

# Isolation, Characterization and Medium Optimization of Halophiles from Arabian-Sea Coast

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## Abstract

An Extremophiles are organisms that thrives in and even may require physically or geochemically extreme conditions that are detrimental to the majority of life on Earth. The versatility of these extremophiles has enable them to populate every type of stable environment on Earth, including salt ponds, the polar regions, the deserts, hot spring, acidic or alkaline waters, and many others. Among them, Halophiles [Greek: Salt-loving] are organisms that thrive in environments with very high concentration of salt. Surface seawater and soil samples were collected from the Arabian coasts, Dandi [21° 20' 0" N, 72° 38' 0" E] and Dumas [21° 6' 0" N, 72° 42' 0" E] Gujarat (India). Four isolates were obtained having high versatility towards various ranges of salt concentrations. These microbes were found to have ability of osmoregulation through which they can maintain an internal osmotic potential that equals their external environment. Their colonial characteristics, motility, and optimization towards various salt concentrations were carried out. Each isolates showed versatility towards 0–22% NaCl concentration. Initially, isolates took 168 hrs to give visible colonies but after optimization, colonies were visible in 24 hrs.

**Keywords :** Halophiles; Salt-tolerance; Sea-water; Medium optimization

## Introduction

Halophiles are extremophile organisms that thrive in environments with very high concentrations of salt. The name comes from Greek for “salt-loving”. While the term is perhaps most often applied to some halophiles classified into the Archaea domain, there are also bacterial halophiles and some eukaryota. Halophiles are categorized slight, moderate or extreme, by the extent of their tolerance towards various salt concentrations. Some well-known species give off a red color from carotenoid compounds due to photosynthetic pigment bacteriorhodopsin.

Hypersaline environment represents an extreme environment that relatively few organisms have been able to adapt to and occupy. In order to survive in the high salinities, halophiles employ two differing strategies to prevent desiccation through osmotic movement of water out of their cytoplasm. In the first “Compatible Solute” strategy, cells maintain low concentrations of salt in their cytoplasm by balancing osmotic potential with organic, compatible solutes, by the synthesis or uptake of compatible solutes such as glycerol, sugars and their derivatives, amino acids and their derivatives & quaternary amines such as glycine betaine. The second, more radical, adaptation involves the selective influx of potassium (K<sup>+</sup>) ions into the cytoplasm. Cells can have internal concentrations that are osmotically equivalent to their external environment. They maintain osmotically equivalent internal concentrations by accumulating high concentrations of potassium chloride. Both strategies work by increasing the internal osmolarity of

the cell. This adaptation is restricted to the moderately halophilic bacterial order Halanerobiales, the extremely halophilic archaeal family Halobacteriaceae. The presence of this adaptation in three distinct evolutionary lineages suggests convergent evolution of this strategy, it is being unlikely to be an ancient characteristic retained in only scattered groups or through massive lateral gene transfer (Santos *et. al.* 2002).

## Materials and methods

Surface seawater and soil samples were collected in plastic containers disinfected with alcohol. Samples were collected from the surface at a depth of 1–1.6 ft from the Arabian coasts, Dandi [21° 20' 0" N, 72° 38' 0" E] (Figure 1) and Dumas [21° 6' 0" N, 72° 42' 0" E] (Figure 2), Gujarat (India). Two samples of 2 liters were taken from each point.

Halobacterium medium (Atlas, 2004) was selected as enrichment medium. The composition of the medium with 24% salt content was (w/v): NaCl, 220; Agar,30; MgSO<sub>4</sub> .7H<sub>2</sub>O,10; Casein hydrolysate (replaced by peptone),5; KCl,5; Disodium citrate, 3; KNO<sub>3</sub>,1; Yeast extract,1; CaCl<sub>2</sub>.6H<sub>2</sub>O,0.2 having pH 7.3 at 25°C. 50 ml enrichment medium supplemented with 5 ml of sample (each) were incubated at 37°C at 120 rpm. Visible turbidity of enrichment was taken into account which was obtained after 120 hrs of incubation. Microscopic observation of isolates was done by performing gram staining and negative staining technique (Benson, 2001). Sterile seawater was used for preparing suspension of isolates, but due to presence of higher amount of particles, it created difficulty during staining procedures, hence it was replaced by

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20% NaCl solution to prevent osmolysis. Motility determination test of each isolates was performed by 'the hanging drop slide' preparation (Benson, 2001). Loopful of suspension of isolates was streaked on Halobacterium medium plates which were subjected to incubation at 32°C for 168 hrs. *In vitro* observations of colonies were done according to study of cultural characteristics (Patel, 2008).

Growth was determined on Halobacterium broth medium (5 ml) having 0%, 2%, 4%, 6%, 8%, 10%, 12%, 14%, 16%, 18%, 20%, 22%, 24% and 26% NaCl concentration supplemented with 1% MgSO<sub>4</sub> .7H<sub>2</sub>O, 0.5% peptone ; 0.5% KCl; 0.3% Disodium citrate; 0.1% KNO<sub>3</sub>; 0.1% Yeast extract; 0.02% CaCl<sub>2</sub>.6H<sub>2</sub>O, 0.2 % having pH 7.3 at 32°C. Growth was estimated by inoculating 0.2 ml of suspension of the strain pre-grown to visible turbidity. Medium was incubated at 32°C. Similar test was carried out with different concentration of MgSO<sub>4</sub> (0.5%, 0.6%, 0.8%, 1.0%, 1.2%, and 1.4%) and KCl (0.5%, 0.6%, 0.8%, 1.0%, 1.2%, and 1.4%). All experiments were performed in duplicates. Observation was made on the basis of visible turbidity.

## Results

Isolates were obtained by spreading 0.3 ml of enriched culture on Halobacterium medium plate by spread plate method (Prescott, 2008). Visible colonies were obtained after incubating plates at 32°C for 168 hrs. Four non-pigmented isolated colonies were selected, designated as H1, H2, H3 and H4 obtained from enriched samples of Dumas and Dandi respectively. Same method was applied for enrichment of evaporated sea water sample (Rodriguez-Valera *et al.*, 1979) but no isolates were obtained after prolonged incubation. The result of each isolates, obtained from enrichment culture is shown below. It was observed that each isolates were gram negative short rods (Figure 3). Results of Negative staining are shown in Figure 4. Each isolates showed pleomorphic characteristics at different salt concentrations. Both motile and non-motile movements were observed with isolates. Information about size, shape, arrangement and motility is presented in Table 1. *In vitro* observation of colonies showed similar cultural characteristics. Cultural characteristics of each isolates are shown in Figure 5.

Four isolates having high versatility in various range of NaCl concentration were isolated. Graph shows the versatility of isolates in different range of NaCl Concentration (Figure 6). Concentrations of all the salts (Figure 7 and 8), except KNO<sub>3</sub> and CaCl<sub>2</sub>.6H<sub>2</sub>O were customized



Figure 1. The Arabian Sea coast, Dandi.

to give the total salt concentration required for the optimum growth of isolates. Pigment production is strongly influenced by salt concentration (Rodriguez-Valera *et al.*, 1979). Observation was done on Halobacterium medium plates with 10–24% salt concentration which showed colourless non-pigmented colonies. Experiments were performed with duplicate. The composition of the optimized medium with 12% salt content was (*w/v*): NaCl, 100; Agar, 30; MgSO<sub>4</sub> .7H<sub>2</sub>O, 6; Peptone, 5; KCl, 8; Disodium citrate, 3; KNO<sub>3</sub>, 1; Yeast extract, 1; CaCl<sub>2</sub>.6H<sub>2</sub>O, 0.2 having pH 7.3 at 32°C. Temperature was modified to 32°C and pH to 7.3. Initially, the isolates took 168 hrs to give visible colony. After the above course of action, results were obtained in 24 hrs.



Figure 2. The Arabian Sea coast, Dumas.

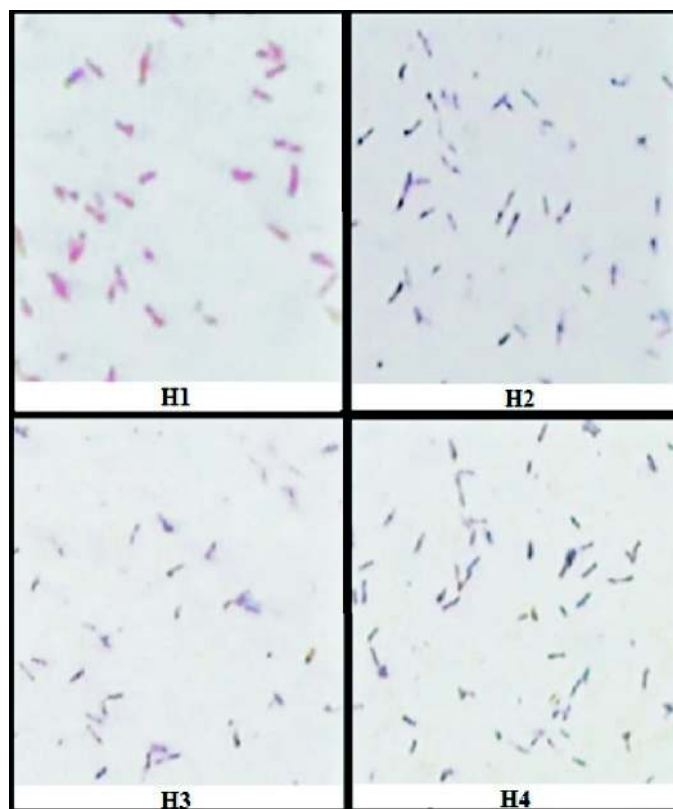


Figure 3. Results of Grams staining of isolates obtained at pH-7.3 with 12% salt concentration

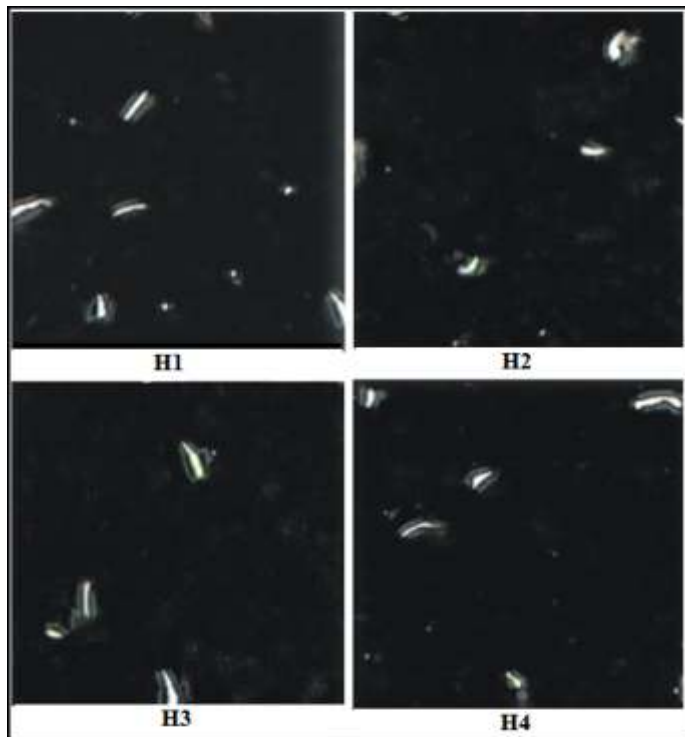


Figure 4. Results of Negative staining of isolates obtained at pH-7.3 with 12% salt concentration

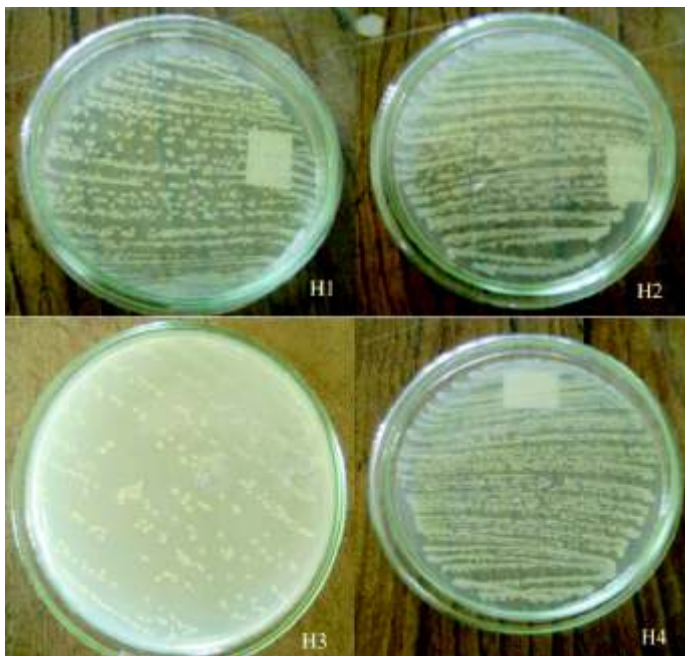


Figure 5. Cultural characteristics of isolates H1, H2, H3 and H4 obtained at pH-7.3 with 12% salt concentration

**Discussion**

H1, H2, H3 and H4 showed similar cultural characteristics. However, they showed different growth patterns in different salt concentrations. Hence, it can be said that they may be of same species but their strain may be different. The features of microorganisms describe here suggest that they have versatile growth characteristics. Our result indicates that these microorganisms have high salt tolerance potential. Their ability to grow

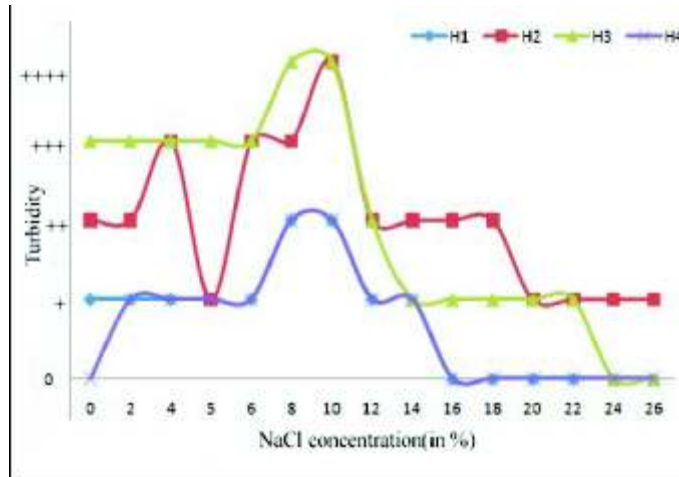


Figure 6. Graph of Turbidity v/s NaCl concentration (%) after 48 hours incubation

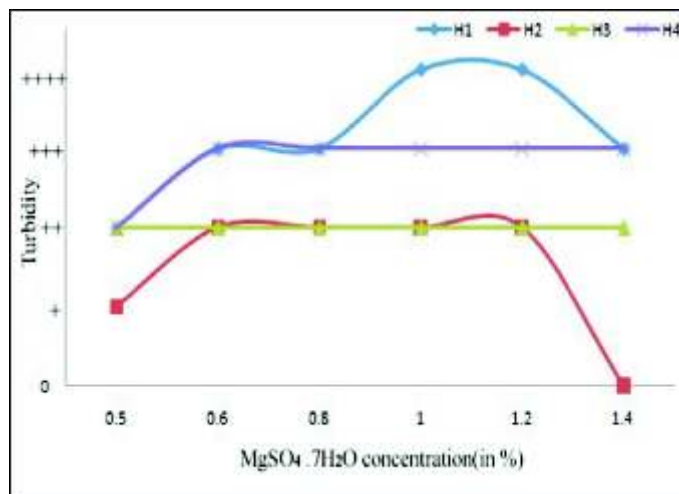


Figure 7. Graph of Turbidity v/s MgSO<sub>4</sub> .7H<sub>2</sub>O concentration (%) after 48 hours incubation

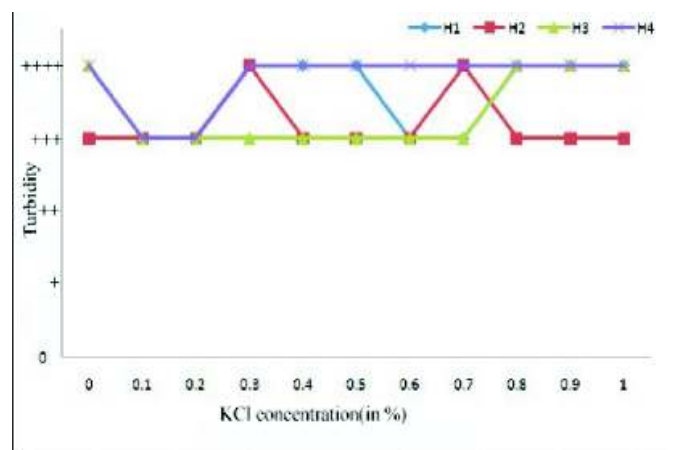


Figure 8. Graph of Turbidity v/s KCl concentration (%) after 48 hours incubation

Keys: + = Minimum turbidity  
 ++ = Moderate turbidity  
 +++ = High turbidity  
 ++++ = Maximum turbidity

	H1	H2	H3	H4
Size	Small	Small	Small	Small
Shape	Short rods	Thick short rods	Short rods	Short rods with tapered ends
Arrangement	Singly	In chains	Clusters	Clusters
Gram's staining	Gram negative	Gram negative	Gram negative	Gram negative
Motility	Motile	Non-motile	Non-motile	Non-motile

Table 1. Morphological characteristics (at pH 7.3 with 12% salt concentration) and result of motility test of isolates.

	Parameters	H1	H2	H3	H4
Colonial characteristics	Size	Intermediate	Small	Small	Small
	Shape	Round	Round	Round	Round
	Surface	Smooth	Smooth	Smooth	Smooth
	Edge	Entire	Entire	Entire	Entire
	Elevation	Low convex	Low convex	Low convex	Low convex
	Opacity	Translucent	Translucent	Translucent	Translucent
	Consistency	Moist	Moist	Moist	Moist
	Pigmentation	Nil	Nil	Nil	Nil

Table 2. Cultural characteristics (at pH 7.3 with 12% salt concentration) of isolates.

even in 2% to 24% salt concentration shows that each isolates are capable of osmoregulation through which they maintain an internal osmotic potential that equals their external environment.

These properties offer significant advantage to study the activity and metabolism of halophiles at various salt concentrations. Such potential halophiles can be used in bioremediation or degradation and transformation of range of organic pollutants in sea water (Margesin *et al.*, 2001). Halotolerance of many enzymes derived from halophilic microorganisms can be exploited wherever enzymatic transformations are required to function at low water activities, such as in the presence of high salt concentrations (Kamekura, 1986). In addition, all exoenzymes excreted by halophiles are active in the presence of high salinities found in their medium, even when the organisms that produce them may maintain low intracellular ionic concentrations (Oren, 2002).

## Conclusion

Halophiles have the distinctive advantage to grow in environment having high salt concentration where other potential microorganisms fail

to survive. This offers a multitude of potential applications in various fields of biotechnology. Their compatible solutes are useful as stabilizers of biomolecules and whole cells, salt antagonists, or stress-protective agents. Biopolymers, such as biosurfactants and exopolysaccharides, are of interest for microbially enhanced oil recovery (MEOR). Enzymes of such organisms such as new isomerases and hydrolases have their own significance due to their potential to remain active and stable in high salt contents. As now the medium composition is optimized, these microorganisms can be used for its various applications in the field of biotechnology.

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