3. Characterization of Strains

3.1 Materials

Chemicals were obtained from Merck India, Hi Media, Mumbai. Glasswares were obtained from Borosil, and specialized chemicals were procured from Sigma.

3.2 Methods

3.2.1 Isolation of strain

*Halomonas sp.* and *Halomonas organivorans* were previously isolated and maintained in our laboratory. Both the strains were isolated from the coastal region of Maharashtra and were used for the present study and further biochemical and microscopic and characterization were carried out.

3.2.2 Sub-culturing and maintenance of isolates

The cultures were preserved and stored in halophilic agar medium, the media constitutes 10% NaCl, 1% Peptone, 1% Yeast Extract, KCl 3.0 g, Trisodium Citrate 1.0 g, MgSO$_4$ 7.0 g and Double distilled water 1000ml pH 7 (Ventosa *et al.*, 1998).

3.2.3 Microscopic Studies

3.2.3.1 Gram’s staining

Heat-fixed smears of test cultures on clear glass slides were prepared and the smears were flooded with crystal violet and allowed to stand for 30 seconds. Rinsed with water for 5 seconds and Gram’s iodine as mordant was added and kept for 1 min. Again rinsed with water for 5 seconds and decolorized with 95% ethanol for 15 to 30 seconds until the crystal violet is destained from the slide. Rinsed with water for 5 seconds and counter stain safranin was added and kept for 30 seconds again.
Rinsed with water, blot and dried with bibulous paper and examined under oil immersion.

3.2.3.2 Motility Test

A small drop of the suspension of test isolate was taken on clear cover glasses and was inverted over a cavity glass slide and was observed under microscope, for the motility.

3.2.3.3 Endospore Staining

The test cultures were aseptically transferred with an inoculating loop on to a slide, air dried and heat-fixed. Malachite green solution was added and allowed it for 5-10 minutes by heating and care was taken that the slide with malachite green solution was not allowed to get dry. The slides were cooled and rinsed with water for 30 sec and counter stain safranin was added and kept for 60 to 90 sec. The slides were rinsed with water and dried with bibulous paper and examined under oil immersion (Bergey’s Manual, 1984).

3.2.4 Biochemical Tests

The isolates were further subjected for different specific biochemical tests for their further characterization.

3.2.4.1 Citrate Utilization Test

A loopful of test isolates were inoculated in Simmon’s citrate agar and incubated at 37°C for 48 h. After incubation, the change in the colour was observed on media from green to blue (Harrigan, 1976) indicating the positive.
3.2.4.2 Oxidase Test

A loopful of 24 h old cultures were inoculated on oxidase discs and observed for the development of blue color within 30 seconds as positive. (Gaby and Hadley, 1957).

3.2.4.3 Gelatin Liquefaction

A loopful of test isolates were inoculated in gelatin broth and incubated for 24 to 48 h at 37°C. Hydrolysis of gelatin was observed as liquid even after keeping the tubes in ice bath, indicating positive test (Bergey’s Manual, 1984).

3.2.4.4 Sugar Fermentation Test

The isolates of halophilic bacteria were further tested for their metabolic activity to characterize and classify them up to species level. A loopful of test isolates were inoculated in nutrient broth containing 3.5% NaCl with different sugars along with bromocresol purple as an indicator at 37°C for 24 h. Based on the production of acid and gas, the characterization of halophilic bacteria was carried out (Harrigan, 1976).

3.2.4.5 Antibiotic Susceptibility Test

Antibiotic resistance response of the microorganisms to different antibiotics was tested on halophilic agar medium. The antibiotics were procured from HiMedia, Mumbai, India. The plates were surface seeded with bacterial suspension and different antibiotic discs with effective concentrations were placed over the plates. Inhibition of growth was depicted by a clear zone formation around the discs indicated sensitive reaction, and the absence indicated the organism was resistant to the antibiotic. Diameter of the inhibition zone was measured with an antibiotic zone scale. Different antibiotics discs used were Amikacin 30mcg, gentamicin 50mcg,
Erythromycin 30mcg, Polymyxin 15 mcg, Rifampicin 25mcg, Cefixime 30mcg, 
Vancomycin 25mcg, Nystatin 30mcg, Methicillin 50mcg, Pepracillin 30mcg, 
Amphotericin 15mcg, Streptomycin 10mcg, Levoflaxacin 15mcg, Oxacillix 20mcg, 
Ciproflaxin 20 mcg, Ampicillin 30mcg, Amoxyclav 50mcg, Cefamandole 30mcg, 
Azithromycin 15mcg, Chlorophenicol 10mcg, Itrconazole 20mcg, Fluconazole 30 
mcg, navobiocin 25 mcg and Neomycin 30mcg.

3.2.5 Heavy metal ion stress

Halophilic media were prepared with 1mM, 2mM, 3mM, 4mM, 5mM of heavy 
metals such as HgCl$_2$, Zinc chloride and Lead Acetate, \textit{Halomonas organivorans} and 
\textit{Halomonas sp.} strain were inoculated and kept for incubation at 37°C, after 12, 18, 24, 
36 hrs of incubation, then the growth was measured by spectrophotometer at 600 nm.

NaCl and common salt tolerance growth of the organisms on nutrient agar medium 
supplemented with different concentration of NaCl. Diluted suspensions of the 
organisms were spotted on the plates, incubated at 30°C for 72 hrs and growth was 
recorded millimolar concentrations.

3.2.6 Genomic studies

3.2.6.1 Isolation of Chromosomal DNA

The isolation of chromosomal DNA was carried out according to the method of 
Sambrook \textit{et al.}, (1989). Cells from overnight cultures were collected by 
centrifugation at 5000 rpm for 5 min were suspended in 400 µl of lysis buffer with 
100 µl of 10 % SDS. The cell suspension was incubated at 60°C for 20 min, in 1.5 ml 
microcentrifuge tube, after incubation 500 µl of saturated phenol was added and 
centrifuged at 10,000 rpm for 10 min at 4°C. The upper layer was recovered and
equal volume of chloroform: isoamylalcohol (24:1) and 100 µl of 3 M sodium acetate (pH-5) was added and centrifuged at 10,000 rpm for 10 min at 4 °C. The upper layer was recovered with fine micropipette and 2 volumes of chilled absolute alcohol was added and kept at -20 °C overnight for the DNA precipitation. DNA was collected by centrifuging at 12,000 rpm for 15 min at 4°C and the DNA pellet was washed with 70 % ethanol and pure ethanol was used to remove the relative salt. DNA pellet was dried at room temperature and re-dissolved in 20 µl of TE-buffer.

3.2.6.2 Purification and Quantification of Chromosomal DNA

To eliminate the RNA contamination from the above DNA preparation, 2 µl of RNase A was added at 50 µg/ml concentration and incubated for 1 hr at 37°C (Sambrook et al., 1989). After RNase treatment, the sample was treated with Proteinase K by the addition of 2 µl of Proteinase K and incubated at 37 °C for 1 hr in microcentrifuge tube to remove protein contamination. After treatment, microcentrifuge tubes were incubated at 60°C for 1 min in water bath. The purity of the DNA samples were determined by measuring the absorbance ratio of light at 260:280 nm in a UV-spectrophotometer (Elico, India). The isolated DNA samples were quantified in solution by reading the absorbance at 260nm.

3.2.6.3 Agarose Gel Electrophoresis

Both the ends of plastic gel casting tray (gel mould) was sealed with an adhesive tape. The agarose mix was prepared in 1X running buffer and boiled in a microwave oven to get a clear homogenous solution and cooled to 55°C and then poured into the gel by placing the combs. Allowed to solidify for at least 30 minutes
and then the comb was removed carefully and peel off the tape from the gel casting tray and the gel was kept in an electrophoresis unit containing 1X running buffer. The sample loading side of the gel should be placed in the cathode side. The gel must be completely submerged in the buffer and the electrodes were connected (black to the negative terminal and red to the positive terminal). Run at 50 mA as a pre-run for about 15 minutes. Add 1/10th volume of tracking dye (10 X stock) to the DNA sample on the surface of a parafilm. Mix thoroughly by pipetting several times and load in the wells and electrophoresis was carried out at 50 volt until the dye reaches the 3/4th of the gel (Sambrook et al., 1989).

The gel was removed from the apparatus and placed in 250 ml of 1X running buffer containing 50 µl of 10 mg/ml ethidium bromide. After half an hour the gel was removed from the solution was replaced with buffer without ethidium bromide. The gel was viewed in an Ultra Violet Transilluminator (Sambrook et al., 1989).

3.2.6.4 PCR Amplification of 16S rRNA Gene Sequence:

The 16S rRNA gene sequence of bacterial strain was amplified by PCR with the following forward and reverse primers:

5'-AGAGTTTGATCATGGCTCAG-3' (position-827) and 5'-CTACGGTTACCTTGTTACGAC-3' (position 1492-1510) respectively.

The PCR conditions were as follow: 50 µl of reaction system, reaction cycles 35 cycles, 94°C pre-denaturation 5 min, 94°C denaturation 1 min, 55°C annealing 30 sec, 72°C extension 1 min, 72°C final extension 8 min, 4°C hold for 4 hrs. The PCR product was purified by DNA gel extraction kit and was sequenced in National Centre for Cell Science (NCCS), Pune.
3.2.7 Protein profiling of *Halomonas organivorans* and *Halomonas sp.*

3.2.7.1 Protein Sample preparation by chemical lysis method:

Cells (~0.1 gm fresh weight) of each treatment with the control were suspended in 1.0 ml lysing buffer. Heated at 100°C for 5 min., centrifuged at 10,000 rpm for 30 min and 50 µl of each extracted protein treatment was used for protein analysis.

3.2.7.2 Purification by Sephadex G-75:

Purification of protein sample was carried out by using sephadex-G-75 (Amersham Pharmacia) gel filtration chromatography column.

**Preparation and packing**

The column used for gel filtration required washing, swelling and conversion of the matrix to the desired “form” of packing material prior to use was prepared. For packing the column, the slurry was prepared by mixing 3gm of Sephadex G-75 in 50ml of distilled water and kept for swelling over night at 4°C and degassed with suction pump. The column was packed evenly with 500ml of 50mM sodium urea buffer and allowed to settle for overnight with constant running of the buffer for equilibrium of the matrix.

**Loading and elution**

Acetone precipitated sample of 2ml were applied on to the top of the Sephadex G-75 column. The sample was eluted using urea buffer at a flow rate of 2 ml/min with the help of the peristaltic pump (Miclins Peristaltic Pump PP10). The fractions of 2ml each were automatically collected up to 30 fractions with the help of the fraction collector (Pharmacia LKB.FRAC-100). Each fraction was subjected for absorbance at OD 280 nm.
3.2.7.3 Protein analysis by Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The protein patterns were analysed using SDS-PAGE according to Laemmli, (1970) in the first dimension.

**SDS-PAGE solutions**

**Stock (1):** Acrylamide (30 grams) and 0.8 gram bis N, N, Methylene bis-acrylamide were dissolved in 100 ml distilled water (each component dissolved separately). The solution was then filtered through glass filter under vacuum and kept at 4 °C in a dark bottle.

**Stock (2):** Tris-HCl (18.2 grams) was dissolved in 50ml distilled water. The pH was adjusted with HCl to 8.8 and the volume was made up to 100 ml with distilled water and the solution was stored in the refrigerator.

**Stock (3):** Tris-HCl (6 grams) dissolved in 50ml distilled water. The pH was then adjusted with HCl to 6.8 after that the volume was completed to 100 ml distilled water and the solution were stored in refrigerator.

**Preparation of SDS:** (SDS 10% w/v): Stock solution was prepared by dissolving 1.0 grams SDS in 10ml distilled water.

**Preparation of APS:** (APS 10 % w/v): (freshly prepared)

A solution of ammonium persulfate was prepared by dissolving 1.0 gram in 10ml distilled water, just before use in gel preparation.

**Procedure**

The gel components were mixed and the acrylamide solutions were quickly poured between the plates. The meniscus of the acrylamide solution should be far enough below the top of the notched plate to allow for the length of the comb 1 cm.
After the gel has been set about 20-30 min, the overlay water poured off and the top of the separating gel was washed several times with distilled water. Excess water was drained with the edge of a paper towel. The stacking gel (5.0 acrylamide) solution was prepared and poured directly into the polymerized separating gel. The appropriate comb was placed into the gel solution without making any bubbles. The comb was cleaned by washing with distilled water and the gel was placed in a vertical position at room temperature. The stacking gel would set in approximately within 15 min.

**Electrode buffer solution (2X):**

Tris-HCl (6 grams), Glycine (28.8 grams) and 2 grams SDS were dissolved in 1000 ml distilled water and adjusted to pH 8.8.

**Loading buffer (2x) (double concentration):** 100 ml 0.125 M (1.512 g/100) Tris-HCl (pH 6.8) – 4 % SDS (W/V) – 20 % glycerol V/V) -10 % 2- mercaptoethanol (v/v) – 0.002 % (w/v) bromophenol blue.

**Protein Analysis:**

**Composition of lysing buffer:**

100 ml 0.625 M (0.706 g/100 ml) Tris-HCl (pH 6.8) – 2 % SDS (w/v) – 10 %

glycerol (v/v) - 5% 2- mercaptoethanol (v/v)

**Sample loading and application:**

After the stacking gel was set, comb was carefully removed. Wells were washed immediately with distilled water to remove unpolymerized acrylamide. Straighten the teeth of the cells. 100µl of each sample was loaded directly into the bottom of the wells, using a microliter syringe washed by electrode buffer after each sample.
Electrophoresis After sample loading, the molded gel was completed with electrode.

Electrophoresis was carried out at (140V) for each well for 120 min.

**Protein staining:**

**Staining solution:**

0.5 gm Commassie R-250 brilliant blue was dissolved in 250 ml methanol, 50 ml Glacial acetic acid, completed by distilled water to 500ml mixed and kept at room temperature.

**Procedure**

Gels were stained overnight in 200 ml of Compassion brilliant blue R-250 solution.

**Destaining solution:**

Destaining of protein was performed in 200 ml of destaining solution which composed of 250 ml methanol, 50 ml glacial acetic acid and 200 ml distilled water with gentle shaking. The destaining solution was changed several times until background colour was removed.

### 3.2.8 Screening of Strains for Extracellular Hydrolytic Enzymes

*Halomonas sp.* and *Halomonas organivorans* were screened for salt requirement as extreme and moderate halophilic bacteria. These were further subjected for the production of different extracellular hydrolases such as protease, amylase, lipases, *β*-galactosidases and *β*-galactosidases on specific media.

#### 3.2.8.1 Extracellular protease production

Proteolytic activities of the isolates were screened using skim milk agar containing 10% (w/v) skim milk, 2% (w/v) agar, supplemented with 10% and 20%
(w/v) NaCl for determining the hydrolytic activity of moderate and extreme halophiles, respectively. Clear zone of hydrolysis was observed after 7 days of incubation indicating positive for the proteolytic activity (Rohban et al., 2008).

3.2.8.2 Extracellular amylase production

The presence of amylolytic activity on plates was determined qualitatively following the method described by Amoozegar et al., 2003, using starch agar medium containing 10% or 20% (w/v) NaCl. After incubation at 30°C for 7 days, the plates were flooded with 0.3% I₂ or 0.6% KI solution.

3.2.8.3 Extracellular lipolytic activity

Lipolytic activity of the isolates was detected by screening of the zone of hydrolysis around the colonies growing in plates containing 1% Tween-80, after 48 h of incubation. Spectrophotometric assay was carried out to determine the lipolytic activity in the culture supernatant with p-nitrophenol esters as substrates. Cleavage of p-nitrophenol esters was measured at 30°C using 0.1 M phosphate- buffer (pH 7.5) according to the Winkler and Struckmann, 1997. One unit was defined as the amount of enzyme which caused the release of 1 μmol of p-nitro phenol per minute under test conditions.

3.2.8.4 Extracellular gelatinase activity

Gelatin (150 g/l) was supplemented to the saline medium of (10% and 20 %) and 2 ml of media was transferred to test tubes inoculated with the test strains and incubated at 30°C for 2-3 days. After the incubation, the cultures were maintained at 4°C for 10mins and the liquefaction of gelatin indicates the production of gelatinase.
3.2.8.5 Extracellular β-galactosidase activity

250ml Erlenmeyer flask containing 100 ml of the medium containing (g/l): lactose, 10; meat extract, 15; biopeptone, 5; yeast extract, 0.5; and sodium chloride, 1.5 (Chakraborti et al., 2000) was inoculated with 1ml of 18 hours old culture was incubated at 30°C for 3days at 120 rpm. Crude enzyme was obtained from the supernatant after centrifugation at 5000 g for 20 min (Mukesh et al., 2012, Nurullah, 2011). Enzyme assay was determined by using 200 µl of crude enzyme with 0.1 M phosphate buffer and 500 µl of 6 mM ONPG (O-Nitrophenyl-β-D-Galactopyrinoside) and the reaction mixture was incubated for 30 minutes at 30°C. The reaction was then stopped by adding 0.5 ml of 1 M sodium carbonate. The amount of ONP (O-Nitrophenol) liberated from ONPG was determined spectrophotometry (Nurullah, 2011).
B Results and Discussions

3.3 Sample collection

The strains for the research work were obtained from our lab, previously isolated and maintained. These cultures possess distinct feature and very limited literature was available on these strains. These two strains were explored for the production and characterization of biomolecules.

3.4 Morphological and Biochemical Characterization

The preliminary identification of the strain was carried out by microscopic, biochemical and 16S rRNA genes has positioned these two isolates in the genus *Halomonas*, Currently more than 90 species have been identified and placed under the *halomonas*, which were isolated from both marine habitat and terrestrial habitat, in all cases the salinity of the habitat found high. The methods used for phenotypic classification have been described detail by Ventosa et al., 1982, the strains formed circular colonies, each 2–3 mm in diameter and colonies were flat/slightly convex with irregular edges. The organisms were identified as *Halomonas organivorans* and *Halomonas sp.* by 16S rRNA sequencing, the Genbank Accession No’s are JQ906721 and JQ906722 respectively.

Conventional methods in identification and classification of microorganisms provide enough data to interpret the microbe family and genera, the numerical and taxonomical data set with nutritional requirement, biochemical, antimicrobial and microscopic data helps to categorize halophiles. Genomic feature and G+C content in
3.4.1 Microscopic and Physicochemical parameters of *Halomonas organivorans & Halomonas sp.*

*Halomonas organivorans* cell colonies are cream coloured, convex and mucoid, Gram negative bacilli, rod shape, non-spore forming and motile microorganism. Measures 1.90µm x 689nm, Halophilic bacteria capable of growing at NaCl concentration range from 3-25 % (w/v), with optimum growth occurring at about 10 % and grows within a temperature range of up to 45°C at pH values of between 5 and 10, the optimum value for pH is 8 as shown in following Table 3.1.

*Halomonas* sp. cells rod shaped elongated, creamy colour colonies appear on the halophilic media, suitably grow between temperature 15-45°C and measures about 1.93 µm x 605.19nm, tolerate the NaCl concentration 3-30%, wide range of pH 5-11 for the growth of the species. The SEM micrographs showed the size of cells of *Halomonas* sp. and *Halomonas organivorans* and their morphology are as shown in Fig.3.1 and 3.2 respectively.

*Halomonas elongata*, was rod shaped, motile, oxidase positive, grows between salt concentration 3.5 to 20% w/v, required optimum 3.5-8% salt and, temperature 15°C - 45°C, pH range between 5-9, G+C is 60.05% (Vreeland, 1980), *Halomonas Subglasiescola* reportedly measure 0.5-1.1X5-10 μM, were rod shaped, creamy colour, motile and oxidase positive, grows between 05-20% NaCl, optimally requires 15%NaCl for the growth between 0-25°C and require pH was 5-9 (Franzmann, 1987),
Halomonas halodurans, optimally grows at 8, and tolerate 3.5-20% NaCl concentrations and temperature 4-37°C growth was observed in pH range 5.5-8.5. Rods, 0.4-0.6 X1.5-2.0 micro m (Hebert and Vreeland, 1987), Halomonas halophila, which is a rod shaped bacteria, measures about 0.3-0.6X0.9-1.3 micro meter, colonies are creamy colour and motile, oxidase positive, grows best at 5-20% Salt (Dobson, Franzmann, McMeekin. 1990),

Halomonas eurihalina, Short rods, non motile, measures about 0.3-0.6X 2.0-2.5, creamy colour, Grows optimally at 7.5% and tolerate between 5-20 % NaCl and 5-45°C temperature, pH 5-10 (Mellado and Ventosa, 1995: Quesada, 1990), Halomonas halophila, rod shaped 0.5-0.7 X 1.5-2.0 creamy colour, motile and tolerate 2-30% NaCl and optimum for the growth is 7.5% and temperature 15-45°C, and pH 5-10 (Quesada, 1984).

H. salina (Dobson and Franzmann. 1996; Woolard, 1992), microscopically observed as short rods measures 0.7–0.8X 3 2–2.5, colonies appear as cream and this bacteria grows normally between 2-20 %, optimum salt required was 5%, and temperature 15-40°C, pH ranges between 6-10 H. halodenitrificans (Dobson, Franzmann. 1990; Ken-Dror, 1986) is a short rods creamy colour colonies appear on halophilic media with varying salt concentration of 3-20% NaCl and growth observed at varying pH range between 5-9, growth was recorded at temperature 15-32°C.

H. variablis (Dobson, 1983, Fendrich, 1988), curved rod cream, optimally growth was observed in media containing 10% Salt and tolerate 7-28%, temperature between
15-37°C, pH for growth was at 6.5-8.5. *H. Canadensis* (Huval Vreeland, 1995) creamy colour colonies on halophilc media, having pH 5-9 supported the growth of bacteria, which were rod shaped, required salt 8% for optimum and between 3-25% temperature 15-30°C.

*H. pantellerience* and *H. israelensis* (Romano, 1991; Huval Vreeland, 1995) are rods, creamy colour colonies and salt between 1.2-15% and 3.5-20%, respectively, 10% and 8% salt is optimum for their growth respectively, temperature between 15-44°C, pH between 7.5-11 and 5-9 respectively. *Chromohalobacter marismortui* (Ventosa, Kocur 1990) typically require alkaline pH 5-11, cells are rod shaped, pigmentation was recorded as creamy, grows on salt between 1-30% and optimum 10%, 15-44°C temperature was required for the growth.
## Table 3.1 Morphological and biochemical characterization of strains

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Characteristics</th>
<th><em>Halomonas</em> sp.</th>
<th><em>Halomonas organivorans</em></th>
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<td></td>
<td>Round</td>
<td>Irregular</td>
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<tr>
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<td>Colony Morphology</td>
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<td>Color</td>
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<tr>
<td>9</td>
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**Morphological tests**

<table>
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<td>Colony Morphology</td>
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**Staining and Biochemical test**

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**Growth at temperature**

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**Growth in medium pH**

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**Growth in the presence of NaCl (w/v)**

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<td>+</td>
</tr>
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<td>7%</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
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<td>10%</td>
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<td>+</td>
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<td>+</td>
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<tr>
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<td>20%</td>
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<tr>
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<td>+</td>
</tr>
<tr>
<td>8</td>
<td>30%</td>
<td>-</td>
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</tr>
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</table>

**Carbohydrate utilization test**

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Carbohydrate</th>
<th><em>Halomonas</em> sp.</th>
<th><em>Halomonas organivorans</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>D-glucose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>L-arabinose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>D-xylose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>D-mannitol</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>D-fructose</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
Fig. 3.1 Electron micrograph of *Halomonas* Sp.

Fig. 3.2 Scanning Electron Micrograph of *Halomonas organivorans*
3.5 16S rRNA sequencing

Halophiles are the biomarkers of the hypersaline environments, since last three four decades almost all salterns were explored, may conventional and molecular methods were used to identify and authenticate the halophilic bacteria. Nucleic acid based information was found very useful in characterization of halophilic strains.

Currently *halomonas* genus has more than 90 species, is a large genus with more moderate halophiles, thus originally Vreeland *et al.*, 1980 proposed *halomonas* genus, constitutes species from marine and terrestrial habitats, with gradient salt concentrations. Species are known for degradation of hydrocarbons and strains are metabolically very versatile (*Ventosa et al.*, 1998).

Universally 16S rRNA sequencing has been the most trusted method in halophiles and other bacteria on the basis of 16S rRNA sequencing results bacteria were identified as *Halomonas organivorans* and *Halomonas* Sp. by using the NCBI Blastn tool. Neighbour Joining method was used to construct the phylogenetic tree (*Saitou- Nei*, 1987) depicts clearly that, strains belongs to family *halomadaceae* (*Dobson & Franzmann*, 1996). The 16S rRNA data shows similarity with the other *halomonas* species between 99 %-98%, these partial sequences are similar with that of *halomonas salina* (98%), *halomonas smyrnesis* strain S54, *halomonas salina* strain muz2N 1913 (98%) (*Arahal et al.*, 2002). *Halomonas Sp.* significantly clustered as single in tree, hence, the further studies with DNA -DNA hybridization yields more information to evidence novelty of this strain. The genomic DNA was isolated and electrophoresed as shown in Fig 3.3a and the 16S rRNA sequences and phylogenetic tree was shown in Fig 3.3b.
**Halonomas organivorans**
Gene Bank Accession ID: JQ906721

Gene Sequences

GAGGCGTCGACGCAGCGACGAGATTGAGATGTAATGCATAGGAATCTGCCCGGTAGTGGGGGATAAC
TTGAGGAAACTCAAGCTAATACCGGATGCTGCTGACCATAGGGGAAACCGAGGCTAGTGGGGGATAAC
TTGAGGAAACTCAAGCTAATACCGGATGCTGCTGACCATAGGGGAAACCGAGGCTAGTGGGGGATAAC

**Halomonas. Sp.**
Gene Bank Accession ID: JQ906722

Gene Sequences

CAGATTGACGCGGAGCGACGAGATTGAGATGTAATGCATAGGAATCTGCCCGGTAGTGGGGGATAAC
TTGAGGAAACTCAAGCTAATACCGGATGCTGCTGACCATAGGGGAAACCGAGGCTAGTGGGGGATAAC
TTGAGGAAACTCAAGCTAATACCGGATGCTGCTGACCATAGGGGAAACCGAGGCTAGTGGGGGATAAC

78
Fig. 3.3a Genomic DNA of *Halomonas* and *Halomonas organivorans*

![Genomic DNA Image]

Note: Lane M= DNA marker ranging from 100bp to 1Kb; Lane 1: Genomic DNA of *Halomonas* sp.; Lane 2: Genomic DNA of *Halomonas organivorans*.

Fig. 3.3b Phylogenetic tree view of *Halomonas* and *Halomonas organivorans*

![Phylogenetic Tree Image]
3.6 Antibiotic susceptibility test

Halophilic bacteria were known to tolerate too many antibiotics, we have used 24 different antibiotics to characterise these strains, and the following pictures shows the antibiogram of strains as shown in Fig 3.4 and 3.5. Eight antibiotics showed resistance, while remaining were sensitive to the halophilic strains. Antibiotics have been used for the purpose of selecting for particular organisms in enrichment or maintenance cultures. Penicillin is most popular, but ampicillin and streptomycin have been used (Torreblanca et al., 1986; Montero et al., 1988; Wais 1988; Kulichevskaya et al., 1992). A combination of penicillin G, erythromycin, and cycloheximide were used to select for archaea at different pH from subzero hypersaline methane species (Niederberger et al., 2010).
Fig. 3.4 Antibiogram of *Halomonas organivorans*

Fig. 3.5 Antibiogram of *Halomonas sp.*
3.7 Screening of Extracellular Hydrolytic Enzymes

The halophilic bacterial enzymes have excellent properties make them more significant, because these are tolerant to salt and heavy metal ions. Enzymes denature or loose activity in presence of salt, but enzymes isolated from the halophiles are active even at higher salt concentrations and metal ions.

*Halomonas sp.* and *Halomonas organivorans* are positive for few hydrolases. Among them protease and amylase positive. The starch hydrolysis by galctosidase is also produced by both the strains and *H. organivorans* and *Halomonas* sp. were also producer of lipases was confirmed.

Studies of Sanchez-Porro and Colleagues (Sanchez-Porro *et al.*, 2002) showed the abundance of five hydrolytic enzymes including protease, amylase, lipase, DNase and pullulanase by moderate halophilic bacteria from saltern samples in Spain. Zavaleta and Colleagues (Zavaleta *et al.*, 2007), determined the production of amylase, lipase and protease among halophilic bacteria isolated from Pilluana brines, Peru. Madalin Enache and colleagues (Roxana *et al.*, 2009), investigated the amylase, gelatinase, lipase, protease, cellulase and xylanase from subterranean salt crystals. Moreno and colleagues (Moreno *et al.*, 2007), determined the diversity of extreme halophiles and potential producers of lipase, protease, amylase and nuclease, in hypersaline environments of South Spain sample. Rohban and colleagues (Rohban *et al.*, 2008), determined the amylase, protease, lipase, DNase, pectinase, inulinase, cellulose, xylanase and pullulanase activity, from Howz Soltan Lake, Iran.

*Halomonas meridiana* this bacterium is able to produce an amylolytic enzyme that has optimal activity at 37°C, pH 7.0, being relatively stable under alkaline
conditions, and 10% NaCl, although activity at salinity as high as 30% salts was reported. The main products resulting from the hydrolysis of starch were maltose and maltotriose (Coronado et al., 2000a).

3.8 Whole Cell Protein Profiling of *Halomonas* sp. and *Halomonas organivorans*

The whole cell protein profiling was carried out by SDS PAGE, Halophilic microorganism’s proteins could be distinguished from non halophilic proteins, and these proteins are highly unstable in low salt or in absence of salt, require minimum concentration of salt for their activity and stability. Hence called these proteins as “Salt in” Proteins.

Under the salt tress these bacterial proteins are acidic in nature, the solubiulization of protein could be done with urea or thiourea was used to store the protein sample. Halophilic proteins, slightly than being unfolded by these situations, appear to be dependent on the presence of salts. In recent years, detailed investigations have tried to unveil the relationships between structure and stability in halophilic proteins. These analyses provided undoubtedly valuable indications on the biophysical and biochemical properties of the halophilic proteins. However, scrutiny of proteome and genomic sequences may not unravel subtle differences at the three-dimensional structural level while structural analysis of a single or few protein families may lack sufficient generalization. For these reasons we report in this work a systematic comparison between the available three-dimensional structures of halophilic enzymes deposited in the data banks and the structure of one of their homologues, to investigate the differences possibly related to shared strategies of structural adaptation to high salt environments. Use of three-
dimensional structure made it possible to investigate subtle modifications of the surface and hydrophobic core of the halophilic proteins especially at the level of conserved hydrophobic contacts.

In particular, these studies suggested that the halophilic proteins bind significant amounts of salt and water in solvent conditions similar to their physiological environment. The peculiar ability of halophilic proteins to bind large amount of salts is largely dependent on the number of acidic amino acids on protein surface.

The protein folding and adequate stability of the native structure in a hypersaline environment may require evolutionary modulation of the hydrophobic interactions occurring at the protein core. Most of the studies carried out to unveil the structural features of halophilic proteins, were based on sequence evaluation at proteome and genome levels or were focused onto single or few protein families. These analyses provided signals on the biophysical and biochemical properties of the halophilic proteins. For these reasons we report in this work a systematic comparison between halophilic proteins
Production and Purification and Characterization of Ectoine form *H. organivorans*

A. Materials and Methods

4.1 Screening, production and characterization of biomolecules

The halophilic bacteria were screened for the production of biomolecules, such as Ectoine, Betaine, Polyhydroxy Butyrate (PHB), Enzymes and synthesis of Silver Nanoparticles from compatible solutes.

4.2 Production of Ectoine

The production of Ectoine by *Halomonas organivorans* was carried out by shake flask for 24 hrs and the bacterial cells were harvested from the culture broth by centrifugation at 5500 g for 10 min as seed culture. The cells were again suspended in 100 ml medium with 10% concentration of NaCl in 500 ml Erlenmeyer flasks for production of ectoine. The culture was incubated for 30 hrs, pH 7 and temperature 37°C at 200 rpm and the ectoine produced was purified and analysed by following the method of Kunte *et al.*, 1993.

4.3 Optimization of growth parameters

4.3.1 Inoculum size and agitation

The production of ectoine was carried out in halobacterium broth in 50ml Erlenmeyer flask kept for the incubation at pH 7, 37°C, for 24 hrs, at 200 rpm. Later the cells were harvested by centrifugation at 5500rpm for 10 minutes at 4°C. 1X10⁵ CFU/ml was inoculated to the fermentation broth with varying percentage from 1-3% v/v and fermentation was carried out for the growth of biomass and production of ectoine was
measured by optical density at 600 nm. Agitation of fermentation broth was monitored from 100 rpm to 250 rpm and the effect of aeration was evaluated.

4.3.2 Effect of pH on growth and production of Ectoine

The production medium (halobacterium medium) was adjusted to different pH ranging from 5 to 13 at a difference of 1pH and temperature 37°C using 1% Glucose was incorporated as a substrate after autoclaving with 10% NaCl for 24 hrs, the growth and production of ectoine was observed by measuring optical density 600nm.

4.3.3 Effect of temperature on growth and production of Ectoine

The optimum temperature for the growth and production ectoine by halophilic bacteria isolated was determined in production medium by keeping it in temperature controlled shaker at 200 rpm range from 35°C to 60°C with an increment of 5°C with optimum pH 7 incubated from 24 hrs and optical density was recorded.

4.3.4 Effect of NaCl on growth and production of Ectoine

The effect of NaCl was studied on the growth and ectoine production using production medium (halobacterium medium) containing different concentrations of NaCl (0, 5,10,15,20 and 30% w/v). This inoculated broth was kept under shaker at optimum temperature 37°C and pH 7 for 24 hrs. and the observations were recorded.

4.3.5 Effect of Heavy Metals on growth and production of Ectoine

A set of conical flasks containing different metal ions such as mercury chloride, Zinc Chloride and Lead acetate (1mM to 5mM) with Glucose (1%) in Halobacterium medium was added after autoclaving with 10% NaCl at pH 7, and incubated at temperature 37°C for 24 hrs. at 200 rpm and the effect of metal ions was evaluated.
4.4 Extraction of compatible solutes

*H. organivorans* cells were permeabilized by hypoosmotic shock using water for the extraction of compatible solutes (Schiraldi *et al.*, 2006). Cells from the culture broth were harvested by centrifugation (6000 rpm for 10 min) and re-suspended in 1.5 ml of H$_2$O. The suspension was allowed to stand for 0.5–2 hrs at room temperature prior to centrifugation and analysis of the osmolyte concentration in the cell-free supernatant was carried out by HPLC.

4.5 Estimation and characterization of ectoine

Ectoine produced was extracted from the cells according to the procedure reported by (Kunte *et al.*, 1993). Ectoine content (weight percent) was calculated as the ratio of dry cell biomass to ectoine concentration by measuring by HPLC and the chemical structure of the compatible solute accumulated in *H. organivorans* was characterized by FTIR.