CHAPTER 3

HALOALKALIPHILIC BACTERIA

PHYLOGENY, DIVERSITY, ENZYMATIC POTENTIAL
3.1 INTRODUCTION

Microbial diversity includes, the diversity of bacteria, protozoan, fungi, and unicellular algae, constitutes the most extraordinary reservoir of life in the biosphere. In a very particular term; diversity is composed of two elements: richness and evenness, so that the highest diversity occurs in communities with many different species present (richness) in relatively equal abundance (evenness) (Huston, 1994). The richness and evenness of bacterial communities reflect selective pressure that shape diversity within communities.

For much of the last century, microbiologists have been aware that we know the nature and identity of only a tiny fraction of the inhabitants of the microscopic landscape. While most people are very familiar with the diversity of life in the plant and animal kingdoms, few actually realize the vast amounts of variability present in the bacterial populations. Interestingly; microorganisms represent the richest repertoire of molecular and chemical diversity in nature as they underlie basic ecosystem processes. The current inventory of the world’s biodiversity is very incomplete and that of microorganisms is especially deficient. Scientists have identified about 1.7 million living species on our planet. Studies indicate that the 5,000 identified species of prokaryotes represent only 1-10% of all bacterial species; therefore we have only a small idea of our true microbial diversity (Bowen et al., 2011).

In particular, extremophiles are organisms able to live in unusual habitats, can potentially serve in a verity of industrial applications (Burg, 2003; Horikoshi, 2008; Horikoshi, 2011). As a result of adaptation to extreme environments, extremophiles have evolved unique properties, which can provide significant biotechnological and commercial opportunities. Major categories of extremophiles include halophiles, alkaliphiles, acidophiles, thermophiles and haloalkaliphiles. The groups of bacteria able to grow under alkaline conditions in the presence of salt are referred as haloalkaliphiles. They possess special adaptation mechanisms for their under salinity and alkaline pH. These properties of dual extremity of halophiles and alkaliphiles make them interesting from both, fundamental research and biotechnological points of view (Dodia et al., 2008a and b; Purohit and Singh, 2011; Romano et al., 2011).
The microbial diversity has focused renewed emphasis and in this regard extremophiles hold great significance among microbial world. Limited studies have identified a huge diversity of extremophiles. Large number of haloalkaliphilic bacterial strains depicted wide diversity, as reflected through microbiological examinations, biochemical characteristics and molecular approaches (Dodia et al., 2008; Joshi et al., 2008; Purohit and Singh, 2011; Siddhpura et al., 2010; Singh et al., 2010 a and b).
3.2 MATERIALS AND METHODS

3.2.1 Sample Collection
For the isolation of the halophilic and haloalkaliphilic bacteria, the soil samples were collected from the different sites along the coast of Gujarat; particularly from saline soil across the coastline and artificial salt pane of Okha Madhi. The samples were collected in sterile plastic bags; the pH and temperature of all the samples were measured manually at the time of the sample collection, and processed within four days after the sample collection. From the total collected samples; two samples (O.M.6.2 and O.M.6.5) were selected for further studies. All the collected samples were stored at 4°C.

3.2.2 Physical and chemical analysis of the samples
Before proceeding for the isolation, the samples were subjected to the physical and chemical analysis, such as pH, temperature, conductivity, total dissolved solids (TDS), turbidity, alkalinity, total hardness and Mg$^{+2}$ concentration as per the method BIS (Bureau of Indian Standards).

3.2.3 Enrichment and isolation
For the isolation of the halophilic and haloalkaliphilic bacteria, 1.0 gm of the soil sample was inoculated into the 100ml of the enrichment medium. The bacteria were isolated by using enrichment culture techniques in Complex Medium Broth (CMB) consisting, (g/liter): Glucose, 10; Peptone, 5; Yeast extract, 5; KH$_2$PO$_4$, 5; with varying concentration of NaCl (10% and 30%, w/v) at different pH (8 and 10). The pH of the medium was adjusted by adding separately autoclaved Na$_2$CO$_3$ (20%, w/v). After inoculation, flasks were incubated on environmental shaker at 37°C with regular monitoring on the turbidity of the enrichment media. After 48-72h of growth, a loop full culture was streaked on the CMB agar (agar, 3%, w/v) plate and incubated at 37°C. After 48h of the incubation, on the basis of colony characteristics, various isolated colonies were selected and pure cultures were obtained by subsequent streaking on the CMB agar plate (Fig.3.2.1).
Fig. 3.2.1: A schematic representation of enrichment and isolation procedure (Joshi, 2006).

3.2.4 Maintenance and preservation

The pure cultures were preserved on the CMB agar media (10% w/v NaCl; and pH 8-10) and stored at 4°C. After screening for the extracellular enzymes, the protease producers were preserved on gelatin agar medium respectively. The cultures were subsequently transferred on fresh CMB agar at 3 months intervals.

3.2.5 Characterization of the organisms

3.2.5.1 Colony characteristics

For the primary characterization, the pure culture of all the isolated bacteria were streaked on the CMB agar plate with corresponding enrichment conditions of the NaCl (10 and 30%, w/v) and pH (8 and 10) and their colony characteristics were observed.

3.2.5.2 Cell morphology and Gram reaction

For the differentiation on the basis of the cell morphology and cell arrangement, individual bacterium was studied for the Gram reaction, in activated culture in CMB at the corresponding enrichment conditions.
3.2.5.3 Biochemical characterization
For further differentiation, the isolates were studied for biochemical and metabolic activities. The biochemical tests included production of catalase, oxidase, \( \text{H}_2\text{S} \), ammonia, indole, hydrolysis of urea, reduction of nitrate and litmus; fermentation of the sugars such as glucose, fructose, sucrose, maltose, lactose and xylose. All the biochemical media and their test reagents were prepared as mentioned by Cappuccino and Sherman (Cappuccino and Sherman, 2004). Because of the halophilic nature of the organisms, all the biochemical media were supplemented with 5% (w/v) \( \text{NaCl} \). The individual isolate was inoculated to the respective biochemical medium and incubated at 37°C for 24-48h and results were subsequently observed.

3.2.6 16S rRNA amplification and nucleotide sequencing
For potential strains; having enzymatic potential to secrete proteases; genomic DNA was isolated from the pure culture of O.M.A18, O.M.E12, O.M.C38 and O.M.C14. The \(~1.5\) kb rDNA fragment was amplified through high-fidelity PCR polymerase by using consensus primers. The PCR product was bi-directionally sequenced by using the forward; 5"-AGAGTTTGATCATGGCTCAG-3’ and reverse primer; 5’-TACGGTTACCTTGTTACGACTT-3’.

3.2.7 Phylogenentic analysis of the 16 S rRNA sequences
The sequence of a selection of published 16S rRNA genes were obtained in aligned form from the Ribosomal Database Project (RDP) (http://rdpwww.life.uiuc.edu, Maidak et al., 1996) using the ‘subalign’ service. The Rt3 sequence was added to this alignment and manually aligned in accordance with RDP “align sequence” report, using the alignment editor AE2 (Larsen Likelihood (ML). The phylogeny of the aligned sequence was obtained using the RDP ‘suggest tree’ service from fast DNAml program (version 1.08).

3.2.8 FAME analysis (Fatty acid based microbial identification software)
The fatty acids are extracted from haloalkaliphiles by a procedure which consists of saponification in dilute sodium hydroxide/methanol solution followed by derivatization with dilute hydrochloric acid/methanol solution to give the respective methyl esters (FAMEs). The FAMEs are then extracted from the aqueous phase by
the use of an organic solvent and the resulting extract is analyzed by GC. Fatty acids were analyzed by Sherlock software which, automates all analytical operations and uses a sophisticated pattern recognition algorithm to match the unknown FAME profile to the stored library entries for identifications.

**3.2.9 Detection of antibiotic resistance and sensitivity**

For the detection of antibiotic resistance and sensitive nature of the isolates, Bauer-Kirby test was performed by using the octadiscs (Hi Media Life science, India) specific for the Gram negative and Gram positive bacteria. The isolates were tested against different antibiotic octadiscs on the basis of its Gram’s reactions. The abbreviations of antibiotics used are: Ampicillin (A); Carbenicillin (Cb); Cephotaxime (Ce); Cholarmphenicol (C); Co-Trimazine (Cm); Gentamicin (G); Norfloxacin (Nx); Oxacillin (Ox); Cephaloridine (Cr); Kanamycin (K); Lincomycin (L); Methicillin (M); Oleandomycin (Ol); Penicillin-G (P); Tobramycin (Tb); Tetracycline (T); Co-Trimaxazole (Co); Cloxacillin (Cx); Cephaladin (Cv); Erythomycin (E); Cefuroxime (Cu); Ceprofloxacin (Cr); Colistin (Cl); Nitrofurantoin (Nf); Steptomycin (S); Cephalexin (Cp); Nalidixic Acid (Na); Furazolidone (Fr); Oxytetracycline (O).

The melted CMB agar medium (10%, NaCl w/v; pH 9) was inoculated with 5% inoculum and poured in sterile plate followed by the addition of antibiotics impregnated octadisc onto the agar surface. The plates were incubated at 37°C for 24-48h. Antibiotic sensitivity was detected by measuring zone of the clearance (zone of inhibition) around the individual antibiotic disc while growth in the vicinity or surrounding the disc indicates the resistance of particular isolate against that antibiotic.

**3.2.10 Screening for extracellular alkaline protease enzyme secretion**

Actively growing cultures of different isolates were prepared in the Complete Medium Broth (CMB) at its optimum NaCl (0-25%); pH (8-10) and used as inoculum ($A_{540}>1.0$) for the primary screening of alkaline protease. The cultures were inoculated in the form of regular spots on gelatin agar medium containing (g/liter); Gelatin, 30; Peptone, 10; NaCl, 100; pH, 8-10 and Agar, 30. The pH of the medium adjusted to 8-10 by adding separately autoclaved 20% Na$_2$CO$_3$ (w/v). The plates were incubated for 48-72h at 37°C and Frazier’s reagent (g/liter: HgCl$_2$, 150g; concentrated
HCl, 200ml) was poured on plate for the gelatin liquefaction. The clear zone surrounding the colony indicated the secretion of extracellular protease. The colony diameter and zone of clearance was measured. The ratio was calculated to assess the relative enzyme secretion as a function of colony size.

### 3.2.11. PCR Amplification of alkaline protease gene

The DNA preparations described above were used as template to amplify region coding alkaline protease. The four pair of primers used for amplification profile was synthetic degenerate oligonucleotides based on the previously known sequence of alkaline protease gene from *Bacillus halodurans*, *Bacillus cerus*, *Oceanobacillus iheyensis* serine proteases and haloalkaliphilic *Bacillus* sp.

<table>
<thead>
<tr>
<th>Primer designation</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPS-1F</td>
<td>5'-gga tcc ttg aac aaa atc att-3’</td>
</tr>
<tr>
<td>SPS-1R</td>
<td>5'-gtc tta aga agc ttt att taa c-3’</td>
</tr>
<tr>
<td>SPS-3F</td>
<td>5'-gga tcc ttg aca aca tca ttg-3’</td>
</tr>
<tr>
<td>SPS-3R</td>
<td>5'-gtc tta aga agc ttt att taa c-3’</td>
</tr>
<tr>
<td>SPS-4F</td>
<td>5'-gga tcc cta ctt gta gta-3’</td>
</tr>
<tr>
<td>SPS-4R</td>
<td>5'-gtc gac atg cat atc gga aaa c-3’</td>
</tr>
<tr>
<td>SPS-5F</td>
<td>5'-gga tcc gcc gcc gag gac gac-3’</td>
</tr>
<tr>
<td>SPS-5R</td>
<td>5'-gta gac atg gga tat tat gac-3’</td>
</tr>
<tr>
<td>SPS-6F</td>
<td>5'-gga tcc gcc gcc gag gac gac-3’</td>
</tr>
<tr>
<td>SPS-6R</td>
<td>5'-gta gac gga cca gag cgt cg-3’</td>
</tr>
<tr>
<td>SPS-7F</td>
<td>5'-cat atg ccc ccc agg ac-3’</td>
</tr>
<tr>
<td>SPS-7R</td>
<td>5'-gtc gac ggc ctc gct gta g-3’</td>
</tr>
</tbody>
</table>

**Table 3.2.1:** Primer sequences used for amplification procedures

The other two primer pairs were designed on the basis of conserved sequences of Haloalkaliphilic *Bacillus species*, by using multiple sequencing tool followed by block generation using degenerate primer designing bioinformatics tool-CODEHOP (Table 3.2.1). To 100 ng of DNA as the template, 25 pmol of each Forward and oligonucleotides primer (Sigma Aldrich, life sciences), 25µl of 2X Red Mix Plus (Merk, Life sciences) were added. Two negative controls; one without template and another without primer, were also included in the PCR reactions to check validity of the experiment. The amplified products were visualized on agarose gel as described above, further purified as discussed below and stored at -20°C till further use.
1. Initial denaturation at 94°C for 2 mins.
2. Denaturation at 94°C for 1 min.
3. Gradient of annealing at 60°C with gradient of 8°C for 45 secs.
4. Extension at 72°C for 1.5 mins.
5. Repeat step 2 to 4 for 29 cycles
6. A final elongation was done at 72°C for 5 min
7. Hold at 4°C
3.3 RESULTS AND DISCUSSION

It has been always fascinating to study the microbial community especially extremophiles in particular. The extreme environments are often more complex and to maintain them under laboratory conditions is a difficult task. So, the development of new strategies of isolation, particularly for the extremophiles, is a challenging issue for the scientists. Furthermore, it is of great value to make available the unexplored world of organisms, as our knowledge is restricted to less than 1-5% of the total microbial population in nature. Up till now majority of the halophiles and haloalkaliphiles have been isolated from athalassohaline environments (Demergasso et al., 2004; Wang et al., 2007), whereas thalassohaline environments have relatively less explored (Munoz et al., 2001; Amoozegar et al., 2003; Guranthan et al., 2010). In view of these facts, we isolated haloalkaliphilic and haloalkalitolerant microbes from the thalassohaline environments.

3.3.1 Sites for sample collection

Around 34 different haloalkaliphilic bacteria were isolated from the saline soil; particularly artificial salt pane samples located near the coastal region of the Western Gujarat (India). The sites of the isolation as depicted in map (Fig. 3.3.1). Several samples were collected as described in Table. 3.3.1. Among them, site designated 6: Okha Madhi; particularly, O.M.6.2 and O.M.6.5 were selected for isolation and enrichment procedures. Selection of site was done on the basis of its physico-chemical properties (Table 3.3.2). Both the sampling sites used for studies were artificial salt pane; having heavy deposition of salt; pane was heavily saturated with salt as well ring of different colors; pink, red and orange were seen around the pane, which could be interesting with diversity view point. The sampling site was around 1.5- 2.0 km long; with several panes located in close proximity with each other. The temperature was around 37°C at the time of sample collection. The salinity and pH of the samples varied from 3.5-4% and 7.8-9, respectively, presence of different color; indicates undissolved salts on the surface. The complete description of the sites, samples, their physical parameters and isolates isolated from each sample along with their enrichment condition is given in Table 3.3.2 and Table 3.3.3.
Fig. 3.3.1: Map of Gujarat displaying site of Isolation, Okha, Gujarat, India.

<table>
<thead>
<tr>
<th>Site of collection</th>
<th>No. of sample</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Okha-Madhi</td>
<td>6.2</td>
<td>Soil collected from red ring from the site, crystalline soil</td>
</tr>
<tr>
<td>Okha-Madhi</td>
<td>6.5</td>
<td>Sticky and smooth mud soil; with high salt concentration</td>
</tr>
</tbody>
</table>

Table 3.3.1: Sample collection details (date of collection: 07-10-07)
Table 3.3.2: Physical and Chemical Properties of Soil {*Tests were performed as per the BIS (Bureau of Indian Standards) IS: 3025}

### 3.3.2 Physical and chemical analysis of the sample

The salinity and alkalinity of the collected soil samples were nearly equal but the values of the turbidity, TDS, total hardness and Mg$^{+2}$ concentrations varied (Table 3.3.2).

### 3.3.3 Enrichment and isolation

In present study, we isolated 34 haloalkaliphilic and haloalkalitolerant bacteria from saline salt pane along the coastal region of Gujarat. Existence of halotolerant, haloalkalitolerant and haloalkaliphilic bacteria clearly indicating the wide spread distribution of such organisms in moderate saline environment beyond the conventionally described habitats of salt lakes, solar salt evaporation ponds and salt deserts. Depending on their optimum growth at 10% (w/v) NaCl and pH 9, the isolated haloalkaliphilic bacteria can be put under the class of moderate haloalkaliphiles. Therefore, in the present thesis the isolates have been referred as haloalkaliphilic instead of halophilic organism. Interestingly, number of isolates from a given site decreased with increasing degree of extremity of salt and pH. From the two soil sample, total 34 different haloalkaliphilic/ haloalkaliphilic bacteria were isolated by using different enrichment conditions of NaCl and pH.
Organisms were preliminary distinguished on the basis of enrichment conditions and colony characteristics. Out of 34 isolates, 16 were isolated at combination of 10% NaCl, \((w/v)\) and pH-8 while 18 organism were isolated at combination of 30% NaCl, \((w/v)\) and pH-10 (Table 3.3.3). Both the combinations were selected for enrichment procedures to isolate both haloalkalitolerant/ haloalkaliphilic bacteria (Fig. 3.3.3). Almost equal numbers of isolates were obtained with both the combinations. The overall profile for the isolation with different enrichment combinations is given in Table 3.3.3 A and B.

<table>
<thead>
<tr>
<th>Designation</th>
<th>Enrichment condition</th>
<th>No. of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>O.M.6.2</td>
</tr>
<tr>
<td>A</td>
<td>pH-8, 10% NaCl</td>
<td>2</td>
</tr>
<tr>
<td>B</td>
<td>pH-8, 30% NaCl</td>
<td>--</td>
</tr>
<tr>
<td>C</td>
<td>pH-10, 10% NaCl</td>
<td>4</td>
</tr>
<tr>
<td>D</td>
<td>pH-10, 30% NaCl</td>
<td>8</td>
</tr>
<tr>
<td>-----</td>
<td>Total isolates</td>
<td>14</td>
</tr>
</tbody>
</table>

**Table 3.3.3A:** Nomenclature of organism isolated from saline soil of coastal Gujarat.

We have noticed by changing growth conditions that there were few isolates growing optimally at 0% NaCl. Along the same line isolates were able to grow at higher concentration i.e. upto 3-4M NaCl concentration. Growth of organism upto 2M NaCl was preliminary characteristic of all isolates. However, along the same line, similar results were not observed with respect to alkaliphiles, organism was able to grow at higher alkaline pH. Maximum amount of organisms were able to grow optimally at pH-9. However the range of growth was quite broad from pH-8 to pH-11. O.M.E_1 and O.M.E_2 were able to grow optimally at pH-11; however there was not much variation in growth from pH-9 or 10. Similiarly this isolates were able to grow upto 20-25% of NaCl, although, few isolates, of interesting features, were isolated from extreme condition. The important point emerged indicated that both diversity and
number of the organisms decreased with the increasing level of extremities, supporting the general view that ultra extreme environments support the growth of true extremophiles only.

<table>
<thead>
<tr>
<th>Site Designation</th>
<th>Site description</th>
<th>Characteristics of samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Physical appearance</td>
<td>pH</td>
</tr>
<tr>
<td>O.M.6.2</td>
<td>Soil with presence of salt crystal</td>
<td>10</td>
</tr>
<tr>
<td>O.M.6.5</td>
<td>Soil with presence of salt crystal and pink pigmentation</td>
<td>10</td>
</tr>
</tbody>
</table>

**Table 3.3.3B:** Site description and characteristics of O.M.6.2 and O.M.6.5 site

**Fig. 3.3.2A:** Distribution of bacteria on the basis of pH profile

**Fig. 3.3.2B:** Isolation and enrichment on the basis of NaCl

**Fig. 3.3.2C:** Isolation and enrichment on the basis of pH
<table>
<thead>
<tr>
<th>Site</th>
<th>No. of isolates</th>
<th>Enrichment conditions</th>
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<tbody>
<tr>
<td>O.M.6.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>O.M.A11</td>
<td>pH 8 NaCl (10)</td>
</tr>
<tr>
<td>2</td>
<td>O.M.A14</td>
<td>pH 8 NaCl (10)</td>
</tr>
<tr>
<td>3</td>
<td>O.M.A16</td>
<td>pH 8 NaCl (10)</td>
</tr>
<tr>
<td>4</td>
<td>O.M.A17</td>
<td>pH 8 NaCl (10)</td>
</tr>
<tr>
<td>5</td>
<td>O.M.A18</td>
<td>pH 8 NaCl (10)</td>
</tr>
<tr>
<td>6</td>
<td>O.M.C11</td>
<td>pH 10 NaCl (10)</td>
</tr>
<tr>
<td>7</td>
<td>O.M.C12</td>
<td>pH 10 NaCl (10)</td>
</tr>
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<td>8</td>
<td>O.M.C13</td>
<td>pH 10 NaCl (10)</td>
</tr>
<tr>
<td>9</td>
<td>O.M.C14</td>
<td>pH 10 NaCl (10)</td>
</tr>
<tr>
<td>10</td>
<td>O.M.D116</td>
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<td>11</td>
<td>O.M.D17</td>
<td>pH 10 NaCl (30)</td>
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<td>12</td>
<td>O.M.D18</td>
<td>pH 10 NaCl (30)</td>
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<td>13</td>
<td>O.M.D114</td>
<td>pH 10 NaCl (30)</td>
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<td>14</td>
<td>O.M.D115</td>
<td>pH 10 NaCl (30)</td>
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<td>15</td>
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<tr>
<td>16</td>
<td>O.M.E11</td>
<td>pH 10 NaCl (30)</td>
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<th>Site</th>
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<th>Enrichment conditions</th>
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<td>O.M.6.5</td>
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<td>1</td>
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<tr>
<td>18</td>
<td>O.M.C28</td>
<td>pH 10 NaCl (10)</td>
</tr>
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</table>

**Table 3.3.4:** Total number of isolates from O.M.6.2 and O.M.6.5 site
3.3.4 Characterization of the organisms

Although, the genetic data and molecular techniques are extensively being used for the identification and phylogenetic relatedness of organisms belonging to prokaryotes and archaeabacteria during the last many years, the traditional classification methods based on phenotypic, morphological and microbiological observation have its own importance in studying.

3.3.4.1 Colony characterization

The isolated haloalkaliphilic bacteria were primarily diversified on the basis of their cultural characteristics, as described in Table 3.3.5. Some common characters are collectively displayed by the majority of isolates from same site as well as those from different sites; such as, round and regular shape with opaque colony, smooth texture and creamish white pigmentation (Fig.3.3.3). The overall impression for the comparison of all the colony characteristics for the isolates of the different sites is described in Fig. 3.3.3A.

For O.M.6.2 site; the differentiation of isolates, was quite evident from the different colony characteristic features (Fig. 3.3.4 A, B, C, D, E, F). On the basis of colony size, among the isolates, around 60% of bacteria, size range between 1-3mm in colony size while about 40% had large (4-6mm) colony size. However, for O.M.6.5 the diversity profile was reversed where around 70% were in the size range of (4-6mm), and only 30% of total isolates were of small size (Fig. 3.3.3B).

With reference to colony shape, for O.M.6.2; no diversity was noticed and all organisms were totally of regular shape; while with respect to O.M.6.5 although majority of them were noticed with regular shape, however 20% were found to be of irregular shape (Fig. 3.3.3C).

With respect to elevation parameter only sheared numbers of isolates in O.M.6.2 and no isolates in O.M.6.5 were noticed with flat elevation. With respect to elevation parameter; in O.M.6.2 around 60% were with raised elevation and 40% were slightly raised. Comparing the other site, exactly reverse side was observed. On studying these parameters it is quite obvious analysis, that although the site of isolation was in close proximity of each other; wide difference was seen with respect to diversity of organisms (Fig. 3.3.3D).
For, colony texture parameter, although majority of organisms were found to be smooth in nature for both the sites; however, ratio was quite different for both site. In O.M.6.5 site around 20% were rough while only 3-4% was found to be of rough texture in O.M.6.2 (Fig. 3.3.3E). There was not much diversity noticed with reference to opacity parameter; and only 2-3% of bacteria were noticed of translucent and rest all were opaque in nature and none of the total collected pool of bacteria has transparent opacity (Fig. 3.3.3F).

Such phenotypic characters were useful for primary characterization, which could be used to assess the initial level diversity among the isolates.
Fig. 3.3.3: Distribution of isolates on the basis of colony characteristics: (A) Percent distribution (B) Colony size (C) Colony shape (D) Colony Elevation (F) Opacity.
Fig. 3.3.4A: Distribution of organism on the basis of different cell morphology.

Fig. 3.3.4B: Distribution of organism on the basis of different cell arrangement.

Fig. 3.3.4C: Schematic distribution of O.M.6.2 organism site on the basis of cell morphology.

Fig. 3.3.4D: Schematic distribution of O.M.6.5 organism site on the basis of cell morphology.
<table>
<thead>
<tr>
<th>Isolates</th>
<th>Size (mm)</th>
<th>Shape</th>
<th>Margin</th>
<th>Elevation</th>
<th>Opacity</th>
<th>Texture</th>
<th>Pigmentation</th>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>5</td>
<td>Round</td>
<td>Regular</td>
<td>Slightly raised</td>
<td>Opaque</td>
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<td>Creamish white</td>
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<td>Smooth</td>
<td>Creamish sticky</td>
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<td>Flat</td>
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<td>Smooth</td>
<td>White</td>
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**Table 3.3.5:** Diversity of the organism on the basis of colony characteristics
Similarly, looking at the gram reactions, Gram positive character was dominated over the Gram negative for the same site. Gram variable characters were much less evident among the isolates (Fig. 3.3.4). On the basis of Gram’s reaction organism were majorly: 75% were gram negative in nature and only 25% were gram positive. While,

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Gram reaction</th>
<th>Size and Shape</th>
<th>Arrangement</th>
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<td>O.M.A.1</td>
<td>Negative</td>
<td>Short thin rod</td>
<td>Singly and in pair</td>
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<td>Negative</td>
<td>Very short thick rod</td>
<td>Singly and in chain</td>
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<td>Variable</td>
<td>Long thin rod</td>
<td>Singly</td>
</tr>
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<td>Negative</td>
<td>Small thin rod</td>
<td>Singly</td>
</tr>
<tr>
<td>O.M.A.8</td>
<td>Negative</td>
<td>Medium thin rod</td>
<td>Singly and in pair</td>
</tr>
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<td>O.M.C1.1</td>
<td>Negative</td>
<td>Small thin rod</td>
<td>Singly and in pair, most of in “V” shape</td>
</tr>
<tr>
<td>O.M.C1.2</td>
<td>Positive</td>
<td>Short thin rod</td>
<td>Singly and in pair</td>
</tr>
<tr>
<td>O.M.C1.3</td>
<td>Positive</td>
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<td>Singly</td>
</tr>
<tr>
<td>O.M.C1.4</td>
<td>Positive</td>
<td>Short thick rod</td>
<td>Singly and in clusters</td>
</tr>
<tr>
<td>O.M.D1.6</td>
<td>Variable</td>
<td>Short thick rod</td>
<td>Singly and in pair</td>
</tr>
<tr>
<td>O.M.D1.7</td>
<td>Positive</td>
<td>Small cocci</td>
<td>Singly and most of in pair</td>
</tr>
<tr>
<td>O.M.D1.8</td>
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<td>Singly</td>
</tr>
<tr>
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<td>Singly</td>
</tr>
<tr>
<td>O.M.D1.15</td>
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<td>Singly and in cluster</td>
</tr>
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<td>O.M. E1.2</td>
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<td>Very small cocci</td>
<td>Singly and in cluster</td>
</tr>
<tr>
<td>O.M. E1.1</td>
<td>Positive</td>
<td>Small cocci</td>
<td>Singly</td>
</tr>
<tr>
<td>O.M.A.2</td>
<td>Negative</td>
<td>Short thick rod</td>
<td>Singly and in pair</td>
</tr>
<tr>
<td>O.M.A.3</td>
<td>Positive</td>
<td>Short thick rod</td>
<td>Singly</td>
</tr>
<tr>
<td>O.M.A.4</td>
<td>Positive</td>
<td>Very short thick rod, with middle spore</td>
<td>Singly and most of in pair and chains</td>
</tr>
<tr>
<td>O.M.A.5</td>
<td>Variable</td>
<td>Small thin rod</td>
<td>Singly and in pair</td>
</tr>
<tr>
<td>O.M.A.7</td>
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<td>Short thick rod</td>
<td>Singly</td>
</tr>
<tr>
<td>O.M. A8</td>
<td>Positive</td>
<td>Small cocci</td>
<td>Singly and in clusters</td>
</tr>
<tr>
<td>O.M. B2.8</td>
<td>Positive</td>
<td>Small oval shape cocci</td>
<td>In tetrad only</td>
</tr>
<tr>
<td>O.M.A1.1</td>
<td>Negative</td>
<td>Thick rod</td>
<td>Singly and in pair</td>
</tr>
<tr>
<td>O.M.A4.1</td>
<td>Positive</td>
<td>Very Short thick rod</td>
<td>Singly</td>
</tr>
<tr>
<td>O.M.A6.1</td>
<td>Positive</td>
<td>Very Short thick rod</td>
<td>Singly</td>
</tr>
<tr>
<td>O.M.A8.1</td>
<td>Positive</td>
<td>Short thick rod</td>
<td>Most of singly and some in pair</td>
</tr>
<tr>
<td>O.M.C1.7</td>
<td>Positive</td>
<td>Long thin rod</td>
<td>Singly</td>
</tr>
<tr>
<td>O.M.C2.1</td>
<td>Positive</td>
<td>Small cocci</td>
<td>Singly</td>
</tr>
<tr>
<td>O.M.C1.3</td>
<td>Positive</td>
<td>Small cocci</td>
<td>In pair and in clusters</td>
</tr>
<tr>
<td>O.M.C1.4</td>
<td>Positive</td>
<td>Very small cocci</td>
<td>Singly and in tetrad</td>
</tr>
</tbody>
</table>

Table 3.3.6: Characterization of organism on the basis of Gram’s reaction
with respect to O.M.6.5; all the 18 isolates were negative and none of them were gram positive. From, majority of the isolates only 10% were of gram variable character for O.M.6.5 while none of such isolate was noticed in O.M.6.5 (Table 3.3.6).

According to literature, moderate halophiles with Gram negative characteristics have been studied in great detailed while information relating to Gram positive is scarce (Mormile et al., 1999). Our studies on O.M.6.2, however, highlighted the dominance of the Gram positive organisms over the Gram negative ones. From the literature, Gram negative haloalkaliphilic bacteria appear to be widely described (Xin et al., 2001; Doronina et al., 2003a; Doronina et al., 2003b; Loiko et al., 2003; Hoover et al., 2003; Banciu et al., 2004; Romano et al., 2002). We found similar results for O.M.6.5 where we have found all the isolates were gram negative in nature. Some of our isolates displayed Gram variable properties. With respect to cell shape, coccid shape was widely observed among the bacteria enriched at higher NaCl concentrations (15-20%); where as the rod shape was frequently distributed at lower NaCl concentration (10%) for O.M.6.2, similar results were obtained by Joshi (2006) from haloalkaliphiles isolated from sea water of coastal Gujarat. However, similar observation was not observed for O.M.6.5.

With respect to cell arrangement; among all the isolates of the O.M.6.2, approx. 55% of total isolates were singly and in pair and 35% were singly and 5% were singly and in clusters and 5% were tetrad in nature, interestingly O.M.C1, displayed very unique pattern of the cell arrangement in “V” shape (Fig. 3.3.4 A and B). Different diversity profile was observed from both the sample. There were not much peculiar characteristic features observed of bacteria isolated from this site. Although the sites of isolation were quite nearby, there is much diversity noticed among the total isolates from both the sites (Fig. 3.3.4 C and 3.3.4 D).

3.3.4.3 Biochemical characterization

In the present day of increasing emphasis on the molecular tools and chronometers, the metabolic and physiological status of the organisms is still important to diversify and differentiate organisms. The microorganisms have their own identifying biochemical characteristics. These biochemical fingerprints are the properties controlled by the cell’s vital molecules and they are responsible for the bioenergetics, biosynthesis and biodegradation.
With these objectives, the biochemical and metabolic activities of all the isolates were studied for the further differentiation and characterization. The detail outline for the biochemical reactions of all the isolates is depicted in Table 3.3.7, Fig. 3.3.5A and 3.3.5E. Among isolated haloalkaliphilic bacteria, approximately 70-75% of the isolates were catalase and oxidase positive, although the extent of the production of catalase varied among the isolates of the same site as well of the other sites (Fig. 3.3.5B). Isolates from O.M.6.5 site were more catalase positive than O.M.6.2 (Fig. 3.3.5C and 3.3.5D). Organisms were also able to utilize and generate diversified result with respect to other biochemical parameters. Maximum organism were able to utilize citrate with 62%, ammonia production was noticed to be 55%, while gelatin utilization was 42%, around 33% of the total isolates were noticed producing \( \text{H}_2\text{S} \) gas. Nitrate reduction and casein hydrolysis was by 22% of the isolates. Indole production was by 10% of the isolates. However extent of utilization was quiet variable among the positive isolates (Fig. 3.3.5E, Table 3.3.7).

On analyzing, the profile of the individual sites for the different biochemical activities, isolates of the O.M.6.2 site were more positive towards catalase with 100%, while for O.M.6.5 it was around 60% (Fig. 3.3.5B). However, oxidase positive were distributed equally. Citrate utilizers were more noticed in O.M.6.5, with 90%; significant numbers i.e. 78% were noticed in the other studied site. Amylase producers were around one-fifth of total isolates in O.M.6.2 and O.M.6.5 judged on the basis of starch liquifications. Overall, 29% isolates were screened as \( \text{H}_2\text{S} \) producers, Significant difference were seen in ammonium production; 90% of the isolates were positive in O.M. 6.5 while in O.M.6.2, its number were reduced to half. While reverse observation was noticed in nitrate reduction, \( \text{H}_2 \text{S} \) gas production and urea utilization, for O.M.6.5 was 62, 42 and 48%, while for O.M.6.5 it was only 10% of isolates able to generate positive results for all the three above mentioned tests. With respect to gelatin liquefaction, which was an important parameter for functional attributes of protease enzyme, it was known that almost half of the isolates were positive. However, for all the positive results, extent of positivity was a variable feature.

With reference to TSI test, maximum alkaline reaction was observed in the slant and butt of isolates of both the sites. However, if we observe the data within the specific site O.M.6.2, slants were more alkali with 64%. 
found 42% of the total isolates which are able to
produce urease activity among 71 extremely halophilic strains but only 4 were able to
secret urease. These results were also supported by Mizuki and his co-workers, who
screened for the urease activity among 71 extremely halophilic strains but only 4 were able to
generate positive reaction in urea.

For the site O.M.6.5, both slants and butt were found to be of alkaline nature with 66 -
68% (Table 3.3.8, Fig. 3.3.6). Although, site of isolation of all isolates is within the
same coastline, the extent of catalase production varied significantly among the
isolates. Organisms from O.M.6.2 were found to be highly aerobic, as all the isolates
were catalase positive and 92% of them were oxidase positive. For, O.M.6.5, we can
say that they were moderate aerobic in nature, as around 40% of the studied isolates
were catalase test negative. The variation in O₂ requirements reflects the differences
in bio-oxidative enzyme systems presents in the organisms. Only 10% of the total
isolates form both the sites were indole positive, which suggest lack of tryptophanase
in these organisms. Similarly, only 10% of organisms from site O.M.6.5 were able to
utilize urea.

These results were also supported by the literature where many moderately halophilic
and alkaliophilic bacteria did not produce indeole or utilized urea (Mota et al., 1997;
Muntyan et al., 2002; Reddy et al., 2003; Romano et al., 2005; Dodia, 2005). The
results were further supported by Mizuki and his co-workers, who screened for the
urease activity among 71 extremely halophilic strains but only 4 were able to secret
the urease (Mizuki et al., 2004). Results of O.M.6.5 contradict to O.M.6.2 as we
found 42% of the total isolates which are able to generate positive reaction in urea.

Table 3.3.7: Biochemical profile of haloalkaliphilic organism (Color Indications: Red
– Negative, Yellow – Partial Positive, Blue – Positive).

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Amonia Production</th>
<th>Nitrate Reduction</th>
<th>Indole Production</th>
<th>H₂S Prod</th>
<th>Urea Hydrolysis</th>
<th>Gelatin Hydrolysis</th>
<th>Casein Hydrolysis</th>
<th>Arsenite Hydrolysis</th>
<th>Citrulate Utilization</th>
<th>Catalase</th>
<th>Oxidase</th>
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73
The isolates varied extensively with respect to \( \text{H}_2\text{S} \) and ammonia production and nitrate reduction, which clearly reflected the metabolic diversity among them. \( \text{H}_2\text{S} \) production was maximally produced by O.M.6.2 site with 42\%, while for the other site, it was only one-tenth of total isolates displaying positive results. In the site O.M.6.5, organisms were more ammonia utilizers with 90\%, while was only 10\% in the site O.M.6.2. However, as revealed in the literature, a number of haloalkaliphilic bacteria possess the ability of nitrate reduction (Vreeland et al., 1980; Mormile et al., 1999; Sorokin et al., 2003a, b). Results of \( \text{H}_2\text{S} \) and ammonia production are of vital importance as they can utilize sulfur-containing amino acids as a carbon source from the protein-rich medium. This may also imply that the concerned habitats are rich in proteinaceous substances occupying the nutritional dynamics where easily utilizable carbohydrates are scares. The high-energy requirement of these organisms could also be attributed to the energy required for the synthesis and transport of compatible solutes to compensate the high osmotic pressure present in the surroundings. In general much of the diversity was observed among the sites, as well characteristic features of the organism with respect to its site of isolation, energy requirement and nutrient parameters differ to an extent.

**Fig. 3.3.5A:** Overall profile of biochemical test for total number of positive isolate

**Fig. 3.3.5B:** Overall % scenario of biochemical test
Fig. 3.3.5C: % of positive isolate for O.M.6.2

Fig. 3.3.5D: % of positive isolate for O.M.6.5

Fig. 3.3.5E: Overall representation of biochemical test
Fig. 3.3.6: TSI Profile of isolates, where slant (■), Butt (■), Gas (■).
Table 3.3.8: Triple Sugar Ion test of haloalkaliphilic organisms

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<tr>
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<th>Butt</th>
<th>Gas</th>
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3.3.4.4 Sugar fermentation

Ability of the organisms to metabolize different sugars for the bioenergetics purpose is one of the approaches to diversify the organisms. The extent of sugar utilization highly varied among the isolates from the same site as well as those from different site. None of the isolates were able to produce gas in Durham’s tube (Table 3.3.9). Similarly, none of them were able to ferment ribose sugar.

For O.M.6.2; around 25% of total isolates were able to utilize glucose and sucrose as a carbon source. Utilization of maltose and manitol was by around 20% of the isolates. Lactose was utilized by half of the total isolates utilizing glucose and
sucrose. Striking point emerged is that only O.M.E1 as a candidate was able to utilize xylose sugar (Fig. 3.3.7A and 3.3.7B). An interesting point was noticed that each isolates as an individual was able to consume only single sugar for their metabolic activities and growth. In much simpler way, e.g. the organism displaying positive test with glucose was displaying negative results with other six sugars tested. This was noticed as a general trend for all the isolates, except O.M.A1, O.M.D18 and O.M.D16. O.M.A1 was able to utilize maltose, lactose, mannitol and sucrose. Among this, four positive sugars with maltose it showed partial positive result, while with other three the utilization was profound (Fig. 3.3.7C and 3.3.7D).

For O.M.6.5; maximum amount of isolates were able to utilize maltose as an energy source with total 55% of total isolates. 15% of isolates were burning glucose for carbon and 10% of isolates used lactose. Only 5% of bacteria used partially mannitol and sucrose. The results were quite contrasting in terms of intake of sugar with respect to O.M.6.2. In this case, utilization of such disaccharides, compared to simple carbon sources, suggested the adaptation of different metabolic pathways for their energy generation. *Spirochaeta americana* sp. nov, a haloalkaliphilic, obligately anaerobic, Gram negative spirochaete (Hoover et al., 2003) and alkaliphilic and moderately halophilic strain *Salinicoccus alkaliphilus* sp. nov. (Zhang et al., 2002) were able to utilized range of sugars such as D-glucose, fructose, maltose, lactose, sucrose, starch and D-mannitol.
### Table 3.3.9: Sugar utilization profile of isolates

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<tr>
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**Fig. 3.3.7A:** Characterization of isolates for sugar utilization for O.M.6.2

**Fig. 3.3.7B:** % of isolates utilizing sugar by O.M.6.2 site

**Fig. 3.3.7C:** Characterization of isolates for sugar utilization for O.M.6.5

**Fig. 3.3.7D:** % of isolate utilizing sugar for O.M.6.5
3.3.5 Phylogenetic identification

3.3.5.1 16S rRNA amplification, nucleotide sequencing and homology prediction

Potential isolate showing interesting results for biocatalytic studies; organism designated as O.M.A18, O.M.E12, O.M.C14 from O.M.6.2 site and O.M.C28 from O.M.6.5 site were identified on the basis of 16S rRNA gene homology. As described in materials and method, the 1500bp rRNA gene was amplified by using forward and reverses primers. Fig. 3.3.8, Table 3.3.10, 3.3.11 provides the aligned sequenced data of 1501 bp. The sequence data were further analyzed for finding the closest homologs for the microbe by comparing gene sequence with reference strains. Using consensus primers, the ~1.5 kb 16S rDNA fragment was amplified using high-fidelity PCR polymerase. The PCR product was bi-directionally sequenced using the forward, reverse and an internal primer. Sequence data was aligned and analyzed for finding the closest homologs for the microbe. The alignment view table (Table 3.3.10); distance matrix (Table 3.3.11) and phylogentic position studied by Mega align software (Fig. 3.3.8).

![Fig.3.3.8A: Phylogenetic Tree made in MEGA 3.1 software using Neighbor Joining method for O.M.A18](image-url)
Fig. 3.3.8B: Phylogenetic Tree made in MEGA 3.1 software using Neighbor Joining method for O.M.E₁₂

Fig. 3.3.8C: Phylogenetic Tree made in MEGA 3.1 software using Neighbor Joining method for O.M.C₈
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<td>0.85</td>
<td>Bacillus halodurans strain C7</td>
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**Table 3.3.10:** Alignment view using combination of NCBI GenBank and RDP database for O.M.A18 and O.M.E12
**Table 3.3.11:** Distance Matrix based on Nucleotide Sequence Homology (Using Kimura-2 Parameter) for O.M.A₁₈ and O.M.E₁₂ (indicates nucleotide similarity (above diagonal) and distance (below diagonal).
**Aligned Sequence Data of O.M.A**: size (1447bp)

GCTGGCGCCTGCTAATCATGCAAGTCGAGCGCAGGAAGTTATCTGATCCTCTTTAGAGGTGACGATAATGGAATGAGCGGCGGACGGGTGAGTAACACGTAGGCAACCTGCCTGTAAGACTGGGATAACTCGTGGAAACGCGAGCTAATACCGGATAACACTTTTCATCTCCTGATGA

GAAGTTGAAAGGCGGCTTTTGCTGTCACTTACA

GATGGGCCTGCGCCTGACTCCTGCTAAGGAAATGCGTAGAGCCGCAACGTGGACCGGATCGAGACCCGGTAAACCTGCTGCAGGCGGTTCTTTAAGTCTGATGTGAAATCTTACGGCTCAACCGTAAACGTGCATTGAGAACTGGGGACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAG

ATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGC

GAACGGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAGGTGTTAGGGGTTTCGATACCCGTAGTGCCAAGCAACGATTAAGACTCCGCCTGGGAGACACCGCAGGTTGAAACTCAAAGGAATGACGGGGCCGCACAACGCCTGGAGCATGTGGTTTAATTCGACGCCACGCGAAGAACCTTACCCGGTCTTGACAACACTCCTCGCCCGCTGGCGCTGGCGTTTTGAGCCAGCCTGGGCAAGGTGGGGCCGGTGATTGGGGTGGAAGTCGTA

GAAACTGCGCAGACTTGGAGTACAGAAAGAGAGATGGGAATTCACCGTGATCGGTGAAATTCGCTAGAGATGCTGAGGGAGGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAGGTGTTAGGGGTTTCGATACCCGTAGTGCCAAGCAACGATTAAGACTCCGCCTGGGAGACACCGCAGGTTGAAACTCAAAGGAATGACGGGGCCGCACAACGCCTGGAGCATGTGGTTTAATTCGACGCCACGCGAAGAACCTTACCCGGTCTTGACAACACTCCTCGCCCGCTGGCGCTGGCGTTTTGAGCCAGCCTGGGCAAGGTGGGGCCGGTGATTGGGGTGGAAGTCGTA

**Aligned Sequence Data of O.M.E**: size (1485 bp)

AGAGTTTGTATCATGCTCGACGACCGCCGACCGTCGCTGCTAATACATGCAACTCGAGCGAACCCGGGGT

GCTTGGCCTGCGCCTGACTCCTGCTAAGGAAATGCGTAGAGCCGCAACGTGGACCGGATCGAGACCCGGTAAACCTGCTGCAGGCGGTTCTTTAAGTCTGATGTGAAATCTTACGGCTCAACCGTAAACGTGCATTGAGAACTGGGGACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAG

ATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGC

GAACGGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAGGTGTTAGGGGTTTCGATACCCGTAGTGCCAAGCAACGATTAAGACTCCGCCTGGGAGACACCGCAGGTTGAAACTCAAAGGAATGACGGGGCCGCACAACGCCTGGAGCATGTGGTTTAATTCGACGCCACGCGAAGAACCTTACCCGGTCTTGACAACACTCCTCGCCCGCTGGCGCTGGCGTTTTGAGCCAGCCTGGGCAAGGTGGGGCCGGTGATTGGGGTGGAAGTCGTA

GAAACTGCGCAGACTTGGAGTACAGAAAGAGAGATGGGAATTCACCGTGATCGGTGAAATTCGCTAGAGATGCTGAGGGAGGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAGGTGTTAGGGGTTTCGATACCCGTAGTGCCAAGCAACGATTAAGACTCCGCCTGGGAGACACCGCAGGTTGAAACTCAAAGGAATGACGGGGCCGCACAACGCCTGGAGCATGTGGTTTAATTCGACGCCACGCGAAGAACCTTACCCGGTCTTGACAACACTCCTCGCCCGCTGGCGCTGGCGTTTTGAGCCAGCCTGGGCAAGGTGGGGCCGGTGATTGGGGTGGAAGTCGTA

85
**Aligned sequence data of O.M.C28** (1450bp)

AAGTTGTGGGACGGCCGAGCCGCGGCTGAGTGGTGAACCGTGGAACCTCGCTGAGTAAACGATGCCTGAACCTGGGATAACTCGCGGAAACGC
AGCTAGGCGCCACCCAGTACCTGACTGAAGCGGCCGAGGCGCAGTACAGGGAACTCTTGCCGCAATGGGAAAACTCTGACAGCAGCAGCC
CGAGTGAGATTAGGTTTTTCTAGGATCTGACAATTCTGTCGTGAGGTTCCGCGCCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCG
AGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGC
CGCGTGAGTGAGATTAGGTTTTTCTAGGATCTGACAATTCTGTCGTGAGGTTCCGCGCCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCG
Based on nucleotides homology and phylogenetic analysis the microbe (Sample: OM C28) was detected to be Haloalkaliphilic bacterium (GenBank accession number: EU118361). Nearest homolog genus-species was found to be *Bacillus pseudofirmus* (accession no. EU090232). Information about other close homologs for the microbe can be found from the alignment view table (Table 3.3.11) its distance matrix is displayed in (Table 12). The relatedness is displayed in Fig.3.3.8.

**Alined sequence data of O.M.C28** (1450bp)

AAGTTGTGGGACGGCCGAGCCGCGGCTGAGTGGTGAACCGTGGAACCTCGCTGAGTAAACGATGCCTGAACCTGGGATAACTCGCGGAAACGC
AGCTAGGCGCCACCCAGTACCTGACTGAAGCGGCCGAGGCGCAGTACAGGGAACTCTTGCCGCAATGGGAAAACTCTGACAGCAGCAGCC
CGAGTGAGATTAGGTTTTTCTAGGATCTGACAATTCTGTCGTGAGGTTCCGCGCCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCG
Based on nucleotides homology and phylogenetic analysis the Microbe (Sample: OM C28) was detected to be *Oceanobacillus oncorhynchi* (GenBank accession number: EU118361). Nearest homolog genus-species was found to be *Oceanobacillus sp.* (accession no. AY553089).
3.3.5.2 FAME Analysis

Microbial fatty acid profiles are unique from one species to another, and this has allowed for the creation of very large microbial libraries.

O.M.A8 Datasheet

Volume: DATA            File: E089028.04A        Samp Ctr: 3                  ID Number: 1257

Type: Samp Bottle: 2                Method: RTSBA6

Created: 9/2/2008 7:35:13 PM

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<th>ECL</th>
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<th>Percent</th>
<th>Comment</th>
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<td>----</td>
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ECL Deviation: 0.003                             Reference ECL Shift: 0.002
Number Reference Peaks: 11                     Totals Named: 200394
Total Response: 202535                          Total Amount: 189750
Percent Named: 98.94%                          
**O.M.E$_1$2 Datasheet**

**Volume:** DATA  **File:** E089028.04A  **Samp Ctr:** 6  **ID Number:** 1260

**Type:** Samp  **Bottle:** 5  **Method:** RTSBA6

**Created:** 9/2/2008 8:00:41 PM

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**Library**  |  **Sim Index**  |  **Entry Name**
---|---|---
RTSBA6  |  6.00  |  Geobacillus-stearothermophilus-GC subgroup A (55C, Bacillus)

---

**RT**  |  **Response**  |  **Ar/Ht**  |  **RFact**  |  **ECL**  |  **Peak Name**  |  **Percent**  |  **Comment**
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0.7344  |  2.224E+9  |  0.017  |  ----  |  6.6441  |  SOLVENT  |  ----  |  < min rt
2.1297  |  4558  |  0.009  |  0.989  |  13.6282  |  14:0 iso  |  6.99  |  ECL deviates
2.2399  |  1620  |  0.009  |  0.976  |  14.0005  |  14:0  |  2.45  |  ECL deviates
2.4371  |  16776  |  0.008  |  0.957  |  14.6311  |  15:0 iso  |  24.89  |  ECL deviates -
2.4663  |  23538  |  0.009  |  0.954  |  14.7247  |  15:0 anteiso  |  34.83  |  ECL deviates
2.7556  |  5719  |  0.009  |  0.933  |  15.6334  |  16:0 iso  |  8.27  |  ECL deviates
2.8732  |  7255  |  0.009  |  0.926  |  16.0000  |  16:0  |  10.42  |  ECL deviates
3.0779  |  1754  |  0.009  |  0.918  |  16.6374  |  17:0 iso  |  2.50  |  ECL deviates
3.1086  |  5184  |  0.009  |  0.917  |  16.7330  |  17:0 anteiso  |  7.38  |  ECL deviates
3.4608  |  785  |  0.011  |  0.915  |  17.8365  |  Sum In Feature  |  1.11  |  ECL deviates -
3.5129  |  814  |  0.009  |  0.916  |  17.9998  |  18:0  |  1.16  |  ECL deviates
3.7341  |  628  |  0.012  |  ----  |  18.7110  |  ----  |  ----
4.0380  |  626  |  0.010  |  ----  |  19.7040  |  ----  |  ----
4.2713  |  1016  |  0.021  |  ----  |  20.4712  |  ----  |  > max rt
----  |  785  |  ----  |  ----  |  Summed  |  1.11  |  18.1 w/c
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Reference ECL Shift: 0.002
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Total Named: 68003
Percent Named: 98.19%
Total Amount: 64479

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3.3.6 Antibiotic resistance and sensitivity

The haloalkaliphilic bacteria were highly diversified in terms of their antibiotic sensitivity and resistance property. The detailed antibiotic profile for all the isolates is depicted in Table 3.3.12. The strategy conceived for antibiotic profiling were gram-positive organisms were checked for antibiotics related to gram positive nature and gram negative were assessed for gram negative features. From, the total isolates from gram reaction it was observed that all the isolates were gram positive in O.M.6.2 site.
and in O.M.6.5 majority of isolates were gram positive; however few isolates were gram negative. The profile of the sensitivity varied among the isolates from the same site and those from the other site. Some of the antibiotics used were specific for both, Gram positive and negative bacteria viz., Tetracyclin, Co-Trimaxazole, Ampicillin, Gentamycin, Cephotaxime and Nalidixic Acid.

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<th>Isolates with gram positive character from O.M.6.5</th>
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**Table 3.3.12A:** Antimicrobial properties of gram positive isolates for O.M.6.5

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**Table 3.3.12B:** Antimicrobial properties of isolates for O.M.6.2 site
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</table>

Table 3.3.12C: Antimicrobial properties of isolates for O.M.6.2 site

**Fig.3.3.9A:** % of isolates displaying positive results in antimicrobial test for O.M.6.5

**Fig.3.3.9B:** Distribution of organism on the basis of antimicrobial test
Fig. 3.3.9C: % of positive tests for O.M.6.2 Gram positive isolates

Fig. 3.3.9D: Distribution of organism on the basis of antimicrobial tests for O.M.6.2

Fig. 3.3.9E: % of positive tests for O.M.6.5 Gram positive isolates

Fig. 3.3.9F: Distribution of organism on the basis of antimicrobial tests for O.M.6.5
Overall, for O.M.6.5 site; the isolates were highly resistance towards Cu, Se, Cw, Ce, Ro, Cf, while in response to Cs and Cq around 14% of isolates were sensitive, however sensitivity towards Ax was more than 50% (Fig. 3.3.9.1). With reference, to gram positive isolates of O.M.6.2; it was observed that all the isolates were having 85% sensitivity towards all the tested antibiotics except Ak and Ce (Fig.3.3.9.2). With respect to gram negative isolates; there was not much diversity noticed and all the isolates were found sensitive to the array of the antibiotics (Fig. 3.3.9.3). In comparison to O.M.6.2, isolates of O.M.6.5 were found more sensitive (Table 3.3.12).

In general, the isolates were sensitive against antibiotics that affect purine biosynthesis and DNA replication. Isolates showed higher degree of resistance against antibiotics which affect the protein synthesis. However, the molecular and genetic basis of the resistance remains to be unexplained and further investigations would be required in this context. Nevertheless, antibiotic resistance genes, in general, are plasmid born (Vargas and Nieto, 2004).

3.3.7 Screening isolates for functional attributes by detecting proteolytic activity

All the isolates earlier studied for other classical and molecular parameters to judge their diversity profile; were also assessed for its proteolytic activity by subjecting them to protein rich medium under sets of conditions as described in materials and methods sections. Efficiency of proteolytic secretion was done at varying physico-chemical parameters; pH, temperature and NaCl (Table 3.3.13).

Among the total isolates screened from both the isolates; it was found that isolates of O.M.6.2 were able to secret protease in large amount as compared to other site. From total isolates screened for protease secretion on gelatin agar plate; total 17 isolates were found protease positive. Within them around 78% were from O.M.6.2 (Fig. 3.3.10.2). In O.M.6.2; it was observed that two isolates O.M.A16 and O.M.A18 were able to produce profound amount of enzyme. All the isolates enriched at pH-10 with NaCl 10% and pH-10 with NaCl-30% were protease positive along with O.M.E12.
Table 3.3.13: Screening for extracellular enzyme (proteases) in isolated cultures; effect of physico-chemical factors (pH, temperature and NaCl) on enzyme secretion. Screening was done on gelatin agar plate.

3.3.7.1 Protease secretion with varying NaCl concentration

Efficiency of protease secretion and growth was judged by varying salt concentration in the range of 0-25%. In detail; isolate O.M.A.6 was able to grow in range of 0-15%; while protease secretion was noticed in 0 and 5% NaCl. Isolate O.M.A.7 was unable to secrete protease however, organism were able to grow upto 20% of NaCl concentration. Similarly, O.M.A.8 was able to secrete enzyme upto 10; however with increase in NaCl, enzyme secretion was reduced and growth was enhanced. It is clearly demonstrated there is not straightforward role between growth and enzyme secretion.
secretion among the isolates, in fact enzyme secretion is noticed only among the early growth. Along the same line; halotolerant organisms would have adopted to some alternate strategy to grow in protein rich medium without secreting protease into the medium. Both O.M.B\textsubscript{2}3 and O.M.B\textsubscript{2}8 were able to grow and secrete protease in range of 0-10% NaCl, O.M.B\textsubscript{2}8 was secreting protease in 5-10% NaCl. As organism was unable to secret enzyme at 0% NaCl concentration; it clearly demonstrates that although organism were not able to secrete enzyme in higher range of concentration; it was mandatory for enzyme secretion, this describes the haloalkaline nature of the studied isolates.

Similar results were obtained for C series of organisms from same site. Within the D series of the isolates; the unique feature which was observed that with increase in NaCl concentration, the growth of organisms were increased 9-10 times in D\textsubscript{1}13, D\textsubscript{1}14; D\textsubscript{1}17; D\textsubscript{1}18.; although the organisms were halotolerant in nature, the increase in growth with increase in NaCl emerged as a noticeable feature (Fig 3.3.10). The moderate haloalkaliphilic organism with unique features was O.M.E\textsubscript{1}2, an organism was able to grow and secrete enzyme in range of 5-20%, with optimum in range of 10-20% NaCl (Table 3.3.13).

**3.3.7.2 Effect of pH on enzyme secretion**

For all the protease producers; effect of pH was assessed on enzyme secretion profile. The variable pH of enzyme; pH-8-11 was set by supplementing 20% Na\textsubscript{2}CO\textsubscript{3}. All the organisms were able to secrete enzyme in alkaline environment. Organisms were able to secret protease in a broader range from pH-8 to 10, for O.M.E\textsubscript{1}2 it was up to 11 pH. Optimum level of secretion was noticed at pH-9 for all isolates, except O.M.E\textsubscript{1}2 and O.M.B\textsubscript{2}3 having optimum pH-11 and 8 respectively for enzyme secretion. There was no variable pattern observed for enzyme secretion for both the sites, no striking diversity was observed with respect to pH (Table 3.3.13).

**3.3.7.3 Effect of Temperature on enzyme secretion**

The effect of temperature was analyzed on the enzyme secretion profile; protease enzymes from all the isolates were having optimum temperature 37°C; except O.M.C\textsubscript{2}3 and O.M.C\textsubscript{2}4 isolates of O.M.6.5 having 50°C. All the isolates were able to secrete enzyme in quite broad range from 37-50°C; however organisms of D series from O.M.6.2 site were able to secrete enzyme only at 37°C (Table 3.3.13).
Fig. 3.3.10: Effect of NaCl on growth and enzyme secretion on different organism of O.M.6.2 and O.M.6.5 site.
Contd...

Colony Diameter (mm) vs. Ratio (m/z)

- O.M.C₂₄
- O.M.C₂₃
- O.M.D₁₄
- O.M.D₄₁₃
- O.M.D₇
- O.M.D₁₈
- O.M.E₁(2)

NaCl (%)
3.3.8 Alkaline protease amplification

All the sets of primers designed for alkaline proteases were used for amplification (Table 3.3.14). The detailed description of designed primer and its specificity is as described in materials and methods. For, amplification of approx. 700-bp to 1.2 kb ORF of the protease gene, the genomic DNA of protease positive strains were isolated. PCR reactions were carried out at three gradient of annealing temperatures using Gradient Thermocycler (Eppendorf). By using different primers, PCR reactions were carried, to ensure complete amplicon generation. For O.M.E12; amplicon size of product obtained from SPS-6 was of aprox. 1kb.

The concentration of product varied with respect to gradient of temperature and primer pair used for the amplification profile generation (Fig.3.3.14). Partial amplified product was generated by SPS- 1, 4, 5. For O.M.A18; amplicon were generated by using three different sets of primer; SPS-3, 4, 7. A quite satisfactory product size was obtained, SPS-3 and SPS-4 generated 1 and 1.1 kb band, however SPS-7 generated 0.8 kb band. For O.M.A16, only partial amplified products were obtained of 0.3 kb with SPS-1 and 0.5 kb with SPS-5 and SPS-7 (Fig 3.3.11). With reference to O.M.C14, SPS-3 generated amplified band with product size of 1kb while SPS-1 generated only partial amplified band of product size 0.5 kb. For O.M.C13 a partial amplified products were generated by SPS-5, 6 and 7. For O.M.C14; SPS-4 generated intense amplified product of 1 kb while SPS-3 generated only partial product.

In general, numbers of amplicon, with product size ranging from 1.2-0.5 kb, were visualized on agarose gel. While one of the reasons for the multiple bands from the site could be due to the annealing of the primer at different sites within the template. The different size of products was tapered with reference to primer pair combinations, which is primarily due to specificity of primer sequence with template sequence at variable positions. The product size was also dependent on Ta; and there was a noticeable change in concentration of product with gradient of annealing temperature. For assessment and quantification of PCR products; amplicons were resolved on an agarose gel. Amplification of template was found with multiple primers.
Fig. 3.3.11: Amplification profile for potential alkaline proteases producers.

3.3.9 Environmental Studies

Sea water and soils are often contaminated with heavy metals or other compounds from anthropogenic manufacturer of chemicals and oil industries Oren et al., 1992; Margesin and Schinner, 2001). Thus, protecting the integrity of our biosphere resources is one of the most essential environmental issues of 21st century. Usually, biodegradation of commercial dyestuff is the friendliest method as it does not require
A large amount of energy and does not generate toxic substances. Conventional microbiological treatment employing normal flora do not function at alkaline pH and high salt concentrations. To cope with this problem, we tried to explore the dye degradation potential of haloalkaliphilic bacteria. Our initial studies on dye decolorization indicated that some of our haloalkaliphilic bacterial strains could decolorize the azo dyes within 6h of incubation. Among 3 isolates studied; O.M.E_12, O.M.A_18 and O.M.C_28 we found that O. M. C_28, was potent candidate for the decolorization of consortium of dyes. In secondary screening we emphasized specially with various parameters as same as before we proceeded for other strain.

In addition to that each parameters where checked for influence of NaCl concentration (0%, 5% and 10%) in dye decolorization. The extent of decolorization enhanced under shaking condition at higher concentrations. Such properties would be useful in biological waste treatment and bioremediation purpose. The progressive growth and decolorization of acidred-4 dye by strain O.M.C_28 under static and shaking condition with respect to incubation time from 0-31 hr were noted for each salt concentration (Table 3.3.15).

<table>
<thead>
<tr>
<th>Time in hr</th>
<th>Incubation in static condition</th>
<th>Incubation in shaking condition</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Growth control (660nm)</td>
<td>Growth (660nm)</td>
</tr>
<tr>
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**Table 3.3.15:** Secondary screening of strain C-28 (5% NaCl concentration) to access the dye (Acidred-4) decolorizing potential in static and shaking flask conditions.
The study highlighted in this chapter, basically focuses on haloalkaliphilic bacterial diversity and phylogeny, the study was planned to assess the microbial heterogeneity among the haloalkaliphilic bacteria isolated from the unexplored habitat of Gujarat by using certain conventional and traditional approaches. Such primary and basic information’s are really helpful to understand the biochemical, physiological and genetic basis of these organisms. Besides, their investigation is likely to generate enough knowledge towards many basics questions of biology. We have attempted to explore some unique and novel properties of the organisms.