Materials & Methods
SAMPLE COLLECTION

Samples were collected at the salt crystallizer pans of Tuticorin coastal region, Tamilnadu, India in sterile polyethylene bags and transferred to the lab.

PHYSICO-CHEMICAL ANALYSIS OF THE SAMPLES

The collected samples were analyzed for pH, EC, salinity and concentrations of ions such Na⁺, K⁺, Ca²⁺, Cl⁻, HCO₃, SO₄²⁻ and Mg²⁺ at The Central Electro-Chemical Research Institute (CECRI), Karaikudi, Tamilnadu, India.

HALOPHILE ISOLATION

Modified nutrient agar supplemented with 15 % NaCl and 0.1 % Mg was used for the isolation of halophiles in situ. The crude samples collected from the brine water at different locations in the same area were cultured (100 µL) using spread plate technique on a modified nutrient agar medium. After incubation for a minimum of one week (to until appearance of visible colonies) at 37°C, the isolated halophiles were selected for purification by repeated subculturing.

Composition of modified nutrient broth

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>5g</td>
</tr>
<tr>
<td>Yeast</td>
<td>3g</td>
</tr>
<tr>
<td>NaCl</td>
<td>150g</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>1g</td>
</tr>
<tr>
<td>Distilled H₂O</td>
<td>1000mL</td>
</tr>
<tr>
<td>pH</td>
<td>7.2</td>
</tr>
</tbody>
</table>

In the case of solid medium, agar at a concentration of 2 % was used.
Halobacterium specific agar

The ready to use medium supplied by HiMedia was used

The medium contained (g/L):

\[
\begin{align*}
\text{NaCl} & \quad 250 \\
\text{KCl} & \quad 2 \\
\text{MgSO}_4 & \quad 20 \\
\text{Tri-Na-Citrate} & \quad 3 \\
\text{FeSO}_4\cdot 7\text{H}_2\text{O} & \quad 0.02 \\
\text{Casamino acids} & \quad 5 \\
\text{MnCl}_2\cdot 4\text{H}_2\text{O} & \quad 0.026 \\
\text{Yeast extract} & \quad 5 \\
\text{Agar} & \quad 20
\end{align*}
\]

The pH was adjusted to 7.0 before autoclaving.

GROWTH CONDITION OPTIMIZATION:

**Determination of salt (NaCl) requirement:** Each of the isolated strains was grown in 100 mL of modified nutrient broth in a 500 mL Erlenmeyer flask containing NaCl concentrations ranging from 0-25 % to determine the salt requirement of the isolates. Cultures were incubated on a shaker water bath (130 rpm) at 35°C for 10 days. Growth was monitored in terms of optical density (A600) measured at every 12 hours with UV-VIS scanning spectrophotometer, UV2101 pc, Shimadzu.

The optimum parameter from each experiment was utilized in the subsequent assay until all the parameters were optimized

**pH and Temperature optimization:** Optimal pH was tested in optimum NaCl containing modified nutrient broth adjusted to pH values of 4.5, 5.0, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0 and 10.0. Optimum temperature for growth of each isolates
was identified by growing each isolate in modified NB with optimum NaCl and incubated at different temperatures (4, 20, 25, 30, 35, 40, 45 and 50°C).

**Pigment extraction and analysis:** The strain MVBDU-1 was selected for pigment study on the basis of high growth and degree of cell pigmentation.

The strain MVBDU-1 was grown in 50 mL of modified nutrient broth containing 15% NaCl, in a 250 mL Erlenmeyer flask and incubated on a rotary shaker at 130 rpm for 4 days at 37°C. When the culture reached the early stationary phase, according to growth curve, cells were harvested by centrifugation (12,000 rpm for 15 min), washed twice with 2.5M NaCl solution at the centrifuge and finally resuspended in 2.5M NaCl solution. The pigment was extracted by acetone-methanol in equal ratio (1:1) and analyzed by scanning the absorbance in the wavelength region of 400-600 nm using UV-VIS scanning spectrophotometer (UV 2101 pc, Shimadzu).

**PHENOTYPIC AND BIOCHEMICAL CHARACTERIZATION OF THE ISOLATES**

**Gram staining** was performed by using the variation described by Dussault (1955), in which air-dried slides are fixed and desalted in 2% acetic acid for 5 min and then dried and stained using standard procedure.

**Motility** assessment was done by microscopic observation of wet mounts.

For all the **biochemical tests** standard biochemical test methods were used with addition of optimal NaCl concentration.

For **Indole** production test, pure isolates were grown in sterile peptone broth (with optimum NaCl) for 24 to 72 h followed by addition of five drops of Kovac’s reagent.
**Materials and Methods**

**Nitrate reduction** was tested in modified nutrient broth supplemented with 0.5 % NaNO₃. Formation of gaseous products from nitrate was detected by the presence of gas bubbles in Durham tubes.

The presence of **catalase** was determined by formation of bubbles on adding 1 % (v/v) H₂O₂ solution to colonies on solid medium.

**Oxidase** was detected by the appearance of pink or violet colour within 30 sec after streaking a colony on oxidase disk (HiMedia).

**Acid production** from glucose, sucrose, lactose, fructose and maltose, was tested in unbuffered growth medium supplemented with 1 % of the above mentioned carbohydrates. The yeast extract concentration in this case was reduced to 0.1 %, and 0.001 % (w/v) phenol red was added as pH indicator (Arahhal et al., 1996).

**Gelatine hydrolysis** was tested in the solid medium containing 1 % gelatine and a zone of clearance around the colonies was observed after flooding the colonies with 1 % amidoblack which stains the un-hydrolyzed gelatin.

**Casein hydrolysis** was determined by observing the formation of clear zones around colonies on a modified nutrient agar medium with 0.15 % (w/v) skim milk powder.

All of the above tests were carried in triplicates.

**FATTY ACID METHYL ESTER (FAME) EXTRACTION AND ANALYSIS BY GC-MS**

All the glass-wares used were detergent free. And the extraction with solvents was carried out in a hood.
Each of the isolated strains was grown independently in 750 mL of modified nutrient broth in a 2 L Erlenmeyer flask, incubated on a rotary shaker at 130 rpm under optimal conditions. The membrane fraction was extracted in chloroform/methanol( 2:1, v/v) as described by Folch et al. (1957) as follows: From 750 mL of cultures (just before reaching stationary phase), cells were collected by centrifugation (12,000 rpm) at 4°C and suspended in 2 mL of 4 M NaCl (2 mL of Water in the case of halophilic eubacteria) and extracted with 7.5 mL of methanol (CH$_3$OH)–chloroform(CHCl$_3$) (2:1, v/v) for 90 min). The extracts were collected by centrifugation and the supernatant was stored at 4°C. The pellets were re-extracted with 9.5 mL of methanol – chloroform – water (2:1:0.8). Then, 5 mL of chloroform and 5 mL of water were added, and the chloroform phase was collected by centrifugation and dried in a stream of nitrogen and stored at -20°C to which 500 µL of chloroform- methanol (2:1) was added, taken to fresh tubes in duplicates and volatilized under nitrogen stream. Then the extract was digested with 500 µL of perchloric acid (HClO$_4$) in sand bath at 175-180°C for 5 hours evaporated under nitrogen stream and stored at -20°C

The total lipid extracts were concentrated under nitrogen. Fatty acid methyl esters were prepared by refluxing with BF3 (20 %, w/v, in methanol; Merck), as described by Morrison & Smith (1964) as follows: To the total lipid extract, 2 mL mixture of (35 % BF$_3$-CH$_3$OH + 30 % benzene + 35 % CH$_3$OH) was added and incubated at 100°C for 90 min. At the end of the incubation period, the mixture was brought to room temperature and 2 mL of water was added and extracted with 4 mL of petroleum ether and was repeated for second time. Petroleum ether phase was transferred to a new tube and washed twice with 2 mL water. Treated with sodium sulphate (Na$_2$SO$_4$) and washed once again with small amount of petroleum ether and Fatty acid methyl esters were analysed by gas-liquid chromatography (Varian 3700) on a 15 % DEGS column (2 m) supported on Chromosorb W, AW, 80/100 at 175 °C. Injector and detector
temperatures were 240 and 270 °C respectively. The flow rate of the carrier gas (N₂) was 30 mL min⁻¹. Fatty acids were identified using authentic standards (fatty acid methyl esters obtained from Poly Science Corporation). Percentage fatty acid composition was calculated by multiplying the retention time by the peak height on the trace.

MOLECULAR CHARACTERIZATION

Isolation of genomic DNA from bacteria: Genomic DNA of eubacterial isolates was extracted according to the method described by Ausubel et al. (1994), which utilized cetyltrimethylammonium bromide (CTAB) after an initial degradation of the cell wall with proteinase K.

The procedure requires the following:

- TE (Tris-EDTA) buffer (pH 8.0):
  - 10 mM Tris-Cl (pH 8.0)
  - 1 mM EDTA (pH 8.0)
- SDS (10 % (w/v))
- Proteinase K (20 mg/mL) (stored in small single use aliquots at -20°C)
- NaCl (5 M)
- CTAB/NaCl (10 % CTAB /0.7 M NaCl):
  - 4.1 g NaCl dissolved in 80mL distilled water
  - 10 g CTAB slowly added while stirring and slow heating.
  - Final volume adjusted to 100 mL
- Chloroform/Isoamyl alcohol (24:1)
- Phenol/Chloroform/Isoamyl alcohol (25:24:1)
- Isopropanol
- Ethanol (70 %)
Lambda DNA (marker) preparation:

- 100 µL Λ-DNA (Hind III digest)
- 400 µL TE-10
- 3 minutes at 60ºC (Thermo-block)
- 100 µL gel loading buffer

The detailed procedure is as follows:

5 mL of bacterial culture was grown to saturation and microcentrifuged. The pellet was suspended in 576 uL of TE buffer by repeated pipeting. Then 30 uL of 10 % SDS and 3 uL of 20 mg/mL proteinaseK were added, mixed and incubated for 1 hour at 37ºC, to which 100 uL of 5 M NaCl was added and mixed thoroughly followed by 80 µL of CTAB/NaCl solution. Mixed and incubated for 10 minutes at 65ºC. Then equal volume of chloroform/isoamyl alcohol was added, mixed and centrifuged at 12,000 rpm for 10 min. To the supernatant, equal volume of phenol/chloroform/isoamyl alcohol mix was added, centrifuged for 5 min at 12,000 rpm and the supernatant was transferred to a fresh tube. 450 µL of isopropanol was added and mixed gently until DNA precipitated, and centrifuged at 6000 rpm for 10 min followed by ethanol wash with 1 mL of 70 % ethanol. Microcentrifuged and ethanol was discarded carefully, dried in room temperature and resuspended in 100 µL sterile distilled water and stored at -20ºC.

**Isolation of genomic DNA from archaea:** Genomic DNA of the strains MVBDU-1, 2 and 7 was extracted directly. A single isolated colony was suspended in 10 µL of distilled water and heated in a boiling water bath for 2-3 min. The content was centrifuged at 10,000 rpm for 3 min and the released DNA was isolated and purified with phenol/chloroform/isoamyl alcohol (25:24:1, v/v) and chloroform/isoamyl alcohol (24:1, v/v).
The purity of genomic DNA was examined by gel electrophoresis on 0.8 % agarose gel prepared in 1X TAE (Tris Actetate EDTA) buffer containing 0.5 μg/mL ethidium bromide. Lambda DNA Hind III digest ladder was used as molecular weight marker for comparison of size of PCR products. The gel was visualized in UV transilluminator followed by gel documentation.

The isolated total DNA of each isolate was used as DNA template for PCR system.

**PCR amplification of 16SrDNA:** Genomic DNA of isolates was used as template for amplification of the 16SrDNA as follows:

**Primers used:**
Universal bacterial primers 8F (5’-AGA GTT TGA TCC TGG CTC AG-3’)
1492R (5’-GGT TAC CTT GTT ACG ACT T-3’)
Halobacterium specific primer 48F (5’-ACU ACG ATT TAG CCA TGC TAG T-3’)
338R (5’- CAU CAG TGT AAA GGT TTC GCG)

**PCR mixture:**
- Genomic DNA: 1-2 μL reaction
- Buffer enzyme: 5 μL (10X)
- MgCl2: 1.5 μL
- DNA polymerase: 0.5 μL
- Primer 1: 2 μL
- Primer 2: 2 μL
- DNTPs: 5 μL
- Water to make up to: 50 μL

**PCR cycle followed:**
The PCR was performed for 35 cycles
Lid temperature: 105°C
Materials and Methods

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
<th>Time</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre amplification denaturation</td>
<td>5 minutes</td>
<td>95°C</td>
<td></td>
</tr>
<tr>
<td>Thermal cycle 1. Denaturation</td>
<td>1 minute</td>
<td>94°C</td>
<td></td>
</tr>
<tr>
<td>2. Annealing</td>
<td>2 minutes</td>
<td>60°C</td>
<td></td>
</tr>
<tr>
<td>3. Extension</td>
<td>3 minutes</td>
<td>72°C</td>
<td></td>
</tr>
<tr>
<td>Final Extension</td>
<td>10 minutes</td>
<td>72°C</td>
<td></td>
</tr>
</tbody>
</table>

**Agarose gel electrophoresis:** The purity and size of the PCR product was examined by gel electrophoresis on 0.8 % agarose gel prepared in 1X TAE (Tris Acetate EDTA) buffer containing 0.5 μg/mL ethidium bromide with Lambda DNA Hind III digest ladder as molecular weight marker for comparison of size of PCR products. The gel was visualized over a UV transilluminator.

**PCR clean up gel extraction:** The PCR amplicon was agarose gel electrophoresed and then purified by gel extraction using Nucleospin Extraction kit. After agarose gel electrophoresis, the amplicon band of size 1.5 Kbp was excised using a clean scalpel and the gel was solubilized with buffer (200 µL/100 mg of gel slice) by incubating at 50°C for 5-10 minutes with brief vortex until the gel dissolved completely. Following solubilization of the gel slice, the DNA was then bound to the silica membrane of Nucleospin extract column. The silica membrane was then washed with the buffer provided and then dried by centrifugation at 11,000 rpm for 2 min. The DNA was then eluted from the membrane to a clean 1.5 mL eppendorf by using the elution buffer provided and incubating at room temperature for 1 min to increase the yield followed by centrifugation at 11,000 rpm for 1 min.

**Ligation:** The purified amplicons of 16S rDNA were ligated to pCR-Blunt vector as follows:

- 10µL ligation reaction mixture
- 1µL pCR-Blunt (25ng)
- 1-5 µL PCR product
- 1 µL 10X ligation buffer with ATP
Make up to 9 µL with sterile distilled water
1 µL T4 DNA Ligase (4U/ µL)

**Transformation:** Following ligation cells of E.coli DH5α were transformed according to Sambrook *et al.* (1989)

**Luria broth** 10 g tryptone, 5 g yeast extract, 10 g NaCl in 1 L water
pH 7.2 and autoclaved.

**Luria agar** Luria broth containing 1.5 % agar and autoclaved.

*E.coli* DH5α from glycerol stock was streaked out on LB plate. An isolated colony was picked and grown as 3 mL culture in LB (without antibiotic) overnight at 37°C with shaking. Next day, overnight culture was inoculated to fresh LB in 3 mL tubes and incubated for 1:20 hours at 37°C with shaking to get the absorbance of 0.2-0.3 at OD600. Spun down 1.5 mL of the culture in sterile eppendorf at 12,000 rpm for 10 min and the pellet was resuspended in 1 mL ice-cold 50 mM CaCl2. Incubated on ice for 30 min, centrifuged at 12,000 rpm for 5 min and resuspended the pellet in 200 µL of 50 mM CaCl2. To that, 5 µL of ligation reaction mixture was added and kept on ice for 30 min followed by 45 seconds of incubation at 44°C and once again transferred to ice for 1 minute. Then, 1200 µL of fresh LB was added, mixed gently and incubated at 37°C for 1 hour. 100 µL was inoculated by spread plate technique on Luria Agar containing Kannamycin, Xgal and IPTG and the plates were incubated at 37°C overnight.

**Plasmid mini-preperation:** After transformation, the positive colonies were selected for plasmid isolation and confirmation of the clone. The plasmid isolation procedure followed is as follows:

<table>
<thead>
<tr>
<th>Solution I</th>
<th>50 mM glucose; 25 mM Tris-HCl (pH 8.0); 10 mM EDTA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solution II</td>
<td>200 mM NaOH and 1% SDS</td>
</tr>
</tbody>
</table>
Plasmid DNA was isolated using alkaline lysis method (Brinboim and Dolly, 1979). 1.5 mL of overnight culture, grown at 37°C in LB containing the antibiotic Kanamycin, was pelleted at 12,000 rpm. Resuspended the pellet in 100 µL of solution I and incubated at room temperature for 5 min to which, 200 µL of solution II was added, mixed gently and incubated on ice for 5 min. 150 µL of solution III was added and mixed gently. After 5 min of incubation on ice, centrifuged for 10 min at 12,000 rpm and the supernatant was carefully collected in a fresh tube. The plasmid DNA was precipitated by 300 µL of isopropyl alcohol, followed by incubation at room temperature for 10 min and centrifugation at 12,000 rpm for 10 min. The pellet was resuspended in 300 µL TE buffer and 0.5 µL RNase and incubated at room temperature for 30 min to which, 30 µL of 3 M sodium acetate and 300 µL of ice cold ethanol was added and incubated at -80°C for 45 min followed by centrifugation at 12000 rpm for 15 min at 4°C. The pellet was then washed with 70 % ethanol, vacuum dried and suspended in 55 µL of sterile distilled water.

**Restriction digestion analysis:**

**Reaction mixture**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>EcoR1</td>
<td>1 µL</td>
</tr>
<tr>
<td>10X reaction buffer</td>
<td>2 µL (1X)</td>
</tr>
<tr>
<td>Substrate DNA</td>
<td>≤ 1 µg</td>
</tr>
<tr>
<td>Sterile distilled water</td>
<td>to make up to 20 µL</td>
</tr>
</tbody>
</table>

Reaction temperature: 37°C for 1 to 1.30 hours.

Agarose gel was electrophoresed to confirm the transformation.
16S rDNA sequence analysis: The cloned 16S rDNA of the isolates were sequenced using an automated DNA sequencer at Macrogen.

BLAST and ClustalW2 analysis: The sequences were analyzed by NCBI BLAST search and were submitted to NCBI GeneBank database. The sequences producing significant alignments were multiple sequences aligned by ClustalW2 and the phylogram was constructed.

OPTIMIZATION OF EXTRACELLULAR PROTEASE PRODUCTION BY Halobacterium SP. MVBDU1 (THE EFFECT OF GROWTH CONDITIONS)

Enzyme production: The medium used for protease production was the modified nutrient agar mentioned above supplied with 1% casein. 250 mL of above medium was taken in a 1000 mL Erlenmeyer flask; autoclaved at 121ºC for 15 min (MgSO₄ and Casein are added after sterilization). Flasks were inoculated with 1mL of a week old culture of MVBDU-1 and incubated at 37ºC in an incubator shaker at 95 rpm. The samples were withdrawn at regular interval and the growth as well as enzyme activity were determined. The growth was monitored by absorbance at 600 nm in UV/Vis Spectrophotometer (Schimadzu). The enzyme production was investigated at various pH, temperature, NaCl concentrations, metal ions, carbon and nitrogen sources, and immobilization.

Protein determination: Total protein was determined by Bradford (1976) assay with BSA (Bovine serum albumin) as the standard. 0.2 mL Bradford reagent was mixed with 0.8 mL of sample. The solution was incubated for 5-30 min at room temperature and the absorbance was measured at 590 nm against a blank containing 0.2 mL Bradford reagent and 0.8 mL buffer.
**Bradford Reagent:** 100 mg of Coomassie Brilliant Blue G-250 was dissolved in 50 mL of 95 % ethanol to which 100 mL of 80 % w/v phosphoric acid was added and diluted with water to 1 L and filtered.

**Enzyme extraction:** The crude supernatant, collected by centrifugation (12,000 rpm) at room temperature, from the exponentially grown culture broth of the strain, MVBDU-1 was used as the crude enzyme.

**Protease Assay:** Determination of protease activity was carried out according to Anson (1938) and Folin and Ciocalteu (1929). The culture was centrifuged at 12,000 rpm for 20 min and the culture supernatant was used as a source of protease. The assay was performed at 37°C using 1 % casein as a substrate. The substrate was prepared in 50 mM Tris-HCl buffer (pH 7.2) containing 2 M NaCl. The concentration of NaCl was set at 2 M in the assay system, as casein is known to lose its original conformation at higher NaCl concentrations (Capiralla et al., 2002). 1 mL of casein buffer solution was preincubated at 37°C for 5 min. The reaction was initiated by adding 1 mL of the enzyme. After incubation for 10 min at 37°C, the reaction was terminated by adding 3 mL of 5% (w/v) trichloroacetic acid (TCA). For blank tubes TCA was added prior to the enzyme. To 0.5 mL supernatant, 2.5 mL of Folin-ciocalteau reagent was added, the reaction mixture was incubated at room temperature for 30 min and then the absorbance was measured at 660 nm with UV-VIS Spectrophotometer.

One unit of enzyme activity was defined as 1 μg of tyrosine released per minute.

**Effect of pH, temperature and NaCl concentrations on protease production:** Determination of optimum pH and NaCl concentrations on the growth and enzyme production was studied by cultivating the organism at different pH
levels (4.0 – 12.0) of the broth, different temperatures (10 – 60°C), and different concentrations of NaCl (10-30 %).

**Effect of Carbon and Nitrogen sources on enzyme production:** The effect of addition of carbon sources (glucose and starch) and nitrogen sources (organic sources like gelatin, casein, peptone, beef extract, yeast extract, and inorganic sources like urea, ammonium nitrate and ammonium chloride) at 1 % concentration on growth and protease production was analysed independently in the growth medium containing only NaCl and Mg$^{2+}$.

**Effect of metal ions on protease production:** Different metal ions (MnSO$_4$, ZnSO$_4$, CaCl$_2$, FeSO$_4$ and MgSO$_4$) at 1.0 mM level were added to the growth medium independently to study their effect on the growth and enzyme production.

**Immobilization:** The mid-exponential phase (2 days old) cell suspension (5 g wet weight) was aseptically added to the sterile sodium alginate solution (100 mL of 2 % W/V) to achieve the required cell/alginate ratio. The mixture obtained was then extruded drop-wise into 50 mM CaCl$_2$ solution and hardened in this solution. The beads were washed twice with 50 mM Tris-HCl buffer (pH 7.5) containing 15 % NaCl and used as inoculum for protease production.

Alginate- and agar-entrapped cells and the enzyme, were assayed independently for protease activity at different temperature ranging from 4º to 90ºC.

The culture free supernatant (12,000 rpm) after 4 days of growth was immobilized as in the case of cell immobilization. The beads were washed twice with 50 mM Tris-HCl buffer (pH 7.5) containing 15 % NaCl and the enzyme activity was assayed at different temperatures from 4º to 90ºC.
EFFECT OF SUBSTRATE CONCENTRATIONS ON PROTEASE ACTIVITY: (MICHAELIS MENTON PLOT)

One of the most fundamental factors affecting the enzyme activity is substrate concentration. In this experiment reaction mixtures were designed varying the substrate concentration (50 to 1000 µg) and the Michaelis Menten’s constant (Km) and the maximum attainable velocity (Vmax) were determined employing Michaelis-Menten’s non-linear regression plot.

Two different substrates casein and BSA were used.

Characterization of extracellular protease from Halobacterium sp. MVBDU-1 (The effect of assay conditions)

Following the optimization of protease production by the strain MVBDU-1, the protease in the culture free supernatant was further characterized with respect to the assay parameters (pH, Temperature, NaCl concentration, Divalent cations, Inhibitors, Detergents and reducing agents).

In the case of pH, different buffers 50 mM sodium acetate (pH 4.0 and 5.0), 50 mM potassium dihydrogen phosphate (pH 6.0), 50 mM Tris-HCl (pH 7.0 to 9.0), 50 mM glycine-NaOH (pH 10.0) and sodium hydroxide (pH 11.0 and 12.0) were used for assay.

To determine the effect of temperature, the assay mixture was incubated under different temperatures (0-100°C). For optimal NaCl concentration in the reaction mixture, the assay mixture with different NaCl concentrations (5-50%) was employed. Divalent cations (ZnCl₂, MgCl₂, MnCl₂ and CaCl₂), at 1 and 2 mM concentrations, were added to the assay mixture independently under optimum conditions of NaCl, pH, and temperature.

The effects of protease inhibitors such as HgCl₂ (cysteine-inhibitor), EDTA (ion chelator/metalloprotease inhibitor), PMSF and DTP (serine-protease
inhibitors) and n-octyl alcohol (aspartate-protease inhibitor) were determined independently by adding them to the assay mixture at a concentration of 1.0 and 2.0 mM.

The compatibility of the MVBDU-1 protease with detergent (SDS, 1%) and reducing agents like urea (1.0 mM), DTT (1.0 mM), and β-mercaptoethanol (1 %) was determined under optimum assay conditions independently.

All the assays were carried out in triplicate and against a control assay without enzyme.

**PURIFICATION AND MOLECULAR CHARACTERIZATION OF EXTRACELLULAR PROTEASE FROM Halobacterium SP. MVBDU1**

**Enzyme Purification:** Enzyme was purified by three step process by following May and Dennis, 1987 with modifications.

The culture supernatant was obtained by centrifugation of 4 days old culture broth (2 L) at 12,000 rpm for 10 min. Ammonium sulphate was added into the culture supernatant and the precipitate obtained at 60 % saturation was collected. The precipitate was dissolved in 50 mM Tris-HCl buffer (pH 7.5) and
was dialyzed against the 15 % NaCl containing 50 mM Tris-HCl buffer (pH7.5) overnight.

The dialyzed protein mixture was then applied to DEAE-sepharose (sigma) column (30mL bed volume) equilibrated with buffer 50 mM Tris-HCl. The unbound proteins were eluted with the same buffer, containing 0 - 1M NaCl, as 3 mL fractions at the rate of 0.5 mL/min. The fractions with metallo-protease activity were pooled, concentrated, dialyzed as before and resuspended in Tris-HCl (pH 7.5) (15 % NaCl).

The active fractions from DEAE-sepharose was then loaded on to gel filtration column of Sephadex G-100 and 3 mL fractions were collected at the rate of 0.5 mL/minute with the same buffer as above.

**SDS – Poly Acrylamide Gel Electrophoresis:** Crude and purified protease was analyzed in SDS-PAGE (on 10 % polyacrylamide gel containing 0.1 % sodium dodecyl sulphate) according to the method of Laemmli (1970) at a constant current of 25 mA in Biorad minigel apparatus.

**Solutions for SDS-PAGE**

1. 30 % acryl-bisacrylamide mixture (29.9 g Acrylamide and 0.8 g NN’-methylene bisacrylamide)
2. Buffer for resolving gel (1.5 M Tris-HCl, pH 8.8 )
3. Buffer for stacking gel (0.5 M Tris-HCl, pH 6.8)
4. 5X SDS-PAGE sample loading buffer (0.0625 M Tris-HCl, pH 6.8; 15 % glycerol; 0.1 % SDS; 1 % β- mercaptoethanol; 0.001 % Bromophenol blue)
5. SDS-PAGE running buffer (14.4 g/L Glycine; 3 g/L Tris; 1 g/L SDS)
6. Staining solution (0.1 % coomassie brilliant blue R-250 in 50 % methanol and 10 % acetic acid)
7. Composition of the separating gel (10 mL) and the stacking gel
### Materials and Methods

<table>
<thead>
<tr>
<th>Solutions (mL)</th>
<th>10 %</th>
<th>Solutions (mL)</th>
<th>6.67 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 % acrylamide</td>
<td>3.33</td>
<td>30 % acrylamide</td>
<td>1.0</td>
</tr>
<tr>
<td>1.5 M Tris, pH 8.8</td>
<td>2.50</td>
<td>0.5 M Tris, pH 6.8</td>
<td>1.0</td>
</tr>
<tr>
<td>Water</td>
<td>4.0</td>
<td>Water</td>
<td>1.94</td>
</tr>
<tr>
<td>10 % SDS</td>
<td>0.1</td>
<td>10 % SDS</td>
<td>0.04</td>
</tr>
<tr>
<td>10 % APS</td>
<td>0.1</td>
<td>10 % APS</td>
<td>0.06</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.005</td>
<td>TEMED</td>
<td>0.005</td>
</tr>
</tbody>
</table>

The gel was washed with deionized water and incubated at room temperature in the staining solution, till the stained bands of protein just started to appear.

**STABILITY CHARACTERIZATION OF Halobacterium SP. MVBDU-1 PROTEASE**

**Determination** of protease stability was carried out by the method followed by Karbalai-Heidari et al. (2007). The thermostability of the MVBDU-1 was measured under standard assay conditions after preincubation of the enzyme in Tris-HCl buffer (pH 7.0) for 30 and 60 min at various temperatures (from 10 to 80°C).

To check the pH stability, the enzyme solution (10 µL) was mixed with 190 µL of each buffer solution and after incubation for 10, 20 and 30 min, protease activity was measured under standard assay conditions.

The effect of NaCl and KCl on MVBDU-1 protease stability was also determined by preincubation of the enzyme in Tris-HCl buffer (pH 7.0) containing different concentrations of NaCl and KCl (5-35 %).
EVALUATION OF POTENTIAL APPLICATIONS

**Dehairing of goat hide:** Dehairing potential of the MVBDU-1 protease was analysed by following the method of Nilegaonkar et al. (2007). The crude enzyme supernatant (12,000 rpm) was applied at 1% concentration to a piece of goat hide (≈5cm) immersed in glass distilled water and kept at 37°C in a dry place. Loosening of hair and epidermis were observed by mechanical shearing at regular time intervals.

**Stain removal:** Washing test was carried out using 1% of the crude enzyme supernatant (12,000 rpm) (Chen and Wang, 2008).
A clean cloth stained with blood was cut into equal piece (≈3cm) and treated with the protease at room temperature for 0, 15, 30 and 45 mins independently, at the end of which the cloth pieces were washed in double glass distilled water.

All the data are mean value of triplicates.