN	Crystal structure of T1 lipase	(Matsumura et al., 2008)
3	4FMP	Crystal structure of thermostable, organic-solvent tolerant lipase from *Geobacillus sp. strain ARM*	(Nisbar, 2013)
4	5O7G	The crystal structure of a highly thermostable carboxyl esterase from Bacillus coagulans	(De Vitis et al., 2018)
5	5H6B	Crystal structure of a thermostable lipase from Marine *Streptomyces*	(Zhao et al., 2017)
6	6A12	X-ray structure of lipase from *Geobacillus thermoleovorans*	(Moharana et al., 2019)
7	5XKS	Crystal structure of monoacylglycerol lipase from thermophilic *Geobacillus sp. 12AMOR*	Wang and Lan, 2018 (To be published)
8	5LK6	Crystal structure of a lipase carboxylesterase from *Sulfolobus islandicus*	Schwarz-Linnet et al., 2017 (To be published)
9	4FDM	Crystallization and 3D structure elucidation of thermostable L2 lipase from thermophilic locally isolated *Bacillus sp. L2*.	(Rahman et al., 2012)

Sr. No.	PDB ID	Title	Reference
Mesophilic			
10	5CRI	Wild-type *Bacillus subtilis* lipase A	(Nordwald et al., 2015)
11	1OIL	Structure of lipase	(Kim et al., 1997)
12	3RM3	Crystal structure of monoacylglycerol lipase from *Bacillus sp. H257*	(Rengachari et al., 2012)
13	2Z8X	Crystal structure of extracellular lipase from *Pseudomonas sp. MIS38*	(Angkawidjaja et al., 2007)
14	1EX9	Crystal structure of the *Pseudomonas aeruginosa* lipase complexed with rc-(rp,sp)-1,2-dioctylcarbamoyl-glycero-3-o-octylphosphonate	(Nardini et al., 2000)
15	3W9U	Crystal structure of Lipk107	Yuan, 2013 (To be published)
16	4BZZ	Complete crystal structure of carboxylesterase Cest-2923 from *Lactobacillus plantarum WCFS1*	(Benavente et al., 2013)
17	1QGE	New crystal form of *Pseudomonas glumae* (formerly *Chromobacterium viscosum* atcc 6918) lipase	(Lang et al., 1999)
18	4KRX	Structure of Aes from *E. coli*	(Schiefner et al., 2014)

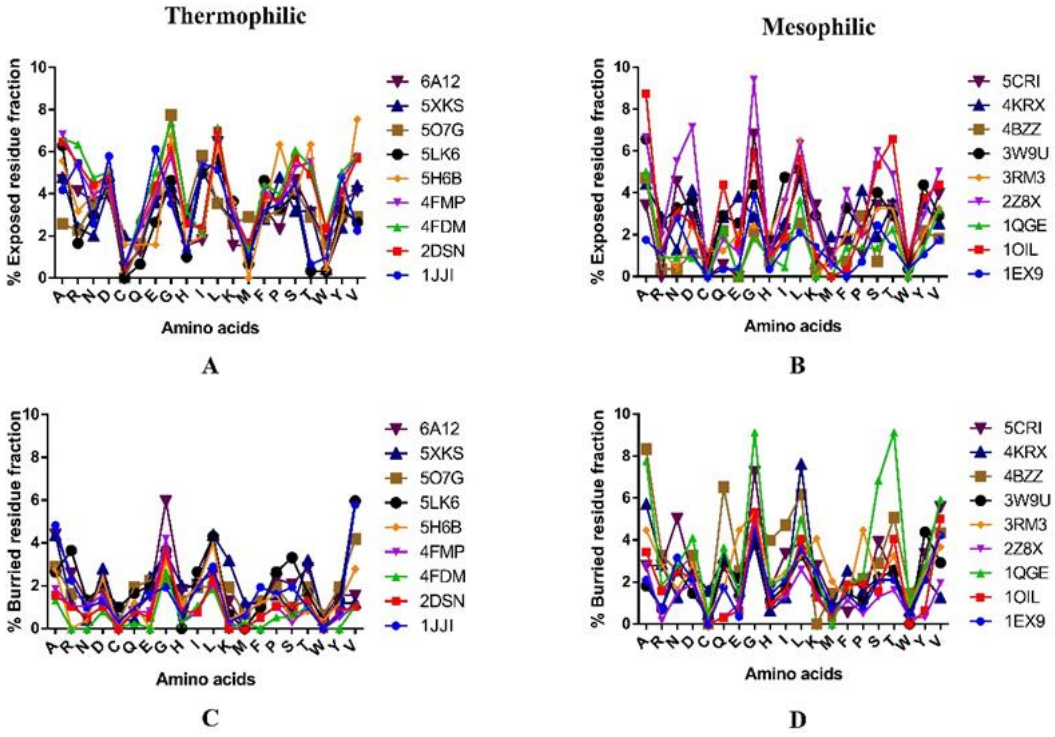


Figure: 4.16 Comparison of solvent accessibility of Lipases structures from Thermophilic and Mesophilic microorganisms. **(A)** and **(C)** represents the % exposed and buried residue fraction, respectively for lipases from thermophilic microorganisms. **(B)** and **(D)** represents the % exposed and buried residue fraction, respectively for lipases from mesophilic microorganisms.

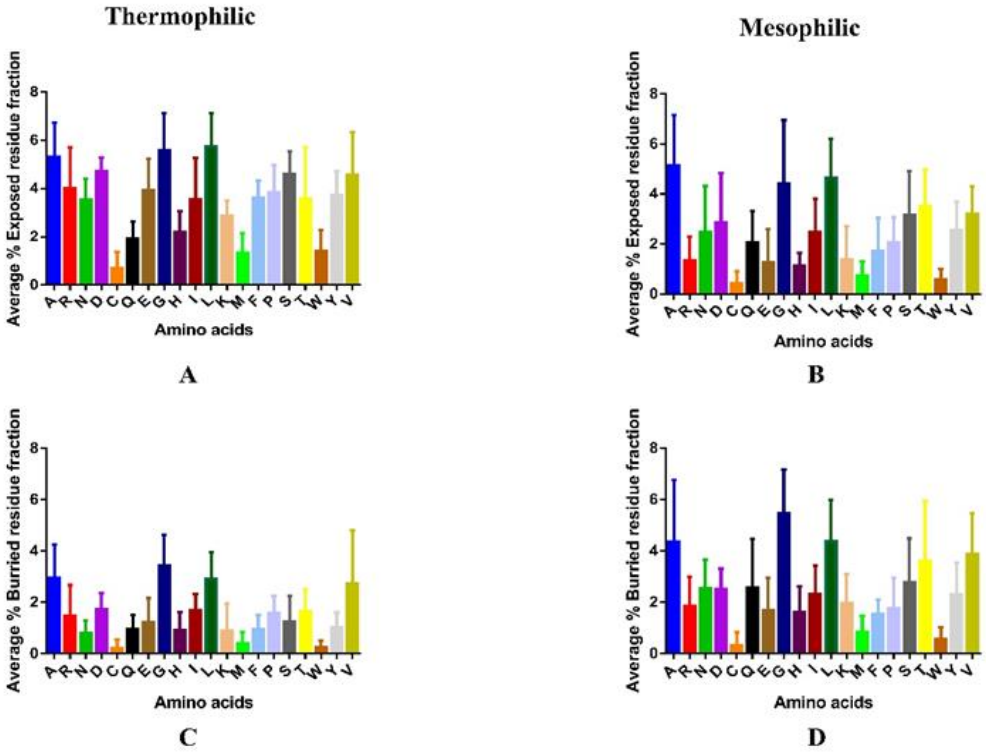


Figure 4.17: Comparison of average of solvent accessibility of Lipases structures from Thermophilic and Mesophilic microorganisms. **(A)** and **(C)** represents the average % exposed and buried residue fraction, respectively for lipases from thermophilic microorganisms. **(B)** and **(D)** represents the average % exposed and buried residue fraction, respectively for lipases from mesophilic microorganisms.

Table 4.3 Comparison of negatively and positively charged residues in thermophilic and mesophilic lipases.

Sr No.	PDB ID (Thermophilic)	% Arg	% Glu	% Negatively charged residues	% Positively charged residues	Sr No.	PDB ID (Mesophilic)	% Arg	% Glu	% Negatively charged residues	% Positively charged residues
1	1JJI	**7.7**	**7.7**	**14.8**	**10.93**	10	5CRI	**2.8**	**1.7**	**6.63**	**8.84**
2	2DSN	**6.5**	**4.9**	**10.85**	**9.3**	11	1OIL	**2.8**	**2.2**	**6.87**	**5**
3	4FMP	**6.3**	**4.7**	**10.76**	**9.71**	12	3RM3	**4.1**	**6.3**	**11.11**	**7.78**
4	5O7G	**4.1**	**5.8**	**13.04**	**8.7**	13	2Z8X	**2.1**	**2.1**	**11.18**	**6.32**
5	5H6B	**3**	**1.9**	**9.1**	**6.8**	14	1EX9	**3.9**	**3.9**	**8.07**	**6.32**
6	6A12	**6.7**	**5.4**	**11.05**	**9.51**	15	3W9U	**3.4**	**4.8**	**10**	**8.28**
7	5XKS	**3.5**	**5.9**	**12.9**	**9.37**	16	4BZZ	**3.5**	**2.5**	**6.74**	**5.32**
8	5LK6	**5.1**	**4.5**	**10.83**	**9.55**	17	1QGE	**2.7**	**1.4**	**6.3**	**4.95**
9	4FDM	**6.3**	**4.8**	**10.65**	**9.14**	18	4KRX	**5.7**	**5.1**	**11.71**	**8.11**

It has also been shown that surface salt bridges make a significant contribution to protein stability. The number of salt bridges in a thermophilic protein is expected to be relatively higher than in the corresponding mesophilic homolog. Salt bridges play an important role in stabilizing helices. An increased frequency of both exposed aromatic and positively charged residues in thermophiles suggests that cation-π interactions might have a significant stabilizing effect in enhancing thermal stability (Chakravarty and Varadarajan, 2002). From our comparative studies also, we found that thermophilic lipases show presence of higher number of salt bridges and higher number of electrostatic interactions (**Tables 4.4 & 4.5**).

Table 4.4: Comparison of Number of salt bridges per 100 residues in thermophilic and mesophilic lipases.

Sr No.	**PDB ID (Thermophilic)**	**No. of salt bridges per 100 residues**	**Sr No.**	**PDB ID (Mesophilic)**	**No. of salt bridges per 100 residues**
1	1JJI	**17.36**	10	5CRI	**7.26**
2	2DSN	**12.66**	11	1OIL	**4.06**
3	4FMP	**11.052**	12	3RM3	**10.44**
4	5O7G	**10.06**	13	2Z8X	**7.14**
5	5H6B	**5.16**	14	1EX9	**5.96**
6	6A12	**12.17**	15	3W9U	**5.94**
7	5XKS	**11.11**	16	4BZZ	**3.62**
8	5LK6	**13.24**	17	1QGE	**1.82**
9	4FDM	**11.11**	18	4KRX	**13.61**

Table 4.5: Comparison of number of electrostatic interactions per 100 residues in thermophilic and mesophilic lipases**.**

Sr No.	PDB ID (Thermophilic)	No. of electrostatic interactions per 100 residues	Sr No.	PDB ID (Mesophilic)	No. of electrostatic interactions per 100 residues
1	1JJI	**9.6463**	10	5CRI	**4.4693**
2	2DSN	**7.772**	11	1OIL	**3.125**
3	4FMP	**6.8421**	12	3RM3	**7.228**
4	5O7G	**6.7742**	13	2Z8X	**4.5528**
5	5H6B	**4.7809**	14	1EX9	**3.1579**
6	6A12	**6.7532**	15	3W9U	**4.2105**
7	5XKS	**7.1429**	16	4BZZ	**2.8986**
8	5LK6	**7.9734**	17	1QGE	**1.8265**
9	4FDM	**6.8966**	18	4KRX	**7.9365**

Table 4.6: Comparison of % secondary structure in thermophilic and mesophilic lipases.

Sr No.	PDB ID (Thermophilic)	% Helix	% Sheets	% Turns	% Coils	Sr No.	PDB ID (Mesophilic)	% Helix	% Sheets	% Turns	% Coils
1	1JJI	**56.91**	**25.72**	**5.15**	**12.22**	10	5CRI	**38.17**	**53.44**	**11.45**	**35.12**
2	2DSN	**30.75**	**47.27**	**8.3**	**13.7**	11	1OIL	**20**	**58.44**	**7.81**	**13.75**
3	4FMP	**37**	**32.28**	**8.92**	**21.79**	12	3RM3	**39.26**	**37.04**	**7.78**	**15.93**
4	5O7G	**29**	**40**	**9.57**	**21.45**	13	2Z8X	**30**	**23.5**	**12**	**34.5**
5	5H6B	**26.42**	**55.1**	**5.28**	**13.21**	14	1EX9	**35.09**	**27.37**	**11.23**	**26.32**
6	6A12	**48.7**	**63.94**	**10.41**	**21.56**	15	3W9U	**42.1**	**31.72**	**8.97**	**17.24**
7	5XKS	**44.58**	**37.08**	**7.5**	**10.83**	16	4BZZ	**36.88**	**48.23**	**4.26**	**10.64**
8	5LK6	**35.03**	**48.73**	**5.41**	**10.83**	17	1QGE	**20.72**	**57.66**	**6.31**	**15.32**
9	4FDM	**31.98**	**46.45**	**8.12**	**13.45**	18	4KRX	**36.64**	**48.35**	**6.31**	**8.71**

Table 4.7: Comparison of number of hydrophobic interactions per 100 residues in thermophilic and mesophilic lipases.

Sr No.	PDB ID (Thermophilic)	No. of hydrophobic interactions per 100 residues	Sr No.	PDB ID (Mesoophilic)	No. of hydrophobic interactions per 100 residues
1	1JJI	**59.4855**	1	5CRI	**50.2793**
2	2DSN	**52.3316**	2	1OIL	**34.6875**
3	4FMP	**49.7368**	3	3RM3	**53.8153**
4	5O7G	**50.6452**	4	2Z8X	**45.5285**
5	5H6B	**45**	5	1EX9	**37.5439**
6	6A12	**52.4675**	6	3WQU	**45.9649**
7	5XKS	**54.3651**	7	4BZZ	**57.971**
8	5LK6	**56.4784**	8	1QGE	**34.2466**
9	4FDM	**52.2546**	9	4KRX	**60.3175**

Table 4.8: Comparison of number of H-bonds per 100 residues in thermophilic and mesophilic lipases.

Sr No.	PDB ID (Thermophilic)	No. of H-bonds per 100 residues	Sr No.	PDB ID (Mesophilic)	No. of H-bonds per 100 residues
1	**1JJI**	**133.4405**	**10**	**5CRI**	**165.9218**
2	**2DSN**	**132.3834**	**11**	**1OIL**	**128.4375**
3	**4FMP**	**132.1053**	**12**	**3RM3**	**128.5141**
4	**5O7G**	**131.6129**	**13**	**2Z8X**	**113.6585**
5	**5H6B**	**111.5538**	**14**	**1EX9**	**135.0877**
6	**6A12**	**132.2078**	**15**	**3WQU**	**122.4561**
7	**5XKS**	**121.4286**	**16**	**4BZZ**	**113.7681**
8	**5LK6**	**117.608**	**17**	**1QGE**	**110.5023**
9	**4FDM**	**133.9523**	**18**	**4KRX**	**142.5397**

4.4 Conclusion

The new lipase enzyme characterized in this study is considerably stable at higher temperatures (having optimal activity at 70^{o}C) and also exhibited unusually high resistance

towards proteolysis by trypsin. The results obtained from the proteolytic cleavage experiments suggest the presence of two independently folding structural domains in the lipase protein. One of the two domains seems to be highly stable to proteolysis. Differential Scanning Calorimetry studies also confirmed the presence of two structural domains out of which one was relatively much more stable at higher temperatures. These results together indicate that one of the structural domains might be the vital contributing factor in over all higher stability of protein towards various parameters. Preliminary data indicate the presence of glycosylation in the lipase enzyme although its origin is a bacterium. The great thermal stability of the enzyme could be attributed to this feature.

Computational analysis of sequence and structural parameters of the lipase and their comparison with other mesophilic and thermophilic lipases suggest that the lipase isolated from a mesophile, possesses greater sequence and structural similarities with thermostable lipases isolated from thermophiles. Several sequence and structural factors have been proposed to contribute towards greater stability of thermophilic proteins. From the comparative study that we carried out here, we again observed that both thermophilic and mesophilic proteins have similar hydrophobicities, compactness, oligomeric states, main-chain and side chain hydrogen bonds, whereas, salt bridges and electrostatic interactions were higher in majority of the thermophilic lipases. The stability features of the lipase identified in this study makes it a potential candidate for use in industry.

Chapter 5

Isolation, Purification, Partial characterization and Cloning of Amylase from *Bacillus atrophaeus*

Isolation, purification, partial characterization and cloning of Amylase from *Bacillus atrophaeus*

5.1 Preamble

Amylases are among the most important commercial enzymes having potential applications in a wide number of industrial processes such as starch-processing, brewing, alcohol production, textile, food industries, fermentation, and pharmaceutical industries. α-Amylases can be obtained from plants, animals and microorganisms, However, enzymes from fungal and bacterial sources have dominated applications in industrial sectors (Hyun and Zeikus, 1985). The work presented here reports purification and characterization of amylase from *Bacillus atrophaeus*, which is an industrially well exploited bacterial species. The robust characteristics of the microorganism is the main reason to purify and characterize extracellular enzyme (amylase) secreted from it. The wild type amylase was cloned into *E.coli* (mesophilic host) to get rapid production of enzyme. The clone could be further used for generating thermostable variants by applying protein engineering methods such as directed evolution.

5.2 Materials and Methods

Bacillus atrophaeus and all microbial media supplements were purchased from HiMedia Laboratories Private Limited (Mumbai, India). DNSA and Sephadex G-75 were purchased from Sigma–Aldrich (St. Louis, MO, USA). Starch, Calcium Chloride, EDTA and Tris base and all

other salts and buffers were purchased from Merck. Restriction enzymes, PCR reagents and aLICator LIC cloning & expression kit were purchased from Thermofisher. All other chemicals and reagents used were of Analytical grade. All buffer solutions were filtered through a 0.22 µm Micro Syringe filter (Millipore, USA). The pH was checked with pHTestr® 3 pH-meter (Oakton).

5.2.1 Revival of bacterial strain and qualitative screening for amylase

Bacillus atrophaeus obtained from American Type Culture Collection (ATCC® 9372™) was revived in nutrient broth as specified by ATCC. It was grown on nutrient agar plates and glycerol stocks were prepared for the long-term preservation of the culture. Gram staining was performed to check morphological features of the strain. To screen extracellular amylase from *B.atrophaeus*, the cells were grown in nutrient broth till saturation and the cell free supernatant was obtained by centrifugation at 7500 rpm for 15 minutes. 50 µl of the cell free supernatant was poured into the wells bored in starch agar plates. The plates were incubated at 37°C for 12 h and then flooded with iodine solution to visualize the zone of hydrolysis.

Further confirmation of extracellular amylase production was done by Starch-iodine tube test. For the assay, three tubes were taken: 1) Negative control, 2) positive control, and 3) Cell free supernatant obtained from the experimental culture. 500 µl of 1% starch solution in 100 mM phosphate buffer, pH 7.0, was taken in each tube. 200 µl water was then added to the negative control tube, 200 µl pure standard amylase was added to the positive control tube and 200 µl cell free supernatant was added to the experimental tube. The mixtures were incubated at 37°C for 2 hours following which 0.5 ml of 0.1 % iodine solution was added in each tube to observe the change in color.

5.2.2 Optimization of media parameters for maximum production of amylase by the microorganism

Different media combinations containing starch as the sole carbon source were tried out to obtain maximum amylase production. The different media were inoculated with 1% inoculum of seed culture and the cells were allowed to grow in Erlenmeyer shake flasks at 35°C. Aliquots were removed from the growing culture at regular intervals to spectrophotometrically measure the cell density and determine amylase production at that specific time point. Cell free supernatant from each aliquot was tested for amylase activity by loading it into wells bored on

starch-agar plates. The plates were incubated at 35°C for 12 h followed by addition of iodine solution to the plates. The zones of hydrolysis were measured and compared to determine which medium gave the maximum amylase production. The media studied to assess the optimum amylase production were, Nutrient broth (growth medium for *Bacillus atrophaeus*), Nutrient broth containing 1% starch and Medium 4 (containing 1% starch, 0.5% peptone, 0.2% yeast extract, 0.5% $MgSO_4$, 0.05% NaCl and 0.015% $CaCl_2$).

The activity of the secreted amylase was determined using the 3,5-dinitrosalicylic acid (DNSA) reagent according to the method of Miller, 1959. DNSA reagent was prepared by mixing 1% DNSA, 1.0 M potassium sodium tartarate (Rochelle salt), and 0.4 M NaOH in ddH_2O. A standard curve was plotted with known concentrations of maltose. 500 µl of each sample was mixed with 1% starch solution in 0.1 M phosphate buffer, pH 7.0 and incubated at 37°C for 1 hour. The reaction was stopped by the addition of 1.0 ml of the DNSA reagent. After 5 minutes of incubation of the samples in a boiling water bath, the absorbance was measured spectrophotometrically at 540 nm.

5.2.3 Molecular weight determination of amylase by SDS-PAGE and zymography

10% Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed according to Laemmli (1970). An identical gel was prepared for zymography (Leber and Balkwill, 1997) to check the in-gel activity of amylase. Electrophoresis was carried out for 1.5 h at 50 mA at room temperature. CBB staining couldn't detect proteins as the enzyme concertation was low, hence silver staining (Chevallet et al., 2006) was used to visualize the amylases on SDS-PAGE for the determination of molecular weight.

5.2.4 Purification of the wild type amylase

For the purification of amylase, *B.atropaheus* was allowed to grow in optimized medium 4 for 48 hours in shake flasks at 35°C. The cells were removed by centrifugation at 4°C at 7500 rpm for 15 minutes. The proteins from the cell free supernatant were concentrated by ammonium sulphate precipitation (to 100% saturation) and dialysed against 25 mM phosphate buffer containing 25 mM NaCl, pH 7.0, to remove excess salt. The crude amylase obtained after dialysis was filtered through 0.22µm PES filter to remove any particulate matter, if present, before proceeding to load into the column for purification.

The column packed with Q-sepharose Fast Flow resin was equilibrated with five column volumes of equilibration buffer (25 mM phosphate buffer, pH 7.0, with 25 mM NaCl). To purify amylase from other secreted proteins, the crude sample was loaded into anion exchange matrix. Unbound proteins were washed out with the same buffer and then elution of the bound protein was carried out by step gradient from 25 mM NaCl to 1000 mM NaCl at a constant flow rate of 1ml/min. All the steps were carried out at 4°C. The column was washed with 4 M NaCl and stored in 20% ethanol at 4°C.

Size exclusion chromatography of amylase, recovered after anion exchange chromatography, was also carried out using Sephadex G-75 packed column. The column was equilibrated with 25 mM phosphate buffer, pH 7.0 containing 25 mM NaCl. The native protein was loaded, allowed to pass through the column and 0.5 ml fractions were collected while maintaining a steady flow rate of 0.1 ml/min. The collected fractions were assayed for amylase activity.

5.2.5 Determination of optimum temperature for amylase activity

The enzymatic activity of the purified amylase was measured at different temperature: 20°C, 30°C, 40°C, 50°C, 60°C, 70°C, 80°C and 90°C. The standard enzyme activity assay (DNSA method) was carried out at the specific temperatures at pH 7.0 in 25 mM phosphate buffer containing 25 mM NaCl. The optimum temperature for amylase activity was determined from the graph of amylase activity as a function of temperature.

5.2.6 Demonstration of optimum pH for amylase activity

Amylase was assayed at different pH values to obtain optimum pH for enzyme. The enzyme activity was measured by the standard assay method in different buffer systems such as citrate buffer (pH 3.0-6.0), phosphate buffer (pH 7.0-8.0) and glycine-NaOH buffer (pH 9.0-10.0). The reaction mixture for different pH were prepared using different buffers and the activity of the enzyme was measured by following the standard enzyme assay at 40°C. pH correction for different buffers was taken into consideration at given temperature.

5.2.7 Ligation independent Cloning of the genes of amylases

5.2.7.1 Genomic DNA isolation & Primer designing for genes of interest

The genomic DNA was isolated from actively growing culture of *B. atrophaeus* according to the method described in Short Protocols in Molecular Biology, Volume 1. The isolated DNA was treated with RNase A, to overcome contamination of residual RNA in next steps. PCR Primer sequences were designed as suggested in aLICator LIC Cloning and Expression Kit 3 (C-terminal His-tag, #K1261) manual.

5.2.7.2 Polymerase chain reaction (PCR)

From the purified genomic DNA, the genes of interest (BA553-1.6 kb and BA666-2.0 kb) were amplified using PCR.

PCR to amplify the genes of amylases was set up as follows:

10x amplification buffer	:	2.5 µl
20 mM solution of four dNTPs (pH 8.0)	:	1 µl
20 µM forward primer	:	1 µl
20 µM reverse primer	:	1 µl
Template DNA	:	0.5 µl (1 ng)
Taq polymerase	:	1.0 µl (1-2units)
Sterile MilliQ water	:	18 µl
Total volume	:	25 µl

PCR was carried out according to the following cycle:

Initial denaturation	:	95°C, 5 minutes
Final denaturation	:	94°C, 1 minute
Annealing	:	55°C, 30 seconds
Extension	:	72°C, 2 minutes 30 seconds
Final extension	:	72°C, 10 minutes
Number of cycles	:	35

PCR products BA553 and BA666 were cleaned using Nucleo-pore (SureExtract® PCR Clean-up/Gel Extraction Kit) and quantified. The amplified products were sent for sequence confirmation. Ligation independent cloning was performed for the purified genes (BA553 and BA666) with pLATE31 vector as recommended by the kit protocol.

5.2.7.3 Preparation of competent cells

A single bacterial colony (2-3 mm in diameter) (from *E.coli* DH5α) was picked from a plate and transferred into 20 ml LB medium. The culture was incubated for 2.5 h at 37°C with vigorous shaking and transferred to 2 ml sterile, disposable, ice-cold microfuge tubes, the cultures were cooled to 0°C by storing the tubes on ice for 10 minutes. The cells were recovered by centrifugation at 2700g (4100 rpm) for 10 minutes at 4°C. The supernatant was decanted and cell pellets were air dried. Each pellet was resuspended in 30 ml ice-cold filter sterile 85 mM $MgCl_2$-$CaCl_2$ solution. The cells were recovered by centrifugation at 2700g (4100 rpm) for 10 minutes at 4°C. The supernatant was discarded and the pellet was resuspended in 2 ml filter sterile ice-cold 0.1 M $CaCl_2$. The cells obtained were used for transformation (Short Protocols in Molecular Biology, Volume 1).

5.2.7.4 Transformation of product in *E.coli* DH5α

To transform the $CaCl_2$ treated cells, 200µl from each suspension of competent *E.coli* DH5α cells was transferred to a sterile, chilled microfuge tube using a chilled micropipette tip. 5µl of the vector-product mixture (for BA553 & BA666) was added to the tube and contents were mixed by swirling gently. The tube was stored on ice for 15 minutes and transferred to a rack placed in a preheated 42°C circulating water bath for 90 seconds. The tube was transferred rapidly to an ice bath and allowed to chill for 10 minutes. 800µl of LB medium was added to the tube. The culture was incubated for 45 minutes in a shaker at 37°C to allow the bacteria to recover and to express the antibiotic resistance marker encoded by the plasmid. 100µl of transformed competent cells were transferred using spread plate method onto agar LB medium containing the appropriate antibiotic (final ampicillin concentration - 100µg/ml). The plate was stored at room temperature until the liquid had been absorbed and incubated at 37°C. (Short Protocols in Molecular Biology, Volume 1). For screening of transformants, 5-7 well separated colonies were selected, half of each colony was used for colony PCR (as described in kit manual) and other half was transferred into LB/Amp medium and allowed to grow overnight at 37˚C. Plasmid isolation from the *E.coli* DH5α cells was carried out by Nucleo-pore (SureSpin® Plasmid Mini Kit).

5.2.7.5 Restriction digestion analysis of plasmid

The isolated plasmids were subjected to double digestion with EcoRI and HindIII (BA553), Xbal and HindIII (BA666).

RE digestion reaction set up was as follow: Solutions were added in the following order:

10X Restriction enzyme buffer	:	2 µl
Sterile distilled water	:	14.5 µl
Plasmid cloned with gene of interest	:	2 µl (1 µg)
Restriction enzymes	:	0.5 µl (10 U)

After mixing all the solutions properly, the reaction mixture was incubated at 37°C in a water bath for 1 h. The restriction enzyme (EcoRI/XbaI) was heat inactivated by incubating the tube at 65°C for 15 minutes. Then, 0.5µl (10 U) Restriction enzyme (HindIII) was added to the above reaction mixture and incubated at 37°C in a water bath for 1 h. The restriction enzyme was heat inactivated by incubating the tube at 65°C for 15 minutes. The protocol used for restriction digestion of the vector is as described in Short Protocols in Molecular Biology, Volume 1. The insert release was checked for both genes on 1% agarose gel. For further confirmation of successful cloning, the plasmids carrying genes of interest were used as the template for PCR amplification of genes.

5.2.8 Expression of amylase in *E.coli* BL21 cells

E.coli BL21 was transformed with the plasmid to get the expression host. Transformed BL21 cells were grown at 37°C overnight and inoculated into fresh LB/Amp medium (dilution ratio 1:50). The cells were allowed to grow at 37ºC in a shaking incubator (220-250 rpm) until the culture reaches an optical density OD600 of 0.5-0.6 (~2 h). To induce expression, IPTG to a final concentration of 1 mM was added and incubation was resumed for 3 h in a shaking incubator. 500µL of culture was harvested by centrifugation before induction and refrigerated at -20 °C to be used as an uninduced control. 250µL of induced culture was harvested by centrifugation for SDS-PAGE analysis. 12% SDS-PAGE was run to check to expression of induced protein.

5.2.9 Purification of recombinant amylase by affinity chromatography

The IPTG induced *E.coli* BL21 cells carrying the gene of interest were subjected to osmotic shock. 10 ml culture was grown overnight. Cells from 2.0 ml culture were pelleted down at 3500 rpm for 5 minutes at 4°C and media was dispensed. The pellet was frozen at -80°C. The frozen cell pellets were thawed by placing on ice for 10 minutes. 500 µl ice-cold hypnotic lysis buffer (20 mM HEPES, pH 7.4, 1 mM DTT, 100 µM PMSF, 1 µg/ml protease inhibitor K) was added on top of each cell pellet. The cell pellet was resuspended to homogeneity by slow, gentle pipetting and incubated on ice for 10 minutes. The sample was centrifuged for 5 minutes at 14000 rpm at 4°C. The supernatant comprised the cytoplasmic fraction. The cytoplasmic fraction was then sonicated for 5 minutes during which cells were incubated on ice and temperature was maintained. Number of short pulses (5-10 sec) with pauses (10-30 sec) were repeated for 5 times. All of the extracts were clarified by centrifugation of lysed suspension for 15 minutes at 20,000 x g at 4°C. The supernatants were recovered and then subjected to further purification. The protein (C-terminal 6xHis-tag) was purified by affinity chromatography using Ni-NTA column. It was allowed to bind to the resin for 2 h and eluted with gradient of 20 mM, 100 mM, 300 mM and 500 mM imidazole. Fractions were collected by gravity flow. 12% SDS-APGE was run to inspect purification pattern and the fractions containing pure enzyme were pooled together.

5.2.10 Confirmation of amylase activity

Amylase activity of the purified protein was assayed using the DNSA method. For DNSA assay 20 ml of 2 N NaOH was prepared. 1 g DNSA was dissolved in it with constant stirring. 30 g Na-K tartarate was dissolved in 50 ml distilled water, which was slowly poured into DNSA in NaOH solution to make volume upto 100 ml. The solution was filtered with Whatman filter paper grade 1. The absorbance was measured at 540 nm by UV-VIS spectrophotometer. (Cary UV-VIS spectrophotometer, Agilent technologies). 1 ml DNS solution was used for 10 ml reaction buffer. A standard curve was constructed for maltose and protein concentration was quantified by Bradford method (Bradford, 1976).

5.2.11 Demonstration of optimum temperature for recombinant amylase

The optimum temperature for amylase activity was determined by conducting enzyme assay at different temperatures ranging from 35°C to 80°C (35°C, 40°C, 50°C, 60°C, 70°C and 80°C), under standard assay conditions.

5.2.12 Demonstration of optimum pH for recombinant amylase

The optimum pH of the amylase was determined by assaying the enzyme using buffers of different pH. Assay was carried out at pH 3.0, pH 4.0, pH 5.0, pH 6.0, pH 7.0, pH 8.0, pH 9.0 & pH 10.0, under standard assay conditions.

5.3 Results and Discussion

5.3.1 Revival of bacterial strain and qualitative screening of amylase

Bacillus atrophaeus purchased from ATCC was inoculated into nutrient broth as per ATCC instructions. The bacterial cell density started to increase 12 hours after inoculation. Gram staining (**Figure 5.1**) confirmed that the mentioned strain is gram positive short rods with singular forms. Extracellular amylase secretion was confirmed with starch iodine tube test and starch iodine plate assay.

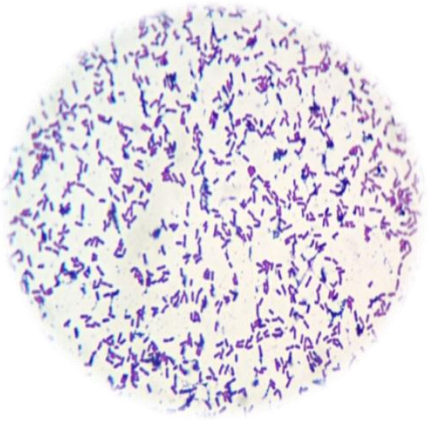
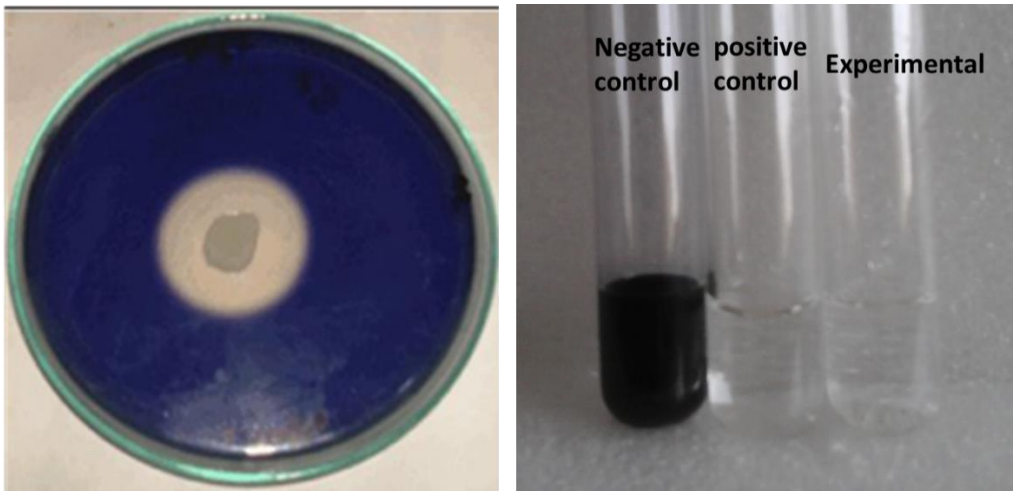


Figure 5.1: Gram staining of *Bacillus atrophaeus*.

Amylase can cleave the glycosidic linkages in starch to release individual glucose molecules. The development of blue color upon addition of iodine indicates presence of starch, while the absence of color indicates that the starch has been hydrolyzed. Clear zones of hydrolysis were obtained after incubation of cell free supernatant in bores in starch agar plate, thereby confirming amylase production by the organism (**Figure 5.2 A**). In Starch-iodine tube test, the negative control gave a dark blue color while the positive control and experimental tube did not show any color due to hydrolysis of the starch by amylase present in the cell free supernatant (**Figure 5.2 B**). These results reaffirmed secretion of active amylase by the *Bacillus atrophaeus* cells.

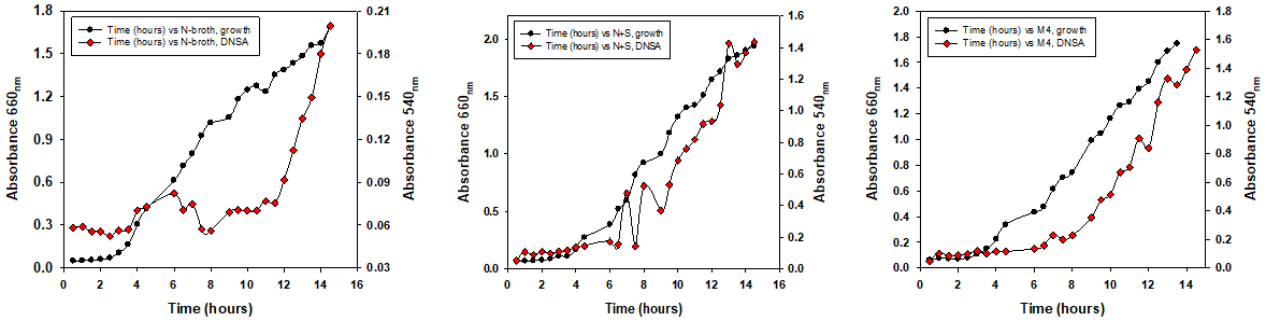


A **B**

Figure 5.2 (A): Zone of starch hydrolysis obtained around well in starch agar plate loaded with cell free supernatant of *Bacillus atrophaeus* culture, **(B)** Starch-iodine tube test showing change in color due to hydrolysis of starch in experimental tube containing cell free supernatant and positive control (a standard amylase), indicating extracellular amylase production by the organism.

5.3.2 Optimization of media parameters for maximum production of amylase by the microorganism

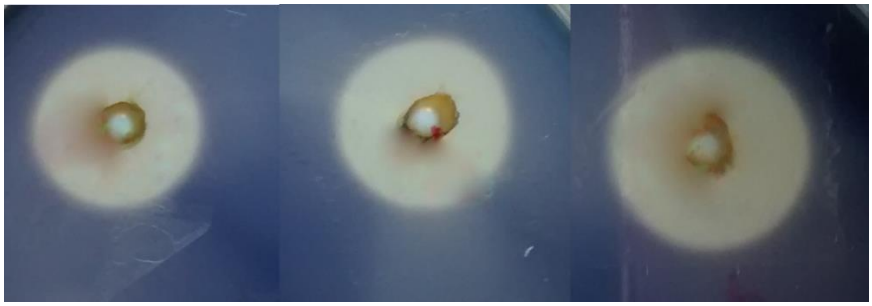
The growth curves of *Bacillus atrophaeus* and the corresponding amylase activity in the culture supernatant in three different media are shown in **Figure 5.3 A, B** & **C.** In all the media log phase of growth started 2 hours after inoculation.



A **B** **C**

Figure 5.3: Secretion of amylase during different phases of growth of *Bacillus atrophaeus* in Nutrient broth **(A)**, Nutrient broth with 1% Starch **(B)** and Medium 4 **(C)**.

Out of all tried media, Medium 4 was found to the most efficient medium for amylase production, giving the maximum amount of amylase. The zones of hydrolysis on starch-agar plates obtained from the cell free supernatants withdrawn from all three media at each time point were also measured and compared. As shown in **Figure 5.4, c**ells grown in Medium 4 gave the largest zone of starch hydrolysis after 48 hours of growth. The enzyme activity was also confirmed by DNSA assay.

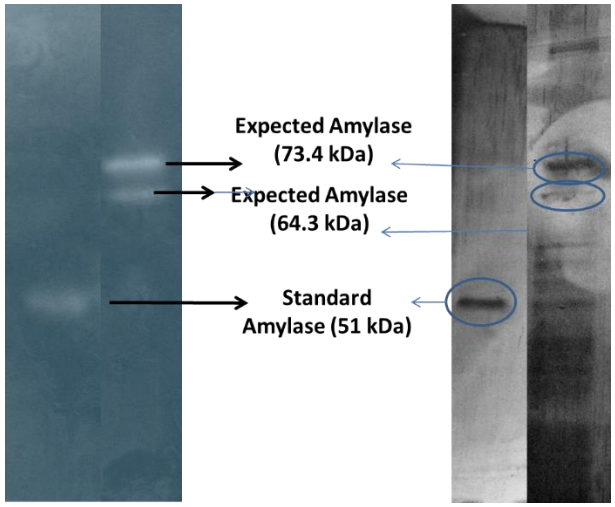


A **B** **C**

Figure 5.4: Starch-Iodine plate assay to check for the optimum amylase production in different media. The zones of hydrolysis by cell free supernatants of the three media, Nutrient broth **(A)**, Nutrient broth with 1% Starch **(B)** and Medium 4 **(C),** after 48 hours of growth are shown here.

5.3.3 Molecular weight determination of amylase by SDS-PAGE and zymography

The crude amylase was loaded into SDS-PAGE and zymography was also done. The SDS-PAGE was silver stained which showed several bands of protein. The amylase bands obtained in zymography corresponded to 73.4 kDa and 63.4 kDa bands seen in the silver stained SDS-PAGE and confirmed the expected molecular weight, when compared to standard amylase of 51 kDa (**Figure 5.5)**.

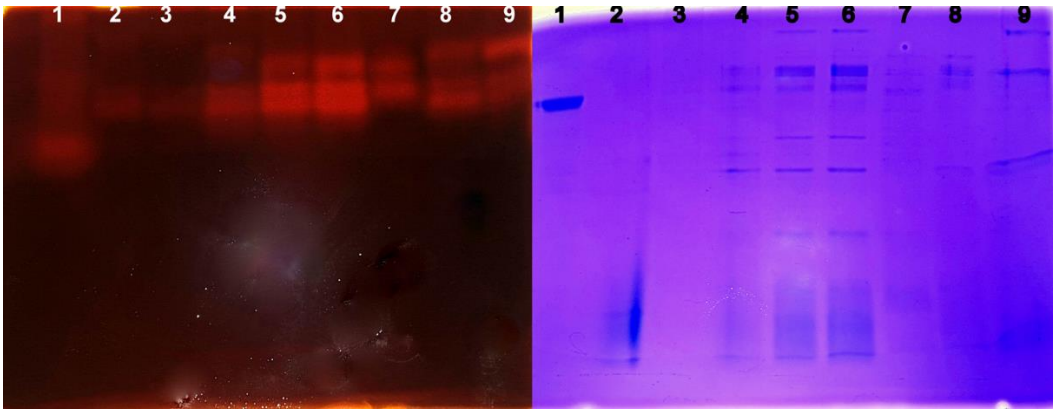


A **B**

Figure 5.5: **(A)** 10 % zymogram of amylases **(B)** 10% SDS-PAGE of amylases (silver staining).

5.3.4 Purification of the wild type amylase

To purify the secreted amylase, the crude sample (cell free supernatant) was loaded onto a Q-sepharose column (anion exchange chromatography). The column was equilibrated with five column volumes of equilibration buffer (25 mM phosphate buffer, pH 7.0, with 25 mM NaCl). The crude sample was loaded into anion exchange matrix. Unbound proteins were washed out with the same buffer and then elution of the bound protein was carried out by step gradient from 25 mM NaCl to 1000 mM NaCl at a constant flow rate of 1ml/min. All the steps were carried out at 4°C. 12% SDS-PAGE was run to assess the pattern of purification from all collected fractions. Separation of the 73.4 kDa amylase from the 63.4 kDa amylase was not achieved in ion exchange chromatography. Both amylases, when analysed, were found to have similar pI values.



A **B**

Figure: 5.6 (A) 12% Zymography (Lane 1: 5 µg standard amylase of 51 kDa, Lane 2: 100 mM NaCl fraction, Lane 3: 150 mM NaCl fraction, Lane 4: 200 mM NaCl fraction, Lane 5: 250 mM NaCl fraction, Lane 6: 300 mM NaCl fraction, Lane 7: 350 mM NaCl fraction, Lane 8: 400 mM NaCl fraction, Lane 9: 450 mM NaCl fraction) **(B)** CBB staining of 12% SDS-PAGE (Lane 1: 5 µg standard amylase of 51 kDa, Lane 2: 100 mM NaCl fraction, Lane 3: 150 mM NaCl fraction, Lane 4: 200 mM NaCl fraction, Lane 5: 250 mM NaCl fraction, Lane 6: 300 mM NaCl fraction, Lane 7: 350 mM NaCl fraction, Lane 8: 400 mM NaCl fraction, Lane 9: 450 mM NaCl fraction).

The fraction containing 350 mM NaCl was taken for further experiments because it showed maximum purity out of all other fractions, but two amylases were still not purified. So, we carried out manual size exclusion chromatography. The column was packed with sephadex-G75 and the flow rate was set constant at 0.1ml/minute. The fractions collected from size exclusion chromatography were further concentrated by ethanol precipitation and loaded into SDS-PAGE. It was observed that even after gel filtration chromatography, effective separation of the two amylases was not achieved. Hence, we carried out ammonium sulphate fractionation to try to separate two amylases. The protein mixture was precipitated out with increasing ammonium sulphate concentrations and activity from each fraction was assayed using starch-agar plate. The samples showing positive amylase activity were loaded to 12% SDS-PAGE. One amylase (63.4 kDa) was separated in the 70%-80% ammonium sulphate precipitated fraction, as observed in **Figure 5.7**.

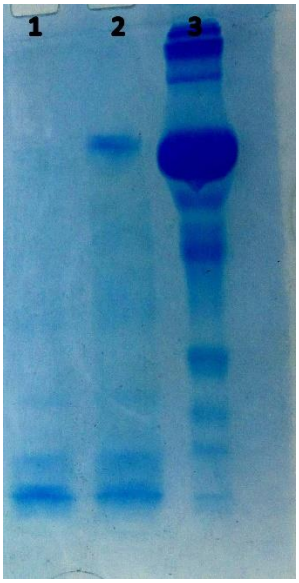


Figure 5.7: 12% SDS PAGE **(**Lane 1:40-50%ammonium suphate fractionated protein, Lane 2: 70-80% ammonium sulphate fractionated protein, Lane 3: BSA-66.4 kDa).

5.3.5 Determination of optimum temperature for native amylase activity

The purified 63.5 kDa amylase was assayed for its starch hydrolytic activity at several different temperatures ranging from 20°C to 90°C. The amylase activity as a function of temperature is shown in **Figure 5.8.** The optimum temperature of the amylase (63.5 kDa) was found to be 40°C as shown in the figure. All the assays were carried out in triplicate and the results shown are an average of the triplicate data.

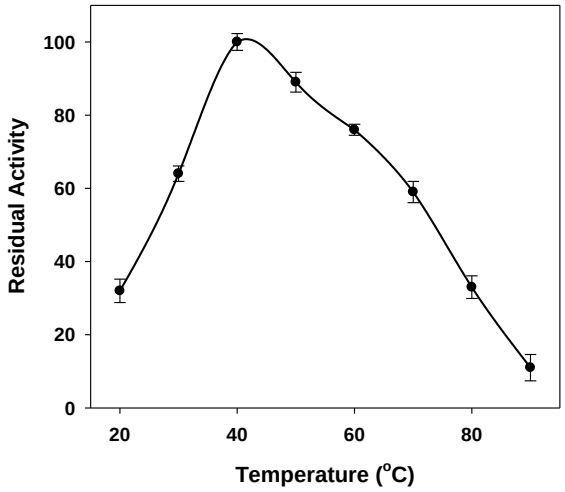


Figure 5.8: Relative activity of purified amylase as a function of temperature. The maximum activity obtained was taken as 100% and the remaining were calculated as a relative percentage of this value. All assay parameters were kept constant except for the temperature which was varied from 20°C to 90°C. Each point is an average of three individual experiments. The optimum temperature was found to be 40°C.

5.3.6 Determination of optimum pH for native amylase activity

The activity assay of the purified amylase was carried out at different pH using buffers of different pH. The optimum activity of amylase was observed at pH 5.0 (as shown in **Figure 5.9**) indicating that the amylase is an acidic amylase. All the assays were carried out in triplicate to reduce possible errors while handling and the data are an average of the triplicate readings.

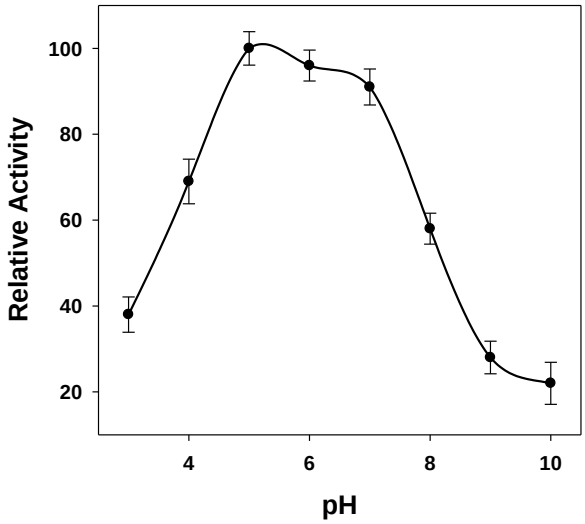


Figure 5.9: Relative Lipase activity as a function of pH (optimum pH 5.0). All the assay parameters except the pH were kept constant for all the assays and the curve obtained is an average of three independent experiments.

5.3.7 Ligation independent Cloning of amylases from *Bacillus atrophaeus*

5.3.7.1 Genomic DNA isolation & Primer designing for genes of interest

The genomic DNA isolated from *Bacillus atrophaeus* was run on 0.8% agarose gel electrophoresis (**Figure 5.10**). The reported genome size of *B.atrophaeus* is 4.13 Mb.

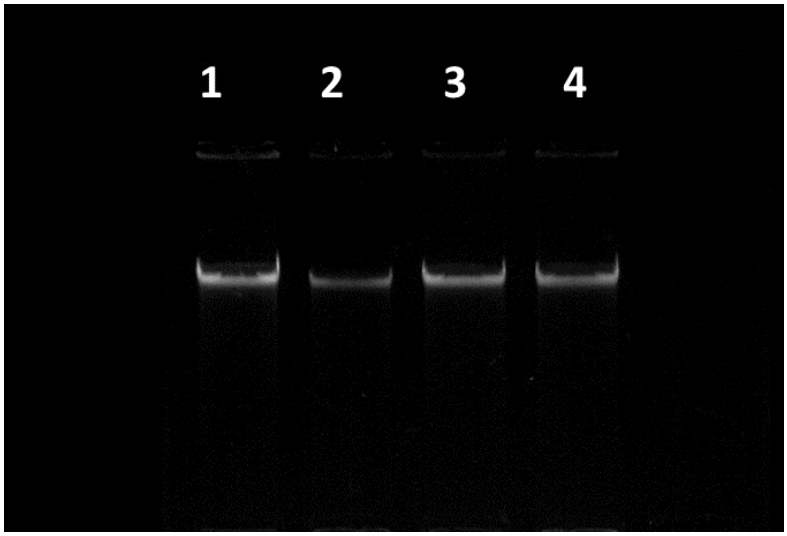


Figure 5.10: Genomic DNA Isolation from *Bacillus atrophaeus* (Lane 1-4 Genomic DNA of *Bacillus atrophaeus*).

The purified genomic DNA was used as template for PCR amplification of the two amylase genes of sizes 1.6 kb (BA553) and 2.0 kb (BA666). The primers designed for the respective genes to be cloned into pLATE31 vector are:

pLATE31 BA553 forward primer:

5'- AGA AGG AGA TAT AAC TAT GGA AAA GGC TTG GTG GAA AGA AGC -3'

pLATE31 BA553 reverse primer:

5'- GTG GTG GTG ATG GTG ATG GCC CCA TAC GTA TAT TCT TGT TTC G -3'

BA553 Amylase gene Sequence

ATGGAAAAGGCTTGGTGGAAAGAAGCGGTGGTATATCAAATTTATCCCCGCAGCTTC
AAGGATTCGAATGGGGATGGAATCGGCGATATCAACGGGATCAGATCAATGCTGCC
TTACATAAAAGATTTGGGGGCGGATGTCATCTGGATATGTCCGGTCTTTGACTCGCC
CAATGCGGATAACGGATATGATATAAGGGATTACAAAAAGATTTTAACTGAGTTTGG
AACCATGGATGATCTTGATAGCCTGCTTAATGAAATACACGAGCTTGGCATGAAGCT
GATTATCGACCTTGTCGTCAACCATACGAGTGATGAGCATCCGTGGTTCATTGAGTC
ACGCTCGGCCTTAGACTCCGAAAAAAGGGACTGGTACATCTGGAAAGATGGAAAAA
ACGGCAAAGAACCGAACAACTGGGAAAGCATTTTCAGCGGATCAGCCTGGCAGTAC
GATCAAACGACAGACCAATACTATCTCCATCTGTTTGACAAAAAACAGCCGGATCTG
AATTGGGAAAACCAAAACATGCGGTTTGCCGTTTATGAAATGGTGAATTGGTGGCTG
GACAAAGGAATTGACGGATTTCGGGTGGACGCCATTTCTCATATCAAGAAAAAGAA
AGGGCTTCCCGATCTGCCGAATCCCGAGGCTCTTCCATATGTCCCTTCTTTTCCGTAC
CATATGAACGTGGAAGGGATCATGGATTTTTTAAGAGAACTGAAAAAAGAAACGTT
TTCCCGCTATCCGATCGTGACAGTCGGAGAGGCGAATGGCGTTACGGCGGACGAAG
CAGCGGATTGGGCCGGAGGAAAATCCGGCATTTTTGATATGATTTTTCAATTTGAAC
ACCTTGGTTTGTGGGATATTGACGCAGATGAGCGCATTGATGTCGCGGAGTTGAAAA
GGATTTTGTCTAAATGGCAAAACAGTTTGGAAGGCGTAGGCTGGAACGCTCTCTTTA
TGGAGAATCACGATCAGCCCCGCTCGGTCTCCGCATGGGGAGACGATCAAACCTATG
TAACTGAAAGCGCAAAAGCACTCGCCGCTATGTACTTTTTGATGAAAGGCACCCCGT
TTATCTATCAGGGACAAGAAATCGGCATGACAAATGTGGCCTTTCCTTCAATTGAAG
ATTACGATGACGTGGCGATGAAACGGTTATACGATATAGAGACGGCCAAAGGGGTT
CCTCATAAAGAAATGATGAAGGTGATCTGGAAAAAGGGAAGAGATAACTCAAGAAC

TCCGATGCAATGGAATGAAAGCAAGTACGCAGGATTTTCGGATGCTCCCCCCTGGAT
CGGCATCAATGACAATTACACATGGCTGAATGCTAAATCACAAATGCAGGATAAAG
CATCCGTTTATCATTTTTATAAAAATCTAATCGCACTCCGACGAAAGTATGATGTCTT
TATTTATGGTTTGTACGATTTGCTTTTGCCGGAAGATAAACAAATCTTTGCCTACTTG
CGAAAGAGCGATCGGCAAACCGCGCTTATCCTGACAAACCTGACGAAGACACCCGC
TCTTTACAGACATCCCGCCTATCCGTTAAGCTCAGATTCGCTCGTCTTATCGAACATC
GAGACAACACATCATCAGCATACCACCTCAATCCTCTTACAACCTTACGAAACAAGA
ATATACGTATGGTAG

pLATE31 BA666 forward primer:

5'- AGA AGG AGA TAT AAC TAT GTT TAA AAA ACG ATT AAA TAC CTC TTT ACT GCC GTT ATT CG -3'

pLATE31 BA666 reverse primer:

5'- GTG GTG GTG ATG GTG ATG GCC ACG GAA TAT CAG -3'

BA666 Amylase gene sequence

ATGTTTAAAAAACGATTAAATACCTCTTTACTGCCGTTATTCGCTGGATTATTATTGC
TGTTCCATTTGATTCTGGCTGGGCCGGTGGCTGTAAATGCAGAGACCGCGAACCAAT
CAAATGAGTATACAGCGCCATCAATAAAGAGCGGAACCATTCTTCATGCTTGGAATT
GGTCGTTCAACACCTTAAAAAATAATATGAAGGATATCCATGATGCAGGATATACTG
CCATTCAGACGTCTCCTATTAATCAAGTAAAGGAAGGAAATAATGGAGATAAAAGC
ATGGGGAACTGGTACTGGCTTTACCAGCCGACCTCTTACCAGATCGGCAACAGGTAC
TTGGGTTCCGAGGAAGAGTTCAAAGAAATGTGTGCGACGGCTGAAGAATATGGTGT
GAAGGTTATTGTCGATGCCGTCATCAATCACACAACCAGTGACTATGCTGCGATTTC
AAATGAAATTAAAAGCATTTCAAATTGGACGCATGGAAACACACAAATTAAAAATT
GGTCCGATCGAAGGGATGTCACGCAGAATTCATTGCTGGGGCTTTATGATTGGAATA
CGCAGAATACACAAGTACAGTCATATCTAAAAAATTTCCTGAAAAGAGTATTGGATG
ACGGTGCCGACGGATTTAGGTATGATGCGGCAAAACATATAGAGCTTCCGGATGATA
GTGATTTTGGCAGTCATTTTTGGCCGAATATCACAGATACATCTGCAGAATTCCAATA
TGGAGAAATATTACAAGACAGTGCCTCCAGAGATGCTGCATATGCGAATTATATGAA
TGTCACAGCATCAAACTATGGGCATTCCATAAGGTCTGCTTTAGTGAATCGTAATTTT
AGTACGTCGAAAATCTCCAATTATAAATCTGATGTGTCTGCAGATGATCTAGTAACA
TGGGTGGAATCACATGATACGTATGCCAATGATGATGAAGAATCAACTTGGATGAGT

GATGATGATATTCGTTTAGGCTGGGCGGTTATTGCTTCTCGTTCAGGGAGTACACCTC
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CATTTCATAATGTAATGACTGGACAGTCTGAGAAACTCTCTAACCCAGATGGAAACA
ATCAGATATTCATGAATCAGCGCGGCTCTGATGGCGTAGTTTTGGCAAATGCATCTT
CATCATCAGTTTCTCTTCATACTTCAACGGATTTACCTGATGGCATCTATGATAATAA
AGCCGGGGACGGTTCGTTTGAAGTAGCGGATAGCAAACTGACAGGTATGATCAGCG
GCAGATCTGTGGTTGTTCTTTATCATGATGATACTGCCAATGCACCTAATGTATTCCT
CGAGAACTATAAAACAGGTGTAACACATCCTTTCTATAATGATCAACTGACGGTAAC
TTTGCGTGCAGATGCTAAAACAACAAATGCTGCTTATCAAATAAATAATGGTCATGA
GACAGAGTTTAAGGATGGAGATCAATTAACGATCGGAACAGGGGATCCATTCAACA
CAACATACAAGTTCACTTTAACAGGAACGAACAGTGAAGGAGTAACCAGGACAAAA
GAATACACTTTTGTAAAAACTGACCCATCTTCGGCGAACATAATTGGTTATCAAAAC
CCAAATAATTGGAGTCAAGTTAATGCATATGTATATAAGGAGAATGGTGGTCAGGCA
ATAGAATTAACCGGATTTTGGCCGGGAAAAGCAATGGCTAAGAATTCAGATGGAAT
TTACACTCTGACGTTACCCACGGGTACTGATACAAAAAACGCCAAAGTGATTTTTAA
TAATGGCAGCGCCCAAGTACCGGGACAGAATCAACCTGGCTTTGATTATGTACAAAA
TGGTTTATATCATGACTCTGGCTTAAATGGTTCTCTGCCTCATTTAGTCCTGATATTCC
GTTAA

5.3.7.2 Polymerase chain reaction (PCR)

The optimum PCR conditions were established by gradient PCR for the efficient amplification of the amylase genes (data not shown) and then subsequently used for amplification. PCR amplification of both amylase genes were successfully carried out. The amplified product was analyzed on a 1% agarose gel (**Figure 5.11**). The amplified genes were at the correct position in gel i.e. 1.6 kb and 2.0 kb.

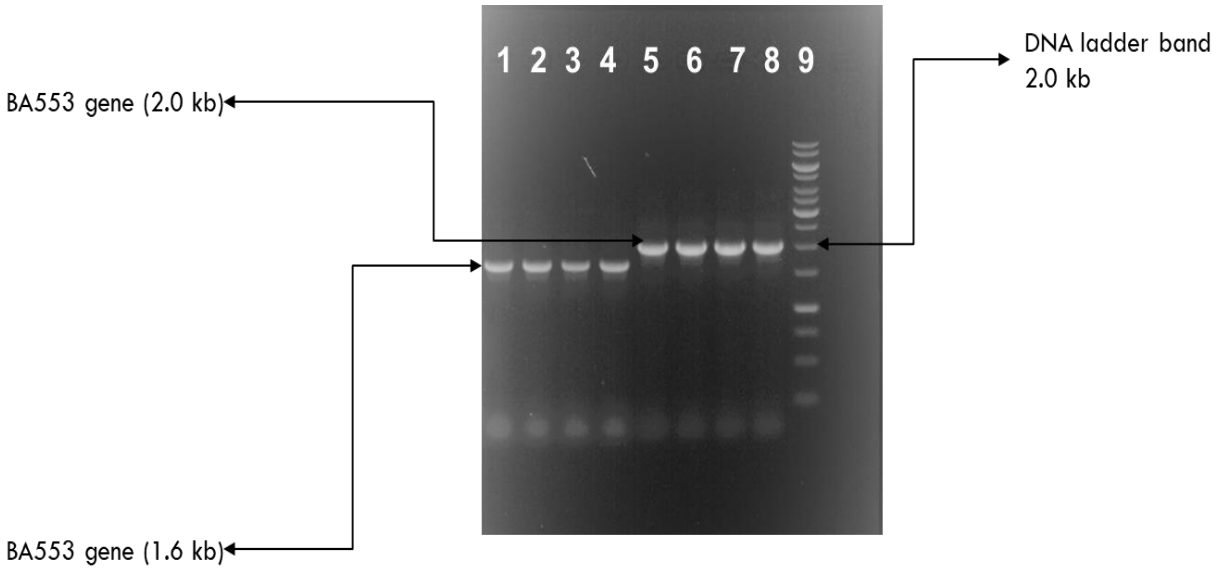


Figure 5.11: PCR amplification of BA553 & BA666 genes (Lane 1-4: PCR product for amylase (BA553-1.6 kb), Lane 5-8: PCR product for amylase (BA666-2 kb), Lane 9: 1kb DNA ladder).

5.3.7.3 Preparation of competent cells

The efficiency of the prepared competent cells was checked by transforming them with pUC19 plasmid and plating them on LB agar plates and on LB/Amp agar plates. The efficiency was found to be 2 x 10^6 cfu/µg.

5.3.7.4 Transformation of *E.coli* DH5α cells

After the transformation of DH5α ells with the vector-product mixture, colonies appeared on the LB/Amp agar plates after 12-16 h of incubation at 37°C. 5-8 isolated colonies were randomly selected for plasmid isolation showed positive transformation of gene into plasmid (**Figure 5.12)**.

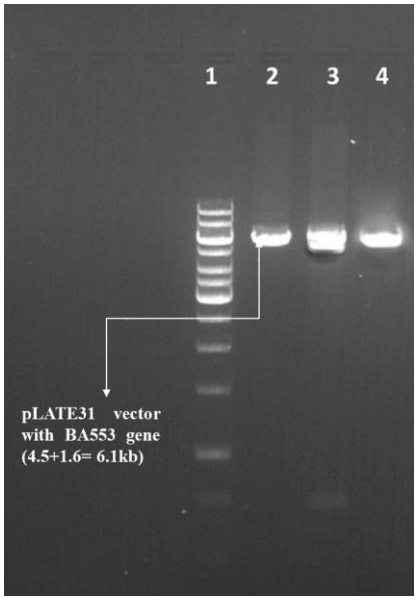


Figure 5.12: pLATE31 plasmid carrying BA553 (1.6 kb) gene isolated from transformed *E.coli* DH5α.

When colonies were selected for plasmid isolation, only half of each colonies were picked up (by sterile tooth pick) and remaining half of the colonies used as PCR template to amplify the transformed gene showed amplification of 1.6 kb amylase gene (BA553) (**Figure 5.13**).

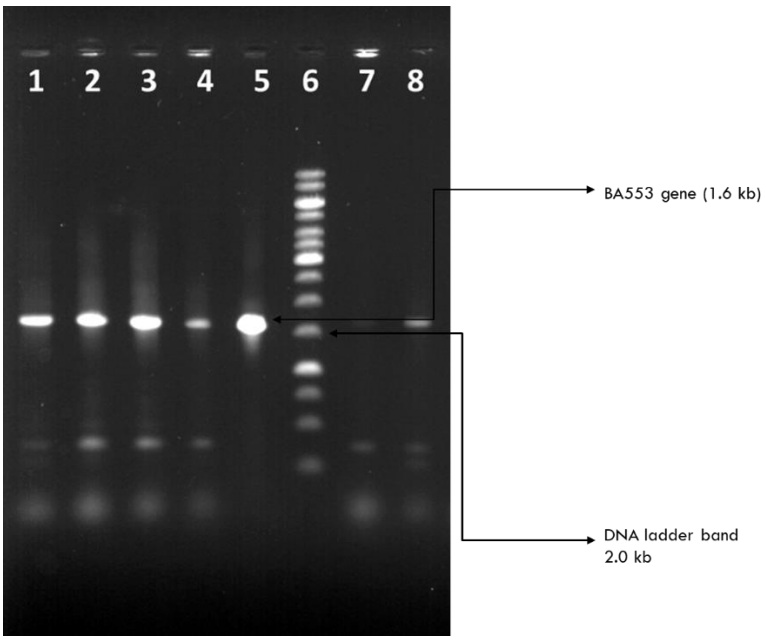


Figure 5.13: Colony PCR performed from transformed *E.coli* DH5α colonies showing amplification of BA553 gene (1.6 kb). (Lane 1-5: colony PCR product of transformed *E.coli* DH5α, Lane 6: 1 kb DNA ladder, Lane 7 & 8: colony PCR product of transformed *E.coli* DH5α).

5.3.7.5 Restriction digestion analysis of plasmid

The amylase gene (1.6 kb) in pLATE31 vector was double digested using the restriction enzymes EcoRI and HindIII (**Fig.5.14**). Bands were obtained at their expected positions on 1% agarose gel, a 4.5 kb vector band and a 1.6 kb insert gene band.

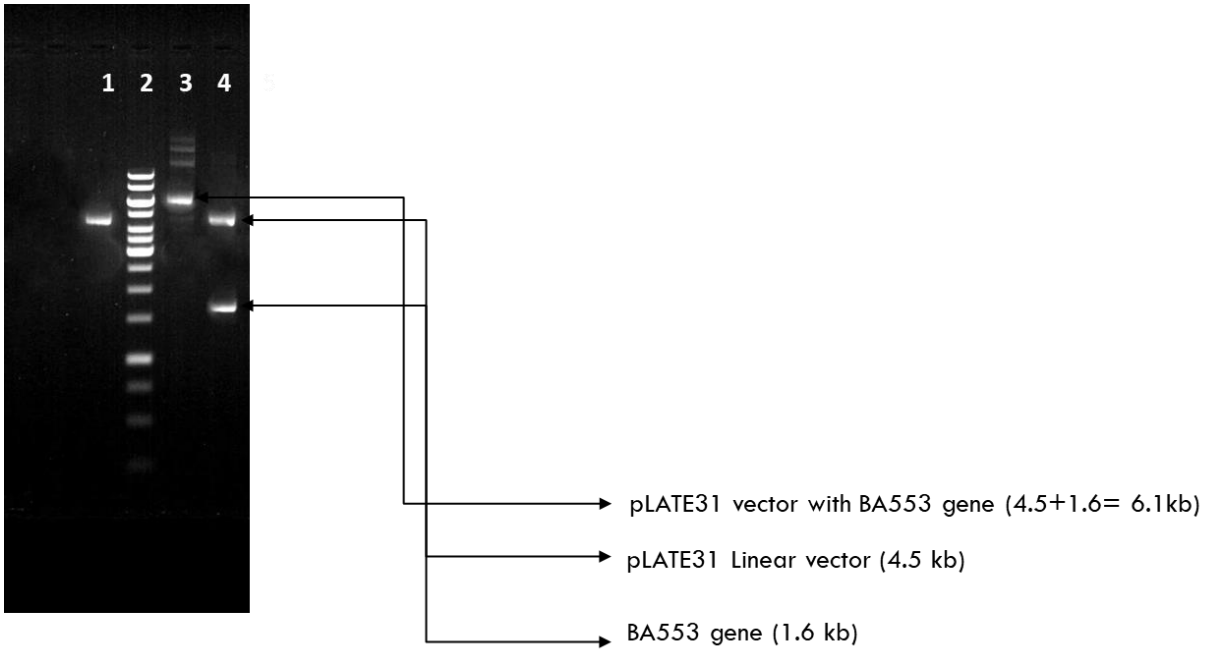


Figure 5.14: 1% agarose gel to check insert release. Lane 1: pLATE31 vector with BA553 gene, Lane 2: 1kb DNA ladder, Lane 3: Linearised pLATE31+BA553 plasmid (4.5+1.6= 6.1kb), Lane 4: Double digested pLATE31+BA553 plasmid showing insert release of correct size.

5.3.8 Expression of amylase in *E.coli* BL21 cells

12 % SDS-PAGE was run to check the expression of recombinant amylase. *E.coli* BL21 cells induced by IPTG showed high protein expression as compared to uninduced cells as shown in **Figure 5.15**.

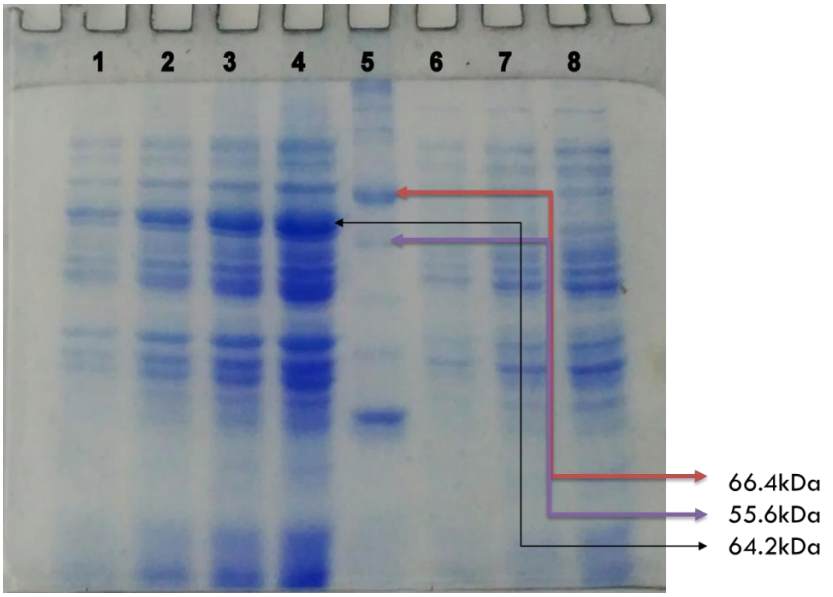
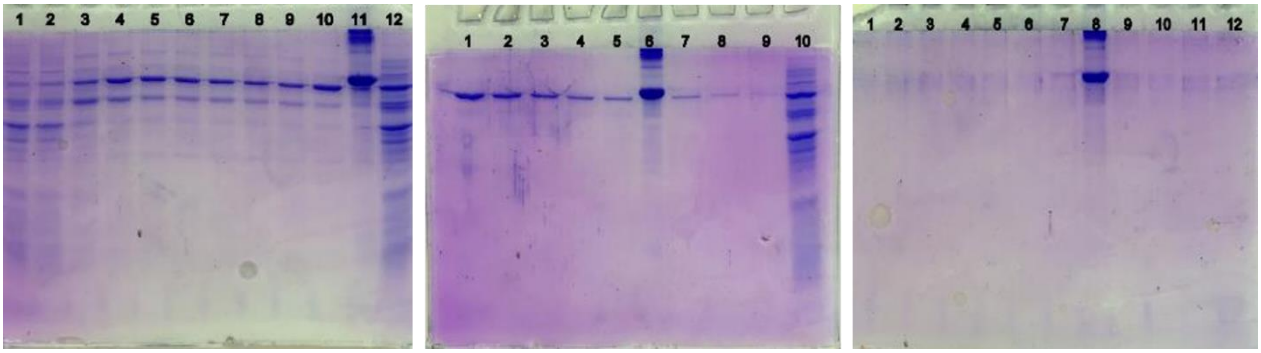


Figure 5.15: 12% SDS-PAGE to check expression of cloned amylase in *E.coli* BL21 cells. The samples loaded are cell lysates of BL21 cells after and before induction with IPTG. Lanes 1-4 contain induced cell lysate, 50 µl, 100 µl, 150 µl and 200 µl; Lane 5: Protein molecular weight marker (NEB); Lanes 6-8 contain uninduced cell lysate, 50 µl, 100 µl and 150 µl.

5.3.9 Purification of recombinant amylase by affinity chromatography

The expressed recombinant amylase containing a C-terminal 6x His-tag was purified by affinity chromatography to high purity using Ni-NTA column. The fractions eluted with 100 mM imidazole to 300 mM imidazole showed presence of pure protein when analyzed on 12% SDS-PAGE (**Figure 5.16**). All fraction containing pure protein were pooled together and found to have functionally active amylase.



A **B** **C**

Figure 5.16: 12% SDS-PAGE showing purification of cloned amylase by affinity chromatography using Ni-NTA column. **(A)** Lane 1: Flow through; Lane 2: Wash; Lanes 3-10: 20 mM Imidazole Fractions; Lane 11: BSA, Lane 12: Induced cell lysate **(B)** Lane 1-5: 100 mM Imidazole Fractions; Lane 6: BSA, Lane 7-9: 300 mM Imidazole Fractions; Lane 10: Induced cell lysate **(C)** Lane 1: 300 mM Imidazole Fraction 17, Lane 2-7: 500 mM Imidazole Fractions Lane 8: BSA, Lane 9-12: 500 mM Imidazole Fractions.

5.3.10 Confirmation of amylase activity

3,5-Dinitrosalicylic acid (DNSA, IUPAC name 2-hydroxy-3,5-dinitrobenzoic acid) is an aromatic compound that reacts with reducing sugars and other reducing molecules to form 3-amino-5-nitrosalicylic acid. In case of glucose, the maximum absorption by DNS can be observed at 540 nm. This method tests for the presence of free carbonyl group (C=O) of reducing sugars. DNSA assay confirmed that the recombinant protein purified by affinity chromatography was functionally active. Amylase activity obtained was 792U/ml from the standard curve of maltose (**Figure 5.17**).

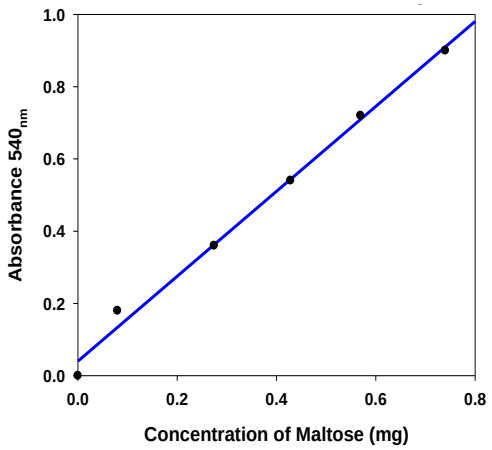


Figure 5.17: Standard curve of maltose by DNSA for estimation of amylase activity.

5.3.11 Determination of optimum temperature for recombinant amylase

Figure 5.18 shows the activity of the recombinant amylase at different temperatures ranging from 35°C to 80°C. The optimum temperature was observed to be 40°C, similar to the native amylase secreted by *Bacillus atrophaeus*.

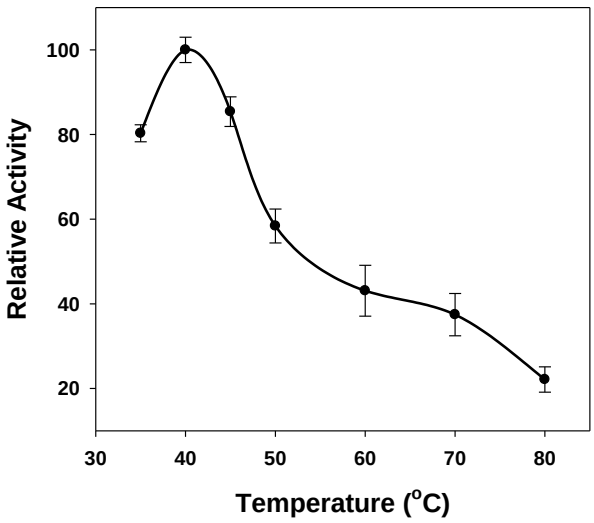


Figure 5.18: Activity of recombinant amylase as a function of temperature. All the assay parameters were kept constant except for the assay temperature. Each data point is an average of three independent experiments.

5.3.12 Determination of optimum pH for recombinant amylase

Figure 5.19 shows the activity of recombinant amylase as a function of pH, when the assays were carried out in different buffers of varying pH. The recombinant protein had an optimum pH of 6.0, just like the native amylase secreted by *Bacillus atrophaeus.* All the experiments were performed in triplicate sets.

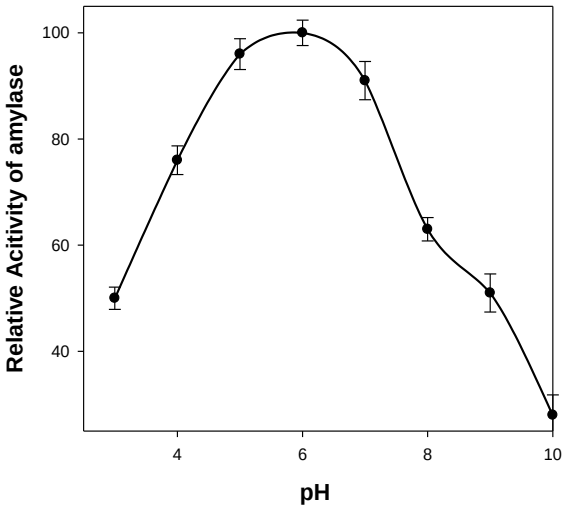


Figure 5.19: Activity as a function of pH to determine optimum pH of recombinant amylase. All the assay parameters were kept constant except for the pH. Each data point is an average of three independent experiments. The optimum pH was found to be 6.0.

5.4 Conclusion:

Amylase from *Bacillus atrophaeus* was screened and purified for characterization. We faced the difficulties in purification of amylase because the bacteria secreted more than two amylases extracellularly and coming from same bacteria they possessed similar pI values. Hence, purification by ion-exchange chromatography was not completely successful. Purification could be achieved by ammonium sulphate fractionation but the yield of purified amylase was poor. The wild type amylase was partially characterized and showed optimum temperature 40°C and optimum pH 5. Cloning was performed for two amylases and one of the amylase gene (1.6 kb) could be successfully used to transform *E.coli* DH5α cells and expressed in *E.coli* BL21 cells. The cloned amylase was partially characterized showing optimum temperature 40°C and optimum pH 6, similar to wild type amylase.

Chapter 6

Summary and Conclusion

Summary & Conclusion

As enzymes have the unique capacity to catalyze reactions with high velocity and unmet specificity under a variety of conditions, it has now become possible to develop new enzymatic processes and this is potentially profitable. There is a continuously growing use of industrial enzymes and this leads to the requirement to develop enzymes with improved performance and reduced cost. This purpose can now be met as there is now a rapidly growing database of natural enzyme diversity, development of better recombinant DNA and fermentation technologies. These enable us to construct this diversity at low cost. Protein engineering methods are also rapidly developing, which can help to produce enzymes with characteristics to fit the requirements of the industrial marketplace. The properties of an enzyme (any one or a combination of properties) may need to be altered for it to be useful for a specific, non-natural application. Hence there is always a search on to identify enzymes which can tolerate these extreme conditions or develop methods which could improve stability of naturally occurring proteins. The problem is that the molecular determinants of protein stability are not yet fully understood by the scientific community although a huge number of scientists from all over the world are engaged in trying to elucidate this.

Isolation and identification of wild bacterial or archaeal strains with novel properties is an on-going mission to meet the biotechnological applications of lipases. Lipases are generally important in industry, but among those, thermostable lipases occupy a prominent position in aqueous and non-aqueous biocatalysis. Medical and pharmaceutical applications of enzymes require highly pure preparations, but partial purification of lipases can be sufficient for general and industrial applications. Thus, characterization with respect to enzyme activity, specificity, thermostability, enantioselectivity and tolerance to various solvent systems are the prerequisite for thermostable lipase selection which make them highly demanding in lipase catalyzed reactions.

The work presented in this thesis reports a newly isolated thermotolerant/thermoactive lipase from *H. shengliensis*, and its enzymatic as well as structural characterization. Although isolated from a moderate haloalkalophile, the lipase shows interesting characteristics like high thermostability, making it a good target to study and understand structure function relationship of proteins as well as makes it a satisfactory and promising candidate for industrial applications. The study of denaturation of proteins is useful in the field of proteomics to understand the mechanism of protein folding and unfolding. Proteins avoid searching irrelevant conformations and fold fast and efficiently by deciding on local independent structures first, followed by final global folding. To investigate the folding process, one needs to understand the microscopic folding pathways to the global minima and how to avoid getting trapped in local minima during conformational changes towards the final folded state. Such fine understanding is accumulated from large number of studies of diverse proteins. Our investigation in this report to understand the lipase stability could be another small step in this direction and we provide evidence for intermediate states in its unfolding of the lipase which is possibly a divergence from the classical two state mechanism. Biophysical, biochemical and computational methods have been employed for this study, where we show that the enzyme is unusually thermostable and active at higher temperatures although it originated from a mesophilic bacterium. It is also tolerant to various organic solvents, surfactants and inhibitors, making it a potential candidate for industrial applications.

Another experimental work was carried out on the isolation, purification and partial characterization of an amylase from *Bacillus atrophaeus*. Amylases are another group of important enzymes that are industrially important and protein engineering to obtain thermostable amylases is in great demand. We started the work with an aim to apply directed evolution method to generate thermostable variants of this mesophilic enzyme and in the process, try to understand the molecular determinants of protein thermostability (Extramural grant from Gujarat State Biotechnology Mission). We have characterized the wild type enzyme and cloned it in a bacterial expression vector that can express high levels of the recombinant protein. The cloned amylase can now be further used by other researchers to carry out protein engineering methods including directed evolution and generate thermostable variants and in the process study the stability of proteins at a molecular level. Engineering proteins for thermostability is a particularly stimulating and challenging field, as it is crucial for expanding the industrial use of recombinant proteins. Biochemical and biophysical characterization opens doors for enzymes to be used in various industries for novel applications.

The whole research work presented in this dissertation was designed and carried out in a flow with the mindset of classic scientific research to answer basic questions i.e. origin of research, defining the problem, proposing the possible solution, working on the solution and gathering the relevant information which could be beneficial to researchers as well as industries. We hope that this work will prove to be another small step towards developing enzymes that meet industrial requirements.

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