Protease Production by haloarchaea Natrinema sp. BTSH10 isolated from salt pan of South India

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CERTIFICATE

This is to certify that the research work presented in this thesis entitled "**Protease Production by haloarchaea *Natrinema* sp. BTSH10 isolated from salt pan of South India"**, is based on the original research work carried out by **Mrs. R. Manjula** under my guidance and supervision at the Department of Biotechnology, Cochin University of Science and Technology, in partial fulfillment of the requirements for the degree of **Doctor of Philosophy** and that no part thereof has been presented for the award of any degree, diploma, associateship or other similar titles or recognition.

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DECLARATION

I hereby declare that the thesis entitled **"Protease Production by haloarchaea *Natrinema* sp. BTSH10 isolated from salt pan of South India"**, is based on the original research work carried out by me for my doctoral degree, under the supervision and guidance of Dr. M. Chandrasekaran, Professor, Department of Biotechnology, Cochin University of Science and Technology and Co-guidance of Dr. Sarita G. Bhat, Associate Professor, Department of Biotechnology, Cochin University of Science and Technology and the thesis or no part thereof has presented for the award of any degree, diploma, associateship or other similar titles or recognition.

Cochin - 22
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मातरं पितरं नत्वा
ईश्वरं हृद्गतं मुदम्।
यत्कृतं सकलं त्वद्य
गुरुपादांबुजेऽर्पये।।

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Abbreviations

%	-	Percentage
°C	-	Degree Celsius
µ	-	Micron
µg	-	microgram
µL	-	microlitre
APS	-	Ammonium persulfate
BLAST	-	Basic Local Alignment Search Tool
BSA	-	Bovine Serum Albumin
cm	-	centimeter
DMSO	-	Dimethyl sulphoxide
DNA	-	Deoxyribonucleic acid
DW	-	Distilled Water
EC	-	Enzyme Commission
EDTA	-	Ethylenediaminetetraacetic acid
Fig.	-	Figure
g	-	grams
g/L	-	grams per litre
h	-	hour
HPLC	-	High Performance Liquid Chromatography
kDa	-	Kilo Dalton
K_m	-	substrate concentration at which the reaction velocity is half Maximum
M	-	Molar
mg	-	milligram
min.	-	minutes
mL	-	millilitre
mm	-	millimetre
mM	-	milli Molar
MW	-	Molecular Weight
NCBI	-	National Centre for Biotechnology Information

OD	-	Optical Density
PAGE	-	Poly Acrylamide Gel Electrophoresis
PCR	-	Polymerase Chain Reaction
PSU	-	Practical salinity units
RNA	-	Ribonucleic Acid
rpm	-	rotations per minute
SDS	-	Sodium Dodecyl Sulphate
SmF	-	Submerged Fermentation
sp.	-	Species
TCA	-	Trichloroacetic acid
TEMED	-	N-N-N'-N'-Tetramethylethylenediamine
U/mg	-	Units / milligram
U/mL	-	Units / millilitre
V_{max}	-	Maximal Velocity
ZA	-	Zobell Agar

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Chapter 1

INTRODUCTION

Extracellular hydrolytic enzymes such as amylases, proteases, lipases, DNases, pullulanases and xylanases have quite diverse potential usages in different areas such as food industry, feed additives, biomedical sciences and chemical industries.Industrial enzymes could be divided into four major categories, based on application - detergent enzymes, technical enzymes, food enzymes and feed enzymes. The technical enzymes could further be divided into textile enzymes, leather enzymes, pulp and paper enzymes, fine chemicals enzymes, fuel ethanol enzymes and others (van Beilen and Li, 2002).

According to the market research report on world enzymes published in 2007, the world market for enzymes is expected to grow at the rate of 7.6% per year to $6 billion in 2011 (David *et al*., 2009). Survey on world sales of enzymes ascribes 31% for food enzymes, 6% for feed enzymes and the remaining for technical enzymes (Berka and Cherry, 2006; Agrahari, 2011).

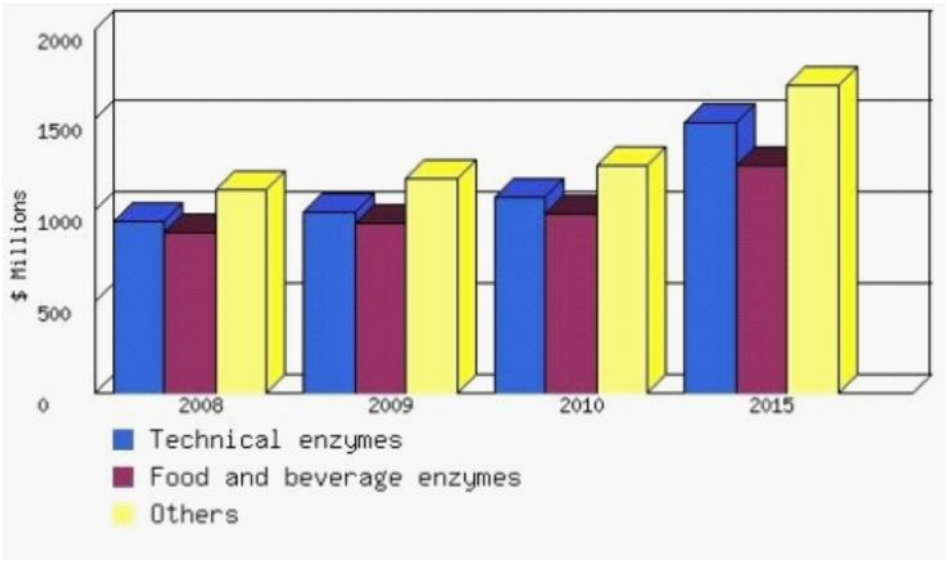


Fig. 1.1. Global Industrial Enzyme Market 2008-2015 (Sarrouh *et al*., 2012)

A report by BCC Research (2011)stated that the global market for industrial enzymes was estimated to reach a value of $3.3 billion in 2010 and is expected to

reach $4.4 billion by 2015, a compound annual growth rate (CAGR) of 6% over the 5year forecast period. Singhal *et al.*(2012),reported that the world enzyme market was at $5.1 billion and was expected to rise by 6.3% annually by 2013. According to Sarrouh *et al*. (2012) technical enzymes, valued at over $1 billion in 2010, were expected to increase at a 6.6% compound annual growth rate (CAGR) to reach $1.5 billion in 2015. The highest sales of technical enzymes occurred in the leather market, followed by the bioethanol market (Fig. 1.1). The food and beverage enzymes segment was speculated to reach about $1.3 billion by 2015, from a value of $975 million in 2010, rising at a compound annual growth rate (CAGR) of 5.1%. Within the food and beverage enzymes segment, the milk and dairy market had the highest sales, with $401.8 million in 2009 (Sarrouh *et al*., 2012).

Major enzyme producers are based in USA, Europe and Japan. Denmark dominates the world enzyme production, with major players like Novozymes and Danisco contributing 45% and 17% respectively; Genencor (USA), DSM (The Netherlands) and BASF (Germany) making up the rest of world market (Binod *et al.*, 2008; BCC-Business Communications Company, Inc., 2009; Agrahari, 2011).

Microorganisms represents the most common source of enzymes as they are relatively more stable and active than the enzymes derived from plant or animal sources. They are preferred due to their broad biochemical diversity, feasibility, mass culture and ease of genetic manipulations. Further microbes serve as a preferred source of these enzymes owing to their rapid growth, requirement of limited space for their cultivation and the ease with which they can be genetically manipulated to generate new enzymes with altered properties that are desirable for their various applications.

1.1.Proteases

Global Strategic Business Report (2012-http://www.strategyr.com/Industrial_Enzymes_Market_Report.asp.), on enzymes market highlighted the fact that proteases constitute the largest product segment in the global industrial enzymes market. Among the various industrial enzymes extracellular proteases are considered as important for the hydrolysis of external proteins. Among the enzymes, microbial proteases account for approximately 60% of

the total enzyme sales in the world (Singh *et al*., 2001, Banik and Prakash, 2004). Proteases enable the cell to absorb and utilize hydrolytic products. Further proteases hydrolyze peptide bonds in aqueous medium and also facilitate synthesis of peptides in low water or non-aqueous medium. In fact, proteases that can be purified easily have been commercially exploited to assist protein degradation in various industrial processes. Thus, proteases are the largest selling industrial enzymes and their sale is projected to increase further in the coming years with anticipated applications in protein processing, peptide synthesis and detergent formulations. Microbial proteases are probably one of the most extensively studied enzymes with wide ranging applications in industries like detergent, pharmaceutical, food, waste management, leather, diagnostics etc. (Tari *et al*., 2006; Bhaskar *et al*., 2007; Dodia *et al*., 2008; Setati, 2010).

Industrial processes are carried out under specific physical and chemical conditions, which cannot always be adjusted to the optimal values required for the activity and stability of the available enzymes. Industrial applications of proteases often require the enzymes to be stable in the presence of organic solvents. These most often act to inactivate the enzymes and retard the rate of reaction. Several physical and chemical methods such as chemical modification, immobilization, entrapment and protein engineering have been employed for the stabilization of enzymes towards organic solvents. However, if enzymes are naturally stable and exhibit high activities in the presence of organic solvents, such stabilization is not necessary. Therefore, it would be of great importance to have available enzymes showing optimal activities at different values of pH, salt concentration, organic solvents and temperatures. In this context, extensive research efforts are being directed to screen new sources of proteases with novel properties.

Despite the facts that many different proteases have been identified and some of them have been used in biotechnological and industrial applications, the present proteases are not sufficient to meet most of the industrial demands. In view of these restrictions, attention to isolation and characterization of proteases from extremophiles is very important. In this context, halophiles are the most likely sources of such enzymes, because not only are their enzymes salt tolerant but many are thermotolerant.

1.2.Applications of Proteases

Proteases are robust enzymes with considerable industrial potential in detergents, leather processing, silver recovery, medical purposes, food processing, meat tenderization, cheese making, dehairing, feeds, and chemical industries as well as waste treatment. These enzymes contribute to the development of high value-added applications or products by using enzyme aided (partial) digestion. Probably the largest application of proteases is in laundry detergents, where they help removing protein based stains from clothing (Banerjee *et al*., 1999). For an enzyme to be used as an detergent additive it should be stable and active in the presence of typical detergent ingredients, such as surfactants, builders, bleaching agents, bleach activators, fillers, fabric softners and various other formulation aids. In textile industry, proteases may also be used to remove the stiff and dull gum layers of sericine from the raw silk fiber to achieve improved luster and softness. Protease treatments can modify the surface of wool and silk fibers to provide new and unique finish. Currently proteases are used in various industries including that of detergents, food, pharmaceuticals, leather industry, basic research and for extraction of silver from used X-ray films.

1.2.1.Detergent Additives

Enzymes used in detergents include proteases, amylases and lipases, of which alkaline proteases hold a lion's share and constitute 60-65% of the global industrial enzyme market (Amoozegara *et al*., 2007). Microbial alkaline proteases dominate commercial applications with a significant share of market owned by subtilisins and /or alkaline proteases from *Bacillus* species for laundry detergent applications. Alkaline proteases added to laundry detergents enable the release of proteinaceous material from stains. The increased usage of these proteases as detergent additives is mainly due to the cleaning capabilities of these enzymes in environmentally acceptable, non phosphate detergents. In addition to improved washing efficiency, the use of these enzymes allows lower wash temperatures and shorter period of agitations, often after preliminary period of soaking. Ideally, proteases and other enzymes used in detergent formulations should have high activity and stability over a broad range of pH and temperature. The enzymes used should be effective at low levels and should also be compatible with various detergent components along with oxidizing and

sequestering agents. Very few published reports are available on the compatibility of the alkaline proteases with detergents. Some cleaning applications are less demanding than others. For instance, presoak formulations and contact lens cleaning solutions do not require the same enzyme thermal stability as an all temperature laundry detergent.

1.2.2.Tannery Industry

Alkaline proteases possessing elastolytic and keratinolytic activity offer an effective biotreatment of leather, especially the dehairing and bating of skins and hides. The alkaline conditions enable the swelling of root hairs and subsequent attack of proteases on the hair follicle protein allow for easy removal of the hair. The bating followed by dehairing process involves the degradation of elastin and keratin, removal of hair residues and the deswelling of collagen, which produce a good, soft leather mainly used for making leather clothes and goods.

1.2.3.Silver Recovery

Alkaline proteases find potential applications in the bioprocessing of used X-ray films for silver recovery. Used X-ray film contains approximately 1.5 to 2.0% (by weight) silver in its gelatin layers. The conventional practice of silver recovery by burning film causes a major environmental pollution problem. Thus, the enzymatic hydrolysis of the gelatin layers on the X-ray films enables not only the silver, but also the polyester film base to be recycled (Ishikawa *et al*., 1993).

1.2.4.Medical Uses

Collagenases with alkaline protease activity are increasingly used for the therapeutic applications in the preparation of slow release dosage forms. A new semi alkaline protease with high collagenolytic activity was obtained from *Aspergillus niger* LCF9. The enzyme hydrolyzed various collagen types without aminoacid release and liberated low molecular weight peptides of potential therapeutic use. Similarly Elastoterase, a preparation with high elastolytic activity from *Bacillus subtilis* 316M, was immobilized on a bandage for the therapeutic applications in the treatments of burns and purulent wounds, furuncles and deep abscesses. Furthermore, *Bacillus* species have been recognized as being safe to humans and an alkaline protease having fibrinolytic activity has been used as a thrombolytic agent. Proteases are also useful and important components in biopharmaceutical products such as

contact-lens enzyme cleaners and enzymic debridement (Anwar and Saleemuddin, 2000). The proteolytic enzymes also offer a gentle and selective debridement supporting the natural healing process in the successful local management of skin ulcerations by efficient removal of the necrotic material (Sjodahl *et al*., 2002).

1.2.5.Food Industry

Alkaline proteases can hydrolyze proteins from plants, fishes or animals to produce hydrolysates of well defined peptide profile. The commercial alkaline protease-Alcalase has a broad specificity with some preference for terminal hydrophobic aminoacids. Neutral proteases have been reported to be used to produce soy sauce and soy products which are less bitter; they are used in brewing industry as they are not sensitive to natural plant proteinase inhibitors (Rao *et al*., 1998).

1.2.6.Waste Treatment

Alkaline proteases from *Bacillus subtilis* was reported to be used for treatment of waste feathers (Dalev, 1994).

1.3.HalophilicArchaea

Halophiles are microorganisms that adapt to moderate and high salt concentrations. Halophiles include a great diversity of organisms, like moderately halophilic aerobic bacteria, cyanobacteria, sulphur oxidizing bacteria, heterotrophic bacteria, anaerobic bacteria, archaea, protozoa, fungi, algae and multicellular eukaryotes. They are found in all three domains of life: Archaea, Bacteria and Eukarya. Halophilic archaea is a member of the Halobacteriaceae family, the only family in the *Halobacteriales* order. Halophilic bacteria grow over an extended range of salt concentrations (3-15% NaCl, w/v and above). The term ‘halobacteria’ refers to the red-pigmented extremely halophilic archaea, members of the Halobacteriaceae family. Halobacteria is phylogenetically distinct from bacteria and eukaryotes, and are classified as archaea. They exhibit features characteristic of the archaea, including eukaryotic-like transcription and translation machinery, ether-linked lipids and like some bacteria, a cell wall S-layer composed of glycoproteins (DasSarma and Arora, 2001). Microorganisms that are able to grow in the absence as well as in the presence of salt are designated as halotolerant and those that are able to grow above approximately 15% (w/v) NaCl (2.5 M) are considered extremely halotolerant.

Extreme halophilic archaea are chemo-organotrophic organisms that satisfy some of their energy requirements with light. These archaea are classified in one order, *Halobacteriales*, and one family, *Halobacteriaceae* (Grant and Larsen, 1989). Thereafter, 16S rDNA sequencing, DNA-DNA hybridization, polar lipid analysis and other studies have recognized 40 genera (Parte, 2013). Some important genera are: *Halobacterium*, *Haloarchaea*, *Haloferax*, *Natronobacterium*, *Natronococcus* (Tindall *et al*., 1984; Torreblanca *et al*., 1986; Tindall, 1992; Grant and Larsen, 1989; *Halorubrum* (McGenity and Grant, 1995), *Halobaculum* (Oren *et al*., 1995), *Natrialba* (Kamekura and Dyall Smith, 1995), *Natronomonas* (Kamekura *et al*., 1997), *Halogeometricum* (Montalvo-Rodriguez *et al*., 1998), *Natrinema* (McGenity *et al*., 1998), *Haloterrigena* (Ventosa *et al*., 1999), *Natronorubrum* (Xu *et al*., 1999) and *Halorhabdus* (Waino *et al*., 2000).

Members of the family *Halobacteriaceae* are characterized by red coloured cells, the colour mainly being due to the presence of C_{50}- carotenoids (bacterioruberins) as the major carotenoids (Ronnekleiv and Liaaen-Jensen, 1995). Some members of the genera *Halobacterium* and *Haloarcula* have been reported to partially produce C_{40}- carotenoids and Ketocarotenoids such as β-carotene, lycopene, 3-hydroxy echinenone and trans- astaxanthin and the minor carotenoids (Calo*et al*., 1995).

Halophiles are microorganisms which grow over an extended range of salt concentration (3-30% NaCl, w/v) and include the halotolerant bacteria and the obligate halophilic archaea. They are found in salt marshes, marine ecosystems, salted meats, hypersaline seas, salt evaporation pools and salt mines. Hypersaline environment originates by the evaporation of sea water and are also called thalassohaline environments. As water evaporates, sodium chloride precipitates and salinity increases above 300 PSU. Despite the prevailing extreme environment, a great diversity of extremophiles especially *Haloarchaea* have been reported in these environments.

Halophiles are categorized as slight, moderate or extreme, by the extent of their halotolerance. Slight halophiles prefer 0.3 to 0.8 M (1.8 to 4.7% - seawater is 0.6 M or 3.5%), moderate halophiles 0.8 to 3.4 M (4.7 to 20%), and extreme

halophiles 3.4 to 5.1 M (20 to 30%) NaCl (Ventosa *et al.*, 1998; Anton *et al.*, 1999). Halophiles require NaCl for growth in contrast to halotolerant organisms, which do not require NaCl but can grow under saline conditions.

Halotolerant bacteria form a versatile group, adapted to life at the lower range of salinities, with the possibility of rapid adjustment to the changes in the external salt concentrations for survival. This property of halotolerant bacteria makes them better candidates for bio-prospecting than their halophilic counterparts.

Halophilic archaea are considered as a potentially valuable resource in the development of novel biotechnological processes and industrial applications in terms of new pharmaceuticals, cosmetics, nutritional supplements, molecular probes, enzymes and fine chemicals. Many of them are known to produce compounds of industrial interests such as enzymes, polymers and osmoprotectants and some also possess useful physiological properties which can facilitate their exploitation for commercial purposes. Recently the biotechnological potential of these members of the archaea has been recognized by researchers because of their unique features, which facilitates many industrial products/ procedures.

Halophilic archaea have also been evaluated for bioremediation in harsh environments for the degradation of organic pollutants (Margesin and Schinner, 2001) and degradation of hydrocarbon by archaeal microbes under anoxic condition (Lovely, 2001). Biosurfactant producing halophilic archaea play a significant role in the accelerated remediation of oil polluted saline environments. Certain strains of halophilic archaea contain membrane bound retinal pigments, bacteriorhodopsin and halorhodopsin, which enable microorganisms to use light energy to derive bioenergetic processes (Oren, 1994; Lanyi, 1995). Furthermore, bacteriorhodopsin can be exploited for the renewal of biochemical energy such as the back conversion of ADP to ATP. A device based on bacteriorhodopsin and ATP synthesis has been developed and patented (Saito *et al.*, 1992). Thus, a wide variety of biotechnological products such as bacteriorhodopsins, halorhodopsins, biopolymers, biosurfactants, exopolysaccharides, polyhydroxyalkonates, flavoring agents, antitumor drugs and enzymes are produced by halophilic archaea.

1.3.1.Potential of Halophiles for Biotechnological Use

Extremozymes have great economic potential in many industrial processes (e.g. agriculture, food, feed, drinks, detergents, textile, leather, pulp and paper). Although there are controversial opinions about the potential of extremophiles, some companies (e.g. Diversa, Genecor International Inc, Novozymes) and several research groups are investing money and time, searching for these microbes and novel applications of extremozymes.

The industrial and environmental applications of halophilic microorganisms have been reviewed by Oren (2010). The review highlights the salient features of halophiles, including their highly successful applications like β-carotene production by *Dunaliella* and ectoine synthesis using *Halomonas* and other moderately halophilic bacteria. BenAmotz and Avron(1989), have reported the use of *Dunaliella* for production of β-carotene which is used as a food colourant, precursor of Vitamin A, additive in cosmetics and preparation of multivitamins and health food preparations. Bacteriorhodopsin the retinal protein proton pump of *Halobacterium* finds applications in holography, artificial retina, neural network and optical computing. Other possible use of halophilic microorganisms includes in the treatment of saline and hyper saline waste waters and production of exopolysaccharides, poly β-hydroxyalkanoate bioplastics and biofuels. Margesin and Schinner (2001) also reported that *H. cutrubrum* was used for liposome production used in medicine and cosmetics to transport compounds to specific target sites. Ectoine and hydroxyetoine produced by *Halomonas elongata* KS3, are used in moisturizers and ectoine is also a stabilizer in PCR (Motitschke *et al.,* 2000). These microorganisms can be used as a source of metabolites, compatible solutes and other compounds of industrial value.

Novel halophilic biomolecules may also be used for specialized applications e.g. bacteriorhodopsin for biocomputing, pigments for food colouring and compatible solutes as stress protectants (DasSarma *et al.*, 2001). Exopolymer poly (γ-D-glutamic acid) produced by *Natrialba* is used as a biodegradable thickener and drug carrier in food or pharmaceutical industry (Kunioka,1997; Hezayen *et al*., 2000). Biodegradation of organic pollutants by halophilic bacteria and archaea has been recently reviewed (Le Borgne *et al*., 2008). These microorganisms are good candidates for the bioremediation of hypersaline environments and the treatment of

saline effluents. *Halobacterium*is used for bioremediation of oil spills in saline environments and degradation of n-alkanes with $C_{10} - C_{30}$ (Kulichevskaya *et al.*, 1992).Yongsawatdigul *et al*. (2007), has reported the use of species of *Halococcus*, *Bacillus* and *Vibriobacillus* for production of Thai fish sauce. Ryu *et al*.(1994) reported the isolation of a serine protease from *Halobacterium halobium* which could be used as a catalyst for the production of glycine containing peptides in presence of organic solvents.

Halophilic bacteria are a potential source of extracellular hydrolases like proteases with a wide array of industrial applications. These enzymes exhibit stability over a range of saline conditions (Shivanand and Jayaraman, 2009). The importance of proteases is highlighted by the fact that they have many practical applications in biotechnology and industry (Rao *et al*., 1998).Halophilic bacteria constitute excellent models for the molecular study of osmoregulatory mechanisms (Ventosa *et al*., 1998).

1.3.2.HalophilicEnzymes - Halozymes

Moderately halophilic bacteria that grow optimally in a media containing 3-15% NaCl are considered as a likely source of such enzymes. Hence, halophilic microorganisms are perceived to be a valuable source of enzymes with unique structural features and properties. In order to survive in saline environments, these organisms accumulate high concentration of salts (most often NaCl or KCl) or osmolytes (e.g. betaine, glycerol) in the cytoplasm (Le Borgne *et al*., 2008). As a consequence, their enzymes are generally salt stable. In terms of water availability, saline environments are similar to non-aqueous systems. Therefore, halophilic enzymes should logically be stable in organic solvents. Although large numbers of salt-stable enzymes have been reported from halophilic sources, stability towards organic solvents has been noticed in only few cases. Further, enzymes from the halophilic archaea tend to be more thermostable than expected from the organism's growth temperature.

Halophiles from the archaeal domain provide the main source of extremely halophilic enzymes. The potentials of halophiles and haloenzymes have been highlighted in literature (Eichler, 2001; Oren, 2002). Extracellular halophilic enzymes such as xylanases, amylases, proteases and lipases has been reported in many

halophiles belonging to the genera *Haloferax, Actinobacter, Halobacterium, Marinococcus, Natronoccus, Halobacillus, Halorhabdus* and *Halothermothrix* (Adams *et al.,* 1995; Sellek and Chaudhari, 1999; Madern *et al*., 2000; Mevarech *et al*., 2000; Eichler, 2001). Halophilic microorganisms produce stable enzymes (including many hydrolytic enzymes such as DNases, lipases, amylases, gelatinase and protease) capable of functioning under high concentration of salt which leads to precipitation or denaturation of most proteins. Most halophilic enzymes are inactivated and denatured at concentration of NaCl below 1M. Examples of halophilic enzymes are serine proteases from the extreme halophilic *Halobacterium halobium* (Izotova *et al*., 1983), DNA topoisomerases from *Methanopyus kandleri* (Kozyavkin *et al*.,1994), extremely halophilic β- galactosidase from *Haloferax alicantei* (Holmes *et al*.,1997), D-hydantoinase from halophilic *Pseudomonas* species (Sudge *et al*.,1998) and halophilic α- amylase from *Nesterenkonia* sp. (Shafiei *et al*., 2012).

1.4.Microbial Gelatinases

Gelatinase is one type of diverse group of protease, an extracellular metallo-endopeptidase or metalloproteinase which is able to hydrolyze gelatin and other compounds such as pheromone, collagen, casein and fibrinogen (Makinen and Makinen, 1994; Makinen *et al*., 1989). Gelatinase and collagenase are important metalloproteases and these are widely used not only in chemical and medical industries but also in food and basic biological sciences (Hisano *et al.,* 1989).

Bacterial metalloproteases are associated with virulence and matrix metalloproteases of eukaryotes play a role in processing of precursors which play modulation roles in tumor formation (Lennarz *et al*., 1991; Makinen and Makenin, 1994). Mazotto *et al*.(2010) have reported the isolation of *Bacillus subtilis* AMR from poultry wastes which could hydrolyze human hair producing serine peptidases with keratinase and gelatinase activity. They suggested that the peptides obtained from enzymatic hydrolysis of hair may be useful for the production of pharmaceutical and cosmetic formulations. Thus, gelatinases could be used for recovery of silver from used photographic films, treatment of waste (poultry and animal waste) and they are medically important as targets for drug development and for design of inhibitors for disease treatment. Mazollo *et al*. (2011) have isolated *Bacillus* sp. capable of acting

on gelatin, keratin and casein from agroindustrial residues in a poultry farm. They observed degradation of feather along with production of enzyme using feather as a cheap eco-friendly substrate.

Gelatinase enzyme produced by microorganism hydrolyze gelatin into its sub compounds (polypeptides, peptides and amino acids) that can cross the cell membrane and be used by the organism. Forms of gelatinases are expressed in several bacteria including *Pseudomonas aeruginosa, Staphylococcus aureus, Clostridium perfringens, Serratia marcescens* and *Bacillus* (Shanmugasundaram *et al.*, 2012). The potential uses of gelatinase and their high demand, the need exists for the discovery of new strains of bacteria that produce enzymes with novel properties.

1.5. Objectives of the Study

Among the proteases 'gelatinases' are those enzymes which cleave gelatin, casein, fibrinogen, etc.to result in polypeptides, peptides and amino acids. They are metalloendopeptidases which have applications in leather industry, production of fish sauce, fish processing, peptide synthesis etc. Whereas literature available on halophilic proteases, particularly gelatinases is rather scanty, while there is more scope in exploring halophiles, as a source of proteases. Considering the potentials of gelatinase for industrial applications and the lack of information available in literature on haloarchaeal gelatinase it was desired to explore the haloarchaea towards isolating potential gelatinase producing halophilic archaea towards their prospective utilization in industry. Hence this study was planned with the following objectives.

Specific objectives of the present study include:

1) Isolation of halophiles and screening of gelatinase.
2) Optimization of bioprocess conditions for gelatinase production under SmF by *Natrinema* sp. BTSH10.
3) Purification of the enzyme.
4) Characterization of the enzyme.
5) Application studies.

Chapter 2

REVIEW OF LITERATURE

2.1.Archaea

Archaea, which are highly adapted to survive in extreme environments, comprise of hyperthermophiles, halophiles and methanogens and are more closely related to the Eukarya than to the Eubacteria (Fig. 2.1) (Bullock, 2000). Based on 16S rDNA analysis, archaea are classified into four major Kingdoms – Crenarchaeota, Euryarchaeota, Korarchaeota (Grant and Larsen,1989) and Nanoarchaeota (Huber *et al*., 2002).

The Kingdom Crenarchaeota comprises organisms that thrive in very hot and very cold environments. Majority of cultured Crenarchaeotes are hyperthermophiles, isolated from geothermally heated soils or wastes containing elemental sulphur and sulphides. Psychrophilic Crenarchaeotes have been identified from community sampling of 16S rRNA genes from many non thermal environments. Marine planktonic Crenarchaeotes have been isolated in large numbers from Antarctic region (Madigan *et al*., 2009).

The Kingdom Euryarchaeota includes thermophilic methanogens, methanogens, halophiles and hyperthermophiles. Methanogens are obligate anaerobes abundantly seen in intestinal tracts of animals, sewage treatment facilities, marine and fresh-water sediments, bogs and deep soils. Extremely halophilic archaea are a diverse group of prokaryotes that inhabit hyper saline environments such as solar salt evaporation ponds, the surfaces of heavily salted foods like certain fish and meats, and natural salt lakes. Extreme halophiles are obligate aerobes with a requirement of high salt concentrations for growth (Madigan *et al*., 2009).

The Kingdom Korarchaeota includes hyper thermophiles growing optimally at 85°C and were originally discovered from iron and sulphur-rich Yellow Stone hot spring, Obsidian Pool (Madigan *et al*., 2009).

Kingdom Nanoarchaeota has only one representative, *Nanoarchaeum equitans* which is an obligatory symbiont on the archaeon *Ignicoccus* (Huber *et al.*, 2002). *Nanoarchaeum equitans* has the smallest archaeal genome (around 500 kb) and the initial studies of single stranded ribosomal RNA indicated a vast difference between this group and the Kingdoms Crearchaeota and Euryarchaeota. Brochier *et al.*(2005) suggested that the initial sample of ribosomal RNA was biased and *Nanoarchaeum* actually belongs to Euryarchaeota.

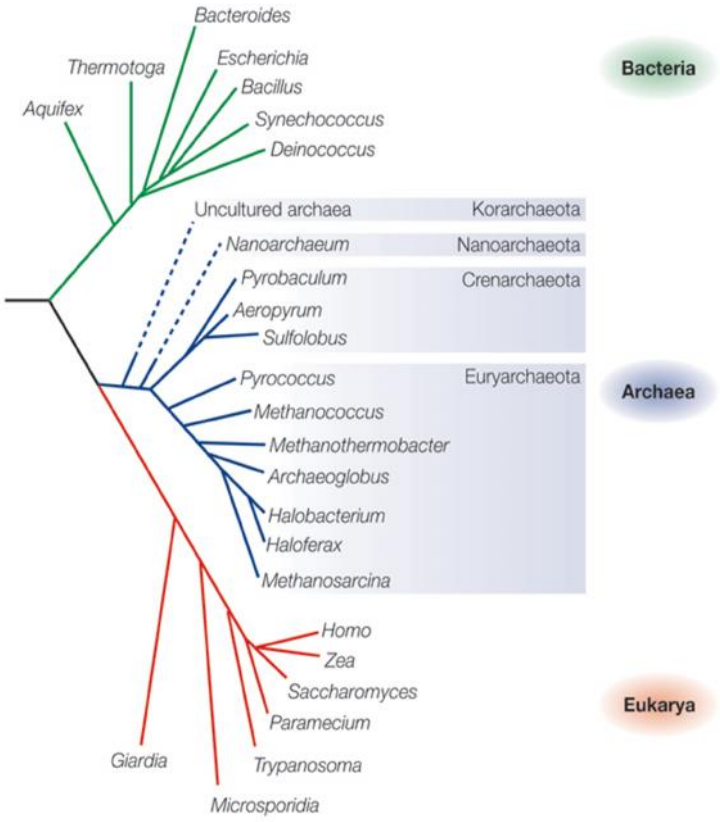


Fig. 2.1. Phylogenetic tree of the three domains of life (Allers & Mevarech, 2005).

The term extremophile collectively applies to a number of bacteria and archaea that grow optimally under 'extreme' conditions such as acidic or alkaline pH, extremes of temperature, extremes of atmospheric pressure and extremes of salt or organic ion concentrations. Extremophiles are best characterised according to their

growth profiles, using marginal data, under certain culture or environmental conditions, such as NaCl ranges ($NaCl_{opt}$, $NaCl_{min}$, $NaCl_{max}$) or temperature profile (T_{opt}, T_{min}, T_{max}) (Mesbah and Wiegel, 2008). Examples of extremophiles include, thermophiles (high temperature), psychrophiles (low temperature), acidophiles (low pH), alkaliphiles (high pH), piezophiles (high pressure, formerly known as barophiles), halophiles (high salt concentration), osmophiles (high concentration of organic solutes), oligotrophs (low concentration of solutes and or nutrients) and xerophiles (very dry environment) (Mesbah and Wiegel, 2008). Extreme environments are proving to be a valuable source of microorganisms that secrete interesting new molecules and these properties seem to offer numerous applications in various fields of industry (Margesin and Schinner, 2001).

2.2.Halophiles

Extremely hypersaline habitats are seen in hot, dry areas of the world. They are of two types: thalassohaline and athalassohaline environments. Hypersaline environments which originate by evaporation of sea water are called thalasssohaline environments (eg. Great Salt Lake). Their salt composition is similar to that of sea water, with the dominating ions being sodium and chloride ions. The pH is near neutral or slightly alkaline. Thalassohaline brines (saltern crystallizer ponds) display bright colouration due to the large numbers of pigmented microorganisms they harbour. In athalassohaline hypersaline environments, like the Dead Sea, the concentration of divalent cations exceeds that of monovalent cations and the pH is around 6. Oren (1988) reported presence of microorganisms in the Dead Sea. Cayol *et al.* (1994), have reported microorganisms capable of tolerating high salt concentrations (200g/L) and high temperatures of around 68°C. Jie Lu *et al*. (2001), isolated an extremely halotolerant *Oceanobacillus ilheyensis* from Ilheya Ridge at a depth of 1050 m below sea level. Oren (2002) reported presence of microbial life in alkaline soda lakes with high pH values of 11 and higher and high salt concentrations about 300g/L. Rohban *et al.*(2009) reported hydrolytic enzyme producing *Oceanobacillus* sp. isolated from Howz Soltan Lake in Iran.

Halophilic archaea have requirement for high concentrations of NaCl (3.5-4.5 M). Some strains may grow at low salt concentrations of 1.5 M NaCl while others grow well in saturated NaCl (5.2 M). Haloarchaea accumulate KCl up to 5 M

(Matheson *et al.*, 1976). It was reported that intracellular enzymes of halophilic archaea have requirement for high levels of KCl (Kushner, 1985). *Halobacillus halophilus* a moderate halophile was shown to use a hybrid strategy for osmoadaptation by accumulating both molar concentrations of chloride and compatible solutes (Hänelt and Müller, 2013). This distinctive feature enables *H. halophilus* to grow over a broad range of salinities (up to 3 M) and to adapt sufficiently to rapidly changing environments. The salinity and growth-phase dependent adaptation of the accumulated solutes is incredible and probably demonstrates a long lasting evolution being optimally prepared for its changing environment. A dominant compatible solute, such as carbon and nitrogen is used to guarantee energy optimization.

According to Grant *et al*. (1998), who reviewed the diversity of halophilic bacteria and archaea, halophilic bacteria include *Chlorobium limnicola*, *Thiocapsa halophila*, species of *Acinetobacter*, *Alteromonas*, *Deleya*, *Flavobacterium*, *Marinomonas*, *Pseudomonas* and *Vibrio*. Species belonging to genera *Marinococcus*, *Bacillus*, *Sporosarcina* and *Salinococcus* have been isolated from saline soils and salterns. Hypersaline waters harbour archaeal genera including *Haloarcula*, *Halococcus*, *Halobaculum*, *Halobacterium*, *Halorubrum*, *Haloferax* and *Haloterrigena*. *Halobacterium salinarum* has been isolated from salted food. Hyper saline lakes also harbour halophilic methanogens like *Methanohalophilus mahii*, *M. halophilus* and *M. evestigatum*. According to Antón *et al.*(2000 and 2002), Eubacteria belonging to genus Halorhodospira (γ-Proteobacteria), the actinomycete *Actinopolyspora halophila* and *Candidatus salinibacter* resemble haloarchaebacteria in their salt requirement.

The classification of halophilic archaea is as follows- Domain: Archaea, Class: Halobacteria, Order: Halobacteriales and Family: Halobacteriaceae. Family Halobacteriaceae comprises of 40 genera encompassing 137 species (Parte, 2013; Minegishi, 2013). Some of the genera include *Halalkalicoccus*, *Halobaculum*, *Halobiforma*, *Halomicrobium*, *Halobacterium*, *Haloarcula*, *Haloferax*, *Halococcus*, *Halorhabdus*, *Halorubrum*, *Halosimplex*, *Halostagnicola*, *Halovivax*, *Natrialba*, *Halogeometricum*, *Haloterrigena*, *Natrinema*, *Natronolimnobius*, *Natronomonas*, *Natronococcus* and *Natronorubrum* (Grant *et al.*,2001; Oren *et al.*,2002; Itoh *et*

al.,2005; Castillo *et al.*,2006a and 2006b; Gutierrez *et al.*,2007). In fact the composition of membrane polar lipids have been used as one of the key chemotaxonomic criteria for the differentiation of haloarchaeal genera (Kamekura and Kates, 1999).

2.3. Natrinema

Natrinema sp. J7, previously named as *Halobacterium salinarum* J7, was isolated from a salt mine in Hubei province, China. It was found that this strain harbors a high copy number plasmid pHH205 and possesses extracellular proteolytic activity(Ye *et al*., 2003). A gene encoding an extracellular protease, *SptA*, was cloned from the halophilic archaeon *Natrinema* sp. J7. The SptA gene was expressed in *Haloferax volcanii* WFD11, and the recombinant enzyme could be secreted into the medium in an active mature form. The N-terminal amino acid sequencing and MALDI-TOF mass spectrometry analysis of the purified *SptA* protease indicated that the 152-amino acid prepropeptide was cleaved and the C-terminal extension was not processed after secretion. The *SptA* protease was optimally active at 50°C in 2.5 M NaCl at pH 8.0. When the twin-arginine motif in the signal peptide of SptA protease was replaced with a twin-lysine motif, the enzyme was not exported from *Hfx. volcanii* WFD11 (Shi *et al*., 2006). A halophilic extracellular serine protease produced by *Natrinema* sp. R6-5 with molecular size 62 kDa was purified using bacitracin-Sepharose 4B chromatography. The protease exhibited optimum activity at NaCl concentration of 3 mol/L. At the optimum NaCl concentration of 3 mol/L, the optimum temperature and the optimum pH were 45°C and 8.0 (Shi *et al*., 2007). *Natrinema* sp., isolated from a hypersaline lake in Iran produced 6 different types of enzymes including protease, lipase, pullulanase, cellulase, chitinase and inulinase (Makhdoumi Kakhki *et al*., 2011). *Natrinema* sp., isolated from Lonar lake in Maharashtra was found to produce amylase, caseinase, cellulase and xylanase (Patil and Bajekal, 2013).

Feng *et al*. (2012) sequenced the complete genome of *Natrinema* sp. J7-2, an extreme haloarchaeon capable of growing on synthetic media without amino acid supplements. The complete genome sequence of *Natrinema* sp. J7-2 was found to be composed of a 3,697,626-bp chromosome and a 95,989-bp plasmid pJ7-I. This was the first report of complete genome sequence of a member of the genus *Natrinema*.

They reconstructed the biosynthetic pathways for all 20 amino acids and discussed a possible evolutionary relationship between the haloarchaeal arginine synthetic pathway and the bacterial lysine synthetic pathway. The genome harboured the genes for assimilation of ammonium and nitrite, but not nitrate, and had a denitrification pathway to reduce nitrite to N_2O. *Natrinema* sp. BTSH10 isolated from saltpan of Kanyakumari, Tamilnadu, India was identified, and medium for enhanced production of halocin SH10 was optimized (Karthikeyan *et al*., 2013).

2.4. Haloenzymes

Halozymes are enzymes produced by the halophilic archaea. Several enzymes isolated from archaea such as xylanases and cellulases could play important roles in the chemical, pharamaceutical, paper pulp or waste treatment industries. Research on hydrolytic enzymes from halophilic organisms was pioneered by Norberg and Hofsten in1969 (Norberg and Hofsten, 1969). Since then, a considerable amount of effort has been directed towards the evaluation of extracellular salt-tolerant enzymes of the moderately halophilic bacteria and the use of such enzymes in biotechnological processes (Ventosa *et al*., 1998; Eichler, 2001; Oren, 2002; Zhang and Kim, 2010). Halophilic enzymes have been suggested for use in biotechnological applications due to their halotolerance, thermostability for long incubation periods and ability to retain activity in presence of high levels of organic solvents (Eichler, 2001; Madern *et al*., 2000). Halophilic bacteria producing alkaline proteases displaying thermostability, activity at high pH, organic solvent stability and detergent compatibility have been reported (Makhija *et al*., 2006). Hydrolases and isomerases from extremely *Haloarchaea* have potential application in several biotransformations in the production of supplements and are exploited in the production of fermented food (Margesin and Schinner, 2001). Extracellular production of halophilic enzymes such as xylanases, amylases, proteases and lipases has been reported from many halophiles belonging to the genera *Haloferax, Halobacterium, Halorhabdus, Marinococcus, Micrococcus, Natronococcus, Halobacillus* and *Halothermothrix* (Eichler, 2001; Zhang and Kim, 2010). Production of a fructose-1,6-biphosphate aldolase from *Haloarcula vallismortis* (Krishnan and Altekar, 1991), lipase by *Natronococcus* sp. (Bhatnagar *et al*., 2005) and β-xylanase by *Halorhabdus utahensis* (Waino and Ingvorsen, 2003) was also reported. In addition, use of halophilic organisms and their enzymes for biodiesel production (Begemann *et al*.,2011) and for

degrading cellulosic biomass with reduced requirement for high temperature and pH neutralization of pretreated biomass before fermentation, have been also reported.

2.5. Proteases

2.5.1. Classification of Proteases

Barrett (2001) has classified proteases based on three criteria:

(i)Type of reaction catalyzed,

(ii) Evolutionary relationship with reference to structure and

(iii) Chemical nature of catalytic site.

Based on their site of action proteases are broadly classified as exopeptidases and endopeptidases. Based on their catalytic mechanism, proteases are classified into four types, (a) serine proteases,(b) aspartic proteases,(c) cysteine proteases and (d) metalloproteases. Depending on the pH of optimal activity, proteases are classified into three types and they are acidic, neutral and alkaline proteases.

2.5.2.Exopeptidases

Exopeptidases cleave peptide bonds near the ends of the polypeptide chain and are further classified as aminopeptidases (acting at amino terminus) and carboxypeptidases (acting at carboxy terminus). Aminopeptidases act at N- terminus of polypeptide chain liberating a single amino acid or a dipeptide or a tripeptide. Carboxypeptidases act at C- terminus of the polypeptide chain liberating a single amino acid or a dipeptide. Based on the nature of the amino acid residues at the active site of the enzymes carboxypeptidases are mainly of three types (Rao *et al*., 1998) and they are: (a) serine carboxypeptidases, (b) metallocarboxypeptidases and(c) cysteine carboxypeptidases,

2.5.3. Endopeptidases

Endopeptidases cleave peptide bond within the polypeptide chain, the presence of free amino or carboxyl group bears a negative influence on enzyme activity. Based on their catalytic mechanism these enzymes are grouped into four types: (a) Serine proteases, (b) Cysteine/thiol proteases, (c) Aspartic proteases and (d) Metalloproteases.

2.5.3.1.Serine Proteases(Cera,2009)

The hall mark of serine proteases is the presence of a serine in their active site (E.C.3.4.21).These enzymes are reported to have esterolytic and amidase activity also. Barett (1994) classified serine proteases into 40 families, which were further subdivided into about 13 clans. He has indicated four separate evolutionary origins for serine proteases because the primary structure of four clans are unrelated (Chymotrypsin (SA), subtilisin (SB), carboxypeptidase C (SC) and *Escherichia* D-Ala-D-Ala peptidase A(SE)). Serine proteases are generally active at neutral and alkaline pH, and the isoelectric points of serine proteases fall within the pH range 4-6. Alkaline serine proteases embody the largest subgroup of serine proteases. They cleave a peptide bond having a tyrosine, leucine or phenylalanine at the carboxyl side of the splitting bond. The optimum pH for activity of alkaline proteases is about 10, with the isoelectric point around pH 9. Serine proteases are irreversibly inhibited by inhibitors like phenylmethylsulfonyl fluoride (PMSF) 3,4-dichloroisocoumarin (3,4-DCI), tosyl-L-lysine chloromethyl ketone (TLCK), L-3-carboxy trans-2,3-epoxypropyl-leucylamido (4-guanidine) butane (E.64) and di-isopropylfluorophosphate (DFP). Some of the serine proteases, having a cysteine residue near the active site are inhibited by thiol reagents such as *p*-chloromercuricbenzoate (PCMB).

2.5.3.2. Aspartic Proteases

Aspartic proteasesalso called acidic proteases, have aspartic acid residues in their catalytic site (E.C.3.4.23). Acidic proteases are placed in clan AA. Barett, (1995), recognized three families - pepsin (A1), retropepsin (A2), and enzymes from pararetroviruses (A3). Most aspartic proteases are active at low pH (pH 3 to 4) and have isoelectric points in the range of pH 3 to 4.5. Inhibitors of aspartic proteases include pepstatin (Fitzgerald *et al*., 1990), diazoketone compounds such as di-azoacetyl-DL-norleucine methyl ester (DAN) and 1,2-epoxy-3-(*p*-nitrophenoxy) propane (EPNP) in the presence of copper ions. Microbial aspartic proteases include (i) pepsin-like enzymes produced by *Penicillium*, *Aspergillus*, *Neurospora* and *Rhizopus*, and (ii) rennin-like enzymes produced by *Mucor* and *Endothia* (Rao *et al*., 1998).

2.5.3.3.Cysteine/Thiol Proteases (Grzonka *et al*., 2001)

As many as 21 different families of cysteine proteases (E.C.3.4.22) have been described, occuring in both prokaryotes and eukaryotes (Grzonka *et al*., 2001). The catalytic activity of cysteine proteases depends on a dyad consisting of cysteine and histidine residues. The order of Cys and His (Cys-His or His-Cys) residues differ among the families (Barett, 1994). Cysteine proteases are active only in the presence of reducing agents such as cysteine or HCN.

They are divided into four groups on the basis of their side chain specificity, (i) papain-like, (ii) trypsin- like with preference for cleavage at the arginine residue, (iii) specific to glutamic acid, and (iv) others. The optimum pH of cysteine proteases is 7, but, lysosomal proteases are optimally active at acidic pH. They are susceptible to sulfhydryl agents such as PCMB but are unaffected by DFP and metal-chelating agents. Mechanism of action of cysteine proteases resembles that of aspartic proteases. Cysteine proteases cause the hydrolysis of carboxylic acid derivatives via a double-displacement pathway involving general acid-base formation and hydrolysis of an acyl-thiol intermediate.

2.5.3.4.Metalloproteases

Metalloproteases (E.C.3.4.24) are a large, diverse group of proteases (Barett, 1995), which require a divalent metal ion for their activity and this accounts for their inactivation by the addition of chelating agents or by dialysis. The ion coordinates to the protein via three ligands (histidine, glutamate, aspartate, lysine or arginine) and a labile water molecule. Rao *et al*. (1998) reported that neutral metalloproteases show specificity for hydrophobic amino acids, while the alkaline metalloproteases have a very broad specificity.

About 61 families of metalloproteases have been recognized (Jisha *et al*., 2013), of which 17 contain only endopeptidases, 12 contain only exopeptidases, and 1 (M3) contains both endo- and exopeptidases (Rao *et al*., 1998). Families of metalloproteases have been grouped into 16 clans based on the nature of the amino acid that completes the metal-binding site; e.g., clan MA has the sequence HEXXH-E and clan MB corresponds to the motif HEXXH-H (Rao *et al*.,1998). The activity of metalloprotease requires the binding of a divalent metal ion to a His-Glu-Xaa-Xaa-His

motif. It is believed that the Glu143 helps the nucleophilic attack of a water molecule on the carbonyl carbon of the scissile peptide bond polarized by the Zn^{2+} ion (Holm and Mathews, 1981).

The HExxH motif forming a α-helix is well conserved active site in many monozinc enzymes as in which the two histidine residues coordinate with the zinc ion. Zinc-binding motifs, of some monozinc proteases are, HxxE(D)-aan-H in the carboxypeptidase family and HxD-aa12-H-aa12-H in the matrix metalloprotease family. Dipeptidyl peptidase (DPP) III has a unique zinc binding HELLGH motif as active site which coordinated with a zinc ion. The motif HELLGH could not be found in any other metalloproteases, it exists in three kinds of monooxygenases (tyrosine, phenylalanine, and tryptophan hydroxylases) as an iron-binding site, as revealed by a search of the NBRF-PIR protein sequence database (Fukasawa *et al*., 2011).

2.6.Gelatinases

Gelatinases are proteases and found in humans as matrix metalloproteinases (MMP 2 & 9) which break down extracellular matrix, playing a role in embryonic development, morphogenesis, reproduction and tissue remodelling as well as in diseases like arthritis, cardiovascular and neurological diseases and also cancer and metastasis hence, they are medically important as targets for drug development (Pacheco *et al*., 1998; Stetler-Stevenson *et al*., 1993; Deryugina and Quigley, 2006; Zitka *et al*., 2010). It has been proved that bacterial metalloproteases are associated with virulence and matrix metalloproteases of eukaryotes play a significant role in processing of precursors which play modulation roles in tumour formation (Lennarz *et al.*,1991; Makinen *et al*.,1994). Metalloproteases have thus attracted considerable attention for development of inhibitors for disease treatment (Tamaki *et al*.,1995). Gelatinases have role in connective tissue degradation associated with tumour metastasis, hence, they are medically important as targets for drug development (Pacheco *et al*., 1998; Stetler-Stevenson *et al*., 1993). Matrix metalloproteinases (MMPs) degrade components of extracellular matrix (Murphy and Docherty, 1992). Gelatinases A and Gelatinase B cleave gelatins and also types IV and V collagens (Collier *et al*., 1988) and elastin (Senior *et al*., 1991). Production and characterization of a collagenolytic serine proteinase by *Penicillium aurantiogriseum* URM 4622 was reported by Lima *et al*.(2011).

Tetracycline antibiotics inhibit mammalian tissue destructive proteinases like collagenases and gelatinases during disease processes like tumour invasion, metastasis, angiogenesis, periodontitis etc. and chemically modified non-antibacterial analogues of tetracycline was reported to inhibit these enzymes without producing the typical side effects induced of antibiotics (Golub *et al*.,1996). Inactivation of gene encoding zinc metalloprotease gelatinase was found to prevent biofilm formation and this enzyme was suggested as a unique target for therapeutic intervention in enterococcal endocarditis (Hancock and Perego, 2004).

Gupta and Ramnani (2006) reported that feather hydrolysates obtained after feather degradation could be used as additives for feedstuffs, fertilizers, glues and films or used for the production of the rare amino acids -serine, cysteine, and proline. Mazotto *et al*.(2010) isolated *Bacillus subtilis* AMR from poultry waste which could hydrolyse human hair and reported that serine peptidases with keratinase and gelatinase activity hydrolysed human hair indicating that the peptides obtained may find applications in pharmaceutical and cosmetic formulations. Mazollo *et al*.(2011) reported the isolation of *Bacillus* sp. from agroindustrial residues in a poultry farm, which could degrade gelatine, keratin, casein etc. They demonstrated that feather waste could be used as a cheap and ecofriendly substrate for the enzyme.

2.7. Sources of Proteases

Microbes are the preferred source of proteases owing to their great biochemical diversity and susceptibility to genetic manipulation. They can be cultured in vast quantities within a short span of time to ensure an abundant supply of proteases. Extracellular nature of microbial proteases simplify the downstream processing of enzyme and have a long shelf life, with less stringent storage requirements. Only non-toxic and non-pathogenic microbes are used for commercial production and referred to as "genetically regarded as safe"(GRAS).GRAS is an acronym forthe phrase Generally Recognized As Safe (http://www.gmo.hr/eng/Additional-content/Glossary/generally-recognized-as-safe-GRAS). Bacterial neutral proteases have low thermotolerance and have an affinity for hydrophobic amino acids. Some of them are metalloproteases with a requirement for divalent metal ions for activity, others are serine proteases. Bacterial alkaline

proteases are characterized by optimal activity at high pH, broad substrate specificity with optimal temperatures around 60°C which make them ideal for use in detergent industry (*http://novozymesbiotech.com). Bacillus* sp. is a potent producer of neutral and alkaline proteases among bacteria and prominent among them are *Bacillus licheniformis, Bacillus subtilis, Bacillus amyloliquefaciens* etc. (Kumar *et al*., 1999a; Rao *et al*., 1998; Schallmey *et al*., 2004; Fujinami and Fujisawa, 2010). *Bacillus subtilis* (MTCC9102) was reported to produce keratinase by solid state fermentation using horn meal as a substrate (Kumar *et al*., 2010). *Pseudomonas* sp. was recognized as potent protease producers (Bayoudh *et al*., 2000). A metalloprotease secreted by *Pseudomonas aeruginosa* MTCC 7926 which was found to be useful for dehairing of animal skin, processing of X-ray film and exhibited antistaphylococcal activity was reported by Patil and Chaudhari (2009). A Ca^{2+}dependent metalloprotease, capable of degrading insect tissues, was purified from entomopathogenic bacterium *Photorhabdus* sp. strain EK1 (PhPrtPI) (Soroor *et al*., 2009). Halophilic proteases have been isolated and characterized from several bacterial species including *Bacillus* species (Shivanand and Jayaraman, 2009). A marine *Exiguobacterium* sp. was reported for concomitant production of protease and antioxidant materials from shrimp biowaste (Anil Kumar and Suresh, 2014). A fibrinolytic enzyme producing *Pseudoalteromonas* sp. IND11 was isolated from the fish scales using cow dung as a substrate by solid-state fermentation (Vijayaraghavan and Vincent, 2014).

Aspergillus sp., (Chakrabarti *et al*., 2000), *Rhizopus* sp. (Banerjee and Bhattacharyya, 1992), *Conidiobolus* sp. (Bhosale *et al*., 1995), *Penicillium* sp. are the different fungi which elaborate proteases. Solid state fermentation for protease production is usually done using fungi. Fungal acid proteases have optimum activity at acidic pH and fungal neutral proteases are usually metalloproteases active at neutral pH. *Candida* sp. (Poza *et al*., 2001) and *Streptomyces* (Petinate *et al*., 1999) are known producers of proteases. Alkaline proteases from yeasts like *Cryptococcus aureus, Aureobasidium pullulans, Issatchenkia orientalis* and *Yarrowia lipolytica* (Li *et al*., 2009) and mushrooms have also been reported (Zhang *et al*., 2010; Zheng *et al*., 2011).

Viruses are known to elaborate serine peptidases, aspartic peptidases and cystine peptidases, all of which are endopeptidases (Rawlings and Barrett, 1993).

Methanococcus jannaschii, a thermophilic methanogen isolated from deep-sea was shown to produce a hyperthermophilic and barophilic protease. The enzyme showed specificity for leucine at the P1 site of polypeptide substrates and the activity and thermal stability of the enzyme increased with increase in pressure (Michels and Clark, 1997). *Pyrococcus furiosus* a hyperthermophilic archeaon was shown to produce intracellular proteases (Halio *et al*., 1997). A moderately thermophilic and halotolerant alkaline protease secreted by *Salinivibrio* sp. strain AF-2004 exhibiting broad pH ranges (5.0–10.0) was described by Amoozegara *et al*. (2007).

A halophilic isolate *Salimicrobium halophilum* strain LY20 producing extracellular amylase and protease was isolated by Li and Ying-Yu (2012). Both enzymes were highly stable over broad temperature range (30 to 80 °C), pH (6.0 to12.0) and NaCl concentration (2.5 to 20%) ranges, showing excellent thermostable, alkalistable, and halotolerant nature with remarkable stability in the presence of water soluble organic solvents. A metalloprotease from *Streptomyces olivochromogenes* was reported to be useful in organic solvent-based enzymatic synthesis and detergent formulation by Simkhada *et al*. (2010). Protease production by solid state fermentation using anchovy waste meal by moderate halophile *Serratia proteamaculans* AP-CMST isolated from fish intestine was reported by Esakkiraj *et al*.(2011). An extracellular organic solvent-tolerant protease, with outstanding activity in organic solvents and imidazolium-based ionic liquids and having potential application in low water synthetic section of industrial biotechnology was purified from a novel moderately halophilic bacterium *Salinivibrio* sp. strain MS-7 (Karbalaei-Heidari *et al*., 2013).

Protease production is constitutive but it may also be inducible. Proteases are mainly secreted during the stationary phase and are related to sporulation in many bacilli (Hanlon and Hodges, 1981). Bierbaum *et al*.(1991), reported that protease production and sporulation are two independent events and extracellullar protease production is a manifestation of nutrient limitation seen in the beginning of stationary phase.

2.8. Protease Production by Halophiles

Many halophilic archaea possess proteolytic activity and some of the extracellular proteases isolated from the halophilic archaea are serine proteases which enable the degradation of proteins and peptides in the natural environment (DeCastro *et al*., 2006; Oren, 2002). Enzymes which exhibit optimal activities at various ranges of salt concentration, pH and temperature are of tremendous importance for industrial processes (Rohban *et al*., 2009).

Despite the growing interest in the use of halozymes for biotechnological applications, only few reports are available regarding the production and characterization of halophilic enzymes (Bhatnagar *et al*., 2005). These include proteases of 40-66 kDa isolated from neutrophilic haloarchaea including the strains of *Halobacterium salinarum* (*H. halobium*) (Ryu *et al*., 1994; Kim and Dordick, 1997), *Nab. asiatica* 172 P1 (Kamekura *et al.,* 1992) and *Haloferax mediterranei* 1538 (Stepanov *et al*., 1992) and R4 (Kamekura and Seno, 1993). A few extracellular proteases from haloarchaea have been characterized and their activity has been found to depend on high salt concentration with high pH (Giménez *et al.,* 2000). Alkaline protease from *Halobacterium halobium* S9 with potential application in debittering of protein hydrolysates was reported by Capiralla *et al*. (2002). Extracellular alkaline proteases from halophilic bacteria with high pH and thermostability, organic solvent stability and compatibility with detergents also have been studied (Makhija *et al*., 2006).

Moderate halophiles producing hydrolases having optimal activities at different salt concentrations could be useful in several industrial processes (Gomez and Steiner, 2004; Sanchez Porro *et al*., 2003a). Many species producing enzymes have been described including *Psuedoaltermonas* species (Sanchez-Porro*etal*., 2003b), *Salinivobrio* species (Amoozegara *et al*., 2007), *Salicola* species (Moreno *et al*., 2009), *Halobacillus* species (Namwong *et al*., 2006; Karbalaei-Heidari *et al*.,2009), *Filobacillus* species (Hiraga *et al*.,2005), *Chromohalobacter* species (Vidyasagar *et al*., 2009), *Nesterenkonia* species (Bakhtiar *et al*., 2005) and *Virgibacillus* species (Sinsuwan *et al*., 2010).

Production of proteases by halophilicarchaea in high salt medium has been reported by several researchers; *Natrinema* sp. R6-5 (Shi *et al*., 2007), *Halobacterium* sp.SP1(1) (Akolkar *et al*., 2008), *Halobacterium salinarum* (Kim and Dordick, 1997), *Natronomona spharonis* (Stan-Lotter *et al*., 1999), *Natrialba magadii* (Giménez *et al*., 2000), *Natronococcus occultus* (Studdert *et al*., 1997), *Halogeometricum borinquense* (Vidyasagar *et al*., 2006), *Halobacterium mediterranei* (Stepanov *et al*., 1992), *Natrialba asiatica* (Kamekura and Seno, 1990) and *Haloferax lucentensis* VKMM007 (Manikandan *et al*., 2009).

2.9. Fermentation Production of Protease

The total cost of enzyme production and downstream processing play a major role in the successful application of technology in enzyme production. Optimization of process parameters assesses the effect of media components on growth of microorganism and enzyme production. Beg *et al*. (2003b), stated that the traditional one-at-a-time approach leads to increase in production, but it does not take into consideration the interaction of different physico-chemical parameters. Other strategies for increasing protease production include screening for strains which produce high amount of proteases, cloning and over-expression, optimization of fermentation media etc. (Gupta *et al*., 2002).

Several statistical and non statistical methods have been used to study the effect of medium components on protease production (Montgomery, 2002; Akolkar, 2009). In order to achieve maximum enzyme production, the composition of media is different for different microorganisms, hence the media constituents and concentrations have to be optimized. Optimum production of enzymes involves efficient and economical process development (Oskouie *et al*., 2008). Stastical optimization of growth medium for protease production by *Haloferax lucentensis* VKMM 007 by response surface methodology was reported by Manikandan *et al*. (2011).

Proteases are usually produced during the stationary phase and their production is regulated by carbon and nitrogen stress. Submerged fermentation (SmF) is used for protease production. Here, the microbes are cultivated in substrates submerged or dissolved in aqueous phase. In this case, the different parameters can

be monitored and controlled and hence, the process of scale up from laboratory scale to industry is much easier (Paul, 2005). In solid state fermentation (SSF), microbes are grown on moist solid substrates without free flowing water. Use of cheap substrates, less requirement for water and production of metabolites in concentrated form resulting in downstream processing which is less expensive and time consuming, make SSF a very economic alternative for enzyme production. Alkaline protease production using *Aspergillus versicolor* PF/F/107 by solid state fermentation was described by Choudhary and Jain (2012).

Production of alkaline proteases have also been carried out using different agro-industrial wastes like green gram husk, chick pea husk, wheat bran, rice husk, lentil husk, cotton stalk, crushed maize, millet cereal, tannery wastes, shrimp wastes, date wastes, grass and potato peel, etc., in order to cut cost of production (Prakasham *et al.*, 2006; Khosravi-Darani *et al.*, 2008; Mukherjee *et al.*, 2008; Nadeem *et al.*, 2008; Akcan and Uyar, 2011; Haddar *et al.*, 2011; Ravindran *et al.*, 2011).

Various factors affect protease production such as the presence of amino acids in the medium, presence of easily metabolizable sugar like glucose (Beg *et al.*, 2002), and metal ions (Varela *et al.*, 1996). Factors like pH, temperature, aeration, inoculum density etc also have a profound effect on protease production (Puri *et al.*, 2002). Protease production by microorganisms is influenced by various nutritional factors such as metal ions, carbon and nitrogen source, easily utilizable sugars like glucose and rapidly metabolizable amino acids. Metal ions such as Fe^{3+}, Ca^{2+}, Mg^{2+} and Zn^{2+} affect protease production in microorganisms (Singh *et al.*, 2001; Thangman and Rajkumar, 2002; Adinarayana *et al.*, 2003).

Members of genus *Halobacterium* utilize amino acids, peptides and proteins as carbon and energy source; carbohydrates are not utilized by these microorganisms (Grant and Larsen, 1989). An alkaline salt tolerant protease produced by *Halomonas marisflava* KCCM 10457 showed highest activity towards the substrate gelatin (In *et al.*, 2005). In the case of *Natrialba magadii* (D'Alessandro *et al.*, 2007) and *Bacillus* sp. (Patel *et al.*, 2005), yeast extract and gelatin increased protease production respectively. Skimmed milk and yeast extract have been reported as good carbon and nitrogen source for haloarchaeal protease production and Ca^{2+} ions are known to

increase the production of haloarchaeal protease (Vidyasagar *et al*., 2006). It was reported that the protease production by *Halobacterium* sp. SP1(1) was highest in presence of protein rich soybean flour (Akolkar, 2009).

Halophilic archaea (extreme halophiles) generally requires 15-30% NaCl for optimum growth, depending on the species, (Oren, 2002). It was reported that 12% NaCl was optimum for protease production by *Salinivibrio* (Lama *et al*., 2005). In the case of *Halogeometricum* sp. TSS101 Ca^{2+} at 200 mM concentration enhanced the protease production and Fe^{2+} was found to be inhibitory (Vidyasagar *et al*., 2006). Production of protease by *Halobacterium* sp. SP1(1) increased on supplementing the medium with $FeCl_3$ and Ca^{2+} at 200 mM concentration inhibited the growth of *Halobacterium* sp. SP1(1) (Akolkar, 2009). Incorporation of Ca^{2+}, Mn^{2+}, Mg^{2+} together (200mM) in growth medium yielded maximum enzyme production in the case of *Halobacterium* sp. (VijayAnand *et al*., 2010)

Surfactants are known to affect cell membrane permeability (Kragh-Hansen, 1998; Helander and Mattila-Sandholm, 2000) leading to increased secretion of extracellular enzymes. Presence of CTAB caused an appreciable increase in activity of serine protease from *Halogeometricum borinquense* strain TSS101(Vidyasagar *et al*., 2006). CTAB had an inhibitory effect on growth and protease production by *Halobacterium*sp. SP1(1) and the enzymatic activity was reduced in presence of CTAB and SDS (Akolkar, 2009). Protease production by *Halobacterium* sp. SP1(1) was increased in presence of dicotylsulfosuccinate, whereas, Triton X-100, Tween 80 and Tween 20 did not affect the production (Akolkar, 2009).

2.10. Gelatinase Assay

Procedure for gelatinase assay was described by Dworschack *et al*.(1952). This method was followed by Pisano *et al.*(1964) while studying the gelatinase production by marine fungi. Purified calfskin gelatin was the substrate for the reaction carried out at 40°C (pH 7.5). The degree of liquefaction was determined viscometrically and amino nitrogen liberated measured by Sorensen's titration as modified by Dworschack *et al*. (1952). A spectrophotmetric assay for gelatinase using succinylated gelatin was described by Baragi *et al*.(2000). This is based on the measurement of primary amines by hydrolysis of substrate by gelatinases, with

sensitivity comparable to that of gelatin zymography. Tran and Nagano (2002) described a gelatinase assay, wherein gelatinase was incubated with gelatin in TrisHCl (pH 7.5) with 12 mM $CaCl_2$. The reaction was stopped using HCl and the released free amino group was measured by ninhydrin method. Gelatinase activity was expressed as µmol of leucine equivalent /min/mL of the culture filtrate (Hamza *et al.*, 2006). The caseinolytic activity of halophilic proteases decreased beyond 2M NaCl concentration because casein loses its original conformation at higher NaCl concentrations limiting the availability of substrate to the enzyme (Capiralla *et al.*, 2002).

2.11.Purification of Protease

Purification of proteases is imperative for the understanding of their properties and action. Most proteases are extracellular in nature. The culture of microorganism is separated from the fermented broth by centrifugation or filtration and the culture supernatant is subjected to different methods of purification. In case the protease is intracellular, appropriate methods for cell disruption are to be adopted (Walsh, 2001). Various methods have been used for downstream processing to purify extracellular proteases from the culture broth. Commonly used methods include ammonium sulphate precipitation followed by affinity chromatography and gel filtration (Kumar *et al.*, 1999a).

Ammonium sulphate is used for precipitating proteases from culture supernatant. Ammonium sulphate is inexpensive, highly soluble and does not denature the proteases, hence this is preferred by many researchers (Adinarayana *et al.*, 2003; Anita and Rabeeth, 2010). Acetone has been used for extraction (Kumar *et al.*, 1999a). Ethanol has also been used for extraction (El-Shanshoury *et al*., 1995). Ultrafiltration is another method used for concentration with no loss of activity (Kang *et al*.,1999). Diafiltration is another method for salt removal or changing salt concentration (Peek *et al.*, 1992). Other methods for concentration include lyophilization (Manonmani and Joseph, 1993) and use of activated charcoal (Aikat *et al.*, 2001).

Different chromatographic techniques used for purification of protease include:

1. Affinity chromatography using adsorbents like dydroxyapatite (Kobayashi *et al.*, 1996), aprotinin-agarose (Petinate *et al*., 1999), casein-agarose (Hutadilok-Towatana *et al.*, 1999) and bacitracin-sepharose (Manikandan *et al.*, 2009) have been described. The labile nature of affinity ligands and high cost of enzyme supports are the major limitations of this method.

2. Ion Exchange chromatography, the matrices for which contain functional groups like Diethylaminoethyl and carboxymethyl which get associated with the charged protein molecules adsorbing them to matrices. The adsorbed protein is then eluted out using gradient change in ionic strength or pH of eluting buffer. Sana *et al.*(2006) has described the use of DEAE cellulose for purification of alkaline protease.

3. Hydrophobic interaction chromatography is based on the binding of hydrophobic amino acid residues on proteins to hydrophobic surfaces. These interactions are strengthened by high temperatures and high salt concentrations and are weakened considerably in presence of miscible organic solvents or detergents. Karan and Khare (2010) have reported the use of phenyl sepharose 6 for hydrophobic interaction chromatography.

4. Gel filtration using Sephacryl (Kumar *et al.*, 1999b), Sepharose (Singh *et al*., 2001) and Sephadex (Adinarayana *et al*., 2003) is used for separation based on size. The desired protein gets diluted and this method has lower capacity for loading proteins.

Affinity precipitation, in which, ligand polymer is added to enzyme solution under conditions favouring binding of the desired protein. The ligand polymer is precipitated and the supernatant is removed. The protein is eluted from the polymer using suitable conditions. This method is used to purify alkaline protease from *B. licheniformis* (Pecs *et al*., 1991).

Other techniques are also used for purification of proteases. Aqueous two phase system of PEG/Citrate was used to purify proteases produced by *Clostridum perfringens* from culture broth (Porto *et al*., 2008). Extraction of alkaline protease from the culture broth of *Nocardiopsis* sp. was done using reverse micelles of sodium di(2-ethyl hexyl) sulfosuccinate (AOT) in isooctane with equal phase volume ratio (Monteiro *et al*., 2005).

Halophilic archaea produce proteases that are active and stable at high salt concentrations (4 to 5 M) and the absence of salt cause irreversible inactivation of

these proteases (Lanyi, 1974). Hence, the conventional methods used for purification of non-halophilic proteases fail to purify haloarchaeal proteases. The presence of high salt in the medium inhibits the precipitation of haloarchaeal proteases by saturating with ammonium sulphate. Ethanol precipitation method has been reported for haloarchaeal proteases by several researchers (Vidyasagar *et al*., 2006). Capiralla *et al.*(2002) have reported the use of ultrafiltration and the use of Bacitracin-Sepharose column for purification of protease by *Halobacterium halobium* S9.

Halozymes are unstable at low concentration of neutral salts, this factor imposes considerable restrictions on the choice of purification techniques (Madern *et al*., 2000). Haloarchaeal proteases were purified by ethanol precipitation followed by affinity chromatography and gel filtration (Gimenez *et al*., 2000; Vidyasagar *et al*., 2006). The extracellular protease from *Halobacterium* sp. SP1(1) was purified by gel permeation using sephacryl S-200 column (Akolkar, 2009).

2.12. Characterization of Protease

Some of the enzymes may display polyextremophilicity. For instance, the enzymes may be *haloalkaliphilic* (Gupta *et al.,* 2005) or *halothermophilic* (Vidyasagar *et al*., 2009). Consequently, halophilic and halotolerant bacteria harbour a pool of proteases that will be more suitable for application in food production processes involved in the production of various protein rich foods including processing of fish and meat-based products and the production of soy sauce (Setyorini *et al.,* 2006). Moreover, the enzymes derived from halophiles make excellent additives for laundry detergent as most of them are either alkali tolerant or alkaliphilic. Some proteases such as those from *Nesterenkonia* species have been reported to display unique substrate specificities which might open up new application opportunities (Bakhtiar *et al*., 2005).

The extracellular proteases isolated from halophilic archaea are serine proteases and are closely related to the subtilisin-like serine protease subfamily S8A and have been denoted as halolysins (DeCastro *et al.,* 2006). Protein sequences of haloarchaeal extracellular proteases have retained the conserved "Ser-His-Asp" catalytic triad (DeCastro *et al.,* 2006). A serine protease isolated from *Halobacterium salinarum* P-535 had maximum activity at 37°C between pH 8-9 (Izotova *et al*.,

1983). A serine protease of *Halobacterium mediterranei* also shows optimum activity at pH 8-8.5 (Stepanov *et al*., 1992).Most of the enzymes from halophilic archaea are stable at high salt concentrations, representing a model for biocatalysis in low water activity media (Sellek and Chaudhary, 1999) and an attractive example of adaptation (Madern *et al*., 2000).

Capiralla *et al*. (2002) reported optimum activity of an extracellular serine protease from *Halobacterium halobium* S9 at 40°C and pH 8.7. Lama *et al*.(2005) noted that the optimum pH for a protease isolated from *Salinivibrio* was 8. These enzymes displayed optimal activity in the presence of NaCl and maintained stability over a wide pH range (pH 5-10). Further, the enzymes were active at temperatures of 40-75ºC, while some of the enzymes displayed an absolute requirement of NaCl (Vidyasagar *et al.*, 2009). The trypsin–like serine protease described by Manikandan *et al*. (2009) showed optimum activity at pH 8 and its optimum temperature was 60°C at 4.3 M NaCl. The optimum temperature for halophilic proteases are varied; 75°C for a protease from *Chromohalobacter* sp. TVSP101 (Vidyasagar *et al*., 2009), 40°C for a protease produced by *Halobacterium* sp. PB407 (Werasit *et al*., 2004), 60°C for extracellular protease from *Natronococcus occultus* (Studdert *et al*., 2001), *Haloferax lucentensis* VKMM007 (Manikandan *et al*., 2009) and *Salinivibrio* (Lama *et al*.,2005).

Thermostability of enzymes is enhanced by stabilizers like PEG, starch, polyhydric alcohols or by manipulating the structure of enzymes by protein engineering. Ca^{2+} ions increase the activity and stability of alkaline proteases at high temperatures (Kumar, 2002) and ions like Mn^{2+}, Ba^{2+}, Mg^{2+} and Zn^{2+} are used for stabilizing proteases (Johnvesly and Naik, 2001). These metal ions prevent thermal denaturation and maintain active conformation of enzymes at high temperatures.

According to DeCastro *et al*. (2006) molecular mass of haloarchael proteases are within the size range 41-66 kDa. Thus, proteases with different molecular masses have been reported. Molecular mass of 49 kDa for protease isolated from *Natronobacterium* sp. (Yu, 1991); a 41 kDa serine protease from *Halobacterium mediterranei* (Stepanov *et al*., 1992); 66 kDa protease from *Halobacterium halobium* (Kim and Dordick,1997), and *Chromohalobacter* sp. TVSP101 (Vidyasagar *et*

al.,2009) were reported. Protease of *Natronococcus occultus* with a size of 130 kDa was the largest among the group (Studdert *et al*., 2001).

Kinetic parameters like K_m, V_{max} and K_{cat} are important for designing enzyme reactors and quantifying applications of enzyme under different conditions. Banerjee and Bhattacharyya (1993) reported that the K_m and V_{max} for an alkaline protease from *Rhizopus oryzae* increased when the temperature increased from 37°C to 70°C. Beg *et al*. (2002), reported that an alkaline protease from *B. mojavensis* the K_m for casein decreased with increase in V_{max} when the reaction temperature was increased from 45°C to 60°C.

Characterization of the biochemical properties in combination with the gene information would be helpful to improve the understanding of halophilic proteases. The mechanism of secretion and activation of extracellular haloarchaeal proteins remains to be elucidated (De Castro *et al*., 2006).

2.13. Molecular Characteristics of Halophilic Proteases

Nucleic acid and amino acid sequences of proteases serve to derive primary structure and identify functional regions of proteases. They serve as a prelude to phylogenetic analysis of proteins and help in predicting secondary structures of proteins and DNA and also elucidate the structure-functional relationship of proteases (Rao *et al*., 1998). Haloarchaea represent suitable model systems to study protein breakdown in archaea. During the last decades several research advances have been made to further our understanding of haloarchaeal proteases. Glutamate residues have high water binding capacity and are found in excess on the surface of halophilic proteases (Dym *et al*., 1995; Britton *et al.,* 2006). Acidic amino acid residues constitute up to 20-23% of halophilic protein (Ishibashi *et al*., 2001; De Castro *et al.*, 2008). Negatively charged amino acid residues in halophilic proteins bind hydrated cations and maintain a surface hydration layer, reducing their surface hydrophobicity and contribute to mutual electrostatic repulsion (Kastritis *et al.,* 2007; Tadeo *et al.*, 2009). These properties prevent the aggregation of proteins at high salt concentration (Elcock and McCammon, 1998). Halophilic proteins lack lysine residues on the protein surface (Kennedy *et al*., 2001; Fukuchi *et al*., 2003; Paul *et al*., 2008). In halophilic proteins, lysine residues are replaced by arginine residues, the greater

hydrophilicity of the guanidinyl side chain plays a role in maintaining active protein structure (Pire *et al.*, 2004; Britton *et al.*, 2006; Esclapez *et al.*, 2007; Kastritis *et al.*, 2007; Tadeo *et al.*, 2009). The number of larger hydrophobic amino acid residues like phenylalanine, isoleucine and leucine was less compared to small amino acid residues like glycine and alanine and borderline hydrophobic amino acid residues like serine and threonine (Fukuchi *et al.*, 2003; Bolhuis *et al.*, 2008; Paul *et al.*, 2008). Another interesting feature of halophilic proteins is that oppositely charged neighbouring residues interact to form salt bridges which play a role in protein folding, structure and oligomerization. An increase in salt concentration decreased electrostatic repulsion by ion binding, stabilising oligomerization necessary for catalytic activity (Bandyopadhyay and Sonawat, 2000). High salt concentration enhances the native conformation and function in halophilic proteins (Jolley *et al.*, 1997; Rao *et al.*, 2009). The salt concentration affects the folding, conformation, subunit structure and kinetics of halophilic proteins, withdrawal of salt results in loss of structure and unfolding of halophilic proteins (Müller-Santos *et al.*, 2009).

Karan *et al.* (2011), cloned and characterized the gene encoding a solvent-tolerant protease from the haloalkaliphilic bacterium *Geomicrobium* sp. EMB2. Primers were designed based on the N-terminal amino acid sequence of the purified EMB2 protease and were used to amplify a 1,505-bp open reading frame that had a coding potential of a 42.7 kDa polypeptide. The EMB2 protein contained a 35.4 kDa mature protein of 311 residues, with a high proportion of acidic amino acid residues. Phylogenetic analysis placed the EMB2 gene close to a known serine protease from *Bacillus clausii* KSM-K16. Primary sequence analysis indicated a hydrophobic inclination of the protein. The 3D structure modelling elucidated a relatively higher percentage of small (glycine, alanine, and valine) and borderline (serine and threonine) hydrophobic residues on its surface. The structure analysis also highlighted the fact that enrichment of acidic residues contributed to salt stability of the enzyme. The solvent stabilities in *Geomicrobium* sp. protease may be accorded to the presence of a number of small hydrophobic amino acid residues on the surface.

Giant proteolytic 'nanocompartments' have been isolated from haloarchaea, including 20S proteosomes (Kaczowka and Maupin-Furlow, 2003) and tetrahedral aminopeptides (TET) (Franzetti *et al.*, 2002). The genome sequences of

Halobacterium sp. strain NRC-1 (Ng *et al*., 2000), and *Haloarculamaris mortui* (Baliga *et al*., 2004) have been completed and many other haloarchaeal genome sequencing projects are undertaken such as *Haloferax volcanii* DS2 (Hartman *et al*., 2010) and *Halobaculum gomorrense, Natrialba asiatica* (Goo *et al*., 2004). General secretory (Sec) and twin-arginine-transport (Tat) pathways for protein secretion are conserved in haloarachaea (Ring and Eichler, 2004; Pohlschroder *et al*., 2005). Rose *et al*.(2002) suggests that Tat pathway is the major protein secretion pathway of *Halobacterium* sp. NRC-1, enabling the proteins to fold in the cytoplasmic milieu of high KCl prior to secretion into high NaCl environment. They suggest that halolysins are secreted by Tat pathway due to the presence of conserved twin-arginine motif within the first 35 amino acid residues.

A serralysin-type metalloproteases from marine bacterium strain YS-80-122 with 463 amino acid residues and ANGTSSAFTQ as the N-terminal amino acid sequence has been reported by Wang *et al*. (2010). An alkaline protease BPP-A from *Bacillus pumilus* strain MS-1consisting of 275 amino acids (mature protein), was reported by Miyaji *et al*. (2006). Amino acid sequence of halotolerant alkaline proteases from *Bacillus subtilis* FP-133 was studied and it was found that the mature protein consisted of 275 amino acid residues with a catalytic triad centre containing Asp, His and Ser residues. Three dimensional modelling suggested that the acidic and polar amino acid residues located on the surface stabilize protein structure in the presence of relatively high NaCl concentrations (Takenaka *et al*., 2011).

Multiple sequence alignment of alkaline protease protein sequence of different *Aspergillus* species revealed a stretch of conserved region for amino acid residues from 69 to 110 and 130 to 204. A signature motif with an amino acid sequence of 50 amino acids was uniformly observed in proteases protein sequences indicating its involvement with the structure and enzymatic function (Morya *et al*., 2012).

2.14. Recombinant Technology

Major biotechnological advances are expected in the area of protein engineering. Identification of structural properties essential for thermal activity and stability will enable development of proteins with the required catalytic and thermal

properties. The halolysin Nep is secreted by the alkaliphilic haloarchaeon *Natrialba magadii*, and the recombinant active enzyme has been synthesized in *Haloferax volcanii*. The study revealed the secretion and maturation of a Tat-dependent halophilic subtilase (Ruiz *et al*., 2012).

2.15.Applications of Proteases

2.15.1.Detergent Industry

Proteases find application in laundry detergent and dish washing detergents and cleaning detergents (Godfrey and West, 1996; Showell, 1999). Burnus the first enzymatic detergent produced in 1913, consisted of crude pancreatic extract and sodium carbonate. BIO-40, the first detergent containing bacterial enzyme was produced in 1956. Novo Industry A/S introduced Alcalase® (produced by *Bacillus licheniformis*) under the trade name BIOTEX. Gist-Brocades introduced a detergent Maxatase. Genencor International and Novo Nordisk are the major suppliers of detergent enzymes in the world market. Kannase, detergent protease active at low temperatures (10-20°C) was introduced by Novo Nordisk Bioindustry, Japan. Several oxidatively stable serine proteases (OSPs) suitable for use in detergents have been isolated from alkaliphilic *Bacillus* strains (Saeki *et al*., 2000). Surgical instruments can be cleaned using Pronod 153L (Gupta *et al*., 2002). Enzymes used for cleaning membrane systems include- P-3 pardigm (Henkel-Ecolab, Dusseldorf, Germany), Terga-zyme (Alconox, New York, U.S.A.) and Alkazym, (Novodan, Copenhagen, Denmark).

2.15.2.Leather Industry

Alkaline proteases possessing keratinolytic and elastolytic activities find use in leather industries. Treatment with proteases removes pigments, albumins and globulins; and clean hide is produced. Chemical processing of leather used chemicals like sodium sulfide which causes pollution and problems of effluent disposal. Treatment of leather using enzymes is ecofriendly and is generally preferred (Andersen, 1998). Alkaline proteases are used in combination with sodium chloride and hydrated lime for dehairing. Alkaline conditions cause swelling of hair roots followed by action of protease on hair follicle protein results in dehairing with considerable reduction in waste water generated. Pyrase, NovoLime, NUE, Aquaderm and Novocor S are different proteases manufactured by Novo Nordisk for

leather processing. A novel protease showing keratinolytic activity from *B. subtilis* which was used for dehairing process of leather industry was described by Arunachalam and Sarita (2009). A protease from *Thermoactinomyces* sp. RM4 was used for dehairing goat hides by Verma *et al*. (2011).

2.15.3.Textile Industry

Proteases find application in processing of wool and degumming of silk. Sericin which constitutes about 25% of weight of raw silk fibres confers a rough texture to raw silk fibres. Conventional methods used to remove sericin from inner core of fibroin is by conducting shrink-proofing and twist-setting for the silk yarns using starch (Kanehisa, 2000). This is an expensive process, and the alternative method is the use of proteases for degumming of silk prior to dyeing (Freddi *et al.*, 2003). Proteases are used to wash printing screens to remove proteinaceous gums used for thickening of printing pastes. Bio-polishing and bio-stoning are the current trends in the area of enzyme processing (Ramachandran and Karthik, 2004).

2.15.4.Pharmaceutical Industry

The immobilized alkaline protease from *Bacillus subtilis* possessing therapeutic properties has been used for development of ointment compositions, soft gel-based medicinal formulas, gauze, non-woven tissues and new bandage materials (Davidenko, 1999). Rao *et al*. (1998) have reported the oral administration of proteases from *Aspergillus oryzae* to correct certain lytic enzyme deficiency syndromes.

Asparagine, seen in the bloodstream in cases of lymphocytic leukemia, is removed using asparaginase produced by *E. coli*. Kim *et al*.(1996) has described an alkaline protease with fibrinolytic activity which could be used as a thrombolytic agent. Elastoesterase, produced from *Bacillus subtilis* 316M was used for treatment of carbuncles, furuncles, deep abscesses, burns and purulent wounds (Kudrya and Simonenko, 1994). Barthomeuf *et al*.(1992), described a collagenase produced by *Aspergillus niger* LCF9 which could hydrolyse collagen to release low molecular weight peptides with potential therapeutic use. Attempts were made to treat some types of lytic enzyme deficiency syndromes by oral use of proteases (Luizym and Nortase) from *Aspergillus oryzae* (Rao *et al*., 1998). Broad spectrum antibiotics in

combination with clostridial collagenase or subtilisin, is used to treat burns and wounds. Aqua-Biotechnology (http://aquabiotechnology.com) has launched a skin care product Zonase X^{TM}, which removes the dead cells in the outer layers of the human skin and accelerates the renewal and healing process of the skin.

2.15.5.Food and Feed Industry

Halophilic proteases are used for preparation of fish sauce and soy sauce as they are prepared in high salt (20-30%) containing brines (Yongsawatdigul *et al.*, 2007). Proteases are used for the manufacture of protein rich therapeutic diets, hypoallergenic infant food formulations and also fortification of fruit juices and soft drinks. Alkaline protease from *Bacillus licheniformis* is used for the production of protein hydrolysate with angiotensin I converting enzyme inhibitor activity from sardine muscles; used in blood pressure regulation (Matsui *et al*., 1993). The brewing industry uses Neutrase (neutral protease) insensitive to natural plant proteinase inhibitors (Rao *et al*., 1998).

Alkaline elastase (Takagi *et al*., 1992) and alkaline protease (Wilson *et al*., 1992) are meat tenderizing enzymes possessing the ability to hydrolyze muscle fibre proteins as well as connective tissue proteins. Alkaline protease (B72) from *Bacillus subtilis* and *B. licheniformis* PWD-1 were used for production of proteinaceous fodder from feather keratin (Cheng *et al*., 1995; Dalev, 1990, 1994)

2.15.6.Peptide Synthesis

Peptide synthesis in organic medium using halophilic proteases is an interesting application. The use of biocatalysis in organic media offers distinct advantages of higher solubility of hydrophobic species, reduced microbial contamination, and reduced water activity which alters the hydrolytic equilibrium (Sellek and Chaudhuri, 1999). Kim and Dordick (1997), have reported the efficient use of extracellular protease from *Halobacterium halobium* for peptide synthesis in water/N -N –dimethylformamide. Okazaki *et al.*(2000) used surfactant-protease complex as a biocatalyst for peptide synthesis in hydrophilic organic solvents. Chen *et al.*(1991) reported the use of Alcalase as a catalyst for the resolution of N-protected amino acid esters.

2.15.7.Silver Recovery

Silver is used in photographic industry in vast quantities. A photographic film is made up of a support layer (glass, plastic sheet, or paper) coated with an emulsion layer consisting of silver halide crystals in gelatin (Moore *et al*., 1996). Proteases can be used for recovery of silver from used X-ray films which contain about 1.5 -2% (w/w) silver in its gelatin layers (Nakiboglu *et al.,* 2001). Silver can be recovered by using chemical solutions to strip gelatin silver layer (Syed *et al*., 2002) and also by oxidation of silver following electrolysis (Ajiwe and Anyadiegwn, 2000). Stripping of gelatin using chemicals is hazardous, not economical and time consuming (Sankar *et al*., 2010). An increase in temperature was reported to cause increase in gelatin hydrolysis by Sankar *et al*. (2010). Nakiboglu *et al*. (2001) reported that 50ºC was optimum for stripping gelatin at the optimum pH 8 by enzyme of *Bacillus subtilis* ATCC 6633. Sankar *et al.*(2010) found that pH 10 is effective. Recovery of silver from used X- ray films by burning causes environmental pollution which can be overcome by the use of microbial enzymes, which also enables the polyester film base to be recycled (Kumar and Takagi, 1999).

2.15.8.Other Applications

In molecular biology, proteases are used in the process of extraction of nucleic acids (Kyon *et al.,* 1994). They are also used in selective delignification of hemp (Dorado *et al.,* 2001) and also in pest control (Kim *et al.,* 1999). Anita and Rabeeth (2010) reported the use of proteases for biodiesel production. Proteases are used for sequencing of proteins and also elucidation of structure- function relationship. Another interesting application of halophilic proteases is in antifouling coating preparations used to prevent biofouling of submarine equipments (http//www.ttc.ust.hk/patents3/index5.htm). The ease in which halophiles are grown and the absence of a necessity for aseptic conditions makes them very attractive for commercial applications including production of biodegradable plastics (Lillo and Rodriguez-Valera, 1990) and cosmetics (Galinski and Tindall, 1992).

Chapter 3

MATERIALS AND METHODS

3.1. Isolation of Halophiles

3.1.1. Sample

Tannery effluent (Sivabalan and Jayanthi, 2009) and food grade table salt crystals were used for isolation of halophiles. The effluent was procured from a tannery located in Erode district, Tamil Nadu, India. Food grade table salt crystals available in local market were purchased and used as samples.

3.1.2. Medium

Zobell's Agar (ZA) medium (HI Media, Mumbai, India) was used for the isolation and cultivation of halophilic bacteria. The ZA medium was supplemented with additional sodium chloride (NaCl) such that the final NaCl concentration in the medium was 15% and suitable for the isolation of halophilic bacteria (Karthikeyan *et al.*, 2013)

3.1.3. Plating Procedures

An aliquot of 100 µL of the tannery effluent sample was spread plated on Zobell's Agar medium. In the case of table salt crystals, a solution of 1g of salt crystals in 1 mL of sterile distilled water was prepared and 100 µL of the same was spread plated on the prepared Zobell's Agar medium. The inoculated plates were incubated at 42°C for 7 days and observed for appearance of bacterial colonies. Those bacterial colonies which appeared on the surface of agar medium were then isolated based on their colony morphology and subcultured in fresh sterile Zobell's Agar medium. All the isolates obtained were repeatedly purified by streak plate method. Single cell colonies obtained after purification were used for preparation of stock cultures which were maintained as agar slope cultures under sterile liquid paraffin. Glycerol stocks were also prepared and stored at -70°C for future studies.

3.1.4. Identification of Bacteria

All the bacterial strains isolated from the tannery effluent and table salt crystals were identified based on polyphasic taxonomy which included morphological, biochemical and physiological characteristics (Gillis *et al*., 2001). Molecular identification of isolates was done by amplification and sequencing of 16S rDNA (Shivaji *et al*., 2000).

3.1.4.1. Determination of Different Characteristics of Isolates

Gram staining of the isolates was performed according to the method described by Dussault (1955). Biochemical characterization of the isolates was done according to Cheesebrough (2006). All the media used for biochemical and physiological characterization were supplemented with 15% NaCl. The tests included IMViC, production of hydrogen sulphide (H_2S), urease, oxidase, catalase, coagulase, fermentation of glucose, sucrose, mannitol, lactose, glycerol, sorbitol, starch hydrolysis (amylase), casein hydrolysis (protease) and tributyrin utilization (lipase). Amylase production was checked on Zobell's Agar supplemented with starch. Addition of I_2-KI solution (0.1% I_2 - 0.2% KI) after growth of bacteria produced a colourless halo around colonies that produced amylase (Gonzalez *et al.*, 1978).

Lipase (Mourey and Kilbertus, 1976) production was indicated by a zone around the colonies (grown in tributyrin agar medium (HI Media, Mumbai, India) supplemented with 15% NaCl) which produced lipase. Protease production was indicated by zones around colonies grown in casein agar plates supplemented with 15 % NaCl (Cojoc *et al.*, 2009). Cultures which showed enzyme production were subcultured, purified and glycerol stocks of isolated colonies were prepared.

3.1.4.2. Molecular Classification of Isolates

Eubacterial universal 16S rDNA primer (Vidyasagar *et al*., 2009) was used. The primer sequence (Sigma Aldrich-India) was as follows. 16F27 (5¹ CCA GAG TTT GAT CMT GGC TCA G 3¹) and 16R1 525XP (5¹ TTC TGC AGT CTA GAA GGA GGT GWT CCA GCC 3¹).

The partial gene sequences of 16S rDNA of the bacterial isolates were amplified by colony PCR (Kwon *et al*., 2004) on a thermocycler MJ Mini (Biorad, USA) using eubacterial primers (Vidyasagar *et al.,* 2009).

PCR process involved the initial denaturation at 95°C for 5 min., denaturation at 95°C for 1 min., primer annealing at 65°C for 1 min., and extension at 72°C for 1 min. After 35 cycles additional extension was done at 72°C for 10 min.

Sequencing of the amplified 16S rDNA was done using Applied Biosystems 3730XL DNA Analyzer, according to Sanger's dideoxy method. The obtained 16S rDNA sequences were subjected to BLAST analysis for confirming DNA homology and identity of the isolates. Sequences were submitted to NCBI (http://www.ncbi.nlm.nih.gov/) (JX975066, JN228200, JN228197, JN228201, JN228199, JN228198, KC019171, KC019170, JX975064, JX975065 and JX975062). A Phylogenetic tree was constructed using MEGA4 (Tamura *et al*., 2007) software and the evolutionary history was inferred using Maximum Parsimony method (Eck *et al*., 1966).

3.2. Screening of Bacteria for Gelatinase Production

All the bacterial strains isolated from tannery effluent and table salts were screened for gelatinase. In addition, halophilic archaeal strains *Natrinema* sp. BTSH10 (NCBI Accession no. JN228202) and *Halorubrum* sp. BTSH03 (NCBI Accession no. JF830242) available in the culture collection of Microbial Technology Laboratory of the Dept. of Biotechnology, Cochin University of Science and Technology Cochin, Kerala, which were recognized to produce halocin as part of another study were also screened for gelatinase production (Kanemitsu *et al.,* 2001).

3.2.1. Media for Screening

Zobell's medium (M385-500G, Hi Media, Mumbai, India) supplemented with 15% NaCl was used as broth for growth of isolates and gelatinase production.

Composition of the medium is as given below:

Ingredients	**g/L**
Peptic digest of animal tissue	5.0
Yeast extract	1.0
Ferric Citrate	0.10
Sodium Chloride	19.45
Magnesium Chloride	8.80
Sodium Sulphate	3.24
Calcium Chloride	1.8
Potassium Chloride	0.55
Sodium Bicarbonate	0.16
Potassium Bromide	0.08
Strontium Chloride	0.034
Boric acid	0.022
Sodium Silicate	0.004
Sodium fluorate	0.0024
Ammonium nitrate	0.0016
Disodium phosphate	0.008

Final pH (at 25°C 7.6 ± 0.2).

Weighed 40.25 g of Zobell's medium (M385-500G, Hi Media) and dissolved in 1L of distilled water and added an additional 109.75 g of NaCl to make final concentration to 15%. Agar (4%) was added to get solid media. The medium was then autoclaved at 121°C at 15 lbs pressure for 15 min. The above said medium was used as broth for preparation of inoculum and mass culture of halobacterial and haloarchaeal isolates towards obtaining crude enzyme for gelatinase test.

3.2.2. Screening of Isolates using Gelatin Media

Gelatinase detection was performed on agar plates containing a medium composed of 0.5% gelatin, 1.5% agar and 15% NaCl. The gelatin was dissolved in little water under boiling conditions and added to the medium. The prepared medium

was autoclaved at 121°C at 15 lbs pressure for 15 min., cooled, and poured into sterile plates aseptically.

3.2.3. Preparation of Crude Enzyme from Halobacterial and Archaeal Isolates for Gelatinase Assay

All the isolates were grown in 100 mL of the Zobell's broth (mentioned in previous section 3.2.1) taken in conical flasks. The prepared broth was inoculated using freshly subcultured agar slope culture as inoculum and incubated at 37°C in a rotary shaker maintained at 125 rpm, for 5 days. After incubation the culture broth was centrifuged at 10,000 rpm at 4°C for 15 min. and the cell free culture supernatant was harvested. This supernatant was used as crude enzyme extract for gelatinase assay.

3.2.4. Gelatinase Assay

Gelatinase was assayed according to a modified method described by Kanemitsu *et al.* (2001). Proteinase K, known to act on gelatin was used as the standard enzyme to estimate haloarchaeal gelatinase activity.

Wells were cut into gelatin agar plates prepared as mentioned in previous section 3.2.2 and Proteinase K was added to each well in different concentrations. The plate was incubated at 37°C for 24 hrs. After incubation 10% TCA was added to precipitate gelatin, which resulted in the formation of zones around the wells containing Proteinase K, as the enzyme could hydrolyze gelatin. The zone diameter was measured and the correlation between the concentration of Proteinase K and zone diameter was determined. The concentration of Proteinase K vs the zone diameter was plotted, and a standard curve with good correlation was obtained (R^2 =0.978). Each mg of the standard Proteinase K contained 30 Units. This standard curve was used for computing gelatinase activity of the haloarchaea.

The crude enzyme extract obtained from haloarchaeal isolates was added to the wells cut out in gelatin plates and the zones formed on addition of TCA, after 24 h incubation at 37°C, was measured. A halo zone size of 1.5 cm diameter was found to be produced by 1 unit of gelatinase which was equivalent to proteinase K activity of

30 x 10^{-3} µg/mL. Gelatinase activity was expressed as U/mL. Using the standard graph, the gelatinase activity of the haloarchaea could be converted to Proteinase K activity and expressed as U/mL.

3.3. Selection of Potential Haloarchaea for Gelatinase Production

Among all the isolates of halobacteria obtained from tannery effluent and table salts besides the 2 stock cultures, the strain belonging to the genus *Natrinema* sp. BTSH10 was found to have the potential for gelatinase production. Hence, the same was selected and used for further studies. The strain was maintained as glycerol stock culture at -70°C, at the Microbial Technology Laboratory, Department of Biotechnology, Cochin University of Science and Technology. The haloarchaeal strain was periodically subcultured at 37°C for 4 days and stored at 4°C.

3.4. Selection of Media for Gelatinase Production

Five different media were evaluated to select the optimal medium that supports maximum gelatinase production. They included the following:

Medium 1 - Medium suggested by Akolkar (2009) was used with modifications as described in 3.4.2.

Medium 2 - Medium suggested by Vidyasagar *et al.* (2006) was modified and used (200 g/L NaCl, 10 g/L $MgCl_2$, 5 g/L KCl, 3 g/L Trisodium citrate and 10 g/L gelatin; pH 7.2).

Medium 3 - Eimhjellen medium (Catherine *et al*., 2001) with yeast extract 5%, $MgSO_4.7H_2O$ 2%, $CaCl_2.2\ H_2O$ 0.5% and NaCl 25%.

Medium 4 - Zobell's medium (M385-500G, Hi Media, Mumbai, India) was prepared as described in section 3.2.1.

Medium 5 - liquid Brown's medium (Birbir *et al*., 2004) containing 5 g/L yeast extract, 3 g/L Trisodium citrate, 20 g/L $MgSO_4.7\ H_2O$, 2 g/L KCl and 250 g/L NaCl.

Each of the media was prepared by dispensing 100 mL of the media in 250 mL Erlenmeyer flask and autoclaved at 121°C at 15 lbs pressure for 15 min.

Among the media evaluated, Medium 1 gave maximum gelatinase production and hence, it was used for optimization of bioprocess conditions towards maximum enzyme production.

3.4.1. Culture Conditions in Liquid Media and Inoculum Preparation

Single cell colony from freshly subcultured *Natrinema* sp. BTSH10 on Zobell's Agar medium was used as inoculum for preparation of a preculture in 10 mL of Zobell's broth. After 2 days of incubation 5 mL of the culture broth was transferred to 100 mL of Zobell's broth and incubated at 37°C in a rotary shaker maintained at 125 rpm for 3 days and the culture broth was used as inoculum. The prepared inoculum was adjusted to 0.1 OD at 600 nm using sterile media.

3.4.2. Inoculation and Incubation

Production of gelatinase by *Natrinema* sp. BTSH10 was carried out using the modified Medium 1, in which gelatin was used as substrate instead of soyabean.

The composition of the medium used is as given below:

Ingredients	**g/L**
NaCl	250
KCl	2
$MgSO_4$	20
Sodium Citrate	3
Gelatin	10

Gelatin was first dissolved in small quantity of distilled water taken in a conical flask under boiling conditions in a water bath and then added to the conical flask in which all the other ingredients were dissolved. Then the pH was adjusted to 7 and autoclaved at 15 lbs pressure for 15 min. 100 mL of the media prepared and taken in a 250 mL Erlenmeyer flask was inoculated with 2% of the prepared inoculum and incubated at 37°C in a rotary shaker maintained at 125 rpm, for 5 days. Samples were withdrawn at regular intervals to check for production of gelatinase.

3.4.3. Recovery of Enzyme

After incubation, the culture broth was centrifuged at 10,000 rpm for 10 min. at 4°C. After centrifugation the supernatant was collected and used as crude enzyme for further studies.

3.5. Analytical Methods

3.5.1. Gelatinase Assay

Gelatinase assay was done according to a modified method described earlier in section 3.2.4.

3.5.2. Protein Estimation

Protein content was estimated according to the method described by Lowry *et al*. (1951) using Bovine Serum Albumin (BSA) as standard. Optical density was measured at 750 nm and concentration of protein was expressed as mg/mL.

Reagents

A. Sodium carbonate in 0.1 N Sodium hydroxide 2% (w/v)
B. Cupric sulphate in water 0.5% (w/v)
C. Sodium potassium tartarate in distilled water 1% (w/v)
D. Working reagent -100 mL of reagent (A) was mixed with 1mL of reagent (B) and reagent (C).
E. 1:1 Folin and Ciocalteu's phenol reagent diluted with distilled water.
(D) and (E) were freshly prepared before use.

Estimation

An aliquot of 100 µL of the sample was made up to 1 mL with distilled water and added to 2.5 mL of freshly prepared reagent (D), mixed and allowed to stand for 10 min. Blank was set up using 1 mL distilled water without sample. 250 µL of reagent (E) was added and incubated for 30 min. The absorbance was measured at 750 nm in a UV-Visible spectrophotometer (Shimadzu, Japan).

3.5.3. Specific Activity

Specific activity was calculated by dividing the enzyme units with protein content and expressed as U/mg protein.

$$\text{Specific activity} = \frac{\text{Enzyme Activity (U/mL)}}{\text{Protein (mg/mL)}}$$

3.6. Growth Curve

Growth curve of *Natrinema* sp. BTSH10 was estimated using Zobell's broth supplemented with 15% NaCl. 100 mL of Zobell's broth was inoculated with 2 mL of inoculum prepared as described under section 3.4.1 and incubated at 37°C in a rotary shaker at 125 rpm. At regular intervals samples were taken and growth was measured in terms of turbidity at 600 nm in a UV-Visible spectrophotometer (Schimadzu, Japan). Uninoculated medium served as control. The optical density values were plotted against time and a growth curve was constructed. Generation time and growth rate constant were calculated.

3.7. Production of Gelatinase by *Natrinema* sp. BTSH10

Media used for production was the same as described in section 3.4.2.

3.7.1. Inoculum Preparation and Incubation

Inoculum preparation and incubation were done as described in section 3.4.1.

3.7.2. Inoculation and Incubation

Inoculation and incubation were done as described in section 3.4.2.

3.7.3. Recovery of Enzyme

Recovery of enzyme was done as described in section 3.4.3.

3.7.4. Optimization of Bioprocess Variables for Gelatinase Production by BTSH10.

Physico-chemical and bioprocess variables that influence gelatinase production were optimized for maximal production under submerged fermentation.

The effects of different parameters were evaluated using 'one-variable at a time' approach. The medium 1 (Akolkar, 2009) as described in section 3.4.2 was used. The different parameters optimized included incubation temperature, initial pH of medium, agitation, effect of different carbon and nitrogen sources, sodium chloride concentration, inoculum age, % of inoculum and incubation time. Preparation of medium, preparation of inoculum, inoculation and incubation and recovery of enzyme were done as described in sections 3.4.1, 3.4.2, 3.4.3 respectively. In each case, enzyme assay and protein estimation was done as detailed in sections 3.2.4 and 3.5.2.

3.7.4.1. NaCl Concentration

Halophiles have requirement for high salt concentration and the concentration of NaCl plays a prominent role in the growth and production of enzymes. Hence enzyme production at different salt concentration (10%, 15%, 20%, 25% 30% and 35 %) were evaluated by adjusting the final salt concentration of the medium and assaying gelatinase. The enzyme production after 120 h of incubation was determined. Preparation of medium, preparation of inoculum, inoculation and incubation and recovery of enzyme were done as described in sections 3.4.1, 3.4.2, 3.4.3 respectively. In each case, enzyme assay and protein estimation was done as detailed in sections 3.2.4 and 3.5.2.

3.7.4.2. Initial pH of Medium

Initial pH of medium that could support maximal protease production was determined by adjusting the pH of the medium from 2 - 12 using 1N NaOH or 1N HCl and assaying the gelatinase produced at the respective pH of the medium. The protease production after 120 h of incubation was determined. NaCl concentration of media adjusted to 25%. Preparation of medium, preparation of inoculum, inoculation and incubation and recovery of enzyme were done as described in sections 3.4.1, 3.4.2, 3.4.3 respectively. In each case, enzyme assay and protein estimation was done as detailed in sections 3.2.4 and 3.5.2.

3.7.4.3. Incubation Temperature

The optimal incubation temperature that supported maximal gelatinase production was determined by incubating the inoculated media at different

temperatures *viz* ; 22°C, 27°C, 32°C, 37°C, 42°C, 47°C and 52°C. The initial pH of media was adjusted to 7 and the salt concentration was 25%. The protease production after 120 h of incubation was determined. Preparation of medium, preparation of inoculum, inoculation and incubation and recovery of enzyme were done as described in sections 3.4.1, 3.4.2, 3.4.3 respectively. In each case, enzyme assay and protein estimation was done as detailed in sections 3.2.4 and 3.5.2.

3.7.4.4. Inoculum Concentration

The optimal concentration of inoculum which supported the maximum enzyme production was evaluated using different concentrations of inocula ranging from 1% - 10%. The initial pH of media was adjusted to 7 and the salt concentration was 25% and the incubation temperature was adjusted to 42°C. The protease production after 120 h of incubation was determined. Preparation of medium, preparation of inoculum, inoculation and incubation and recovery of enzyme were done as described in sections 3.4.1, 3.4.2, 3.4.3 respectively. In each case, enzyme assay and protein estimation was done as detailed in sections 3.2.4 and 3.5.2.

3.7.4.5. Inoculum Age

The age of inoculum affects the enzyme production and hence inoculum with age of 24 h, 36 h, 48 h, 60 h, 72 h and 90 h were used. The enzyme production after 120 h of incubation was determined. The initial pH of media was adjusted to 7 and the concentration of inoculum was 2%. The salt concentration was 25% and the incubation temperature was adjusted to 42°C. Preparation of medium, preparation of inoculum, inoculation and incubation and recovery of enzyme were done as described in sections 3.4.1, 3.4.2, 3.4.3 respectively. In each case, enzyme assay and protein estimation was done as detailed in sections 3.2.4 and 3.5.2.

3.7.4.6. Agitation

The effect of agitation on enzyme production was studied by incubating the inoculated conical flasks at different agitation rates 0 rpm, 50 rpm, 100 rpm, 150 rpm, 200 rpm and 250 rpm. The enzyme production after 120 h of incubation was determined. The initial pH of media was adjusted to 7 and the concentration of inoculum was 2%. The age of inoculum was adjusted to 36 h. The salt concentration

was 25% and the incubation temperature was adjusted to 42°C. Preparation of medium, preparation of inoculum, inoculation and incubation and recovery of enzyme were done as described in sections 3.4.1, 3.4.2, 3.4.3 respectively. In each case, enzyme assay and protein estimation was done as detailed in sections 3.2.4 and 3.5.2.

3.7.4.7. Additional Carbon Sources

Additional carbon sources (at a concentration of 0.1M) *viz.*, dextrose, sucrose, cellobiose, maltose, fructose, galactose, mannitol, mannose, lactose and sorbitol were used to evaluate the suitable carbon source that supported the maximum enzyme production. The enzyme production after 120 h of incubation was determined. The initial pH of media was adjusted to 7 and the salt concentration was 25%. The concentration of inoculum was 2% and the inoculum age was adjusted to 36 h. The flasks were incubated at 42°C on a rotary shaker at 150 rpm. Preparation of medium, preparation of inoculum, inoculation and incubation and recovery of enzyme were done as described in sections 3.4.1, 3.4.2, 3.4.3 respectively. In each case, enzyme assay and protein estimation was done as detailed in sections 3.2.4 and 3.5.2.

3.7.4.8. Additional Nitrogen Sources

Both organic and inorganic nitrogen sources were evaluated for maximum enzyme production.

3.7.4.8.1. Inorganic Nitrogen Sources

Inorganic nitrogen sources like ammonium ferrous sulphate, ammonium dihydrogen phosphate, ammonium hydrogen carbonate, ammonium acetate, ammonium chloride, ammonium oxalate, ammonium nitrate, ammonium sulphate, Di ammonium hydrogen phosphate and sodium nitrate at a concentration of 0.1M were evaluated by incorporating into the medium as additional nitrogen source.

3.7.4.8.2. Organic Nitrogen Sources

Organic nitrogen sources like soyabean casein digest, beef extract, soyabean meal, yeast extract, peptone and skimmed milk powder at a concentration of 0.5% were evaluated by incorporating into the medium as additional nitrogen source.

The protease production after 120 h of incubation was determined. The initial pH of media was adjusted to 7 and the salt concentration was 25%. The concentration of inoculum was 2% and the inoculum age was 36 h. Media contained 0.1M sorbitol was used. The flasks were incubated at 42°C on a rotary shaker at 150 rpm. Preparation of medium, preparation of inoculum, inoculation and incubation and recovery of enzyme were done as described in sections 3.4.1, 3.4.2 and 3.4.3 respectively. In each case, enzyme assay and protein estimation was done as detailed in sections 3.2.4 and 3.5.2.

3.7.4.9. Detergents

Impact of different detergents (0.2g/L) including CTAB (cetyltrimethylammonium bromide), Sodium taurocholate, TritonX100, Tween 20, Tween 80 and sodium lauryl sulphate on gelatinase production was evaluated. The enzyme production after 120 h of incubation was determined. The initial pH of media was adjusted to 7 and the salt concentration was 25%. The concentration of inoculum was 2% and the inoculum age was 36 h. Media containing 0.1M sorbitol, 0.5% skimmed milk and 0.1 M ammonium sulphate was used. The flasks were incubated at 42°C on a rotary shaker at 150 rpm. Preparation of medium, preparation of inoculum, inoculation and incubation and recovery of enzyme were done as described in sections 3.4.1, 3.4.2, 3.4.3 respectively. In each case, enzyme assay and protein estimation was done as detailed in sections 3.2.4 and 3.5.2.

3.7.4.10. Time Course Study under Optimal Conditions

Time course Study was done with optimized conditions determined after optimization of different variables. The conditions included

1. 2% inoculum concentration
2. Agitation at 150 rpm
3. 25% NaCl concentration
4. pH 7
5. 0.1M sorbitol, 0.1 M ammonium sulphate and 0.5% skim milk powder.
6. Incubation temperature 42°C
7. 36 h old inoculum

Preparation of medium, preparation of inoculum, inoculation and incubation and recovery of enzyme were done as described in sections 3.4.1, 3.4.2, 3.4.3 respectively. In each case, enzyme assay and protein estimation was done as detailed in sections 3.2.4 and 3.5.2.

3.8. Purification of Enzyme

The culture supernatant obtained after centrifugation at 10,000 rpm for 10 min. at 4°C was purified as detailed below:

3.8.1. Filtration

The crude enzyme was first subjected to filtration using Amicon filters (Millipore) with a cut off size 10 kDa. The fraction containing proteins below 10 kDa size did not show enzyme activity and hence the fraction was discarded. The fraction containing proteins with size greater than 10 kDa showed enzyme activity and hence further studies were done using this fraction. This fraction obtained was again subjected to filtration using Amicon filters (Millipore) with a cut off size 30 kDa. The fraction containing protein with size ranging from 10 kDa to 30 kDa showed enzyme activity.

3.8.2. Gel Filtration Chromatography

Sephadex G-50 (Sigma-Aldrich) which can be used to separate proteins ranging in size from 50 kDa to 3 kDa was used for gel filtration chromatograhy. Sephadex G-50 column packed in Bio-Rad column of 55 cm height and 1 cm diameter. The buffer used was Tris-HCl buffer 0.05 M (pH 8) with 2M NaCl.

3.8.2.1. Preparation of Column

a. 5 g of sephadex G-50 (Sigma-Aldrich) was suspended in 0.05 M Tris-HCl pH 8.0 (with 2M NaCl) and hydrated for 15 min. at 80°C in a water bath and the fine particles were removed.
b. To the hydrated sephadex suspension some more buffer was added.
c. The glass column was filled with the column material without air bubble formation maintaining a slow flow rate so that the column settled under gravity.

d. The column was equilibrated by allowing 450 mL of 0.05 M Tris-HCl pH 8.0 (with 2M NaCl) buffer to pass through the column.

3.8.2.2. Sample Preparation and Application on the Column

A total volume of 1.6 mL of concentrated 10- 30 kDa molecular weight cut off sample was loaded on to a packed column (55 cm x 1 cm). The sample was completely dissolved and applied on column. Initial 10 mL of the buffer was collected as a single fraction and then fractions of 1 mL/4 min. were collected and OD of the fractions were checked at 280 nm using UV-Visible spectrophotometer (Shimadzu, Japan). Gelatinase activities in the fractions were checked and all the fractions having the activities were pooled together.

3.8.3. Dialysis

The lyophilized protein fraction with size ranging from 10-30 kDa was dialysed against Tris- HCl buffer 0.05 M (pH 8). Dialysis tube (Sigma –Aldrich) was first subjected to pre-treatment to remove glycerin and sulphur compounds associated with it and to clear the pores of the tube. The process involved washing the tube for 3-4 h in running water, dipping in 0.3% (w/v) sodium sulfide for 1min. at 80°C, washing with hot water (60°C) for 2 min. This was followed by rinsing in 0.2% (v/v) sulphuric acid and rinsed with distilled water. The protein was dialysed using pretreated dialysis tube at 4°C for about 48 h with several changes of buffer. Gelatinase activity, protein content, specific activity and yield were calculated. The treated tube retained proteins of molecular weight 12 kDa and more.

The dialysis was repeated using Tris HCl buffer 0.05 M (pH 8) with 2M NaCl. All the other procedures were done as described earlier.

3.8.4. High Performance Liquid Chromatography

Crude enzyme, gel filtered fraction which showed activity and the dialysed 10-30 kDa fractions were subjected to HPLC (Schimadzu LC - 6 AD). Semi-preparative column C 18 was used. The column was prior run with methanol followed methanol (with 0.1% TFA) and the samples were injected. The solvent system used

for separation was methanol (with 0.1% TFA) and water. The procedure was completed in about 70 min. with a flow rate of 2 mL/min.

3.8.5. Polyacrylamide Gel Electrophoresis

3.8.5.1. Reagents for Polyacrylamide Gel Electrophoresis

1. Stock acrylamide solution (30:0.8)

Acrylamide (30%)	-	60.0 g
Bis-acrylamide (0.8%)	-	1.6 g
Distilled water (DW)	-	200.0 mL

The reagent prepared was stored at 4°C in amber coloured bottle.

2. Stacking gel buffer stock (0.5 M Tris-HCl, pH 6.8)

Tris buffer	-	6 g in 40 mL DW

Titrated to pH 6.8 with 1M HCl and made up to 100 mL with DW.

The reagent was filtered using Whatman No: 1 filter paper and stored at 4°C.

3. Resolving gel buffer stock (3 M Tris-HCl, pH 8.8)

Tris buffer	-	36.3 g

Titrated to pH 8.8 using 1M HCl and made up to 100 mL with DW.

The reagent was filtered using Whatman No:1 filter paper and stored at 4°C.

4. Reservoir buffer for Native- PAGE (pH 8.3)

Tris buffer	-	3.0 g
Glycine	-	14.4 g

Dissolved and made up to 1 L with DW.

The reagent is prepared in 10X concentration and stored at 4°C.

5. Reservoir buffer for SDS- PAGE (pH 8.3)

Tris buffer	-	3.0 g
Glycine	-	14.4 g
Sodium dodecyl sulfate (SDS)	-	1.0 g

Dissolved and made up to 1L with DW. The reagent was prepared in 10X concentration and stored at 4°C.

6. Sample buffer for Native -PAGE

Tris-HCl (pH 6.8)	-	0.0625 M
Glycerol (optional)	-	10% (v/v)
Bromophenol blue	-	0.01%

The reagent is prepared in 2X concentration and stored at 4°C.

7. Sample buffer for Reductive SDS -PAGE

Tris- HCl (pH 6.8)	-	0.0625 M
Glycerol (optional)	-	10% (v/v)
SDS	-	2%
Dithiothreitol	-	0.1 M
Bromophenol blue	-	0.01%

Prepared in 2X concentration and stored at 4°C

8. SDS (10%)

Sodium dodecyl sulfate (SDS) - 1 g in 10 mL DW

9. Sucrose (50%)

Sucrose - 5 g in 10 mL DW, autoclaved at 121°C for 15 min. and stored at 4 °C.

10. Silver Staining

a. Methanol : acetic acid: water (50:10:40, v/v).	-	100 mL
b. 5% methanol	-	100 mL
c. 0.02% sodium thiosulphate	-	100 mL
d. 0.2% silver nitrate	-	100 mL
e. Developing solution :- 2 mL of 0.02% sodium thiosulphate and 50 µL of formaldehyde in 100 mL 3% sodium carbonate	-	100 mL
f. 1.4% EDTA	-	100 mL

11. Protein Marker (Biorad) for Native-PAGE

Components	MW kDa
Phosphorylase B	97.4
BSA	66.2
Ovalbumin	45
Carbonic anhydrase	31
Trypsin inhibitor	21.5
lysozyme	14.4

12. Protein Marker for reductive SDS-PAGE

Low molecular weight marker mix (Amersham Pharmacia) was used. Lyophilized marker mix was reconstituted in 1X sample buffer for reductive SDS-PAGE; boiled for 5 min. and 5 µL of marker was loaded on to gel. Composition of marker mix is given below.

Components	MW kDa
Phosphorylase B	97.0
BSA	66.0
Ovalbumin	45
Carbonic anhydrase	30
Trypsin inhibitor	20.1
α-lactalbumin	14.4

3.8.5.2. Native - Polyacrylamide Gel Electrophoresis (Native -PAGE)

3.8.5.2.1. Gel preparation

Resolving Gel (8%)

Acrylamide: Bis-acrylamide (30:0.8)	-	1 mL.
Resolving gel buffer stock	-	1.56 mL.
Ammonium persulphate (APS)	-	75 µL
Water	-	3.318 mL.
TEMED	-	15 µL.

Stacking Gel (4%)

Acrylamide:Bis-acrylamide (30:.08)	-	0.67 mL.
Stacking gel buffer stock	-	1.25 mL
Ammonium persulphate (APS)	-	40 µL.
Water	-	3.075 mL.
TEMED	-	12 µL.

3.8.5.2.2. Sample Preparation

The lyophilized sample was mixed with 50 mM Tris-HCl buffer (pH 8) and 15 µL of sample with 3 µL sample buffer for Native- PAGE was loaded on to the gel. 1 µL marker with 3 µL sample buffer for Native -PAGE was made up to 18 µL with 50 mM Tris-HCl buffer (pH 8) and loaded on to the gel.

3.8.5.2.3. Procedure

a. The gel plates were cleaned and assembled**.**
b. Resolving gel - All the components of the resolving gel were mixed gently and the mixture was poured into the cast and a layer of butanol was poured over the gel and allowed to solidify for at least 1 h.
c. Stacking gel - The butanol layer was poured off. The components were mixed gently and poured into the cast above the resolving gel. The comb was inserted between the glass plates and allowed to solidify for 30 min.
d. The gel was placed in the electrophoresis unit and the reservoirs, upper and lower were filled with reservoir buffer for Native-PAGE.
e. The gel was loaded with sample
f. The gel was run at 80 V until the sample entered the resolving gel.
g. The current was increased to 100 V.
h. The current was switched off when the dye front reached about 1 cm above the lower end of glass plate.
i. The gel was removed and stained using silver staining method.

Modified method of Sambrook and Russel (2001) was employed to visualize the protein bands on gel. The gel was immersed in fixing solution comprising of

methanol: acetic acid: water (50:10:40, v/v) for 30 min. Discarded the fixing solution, and the gel was immersed in 5% methanol for 15 min. The gel was washed with water thrice for 5 min. each. The gel was immersed in 0.02% sodium thiosulphate for 1 min. The gel was washed with water thrice for 30 sec. each and stained using 0.2% silver nitrate for 25 min. Washed again with water thrice for 1 min. each. Developing solution (2 mL of 0.02% sodium thiosulphate and 50 µL of formaldehyde in 100 mL 3% sodium carbonate) was added. After 10 min, the reaction was stopped by adding 1.4% EDTA.

3.8.5.3. Reductive SDS-PAGE.

3.8.5.3.1. Gel Preparation

Resolving Gel (8%)

Acrylamide: Bis-acrylamide (30:0.8)	-	1 mL.
Resolving gel buffer stock	-	1.56 mL.
10% SDS	-	0.6 mL
Ammonium persulphate (APS)	-	75 µL
Water	-	3.318 mL.
TEMED	-	15 µL.

Stacking Gel (4%)

Acrylamide: Bis-acrylamide (30:0.8)	-	0.67 mL.
Stacking gel buffer stock	-	1.25 mL
Ammonium persulphate (APS)	-	40 µL.
10% SDS	-	50 µL.
Water	-	3.075 mL.
TEMED	-	12 µL.

Sample Buffer (1X)

SDS-PAGE sample buffer (2X)	-	1 mL
50% sucrose	-	0.4 mL
DW	-	0.6 mL

3.8.5.3.2. Sample Preparation

The gelatinase enzyme was mixed with the sample buffer and boiled for 5 min. in a water bath, cooled to room temperature and 20 µL was loaded to gel. 5 µL of protein marker was loaded to the gel.

3.8.5.3.3. Procedure

Procedures for electrophoresis and staining were the same as described in section 3.8.5.2.3, and the reservoir buffer used was that of SDS-PAGE.

3.8.5.4. Zymogram

Gelatinase activity of the purified enzyme protein band was confirmed by performing zymogram. The method described by Raut *et al*. (2012) was modified by addition of more NaCl and $ZnSO_4$. Gelatin was incorporated into gel (500 mL of resolving gel contained 500 µL of gelatin solution (0.1 g/10 mL)) and Native-PAGE was performed. After running the protein, it was washed with water and immersed in a developing solution which contained 50 mM Tris (pH 8) with 1 mM $ZnSO_4$ and 15 % NaCl, and incubated at 37°C overnight. The gel was then dipped in Coomassie brilliant blue stain for 1 h and destained using protein destaining solution.

Protein staining solution

Coomassie brilliant blue (0.1%)	-	100 mg
Methanol (40%)	-	40 mL
Glacial Acetic acid	-	10 mL
DW	-	50 mL

Protein destaining solution

Methanol (40%)	-	40 mL
Glacial Acetic acid	-	10 mL
DW	-	50 mL

3.8.6. Analytical Methods

Gelatinase activity, protein content and specific activity were determined as described in 3.2.4 and 3.5.2 and 3.5.3 and expressed as U/mL, mg/mL and U/mg protein respectively.

3.8.7. Calculation of Yield of Protein, Yield of Enzyme Activity and Fold of Purification

Yield of protein of each fraction during purification is the percentage obtained

by dividing the total protein content of the fraction with the total protein content of the crude extract. Yield of enzyme activity of each fraction is the percentage activity obtained by dividing the activity of the fraction by the activity of the crude extract.

Fold of purification was calculated by dividing the specific activity of the fraction with that of the crude extract.

$$\text{Yield of Protein} = \frac{\text{Total protein content of fraction} \times 100}{\text{Total protein content of the crude extract}}$$

$$\text{Yield of Activity} = \frac{\text{Total activity of fraction} \times 100}{\text{Total activity of the crude extract}}$$

$$\text{Fold of Purification} = \frac{\text{Specific activity of fraction} \times 100}{\text{Specific activity of the crude extract}}$$

3.9. Characterization of Purified Enzyme

3.9.1. Optimal pH for Gelatinase Activity.

Optimal pH for gelatinase activity was evaluated by assaying gelatinase in plates with media in the pH range 2 to 13 as described in 3.2.4. The buffer systems used included HCl-KCl buffer (pH 2), Citrate buffer (pH 3 to 5), Phosphate buffer (pH 6 and 7), Tris-HCl buffer (pH 8), Glycine-NaOH buffer (pH 9), Bicarbonate buffer (pH 10), disodium hydrogen phosphate-sodium hydroxide buffer (pH 11 and 12) and KCl-NaOH buffer (pH 13). Enzyme activity was calculated as described in section 3.2.4.

3.9.2. Stability of Gelatinase at Different pH

Stability of gelatinase at different pH conditions was evaluated by incubating the enzyme in different buffer systems of varying pH (pH 2 to 13) for 24 h at 4°C and conducting gelatinase assay. The buffer systems used included HCl-KCl buffer (pH 2), Citrate buffer (pH 3 to 5), Phosphate buffer (pH 6 and 7), Tris-HCl buffer (pH 8), Glycine-NaOH buffer (pH 9), Bicarbonate buffer (pH 10), disodium hydrogen phosphate-sodium hydroxide buffer (pH 11 and 12) and KCl-NaOH buffer (pH 13). After incubation, assay was done for gelatinase activity as described in section 3.2.4.

Residual activity was determined as described in section 3.9.12.1 and enzyme activity at pH 8 was taken as control.

3.9.3. Optimal Temperature for Gelatinase Activity

Optimal temperature for gelatinase activity was evaluated by assaying gelatinase in plates with media at different temperatures from 5°C to 47°C. Enzyme activity was determined as described in section 3.2.4.

3.9.4. Stability of Gelatinase at Different Temperatures

Stability of gelatinase at different temperatures was determined by incubating the enzyme at different temperatures ranging from 20°C to 80°C. The assay was conducted at 30 min., 1 h, 2 h, 4 h, 6 h, 8 h, 10 h, 12 h and 24 h., as described in section 3.2.4.

Enzyme activity of the sample kept at 4°C was taken as control. Residual activity of the enzyme was calculated as described in section 3.9.12.1**.**

3.9.5. Effect of Inhibitors on Gelatinase Activity

Effect of inhibitors on gelatinase was evaluated using 0.2 mM to 1 mM Aprotinin; 10 mM to 50 mM EDTA and 10 mM to 50 mM Iodoacetamide. The inhibitors were added to the purified enzyme and incubated at room temperature for 30 min. After incubation enzyme assay was done as described in section 3.2.4. Residual activity of the enzyme was calculated as described in section 3.9.12.1 and expressed in percentage. Enzyme in the absence of inhibitors was used as control.

3.9.6. Substrate Specificity

Substrate specificity was determined by conducting the assay using plates of gelatin, skim milk, Bovine serum albumin and haemoglobin (0.5% w/v). The enzyme activity was done as described in section 3.2.4. and expressed in U/mL.

3.9.7. Kinetic Studies

Gelatinase was subjected to kinetic studies to determine the K_m and V_{max}. K_m, the substrate concentration at which reaction velocity is half maximum and V_{max} the maximum velocity of the enzyme reaction, were determined by conducting enzyme

assay using plates containing different concentrations of gelatin (0.2 -20 mg/mL) as described in section 3.2.4.

3.9.8. Effect of Various Metal ions on Gelatinase Activity

The effect of various metal ions on gelatinase activity was studied by incubating the enzyme along with different concentrations of metal ions for 30 min., followed by measuring the residual activity (section 3.9.12.1.). The metals studied included 1 mM, 5 mM, 10 mM, 20 mM and 30 mM final concentrations of $CaCl_2$, $MgSO_4$, $ZnSO_4$, $CuSO_4$, $FeSO_4$, $MnCl_2$, $NiSO_4$, $CoCl_2$, $BaCl_2$, $CdCl_2$ and $Al_2(SO_4)_3$, which contributed the metal ions, Ca^{2+}, Mg^{2+} Zn^{2+}, Cu^{2+}, Fe^{2+}, Mn^{2+}, Ni^{2+},Co^{2+},Ba^{2+} Cd^{2+} Al^{3+} respectively. Enzyme in the absence of metal ions was used as control.

3.9.9. Effect of Various Detergents on Gelatinase Activity

The effect of various detergents on enzyme activity was evaluated by incubating the enzyme in different concentrations of each detergent viz., 0.2 %, 0.4%, 0.6%, 0.8%, 1% and 5% for 30 min. and the residual enzyme activity was determined as described in section 3.9.12.1. The detergents tested included CTAB (cetyltrimethylammonium bromide), Sodium Lauryl Sulphate, Triton X 100, Tween 20 and Tween 80. Enzyme in the absence of detergents was used as control.

3.9.10. Effect of Various Concentrations of NaCl on Gelatinase Activity

Gelatin plates containing 0%, 5%, 10%, 15%, 20% and 25% NaCl were prepared and assay was done as described in section 3.2.4. Gelatin plates with concentration of NaCl above 25% could not be prepared as NaCl crystalizes on solidification.

3.9.11. Effect of Organic Solvents on Gelatinase Activity

Effect of organic solvents on gelatinase activity was evaluated by incubating the enzyme with the solvent for 30 min and the residual activity was assayed as described in section 3.9.12.1. The organic solvents used included acetone, ethanol, methanol, dimethyl sulphoxide, diethyl ether, acetonitrile amd isopropanol in the concentrations 1%, 2%, 5%, 10% and 20% (v/v). Enzyme in the absence of organic solvents was used as control

3.9.12. Analytical Methods

Gelatinase activity, protein content and specific activity were determined as described in 3.2.4, 3.5.2 and 3.5.3 and expressed as U/mL, mg/mL and U/mg protein respectively.

3.9.12.1. Residual Activity

Residual activity is the percent enzyme activity of the sample with respect to the enzyme activity of the control sample.

$$\text{Residual Activity} = \frac{\text{Activity of sample (U/mL) x 100}}{\text{Activity of Control (U/mL)}}$$

3.9.12.2. Relative Activity

Relative activity is the percent enzyme activity of sample with respect to the sample for which maximum activity was obtained.

$$\text{Relative Activity} = \frac{\text{Activity of sample (U/mL) x 100}}{\text{Activity of maximal enzyme activity obtained sample (U/mL)}}$$

3.9.13. Application Studies

3.9.13.1. Decomposition of Gelatin layer on X-ray film

Ability of gelatinase to hydrolyse gelatin layer of the X-ray film for recovery of silver was studied by incubating the enzyme (12 U) with 2 g of X-ray film (Kumar and Takagi, 1999).

The following sets were prepared and studied.

1. 19 mL Tris-HCl buffer (pH 8) with 15% NaCl + 2 g X-ray film + 1 mL gelatinase enzyme (12 U).

2. 19 mL Tris-HCl buffer (pH 8) with 15% NaCl + 2 g X-ray film + 1 mL Proteinase K enzyme (12 U).
3. 20 mL Tris-Hcl buffer (pH 8) with 15% NaCl + 2 g X-ray film.

The flasks were kept in a rotary shaker at 120 rpm at 37°C. After 3 h, the X-ray film was removed and rinsed with tap water and examined visually. Protein released by the action of enzyme into the supernatant was estimated by the method as described in section 3.5.2 (Lowry *et al.*, 1951).

3.10. Statistical Analysis

Statistical analysis was done using Microsoft Excel and average of five values were taken.

Chapter 4

RESULTS

4.1.1. Isolation and Identification of Halophiles

Halophilic bacteria associated with tannery effluent and commercially available food grade table salt crystals were isolated using Zobell's medium employing standard plating procedures. Several colonies which were very similar and identical in their morphological characteristics were noted on the plates. Hence, representative colonies that showed identical morphological characteristics were picked randomly and sub cultured. Thus, six representative colonies designated as BTMT01, BTMT02, BTMT03, BTMT04, BTMT08 & BTMT10 were isolated from tannery effluent. Similarly, five representative colonies designated as BTMT05, BTMT06, BTMT11, BTMT12 and BTMT13 could be isolated from food grade table salt crystals.

All the isolates obtained were studied for their cell characteristics, biochemical and physiological characteristics, and 16S rDNA sequencing was done for identification, as described under the materials and methods section. Interestingly, all the isolates obtained from tannery effluent were found to be Gram positive while the isolates obtained from food grade table salt crystals were Gram negative bacilli.

Among the isolates tested for production of different hydrolytic enzymes, isolates BTMT01 and BTMT03 obtained from tannery effluent were observed to produce large quantities of lipase and protease. However, the isolates BTMT08 and BTMT10 did not show lipase production. All the isolates obtained from food grade table salt crystals were observed to produce lipase, amylase and protease, although the levels of the enzyme production were very less (refer Appendix-I).

4.1.2. Molecular classification of Isolates.

The partial gene sequences of 16S rDNA of the bacterial isolates were amplified by colony PCR using universal eubacterial primers and the ~ 1kb amplicons were sequenced. The identity of the nucleotide sequence was confirmed by BLAST analysis and DNA homology was ascertained. Based on the information obtained, the

identity of the isolates were inferred. The partial gene sequences of 16S rDNA were submitted to NCBI and the details of the identity and NCBI Accession Numbers for the halophilic bacterial strains isolated from tannery effluent and table salt crystals are shown in Table 4.1.

Table 4.1.NCBI Accession Numbers for halophilic bacteria isolated from tannery effluent and table salt crystals

Source	Strain No.	Bacteria	NCBI Accession Number	Organism showing closest match	NCBI Accession Number	% similarity
Tannery effluent	BTMT01	*Oceanobacillus* sp.	JX975066	*Oceanobacillus* sp. H-82	KF021765	99
	BTMT02	*Staphylococcus arlettae*	JN228200	*Staphylococcus arlettae* strain VIT-RJ1	KJ716448	99
	BTMT03	*Oceanobacillus* sp.	JN228197	*Oceanobacillus* sp. 803(2012)	JN039425	99
	BTMT04	*Staphylococcus arlettae*	JN228201	*Staphylococcus arlettae* strain NS2	KP279979	98
	BTMT08	*Salimicrobium* sp.	JN228199	*Salimicrobium album* strain NBRC 102360	NR114064	99
	BTMT10	*Salimicrobium* sp.	JN228198	*Salimicrobium album* strain NBRC 102360	NR114064	99
Salt crystals	BTMT05	*Halomonas elongata*	KC019171	*Halomonas elongata* strain BK-AG18	KJ185382	99
	BTMT06	*Halomonas elongata*	KC019170	*Halomonas elongata* strain BK-AG18	KJ185382	99
	BTMT11	*Chromohalobacter salexigens*	JX975064	*Chromohalobacter salexigens* strain DSM3043	NR074225	98
	BTMT12	*Halomonas elongata*	JX975065	*Halomonas elongata* strain BK-AB8	KJ185379	99
	BTMT13	*Halomonas elongata*	JX975062	*Halomonas elongata* strain BK-AG18	KJ185382	99

From the results presented in Table 4.1., it was inferred that only species of *Oceanobacillus, Staphylococcus and Salimicrobium* were associated with tannery effluent while food grade table salt crystals harbored species of *Halomonas* and

Chromohalobacter salexigens. Further, it was observed that *Halomonas elongata* was dominant among the bacteria associated with the food grade table salt crystals. It was also observed that both the samples of tannery effluent and food grade table salt crystals harbored very different species of halophilic bacterial flora indicating that source of sample influenced species diversity.

4.1.3. Phylogenetic Tree of the Halophiles Isolated from Tannery Effluent and Commercially Available Salt Crystals

A phylogenetic tree was constructed using MEGA4 software and the evolutionary history was inferred using Maximum Parsimony (MP) method (Eck and Dayhoff,1966). Tree #12 out of 15 most parsimonious trees (length = 375) is shown. The consistency index (0.955556), the retention index (0.986051), and the composite index 0.943979 (0.942226) for all sites and parsimony-informative sites (presented in parentheses) were computed. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown above the branches (Felsenstein, 1985). The MP tree was obtained using the Close-Neighbor-Interchange algorithm (Nei and Kumar, 2000) with search level 3 (Felsenstein, 1985; Nei and Kumar, 2000) in which the initial trees were obtained with the random addition of sequences (10 replicates). The tree is drawn to scale; with branch lengths calculated using the average pathway method (Nei and Kumar, 2000) and are in the units of the number of changes over the whole sequence. All positions containing gaps and missing data were eliminated from the dataset (Complete Deletion Option). There were a total of 525 positions in the final dataset, out of which 311 were parsimony informative.

Although all the isolated bacteria were halotolerant, the phylogenetic tree (Fig. 4.1) showed the bacteria isolated from tannery effluent and those isolated from food grade table salt crystals in different clusters. Similar organisms are paired on the same branch. *Halomonas elongata* and *Chromohalobacter salexigens* isolated from salt crystals are seen in two branches, while species of *Halomonas elongata* are clustered in a single branch, reflecting their similarity.

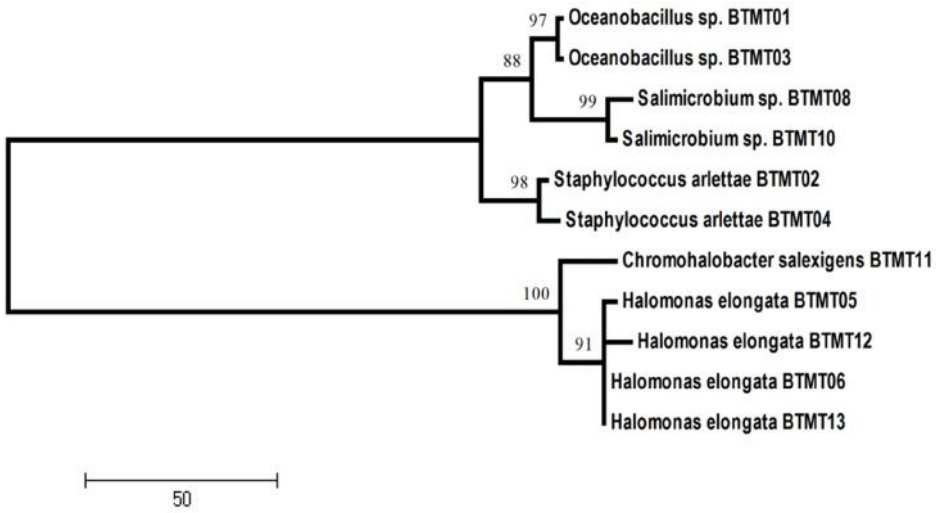


Fig. 4.1.Phylogenetic tree of the halophiles isolated from tannery effluent and commercially available salt crystals. (Evolutionary relationships of 11 taxa.Phylogenetic analyses were conducted with MEGA4 software)

4.2. Screening and Selection of Potential Haloarchaebacteria for Gelatinase Production

All the bacterial strains isolated from tannery effluent and table salts were screened for gelatinase production along with haloarchaea *Natrinema* sp.BTSH10 and *Halorubrum* sp.BTSH03 using Zobell's medium supplemented with 0.5% gelatin and 15% NaCl. Gelatinase activity was detected on gelatin supplemented agar medium in terms of clearing zone produced around the inoculated colonies on addition of 10% TCA after growth followed by computation of gelatinase enzyme units as described under the materials and methods section 3.2.4.

From the results presented in Fig. 4.2, it was evident that *Natrinema* sp. BTSH10 produced maximal quantities (11U/mL) of gelatinase, followed by *Halorubrum* sp.BTSH03 (9 U/mL), *Oceanobacillus* sp. BTMT01 (5.9 U/mL) and *Oceanobacillus* sp. BTMT03 (5.8 U/mL) while all other isolates produced only lesser amounts of enzyme. Gelatinase activities demonstrated by three strains which showed relatively maximal enzyme activities are shown in Fig. 4.3.

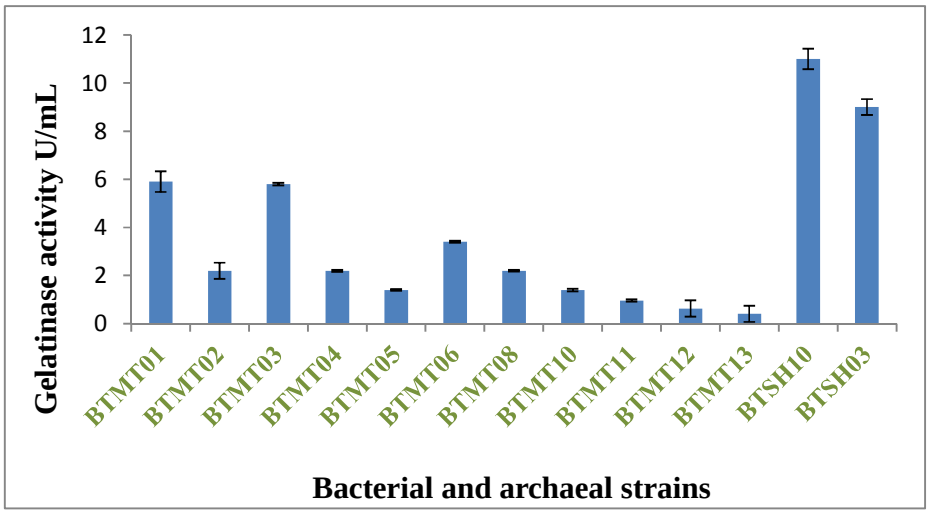
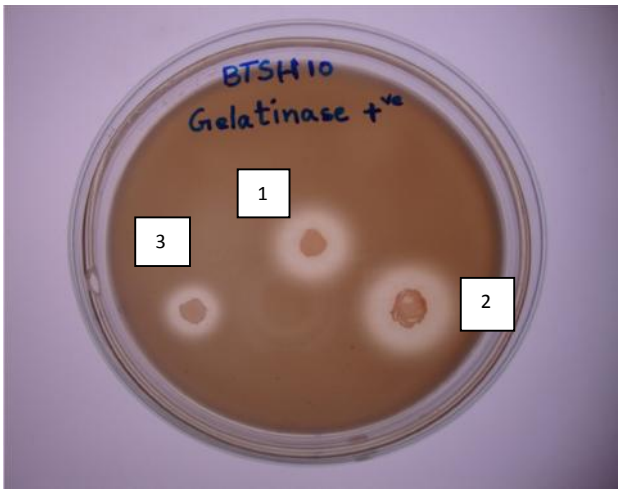


Fig. 4.2.Gelatinase production by BTSH10, BTSH03 and bacteria isolated from tannery effluent and food grade salt crystals



1. *Halorubrum*sp.BTSH03
2. *Natrinema* sp.BTSH10
3. *Oceanobacillus* sp. BTMT01

Fig. 4.3. Gelatin plate with bacteria showing gelatinase production

Based on the results obtained, *Natrinema* sp.BTSH10 was selected for further optimization of bioprocess conditions for gelatinase production. Typical growth of *Natrinema* sp.BTSH10 on Zobell's agar medium supplemented with 15% NaCl is shown in Fig.4.4.

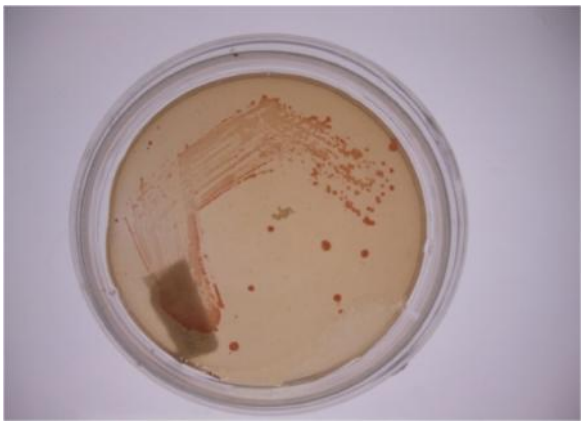


Fig.4.4.Typical growth of *Natrinema* sp. BTSH10 on Zobell's agar medium supplemented with 15% NaCl

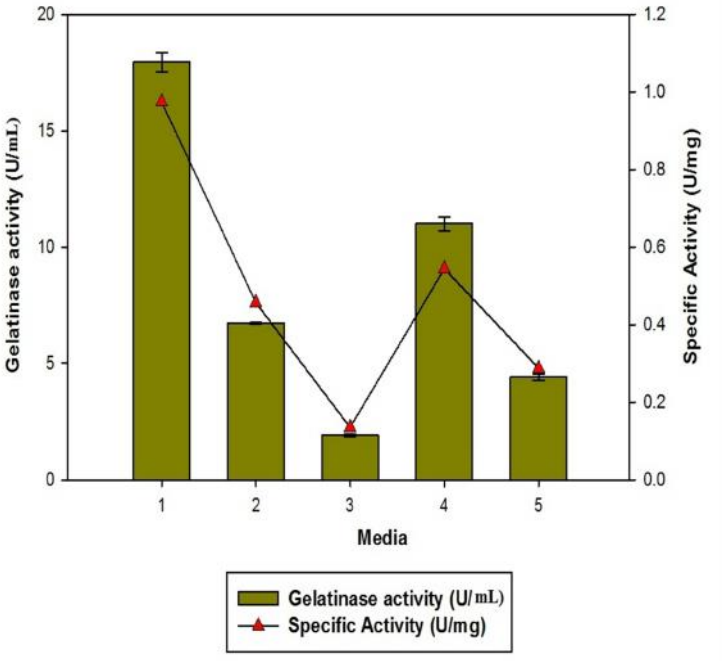
4.3.Selection of Media for Gelatinase Production

Five different media were evaluated to select the optimal medium that supported maximum gelatinase production by the selected potential haloarchaeal strain. From the results presented in Fig. 4.5, it was inferred that among the five different media tested, Medium 1(Akolkar, 2009) supported maximal production of gelatinase enzyme (17.9 U/mL) followed by Medium 4[Zobell Marine Broth (11 U/mL)], Medium 2[Vidyasagar *et al*., 2006(6.7 U/mL)] andMedium 5[Liquid Brown Medium(4.4U/mL)]. Medium 3[EimhjellenMedium (Catherine *et al*., 2001)] supported only 1.9 U/mL of gelatinase production. Hence, Medium 1 was selected for further optimization of bioprocess parameters towards obtaining maximal gelatinase production.

4.4. Growth Curve of *Natrinema* sp. BTSH10

Growth curve of *Natrinema* sp. BTSH 10 was estimated using Zobell's broth supplemented with 15% NaCl and is presented as Fig. 4.6. It is evident from the results presented that the archaea is slow growing and took a long time to acclimatize to the medium since, it remained in the lag phase upto 40 h. The archaea was observed to enter the logarithmic phase after 40 h. In fact, an active progressive log phase was noted during the period 60-100 h after which it entered the stationary phase and remained in the same phase until 170 h. Mid log phase was noted during 60-90 h. The

doubling time and growth rate constant of *Natrinema* sp. BTSH10 was 24 h and 0.0416 respectively.



Medium 1:(Akolkar, 2009)
Medium 2: (Vidyasagar *et al*., 2006)
Medium 3: Eimhjellen medium (Catherine *et al*., 2001)
Medium 4:Zobell's medium
Medium 5: liquid Brown's medium (Birbir *et al*., 2004)

Fig. 4.5. Selection of media for gelatinase production

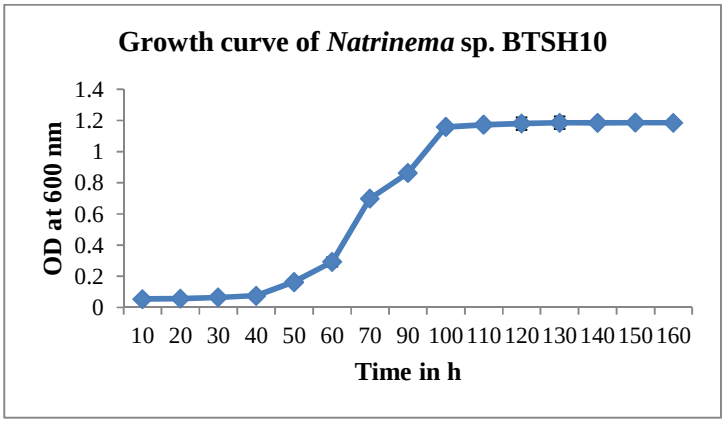


Fig. 4.6. Growth curve of *Natrinema* sp. BTSH10

4.5. Optimization of Bio-process Conditions for Gelatinase Production by BTSH10 *Natrinema* sp. under Submerged Fermentation.

Different physico-chemical and bioprocess variables that influence gelatinase production by *Natrinema* sp. BTSH10 under submerged fermentation were optimized for maximal production using the Medium 1(Akolkar, 2009) employing " one-variable-at-a-time" approachand the results obtained are presented below.

4.5.1. Optimization of NaCl Concentration

Halophiles have a requirement for high salt concentration for growth and enzyme production. Hence, optimal requirement of salt in terms of NaCl for maximal enzyme production was determined by supplementing the fermentation medium with different concentrations of NaCl and assaying enzyme yield. From the data presented in Fig. 4.7, it was found that the archaeal strain required 25% NaCl for effecting maximal gelatinase (22.4 U/mL). Nevertheless 20% NaCl concentration also supported considerable level of gelatinase production (14.8U/mL) compared to other concentrations of NaCl. It was also observed that specific activities for gelatinase also showed exactly the same trend which was observed for the enzyme activity with respect to different concentrations of NaCl.

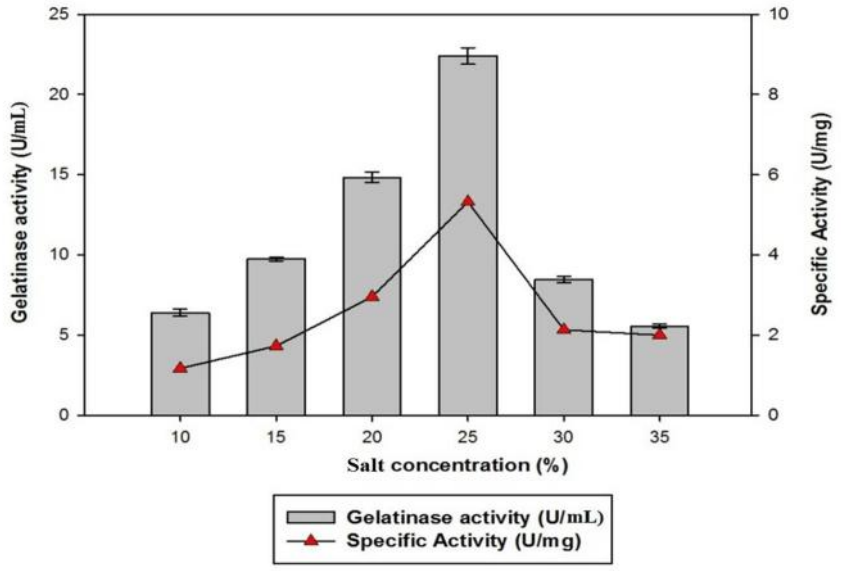


Fig. 4.7. Optimization of NaCl concentration for gelatinase production by *Natrinema* sp. BTSH10.

4.5.2. Optimization of Initial pH of Medium

Initial pH of the medium often influences the course of fermentation and rate of enzyme production. Hence, optimal pH required for maximal enzyme production was determined by adjusting the initial pH of the fermentation medium to varying pH levels and assaying enzyme yield after fermentation. From the results documented in Fig. 4.8.it was inferred that pH 7.0 is the optimal requirement for the archaea to record maximum gelatinase (30.6 U/mL) production. It was observed that very little gelatinase activities were supported by initial pH 2-5 while neutral pH supported considerable levels of gelatinase production compared to alkaline pH conditions. Further increase in pH above 9 did not support gelatinase production by the archaeal strain. It was also observed that specific activities for gelatinase also showed exactly the same trend which was observed for the enzyme activity with respect to different levels of initial pH in the medium.

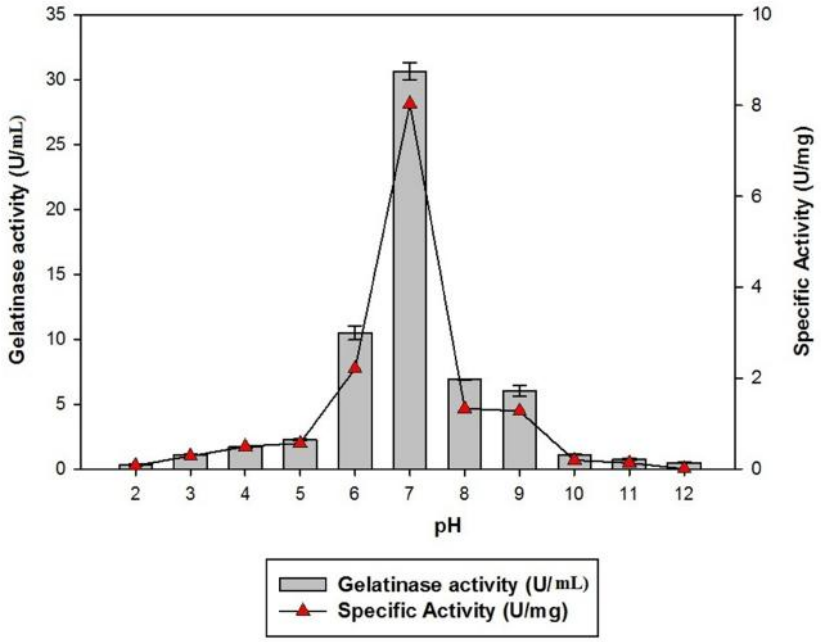


Fig.4.8. Optimization of initial pH of medium for gelatinase production by *Natrinema* sp. BTSH10

4.5.3. Optimization of Incubation Temperature

Optimal incubation temperature requirement of the haloarchaea for maximal gelatinase production was determined by incubating the inoculated medium at different temperatures and assaying gelatinase yield. From the data presented in

Fig.4.9., it was found that this archaeal strain preferred 42°C for recording maximal gelatinase production (37 U/mL), compared to 37°C (15 U/mL) which was commonly used for cultivation of the archaeal strain. Further, it was also observed that increase in incubation temperatures above 42°C and incubation at temperatures below 37°C resulted in reduced levels of gelatinase. It was observed that profile of specific activities for gelatinase also demonstrate similar pattern which was observed for the enzyme activity with respect to different incubation temperatures.

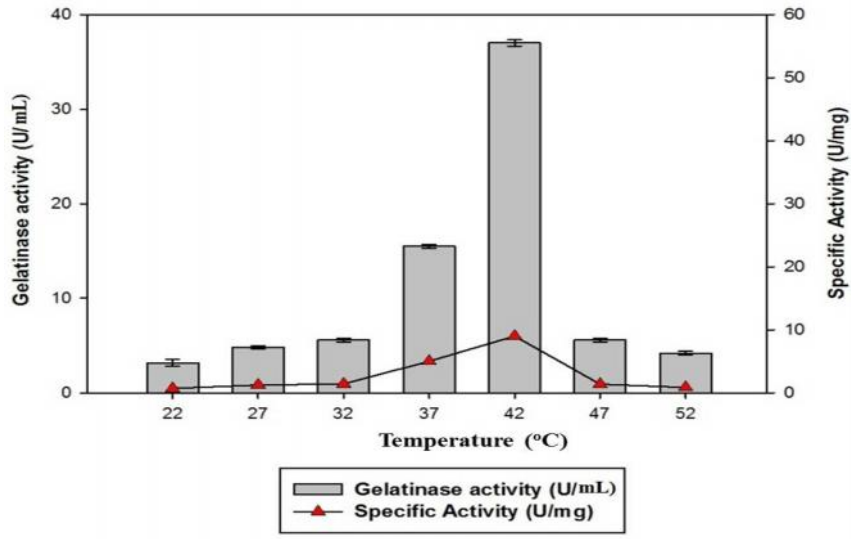


Fig. 4.9.Optimization of incubation temperature for gelatinase production by *Natrinema* sp. BTSH10

4.5.4. Optimization of Inoculum Concentration

Optimal inoculum concentration required for maximal gelatinase production by *Natrinema* sp. BTSH10 was determined by inoculating the medium with different concentration of inocula and assaying enzyme yield. From the results presented in Fig. 4.10., it was evident that 2% inoculum concentration was optimum since maximal enzyme (45 U/mL) yield was obtained when compared to other concentrations tested. Concentrations above 2% recorded a decline in the gelatinase production. 1% inoculum did not support enhanced level of enzyme production. In a similar manner, the specific activities for gelatinase also followed exactly the same trend which was observed for the enzyme activity with respect to different inoculum concentrations.

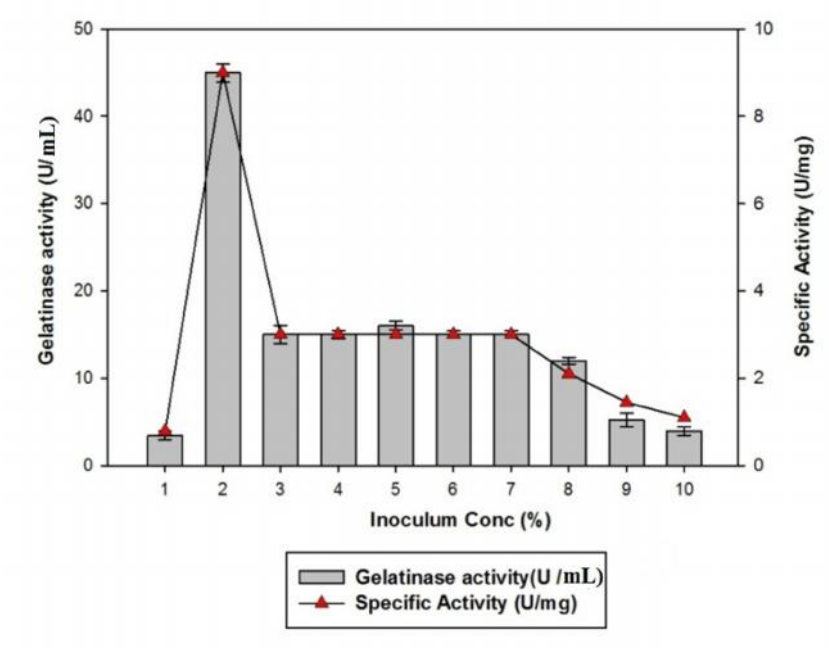


Fig. 4.10.Optimization of inoculum concentration for gelatinase production by *Natrinema* sp.BTSH10

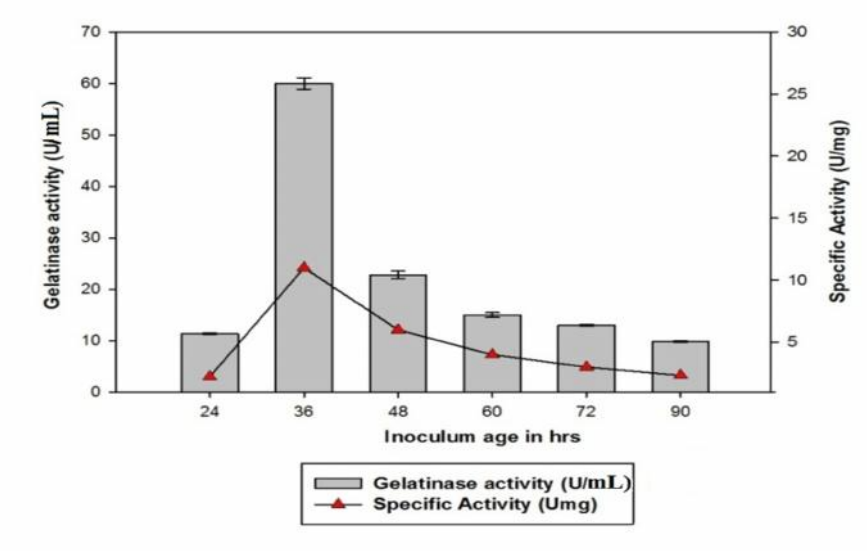
4.5.5. Optimization of Inoculum Age

Impact of age of inocula used for inoculation on gelatinase production was evaluated and the results are presented in Fig. 4.11. It was found that inoculation of enzyme production media with inoculum age of 36 h supported maximal gelatinase yield (60 U/mL). When inocula of 24 h age were used, only 11.2 U/mL of gelatinase could be obtained. However, a yield of 22.7 U/mL could be obtained when 48 h inoculum was used. Similarly inoculum of age above 36 h did not support enhanced levels of gelatinase production since increase in the inoculum age was found to result in a decline in the levels of gelatinase production. It was observed that pattern of specific activities for gelatinase also showed exactly the same trend which was observed for the enzyme activity with respect to different inoculum ages.

4.5.6. Effect of Agitation

Impact of agitation and optimal rate of agitation required for maximal gelatinase production by *Natrinema* sp. BTSH10 was determined by incubating the inoculated media in a rotary shaker at different rates of agitation and assaying enzyme yield. From the data presented in Fig. 4.12, it was inferred that rate of agitation

influenced the rate of enzyme production during incubation. Thus, under static conditions, the enzyme production was only 2 U/mL which increased to only 3 U/mL when it was subjected to 50 rpm. When the rate of agitation was enhanced to 100 rpm, the gelatinase production increased to 55 U/mL, which increased to a maximum of 75 U/mL when the agitation rate was increased to 150 rpm. However, further increase in agitation rates, did not enhance level of enzyme production but instead led to a decline in the rate of gelatinase production. It was observed that specific activities for gelatinase also showed exactly the same trend which was observed for the enzyme activity with respect to different rates of agitation.



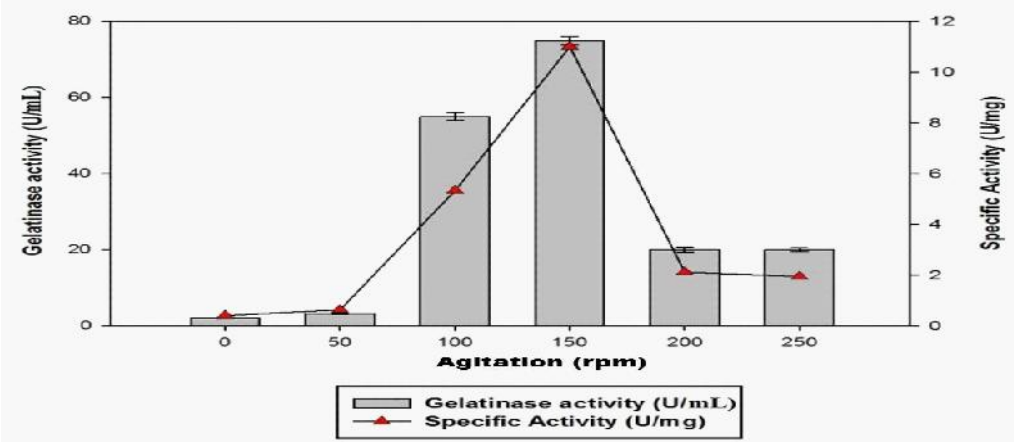


Fig .4.12. Effect of agitation on gelatinase production by *Natrinema* sp. BTSH10

4.5.7. Effect of Additional Carbon Sources

Effect of additional carbon sources in the medium on gelatinase production was evaluated using different carbon sources. From the results presented in Fig. 4.13., it was found that 0.1M sorbitol was the preferred additional source of carbon in the medium since maximal gelatinase (95 U/mL) was recorded with this source among the various carbon sources tested. Further, it was noted that dextrose, maltose, fructose and lactose did not favour gelatinase production. Next to sorbitol, mannitol recorded considerable level of gelatinase (55U/mL) followed by mannose (39.8U/mL), cellobiose (39 U/mL), sucrose (37.4 U/mL)and galactose (32.6 U/mL). It was observed that profile of specific activities for gelatinase also showed exactly the same trend which was observed for the enzyme activity with respect to different carbon sources.

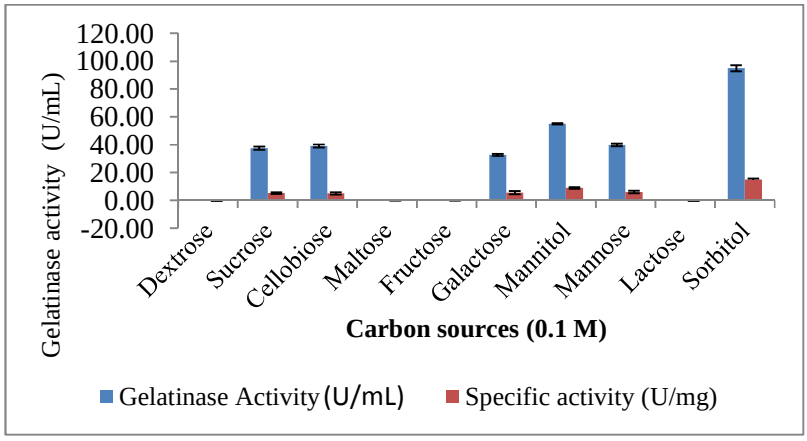


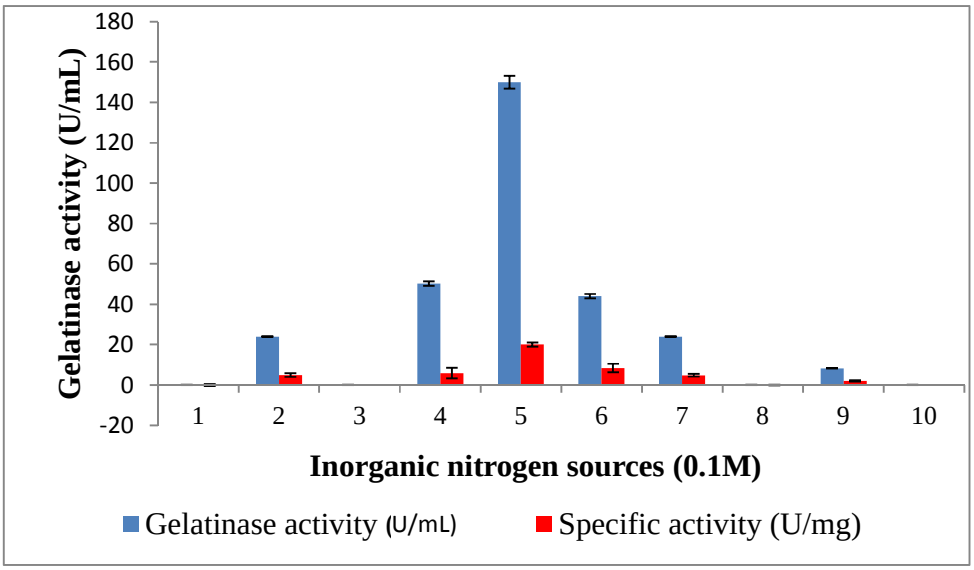
Fig.4.13.Effect of additional carbon sources on gelatinase production by *Natrinema* sp. BTSH10.

4.5.8. Effect of Additional Nitrogen Sources

4.5.8.1. Effect of Additional Inorganic Nitrogen Sources

Effect of additional inorganic nitrogen sources on gelatinase production was evaluated using different inorganic nitrogen sources. It was inferred from the results presented in Fig. 4.14a.that addition of 0.1M ammonium sulphate to the medium enhanced gelatinase production (150 U/mL) compared to all other inorganic nitrogen sources. Addition of ammonium acetate resulted in the production of 50 U/mL of

gelatinase and incorporation of ammonium chloride yielded 44 U/mL. Addition of sodium nitrate, ammonium nitrate and ammonium ferrous sulphate resulted in reduced levels of gelatinase production. Interestingly, it was observed that addition of ammonium dihydrogen phosphate, ammonium hydrogen carbonate, ammonium oxalate and diammonium hydrogen phosphate resulted in inhibition of gelatinase production. Specific activities for gelatinase recorded with different inorganic nitrogen sources also showed exactly the same trend which was observed for the enzyme activity with respect to different inorganic nitrogen sources.



1 - $NH_4H_2PO_4$ 6 - NH_4Cl
2 - $NaNO_3$ 7 -NH_4NO_3
3 - NH_4HCO_3 8 -$(NH_4)_2C_2O_4$
4 -$NH_4C_2H_3O_29$ -$(NH_{4)2}SO_4FeSO_4$
5 - $(NH_4)_2SO_4$ 10 -$(NH_4)_2HPO_4$

Fig. 4.14a.Effect of additional inorganic nitrogen sources on gelatinase production by *Natrinema* sp. BTSH10

4.5.8.2. Effect of Additional Organic Nitrogen Source

Impact of additional organic nitrogen sources on gelatinase production was evaluated using different organic nitrogen sources. It was inferred from the results presented in Fig.4.14b., that addition of skim milk powder to the medium enhanced maximal gelatinase production (180 U/mL) compared to all other organic nitrogen sources. Soya bean meal (100 U/mL) followed by peptone (85 U/mL), soya casein digest (62 U/mL) and beef extract (24U/mL) also recorded gelatinase production.

However, yeast extract did not support gelatinase production. Further, it was noted that specific activities for gelatinase also showed similar trend as that observed for the enzyme activity with respect to different organic nitrogen sources.

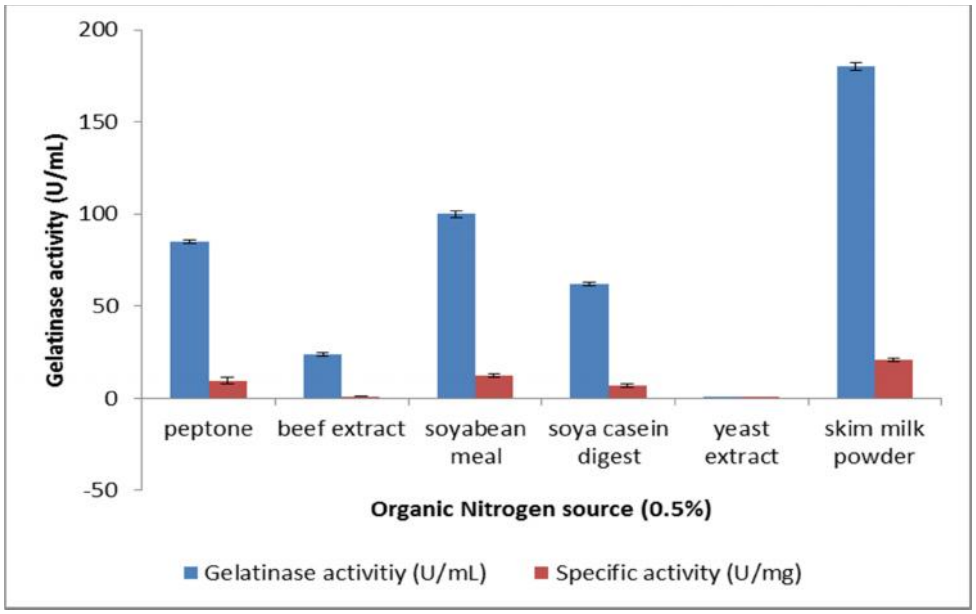


Fig. 4.14b. Effect of additional organic nitrogen source on gelatinase production by *Natrinema* sp. BTSH10

4.5.9. Effect of Detergents

Detergents affect the permeability of cell membranes and leads to increased secretion of extracellular enzymes into the fermentation medium. Hence, impact of different detergents in the fermentation medium on enzyme yield was evaluated. From the results presented in Fig. 4.15, it was found that among the 6 detergents tested, addition of CTAB, sodium taurocholate and sodium lauryl sulphate to the production media resulted in inhibition of gelatinase production by the archaea. Incorporation of Tween-80 led to maximum enzyme activity (190 U/mL) in the fermented medium compared to Tween-20 (100 U/mL) and TritonX100 which showed meagre levels of enzyme production (10 U/mL). From the results, it was inferred that Tween-80 could be used to get maximal enzyme yield from the fermented medium. It was observed that specific activities for gelatinase recorded respective levels for the different detergents along with increase in enzyme activity.

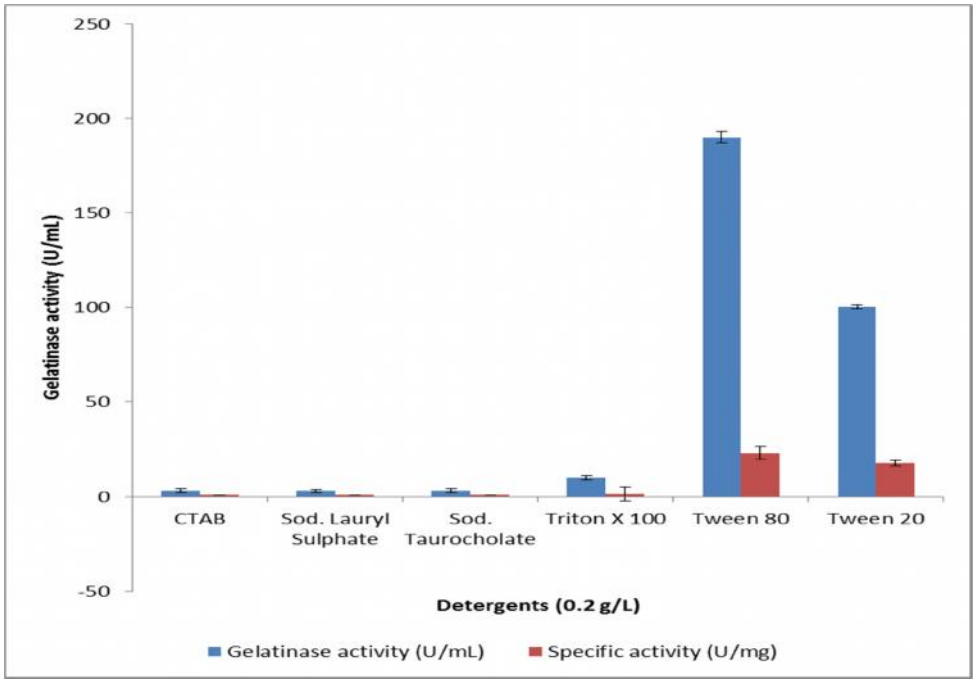


Fig. 4.15. Effect of detergents on gelatinase production by *Natrinema* sp. BTSH10

4.5.10. Time Course Experiment

A time course experiment was conducted after optimizing all the bioprocess variables for obtaining maximal gelatinase production by *Natrinema* sp.BTSH10 during submerged fermentation over a period of 168 h under optimized conditions. The results obtained are documented in Fig.4.16. From the results, it was evident that gelatinase production by the archaeal strain commenced after 48 h growth and reached a maximum after 120 h (229.4 U/mL). It was noted that there were very low levels of gelatinase production at 72 h and 96 h which rapidly increased during the period between 96h and 120 h. It is assumed that maximal gelatinase production has taken place during late logarithmic phase of the growth curve compared to mid log phase. Further, it was also noted that during incubation beyond 120 h the enzyme yield decreased progressively along with increase in incubation time.It was observed that specific activities for gelatinase also showed exactly the same trend which was observed for the enzyme activity.

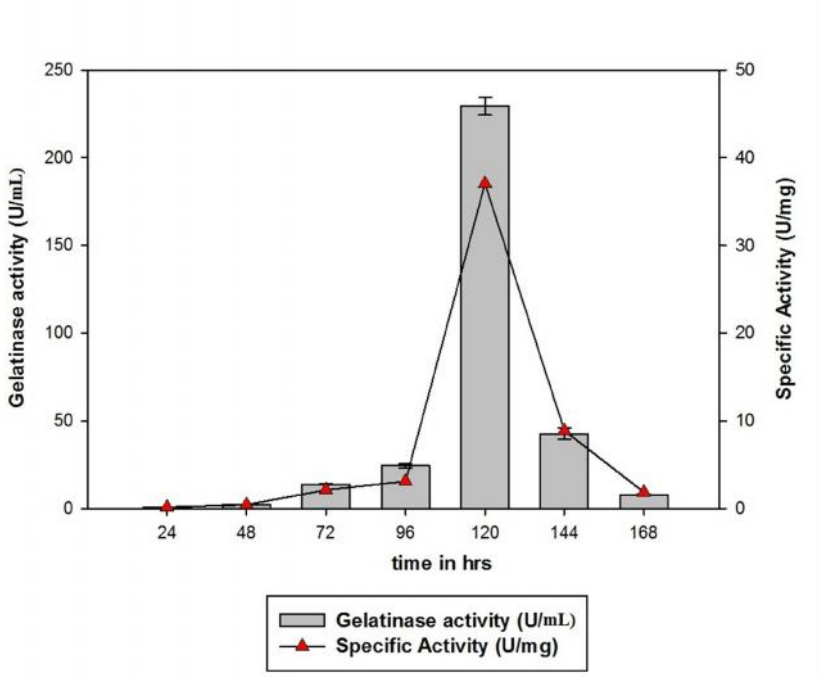


Fig. 4.16. Time course study on gelatinase production by *Natrinema* sp. BTSH10 under optimal conditions

4.6. Purification of Gelatinase

The culture supernatant obtained after centrifugation at 10,000 rpm for 10 min at 4°C, followed by removal of archaeal cells was considered as crude gelatinase enzyme which was subjected to purification employing Membrane Filtration, Gel Filtration Chromatography and Dialysis followed by HPLC. The results obtained are presented in Table4.2.

4.6.1. Filtration

The crude enzyme prepared was initially subjected to filtration at 4°C using Amicon filters with a cut–off size 10 kDa and all the fractions were analyzed for presence of enzymes. From the results obtained, it was observed that only those fractions comprising proteins with a size greater than 10 kDa showed enzyme activity. Hence, these fractions were further subjected to filtration using Amicon filters with a cut off size 30 kDa. It was found that the fractions consisting of proteins between 10 - 30 kDa showed gelatinase activities and these fractions were used for further purification. Results presented in Table 4.2 show the efficiency of purification by filtration.

Table 4.2. Yield and fold of purification of gelatinase

Sample	Volume (mL)	Protein (mg)	Activity (U)	Specific activity (U/mg)	Yield of protein (%)	Yield of activity (%)	Fold of purification
Crude	1000	4500	11203.66	2.48	100	100	1
Filtration 10 kDa cut off (>10 kDa fraction)	550	2447.5	9385.85	3.83	54.38	83.77	1.54
Filtration 30 k Da cut off (10-30 kDa fraction)	330	1212.75	8577.80	7.07	26.95	76.56	2.84
Gel filtration	2	3.1	79	25.48	0.06	0.70	10.23
HPLC	1	2.1	60.3	28.71	0.04	0.53	11.53

4.6.2.Gel Filtration Chromatography

Gel filtration chromatograhy was performed using Sephadex G-50 (Sigma-Aldrich) to separate proteins ranging in size from 50 kDa -3 kDa. From the data presented in Fig. 4.17., it was inferred that the fractions numbered 18 to 24 contained gelatinase enzyme. These fractions were pooled, lyophilised and used for dialysis of the enzyme fractions towards further purification.

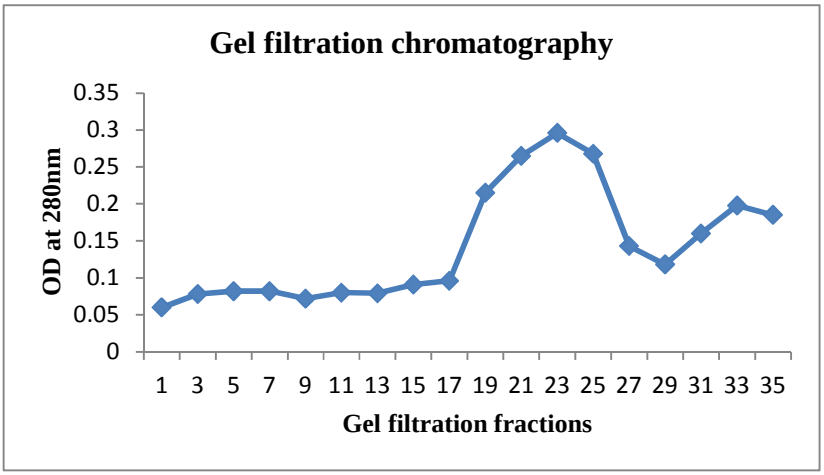


Fig. 4.17. OD at 280 nm of gel filtration fractions

4.6.3. Dialysis

The lyophilised protein fraction with size ranging from 10-30 k Da, obtained after gel filtration chromatography was dialysed against Tris-HCl buffer 0.05M (pH 8). It was observed that the dialysed enzyme fraction did not show gelatinase activity. From this observation, it was inferred that since the halozymes require high salt concentration for their activity, the dialysed fractions did not show any enzyme activity. However when the dialysis process was repeated using buffer supplemented with2M NaCl, the resultant dialysed fraction showed gelatinase activity.

4.6.4. HPLC

HPLC of enzyme fractions was performed using semi-preparative column C 18 (Schimadzu LC -6 AD) and the results are presented in Fig. 4.18., Fig.4.19.and Fig. 4.20. Data obtained from the HPLC analysis of crude enzyme are presented in Fig. 4.18and it showed many peaks viz:6.125 m, 10.2 m, 15.9 m, 21.2 m, 24 m. Among the peaks the one obtained at 10.2 m was very prominent. These peaks indicated presence of more than one protein along with the target gelatinase in the fractions collected as crude enzyme.

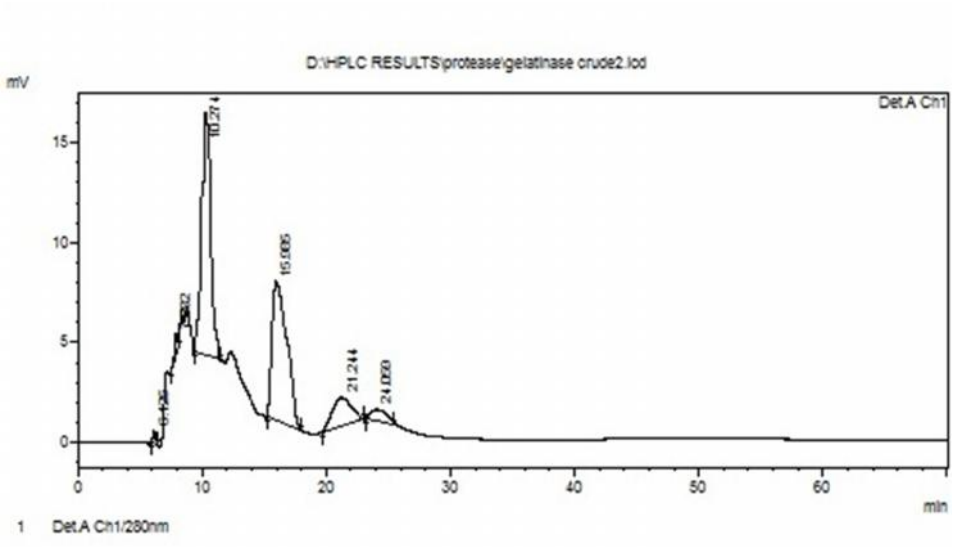


Fig.4.18. HPLC of crude sample

The enzyme fractions obtained after gel filtration chromatography, when subjected to HPLC, showed a single peak (6.828 m) indicating the effectiveness of purification by gel filtration chromatography and the purity of enzyme. It was also noted that this single peak was observed in the fraction collected during 6.5-7.5 m(Fig. 4.19.), when the concentration of methanol was between 9.3 % and 10.7 % methanol.

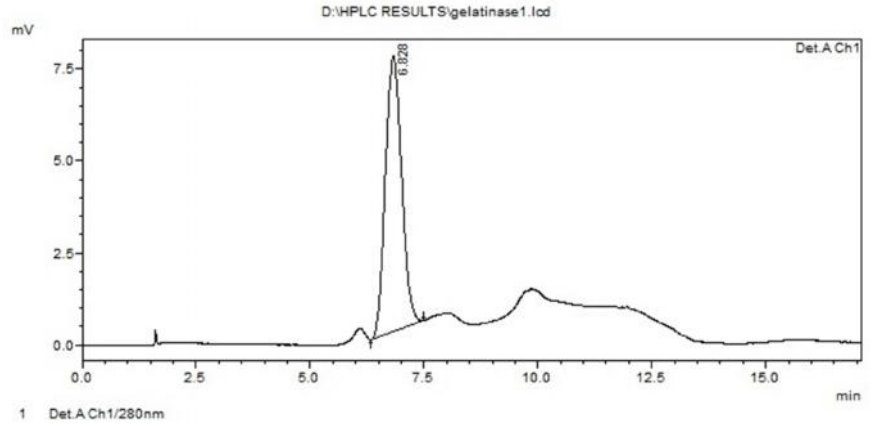


Fig.4.19. HPLC of gel filtration chromatographic fraction

From the data depicted in Fig. 4.20. it was inferred that HPLC purification of the dialysed sample (using buffer containing 2M NaCl) resulted in a single peak which was observed at 6.132 m. The efficiency of the purification in terms of yield and fold are presented in table 4.2.

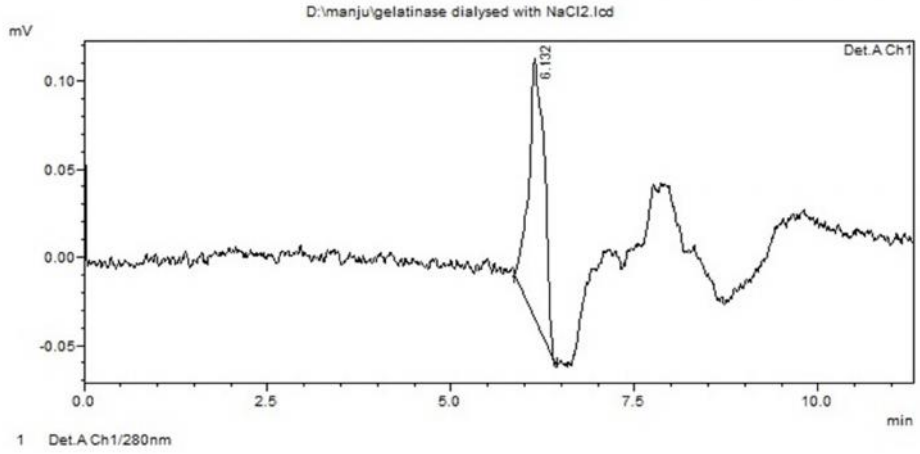


Fig.4.20. HPLC of dialysed sample

4.6.5. Polyacrylamide Gel Electrophoresis (PAGE)

4.6.5.1.Native Polyacrylamide Gel Electrophoresis

Purified enzyme obtained after HPLC was subjected to native PAGE for confirming the purity of enzyme protein and its approximate size. Result presented in Fig. 4.21., showed the presence of a single band on the gel and the molecular mass of was inferred as 21 kDa.

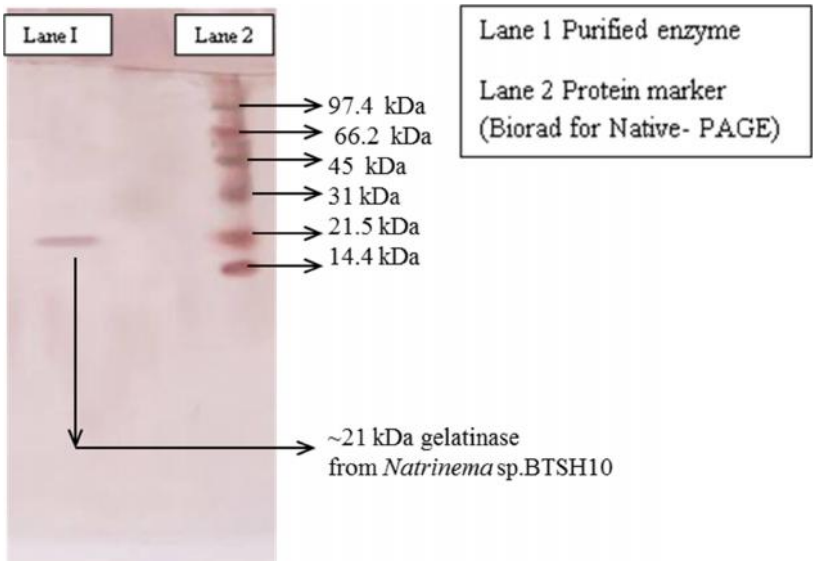


Fig. 4.21. Native PAGE of purified enzyme sample

4.6.5.2.Reductive SDS-PAGE

Further, the purified enzyme obtained after HPLC was also subjected to reductive SDS-PAGE for confirming the probable number of subunits of the purified enzyme protein and their approximate size. From the data presented in Fig. 4.22., it was observed that the enzyme has a homogenous single unit since only a single band of approximately 19 kDa size was obtained after reductive SDS-PAGE.

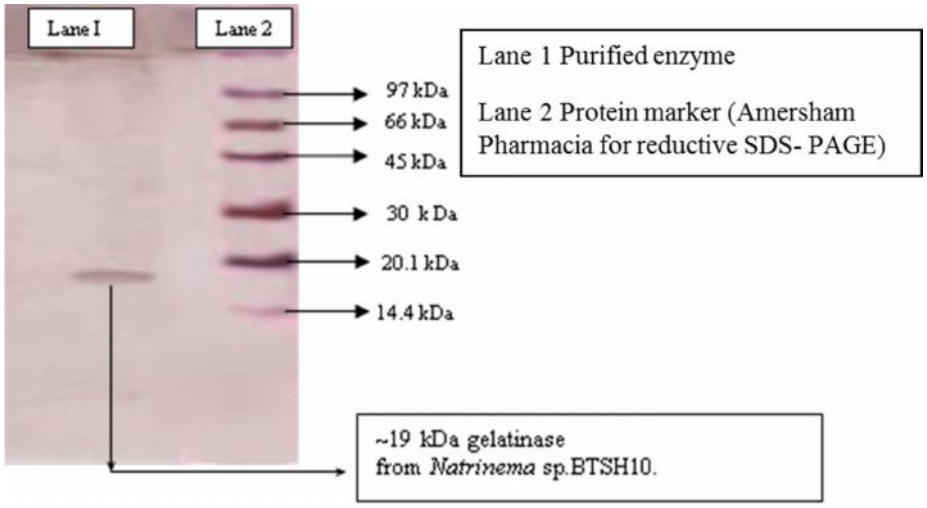
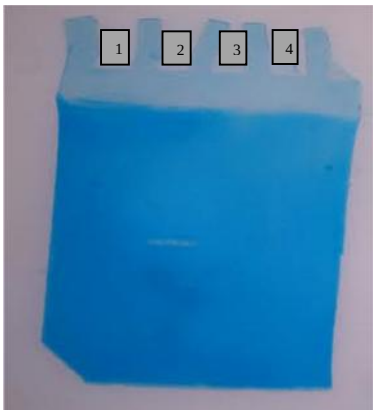


Fig. 4.22. Reductive SDS-PAGE of purified enzyme sample

4.6.5.3.Zymogram

Gelatinase activity of the purified enzyme protein band was confirmed by performing zymogram analysis. The results presented in Fig.4.23., indicate that the purified enzyme protein showed good gelatinase activity since a clear zone in blue coloured gel was observed around the site of protein band. The results confirmed that the purified enzyme protein was gelatinase.



Clearing seen in lane 2

Fig. 4.23.Zymogram analysis of gelatinase

4.7. Characterization of the Purified Enzyme

4.7.1. Optimal pH for Gelatinase Activity.

Results obtained for the studies on determination of optimal pH for gelatinase activity evaluated by assaying gelatinase in plates with media in the pH range 5 – 13 is presented in Fig 4.24. There was no enzyme activity in the pH range2-6. From the results, it was inferred that the optimal pH for maximal activity of the enzyme was pH 8 (about 68.8 U/mL).Whereas at pH 9.0, it was observed that the maximal enzyme active was reduced to half when compared to that observed at pH 8.0. At neutral pH 7.0 and at alkaline pH (pH 10) there was reduced enzyme activity (14 U/mL). The enzyme activity reduced beyond pH 11 and at pH 13, it was reduced to about 6 U/mL.

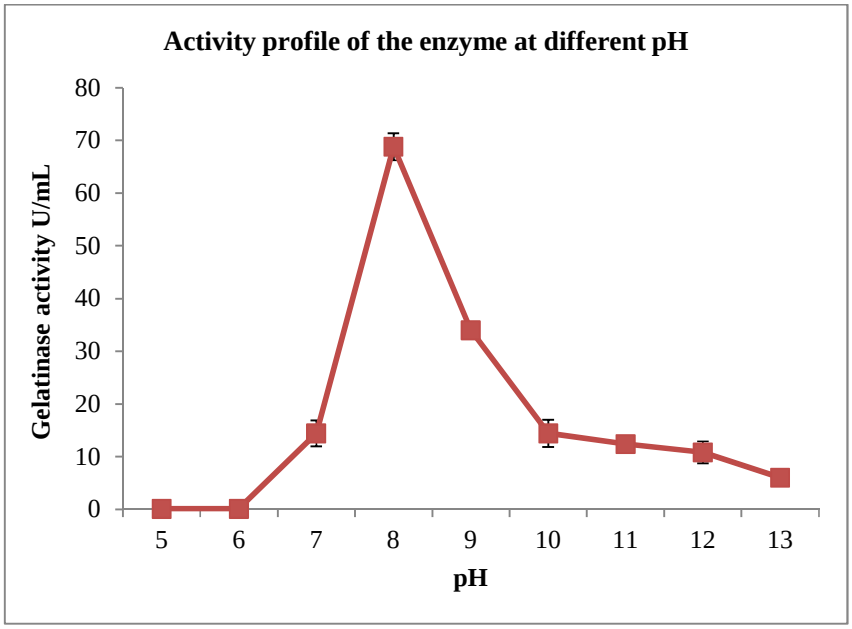


Fig.4.24. Activity profile of gelatinase at different pH

4.7.2. Stability of Gelatinase at Different pH

Stability of gelatinase at different pH conditions was evaluated by incubating the enzyme in different buffer systems prepared in the range of pH 2 to pH 13 for 24 h at 4°C and assaying the residual gelatinase activity. Results presented in Fig. 4.25. very clearly indicated that there was no residual enzyme activity at pH 2 to 5

indicating that the enzyme was not stable under acidic conditions when compared to alkaline pH conditions (pH 9 to pH 13). The residual activities were above 56% for pH 12 and 49% for pH 13, about 86% for pH 9 to 11 range. At neutral pH 7, 65% residual activity was recorded. Results indicated that probably the enzyme is alkalophilic in nature.

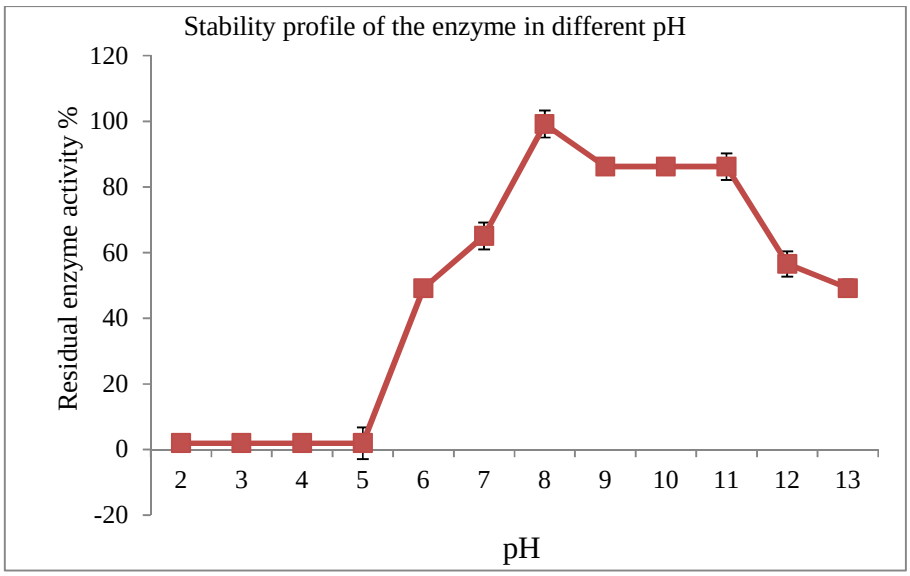


Fig.4.25. Stability of gelatinase at different pH

4.7.3. Optimal Temperature for Gelatinase Activity

Optimal temperature required for gelatinase activity was evaluated by assaying gelatinase by incubation at different temperatures varying from 5°C to 47°C. Data presented in Fig. 4.26.indicated that the optimum temperature required for maximal enzyme activity was 37°C. It was also observed that temperatures above 42°C and below 27°C led to a steep decline in activity. However, moderate levels of enzyme activities, almost less than 50% of the level recorded at 37°C could be observed at 32°C and 42°C.

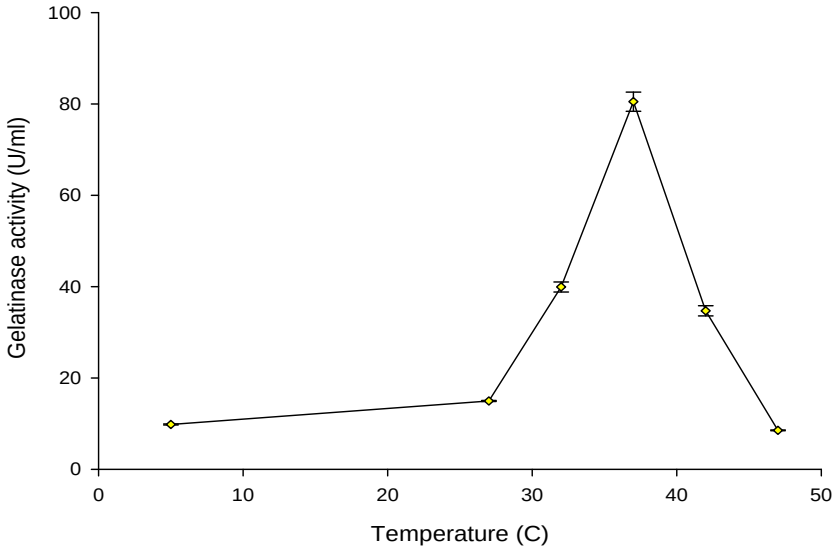


Fig.4.26. Effect of temperature on gelatinase activity

4.7.4. Stability of Gelatinase at Different Temperatures

Stability of gelatinase at different temperatures was determined by incubating the enzyme at different temperatures ranging from 20°C to 80°C and assaying residual enzyme activity after 30 m, 1 h, 2 h, 4 h, 6 h, 8 h, 10 h, 12 h and 24 h. From the results presented in table 4.3, it was inferred that the enzyme was stable without losing activity (about 99% residual activity) at 40°Cupto 4 h and upto 30 min. at 20°C, 30°C, 50°C and 60°C. The enzyme showed a residual activity of 63% upto 24 h at temperatures of 20°C and 30°C; up to 12h at 40°C, upto 6h at 50°C and upto 4h at 60°C. Extended incubation at higher temperatures resulted in loss of activity since the residual activity declined to about 43%. At higher temperatures of 70°C and 80°C, there was no residual activity indicating loss of enzyme activity.

Table. 4.3. Residual activity of gelatinase at different temperatures

Time	Residual activity (%) at different temperatures (°C)						
	20	**30**	**40**	**50**	**60**	**70**	**80**
30min	99.0± 4.30	99.66 ± 2.48	99.66± 2.48	99.0± 4.3	98.66± 4.96	12.0± 4.3	1.1± 1.13
1h	66.66 ± 8.95	66.66 ± 6.57	99.66± 2.48	67.0± 8.6	65.66± 2.48	0.96± 0.24	0.96± 0.24
2h	65.33 ± 2.48	65.66 ± 2.48	99.0± 4.30	65.66± 2.48	64.66± 2.48	0.93± 0.24	0.96± 0.24
4h	65.0± 4.30	65.33± 2.48	98.66± 4.96	64.33± 4.96	64.33± 2.48	0.96± 0.24	0.96± 0.24
6h	64.66 ± 2.48	65.33 ± 2.48	66.0± 4.30	64.0± 4.3	43.66± 2.48	0.96± 0.24	0.96± 0.24
8h	64.33± 2.48	64.66 ± 2.48	65.66± 2.48	42.0± 7.45	43.66± 2.48	0.96± 0.24	0.96± 0.24
10h	64.0± 4.30	64.33± 2.48	65.66± 2.48	42.0± 6.57	42.0± 4.3	0.96± 0.24	0.96± 0.24
12h	64.33 ± 2.48	63.66 ± 4.96	64.33± 2.48	28.33 ± 2.48	19.66 ± 6.57	0.93± 0.24	0.96± 0.24
24h	64.0 ± 4.30	63.66 ± 4.96	29.0± 4.3	27.0± 4.3	18.66± 2.48	0.93± 0.24	0.96± 0.24

95% confidence interval for residual activity

4.7.5. Effect of Inhibitors on Gelatinase Activity

Effect of inhibitors on gelatinaseactivity was evaluated using Aprotinin, EDTA and Iodoacetamide, and the results obtained are presented in table 4.4. From the residual enzyme activities recorded for the various inhibitors it was found that the enzyme showed marked decline in enzyme activities along with increase in the concentration of the enzyme inhibitors in all the three cases. Among the three

inhibitors tested, maximal inhibition was effected by EDTA which is a metallo-protease inhibitor. Even at the lowest concentration 10 mM, marked inhibition (20%) of the gelatinase was observed which declined rapidly to 3% in presence of 50 mM EDTA. Whereas in the presence of aprotinin, the gelatinase showed a residual activity of 75% at 0.2 mM which decreased to 49% when the concentration was increased to 0.6 mM. Further increase in inhibitor concentration to a maximum of 1 mM led only to a marginal decline from 49% to 43%. In the case of Iodoacetamide 43% residual activity was noted for the lesser concentrationsof 10mM and 20 mM indicating more than 50% enzyme inhibition, while 27% residual activity was observed in the presence of 50mM concentrations.

Table. 4.4. Effect of inhibitors on gelatinase activity.

Name of inhibitor (class) used	**Concentration (mM)**	**Residual Activity (%)**
Aprotinin (Serine protease)	0.2	75.17 (± 4.50)
	0.4	65.88 (± 4.38)
	0.6	49.19 (± 4.54)
	0.8	43.03 (± 4.31)
	1.0	43.70 (± 2.62)
EDTA (Metallo-protease)	10	20.12 (± 5.13)
	20	18.52 (± 2.16)
	30	8.73 (± 2.78)
	40	5.41 (± 2.23)
	50	3.31 (± 1.18)
Iodoacetamide (Cysteine protease)	10	43.03 (± 4.31)
	20	43.70 (± 2.23)
	30	29.09 (± 3.68)
	40	27.76 ± 2.92
	50	27.43 ± 3.22

95% confidence interval for residual activity

4.7.6. Substrate Specificity

Substrate specificity was determined by conducting the enzyme assay using gelatin, skim milk, BSA and haemoglobin as substrates in agar plates incorporated with respective substrates. From the results presented in Fig. 4.27., it was found that

the ideal substrate that supported maximal activity was skim milk followed by gelatin. Use of BSA and haemoglobin as substrates did not yield appreciable activity.

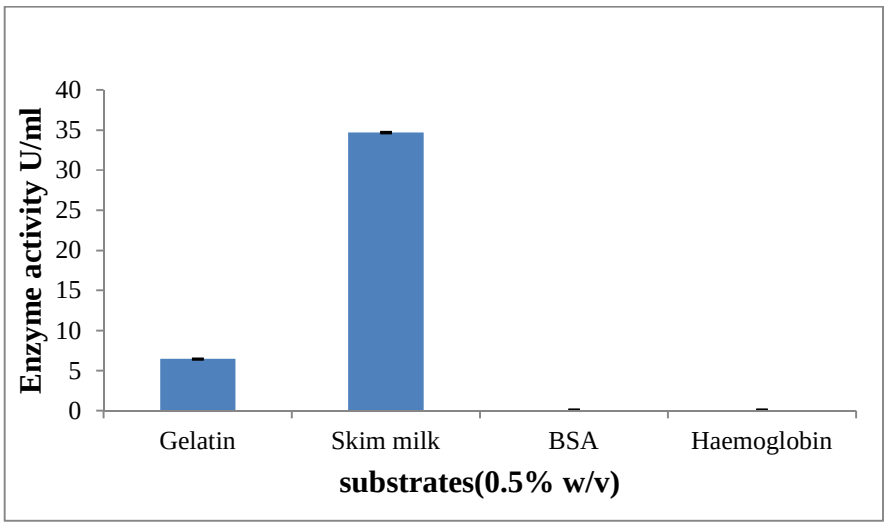


Fig.4.27. Substrate specificity of gelatinase enzyme

4.7.7. Kinetic Studies

Enzyme kinetic studies were conducted to determine the K_m, the substrate concentration at which reaction velocity is half maximum and V_{max} the maximum velocity of the enzyme reaction for gelatinase. K_m, the substrate concentration at which reaction velocity is half maximum and V_{max} the maximum velocity of the enzyme reaction, were determined by conducting enzyme assay using different concentrations of gelatin. From the results obtained (Fig. 4.28) it was found that K_m of gelatinase was 21.38 mg/mL and V_{max} was recorded as 55.55 U/mL.

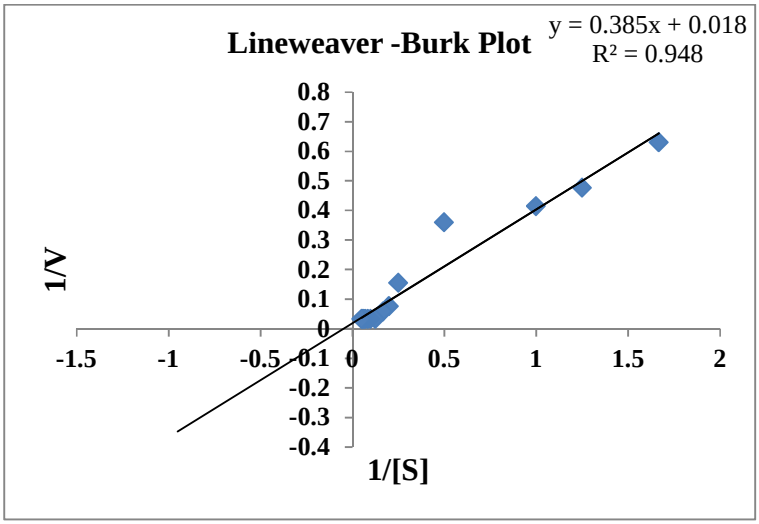


Fig.4.28.Lineweaver- Burk Plot

4.7.8. Effect of Various Metal ions on Gelatinase Activity

The effect of $CaCl_2$, $MgSO_4$, $ZnSO_4$, $CuSO_4$, $FeSO_4$, $MnCl_2$, $NiSO_4$, $CoCl_2$, $BaCl_2$, $CdCl_2$ and $Al_2(SO_4)_3$, which contributed the metal ions, Ca^{2+}, Mg^{2+} Zn^{2+}, Cu^{2+}, Fe^{2+}, Mn^{2+}, Ni^{2+},Co^{2+},Ba^{2+} Cd^{2+}andAl^{3+} respectively on gelatinase activity was studied by measuring the residual activities after incubating the enzyme along with different concentrations of metal ions for 30 m.Results obtained are presented in table 4.5. In general $ZnSO_4$ (149%) $CuSO_4$ (145%), $FeSO_4$ (115%) and$MnCl_2$ (130%) wereobserved to enhance residual enzyme activities to levels above their original activities (100%) at 1 mM. Whereas at other concentrations of these metals except $ZnSO_4$ (149% at 5 mM) recorded reduced levels of residual activities indicating inhibition at concentration of 5 mM and above. All other metals showed marked inhibition of enzyme activities indicated by reduced levels of residual activities at all the concentrations of the metals tested.$CaCl_2$, $MgSO_4$,$BaCl_2$, $CdCl_2$ and $Al_2(SO_4)_3$ showed marked inhibition of enzyme activities when compared to other metals at all concentrations except $Al_2(SO_4)_3$ which showed 66% residual activity at 1mM. Interestingly, $MnCl_2$ was observed to have relatively reduced effect on inhibition of gelatinase at all concentrations tested since up to 72% residual activity was recorded even at the maximum concentration of 30mM. It must be also noted that the reduction in residual enzyme activity levels gradually declined from 97% at 5mM to 85% at 10mM and 20 mM. Further it was also noted that $NiSO_4$, did not inhibit enzyme

activity drastically since the residual enzyme activities were 99% at 1 mM and 5mM, 84% at 10mM and 55% at 20mM. In the case of $CoCl_2$, it was noted that $CoCl_2$ did not inhibit enzyme activity at 1 mM while almost complete inhibition was observed at higher concentrations.

Table.4.5. Effect of metal ions on gelatinase activity

Metal ions	**Concentration of metal ions used (mM)**				
	1	**5**	**10**	**20**	**30**
$CaCl_2$	47.0 ± 11.38	40.33 ± 6.57	37.33 ± 2.48	37.66 ± 2.48	37.66 ± 2.48
$MgSO_4$	26.0 ± 8.60	16.33 ± 2.48	16.33 ± 2.48	16.33 ± 2.48	16.33 ± 2.48
$ZnSO_4$	149.33 ± 8.95	149.33 ± 8.95	0.93 ± 0.24	0.93 ± 0.24	0.93 ± 0.24
$CuSO_4$	145.0 ± 4.30	0.96 ± 0.24	0.96 ± 0.24	0.96 ± 0.24	0.93 ± 0.24
$FeSO_4$	115.0 ±4.3	86.0 ± 8.60	0.96 ± 0.24	0.96 ± 0.24	0.96 ± 0.24
$MnCl_2$	130.33 ± 8.95	97.66 ± 6.57	85.66 ±6.57	85.33 ± 4.96	72.33 ± 8.95
$NiSO_4$	99.0 ± 4.30	98.0 ± 4.30	84.33 ± 8.95	55.66 ± 6.57	37.66 ± 4.96
$CoCl_2$	98.0 ±11.38	56.33 ± 6.57	37.66 ± 4.96	37.66 ± 4.96	23.0 ±4.30
$BaCl_2$	36.66 ± 6.57	36.66 ± 6.57	31.0 ± 4.30	20.66 ± 2.48	20.66 ± 2.48
$CdCl_2$	37.66 ± 4.96	37.66 ± 4.96	37.66 ± 4.96	37.66 ± 4.96	36.33 ± 4.96
$Al_2(SO_4)_3$	66.66 ± 8.95	0.93 ± 0.24	0.93 ± 0.24	0.93 ± 0.24	0.93 ± 0.24

95% confidence level for residual activity

4.7.9. Effect of Various Detergents on Gelatinase Activity

The effect of various detergents CTAB (cetyltrimethylammonium bromide), Sodium Lauryl Sulphate, Triton X 100, Tween-20 and Tween-80 on enzyme activity was evaluated by determining the residual enzyme activity after incubation of the enzyme with different concentrations of detergents. Results presented intable 4.6., indicated that all the detergents showed considerable level of inhibition of enzyme activity at higher concentrations above 1%, except sodium lauryl sulphate which remained stable up to 1% without loss of activity. In the case of Tween- 20, there was a decrease in activity with increasing concentration of detergent from 0.2% onwards with almost no activity at a concentration of 5%.

Table. 4.6. Effect of detergents on gelatinase activity

Detergents	**Residual activity of gelatinase at different concentrations of detergents**					
	0.2%	**0.4%**	**0.6%**	**0.8%**	**1%**	**5%**
Tween 20	66.66 ± 6.57	65.33 ± 2.48	53.33 ± 2.48	52 ± 7.45	28 ± 4.30	0.96 ±0.24
Triton X 100	98.66 ± 6.57	99.66 ± 2.48	99 ± 4.3	65.33 ± 2.48	56.33 ± 4.96	14 ± 4.30
Tween 80	98 ± 11.38	98.66 ± 6.57	51.33 ± 6.57	44 ± 4.30	42.66 ± 2.48	42 ± 4.30
CTAB	98.33 ± 6.57	97.66 ± 8.95	99.66 ± 2.48	97.33 ± 9.93	51.66 ± 9.93	0.96 ± 0.24
Sodium Lauryl sulphate	99.66 ± 2.48	99 ± 4.30	98.66 ± 6.57	98 ± 8.60	97 ± 7.45	65 ± 8.60

95% confidence level for residual activity

4.7.10. Effect of Various Concentrations of NaCl on Gelatinase Activity

Results obtained for the study on the effect of various concentrations of NaCl on gelatinase activity determined using 0%, 5%, 10%, 15%, 20% and 25% NaCl are

presented in Fig. 4.29. Gelatin plates with concentration of NaCl above 25% could not be prepared as NaCl crystalizes on solidification. From the data, it was found that NaCl concentration had a profound effect on enzyme activity. At 15% NaCl, the enzyme showed maximum activity which declined on increase of salt concentration. At concentrations below 10%, there was no enzyme activity.

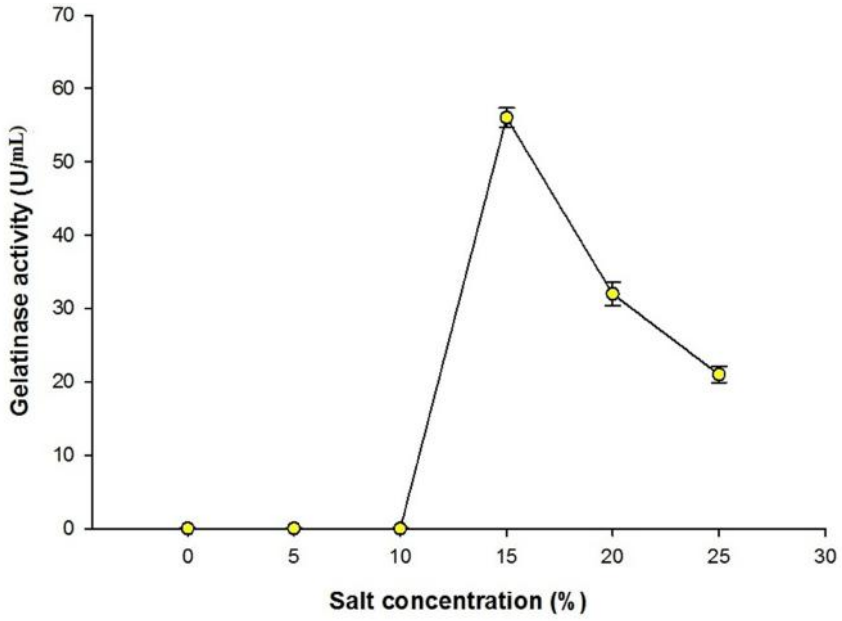


Fig.4.29. Effect of different concentrations of NaCl on gelatinase activity

4.7.11. Effect of Organic Solvents on Gelatinase Activity

Effect of organic solvents on gelatinase activity was evaluated by incubating the enzyme with the organic solvents such as acetone, ethanol, methanol, dimethyl sulphoxide, diethyl ether, acetonitrile and isopropanol at concentrations of 1%, 2%, 5%, 10% and 20% (v/v) for 30 min and the residual activities were assayed. In general, it was observed that the gelatinase was inhibited by all the organic solvents although the percent level of inhibition varied for the solvents. It may be noted from the results presented in table 4.7.that among the different solvents only ethanol registered relatively higher levelof residual activities (65%) at a concentration of 1%-2% indicated lesser levels of inhibition. Further increase in concentration of ethanol resulted in more than 50% enzyme inhibition since there was a decline in residual activities (43%) and a total inhibition was recorded at 20%. All the other organic solvents showed more than 50% enzyme inhibition since they recorded residual

activities varying from 20% (acetonitrile and isopropylalcohol) to 43% (diethyl ether) 44% (acetone) 45% (methanol) even at 1% of organic solvent in the reaction mixture. Isopropylalcohol (20-12%) and DMSO (29-17%) recorded lesser levels of residual activities indicating maximal enzyme inhibition for the various concentration tested when compared to other solvents (table 4.7.). It was also observed that at a concentration of 20%, only 1% residual activity of the enzyme was recorded in the case of methanol, ethanol and diethyl ether indicating almost complete enzyme inhibition.

Table. 4.7.Effect of organic solvents on gelatinase activity

Solvents	Concentration of organic solvents used				
	1%	**2%**	**5%**	**10%**	**20%**
Acetone	44.66 ± 6.57	43.33 ± 2.48	43.0 ± 4.30	42. 0 ± 4.30	19.66 ± 6.57
Ethanol	66.33 ± 6.57	65.33 ± 2.48	43.33 ±2.48	42.0 ± 4.30	1.03 ± 0.65
Methanol	45.0 ± 8.60	19.66 ± 6.57	17.66 ± 6.57	11.0 ± 4.30	0.96 ± 0.24
DMSO	29.0 ± 4.30	26.66 ± 6.57	19.66 ± 6.57	18.33 ±2.48	17.66 ± 2.48
Diethyl ether	43.66 ±13.14	28.66 ± 4.96	28.33 ± 2.48	16.66 ±6.57	1.03 ± 1.08
Acetonitrile	20.66 ±10.82	18.0 ± 4.30	13.33 ± 6.57	13.0 ± 4.30	11.66 ± 2.48
Isopropylalcohol	20.66 ± 6.57	20.33 ± 4.96	11.33 ±2.48	11.66 ±2.48	12.0 ±4.30

95% of confidence interval for residual activity

4.8. Application Studies

4.8.1. Decomposition of Gelatin Layer on X-ray film

Potential of gelatinase enzyme purified from *Natrinema* sp.BTSH10 to degrade the protein layer of the X-ray film for recovery of silver was studied by incubating the enzyme (12U) with 2g of X-ray film. Results presented in Fig. 4.33

indicated, when compared to the control without enzyme where the protein content of the supernatant was only 0.425 mg/mL (Fig. 4.30.), there was considerable level of degradation of protein on the protein layer of X-ray film since the supernatant estimated after incubation with gelatinase enzyme recorded 8.9 mg of protein per mL (Fig. 4.31).Similarly incubation with Proteinase K yielded 11 mg/mL protein in the supernatant (Fig. 4.32). Further visual examination of the X-ray film also indicated that the enzyme could effectively decompose the gelatin layer.

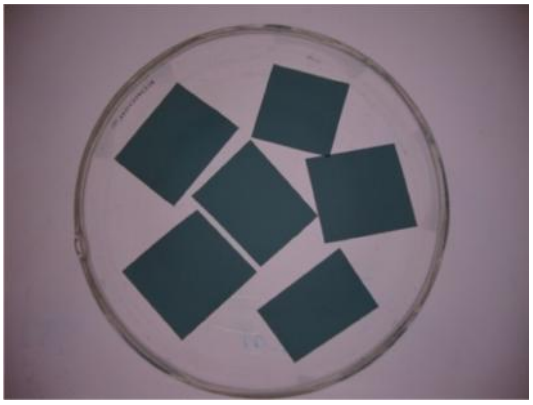


Fig. 4.30. Untreated X-ray film

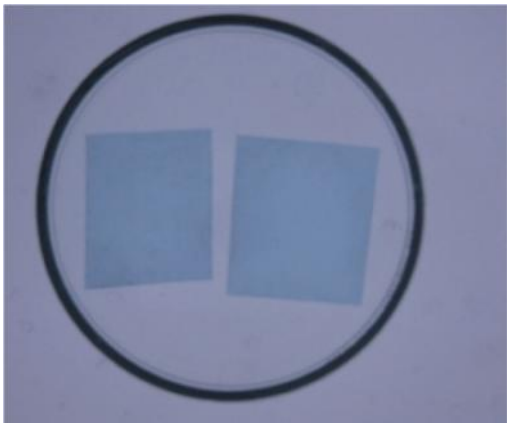


Fig. 4.31. X-ray film treated with gelatinase from *Natrinema* sp. BTSH10

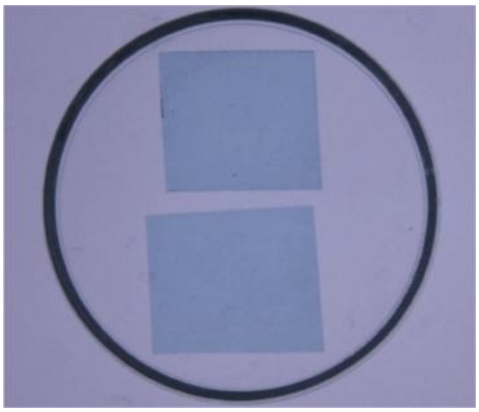


Fig. 4.32. X-ray film treated with Proteinase K

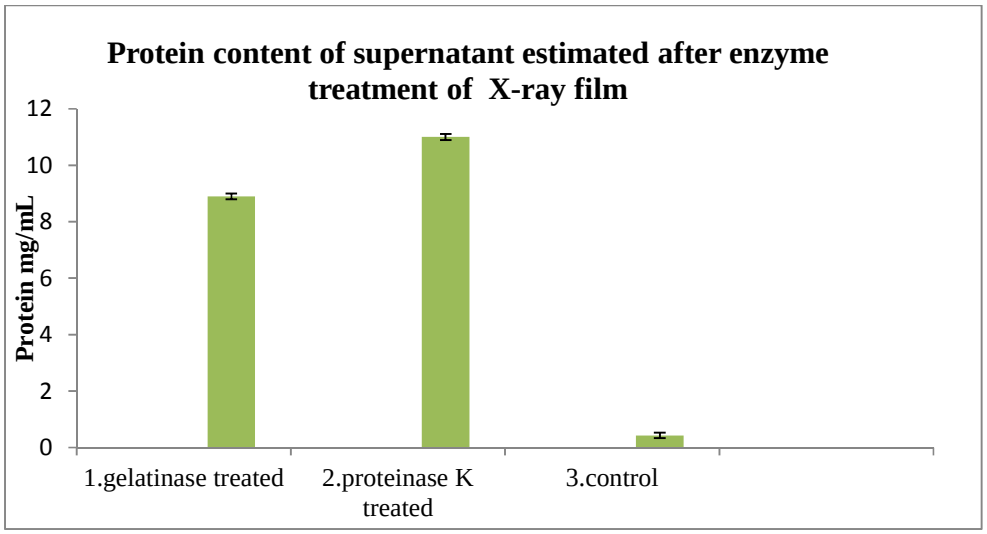


Fig. 4.33. Protein content of supernatant after enzyme treatment on X-ray film.

Chapter 5

DISCUSSION

Halophilic bacteria associated with tannery effluent and commercially available food grade table salt crystals were isolated using Zobell's medium employing standard plating procedures. Interestingly, all the isolates obtained from tannery effluent were found to be Gram positive while the isolates obtained from food grade table salt crystals were Gram negative bacilli. From the results of the present study, it was inferred that only species of *Oceanobacillus, Staphylococcus and Salimicrobium* were associated with tannery effluent while food grade table salt crystals harbored species of *Halomonas* and *Chromohalobacter salexigens* which are Gram negative. Further, it was observed that *Halomonas elongata* was dominant among the bacteria associated with the food grade table salt crystals. It was also observed that both the samples of tannery effluent and food grade table salt crystals harbored very different species of halophilic bacterial flora indicating that the source of sample influenced species diversity. This is evidently seen in the phylogenetic tree which clearly reflects the fact that the bacteria isolated from tannery effluent and those isolated from food grade table salt crystals are seen in different clusters. Similar organisms are paired on the same branch. *Halomonas elongata* and *Chromohalobacter salexigens* isolated from salt crystals are seen in two branches.

In the present study, *Halomonas* sp. produced amylase, lipase and protease and *Chromohalobacter* sp. produced lipase. *Staphylococcus arlettae* isolated from tannery effluent could elaborate coagulase, an enzyme which is usually seen in pathogenic species of *Staphylococci*. *Salimicrobium* sp. isolated from tannery effluent could not produce lipase. *Oceanobacillus* sp. isolated from tannery effluent produced large quantities of lipase and protease. All the bacteria isolated from food grade table salts were also observed to produce lipase, amylase and protease, albeit, in small amounts. It was reported that almost 10% of moderately halophilic bacteria isolated from solar salterns and saline soils of Isla Cristina (Spain) secrete proteases; while other isolates secrete enzymes like lipase amylase etc. (Sanchez –Porro *et al*., 2003a). Birbir *et al*. (2004) isolated extremely halophilic archaebacteria from Tuzkoy Salt Mine, Turkey which included species belonging to *Haloarcula*, *Halobacterium*, *Natrinema* and

Halorubrum. Most of them produced enzymes capable of degrading macromolecules including casein, gelatin, starch, cellulose etc. They observed that since the salt is used for preservation of hides and food, the presence of halophilic archaeal community capable of elaborating hydrolytic enzymes in the salt, could lead to the deterioration of the salted products.

In the present study, during screening and selection of potential haloarchaebacteria for gelatinase production it was found that *Natrinema* sp. BTSH10 produced gelatinase in maximal quantities compared to *Halorubrum* sp.BTSH03 and all other isolates obtained from tannery effluent and table salt crystals. Hence, this *Natrinema* sp. BTSH10 was selected for further studies. Further, it was also noted that this strain was observed to grow well in Zobell's agar medium supplemented with 15% NaCl. However, it was observed to be slow growing and took a long time to acclimatize to the medium since it remained in the lag phase upto 40 h. Nevertheless the archaea showed an active progressive log phase during the period 60-100 h after which it entered the stationary phase and remained in the same phase until 170 h. Karthikeyan *et al*. (2013) during the time course study on production of halocin SH10 by *Natrinema* sp. BTSH10 observed that it remained in lag phase up to 40h and recorded logarithmic phase only during 60-100 h during which maximal halocin was secreted.

In the present study, the enzyme showed optimum activity at 15% NaCl and no activity at 10% NaCl; indicating that at higher concentrations of NaCl, probably, the configuration of casein was affected leading to its non availability to the enzyme. Even if small amounts of tyrosine were produced on hydrolysis of casein, it was probably masked due to the presence of high concentration of salt. Hence, liquid assay using casein employing spectrophotometric method could not be standardized. This led to the choice of the specific protease – gelatinase which could be assayed as described by Kanemitsu *et al.*(2001). After adding gelatinase to the gelatin plates and incubation, 10% TCA was added. The zone was visible even when gelatin plates containing 15% NaCl was used. In this context, it may also be noted that the caseinolytic activity of halophilic proteases decreased beyond 2 M NaCl concentration because casein loses its original conformation at higher NaCl concentrations limiting the availability of substrate to the enzyme (Capiralla *et al*., 2002).

5.1.Gelatinase Production

Five different media were evaluated to select the optimal medium that supported maximum gelatinase production by the selected potential haloarchaeal strain *Natrinema* sp. BTSH10, as described under materials and methods section. Among the different media tested, modified Medium 1 (Akolkar, 2009) was found to support maximal production of gelatinase enzyme compared to other media. Probably the suitability of modified Medium 1 (Akolkar, 2009) could be attributed to the composition of the medium with respect to 25% of NaCl and presence of sodium citrate compared to the presence of trisoidum citrate and yeast extract in other media. Of course, no conclusive reasons could be ascribed since optimal requirement of various media ingredients by *Natrinema* sp. BTSH 10 could not be studied in detail towards a comparative evaluation of various media. Further, it was out of scope of the present study.

Various bioprocess variables that influence maximal production of gelatinase by *Natrinema* sp. BTSH 10 were studied and very interesting results were obtained. In fact, halophiles have a requirement for high salt concentration for growth and also enzyme production. From the data obtained in the present study, it was found that this archaea required 25% for effecting maximal gelatinase production compared to the levels of NaCl tested. Nevertheless 20% NaCl concentration also supported considerable level of gelatinase production compared to other concentrations of NaCl. Further, it was observed that specific activities of gelatinase also showed exactly the same trend which was observed for the enzyme activities with respect to different concentrations of NaCl. Even during the course of optimization of medium for gelatinase production, it was observed that medium containing 25% NaCl alone supported maximal gelatinase. The results evidently confirmed the halophilic nature of *Natrinema* sp BTSH 10 and hence, there is requirement for high concentration of NaCl. In a similar study reported by Lama *et al*. (2005) it was reported that 12% NaCl was optimum for protease production by *Salinivibrio*.

Initial pH of the medium often influences the course of fermentation and rate of enzyme production, since pH of the culture media drastically affects the conformation of the plasma membrane, and consequently affects the membrane bound ribosomes involved in protein synthesis (Razak *et al*., 1997). Hence, optimal pH requirement for maximal enzyme production by microorganism is usually determined before finalization of enzyme production medium. Thus, in the

present study optimal pH requirement for gelatinase production by *Natrinema* sp.BTSH 10 was studied. From the results, it was inferred that pH 7.0 is the optimal requirement for *Natrinema* sp. BTSH10 to record maximum gelatinase production. Further, it was observed that very meagre gelatinase activities were supported by initial pH 2-5 and pH levels above pH 9 did not support gelatinase production. In fact, neutral pH supported considerable levels of gelatinase production by *Natrinema* sp. BTSH 10 compared to alkaline pH conditions. It was also noted that specific activities of gelatinase also showed exactly the same trend which was observed for the enzyme activity with respect to different levels of initial pH in the medium. The results of the present study are in agreement with protease production reported for *Halobacterium* sp .Js1(VijayAnand *et al*., 2010). Whereas, the same *Natrinema* sp. BTSH 10 was reported to record enhanced growth and maximal production of halocin, at pH 8.0 compared to other pH levels (Karthikeyan *et al.*,2013).

Incubation temperature significantly affects the properties of the cell membrane and thereby influences the secretion of extracellular enzymes since temperature probably controls enzyme synthesis at transcriptional and translational levels (Votruba *et al*., 1991). Further, the incubation temperature also profoundly affects the duration of enzyme synthesis phase and the enzyme yield (Ramesh and Lonsane, 1987; Ray *et al*., 1992).Hence, the incubation temperature for maximal gelatinase production by *Natrinema* sp. BTSH 10 was evaluated. From the data obtained, it was found that this archaea preferred 42°C for recording maximal gelatinase compared to that obtained with 37°C which was commonly used for cultivation. Further, it was observed that increase in incubation temperatures above 42°C and incubation at temperatures below 37°C resulted in reduced levels of gelatinase production. Profile of specific activities of gelatinase also demonstrated similar pattern which was observed for the enzyme activities with respect to different incubation temperatures. Earlier, an optimum temperature of 40°C was reported for protease production by *Chromohalobacter* sp. TVSP101 (Vidyasagar *et al*., 2007) while 37°C was noted for protease production by *Halobacterium* sp. SP1 (1) (Akolkar, 2009). Optimal requirement for incubation temperature may be species specific and hence may vary for different organisms.The same organism was reported to record enhanced growth and maximal production of halocin, at 42°C compared to other temperatures (Karthikeyan *et al*., 2013).

Increase in inoculum size may adversely affect production of enzyme as it causes nutrient depletion and lack of oxygen in the culture media (Rahman *et al*., 2005). Hence, in the present study optimal requirements for inoculum concentration and age of inocula were determined. From the results obtained, it was noted that 2% inoculum concentration was optimum for *Natrinema* sp. BTSH 10 since maximal enzyme yield was obtained compared to other concentrations tested. It was noted that concentrations above 2% led to a decline in the gelatinase production while 1% inoculum did not support enhanced level of enzyme production. Further, it was found that inoculation of enzyme production media with inoculum age of 36 h supported maximal gelatinase yield compared to decreased levels of gelatinase recorded with young age of inocula. Also, it was noted that inoculum with age above 36 h did not support enhanced levels of gelatinase production. Sehar and Hameed (2011) reported that maximum extracellular alkaline protease was produced by *Bacillus* sp. with 24 h incubation and 10% inoculum concentration. In the present study, a reduced level of inoculum concentration of 2% gave best results.

At optimal agitation, increase in aeration of media caused increased nutrient uptake which probably led to increased protease production (Beg *et al*., 2003b). At low rpm rates, lesser availability of dissolved oxygen and low mixing rates cause decrease in protease production (Potumarthi *et al*., 2007). Higher agitation rates of 200 rpm or more could lead to denaturation of enzymes and hence cause fall in production (Burkert *et al*., 2005; Shafee *et al*., 2005). It was reported that 150 rpm was optimum for protease production by *Bacillus* sp. isolated from Khewra salt range, Pakistan (Sehar and Hameed, 2011). Hence, in the present study optimal agitation rate for maximal gelatinase production was studied. From the data obtained, it was inferred that rate of agitation influenced the rate of enzyme production by *Natrinema* sp. BTSH 10 during incubation. Thus, under static conditions, the enzyme production was only 2 U/mL which increased to a maximum of 75 U/mL when the agitation rate was increased to 150 rpm. However, further increase in the rate of agitation rates, did not enhance level of enzyme production and instead led to a decline in the rate of gelatinase production. It was also observed that specific activities of gelatinase also showed exactly the same trend which was observed for the enzyme activity with respect to different rates of agitation. The same organism was however reported to record enhanced growth and maximal production of halocin, at 200 and 250 rpm compared to lesser agitation rates (Karthikeyan *et al.*, 2013).

Often additional carbon sources other than the substrate are required for maximal production of any metabolite or enzyme. Hence, requirement for additional carbon sources by *Natrinema* sp. BTSH 10 for gelatinase production was studied using several carbon sources. From the results obtained it was observed that 0.1M sorbitol was the preferred additional source of carbon in the medium for maximal gelatinase production compared to other carbon sources tested. Further, it was noted that dextrose, maltose, fructose and lactose did not favour gelatinase production. Next to sorbitol, mannitol recorded considerable level of gelatinase production, followed by mannose, cellobiose, sucrose and galactose. It was also observed that the profile of specific activities of gelatinase also showed exactly the same trend with respect to different carbon sources. Preference of carbon sources by any microorganism is species specific and may vary for the organisms as well as the product of interest. Thus, *Natrinema* sp. BTSH10 was reported to prefer galactose followed by sorbitol maltose, glycerol and glucose for halocin SH10 production (Karthikeyan *et al*., 2013).

Nitrogen sources, inorganic or organic, in the medium significantly influence the cellular biosynthesis and secretion of metabolites and enzymes into the medium. Hence, optimal requirements of additional nitrogen sources in the enzyme production medium were evaluated using different inorganic nitrogen and organic nitrogen sources. In the present study, from the results obtained it was inferred that addition of 0.1M ammonium sulphate to the medium enhanced maximal gelatinase production compared to all other inorganic nitrogen sources tested. Whereas sodium nitrate, ammonium nitrate and ammonium ferrous sulphate resulted in reduced levels of gelatinase production. Interestingly, it was observed that addition of ammonium dihydrogen phosphate, ammonium hydrogen carbonate, ammonium oxalate and diammonium hydrogen phosphate resulted in inhibition of gelatinase production. Whereas addition of ammonium to the medium was earlier reported to decrease enzyme activity, in the case of *Pseudoalteromonas* sp. strain CP76 (Sánchez-Porro *et al.*, 2003b) and *Aeromonas hydrophila* (O'Reilly and Day, 1983). Further, it was also reported that inorganic nitrogen sources like urea, $NaNO_3$, NH_4Cl, and $(NH_4)_2SO_4$ did not support enzyme production in *Halobacterium* sp. (VijayAnand *et al.*, 2010). This observation with respect to *Natrinema* sp. BTSH10 may be attributed to the preferential requirement of inorganic nitrogen sources by microorganism which could be

species specific and may vary for the organisms as well as with the product of interest similar to that of carbon sources.

Protease production is usually constitutive and in some cases, it may be inducible (Kalisz, 1988). Proteases are maximally produced during the stationary phase and production is regulated by nitrogen and carbon stress (Hölker *et al.*, 2004). Incorporation of gelatin in medium increased protease production by *Salinivibrio* (Lama *et al*., 2005). In the case of *Natrialba magadii* (D'Alessandro *et al*., 2007) and *Bacillus* sp. (Patel *et al*., 2005) yeast extract and gelatin increased protease production, respectively. Addition of casein was also reported to promote protease production by *Bacillus clausii* under solid state fermentation using rice bran (Sumantha *et al*., 2006). Protease production by *Halobacterium* sp. SP1 (1) was highest in presence of protein rich soybean flour (Akolkar, 2009). Maximum proteolytic activity by *Halobacterium* sp. Js1was obtained when medium containing combination of skim milk powder (1%) and peptone (1%) were used. While poor protease secretion was noted when medium contained gelatin, corn steep, liquor, beef extract and yeast extract (VijayAnand *et al*., 2010). Whereas, in the present study, skim milk powder followed by soy bean meal were noted as good organic nitrogen sources for gelatinase production. It was inferred from the results that addition of skim milk powder to the medium enhanced maximal gelatinase production compared to all other organic nitrogen sources. Soya bean meal followed by peptone, soya casein digest and beef extract also recorded gelatinase production. However, yeast extract did not support gelatinase production. Further, it was noted that specific activities of gelatinase also showed similar trend as that observed for the enzyme activity with respect to different organic nitrogen sources.

Surfactants are known to affect cell membrane permeability leading to increased secretion of extracellular enzymes (Helander and Mattila-Sandholm, 2000). Protease production by *Halobacterium* sp. SP1 (1) was increased in presence of dicotylsulfosuccinate, whereas, Triton X-100, Tween-80 and Tween-20 did not affect the production (Akolkar, 2009). These results contradict earlier reports on extracellular protease production by *Bacillus cereus* and *Rhizopus oryzae* which was enhanced in the presence of Triton X-100 and Tween-80 (Esakkiraj *et al*., 2009). In the present study, it was inferred that Tween- 80 could be used to get maximal enzyme yield from the fermented medium compared to Tween-20 and TritonX-100 which showed meagre levels of enzyme production by

Natrinema sp. BTSH10. CTAB had an inhibitory effect on growth and protease production by *Halobacterium* sp. SP1 (1) and the enzymatic activity of the protease was reduced in presence of CTAB and SDS (Akolkar, 2009). Presence of CTAB caused an appreciable increase in activity of serine protease from *Halogeometricum borinquense* strain TSS101 (Vidyasagar *et al*., 2006). Whereas, in the present study, it was found that addition of CTAB, sodium taurocholate and sodium lauryl sulphate to the production media resulted in inhibition of gelatinase production by the archaea. It was also observed that for the different detergents used, specific activities of gelatinase recorded levels corresponding to the increase in enzyme activity.

A Time course experiment was conducted after optimizing all the bioprocess variables for obtaining maximal gelatinase production by *Natrinema* sp.BTSH10 during submerged fermentation over a period of 168 h under optimized conditions. From the results, it was evident that gelatinase production commenced after 48 h growth and reached a maximum after 120 h (229.4 U/mL). It is assumed that maximal gelatinase production has taken place during late logarithmic phase of the growth curve compared to mid log phase. Further, it was also noted that during incubation beyond 120 h the enzyme yield decreased progressively along with increase in incubation time.

5.2.Enzyme Characteristics

Strong acidic and alkaline conditions change the surface structure of enzymes, which has an impact on the interactions between active site and substrate. Thus, enzyme activity may be lost due to enzyme denaturation. Gelatinase produced by *Natrinema* sp. BTSH10 was active at pH 8 with sharp decline in activity at pH levels both above and below pH 8.0. Further, the gelatinase was observed to be stable in the pH range 7-11 although further increase in pH above pH 11 led to loss of activity. It was also observed that in acidic pH conditions up to pH 6, there was no activity. The optimum pH for a protease isolated from *Salinivibrio* was also reported as pH 8 (Lama *et al*., 2005). The trypsin–like serine protease isolated from from *Haloferax lucentensis* VKMM007 also showed optimum activity at pH 8 (Manikandan *et al*., 2009). The optimum activity of an extracellular serine protease from *Halobacterium halobium* S9 was observed at pH 8.7 (Capiralla *et al*., 2002). Results very clearly indicated that there was no residual enzyme activity at pH 2 to 5, indicating that the

gelatinase enzyme was not stable under acidic conditions when compared to alkaline pH conditions (pH 9 to 13).

In the present study, results obtained indicated that the optimum temperature required for maximal enzyme activity by gelatinase was 37°C and temperatures above 42°C and below 27°C led to a steep decline in activity. However, moderate levels of enzyme activities, almost less than 50% of the level recorded at 37°C could be observed at 32°C and 42°C. Similar observations were reported earlier in literature. Optimum activity of an extracellular serine protease from *Halobacterium halobium* S9 was observed at 40°C (Capiralla *et al*., 2002). Proteases from *Halobacterium halobium* ((Izotova *et al*., 1983) and *Bacillus* sp. (Patel *et al*., 2006) were reported to have an optimum temperature of 37°C. Moreover from the present study, it was inferred that the gelatinase enzyme was stable without losing activity (about 99% residual activity) at 40°C upto 4 h and upto 30 min at 20°C, 30°C, 50°C and 60°C. Extended incubation at higher temperatures resulted in loss of activity and at 70°C and 80°C there was no residual activity indicating loss of enzyme activity.

Impact of EDTA on enzyme is often studied since it is generally considered as an enzyme inhibitor and EDTA inhibition of enzyme activity is assumed to be the characteristic feature of metalloprotease. From the results obtained for the experiments conducted with various inhibitors, it was found that the enzyme showed marked decline in enzyme activities along with increase in the concentration of the enzyme inhibitors and maximal inhibition was effected by EDTA. Even at the lowest concentration of 10 mM EDTA, marked inhibition of the gelatinase was observed. It was reported earlier that serine proteases can be inhibited by metal chelating agents like EDTA (Izotova *et al*., 1983) and protease from *Salinivibrio* sp., which had an optimum temperature of 65ºC and optimum pH of 8.5, was reported to be inhibited by EDTA(Karbalaei-Heidari *et al*., 2007). Similarly protease isolated from *Halobacillus karajensis* was also inhibited to 80% by 1mM EDTA (Karbalaei-Heidari *et al*., 2009).

It was observed, in the present study, that the best substrate for gelatinase activity was skim milk and the second best substrate was gelatin. Use of BSA and haemoglobin as substrates did not yield appreciable activity. In fact, according to Grebeshova *et al*. (1999) protease potency is indicated by ability to hydrolyse several substrates and alkaline proteases were reported to show highest activity

towards casein, compared to other proteins (Kumar *et al*., 1999b). It may be noted that skim milk powder contains casein and thus it has served as an ideal substrate for gelatinase produced by *Natrinema* sp. BTSH10 and the present results are in agreement with earlier reports.

In the present study, the presence of $ZnSO_4$, $CuSO_4$, $FeSO_4$ and $MnCl_2$ were observed to enhance residual enzyme activities of gelatinase to levels above their original activities (control values) at 1 mM while at other concentrations, except $ZnSO_4$ at 5 mM, reduced levels of residual activities were recorded indicating inhibition at concentration of 5 mM and above. $CaCl_2$, $MgSO_4$, $BaCl_2$, $CdCl_2$,and $Al_2(SO_4)_3$were observed to show marked inhibition of enzyme activities when compared to other metals at all concentrations except $Al_2(SO_4)_3$ which showed 66% residual activity at 1mM. Present results contradict many of the earlier reports on protease. Presence of 5mM concentrations of Ca^{2+} and Mn^{2+} was reported to cause an increase in activity of serine protease of *Bacillus subtilis* PE 11 (Adinarayana *et al*., 2003). Fe $^{2+}$ and Mn^{2+} were shown to increase the protease activity in *Bacillus mojavensis* (Beg *et al*., 2003a). Zn^{2+} ions at high concentrations were reported to inhibit metalloproteases (Teo *et al*., 2003) by forming zinc monohydroxide which bridges the catalytical zinc ion to the side chain of the active site of the enzyme (Larsen and Auld, 1991). Serine protease was reported to show increased activity in presence of Ba^{2+} and Ca^{2+}, whereas, Zn^{2+}, Hg^{2+}, Co^{2+} inactivated the enzyme (Sana *et al*., 2006). At 1 and 5 mM concentrations, Ni^{2+} could inhibit the enzyme isolated from *Salinivibrio* sp. (Karbalaei-Heidari *et al*., 2007). Presence of metal ions $Ca^{2+,}$ K^{+}, $Mg^{2+}$was observed to enhance protease enzyme activity while Zn^{2+}, Mn^{2+} and Cu^{2+} inhibited activity (Manikandan *et al*., 2009). Protease production was increased in presence of Ca^{2+} and Mg^{2+} ions and decreased in presence of Cu^{2+} and Zn^{2+} ions (Sehar and Hameed, 2011).

According to Wyman and Gill (1990), metals bind to the active site of the enzyme, thereby imparting stability to the protein. James *et al*. (1991) reported that binding of Ca^{2+} ions prevent unfolding of protease at higher temperatures. Ca^{2+} strengthens the interactions inside the molecule by binding to the autolytic sites and inner surface of protease (Ghorbel *et al*., 2003). Cu^{2+} ions lead to denaturing of protease enzyme (Demina and Lysenko, 1995). The differential observation made with *Natrinema* sp. BTSH10 with respect to effect of different metal ions on gelatinase may be species specific, particularly for haloarchaea. No

conclusive inference could be made on probable reason on this varied response and further specific experiments on the effect of metals on gelatinase may throw more light on this aspect.

In the present study, the enzyme showed maximum activity at 15% NaCl which decreased on increasing the concentration. At concentrations lower than 15%, there was a decline in enzyme activity. Gelatin plates with concentration of NaCl above 25% could not be prepared as NaCl crystalises on solidification. From the data obtained, it was found that NaCl concentration had a profound effect on enzyme activity. The protease secreted by *Haloferax mediterranei* was completely inactivated at low salt concentrations (Nolasco *et al*., 2002) and the denaturation was irreversible as was seen in the case of other halophilic proteins (Kamekura and Seno, 1990). In fact, extremely halophilic bacteria which have optimal salt concentration of 20% have been reported to have enzymes which are inhibited by salt concentrations greater than 3% (Louis and Fitt, 1971). Collagenolytic activities of halophiles isolated from cured hides were reported to be inhibited at 7% NaCl, probably due to inactivation of enzyme by salt or due to inability to produce the enzyme at that salt concentration (Thomson *et al.,* 1972). A halothermophilic serine protease purified from *Chromohalobacter* sp. isolated from solar saltern samples was reported to retain 100% stability in the absence of NaCl (Vidyasagar *et al*., 2009). In the present study, *Natrinema* sp. BTSH10 isolated from similar saltern ponds however required NaCl above 15%, to have good growth and enzyme activity. These observations strongly indicate the true halophilic nature of *Natrinema* sp. BTSH10 and suggest gelatinase as a halozyme.

Enzyme inhibition at a range of concentrations of detergents may be attributed to changes in the tertiary structure due to reduction in hydrophobic interactions and also direct interactions with the protein molecule (Creighton, 1989). According to Bressollier *et al*. (1999), increased substrate accessibility may contribute to increased enzyme activity in the presence of detergents. In the present study the results obtained for the experiments on the effect of detergents on gelatinase activity indicated that all the detergents showed considerable level of inhibition of enzyme activity at higher concentrations above 1%, except sodium lauryl sulphate which remained stable up to 1% without loss of activity. In the case of Tween -20, there was a decrease in activity with increasing concentration of detergent from 0.2% onwards with almost no activity at a concentration of 5%.

In the case of CTAB, there was a decrease in activity at lower concentrations which was regained at 0.6% concentration, followed by decrease in activity.

Organic solvents have a stabilizing effect on protease due to the replacement of water molecules in enzyme with organic molecules stabilizing the enzyme structure (Frikha *et al*., 2005).Kim and Dordick (1997) reported an organic solvent tolerant halophilic protease from *Halobacterium halobium*. Kumar and Bhalla (2004) reported a thermostable protease from *Bacillus* sp. which showed stability in the presence of methanol, petroleum and ethanol. A solvent stable endopeptidase from a marine crab which showed increased activity in presence of 2-propanol was reported by Saborowski *et al*. (2004). In the present study it was observed that the gelatinase was inhibited by all the organic solvents tested although the percent level of inhibition varied for the solvents. Among the different solvents only ethanol registered relatively higher level of residual activities at a concentration of 1% to 2% indicating lesser levels of inhibition. Further increase in concentration of ethanol resulted in more than 50% enzyme inhibition. Based on the results it was inferred that gelatinase from *Natrinema* sp. BTSH10 is not tolerant to organic solvents, except to ethanol.

Akolkar (2009) studied the protease produced by *Halobacterium* sp. SP1(1) and showed that the K_m and V_{max} values as determined by Lineweaver–Burk plot for hydrolysis of casein in presence of 2M NaCl at 37°C and pH 7.2 were 0.262 mg/mL and 40.984 U/mL, respectively. In the presence of Na-glutamate, the values of K_m and V_{max} for casein hydrolysis at 37°C increased to 1.266 mg/mL and 106.4 U/mL, respectively. Whereas, in the present study,K_m for gelatinase from *Natrinema* sp. BTSH 10 was found to be 21.38 mg/mL and V_{max} was recorded as 55.55 U/mL. The variations in values of K_m and V_{max} observed with *Natrinema* sp. BTSH 10 could be species specific and enzyme specific and hence no comparisons could be made.

DeCastro *et al*. (2006) have suggested that haloarchaeal proteases are within the size range 41-66 kDa. The proteases produced by *Chromohalobacter* sp. TVSP101 (Vidyasagar *et al*., 2009) and *Halobacterium halobium* (Kim and Dordick, 1997) were reported to have a molecular mass of 66 kDa. Molecular mass of protease of *Natronococcus occultus* was reported to be 130 kDa and was the largest among the group (Studdert *et al*., 2001). Whereas in the present study

the gelatinase of *Natrinema* sp. BTSH10 was found to have a molecular mass of, about 19 kDa in size and is comprised of single polypeptide chain.

X-ray or photographic waste films contain 1.5 to 2.0% silver by weight in their gelatin layer, which can be used as a good source of silver for a variety of purposes, if appropriately recovered. Conventionally, silver is recovered by burning the used X-ray films, which causes undesirable environmental pollution. Enzymatic hydrolysis of gelatin not only helps in extracting silver, but also enables recycling and reuse of the polyester film base. Hence, in the present study hydrolytic activity of the gelatinase of *Natrinema* sp. BTSH 10 on the gelatin layer of the X-ray film was evaluated by incubating the X-ray film in enzyme solution. Results presented indicated, there was considerable level of degradation of protein on the protein layer of X-ray film compared to the X-ray film in the control (without enzyme) and was almost similar to that of Proteinase K indicating that the gelatinase of *Natrinema* sp. BTSH10 could effectively decompose the gelatin layer. Further, visual observation of the film conclusively testified the ability of the enzyme to decompose the gelatin layer of the X-ray film. These observations corroborate well with the reports made earlier for the alkaline proteases from *Bacillus* sp. B21-2 (Ishikawa *et al*., 1993), *Bacillus* sp. B18' (Fujiwara *et al.*, 1991), *B. subtilis* (Fujiwara *et al.*, 1989), and *B. coagulans* PB-77 (Gajju *et al.*, 1996) and fungus *Engyodontium album* (Chellappan, 2005) which were observed to be efficient in decomposing the gelatinous coating on used X-ray films from which the silver could be recovered. It is inferred that the gelatinase of *Natrinema* sp. BTSH10 has potential for application towards the recovery of silver and recycling and reuse of the films.

Chapter 6

SUMMARY AND CONCLUSIONS

Halophiles are potential source of extracellular hydrolases like proteases with a wide array of industrial applications. These enzymes exhibit stability over a range of saline conditions. 'Gelatinases' are those protease enzymes which cleave gelatin, casein, fibrinogen, etc. to result in polypeptides, peptides and amino acids. They are metalloendopeptidases which have applications in leather industry, production of fish sauce, fish processing, peptide synthesis etc. Literature available on halophilic proteases, particularly gelatinases is rather scanty, hence there is more scope in exploring halophiles as source for proteases. Hence, this study was planned to isolate gelatinase from haloarchaeal strain, purify and characterize the same.

Halophilic bacteria were isolated from samples of tannery effluent and food grade table salt crystals. Six isolates were obtained from tannery effluent and five isolates were obtained from food grade table salt crystals. The isolates associated with tannery effluent were identified as species of *Oceanobacillus*, *Staphylococcus and Salimicrobium* while species of *Halomonas* and *Chromohalobacter salexigens* were found to be present in food grade table salt crystals. It was also observed that *Halomonas elongata* was dominant among the bacteria associated with the food grade table salt crystals. All the eleven isolates obtained were evaluated for gelatinase production along with *Natrinema* sp. BTSH 10 and *Halorubrum* sp. BTSH 03 which were isolated from saltern ponds in an earlier study and available as stock cultures in the Department of Biotechnology, Cochin University of Science and Technology, Cochin. Among the strains tested, *Natrinema* sp. BTSH 10 was found to produce gelatinase in maximal quantities, and hence it was selected for further studies.

Among the various media evaluated, the medium suggested by Akolkar (2009) was observed to support maximum gelatinase production by *Natrinema* sp. BTSH10 when gelatin was used as substrate instead of soyabean. The optimal conditions of different bioprocess variables that supported maximal gelatinase production under submerged fermentation was determined employing 'one factor at a

time approach'. The optimized variable was incorporated in the subsequent experiments.

Maximal gelatinase production was recorded with 25% NaCl (22.4 U/mL), pH 7 (30.6 U/mL), incubation at 42°C (37 U/mL), 2% inoculum concentration (45 U/mL), 36 h of inoculum age (60 U/mL), agitation rate of 150 rpm (75 U/mL), sorbitol as additional carbon source (95 U/mL), addition of ammonium sulphate as additional inorganic nitrogen source (150 U/mL), and skim milk powder as additional organic nitrogen source (180 U/mL). Time course study conducted employing optimized conditions of different variables resulted in an enhanced level of gelatinase (229.4 U/mL).

The crude enzyme obtained as the culture free supernatant after submerged fermentation was purified by filtration, gel filtration, dialysis and HPLC. The purified enzyme fraction consisting of proteins between 10 - 30 kDa molecular mass showed gelatinase activity. During purification, it was noted that dialysis against buffer without salt resulted in complete loss of activity.

The purified gelatinase enzyme fraction, when subjected to reductive SDS PAGE, showed only a single band indicating that the enzyme had a single unit with a molecular mass of 19 kDa. Zymography also revealed a single band of clearance in the gel, which appeared as a clear zone in blue coloured gel.

The purified enzyme was characterized for its optimal activities in response to various physico-chemical variables. The optimal pH of the purified enzyme was found to be pH 8 although it showed considerable level of activity in the pH range 7 to 10. It was also found to be stable at pH levels in the pH range 6 to13. The optimum temperature for the enzyme activity was noted as 37°C.

EDTA which is a metallo-protease inhibitor was observed to effect marked inhibition of the gelatinase. Thus, only 20% residual activity was noted in the presence of 10 mM which declined to 3% in the presence of 50 mM EDTA.

Kinetic studies on gelatinase conducted using gelatin as the substrate indicated that the K_m for the gelatinase was 21.38 mg/mL, and V_{max} was 55.55 U/mL.

A marked increase in residual activity of gelatinase at lower concentrations of $ZnSO_4$ (1 mM and 5 mM) was observed. Similarly in the presence of $CuSO_4$ (1 mM) and $FeSO_4$ (1 mM) also there was an increase in residual activity which decreased along with the increase in the concentration of the metal ions.

The enzyme was found to show a maximum activity at 15% NaCl, which declined on further increase of NaCl concentration. Skim milk powder was observed as the ideal substrate for gelatinase activity, followed by gelatin.

In the presence of higher concentration of detergents, the gelatinase recorded a decline in residual activity. With Tween-20 (0.2%), the residual activity was recorded as 66%. Whereas in the presence of increasing concentration of solvents, there was a decline in residual activity with ethanol recording 65% residual activity at 2% concentration.

Application studies conducted with X-ray film indicated that the purified gelatinase enzyme could degrade the protein layer of the X-ray film, on incubation of the X-ray film in enzyme solution. Results indicated scope for application of this enzyme in removal of gelatin from X- ray film and recovery of silver.

Conclusions

The present study led to the recognition of *Natrinema* sp. BTSH 10 isolated from saltern ponds, as an ideal candidate species for production of gelatinase, which was noted as a halozyme capable of showing enzyme activity in the presence of 15% NaCl. Results obtained during the course of the present study indicated potential for application of this enzyme in industrial catalysis that are performed in the presence of high concentrations of salt. The enzyme characteristics noted with this gelatinase also indicate the scope for probable applications in leather industry, meat tenderization, production of fish sauce and soy sauce. Since halophilic proteases are tolerant to organic solvents, they could be used in antifouling coating preparations used to prevent biofouling of submarine equipments. The gelatinase from haloarchaea could be considered as a probable candidate for peptide synthesis. However, further studies are warranted on this haloarcheal gelatinase particularly on structure elucidation and

enzyme engineering to suit a wide range of applications. There is immense scope for developing this halozyme as an industrial enzyme once thorough biochemistry of this gelatinase is studied and a pilot scale study is conducted towards industrial production of this enzyme under fermentation is facilitated. Based on the present study it is concluded that haloarchaea *Natrinema* sp. that inhabit solar saltern ponds are ideal source for deriving industrially important halozymes and molecular studies on enzymes are prerequisite for their probable industrial applications. This is the first time this species of archaea is recognized as a source of gelatinase enzyme that has potential for industrial applications.

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LIST OF PUBLICATIONS

R. Manjula, P. Karthikeyan, P.C. Cikesh, E.S. Bindiya, Sarita G Bhat, and Chandrasekaran, M. (2014). Halophiles and Halozymes from Tannery effluent as well as food grade table salt crystals. *J. Pure and Appl. Microbio.* **8**(1): 707-713.

GenBank Submissions

1. **Manjula, R**., Karthikeyan, P., Sarita, G.B. and Chandrasekaran, M. (2012). JX975066 – *Oceanobacillus* sp. strain BTMT01 16S ribosomal RNA gene, partial sequence.
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4. **Manjula, R**., Karthikeyan, P., Sarita, G.B. and Chandrasekaran, M. (2011). JN228201 - *Staphylococcus arlettae* strain BTMT04 16S ribosomal RNA gene, partial sequence.
5. **Manjula, R**., Karthikeyan, P., Sarita, G.B. and Chandrasekaran, M. (2012). KC019171 - *Halomonas* sp. strain BTMT05 16S ribosomal RNA gene, partial sequence.
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12. **Manjula, R**., Karthikeyan, P., Sarita, G.B. and Chandrasekaran, M. (2012). JX975065 - *Halomonas* sp. strain BTMT12 16S ribosomal RNA gene, partial sequence.
13. **Manjula, R**., Karthikeyan, P., Sarita, G.B. and Chandrasekaran, M. (2012). JX975062 - *Halomonas* sp. strain BTMT13 16S ribosomal RNA gene, partial sequence.

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APPENDIX-I

Table -1. Morphological & biochemical characteristics of bacterial isolates from tannery effluent and table salt crystals.

Characteristics	BTMT 01	BTMT 02	BTMT 03	BTMT 04	BTMT 05	BTMT 06	BTMT 08	BTMT 10	BTMT 11	BTMT 12	BTMT 13
Gram staining	positive	positive	positive	positive	negative	negative	positive	positive	negative	negative	negative
Morphology	bacilli	cocci	bacilli	cocci	bacilli	bacilli	cocci	cocci	bacilli	bacilli	bacilli
Indole	-	-	-	-	-	-	-	-	-	-	-
MR	+	+	+	+	-	-	+	+	-	-	-
VP	-	-	-	-	-	-	-	-	-	-	-
Citrate	-	-	-	-	+	+	-	-	+	+	+
H_2S	+	-	+	-	+	+	-	-	+	+	+
Sugar fermentation											
sucrose	-	-	-	-	+	+	-	-	+	+	+
mannitol	-	-	-	-	+	+	-	-	+	+	+
glycerol	-	-	-	-	+	+	-	-	+	+	+
sorbitol	-	-	-	-	+	+	-	-	+	+	+
lactose	-	-	-	-	+	+	+	+	+	+	+
glucose	-	+	-	+	+	+	+	+	+	+	+
Enzyme activity											
Urease	+	-	+	-	+	+	-	-	-	+	+
catalase	+	+	+	+	-	-	+	+	+	-	-
oxidase	+	-	+	-	-	-	+	+	-	-	-
coagulase	-	+	-	+	-	-	-	-	-	-	-
Amylase (starch)	+	+	+	+	+	+	+	+	+	+	+
Lipase (tributyrin)	+	+	+	+	+	+	-	-	+	+	+
Protease (casein)	+	+	+	+	+	+	+	+	+	+	+

(+ indicates positive result and – indicates negative result)