ISOLATION AND PHYLOGENETIC CHARACTERIZATION OF EXTREMOPHILES FROM MARAKANAM SALTERNS

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ABSTRACT: Salterns are highly saline environments, where salt concentrations inhibit growth of most of the organisms. Recent studies have revealed that these environments are loaded with a variety of microbial life. In the present study, sediment samples were collected from Salterns of Marakanam, Tamil Nadu and were analyzed for presence of any halotolerant bacteria in them. Twelve isolates were obtained after processing the samples and were characterized. Two isolates among the total were selected based on unique biochemical characteristics and were further studied. The 16S rRNA gene sequencing revealed that these isolates are *Pontibacillus chungwensis* (P1) and *Bacillus barbaricus* (P2). The isolate P2 has not yet been reported to be isolated from such an environment and no reports of salt tolerance characteristics. In addition to that the 16S rRNA gene analysis results showed the sequence of strain to be located in a different domain well away from *Bacillus barbaricus* type strain. Further work on the bacterium might result in the strain P2 being claimed as novel. Any study on the microbial life of these salterns is helpful in extending our knowledge on the microorganisms present in such environments and their physiology which might be useful in agriculture, industry and Costal areas.

Key words- *B.barbaricus, P.chungwensis*, salterns, halophiles, Marakanam coast

INTRODUCTION

A wide range of microbes habitats and be an imperative part of all environment ranging from moderate to harsh conditions to live like hot water springs, hydrothermal vents, salterns, and other such environments with extreme conditions, where ordinary life conditions are not observed.(Madigan *et al.,* 1997) Astonishingly, these extreme environments have been found to contain organisms that developed strategies or live optimally in such environments and lives occurring in such environments have found to possess an elite biotechnological potential.(Dave *et al.,* 2006) High amount of salts (more than 3%) in the environment is a source of stress and threat to life in such severe environment. Artificial solar salterns usually consist of a series of shallow evaporation ponds connected by pipes and canals, and as evaporation occurs, brine is directed into ponds and salt is precipitated.(Oren, 2006) Halophiles are basically salt-loving organisms that inhabit hypersaline environments which include a variety of heterotrophic and methanogenic archaea, photosynthetic, lithotrophic and heterotrophic bacteria and photosynthetic and heterotrophic eukaryotes, which balance the osmotic pressure from the harsh saline environment. (Das Sarma *et al.,* 2001) Halophilic bacteria can be classified according to their salt requirement and growth pattern. Slight halophiles show optimum growth at 2–5% NaCl, moderate halophiles at 5–20% NaCl, and extreme halophiles show 20–30% NaCl respectively. Many halophiles and halotolerant microorganisms can grow over a wide range of salt concentrations with occasionally depending on environmental and nutritional factors for the growth and tolerance. (Surajit Das *et al.,* 2006).

Studies on the microbial life at salterns have revealed that these environments nurture an array of organisms includes macroscopic organisms like multicellular eukaryotes, algae, fungi, and prokaryotic microscopic population consists of cyanobacteria, various sulfur-oxidizing bacteria, phototrophic bacteria and a large range of archea. (Das Sarma *et al.,* 2001) Study on the microbial life of salterns will be very much useful as it would result in discovery of novel microorganisms or novel products. Such studies will also help in understanding the mechanisms behind the survival of life under extreme conditions. Therefore research on such area must be emphasized upon. In the current study an attempt was taken to isolate the bacterial population from the saltern sediments collected from Marakanam and were studied for their biochemical and taxonomic status of two strains through 16S rRNA gene sequencing.

MATERIALS AND METHODS

Collection of samples:

The sediment samples were collected from the saltern zone Kaipenikuppam near Marakkanam, Tamil Nadu in the month of February and March of 2009, temperature ranging 40-450c, pH-8 respectively. The samples were collected using a sterile container and were transferred to laboratory, processed within 24 hours of collection. All the the samples were stored in the refrigerator at 70. Serial Sample dilution was carried out using sterile saline which contain 8.5 gm NaCl in 1000 ml. Dilutions up to 10-7 were made to perform Spread plate technique. All the samples were plated on Modified Nutrient Agar, Starch Caesin Agar, and Ken Knight's Agar prepared with 50% natural sea water and supplemented with additional NaCl of 7gms/100mL. Artificial sea water was used in absence of seawater

Biochemical Characterization:

Gram staining was performed to study the morphological characteristics of the selected isolates. Various biochemical test were performed *viz.,* citrate utilization, production of
Catalase, oxidase, indole, urease, acetoin or acid production and gelatin liquefaction. Various carbon sources were tested Sucrose, Glucose, Arabinose, Maltose and Lactose. (Cappuccino J G, 2006)

Salt tolerance:

All isolates were screened for their salt tolerance efficacy level by plating them on to nutrient agar plates amended with concentrations of salt (NaCl) ranging from 0% to 25% (Rohban.R *et al*., 2009)

pH Range

Modified Nutrient broth was prepared at different pH range to detect the tolerance level of the selected strains P1 and P2. The flasks were incubated in a rotatory shaker for
24 hrs at 28 ± 2^0 c and the OD value for each pH flask was noted. (Rohban.R *et al*., 2009)

Proteolytic Assay:

The proteolytic activity of the organism was checked using Modified Skim Milk Agar. (Malka Halpern *et al,* 2007) Skim Milk Agar plates were prepared and the isolates were inoculated. The plates were incubated at room temperature for 48 hours for the zone of clearance around the colonies.

Lipolytic Assay:

The lipolytic activity of the organism was checked using Modified Tributyrin Agar. (Mielmann *et al,* 2006) Tributyrin Agar plates were prepared and the organism was inoculated on to it. The plates were incubated at room temperature for 48 hours. The dark blue zones around the colonies were expected.

Starch hydrolysis:

Modified Starch Agar was prepared for the detection of starch hydrolysis by the test isolates. (Hugo *et al*, 2003) Starch Agar plates were prepared and inoculated with the isolated for 24 hours at RT. Iodine was poured in drops on the plate.

Phosphate solubilization:

Phosphate solubilising assay for the test isolates were carried out using Modified Pikovskaya's Agar. (Husen, 2003) Modified Pikovskaya's Agar plates was streaked with the isolates and incubated at room temperature for 48 hours and the plates were observed for the zone of solubilisation.

Siderophore Analysis:

The siderophore producing ability of the organism was checked using the universal CAS assay. (Shwyan and Neiland 1987)The blue colour plates were incubated for 24-48hrs at RT for the change of colour from blue to orange or yellow.

Heavy Metal tolerance:

The strains P1 and P2 were analyzed for their tolerance to heavy metals like Zinc and Lead. LGI broths containing 1000ppm of the mentioned metals were prepared and the strains were inoculated. The broth was observed for any growth and solublization of the metals. (Saravanan *et al*., 2007)

16S rRNA Gene sequencing

Bacterial DNA from cells cultured on nutrient agar was extracted according to the sambrook et al. The strains were amplified using the following universal primers 27F (AGAGTTTGATCCTGGCTCAG), 1492R (GGTTACCTTGTTACGACTT) and the Sequences were identified by PCR direct sequencing using big-dye primer method using an automated DNA sequencer (ABI Prism 310 Genetic Analyser, Tokyo, Japan). (Reference)

RESULTS AND DISCUSSION

In the present study the Sediment samples from Marakanam solar salterns were collected and were serially diluted in saline and plated onto Modified Nutrient Agar medium. Dilutions from 10^{-4} to 10^{-8} were used for plating. The media was prepared using 50% (v/v) natural sea water and 7% (w/v) NaCl with other common ingredients of Nutrient Agar medium. The subcultered colonies showed interesting characteristics like pigment production (yellow colour) and high salt tolerance up to 25%. Finally 12 strains were isolated and maintained on Modified Nutrient Agar medium at 37^{o}C.The isolates were analyzed for standard biochemical tests like Citrate utilization, Urease Production, Indoleproduction, Methyl red, Voges proskauer, Gelatin, Nitrate reduction, Catalase and Oxidase production. All the results were tabulated in Table1

TABLE 1: Biochemical Characterization

Sl. No	Strain	Citrate	Urease	Indole	MR	VP	Nitrate reduction	Catalase	Oxidase	Gelatin
1	P1	-	-	-	-	-	-	+	-	-
2	P2	-	-	-	-	-	-	+	-	-
3	P3	+	+	-	-	-	+	+	+	-
4	P4	+	+	-	-	-	-	+	+	-
5	P5	+	+	-	-	-	-	+	-	-
6	P6	+	-	-	-	-	+	+	+	-
7	P7	-	+	-	-	-	-	+	-	-
8	P8	-	-	-	-	-	-	+	-	-
9	P9	-	-	-	-	-	+	-	+	-
10	P10	-	-	-	-	-	+	-	+	-
11	P11	-	+	-	+	-	+	+	-	-
12	P12	-	-	-	-	-	+	+	-	-

Two strains were selected based on their unique biochemical characteristics and were further studied. Further studies included plotting the proteolytic, lipolytic assays, hydrolysis of starch and gelatin, phosphate solublization, production of siderophores, and heavy metal tolerance. The strains P1 and P2 selected for study were unable to possess the proteolytic and Lipolytic activity. The isolates were analyzed for the utilization of various sugars as carbon sources and the results were tabulated in Table 2. The sugars used were glucose, arabinose, lactose, maltose, and sucrose. The Starch hydrolysis test for the selected strains P1 and P2 on Starch agar plates with incubation of 24 hrs were found to hydrolyze the starch forming zone on the iodine amended plate.

Siderophore is an iron chelating molecule that are utilized by the beneficial microbes in order to bypass iron deficiency, strains P1 and P2 were inoculated in Modified Fiss Minimal Medium and then incubated at room temperature for 48 hours in the shaker. The inoculated medium was centrifuged at 13,500 rpm for 10 minutes after incubation. The supernatant was collected in a sterilized tube. The CAS agar plates were prepared and wells were made in the medium using cork borer, the supernatant (60µL) was poured in to it and incubated for 8-12 hours. The lack of color change of the medium may be due to non requirement of the iron molecule. The isolates were found to grow in the presence of heavy metals (zinc and lead) at 1000 ppm level. This shows Heavy metal tolerance and that the isolates can even grow at metal contaminated sites. Further studies on metal solublization or degradation can result in potential application on remediation of soils having high salinity conditions and metal contamination if proved to be positive for such properties.

The isolates were analyzed for their salt tolerance level and the tolerance ranges of the isolates were tabulated in Table 3.

TABLE 2: Utilization of carbon sources

Sl.No	Strains	Utilization of Carbon Source				
		Glucose	Arabinose	Lactose	Maltose	Sucrose
1	P1	-	-	-	-	-
2	P2	-	-	-	-	-
3	P3	-	-	-	-	-
4	P4	-	-	-	-	-
5	P5	+	-	-	+	+
6	P6	-	-	-	-	-
7	P7	+	-	-	+	+
8	P8	+	-	-	+	-
9	P9	-	-	-	-	-
10	P10	-	-	-	-	-
11	P11	+	-	-	+	+
12	P12	-	-	-	-	-

16S rRNA gene sequence analysis:

The 16S rRNA sequence analysis showed that the isolates P1 to be *Pontibacilli chungwensis* and P2 to be *Bacillus barbaricus*. The rRNA sequence of the isolate P1 showed that the isolate belonged to *Pontibacillis chunwensis* which has been already isolated and has been well described. Given below is the phylogenetic tree showing the relation of P1 with its top 20 matches in their rRNA gene sequence. The Phylogenetic tree is based on 16S rRNA gene sequence of **P1**, which is *Pontibacillus chungwensis* with certain related genera. The strain analysed in this study is highlighted. The numbers at the nodes indicate the levels of the bootstrap support [high bootstrap values (close to 100%) mean uniform support] based on a neighbor-joining analysis of 1,000 re-sampled data sets. The bootstrap values below 50% were not indicated. Bar 0.005 substitutions per site.

TABLE 3: Salt tolerance level

Sl.No.	Strains	NaCl concentration in %										
		0	3	5	7	10	12	15	17	20	22	25
1	P1	+	+	+	+	+	+	+	+	+	+	-
2	P2	+	+	+	+	+	+	-	-	-	-	-
3	P3	+	+	+	+	+	+	+	+	+	+	-
4	P4	+	+	+	+	+	+	+	+	+	+	+
5	P5	+	+	+	+	+	+	-	-	-	-	-
6	P6	+	+	+	+	+	+	+	+	+	+	-
7	P7	+	+	+	+	+	+	-	-	-	-	-
8	P8	+	+	+	+	+	+	-	-	-	-	-
9	P9	+	+	+	+	+	+	+	+	+	+	-
10	P10	+	+	+	+	+	+	+	+	+	+	+
11	P11	+	+	+	+	+	+	-	-	-	-	-
12	P12	+	+	+	+	+	+	+	+	+	+	+

Given below is the phylogenetic tree showing the relation of P2 with its top 20 matches in their rRNA gene sequence. Again the phylogenetic tree is done by a neighbor joining tool, which shows the relation between the isolate P2 and its neighbor strains along with their respective distances. The Phylogenetic tree is based on 16S rRNA gene sequence of **P2**, which is *Bacillus barbaricus* with its related genera. The strain analysed in this study is highlighted. The numbers at the nodes indicate the levels of the bootstrap support [high bootstrap values (close to 100%) mean uniform support] based on a neighbor-joining analysis of 1,000 re-sampled data sets. The bootstrap values below 50% were not indicated. Bar 0.001 substitutions per site

It was observed that the strain P2 bifurcated from the already type described species in the site of sample collection and salinity tolerance level, thus creating a possibility that the isolated culture might be a different from the already described species. The phylogenetic tree was constructed using neighbor joining tool, which shows the relationship between the isolate P1 and its neighbour strains along with their respective distances.

Molecular analysis of the ribosomal RNA showed that the strains as P1- *Pontibacillus chungwensis* and P2 – *Bacillus barbaricus*. Pontibacillus has already been reported to be isolated from salterns of Korea, but Bacillus barbaricus is being first time reported in this work as an isolate from saltern environment. In addition to this the 16S rRNA gene sequencing studies show that the position of the sequence bifurcates from its domain and might be described as a novel species if further studied and characterized in detail. The two strains have shown to tolerate extreme conditions as they can grow at high pH, high salt levels and can also grow in medium containing heavy metals. Further studies have to be conducted to exploit the isolates for using them in various applications of bioremediation and costal area irrigation.

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