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ISOLATION AND CHARACTERIZATION OF HALOPHILES FROM SOLAR SALTERNS OF BHAVNAGAR

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ABSTRACT

Halophiles are organisms that thrive in high salt concentration. They are type of extremophiles. Halophiles are categorized as slight, moderate or extreme by the extent of their halotolerance that requires NaCl for growth. Most Halophiles are unable to survive outside their high salt native environment. Majority of extreme Halophiles belong to the Archaea domain. Total 11 salt samples were studied. Throughout the work of this study total seven isolates were isolated & analyzed. Total 7 colonies were isolated of 3 were Gram –ve and 4 were Gram +ve. For most isolates growth occurred at 30-33 °C, pH 7± & 20% (w/v) NaCl, but not at 28% NaCl, suggesting that most of these isolates were moderately halophilic. The community of isolated bacteria in this study was compared with those of recent study on Halophilic bacteria isolated from the saline environment.

KEY WORDS

Halophiles, Extremophiles, Halotolarence, Anthropogenic

INTRODUCTION

Soil microbiology is the study of organisms in soil, their functions, and how they affect soil properties. Microorganisms in soil are important because they affect soil structure and fertility. Soil microorganisms can be classified as bacteria, actinomycetes, fungi, algae and protozoa. These groups has characteristics that define them and their functions in soil [8]. Up to 10 billion bacterial cells inhabit each gram of soil in and around plant roots, a region known as the rhizosphere. In 2011, a team detected more than 33,000 bacterial and archaeal species on sugar beet roots [12]. An extreme environment contains conditions that are hard to survive for most known life forms. These conditions may be extremely high or low temperature or pressure; high or low content of oxygen or carbon dioxide in the atmosphere; high levels of radiation, acidity, or alkalinity; absence of water; water containing a high concentration of salt or sugar; presence of sulphur, petroleum, and other toxic substances. The distribution of extreme environments on earth has varied through geological time. Humans generally do not inhabit extreme environments. There are organisms referred to as extremophiles that do live in such conditions and are so well-adapted that they readily grow and multiply. Among extreme environments are like, acidic, extremely cold, extremely hot, hyper-saline, places altered by humans, such as mine tailings or oil impacted habitats [9].

- **Alkaline:** natural habitats above pH 9 whether persistently, or with regular frequency or for protracted periods of time.
- **Acidic:** natural habitats below pH 5 whether persistently, or with regular frequency or for protracted periods of time.
- **Extremely cold:** Broadly conceived habitats periodically or consistently below -17 °C either persistently, or with regular frequency or for protracted periods of time. Includes mountain sites, polar sites, and deep ocean habitats.

- **Extremely hot:** Broadly conceived habitats periodically or consistently in excess of 40 °C either persistently, or with regular frequency or for protracted periods of time. Includes sites with geological thermal influences such as Yellowstone and comparable locations worldwide or deep-sea vents.
- **Hypersaline:** (High salt) Environments with salt concentrations greater than that of seawater, that is, >3.5%. Includes salt lakes.
- **Under pressure:** habitats under extreme hydrostatic pressure — i.e aquatic habitats deeper than 2000 meters and enclosed habitats under pressure. Includes habitats in oceans and deep lakes.
- **Radiation:** habitats exposed to abnormally high radiation or of radiation outside the normal range of light. Includes habitats exposed to high UV and IR radiation.
- **Without water:** habitats without free water whether persistently, or with regular frequency or for protracted periods of time. Includes hot and cold desert environments, and some endolithic habitats.
- **Without oxygen:** habitats without free oxygen - whether persistently, or with regular frequency, or for protracted periods of time. Includes habitats in deeper sediments.
- **Altered by humans,** i.e anthropogenically impacted habitats. Includes mine tailings, oil impacted habitats, and pollution by heavy metals or organic compounds [7].

Hypersaline**:** (High salt) Environments with salt concentrations greater than that of seawater, that is, >3.5% includes salt lakes, marine salterns. Halophiles are extremophiles that thrive in high salt concentration requiring at least 0.2M concentrations of salt (NaCl) for growth. [2]**.** The word Halophiles comes from the Greek word for "salt-loving". While most halophiles are classified into the Archaea domain, there are also bacterial halophiles and some eukaryota, such as the alga *Dunaliella salina* or fungus *Wallemia ichthyophaga*. Halophiles are categorized as slight, moderate, or extreme, by the extent of their halotolerance. Slight halophiles prefer 0.3 to 0.8 M (1.7 to 4.8% — seawater is 0.6 M or 3.5%), moderate halophiles 0.8 to 3.4 M (4.7 to 20%), and extreme halophiles 3.4 to 5.1 M (20 to 30%) salt content**.** [1]**.** Halophiles require sodium chloride (salt) for growth, in contrast to halotolerant organisms. Most halophilic and all halotolerant organisms expend energy to exclude salt from their cytoplasm to avoid protein aggregation ('salting out'). To survive the high salinities, halophiles employ two differing strategies to prevent desiccation through osmotic movement of water out of their cytoplasm. A thorough study of salt-crystallizing ponds from several places around the world by Oren [6] showed consistent communities between salterns ponds. The genera isolated were primarily *Haloferax, Halogeometricum, Halococcus, Haloterrigena, Halorubrum, Haloarcula, Halobacterium,* and *Natronococcus*. Halophilic bacteria have been found to perform fermentation, acetogenesis, sulfate reduction, phototrophy, and methanogenesis. [4]. Halophilic microorganism used in the treatment of saline and hypersaline wastewater and the production of exopolysaccharide bioplastics and biofuel are being investigated [5].

MATERIALS AND METHODS

Sample Collection

A total 11 salt samples, 2 soil samples, and 1water samples were collected from the Sharda and Khodiyar salt companies (which are located in village Goga, Dist. Bhavnagar, Gujarat, India). Soil Sample collected in sample collection bag and water sample was collected in sterile glass bottle. Sample stored in refrigerator.

Sample Preparations

2g sample was weighed from each of the 11 salt samples and the two soil samples similar volume was added to 2ml water sample.

Composition of MSW agar medium (Minimal Salt Water Medium)

MSW	4 (W/V %)
NaCl	20
$MgSO_4.7H_2O$	2.0
KCL	0.5
Tryptone	0.3 - 0.5
Yeast extract	0.3 - 0.5
Agar	2 - 3%
pH	7.0

Spread Plate Technique

0.1 ml sample was spread in to the MSW4 medium (Supplemented with varying Concentration of NaCl) in pre-labeled Petri dishes. The inoculums were spread over the entire surface of media with the help of bent glass rod that sterilized by dipping in 95% ethanol and quickly flamed to remove the ethanol. Inoculated broth were plates were incubated for the 4 to 7 days at 30° C in the incubator.

Sample Preparation (Liquid Media)

1 g of sample was Weighed from each 13 soil samples and Dissolved in 9ml sterile distilled water with the help of sterile spatula. 1ml of this prepared was added to 100ml sterile MSW4 broth. Add the sample with help of sterile micro pipette. All of the 14 flasks were incubated in rotary shaker for 4 to 7 days at 30° C temperature.

NaCl Variation study

2.0 ml culture was added in MSW 4 broth with varying concentrations of NaCl (14%, 16%, 18%, 20%, 22%, 24%, 26%, 28%). All the MSW 4 broth was incubated in rotary shaker at 30°C temperature.

pH Variation study

2.0 ml culture was added to 10 ml sterile MSW4 broth with varying which contains different variation of pH (3, 4, 5, 6, 7, 7.5, 8, 9, 10, 11). The sample was added with help of sterile micro pipette. All the M.S.W.-4 broth was incubated in rotary shaker at 30° C. After incubation the optical density of the broth was determined with the help of colorimeter at 570 nm.

Identification by Colony characteristics: Well isolated colony characteristics Were studied for their shape, size, margin, elevation, texture, consistency, pigmentation, opacity.

RESULT AND DISCUSSION

In the past few years, much attention has been paid to halophilic bacteria especially to moderately halophilic bacteria. Several studies have been conducted on their biotechnological applications such as production of bioactive compounds (Antibiotic) as well as their ecologic and phylogenetic characteristics [11]. Bhavnagar, Ghogha is one of the most permanent hypersaline salterns in the Gujarat and it is oligotrophic solar saltern with Thalassohaline origin. The present study made a first attempt to investigate halophilic bacteria. Apart from solar salterns of any previously published article on its bacterial community, subjection of the solar salterns to high salinity and dryness were the other reasons for this study.

Throughout the course of this work, total seven colonies were isolated which was predominantly Gram negative 3 and Gram positive 4 (Table 1.1). For most of the growth occurred at 30-33˚C, pH 7±0.5 and 20% (w/v) NaCl, none of the isolate could grow at 28% NaCl. Seven isolates were obtained and labeled as C_1 to C_7.

C_1 had the capability to ferment glucose with acid and gas production, it hydrolyzed urea and produced ammonia.

According to the results of biochemical tests for the isolate C_2, acid and gas production is observed in lactose, mannitol, maltose and sucrose Broth tubes also utilized urea and ammonia production test is positive.

The isolate **C_3** gives acid and gas production in sucrose broth also utilizes urea and ammonia production shows test is positive.

The C_4 gave gas and acid production in glucose broth, lactose and maltose broth, it utilizes urea, production of ammonia shows test is positive.

C_5 isolate gives positive result with acid and gas production in lactose, mannitol and sucrose broth and Methyl red, urea hydrolyses, and ammonia production tests were also positive test.

The isolate C_6 produced acid and gas with lactose, xylose, maltose and sucrose, gave following: urea hydrolysis and dehydrogenase tests were positive.

The following tests were positive for the C_7 isolates that produce acid and gas production in glucose, it also gave positive result with indole production test, methyl red test, urea hydrolysis test, motility test and gelatin liquification test, suggesting that most of these isolates considered moderately halophilic according to the definition by [10] (Table 1.5). The community of the isolated bacteria in this study was compared with those of recent studies on halophilic bacteria isolated from Urmia lake, Iran.

Table: 1 Morphological characteristics of different samples on MSW4 plates

Colony Characteristics	S-10 C-1	S-1 C-2	S-9 C-3	S-12 C-4	S-H soil C-5	S-6 C-6	S-5 C-7
Shape	Round	Round	Irregular	Round	Round	Round	Round
Size	Small	Small	Small	Small	Small	Small	Small
Margin	Even	Entire	Even	Entire	Entire	Entire	Entire
Texture	Rough	Smooth	Smooth	Rough	Smooth	Rough	Smooth
Opacity	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque
Pigment	White	White	White	White	Creamy White	White	White
Elevation	Flat	convex	Flat	Convex	Convex	convex	Convex
Consistency	Dry	moist	moist	Dry	Moist	dry	Moist

MORPHOLOGICAL IDENTIFICATION BY GRAM STAINING

Table: 1.1 Result of Gram's Staining

Sr. No.	Colony/ sample	Size.	Shape	Arrangement	Gram's reaction
1	S-10 C-1	Small	Rod	Single/chain	-ve
2	S-1 C-2	Small	Round	Single/cluster	+ve
3	S-9 C-3	Small	Rod	Single/cluster	-ve
4	S-12 C-4	Small	Rod	Single/chain	+ve
5	S-Soil 1 C-5	Small	Round	Single/cluster	+ve
6	S-6 C-6	Small	Rod	Single/chain	-ve
7	S-5 C-7	small	Round	Cocci/bunch	+ve

Table: 1.3 Biochemical results

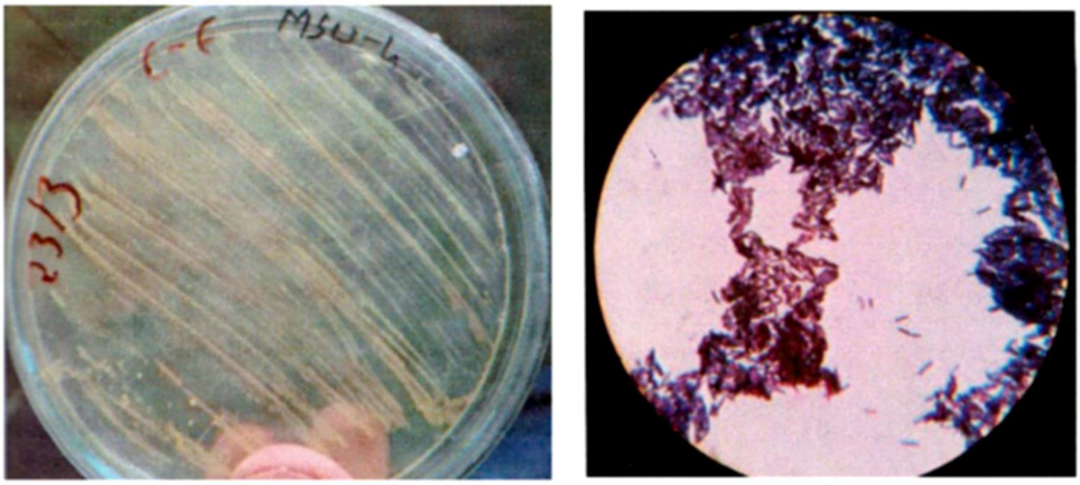
Test name		C-1	C-2	C-3	C-4	C-5	C-6	C-7
Carbohydrate fermentation test	Glucose	+	+	-	+	-	-	+
	Lactose	-	+	-	+	+	+	-
	Xylose	-	-	-	-	-	+	-
	Mannitol	-	+	-	-	+	-	-
	Maltose	-	+	-	+	-	+	-
	Sucrose	-	+	+	-	+	+	-
Indole production test		-	-	-	-	-	-	+
H_2S production test		-	-	-	-	-	-	-
M-R Test		-	-	-	-	+	+	+
V-P Test		-	-	-	-	-	-	-
Citrate utilization test		-	-	-	-	-	-	-
Urea hydrolysis Test		+	+	+	+	+	+	+
Catalase Test		-	-	-	-	-	-	-
Motility Test		-	-	-	-	+	-	+
Ammonia production Test		+	+	+	+	+	-	-
Dehydrogenase Test		-	-	-	-	-	+	-
Starch hydrolysis Test		-	-	-	-	-	-	-
Lipid hydrolysis Test		-	-	-	-	-	-	-
TSI Slant		+	-	+	-	-	+	+
Gelatin liquification Test		-	-	-	-	-	-	+
Casein hydrolysis Test		-	-	-	-	-	-	-
Amino acid Determination Test		-	-	-	-	-	-	-

Table: 1.4 Result on TSI agar Slant

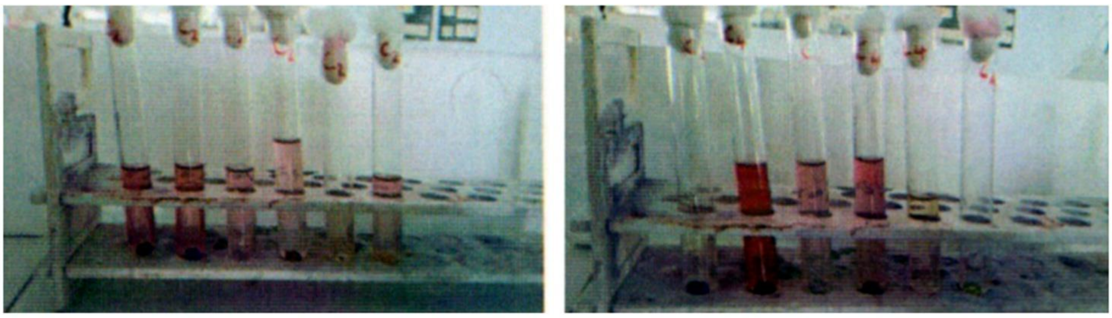
Isolates	Slant (Acidic / Alkaline)	Butt	Gas Production	H_2S Production
C_1	No change	No change	No change	No change
C_2	No change	No change	No change	No change
C_3	Alkaline	Alkaline	No change	No change
C_4	No change	Acidic	No change	No change
C_5	Alkaline	Alkaline	No change	No change
C_6	Alkaline	Alkaline	No change	No change
C_7	Alkaline	Alkaline	No change	No change

Table: 1.5 Halophilic bacteria isolated from saline environments

Isolated from	pH	NaCl concentration	References
Urmia lake, Iran	6 to 9	20%	**Vahed S.Z et al. 2011**
Bhavanagar, India	7±0.5	20%	Present study



Gram Staining of isolates C_6



Biochemical Test : Carbohydrates utilization

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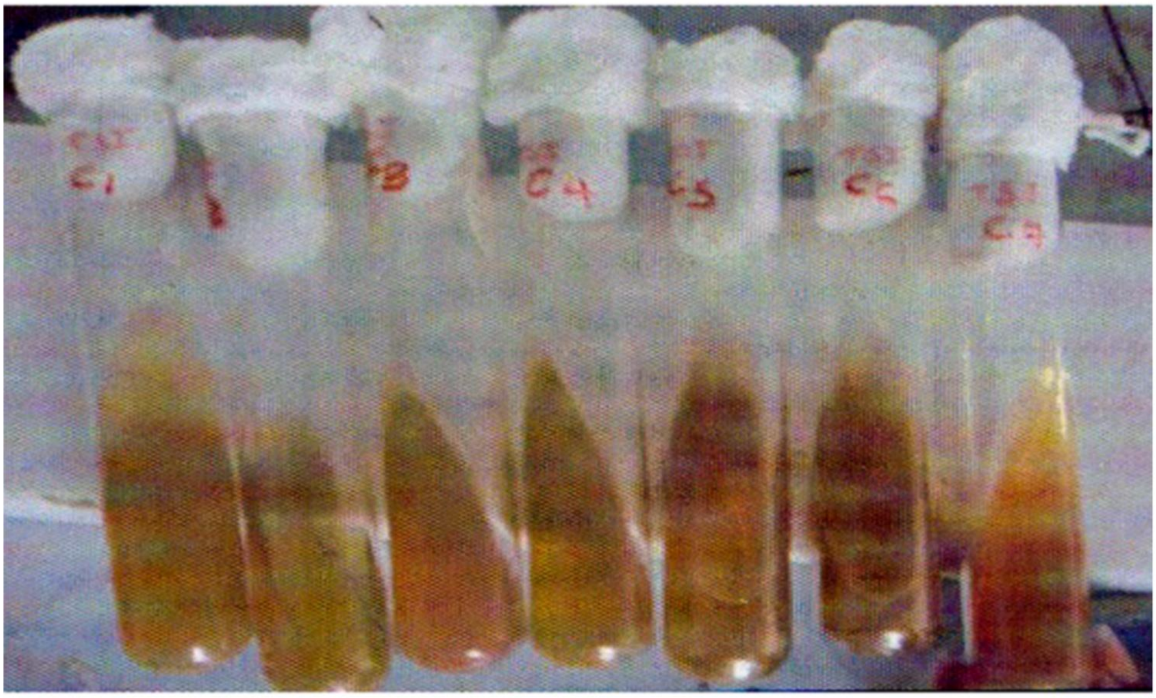
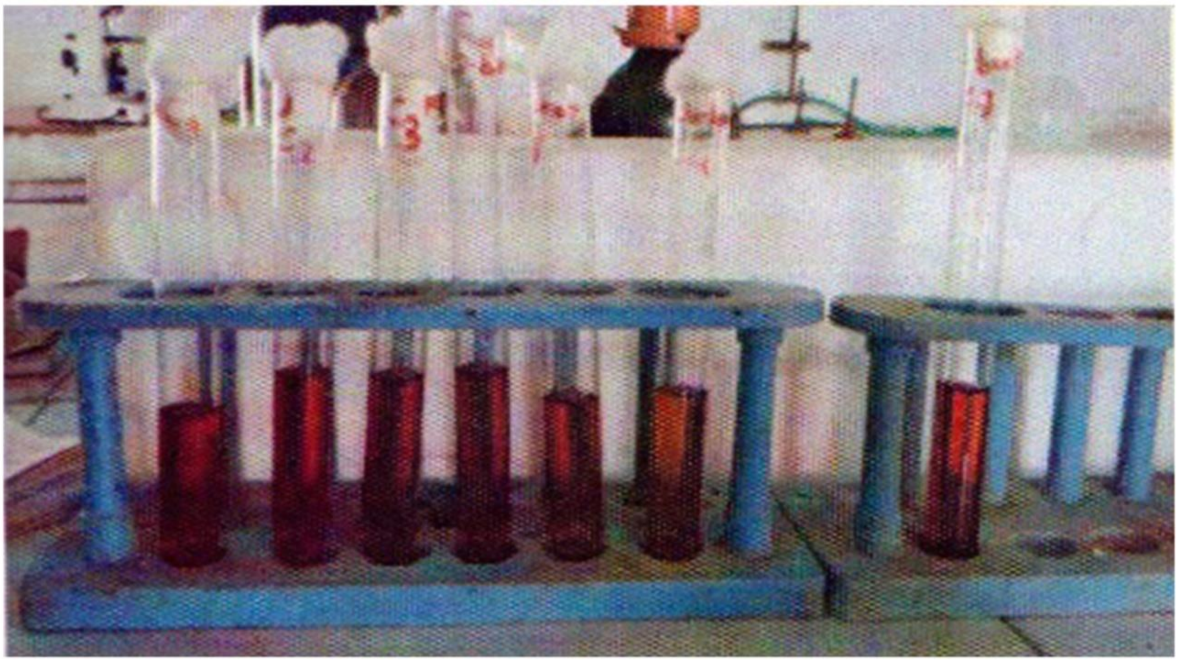


Biochemical Test : TSI



Biochemical test : Urea utilization

Sample collection site, Ghoga, Bhavnagar

CONCLUSION

Seven isolates obtained from salt sample were subjected to morphological and biochemical characterization along with pH and NaCl optimum, and it is concluded that the isolates were found to be three as Gram negative and four as Gram positive, their colony characteristics ranging from round to irregular, rough to smooth, moist to dry. The optimum pH was 7 to 7.5 and the NaCl requirement was 20% to 22%.

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