Screening and characterization of an extracellular protease enzyme from a halophilic bacterium

Broad Area

Enzyme Technology

Synopsis of Ph. D. Thesis

Submitted by

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Enrolment No. 079253

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Introduction

Hypersaline environments on the earth occur either naturally as permanent saline lakes and ephemeral salt pans or artificially as solar salterns. Hypersaline ecosystems show considerably rich diversity of microbes and are biologically very productive. Some of the microbes isolated from hypersaline environments show polyextremophilicity thus the enzymes from these microbs are also polyextremophilic. These enzymes remain active and stable in high salt conditions and are also reported to be thermo tolerant and alkaliphilic (Moreno et al. 2009).These properties made halophilic enzymes attractive for various biotechnological, industrial applications as they can catalyze reactions under harsh reaction conditions. Among all the extremozymes, halophilic proteases are the most widely exploited enzymes in industry (Gupta R. et al. 2002). Industrially, it is desirable to have an enzyme that can withstand non-aqueous conditions. In aqueous environment, proteases hydrolyse peptide bonds but in non-aqueous environment they catalyze the reverse reaction i.e. synthesis of peptide bond (Gupta M.N. et al.2004). The increasing concentration of organic co-solvent would always lower the enzymatic activity due to direct contact of the organic solvent with the enzyme. Several methods have been used for stabilizing proteases in organic solvent media such as chemical modification (Takahashi et al. 1984), immobilization (Kise et al. 1990).If the enzyme is naturally solvent tolerant then no such modification is required.

In this work, a new haloalkalliphilic bacterium *Halobiformalacisalsi* strain BNMIITR is isolated and its physiological, biochemical and genotypic characterizations along with its potential to produce a highly solvent tolerant extracellular protease and further characterization of enzyme are reported. The enzyme also showed significant organic solvent stability in polar and non polar solvents.

Objectives;

Major objective of this research work are as follows;

- Isolation and characterization of halophilic bacteria from Sambhar lake Rajasthan.
- Screening of extracellular proteases from halophilic bacteria
- Optimization of enzyme production
- Purification of the enzyme
- Characterisation and potential application of extracellular protease

Work done

Objective 1:

Isolation and characterization of halophilic Bacteria from Sambhar lake Rajusthan

Four soil and one salt samples were collected from Sambhar lake Rajasthan located in Northern India. Sambhar lake is the largest inland salt lake predominated by sodium chloride, sodium carbonate, sodium bicarbonate sodium sulphate and lacked divalent cations (calcium and magnesium), pH ranges between 9.5-9.7 and temperature 37-45 ºC (Upasani and Desai).

1.1. Isolation of the halophilic bacteria

Initially soil sample was incubated at 45 ºCon a rotatory shaker (180 rpm) in a complex halophilic medium, (Asker and Ohta. 1999). The pH of the medium was adjusted to 9.0 with 1 M potassium hydroxide (KOH). After 8 days of incubation it was further sub cultured in the same liquid medium under similar experimental conditions. The isolates were purified by serial dilutions and repeated streaking on agar plate having same medium composition as liquid complex medium. Sixteen isolates were isolated from soil and salt samples and further characterized.

Isolates								
Characteristics	MSL-1	MSL-2	MSL-3	MSL-4	MSL-5	MSL-6	MSL-7	MSL-8
Cell shape	Long Rods to cocuss (pleomor phic)	cocuss	cocuss	cocuss	Short rods	Short rods	Short rods	Long rods
Pigmentation	Dark Orange	Orange	Light orange	White	White	Pale	Pale	Transpar ent
Gram's Nature	Negative	Positive	Positive	Positive	Positive	Positive	Positive	Positive
Range of NaCl (%w/v)concentration which supports growth	2-5 M	2-5 M	2-5 M	2-5 M	1-4 M	1-4 M	1-4 M	1-4 M
Range of KCl (%w/v)concentration which supports growth	1-4 M	1-4 M	1-4 M	1-4 M	1-3 M	1-3 M	1-3 M	1-3 M
Range of temperature which supports growth	18-55 ^{0}C	18 -55 ^{0}C	18-55 ^{0}C	18-55 ^{0}C	18-50 ^{0}C	18-50 ^{0}C	18-50 ^{0}C	18-50^{0}C
Range of pH which supports growth	6-10	6-11	6-11	6-10	6-11	6-11	6-11	6-10
Optimum NaCl(% w/v) concentration for growth	3 M	3 M	3 M	3 M	2 M	2 M	2 M	2 M
Optimum KCl(% w/v) concentration for growth	3 M	3 M	3 M	2 M	2 M	2 M	2 M	2 M
Optimum temperaturefor growth	45 ^{0}C	45 ^{0}C	45 ^{0}C	45 ^{0}C	37 ^{0}C	45 ^{0}C	45 ^{0}C	37 ^{0}C
Optimum pH for growth	8-8.5	9	9	8	8	8	8	8
Catalase	Positive	Negative	Negative	Positive	Positive	Positive	Positive	Positive
Oxidase	Negative	Negative	Negative	Positive	Positive	Negative	Negative	Negative
Spore	-	-	-	-		Terminal oval	Terminal oval	Terminal oval
Isolates								
Characteristics	MSL-9	MSL-10	MSL-11	MSL-12	MSL-13	MSL-14	MSL-15	MSL-16
Cell shape	Short rods	Long Rods to cocuss (pleomor phic)	cocuss	Long rods	Long rods	Long rods	Long rods	Long rods

Pigmentation	Pale	Light Orange	Light orange	Pale	Pale	Transparent	Transparent	Transparent
Gram's Nature	Positive	Negative	Positive	Positive	Positive	Positive	Positive	Positive
Range of NaCl (%w/v)concentration which supports growth	1-3 M	2-5 M	2-5 M	1-5 M	1-5 M	1-4 M	1-4 M	1-4 M
Range of KCl (%w/v)concentration which supports growth	1-3 M	1-4 M	1-4 M	1-4 M	1-4 M	1-3 M	1-3 M	1-3 M
Range of temperature which supports growth	18-50 ^{0}C	18 -55 ^{0}C	18-55 ^{0}C	18-50 ^{0}C	18-50 ^{0}C	18-50 ^{0}C	18-50 ^{0}C	18-50 ^{0}C
Range of pH which supports growth	7-10	6-10	6-11	6-10	6-10	6-10	6-10	6-10
Optimum NaCl(% w/v) concentration for growth	1 M	4 -5 M	3 M	2 M	2 M	2 M	2 M	2 M
Optimum KCl(% w/v) concentration for growth	1 M	3 M	3 M	2 M	2 M	2 M	2 M	2 M
Optimum temperaturefor growth	37 ^{0}C	45 ^{0}C	45 ^{0}C	37 ^{0}C	37 ^{0}C	37 ^{0}C	37 ^{0}C	37 ^{0}C
Optimum pH for growth	8	9	9	8	8	8	8	8
Catalase	Positive	Positive	Negative	Positive	Positive	Positive	Positive	Positive
Oxidase	Positive	Negative	Negative	Positive	Positive	Positive	Negative	Negative
Spore	-	-	-	Terminal oval	Terminal oval	Terminal oval	Terminal oval	Terminal oval

Table-1: Characterization of isolates on the basis of morphological and physiological characters

1.2 Morphology of halophilic isolates

The cellular morphology of the isolate was studied by using bright field microscopy under 100 X magnification. Gram staining was performed according to Dussault (1955). Out of 16 isolates two were found pleomorphic (from rod to coccus), four coccus (Gram positive) and other are gram positive rods.

1.3 Physiological characterization

1.3. 1 Salt requirement

To ascertain the halophilic nature, isolates were grown in complex halophilic media with salt concentrations ranging from 1-5 M for NaCl and 1–4 M for KCl at 45 ºCon a rotatory incubator shaker (180 rpm). Final pH was adjusted to 9.0. Growth was measured in terms of optical density at 600 nm. Out of 16 isolates, six are extremely halophilic and nine are moderately halophilic and one is halotolerant.

1.3.2 pH and Temperature profiles

The temperature and pH ranges for growth was determined by incubating agar plates containing complex halophilic medium, in temperature range between 16-60 ºC and pH range 5-12 respectively. Results have been summarized in the following table

1.3.3 Lipid and cellular fatty acid analysis

Total lipid analysis was done using thin layer chromatography (TLC) [Elevi et al. (2004).

1.3. 4 Sensitivity to antimicrobial agents

Antibiotic sensitivity test was performed by streaking the log phase culture on solid complex halophilic medium with 2 M or 3 M NaCl (according to halophilic nature of isolates) and applying disks (HIMEDIA octa - disks) impregnated with the various antimicrobial agent.

MSL-1, MSL-2, MSL-3, MSL-4, MSL-5, MSL-10 and MSL-11 show resistance towards many antibiotics while others are sensitive to them.

1.3. 5 Pigment profile

Pigment was extracted from cell pellet with methanol/acetone (1:1 v/v) and the extract was analyzed by scanning the absorbance in the wavelength region of 300–800 nm using Varian UV-Vis spectrophotometer against the same solvent as blank (Oren et al. 2002).

Out of 16 isolates five (MSL-1, MSL-2, MSL-3, MSL-10 and MSL-11) are found to possess carrotenoid pigment barring MSL-1 which was found to have multiple pigments.

1.4 Biochemical characterization

BIOLOG experiment was carried out by using 12 and 15 % salt in universal inoculating fluid IF-A. It was found that Gram positive rod shaped bacteria with low NaCl requirement (moderate halophiles and halotolerant) can utilize more carbon source than extreme halophilic bacteria.

1.5 Screening of isolate for extracellular protease activities

Five isolates (MSL-1, MSL-4, MSL-5, MSL-12 and MSL-13) were found to have protease activity in agar plate (Amoozegar, M. A et al 2007).

Objective 2

2.1 Protease production by the selected isolates

The isolates showing significant zone of clearance in skimmed milk agar plate were further analysed for the amount of protease produced by them. The isolates were grown in the production medium at 45 °C for 160 h under shaking conditions (200 rpm) and the enzyme activity was estimated at interval of 12 h. On the basis of higher enzyme activity and specific activity isolate (MSL-1) was selected and processed further.

2.2 Genotypic analysis of screened strain

Extracted DNA (Mak and Ho 1992) used for amplification of the gene 16S rRNA by polymerase chain reaction (PCR). Further 16S rRNA gene sequence was determined by Eurofins Genomics India Pvt Ltd. The 16S rRNA gene sequence was aligned and compared with available sequences of gene bank at National Centre for Biotechnology Information (NCBI) using Basic Local Alignment Search Tool (BLAST). The phylogenetic tree was constructed by the neighbour-joining method. Bootstrap analysis was used to evaluate the tree topology of the neighbour-joining data by performing 1,000 resampling. Sequence was submitted to the NCBI Gene Bank under the accession number **KF424537**.

2.3 Optimization of reaction conditions

Optimum pH for proteolytic activity was checked from pH 6 to pH 12 at 45 ºC with cell free extract. The enzyme was remarkably active in alkaline pH 9 and broad range of temperature (30– 70 ºC).

2.4 Effect of additives, surfactants and solvents on protease activity

The effects of PMSF, EDTA and urea on crude protease activity were determined by including them in reaction mixture at a concentration of 10mM and 50mM. The effects of surfactants tritonX-100, tween 80, tween 40, Sodium dodecyl sulphate (SDS) and cetyl trimethylammonium bromide (CTAB) on enzyme activity were studied at concentrations of 0.5 and 1%. The activity was remained unaffected by DTT and urea. Minor reductions of activity by CTAB and SDS were observed. Interestingly, it was increased in presence of Tween 20 and Tween 80. Extracellular protease ofIsolate *Halobiforma lacisalsi*strain BNMIITR showed high solvent tolerance towards 50% n-hexane, DMF and DMSO.

Objective 3:

Production optimization of extracellularprotease from Isolate *Halobiforma lacisalsi* strain BNMIITR in shake flask.

3.1 Effect of Salinity:

Isolate *Halobiforma lacisalsi* strain BNMIITR can grow in a broad range of salinity (2-5 M).Effect of salinity on protease production was examined by growing the isolate *Halobiforma lacisalsi* strain BNMIITR at a salinity range 2-5 M at 45 ºC with pH 9.0 on a rotatory shaker (180 rpm).Effects of various salts on protease production were also evaluated similarly and NaCl was found to be the most suitable salt for protease production when used at 3 M concentration.

3.2 Effect of pH:

The isolate *Halobiformalacisalsi* strain BNMIITR can grow in a broad range of pH(6-11).Effect of pH on protease production was examined by growing the Isolate *Halobiforma*

lacisalsi strain BNMIITR in a pH range 7-11at 45 ^{0}Cwith 3 M NaCl on a rotatory shaker (180 rpm).

3.3 Effect of Temperature:

The isolate *Halobiformalacisalsi* strain BNMIITR can grow in a broad range of temperature.Effect of temperature on protease production was examined by growing the isolate *Halobiformalacisalsi* strain BNMIITR in a temperature range 30-55 ^{0}C at pH 10.0 with 3 M NaCl in anincubator shaker (180 rpm).

3.4 Effects of various carbon sources on protease production;

The growth medium was supplemented with different carbon sources containing 1% (w/v) glucose, fructose, sucrose, lactose, galactose, glycerol, mannitol, starch, xylose, trisodium citrate, citric acid and different low cost agro industrial byproducts such as wheat bran ,wheat flour to investigate theireffect on protease production.Very little protease production was observed when low cost agro industrial byproducts were present in production medium while it was maximum when xylose was used as a carbon source

3.5 Effects of Various Nitrogen Sources on protease production;

The growth mediumwas supplemented with different nitrogen sources containing 1% (w/v) peptone, casein, gelatin, peptone, yeast extract, soya peptone, soya bean, casein digest, cassamino acid and skim milk powder, inorganic nitrogen sources such as NH_4NO_3, NH_4Cl, $NaNO_3$, $(NH_4)_2SO_4$ and urea, different low cost agricultural byproducts such as soybean meal, soya bean husk, chick pea flour and chick pea husk to investigate theireffect on protease production. No protease production was observed when inorganic nitrogen sources were present in production medium while it was maximum when low cost agricultural byproducts were used as nitrogen sources.

3.6 Effect of various amino acids on protease production;

Amino acids are known to catabolically repress the protease production. The effect of amino acids on protease production was checked by supplementing the medium with various amino acids at concentrations (0.1, 0.5, and 1 %). Asparagine and proline have no effect on protease production while other amino acids repress protease production at different magnitude.

3.7 Effect of various surfactants on protease production;

The effect of surfactants on protease production was checked by supplementing the medium with various surfactants at concentrations (0.1, 0.5, and 1 %). Different surfactants repress protease production and the extent of repression was varied with different surfactants.

Objective 4

Purification of extracellularprotease from isolate *Halobiforma lacisalsi* strain BNMIITR

4.1 Ultra filtration and solvent precipitation

Two litres of crude enzyme extract was concentrated by tangential flow filtration system using 10 kD membrane. Concentrated enzyme was precipitated by adding pre chilled acetone at 30 % saturation and extra proteins were removed. This supernatant was precipitated again with pre chilled acetone up to 70 % saturation.Precipitate was pelleted at 8000 g for 10 min and re suspended in tris buffer pH 9.0 (1.2 % NaCl). Precipitated enzyme was further concentrated by using centrifugal concentrators (Amicon10 kD).

4.2 Hydrophobic interaction chromatography

The concentratedenzyme was further purified by hydrophobic interaction chromatography (HIC) on a Phenyl Sepharose 6 Fast Flow column(1 cm × 24cm), equilibrated with Tris buffer pH 9.0 with 25% NaCl. The column was washed with equilibrating buffer until the washings were free of proteins. Further elution was done by a linear gradient of NaCl (25 %-0%) in Tris buffer pH 9.0. The enzyme was eluted at 11-10 % NaCl in Tris buffer pH 9.0.

Active fractions were pooled. Using this method 31 fold purification of protease was achieved.

4.3 Polyacrylamide gel electrophoresis

Native and sodium dodecyl sulphate polyacrylamide gelelectrophoresis (native PAGE and SDS-PAGE) were carried out using 12% cross-linked polyacrylamide gel on a gel electrophoresis unit according to the method of Laemmli (1970). Casein zymography was done according to Meera Venugopal etal 2006.Gel was stained with 0.1%Coomassie blue R-250 (in methanol–acetic acid–water, 40:10:50) followed by destaining with methanol–aceticacid–water (5:10:85). SDS PAGE analysis revealed a single band with a molecular mass of 21 kD.

Objective 5

Characterization and potential application of purified extracellularprotease from isolate*Halobiformalacisalsi* strain BNMIITR

Purified enzyme obtained after HIC was found to lost its 95 % activity in the absence of NaCl it requires minimum 1-2 % NaCl to retain its activity. Thus enzyme was desalted by using centrifugal concentrators (amicon10 kD) with 50mM tris buffer pH 9.0 and 1.2 % NaCl. Further characterization of purified enzyme (in 50mMTris buffer, pH 9.0, containing 1.2 % NaCl) was done with casein (final concentration in reaction mix. was 1 %) as substrate in 50mMglycine NaOH buffer pH 10.0 and 45 ^{0}C.

5.1 Determination of optimum pH

Effect of pH on protease(in 50mMTris buffer pH 9.0, containing 1.2 % NaCl) was evaluated by assaying it with casein in 50 mM buffers (containing 1% NaCl)of different pH, potassium phosphate (pH 6.0–7.5), Tris (pH 8.0–9.5), glycine NaOH(pH 10 –12). Enzyme was found to have maximum activity at pH 10.0.

5.2 Determination of optimum temperature

The optimum temperature of the protease was determined by incubating it with casein solution in 50 mM glycine NaOH buffer, pH 10.0. The mixture was incubated at different temperatures (20–70 ^{0}C).Although optimum temperature of the enzyme was 50 ^{0}C but enzyme was very unstable at this temperature, further characterization was done at 45 ^{0}C.

5.3 Determination of Substrate specificity

Protease activity of purified enzyme was analyzed with different substrates such as casein, BSA, gelatin, N-Succinyl-Ala-Ala-Ala-*p*-nitroanilide, Paranitrophenylactetate. Enzyme has maximum activity towards casein and besides having proteolytic activity it also has esterase and elastase activities.

5.4 Effect of metal ions on enzyme activity

Effect of various metal ions on protease activity was studied by incubating the enzyme at 45 ^{0}C in glycine NaOH buffer (pH 10) with various metals at 5 and 10 mM concentrations. Enzyme retains its complete activity in presence of most of the metals viz. Barium, calcium, magnesium, Iron, cobalt but it was found to be sensitive towards nickel and zinc.

5.5 **Effect of additives and solvents on enzyme activity**

The effects of PMSF, EDTA and urea on crude protease activity were determined by including them in reaction mixture at concentrations of 5mM and 10 mM. The effects of surfactants tritonX-100, tween 80, tween 40, Sodium dodecyl sulphate (SDS) and cetyl trimethylammonium bromide (CTAB) on enzyme activity were studied at a concentration of 0.1 % and 0.5 %.The relative activities were measured. The activity of the enzyme measured in absence of any additive was taken as 100% (control).

Enzyme activity was reduced in the presence of additives like CTAB, EDTA, SDS and completely inhibited by PMSF which shows that it is a serine protease. Enzyme was unaffected by Surfactants such as Triton X-100. Interestingly, Tween 20, Tween 40 and Tween 80 had positive effects on activity.

5.6 Effects of Organic solvents on protease activity;

The crude enzyme activity was determined in the presence of 10 % 30% and 50% (v/v) of various organic solvents (ethanol, methanol, hexane DMF, DMSO heptane and xylene. The enzyme retains its 100 % activity in non polar solvents and more than 60 % activity in the presence of polar solvents. DMF and DMSO have positive effects on activity.

5.7 Protease stability in organic solvents;

One ml of protease along with 1 ml of organic solvents incubated at 30 ^{0}C and 45 ^{0}C with constant shaking at 180 rpm. Samples were withdrawn after 24 h, and 48 h. Residual protease activity was determined. The enzyme incubated without solvent was treated as the control. Enzyme was considerably stable in polar as well as non polar solvents and it shows activity even after 48 h of incubation at 30 ° C.

5.8 Effect of osmolytes on enzyme activity

The effects of different concentrations (1-10 %) of various osmolytes, KCl, glycerol, mannitol and sucrose on the activity of purified enzyme wereexamined. Activity was increased when 1 % sucrose and 2 % glycerol were present in the reaction mix.

5.9 Thermostabilityof the enzyme

Stability of enzyme at different temperatures was determined. Although optimum temperature of the enzyme was 50 ^{0}C but enzyme was very unstable at this temperature. After 24 hours enzyme lost more than 50 % activity at 45 ^{0}C in the presence of 1.2 % NaCl while it retain more than 70 % activity at 20 ^{0}C after 48 h.

5.9.1 Effect of NaCl on thermal stability of enzyme

Enzyme was incubated at different temperatures with different NaCl concentrations and it was found that at 10-12 % NaCl concentration enzyme retain more than 90 % activity after 24 h at 45 ^{0}C. Thus enzyme does not require NaCl for activity but it provides stability to the enzyme.

5.10 Detergent compatibility of the enzyme

The protease was found to be stable and compatible with detergents, as it retained activity even after 24 h incubation with the commercial detergents (1.0%, w/v) Surf excel, Ariel, Tide, Rin and Wheel.

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ABSTRACT

In aqueous medium Proteases hydrolyze peptide bonds but in non-aqueous they catalyze the reverse reaction and catalyze the synthesis of peptides. For Industrial applications organic solvent stable proteases are required. Most of the salt stable halophilic protein can work well in non-aqueous conditions. Thus there is a continuous demand of new microbes which can produce novel enzymes. In the present study four soil and one salt samples were collected from Sambhar salt lake Rajasthan. Complex halophilic medium with 4 M NaCl at pH 9.0 and 45 ºC were used to study bacterial diversity of the samples. Sixteen purified cultures were obtained from four soil and one salt samples. These isolates were colorless transparent to red orange in color and showed rod, cocci and pleomorphic type of cellular morphology when seen under 100 X magnification of phase contrast microscope. On the basis of salt requirement isolates were extreme halophiles, borderline extreme halophiles and moderately halophilic. All the isolates showed better growth in NaCl medium as compared to that in KCl medium. All the isolates can grow at a wide range of temperature and pH and some of them had potential to produce industrially important hydrolytic enzymes. Extremely halophilic and borderline extremely halophilic isolates showed resistance towards many antimicrobial agents while all the moderately halophilic isolates were found to sensitive for them. Moderately halophilic isolates were found to utilize more carbon sources in comparison to extremely halophilic and borderline extremely halophilic isolates. Five isolates out of sixteen were found to produce extracellular protease. On the basis of maximum protease activity isolate MSL-1 was selected and further characterize. 16S rRNA gene sequence of MSL-1 showed 98 % similarity with *Halobiforma lacisalsi*, 97 % with *Hbr. haloterrestris* and 96 % with *Hbr. nitratireducens.* Thus the strain MSL-1 phylogenetically belongs to genus *Halobiforma* and named as *Halobiforma* sp. strain BNMIITR. It had six different types of lipids

with two glycolipids and one phospholipid. Major cellular fatty acids were $C_{14:0}$, $C_{15:0}$ iso, $C_{15:0}$ anteiso, $C_{16:0}$, $C_{17:0}$ iso, $C_{17:0}$. Further protease production was affected by nutritional factors and culture conditions. NaCl was found most suitable salt for protease production at pH 10.0 and 45 ºC temperature. Protease production was considerably increased in the presence of low cost agro industrial byproducts like soybean husk, soybean flour, chick pea flour and chick pea husk. Protease production was catabolically repressed by many amino acids with positive effect of proline. Protease production was reduced by many surfactants while it was completely inhibit by SDS. Enzyme purification was done by solvent precipitation followed by HIC. Enzyme was a monomer with a molecular mass 21 kDa. Protease of *Halobiforma sp.* strain BNMIITR can work at wide range of pH (6-12). Enzyme also showed broad range of substrate specificity (caseinolytic, esterase and elastase activities). Enzyme was very stable in presence of many metals, surfactants and oxidizing agents but activity was reduced in the presence SDS and EDTA with complete inhibition by PMSF thus a serine kind of protease. Thermal stability of the enzyme was affected by salt concentration. Enzyme was stable in polar as well as non polar solvents with considerably high stability in nonpolar solvents at 30 ^{o}C. Enzyme was stable and active in the presence of various commercially available detergents. Interestingly enzyme activity was increased in the presence of DMF and DMSO.

1. INTRODUCTION

Extreme environments include acidic environment, hot and cold environments, dry and hypersaline environments are distributed all over the earth. Hypersaline environments can be thassohaline or athassohaline. In India, many hypersaline sites are known, among them Sambhar lake is largest inland salt lake. Microorganisms found in hypersaline environment are known as halophilic (salt loving). Halophilic microorganisms generally belong to archaea, eubacteria and eukarya. Halophilic archaea are members of family Halobacteriaceae. Most of the members of the family are orange to red in colour because they have carotenoids. These carotenoids play an important role in photo repair of thymine dimers by absorbing extra heat (Joo and Kim 2005). These members also produce gas vesicles which are proteinaceous gas filled structures which provide buoyancy to the bacteria in aquatic system (walsby 1994). Some biosurfactants has also been reported from halophiles. These biosurfactants can be used in bioremediation of oil contaminated soil. Archaeal bacterial cell membranes are characterized by the presence of ether linked fatty acids in place of normal ester linked fatty acids in their counterparts. Thus they are resistant to esterases. Liposome derived from ether linked fatty acids are more stable and had potential use in cosmetics and medicines for drug delivery (Gambacorta et al. 1995). Structure of small subunit of rRNA of archaea is different from that of eukarya and eubacteria. Archaea have some unique structures in the region between nucleotide base positions 180 and 197 or 405 and 498 (Woese et al. 1990). Archaea has similar machinery for DNA replication, glycosylation and transcription as that of eukarya (White 2003).

Enzymes from these halophilies have some unique characteristics which could be commercially exploited. One of the unique characteristics of halophilic enzymes which is of interest to biochemists is their ability to retain activity in non-aqueous organic solvent. Biocatalysis serves as an alternate tool for the synthesis of agrochemical intermediates and pharmaceutical chemicals. Biocatalytic transformations have been proved as a green, sustainable environment friendly methodology in chemical synthesis. Enzymes are natural catalyst, economic and very specific for the reaction they catalyze. Thus there is a continuous demand of new enzymes.

Among enzymes, hydrolases predominate in bioprocesses. Among hydrolases, proteases are most industrially used (60 %) enzyme. Wide applications of protease in leather, food, ultra filtration membrane cleaning, pharmaceuticals, meat tendering, detergent and extraction of silver from used X-ray Film had already been reported in literature (Gupta et al.2002). Medically, protease serves as

an agent for treatment of, inflammation, pain, wound healing and arthritis (Rawat et al. 2007). Normally protease work in aqueous conditions but in the presence of organic solvents they catalyze the synthesis of esters and peptides. For potential new application, protease should be stable in organic solvent, detergent and oxidizing agent (Gupta et al. 2002). Some synthetic reactions require the presence of organic solvents while others can be done in aqueous systems. Polar and non polar solvents modify efficiency and selectivity of the enzyme. On the basis of the miscibility of solvent with water, organic solvent systems are of three types 1) Co solvent which is miscible solvent system 2) Biphasic immiscible solvent system 3) nearly anhydrous solvent system (Doukyu and Ogino 2010). An organic solvent tolerant enzyme is desirable to make the synthesis reactions easy and economical. Several protein engineering methods have been used to increase stability and activity of enzyme in organic solvents. If the enzyme is naturally organic solvent tolerant then no such modification is needed. An artificial low-calorific sweetener, aspartame has been produced using a thermo-stable metalloprotease, thermolysin in the presence of solvent (Ogino et al. 2007). Peptide and ester synthesis reactions take place in cosolvent system but at higher solvent concentration contact of solvent with enzyme increase which in turn reduce enzyme activity (Doukyu and Ogino 2010). Microorganisms are good source of enzymes because of short generation time, easy genetic modification which are useful for bulk production (Joo et al. 2002). First organic solvent tolerant enzyme has been reported from *Pseudomonas aeruginosa* was a lipolytic enzyme (Ogino et al. 1994). Many extremophilic bacteria such as thermophiles and halophiles serve as a good source of organic solvent tolerant enzyme besides their counterpart mesophiles (Doukyu and Ogino 2010). Halophiles are adapted to grow at high salt conditions thus the enzymes from halophiles require salt for activity and stability. High salt environments are low water environments thus halophiles are adapted to cope with low water activity. High salt environment mimic with organic solvent environment. High salt forms a shield around halophilic enzyme and protect them from water damage of protein. Halophilic proteins have high amount of acidic amino acid like glutamic acid and aspartic acid residues and excess negative charge on the surface (Lyani et al. 1974). Halophilic archaea accumulate large concentration. Halophilic proteins are resistant to high salt concentration which cause structural disruption in mesophilic bacteria. During the synthesis of halophilic proteins many hydrophobic amino acid residues which forms the interior of the enzyme become exposed at high salt conditions which in turn result in to the formation of many inter and intra molecular bonds with amino acid side chains. Thus halophilic proteins require high salt concentration for active and stable conformation. Halophiles can also use

compatible solute like ectoins and betain to stabilize the protein at high salt concentrations (Joo and Kim 2005). Besides being salt tolerant halophilic proteins can also work at elevated temperature and pH. A chymotrypsinogen of the archaeon *Natronomonas pharaonis,* had maximum activity at 61°C and pH 10 (Stan-Lotter et al. 1999). Lipolytic enzymes from halophiles have been used in food, paper and detergent industry. A lipolytic enzyme from halophilic bacterium *Marinobacter lipolyticus* SM19 had maximum activity at 80 ^{o}C. This enzyme can also work on chiral and prochiral esters (Perez et al. 2011). Alpha amylase from *Thalassobacillus* sp. had maximum activity at 70 ^{o}C and pH 9.0 (Li X et al .2012).

Proteases can also play an important role in reducing biofouling of marine structures. Biofouling impose a huge economic loss and higher environmental pollution. Till 2008, Tri-n-butyl tin (TBT) has been extensively used as antibiofouling agent in marine paint industry. TBT has adverse effect on marine ecological diversity. Thus paints containing TBT has been banned. Haloarchaeal proteases serve as a better alternative in antifouling coating. As most of the coating materials are suspended in organic solvent, there is urgent need to have organic solvent compatible protease and other related enzymes. Organic solvents reduce water activity thus most of the salt stable halophilic enzyme remain active and stable in the presence of organic solvents. Besides conventional protease may also work suboptimally in saline water condition which is the most important criteria for application of enzyme as antifoulant. Archaea are also important to understand life as halites have been found from mars also (Gooding 1992).

Aim and scope of the study

Biotransformation serves as an alternative tool to the chemical synthesis. Proteases are hydrolytic enzymes with high selectivity which does not need any expensive cofactors and can be used as synthetic tools. Most of the synthetic reactions are carried out in presence of organic solvents. Proteases in organic solvents can catalyze reactions such as esterification and peptide synthesis. Unfortunately most of the protease lose their activity in the presence of organic solvents thus there is a continuous demand of microorganisms that can produce solvent tolerant enzymes. New application of protease as antifouling agent needs organic solvent tolerance and high activity in saline sea water. In comparison to lipase which has been most extensively studied hydrolytic enzyme for synthesis reaction there are very few organic solvent tolerant proteases available. Thus there is an urgent need to isolate an organic solvent tolerant protease from halophilic organism.

The present work deals with:

- Isolation and characterization of halophilic bacteria from Sambhar lake Rajsthan India
- Screening of isolates for the production of extracellular protease
- Production optimization of extracellular protease in shake flask
- Purification of extracellular protease
- Characterization and potential application of extracellular proteases

2. REVIEW OF LITERATURE

2.1 Extreme environments

The word "extreme" has been derived from latin word "extremus" which means on the outside (Rothschild and Mancinelli 2001). Extreme environments are characterized by conditions challenging for most life forms. Most extremophiles conjures up the image of unicellular organisms' i.e protists, bacteria and archaea. With exception of a few animals which are capable of surviving in harsh conditions of particular extreme environments, multicellular organisms do not exhibit much diversity (Rothschild and Mancinelli 2001). Extreme environments can be categorized into following on the basis of:

1. pH: a. acidic environments b. alkaline environments
2. Temperature: a. Extremely hot environment b. Extremely cold environment
3. Desiccation: Extremely dry environment
4. Salinity: Hypersaline environment

2.1.1 Acidic environments

Acidic environments are characterized by pH below 5 e.g. sulphuric pools and acid mine drainage etc. Organisms harbouring in such acidic conditions are known as acidophiles (Baker and Dopson 2007). For example *Acidithiobacillus*, *Ferroplasma acidophilum, Cyanidium caldarium*, *Dunaliella acidophila*, *Trichosporon cerebriae* and *Picrophilus* (Rothschild and Mancinelli 2001). The strategies adopted to thrive in such environments include reinforcement of the cell membrane, limiting proton diffusion into the cell and secretion of extracellular polymeric substances (EPS) etc. They are also known to secrete buffer molecules and basic amino acids. (Rothschild and Mancinelli 2001).

2.1.2 Alkaline environments

Alkaline environments broadly conceived as natural habitats having pH above 9 like many soda lakes and hydrothermal springs. Organisms surviving in such environments are named as alkaliphiles (Horikoshi 1998) e.g. *Natronococcus occultus* (Tindall et al. 1984) and *Bacillus alcalophilus* (Horikoshi 1998). Acquired adaptation include, use of polyamines which are rich in positively charged amino acids to buffer cell cytoplasm, introducing acidic compounds like uronic acids, teichoic acid and acidic amino acids for striking a balance between internal neutral and

external alkaline environment and synthesis of high pH resistant specific enzymes (Horikoshi 1998).

2.1.3 Hypersaline environments

These environments possess salt concentrations greater than that of seawater (3.5 %). Organisms living in such environments are called as halophiles e.g archaea, bacteria, and eukarya (Gunde-Cimerron et al. 2005). More detailed discussion on halophiles and hypersaline environments will be provided later.

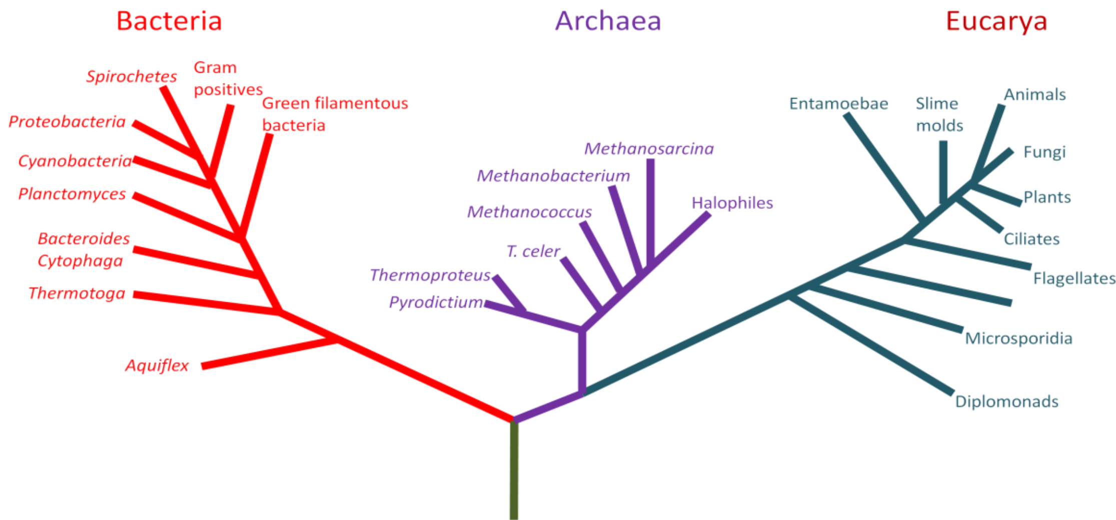


Fig. 2.1: Phyllogenetic tree of life (Olsen and Woese 1993).

2.1.4 Extremely hot environments

Hot springs, geysers and hydrothermal vents in the ocean floor are considered as extremely hot environments where temperature is periodically or consistently above 40 °C (Stetter 1998) and organisms survived in these habitants are called thermophile. This includes phototrophic bacteria, eubacteria, actinomycetes, spirochetes and archaea etc. The adaptations to avoid thermal denaturation of cellular biomacromolecules include stabilization of proteins by formation of new disulfide bridges, hydrogen bonds, ionic and hydrophobic interactions (Stetter 1998).

2.1.5 Extremely cold environments

These environments are characterized by temperatures below 5 °C. Ice caps, snow fields and sea ice found in the Arctic, Antarctic glaciers are some examples of extremely cold environments. Organisms harbored in such settings are called psychrophiles (Morita1975). Psychrophiles include algae, fungi, bacteria and archaea (D Amico et al. 2006). To overcome challenges posed by

low temperatures, psychrophilic organisms have successfully evolved strategies such as they synthesize high amount of unsaturated, branched or short-chain fatty acids with polar and large head groups (Chintalapati et al. 2004). Psychrophiles also synthesize extracellular polymeric substances (EPS) and trehalose to protect the DNA at extremely low temperatures (D'Amico et al.2006).

2.1.6 High-pressure environments

Environments settings where pressure is greater than atmospheric pressure (Yayanos 1998) are called high-pressure environments. Organisms capable to survive in these atmospheres are termed as piezophiles. Adaptations acquired to circumvent the problems caused by high pressure include metabolic versatility and high content of unsaturated fatty acids in their cell membranes (Lauro and Bartlett 2008).

2.1.7 Extremely dry environments

Extremely dry environments are those where availability of free water is scarce e.g some terrestrial endolithic habitats, the hot and cold deserts. Organisms requiring such dry environments for their survival are called xerophiles. Xerophiles include a diversity of bacteria, fungi, yeast, plants, nematodes and insects (Wright 1989; Glasheen and Hand 1988). Table 2.1 is summarizing the different habitats along with their peculiar characteristics.

Table 2.1: Extreme environments along with their peculiar characteristics (Rothschild and Mancinelli 2001)

Environmental parameter	Type	Definition	Examples
Temperature Growth	Hyperthermophile	>80°C	*Pyrolobus fumarii*
	Thermophile	Growth 60–80°C	*Synechococcus lividis*
	Mesophile	20 and 45°C	*Staphyloccus aureus*
	Psychrophile	<15°C	*Psychrobacter*

pH	Alkaliphile	pH > 9	*Natronobacterium,*
	Acidophile	pH< 5	*Cyanidium caldarium*
Salinity	Moderate halophile	0.4-3.4 M	*Alkalibacillus silvisoli*
	Halotolerant	>0.2 M	*Halomonas subglaciescola*
	Extreme halophile	2.0-5.2 M	*Halococcus sp*
Desiccation	Xerophiles	Anhydrobiotic	*Artemia salina*

2.2. Hyper saline environments

The two major factors which define hyper saline environments are total salt concentration and ionic composition. Based on ionic compositions terms assigned to such environments are thassohaline and athassohaline. Thassohalines are those brines which have come into existence by evaporation of sea water. Here the predominant ions are sodium and chloride ions and pH ranges from near neutral to slight alkaline. Throughout evaporation ionic composition undergoes changes which lead to a relative increase in potassium and magnesium ions concentration. Among other minerals, $CaCO_3$ and $CaSO_4.2H_2O$ precipitate at a total salinity above 100-120 g/l. Major examples of such system are hypersaline marine lagoons and solar saltern evaporation ponds (Rodriguez-Valera 1988; Grant 1990). Athassohaline are characterized by ionic composition which is influenced by surrounding geology, topography, climatic conditions, dissolution of mineral deposits and it varies widely from sea water (Rodriguez-Valera 1988; Javor 1989; Grant 1990). Here dominating ions are divalent cation such as Mg^{2+} and Ca^{2+} and anions such as Cl^- and Br^- with pH ranges from slightly acidic to neutral. Typical examples are alkaline Soda lakes and the Dead Sea (Oren A 2006). Some major hypersaline environments and their characteristics have been summarized in Table 2.2

Table 2.2: Hypersaline environments of the world and their characteristics

Hypersaline environments	characteristics	Predominant Ionic composition	Microbial diversity	Reference
Great salt lake, Utah, North America	thassohaline	Na^+, Cl^-	Archaea- *Halorhabdus utahensis* bacteria - *Halomonas variabilis* red algae *Dunaliella salina*	Aldrich and Paul 2002, Waino et al. 2000, Dobson and Franzmann 1996
Dead Sea	athassohaline	Ca^{2+}, Mg^{2+}	Archaea- *Haloferax volcanii* Bacteria - *Orenia marismortui*, *Algae-Dunaliella viridis*	Mullakhanbhai and Larsen 1975, Oren et al. 1987, Elazari-Volcani 1940b
Solar Salterns	artificial shallow ponds with increasing salinity	Na+, Cl^-	Archaea- *Natrialba aegyptiaca* Bacteria- *Thermohalobacter berrensis* Algae- *Dunaliella salina*	Anton et al. 2000, Hezayen et al. 2001 Cayol et al. 2000
Alkaline Hypersaline Lakes	most stable at high pH (8-12)	$CO_3^{\ 2-}$ $HCO_3^{\ 2-}$ and Cl^-.		

• Wadi Natrun, Egypt	shallow lakes pH is around 11 divalent cations like Mg^{2+} and Ca^{2+} scarce	Na^{+} and Cl^{-}	Archaea- *Methanosalsus zhilinae* Bacteria-*Bacillus haloalkaliphilus* *green algae*	Mathrani et al. 1988, Fritze 1996
• Lake Magadi Kenya	containing a precipitate of trona i.e sodium sesquicarbonate	Na^{+}	Archaea - *Natrialba magadii* Bacteria- *Natroniella acetigena*	Tindall et al. 1984 Zhilina et al. 1996a
• Sambhar Lake, Rajasthan, India	The salinity fluctuates widely from 70 g/l to over 300 g/l with climactic change	CO_3^{2-} and SO_4^{2-}	Archaea - *Natronobacterium*	Upasani and Desai 1990, Sahay et al. 2012
• Monolake, California	pH is in the range 9.7-10	Na^{+}	diatoms: *Navicula crucialis,* coccoid cyanobacteria *:* *Oscillatoria sp*. green algae*:* *Picocystis*	Kociolek and Herbst 1992, Herbst and Blinn 1998, Roesler et al. 2002

2.2.1 Sambhar lake Rajsthan, India

The Sambhar salt lake (longitude 75° 05 ′E , Latitude 26° 58 ′N), India's largest inland salt lake is located 96 km south west of the city of Jaipur (Northwest India). The chemical composition of the brine and ecological diversity is unusually different from Wadi Natrun, Egypt (Imhoff et al. 1979) and Lake Magadi, Kenya (Tindall et al. 1980). The pH ranges 9 - 9.5 or higher. The brines have high concentrations of carbonate and sulphate and low concentration of divalent cation Mg^{2+} and Ca^{2+}. The salinity fluctuates widely from 70 g/l to over 300 g/l with climate change. The alkaliphilic members of *Halobacteriacea* found here have high requirement of NaCl and pH (8.5-11) but low concentration of Mg^{2+} (below 10 mM) (Upasani 2008). *Natronobacterium* like strains belonging to red, extremely haloalkaliphilic archaea reported from Sambhar lake (Upasani and Desai 1990). Recent exploration of the Sambhar lake brines led to the isolation of more than 93 haloalkaliphilic bacteria which require 2– 25 % salt and 6–12 pH for optimal growth (Sahay et al. 2012). Majority of the isolates were found to bear similarities with the characteristics of phylum firmicutes, proteobacteria (40.63 %) and actinobacteria (6.25 %).

2.3 Halophilic microorganisms

Organisms growing at extreme environments are called extremophiles. Halophiles are a class of extremophile which grow in extremely high salt concentrations. According to the salt requirement, halophilic microorganisms can be classified into following categories: (Kushnaer and Kamekura 1988)

1. Slight halophiles: They grow optimally in the range 0.2- 0.5 M.
2. Moderate halophile: The range of salt concentration for growth is 0.4- 3.4 with optimum growth at 0.5- 2 M.
3. Halotolerant: The Optimum salt concentration for growth is below 0.2 M.
4. Borderline extreme halophiles: Optimum growth is observed in the range 2.0- 3.0 M.
5. Extreme halophile: These halophiles are characterised by high salt requirement in a range 2.0- 5.2 M with an optimum growth at or above 3 M.

These kinds of organisms found in archaea, bacteria and eukarya as shown in Fig. 2.1.

2.3.1 Halophilic archaea

The word archaea originated from the Greek word άρχαι α, which means “ancient things” (http://www.merriam-webster.com/dictionary/archaea). All the prokaryotes were initially placed in

a single group based on their morphology, metabolism and biochemistry. However in 1977, based on rRNA sequencing, archaea were first separated from prokaryotes by Carl Woese and George E. Fox in evolutionary phylogenetic trees (Woese et al. 1977). Halophilic archaea are widely distributed and are considered to be primitive. They have adapted to cope with extreme environments and are divided into three broad groups: hyperthermophiles, halophiles and methanogens. Halophilic archaea can be further subdivided into three families Halobacteriaceae, the Methanospirillaceae and the Methanosarcinaceae. Order Halobacteriales include family Halobacteriaceae which consist of most of the halophiles and halotolerant species Major morphological forms observed are Rod (*Halobacterium)*, cocci (*Halococcus, Natronococcus),* flat to pleomorphic (*Haloferax)*, (Mullakhanbhai and Larsen 1975) and triangular, trapezoid cells *Haloarcula japonica* (Takashina et al. 1990).

The cell wall of methanogenic archaea consists of pseudopeptidoglycan and polysaccharide (N-acetyl glucosamine β1-3 N-acetyl talosaminuronic acid β1-3) which is resistant to hydrolysis by lysozymes. Most peculiar feature of archaeal membrane lipid is that they contain ether linkages (-CH_2-OCOR) between glycerol and fatty acids in place of normal ester linkages as shown in Fig. 2.2.

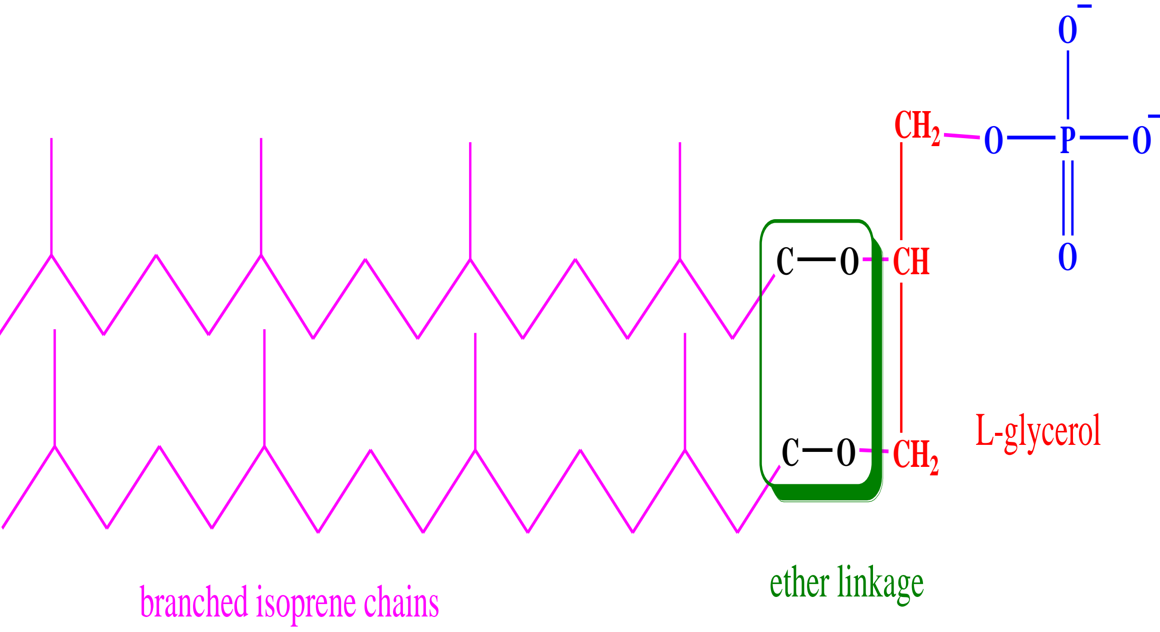


Fig. 2.2: Archaeal membrane lipid showing ether linkage (Bullock 2000).

The cell wall of the species of Halobacteriaceae lack normal cell wall component like D amino acids or teichoic acid (Kushner and Onishi 1968). Structural stability of the glycoprotein cell wall is maintained by combination of high NaCl concentration and divalent cations like magnesium that stabilize the charge shielding effects and hydrophobic interactions involved in the maintenance of the structure of cell envelope. Optimal salt concentration required by Haloarchaea is around 3.5–4.5 M NaCl (Grant et al. 2001). Haloarchaea contain C 50 carotenoids called bacterioruberins, responsible for red to pink pigmented colonies and bacteriorhodopsin (Ventosa A. 2006). Phyllogenetically archaea is divided into following four kingdoms:

1) **Crenarchaeota:** includes the organisms that are found at very high or low temperature
2) **Euryarchaeota:** includes hyperthermophiles, methanogens, halophiles and thermophilic methanogens
3) **Korarcheota:** includes hyperthermophilic
4) **Nanaoarcheota:** includes hyper thermophile (Madigan et al. 2009)

2.3.2 The halophilic and halotolerant bacteria

Halophiles are widely distributed in nature. Based on their varying physiological properties they could be aerobic or anaerobic chemoheterotrophs, photoautotrophic or photoheterotrophic species and chemolithotrophs (Oren 1999). *Halospirulina tapeticola* is an example of halophilic cyanobacteria (Nubel et al. 2000). They are found in high salinity places like hypersaline lakes where salt concentration is 200- 250 g/l or higher. The halophilic anoxygenic photosynthetic sulfur bacteria include extremely halophilic alkaliphilic bacteria *Halorhodospira* and *Ectothiorhodospira* (Imhoff 2001). Aerobic moderate halophiles include metabolically versatile species of the family Halomonadaceae (Rainey et al. 1995). The cellular structures of halophilic bacteria resemble with that of non halophilic members.

2.3.3 The halophilic and halotolerant eukarya

The halophilic and halotolerant eukarya grow at high salinities eg. algae *Dunaliella salina*, *Dunaliella bardawil* of the genus *Dunaliella* (Olmos et al. 2000; Olmos-Soto et al. 2002), *Picocystis salinarum* ((Roesler et al. 2002), protozoa, fungi *Trimmatostroma salinum* (Zalar et al. 1999) and black yeasts (Gunde-Cimerman et al. 2000). *Dunaliella* lack rigid cell wall and contain β-carotene globules.

2.4 Osmotic adaptation of halophiles

All biological membranes are selectively permeable but they all are permeable to water. To survive at high salt concentration, all microorganisms are required to keep their cytoplasm at least iso-osmotic with respect to the osmotic pressure of the surrounding environment. In such conditions turgor pressure is maintained by keeping the cytoplasm hyperosmotic with respect to the outer medium. Hence the halophiles have undergone osmotic adjustment or osmoadaptation (term "osmoregulation") (Reed 1984). Halophiles have adopted different strategies to cope with their hyper saline environment. One strategy involves intracellular accumulation of inorganic ions (K^+ and Cl^-) which provide osmotic balance at high salt concentration. For example a member of halophilic archaea belonging to order Halobacteriales accumulate as much KCl as NaCl in the surrounding medium. Within bacteria, the members of order Haloanaerobiales including fermentative or homoacetogenic anaerobes have exhibited such "salt in" property. Bioenergetically it costs relatively little energy. However high amount of KCl in the cytoplasm has its own limitations and to overcome those, far-reaching adaptations are required (Oren 1999a; Oren 2000b). Another strategy involves use of organic "compatible" solutes and excluding salts from the cytoplasm as much as possible to increase the osmotic pressure of the cytoplasm. Glycerol, amino acids and derivatives like betaine, ectoine, glycine and simple sugars like sucrose and trehalose can be used as compatible solutes (Galinski 1995). This strategy provides osmotic balance and maintains proper tugor pressure.

Compared to the first strategy, adaptations requirement for the intracellular enzymatic machinery are much less in case of organic osmotic solutes. However, bioenergetically organic osmotic solute production is expensive (Oren.1999). Some examples of compatible solutes accumulated by microorganisms are given in Table 2.3.

Table 2.3: Compatible solutes accumulated by halophilic microorganisms (Madigan et al. 2009)

Taxonomic group	Major solutes accumulated	Reference
Archaea		
Aerobic	2-sulfotrehalose	Desemarais et al. 1997
Anaerobic	Glycine betaine, β-glutamine, β-glutamate	Lai et al. 1999, 2000, Lai and Gunsalus 1992, Menaia et al.1993, Robertson et al. 1990a, 1990b.
Bacteria		
Photosynthetic (oxygenic)	Glycine betaine, glucosylglycerol, sucrose, trehalose	Mackay et al. 1984
Photosynthetic (anoxygenic)	Glycine betaine, ecotine, trehalose, Nα-carbamoyl- glutamine amide, sucrose, N-acetyl-glutaminyl-glutamineamide	Galinski and Truper, 1982, Galinski et al 1995,Galinski and Oren 1991,Severin et al. 1992
Heterotrophic aerobic	Ecotine, hydroxyecotine, proline, Nε - acetyllysine, glutamate, 5-oxoproline	Wohlfarth et al. 1990, 1993, Louis and Galiniski 1997, Khulman and Bremer 2002, Trotsenko and Khmelenina 2002, Severin et al. 1992
Heterotrophic anerobic	Glycine betaine, trehalose	Welsh et al. 1996
Eukarya		
Photosynthetic	Glycerol, Dimethylsulfonio-propionate	Ben-Amotz and Avron 1973, Wegmann et al. 1980, Edwards et al. 1987
Hetrotrophic	Glycerol, Arabitol	Brown 1990

2.5 Properties of halophilic proteins

Usually proteins and other macromolecules denature in the presence of high salt concentration.

Salts denature proteins and enzymes by three ways:

1. Enhancement of hydrophobic interactions which cause disruption of the tertiary structure.
2. By charge shielding: it causes interference in electrostatic interactions (inter and intra) of macromolecules.
3. Salt ion hydration: it causes reduction in free water availability which is required to maintain biological activity (Madern et al. 2000, Zaccai and Eisenberg 1991).

Due to these challenges posed by high intracellular salt concentrations, enzymatic adaptations towards high salt concentration are required. However, these adaptations pose newer challenges. Now the cells require continuous presence of high salt for the crucial biological processes (Ebel et al. 1999; Lanyi 1974). The halophilic members of order Halobacteriales, Halanaerobiales, and the aerobic red halophilic bacterium *Salinibacter* are few examples of halophiles which use inorganic ions to produce salt-adapted and salt-dependent enzymes. The extracellular enzymes produced by these halophiles remain active and stable at high salt concentration. It has been found that for most enzymes and other proteins of the archaea *Halobacteriales* (*Halobacterium Salinarum*) get denatured when exposed to solutions containing less than 1- 2 M salt. Other enzymes like lactate dehydrogenase, fumarate dehydrogenase, malate dehydrogenase and cytochrome oxidase function equally well in the presence of KCl and NaCl (Lanyi 1974). It was found that "salting-out" salts stabilize the halophilic proteins while "salting-in" salts have adverse effect on halophilic proteins. Reistad 1970 reported high aspartate and glutamate content in halophilic proteins. Excess negative charges due to the acidic amino acids on the protein surface bring about repulsion between the side groups thereby rendering the structure unstable. This effect can be counteracted by adding high concentrations of cations which reduces the negative charges and maintain structurally active and stable conformation of protein. All aerobic halophilic bacteria can tolerate high salt concentrations except *Salinibacter ruber* (Oren and Mana 2002). Halophilic bacterial cells exclude salt and prefer using organic osmotic solutes for maintaining osmotic balance. Halophilic bacterial enzymes can be of three types: a) true intracellular enzymes unexposed to high saline medium b) enzymes performing membrane-bound activities of transporters which may sense both low ion containing intracellular environment and highly saline external medium c) extracellular enzymes secreted to outer hypersaline environment.

The earliest studies have shown that intracellular enzymes like malate dehydrogenase and isocitrate dehydrogenase from *Halomonas halodenitrificans* can function at low salt conditions. This trend changes for membrane bound enzymes of the same organism such as cytochrome oxidase and lactate dehydrogenase which remain active at high salt conditions (Baxter and Gibbons 1956). Extracellular enzymes of halophilic bacteria are adapted to function at high salt conditions that existed outside the cell. The adaptation could be attributed to the presence of an excess of acidic amino acids on the surface of proteins. Salt adapted enzymes of fermentative anaerobic member of the order Halanaerobiales are characterized by high intracellular ionic concentration and remain active and stable at high salt concentrations (Oren and Gurevich 1993). Halophilic eukarya like *Dunaliella* are characterized by salt sensitive enzymes. The intracellular ion concentration is low and accumulated osmotic solute like glycerol. Interestingly, unlike other cases where cations sodium and potassium determine activity of an enzyme, in *Dunaliella* Cl^- determines activity (Gimmler et al. 1984).

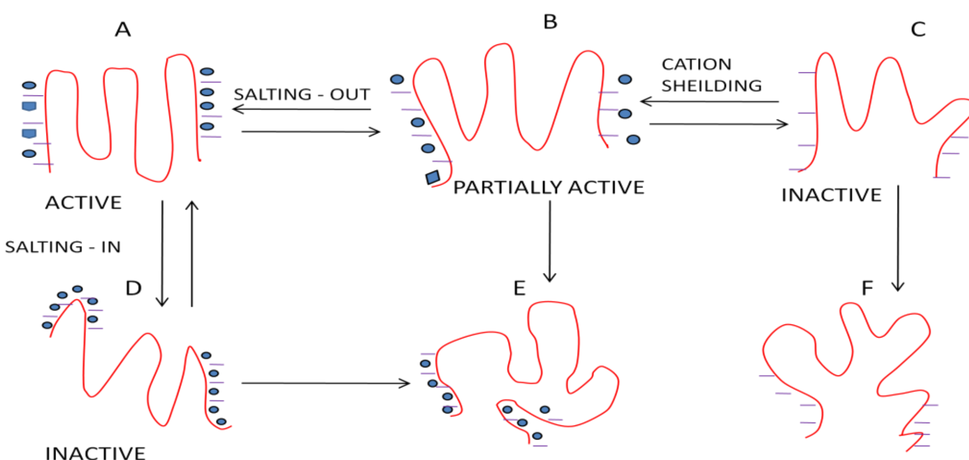


Fig. 2.3: Mechanism of salt tolerance of halophilic enzymes (Lanyi 1979).

2.6 Potential biotechnological applications of halophilic microorganisms

The halophilic microorganisms are more robust as compared to their non-halophilic counterpart due to their ability to adapt towards high salinity condition. Halophilic microorganisms present a diverse array of commercially viable opportunities for producing valuable enzymes which can function at low water activity.

2.6.1 Application of halophilic archaea

Halobacterium and *Halococcus*, belong to the family Halobacteriaceae, have been used in the production of certain traditional fermented foods ("nam pla" a fermented fish sauce, Thiland) in the Far East. It is assumed that the halobacterial proteases are also involved in the fermentation process (Thongthai et al. 1992). Some metabolic products of archaea provide specific aroma to the sauce (Lopetcharat et al. 2001). The isoprenoid diether lipids of halophilic archaea have been investigated for its suitability as a food additive, low-calorie fat substitute and emulsifier (Post and Collins 1982).

Halophilic archaea can be exploited for its biodegradative potential. A hydrocarbon-degrading halophilic archaea was isolated from a saltern pond in France which is able to degrade 48- 88 % of the straight-chain hydrocarbons and near about 19- 24 % of the aromatic hydrocarbons (Bertand et al. 1990). Some of the other important examples include hydrocarbon-degrading red archaea (Kulichevskaya et al. 1991). *Haloferax* strain D1227 was found to grow on aromatic compounds like cinnamate, benzoate and 3-phenylpropionate (Emerson et al. 1994). Dense communities of red halophilic archaea absorb solar radiation and raise the temperature of the brine in the saltern crystallizer ponds. This in turn increases salt production (Javor 2002). Another promising field in applied research is the use of proton pump of *Halobacterium salinarum*, bacteriorhodopsin for the development of photoelectric converters and building block of computer memories and processing units. Several halophilic archaea are known to synthesize biodegradable polymer like poly β-hydroxyalkanoate which is very useful in the commercial production of biodegradable plastics (Rodriguez Valera et al. 1991). The advantages halophilic archaea over already commercially exploited *Ralstonia eutropha*, for the production of poly β-hydroxyalkanoate is that halophiles can use cheap substrate such as starch for growth. They are genetically more stable and being an extremophile possesses reduced danger of contamination. Archaeal cells easily lyse in water, this property can be utilized for easier product purification and downstream processing (Ventosa and Nieto 1995). The polysaccharides produced by halophilic archaea *Haloferax mediterranei, Haloferax volcanii* and *Haloferax gibbonsii* can be of considerable biotechnological potential (Paramonov et al. 1998). These polymers have high viscosity even at low concentrations, and were resistant to extreme pH, temperature and salt concentration and can be used for the modification of rheological properties as gelling agents, emulsifiers and thickening agents (Ventosa and Nieto 1995). Another halophilic archaea *Natrialba aegyptiaca* produces an extracellular polymer poly γ-

D-glutamic acid which can find its application as a biodegradable thickener, humectants or a drug carrier in pharmaceutical and food industries. Many extracellular enzymes of halophilic archaea like proteases, amylases and lipases have potential to work at high salt conditions and are useful for biotechnological processes (Ventosa and Nieto 1995). *Natronomonas pharaonis* produce a chymotrypsinogen–B like protease which can be used as additive in detergent industry (Stan-Lotter et al.1999). Productions of a novel enzyme called cyclophilin type peptidyl prolyl cis-trans isomerase which catalyze the regeneration of denatured protein are useful in stabilization of proteins and for recombinant proteins production (Lida et al. 1997).

2.6.2 Applications of halophilic bacteria

Pediococcus halophilus is found to be associated with salted fish (Villar et al. 1985). They are known to be involved in the production of traditionally fermented salted foods in the far East e.g "Nukazuke" a paste of fermented fish in bran that contains between 10 and 15 % salt (Kuda et al. 2001). Flavouring agents 5'-guanylic acid (5'-GMP) and 5'-inosinic acid have been obtained from halophilic nucleaseH of *Micrococcus varians* subsp. *Halophilus* (Kamekura et al. 1982). Wastewaters containing high salt, toxic organic and inorganic compounds are generated during various industrial processes such as manufactures of pesticides, pharmaceuticals, oil and gas recovery. For treatment of such environmentally hazardous wastes, biodegradative potential of halophilic bacteria can be utilized. Toxic compounds such as formaldehyde (Oren et al. 1992) can be degraded at high salt concentrations by halophilic bacteria. Benzoate and other aromatic compounds can be degraded by halophilic bacteria *Halomonas halodurans* (Rosenberg 1983). A member of family Halomonadaceae was found to degrade herbicide 2, 4-dichlorophenoxyacetic acid (2, 4-D), aromatic compounds like 3-chlorobenzoic acid, benzoic acid and 4-chlorophenol (Oriel et al. 1997). Halophilic bacteria produce organic solutes such as tetrahydropyrimidines, ectoine and hydroxyectoine .These solutes have a strong in vitro stabilizing action thus increases the activity of enzyme, membranes, DNA and even whole cells against high salinity, desiccation, thermal denaturation and freezing (Margesin and Schinner 2001). Ectoine industrially produced from *Halomonas elongata* and its derivatives hydroxyectoine from *Marinococcus* M52 are commercially exploited by Bitop (Witten, Germany). Ectoine can be used as cosmetic ingredients and moisturizers (Motitschke et al. 2000). Halobacterial salt resistant exopolymers can be used as bioemulsifying and surfactant agents in enhanced oil recovery processes from saline to hypersaline oil deposits. Halophilic cyanobacteria (Sudo et al. 1995) have also found to produce ample

amounts of exopolysaccharides of biotechnological potential. Extra and intracellular enzymes including amylases, nucleases, phosphatases, proteases and esterases from halophilic bacteria have been characterized for their potential applications (Kamekura 1986; Onishi and Sonoda 1979; Onishi et al. 1983; Hinrichsen et al. 1994).

2.6.3 Application of halophilic eukarya

Halophilic unicellular eukarya green alga *Dunaliella* (*Dunaliella salina*, *Dunaliella bardawil*) biomass is a source of β-carotene. β-carotene has antioxidant properties and can be used as a pro-vitamin A (retinol), food colouring agent, as a health food and as additive to cosmetics (Borowitzka 1986). Other uses which are explored include the production of glycerol and oil which is produced from pyrolysis of *Dunaliella*. *Dunaliella* protein is easily digestable and can be used as feedstock in mariculture (crab, shrimp, and shellfish) and for livestock such as chickens (Galinski and Tindall 1992).

2.7 Extracellular enzymes from halophiles

Halophilic microorganisms produce unique enzymes on account of their ability to thrive in extreme environments such as ecological niches in saline and hypersaline environments.

2.8 Protease

Proteases, also known as peptidases, proteolytic enzymes or proteinases are the hydrolases (E.C 3.4) which cleave amide (peptide) bond in protein or peptide substrates. This enzyme is ubiquitous in all kingdoms for instance, plant, animal and microorganism. The vast diversity of proteases, in contrast to the specificity of their action, has attracted worldwide attention on exploiting their physiological and biotechnological applications (Fox et al. 1991; Poldermans B. 1990). Microbial proteases are most widely applied as additives in food processing, detergents, pharmaceuticals, diagnostic reagents, leather and waste management as well as silver recovery (Amoozegar et al. 2007). Halophilic proteases have high stability at saturated salt concentrations or organic solvent (Akolkar et al. 2008). They have been purified and characterized from *Halobacillus* sp. (Karbalaei-Heidari et al. 2009), *Bacillus* sp. (Kamekura and Onishi 1974), *Salicola* sp. (Moreno et al. 2009), *Pseudoaltermonas* sp. (Sanchez-Porro et al. 2003), *Salinivobrio* sp. (Amoozegar et al. 2007), *Halobacterium salinarum, Natrialba asiatica* (Kamekuera et al. 1992), *Haloferax mediterranei* (Stepanov VM et al. 1992, kamekura et al. 1996). Based on their amino acid sequences, proteases are classified into different families with a code letter denoting the type of

catalysis i.e. S, C, A, M, or U for serine, cysteine, aspartic, metallo or unknown type respectively. Each family is again subdivided into “clans (Rao et al. 1998).

2.8.1 Classification

Proteases are classified according to three criteria:

1. Site of action
2. Reaction mechanisms and nature of catalytic active site residues involved in mechanism
3. The evolutionary relationship, as revealed by the structure

2.8.1.1 Site of action

Based on the site of action, peptidases can be classified into two broad categories namely exopeptidases and endopeptidases.

2.8.1.1.1 Exopeptidases

The exopeptidases cleave near the ends of polypeptide chains. Based on the location of the enzymatic action either at the amino (N) or carboxy (C) terminus and the liberated residue they can be classified further into aminopeptidase and carboxypeptidase.

2.8.1.1.1.1 Aminopeptidase

Those acting at a free N terminus release a single amino acid residue are called aminopeptidases. Whereas those liberating a dipeptide or a tripeptide are termed as dipeptidyl- peptidases and tripeptidyl- peptidases, respectively. Aminopeptidases plays an essential role in many cellular functions. Many of these enzymes are zinc metalloenzymes and are widely distributed in a number microbial species which includes bacteria and fungi. Although generally intracellular in nature, an extracellular aminopeptidase has been reported from *A. oryzae*. Aminopeptidase I of *Escherichia coli* is a large protease of molecular weight around 400 KDa requires Mg^{2+} or Mn^{2+} for activity and optimum pH ranges from 7.5 to 10.5 (De Marko and Dick 1978). The Aminopeptidase II of *B. stearothermophilus* is a dimer with 80 KDa to 100 KDa molecular mass (Stoll 1976) and requires Zn^{2+}, Mn^{2+} or Co^{2+} ions for activation (Rao et al. 1998).

2.8.1.1.1.2 Carboxypeptidase

The exopeptidases cleaving peptide bond at a free C terminus to release a single residue are known as carboxypeptidases. Carboxypeptidases liberating dipeptides are called peptidyl-dipeptidases.

Other exopeptidases include are dipeptidases and omega peptidases which remove specifically the dipeptides and peptide linkages other than those of α-carboxyl to amino groups respectively. On the basis of nature of the amino acid present at the active site carboxypeptidases can be serine carboxypeptidases, metallocarboxypeptidases and cysteine carboxypeptidases e.g carboxypeptidases isolated from *Saccharomyces* sp., *Penicillium* sp., and *Aspergillus* sp. are some examples of serine carboxypeptidases. Examples of metallocarboxypeptidases are caboxypeptidases obtained from *Saccharomyces* sp. (Felix and Brouillet. 1966) and *Pseudomonas* sp. (Lu et al. 1969).

2.8.1.1.2 Endopeptidases

Endopeptidases cleave peptide bonds internally away from N and C termini of the protein substrate. Unlike exopeptidase it cannot break down a protein into monomers. Certain endopeptidases are very specific for some amino acids. The endopeptidases are classified into four subgroups based on catalytic mechanism (i) serine proteases (ii) aspartic proteases (iii) cysteine proteases and (iv) metalloproteases (Rao et al. 1998).

2.8.1.1.2.1 Serine protease

Serine proteases are a group of ubiquitous protease with serine in their active site. Molecular masses of these proteases range between 18 and 35 kDa with the exception of the *Blakeslea trispora* serine protease whose molecular mass is 126 kDa (Govind et al. 1981). Serine proteases are present in all peptidase groups namely exopeptidase, endopeptidase, oligopeptidase and omegapeptidase. Based on their structural similarities, serine proteases have been classified into 20 families which have been further subdivided into about six clans which have diverged from a common ancestor (Barett 1994). The primary structures of four clans chymotrypsin (SA), subtilisin (SB), carboxypeptidase C (SC) and *Escherichia* D-Ala–D-Ala peptidase A (SE) are unrelated. It could be suggested that serine proteases have been originated from four separate evolutionary origins. Clans SA, SB, and SC presents a case of convergent evolution where proteins possess dissimilar protein fold but similar reaction mechanism. The reaction mechanism consists of a common Ser-His-Asp catalytic triad. SE clan lacks the catalytic triad, hence different. Serine proteases are inhibited irreversibly by L-3-carboxytrans, 2, 3-epoxypropyl-leucylamido (4-guanidine) butane (E.64), 3, 4-dichloroisocoumarin (3, 4-DCI), di-isopropylfluorophosphate (DFP), tosyl-L-lysine chloromethyl ketone (TLCK) and phenylmethylsulfonyl fluoride (PMSF).

Serine proteases are generally active within the pH range of 7- 11. Substrate specificities include esterolytic and amidase activity. The isoelectric points were found nearly between pH 4- 6 for serine proteases. Serine alkaline proteases is the largest subgroup of serine proteases (15-30 kDa) produced by several yeasts, molds, bacteria and fungi with optimal pH around 10 and isoelectric point around pH 9. eg. protease from *Arthrobacter*, *Streptomyces* and *Flavobacterium* sp. (Rao et al. 1998).

2.8. 1.1.2.2 Aspartic proteases

Aspartic acid proteases (30 to 45 kDa) are acidic proteases (pH 3- 4) with aspartic acid residues in their catalytic site. Isoelectric points range for most aspartic proteases is between pH 3- 4.5. The active-site aspartic acid residue is located within the motif Asp-Xaa-Gly in which Xaa can be serine or threonine. Microbial aspartic proteases further divided into, (i) pepsin-like enzymes produced by *Rhizopus*, *Aspergillus* and *Penicillium* (ii) rennin-like enzymes produced by *Mucor* sp. The aspartic proteases are inhibited by pepstatin (Fitzgerald 1990).

2.8.1.1.2.3 Cysteine/ thiol proteases

These kinds of proteases active only in the presence of reducing agents such as hydrogen cyanide (HCN) or cysteine and contain cysteine/ thiol in their active site. The reaction mechanism involves catalytic dyad consisting of cysteine and histidine. They are further classified on the basis of their side chain specificity into four groups: (i) papain-like (ii) trypsin-like with preference for cleavage at the arginine residue iii) specific to glutamic acid and (iv) others e.g papain. Cysteine proteases are mostly active in neutral pH except a few which are active in acidic pH e.g lysosomal protease. They are inhibited by sulfhydryl agents like PCMB but are unaffected by metal-chelating agents and DFP (Rao et al. 1998).

2.8.1.1.2.4 Metalloproteases

Metalloproteases require divalent metal ion for their activity. They form the most diverse catalytic types of proteases (Barett 1995) e.g collagenases from higher organisms, thermolysin from bacteria and hemorrhagic toxins from snake venoms (Hibbs 1985; Okada 1986; Shannon 1989; Weave 1977; Wilhelm 1987). Metalloproteases are present in both endopeptidases and exopeptidases. There are 30 families of metalloproteases which have been subdivided into different clans on the basis of the nature of the amino acid that completes the metal-binding site. An alternative

classification is based on the specificity of their action: (i) neutral (ii) alkaline (iii) Myxobacter I and (iv) Myxobacter II (Rao et al. 1998).

Table 2.4: Specificity of proteases on the basis of site of action (Rao et al. 1998). Site of protease action is indicated by arrow and Xaa means any of the amino acid.

Enzyme	Peptide bond cleaved
Trypsin	Lys (or Arg) 2 ↓ — — — —
Chymotrypsin, subtilisin	Trp (or Tyr, Phe, Leu) 2 ↓ — — — — —
Staphylococcus V8 protease	-Asp (or Glu) 2 ↓ — — — — —
Papain	-Phe (or Val, Leu)-Xaa2 ↓ — — — — —
Thermolysin	— — — _ ↓ 2- Leu (or Phe) ------
Pepsin	- Phe (or Tyr, Leu)2 ↓ Trp (or Phe, Tyr)

Table 2.5: Classification of proteases (Rao et al. 1998). Site of protease action is indicated by arrow open circles indicates amino acids in peptide chain while terminal amino acid is representeby solid circle and star represents block terminus.

Protease	Mode of action	EC no.
Exopeptidase		
Amino Peptidase		3.4.11
Dipeptidyl Peptidase		3.4.14
Tripeptidyl Peptidase		3.4.14
Carboxypeptidase		3.4.16-3.4.18
Serine type protase		3.4.16
Metalloprotease		3.4.17
Cysteine type protase		3.4.18
Peptidyl dipeptidases		3.4.15 3.1.13
Dipeptidases		
Omega peptidases		3.4.19
		3.4.19
Endopeptidases		
Serine protease		3.4.21
Cysteine protease		3.4.22
Aspartic protease		3.4.23
Metalloprotease		3.4.24
Endopeptidases of unknown catalytic mechanism		3.4.99

2.8.1.2 Reaction mechanisms and nature of catalytic active site residues involved in mechanism

The catalytic site of proteases is sandwiched between specificity subsites. These subsites accommodate the side chain of a single amino acid residue from the substrate. The numbering for these sites is done from the catalytic site S1 through SN toward the N terminus of the structure and Sl' through SN' toward the C terminus. Numbering for residues accommodated from the substrate are numbered Pl through PN toward the N terminus and P1' through PN' toward the C terminus (Rao et al. 1998).

2.8.1.2.1 Serine proteases

Serine proteases have serine in its active site and catalyse the formation of a negatively charged tetrahedral transition intermediate between the substrate and the serine leading to the cleavage of peptide bond. The peptide is liberated once the acyl-enzyme intermediate is hydrolyzed by a molecule of water. This restores the Ser-hydroxyl of the enzyme.

This class has two families: the chymotrypsin family includes all the mammalian protease enzymes like, trypsin or elastase or kallikrein and chymotrypsin the substilisin family contains bacterial enzymes such as subtilisin. The 3D structure of protease from two families is different but they have the same geometry of active site and same catalytic mechanism. The serine proteases have different substrate specificities due to substitution of amino acid in subsites which interact with the substrate residues (Rao et al. 1998).

2.8.1.2.2 Cysteine protease

The cysteine proteases belong to a large superfamily of thiol-dependent endopeptidases (Barrett et al. 2004) which includes the plant proteases such as papain, several mammalian lysosomal calcium-activated cathepsins as well as several parasitic proteases like the serine proteases catalysis proceeds through a three step acyl enzyme intermediate involving a catalytic Cys-His-Asn triad analogous to the Ser-His-Asp triad of the serine proteases. Catalysis by cysteine proteases involves the hydrolysis of carboxylic acid by double-displacement pathway which involves general acid- base formation and hydrolysis of an acyl- thiol. A key difference is that in the cysteine proteases, a thiolate ion plays the role of nucleophile which form a histidine imidazolium/ cysteine thiolate ion-pair whereas in the serine proteases, nucleophile is a hydroxyl group generated by His-Im (Rao et al. 1998).

2.8.1.2.3 Aspartyl protease

Most of aspartic proteases are members of the pepsin family which includes digestive enzymes such as pepsin, a lysosomal cathepsins and chymosin D, processing enzymes such as renin and fungal proteases (endothiapepsin penicillopepsin and rhizopuspepsin). These are endopeptidases with aspartic acid residues at their catalytic site. A general base catalytic mechanism has been proposed for the hydrolysis of proteins by fungal aspartic proteases (Pearl 1987). Crystallographic studies have revealed that these enzyme molecules possess active site in between two homologous lobes with Asp-Thr-Gly-Xaa motif in both the C- and N- terminus of the enzyme, having arisen by gene duplication. Xaa is Ser or Thr whose side chains can form hydrogen bond to Asp. Each lobe contributes one aspartate residue of the catalytically active diad of aspartates as the activity requires the formation of a noncovalent homodimer (Miller et al. 1989). At optimum pH range of 2- 3, one of the two aspartate is ionized and another unionized which is required for the formation of a dimer essential to form an active enzyme. The catalytic mechanism of aspartic proteases involves a noncovalent tetrahedral intermediate although unlike serine and cysteine proteases the peptide bond cleavage is achieved by the nucleophilic attack accomplished by two simultaneous proton transfer: one from a water molecule to the diad of the two carboxyl groups and a second one from the diad to the carbonyl oxygen of the substrate. This general acid-base catalysis called a "push-pull" mechanism which leads to the formation of tetrahedral intermediate (Laszlo Polgar 1987).

2.8.1.2.4 Metallo proteases

The metallo proteases are found in higher organisms, bacteria and fungi. They are different in their sequences and their structures. The mode of action of metalloproteases depends upon the presence of bound divalent cations (Zn^{2+}, Co^{2+}, Ni^{2+}) to their active site and can be inhibited by chelating agents and dialysis e.g. crystal structure of bacterial thermolysin showed that zinc is flanked by two histidines and one glutamic acid. On the basis of X-ray studies of the complex with a hydroxamic acid inhibitor, it has been proposed that water molecule acts as a nucleophile assisted by Glu143 on the carbonyl carbon of the peptide bond, which is polarized by the Zn^{2+} ion to form a non covalent tetrahedral intermediate (Holmes and Matthews 1981). This intermediate is further degraded by transfer of the proton from glutamic acid to the leaving group (Holmes and Matthews 1987). Most of the metalloproteases enzymes were found to have motif His-Glu-Xaa-Xaa-His (HEXXH).

2.8.1.3 Classification by evolutionary relationship

Group of peptidases possessing structural similarities are believed to evolved by divergent evolution from a single ancestral protein and are thus homologous as defined by Reeck et al. 1999. On the basis of structure, mechanism and catalytic action, proteases are classified firstly into 'clan' or alternatively known as super family. Within each 'clan', peptidases sharing similar catalytic mechanism, biological functions and other properties are included into a family. The members of families show statistically significant relationship in amino acid sequence. Each family may contain many related proteases (e.g. trypsin, elastase, thrombin and streptogrisin within the same family). More than 50 clans have been reported till date indicating an independent evolutionary origin of proteolysis. Rawlings established a numbering system in which a code was assigned to each family of peptidases denoting the catalytic type (C, A, S, M, or U, for cysteine, aspartic, serine, metallo or unknown respectively) followed by an arbitrarily assigned number (Rawlings 2010).

2.9 Extracellular protease from halophiles

All of the haloarchaeal extracellular proteases are serine protease. These include proteases (40-60 kDa) from neutrophilic haloarchaea *Halobacterium salinarum* (Ryu et al. 1994; Kim and Dordick 1997), *Haloferax mediterranei* 1538 (Stepanov et al. 1992) and *Nab. asiatica* 172 P1 (Kamekura et al. 1992). Haloalkaliphilic archaea secreting extracellular protease has been repoted from an unidentified strain A2 (Yu 1991), *Natrialba magadii* (Gimenez et al. 2000) and from *Natronococcus occultus* (Studdert et al. 1997, 2001). A brief detail about halophilic proteases along with their optimum pH, salt concentration, molecular weight and substrate specificity is given in Table 2.6

Table 2.6: Extracellular protease from halophiles

Haloarcheaon	Optimum NaCl concentration	Optimum pH	Molecular weight	Substrate	References
		Haloneutrophilic			
Halobacterium Salinarum p-535	>2 M	8-9	41 kDa	azocasein	Izotova et al. (1983)
Haloferax mediterranei VKM-B 1538	>2 M	8-8.5	41 kDa	insulin b-chain, CL	Stepanov et al. (1992)
Natrialba asiatica 172 P1	2 M	10.7	46 kDa	azocasein, CL	Kamekura and Seno (1990) Kamekura et al. (1992)
		Haloalkaliphilic			
Haloalkaliphilic strain A2	>0.3	9	49 kDa	nd	Yu (1991)
Natrococcus occultus	1– 2 M	7–9	130 kDa	azocasein, gelatin,CL	Studdert et al. (1997, 2001)
Natrialba magadii	1.5 M	8–10	45 kDa	azocasein, gelatin,CL	Gim ´ enez et al. (2000)

Haloferax lucentensisVKMM007	(0.85–5.13 M)	(5.0–9.0)	57.8 kDa		Manikdan et al. 2009
Halogeometricum Borinquense TSS101	20–25 %	6.0 to 10.0	86 kDa.	azocasein	Vidyasagar et al. 2006
Halobacterium sp. SP1 (1)	25 %	8	42.1 kDa	casein	Akolkar et al. 2008
Natrinema sp. R6-5	3 mol/L	8	62 kDa		Shah et al. 2007
Chromohalobacter Sp.	4.5 M	8	66 kDa	azocasein	Vidyasagar et al. 2007
Pseudoalteromonas ruthenica	0	8.8	38 kDa	nd	Porro et al. 2009

Where: Nd: not determined; CL: chymotrypsin-like activity.

2.10 Application of protease

Proteases have found application in food industry as well as in detergent, leather and pharmaceutical industries. Major applications in food industry include baking, cheese making, meat tenderization and preparation of soya hydrolysates. Further industrial application of proteases are summarized in Table 2.7. Proteases are one of the major additives in detergents. In detergent industry, proteases accounts for approximately 25 % of total sale with a 13 billion tons worldwide production per year. The first commercial detergent BIO-40 containing bacterial protease was introduced in 1956. In another detergent BIOTEX marketed by Novo Industry A/S in 1960 contained alcalase, produced by *Bacillus licheniformis*. All detergent proteases presently available

in the market are serine proteases produced by *Bacillus* strains (Rao et al. 1998). The total enzyme sale is given in Fig. 2.4.

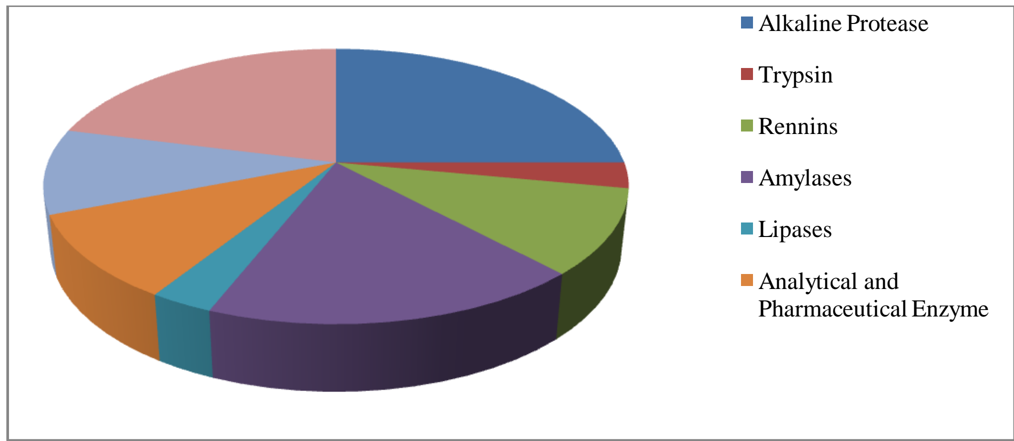


Fig. 2.4: Distribution of protease and other enzymes in total enzyme sales of the world (Godfrey and West 1996).

2.10.1 Leather industry

Major components skin and hair are made up of proteins. Proteases hydrolyze noncollagenous tissues of the skin selectively for removl of globular proteins such as albumins and globulins. This property is exploited in various steps of leather processing which includes soaking, dehairing, bating and tanning. Protease has replaced alkali used in traditional method by ensuring faster absorption of water and considerable reduction of time to soak the hide. The conventional method of dehairing and dewooling also involves treatment with sulfide to solubilise the proteins of the hair root in an extremely alkaline condition. Use of protease has replaced the need for such hazardous chemicals which cause environmental pollution and effluent disposal. At the same time enzymatically treated leather quality is much better compared to its conventional counterpart. Commercial proteases, NUE, Aquaderm, and Pyrase are used for dehairing, soaking, and bating respectively are marketed by Novo Nordisk (Rao et al. 1998).

2.10.2 Food Industry

Cheese is the product formed after coagulation of milk protein caesin. Proteases hydrolyze the specific peptide bond between the phenylalanine- methionine in casein to generate para-k-casein and macropeptides. These are acid aspartate proteases (30 kDa- 40 kDa) found in animal and microbe. Traditionally in cheese industry chymosin is preferred because of high specificity

towards casein. Chymosin is obtained from rennet extracted from the fourth stomach of unweaned calves. The method of procurement is neither commercially viable nor ethical. This has initialized the search for alternative source of protease e.g. protease produced from *Mucor michei*, *Bacillus subtilis* and *Endothia parasitica*. Recombinant protease produced in 1988 was first to be introduced in cheese making (Rao et al. 1998). Aspartame is a dipeptide composed of L-aspartic acid and the methyl ester of L-phenylalanine is used as a non calorific artificial sweetener. Under certain kinetically controlled conditions, protease (An immobilized preparation of thermolysin) of *Bacillus thermoprotyolyticus* can be used to synthesize aspartame. Major commercial producer of aspartame are e.g. Toya Soda (Japan) and DSM (The Netherlands) (Rao et al. 1998).

2.10.3 Baking industry

Endo and exoproteinases from *Aspergillus oryzae* have been used to modify an insoluble protein called gluten present in wheat flour. The modification alters the dough preparation e.g reducing mixing time, increased loaf volumes, improved strength and extensibility. The handling and machining of dough due to modification in properties brought by enzymatic treatment is easier which permits the production of a wider range of products. Proteases are also used in preparing soy sauce and other soy products from soybeans rich in good-quality protein. Proteolytic modification of soy proteins improves their functional properties e.g soluble hydrosylates with good protein yield, high solubility and low bitterness. Commercially, the soy hydrosylates find their application in protein fortified soft drinks and clinical nutrition supplements and as flavoring agents (Rao et al. 1998).

2.10.4 Medicinal use

Proteases are diverse and specific hydrolytic enzyme whose properties can be exploited for production of pharmaceuticals. Proteolytic enzymes can be used as treatment of pain, atherosclerosis, arthritis, inflammation and wound healing (Rawat et al. 2007; Gomes et al. 2009) Certain lytic enzyme deficiency syndrome can be corrected by oral administration of proteases from *Aspergillus oryzae* (Rao et al. 1998).

2.10.5 Marine industry

Undesirable attachment and accumulation of phytoplankton zooplankton and other microorganisms on ship surface is termed as fouling. Microbial biofouling occur in many steps which firstly involves the formation of a conditional layer than unicellular microorganisms of marine ecosystem attached to it and lastly large multicellular organisms attached to it and cause biofouling (Banerjee et al. 2010). This process is very much similar to bacterial biofouling of implants, when an implant is placed in the body proteins and other macromolecules adsorbed on the surface of implant and forms a conditional layer, eventually this conditional layer is colonized by neutrophills and macrophages. Colonization is followed either by collagen encapsulation or bacterial infection (Fig. 2.5). If bacterial infection takes place before encapsulation than it is impossible to cure infection (Castner et al. 2002).

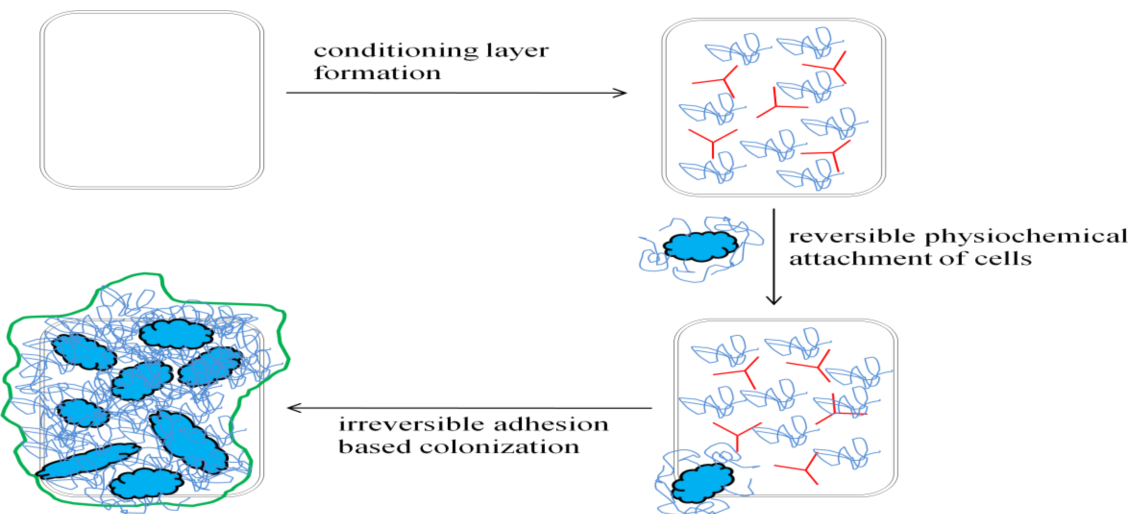


Fig. 2.5: Schematic representation of steps involved in biofouling.

Several antifouling strategies have been used to overcome such problems which include the preparation of anti-adhesion coatings by chemical or physical adsorption of hydrophilic polymer molecules that can work as a steric and/ or hydration barrier between the underlying surface and the proteins and/ or glycoproteins of the cells thus prevents the initial attachment. Several biomolecules such as Bovine Serum Albumin (BSA), dextran, hyaluronic acid, chitosan, alginate, and mannitol were used as anti- adhesive (Bongaerts et al. 2009; Luk et al. 2000). Besides these polymers such as Poly ethylene glycol (PEG), polyacrylamides were also used as anti-adhesive (Buddy et al. 1996; Callow et al. 2011). Several anti fouling coatings were used to stop fouling in

marine industry which involves use of tributyltin self-polishing copolymer (TBT-SPC) in paints. TBT-SPC was an environmental threat thus it was completely banned from January 2008. Use of enzymes in paints has provided an alternative and environmental friendly way to overcome fouling. Enzymes present in the paint directly interact with glycoproteins of microorganism thus reduces attachment to the ship surface. Organic solvents are the essential component of paints thus it is mandatory to use an organic solvent stable enzyme in such preparations. Organic solvent tolerant and stable proteases from different bacteria sources have proved beneficial in marine industry to stop fouling (Yebra et al. 2004). In industrial bio catalysis, cross-linked enzyme aggregates (CLEAs) are very beneficial in terms of economy and environment. CLEAs are easily obtained from crude enzyme thus economic over immobilization through protein engineering.

2.10.5.1 Preparation of CLEAs

General mode of preparation of CLEAs is given in Fig. 2.6 glutaraldehyde is used as cross-linking agent for decades. Glutaraldehyde bring about inter and intramolecular aldol condensations reaction between the free amino groups of lysine residues, on the surface of neighbouring enzyme molecules which involves schiff's base formation and Michael-type 1,4 addition to a, β-unsaturated aldehyde moieties resulting the formation of CLEAs (Migneault et al. 2004).

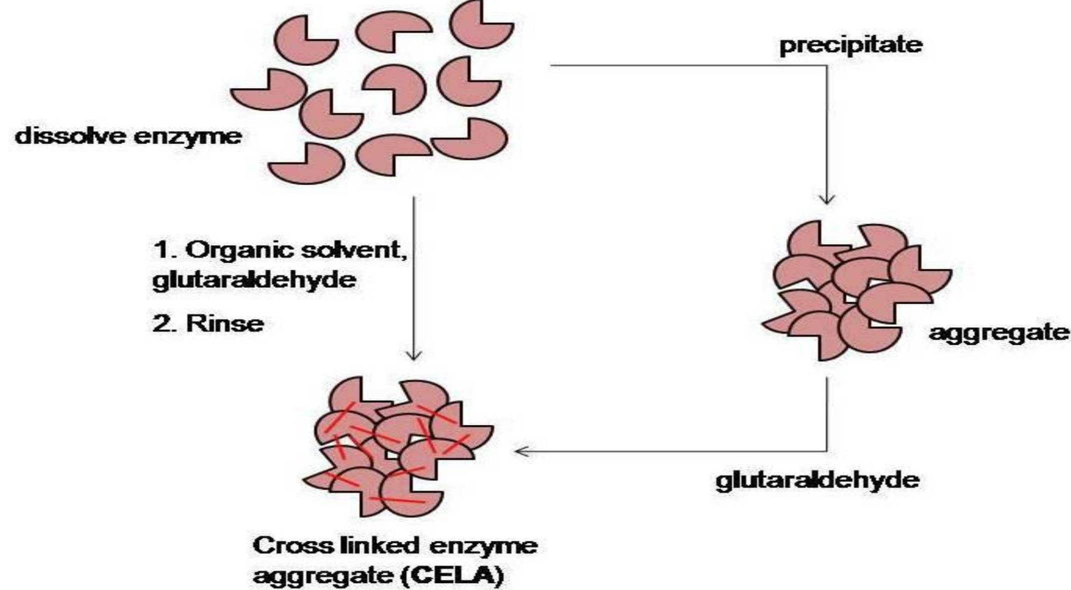


Fig. 2.6: Schematic representation of CLEA preparation (Migneault et al. 2004).

CLEAs can also be prepared by the cross- in the presence of a siloxane e.g. $(MeO)_4Si$, resulting the formation of CLEA- silica composite (Schoevaart et al. 2006). Parameters such as, pH, temperature stirring rate, precipitant, additives and cross-linking agent play an important role in CLEAs making. Extensive cross linking beyond a certain limit can result into complete loss of enzymatic activity thus enzyme and cross-linking agent ratio should be optimized. Major advantage of using CLEAs is that they can be recycled. Immobilizations of enzyme as CLEAs increase stability at high temperatures (Sheldon 2011). Skovgard et al. 2010 stabilized subtilisins, from different bacterial sources, by converting them in cross-linked enzyme aggregates- CLEAs. Protease activity of CLEAs in artificial seawater (ASW) was tested to find out their stability towards marine conditions furthermore they incubated the CLEAs in xylene an important component of ship paint. They found that catalytic activity was increased as compared to the initial catalytic activity in ASW for 7 days. A possible explanation is that continuous hydration of paint increases in seawater which leads to an increased amount of molecules leaching from the paint surface. Silicates can be used as matrices for enzyme immobilization. The silica-based materials entrap which involves conventional sol-gel processing (Ellerby et al. 1992).

Table 2.7: Industrial application of protease

Use	Source	Reference
Detergent additive	*Bacillus* sp.	Ward, 1985
Cleaning of ultrafiltration reverse osmosis membranes	*Thermus* sp. Rt41A *Bacillus* sp. Strain MK5-6	Coolbear et al. 1992; Kumar et al. 1997
Leather industry	*Aspergillus flavus, Streptomyces* sp. *B. amyloliquefaciens, B. subtilis*	Mukhopadhyay and Chandra 1993; Hameed et al. 1996; Varela et al. 1997; Malathi and Chakraborty 1991; George et al. 1995
Bioprocessing of used X-ray films or silver recovery	*Bacillus* sp. B21-2, *Bacillus* sp. B18' and *B. coagulans* PB-77	Fujiwara et al. 1991; Fujiwara and Yamamoto 1987; Gajju et al. 1996
Medical use: Treatment of burns and wounds purulent carbuncles, furuncles and deep Abscesses, Thrombolytic agent	*Aspergillus niger* LCF9, *B. subtilis* 316M, *Bacillus* sp.	Barthomeuf et al. 1992, Kudrya and Simonenko 1994, Kim et al. 1996
Food industry: Production protein hydrolysate used in hypoallergenic infant food formulations,	*B. amyloliquefaciens, B. licheniformis*	Adler-Nissen 1986; Nakamura 1993 George, 1997, Matsui et al. 1993

fortification of fruit juices or soft drinks and manufacture of protein rich therapeutic diet		
Tenderization of beef canned meat products and as a flavouring agent	*Bacillus* sp.	Takagi 1992, O'Meara and Munro 1984a and 1984b
Waste treatment: Processing of waste feathers: used as feed additive	*B. subtilis*	Dalev 1994
Chemical industry: biodegradable sucrose-polyester synthesis Catalyst for resolution of N-protected amino acid esters	*Bacillus sp.*	Patil et al. 1991**;** Chen et al. 1991
Substitute for trypsin used in preparation of animal cell culture	*Conidiobolus sp*	Chiplonkar 1985
Alternative for proteinase K in DNA isolation	*Vibrio metschnikovii* RH530	Kwon 1994

2.11 Organic solvent tolerance

Halophiles serve as a good source of salt tolerant enzymes (Ogino 2008). Organic solvent-tolerant enzymes are advantageous in synthetic biotechnology, due to their industrial advantage over the aqueous system. Protease enzyme has potential use in peptide synthesis, hypersaline waste treatment and detergents (Ray 2012). It is desirable to have a protease that is stable at wide range of pH, temperature, surfactants, oxidizing agents and solvent for its efficient use in industry (Bhunia et al. 2011). Organic solvents as reaction media for enzymatic reaction present several commercial advantages as most chemical reactions are not feasible in aqueous system. The advantages include: water-dependent side reactions become suppressed; substrate specificity and enantioselectivity get changed and improved downstream processing. Advantages of using enzymes are summarized in Table 2.8.

Table 2.8: Advantages of using enzymes in organic media Serdakowski et al. 2008.

Advantages of using enzymes in organic media
➢ solubility of nonpolar substrates increased ➢ Synthetic reactions favoured over hydrolysis ➢ Enzyme recovery become easy by filtration or centrifugation ➢ Thermostability increases ➢ Low risk of microbial contamination ➢ Enzymes can be used directly in a chemical process

The increasing concentration of organic co-solvent lowers the enzymatic activity. In cosolvent system solvent make direct contact with enzyme when used at higher concentration. Several methods have been used for protease stabilization in organic solvent system such as chemical modification (Takahashi et al. 1984), immobilization (Kise et al. 1990). Covalently modified systems suffer from drawbacks such as more labour and cost-intensive preparation of biocatalysts, mass-transfer limitations. If the enzyme is naturally solvent tolerant then no such modification is required. Proteases catalyze transestrification reactions i.e synthesis of peptide in cosolvent system.

An organic solvent-tolerant bacterium *P. aeruginosa* PST-01 was isolated as the source of potent organic solvent-stable protease producer (Ogino et al. 1999). A protease from a moderately halophilic *Bacillus* sp. strain isolated from sea water has maximum activity at pH optimum 9.0, $t_{1/2}$ 190 min at 60 ^{o}C and 1 % (w/v) NaCl. The protease shows stability in polar and nonpolar solvents at high concentrations (Sinha and Khare 2013). Halophilic enzymes are adapted to high salt conditions which reduces water activity. This high salt environment mimic with organic solvent systems thus halophilic enzymes are considered to be used in biocatalysts in aqueous organic media. A protease from *gamma-Proteobacterium* was found to be stable in various solvents (Sana et al. 2006). The protease from haloalkaliphilic archaeon *Natrialba magadii* required high salt concentration for stability in organic solvents (Ruiz et al. 2007). There are three types of solvent systems 1) cosolvent system (water + water-miscible organic solvent) 2) biphasic system (water + water-immiscible organic solvent) 3) anhydrous organic solvent system (Doukyu and Ogino 2010). The cosolvent system is more suitable for organic synthesis because thermodynamic equilibrium favours synthesis over hydrolysis (Doukyu and Ogino 2010). Enzymes are relatively more stable in the biphasic system because direct contact between enzyme and organic solvent is minimized. Most of the solvent tolerant enzymes which are stable and active in water immiscible solvents lost their activity (completely or partially) in polar solvents. Polar solvents can penetrate the protein and bring about structural changes in proteins. This is perhaps not surprising because enzymes have evolved to function in aqueous environment. Protein structure is maintained by a balance between hydrogen bonds, hydrophobic, ionic interactions and vander wall forces, water stabilize the balance and thus active confirmation of protein. In the presence of solvents such balance is lost and brings about protein unfolding (Singer et al. 1962). Although enzyme does not alter the thermodynamics of reaction but in the presence of solvent, solubility of reactants increase which leads to thermodynamic stabilization of ground state of reactants which slows down the original catalytic activity (Serdakowski et al. 2008). Water is required to maintain protein active confirmation; organic solvents remove water and thus disrupt the active confirmation of protein. Water miscible solvents remove more water than water immiscible solvents (Gorman et al. 1992). The utility of protease of *B. licheniformis* RSP-09-37 was demonstrated in trans-esterification reaction. The enzyme activity was highest in isooctane. In the presence of DMSO transesterification activity was not observed (Sareen and Mishra 2008). Organic solvent stability of an enzyme depends upon its secondary structure. Most of organic solvent tolerant enzymes have relatively more β sheats as compared to α helix (Doukyu and Ogino 2010). A protease from

moderately haloalkaliphilic bacterium, *Geomicrobium* sp. EMB2 can withstand range of solvents having a log*P* >2 but show less activity in cosolvent systems (Karan and. Khare. 2010). Some enzymes show enhancement in activity when present in organic solvents (solvent activation), a lipase from *P. Aeruginosa* activated in polar solvents like 2- propanol (Lima et al. 2004). Here, the solvent activation of the enzyme is due to the resistance of the enzyme towards denaturation by formation of multiple hydrogen bonds with water for structural flexibility. A solvent-stable protease from *Pseudomonas aeruginosa* strain K was found to be activated by water immiscible solvents. Organic solvent have low dielectric constant in comparison to water thus helps to form stronger intra- protein electrostatic interactions (Zaliha et al. 2006). An elastase from strain K was activated by both water miscible and water immiscible solvents, 17 % enhancement of the activity was observed by DMSO (Di methyl sulfoxide) (Zaliha et al. 2011). DMSO is used to catalyze synthesis reactions in homogenous organic-aqueous mixtures, thus the activity of the protease in the presence of 30–50 % DMSO suggest its further application in organic synthesis reactions. An extracellular protease from haloalkaliphilic archaeon *Natrialba magadii* show >75 % activity in 15 % (v/v) of DMSO and DMF (di methyl formamide) and at 30 % (v/v) concentration, activity was >70 % but activity was reduced at higher solvent concentration (Ruiz et al. 2007). Tsuchiyama et al. 2007 used organic-solvent-stable PST-01 protease in cosolvent systems (buffer and DMSO 1:1 v/v) to synthesize aspartame. Dobretsov et al. 2007 reported that a solvent stable protease, from *Pseudoalteromonas* sp., can be used as antifouling agent in paints to reduce larval attachment. A protease from *Bacillus cerus* showed higher activity due to the solvent activation by non polar solvents. In the presence of solvent, enzyme was resistant to denaturation by water with formation of multiple hydrogen bonds (Shah et al. 2010). Organic solvent tolerant protease from different bacterial sources has been summarized in Table 2.9.

Table 2.9: Organic solvent tolerant protease from different microorganisms (Ogino et al. 2010)

Source	Incubation Condition	Stability	Unstable in the presence	References
Pseudomonas aeruginosa K	37 °C, 14 days	25 % (v/v) Deccane, Octane	5 % (v/v) Benzene, Heptane, Xylene	Geok et al. 2003
Pseudomonas aeruginosa PST-01	30 °C, 15 days	25 % (v/v) Ethanol, methanol, DMSO, Octanol, Butanol	25 % (v/v) Benzene, Haptane, Xylene	Ogino et al. 1999
Pseudomonas aeruginosa PseA	30 °C, 72 h	25 % (v/v) Benzene, Heptane, Hexane, Toluene	25 % (v/v) Butanol	Gupta et al.2005
Pseudomonas aeruginosa PT121	30 °C, 5 or 14 days	50 % (v/v) Benzene, Heptane, Hexane, Toluene and DMSO	50 % (v/v) DMF, Ethanol,	Tang et al. 2008
Pseudomonas aeruginosa san-ai	30 °C, 10 days	25 % (v/v) DMF	25 % (v/v) Hexane, Benzene, Acetone	Karadzic et al. 2004
Bacillus sp. APR-4	4 °C, 24 h	50 % (v/v) Ethanol, Methanol Benzene, Butanol	50 % (v/v) Acetone	Kumar et al. 2005
Bacillus cereus BG1	30 °C, 1–55 days	25 % (v/v) DMSO, Ethanol, methanol	25 % (v/v) Acetonitrile	Ghorbel et al. 2003

gamma-Proteobacterium	30° C, 10 days	33 %(v/v) Ethanol, Methanol Butanol DMSO, Xylene		Sana et al. 2006
Natrialba magadii	30° C, 24 h, 1.5 M NaCl	15 % or 30 % (v/v) DMSO	15 %(v/v) Acetone, Ethanol,Acetonitrile	Ruiz and De Castro et al. 2007
Halobacterium sp. SP1	20° C, 30 min	33 % (v/v) Toluene, Xylene		Akolkar et al. 2008
halophilic *Bacillus* sp.	30° C, 24 h	50 % (v/v) Ethanol, Methanol	50 % (v/v) Benzene, Toluene	Sinha and Khare 2013
Geomicrobium sp. EMB2	30° C, 24 h	50 % (v/v) Toluene, Butanol, Heptane, Hexane,Benzene		Karan and Khare 2012
Staphylococcus aureus strain MSSA 476		Toluene, xylene and cyclohexane		Nielsen et al., 2005
Rhodococcus sp.		Benzene		Paje et al., 1997
Haloferax volcanii JCM 8879 *Natrialba asiatica* JCM 9576		Hexylether and n-decane		Usami et al., 2003
Oceanobacillus sp.		Isooctane		Pandey et al. 2012

3. MATERIALS AND METHODS

3.1 Materials

Four soil and one salt sample were collected from Sambhar salt lake Rajasthan situated in Northern India. Soil samples were collected from four different locations of Sambhar lake namely Main lake, Gudha Kyar, Jhapog kyar, Deodani kyar. Salt sample was collected from Sambhar salt industry (Table 3.1).

Table 3.1: Different soil and salt samples collected from Sambhar salt lake Rajasthan.

S. No.	Source	pH	Temperature
1.	Main lake	9.93	39 ^oC
2.	Gudha kyar	9.63	39 ^oC
3.	Jhapog kyar	9.61	40 ^oC
4.	Deodani kyar	10.01	40 ^oC
5.	Waste salt of Sambhar salt industry	9.0	39 ^oC

DNase test agar medium and pectin were purchased from SRL Pvt. Ltd. Manganese chloride, magnesium sulphate heptahydrate, potassium chloride, xylose, casein, phenylmethylsulphonyl fluoride (PMSF) and reagents for PAGE (Poly Acrylamide Gel Electrophoresis) were procured from Himedia laboratories (India). SDS PAGE (Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis) protein molecular mass markers were purchased from BIORAD. Phenyl Sepharose 6 Fast Flow resins, N-Succinyl-Ala-Ala-Ala-*p*-nitroanilide were procured from Sigma – Aldrich (USA). Amicon ultra (10 kDa) were procured from Millipore (USA). Other medium components and solvents were purchased from S.D. Fine Chem. (India). All the other chemicals were procured from different commercial sources.

3.2 Habitat

Sambhar lake is the largest inland saline wetland predominated by sodium chloride, sodium carbonate, sodium bicarbonate, sodium sulphate and lacked divalent cations (calcium and magnesium) with pH ranges from 9.5- 9.7 and temperature 37- 45 ºC (Upasani and Desai). Sambhar lake is located 96 km south west of the city of Jaipur (Northwest India) and 64 km north east of Ajmer.

3.3 Isolation of halophilic bacteria

To isolate halophilic bacteria, all soil and salt samples were incubated at 45 ºC on a rotatory incubator shaker (180 rpm) in a complex halophilic medium (Asker and Ohta. 1999). Briefly, 0.5g soil sample was inoculated in 50 ml complex medium in 250 ml Erlenmeyer flask. Composition of the complex medium is given in Table 3.2. The pH of the medium was adjusted to 9.0 with 1 M potassium hydroxide (KOH). After 8 days of incubation, 0.5 ml of this broth was further sub cultured in to 50 ml of same liquid medium under same experimental conditions. The medium was sterilized by autoclaving at 121 ^{o}C, 15 psi for 20 min in which salts (magnesium sulphate heptahydrate, sodium chloride and ferrous sulphate in separate flasks) were separately autoclaved at 121 ^{o}C, 15 psi for 20 min, cooled and then mixed with cassamino acid, yeast extract-agar mixture to avoid precipitation. After sub-culturing three times, isolates were purified by serial dilutions and repeated streaking on agar plate having same medium composition as liquid medium.

Table 3.2: Composition of the complex medium.

S.No	Media Component	Concentration (g/l)
1	Cassamino acid	7.5 g
2	Yeast Extract	10 g
3	Tri-sodium Citrate	3 g
4	Potassium Chloride	2 g
5	Magnesium Sulphate Heptahydrate	20 g

6	Ferrous Sulphate	1.6 mg
7	Manganese Chloride	0.36 mg
8	Sodium chloride	240 g

3.4 Characterization of isolates

Characterization of isolates involved morphological, physiological and biochemical characterization.

3.4.1 Morphological characterization

3.4.1.1 Morphology of halophilic isolates

Colony morphology and pigmentation were examined on complex agar plates. The cellular morphologies of all isolates were studied by using bright field microscopy under 100 X magnification. Gram staining was performed according to Dussault (1955).

For Gram staining a single colony of culture was suspended in sterile 2M NaCl solution. This suspension was used to prepare smear on a clean microscopic slide. After air drying and heat fixing, smear was washed with 5 % acetic acid for 5 minutes and dried. The slide was then stained with Gram's crystal violet followed by Gram's iodine then decolourization with Gram's decolourizer for 5 seconds and lastly slide was counterstained with Gram's safranin and washed with distilled water, air dried and observed under 100 X magnification of phase contrast microscope with oil immersion.

3.4.1.2 Spore formation

Isolated strains were also examined for the formation of spores. Spore formation was investigated by spore staining. For spore staining, a single colony of late stationary phase culture was suspended in sterile NaCl solution containing 2 M NaCl. This suspension was used to prepare a smear on a clean microscopic slide. The slide was then kept for air drying followed by heat fixing of smear. Now smear was washed with 5 % acetic acid for 5 minutes and dried. A small piece of blotting paper was adjusted on the slide and slide was placed on a beaker that has been placed in heating mantle with boiling water. Now malachite green was applied drop by drop to completely cover the blotting paper and this step was repeated again and again to prevent the blotting paper

from drying and the process continued for 10 min. After then the slide was cooled for 2 min and then washed with distilled water. Slide was then counterstained with safranin, washed with water; air dried and then observed under 100 X magnification of phase contrast microscope with oil immersion.

3.4.2 Physiological characterization

3.4.2.1 Salt requirement

To ascertain the halophilic nature, isolates were grown on complex halophilic agar plates having NaCl concentration 0- 5 M. Further, all the isolates were grown in complex halophilic liquid medium with salt concentrations ranging from 2- 5 M NaCl with KCl at 2 g/l concentration for extreme and borderline extremely halophilic isolates and 1- 4 M NaCl with KCl at 2 g/l concentration for moderately halophilic isolates. KCl was also used at 1- 4 M in the growth medium for all the isolates with NaCl at 2 g/l concentration. Initial pH was adjusted to 9.0 with

1 M KOH. Inoculated flasks were incubated at 45 ºC on a rotatory incubator shaker (180 rpm). Growth was measured in terms of optical density (OD) at 600 nm in UV-Vis spectrophotometer (Varian Cary Win UV/Vis).

3.4.2.2 pH and temperature profile

The temperature range for growth was determined by incubating agar plates containing complex halophilic medium with 2 M or 3 M NaCl (according to halophilic nature of isolates) at temperature range between 16- 60 ºC. The optimum pH for growth was determined by incubating agar plates at 45 ºC with pH range 5-12.

3.4.2.3 Sensitivity to antimicrobial agents

Antibiotic sensitivity test was performed by streaking the log phase culture on solid complex halophilic medium with 2 M or 3 M NaCl (according to halophilic nature of isolates) and applying disks (Himedia octa - disks) having antimicrobial agents- ampicillin 10 µg, penicillin-G 2 unit, gentamicin 10 µg, tetracycline 10 µg, cephalothin 30 µg, chloramphenicol 30 µg, clindamycin 2 µg, erythromycin 15 µg, oxacillin 1 µg, vancomycin 30 µg, co-trimoxazole 10 µg, cloxacillin 5 µg, cefradine 30 µg, lincomycin 10 µg and cefuroxime 30 µg. The isolates were incubated at 45 ºC for 7- 10 days. The sensitivity was reported based on the presence of zone of growth inhibition.

3.4.2.4 Pigment profile

Out of sixteen isolates, five were found to produce pigment. All pigment producing isolates were grown in liquid medium as described in 3.2. Pigment was extracted according to Oren 2001. Briefly, 10 ml log phase culture was taken and centrifuged at 10,000 *g* for 10 min (4 ºC). The pellet was washed with 4 M NaCl two times after then pigment from cell pellet was extracted with 1 ml methanol/acetone 1:1 (v/v) and vortexed for 4 hr and the extract was analyzed by scanning the absorbance in the wavelength region of 300- 800 nm using Varian UV-Vis spectrophotometer against the same solvent as a blank.

3.4.3 Biochemical characterization

Carbon substrate usage by isolates was determined using the BIOLOG Phenotype GEN III plates, (MicroStation™ System/MicroLog Hayward, USA). Each 96 well microtiter plate has 94 wells that contains a single substrate per well with one negative and one positive control well. Substrate usage was evaluated colorimetrically along with spectrophotometric cellular growth measurement. BIOLOG experiment was carried out by using 12 % (moderately halophilic isolates) and 15 % (extreme and borderline extremely halophilic isolates) salt in universal inoculating fluid IF-A. Isolates were incubated for 24 hr. to 7 days depending on the growth.

3.4.3.1 Catalase test

Isolates were also examined for checking catalase activity. Single isolated colony was picked from log phase complex medium agar plate and suspended in 2 M NaCl solution. This suspension was used to form a smear on clean microscopic slide. Now a drop of 3 % hydrogen peroxide was placed on the smear. Formation of bubbles was taken as the indication of catalase reaction.

3.4.3.2 Oxidase test

Isolates were also examined for oxidase activity. Single isolated colony was picked from log phase complex medium agar plate and suspended in 2 M NaCl solution. Now culture suspension was spreaded on oxidase disk from Himedia Ltd., blue colour development within 5- 10 seconds was taken as the positive indication of oxidase reaction.

3.4.3.3 Screening of isolates for extracellular hydrolytic activities

In order to detect the production of extracellular hydrolases, different enzymatic agar plate assays were performed according to Onishi et al. 1983 for DNase, Marcia et al. 1999 for pectinase, Amoozegar et al. 2003 for amylase and Amoozegar et al. 2008 for protease.

3.4.3.3.1 Screening of isolates for extracellular protease production

To detect the protease activity, skimmed milk complex agar plates were prepared (Amoozegar et al. 2008). 10 % skimmed milk stock solution was prepared and then sterilized by autoclaving. Complex agar medium was prepared as described earlier with 2 M and 3 M NaCl (for halotolerant and strict halophiles respectively). After autoclaving, skimmed milk stock solution added to complex medium at a final concentration of 1 %. All isolates were streaked on skimmed milk complex agar plates and incubated at 45 °C for 7- 9 days. Visible clear zone around the colony on skimmed milk complex agar plates was taken as the evidence of protease production.

3.4.3.3.2 Screening of isolates for extracellular amylase production

To detect the amylase activity, complex starch agar plates were prepared with 1 % starch with 2 M and 3 M NaCl (for halotolerant and strict halophiles respectively). In complex medium (Table 3.1) tri sodium citrate was replaced with starch. The medium was sterilized by autoclaving at 121 ^{o}C, 15 psi for 20 min in which salts were autoclaved separately, cooled and mixed with complex mixture to avoid precipitation. All isolates were streaked on starch agar plates and incubated at 45 °C for 7- 9 days. Then plates were flooded with 0.3 % I_2 – 0.6 % KI solution (Amoozegar et al. 2003). A clear zone around colonies on complex starch agar plates was taken as indication of amylase production.

3.4.3.3.3 Screening of isolates for extracellular pectinase production

To detect the extracellular pectinase activity, complex agar plates were prepared with 1 % pectin, 0.5 % yeast extract, 2 M and 3 M NaCl (for halotolerant and strict halophiles respectively). In complex medium (Table 3.1) tri sodium citrate was replaced with pectin. The medium was sterilized by autoclaving at 121 ^{o}C, 15 psi for 20 min in which salts were autoclaved separately, cooled and mixed with complex mixture to avoid precipitation. All isolates were streaked on plates and incubated at 45 °C for 7- 9 days. After then plates were flooded with 0.3 % I_2 – 0.6 % KI solution (Marcia et al. 1999). A clear zone around colonies on agar plates was taken as indication of extracellular pectinase production.

3.4.3.3.4 Screening of isolates for extracellular DNase production

DNase activity of the strains was detected by using 42 g/1 of DNase test agar medium (SRL Ltd.) with 2 M and 3 M NaCl (for halotolerant and strict halophiles respectively) 2 g/l KCl and 10 g/l magnesium sulphate heptahydrate. All isolates were streaked on plates and incubated at 45 °C for 7- 9 days. After then plates were flooded with 1 N HCl solution. Clear zone around the grown colony was taken as measure of DNase activity.

3.5 Protease production by the selected isolates

Isolates showing significant zone of clearance on skimmed milk agar plate were further used for protease production in liquid medium. All the isolates were grown in protease production medium having same composition as that of growth medium but cassamino acid was replaced with casein protein (10 g/1). Final pH of production medium was set at 9 by 1 M KOH. Samples were withdrawn aseptically at different time intervals for determination of protease activity. The cell free supernatant was used as the crude enzyme source.

3.5.1 Protease activity assay

The culture was centrifuged at 10,000 *g* for 10 min (4 ºC) and the culture supernatant was used as a source of protease. The proteolytic activity of the crude enzyme, with casein as the substrate, was determined by the modified method of Kunitz (1947). Briefly, 0.2 mL of crude enzyme was added to 0.8 mL of substrate solution (50 mM Tris buffer, pH 9.0, concentration of casein was 1 % in final reaction mixture) and the mixture was incubated at 45 ºC for 30 min. The reaction was stopped by adding 1.0 mL of 10 % trichloroacetic acid (TCA) and kept at room temperature. After 15 min, the supernatant was separated by centrifugation at 12,000 *g* for 15 min and the absorbance was recorded spectrophotometrically at 280 nm with tyrosine as the standard. The blank was prepared in which 1.0 mL of TCA was added before enzyme addition. One unit of protease activity was defined as the amount of enzyme required to produce 1 μg of tyrosine per minute. Standard graph for tyrosine estimation is given in Fig. 3.1. For standard preparation, a stock solution of tyrosine (1 mg/ml) was prepared in 50 mM Tris buffer, pH 9.0 and was diluted with the same buffer in a concentration range of 50 microgram/ml to 1 mg/ml and then 1 ml of 10 % trichloroacetic acid was added to 1 ml of tyrosine standard solution kept at room temperature. After 15 min, the supernatant was separated by centrifugation at 12,000 *g* for 15 min and the

absorbance was recorded spectrophotometrically at 280 nm. Absorbance at 280 nm was plotted against tyrosine concentration in µg/ml.

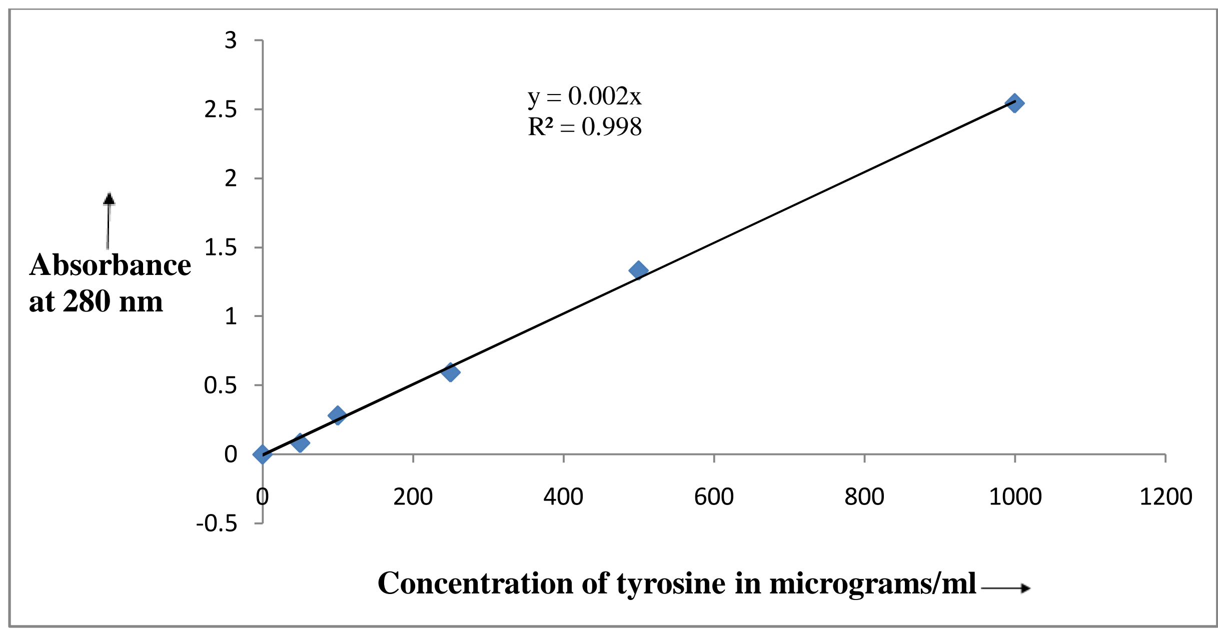


Fig. 3.1: Standard graph for enzyme activity (Caseinolytic).

3.5.2 Determination of specific activity of enzyme

Specific activity of an enzyme is defined as total enzyme units present per milligram of protein. Here specific activity is defined in terms of enzyme U/ mg of protein. Protein estimation was done by Bradford reagent. Bradford reagent was prepared by dissolving Coomassie Brilliant Blue G 250 dye in 10 ml ethyl alcohol and 20 ml of 85 % phosphoric acid then final volume was adjusted to 200 ml with distilled water. Finally it was filtered through Whatman filter paper no. 1 and kept at 4 ºC in an amber coloured bottle. For protein estimation, briefly 0.2 ml of sample solution was mixed with 1 ml of Bradford reagent and absorbance was taken at 540 nm. For blank 50 mM Tris buffer, pH 9.0 with 3 M NaCl was used in place of sample solution. Standard graph for protein estimation by Bradford reagent using BSA is given in Fig. 3.2. For standard preparation, a stock solution of BSA (1 mg/ml) was prepared in 50 mM Tris buffer, pH 9.0 with 3 M NaCl and was diluted with the same buffer at different concentration (0.02- 0.5 mg/ml). One ml of Bradford reagent was added to 0.2 ml of each protein solution and absorbance was taken at 540 nm. Absorbance at 540 nm was plotted against protein concentration in mg/ml.

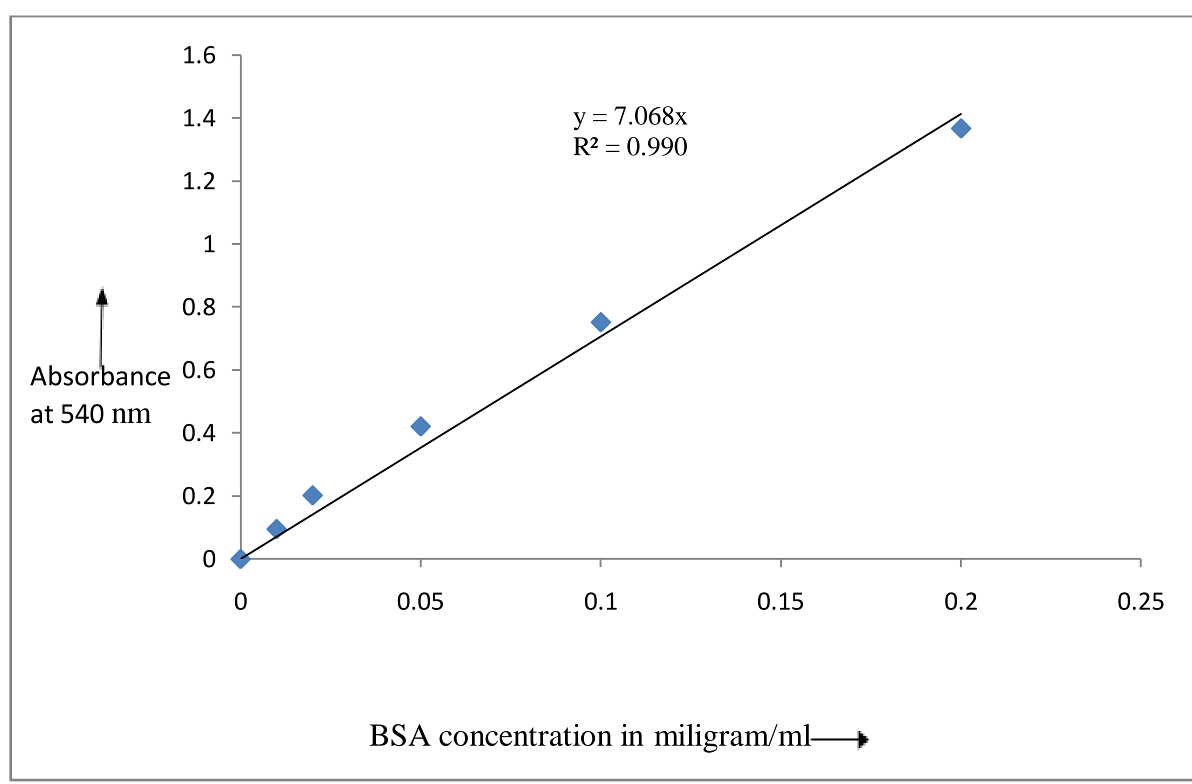


Fig. 3.2: Standard graph for protein estimation by Bradford reagent using BSA.

On the basis of higher enzyme and specific activities, one isolate (MSL-1) was selected and used in further studies.

3.6 Genotypic characterization of selected isolate

For genotypic characterization, genomic DNA of MSL-1 was isolated using DNA extraction kit of Himedia ltd. The gene encoding 16S rRNA was amplified by polymerase chain reaction (PCR). Sequence of primers used is given in Table 3.3.

Table 3.3: Sequence of primers used to amplify 16S rRNA gene of isolate MSL-1.

S. No.	Primer Sequence	Type
1.	27f - 5'-AGAGTTTGATCATGGCTCAG-3' 1492r- 5'-GGTTACCTTGTTACGACTT-3	Universal Eubacterial
2.	Arc21F 5'-GCCGGAGGTCATTGCTAGTGGAGTC-3' Univ 529R 5'-AGGAGGTGATCCAGCCGCAGATTCC-3'	Universal Archaeal
3.	A571F 5'- GCYTAAAGSRICCGTAGC -3' UA1204 R5'- TTMGGGGCATRCIKACCT -3'	Universal Archaeal

50 µL of PCR reaction cocktail **was** used with composition listed in Table 3.4.

Table 3.4: Composition of 50 µL PCR reaction cocktail.

Reagents	Stock concentration	Final concentration	Amount in 50 µL reaction
Pfu buffer with $MgSO_4$	10 X	1X	5 µL
dNTPs	10 mM	0.2 mM	5 µL
Forward primer	10 µM	0.4 µM	2 µL
Reverse primer	10 µM	0.4 µM	2 µL
DNA polymerase	2.5 U/µL	5 U/µL	2 µL
Template DNA	20 ng/µL	20 ng/µL	1 µL
Nuclease free water	-	-	33 µL

3.6.1 PCR programme

PCR programme used to amplify 16S rRNA gene has been given below in Table 3.5.

Table 3.5: PCR programme used to amplify 16S rRNA gene of isolate MSL-1.

94 ºC	4 min	Initial denaturation
94 ºC	30 cycles of 60 s	Denaturation
55 ºC	30 s	Primer annealing
72 ºC	60 s	Primer extension
72 ºC	10 min	Final extension

3.6.2 Agarose gel electrophoresis

The purity and size of genomic DNA and amplified PCR products were analyzed by gel electrophoresis on 0.8 % agarose gel prepared in 1 X TAE (Tris Actetate EDTA) buffer containing 0.5 µg/ml ethidium bromide. 1 kb plus ladder was used as a molecular weight marker. The gel was visualized in gel documentation system (BIORAD).

Tris Actetate EDTA (TAE) buffer: A 10 X stock of TAE buffer was prepared by dissolving Tris-base 48.4 g, glacial acetic acid 10.9 g, EDTA 2.92 g in one litre of distilled water.

Ethidium Bromide (EtBr) stock solution (10 mg/ml): 100 mg EtBr was dissolved in 10 ml of distilled water to prepare stock solution of ethidium bromide.

Gel Loading dye: 30 % (v/v) glycerol, 0.25 % (w/v) Bromophenol blue.

0.8 % (w/v) agarose gel was prepared by dissolving 0.4 g agarose in 50 ml of 1 X TAE buffer and heated in microwave till the solution become transparent. Solution was cooled up to 50 ^{o}C and then 2.5 µl of EtBr stock solution was added in to it to get a final concentration of 5 µg/ ml. Warm agarose mix was poured in to horizontal gel casting tray containing a gel comb at its proper place. The gel was allowed to solidify on room temperature after than comb was removed carefully and the gel tray along with gel was submerged in tank buffer (1 X TAE). The DNA samples were prepared by adding 1 µl of gel loading dye to 5 µl of DNA solution and were loaded into the well along with 1 kb plus DNA marker to determine the size of amplified fragment. The electrodes present at two sides of the tank were connected to power supply and 50 V direct current was

applied. When dye migrated to 2/3 rd of the gel, the electric current was turned off and DNA was visualized in gel documentation system (BIORAD).

3.6.3 Phylogenetic analysis and strain submission

16S rRNA gene sequence was determined by Eurofins Genomics India Pvt Ltd. The 16S rRNA gene sequence was aligned and compared with available sequences of gene bank at National Centre for Biotechnology Information (NCBI) using Basic Local Alignment Search Tool (BLAST). The phylogenetic tree was constructed by the neighbour-joining method. Bootstrap analysis was used to evaluate the tree topology of the neighbour-joining data by performing 1,000 re-sampling. Sequence was submitted to the NCBI Gene Bank under the accession number **KF424537**.

3.7 Lipid and cellular fatty acid analysis

Total lipid analysis was done using thin layer chromatography (TLC). Lipid types were analyzed after separation on TLC plates coated with silica gel. Total lipid extraction and polar lipid analysis on silica gel G plates was followed according to Elevi et al. (2004). Cellular fatty acid analysis was done by Royal Life Sciences Pvt. Ltd. (India).

3.7.1 Extraction of membrane polar lipids

For lipid extraction, all the isolates were grown in liquid medium containing 2 and 3 M NaCl for halotolerant and strict halophiles respectively. Briefly 20 ml of liquid broth of the isolate was centrifuged at 10,000 g for 15 min and the pellet was suspended in 1 ml water. Total lipid extraction was done with 3.75 ml chloroform-methanol (1:2 v/v) for 4 h. After collection of extract, lipids were re-extracted from pellet with 4.75 ml methanol-chloroform-water (2:1:0.8 v/v). Both the supernatants were mixed and then chloroform and water (2.5 ml each) were added. After centrifugation, phase separation was done. Chloroform phase was collected and kept for drying. Lipids obtained after drying were then suspended in small volume of chloroform.

3.7. 2 Thin layer chromatography (TLC) of lipids

Lipid extracts were directly applied to silica gel TLC plates. For separation of total lipids the mobile phase used was hexane/diethyl ether/formic acid (80: 20: 1 v/v/v). Visualization was done with 5 % concentrated sulphuric acid in 95 % ethanol (Buckner et al. 1999). For separation of polar lipids mobile phase used was chloroform-methanol-acetic acid-water (85: 22.5: 10: 4 v/v).

Plates were then sprayed with 0.5 % α- naphthol in 50 % methanol. After drying, plates were again sprayed with 5 % H_2SO_4 in ethanol and heated at 150 °C. Glycolipids were developed as blue-purple spots.

3.8 Characterization of crude enzyme

3.8.1 Effect of pH on protease activity

The effect of pH on the activity of crude enzyme was studied by carrying out the reaction at pH values ranging from 7.0 to 12.0. To maintain the reaction mixture at various pH values following buffers were used: 50 mM phosphate (pH 7.0), 50 mM Tris–buffer (pH 8.0–9.0), 50 mM glycine-NaOH (pH 10.0–12.0). Substrate was prepared in the above mentioned buffers and 0.2 mL of crude enzyme was added to 0.8 mL of substrate solution (casein concentration was 1 % in final reaction mixture) and the mixture was incubated at 45 ºC for 30 min.

3.8.2 Effect of temperature on activity of enzyme

The effect of temperature on the activity of crude protease was examined by incubating the reaction mixture within the temperature range 30- 80 ºC. Briefly 0.2 mL of crude enzyme was added to 0.8 mL of substrate solution (50 mM Tris buffer, pH 9.0, concentration of casein was 1 % in final reaction mixture) and the mixture was incubated at different temperatures for 30 min.

3.8.3 Effect of additives and surfactants on protease activity

The effects of phenylmethylsulphonyl fluoride (PMSF), ethylenediaminetetraacetic acid (EDTA) and urea on crude protease activity were determined by including them in reaction mixture at a concentration of 10 and 50 mM respectively. The effects of surfactants Triton X-100, Tween 80, Tween 20, sodium dodecyl sulphate (SDS) and cetyltrimethylammonium bromide (CTAB) on enzyme activity were studied at a concentration of 0.5 and 1 % (w/v). The activities were determined and presented as relative activities. The activity of the enzyme, incubated without any additive or surfactant, was taken as 100 % (control). 0.2 mL of crude enzyme was added to 0.8 mL of substrate solution (50 mM Tris buffer, pH 9.0, concentration of casein was 1 % in final reaction mixture) containing additive at required concentration and the mixture was incubated at 45 ºC for 30 min.

3.8.4 Effects of organic solvents on protease activity

The crude enzyme activity was determined in the presence of 30 % and 50 % (v/v) of various organic solvents (ethanol, methanol, hexane DMF and DMSO). The activities were measured and presented as relative activities. The activity of the enzyme measured in substrate solution (50 mM Tris buffer, pH 9.0, concentration of casein was 1 % in final reaction mixture) without any organic solvent was taken as 100 % (control). Reaction mixture was incubated at 45 ºC for 30 min.

3.9 Production optimization of extracellular protease from isolate *Halobiforma* sp. strain BNMIITR in shake flask

For protease production, 1 mL of 96 hour grown culture (in complex medium) was used as an inoculum in 100 mL of production medium containing composition as given in Table 3.6. Initial pH of the production medium was set to 9.0 by 1 M KOH. Samples were withdrawn aseptically at different time intervals for determination of protease activity. The cell free supernatant was used as the crude enzyme source.

Table 3.6: Composition of protease production medium.

S.No	Media Composition	Concentration (g/l)
1.	Sodium Chloride	180 g
2.	Magnesium Sulphate Heptahydrate	20 g
3.	Potassium Chloride	2 g
4.	Trisodium Citrate	3 g
5.	Casein	10 g
6.	Manganese Chloride	0.36 mg
7.	Ferrous Sulphate	1.6 mg

3.9.1 Effect of Salinity

Isolate *Halobiforma* sp. train BNMIITR can grow in a wide range of salinity (2- 5 M NaCl and 1-4 M KCl). Effect of NaCl on protease production was examined by growing the isolate *Halobiforma* sp. strain BNMIITR at a NaCl range of 2- 5 M in the production medium having initial pH 9.0. Incubation was carried out at 45 ^{o}C on a rotatory incubator shaker (180 rpm). Effects of various salts such as KCl, Na_2SO_4, Na_2PO_4, $NaNO_3$ and CH_3COONa on protease production were also evaluated similarly.

3.9.2 Effect of pH

The isolate *Halobiforma* sp. strain BNMIITR can grow in a broad range of pH (6- 11). Effect of pH on protease production was examined by growing the isolate *Halobiforma* sp. strain BNMIITR in a pH range 7- 11 with 3 M NaCl in the production medium. Incubation was carried out at 45 ^{o}C on a rotatory incubator shaker (180 rpm). After 24 hours, samples were taken at regular interval of 12 hour and protease activity assay was done as mentioned in section 3.5.1.

3.9.3 Effect of temperature

The isolate *Halobiforma* sp. strain BNMIITR can grow in a broad range of temperature. Effect of temperature on protease production was examined by growing the isolate *Halobiforma* sp. strain BNMIITR in a temperature range 30- 55 ^{o}C with production medium having initial pH 10.0 and 3 M NaCl. Incubation was carried out at 45 ^{o}C on a rotatory incubator shaker (180 rpm). After 24 hours samples were taken at regular interval of 12 hour and protease activity assay was done as mentioned in section 3.5.1.

3.9.4 Effect of various carbon sources on protease production

Effect of carbon sources on protease production was studied by replacing tri sodium citrate from the production medium mentioned in Table 3.4 with different carbon sources at 1 % (w/v) concentration. The production medium having initial pH 10 and 3 M NaCl was used. Incubation was carried out at 45 ^{o}C on a rotatory incubator shaker (180 rpm). Different carbon sources used were glucose, fructose, sucrose, lactose, galactose, glycerol, mannitol, starch, xylose, tri sodium citrate, citric acid and low cost agro industrial byproducts like wheat bran, wheat flour. After 24 hours, samples were taken at regular interval of 12 hour and protease activity assay was done as mentioned in section 3.5.1.

3.9.5 Effect of various nitrogen sources on protease production

Effect of nitrogen sources on protease production was studied by replacing casein from production medium mentioned in Table 3.4 with different nitrogen sources at 1 % (w/v) concentration. The production medium having initial pH 10 and 3 M NaCl was used. Incubation was carried out at 45 ^{o}C on a rotatory incubator shaker (180 rpm). Peptone, casein, gelatin, yeast extract, soy peptone, soy bean, casein digest, cassamino acid and skim milk powder, inorganic nitrogen sources such as NH_4NO_3, NH_4Cl, $NaNO_3$, $(NH_4)_2SO_4$, urea and low cost agricultural byproducts such as soybean meal, soy bean husk, chick pea flour and chick pea husk were used to investigate their effects on protease production. After 24 hours, samples were taken at regular interval of 12 hour and protease activity assay was done as mentioned in section 3.5.1.

3.9.6 Effect of various amino acids on protease production

Amino acids are known to catabolically repress the protease production. The effect of amino acids on protease production was checked by supplementing the medium with various amino acids at concentrations of 0.1, 0.5 and 1 %. The production medium having initial pH 10 and 3 M NaCl was used. Incubation was carried out at 45 ^{o}C on a rotatory incubator shaker (180 rpm). After 24 hours samples were taken at regular interval of 12 hour and protease activity assay was done as mentioned in section 3.5.1.

3.9.7 Effect of various surfactants on protease production

The effect of surfactants on protease production was checked by supplementing the medium with various surfactants at concentrations of 0.1, 0.5 and 1 %. The production medium having initial pH 10 and 3 M NaCl was used. Incubation was carried out at 45 ^{o}C on a rotatory incubator shaker (180 rpm). After 24 hours, samples were taken at regular interval of 12 hour and protease activity assay was done as mentioned in section 3.5.1.

3.10 Enzyme purification

3.10.1Crude enzyme production

One mL of 96 hour grown culture (in complex medium) was inoculated into 100 mL (500 ml Erlenmeyer flask) of production medium containing composition as given in Table 3.4 except tri sodium citrate was replaced with xylose. Initial pH of the production medium was set to 10 by 1 M KOH. Twenty Erlenmeyer flask of 500 ml containing 100 ml medium were incubated at 45 ^{o}C for

96- 120 hours. Two liter supernatant was obtained by centrifugation of culture at 12,000 g for 20 min (4 ºC). The culture supernatant was used as a source of crude enzyme. The caseinolytic activity of crude enzyme was determined. Two liter of crude enzyme was concentrated to 200 ml by using Pall advanced sepration system (Manual centramate system) with 10 kDa membrane cassettes (Pall omega 10 kDa T series).

3.10.2 Enzyme precipitation

Precipitation of crude enzyme was carried out by using sodium sulphate and ammonium sulphate. Crude enzyme solution contained high NaCl concentration (3 M). For precipitation, sodium sulphate was added to crude enzyme solution up to 20 % (w/v) and further increase in concentration of sodium sulphate couldn't be achieved as it could not be dissolved in the crude enzyme solution. Precipitate was collected by centrifugation at 12,000 g for 10 min and protease activity was checked using standard assay protocol. No protease activity was found in the precipitate. Again precipitation was done with ammonium sulphate but due to the presence of high NaCl it was not possible to dissolve ammonium sulphate beyond 40 % saturation. Precipitate was collected and checked for protease activity but no activity was found. Thus enzyme precipitation was done with pre chilled acetone. Acetone precipitation was done in three different methods:

Method I: Pre chilled acetone was added to crude enzyme up to 30 % saturation and the precipitate (A1) was collected by centrifugation at 8,000 g for 10 min, then supernatant was again precipitated with gradual addition of pre chilled acetone up to 60 % saturation and kept at -20 ^{o}C for one hour and precipitate (A2) was collected, supernatant was again precipitated with gradual addition of pre chilled acetone to 85 % saturation and kept at -20 ^{o}C for one hour and precipitate (A3) was collected. All the fractions A1, A2 and A3 were checked for protease activity.

Method II: Pre chilled acetone was added to crude enzyme up to 40 % saturation and precipitate (B1) was collected, then supernatant was again precipitated with gradual addition of pre chilled acetone to 70 % saturation and kept at -20 ^{o}C for one hour and precipitate (B2) was collected, supernatant was again precipitated with gradual addition of pre chilled acetone to 85 % saturation) and kept at -20 ^{o}C for one hour and precipitate (B3) was collected. All the fractions B1, B2 and B3 were checked for protease activity.

Method III: Pre chilled acetone was added to crude enzyme up to 50 % saturation and precipitate (C1) was collected, then supernatant was again precipitated with gradual addition of pre chilled

acetone 70 % saturation saturation and kept at -20 ^{o}C for one hour and precipitate (C2) was collected, supernatant was again precipitated with gradual addition of pre chilled acetone to 85 % saturation and kept at -20 ^{o}C for one hour and precipitate (C3) was collected. All the fractions C1, C2 and C3 were checked for protease activity. Among all the above mention methods, maximum activity and specific activity was found when precipitation was preceded according to method II. Firstly pre chilled acetone was added to concentrated enzyme up to 40 % (v/v) and kept at -20 ^{o}C for one hour and precipitate was collected by centrifugation at 8,000 g for 10 min then this precipitate was discarded as it contain undesired extra proteins. Now supernatant was again precipitated with gradual addition of pre chilled acetone upto 80 % saturation and kept at -20 ^{o}C for one hour and precipitate was collected by centrifugation at 8,000 *g* for 10 min. The precipitate was dissolved in 50 ml of 50 mM tris buffer pH 9.0 containing 1.2 % NaCl. The solution was further concentrated to 5 ml using centrifugal concentrators (Amicon ultra Milipore 10 kDa) in centrifuge at 5000 *g* and 4 ^{o}C.

3.10.3 Hydrophobic interaction chromatography

The concentrated enzyme was further purified by hydrophobic interaction chromatography (HIC). HIC purification was carried out under gravity flow mode. Two ml of concentrated enzyme solution was loaded on a Phenyl Sepharose 6 Fast Flow column (1 cm × 24 cm bed volume 20 ml), which was equilibrated with 60 ml tris buffer pH 9.0 containing 25 % NaCl. The column was washed with 50 ml equilibrating buffer until no protein was detected in the washings. Further elution, was done by applying a linear gradient of NaCl (20 %- 0 %) in 50 mM tris buffer pH 9.0 (20- 0 %). The enzyme was eluted at 11- 10 % NaCl in tris buffer 50 mM pH 9.0. Fractions having protease activity were pooled together for further characterization.

3.10.4 Ultrafiltration

Pooled fractions were concentrated using centrifugal concentrators (Amicon ultra Milipore 10 kDa) in centrifuge at 5000 *g* and 4 ^{o}C. The concentrated protein sample was than buffer exchanged with 50 mM tris buffer pH 9.0 and 1.2 % NaCl (40 ml) using centrifugal concentrators (Amicon ultra Milipore 10 kDa) in centrifuge at 5000 *g* and 4 ^{o}C. Buffer exchange with 50 mM tris buffer pH 9.0 containing 1.2 % NaCl was repeated three times. Final enzyme in 50 mM tris buffer pH 9.0 containing 1.2 % NaCl was used for all further studies.

3.10.5 Polyacrylamide gel electrophoresis

3.10.5.1 Casein zymography

Casein zymography was done according to Venugopal et al. 2006 with minor modifications. Casein was co polymerized with 10 % polyacrylamide gel at a final concentration of 1 %. The purified enzyme sample was applied in non reducing conditions at 90 V with running buffer having 20 mM NaCl. After electrophoresis, gel was transferred to 50 mM tris buffer having 1.2 % NaCl pH 9.0 and incubated at 45 ^{o}C for 12 hours. Stained with 0.1 % Coomassie blue R-250 (in methanol–acetic acid–water, 40: 10: 50) for 6 hours followed by destaining with methanol-aceticacid-water (5: 10: 85) for 18 hours.

3.10.5.2 SDS PAGE

Further molecular weight and purity of the enzyme was confirmed by SDS PAGE analysis.

Reagents for PAGE

Solution A: A stock solution of 29 % (w/v) acrylamide and 1 % (w/v) bisacrylamide premix was used from Himedia.

Solution B: Resolving gel buffer, 1.5 M tris HCl pH 8.8

Solution C: Stacking gel buffer, 1.0 M tris HCl pH 6.8

Solution D: 10 % (w/v) SDS in distilled water

Solution E: 10 % ammonium per sulphate (APS freshly prepared) in distilled water

Solution F: N, N, N,'' N''- tetramethylenediamine TEMED

Reagents	Resolving gel 12 % ml	Stacking gel (5 %) ml
Solution A	4	0.5
Solution B	2.5	
Solution C		0.38
Solution D	0.1	0.02
Solution E	0.1	0.02

Solution F	0.004	0.003
Distilled water	3.3	2.5
Total volume (ml)	10	3

3.10.5.2.1 Casting of gel

Gel electrophoresis was done using BIORAD Mini format 1 D electrophoresis system (tetra cell). A sandwich of glass plates were assembled and were held by using clamps. Resolving gel was prepared by mixing all the components followed by the addition of APS and TEMED solutions. The mixture was gently mixed and poured between the plates using 1 ml micro-pipette and leaving the adequate space at the top for pouring the stacking gel after polymerization. Resolving gel was overlaid by distilled water to prevent oxygen from diffusing into the gel which inhibits polymerization. After completion of polymerization, overlay was poured-off and top of gel was washed several times with de-ionized water to remove any un-polymerized acrylamide. Stacking gel was poured similarly as the resolving gel and comb immediately inserted into the mixture to form wells. After polymerization of stacking gel, comb was carefully removed and formed wells were rinsed with de-ionized water and polymerized gel was used for electrophoresis.

3.10.5.2.2 Sample preparation

Sample buffer was prepared for zymography and SDS PAGE having following composition

Reagents	**Gel loading buffer for SDS PAGE Gel**	**Gel loading buffer for zymogram**
Distilled water	4 ml	6.5 ml
0.5 M Tris HCL	1.25 ml	1.25 ml
50 % glycerol	2 ml	2 ml
10 % SDS	2 ml	
Bromophenol blue	1.5 mg	1.5 mg
β- mercaptoethanol	0.5 ml	

Protein samples were diluted with sample buffer at 2:1. For zymogrphy diluted samples were directly loaded to the gel whereas for SDS PAGE, samples were first denatured at 95 ^{o}C for 5 minutes and then centrifuged 12,000 *g* for 5 minutes to remove debris and then supernatant was loaded to the gel.

3.10.5.2.3 Electrophoresis

Tris- glycine buffer for zymogrphy (1 X): 25 mM Tris, 250 mM glycine (pH 8.3)

Tris- glycine buffer for SDS PAGE 1 X): 25 mM Tris, 250 mM glycine (pH 8.3) and 0.1 % SDS

Gel was placed in electrophoresis system and chamber was filled with tris- glycine buffer. Total 30 µl of sample was loaded on the gel using micropipette. Electrophoresis was carried out at a constant voltage of 100 volt for 2 hours until dye reached to the bottom. Gel after electrophoresis was removed and stained for 4 hours with gentle shaking in 0.25 % Coomassie Brilliant blue R-250 (w/v) in methanol: glacial acetic acid: water (50: 10: 40) at room temperature. Gel was de stained by destaining solution methanol: glacial acetic acid: water (50: 10:40) for 6 hours with gentle shaking on gel rocker. This destaining procedure was repeated many times to get protein bands over a clear background.

3.11 Characterization and application of purified extracellular protease from isolate *Halobiforma* sp. BNMIITR

Purified enzyme was (50 mM Tris buffer pH 9.0, 1.2 % NaCl) buffer exchanged with50 mM tris buffer pH 9.0 containing salt concentration ranging from (0-10 %). Relative activities were calculated and activity at 1.2 % NaCl was taken as 100 %. Purified enzyme obtained after HIC was found to lose its 80 - 90 % activity in the absence of NaCl and it requires minimum 1- 2 % NaCl to retain its activity. Thus enzyme was buffer exchanged with 50 mM tris buffer pH 9.0 containg 1.2 % NaCl using centrifugal concentrators (amicon10 kD). Further characterization of purified enzyme (in 50 mM Tris buffer, pH 9.0, containing 1.2 % NaCl) was done with casein (final concentration in reaction mix. was 1 %) as substrate in 50 mM glycine NaOH buffer pH 10.0 and at 45 ^{o}C.

3.11.1 Determination of optimum pH

Effect of pH on purified enzyme activity was analyzed by assaying it with casein (final concentration in reaction mix. was 1 %) in 50 mM buffers of different pH, potassium phosphate (pH 6.0–7.5), Tris (pH 8.0–9.5), glycine NaOH (pH 10-12) at 45 ^{o}C for 30 min. Activities were determined and then relative activities were calculated based on the activity of the enzyme at pH 10.0 (Optimum pH found to be 10).

3.11.2 Determination of optimum temperature

Effect of temperature on purified enzyme was determined by incubating it with casein solution (final 1 % concentration) in 50 mM glycine NaOH buffer, pH 10.0. The mixture was incubated at different temperatures (20- 70 ^{o}C) for 30 min. Relative activities were presented. The activity of the enzyme measured at 50 ^{o}C was taken as 100 % as maximum activity was obtained at this temperature. Enzyme was very unstable at 50 ^{o}C thus further characterization was done at 45 ºC.

3.11.3 Determination of substrate specificity

Substrate specificity of purified enzyme from *Halobiforma* sp. strain BNMIITR was determined by using substrates such as casein, Bovine serum albumin (BSA) and gelatine. Briefly, 0.2 mL of purified enzyme (in 50 mM Tris buffer pH 9.0, containing 1.2 % NaCl) was added to 0.8 mL of substrate solution (50 mM glycine NaOH buffer, pH 10.0, final concentration of substrate in reaction mixture was 1 %) and the mixture was incubated at 45 ºC for 30 min.

3.11.3.1 Elastase activity

Elastase activity of extracellular protease from *Halobiforma* sp. BNMIITR was determined according to Skovgaard et al. 2010 using N-Succinyl-Ala-Ala-Ala-*p*-nitroanilide as a substrate. Briefly, 0.2 mL of purified enzyme (in 50 mM Tris buffer pH 9.0, containing 1.2 % NaCl) was incubated with 0.8 ml of substrate solution (N-Succinyl-Ala-Ala-Ala-*p*-nitroanilide in 50 mM glycine NaOH buffer, pH 10.0 at final concentration 0.2 % in reaction mixture) at 45 ^{o}C for 30 minute and released amount of p-nitroaniline was determined spectrophotometrically by taking O. D. at 405 nm. For blank 0.2 ml of 50 mM glycine NaOH buffer, pH 10.0 was added instead of enzyme solution. One unit of enzyme was determined as amount of enzyme required to produce 1 µmole of p-nitroaniline per min. A standard graph for estimation of p-nitroaniline concentration is given in Fig. 3.3. For standard preparation, a stock solution of p-nitroaniline (10 mili mole/ml) was prepared in DMSO and was diluted with 50 mM glycine NaOH buffer, pH 10.0 at different

concentrations (12.5- 250 micro mole/ml) and absorbance was taken at 405 nm. Absorbance at 405 nm was plotted against p-nitroaniline concentration in micro mole /ml.

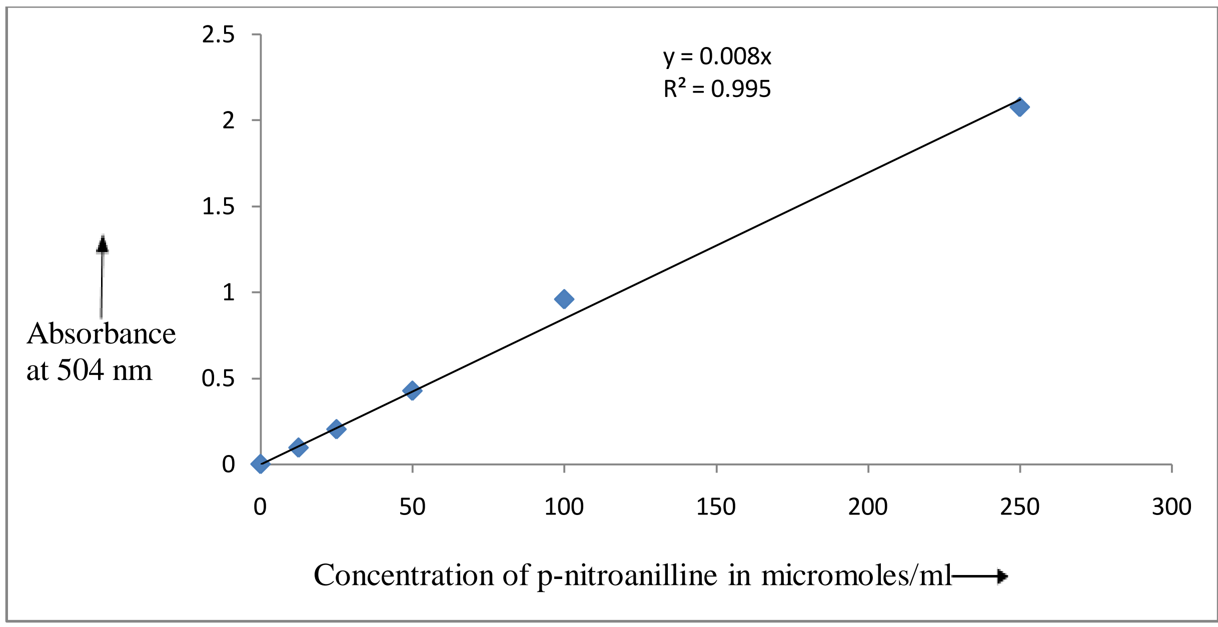


Fig. 3.3: Standard graph for elastase activity.

3.11.3.2 Esterase activity

Esterase activity of enzyme was tested by using p-nitrophenyl actetate (pNPA) as substrate according to Oceguera-Cervantes et al. (2007). 20 mM stock of pNPA in acetonitrile was prepared. 0.250 ml pNPA substrate from stock solution, 0.55 ml 50 mM glycine NaOH buffer, pH 10.0 and 0.2 mL of purified enzyme (in 50 mM Tris buffer pH 9.0, containing 1.2 % NaCl) were incubated at 45 ^{o}C for 10 min. After 10 minute, reaction was stopped by keeping the reaction mix in ice and released amount of p-nitrophenol (pNP) was determined spectrophotometrically by taking O.D. at 405 nm. For blank 0.2 ml of 50 mM glycine NaOH buffer, pH 10.0 was added instead of enzyme solution. One unit of enzyme was determined as amount of enzyme required to produce 1 μmole of pNP in aqueous medium per min. The standard graph for estimation of p-nitrophenol is given in Fig. 3.4 For standard preparation, a stock solution of p-nitrophenol (10 mili mole/ml) was prepared in DMSO and was diluted with 50 mM glycine NaOH buffer, pH 10.0 at different concentrations (20- 100 micro mole/ml) and absorbance was taken at 405 nm. Absorbance at 405 nm was plotted against p-nitrophenol concentration in micro mole/ ml.

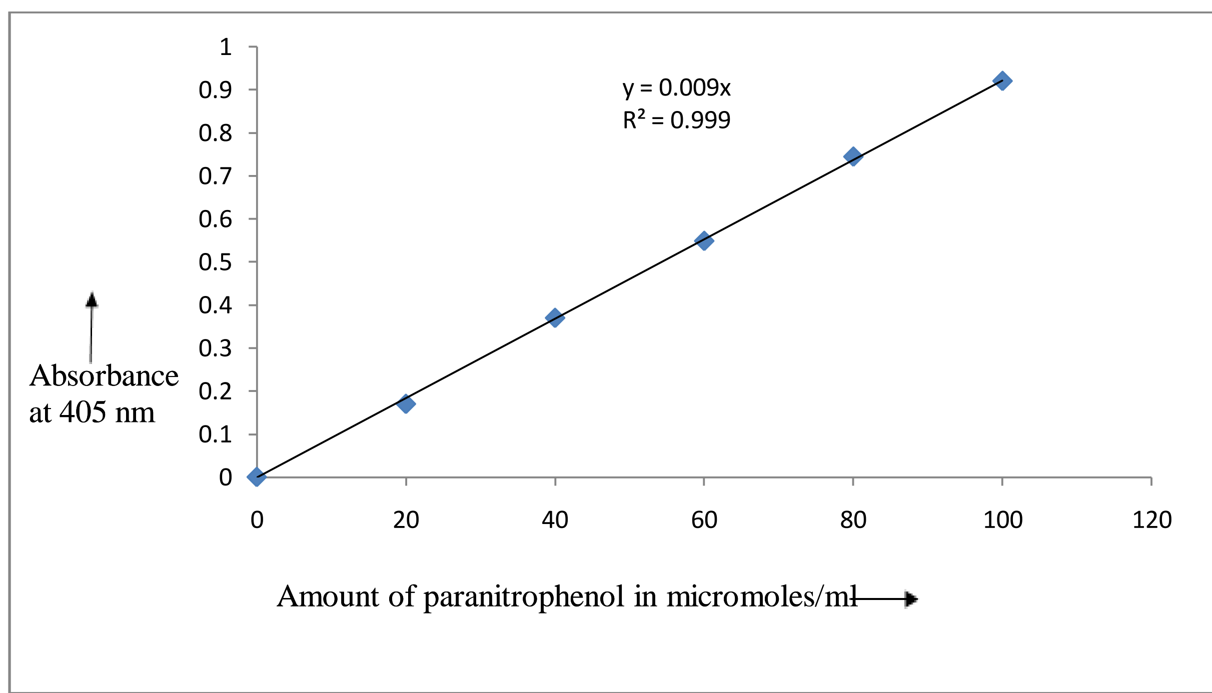


Fig. 3.4: Standard graph for esterase activity.

3.11.4 Effect of metal ions on enzyme activity

Effect of various metal ions on purified enzyme of *Halobiforma* sp. BNMIITR (in 50 mM Tris buffer pH 9.0, containing 1.2 % NaCl) was studied by determining the enzyme activity at 45 ^{o}C in glycine NaOH buffer (pH 10) with various metals at 5 and 10 mM concentrations. Activities were determined according to the standard enzyme assay protocol. The activity of the enzyme measured in absence of any metal was taken as 100 % (control).

3.11.5 Effect of osmolytes on enzyme activity

Effect of different concentrations (1- 10 %) of various osmolytes like KCl, glycerol, mannitol and sucrose on the activity of purified enzyme were examined by including them in reaction mixture and following standard enzyme assay protocol. Relative activities were calculated and activity of enzyme without osmolyte (in 50 mM tris buffer pH 9.0) was taken as 100 %. Effect of osmolytes on renaturation of enzyme was also checked. Purified enzyme of *Halobiforma* sp. BNMIITR (in 50 mM Tris buffer pH 9.0, containing 1.2 % NaCl) was equilibrated with 30 ml 50 mM tris buffer pH 9.0 with 12 and 20 % of sucrose, mannitol and glycerol using centrifugal concentrators (amicon10 kD). Relative activities were calculated and activity of purified enzyme in 50 mM Tris buffer pH 9.0, containing 1.2 % NaCl was taken as 100 %.

3.11.6 Thermo stability of the enzyme and effect of NaCl on thermo stability of the enzyme

Stability of enzyme at different temperatures was determined by incubating the enzyme (in 50 mM tris buffer pH 9.0 with 1.2 % NaCl) at different temperatures (50 ^{o}C, 45 ^{o}C, 30 ^{o}C and 20 ^{o}C) for 24 hours. Samples were taken at regular intervals. Relative activities were calculated and initial activity was taken as 100 %. Effect of NaCl on thermo stability of the enzyme was determined by incubating the enzyme with different salt concentrations (0- 12.6 %) at 45 ^{o}C. One ml of enzyme (in 50 mM tris buffer pH 9.0 with 1.2 % NaCl) was diluted with one ml of 50 mM tris buffer pH 9.0 containing different NaCl concentrations. Enzyme was incubated at 45 ^{o}C in 50 mM tris buffer pH 9.0 and final NaCl concentrations were 0.6%, 1.2 %, 3.6 %, 5.6 %, 10.6 % and 12.6 % for 24 hours. Samples were taken at regular intervals. Relative activities were calculated and initial activity of (in 50 mM tris buffer pH 9.0) enzyme was taken as 100 %.

3.11.7 Effects of additives on enzyme activity

Effects of various additives on purified enzyme (in 50 mM tris buffer pH 9.0, containing 1.2 % NaCl) of *Halobiforma* sp. BNMIITR were studied by determining enzyme activity at 45 ^{o}C in glycine NaOH buffer (pH 10) with various additives like serine protease inhibitor phenylmethylsulphonyl fluoride (PMSF), ethylenediaminetetraacetic acid (EDTA), and Phenanthrolene at 5 mM and 10 mM concentrations, cationic detergent cetyl trimethyl ammonium bromide (CTAB), anionic detergent sodium dodecyl sulphate (SDS) and neutral detergents like Tween-20, Tween-40, Tween-80, Triton X-100, sodium cholate and sodium deoxycholate at 0.1 and 0.5 % concentrations, reducing agents as Dithiotheritol (DTT), oxidizing agents hydrogen per oxide, Ammonium persulphate (APS) at 5 and 10 mM concentrations. Activities were determined according to the standard enzyme assay protocol. The activity of the enzyme measured in absence of any additive was taken as 100 % (control).

3.11.8 Effects of Organic solvents on protease activity

Effects of various solvents on purified enzyme of *Halobiforma* sp. BNMIITR (in 50 mM Tris buffer, containing 1.2 % NaCl) were studied by determining enzyme activity at 45 ^{o}C in glycine NaOH buffer (pH 10) with various non polar solvents like toluene, n-hexane, n-heptane, xylene, butanol, dichloromethane, octane and polar solvents like DMF, DMSO, methyl alcohol, ethyl alcohol, isopropanol, at 10 %, 30 % and 50 % (v/v) concentrations. Activities were determined

according to the standard enzyme assay protocol. The activity of the enzyme measured in absence of solvent was taken as 100 % (control).

3.11.9 Protease stability in organic solvents

One ml of protease along with 1 ml of organic solvents incubated at 30 ^{o}C and 45 ^{o}C with constant shaking at 180 rpm. Samples were withdrawn after 24 h, and 48 h. Residual protease activities were determined according to the standard protocol. The enzyme incubated without solvent was treated as the control.

3.12 Detergent compatibility of the enzyme

To find out the detergent compatibility of the enzyme, one ml enzyme (in 50 mM tris buffer, pH 9.0, containing 1.2 % NaCl) was incubated with commercial detergents such as Surf excel, Ariel, Tide, Rin and Wheel at 1.0 % w/v. After four hours, residual activity was checked and the activity without detergent was taken as 100%.

4. RESULTS AND DISCUSSION

4.1 Isolation of halophilic bacteria

Naturally occurring hypersaline environments are known as soda lakes (Jones et al. 1994). In India, there are about 10 thousand hypersaline sites which include Sambhar salt lake in Rajasthan, coastal regions of Maharashtra, Gujarat (Kutch Saurashtra and Bhavnagar), Orissa (Chilka lake), West Bengal and Andhra Pradesh. Several halophilic isolates belonging to archaea, eubacteria and eukarya have been isolated from these hypersaline sites. Sambhar lake, which means salt of earth, is the largest inland salt lake of India. Bacterial isolates from Sambhar lake Rajsthan belong to class Firmicutes, Proteobacteria, Gammaproteobacteria, Betaproteobacteria, Actinobacteria and Halobacteriaceae (Sahay et al. 2012). These microbes have potential biotechnological applications for production of osmolytes, PHB (polyhydroxybutyrate), pigments and industrially important enzymes. In the present study, four soil and one salt sample were collected from Sambhar salt lake Rajasthan which is located in Northern India. Initially, soil and salt samples were added in a complex halophilic medium and incubated at 45 ℃, pH 9.0 on a rotatory incubator shaker (180 rpm). After three subcultures, pure cultures were obtained by serial dilutions and repeated streaking on agar plate having same medium composition as liquid medium. A total of sixteen different bacterial isolates were isolated from four soil and one salt sample of Sambhar salt lake, Rajasthan, out of which five isolates were obtained from soil sample of Main lake, three from soil sample of Gudha kyar, three from soil sample of Jhapog kyar, three from soil sample of Deodani kyar and two from waste salt of Sambhar salt industry. Isolates obtained from different samples have been listed in Table 4.1. Although, initial screening was done at 4 M NaCl concentration however after getting purified isolates, all the isolates were maintained on complex medium agar plates containing 2 and 4 M NaCl according to their halophilic nature.

Table 4.1: Isolates obtained from soil samples of various salt kyars and waste salt of Sambhar salt industry.

S. No.	Source	pH	Temperature	Isolates
1.	Main lake	9.93	39 ^{o}C	MSL-1, MSL-2, MSL-6, MSL-8, MSL-9
2.	Gudha kyar	9.63	39 ^{o}C	MSL-5, MSL-7, MSL-16
3.	Jhapog kyar	9.61	40 ^{o}C	MSL-10, MSL-4, MSL-13
4.	Deodani kyar	10.01	40 ^{o}C	MSL-12, MSL-15, MSL-14
5.	Waste salt of Sambhar salt industry	9.0	39 ^{o}C	MSL-3, MSL-11

4.2 Characterization of isolates

4.2.1 Morphological characterization

4.2.1.1 Morphology of isolates

Colony morphologies of all the isolates on agar plate are shown in Fig 4.1. According to the observations, colony morphologies of all these isolates on plate were

MSL-1: Small, round, smooth colonies, shining dark orange in colour

MSL-2: Small, round, rough colonies, orange in colour

MSL-3: Small, round, rough colonies, light pink in colour

MSL-4: Small, round, smooth slimy colonies, white in colour

MSL-5: Small, round, smooth turbid colonies, white in colour

MSL-6: Small, round, smooth colonies, pale in colourMSL-7: Small, round, smooth colonies, pale in colour

MSL-8: Small, round, smooth colonies, transparent in colour

MSL-9: Broad, rough colonies with discreet margins, dark pale in colour

MSL-10: Small, round, smooth colonies, shining light orange in colour

MSL-11: Small, round, rough colonies, light pink in colour

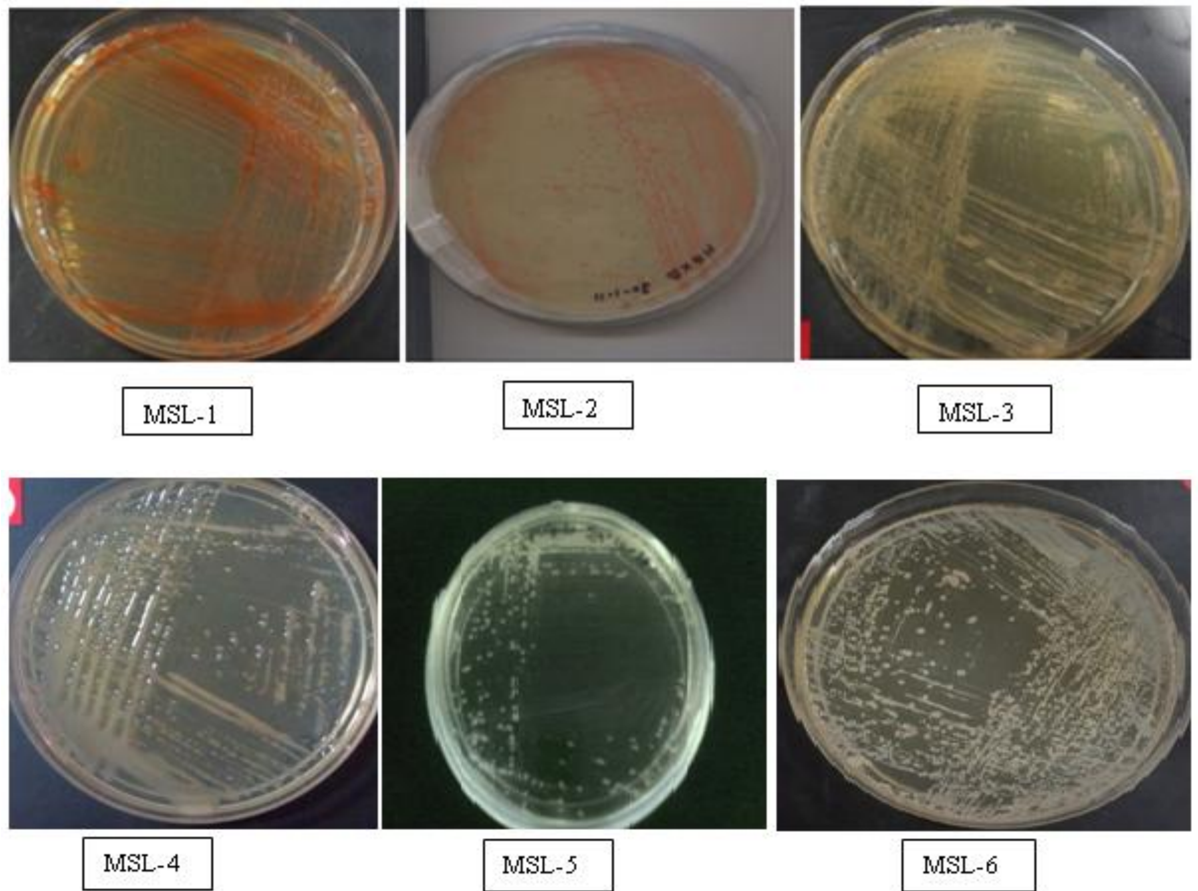
MSL-12: Broad, rough colonies, dark pale in colour

MSL-13: Broad, rough colonies, dark pale in colour

MSL-14: Small, round, smooth colonies, pale in colour

MSL-15: Small, round, smooth colonies, transparent in colour

MSL-16: Small, round, smooth colonies, transparent in colour



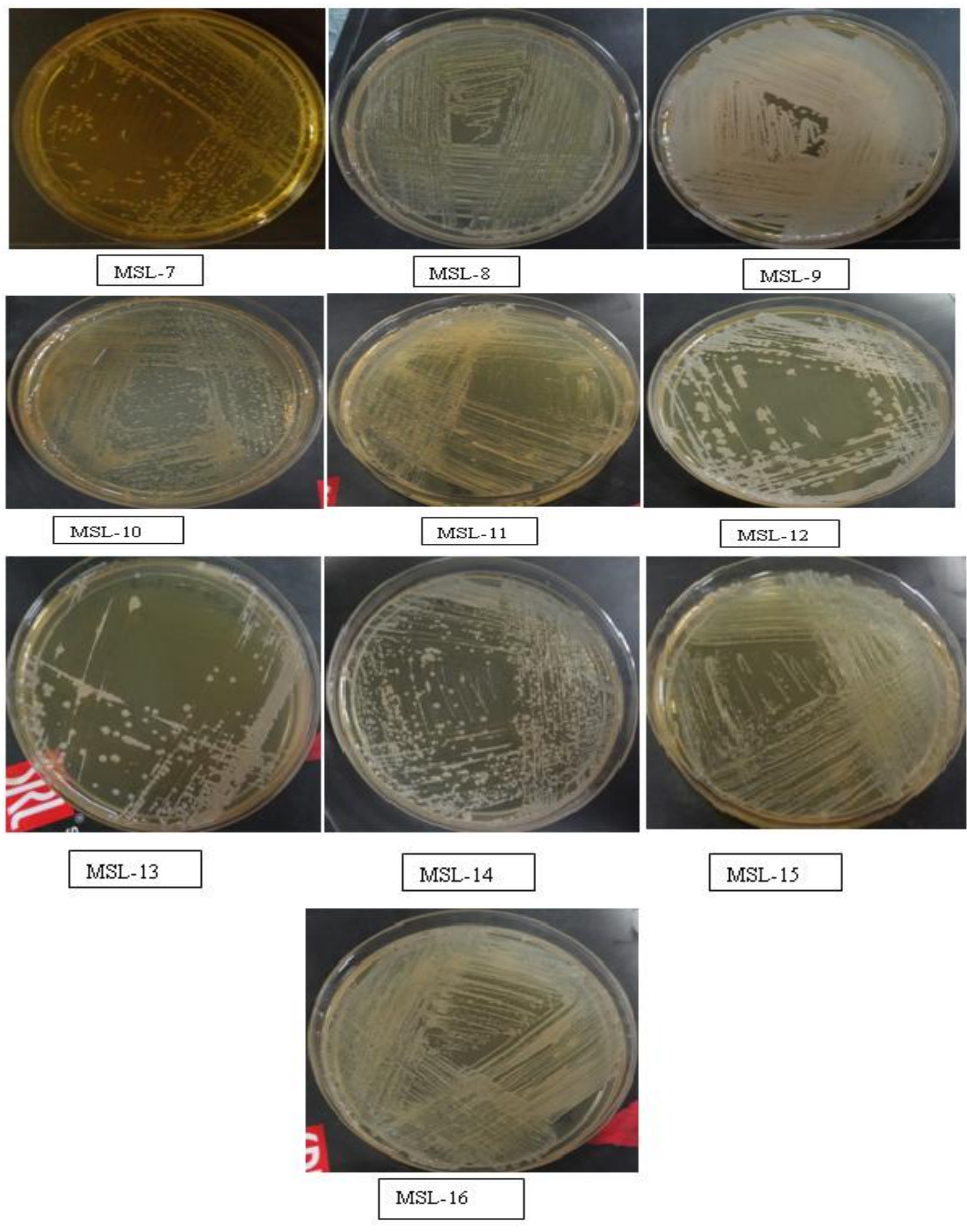
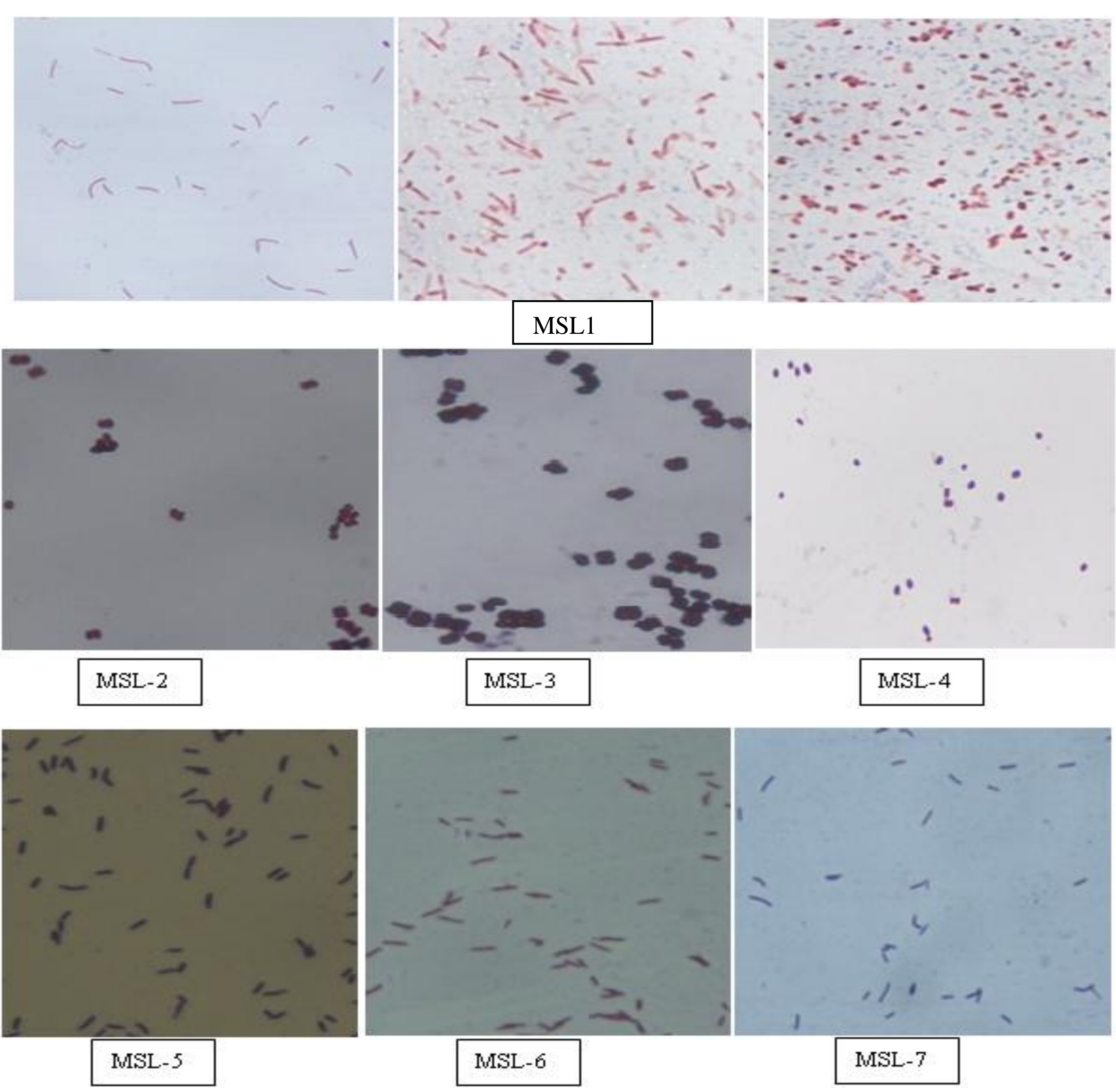


Fig. 4.1: Colony morphology of isolates on complex medium agar plate (MSL-1 to MSL-16).

On the basis of cellular morphology (phase-contrast micrograph under 100 X magnification) isolates were divided in three groups 1) rods 2) cocci 3) pleomorphic. Out of 16 isolates two were found to be pleomorphic (from rod to cocci), Gram negative (MSL-1 and MSL-l0), four cocci Gram positive (MSL-2, MSL-3, MSL-4 and MSL-11) and others were Gram positive rods (MSL-5, MSL-6, MSL-7, MSL-8, MSL-9, MSL-12, MSL-13, MSL-14, MSL-15, MSL-16). Phase-contrast micrographs of all isolates under 100 X magnification are shown in Fig. 4.2.



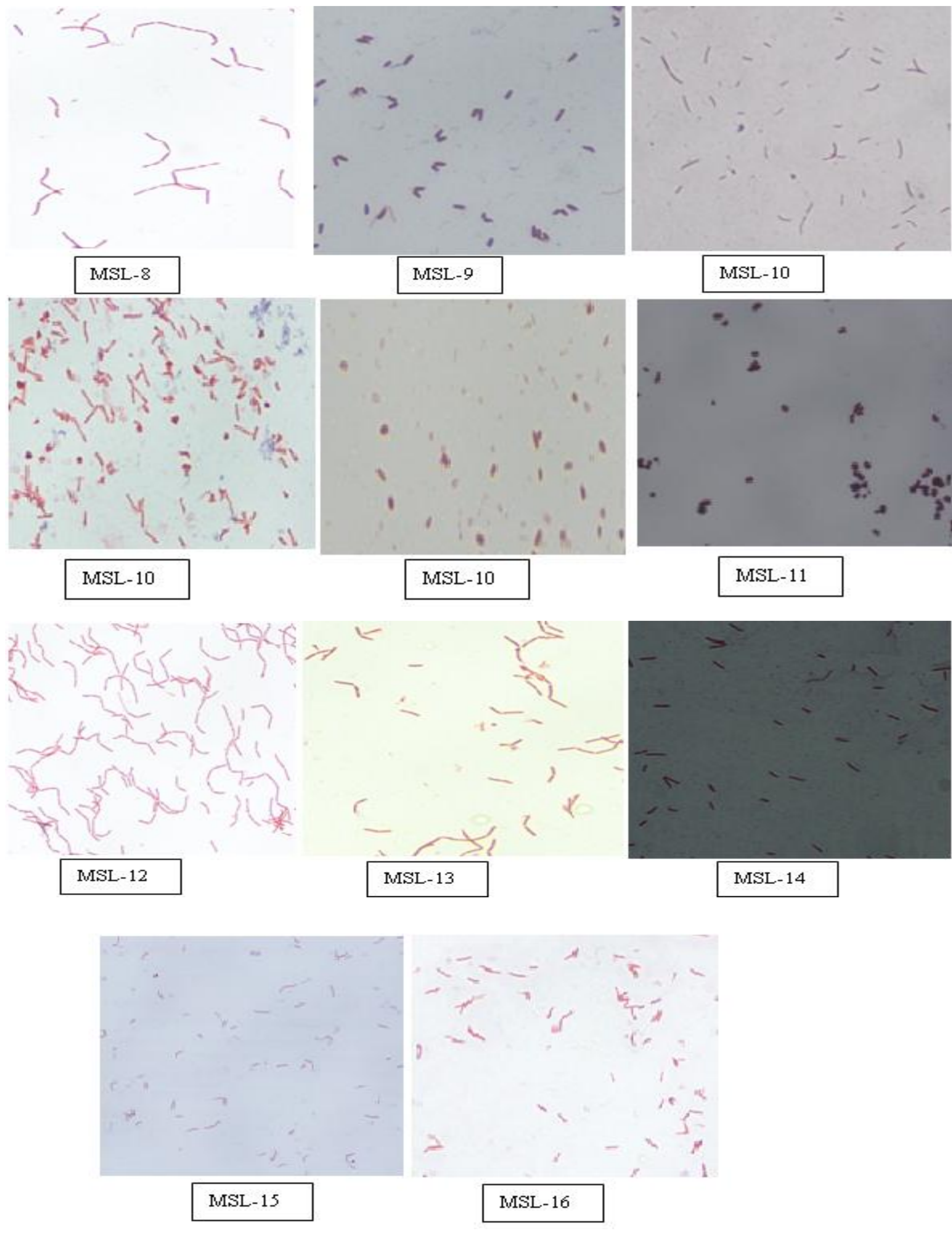


Fig. 4.2: Phase-contrast micrograph of all isolates (MSL-1- MSL-16) under 100 X magnification after Gram Staining.

4.2.1.2 Spore formation ability

Out of sixteen isolates, eight isolates (MSL-6, MSL-7, MSL-8, MSL-12, MSL-13, MSL-14, MSL-15, MSL-16) were found to form spores. All the observed spores were terminal in position and oval in shape (Fig. 4.3). Terminal and spherical spores have been reported in family Firmicutes which mainly includes moderately halophilic Gram positive rods (Wang et al. 2009).

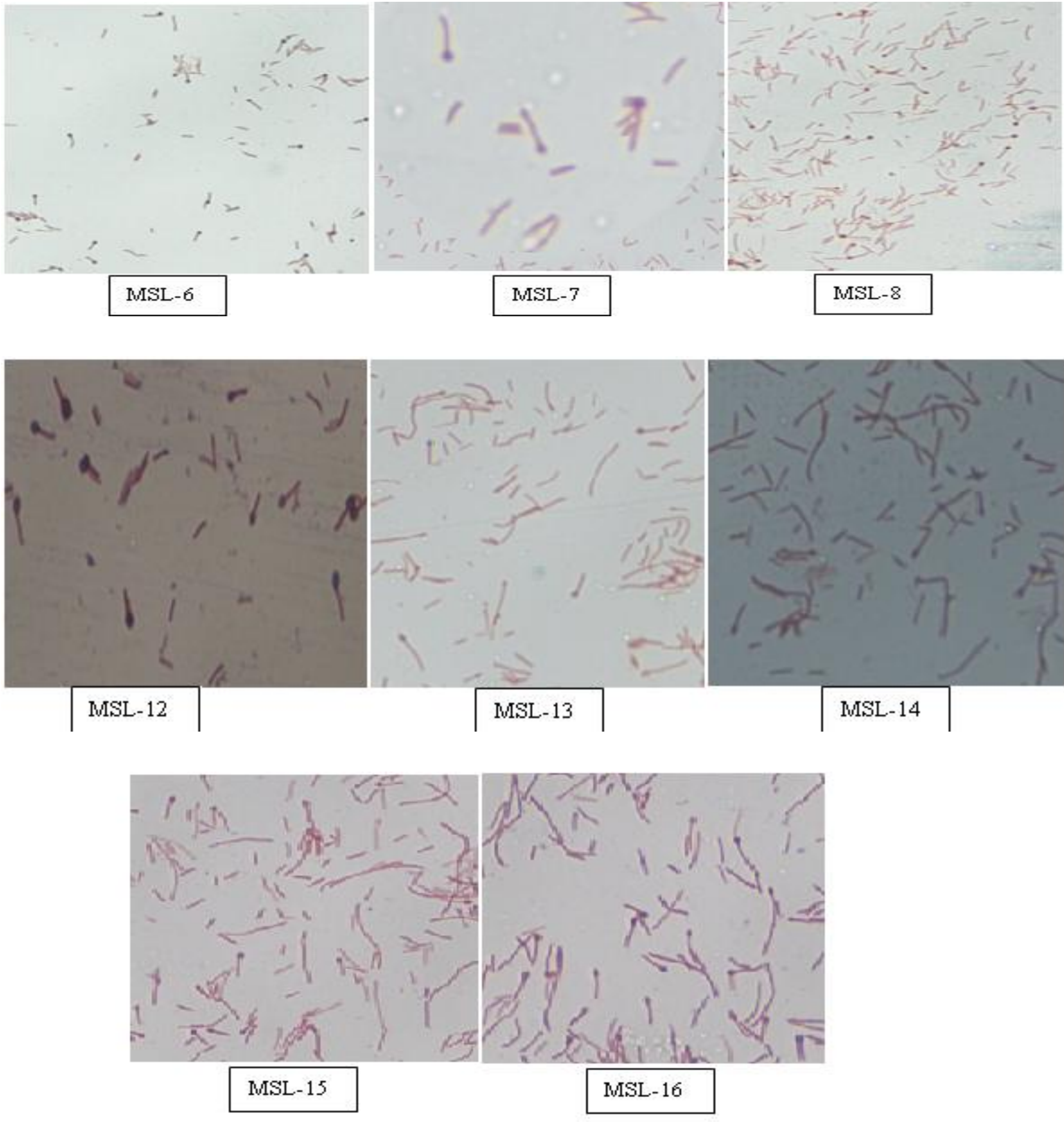


Fig. 4.3: Shape and position of spores produced by different isolates obtained from soil and salt samples.

4.2.2 Physiological characterization

4.2.2.1 Salt requirement of isolates

To ascertain the halophilic nature, isolates were grown on complex halophilic agar plates having NaCl concentration in the range of 0- 5 M. No isolate was able to grow in a medium without NaCl. All isolates can grow at varying concentration of NaCl and KCl. Minimum and optimum salt requirements for growth was different for different isolates. On the basis of plate study, isolates were divided in two groups.

Group 1: isolates which can grow at 2-5 M salt concentration (MSL-1, MSL-2, MSL-3, MSL-4, MSL-11 and MSL-10). These isolates require minimum 2 M NaCl for visible growth.

Group 2: isolates which can grow at 1- 4 M salt concentration (MSL-5, MSL-6, MSL-7, MSL-8, MSL-9, MSL-12, MSL-13, MSL-14, MSL-15 and MSL-16).

Further confirmation of the halophilic nature of all isolates was done by growing them in liquid medium having salt concentration 2- 5 M for Group 1 isolates and 1- 4 M for Group 2 isolates. On the basis of salt requirement and growth profile (Fig 4.4- 4.19) in the presence of NaCl and KCl, isolates were divided into three groups according to Kushnaer and Kamekura 1988.

Extreme halophiles: Only one isolate, MSL-10 could grow at NaCl concentration range of 2- 5 M with an optimum growth at 4- 5 M NaCl. Growth was five times less when NaCl was replaced with KCl.

Borderline extreme halophiles: Five isolates out of sixteen (MSL-1, MSL-2, MSL-3, MSL-4, MSL-11) could grow at NaCl concentration range of 2- 5 M with an optimum growth observed in the range 2- 3 M NaCl concentration. Again all of these isolates showed 5- 10 times less growth in a medium containing KCl in place of NaCl.

Moderately halophilic: Ten isolates out of sixteen (MSL-5, MSL-6, MSL-7, MSL-8, MSL-9 MSL-12, MSL-13, MSL-14, MSL-15, MSL-16) could grow at NaCl concentration range of 1- 4 M with an optimum growth observed at 2 M NaCl concentration. Moderately halophilic isolates show better growth in KCl in comparison to borderline and extreme halophiles. MSL-5 and MSL-9 grow equally well in NaCl and KCl with optimum growth at 2 M NaCl and 1 M KCl for MSL-5 and 1 M NaCl and 1 M KCl for MSL-9. MSL-6 and MSL-7 showed optimum growth at 2 M KCl which was 2- 3 times less than that of NaCl. MSL-8 showed very less growth in 2 M KCl (five times

less). MSL-12 and MSL-13 grow equally well in 2 M NaCl and 2 M KCl. MSL-14, MSL-15, MSL-16 grow optimally at 2 M NaCl and 2 M KCl but the growth was 1.2- 2 times less in KCl medium.

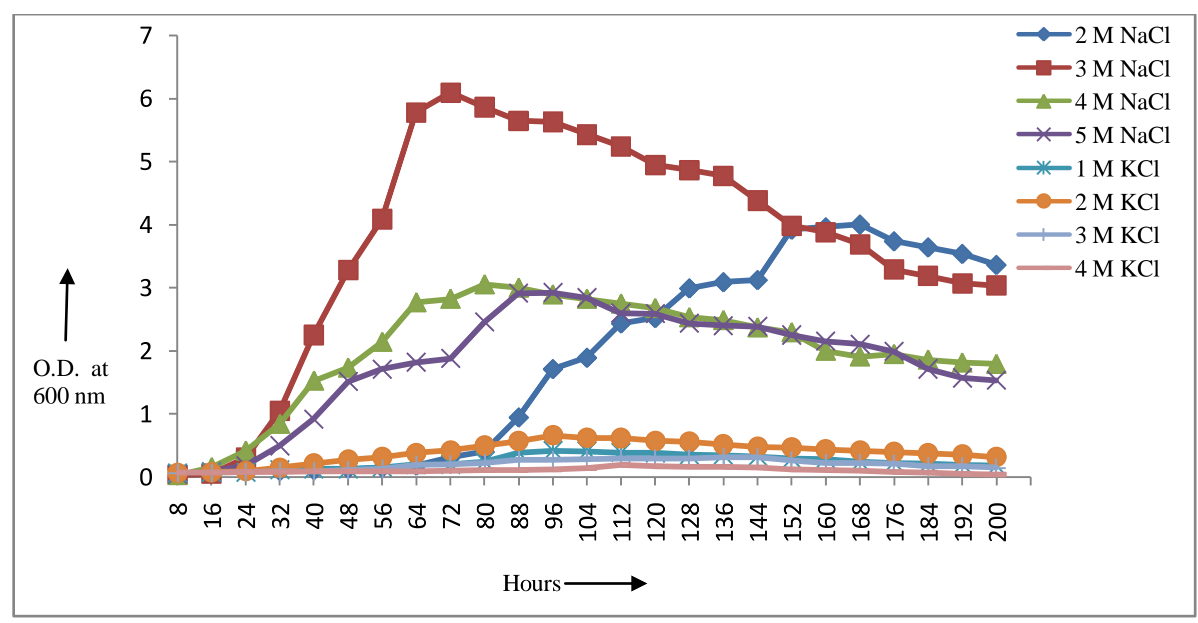


Fig. 4.4: Growth profiles of MSL-1 in the presence of 2- 5 M NaCl and 1- 4 M KCl.

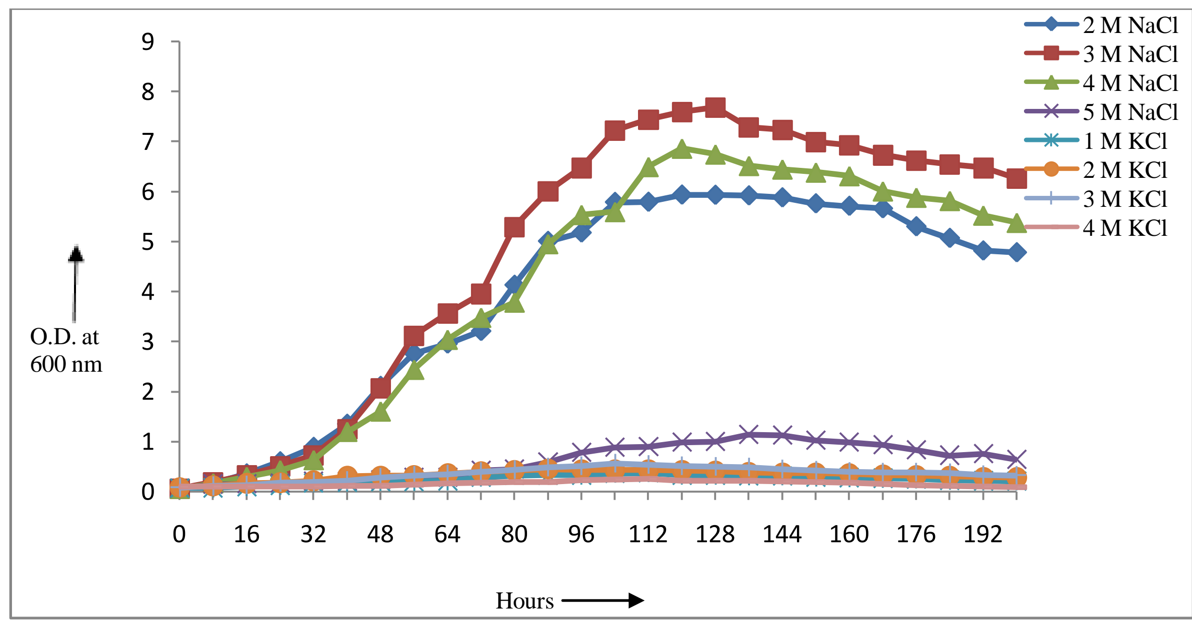


Fig. 4.5: Growth profiles of MSL-2 in the presence of 2- 5 M NaCl and 1- 4 M KCl.

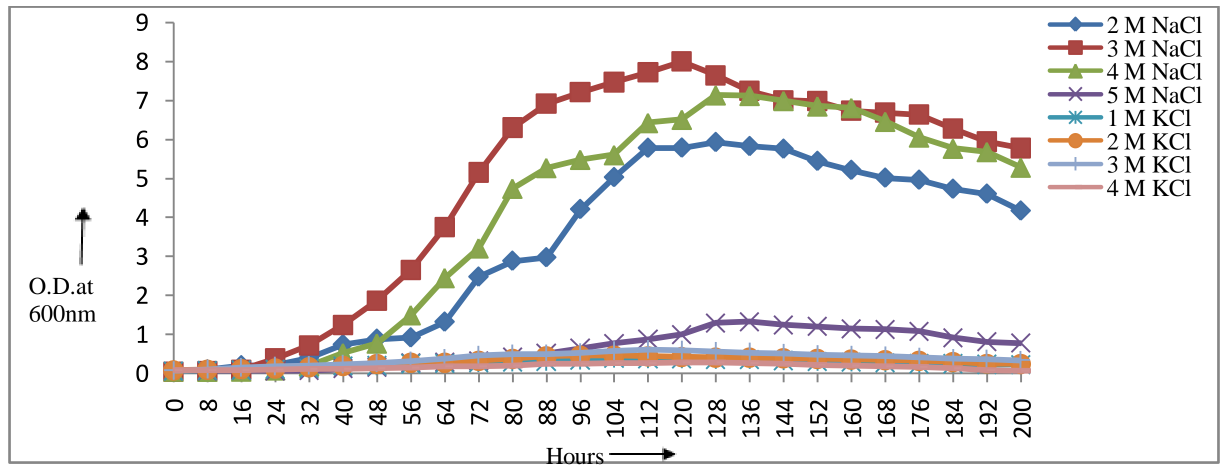


Fig. 4.6: Growth profiles of MSL-3 in the presence of 2- 5 M NaCl and 1- 4 M KCl.

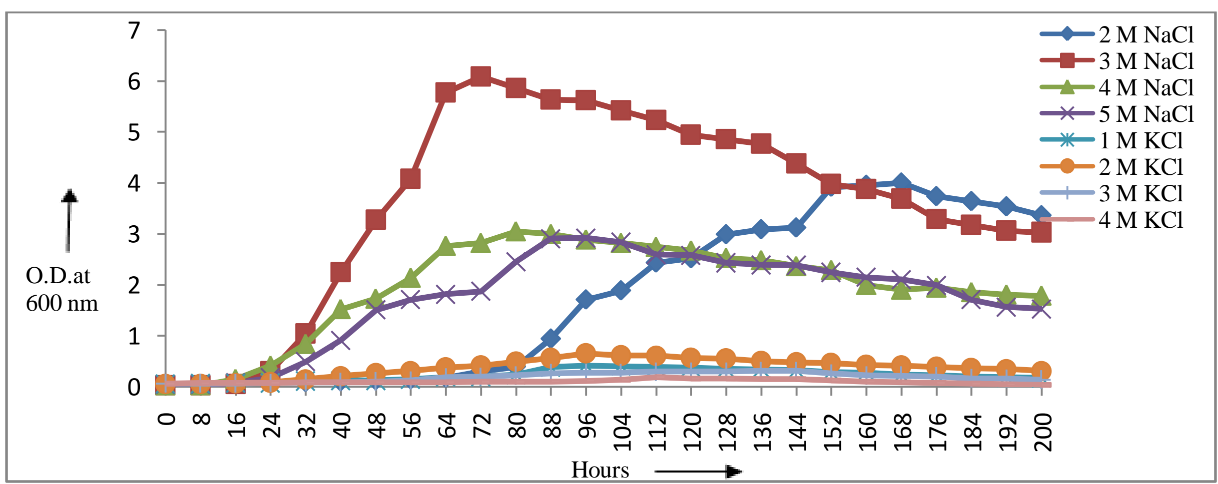


Fig. 4.7: Growth profiles of MSL-4 in the presence of 2- 5 M NaCl and 1- 4 M KCl.

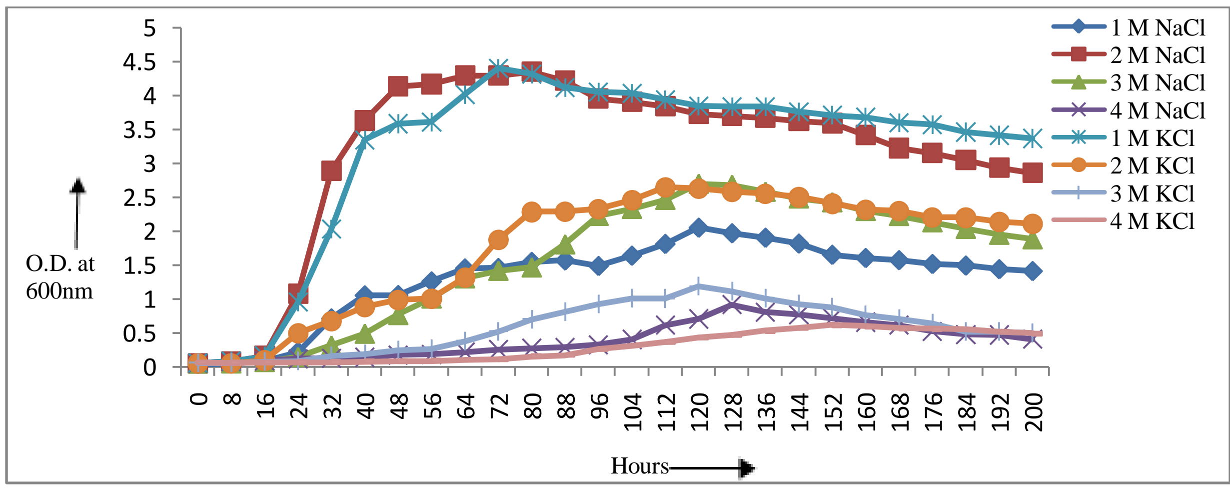


Fig. 4.8: Growth profiles of MSL-5 in the presence of 1- 4 M NaCl and 1- 4 M KCl.

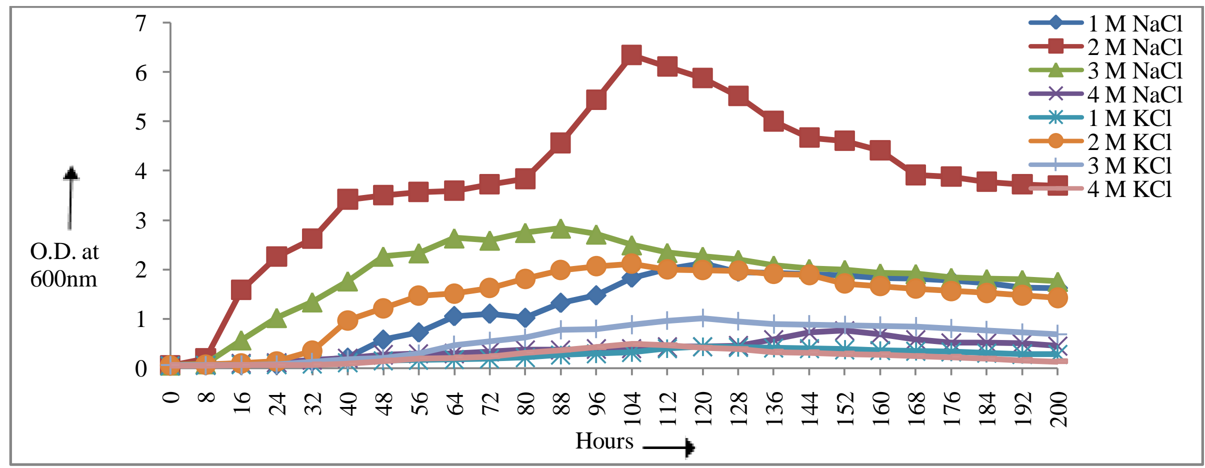


Fig. 4.9: Growth profiles of MSL-6 in the presence of 1- 4 M NaCl and 1- 4 M KCl.

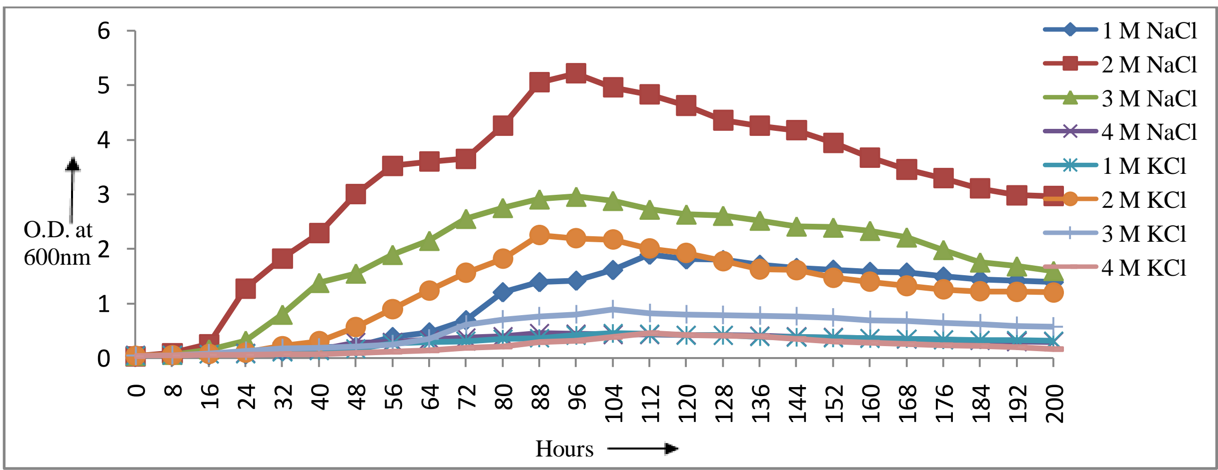


Fig. 4.10: Growth profiles of MSL-7 in the presence of 1- 4 M NaCl and 1- 4 M KCl.

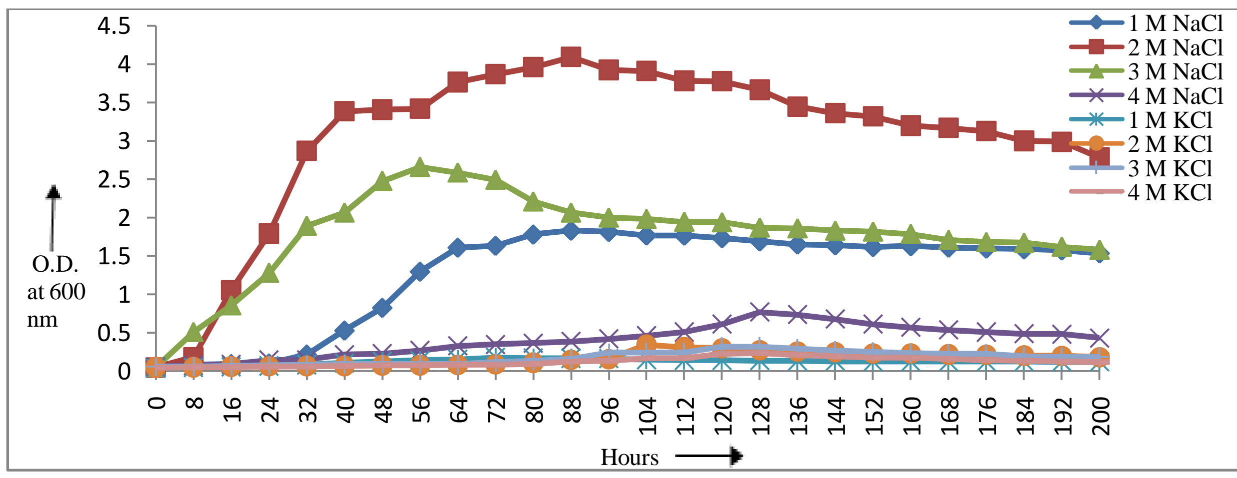


Fig. 4.11: Growth profiles of MSL-8 in the presence of 1- 4 M NaCl and 1- 4 M KCl.

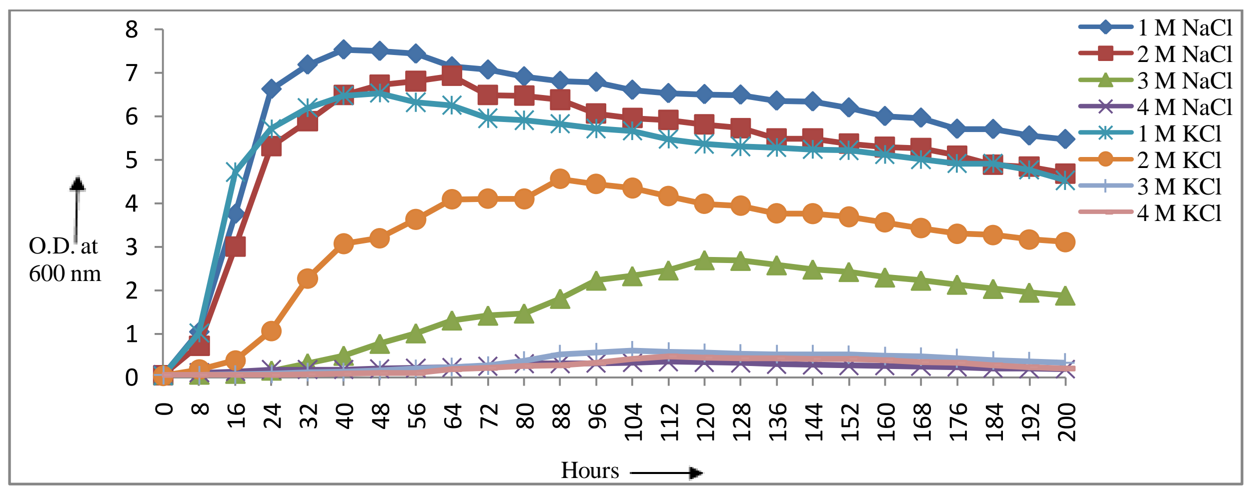


Fig. 4.12: Growth profiles of MSL-9 in the presence of 1- 4 M NaCl and 1- 4 M KCl.

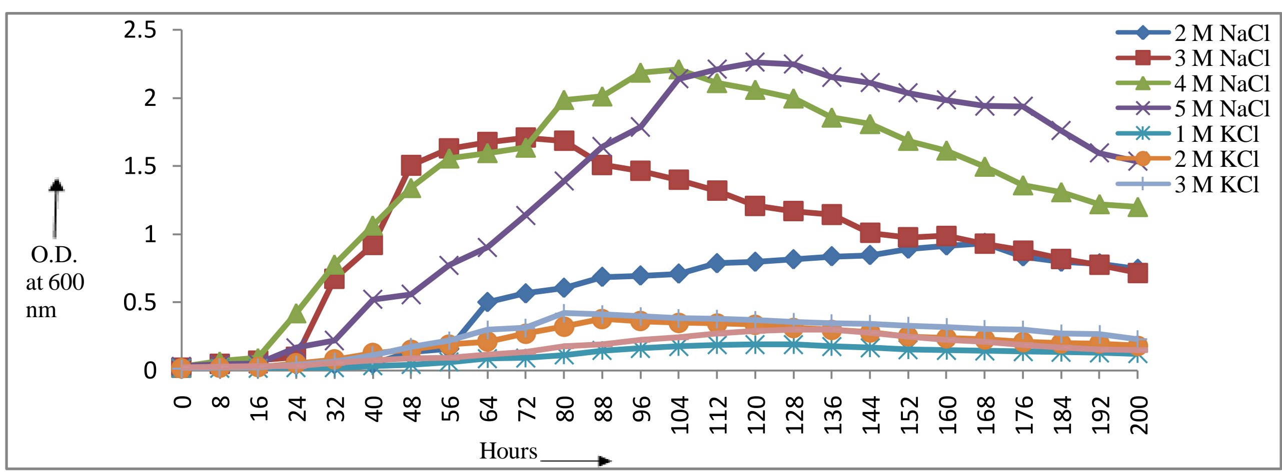


Fig. 4.13: Growth profiles of MSL-10 in the presence of 2- 5 M NaCl and 1- 4 M KCl.

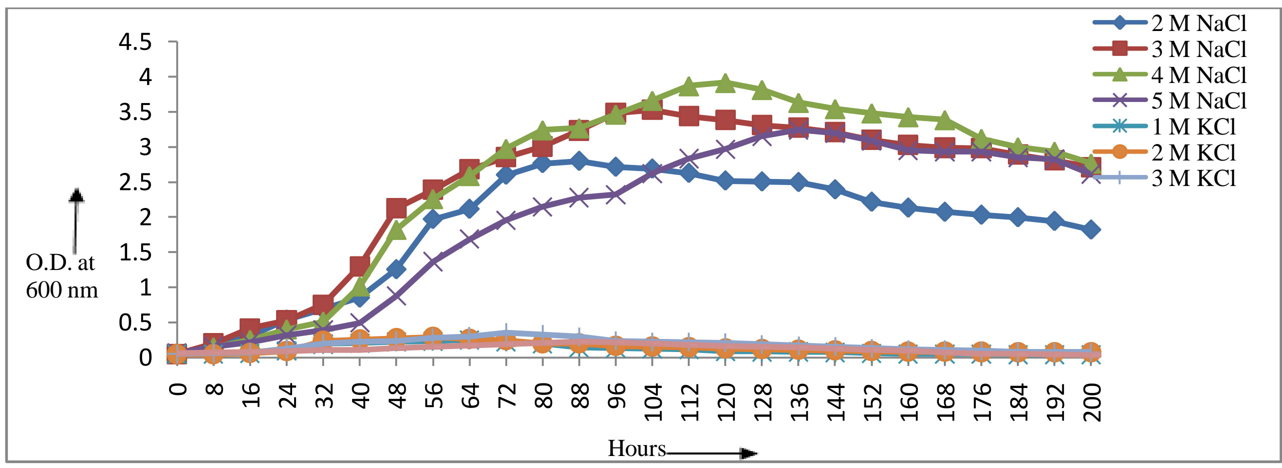


Fig. 4.14: Growth profiles of MSL-11 in the presence of 2- 5 M NaCl and 1- 4 M KCl.

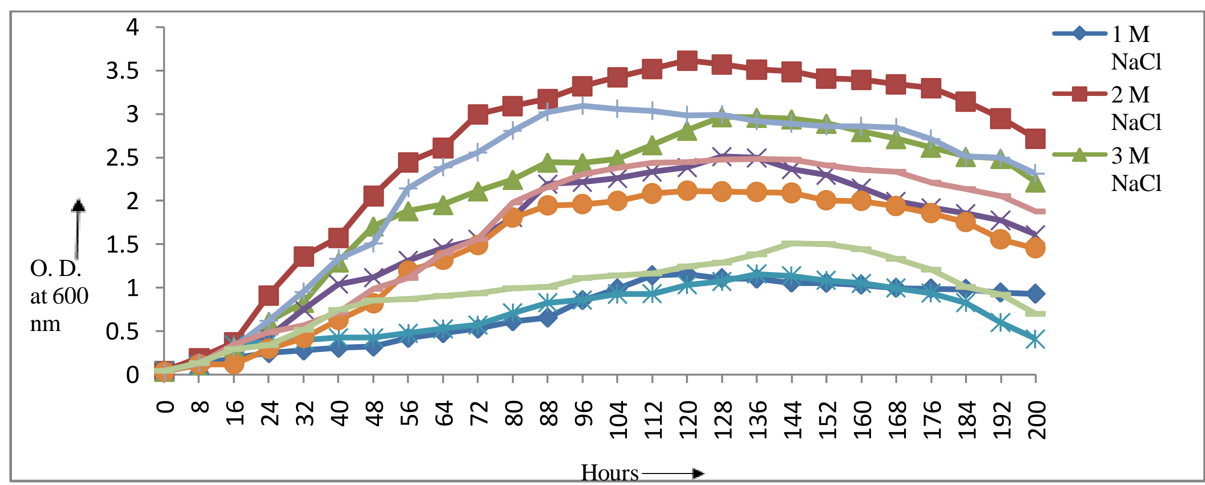


Fig. 4.15: Growth profiles of MSL-12 in the presence of 1- 5 M NaCl and 1- 4 M KCl.

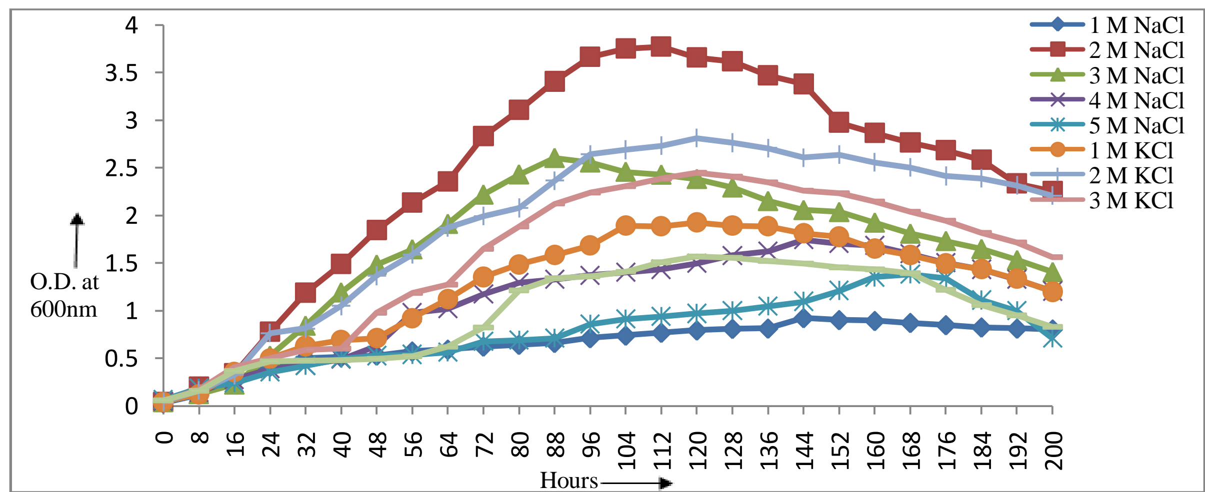


Fig. 4.16: Growth profiles of MSL-13 in the presence of 1- 5 M NaCl and 1- 4 M KCl.

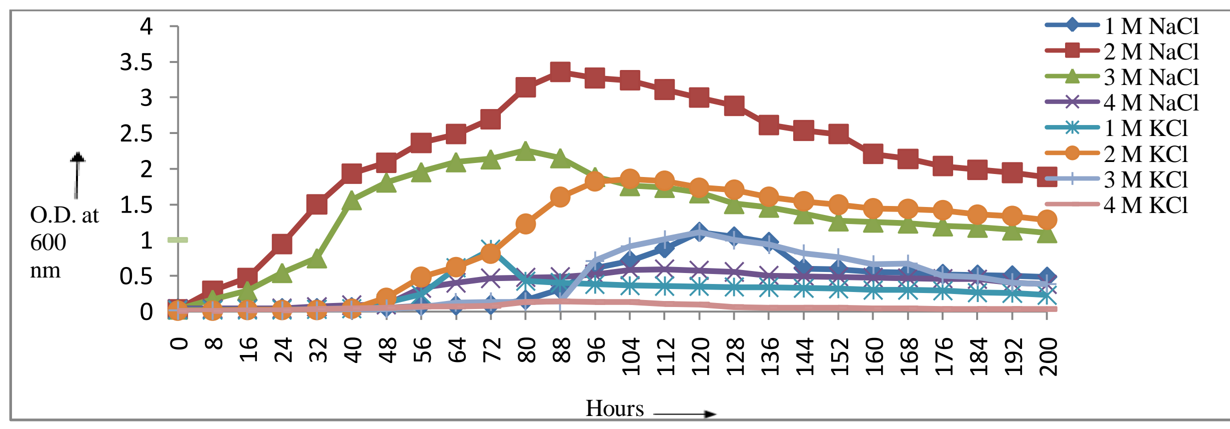


Fig. 4.17: Growth Profile of MSL-14 in the presence of 1- 4 M NaCl and 1- 4 M KCl.

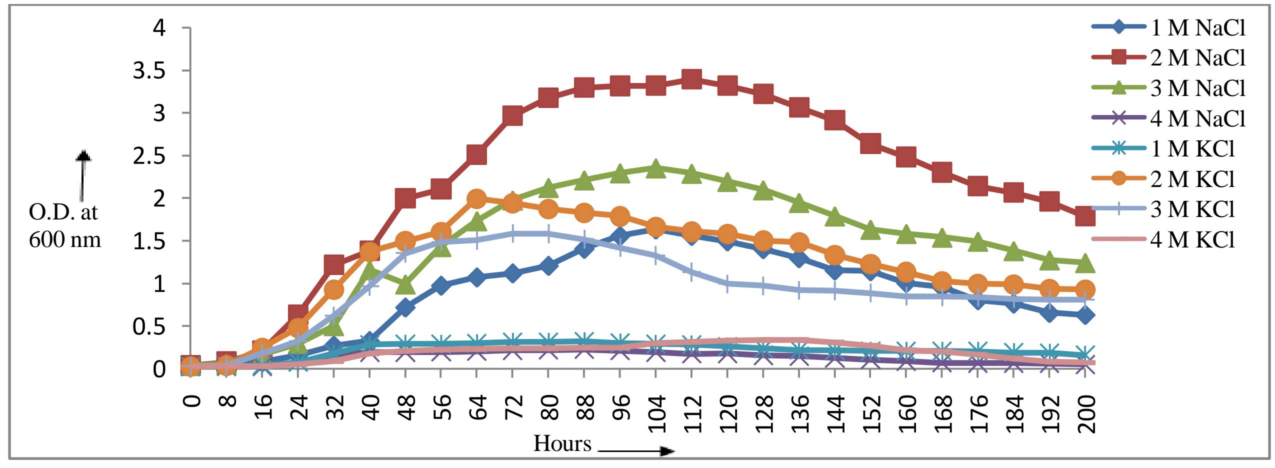


Fig. 4.18: Growth Profiles of MSL-15 in the presence of 1- 4 M NaCl and 1- 4 M KCl.

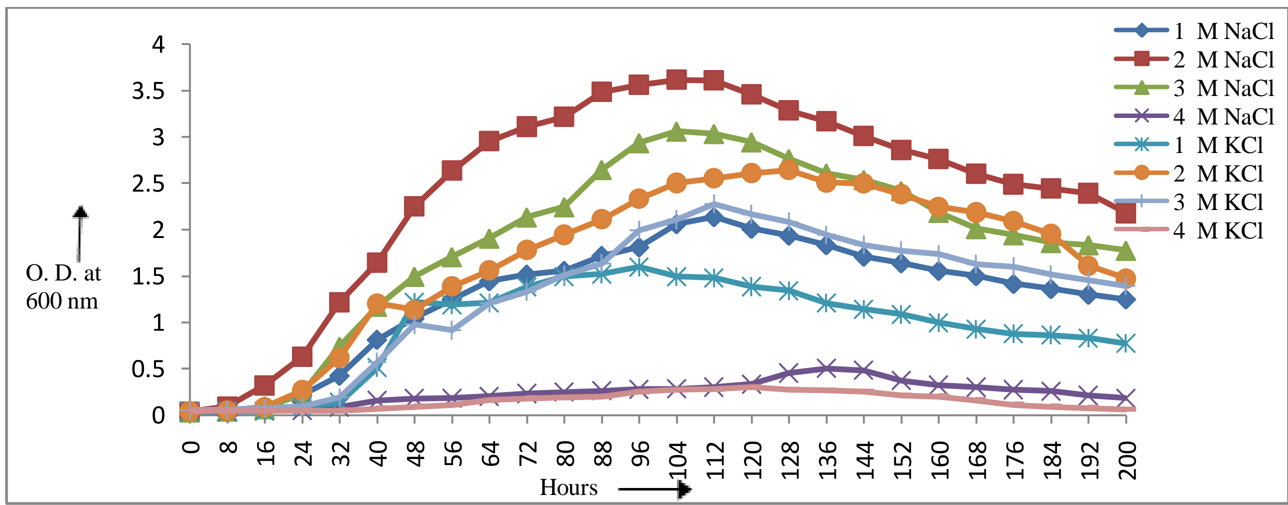


Fig. 4.19: Growth Profiles of MSL-16 in the presence of 1- 4 M NaCl and 1- 4 M KCl.

4.2.2.2 Determination of optimum pH and temperature for growth of isolates

Range of temperature and pH that supports growth was studied as mentioned in section 3.3.2.2. On the basis of plate study it was concluded that all the isolates have potential to grow at a wide range of temperature and pH. All moderately halophilic isolates can grow up to 50 ^{o}C. Unlike moderately halophilic isolates, borderline and extremely halophilic isolates were found to grow at higher temperature of 55 ^{o}C. pH range that supports growth was also different for isolates. Isolate MSL-1, MSL-4, MSL-8, MSL-10, MSL-12, MSL-13, MSL-14, MSL-15 and MSL-16 can grow at a pH range 6- 10 while MSL-2, MSL-3, MSL-5, MSL-6, MSL-7 and MSL-11 can grow at a pH range 6-11. MSL-9 was the only isolate that can grow at a pH range 7-10. Further characters of all the isolates have been summarized in Table 4.2.

Table 4.2: General characteristics of isolates from Sambhar salt lake, Rajasthan.

Characteristics	MSL-1	MSL-2	MSL-3	MSL-4	MSL-5	MSL-6	MSL-7	MSL-8
Cell shape	Long Rods to cocci (pleomorphic)	Cocci	Cocci	Cocci	Short rods	Short rods	Short rods	Long rods
Pigmentation	Dark Orange	Orange	Light orange	White	White	Pale	Pale	Transparent
Gram's nature	Negative	Positive	Positive	Positive	Positive	Positive	Positive	Positive
Range of NaCl concentration which supports growth	2- 5 M	2- 5 M	2- 5 M	2- 5 M	1- 4 M	1- 4 M	1- 4 M	1- 4 M
Range of KCl concentration which supports growth	1- 4 M	1- 4 M	1- 4 M	1-4 M	1-3 M	1-3 M	1-3 M	1-3 M
Range of temperature which supports growth(^{O}C)	18-55	18 -55	18-55	18-55	18-50	18-50	18-50	18-50
Range of pH which supports growth	6-10	6-11	6-11	6-10	6-11	6-11	6-11	6-10
Optimum NaCl concentration for growth	3 M	3 M	3 M	3 M	2 M	2 M	2 M	2 M
Optimum KCl concentration for growth	3 M	3 M	3 M	3 M	1 M	2 M	2 M	2 M
Optimum temperature for Growth	45 ^{o}C	45 ^{o}C	45 ^{o}C	45 ^{o}C	37 ^{o}C	45 ^{o}C	45 ^{o}C	37 ^{o}C
Optimum pH for growth	8	9	9	8	8	8	8	8
Catalase	Positive	Negative	Negative	Positive	Positive	Positive	Positive	Positive
Oxidase	Negative	Negative	Negative	Positive	Positive	Negative	Negative	Negative
Spore	-	-	-	-		Terminal oval	Terminal oval	Terminal oval

Characteristics	MSL-9	MSL-10	MSL-11	MSL-12	MSL-13	MSL-14	MSL-15	MSL-16
Cell shape	Short rods	Long Rods to cocci (pleomorphic)	Cocci	Long rods	Long rods	Long rods	Long rods	Long rods
Pigmentation	Dark Pale	Light Orange	Light orange	Dark Pale	Dark Pale	Pale	Transparent	Transparent
Gram's nature	Positive	Negative	Positive	Positive	Positive	Positive	Positive	Positive
Range of NaCl concentration which supports growth	1-4 M	2-5 M	2-5 M	1-4 M	1-4 M	1-4 M	1-4 M	1-4 M
Range of KCl concentration which supports growth	1-3 M	1-4 M	1-4 M	1-3 M	1-3 M	1-3 M	1-3 M	1-3 M
Range of temperature which supports growth (^{o}C)	18-50	18 -55	18-55	18-50	18-50	18-50	18-50	18-50
Range of pH which supports growth	7-10	6-10	6-11	6-10	6-10	6-10	6-10	6-10
Optimum NaCl concentration for growth	1 M	4-5 M	3 M	2 M	2 M	2 M	2 M	2 M
Optimum KCl concentration for growth	1 M	3 M	3 M	2 M	2 M	2 M	2 M	2 M
Optimum temperature for growth	37 ^{o}C	45 ^{o}C	45 ^{o}C	37 ^{o}C	37 ^{o}C	37 ^{o}C	37 ^{o}C	37 ^{o}C
Optimum pH for growth	8	9	9	8	8	8	8	8
Catalase	Positive	Positive	Negative	Positive	Positive	Positive	Positive	Positive
Oxidase	Positive	Negative	Negative	Positive	Positive	Positive	Negative	Negative
Spore	-	-	-	Terminal oval	Terminal oval	Terminal oval	Terminal oval	Terminal oval

On the basis of salt requirement, halophiles can be subdivided into extreme halophiles, halo tolerant and moderately halophilic (Kushnaer and Kamekura 1988). On the basis of pH they are either haloalkaliphilic or haloacidophilic (Bower et al. 2011). A very few halophiles can grow at alkaline pH and elevated temperature. If a combination of two or more extreme conditions are required for optimum growth than the isolates are termed as poly-extremophiles. According to Bower et al. 2010, out of sixty validly published halophilic species nineteen species had optimum pH at 8.5 and out of these nineteen only ten species had NaCl requirement at or above 2 M. In the present study, sixteen isolates (Table 4.1) have been isolated from four soil and one salt sample from Sambhar lake Rajasthan. Owing to the screening conditions which involve high salt 4 M NaCl, alkaline pH (9.0) and elevated temperature 45 ^{o}C, only sixteen isolates in complex medium were obtained. All the isolated strains were alkaliphilic (pH optimum 8- 9) and can survive in combination of at least two extreme conditions (2- 4 M NaCl and alkaline pH). Out of sixteen isolates one (MSL-10) was extremely halophilic, alkaliphilic and thermo tolerant (Fig. 4.13), four (MSL-1, MSL-2, MSL-3, MSL-4 and MSL-11) were borderline extremely halophilic (Fig 4.4- 4.7 and 4.14), alkaliphilic and thermo tolerant, two (MSL-6 and MSL-7) were moderately halophilic (Fig. 4.9 and 4.10), alkaliphilic and thermo tolerant and seven (MSL-5 MSL-8 MSL-12 MSL-13 MSL-14 MSL-15 MSL-16) were moderately halophilic (Fig. 4.8, 4.11, 4.15- 4.19) and alkaliphilic and one (MSL-9) was moderately halophilic (Fig. 4.12) and alkali tolerant.

Table 4.3: Characterization of isolates on the basis of extremities.

Isolate	Salt Requirement	pH	Temperature
MSL-1, MSL-2, MSL-3, MSL-4 and MSL-11	Borderline extremely halophilic optimum growth at 3 M NaCl	Alkaliphilic optimum growth at pH 8- 9	Thermo tolerant optimum growth at 45 ^{o}C
MSL-10	Extremely halophilic optimum growth at 4- 5 M NaCl	Alkaliphilic optimum growth at pH 8- 9	Thermo tolerant optimum growth at 45 ^{o}C
MSL-6 and MSL-7	Moderately halophilic optimum growth at 2M NaCl	Alkaliphilic optimum growth at pH 8- 9	Thermo tolerant optimum growth at 45 ^{o}C

MSL-5, MSL-8, MSL-12, MSL-13, MSL-14, MSL-15and MSL-16	Moderately halophilic optimum growth at 2 M NaCl	Alkaliphilic optimum growth at pH 8- 9	Optimum growth at 37 ^{o}C
MSL-9	Moderately halophilic optimum growth at 1M NaCl	Alkalitolerant optimum growth at pH 7	Optimum growth at 37 ^{o}C

4.2.2.3 Sensitivity towards antimicrobial agents

Extremely halophilic and borderline extremely halophilic isolates showed resistance towards many antimicrobial agents while moderately halophilic isolates were found to be sensitive towards all the antimicrobial agents used except MSL-5 which showed resistance towards erythromycin, gentamycin and oxacillin. Extremely halophilic and borderline extremely halophilic isolates showed resistance towards ampicillin, cephalothin, chloramphenicol, clindamycin, erythromycin, gentamycin, oxacillin and penicillin- G (Fig. 4.20). All isolates were found to be sensitive for co-trimoxazole, cloxacilin and cefradine (Fig. 4.20). Further sensitivity towards antimicrobial agents has been summarized in Table 4.4 and 4.5. Upasani and Desai 1990 isolated six isolates (archaea) from Sambhar lake and all were sensitive to co-trimoxazole and resistant to ampicillin, and penicillin. Similarly all the isolates in the present study were sensitive to co-trimoxazole whereas borderline and extreme halophiles were resistant to ampicillin and penicillin. Most of the extremely halophilic bacteria belong to domain, archaea. Archaea have been characterized by the absence of peptidoglycan in their cell wall. Thus they are resistant to antibiotics which target peptidoglycan (Holmes and Dyall-Smit1991).

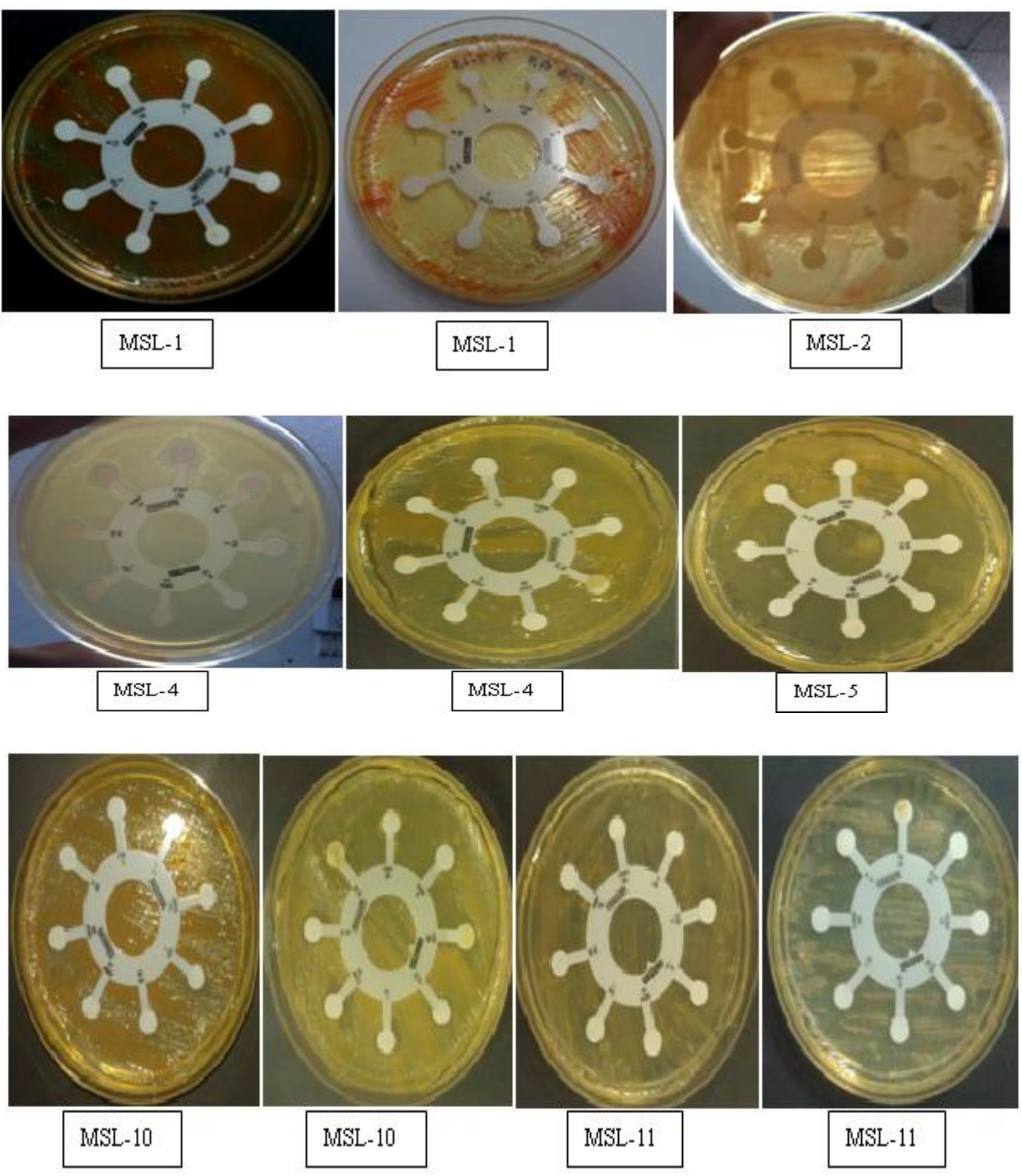


Fig. 4.20: Sensitivity of isolates (MSL-1, MSL-2, MSL-3, MSL-4, MSL-5, MSL-10 and MSL-11) towards antimicrobial agents.

Table 4.4: Sensitivity of isolates MSL1- MSL8 obtained from Sambhar salt lake Rajasthan, towards antimicrobial agents.

	Isolates							
Antibiotic sensitivity	MSL-1	MSL-2	MSL-3	MSL-4	MSL-5	MSL-6	MSL-7	MSL-8
Ampicillin	R	R	R	R	S	S	S	S
Cephalothin	R	R	R	R	S	S	S	S
Chloramphenicol	R	R	R	R	S	S	S	S
Clindamycin	R	R	R	R	S	S	S	S
Erythromycin	R	R	R	R	R	S	S	S
Gentamycin	R	R	R	R	R	S	S	S
Oxacillin	R	R	R	R	R	S	S	S
Vancomycin	R	R	R	R	S	S	S	S
Co-trimoxazole	S	S	S	S	S	S	S	S
Cloxacilin	S	S	S	S	S	S	S	S
Cefradine	S	S	S	S	S	S	S	S
Linomycin	S	R	R	R	S	S	S	S
Cefuroxime	S	R	R	R	S	S	S	S
Penicillin-G	R	R	R	R	S	S	S	S
Tetracycline	R	S	S	R	S	S	S	S

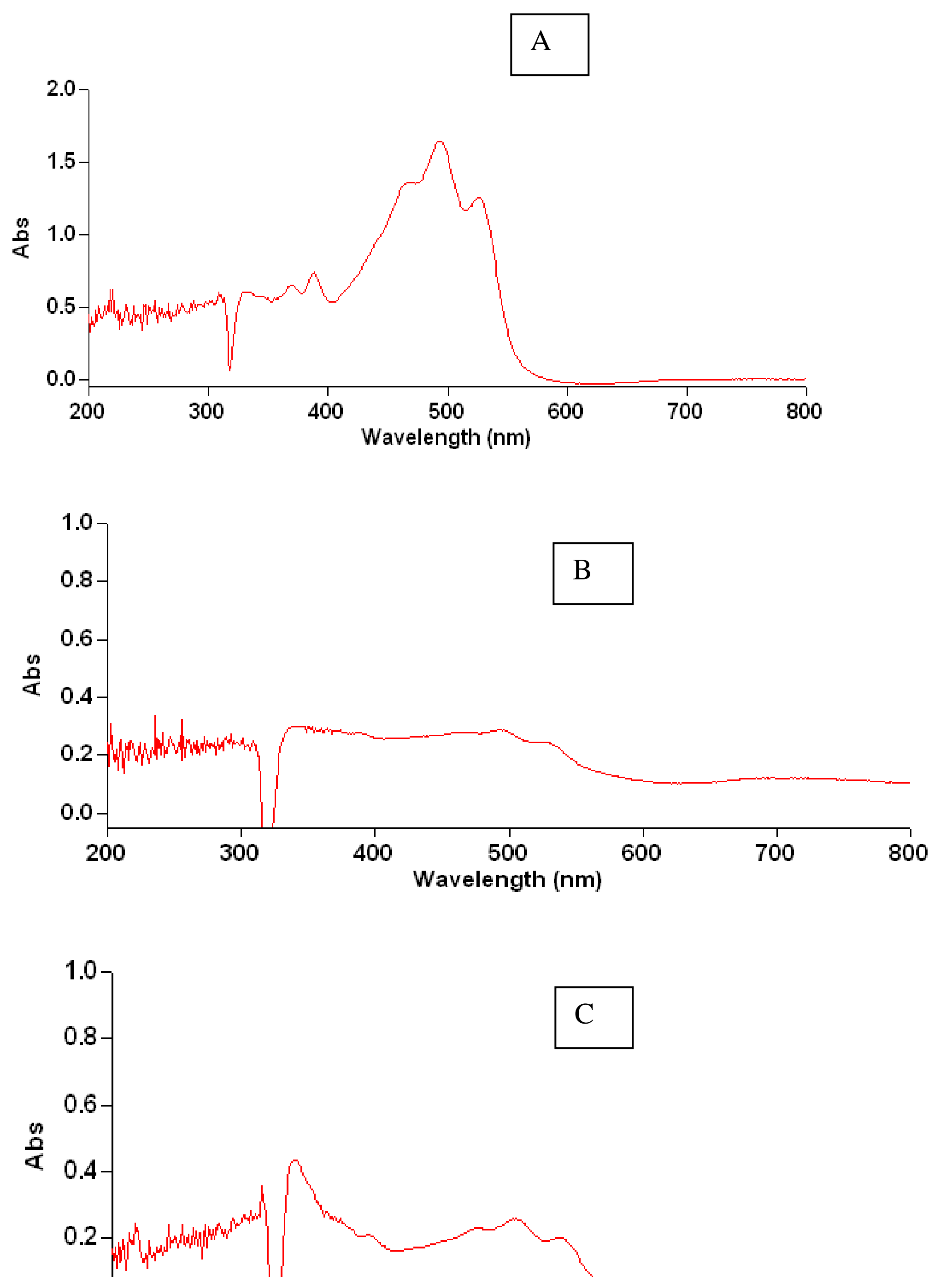
Table 4.5: Sensitivity of isolates MSL9- MSL-16 obtained from Sambhar salt lake Rajasthan, towards antimicrobial agents.

	Isolates							
Antibiotic sensitivity	MSL-9	MSL-10	MSL-11	MSL-12	MSL-13	MSL-14	MSL-15	MSL-16
Ampicillin	S	R	R	S	S	S	S	S
Cephalothin	S	R	R	S	S	S	S	S
Chloramphenicol	S	R	R	S	S	S	S	S
Clindamycin	S	R	R	S	S	S	S	S
Erythromycin	S	R	R	S	S	S	S	S
Gentamycin	S	R	R	S	S	S	S	S
Oxacillin	S	R	R	S	S	S	S	S
Vancomycin	S	R	R	S	S	S	S	S
Co-trimoxazole	S	S	S	S	S	S	S	S
Cloxacilin	S	S	S	S	S	S	S	S
Cefradine	S	S	S	S	S	S	S	S
Linomycin	S	R	R	S	S	S	S	S
Cefuroxime	S	R	R	S	S	S	S	S
Penicillin-G	S	R	R	S	S	S	S	S
Tetracycline	S	S	S	S	S	S	S	S

Note: R: resistant; S: sensitive.

4.2.2.4 Pigment profile

Out of sixteen isolates, five were found to produce pigment in liquid medium. Out of these five, one (MSL-1) was dark shining orange in colour, MSL-2 was orange in colour, MSL-3 and MSL-11 were pink in colour and MSL-10 was light orange in colour. Pigment extracted in methanol / acetone (1:1 v/v) showed an absorption maxima at 526, 494, 388 nm, and a shoulder at 470- 475 nm (Fig. 4.21 A-E) thus according to Cuadros-Orellanaa et al. 2006 study, all five isolates seems to have carotenoid pigment. MSL-1 was found to have additional minor pigments with minor peaks at 350, 355, 360, 368 and 388 nm (Fig. 4.21 A). Thus all extremely halophilic and borderline extremely halophilic isolates except MSL-4 were pigmented. Members of family Halobacteriaceae are halophiles par excellence and have pigment that provide specific red orange colour to the brine (Oren 2001). Most of the members of family Halobacteriaceae have been found to have C-50 carotenoid pigments like bacterioruberin and its derivatives. These pigments provide heat protection by increasing rigidity and mechanical strength of cell membrane (Fang et al. 2010). Previously red colour of the members of family Halobacteriaceae was taken as a recognizable factor to differentiate between archaea and eubacteria. But it is not reliable because many colourless members are known in Halobacteriaceae as well as red colored *Salinibacter* is a true bacteria (Oren 2001). Most of the members of Halobacteriaceae have single pigment ie, carotenoid (Oren 2002) but in the present study MSL-1 was found to have some minor pigments. Possibly these pigments have developed by lateral gene transfer as in *Salinibacter* (Sharma et al. 2007).


A
2.0
1.5
1.0
0.5
0.0
Abs
200
300
400
500
600
700
800
Wavelength (nm)
B
1.0
0.8
0.6
0.4
0.2
0.0
Abs
200
300
400
500
600
700
800
Wavelength (nm)
C
1.0
0.8
0.6
0.4
0.2
0.0
Abs
200
300
400
500
600
700
800
Wavelength (nm)

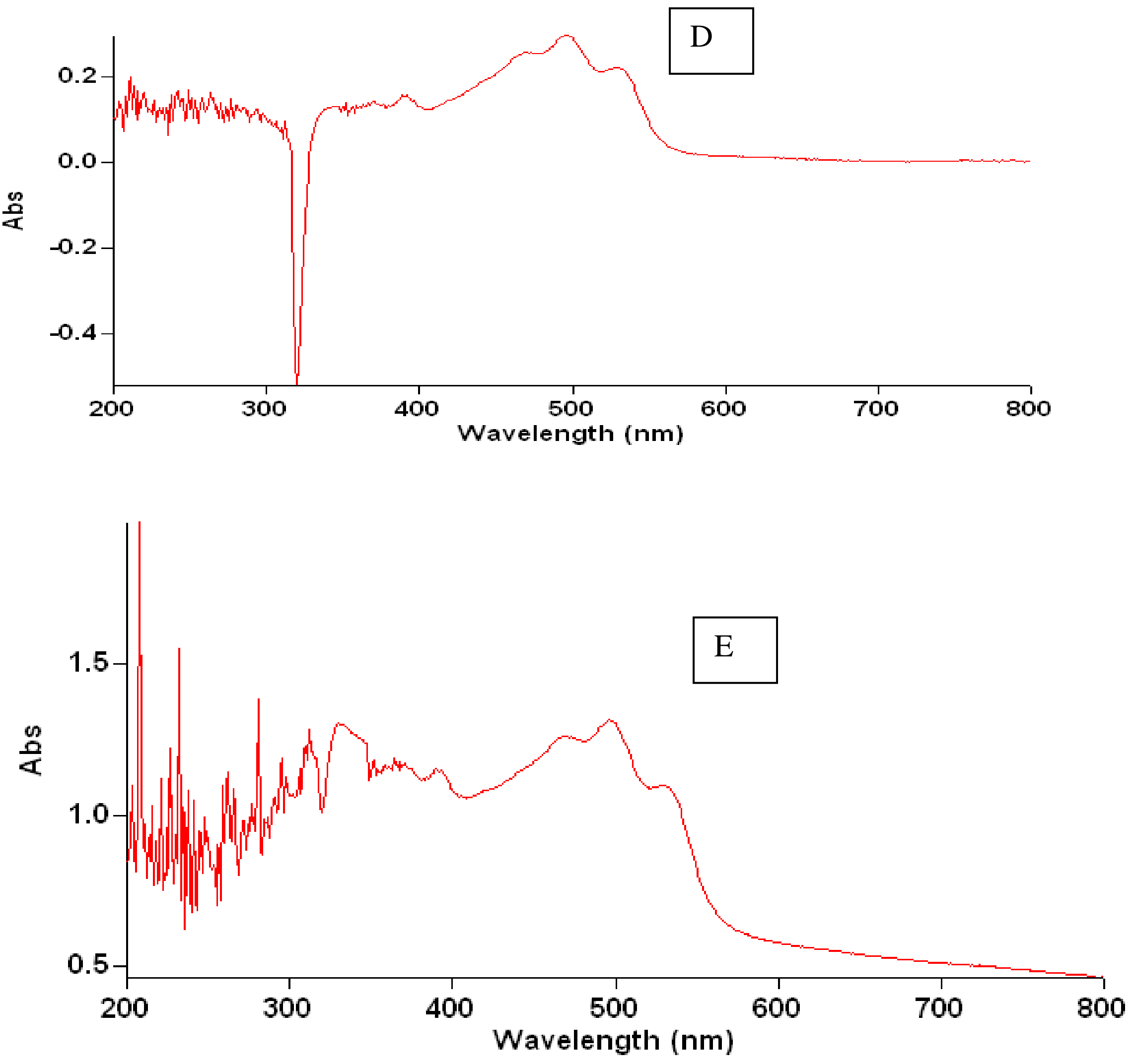


Fig. 4.21(A-E): Absorption spectra of pigment from isolate A) MSL-1 B) MSL-2 C) MSL-3 D) MSL-10 E) MSL-11 extracted in methanol/acetone (1:1, v/v).

4.2.3 Biochemical characterization

Carbon substrate utilization by all the isolates is given in Table 4.6

Table 4.6: Biochemical characterization of isolates based on carbon source utilization via BIOLOG GEN III plates.

	MSL 1	MSL 2	MSL 3	MSL 4	MSL 5	MSL 6	MSL 7	MSL 8	MSL 9	MSL 10	MSL 11	MSL 12	MSL 13	MSL 14	MSL 15	MSL 16
D-Raffinose	BL	BL	BL	-	+	BL	BL	BL	+	BL	BL	BL	BL	BL	BL	BL
α-D-Glucose	BL	BL	BL	-	+	BL	BL	-	+	BL	BL	+	+	+	+	BL
D-sorbitol	BL	BL	-	-	-	-	-	-	+	-	-	-	-	BL	BL	BL
Gelatin	-	-	BL	-	-	-	-	-	BL	-	-	-	-	BL	-	-
Pectin	BL	BL	-	-	-	-	-	-	+	BL	-	BL	BL	-	BL	-
4-Hydroxyphenylacetic acid	-	-	-	-	-	-	-	BL	-	-	-	-	-	BL	-	-
Tween 40	-	-	-	-	BL	-	-	-	-	-	-	BL	BL	BL	BL	-
Dextrin	+	+	+	-	BL	-	-	-	-	-	+	BL	BL	BL	BL	BL
α-D-Lactose	BL	+	BL	-	+	BL	BL	-	+	BL	BL	-	-	-	-	-
D-Mannose	BL	BL	BL	-	+	+	+	BL	+	BL	BL	+	+	BL	+	+
D-Mannitol	+	BL	BL	-	+	+	+	-	+	-	BL	-	-	-	+	-
Glycyl-L-proline	-	-	BL	-	-	BL	BL	-	BL	-	-	-	-	-	BL	-
D-Galacturonic acid	+	BL	+	+	+	+	+	+	BL	+	+	+	+	BL	+	+
Methyl pyruvate	-	-	BL	-	-	-	-	-	-	-	-	-	-	-	BL	-
¥-Amino butyric acid	-	-	-	-	-	-	-	-	-	-	-	-	-	BL	-	-
D-Maltose	+	BL	BL	-	+	BL	BL	-	+	BL	BL	+	+	BL	-	-
D-Melibiose	BL	BL	BL	-	BL	BL	BL	-	BL	BL	BL	BL	BL	-	BL	BL
D-Fructose	+	+	BL	BL	+	+	+	BL	+	BL	BL	-	-	-	+	BL
D-Arabitol	BL	-	BL	-	+	BL	BL	-	BL	-	BL	-	-	-	BL	BL
L-Alanine	-	-	-	-	+	+	+	-	+	-	-	-	-	-	+	BL
L-Galactonic acid lectone	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Lactic acid methyl ester	-	-	-	-	BL	BL	BL	-	+	-	-	-	-	-	BL	-
α -Hydroxy butyric acid	-	-	-	-	BL	-	-	-	-	-	-	BL	BL	-	-	-
D-Trehalose	BL	BL	BL	-	+	+	+	BL	+	BL	BL	+	+	BL	BL	-
β-Methyl D-glucoside	BL	BL	BL	-	+	BL	BL	-	+	-	BL	-	-	-	BL	BL
D-Galactose	BL	BL	BL	BL	+	+	+	BL	+	+	BL	BL	BL	-	BL	BL
Myoinositol	BL	BL	BL	-	-	+	+	-	+	-	BL	BL	BL	-	BL	BL

	MSL 1	MSL 2	MSL 3	MSL 4	MSL 5	MSL 6	MSL 7	MSL 8	MSL 9	MSL10	MSL11	MSL12	MSL13	MSL14	MSL15	MSL16
L-Arginine	-	-	-	-	-	+	+	-	BL	-	-	-	-	-	-	-
L-Gluconic acid	-	-	-	-	+	-	-	-	+	-	-	-	-	-	+	-
L-Lactic acid	-	-	-	-	+	+	+	-	+	-	-	-	-	-	+	-
β-Hydroxy-D,L butyric acid	-	-	-	-	BL	-	-	-	+	-	-	-	-	-	-	-
D-Cellobiose	+	BL	+	-	+	+	+	-	BL	BL	+	+	+	-	BL	-
D-Salicin	-	-	-	-	+	+	+	-	BL	-	-	-	-	-	BL	BL
3-Methyl glucose	BL	BL	BL	-	BL	BL	BL	-	+	BL	BL	-	-	-	BL	-
Glycerol	BL	-	-	-	+	+	+	-	+	-	-	-	-	-	-	-
L-Aspartic acid	BL	-	-	-	-	+	+	-	BL	-	-	-	-	-	BL	-
D-Glucuronic acid	+	+	+	+	+	+	+	+	BL	+	+	+	+	+	+	+
Citric acid	-	-	-	-	+	+	+	-	+	-	-	-	-	-	-	-
α-Ketobutyric acid	BL	-	-	-	-	-	-	-	-	BL	-	-	-	BL	BL	-
Gentiobiose	+	+	BL	-	+	-	-	-	-	BL	BL	+	+	-	BL	BL
N-Acetyl D-glucosamine	BL	BL	BL	-	+	+	+	-	+	-	BL	-	-	-	+	+
D-Fucose	BL	BL	BL	BL	BL	BL	BL	BL	+	BL	BL	-	-	-	BL	BL
D-Glucose-6-PO_4	BL	BL	BL	BL	BL	+	BL	BL	BL	BL	BL	BL	BL	BL	BL	BL
L-Glutamic acid	-	-	-	-	BL	+	BL	-	-	-	-	-	-	-	BL	-
Glucuronamide	+	+	+	+	BL	+	+	+	BL	+	+	+	+	+	+	+
α-Ketoglutaric acid	BL	-	-	-	-	BL	BL	-	+	BL	-	-	-	-	BL	BL
Acetoacetic acid	+	BL	BL	BL	BL	+	+	+	+	BL	BL	BL	BL	BL	BL	BL
Sucrose	BL	BL	BL	-	+	+	+	-	+	BL	BL	-	-	+	-	-
N-Acetyl β-D-mannosamine	BL	BL	BL	-	BL	+	+	-	+	-	BL	-	-	-	+	BL

	MSL1	MSL2	MSL3	MSL4	MSL5	MSL6	MSL7	MSL8	MSL9	MSL10	MSL11	MSL12	MSL13	MSL14	MSL15	MSL16
L-Fucose	BL	BL	BL	BL	BL	BL	BL	BL	+	+	BL	BL	BL	-	BL	BL
D- Fructose-6-PO_4	+	BL	BL	+	BL	+	+	+	-	BL	BL	+	+	BL	+	+
L-Histidine	BL	-	-	-	-	+	+	-	+	-	-	BL	BL	-	BL	-
Mucic acid	-	-	-	-	+	+	+	-	BL	-	-	-	-	-	-	-
D-Malic acid	-	-	-	-	+	BL	BL	-	+	-	-	-	-	-	+	-
Propionic acid	BL	BL	BL	-	+	BL	BL	-	-	BL	BL	+	+	BL	-	-
D-Turanose	BL	BL	BL	-	+	BL	BL	-	+	BL	BL	+	+	BL	BL	BL
N-Acetyl D-galactosamine	BL	BL	BL	-	-	+	+	-	+	-	BL	-	-	BL	+	BL
L-Rhamnose	+	+	+	BL	+	+	+	BL	BL	BL	+	BL	BL	-	BL	BL
D-Aspartic acid	-	-	-	-	-	BL	BL	-	BL	-	-	-	-	-	-	-
L-Pyroglutamic acid	-	-	-	-	-	BL	BL	-	+	-	-	-	-	-	-	-
Quinic acid	-	-	-	-	+	+	+	-	BL	-	-	-	-	-	-	-
L-Malic acid	-	-	-	-	+	BL	BL	-	+	-	-	-	-	-	-	-
Acetic acid	+	BL	BL	BL	+	+	+	-	+	BL	BL	BL	BL	BL	+	+
Stachyose	BL	BL	BL	-	+	BL	BL	-	+	BL	BL	-	-	BL	-	-
N-acetyl Neuraminic acid	-	-	-	-	-	+	+	-	+	+	-	-	-	BL	+	-
Inosine	-	-	-	-	-	+	+	-	+	BL	-	-	-	BL	+	+
D-Serine	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
L-Serine	BL	-	-	-	-	+	+	-	BL	-	-	-	-	BL	-	-

	MSL1	MSL2	MSL3	MSL4	MSL5	MSL6	MSL7	MSL8	MSL9	MSL10	MSL11	MSL12	MSL13	MSL14	MSL15	MSL16
D-Saccharic acid	BL	-	-	-	+	+	+	-	+	-	-	-	-	-	-	-
Bromo succinic acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Formic acid	-	-	-	-	-	-	-	-	-	BL	-	-	-	+	-	-
1% Sodium lactate	BL	-	-	+	+	+	+	BL	+	BL	-	+	+	+	+	+
Troleandomycin	BL	-	-	-	+	+	+	BL	-	BL	-	-	-	BL	BL	BL
Lincomycin	BL	BL	BL	+	+	BL	BL	BL	-	BL	BL	BL	BL	BL	BL	BL
Vancomycin	+	+	+	+	-	-	-	BL	-	BL	+	BL	BL	BL	BL	BL
Nalidixic acid	+	+	+	+	+	+	+	+	+	BL	+	+	+	+	+	+
Aztreonam	+	BL	BL	+	+	+	+	BL	-	BL	BL	BL	BL	BL	BL	BL
Fusidic acid	+	-	-	-	-	-	-	BL	-	+	-	-	-	BL	BL	BL
Rifamycin SV	BL	-	-	+	-	-	-	+	-	BL	-	-	-	+	BL	+
Guanidine HCL	BL	BL	BL	BL	-	+	+	BL	+	BL	BL	+	+	BL	+	BL
Tetrazolium violet	BL	-	-	BL	BL	BL	BL	BL	BL	BL	-	BL	BL	-	-	BL
Lithium chloride	+	BL	BL	+	+	+	+	+	+	+	BL	+	+	+	+	+
Sodium butyrate	+	+	+	+	+	+	+	+	BL	+	+	+	+	+	+	+
Minocycline	+	BL	BL	+	+	+	+	+	+	+	BL	+	+	BL	+	+
Niaproof 4	BL	BL	BL	-	-	-	-	BL	-	BL	BL	BL	BL	BL	BL	BL
Tetrazolium blue	+	BL	BL	-	BL	BL	BL	BL	BL	BL	BL	BL	BL	-	BL	BL
Potassium tellurite	BL	BL	BL	-	-	-	-	BL	+	+	BL	BL	BL	BL	-	-
Sodium bromate	BL	BL	BL	BL	-	-	-	-	-	BL	BL	BL	BL	BL	-	BL

Where (+) = positive, (-) = negative, BL = borderline.

Out of all isolates, MSL-9 was found to utilize maximum numbers of carbon sources and MSL-4 utilized minimum number of carbon sources. Glucuronamide was the only carbon source that was universally utilized by all the isolates. Upasani and Desai 1990, reported that none of their isolates could utilize Tween 40, similarly in the present study all isolates either showed negative or borderline reaction with Tween 40. Except MSL-5 and MSL-9 which showed positive reaction, all the isolates exhibited borderline reaction for D-raffinose. Isolates MSL-1, MSL-2, MSL-3, MSL-6, MSL-7, MSL-10, MSL-11, MSL-16 showed borderline reaction for glucose. Whereas MSL-5, MSL-9, MSL-12, MSL-13, MSL-14, MSL-15 showed positive reaction for it. Sorbitol and pectin were not utilized by any of the isolate except MSL-9 whereas MSL-10 showed borderline reaction to it. All extremely halophilic and borderline extremely halophilic isolates showed borderline reaction for mannose but all moderately halophilic isolates had positive response to it except MSL-8. MSL-8 was the isolate whose carbon substrate utilization was very similar to that of extremely halophilic and borderline extremely halophilic isolates. Methyl pyruvate was not utilized by any of the isolate except MSL-3 which showed borderline reaction to it. All the isolates showed borderline reaction for glucose-6-phosphate except MSL-6 which showed positive reaction for it. Lactic acid and citric acid were utilized only by MSL-5 MSL-6, MSL-7 and MSL-9. Fructose was not utilized by MSL-12, MSL-13, MSL-14 where as MSL-14 was the only isolate that could not utilize galactose. Sugar utilization was more in comparison to acids and amino acids as carbon sources. MSL-6 and MSL-7 were found to use amino acids like alanine, serine, arginine and histidine. MSL-5 was found to utilize most of the acids like lactic acid, citric acid, qunic acid, acetic acid and saccharic acid. Rhamnose was utilized by all the isolates except MSL-14.

4.2.3.1 Oxidase and catalase tests

Thirteen (MSL-1, MSL-4, MSL-5, MSL-6, MSL-7, MSL-8, MSL-9, MSL-10, MSL-12, MSL-13, MSL-14, MSL-15 and MSL-16) isolates were found to be catalase positive (Fig. 4.22). Six isolates (MSL-4, MSL-5 MSL-9 MSL-12, MSL-13 and MSL-14) were found to be oxidase positive.

Fig. 4.22: Isolates showing catalase reaction.

4.2.3.2 Screening of isolate for extracellular hydrolytic activities

4.2.3.2.1 Screening of isolates for extracellular protease production

On the basis of plate assay (as mentioned in materials and method) five (MSL-1, MSL-4, MSL-5, MSL-12 and MSL-13) out of sixteen isolates were found to produce extracellular protease Fig. 4.23.

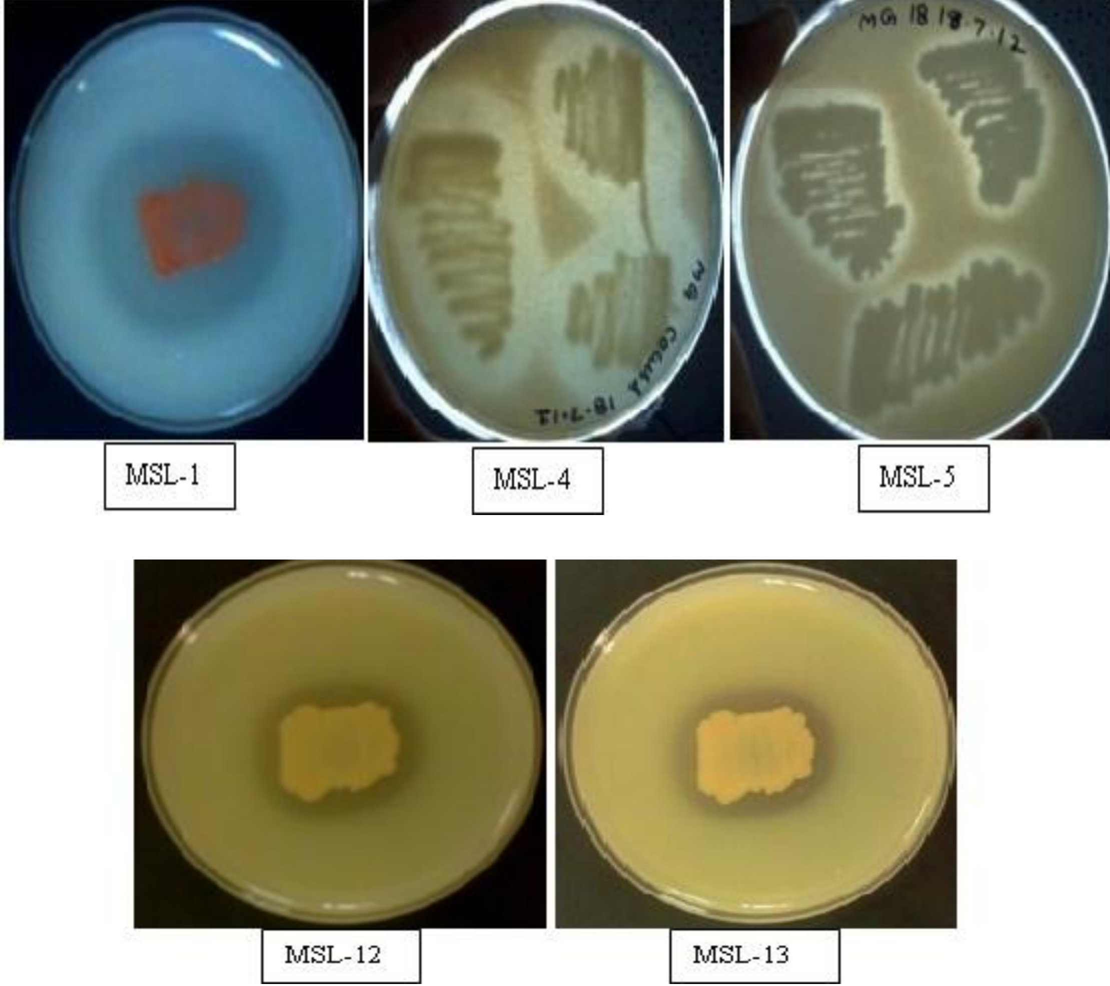


Fig. 4.23: Protease production by isolates on skimmed milk agar plates.

4.2.3.2.2 Screening of isolates for extracellular amylase production

On the basis of plate assay (as mentioned in materials and method section 3.4.3.3.2). MSL-9 and MSL-10 out of sixteen isolates were found to produce extracellular amylase Fig. 4.24.

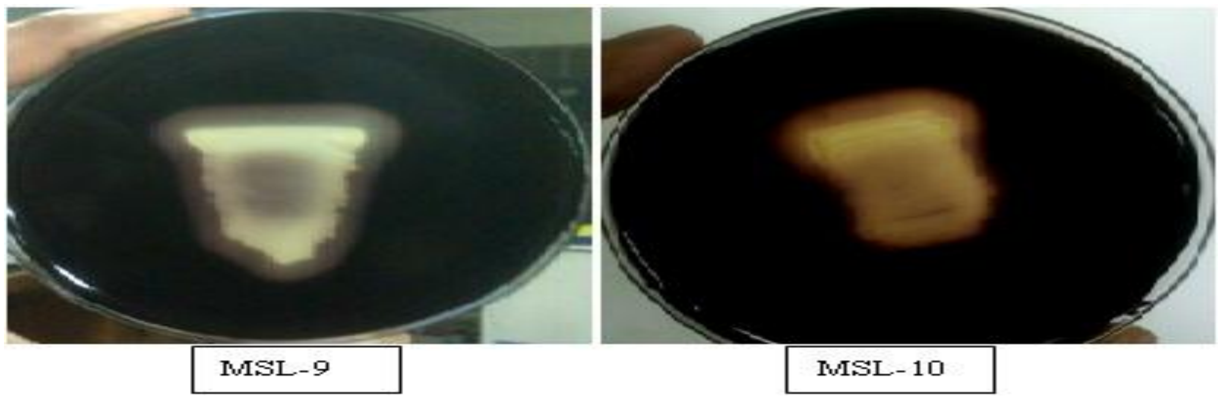


Fig. 4.24: Amylase production by isolates on starch agar plat**4.2.3.2.3 Screening of isolates for extracellular pectinase production**

On the basis of plate assay (as mentioned in materials and method) two (MSL-9 and MSL-10) out of sixteen isolates were found to produce extracellular pectinase Fig. 4.25.

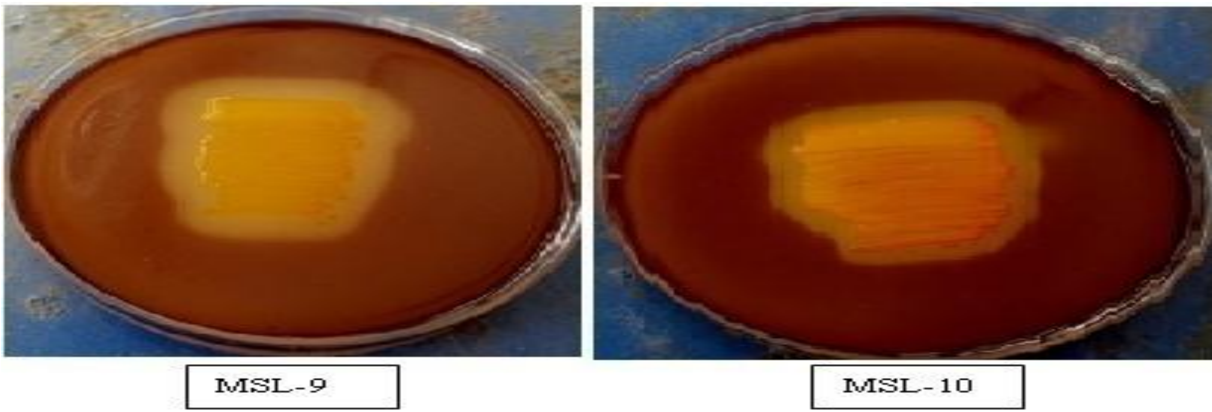


Fig. 4.25: Pectinase production by isolates on pectin agar plates.

4.2.3.2.4 Screening of isolates for extracellular DNase production

On the basis of plate assay (as mentioned in materials and method) seven (MSL-3, MSL-4, MSL-9, MSL-12, MSL-13, MSL-15 and MSL-16) out of sixteen isolates was found to produce extracellular DNase Fig. 4.26.

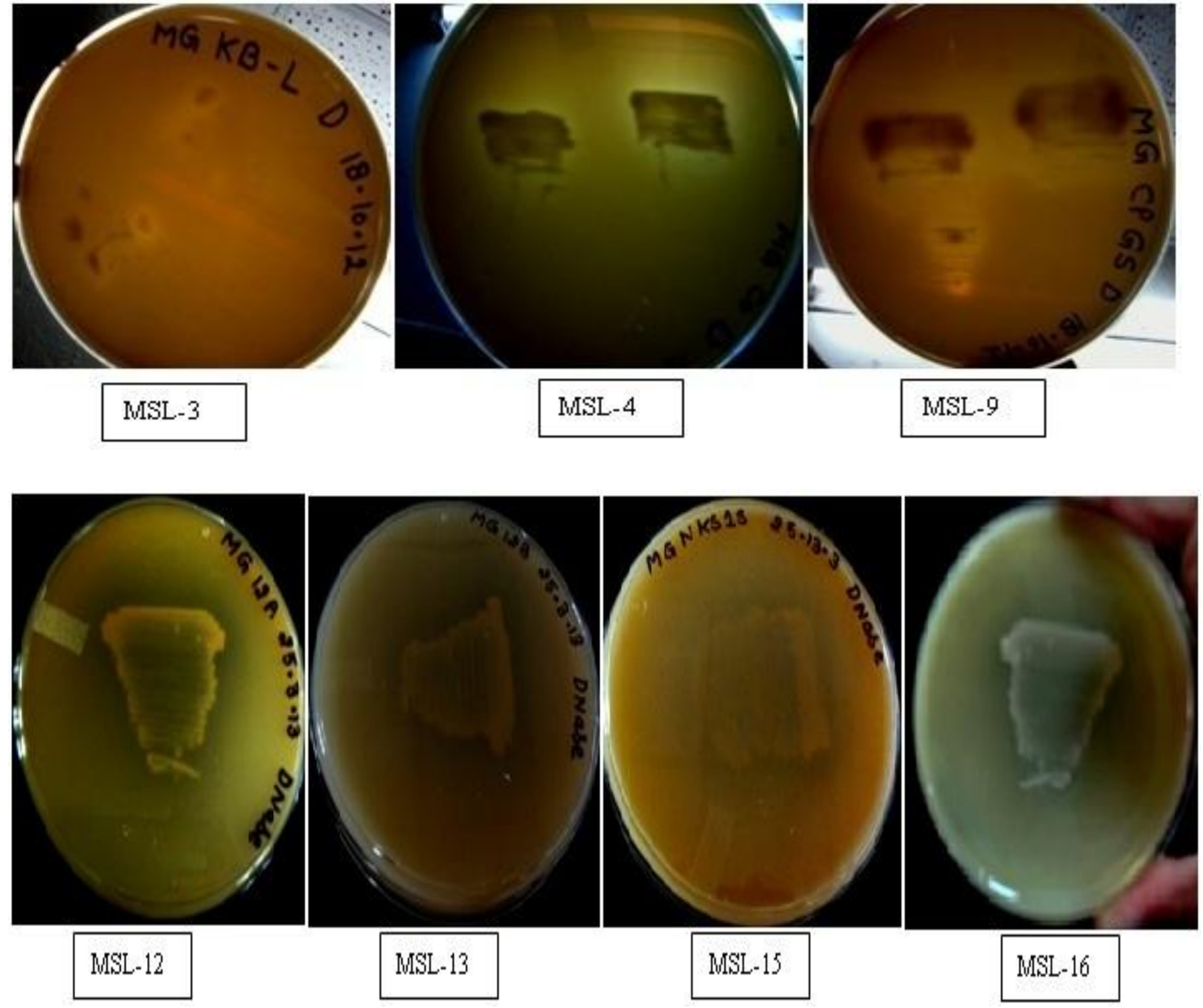


Fig. 4.26: DNase production by isolates on DNase test agar plates.

Total hydrolytic enzyme activities found in different isolates have been summarized in Table 4.7.

Table 4.7: Total hydrolytic enzyme activities found in different isolates from Sambhar salt lake, Rajasthan.

	Hydrolytic Enzyme				
Isolates	**Protease**	**Amylase**	**Pectinase**	**DNase**	**Total**
MSL-1	+	-	-	-	1
MSL-2	-	-	-	-	0
MSL-3	-	-	-	+	1
MSL-4	+	-	-	+	2
MSL-5	+	-	-	-	1
MSL-6	-	-	-	-	0
MSL-7	-	-	-	-	0
MSL-8	-	-	-	-	0
MSL-9	-	+	+	+	3
MSL-10	-	+	+	-	2
MSL-11	-	-	-	-	0
MSL-12	+	-	-	+	2
MSL-13	+	-	-	+	2
MSL-14	-	-	-	-	0
MSL-15	-	-	-	+	1
MSL-16	-	-	-	+	1

Where (+) = positive and (-) = negative.

On the basis of plate assays performed for hydrolytic enzymes it was observed that protease activity was found in five isolates (MSL-1, MSL-4, MSL-5, MSL-12 and MSL-13) out of sixteen isolates, extracellular amylase and pectinase activities were found only in two isolates (MSL-9 and MSL-10) and DNase activity was found in seven isolates (MSL-3, MSL-4, MSL-9, MSL-12, MSL-13, MSL-15 and MSL-16). In turn, isolate MSL-9 was found to have maximum hydrolytic activities ie three (amylase, pectinase and DNase) followed by MSL-10 with two hydrolytic activities (amylase and pectinase), MSL-4, MSL-12 and MSL-13 with two hydrolytic activities (protease, and DNase), MSL-3, MSL-15 and MSL-16 had only one hydrolytic activity (DNase),

MSL-1 and MSL-5 had only one hydrolytic activity (protease). Out of four hydrolases no hydrolytic enzyme could be detected in MSL-2, MSL-6, MSL-7, MSL-8, MSL-11 and MSL-14.

4.3 Protease production by the selected isolates

All five isolates showing extracellular protease activity on skimmed milk agar plate were grown in liquid medium and protease activity was checked at regular intervals. Out of five isolates two isolate MSL-12, MSL-13 produced extracellular protease after 108 hours and the estimated activities were 7.6 and 6.2 U/ml respectively. Protease production by MSL-5 was found after 86 hours and maximum activity was 15.90 U/ml. In MSL-1 and MSL-4 protease was produced after 36 hours and continues till 120 hours and maximum activities were 40.05 and 29.73 U/ml respectively (Fig. 4.27). Specific activities of MSL-1, MSL-4, MSL-5, MSL-12 and MSL-13 were 765.01 ± 8.5, 445.60 ± 9.28, 175.07±10.0, 7.05 ±0.77 and 6.65 ±.77 U/mg (Fig. 4.28). On the basis of these results isolate MSL-1 was selected and characterized further.

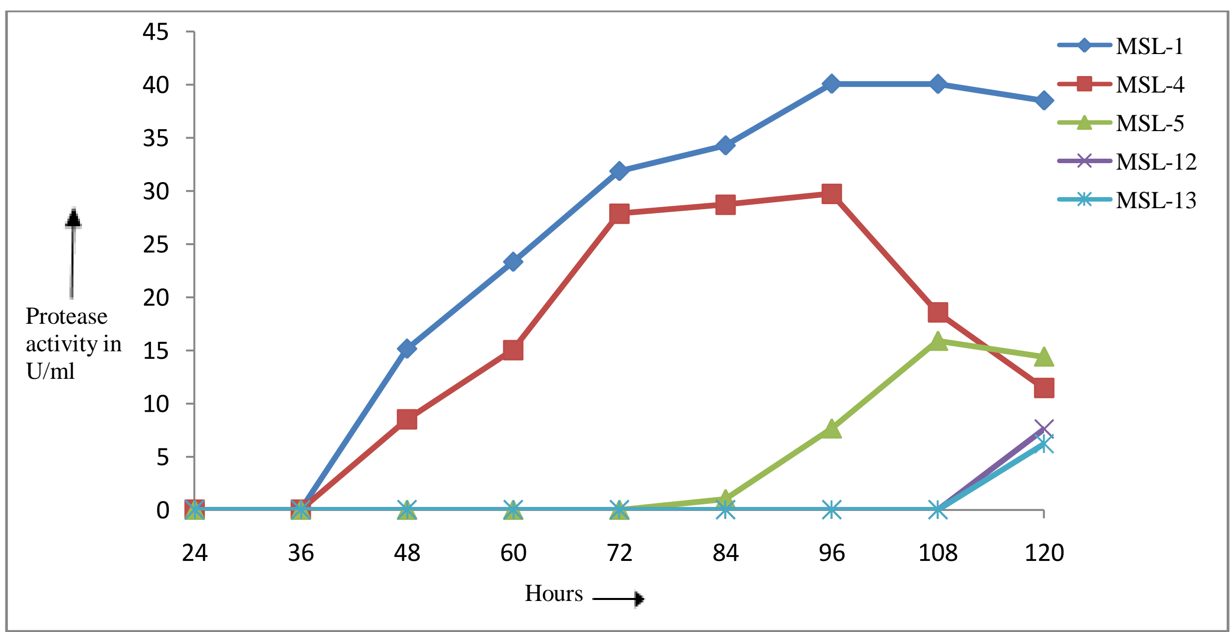


Fig. 4.27: Protease activity profiles of all protease producing isolates, all the isolates grown at 45 °C and pH 9.0 crude enzyme was used as protease source and caseinolytic activity was checked at 45 °C and pH 9.0 for 30 min.

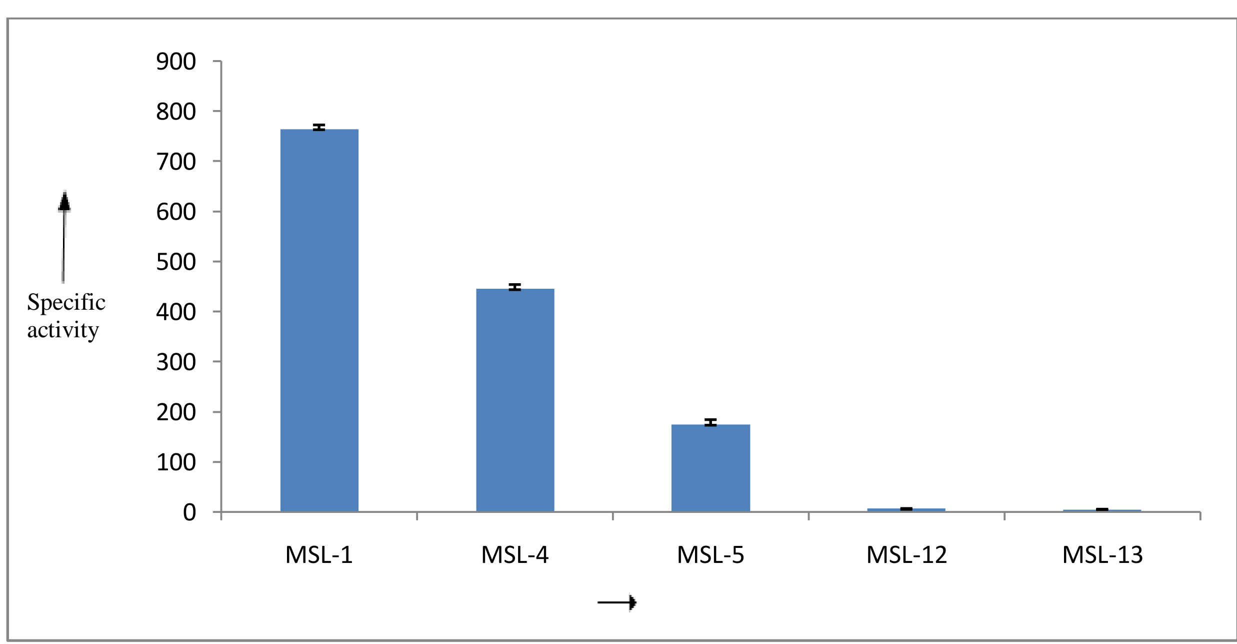


Fig. 4.28: Specific activities of all protease producing isolates, caseinolytic activity was checked at 45 °C and pH 9.0 for 30 min and protein estimation was done by dye binding method.

4.4 Genotypic characterization of selected isolate

The gene encoding 16S rRNA was amplified by polymerase chain reaction (PCR) with the universal archaeal primers (A571F and UA1204R, Arc21F and Univ 529R) and universal eubacterial primers (27F and 1492R). Amplification was detected only when archaeal primers were used. Size of amplified product was found to be around 700 bp (Fig. 4.29). 16S rRNA gene sequence showed 98 % similarity with *Halobiforma lacisalsi*, 97 % with *H. haloterrestris* and 96 % with *H. nitratireducens* (Fig. 4.30). Thus the strain was phylogenetically related to genus *Halobiforma* and closest to *Halobiforma lacisalsi* hence named as *Halobiforma* sp. strain BNMIITR. Comparison of isolate *Halobiforma lacisalsi* strain BNMIITR with other members of the genera given in Table 4.8.

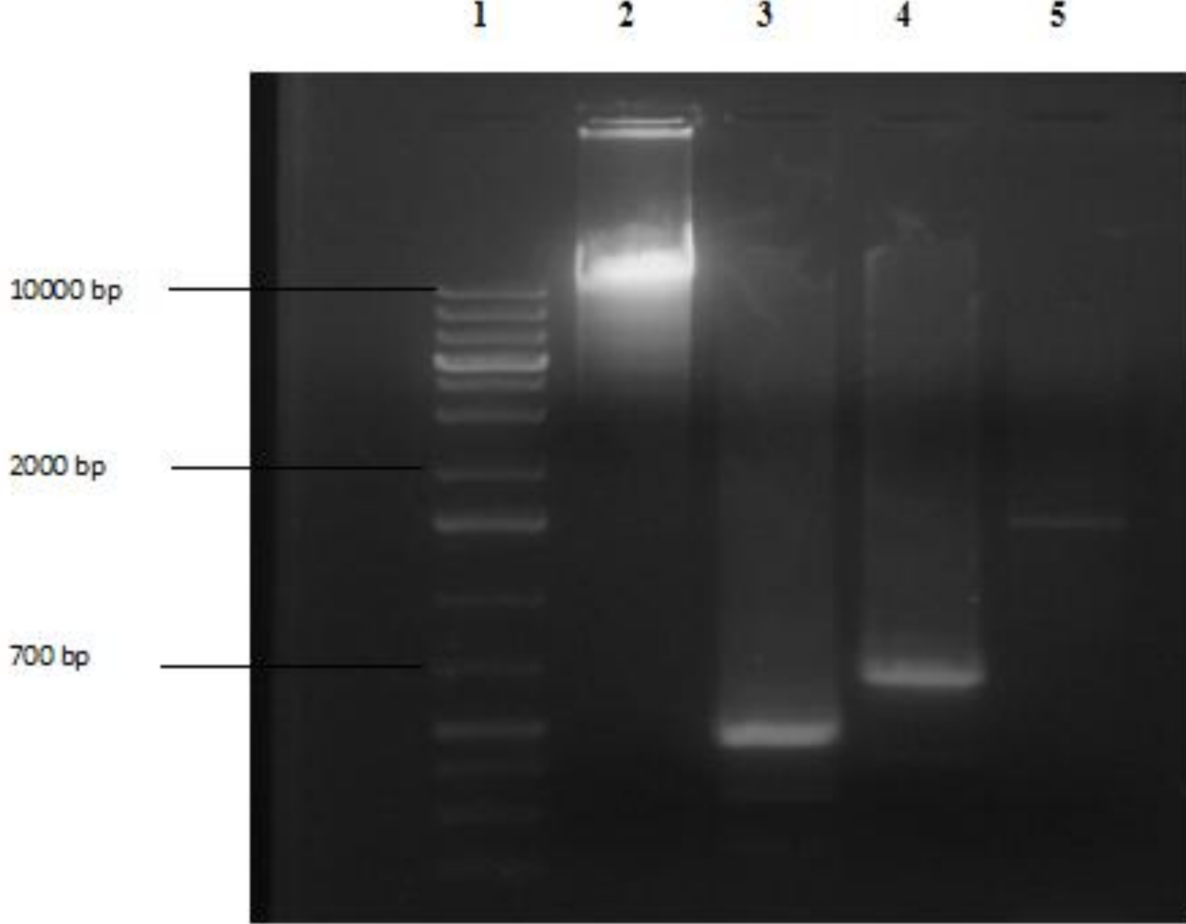


Fig. 4.29: Amplification of 16S rRNA gene of *Halobiforma* sp. strain BNMIITR by PCR using archaeal and eubacterial primers Lane 1: 1 Kb molecular wt standard. Lane 2: Purified genomic DNA of strain *Halobiforma* sp. strain BNMIITR. Lane 3: Amplification using archaeal primer set A571F and UA1204R. Lane 4: Amplification using archaeal primer set Arc21F and Univ529R Lane 5: Amplification using universal Eubacterial primers 27F and 1492R.

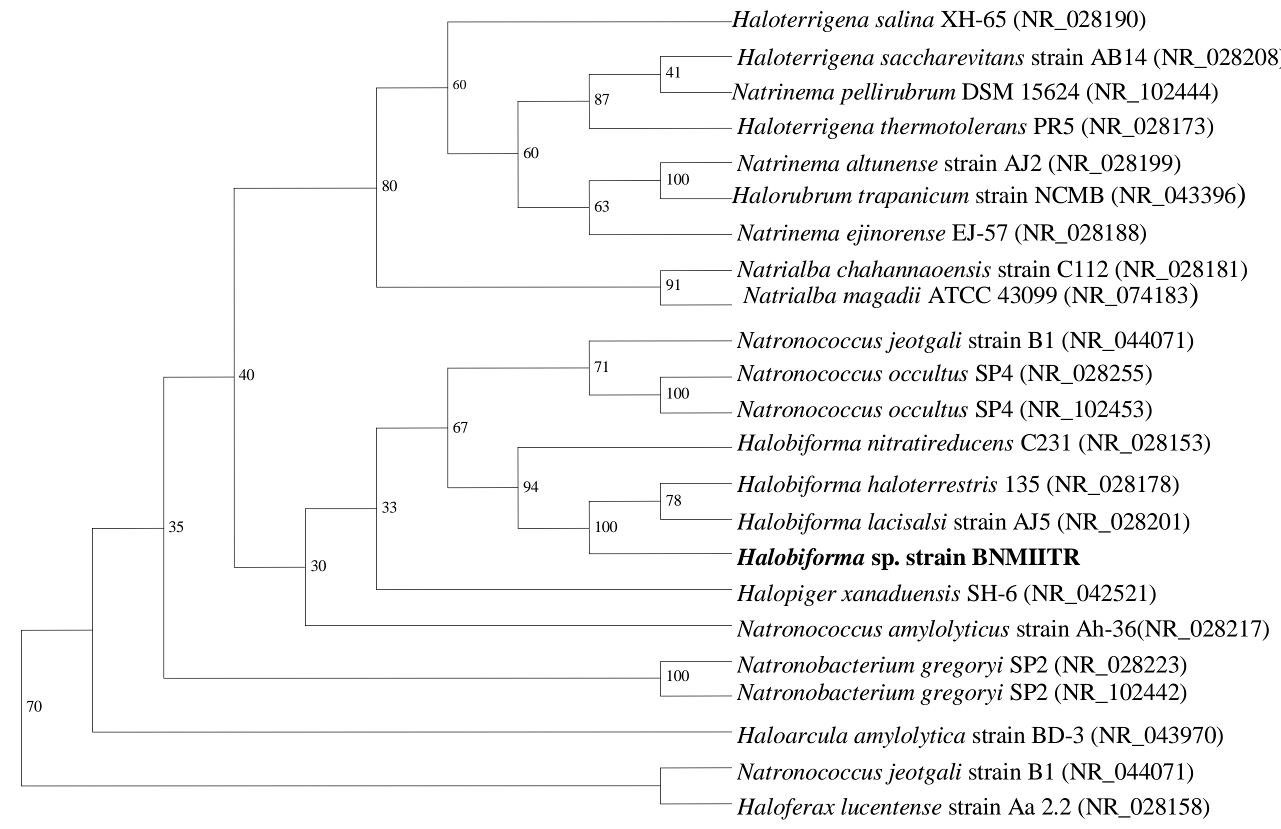


Fig. 4.30: Phylogenetic tree, based on 16S rRNA gene sequence data and neighbour-joining showing the phylogenetic positions of isolate *Halobiforma* sp. strain BNMIITR and some other related taxa. Bootstrap values are based on 1000 replicates and are shown at each node.

Table 4.8: Comparision of isolate *Halobiforma* sp. strain BNMIITR with other members of genera *H. Haloterrestris* according to Hezayen et al. 2002, *Halobiforma lacisalsi* according to Xu et al. 2005 and *H. nitratireducens* according to Xin et al. 2001.

Characteristics	***H. haloterrestris***	***Halobiforma lacisalsi***	***H. nitratireducens***	***Halobiforma* Strain BNMIITR**
NaCl range (M)	>2.2	>1.7	2.5-5.2	2-5
NaCl optimum	3.4 M	2.5-4.3 M	3.5 M	3 M
pH range	6-9.2	6.5-9.0	8-10.5	6-10
pH optimum	7.5	7.5	8.9	8-8.5
Temperature range	Up to 58 ^{o}C	24-57 ^{o}C	26-44 ^{o}C	20-55 ^{o}C

Temperature optimum	42 °C	42-45 °C	36-41 °C	45 °C
Mg^{+2} required for growth (mM)	0	0.2	0.4	0
Catalase	+	+	+	+
Oxidase	+	+	+	-
Hydrolysis of casein	+	-	-	+
Fructose	-	+	-	+
Mannitol	NR	-	+	+
Maltose	+	W	-	+
Sucrose	W	-	-	W
Glycerol	W	+	-	W
Glucose	W	+	W	W
Gelatin	+	+	-	-
Tween-40	+	+	NR	-
Penicillin G	R	NR	R	R
Chloramphenicol	R	NR	R	R
Tetracycline	R	W	NR	R
Fusidic acid	S	NR	NR	R
Nalidixic acid	R	NR	NR	R
Erythromycin	R	NR	S	R
Vancomycin	NR	S	NR	R
Ampicillin	NR	S	R	R
Carotenoid	494, 528, 470	NR	NR	469, 493, 527

Minor pigments	370, 390	NR	NR	350, 355, 360, 368, 388
Total lipids	4	NR	NR	6
Glycolipids	sulfatedtriglycosyl diether and a triglycosyl diether were produced as sole glycolipids	NR	NO	2
Major fatty acids	NR	NR	NR	$C_{14:0}$,$C_{15:0}$ iso, $C_{15:0}$ anteiso, $C_{16:0}$, $C_{17:0}$ iso, $C_{17:0}$ anteiso, $C_{20:2}$ w6,9c

Symbols (+): positive; (-): negative; (W): weak; (NR): not reported; (NO): not present.

On the basis of sequence similarity, strain was phylogenetically related to genus *Halobiforma* and was closest to *Halobiforma lacisalsi*. *Halobiforma* Sp. strain BNMIITR was isolated from the soil sample of Sambhar lake, Rajasthan India. Genus *Halobiforma* has been reported from soda lakes of China and Egypt. This is the first report on isolation and characterization of genus *Halobiforma* from Sambhar lake, India. All the three representative species of genus *Halobiforma* are catalase and oxidase positive; however strain BNMIITR is catalase positive but oxidase negative. Temperature profile of strain BNMIITR is similar to the other members of the genus. Optimum pH for growth of *H. haloterrestris, Halobiforma lacisalsi* is 7.5 but strain BNMIITR show pH optimum at 8.5 which is similar to that of *H. nitratireducens* (8.7) to which it shows 96 % similarity with 16S rRNA gene sequence. All three representative members of *Halobiforma* genus can hydrolyze Tween 40 whereas strain BNMIITR cannot hydrolyze it. Detailed comparison of strain with other members is given inTable 4.8. The 16S rRNA gene similarity of isolate indicates that it is closest to *Halobiforma lacisalsi*. *Halobiforma lacisalsi* is sensitive to tetracycline and vancomycin whereas strain BNMIITR was found resistant to both. Likewise *Halobiforma lacisalsi* can use amino acids such as glutamate and arginine but strain BNMIITR could not utilize them (biochemical characterization Table 4.6 and 4.8). *Halobiforma lacisalsi* cannot hydrolyze casein but strain BNMIITR could use it (Table 4.6 and 4.8). Mannitol and sucrose cannot be utilized by *Halobiforma lacisalsi* whereas strain BNMIITR can utilize both of them as carbon sources. On the basis of the all physiological, biochemical, phylogenetic analysis it was concluded that the strain

BNMIITR should be treated as new haloalkaliphilic bacteria but it is closest to *Halobiforma* thus this strain is referred as *Halobiforma* sp. BNMIITR in all further studies.

4.5 Lipid and cellular fatty acid analysis

In taxonomy of halophilic archaea, glycolipids have been used as taxonomic markers. Thus total lipid of the isolate was extracted and TLC was done for both total lipids and polar lipids. Glycolipids were developed as blue-purple spots. Thin-layer chromatogram of total lipids from *Halobiforma sp.* strain BNMIITR showed the presence of six different types of lipids where as Hezayen et al. (2002) in their studies reported the presence of 4 different types of lipids in *Halobiforma haloterrestris*. Polar lipid profile showed the presence of two glycolipids and one phospholipid (Fig. 4.31). A sulphated triglycosyl diether and a triglycosyl diether were produced as a sole glycolipid in *Halobiforma haloterrestris* which are absent in the majority of neutrophilic and alkaliphilic members of the family *Halobacteriaceae* (Hezayen et al. 2002). No glycolipid was detected in *H. nitratireducens* (Xin et al. 2001). Major cellular fatty acids, found after GC (Gas Chromatography) analysis (Fig.4.32 and Table 4.9) were $C_{14:0}$, $C_{15:0}$ iso, $C_{15:0}$ anteiso, $C_{16:0}$, $C_{17:0}$ iso, $C_{17:0}$ anteiso, $C_{20:2}$ w6, 9c. Fatty acid synthetase reported to be sensitive to high salt concentrations hence fatty acids are not usually a part of archaeal membrane lipids, however small amounts of fatty acids (C_{14}, C_{16}, C_{18}) have been detected in membrane proteins of *H. salinarum* (Pugh and Kates. 1994). Fatty acid composition of the genera *Halobiforma* has not been reported previously and thus it is not possible to compare with the present isolate.

Table 4.9: Major fatty acids found in *Halobiforma sp.* strain BNMIITR

Fatty acid	Percent
14:0	2.73
15:0 iso	11.10
15:0 anteiso	6.73
16:0	8.32
17:0 iso	3.13
17:0 anteiso	3.67
19:0 anteiso	Nil
20:2 w6, 9c	6.01

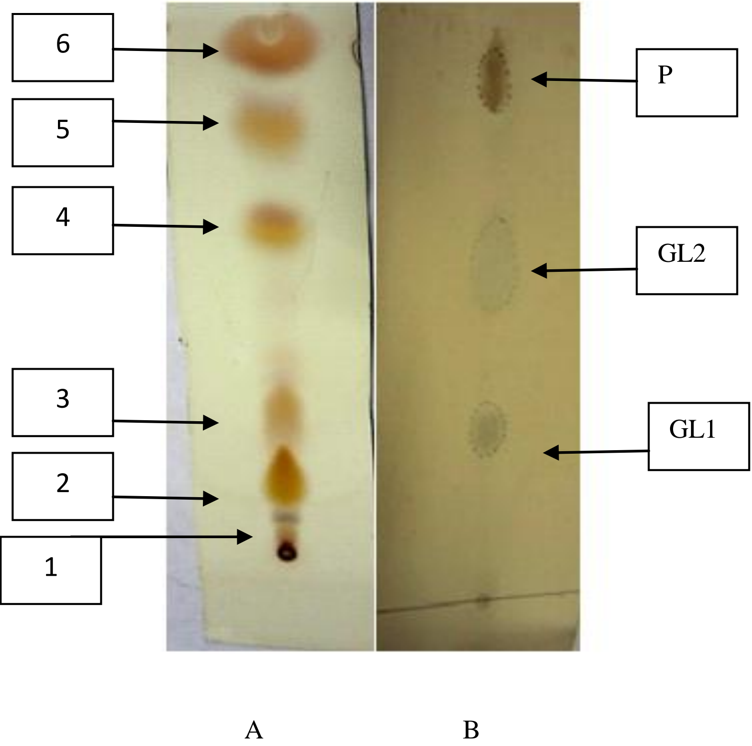


Fig. 4.31(A-B): Thin-layer chromatogram of **A:** Total lipids (1-6), **B:** Polar lipids from *Halobiforma* sp. BNMIITR showing glycolipids (GL1 and GL2) and Phospholipid (P)

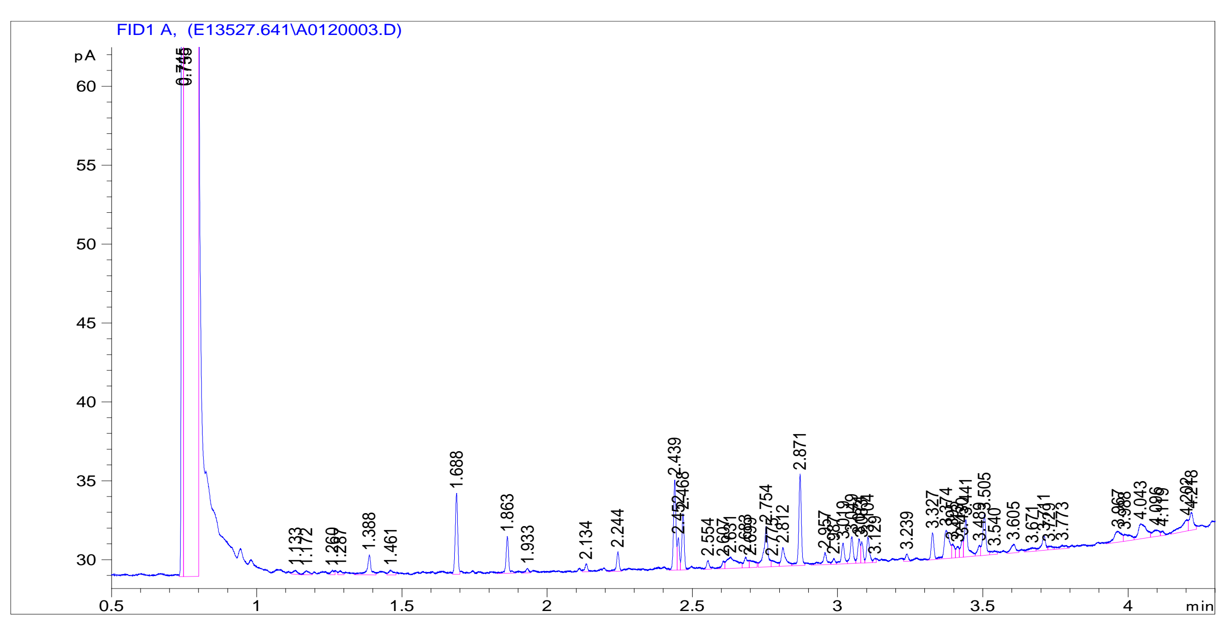


Fig. 4.32: Fatty acid analysis by Gas chromatograph.

4.6 Characterization of crude enzyme

4.6.1 Effects of pH and temperature on protease activity

The effect of pH on protease activity of strain BNMIITR was examined at various pH values at 45 ºC (Fig. 4.33). The crude enzyme was highly active at pH range 6- 12 with optimum pH at 9.0. The protease activity was also examined at different temperatures (30- 80 ºC). The enzyme was active within a wide range of temperature, with an optimum at 55 ºC (Fig. 4.34).

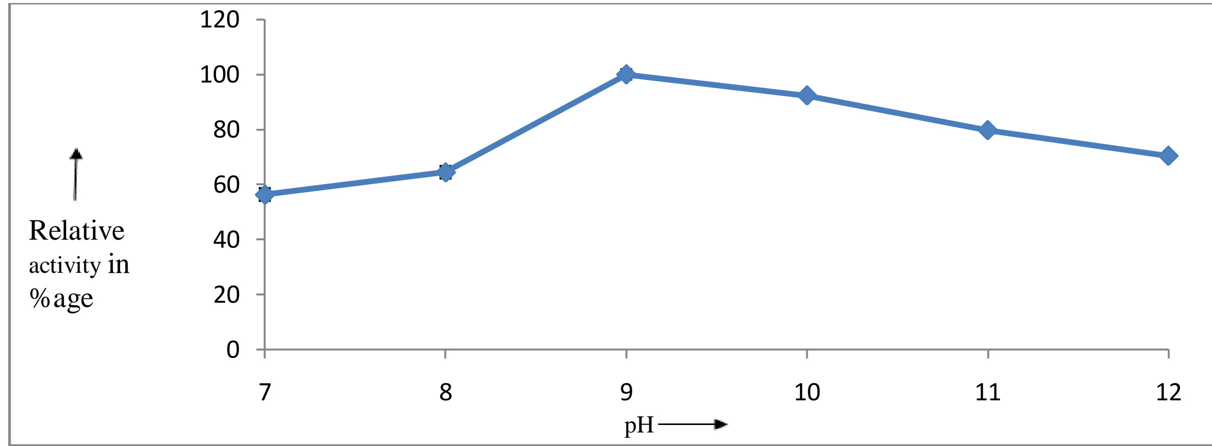


Fig. 4.33: Effect of pH (at 45 ^{o}C) on caseinolytic activity of crude enzyme extract of isolate *Halobiforma sp.* strain BNMIITR. Values presented are the mean ± standard deviation where $n = 3$.

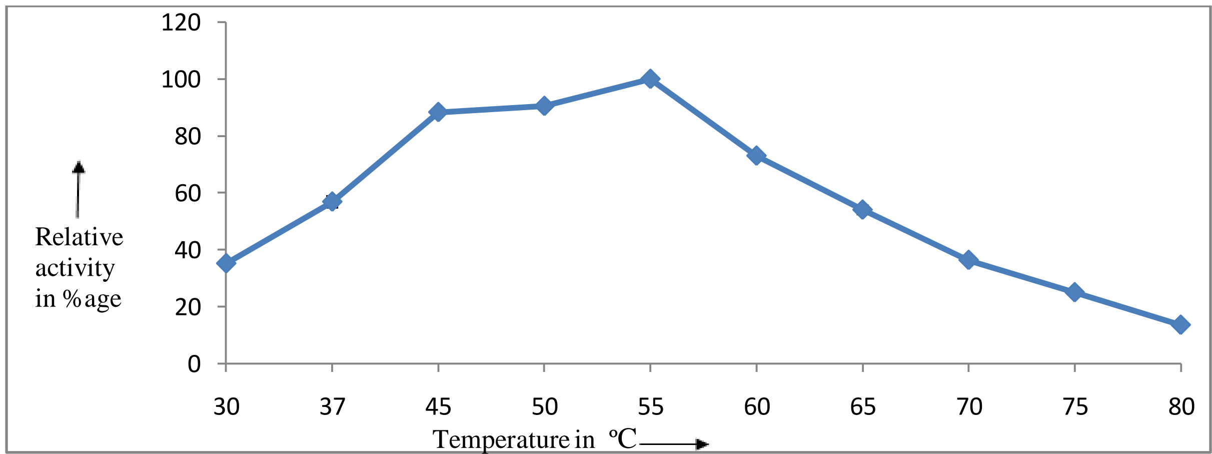


Fig. 4.34: Effect of temperature (at pH 9.0) on caseinolytic activity of crude enzyme of isolate *Halobiforma* sp. strain BNMIITR. Values presented are the mean ± standard deviation where $n = 3$.

Crude enzyme of the isolate was active in a wide range of pH (7- 12) and temperature (30- 80 ºC) with optimum activity at pH 9.0 and 55 ºC temperature (fig 4.35 and 4.36). Protease from *Geomicrobium* sp. EMB2 was stable at a pH range 6.0- 12.0 and temperatures upto 60 ºC with optimum pH at 10.0 and 50 ºC (Karan and Khare. 2010). Protease from *Halobacillus karajensis* undergoes thermal activation above 30 ºC with maximum activity between 45 ºC and 55 ºC followed by thermal deactivation above 60 ºC (Hamid Reza et al. 2009).

4.6.2 The effect of inhibitors and surfactants on the protease

The effect of different enzyme inhibitors such as group specific reagents and chelating agents on the enzyme activity were examined. The enzyme was found to be completely inhibited by the serine protease inhibitor (PMSF 10 mM) indicating the possibility of a serine-type protease. Furthermore, the activity of crude enzyme was also reduced by metallo protease inhibitor EDTA and SDS. The activity remained unaffected by urea. Minor reductions of activity by Triton X-100, CTAB and DTT were observed. Interestingly, it was increased in presence of Tween 20 and Tween 80 (Fig. 4.35).

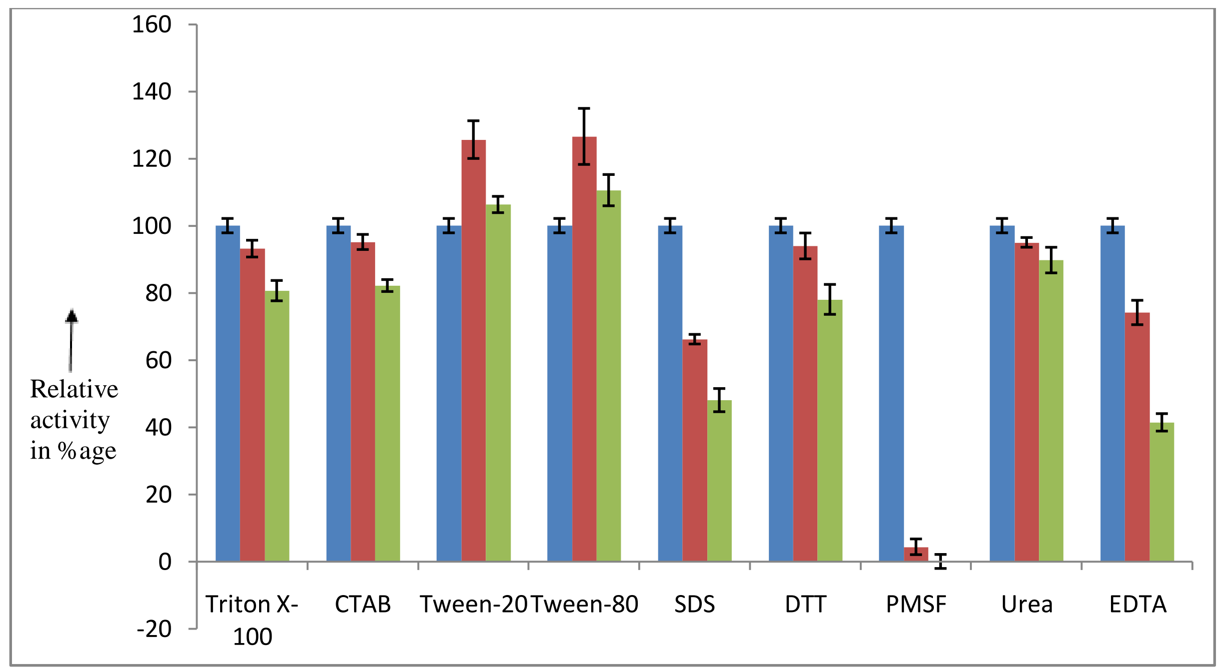


Fig. 4.35: Protease activity of crude enzyme in control (blue bar 100 %), in the presence **1:** Triton X- 100: 0.5 and 1 % (red and green bar respectively.) **2:** CTAB: 0.5 and 1 %_(red and green bar respectively), **3:** Tween 20: 0.5 and 1 % (red and green respectively), **4:** Tween 80: 0.5 and 1 % (red and green bar respectively),**5:** SDS: 0.5 and 1 % (red and green bar respectively), **6:** DTT: 10 and 50 mM (red and green bar respectively), **7:** PMSF: 10 and 50 mM (red and green bar respectively) **8:** Urea: 10 and 50 mM (red and green bar respectively), **9:** EDTA: 10 and 50 mM (red and green bar respectively). Values presented are the mean ± standard deviation where $n = 3$.

4.6.3 Effects of organic solvents on protease activity

Usually enzymes become inactivated or show very little activity in the presence of polar organic solvents (log p value < 1) due to the structural disruptions. Activity of crude enzyme of *Halobiforma sp.* strain BNMIITR was examined in presence of 30 % (v/v) and 50 % (v/v) organic solvents. The relative activities were 72.47 %, 33.45 % and 83.78 %, 33.61 % in the presence of 30 % and 50 % methanol and ethanol respectively. Interestingly activity of crude enzyme was increased in presence of DMF and DMSO. The relative activities were 168.08 % and 122.40 % in presence of 30 % and 50 % DMF. Similarly these were 161.61 % and 131.97 % in the presence of 30 % and 50 % DMSO. The activity remained unaffected in the presence of hexane (nonpolar solvent). In all these studies the activity of the crude enzyme in the absence of any organic solvent was taken as 100 % (Fig. 4.36).

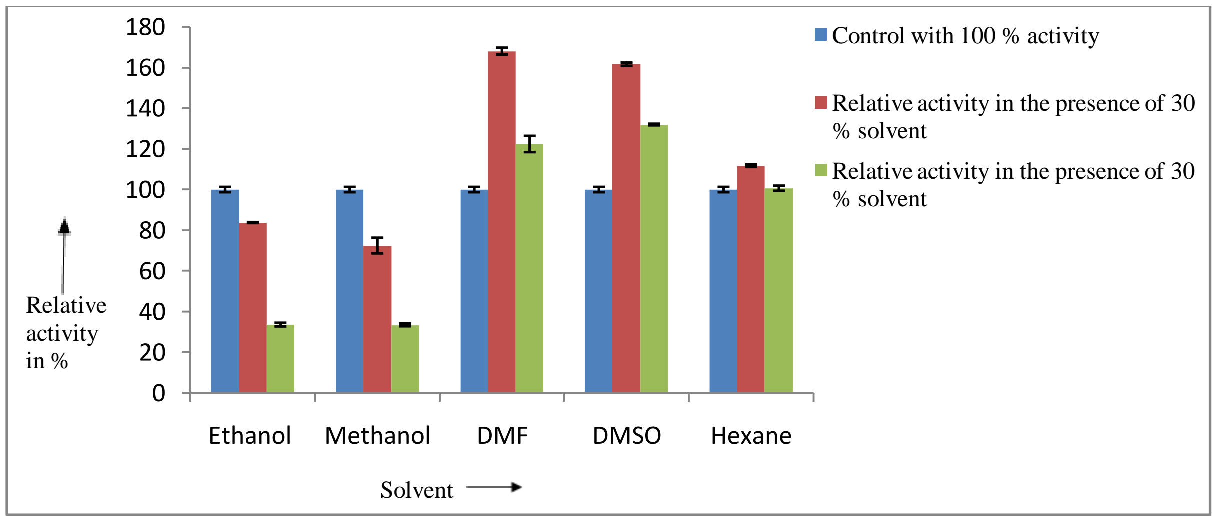


Fig. 4.36: Protease activity of crude enzyme in control (blue bar 100 %), in the presence of 30 % (red bar) and 50 % (green bar) organic solvents v/v. Values presented are the mean ± standard deviation where $n = 3$.

The activity of crude enzyme was very high in presence of DMF and DMSO. A protease from moderately haloalkaliphilic bacterium, *Geomicrobium* sp. *EMB2* reported to withstand range of solvents having a log *P* >2 but show less activity in co solvent systems (Karan and. Khare. 2010). Some enzymes show enhancement in activity in the presence of organic solvents (solvent activation), a lipase from *P. aeruginosa* activated in the presence of polar solvents such as 2-propanol (Lima et al. 2004). The high activity of the enzyme in the presence of solvents was due to the solvent activation of the enzyme. This property of enzyme can be further explored in organic synthesis. In aqueous system water forms multiple hydrogen bonds with enzyme and brings about the structural flexibility. Water activity is reduced in the presence of organic solvents thus organic solvents provide better stability to the enzymes.

4.7 Protease production in shake flasks

Halophilic archaea bacteria are adapted to hypersaline environment and show a slow growth rate which is a limiting factor for their industrial applications. Nutritional factors like nitrogen and carbon sources, amino acids, surfactants and culture conditions such as pH, temperature, agitation are known to effect the protease production (Gupta et al. 2002). Thus optimization of media components and culture conditions are necessary to achieve maximum protease production. Unfortunately very little information is available on optimization of protease production. Several statistical and non-statistical methods are used to optimize such parameters. One factor at a time approach is advantageous to identify the role of newer components on the production of extracellular enzymes (Panda et al. 2007). Role of signal peptides in protease production was studied by Degering et al. 2010. Initial protease production by isolate *Halobiforma* sp. strain BNMIITR, at 3 M NaCl, pH 9.0 and 45 ^{o}C was 40.84 ±0.5957 U/ml and corresponding specific activity was 749.50 ±12.01 U/mg. Maximum protease production was observed after 96 h. Effects of nutritional factors and culture conditions on protease production by isolate *Halobiforma* sp. strain BNMIITR was optimized as follows:

4.7.1 Effect of Salinity

Extreme halophilic isolates are adapted to hypersaline environment and show maximum growth at high salt concentration 3- 5 M and a lower salt concentration below 2 M NaCl result in reduction of growth and protease production (Danson et al. 1977). Isolate *Halobiforma* sp. strain BNMIITR

can grow in a broad range of salinity (2- 5 M). Effect of salinity on protease production was examined and maximum production was found at 3 M NaCl, 40.84 ±0.5957 U/ml and specific activity was 749.50 ±12.01 U/mg (Fig. 4.37 and 4.38). Thus 3 M NaCl was found to be optimum for growth (Fig 4.4) as well as protease production. Similarly, effects of various salts on protease production were also studied in shake flask level. Growth was very less and no protease production was observed in the presence of KCl. Whereas in the presence of Na_2PO_4, $NaNO_3$ and CH_3COONa activities were 5.0125 ± 0.72478, 8.2958 ± 0.00589 and 6.6041 ±0.736 U/ml respectively. Isolate cannot grow in the presence of Na_2SO_4. Thus NaCl was found to be most suitable salt for protease production at 3 M concentration (Table 4.10).

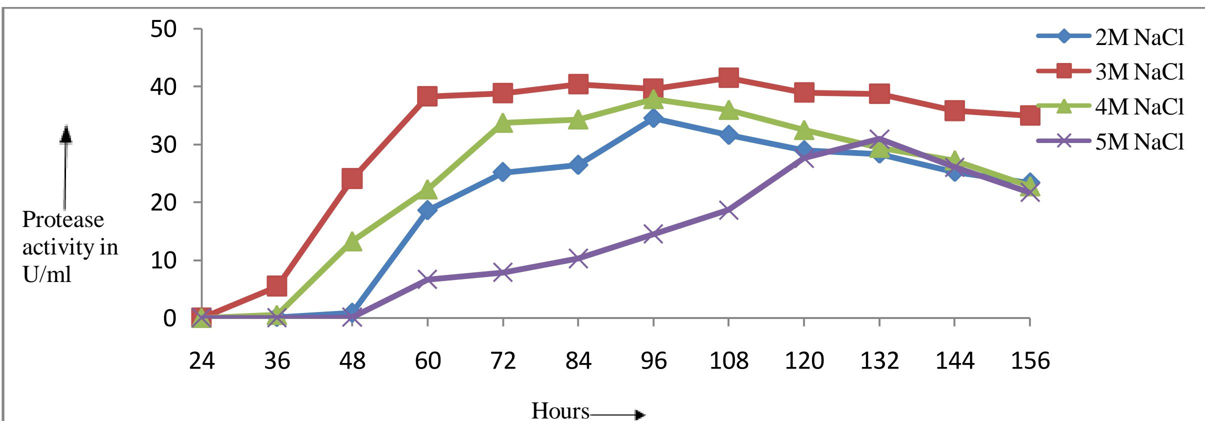


Fig.4.37: Effect of NaCl at 2-5 M concentration on protease production from isolate *Halobiforma* sp. strain BNMIITR. Caseinolytic activity was checked at 45 °C and pH 9.0 for 30 min.

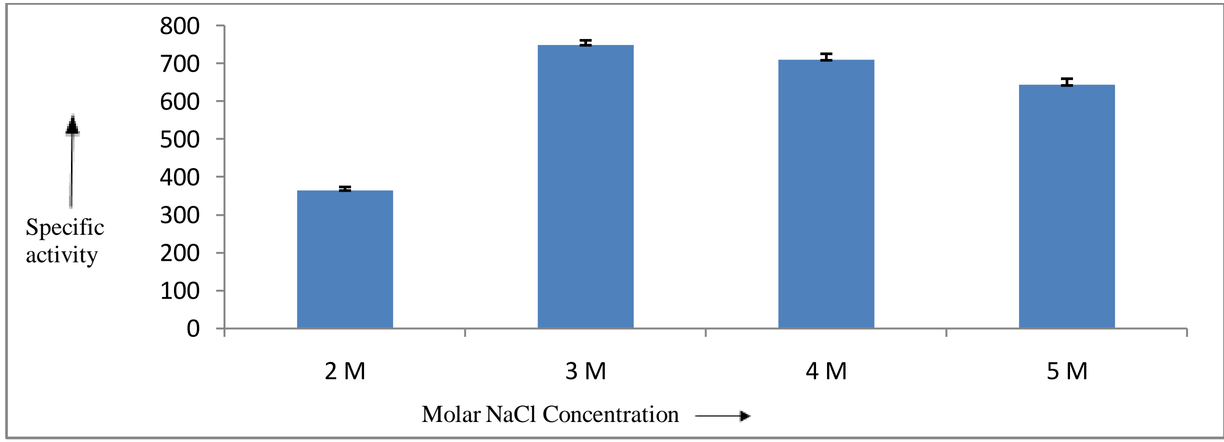


Fig.4.38: Specific activities of protease at 2- 5 M NaCl concentration in the growth medium caseinolytic activity was checked at 45 °C and pH 9.0 for 30 min. and protein estimation was determined by dye binding method.

Table 4.10: Effect of various salts on protease production from isolate *Halobiforma* sp. strain BNMIITR. Caseinolytic activity was checked at 45 ^{o}C and pH 9.0 for 30 min. and protei estimation was done by dye binding method.

S.N.	Salt	Enzyme activity in U/ml	Specific activity U/mg
1	NaCl		
	12 %		
		32.35 ± 1.90	365.22 ± 8.59
	18 %	40.84 ± 0.59	749.50 ±12.01
	24 %	35.38 ± 0.91	709.73 ±16.19
	30 %	27.41 ± 0.56	643.22 ± 16.82
2	KCl		
	12 %	0	0
	18 %	0	0
	24 %	0	0
	30 %	0	0
3	Na_2SO_4		
	18 %	0	0
4	Na_2PO_4		
	18 %	5.012 ± 0.724	50.90 ± 6.01
5	$NaNO_3$		
	18 %	8.29 ± 0.0058	298.85 ± 11.19
6	CH_3COONa		
	18 %	6.60 ± 0.736	76.21 ± 4.38

4.7.2 Effect of pH

Isolate *Halobiforma* sp. strain BNMIITR can grow in a broad range of initial pH (6- 11). pH works as a critical factor in microbial process. Maximum protease activity (48.75 ± 0.3871 U/ml and specific activity 864.50 ± 19.99 U/mg) was found when initial pH was10. This pH was reduced to 8 in late log phase of growth. Effect of pH on protease production from isolate *Halobiforma* sp. strain BNMIITR is presented in Fig. 4.39 and 4.40

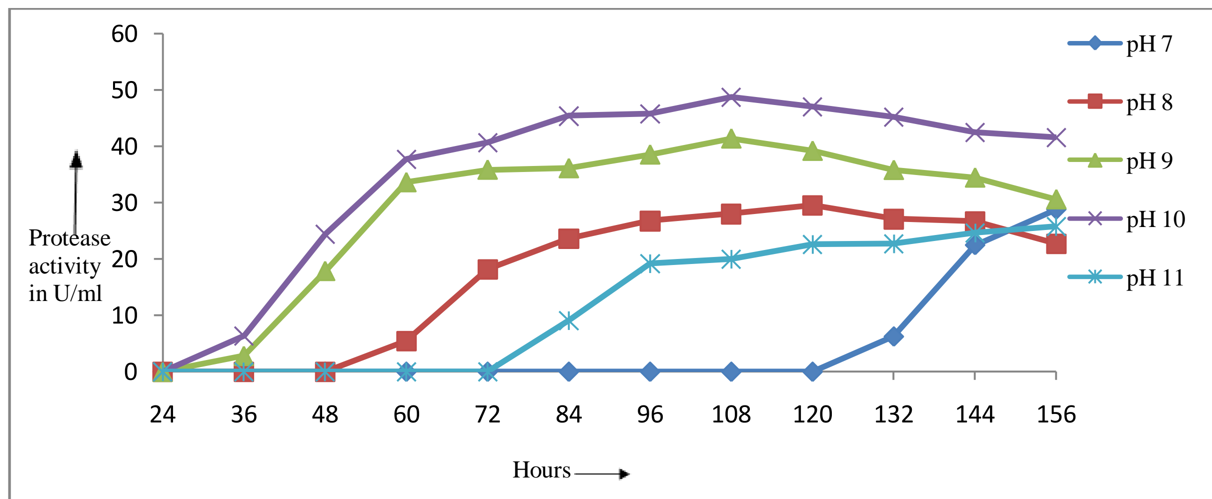


Fig.4.39: Effect of initial pH on protease production from isolate *Halobiforma* sp. strain BNMIITR. Caseinolytic activity was checked at 45 ^{o}C and pH 9.0 for 30 min.

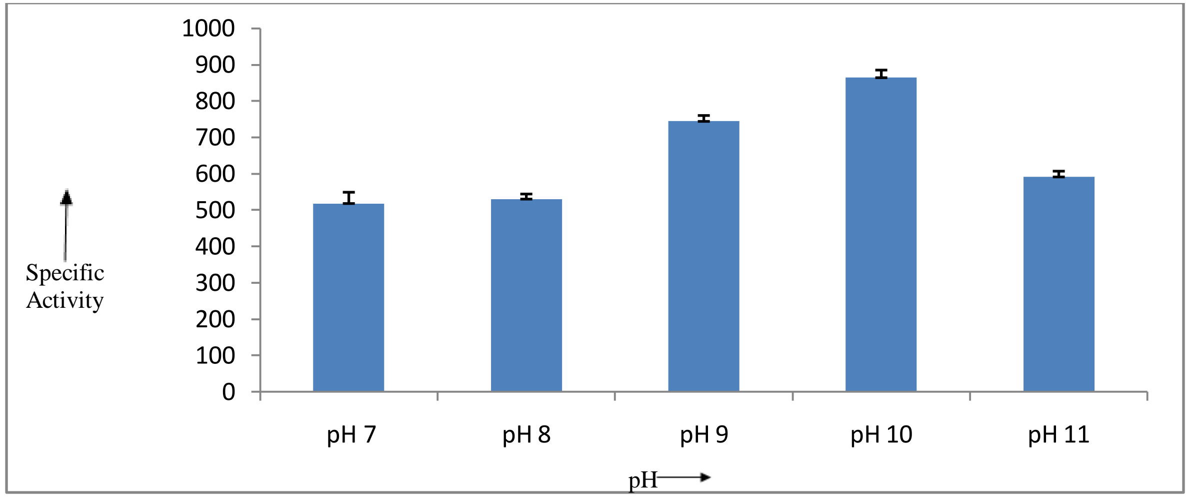


Fig. 4.40: Specific activities of protease produced at different initial pH values of growth medium. Caseinolytic activity was checked at 45 ^{o}C and pH 9.0 for 30 min and protein estimation was done by dye binding method.

4.7.3 Effect of Temperature

Like pH, temperature also works as a critical factor in microbial process. Temperature affects the production of protease by changing the physical properties of cell membrane. Enzyme production was found to be maximum at 45 °C (Fig. 4.41 and 4.42).

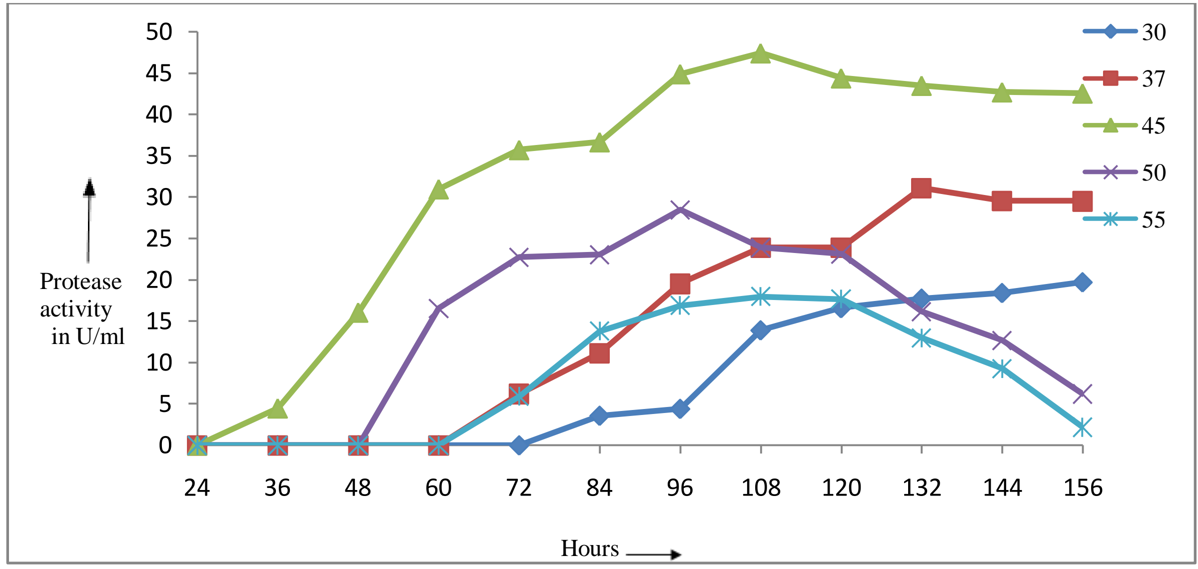


Fig. 4.41: Effect of temperature on protease production from isolate *Halobiforma* sp. strain BNMIITR. Caseinolytic activity was checked at 45 °C and pH 9.0 for 30 min.

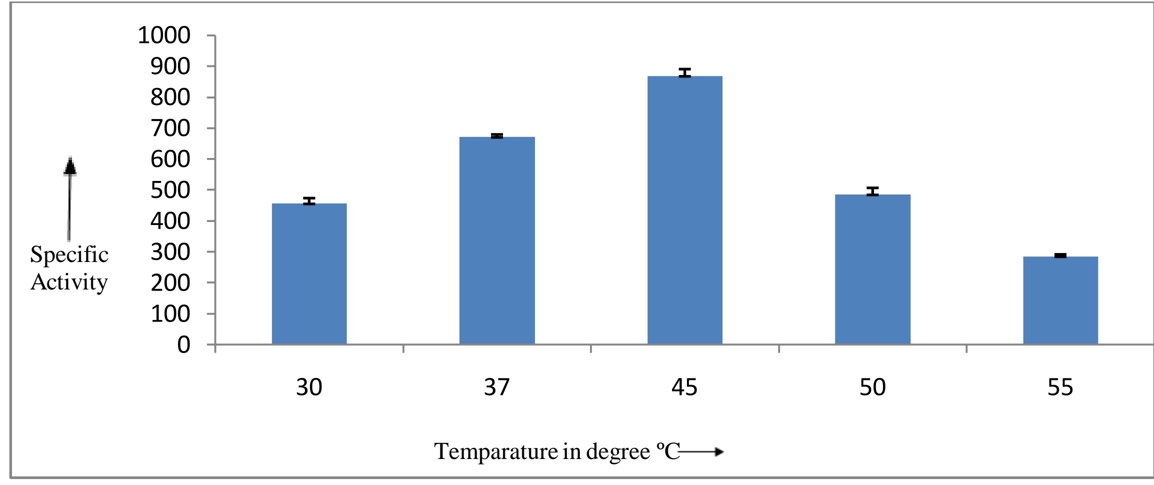


Fig. 4.42: Specific activities of protease produced at different growth temperatures. Caseinolytic activity was checked at 45 °C and pH 9.0 for 30 min and protein estimation was done by dye binding method.

Aunstrup 1980 reported that protease production was maximum when pH of the medium was maintained above 7.5. Most of the halophilic enzymes are optimally produced at temperature range of 37 - 55 ^{o}C. The production of a protease from of *Halobacterium* sp. was maximized at temperature range 35- 45° C, with optimum at 40 °C (Vijay Anand et al. 2010).

4.7.4 Effects of various carbon sources on protease production

Effects of different carbon sources on protease production have been summarized in Table 4.11. Isolate was able to utilize all carbon source used in the present study. Maximum production (activity 75.28 ± 1.69 U/ml and specific activity 1284.86 ± 20.76U/mg) was found when xylose (Table 4.11) was used as a carbon source. Activity was reduced when low cost agro industrial byproducts like wheat bran and wheat flour (14.81 ± 1.30 and 22.77 ± 2.28 U/ml) were used in the production medium. In the present study, protease production was increased by 1.71 fold at 3 M NaCl, pH 10, 45 ^{o}C and in the presence of xylose and casein as carbon and nitrogen sources in comparison to initial production medium (3 M NaCl, pH 9.0, 45 ^{o}C and in the presence of tri sodium citrate and casein as carbon and nitrogen sources). Glucose is known to catabolically repress the production of many enzymes including protease (Patel et al. 2005) but in the present study it was observed that all the sugars have positive effects on protease production. Highest protease production was observed in the presence of lactose from *Halobacillus karajensis (*Karbalaei-Heidari et al. 2009). Protease productions were high in *Bacillu*s sp and *Bacillus cereus* strain 146 when starch and glucose were used as carbon source (Shafee et al. 2005).

Table 4.11: Effect of various carbon sources on protease production from isolate *Halobiforma* sp. strain BNMIITR. Caseinolytic activity was checked at 45 ^{o}C and pH 9.0 for 30 min and protein estimation was done by dye binding method.

S. No.	Carbon Source	Enzyme activity in U/ml	Specific activity U/mg
1	glucose	51.79 ± 2.35	908 ±19.04
2	fructose	58.24 ± 3.47	927 ± 23.92
3	sucrose	54.60 ± 4.12	900.22 ± 29.39

4	lactose	56.57 ± 3.87	985.92 ± 26.46
5	galactose	64.73 ±1.49	1027.78 ± 14.64
6	glycerol	51.88 ± 0.79	853.95 ± 13.72
7	mannitol	55.29 ± 2.24	933.98 ± 13.63
8	starch	49.89 ± 0.64	900.77 ± 16.49
9	xylose	75.28 ± 1.69	1284.86 ± 20.76
10	Trisodium citrate	45.68 ± 0.43	861.86 ± 20.49
11	Citric acid	49.62 ± 0.58	881.64 ± 24.04
12	Wheat flour	14.81 ± 1.30	183.95 ± 14.32
13	Wheat bran	22.77± 2.28	255.87 ± 23.92

4.7.5 Effects of Various Nitrogen Sources on protease production

Effects of nitrogen sources on protease production have been summarized in Table 4.12. Protease production was completely inhibited when inorganic nitrogen sources were used in the production medium. Very poor production was observed when organic nitrogen sources such as peptone and yeast extract were used. Considerable protease production was observed only when proteins were used as nitrogen sources, which was 70.22 ± 1.78 U/ml in the presence of casein. So it can be concluded that the protease was inducible in nature. Protease production was considerably increased when low cost agro industrial byproducts like soybean meal, soybean husk, soybean flour, chick pea flour and chick pea husk were used as nitrogen sources. Protease activity was found to be 123.08 ± 2.82 U/ml and 184 ± 7.65 U/ml in the presence of soybean flour and chick pea flour respectively (Table 4.12). The only limitation of using low cost agro industrial byproducts was delayed growth and delayed production of enzyme. In the presence of casein and other proteins maximum production was achieved with in four days but when low cost agro industrial byproducts were used maximum production was achieved after 8 to 10 days. In present study, the protease production was increased by 2.94 fold and 2.17 fold when chick pea flour and

soybean flour were used as nitrogen source as compared to initial production medium (3 M NaCl, pH 9.0, 45 ^{o}C and in the presence of tri sodium citrate and casein as carbon and nitrogen source). At present efforts were made to reduce cost of industrial protease production by using cheap raw materials. India is a country which has huge agro-industrial residues. So it is economical to use such cheap agro -industrial residues as carbon and nitrogen sources for the production of desired product. Sonleither (1983) have reported that soy meal, lactose, sucrose, and starch can be used as good source for industrial protease production. Protease production by isolate *Halobiforma* sp. strain BNMIITR was found to be very less in the presence of yeast extract and peptone but it was considerably increased in the presence of casein and other proteins which showed that enzyme was inducible in nature. Production of haloalkalophilic protease by *Bacillus clausii* was increased considerably in the presence of casein (Ganesh Kumar et al. 2003). Casein was found as best inducer in many bacterial strains. Casein promoted protease production in *Bacillus licheniformis* MIR (Sumantha et al. 2006). Poor enzyme production was also observed in the presence of yeast extract and beef extract in *Bacillus clausii* (joo et al. 2003). Inorganic nitrogen sources also reported to inhibit enzyme production and similar phenomenon was also observed in the present finding. No protease production was observed in the presence of inorganic nitrogen sources in *Halobacterium* sp. (VijayAnand et al. 2010). A protease from *Chromohalobacter* sp. TVSP101 was also inhibited in the presence of inorganic nitrogen source (Vidyasagar et al. 2007). According to D'Alessandro et al. 2006, inhibition of protease production by inorganic nitrogen compounds like ammonium salts indicate that it may be controlled by nitrogen catabolite repression. A protease produced by haloarchaeon *Halogeometricum* sp TSS101 is inhibited by yeast extract and ammonium (Vidyasagar et al. 2006). In the present work protease production was high in the presence of soybean flour but it was considerably decreased in the presence of soy peptone and inhibited in the presence of soybean casein digest.

Table 4.12: Effects of various nitrogen sources on protease production from isolate *Halobiforma* sp. strain BNMIITR. Caseinolytic activity was checked at 45 ^{o}C and pH 9.0 for 30 min and protein estimation was done by dye binding method.

S.No	Nitrogen Source	Enzyme activity in U/ml	Specific activity U/mg
1	NH_4NO_3	0	0
2	$(NH_4)_2SO_4$	0	0
3	$NaNO_3$	0	0
4	NH_4Cl	0	0
5	Urea	0	0
6	Peptone	2.28 ± 0.12	71.82 ± 1.33
7	Yeast extract	5.90 ± 0.40	176.47 ± 4.89
8	Cassamino Acid	0	0
9	Casein	70.22 ±1.78	1307.08. ± 7.08
10	Skimmed milk	64.89 ± 2.88	1024.91 ±2 8.58
11	Gelatin	52.62 ±1.56	1104.42 ± 6.33
12	Chick pea flour	184 ±7.65	2203.22 ± 14.04

13	Chick pea husk	96.47 ± 2.78	1453.22 ± 15.80
14	Soy bean flour	123.08 ± 2.82	1631.27 ± 6.94
15	Soy bean husk	66.51 ± 0.94	950 ± 10.74
16	Soy Peptone	19.75 ± 1.44	223.24 ± 14.49
17	Soy bean casein digest	0	0
18	Urea	0	0

4.7.6 Effect of various amino acids on protease production

Amino acids are known to catabolically repress protease production (Winderickx et al. 2003). Effects of amino acids on protease production were examined by supplementing the medium with various amino acids at different concentrations (0.1, 0.5, and 1 %). Asparagine and proline had positive effects on protease production. Protease production is catabolically repressed by amino acids in the yeast *Saccharomyces cerevisiae, Pseudomonas maltophilia,* and *Bacilllus intermedius.* Amino acids reported to repress the expressions of genes encoding transporters and proteases (Balaban et al. 2004). Lysine, Arginine and histidine inhibited the protease production even at 0.1 % concentration. Lysine, Arginine and histidine belong to positively charged amino acid group. Thus protease production in *Halobiforma* sp. strain BNMIITR was probably inhibited by positively charged amino acids. Protease production in *Halobiforma* sp. strain BNMIITR was not affected by negatively charged amino acids but concentration above 0.1 % had negative effects on the production. Aspartic acid has been reported to have inhibitory effect on protease production in marine bacterium (Daatselaar et al. 1974). All aromatic amino acids except tyrosine at 0.1 % concentration had negative effects on protease production. Phenylalanine at 1 % and tryptophan and methionine at 0.1 % concentration completely inhibited the protease production. Phenylalanine was found to completely inhibit protease production in *Streptomyces clavuligerus* (Thumar and Singh 2009). In the present study, glycine was found to completely inhibit protease production at

1 % concentration. Activities were 80.83 %, 63.23 %. 55.61 % in the presence of 0.1, 0.5 and 1 % alanine whereas it was 50.08 %, 39.81 %, 26.48 % in the presence of 0.1, 0.5 and 1 % serine (Table 4.13).

Table 4.13: Effects of various amino acids on protease production by isolate *Halobiforma* sp. strain BNMIITR at different concentrations (0.1, 0.5, and 1 %), where production medium without amino acids was taken as positive control. Caseinolytic activity was checked at 45 ^{o}C and pH 9.0 for 30 min and protein estimation was done by dye binding method.

S.N.	Amino acid	Enzyme activity in U/ml	Specific activity U/mg	Relative Enzyme activity in U/ml	Relative Specific activity U/mg
1	Positive Control	70.44 ± 2.87	1288.06 ±19.32	100 %	100 %
2	Glutamate				
	0.1 %	68.73 ± 1.70	1232.44 ± 32.30	97.57 %	95.68 %
	0.5 %	47.55 ± 1.89	1007 ± 13.74	67.34 %	78.19 %
	1 %	16.48 ± 1.01	297.33 ± 11.7	23.39%	23.08 %
3	Asparagine				
	0.1 %	75.18 ± 4.40	1252.04 ± 18.08	106.72 %	97.20 %
	0.5 %	80.42 ± 2.18	1394.73 ± 24.74	114.16 %	108.28 %
	1 %	78.47 ± 1.43	1387.27 ± 37.65	111.39 %	107.70 %
4	Lysine				
	0.1 %	16.05 ± 1.19	332.47 ± 21.92	22.78 %	25.81 %
	0.5 %	10.81 ± 0.94	253.27 ± 7.61	15.346 %	19.66 %
	1 %	7.65 ± 0.53	174.02 ± 9.21	10.86 %	13.51 %
5	Proline				
	0.1 %	81.86 ± 3.09	1394.44 ± 22.38	116.21 %	108.25 %
	0.5 %	79.10 ± 1.20	1359.14 ± 20.70	112.29 %	105.51 %
	1 %	71.73 ± 3.17	1278.34 ± 16.21	101.78 %	99.24 %

6	Threonine				
	0.1 %	17.02 ± 1.03	457.65 ± 18.60	24.16 %	35.53 %
	0.5 %	9.8 ± 0.94	303.66 ± 15.81	13.91 %	23.57 %
	1 %	2.28 ± 0.11	81.99 ± 1.91	3.23 %	6.36 %
7	Tyrosine				
	0.1 %	70.07 ± 1.72	1311.86 ± 17.75	99.47 %	101.84 %
	0.5 %	63.5 ± 2.3	1132.79 ± 37.5	90.14 %	87.94 %
	1 %	42.06 ± 0.19	813.22 ± 7.01	59.71 %	63.13 %
8	Tryptophane				
	0.1 %	0	0	0	0
	0.5 %	0	0	0	0
	1 %	0	0	0	0
9	Arginine				
	0.1 %	60.97 ± 3.28	1193.18 ± 27.11	86.55 %	93.97 %
	0.5 %	35.49 ± 0.81	553.51 ± 15.4	50.38 %	42.97 %
	1 %	5.522 ± 0.39	104.48 ± 7.9	7.83 %	8.11 %
10	Glycine				
	0.1 %	51.73 ± 1.57	1086.05 ± 35.18	73.44 %	84.31 %
	0.5 %	8.019 ± 0.10	212.60 ± 6.6	11.38 %	16.50 %
	1%	0	0	0	0
11	Histidine				
	0.1 %	24.96 ± 1.11	527.45 ± 15.97	35.43 %	40.94 %
	0.5 %	12.90 ± 1.00	288.69 ± 26.22	18.31 %	22.41 %
	1 %	0	0	0	0

13	Methionine				
	0.1 %	0	0	0	0
	0.5 %	0	0	0	0
	1 %	0	0	0	0
14	Alanine				
	0.1 %	54.96 ± 0.48	1041.13 ± 19.90	78.023 %	80.82 %
	0.5 %	41.66 ± 1.87	814.50 ± 36.03	59.14 %	63.23 %
	1 %	39.33 ± 0.65	716.29 ± 25.20	55.83 %	55.60 %
15	Aspartic Acid				
	0.1 %	64.20 ± 1.96	1151.73 ± 30.60	91.14%	89.41 %
	0.5 %	61.56 ± 1.01	1130.43± 9.07	87.39 %	87.76 %
	1 %	50.57 ± 1.36	936.23± 21.52	71.79%	72.68 %
`16	Phenylalanine				
	0.1%	21.91 ± 2.67	457.52 ± 32.22	31.10 %	35.51 %
	0.5%	9.520 ± 0.10	254.88 ± 5.02	13.51 %	19.78 %
	1%	0	0	0	0
17	Serine				
	0.1%	32.18 ± 2.15	645.14 ± 24.44	45.68 %	50.08 %
	0.5 %	25.21 ± 0.67	512.87 ± 1.98	35.78 %	39.81 %
	1 %	15.40 ± 0.08	341.16 ± 4.45	21.86 %	26.48 %

4.7.7 Effects of various surfactants on protease production

The effect of surfactants on protease production was ascertained by supplementing the medium with various surfactants at concentrations of 0.1, 0.5, and 1 % (w/v). Protease production was 85 % lower in the presence of 0.1 % SDS and 82 % lower in the presence of 0.1 % triton-X 100. Isolate couldn’t grow in the presence of 0.5 and 1 % triton-X 100 and SDS. Activities were found to be 69.43 %, 31.8 % and 10.57 % in the presence of 0.1, 0.5 and 1 % Tween 80 respectively. It was also observed that 0.1, 0.5 and 1 % Tween 20 also reduced activities to 66.96 %, 35.44 % and 15.61 % respectively (Table 4.14). In all these studies protease production without surfactant was

taken as 100 %. Akolkar et al. 2009 reported that surfactant affect the membrane permeability which eventually leads to increased secretion of extracellular enzymes.

The production of protease from *Halobacterium* sp. SP1 was increased in the presence of dioctylsulfosuccinate whereas tween 80, triton X-100, and tween 20 had no effects on production. Productions of extracellular protease from *Bacillus cereus* and *Rhizopus oryzae* were enhanced in presence of triton X-100 and tween 80 (Esakkiraj et al. 2008; Banerjee and Bhattacharya 1992).

Table 4.14: Effects of surfactants on protease production by isolate *Halobiforma* sp. strain BNMIITR at different concentrations (0.1, 0.5, and 1 %), where production medium without surfactants was taken as positive control. Caseinolytic activity was checked at 45 ^{o}C and pH 9.0 for 30 min and protein estimation was done by dye binding method.

S. N.	Surfactant	Enzyme activity in U/ml	Specific activity U/mg	Relative Enzyme activity in U/ml	Relative Specific activity U/mg
1	Positive Control	70.44 ± 2.87	1288.0 ± 19.32	100 %	100 %
2	SDS				
	0.1 %	7.88 ± 0.32	193.32 ± 3.13	11.18 %	14.79 %
	0.5 %	0	0	0	0
	1 %	0	0	0	0
3	Triton-X-100				
	0.1 %	10.05 ± 0.69	231.42 ± 13.45	14.26 %	17.70 %
	0.5 %	0	0	0	0
	1 %	0	0	0	0
4	Tween -80				
	0.1 %	48.91 ± 1.75	857.18 ± 25.87	69.43 %	65.58 %
	0.5 %	22.43 ± 1.69	368.06 ± 26.54	31.84 %	28.16 %
	1 %	7.45 ± 1.38	114.71 ± 19.1	10.57 %	8.77 %
5	Tween -20				
	0.1 %	47.17 ± 2.94	794.67 ± 22.80	66.96 %	60.79 %
	0.5 %	24.97 ± 1.05	364.35 ± 12.70	35.44 %	27.87 %
	1 %	11.00 ± 1.84	157.88 ± 22.11	15.61 %	12.0 %

4.8 Purification of extracellular protease from isolate *Halobiforma* sp. strain BNMIITR

Most of the extracellular proteases from haloarchaea belong to serine protease and require NaCl for activity. They remain active in temperature range of 37- 50 °C or higher and neutral to basic pH (Capiralla et al. 2002). Halophilic enzymes have high negative charge on the surface which is shielded by high salt to maintain conformation. Less information is available on proteases of halophilic archaea. Halophilic archaea grow in extreme salt conditions at 3-5 M salt. Thus enzymes of halophilic archaea remain active and stable at high salt conditions and become inactive in the absence of salt (Layni, 1974). Most of the methods used to purify non-halophilic proteins are not suitable for haloarchaeal counterparts. Purification of haloarchaeal proteases are generally carried out by solvent precipitation followed by affinity chromatography (Gimenez et al. 2000; Vidyasagar et al. 2006).

4.8.1 Enzyme precipitation

Enzyme precipitation was done with pre chilled acetone. Acetone precipitation was done in three different methods:

Fractionation of protein was carried out by gradual addition of pre-chilled acetone at various concentrations. In Method I, first fraction was obtained up to 30 % saturation and thereafter acetone was increased up to 60 % saturation to obtain the second fraction. Finally third fraction was obtained by gradual increase of acetone up to 85 % saturation. All fractions (precipitate) were analyzed for protease activity and protein concentration. In method II, first fraction was obtained up to 40 % saturation and subsequent fractions were obtained up to 70 % and 85 % saturation. In method III, first fraction was obtained up to 50 % saturation and subsequent fractions were obtained up to 70 % and 85 % saturation. The results have been summarized in Table 4.15.

Table 4.15: Precipitation of crude protease from isolate *Halobiforma* sp. strain BNMIITR using different saturation of acetone, precipitate obtained in each step was analyzed for protease activity and protein concentration.

Method	First fraction		Second fraction		Third fraction	
	Activity (U/ml)	Specific activity (U/mg)	Activity (U/ml)	Specific activity (U/mg)	Activity (U/ml)	Specific activity (U/mg)
I	1.36	11.88	142.51	3040.58	22.95	1273.44
II	11.34	79.57	150.96	3732.83	4.95	568.03
III	67.7	429.99	94.57	2831.58	8.45	953.43

Among all the above mentioned methods, maximum activity and specific activity were found when precipitation was carried out according to method II. After precipitation study it was decided that modified method II will be followed for large amount of protein solution precipitation. In the modified method, two stages precipitation were followed with first stage precipitation carried out up to 40 % saturation and then followed by increasing the acetone concentration up to 80 % saturation in the supernatant (obtained from 40 % precipitation stage). First of all 2 liter of crude enzyme was concentrated to 200 ml by using Pall advanced separation system (Manual centramate system) with 10 kDa membrane cassettes (Pall omega 10 kDa T series) and this resulted in 2 fold purification. Thereafter, pre chilled acetone was added to the concentrate up to 40 % saturation and precipitate was discarded as it contain mostly undesirable proteins. Now supernatant was again precipitated with gradual addition of pre chilled acetone up to 80 % saturation. Precipitate was dissolved in 50 ml of tris buffer 50 mM pH 9.0 containing 1.2 % (w/v) NaCl. Precpitated enzyme was further concentrated to 5 ml using centrifugal concentrators (Amicon ultra Milipore 10 kDa) in a centrifuge at 5000 *g* and 4 ^{o}C and 8.03 fold purification was obtained after this step. Solvent precipitation is most commonly used method for haloarchaeal protease purification. Extracellular protease of *Natrialba magadii* was purified using ethanol precipitation (Maria et al. 2000) and of *Salinivibrio* sp. strain AF-2004 using acetone precipitation (Karbalaei-Heidari et al. 2007). Similarly, a protease of *gamma-Proteobacterium* was precipitated using acetone (Sana et al. 2005). All the extracellular protease isolated from halophilic archaea are serine proteases, thermo tolerant and require salt for activity and stability (Gimenez et al. 2000).

4.8.2 Hydrophobic interaction chromatography

The concentrated enzyme was further purified by hydrophobic interaction chromatography (HIC) using Phenyl Sepharose 6 Fast Flow column (gravity flow) which was equilibrated with tris buffer pH 9.0 with 25 % NaCl. Further elution was done by applying a linear gradient of NaCl (20 % - 0 %) in 50 mM tris buffer of pH 9.0. The enzyme was eluted at 11- 10 % NaCl in 50 mM tris buffer of pH 9.0. Purification pattern of HIC is shown in Fig. 4.43. Total four peaks were obtained and pooled fractions were analyzed for protease activity (Table 4.16). Peak one was found to have protease activity. Specific activity of the enzyme after HIC was 40919.55 U/mg and thus overall 31.67 fold purification was achieved after this step with a final yield of 10.62 %. Results of all purification steps have been summarized in Table 4.17. In the presence of high salt, non-polar interactions between matrix and protein is generally increased thus HIC have been proved to be a good method to purify halophilic proteins (Karan and Khare. 2010). An alkaline serine protease from haloalkaliphilic *Bacillus* sp was purified in single step using Phenyl Sepharose 6 Fast Flow column and reported molecular mass of the enzyme was 29 kDa (Gupta et al. 2005).

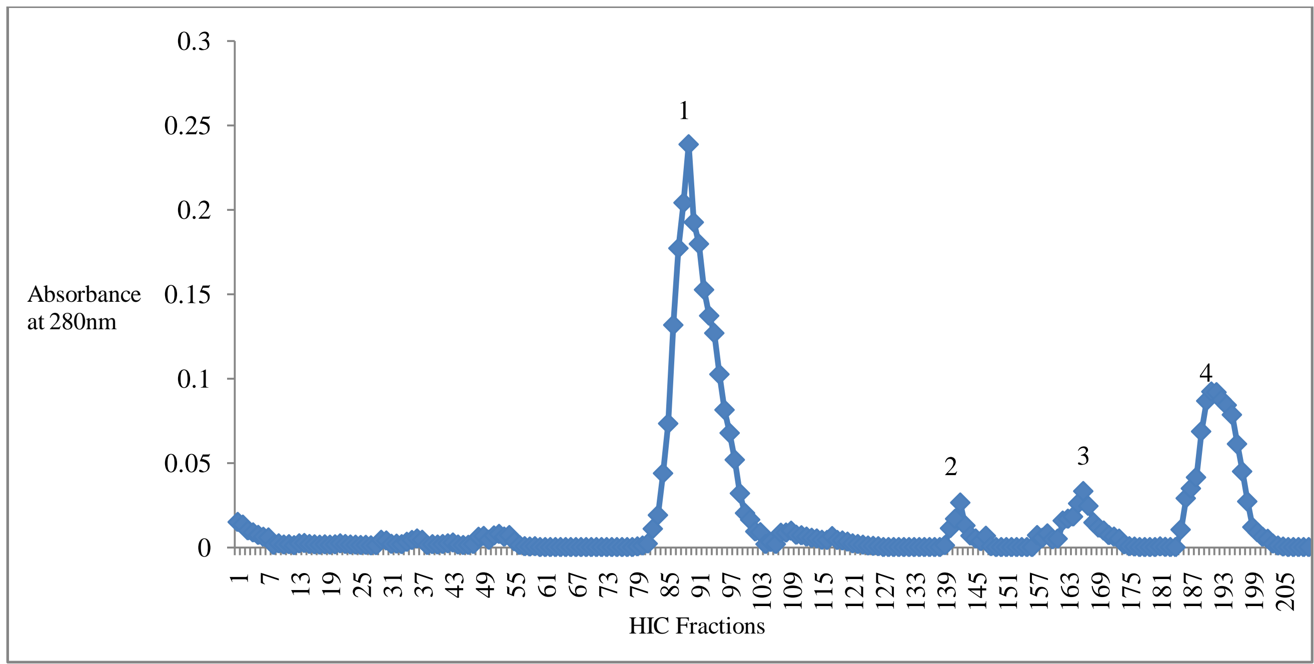


Fig.4.43: Purification of extracellular protease from isolate *Halobiforma* sp. BNMIITR by hydrophobic interaction chromatography on Phenyl Sepharose 6 Fast Flow column.

Table 4.16: Protease activity profile and protein concentration of peaks obtained after HIC using Phenyl Sepharose 6 Fast Flow column.

Peak	Protein in milligram/ml	Protease activity in U/ml	Specific activity in U/mg
1	0.0045	189.85	40919.55
2	0.0011	0	0
3	0.0016	0	0
4	0.00227	0	0

Table 4.17: Purification of extracellular protease of isolate *Halobiforma* sp. BNMIITR. Protease activity and specific activity profile at different steps of purification.

Purification step	**Volume**	**Total protein in mg**	**Specific activity (U /mgprotein)**	**Purification fold**	**Total activity units**	**Yield in %**
Culture medium supernatant	2000	109.1	1292.52	1	140000	100 %
Protein concentrator (10 kD)	200	40.6	2700.55	2.0	109800	78.42 %
Acetone precipitate	50	3.7	10380.81	8.034	38683	27.63 %
HIC	80	0.36	40919.55	31.67	148712	10.62 %

Purified enzyme was buffer exchanged with 50 mM Tris buffer pH 9.0, with salt concentration ranging from (0- 10 %). Results have been given in Fig. 4.44. It can be concluded from the results that enzyme require minimum 1- 2 % NaCl to maintain it's activity thus in all further studies,

enzyme was buffer exchanged with 50 mM tris buffer pH 9.0 with 1.2 % NaCl after purification. In halophilic proteins at high salt concentrations weak hydrophobic core stabilize the protein which become destabilize below 0.2 M NaCl concentration (Kim et al. 1997). A protease from *Geomicrobium* sp. EMB2 required minimum 5 % NaCl to maintain complete activity ((Karan and Khare. 2010).

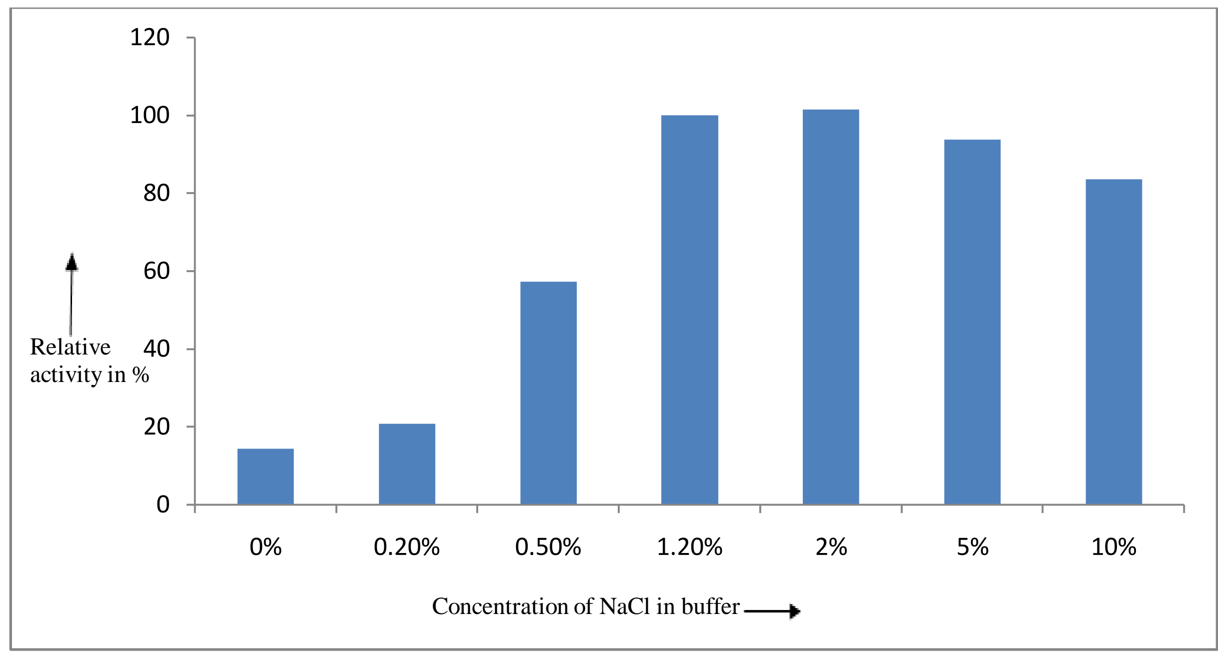


Fig. 4.44: NaCl requirement of the purified enzyme. Enzyme was equilibrated with 50 mM Tris buffer of pH 9.0, with salt concentration ranging from (0- 10 %). Caseinolytic activity was checked at 45 °C and pH 9.0 for 30 min.

4.8.3 Casein zymography and SDS PAGE

Casein zymography and SDS PAGE were done as described in materials and methods section 3.10.5.1 and 3.10.5.2. All protein samples from different purification steps were analysed by SDS PAGE. Purified enzyme after HIC was found to be a monomer and showed a single band whose molecular weight corresponds to 21 kDa (Fig. 4.45). This was further confirmed by casein zymography. Many serine proteases have been reported from haloarchaea with molecular mass in a range of 40- 66 kDa (*Halobacterium salinarum* (Izotova et al.1983) *Haloferax mediterranei* 1538 (Stepanov et al. 1992), halo alkaliphilic archaea *Natrialba magadii (*Gimenez et al. 2000)). A 130 kDa unusual protease have been isolated from *Natronococcus occultus* (Studdert et al. 2001). A haloprotease CPI was produced by moderately halophilic bacterium *Pseudoalteromonas ruthenica* has a molecular mass of 38.0 kDa (Poro et al. 2009).

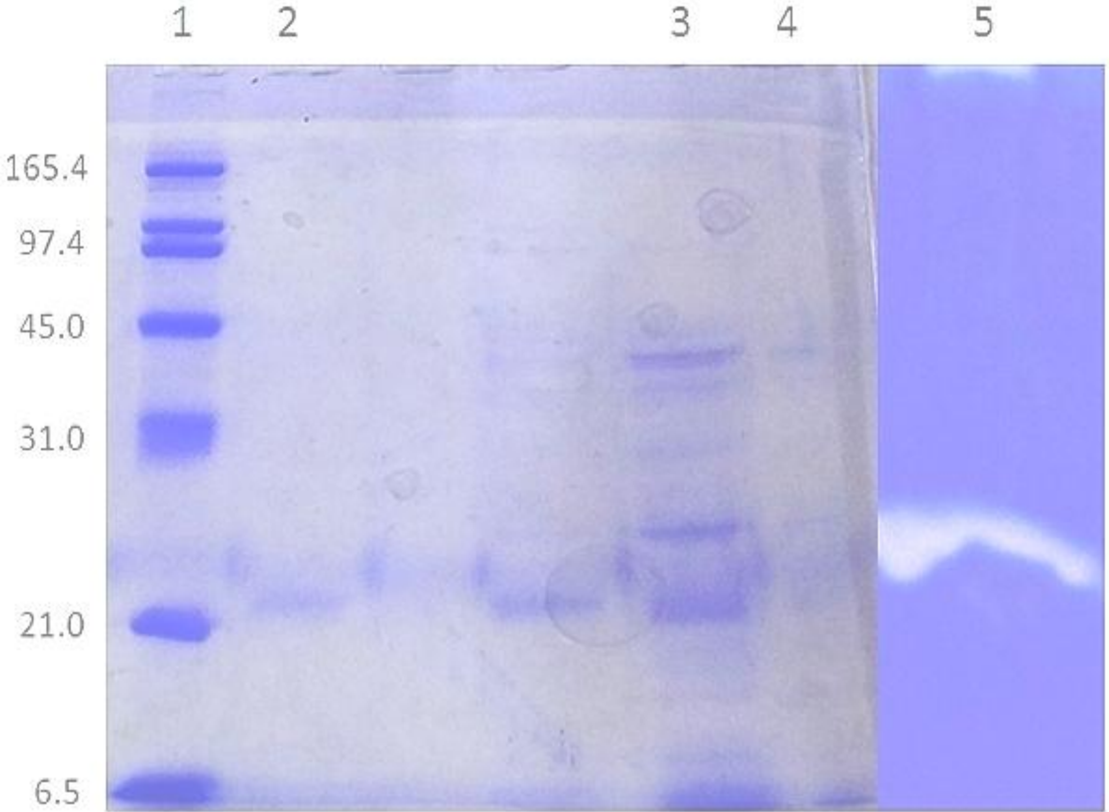


Fig. 4.45: Sodium dodecyl sulphate polyacrylamide gel electrophoresis of extracellular protease from isolate *Halobiforma* sp. BNMIITR, Lane 1: wide range protein molecular weight marker (BIORAD) Lane 2: the purified protease after HIC and Lane 3: protease after acetone precipitation Lane 4: the crude protease Lane 5: shows casein zymogram.

Further characterization of purified enzyme (in 50 mMTris buffer, pH 9.0, containing 1.2 % NaCl) was done with casein (final concentration in reaction mixture was 1 %) as a substrate in 50 mM glycine NaOH buffer pH 10.0 and 45 °C. Enzyme of 150-170 U/ml in (50 mM Tris buffer, pH 9.0, containing 1.2 % NaCl) was used for further characterization.

4.9 Characterization and potential application of purified extracellular protease of isolate *Halobiforma* sp. BNMIITR

4.9.1 Optimization of pH and temperature

Effect of pH on caseinolytic activity of protease (in 50 mM Tris buffer pH 9.0, containing 1.2 % NaCl) was analyzed in 50 mM buffers of different pH, potassium phosphate (pH 6.0–7.5), Tris (pH 8.0–9.5), glycine NaOH (pH 10- 12) at 45 °C for 30 min. It was found that enzyme could work at a wide pH range of 6-12 and the optimum pH was found to be 10 (Fig. 4.46). The optimum temperature of the protease (in 50 mM Tris buffer pH 9.0, containing 1.2 % NaCl) was determined by incubating it with casein in 50 mM glycine NaOH buffer, pH 10.0. The mixture was incubated at different temperatures (20-70 °C). It was found that protease of *Halobiforma* sp. BNMIITR can work at a wide temperature range 20-70 °C with the optimum activity at 50 °C (Fig. 4.47). Protease of *Geomicrobium* sp. EMB2 was stable at a pH range 6.0-12.0 and temperature upto 60 ºC with optimum pH at 10.0 and temperature of 55 ºC (Karan and Khare. 2010). Protease from *Halobacillus karajensis* undergoes thermal activation above 30 ºC with maximum activity between 45 ºC and 55 ºC, followed by thermal deactivation above 60 ºC (Hamid Reza et al. 2009). Izotova et al. (1983) reported that a subtilisin-like serine protease from *Halobacterium salinarum* which had optimum activity at pH 8-9 and temperature of 37 °C. An extracellular serine protease from *Halobacterium halobium* S9 had maximum activity at 40 °C and alkaline pH 8.7. A protease from *gamma-Proteobacterium* was found to be active at a pH range (6.0–11.0) and 30 to 70 °C with optimum at pH 9.0 and 40 °C (Sana et al. 2005). An extracellular protease of *Natrialba magadii* had highest activity at pH 8.0 and temperature 60 °C (Gimenez et al. 2000). An extracellular serine protease from the extremely halophilic archaea bacterium *Halogeometricum borinquense* strain TSS101 had optimum activity at 60 °C and pH 10.0 (Vidyasagar et al. 2006).

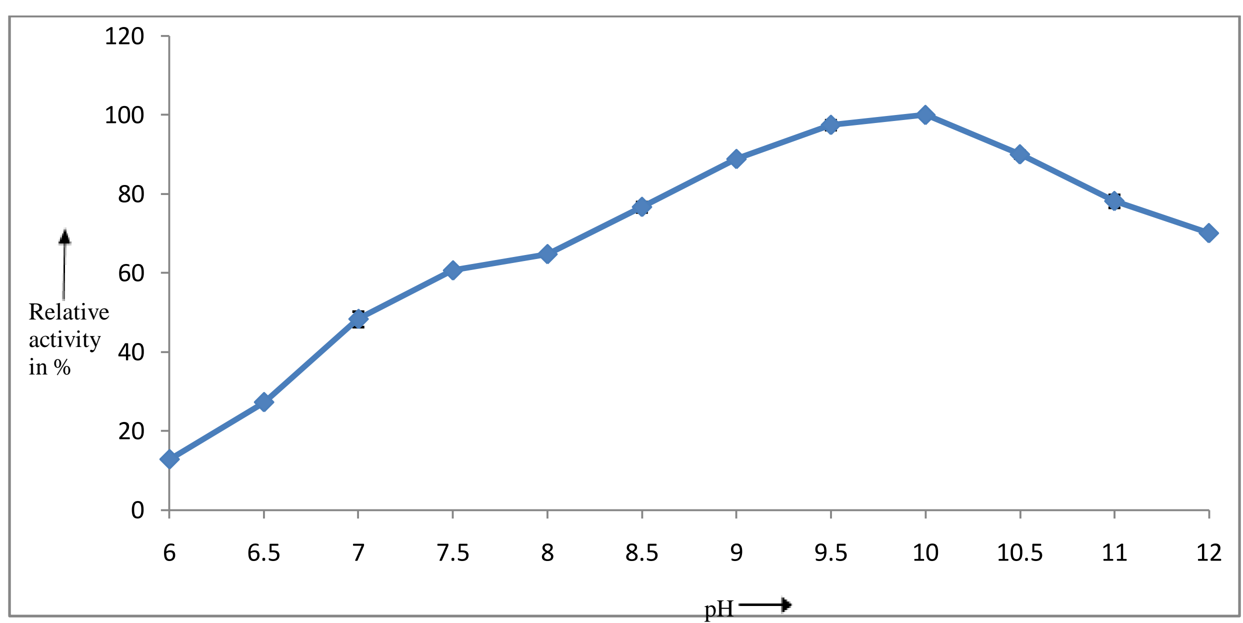


Fig. 4.46: Effect of pH (at 45 ^{o}C) on caseinolytic activity of purified enzyme in (50 mM Tris buffer pH 9.0, containing 1.2 % NaCl) from *Halobiforma* sp. BNMIITR. Values presented are the mean ± standard deviation where $n = 3$.

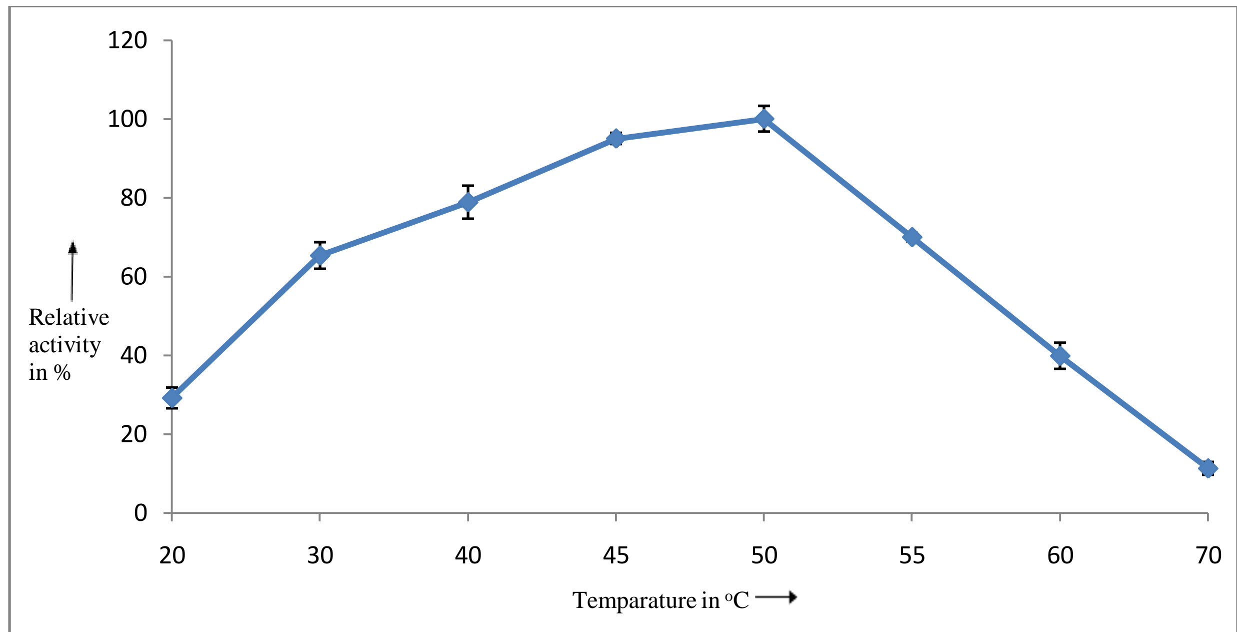


Fig. 4.47: Effect of temperature (at pH 10.0) on caseinolytic activity of purified enzyme in (50 mM Tris buffer pH 9.0, containing 1.2 % NaCl) from *Halobiforma* sp. BNMIITR. Values presented are the mean ± standard deviation where $n = 3$.

Enzyme was found to be unstable at 50 °C thus further characterization was done at 45 °C (Section 4.9.5).

4.9.2 Determination of Substrate specificity

Protease activity of purified enzyme was analyzed with different substrates such as casein, BSA and gelatine. Elastase activity of extracellular protease of *Halobiforma* sp. BNMIITR was determined using N-Succinyl-Ala-Ala-Ala-*p*-nitroanilide as a substrate. Esterase activity of enzyme was tested by using p-nitrophenyl actetate (pNPA) as a substrate. Results of substrate specificity have been summarized in Table 4.18. Enzyme was found to have maximum activity with casein (100 %) followed by BSA 23.83 % and very less activity was observed with gelatine (2.26 %). Besides having caseinolytic activity, it also had esterase and elastase activities which are rarely reported in proteases. Esterase and elastase relative activities were 26.65 % and 36.79 % respectively (100 % as caseinolytic activity). No elastase and esterase activities were found in protease of *Halobacterium* sp. SP1. A protease of *Geomicrobium* sp. EMB2 reported to have 56 %, 61 % of maximum activities with BSA and gelatin respectively but no elastase and esterase activities were reported (Karan and Khare. 2010). An extracellular protease of *Halobacterium halobium* (ATCC 43214) was found to have esterase activity (Ryu et al. 1994). A detergent-stable serine protease of *Bacillus pumilus* CBS had both esterase and elastase activities which were 75 % and 26 % in comparison to 100 % caseinolytic activity respectively (Jaouadi et al. 2008). Marginal elastase activity was found in alkaline protease from alkaliphilic feather-degrading *Nesterenkonia* sp. AL20 (Bakhtiar et al. 2005).

The protease in the present study can use casein and BSA as substrates and had negligible activity with gelatin. However, besides caseinolytic activity it also has elastase and esterase activites which is rarely reported reported in many proteases.

Table 4.18: Substrate specificity of purified enzyme (in 50 mM Tris buffer pH 9.0, containing 1.2 % NaCl) was checked at 45 ^{o}C and pH 10 in 50 mM glycine NaOH buffer.

S.No.	Substrate	Enzyme activity in U/ml	Relative Activity in %
1	Casein	158.52 ± 3.59	100 %
2	Bovine Serum Albumin (BSA)	37.788 ± 2.71	23.83 %
3	Gelatin	3.59 ± 0.65	2.26 %
4	N-Succinyl-Ala-Ala-Ala-*p*-nitroanilide (Elastase)	58.32 ± 0.190	36.79 %
5	Paranitrophenyl actetate (Esterase)	47.5 ±2.63	26.65 %

4.9.3 Effects of metal ions on enzyme activity

Effects of various metal ions on protease activity were studied by incubating the enzyme at 45 ^{o}C in glycine NaOH buffer (pH 10) with various metals at 5 and 10 mM concentrations. Activity remain unaffected by barium, calcium, copper and cobalt when used at 1 mM concentration but activity was reduced to 77.93 %, 89.42 %, 94.57 % and 78.47 % respectively when used at 5 mM concentration. Activity showed considerable decrease in the presence of nickel and zinc. The relative activities were 79.62 % and 52.63 % in the presence of nickel and zinc at 1 mM concentration and 37.00 % and 40.31 % in the presence of nickel and zinc at 5 mM concentration. Activity was not affected by manganese even when used at 5 mM concentration. Iron and magnesium have stimulatory effects on extracellular protease of *Halobiforma* sp. BNMIITR. The estimated relative activities were 114.15 % and 105.46 % in the presence of 1 mM iron and magnesium respectively whereas they were 102.99 % and 111.17 % in the presence of 5 mM iron and magnesium respectively (Fig. 4.48). The activity, of a protease of haloalkaliphilic *Bacillus* sp was reduced by Ni^{2+} at 1 mM and 5 mM concentrations (75 % and 50 %, respectively), whereas

activity remain unaffected by Mn^{2+} , Zn^{2+} and Mg^{2+}. But activity was enhanced by Ca^{2+} (Gupta et al. 2005). A protease from moderately halophilic isolate *Pseudomonas* sp. was activated by Ca^{2+} and Mg^{2+} (Qua et al. 1981). The activity, of a protease from moderately halophilic bacterium *Salinivibrio* sp. strain AF-2004 was enhanced by Ca^{2+} and Mg^{2+} whereas it was reduced to 66 % in the presence of 5 mM zinc and to 16 % in the presence of 5 mM nickel (Karbalaei-Heidari et al. 2007). Activity of an extracellular serine protease from the extremely halophilic archaea bacterium *Halogeometricum borinquense* strain TSS101 006 stimulated by Ca^{2+} and completely inhibited by Cu^{2+}, Zn^{2+}, Co^{2+} where as Ba^{2+}, Mg^{2+} had no effects on the activity of the enzyme (Vidyasagar et al. 2006). Thus it supports the present finding that the activity of protease of *Halobiforma* sp. was inhibited by Zn^{2+}, enhanced by Mg^{2+} where as Co^{2+}, Ba^{2+}, Ca^{2+} had no significant effects. Enhancement in protease activity by Mg^{2+} and Ca^{2+} has been reported in many cases (Thangam et al. 2002). Inhibitions of protease by Zn^{2+} and heavy metal like Ni^{2+} have also been reported (Miyoshi et al. 2002). Thus the enzyme in the present study showed resistance towards alkaline earth metal and heavy metals like iron, manganese, copper and cobalt but was sensitive to nickel and zinc.

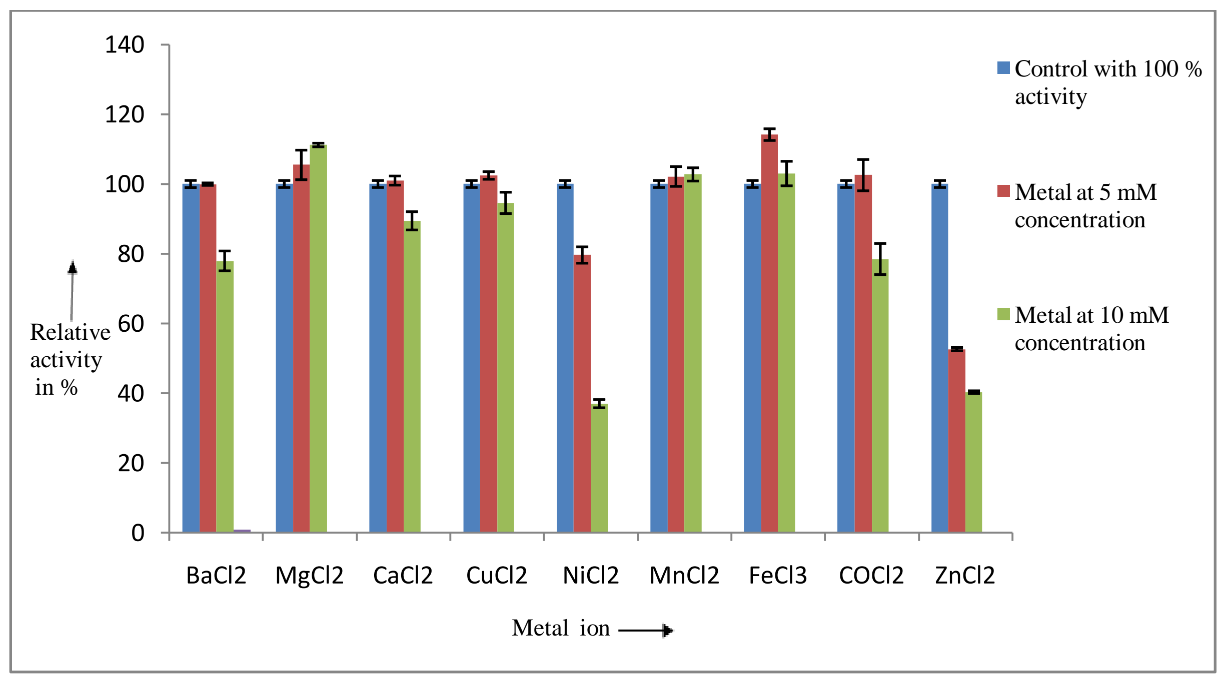


Fig. 4.48: Effects of metal ions on enzyme (in 50 mM Tris buffer pH 9.0, containing 1.2 % NaCl) activity was checked at 45 °C and pH 10 in 50 mM glycine NaOH buffer for 30 min. Blue bar showing control (100 %), red bar showing relative activity in the presence of metal at1 mM

concentration, green bar showing relative activity in the presence of metal at 5 mM concentration Values presented are the mean ± standard deviation where $n = 3$

4.9.4 Effects of osmolytes on enzyme activity and renaturation

Effects of different concentrations (1- 10 %) of various osmolytes like glycerol, mannitol, sucrose and KCl on the activity of purified enzyme were examined. Relative activities have been summarized in Table 4.19. The activity remains unaffected in the presence of mannitol (1- 10 %), whereas there was a continuous decrease in activity in the presence of KCl. Relative activities in the presence of KCl were 94.83 %, 92.97 %, 92.97 %, 84.99 %, 71.64 at1 %, 2%, 3 %, 5% and 10 % concentrations respectively. There was a slight increase in activity in the presence of sucrose and relative activities were 102.24 %, 101.55 % in the presence of 1 and 2 % sucrose respectively and 103.56 % in the presence of 1 % glycerol. There was no considerable change in activity at higher concentrations of sucrose and glycerol. To check the effect of osmolytes on enzyme renaturation, deactivated enzyme (in 50 mM tris buffer pH 9.0 without NaCl) was equilibrated with tris buffer having osmolytes (mannitol, sucrose, and glycerol) at concentration 12 and 20 %. Only 26 % activity was recovered in the presence of 20 % mannitol (Table 4.20). Thus it can be concluded that none of the used osmolytes can be an effective renaturation agent for the protease.

Table 4.19: Effects of osmolytes on enzyme (in 50 mM Tris buffer pH 9.0, containing 1.2 % NaCl) activity was checked at 45 ^{o}C and pH 10 in 50 mM glycine NaOH buffer for 30 min.

Osmolyte	Relative activity at 1 % concentration	Relative activity at 2 % concentration	Relative activity at 3 % concentration	Relative activity at 5 % concentration	Relative activity at 10 % concentration
KCl	94.83% ± 2.25	92.97% ± 0.70	92.97% ± 2.23	84.99 % ± 0.27	71.64 % ±3.21
Mannitol	95.19% ± 1.05	99.91% ± 0.15	99.54% ± 0.88	98.54% ±0.33	97.31% ± 0.77

Sucrose	102.24% ± 4.74	101.55% ± 2.06	98.95% ± 1.63	95.55% ± 1.83	94.78% ± 0.92
Glycerol	103.56% ± 3.75	99.53% ± 2.02	94.47% ± 0.35	93.61% ± 0.82	91.11% ± 1.12

Table 4.20: Effects of osmolytes on enzyme renaturation.

Osmolyte	% activity recovered in the presence of 12 % osmolyte	% activity recovered in the presence of 20 % osmolyte
Mannitol	17.44	26.15
Sucrose	18.15	20.95
Glycerol	11.55	18.17

4.9.5 Thermo-stability of the enzyme and effect of NaCl on thermo stability of the enzyme

Stability of an enzyme is a very important parameter for industrial application of enzyme. Several methods have been developed to stabilize the enzymes at higher temperatures. Stability of enzyme at different temperatures was determined by incubating the enzyme (in 50 mM tris buffer pH 9.0 with 1.2 % NaCl) at different temperatures (50 ^{o}C, 45 ^{o}C, 30 ^{o}C and 20 ^{o}C) for 24 hours. Enzyme was very unstable at 50 ^{o}C retain only 22.91 and 0.84 % activities after 2 and 6 hours of incubation. Relative activities at 45°C were 84.08 %, 63.744 %, 45.33 % and 37.20 % after 2, 6, 12 and 24 hours of incubation (Fig. 4.49). Enzyme was found to retain more than 83 % activity at 30 ^{o}C after 24 hours of incubation whereas as it was found to retain more than 96 % activity at 20 ^{o}C after 24 hours of incubation. A protease enzyme from an extremely halophilic archaeon *Halogeometricum borinquense* strain TSS101 was stable at 60 ^{o}C only for two hours (Vidyasagar et al. 2006). A protease from *Natrialba magadii* showed fast inactivation at high temperatures and was very unstable at 60 ^{o}C (Gimenez et al.2000). Effect of NaCl on thermo stability of the enzyme was determined by incubating the enzyme at different salt concentrations (0- 12.6 %). Maximum stability was found at 10.6 – 12.6 % NaCl concentration and at this concentration enzyme retained it's complete activity even after 24 hours of incubation at 45 ^{o}C where as it was 37.82 % , 43.30 %

and 63.84 % at 1.2, 3.6 and 5.6 % NaCl concentration after 24 hours of incubation at 45 °C (Table 4.21). A protease of *Halobacterium* sp. SP1 (1) was highly stable in the presence of 30 % Na-glutamate, followed by 4 M NaCl. However the optimum NaCl concentration for activity was 2 M but at this concentration enzyme stability was minimum (Akolkar et al. 2009). Similarly in present study, the enzyme reported required 1- 2 % NaCl for optimum activity but at this NaCl concentration enzyme stability was minimum and require 10-12 % NaCl for stability at higher temperature. At high salt concentration, enzyme is shielded by a hydrophobic layer formed by salt which prevents electrostatic repulsion and unfolding of the protein, thus stabilize the protein (Joo and Kim 2005).

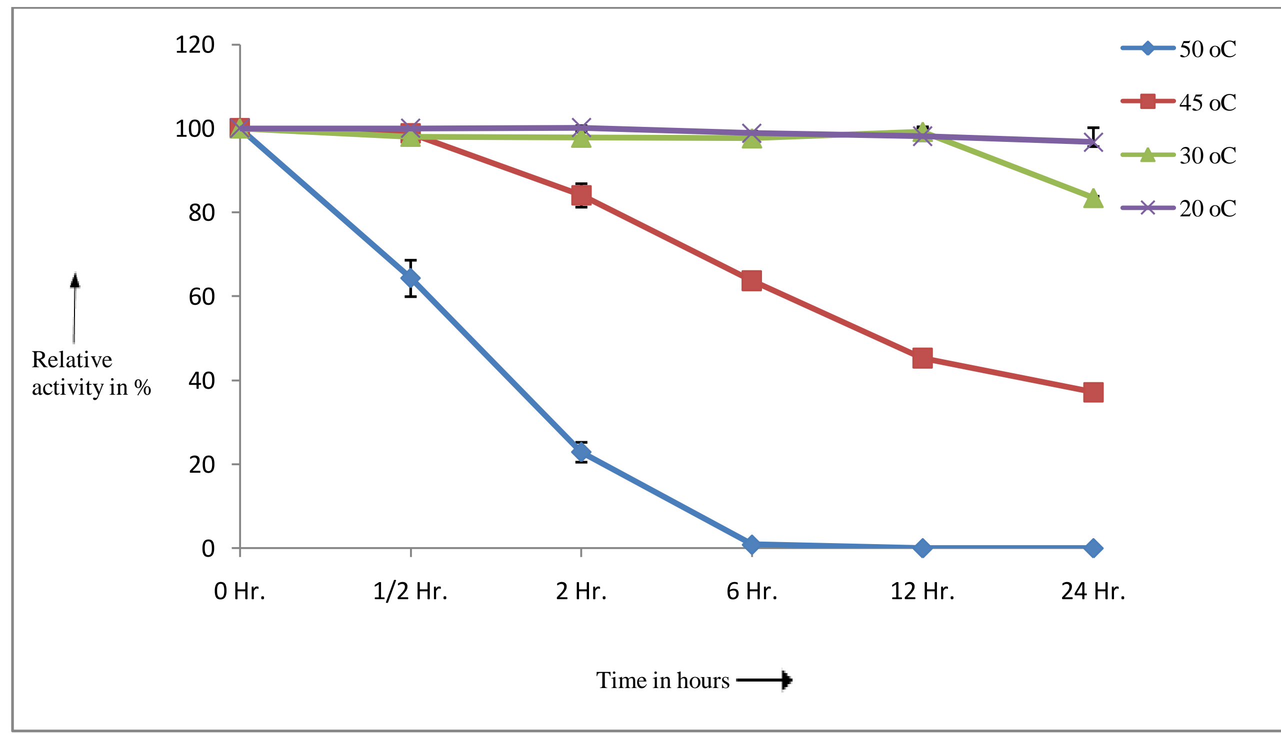


Fig. 4.49: Thermal stability of enzyme (in 50 mM tris buffer pH 9.0 with 1.2 % NaCl) at different temperatures (50 °C, 45 °C, 30 °C and 20 °C) for 24 hours.

Table 4.21 : Effect of NaCl on thermo stability of the enzyme at 45 ^{o}C.

Hours	Relative activity in % at 0.6 % NaCl	Relative activity in % at 1.2 % NaCl	Relative activity in % at 3.6 % NaCl	Relative activity in % at 5.6 % NaCl	Relative activity in % at 10.6 % NaCl	Relative activity in % at 12.6 % NaCl
0	100 % ± 1.57	100% ± 0.79	100% ± 0.79	100% ± 0.79	100% ± 0.79	100% ± 0.79
2	16.96 % ±1.042	85.50% ± 2.79	98.83% ± 1.68	101.11% ± 0.48	102.19% ± 0.66	101.59% ± 0.48
6	0	64.81% ± 1.44	83.17% ± 1.26	96.24% ± 0.69	101.56% ± 2.33	98.50% ± 4.08
12	0	46.09% ± 0.33	53.91% ± 0.07	79.21% ± 1.53	100.18% ± 2.10	100.24% ± 0.40
24	0	37.82% ± 0.69	43.30% ± 0.09	63.84% ± 0.27	100.11% ± 0.64	99.54% ± 0.02

4.9.6 Effects of additives on enzyme activity

Effects of PMSF, EDTA and phenanthrolene on protease activity were determined by including them in the reaction mixture at concentrations of 5 mM and 10 mM. Effects of surfactants (SDS), Tween-20, Tween-40, Tween-80, Triton X-100, sodium cholate, sodium deoxycholate and cetyl trimethylammonium bromide (CTAB) on enzyme activity were studied at concentrations of 0.1 % and 0.5 %. Effects of reducing agents such as Dithiotheritol (DTT), oxidizing agents like hydrogen peroxide, Ammonium persulphate (APS) at 5 and 10 mM concentrations were studied by measuring activity in their presence. The activity of the enzyme measured in absence of any

additive was taken as 100% (control). Activity of extracellular protease of *Halobiforma* sp. BNMIITR remained unaffected by Tween-20, Tween-40, Tween-80 at 0.1 and 0.5 % concentrations whereas it was slightly decreased by Triton X-100 to 90.90 % at 0.5 % concentration. Activity was reduced in the presence of CTAB, SDS, sodium cholate and sodium deoxycholate and relative activities were 93.93 %, 48.30 %, 83.50 % and 88.50 % at 0.1 % concentration and 63.63 %, 37.73 %, 59.79 % and 65.57 % at 0.5 % concentration respectively. Activities were reduced to 66.36 %, 50.50 %, and 65. 95 %, 54.51 % in the presence of 5 mM, 10 mM of phenanthrolene and EDTA respectively (Fig. 4.50). Activity was completely inhibited by 5 mM PMSF which showed that it was a serine protease. PMSF bind specifically to serine residue present at the active site of the serine proteases which results in the inhibition of serine proteases (Bone et al.1987). Most of the serine proteases are inhibited by EDTA. Activity of an extracellular serine protease from the extremely halophilic archaea *Halogeometricum borinquense* strain TSS101 006 was inhibited to 65 % in the presence of 1mM EDTA, to 38 % in the presence of 10 mM EDTA and to 53 % in the presence of 0.1 % SDS (Vidyasagar et al. 2006). Activity of an extracellular serine protease of *Geomicrobium* sp. EMB2 remained unaffected by CTAB, SDS, Triton X-100 and Tween-80 (Karan and Khare. 2010). In the present study, activity of protease of *Halobiforma* sp. remain unaffected by Triton X-100, H_2O_2,Tween-80, Tween-40, Tween-20 and inhibited by CTAB, SDS, phenanthrolene, EDTA, sodium cholate and sodium deoxycholate, whereas it was stimulated by DTT. A protease from *Natrialba magadii* was sensitive to SDS whereas activity was enhanced to 166 % in the presence of 1mM DTT and to 210 % in the presence of 10 mM DTT (Gimenez et al.2000). A protease from *Salinivibrio* sp. strain AF-2004 was strongly inhibited by EDTA and 1, 10-phenanthroline (Karbalaei-Heidari et al.2007). The enzyme reported in the present study was active in the presence of various surfactants, oxidizing and reducing agents which showed that it can be further used in the detergent industry.

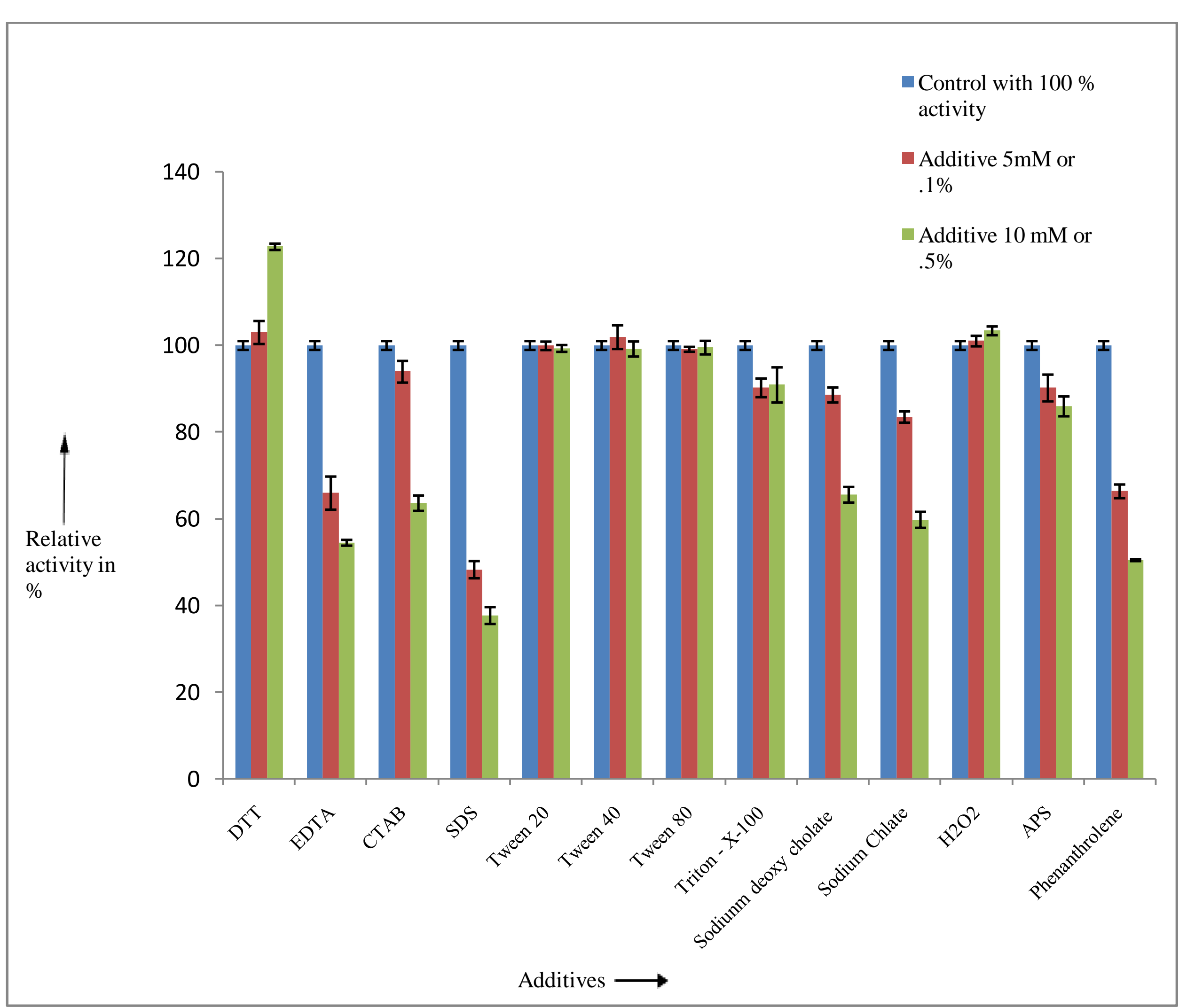


Fig.4.50: Effects of additives on enzyme (in 50 mM Tris buffer pH 9.0, containing 1.2 % NaCl) activity was checked at 45 ^{o}C and pH 10 in 50 mM glycine NaOH buffer for 30 min. Blue bar showing control (100 %), red bar showing relative activity in the presence of additive at 0.1 % or 5 mM concentration, green bar showing relative activity in the presence of at 0.5 % or 10 mM concentration. Values presented are the mean ± standard deviation where $n = 3$.

4.9.7 Effects of organic solvents on protease activity and protease stability in organic solvents

Effects of various solvents on purified enzyme of *Halobiforma* sp. BNMIITR (in 50 mM Tris buffer pH 9.0, containing 1.2 % NaCl) were studied by determining enzyme activity at 45 ^{o}C in glycine NaOH buffer (pH 10) with various non polar solvents such as toluene, n-hexane, n-heptane, xylene, butanol, dichloromethane, octane and polar solvents as DMF, DMSO, methyl

alcohol, ethyl alcohol, isopropanol, at 10 %, 30 % and 50 % (v/v). The relative activities were presented after determining the activities. Activity of extracellular protease of *Halobiforma* sp. BNMIITR remained unaffected by polar solvents at 10 % v/v concentration, but activity was reduced to 64.22 %, 78.70 %, and 62.39 % in the presence of 30 % methanol, ethanol and isopropanol respectively and to 44.40 %, 58.49 %, 47.60 % in the presence of 50 % methanol, ethanol and isopropanol respectively. Enzyme retained more than 86 % activity in the presence of nonpolar solvents (toluene, hexane, heptane, xylene) even at 50 % concentration. Activities were reduced to 92.45 %, 89.93 %, 76.05 % in the presence of 10, 30 and 50 % butanol respectively whereas it was 88.22 %, 55.13 %, 51.48 % in the presence of 10, 30 and 50 % dichloromethane respectively. Interestingly activities were increased to 103.91 %, 114.17 % and 127.59 % in the presence of 10, 30 and 50 % DMF respectively and to 105.60 %, 116.33 % and 136.78 % in the presence of 10, 30 and 50 % DMSO respectively (Fig. 4.51). This property of enzyme can be further explored in organic synthesis. Some enzymes show enhancement in activity in the presence of organic solvents (solvent activation), a lipase from *P. aeruginosa* activated in the presence of polar solvents such as 2- propanol (Lima et al. 2004). The activity of the enzyme measured in absence of solvent was taken as 100 % (control) in the resent case. To find out solvent stability of enzyme, 1 ml of protease along with 1 ml of organic solvents incubated at 30 ^{o}C and 45 ^{o}C with constant shaking at 180 rpm. Samples were withdrawn after 24 h, and 48 h. Residual protease activity was determined. The enzyme incubated without solvent was treated as the control. Enzyme was considerably stable in polar as well as non polar solvents and it shows activity even after 48 h of incubation at 30 °C (Fig. 4.52).

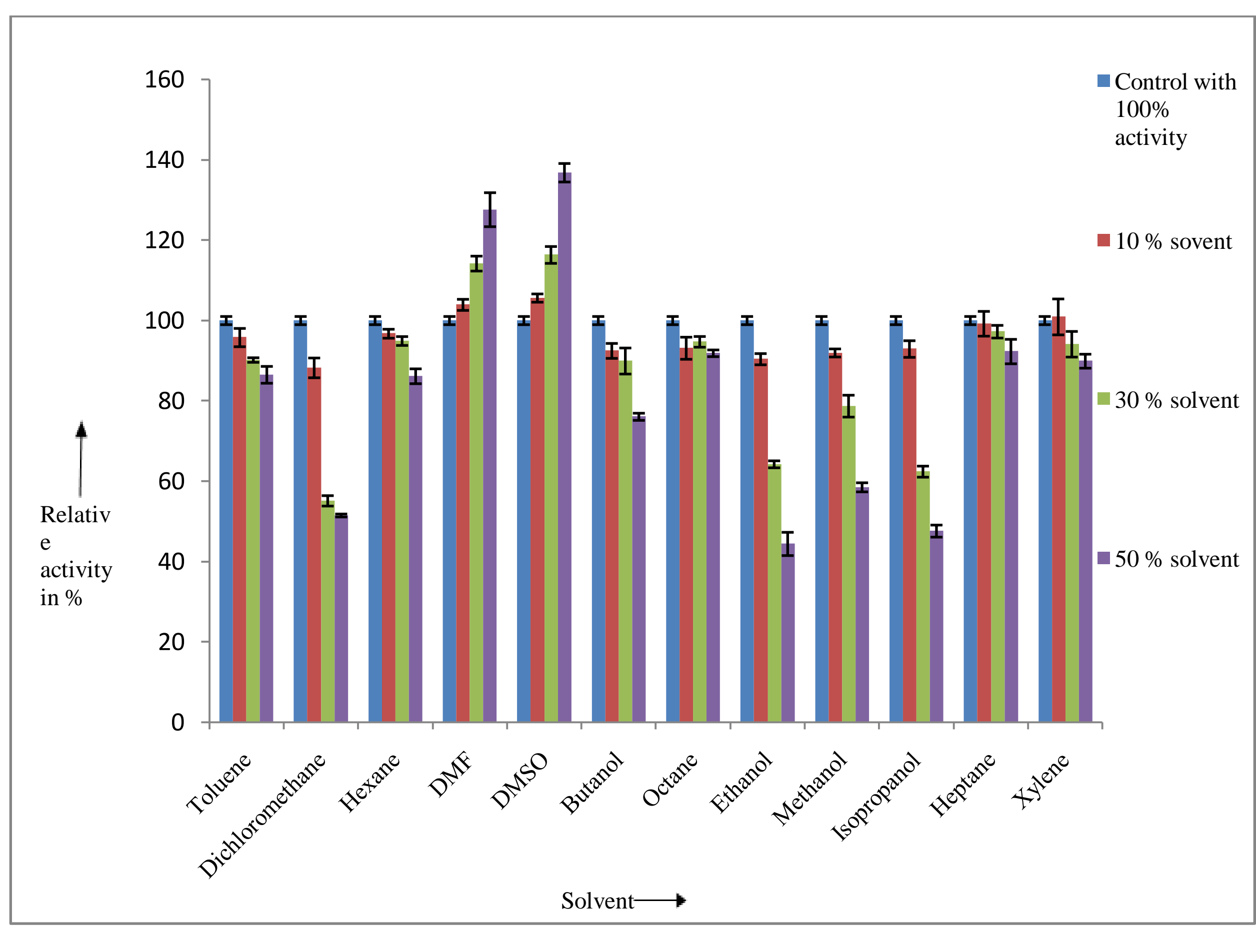


Fig 4.51.: Effects of organic solvents on enzyme (in 50 mM Tris buffer pH 9.0, containing 1.2 % NaCl) activity was checked at 45 ^{o}C and pH 10 in 50 mM glycine NaOH buffer for 30 min. Blue bar showing control (100 %), red bar showing relative activity in the presence of 10 % solvent , green bar showing relative activity in the presence of 30 % solvent, purple bar showing relative activity in the presence of 50 % solvent. Values presented are the mean ± standard deviation where $n = 3$.

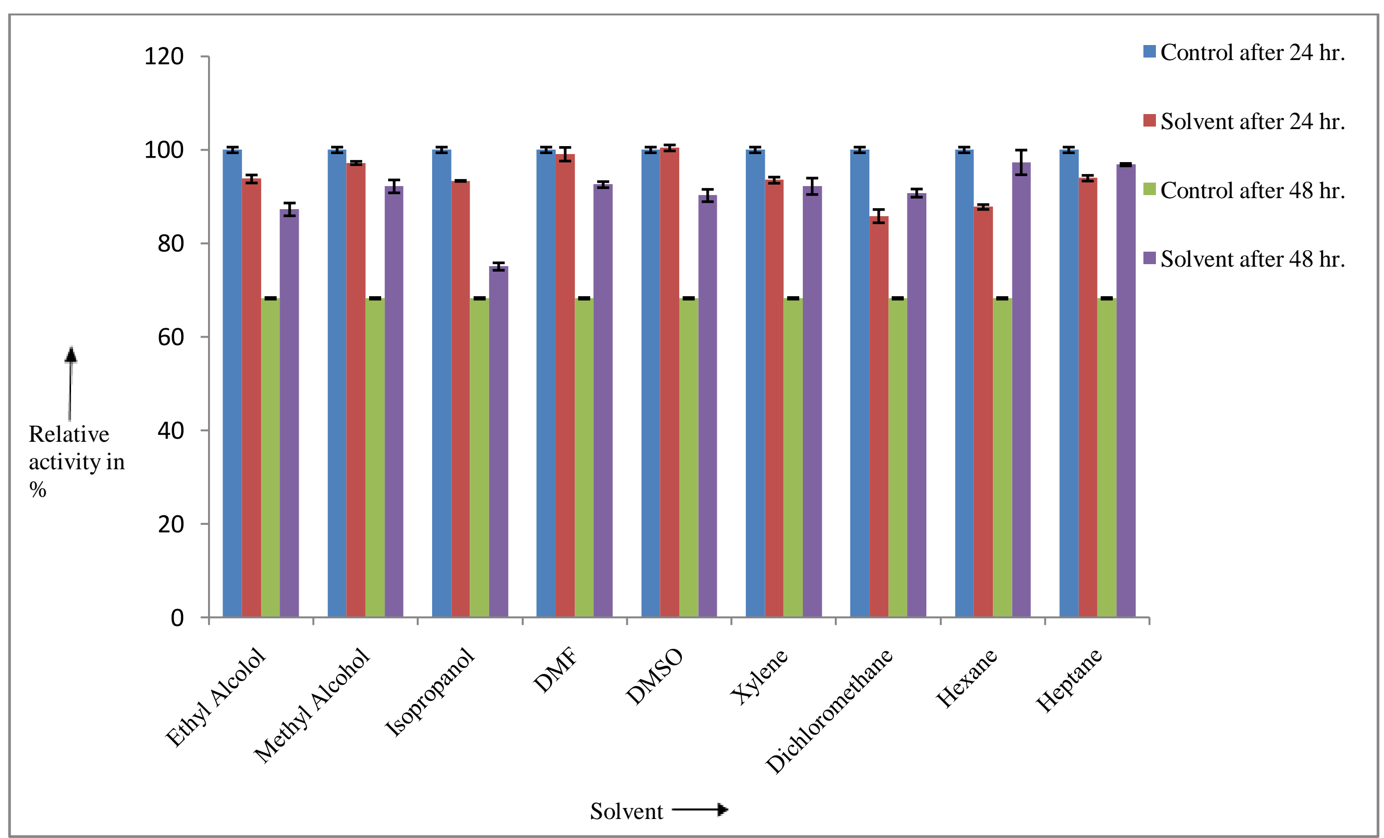


Fig. 4.52: Enzyme (in 50 mM Tris buffer pH 9.0, containing 1.2 % NaCl) stability in the presence of organic solvents at 30 °C blue bar showing control (100 %), red bar showing relative activity in the presence of 50 % solvent after 24 hours, green bar showing relative activity of control after 48 hours, purple bar showing relative activity in the presence of 50 % solvent after 48 hours solvent. Values presented are the mean ± standard deviation where $n = 3$. Relative activities were calculated with reference to aqueous phase (1 ml enzyme + 1 ml 50 mM Tris buffer pH 9.0, containing 1.2 % NaCl) buffer after 24 hours (100 %). Activity of aqueous phase was found 134.29 U/ml whereas initial activity was 158.47 U/ml.

Extracellular protease of *Halobiforma* sp. BNMIITR was very stable in the presence of 50 % polar as well as nonpolar solvents. It retained more than 88 % activity in polar and non polar solvents after 24 hours of incubation at 30 °C in comparison to control after 24 hours. After 48 hours at 30 °C, activities were recorded as 50.87 %, 65.02 %, 68.69 %, 55.93 %, 68.96 %, 67.23 %, 68.70 %, 67.62 %, 72.50 % and 72.15 % in the presence of buffer, ethanol, methanol, isopropanol DMF, DMSO, xylene, dichloromethane, hexane and heptane respectively when compared to initial activity of enzyme (in 50 mM Tris buffer pH 9.0, containing 1.2 % NaCl) at zero hour. After 24 hours of incubation at 45 °C enzyme was found to have 30.64 %, 41.26 %, 37.12 %, 65.42 %,

69.28 %, 62.26 %, 51.35 %, 70.77 % and 61.66 % (Table 4.22) in the presence of ethanol, methanol, isopropanol DMF, DMSO, xylene, dichloromethane, hexane and heptane respectively when compared to initial activity of enzyme (in 50 mM Tris buffer pH 9.0, containing 1.2 % NaCl) at zero hour. Structure of a protein is maintained by a balance between hydrogen interactions Vander wall forces and electrostatic interactions. In aqueous system water forms weak bonds with the enzyme which bring about the destabilization of protein. In the presence of organic solvent water activity minimized thus stability of the protein in the presence of organic solvent was increased (Doukyu and Ogino 2010).

Table 4.22: Enzyme (in 50 mM Tris buffer pH 9.0, containing 1.2 % NaCl) stability in the presence of organic solvents at 45 ^{o}C after 24 hours .Values presented are the mean ± standard deviation where *n* = 3. Relative activities were calculated with reference to aquous phase (1 ml enzyme + 1 ml 50 mM Tris buffer pH 9.0, containing 1.2 % NaCl) at 0 hour.

S.No.	Organic solvent	Enzyme activity in U/ml after 24 hours at 45 ^{o}C	Relative activity in % in comparision to initial 0 hour activity
1	Control	61.86 ± 0.54	44.19 %
2	Ethyl alcohol	42.90 ± 0.83	30.64 %
3	Methyl alcohol	57.76 ± 0.76	41.26 %
4	Isopropanol	51.97 ± 3.0	37.12 %
5	DMF	91.59 ± 2.82	65.42 %
6	DMSO	96.99 ± 0.55	69.28 %
7	Xylene	81.17 ±1 .49	62.26 %
8	Dichloromethane	71.89 ± 3.90	51.35 %
9	Hexane	99.09 ± 0.43	70.77 %
10	Heptane	86.32 ± 3.34	61.66 %

Organic solvents produced from crude oil, probably serves as natural extreme environments and many halophilic archaea have been reported from crude oil polluted soil (Raghavan et al., 2000). Organic solvent tolerant enzymes have been reported from many genera as *Pseudomonas, Bacillus, Enterobacter, Rhodococcus* and *Geomicrobium* (Dalfard et al. 2012). Most of the enzymes got destabilized in organic solvents because organic solvents disrupt the aqueous shield surrounding the enzyme (Ogino et al. 2001). A protease from *Bacillus* sp. was found to be activated up to 10 and 30 % in the presence of 5 and 10 % isopropanol and DMF, respectively. A protease from *Bacillus* sp. retained 90 % activity after 48 hours when incubated in the presence of 20 % DMSO, DMF (Dalfard et al. 2012). Salts are known to reduce the water activity thus high salt environment mimic with non-aqueous environments. Most of the halophilic proteases are found to be active and stable at high salt conditions thus can be used in organic solvents systems. A serine protease from C*hromohalobacter* sp. was found to retain its 100 % activity in the presence of 10 % (v/v) DMSO but 25 % activity was lost in the presence of 10 % (v/v) DMF (Vidyasagar et al.2009). Protease of *Geomicrobium* sp. EMB2 was found to be stable in the presence of non polar solvents (Karan and Khare. 2010). A protease of halophilic *Bacillus* sp. showed enhanced activity in polar solvents but activity was highly decreased in nonpolar solvents by more than 80 % in 50 % toluene after 24 hours (Sinha et al. 2013). A protease from marine *Saccharopolyspora* sp. A9 had more than 70 % activity in non polar solvents but activity was 21-23 % in polar solvents after 48 hours (Raut et al. 2012). Organic solvents systems are industrially more advantageous over aqueous systems as most of the non polar substrates get dissolved only in organic solvents and organic solvents systems may also reduces water based side reactions and microbial contamination (Doukyu and ogino 2010). Protease catalyzes peptide synthesis reaction in the presence of DMSO. Organic solvent compatible proteases may play an important role in marine industry by reducing biofouling thus organic solvent tolerant and stable enzymes are industrially more desirable (Doukyu and ogino 2010). The enzyme reported in the present study is very stable in polar as well as non polar solvents. Activity was considerably high in the presence of non polar solvents. It remained stable in the presence of non polar and polar solvents at 30 ^{o}C till 48 hours. Besides this enzyme had tolerance towards metals and had elastase and easterase activities thus it has potential uses as antifoulant in marine paint industry. DMF and DMSO are the solvents used in protease catalyzed transesterification reactions. Most of the protease reported, lost their activity in the presence of DMF and DMSO. A Protease from *Bacillus cereus* showed high activity in the presence of non polar solvents (toluene and cyclohexane) as compared to that of control and enzyme remain active

even after 144 hr of incubation whereas in polar solvents such as DMSO and ethanol it lost 70-80 % activity after 1 hour (Shah et al. 2010). The protease in the present study retained more than 65 % of initial activity in the presence of 50 % DMF and DMSO at 45 ^{o}C after 24 hours and more than 57 % of initial activity in the presence of 50 % DMF and DMSO at 30 ^{o}C after 48 hours which shows its further application in chemical synthesis.

4.10 Detergent compatibility of the enzyme

The protease was found to be stable and compatible with detergents, as it retained more than 95 % activity after 4 hr incubation with the commercial detergents (1.0 % w/v) Surf excel, Ariel, Tide, Rin and Wheel. Results have been summarized in Table 4.23. Enzyme retained more than 96 % activity in all the detergents used and thus it can be further used in detergent formulations.

Table 4.23: Detegent compatability of the enzyme at 45 ^{o}C after 4 hours.

S.No.	Commercial detergent	Enzyme activity in U/ml after 4 hours at 45 ^{o}C	Relative activity in %
1	Control	186.30 ± 3.175	100 %
2	Surf Exel	180.66 ± 2.59	96.96 %
3	Rin	194.13 ± 3.19	104.20 %
4	Nirma	191.87 ± 2.43	102.98 %
5	Wheel	188.05 ± 2.77	100.93 %
6	Tide	178.80 ± 1.60	95.97 %
7	Ariel	187.62 ± 3.64	100.70 %

Protease from the *Geomicrobium* sp. EMB2 was found to be stable in the presence various commercially available detergents (Karan and Khare. 2010). Similarly a protease from halophilic *Bacillus* sp. retained its activity in tween 80 and was found compatible with commercially available detergents (Sinha et al. 2013). Thus Extracellular protease of *Halobiforma* sp. BNMIITR can be used in detergent formulation for biological and non biological stain removal.

5. SUMMARY AND CONCLUSION

Halophiles are extremophilic bacteria which require high salt concentration for growth and are industrially very important. In present time, biocatalysis has been proved as a green, sustainable environment friendly methodology for chemical synthesis. Most of the chemical reactions take place in the presence of organic solvent. In the presence of organic solvent, protease catalyzes peptide synthesis reaction. Thus organic solvent tolerant enzymes are industrially desirable. Most of the enzymes destabilize in the presence of organic solvents.

Halophic enzyme remains active and stable in the presence of high salt concentration thus serves as alternative tool in chemical synthesis. Thus there is a continuous demand of new halophilic bacteria those can produce enzyme with novel properties.

In the present study, four soil and one salt samples were collected from Sambhar salt lake Rajasthan which is the largest inland salt lake of India. Sambhar salt lake is predominated by sodium chloride, sodium carbonate, sodium bicarbonate, sodium sulphate and lacked divalent cations (calcium and magnesium) with pH ranges from 9.5- 9.7 and temperature 37- 45 ºC. All soil and salt samples were incubated at 45 ºC and pH 9.0 on an orbital incubator shaker (180 rpm) in a complex halophilic medium.

Total sixteen isolates were obtained from four soils and one salt sample. Isolates were purified by serial dilutions and repeated streaking on agar plate. Out of 16 isolates, two were found to be pleomorphic (from rod to cocci), Gram negative (MSL-1 and MSL-l0), four cocci Gram positive (MSL-2, MSL-3, MSL-4 and MSL-11) and others are Gram positive rods (MSL-5, MSL-6, MSL-7, MSL-8, MSL-9, MSL-12, MSL-13, MSL-14, MSL-15, MSL-16). All the isolates were checked for spore formation and it was found that out of sixteen isolates, eight isolates (MSL-6, MSL-7, MSL-8, MSL-12, MSL-13, MSL-14, MSL-15, MSL-16) form spores. All the spores were terminal in position and oval in shape. On the basis of halophilic nature and salt requirement isolates were further divided into

Extreme halophiles: salt requirement in a range 2- 5 M with an optimum growth at 4- 5 M NaCl (MSL-10).

Borderline extreme halophiles: salt requirement in a range 2-5 M with an optimum growth at 2-3 M NaCl (MSL-1, MSL-2, MSL-3, MSL-4, MSL-11).

Moderately halophilic: salt requirement in a range 1- 4 M with an optimum growth at 1- 2 M NaCl (MSL-5, MSL-6, MSL-7, MSL-8, MSL-9 MSL-12, MSL-13, MSL-14, MSL-15, MSL-16).

Borderline and extreme halophiles showed very less growth in medium from which NaCl is replaced with KCl whereas moderately halophilic isolates show better growth in KCl medium. All the isolates were found to grow in a wide range of pH and temperature. All the isolates except MSL-9 were alkaliphilic and can grow at a wide range of temperature and pH. Borderline and extreme halophiles can grow at higher temperature of 55 ^{o}C. Extremely halophilic and borderline extremely halophilic isolates showed resistance towards ampicillin, cephalothin, chloramphenicol, clindamycin, erythromycin, gentamycin oxacillin and penicillin-G. All isolates were found to be sensitive for co- trimoxazole, cloxacilin and cefradine. Out of sixteen isolates, five (MSL-1, MSL-2, MSL-3, MSL-11 and MSL-10) were found to produce pigment. A pigment profile showed that all five isolates have carotenoid pigment and MSL-1 was found to have some minor pigments.

Carbon substrate utilization by isolates was determined by using the BIOLOG Phenotype GEN III plates. Among all the isolates MSL-9 could utilize maximum numbers of carbon sources and MSL-4 utilized minimum number of carbon sources. Borderline and extreme halophiles were found to use less carbon sources in comparison to moderately halophilic isolates. On the basis of plate assay, five isolates (MSL-1, MSL-4, MSL-5, MSL-12 and MSL-13) out of sixteen isolates were found to produce extracellular protease. All five isolates showing protease activity on plate were grown in liquid medium and protease activity was checked at regular intervals. On the basis of activity and specific activity of the crude enzyme MSL-1 was selected and studied further.

Genomic DNA of MSL-1 was isolated and amplified with universal archaeal and universal eubacterial primers. Amplification was observed only with archaeal primers. Complete 16S rRNA gene sequence showed 98 % similarity with *Halobiforma lacisalsi*, 97 % with *H. haloterrestris* and 96 % with *H. nitratireducens.* Thus the strain MSL-1 phylogenetically belongs to genus *Halobiforma* and closest to *Halobiforma lacisalsi* hence named as *Halobiforma* sp. strain BNMIITR.

Thin-layer chromatogram of total lipids from *Halobiforma sp.* strain BNMIITR showed the presence of six different types of lipids where as polar lipid profile showed the presence of two glycolipids and one phospholipid but only of 4 different types of lipids are present in *Halobiforma*

haloterrestris. Major cellular fatty acids from *Halobiforma sp.* strain BNMIITR were $C_{14:0}$, $C_{15:0}$ iso, $C_{15:0}$ anteiso, $C_{16:0}$, $C_{17:0}$ iso, $C_{17:0}$ anteiso, $C_{20:2}$ w6, 9c.

The crude protease enzyme was active at pH range 6- 12 and temperature (30- 80 ºC). The crude enzyme was found to be active in the presence of various polar and non polar solvents.

Nutritional factors and culture conditions affect the protease production. NaCl was found to be most suitable salt for protease production at 3 M concentration (40.84 ± 0.5957 U/ml). Protease production was not observed in the presence of KCl and Na_2SO_4 whereas it was 5- 8 times less in the presence of other salts (Na_2PO_4, $NaNO_3$ and CH_3COONa). Similarly maximum protease production was found at pH 10 and 45 ^oC temperature. Out of various carbon sources used maximum production (activity 75.28 ± 1.69 U/ml and specific activity 1284.86 ± 20.76 U/mg) was found when xylose was used as a carbon source.

Low cost agro industrial byproducts like wheat bran and wheat flour were not found to be very promising for protease production as activity was reduced (14.81 ± 1.30 and 22.77 ±2.28 U/ml). Protease production was considerably increased in the presence of low cost agro industrial byproducts like soybean husk, soybean flour, chick pea flour and chick pea husk. Protease activity was found to be 123.08 ± 2.82 U/ml and 184 ± 7.65 U/ml in the presence of soybean flour and chick pea flour respectively. There was 2.94 and 2.17 folds increased protease production when chick pea flour and soybean flour were used as nitrogen source as compared to initial production medium but in the presence of chick pea flour and soybean flour protease production was delayed from 4 days to 8-10 days. Protease production was not observed in the presence of inorganic nitrogen sources, urea and cassamino acids. Protease production is catabolically repressed by amino acids.

Protease production of *Halobiforma sp.* strain BNMIITR was inhibited by positively charged amino acids whereas negatively charged amino acids have no effect when used at 0.1 % concentration. Phenylalanine at 1 % and tryptophan and methionine at 0.1 % concentration completely inhibited the protease production whereas protease production was enhanced in the presence of asparagine and proline. Protease production was reduced to 14.79 % and 17.70 % (in comparison to control without surfactants) in the presence of 0.1 % SDS and Triton-X-100 respectively. *Halobiforma sp.* strain BNMIITR could not grow at higher concentration of SDS and

Triton-X-100 in the production medium. Whereas protease production was reduced to 8.77% and 12.0 % in the presence of 1 % Tween -80 and Tween -20 in the production medium respectively.

Two litre of protease was obtained by growing *Halobiforma sp.* strain BNMIITR in optimized production medium. Crude enzyme was concentrated by using Pall advanced sepration system with 10 kDa membrane. Concentrated enzyme was fractionated by acetone precipitation and further purified by HIC using Phenyl Sepharose 6 Fast Flow column, equilibrated with50 mM tris buffer pH 9.0 with 25 % NaCl. Further elution was done by applying a linear gradient of NaCl (25 %- 0%) in 50 mM tris buffer of pH 9.0. The enzyme was eluted at 11-10 % NaCl. After this step 31.67 fold purification was achieved with specific activity of 40919.55 U/mg. Like other halophilic enzymes, protease of *Halobiforma sp.* strain BNMIITR was found to lose its 90 - 95 % activity in the absence of salt and required minimum 1- 2 % NaCl to retain activity. Purity of the enzyme was confirmed by SDS PAGE. Enzyme was a monomer with a molecular mass 21 kDa which was further confirmed by casein zymography.

Protease of *Halobiforma sp.* strain BNMIITR can work at wide range of pH (6-12) with optimum activity at pH 10 and 50 ^{o}C but enzyme was found to be unstable at 50 ^{o}C. Protease of *Halobiforma sp.* strain BNMIITR had maximum activity when casein was used as a substrate while it was 23.83 % and 2.26 % for BSA and gelatin respectively. Besides caseinolytic activity, it also had 26.65 % and 36.79 % esterase and elastase activities respectively which are less common in proteases.

Protease was found to be stable in the presence of alkaline earth metals and heavy metals but activity was reduced by nickel and zinc. Protease activity was enhanced in the presence of magnesium. Enzyme was less stable at high temperature and retained 0.84 % activity at 50 ^{o}C after six hours whereas it was 37.20 % at 45 ^{o}C after 24 hours. Enzyme stability at high temperature is affected by salt concentration, enzyme was found to retain 100 % activity at 45 ^{o}C after 24 hours when incubated with 10.6 % NaCl.

Enzyme was active in the presence of various surfactants, oxidizing and reducing agents which showed that it can be further used in the detergent industry but the activity was completely inhibit by PMSF which showed that it was a serine protease. Activity of protease of *Halobiforma* sp. remain unaffected by Triton X-100, H_2O_2,Tween-80, Tween-40, Tween-20 and inhibited by

CTAB, SDS, phenanthrolene, EDTA, sodium cholate and sodium deoxycholate, whereas it was stimulated by DTT.

Organic solvents systems are industrially more advantageous over aqueous systems as synthetic reactions favoured over hydrolysis, thermo-stability increases, low risk of microbial contamination, organic solvents systems may also reduces water based side reactions (Doukyu and ogino 2010). Protease catalyzes peptide synthesis reaction in the presence of DMSO. Activity of extracellular protease of *Halobiforma* sp. BNMIITR remained unaffected by polar solvents at 10 % v/v concentration, but activity was reduced in the presence of 30 % and 50 % methanol, ethanol and isopropanol. Enzyme retain more than 65 % of initial activity in the presence of 50 % DMF and DMSO at 45 ^{o}C after 24 hours and more than 50 % of initial activity in the presence of 50 % DMF and DMSO at 30 ^{o}C after 48 hours of incubation which shows its further application in chemical synthesis. Enzyme retains more than 50 % activity in the presence of nonpolar solvent and more than 30 % activity in polar solvents at 30 ^{o}C after 48 hours of incubation whereas it retained more than 45 % and 55 % activities in the presence of polar and non polar solvents at 45 ^{o}C after 24 hours. Activity and stability of the enzyme was increased in the presence of DMF and DMSO which showed its potential use in chemical synthesis.

The protease was found to be stable and compatible with commercially available detergents Surf excel, Ariel, Tide, Rin and Wheel, and retained more than 95 % activity after 4 hr incubation with 1.0 %, w/v detergents. Thus Extracellular protease of *Halobiforma* sp. BNMIITR can be used in detergent formulation for biological and non biological stain removal.

Undesirable attachment of microorganisms, plants, and animals on ship surfaces is referred as fouling. In the past tributyltin self-polishing copolymer (TBT SPC) paints were used to reduce fouling. TBT-SPC paints were completely banned from January 2008 to protect the environment. One of the most promising approaches is the use of enzymes which can interact directly with microorganisms on the surface. For successful use in paints enzyme must possess solvent stability and should function in saline conditions. Most of the solvent stable protease show less activity in artificial sea water due to inhibition by NaCl, Mg^{+2} and Ca^{+2}. Most halophilic proteins are not suitable for such environment because they require high sat concentration for activity.

The protease in the present study is considerably stable in the presence of nonpolar solvents like xylene (important constituent of paints). Besides this protease is also suitable to work in saline conditions as it require less NaCl for activity and activity increased in the presence of Mg^{+2} with no effect in the presence of Ca^{+2}. Microbial bio fouling firstly involves the formation of a conditional layer than unicellular microorganisms of marine ecosystem attached to it. Enzyme in the present syudy had broad range of substrate specificity. So it can degrade wide range of proteins and lipo proteins. Proteins are involved in the formation of conditional layer. The protease also remains stable in the presence of surfactants. Thus it can degrade wide range of proteins in sea water and can be further used in marine paint industry.

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PUBLICATIONS

1) Shilpi Agarwal, Meenu Gupta, Bijan Choudhury. Solvent free biocatalytic synthesis of isoniazid from isonicotinamide using whole cell of Bacillus smithii strain IITR6b2. Journal of Molecular Catalysis B: Enzymatic (2013) 97:67–73.

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3) Vikash Babu, Shilpi, Meenu Gupta, Bijan Choudhury. Opportunities and challenges for enzymatic surface modification of synthetic polymers. Green chemistry for environmental sustainability.(2010) Taylor and francis group CRC press.LLC, Florida, Boca Raton, USA, pp 93 -104.

*This work is not included in this thesis.