

SHORT REPORT

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Culturable halophilic archaea at the initial and crystallization stages of salt production in a natural solar saltern of Goa, India

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Abstract

Background: Goa is a coastal state in India and salt making is being practiced for many years. This investigation aimed in determining the culturable haloarchaeal diversity during two different phases of salt production in a natural solar saltern of Ribandar, Goa. Water and sediment samples were collected from the saltern during pre-salt harvesting phase and salt harvesting phase. Salinity and pH of the sampling site was determined. Isolates were obtained by plating of the samples on complex and synthetic haloarchaeal media. Morphology of the isolates was determined using Gram staining and electron microscopy. Response of cells to distilled water was studied spectrophotometrically at 600nm. Molecular identification of the isolates was performed by sequencing the 16S rRNA.

Results: Salinity of salt pans varied from 3-4% (non-salt production phase) to 30% (salt production phase) and pH varied from 7.0-8.0. Seven haloarchaeal strains were isolated from water and sediment samples during non-salt production phase and seventeen haloarchaeal strains were isolated during the salt production phase. All the strains stained uniformly Gram negative. The orange-red acetone extract of the pigments showed similar spectrophotometric profile with absorption maxima at 393, 474, 501 and 535 nm. All isolates obtained from the salt dilute phase were grouped within the genus *Halococcus*. This was validated using both total lipid profiling and 16S rRNA data sequencing. The isolates obtained from pre-salt harvesting phase were resistant to lysis. 16S rRNA data showed that organisms belonging to *Halorubrum*, *Haloarcula*, *Haloferax* and *Halococcus* genera were obtained during the salt concentrated phase. The isolates obtained from salt harvesting phase showed varied lysis on suspension in distilled water and /or 3.5% NaCl.

Conclusion: Salterns in Goa are transiently operated during post monsoon season from January to May. During the pre-salt harvesting phase, all the isolates obtained belonged to *Halococcus sp.* During the salt harvesting phase, isolates belonging to *Halorubrum*, *Haloarcula*, *Haloferax* and *Halococcus* genera were obtained. This study clearly indicates that *Halococcus sp.* dominates during the low salinity conditions.

Keywords: Archaea, Haloarchaea, Hypersaline, Solar saltern

Findings

Marine solar salterns are thalassohaline hypersaline environments located in tropical and subtropical areas worldwide, consisting of shallow ponds for the production of common salt from seawater during summer. The method of making salt through natural evaporation dates back to pre-historic times. This traditional approach of

salt production involves construction of series of rectangular ponds, each connected to the other through a common opening [1-4].

Goa (15°34'60N, 74°0'0E) is a coastal state in India and salt making is being practiced for many years. Salt pans are found in Pernem, Bardez, Tiswadi and Salcete talukas of Goa. Salt pans are inundated by sea water from estuaries during high tides. Sea water is retained in every pond for certain time to facilitate evaporation. As concentration of NaCl gradually increases, first component to precipitate is

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calcium ion (Ca^{2+}) in the form of gypsum. Then the concentrated sea water is allowed into final crystallizer pond, where NaCl crystals precipitate out [1]. The whole process of concentrating sea water begins usually in January - February and salt crystals are harvested during March - May. During the remaining months of June - December, ponds are inundated by sea/rain water. The crude salt produced from these salt pans is being used domestically for cooking, ice plants, as fertilizers, as termite repellent and for curing dry fish.

Microbial life is found in various extreme environments and salt pans are no exception. Depending on salt concentration, salt pans are inhabited by different groups of microbes thriving symbiotically [5]. Various microbes that inhabit the salt pans range from prokaryotes like Bacteria (*Salinibacter* spp.) and Archaea (*Halobacterium* spp.) to eukaryotes like Fungi (*Hortaea* spp.) and Algae (*Dunaliella* spp.) [6,7].

Haloarchaea are a group of extreme halophiles which require at least 2.5 M NaCl for their growth and are placed in the order *Halobacteriales* under family *Halobacteriaceae* [8]. At the time of writing, family *Halobacteriaceae* accommodated 36 recognized genera, members of which inhabiting both thalassohaline and athalassohaline environments [9,10]. Many studies have shown that there is a great variation in the diversity and dominance of haloarchaeal genera within various geographical locations [11-16]. Novel haloarchaeal microorganisms have been isolated from various niches such as acidic and alkaline regions, animal hides, salted fishes and also from commercial salt [17-23].

The aim of the present investigation was to evaluate the diversity of culturable haloarchaeal members in salt pans of Goa during two stages of salt production. For this investigation, the diversity of haloarchaea found within a single solar saltern at Ribandar (15°30'N, 73°51'E), Goa was examined.

Ribandar salterns are located on the banks of the river Mandovi, surrounded by mangrove vegetation in the adjoining marshy area. These salt pans cover an area about 12 dm² and lie between the cities of Panaji and Old Goa (Figure 1). Ribandar experiences tropical monsoon climate with maximum temperature around 30-36°C in summer and minimum around 20-28°C in winter. This region receives a heavy monsoon rainfall averaging around 300 cm. The salt pans are located at 9 ft above sea level and experience strong coastal winds during summer facilitating the evaporation of water in salterns. The salt pans are surrounded by raised mud borders called as *bunds* (dykes). These *bunds* help in containing the sea water within the pan. The sluice gate at the inlet regulates the inflow of water from the Mandovi estuary (Additional file 1: Figure S1, supplementary data). The salterns are seeded with crude salt to speed up the

crystallization process. During April and May the salt is harvested daily from these salterns.

Water samples and sediment samples were collected from 0-10 cm distance from the surface. Sampling was carried out twice during the two phases of salt production. First sampling was carried out in February 2010, when the salt pans are full of sea water. The second sampling was carried out in April 2010, when salt harvesting is at its peak. Water and sediment samples were collected by scooping from the surface as well as at a depth of 10 cm. Salinity and pH of sampling sites were measured using conductivity meter (EQUIP-TRONICS MODEL EQ-682) and pH meter (EQUIP-TRONICS MODEL EQ-632). Conductivity was correlated to salinity using the equation given by Williams, 1986 [24].

$$S = 0.4665 \times 1.0878^C \quad (r^2 = 0.98799)$$

The samples were collected in sterile tubes, sterile 1 L bottles and stored at 4°C and processed within 48 h.

Direct plating and enrichment techniques were employed for isolation of haloarchaeal organisms from water and sediment samples. Two media were used in the study, NaCl Tryptone Yeast Extract (NTYE) medium and NaCl Tri-Na-citrate (NT) medium [25-27], both containing 25% NaCl. The main difference with both the media is the presence of trisodium citrate in NT medium, which can support the growth of fastidious organisms as compared to NTYE medium. In direct plating method, one hundred µl of water sample or a loopful of sediment sample was directly spread plated on media. In the enrichment technique method, one ml of water sample or one gram of sediment sample was aseptically transferred to 50ml media and incubated at 37°C for up to 5 days. Then ten µl aliquots were plated on media. Plates were incubated at room temperature (30°C) for 30 to 45 days until red-orange pigmented colonies appeared. Colonies were selected based on their morphology and/or pigmentation and purified through repeated sub-culturing.

Morphology of the isolates was determined using Gram staining and electron microscopy. Cell suspensions were prepared on glass slides in a drop of 15% (w/v) NaCl solution and air-dried. The cells were desalted with 2% acetic acid followed by Gram staining and observed using phase-contrast microscope (Olympus BX41). For electron microscopy, the cell pellets were dispensed in NaCl Synthetic Media (NSM) to an absorbance of 0.8 at 600nm [28]. One hundred µl of suspension was mounted onto circular glass cover slips, fixed with 2.0% glutaraldehyde fixative (prepared in NSM) at room temperature (30°C) overnight. The coverslips were then exposed, to a series of increasing gradient of acetone-water, corresponding to 30%, 50%, 70%, 90% for 10 min respectively and finally in 100%

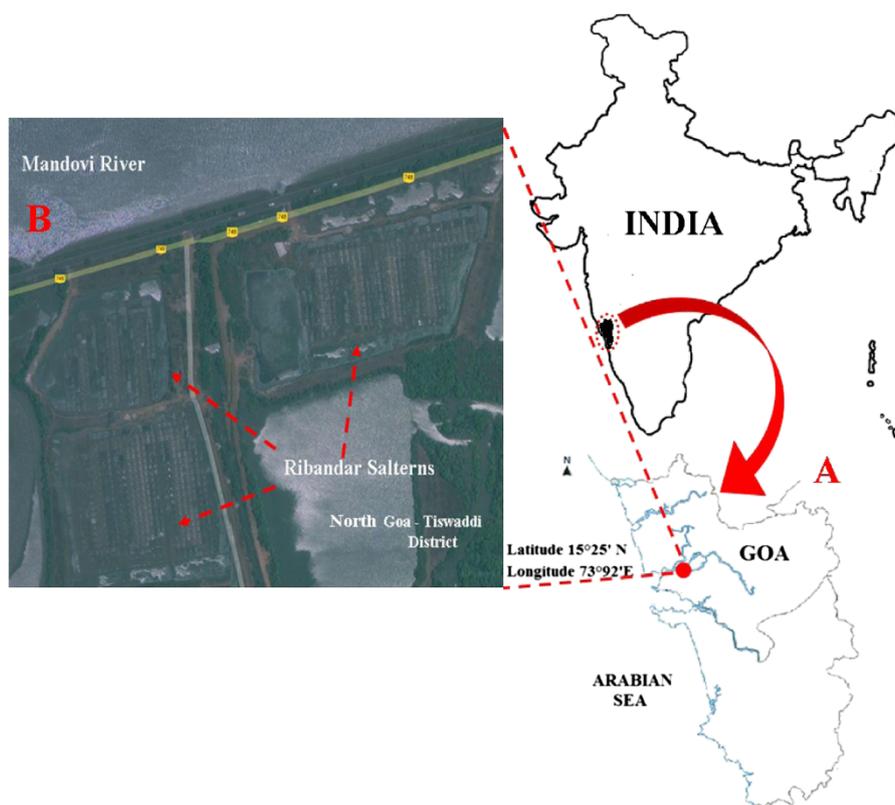


Figure 1 Solar Salterns of Ribandar, Goa. A) Location of Ribandar Salterns in Goa. B) Aerial image of the Ribandar Salterns. [source: Google Earth, 2010].

acetone, for 30 min, air dried and then viewed under scanning electron microscope (JEOL-5800 LV SEM).

Pigments were extracted by sonicating the cells for 30 min at a pulse rate of 0.5 s in acetone or chloroform:methanol (2:1). Identification of pigment was done through spectrophotometric scans in both UV and visible range (190-800nm). Total polar lipids were analysed as described by Litchfield et al in 2000 and Oren et al in 1996 [29,30].

Response of cells to distilled water was studied spectrophotometrically at 600nm (Shimadzu, Japan). Cell viability assay was performed by resuspending the cells in distilled water, 3.5% NaCl and 30% NaCl and then incubating them for time periods of 24 h to 10 days respectively [31]. The cell suspensions were then plated and observed for viable colony formation.

Molecular characterization of the isolates was performed by extracting genomic DNA using phenol-chloroform method and amplifying gene for 16S rRNA with primers A109 (F) AC(G/T)GCTCAGTAACACGT and 1510(R) GGTTACCTTGTTACGACTT [32]. Each PCR reaction contained 2 U Taq Polymerase, 10X Taq buffer, 2 mM MgCl₂, 10 mM of dNTPs (Sigma), 10 μM of each primer (IDT technologies) and 1 μl of template DNA. Final reaction was made up to 50 μl with ultra-pure distilled water. The amplification was performed under the following conditions:

Initial denaturation for 5 min at 94°C, denaturation for 30 s at 94°C, annealing for 40 s at 53.5°C, elongation for 60 s at 68°C (35 cycles) and final elongation at 68°C for 5 min. Amplified products were purified and then sequenced using an automated DNA sequencer (Applied Biosystems). The sequencing results of the amplified 16S rRNA fragments were subjected to BLAST analysis. Multiple sequence alignment was done out using MUSCLE and phylogenetic tree was constructed with MEGA 5.0 by neighbor-joining method with bootstrap analysis using 1000 replicates [33,34].

Specific conductance (conductivity) is a measure of the electrical current of a solution. The greater the salinity, greater is the conductivity. Salinity of salt pans varied from 3-4% (non-salt production phase) to 30% (salt production phase) and pH varied from 7.0-8.0. To determine the nature of elements contributing to the salinity, total chemical analysis of the brine water revealed that Na⁺ and Cl⁻ are the dominant ions. This indicates the thalassohaline nature of the brine. The main cations were Na⁺ (747 g/l), Ca²⁺ (121 g/l), K⁺ (80 g/l) and Mg²⁺ (171 g/l) and the main anion was Cl⁻ (611 g/l).

Plating of sediments and/or water samples on NTYE agar plates, resulted in 30 cream/yellow/white and 14 orange/red colored colonies during the salt dilute phase and about 60-920 cream/yellow/white and 70 - 110 orange/red colonies during salt harvesting phase, on

incubation of the plates for 20-30 days at room temperature (30°C). On NT medium during pre-salt harvesting phase matt growth of cream/yellow/white and pale pink colonies were obtained. During salt harvesting phase about 20 cream colonies appeared within 24 h of incubation. On further incubation of NT media plates for 8-20 days, light orange, orange red to brick red pigmented colonies appeared. Some of the orange-red colonies growing on NTYE and NT plates were accompanied by white/cream colonies, which persisted even after several subcultures. However these white/cream colonies were eliminated when streaked on NTYE and NT plates containing 50 µg/ml of ampicillin, indicating their bacterial origin (Additional file 1: Figure S2, supplementary data).

Since carotenoid or bacterioruberin pigment is one of the characteristic features of haloarchaea, orange-red colored colonies were selected on basis of their difference in colony morphology and pigmentation.

During the pre-salt harvesting phase, orange-red colonies appeared after 20 -30 days of incubation. These colonies appeared as a uniform pure culture in all the water/sediment samples plated. However based on visual differentiation seven different types of orange-red isolates were picked from NTYE medium and were designated as BK3, BK6, BK7, BK11, BK18, BK19 and BK20. The isolates were maintained on NTYE agar slopes.

During the salt harvesting phase, fifteen visually different pigmented isolates were obtained on NT agar medium and were designated as BS1, BS2, BS3, BS4, BS5, BS6, BS7, BS8, BS11, BS13, BS15, BS16, BS17, BS19 and BS20. Two strains BBK1 and BBK2, orange/red in color, were isolated from NTYE agar medium (Table 1).

All the strains stained uniformly Gram negative. All of the BK and BBK series cultures appeared as cocci either as singles, pairs, chains or groups. Cultures in BS series (BS1, BS2, BS3, BS5, BS6, BS7, BS13, BS15 and BS20) appeared as tiny cocci whereas cultures BS4, BS11, BS17 and BS19 appeared as short rods. The coccoid morphology of the cultures was further confirmed by scanning electron microscopy. Most of the coccoid isolates appeared as single cells or diplococci with exception of BK19 which exhibited classical *Sarcina* like packets. The isolates BBK2 and BS16 exhibited unique morphology which appeared as flattened involuted discs (Figure 2).

The orange-red acetone or chloroform: methanol extract of the pigment showed similar spectrophotometric profile with absorption maxima at 393, 474, 501 and 535 nm. These peaks correspond to bacterioruberin pigments which are typical pigments of haloarchaea [35,36].

The BLAST analysis of the 16S rRNA gene fragments of the six isolates BK3, BK6, BK7, BK11, BK18 and BK19 obtained from the pre-salt harvesting phase showed 98-99% similarity to *Halococcus salifodinae* and *Halococcus saccharolyticus* (Figure 3). Among isolates from the salt

harvesting phase, only BBK1 (AB588755) was very close to *Halococcus sp.* with 98-99% similarity whereas cultures BS1, BS2, BS3, BS5, BS6 and BS7 were assigned to the genera *Haloarcula* with similarity of 98-99%. BBK2 and BS16 showed highest similarity to *Haloferax alexandrinus* of about 97-98% and the 16S rRNA sequence of BS17 and BS19 was closely related to the genera *Halorubrum* with 98% similarity.

As noted earlier, orange – red colonies from pre-salt harvesting phase were almost uniform and appeared to be in pure form. All these isolates showed 99% similarity to the genera *Halococcus*. It was of interest to see if there were any differences in these strains. Hence the total polar lipids were studied. The lipid profile obtained showed 2 spots of phosphatidylglycerol (PG), methyl ester of phosphatidyl glycerophosphate (PGP-Me) corresponding to R_f values 0.828 and 0.517 respectively which are the signature polar lipids of haloarchaea. The glycolipid, sulfated diglycosyl diether lipids (S-DGD) having an R_f of 0.368 was seen in all the strains of BK and BBK series which is typical of genus *Halococcus* and *Haloferax* [37]. No differentiation at the strain level could be done based on the lipid profile. Most likely it was the same strain that had been isolated multiple times, due to its abundance in the environment.

The isolates obtained from pre-salt harvesting phase (BK series) were resistant to lysis in distilled water and 3.5% NaCl. However isolates obtained from salt harvesting phase showed varied lysis on suspension in distilled water with the exception of BBK1 which was resistant to lysis. The isolate BBK2 lysed immediately, whereas BS4 and BS5 were observed to have delayed lysis. Viability assay was performed for BK6 obtained during pre-salt harvesting phase and BBK2 obtained during salt harvesting phase. On suspension of cells in distilled water, 3.5% NaCl and 30% NaCl, followed by plating revealed that isolate BK6 was viable even after 10 days on suspension in distilled water. Cells of BK6 also retained their coccoid shape, when observed microscopically. The isolate BBK2 survived for up to 24 h in 3.5% NaCl, but lysed immediately in distilled water showing no growth on plating.

Interestingly the isolates of the BK series failed to lyse in acetone for extraction of pigment even on sonication but lysed on suspension in Chloroform : methanol (2:1 v/v).

It is interesting to see that Halococci were recovered almost as a pure culture in culturable form during the salt dilute phase (whereas other culturable genera could be recovered during the salt concentrated phase). Recent studies have shown that haloarchaea are being isolated from less hypersaline environments. Salt-marsh sediments, sulfur-rich spring and deteriorated ancient wall paintings has been investigated and found to have haloarchaeal members, predominantly belonging to the genera *Halococcus*, *Haloferax* and *Halogeometricum* [38-41]. A

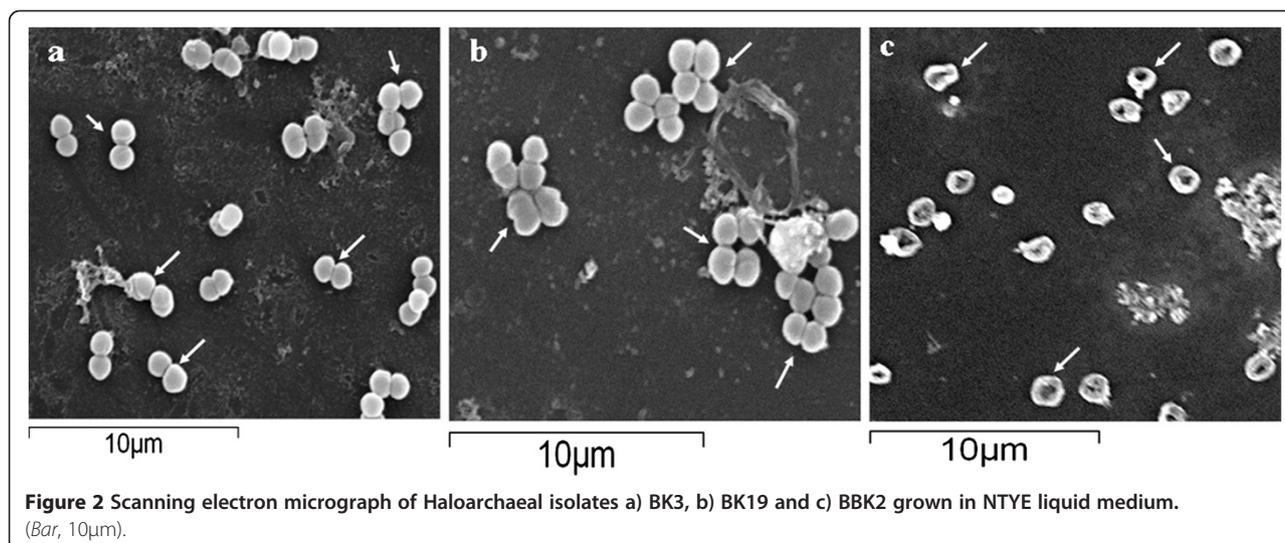
Table 1 Halophilic archaeal isolates obtained from Ribandar solar salterns of Goa, India

Saltern phase and econiche	Isolates	Pigmentation	Gram character and morphology	Lysis in		Identification	Accession No.
				Distilled water	3.5% salt solution		
Pre salt harvesting phase/Initial Stage/Salt Dilute Stage							
Water samples	BK3	Bright Orange-red	Gram negative cocci	-	-	<i>Halococcus salifodinae</i>	HQ455793
	BK6	Bright Orange-red	Gram negative cocci	-	-	<i>Halococcus salifodinae</i>	AB588757
	BK7	Bright Orange-red	Gram negative cocci	-	-	<i>Halococcus salifodinae</i>	HQ455794
	BK11	Bright Orange-red	Gram negative cocci	-	-	<i>Halococcus salifodinae</i>	HQ455795
Sediment samples	BK18	Orange	Gram negative cocci	-	-	<i>Halococcus salifodinae</i>	HQ455796
	BK19	Bright Orange-red	Gram negative cocci	-	-	<i>Halococcus salifodinae</i>	AB588758
	BK20	Light Orange	Gram negative cocci	-	-	Not sequenced	Not sequenced
Salt harvesting phase/Crystallization Stage							
Brine samples	BBK1	Orange	Gram negative cocci	-	-	<i>Halococcus salifodinae</i>	AB588755
	BBK2	Orange	Gram negative cocci	+	+/-	<i>Haloferax volcanii</i>	AB588756
	BS1	Bright red	Gram negative cocci	+	+/-	<i>Haloarcula argentinensis</i>	HQ455797
	BS2	Bright red	Gram negative cocci	+	+/-	<i>Haloarcula japonica</i>	HQ455798
	BS3	Bright red	Gram negative cocci	+	+/-	<i>Haloarcula sp.</i>	HQ455799
	BS5	Bright red	Gram negative pleomorphic	+	+/-	<i>Haloarcula argentinensis</i>	AB588759
	BS6	Bright red	Gram negative short rods	+	+/-	<i>Haloarcula hispanica</i>	HQ455801
	BS7	Bright red	Gram negative pleomorphic	+	+/-	<i>Haloarcula japonica</i>	HQ455802
Sediment samples	BS17	Bright red	Gram negative cocci	+	+/-	<i>Halorubrum sp.</i>	ND
	BS16	Light Orange	Gram negative cocci	+	+/-	<i>Haloferax alexandrinus</i>	HQ455803

"-" No Lysis; "+" Lysis; "ND" Not Deposited.

study by Fukushima et al. (2007), showed that the cells of *Halococcus* survived in sea water (salinity of which is 3.5%) upto 9 days without losing its cell rigidity. It is also possible that haloarchaea are trapped in the salt crystals and get deposited in the sediments. The salinity of sediments obtained at about 10 cm was 6-10%. Therefore it is quite possible that even though the haloarchaeal members

cannot flourish they can still retain their viability. Another possibility is presence of clay in these salterns. These clay particles have micropores on which the salt fluid gets filled along with the haloarchaeal members. These micropores could serve as a salt rich environment for the survival of haloarchaea [42-44]. Diversity studies of hypersaline areas around the world have indicated that *Halorubrum*



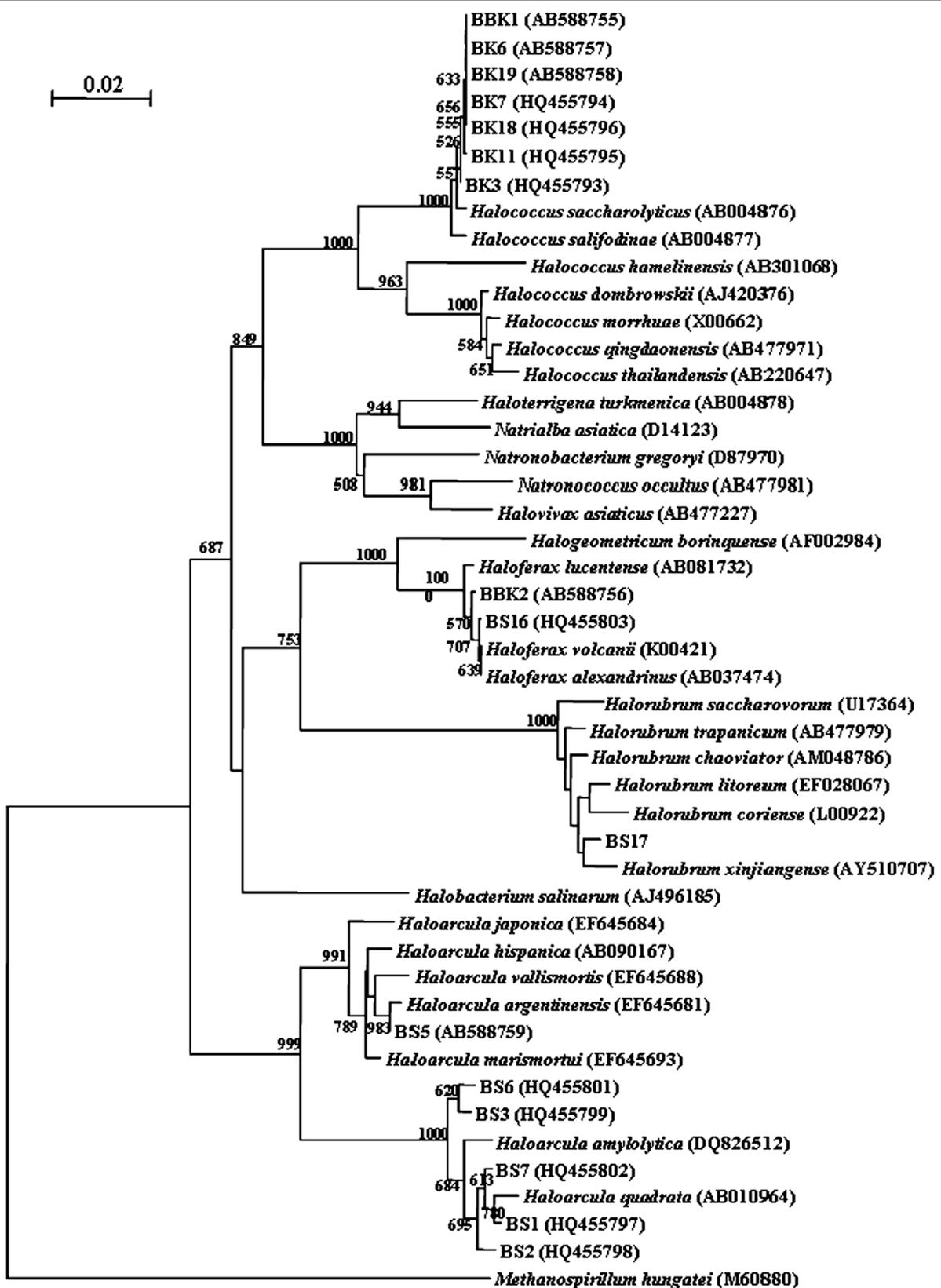


Figure 3 Phylogenetic tree showing the positions of isolated haloarchaeal strains constructed with MEGA 5.0 by using NJ method. *Methanospirillum hungatei* was used as an outgroup.

and *Haloarcula* are the dominant culturable members of haloarchaea [45-50]. This investigation revealed that haloarchaeal members belonging to the genus *Halococcus*, thrive in less saline environments and are the dominant culturable haloarchaea during the pre-salt harvesting phase or the salt dilute phase.

The salt pan under study is transient and operated only during the post monsoon season of January to May. During monsoons, the salt pans are inundated with sea water as well as rain water. Most of haloarchaeal strains are known to lyse in distilled water as they require at least 10% NaCl to maintain the integrity of their outer membrane. It is interesting to note that Halococcal microorganisms were most abundant, during the pre-salt harvesting phase as they are resistant to lysis in lowering salinities than their counterparts. However, as salinity gradually increases, other members of haloarchaeal community start to colonize the salt pans. Of the thirty six defined genera in the family *Halobacteriaceae*, four different genera namely, *Halococcus*, *Haloferax*, *Haloarcula* and *Halorubrum* were found to be represented in this study. This investigation provides valuable information about the change in culturable haloarchaeal diversity under variable salt conditions.

Nucleotide sequence data can be accessed from DDBJ and NCBI database under the accession numbers HQ455793 - HQ455803 and AB588755 - AB588759.

Additional file

Additional file 1: Figure S1. Solar Salt production at the Ribandar salterns a) bed preparative stage showing series of rectangular beds (January) b) Sluice gate / inlet point for entry of saline water c-d) rectangular beds inundated with saline water (February - March) e) tool used for extracting salt f) crude salt heaped up at the corners of the *bandhs* g) collection of brine sample h) crude salt collected and piled up on the sides i) collection of sediment sample. Figure S2. Media plates (NTYE and NT) showing diversity of halophilic microorganisms obtained during initial stage and crystallization stages of salt production. Interested bright orange culture obtained on media containing ampicillin.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

JB conceived the idea and designed the study. KM, BBS and JB conducted the field operations. KM carried out the laboratory work of physico-chemical analysis of the samples, molecular characterisation of the isolates and drafted the manuscript. BBS isolated the haloarchaeal strains and performed pigment characterisation and cell viability assays. KM and BBS carried out the SEM studies. JB edited and revised the manuscript. All authors have read and approved the final manuscript.

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