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Biodiversity of Extremely Halophilic Bacterial Strains Isolated from Solar Salterns of Tuticorin, Tamilnadu, India

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Abstract: Tuticorin solar salterns located in the coastal area of Tamilnadu, India are composed of two main ecosystems (i.e. salt ponds and microbial mats). Even though these locations are characterized by high solar radiation (mean light intensity of 45 mol photons m^{-2} d^{-1}) they harbor a diverse microbial life. Two salt and two brine samples were selected for physico-chemical and microbiological analyses. The total number of extremely halophilic bacteria in salt samples (S1, S2) was higher than the brine samples (S3, S4). Most of the strains were extremely pleomorphic, appearing as irregular, short long and swollen rods. Colony pigmentation was in the shades of orange red to pale pink and yellowish cream to dark cream. None of the isolates grew at less than 10% (W/V) NaCl concentration. The pH values above 6.0 were suitable for all the strains. Six strains were sensitive to ampicillin, 15 strains were resistant to erythromycin and 12 strains were resistant to tetracycline and chloramphenicol. Interestingly four strains including BB_1, BC_3, JS_3 and JS_4 were found to be resistant to all antibiotics tested. The results of this study were discussed in terms of the ecological significance of these microorganisms in their potential for industry applications.

Key words: Athalassohaline · Bacteriorhodopsin · Hypersaline · Solar Salterns · Thalassohaline

INTRODUCTION

Living Organisms can be found over a wide range of extreme conditions. Most of the organisms living in extreme environments (extremophiles) belong to prokaryotes, especially to the domain Archaea [1]. Hypersaline environments are widely distributed in a variety of aquatic and terrestrial ecosystem [2]. Hypersaline environments can be divided into two broad categories: thalassohaline and athalassohaline [3]. Thalassohaline water bodies have similar salt composition to seawater with sodium and chloride being the dominant ions; common examples include the Great Salt Lake in Utah, USA brine springs from underground salt deposits and solar salterns [4]. In contrast, athalassohaline water bodies such as the Dead sea, Lake Magadi in Kenya, Wadi Natrun in Egypt, the soda lakes of Antarctica and Big Soda Lake and Mono Lake in California are dominated by potassium, magnesium, or sodium [3, 4]. Hypersaline water bodies are commonly 9-10 times more concentrated than sea water. Despite the prevailing extreme environment, a great diversity of microbial life has been observed in both natural and artificial hypersaline environments [5]. Aerobic, anaerobic and facultative anaerobic microbes belonging to domains archaea, bacteria and eukarya have been recovered from these environments, where they participate in overall organic matter oxidation. Members of the family Halobacteriaceae are the dominant microorganisms in hypersaline ecosystems (NaCl concentrations greater than 25%) [6, 7].

The most widely studied ecosystems are the Great Salt Lake (Utah, USA), the Dead Sea, the alkaline brines of Wadi Natrun (Egypt) and Lake Magadi (Kenya) [3]. It is noteworthy that low taxonomic biodiversity is observed in all these saline environments [8, 9], most probably due to the high salinity measured in these environments. Microorganisms that thrive in these environments have been broadly classified into halophilic and halotolerant microorganisms. Halophiles can be further divided into slight halophiles that grow optimally in 3% (w/v) total salt, moderate halophiles that grow optimally at 3-15% (w/v) salt and extreme halophiles that grow optimally at 25%, (w/v) salt [10]. The halophilic microorganisms are being the object of basic studies in relation to the origin of life in our planet and the molecular mechanisms of adaptation to saline and hypersaline conditions [11].

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Apart from their evolutionary and ecological significance, halophiles have promising biotechnological applications including food industry pigments, organic osmotic stabilizers, surfactants, enzymes able to function at low water activities, hydrolases (halozymes), production of renewable energy, bacteriorhodopsin applications including halography, optical computer and optical memory [3, 12, 13]. Besides these metabolic and physiological features, halophilic microorganisms are known to play important roles in fermenting fish sauces, degrading and transforming waste and organic pollutants in saline water, decolorization of textile dye effluents, modifying food textures and flavors [14]. South India features numerous ecosystems including extreme hypersaline environments in which microbial diversity has been poorly studied. Therefore the present study was intended to evaluate the physico-chemical properties of the salt and brine samples, to isolate various halophilic bacteria and examine their cultural, physiological, biochemical characters and antibiotic sensitivity pattern.

MATERIALS AND METHODS

Site Description: The study area was solar salterns of Tuticorin, located in the coastal area of Tamilnadu, India. The total area of the solar salterns is around 100 acres. The solar saltern was characterized by a high solar radian (45 mol photons m^{-2} d^{-1}), low precipitation (50 mm) and salinity (upto 600 PSU). Pluvial precipitation in the salterns varies depending on the time of the year, a dry period with a mean precipitation of 50mm runs from April to November. The climate on an average is generally warm and humid, fluctuating from a minimum of 20°C in the month of December and maximum of 42°C in May.

Sample Collection: Samples were collected from the solar salterns at 4 different locations. Samples were collected at the surface and various depths (0.1, 0.2 and 0.3 m) in each site. All the samples were collected in sterile polythene bags to prevent direct contact with air and transported to the laboratory in an ice box for physico-chemical and microbiological analysis. Details of the four different locations were as follows pink-red colored salt samples (S_1), brown colored salt samples from salt-depositing area (S_2), brine samples near waste disposal area (S_3) and brine samples (non salted fields) (S_4).

Physico-Chemical Analysis of the Samples: The pH, moisture content and Na^+, Ca^{2+}, Mg^{2+} and Cl^- contents of the salt and brine samples were measured according to the standard methods of Trussel *et al*. [15]. Cl^- was quantified by titration with $AgNO_3$, Mg^{2+} was quantified by atomic absorption spectrophotometry, Na^+ was quantified by flame spectrophotometry and Ca^{2+} was quantified by complexometry using EDTA. Temperature and pH were measured *in situ*.

Enrichment and Isolation of Halophilic Bacteria: Nearly 130 samples were collected from the above mentioned four different locations (Thalassohaline environment). Enrichment and isolation procedure to recover aerobic or facultative anaerobic moderate to extremely halophilic microorganisms were performed in basal halophilic medium [3] containing (per liter): NaCl, 200g; Casaminoacids, 7.5g; Yeast extract, 10g; Trisodium citrate, 3g; KCl, 2g; and $MgSO_4.7H_2O$, 20g. The pH was adjusted to 7.0 before autoclaving. Enrichment cultures were subcultured several times under the same conditions. Different colonies were picked and restreaked several times to obtain pure cultures. The pure cultures were stored at -20°C in the isolation medium supplemented with 50% glycerol.

Identification of the Isolates

Colony Morphology: The colony morphology was investigated by using standard microbiological criteria, with special emphasis on pigmentation, diameter, colonial elevation, margin, consistency and opacity [16]. The NaCl requirement for bacterial growth was determined in halophilic medium with 0, 5, 10, 15, 20, 25 and 30 % (w/v) total salts. The pH tolerance of each isolate was tested with pH values of 4.5, 6.0, 7.0 and 7.5.

Microscopic Examination: Cell morphology was examined by light microscopy of the exponentially growing liquid cultures. Gram's staining was performed by using acetic-acid fixed samples as described by Dussault [17]. Isolated strains were examined for motility and morphological features in wet mounts. Motility test was performed according to Vreeland [18].

Biochemical Tests: Inoculants for various biochemical tests were prepared by growing the isolates in sterilized Erlenmeyer flasks containing the complex growth medium at 37°C for 7 days. The optimal ionic content (per liter: tri sodium citrate, 3.0 g; KCl, 2.0 g, $MgSO_4.7H_2O$, 20 g and NaCl, 200 g) was added to all the biochemical test media. Gelatinase, β-galactosidase and cellulase activities, tween-80 hydrolysis, indole production, methyl red and voges-proskauer tests were performed using standard procedures [21]. The results were recorded after 21 days of incubation at 37°C.

Antibiotic Sensitivity Test: The antibiotic sensitivity of the halophilic strains was examined by spreading 0.1 ml of bacterial suspension on agar plates containing the above mentioned salt medium and applying discs of ampicillin (10 mg), erythromycin (15 mg), chloramphenicol (30 mg), streptomycin (25 mg), penicillin (10 mg), bacitracin (10 mg), novobiocin (30 mg) and tetracycline (30 mg). The results were recorded in terms of sensitivity or resistance after 14 days of incubation at 37°C, with sensitivity being defined as the appearance of a zone of inhibition extending at least 2mm beyond the antibiotic disc [19-21].

RESULTS AND DISCUSSION

Physico-Chemical Analysis: The average temperature at the sampling sites was 35°C at noon. The physico-chemical characteristics of the saltern samples (salt and brine) were shown in Table 1. Therefore, on the basis of their salt compositions, Tuticorin solar salterns, Tamil Nadu, India was categorized as Thalassohaline environment. Similarly, Maras saltern located in Peruvian Andes, Southern Peru was classified as Thalassohaline [22]. However, El-Djerid salt lake in southern Tunisia was considered athalassohaline because its salt composition

Table 1: Physico-chemical properties of different brine and salt samples (S_1-S_4)

Sampling site	Colour of sample	pH	Ca^{2+} (g/1)	Mg^{2+} (g/1)	HCO_3^- (g/1)	Cl^- (g/1)	Na^+ (g/1)	K^+ (g/1)	Total
S_1	Pink Red	8.20	1.6	1.22	34.16	528.3	324.6	0.3	908.18
S_2	Reddish Brown	8.15	4.0	1.94	39.04	534.4	346.6	0.4	926.38
S_3	Pale Brown	7.20	2.8	17.50	20.74	121.3	78.7	6.7	247.74
S_4	Dark Cream	7.32	1.4	12.15	19.52	137.1	86.5	7.7	264.37

Table 2: Cell and colony morphology of the isolated strains of halophilic bacteria

Strain	Cell Shape	Motility	Cell Arrangement	Colony Morphology	Colony Size	Colony Elevation	Colony Density	Colony Edge	Pigmentation
JS_1	Pleomorphic rod	++	Single and paired cells	Circular	2.5mm	Convex	Transparent and glistening	Entire	Pinkish red
JS_2	Pleomorphic cells	++	Single, paired and regularly clustered cells	Circular	1-3mm	Slightly raised	Transparent and glistening	Entire	Blood red
JS_3	Cocci	-	Single, paired and long chains	Circular	2.0mm	Convex	Translucent and matt	Entire	Yellowish cream
JS_4	Cocci	-	Single, paired and irregularly clustered cells	Circular	0.5-1.5mm	Slightly convex	Transparent and glistening	Entire	Dark cream
VPN-1	Pleomorphic rod	+	Single, paired and short chains	Square	1.5-2.5mm	Slightly raised	Transparent and glistening	Entire	Brick red
VPN-2	Pleomorphic rod	++	Single and paired cells	Circular	2mm	Convex	Transparent and glistening	Entire	Pink
VPN-3	Pleomorphic cells	+	Single, paired and irregularly clustered cells	Circular	1.5mm	Flat	Opaque matt	Entire	Dark cream
VPN-4	Pleomorphic rod	++	Single, paired and long chains	Circular	2mm	Convex	Translucent and matt	Entire	Cream
VPN-5	Cocci	-	Single and paired cells	Circular	1mm	Slightly raised	Transparent and glistening	Irregular and wavy	Yellowish cream
BB_1	Pleomorphic rods	+	Single, paired and long chains	Circular	3mm	Convex	Translucent and matt	Entire	Orange
BB_2	Cocci	-	Single, paired and long chains	Circular	2mm	Slightly raised	Opaque matt	Entire	Pale pink
BB_3	Pleomorphic rods	+	Single, paired and irregularly clustered cells	Circular	2.5mm	Convex	Transparent and glistening	Irregular and wavy	White
BC_1	Pleomorphic rods	-	Single and paired cells	Circular	1.5mm	Convex	Translucent and matt	Entire	Pale pink
BC_2	Pleomorphic rods	+	Single and paired cells	Circular	2.5mm	Slightly sonvex	Transparent and glistening	Entire	White
BC_3	Cocci	-	Single, paired and long chains	Circular	2mm	Flat	Translucent and matt	Entire	Dark cream
BC_4	Pleomorphic cells	+	Single and paired cells	Circular	1mm	Convex	Transparent and glistening	Irregular and wavy	Pink

has derived from the dissolution of minerals of continental origin [1]. Similar to other hypersaline environments, Tuticorin solar saltern is characterized by high salinity (> 25% w/v NaCl concentration), low oxygen concentrations, high light intensity, ample nutrient availability and neutral pH [13], which makes it an ideal target for many researchers. The pH values of the salt samples (S_1 and S_2) were higher than those of brine samples (S_3 and S_4). However, the pH of WadiNatrun lake and Magadi lake (Kenya) was highly alkaline (pH 11.0) [23, 24]. The highest HCO_3^- content was found in the S_2 salt samples. Both salt (S_1, S_2) and brine (S_3, S_4) samples, with the presence of Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^- and HCO_3^- were expected to support the growth of extremely halophilic bacteria (Table 1).

Microbiological Analysis: The total number of extremely halophilic bacteria in salt samples (S_1, S_2) was higher than the brine samples (S_3, S_4). Screening of extreme halophiles from different sampling sites (hypersaline environments) of Tuticorin solar salterns has led to the isolation of 16 extremely halophilic bacterial strains.

Cell and Colony Morphology: Six different morphological varieties of extremely halophilic bacterial strains were observed (Table 2). Most of the isolates were found to be extremely pleomorphic, appearing as irregular, short, long and swollen rods, bent rods, clubs, spheres and triangles that occurred in singles, pairs or short chains. The dominant bacterial population comprised of Gram-negative, motile and pleomorphic rods. As anticipated by Oren and Rodriguez [28], hypersaline environments at different geographical locations may indeed harbor microbial communities with different morphological structures. Colony Pigmentation was in the shades of red (brick-red, blood red), pink (pale pink, pink), cream and yellow. Both salt and brine samples were found to be dominated by extremely halophilic bacterial strains (63%) producing red-pink shaded colonies on halophilic agar plates (Fig. 1). Red pigmentation may be due to a high content of C-50 carotenoid pigments (α-bacterioruberin and derivatives) in their membrane, in some cases accompanied by the purple retinal pigment (bacteriorhodopsin). High carotenoid pigments present in halobacterial cells makes their habitat bright red in color, as noticed in case of Great Salt Lake (Utah), crystallizer ponds of solar salterns, hypersaline soda lake such as Lake Magadi (Kenya) and the Dead Sea [29].

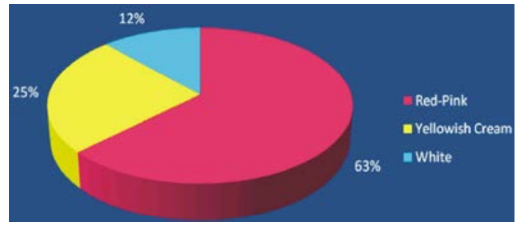

Fig. 1: Chromogenicity of the isolated halophilic bacterial strains

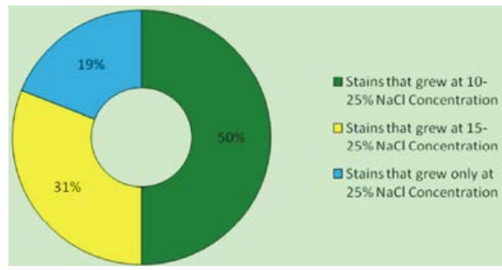

Fig. 2: NaCl requirements of the isolated halophilic bacterial strains

Salt and pH Tolerance: One of the principal findings of the present study was the complete absence of halotolerant microorganisms. Most of the isolates required at least 10% salt (NaCl) to grow and the entire bacterial community could not grow without NaCl. Since almost all the strains had salt requirements above 10% and grew optimally in between 20-25% (w/v) NaCl concentrations, they can be categorized as "Extremely halophilic bacteria "based upon Larsen's definitions [30] (Fig. 2). In general, the halobacterial strains require high salt concentration for cell growth and integrity. A decrease in salt concentration (less than 1 M) may lead to reduction of growth [31]. They, with some exceptions, lyse or lose viability in low salt concentrations, or distilled water. For instance, cells of *Haloarcula japonica* lyse upon suspension in 5% NaCl solution [32]. A "heat-shock" like response to salt dilution has been reported earlier in *Haloferax volcanii* cells [33]. The strains were tested for growth potential with respect to various pH ranges and NaCl concentrations in halophilic medium. The pH values above 6.0 were suitable for all the strains, however growth at pH 7.5 was found to be optimal. None of the isolates exhibited growth at pH 4.5 (Table 3).

Biochemical Characteristics: Out of 16 extremely halophilic bacterial strains, ten isolates produced indole from tryptophan. All the isolates showed positive methyl red reaction except BB_2 (Table 4). Whereas, only one isolate (BB_2) showed positive Voges Proskauer's reaction.

Table 3: Salt and pH tolerance of the isolated strains of halophilic bacteria at 40°C

	Growth at various NaCl (w/v) concentrations						Growth at various pH			
Strain	0% NaCl	5% NaCl	10% NaCl	15% NaCl	20% NaCl	25% NaCl	4.5	6	7	7.5
JS_1	-	-	+	+	+	+	-	+	++	++
JS_2	-	-	-	+	+	+	-	+	+	++
JS_3	-	-	-	-	-	+	-	+	+	++
JS_4	-	-	-	+	+	+	-	+	++	++
VPN-1	-	-	-	-	-	+	-	+	+	++
VPN-2	-	-	+	+	+	+	-	+	++	++
VPN-3	-	-	-	-	-	+	-	+	+	++
VPN-4	-	-	+	+	+	+	-	+	++	++
VPN5	-	-	-	+	+	+	-	+	+	++
BB_1	-	-	+	+	+	+	-	+	+	++
BB_2	-	-	+	+	+	+	-	+	+	++
BB_3	-	-	+	+	+	+	-	+	+	++
BC_1	-	-	+	+	+	+	-	+	+	++
BC_2	-	-	-	+	+	+	-	+	++	++
BC_3	-	-	+	+	+	+	-	+	+	++
BC_4	-	-	-	+	+	+	-	+	+	++

Table 4: Biochemical characteristics of the isolated halophilic bacterial strains

								CHO fermentation with acid production		
Strain	Oxidase	Catalase	Nitrate reduction	Indole	Methyl red	Voges -Proskauer's	Citrate	Glucose (1%)	Fructose (1%)	Maltose (1%)
JS_1	+	+	+	-	+	-	+	-	+	+
JS_2	-	+	+	+	+	-	+	+	-	+
JS_3	+	+	-	-	+	-	+	+	-	-
JS_4	-	+	+	+	+	-	+	+	-	-
VPN-1	+	+	-	+	+	-	+	-	+	+
VPN-2	+	+	+	-	+	-	+	+	-	+
VPN-3	-	+	+	+	+	-	+	+	-	-
VPN-4	+	+	+	+	+	-	+	-	+	+
VPN-5	-	+	+	-	+	-	-	+	-	-
BB_1	-	+	+	+	+	-	+	-	-	-
BB_2	+	+	+	+	-	+	+	-	+	+
BB_3	+	+	+	-	+	-	+	+	-	-
BC_1	-	+	+	-	+	-	+	-	+	+
BC_2	+	+	-	+	+	-	+	-	-	-
BC_3	+	+	+	+	+	-	+	+	+	+
BC_4	+	+	-	+	+	-	+	+	-	-

75% of the isolates showed their ability to reduce nitrate into nitrite and 10 isolates showed positive oxidase reactions. All the 16 isolates produced catalase enzyme. All isolates except VPN-5 utilized citrate as their carbon source. About 56% of the isolates fermented glucose with acid production. Different halophilic bacteria such as *Haloferax sulfurifontis* [34] and *Oceanobacillus aswenensis* FS10 [35] fermented glucose with acid production. Nearly 37% of the isolates fermented fructose with acid production. Fructose fermentation has been reported in *Halobacterium mediterranei* [36].

Antibiotic Sensitivity Test: Antibiotic sensitivity pattern of the 16 halophilic bacterial strains is shown in Table 5 which revealed that, 6 strains were sensitive to ampicillin, 15 strains were resistant to erythromycin, 13 strains were resistant to penicillin, 7 strains were sensitive to novobiocin and bacitracin, 12 strains were resistant to streptomycin, tetracycline and chloramphenicol. Interestingly three strains including BB_1, JS_3 and JS_4 were found to be resistant to all the antibiotics tested. In general, bacterial strains producing cream and yellowish cream colonies were found to be sensitive to ampicillin,

Table 5: Antibiotic sensitivity of the isolated halophilic bacteria

Strain	Ampicillin	Erythromycin	Chloramphenicol	Streptomycin	Penicillin	Tetracycline	Bacitracin	Novobiocin
JS_1	R	R	R	R	R	R	S	S
JS_2	R	R	S	R	R	S	R	R
JS_3	R	R	R	R	R	R	R	R
JS_4	R	R	R	R	R	R	R	R
VPN-1	R	R	R	R	R	R	S	S
VPN-2	R	R	R	R	R	R	S	S
VPN-3	R	R	R	R	R	R	S	S
VPN-4	S	S	S	S	S	S	R	R
VPN-5	S	R	R	S	R	R	R	R
BB_1	R	R	R	R	R	R	R	R
BB_2	S	R	R	R	S	R	S	S
BB_3	R	R	R	R	R	R	R	R
BC_1	S	R	S	R	R	R	S	S
BC_2	S	R	S	S	R	S	R	R
BC_3	S	R	R	S	S	S	R	R
BC_4	R	R	R	R	R	R	S	S

R-Resistant, S-Sensitive

erythromycin, penicillin, chloramphenicol and tetracycline but resistant to novobiocin and bacitracin. Whereas, the strains producing red-pink colonies were found to be resistant to ampicillin, penicillin and chloramphenicol, but sensitive to novobiocin and bacitracin. According to these antibiotic sensitivity results, it was thought that cream-yellowish colonies were the members of eubacteria and red-pink colonies were the members of archaea bacteria (21). The characteristic antibiotic sensitivity pattern of these strains may serve as a guideline in the search for further biochemical and molecular differences between archaebaceria and eubacteria and it may also be helpful in identifying new species in the solar salterns [3, 21].

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