# Microbial diversity at marine salterns near Bhavnagar, Gujarat, India

Marine salterns are artificial, extremely thalassohaline environments consisting of a discontinuous salinity gradient, which are commercially operated for producing NaCl[1–3]. Different marine salterns have been studied throughout the world, including salterns in Alicante, Spain; Mexico, San Diego and Newark, California; Bonaire, the Netherlands; Eilat, Israel and Margherita di Savoia, Italy, etc.[2–5]. The red-orange colour of the crystallizer ponds is due to the presence of both red halophilic Archaea and *Dunaliella*[4]. The main genera of the bacteria isolated from salterns located near Huelva, SW Spain, facing the Atlantic Ocean include non-halophilic *Staphylococcus*, *Bacillus*, *Micrococcus*, moderately halophilic *Deleya*, *Flavobacterium*, *Acinetobacter*, extreme halophilic *Halobacterium*, *Haloarcula*, *Halococcus* and marine halophiles *Pseudomonas*, *Alteromonas*, *Deleya*, *Micrococcus*, *Staphylococcus*, *Bacillus* and *Vibrio*[6].

Major saline sites studied in India are Sambhar salt lake, Rajasthan; coastal regions of Gujarat, Tamil Nadu, Maharashtra, Andhra Pradesh, Orissa and West Bengal. There are about 10,000 salt-production sites all over the country. One of the largest marine chemical complexes in Southeast Asia is at Tata Chemicals Limited (TCL), Mithapur, located on the west coast of Gujarat. Major salt-producing sites in Gujarat are coastal regions of Kutch and Saurashtra at Kandla, Jamnagar, Maliya, Mithapur, Porbandar and Bhavnagar[7–9]. The marine salterns located between Dholera and Bhavnagar, Gujarat, have not been given much attention even though they are being used for salt production for several decades. The present research is an effort to study the microbial diversity of this site.

Samples were collected from marine salterns located at 22°10′N and 72°15′E on western and northern coasts of the Gulf of Khambhat, Arabian Sea, about 25–30 km north of Bhavnagar[10]. Samples were collected in May 2003 when mean day temperature was 40 ± 2°C. The water, salt, stone and soil with salt samples were collected in sterile plastic screw-cap bottles or plastic bags by inserting the sterile stainless steel sampler at least 10 cm deep into the soil containing salt. Within 24 h of collection, the samples were brought to the laboratory. One gram weight of each sample was mixed to get a composite sample in sterile 100 ml distilled water having pH 7.4. Samples were thoroughly mixed and allowed to settle for 60 min. Physico-chemical parameters like pH, redox-potential, conductivity, TDS and salinity of composite sample were measured using portable instruments (Eutech Cybernetics). Chloride was measured using titrimetric method[11].

From the composite sample, 0.1 ml supernatant was spread in aseptic condition on M1 (ref. 12) and Sabouraud's agar plates having different NaCl concentrations, viz. 5, 10, 15, 20, 25% for the isolation of halotolerant and halophilic bacteria and fungi. The plates were covered in clean plastic bags and incubated at 37.0 ± 2.0°C for three weeks. Colony forming units were counted and grouping was done on the basis of pigmentation. Electron microscopy of selected isolate was done at SICART, Vallabh Vidhyanagar, Gujarat. Diversity, evenness and richness indices were calculated using standard formula[13,14] with data of colony counts at various NaCl concentrations.

Specific biochemical tests for halophilic bacteria were done according to the Bergey's manual[15]. Young, actively growing cultures were spreaded on M1 agar plate containing 20% NaCl and antibiotic discs (Hi media) were placed on inoculated plates immediately. Plates were incubated for 5–7 days at 37.0 ± 2.0°C. Lipid analysis was done by Bligh and Dyer method as modified by Kates[9]. 16S rRNA analysis was done for isolate 67.

The pH, redox potential, conductivity, TDS, salinity and chloride of the composite samples were found to be 7.5, 165 mV, 135 mS, 78 ppt, 52.5 ppt and 35 mg/ml respectively. These parameters are suggestive of the presence of dissolved solids, salts and impurities in the form of hydroxide, carbonate and bicarbonates along with sodium chloride imparting slight alkaline nature to the habitat.

Colony count and pigmentation at various NaCl concentrations showed that the increase in NaCl concentration in the medium favoured growth of pigment producers as well as pigment intensity (Table 1). Red and orange pigmented colonies dominated at 25% NaCl, pink at 20%, yellow at 15%, while colourless colonies dominated at 10% NaCl concentration. In a study of salterns in Alicante, Spain, scores of colony pigmentation were used as an indicator of bacterial and archaeal halophiles. Red to pink pigmented colonies were scored as halobacteria. Non-pigmented organisms were shown to be more common in ponds containing up to 14% salt, whereas red-pigmented colonies began to appear at about 18% salt

**Table 1.** Colony count and diversity indices at various NaCl concentrations

| | Grouping of pigmented colonies | | | | | | Diversity determination | | |
|---|---|---|---|---|---|---|---|---|---|
| NaCl concentration (%) | Total colonies (1 ml) | FR/R/RO | O | Y/GY/FO | OW/F/FP/WP/PO | Colourless | Diversity $D_{Shannon}$ (H′) | Evenness $E_{Pielou}$ | Richness $R_{Margalef}$ |
| 5 | 9570 | – | – | 80 | – | 9490 | 0.048 | 0.070 | 0.621 |
| 10 | 3040 | – | – | 80 | – | 2960 | 0.122 | 0.176 | 0.621 |
| 15 | 2420 | – | 30 | 380 | 270 | 1740 | 0.827 | 0.597 | 1.864 |
| 20 | 2320 | 80 | 190 | 240 | 750 | 1060 | 1.279 | 0.794 | 2.485 |
| 25 | 2200 | 260 | 540 | 350 | 50 | 1000 | 1.334 | 0.829 | 2.485 |

FR, Fed red; R, Red; RO, Reddish-orange; O, Orange; Y, Yellow; GY, Golden-yellow; FO, Faint orange; OW, Off white; P, Pink, FP, Faint pink; WP, Whitish-pink; PO, Pinkish-orange.

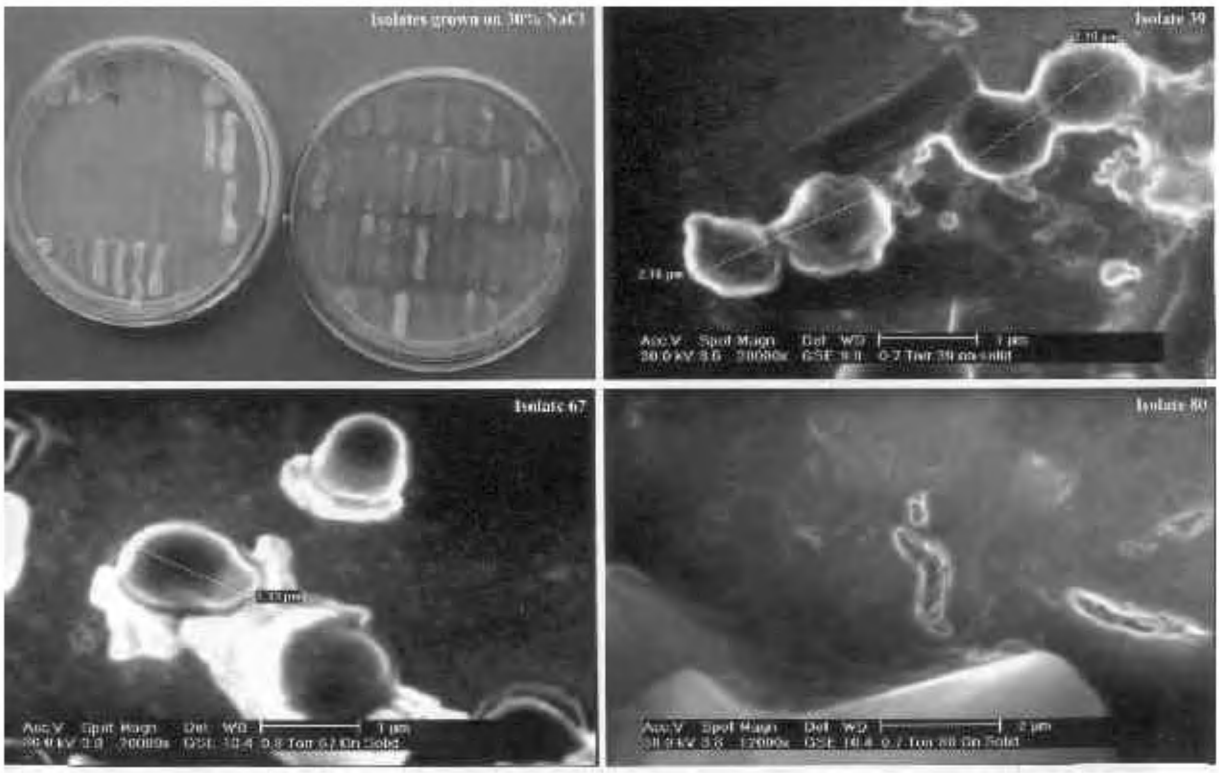

**Figure 1.** Growth of various pigmented isolates on 30% NaCl containing medium and electron photomicrograph of isolate numbers 39, 67, 80.

and were dominant between 27 and 30% salt. Isolated non-pigmented strains grew in 2–10% salt, and predominant genera were *Salinivibrio*, *Flavobacterium*, *Alcaligenes* and *Chromohalobacter*. On the other hand, red-pigmented strains required at least 20% salt[6].

Total microbial count as well as overall diversity were found to decrease with increasing NaCl concentration in the medium. However, amongst the organisms that grew at the respective NaCl concentration, diversity, evenness as well as richness increased with increasing NaCl concentration (Table 1). This means that the site has provided a specific niche and ecosystem for growth and propagation of halotolerant and halophilic microorganisms as it is used for salt production from decades.

Isolation of halotolerant fungi was successfully done incorporating 15% NaCl in Sabouraud's agar medium. Growth of fungi was not found beyond 15% NaCl concentration. On the basis of morphological and colonial characters, the four isolates were identified as three species of *Aspergillus*, and one of *Paecilomyces*. Halophilic fungi of genera *Hypomycete* and *Cladosporium* have been reported in the Great Salt Lake[3].

Out of randomly selected 73 bacterial isolates, 41 stained Gram-negative, 14 stained Gram-variable and 12 stained Gram-positive, whereas Gram reaction was not confirmed for six isolates. It was found that at 10–15% NaCl concentration rod-shape bacteria were dominating, whereas at 20% NaCl concentration there was a mixture of rod and pleomorphic cells and at 25% NaCl concentration pleomorphic shape was dominating (data not shown). Out of 73, 68 isolates grew up to 30% NaCl concentration (Figure 1). The isolates showed the shapes of rods, cocci, disc, box and pleomorphic forms. This finding gives support to the fact that 68 isolates have cellular characters like halobacteria. At 20–25% NaCl concentration, colonies were gummy and their size was larger than the colony that grew below 20% NaCl.

Isolate numbers 23, 39, 67, 79, 80 and 82 were selected for further studies because of their high pleomorphism and high salt tolerance. They grew at pH ranging from 5.5 to 11.0 and NaCl concentration 15–35% and showed overall cell size of 1.0–11.6 µm length and 0.7–1.5 µm width. Electron photomicrograph shows pleomorphism of selected isolates (Figure 1). As reported in the literature 'extreme pleomorphism' is a characteristic feature of halobacteria and many members of Halobacteriales exhibit extreme pleomorphism[15,16]. Halobacteria are known for their astonishing array of cell sizes: rods of *Halobacterium salinarum* range in size from 1 to 6 µm, whereas pleomorphic cells of *Halorubrum* vary in size[15] from 1 to 12 µm. As can be seen from Table 2, all the six selected isolates were found motile, catalase-positive and were able to grow anaerobically in presence of L-arginine, but only two of them were found oxidase-positive. Aerobic nitrate reduction was observed in isolate numbers 39, 67, 80 and 82, whereas only isolate numbers 67 and 80 were able to reduce nitrate anaerobically. All members of Halobacteriales are aerobes, but many are able to grow anaerobically using alternative electron acceptors such as nitrate, DMSO, TMAO or by fermenting L-arginine[16]. Different substrates like starch, casein, gelatin and Tween 80 were also degraded by six selected isolates. They were found to be potential producers of amylase, protease and esterase. The diversity of sugar utilization is shown in Table 2. Xylose was fermented by all the isolates with acid production, except isolate number 80. Isolate numbers 79, 80 and 82 fermented > 50% of the sugars as well as organic acid Na-acetate with alkali production. Growth of isolate number 23 was observed in terms of increasing tur-

**Table 2.** Biochemical characters of six selected isolates

| | Isolate number | | | | | |
|---|---|---|---|---|---|---|
| | 23 | 39 | 67 | 79 | 80 | 82 |
| Oxidase | + | + | – | – | – | – |
| Catalase | + | + | + | + | + | + |
| Aerobic nitrate reduction | – | ++++ | +++ | – | ++ | ++++ |
| Anaerobic nitrate reduction | – | – | ++++ | – | ++++ | – |
| Starch hydrolysis | + | + | – | + | + | + |
| Gelatin hydrolysis | + | – | – | + | – | – |
| Casein hydrolysis | + | + | – | – | – | – |
| Tween 80 hydrolysis | – | + | + | + | + | – |
| $H_2S$ production from Na-thiosulphate | – | – | +++ | – | + | ++ |
| Indole production | – | – | +++ | + | – | – |
| L-arginine (anaerobic) | + | + | + | + | + | + |
| Carbon source utilization | | | | | | |
| Dextrose | U | U | – | +g | U | ++g |
| Galactose | – | – | – | + | U | – |
| Fructose | U | U | – | – | U | P |
| Inositol | – | – | – | P | P | P |
| Lactose | – | – | – | ++g | P | ++ |
| Maltose | – | – | – | ++ | P | + |
| Mannose | U | – | – | – | Nd | P |
| Sorbitol | – | – | – | P | P | P |
| Sucrose | U | – | – | P | P | P |
| Xylose | + | + | + | +++ | – | +++ |
| Arabinose | + | Nd | – | P | P | P |
| Glycerol | U | U | – | P | P | P |
| Raffinose | U | – | – | P | P | P |
| Na-acitate | – | – | – | P | P | P |

Nitrate reduction in terms of formation of red colour on addition of α-naphthyl amine and suphanilic acid: ++++, Pinkish-red, +++, Reddish-brown, ++, Brownish-red; +, Marginal-brown; –, No colour formation. Hydrolysis of substrates: +, Hydrolyses the given substrate; –, Does not hydrolyse the given substrate; nd, Not determined. $H_2S$ production: +++, High amount of blackening; ++, Moderate blackening; +, Slight blackening. Indole production in terms of turning of yellow Kovac's strip into pink colour: +++, Whole strip turns pink-red; ++, Pink-red colour on half of the strip; +, Marginal pink colour at bottom of strip. Utilization of carbon source: –, No change; nd, Not determined; g, Gas production in Durham's vial; U, Growth observed in terms of increasing turbidity without noticeable acid or alkali production; +, Slight acid production in some parts of medium; ++, Acid production in half of the medium or light yellow colour throughout the medium; +++, Dark yellow colour throughout the medium; P, Alkali production or pink colour throughout the medium.

bidity without noticeable acid or alkali production with dextrose, fructose, mannose, sucrose, glycerol and raffinose. Similar result was found for isolate number 39 with dextrose, fructose and glycerol. All halophilic microorganisms are chemoorganotrophs, and use amino acids or carbohydrates as carbon source[15]. Fifty per cent of the isolates showed $H_2S$ production from Na-thiosulphate and indole was produced by isolate number 67 in largest amount and marginally by isolate number 79 in SIM medium (Hi-Media). The optimum temperature for all the six isolates was found in the range of 35 to 40°C. This temperature allowed both optimum growth as well as optimum pigmentation. Out of six isolates 50% isolates showed pH 7.0–9.0 as their optimum value, whereas 50% showed 7.0 as their optimum pH. NaCl concentration of 15% was found to be optimum for isolate numbers 23 and 39, whereas 15 to 20% was optimum for isolate numbers 67 and 80. Twenty per cent NaCl was optimum for isolate number 79, whereas for isolate 82 it was 20–25%. All isolates except isolate number 67 grew even below 10% NaCl concentration. The extreme halophiles require 20 to 25% NaCl for their optimum growth[2,3,6] and some true halophiles do not grow below their minimum required NaCl concentration. Halobacteriales require high salt concentration for structural stability and most non-coccoid forms lyse below 10% NaCl concentration. Certain *Haloferax* and *Haloarcula* species require relatively high divalent cation concentrations in addition to high overall salinity to maintain their native pleomorphic shape and lowering the magnesium concentration may lead to the immediate formation of spheroplasts[16]. The other isolates, characterized as halotolerant, tolerate significant amount of NaCl. They may not be truly halophilic, as they showed growth even below 5% NaCl concentration. Moreover, they did not show appreciable cell lysis in the presence of distilled water. Isolate number 67 was the slowest growing one among the selected isolates, which took minimum 5–7 days on solid medium and at least 3–4 days in liquid medium.

All the six isolates were tested for 11 antibiotics. Isolate number 67 was resistant to more than 50% antibiotics, including Penicillin-G, Ampicillin and Vancomycin (data not shown). These antibiotics affect cell wall synthesis[17], as resistance of

halophilic archaea towards antibiotics affecting cell-wall synthesis is consistent with the lack of cell envelope peptidoglycan. Isolate number 67 showed presence of phosphatidyl glycerol methyl phosphate (PGP-Me) phospholipids, diglycosyl diphytanylglycerol (DGD-1) glycolipids, GL-2 unidentified and an unknown glycolipid[18,19]. PGP-Me and DGD-1 are archaeal polar lipids[5]. The presence of these lipids, lysis in distilled water, growth only above 10% NaCl concentration, resistance towards antibiotics affecting cell-wall synthesis, and presence of pigment of orange with reddish shade in isolate number 67 indicate the characteristic of true halophiles[15,16].

Analysis of 16s rRNA of isolate number 67 was done. It was confirmed that it is haloarchaea and was identified as *Natrinema thermotolerant*, a member of family Halobacteriaceae. This culture grew up to 50°C and tolerated 60°C temperature. Although the rest of the isolates showed presence of phosphatidic acid, they showed more halotolerant characters, rather than halophilic.

Thus if facility is not available for molecular studies, morphological, biochemical and physiological studies can also provide sufficient data for evaluating bacterial diversity from marine saltern ecosystem. Indian marine salterns are neglected for biodiversity studies. Thus this attempt provides considerable data regarding microbial diversity in such ecosystem. The study also provides information regarding applied value of halotolerant and halophilic isolates as they have industrial importance. Out of 73 isolates, only six were studied; the remaining 67 isolates that tolerated up to 30% salt need further study.


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# Source of dissolved sulphate in the Alakananda–Bhagirathi rivers in the Himalayas

The natural sources of sulphate in river water are rainfall, groundwater and weathering of sulphide-rich minerals. In addition, human activities through addition of air pollutants, mining and processing of sulphide ores, petroleum refineries and chemical industries contribute enormously to dissolved sulphate content in river water. These multiple sources of sulphate can be distinguished by their specific isotopic signatures. For example, river sulphate derived from dissolution of evaporites has positive $\delta^{34}S$ values (10 to 30‰), whereas sulphate derived from oxidation of sulphides or from biogenic emissions may have strongly negative $\delta^{34}S$ values[1,2] (–25 to –5‰) ($\delta^{34}S$ denotes $[\{(^{34}S/^{32}S)_{sample}/(^{34}S/^{32}S)_{standard}\}-1] \times 1000$). According to Ivanov *et al.*[3], the annual sulphate flux from continents to oceans is $6.8 \times 10^{12}$ mol, with contributions from rivers, anthropogenic emissions and groundwater of 48, 48 and 4% respectively. Berner and Berner[4] suggest that the significant sources of sulphate in river water in general, are rock weathering (33%) and pollution (43%). Other sources could be natural biogenic-derived sulphate in rain (17%) and a minor fraction (5%) coming from volcanic activity.

In spite of the importance of rivers on global sulphur cycle, relatively little work has been reported on dissolved sulphur isotopes in the watersheds of both major and minor rivers of the world. In the present study, we have attempted to present data to understand the sources of dissolved sulphate in the Alakananda and Bhagirathi rivers in the Himalayas.