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Original Research Article

Diversity of Moderate Halophilic bacteria from Sambhr Salt Lake

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A B S T R A C T

Sambhar lake (27o 58 N, 75o 55 E) is the largest inland halo-alkaline lake of India. Salinity of lake fluctuated from hypo to hypersaline condition (5 to 35%). Lake brine was always alkaline in nature with pH values from 7.15 to 9 during year 2013-14. The lake dried in May-June 2013 resulting in formation of thin salt incrustation on the dry lake bed. Color of lake brine changed from yellow-green to red-orange with a gradual increase of water density. At high density the lake brine was found devoid of any life form other than halophiles.The present study was taken up mainly to study moderate halophiles of the lake. Total Eight moderate Halophilic strains (SL-1 to SL-8) with salt requirement between 3-15% were isolated. All the strains were phenotypically and metabolically diverse. The biochemical parameters like nitrate reduction, H2S production, citrate utilization, oxidase, catalase, acid production from various sugars, were tested to screen the diversity of all halophilic bacteria. All the isolates were also screened for exo-enzyme production by assessing starch, gelatin and lipid hydrolysis.

Keywords

Halophiles, Metabolic diversity, Exo-enzymes

Introduction

Halophiles are salt-loving organisms that inhabit hypersaline environments and are well equipped to balance the osmotic pressure of the environment and resist the denaturing effects of salts. Halophiles can be grouped as slightly, moderately or extremely halophilic, depending on their requirement for NaCl. Moderately halophilic bacteria are microorganisms able to grow optimally in media containing 3 to 15% (wt/vol) salts (Kushner, 1993). This group is very heterogeneous taxonomically and includes a great variety of species belonging to Archaea (Mathrani *et al.*,1988) and Bacteria (Ventosa, 1994).

Halophiles are found distributed all over the world in hypersaline environments, many in natural hypersaline brines in arid, coastal, and even deep sea locations, as well as in artificial salterns used to mine salts from the sea. Their novel characteristics and capacity for large-scale culturing make halophiles potentially valuable for biotechnology (DasSarma and Arora (2001). Halophiles produce a large variety of stable and unique biomolecules including hydrolytic enzymes (Birgul Ozcan *et al*, 2009). Recent decades have seen a surge in studies on extreme environments including hypersaline ecosystems. Major emphasis is on identification of extremophiles based on both molecular and biochemical basis.

Sambhar salt lake being a hypersaline lake is home to several halophiles. Though the lake has gained international importance as a waterfowl habitat yet there is a derth of studies on its unique microbial ecology. Hence an attempt is being made through this work to assess the microbial diversity of sambhar salt lake and to isolate microbes with potentials or highly stable hydrolytic enzymes.

Materials and Methods

Study Site

Located about 80 km nonhwest of Jaipur in central Rajasthan (26°58′N75°05′E) Sambhar is a shallow lake, reaching only about 3m at its deepest. The area of Sambhar lake varies from 190-230 Sq. km and it receives run-off from a catchment area of about 552,000 ha and has no outlet. Sambhar is fed by four main streams: Roopnagar, Mendha, Kharian, and Khandel.

Sample collection and Enrichment

The red-orange and red-purple brine samples were collected from Jhapok dam and Guda area of the lake in sterile bottles. The water sample was enriched in Halophilic broth (10% salt) in 250ml Erlenmeyer flask at 150 rpm and 35oC for 7 days. Enriched broth was poured on Nutrient Agar (NA) plates with 10% NaCl and Halophilic Agar and Incubated for 2-3 days. Post incubation several colonies with a variety of pigmentation appeared. Each colony was pure cultured on a new NA plate.

Growth Range

In order to determine the growth on a range of salt, each isolate was streaked on the NA plates containing salt concentration from 0-30% and incubated at 35oC for 2-3 days. Visible and luxurious growth after twice streaking on plates was considered positive result. pH growth range was also determined for each isolate.

Biochemical characterization

Biochemical tests like nitrate reduction, H2S production, carbohydrate utilisation, oxidase, Catalase, motility were carried out as per standard methods (Cappuccino, 1999).

Screening for Hydrolytic Exo-enzymes

Plate Assay of enzyme for amylase activity

To determine the amylolytic activity, the petriplate containing nutrient agar with 0.2% soluble starch at neutral pH 7.0 were prepared. The plates were inoculated in the centrewith pinpoint inoculum of the test b acteria and incubated at room temperature for 24 to 48 hr. Extracellular enzymes activity was visualized by flooding the plates with iodine solution. The formation of a clear zone around the colony indicated the hydrolysis of starch.

Plate Assay of enzyme for protease activity

The Petri-plates containing nutrient agar with 2% gelatin at neutral pH 7.0 were prepared. The plates were inoculated and incubated at room temperature for 24 to 48 hr. Protease enzyme activity was visualized by flooding the plates with 0.1% mercuric chloride solution. The formation of a clear zone around the colony is an indication of gelatin hydrolysis.

Plate Assay of enzyme for Lipase activity

Lipase production was assessed by inoculating isolates on Nutrient agar containing 2% Tween 20 and Tween 80 and methyl red indicator. Lipolytic activity was determined by the formation of the zone of intensification of the indicator colour after 24-48 hr.

Results and Discussion

The Sambhar, a recognized saline lake and Ramsar site, boasts peculiar physical and chemical characteristic. It is considered athalassohaline as its salt composition derives from the dissolution of minerals of geological origin (Rodriguez-Valera, 1993).

Similarly to other hypersaline ecosystems, the lake is subjected to drastic physicochemical conditions including high salinity, high radiation (UV) and strong changes in temperatures and dryness which make it a relevant study target for microbiologists.

Study on the Sambhar lake halophiles was carried out for year 2013-14. Lake brine samples were also tested for Temperature, pH, Nitrate, Phosphate, Hardness and Chloride contents and the result revealed a highly alkaline and saline nature of the lake.

Nutrients were found in low concentrations. Hardness studies revealed that the lake is poor in Mg2 + and Ca2 + contents, therefore it falls in soft water category. Upasani and Desai (1990) observed similarity in chemical composition and microbial ecology of Sambhar lake to the brines from Wadi Natrun, Egypt (Imhoff *et al*. 1979) and Lake Magadi, Kenya (Tindall *et al*. 1980) as they lack the divalent cations Mg2 + and Ca2 +,and have a high pH (> 9.0), and high contents of carbonates and sulphates. The lake remains hyposaline post monsoon and very soon it attains the status of hypersaline water body, thus reaching a extreme environment. During its concentrated state the lake gains a red-pink coloration and inhibits varied halophiles. Eight moderate halophiles were isolated during the course of study. Isolates were named after the Sambhar lake (SL 1- SL-8).

Morphological and biochemical tests

Isolates were examined for motility, morphological features and pigmentation after growth for 5-7 days on NA with 10% Salt. All isolates formed circular, convex, entire, 4 and smooth colonies. Coloration of colonies ranged from Dull cream, Cream to Yellow and red-orange. The pigmentation intensity lowered with salt contents exceeding their optimum range. The isolates grew well on alkaline medium. Isolate SL-7 formed cream colored, convex and mucoid colonies with entire margins. This isolate showed a wide range of salt and pH tolerance (5-20% and 7-9 respectively). Only this isolate was citrate +ve. Gram staining was carried out as described by Dussault (1955). All isolates were gram negative. During the study all the isolated were tested for various biochemical properties in 10% salt medium. All the isolates were aerobic and tested Catalase and Oxidase positive. None of the isolate tested positive for H2S production on TSI medium.

Growth range

Each of the fresh isolates was tested for ability to grow at Range of salt concentrations and at pH 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0. The salt range tested was 0-30%. None of the isolate could grow in media containing above 15% salt, except SL-7. Isolate SL-7 showed delayed growth on medium containing 20% salt. Optimum growth of all the isolates was observed between 7-15% salt range, thus showing their moderate halophilic nature. Moderately halophilic bacteria constitute the most versatile group of microorganisms that could be used as a source of salt-adapted enzymes. These have advantage over extreme halophiles in that they do not have a strict salt requirement and grow in wide salt range (Kumar *et al*., 2012).

At the time of testing salt range natural brine of various densities was also used in culture medium. According to Javor (1984) the natural brines of 18 to 22 Be, in which the extreme halophiles demonstrates the greatest growth potential also supports fair growth in the moderate halophiles. while in the NaCl saturated brines (≥25.5 Be) moderate halophiles fail to demonstrate appreciable growth. However in the present study the use of brine yielded poor growth of halophiles in laboratory conditions.

Among halophiles "polyextremophilic" types are found that are adapted to grow at combination of extreme conditions (Oran, 2011). The bacterial strains isolated can be considered so as they were able to grow in both Salt and alkaline conditions. Upasani and Desai (1990) also isolated haloalkaliphilic archae-bacteria from Sambhar Salt Lake, India.

Screening of hydrolase activities

Halophilic microorganisms have developed various biochemical strategies, including exo-enzymes production to adapt to hypersaline condition. (Mevarech *et al*.,2000). All the eight isolates were tested for extra-cellular hydrolase producing activity by plate assay on starch, tributyrin and gelatin agar plates for amylase, lipase and protease respectively. All the isolates showed strong amylase activity, while none of the isolate was able to hydrolyze lipid. Only, SL-2, Sl-4 and SSl-8 produced protease enzyme.

Halophiles have been perceived as a potential source of industrially useful enzymes endowed with exceptional stabilities. Kumar *et al.*, (2012) reported 20 strains of moderate halophiles from Sambhar salt lake with the amylase, lipase and protease activity. Sahay *et al*. (2012) studied various saline habitats of India and isolated moderate Halophilic bacterial strains with protease (56 %), cellulase (40 %), and amylase (37 %) producing activities.

Antibiotic Sensitivity test

Javor (1984) reported that moderate halophiles can not grow in the presence of 500 U of penicillin G or 300 U of polymyxin B. In the present study too growth of all the isolates was observed in the presence of these antibiotics and none of them was able to form colony. Further the categorization into archaebacterial and eubacterial strains is also confirmed by their growth in the presence of above mentioned antibiotics. Since the isolates failed to grow on Penicillin and polymyxin, thay fall in moderate Halophilic eubacterial group. It is noteworthy that low taxonomic biodiversity is observed in all the saline environments (Oren 1994; Kamekura, 1998), yet the metabolic diversity of halophiles is great. The diversity of metabolic types encountered decreases with salinity (Oran, 2002).

The distribution and abundance of microbial population to a permanently saline environment includes optimization of basic cell processes, enzyme modulation, nutrient transport and cell membrane function. The bacterial isolates from hypersaline condition also showed the ability to tolerate a wide range of salinity, pH and temperatures, and presented combined hydrolytic activity, which provides the advantage of uses in various industrial processes (Mellado and Ventosa 2003).

Sambhar Salt lake is a unique ecosystem and heaven for halophiles that belong to extremophile group. The present study revealed morphological and metabolic diversity among moderate halophiles of the lake. The lake is a ideal site for halophile studies. Further exploration of the microbial ecology of this lake would yield fruitful data on thediversity of life in this extreme environment.

Table.1 Main characteristics of the isolates

Isolate No.→	**SL-1**	**SL-2**	**SL-3**	**SL-4**	**SL-5**	**SL-6**	**SL-7**	**SL-8**
	Aerobe	Aerobe	Aerobe	Aerobe	Aerobe	Aerobe	Aerobe	Aerobe
Colony pigentation	Cream	Lemon yellow	Greyish Cream	Red-orange	Cream	Yellowish orange	Dull Cream	Red-orange
Elevation	Flat	convex	Flat	convex	convex	convex	Convex	Convex
	Glossy	Glossy	Non-glossy	Glossy	Translucent	Glossy	Non-glossy Mucoid	Glossy
Motility	+	+	+	+	+	+	+	+
Gram reaction	+	+	+	+	+	+	+	+
Growth range								
Salt (%)	0-10	3-10	3-12	5-15	5-15	3-15	1-10	10-20
pH	6-7.5	7-8	7-8	7-8.5	7-8	7-8	6-8	7-9
Oxidase	+	+	+	+	+	+	+	+
Catalas	+	+	+	+	+	+	+	+
NO_3 Reduction	+	+	+	+	+	+	+	+
NO_2 reduction								
Citrate Utilization	–	–	–	–	–	–	–	+
H_2S production	–	–	–	–	–	–	–	–
Hydrolysis of								
Urea	-	-	-	-	-	-	-	-
Starch	+	+	+	+	+	+	+	+
Casein	-	-	-	-	-	-	-	-
Gelatin	_	_	_	_	_	_	_	_
Tween 20	-	-	-	-	-	-	-	-
Tween 80	-	-	-	-	-	-	-	-
Acid production from								
Glucose	+	+	+	+	+	+	+	+
Fructose	+	+	+	+	+	+	+	+
Lactose	-	-	-	+	-	-	-	+
Sucrose	+	+	-	-	-	-	-	+
Arabinose	-	-	-	-	-	-	-	-
Trehalose	-	-	-	-	-	-	-	-

Plate-I; Phenotypic and Pigment variations of all the isolates

Plate-II; Results of Nitrate reduction Test

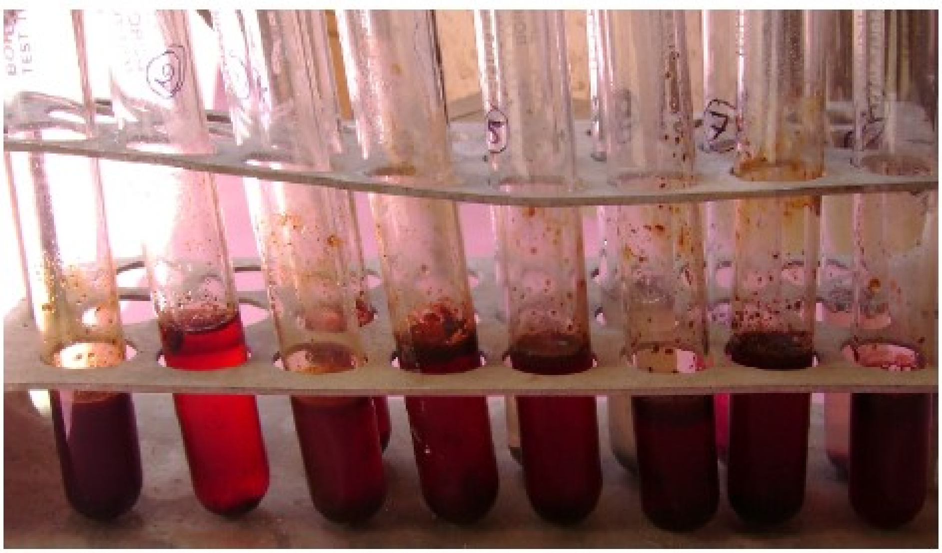


Plate-III Results of Citrate utilization test (SL-7 +ve)

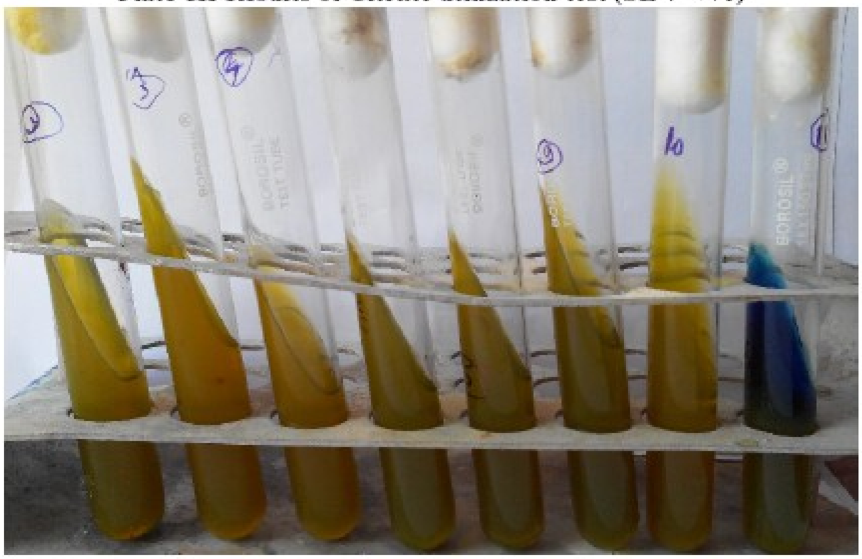
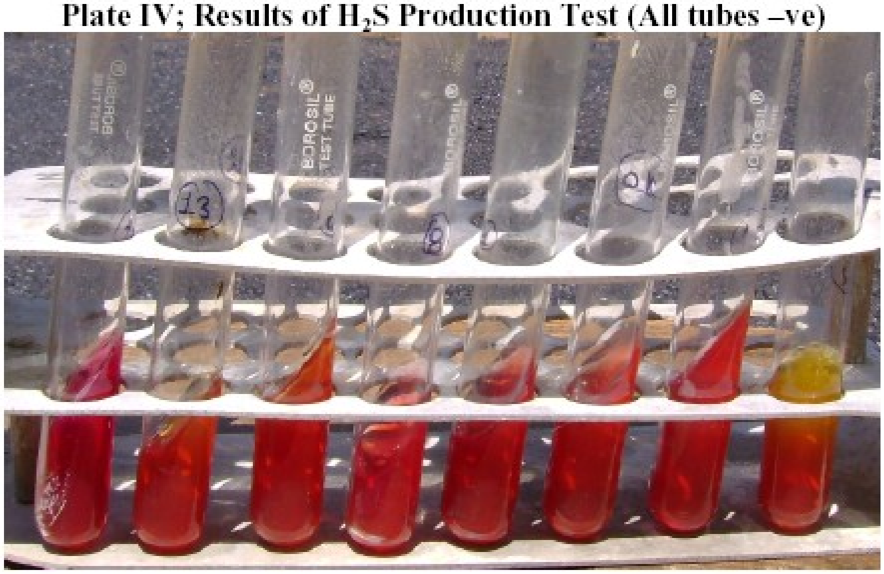


Plate IV; Results of H_2S Production Test (All tubes –ve)



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