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EXPERIMENTAL ARTICLES

A Study on the Halophilic Archaeal Diversity from the Food Grade Iodised Crystal Salt from a Saltern of India

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Abstract—Present study aimed to evaluate culturable halophilic archaeal diversity of iodised crystal salt, obtained from the solar salterns of Vedaranyam, Tamil Nadu, India. Bright yellow-orange-red pigmented colonies were obtained, by surface spreading of the saturated salt crystal suspension, on complex media such as NTYE (NaCl Tryptone Yeast Extract) and EHM (Extremely Halophilic Medium), followed by incubating the plates for 30–45 days. The viable halophilic pigmented/non-pigmented microbial counts were determined as colony forming units (CFU/g), which were ~1.1×10^4/5.5×10^3 and ~1.0×10^4/4.0×10^3 on NTYE and EHM, respectively. Six morphologically distinct isolates were purified and identified. Morphological characteristics of the isolates revealed the cells to be cocci and involuted/cup shape, whereas chemotaxonomic characteristics revealed the presence of bacterioruberin pigment. Small subunit ribosomal RNA (16S rRNA) gene analysis revealed that, majority of the isolates belonged to genus *Halococcus*, except one belonged to genus *Halorubrum*. To the best of our knowledge, this is the first study reporting, prevalence of *Halococcus* in iodised crystal salt, of Vedaranyam, Tamil Nadu, India.

Keywords: haloarchaea, scanning electron microscopy, diversity, crystal salt, phylogenetic analysis

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Solar salt production is mainly carried out on the coastal areas, as there is an easy accessibility to sea water (Oren, 2014). The solar salterns or salt pans are, shallow ponds with increasing salinity gradients (3–30% NaCl w/v) that are artificially designed for the commercial production of salt (NaCl). India is the third largest producer of salt across the globe, with the leading producer being China, followed by United States. Among the 29 states of India, Gujarat is the leading producer of salt (~180.96 lakh tonnes) followed by Tamil Nadu, Rajasthan, Andhra Pradesh, Maharashtra, Odisha, Karnataka, West Bengal and Goa (Mani et al., 2012b).

Solar salterns besides being as mere sites for salt production, is also an excellent ecosystem for the halophilic organisms to thrive in a range of NaCl concentrations (3–30%). Among the halophiles, halophilic microorganism belonging to domain *Archaea* and Bacteria form an integral part of these ecosystems (Oren, 2014). Salt tolerance by halophilic microorganism increases with increasing salinity, with extreme halophiles usually belonging to *Archaea* mostly dominating in the crystalliser pans where the salinity is above 25%. When the salt crystals are formed in the crystalliser pans, halophilic microorganisms get entrapped in the fluid inclusions and therefore remain trapped in the salt crystals. Studies have shown that these trapped organisms can remain viable for millions of years (Stan-Lotter et al., 2002).

Salt forms an integral part of cuisine apart from providing much needed sodium and chloride to the human body. Since ancient times, salt crystals obtained through natural evaporation have traditionally been used in the fermented food preparation throughout South East Asia, for the prevention of undesired microbial activity (non-halophilic) thereby preventing food spoilage. Some of the traditionally fermented foods employing 2.5–10% salt include burong mustala, dakguadong, jiang-gua, sauerkraut, tempoyak and kimchi (Swain et al., 2014). Since, salt crystals harbor numerous halophilic microorganisms, these fermented foods can serve as source for the isolation of novel strains of halophilic bacteria and archaea. Some of the isolated novel halophilic archaeal strains include *Halococcus jeotgali, Haloru-*

Abbreviations: NTYE NaCl Tryptone Yeast Extract; EHM Extremely Halophilic Medium; NH Norberg and Hofstein; rpm revolutions per minute; DDBJ DNA Data Bank of Japan; MEGA Molecular Evolutionary Genetics Analysis; SEM Scanning Electron Microscopy; MUSCLE Multiple Sequence Comparison by Log-Expectation; NCBI National Center for Biotechnology Information.

brum cibi, Halorubrum halophilum, Haloterrigena jeotgali, Natronococcus jeotgali, Haloarcula salari, Haloarcula tradensis. Moreover, a myriad of other known halophilic archaea, belonging to the class *Halobacteria* have been reported from commercial salt obtained from seawater bordering various countries such as Japan, Philippines, Australia, China, Mexico, Korea, Indonesia, etc (Shimane et al., 2010; Kondo et al., 2016; Minegishi et al., 2016; Song et al., 2016; Yamauchi et al., 2013).

Consumption of the fermented food with salt crystals has long been suspected with the association of halophilic archaea with gastro intestinal health. In a study published in 2010 by Oxley et al., the presence of 16S rRNA sequences related to halophilic archaea in the human intestinal mucosa was reported. Confirming the report, a novel halophilic archaea, *Haloferax masiliensis* has been isolated from the human gut recently. These studies suggest a possible association of halophilic archaea with the human health alongside methanogenic archaea, thereby needing a far more attention in studying association of archaea in humans. Apart from their possible human health influencing properties, bio-prospecting of halophilic archaeal strains have proved their potential in bioremediation of saline areas, food industry, pharmaceuticals, bioplastic production, etc. (Antunes et al., 2017).

There is a clear indication on the possible association of halophilic archaea with human health and one of possible entry routes for halophilic archaea to human gut would be through the consumption of food substances enriched with solar salt crystals. Moreover, salterns of Vedaranyamare being operated for hundreds of years thereby providing an opportunity for rich halophilic archaeal diversity to develop. Therefore, it is the need of the hour to study the halophilic archaeal diversity of salt crystals for an understanding the role played by halophilic archaea in human health.

MATERIALS AND METHODS

Iodised crystal salt. The iodised crystal salt samples were obtained from a saltpans located at Verdaranyam in the coastal state of Tamil Nadu, India. The manufacturer's label indicated that, the iodine content of the salt was 30 ppm.

Isolation and purification of halophilic microorganisms. The culturable halophilic microbial diversity of crystal salt was studied using direct surface spread plate technique, as described by Yamauchi et al. (2013) with slight modification. Briefly, ~1 g of crystal salt was dissolved in 10 mL of 15% (w/v) sterile NaCl solution. Hundred μL of the suspension was surface spread plated on Petri plates containing two complex media, (a) NaCl Tryptone Yeast Extract (NTYE) medium and (b) Extremely Halophilic Medium (EHM). The compositions of the media are as described elsewhere (Mani et al., 2012a; Salgaonkar et al., 2019). The plates were incubated in self-sealing plastic bags at 28°C in incubator (REMI RIS-24 PLUS) for up to 30–45 days until various shades of pigmented colonies appeared.

The extremely halophilic counts (CFU/g) of pigmented and non-pigmented colonies, on NTYE and EHM were determined after 7, 15 and 45 days of incubation. Individual colonies were selected based on their pigmentation, size, shape, texture, margin and were transferred to fresh media plates. The cultures were purified by repeated streaking on the respective complex media using quadrant streak technique and maintained on NTYE/EHM agar slopes/plates at room temperature (28°C) and subcultured after 45 days interval. The purified halophilic isolates were characterized based on polyphasic taxonomy analysis.

Morphological characterization. Phenotypic characterization was performed according to Oren et al. (1997). Colony morphology of the pure isolates was analyzed using Stereomicroscope, by observing the isolated colonies for their size, shape, margin, consistency, elevation, opacity and pigmentation. Cell morphology was analyzed by Gram staining as described by Dussault et al. (1955) and observed under the oil immersion objective (100×) of the phase contrast microscope (AXIOM CL2O). Scanning Electron Microscopy (SEM) was performed to confirm the cell morphology. Briefly, smears of the log phase cultures were prepared on a clean grease free glass coverslips using sterile 15% NaCl solution. The coverslips were air dried and desalted with 2% acetic acid. The smears were air dried and fixed with 2% (v/v) glutaraldehyde overnight (10 h) at room temperature (28°C), followed by exposure to a gradient of increasing concentration of acetone, i.e., 30, 50, 70, 90% each for 10 min and finally to 100% for 30 min. The smears were air dried and the cell morphology of each isolate was analysed using Scanning Electron Microscope (SEM) (Carl-Ziess).

Chemotaxonomic characterization. (a) Pigments analysis. Bright-orange-red pigments from the isolates were characterized by extracting it in chloroform: methanol (2 : 1 v/v) and analyzed using UV-spectrophotometer. Cells from 10 mL of the early stationary phase (8–10 days) culture were harvested by centrifuging at 8000 rpm and to each of the cell pellets, 2.5 mL of methanol was added. The methanol pellet suspension was vortexed and sonicated at a pulse rate of 0.5 s, for 5 min using the medium probe of the sonicator (B.BRAUN LABSONIC®U B. Braun Biotech International). To this 5 mL of chloroform was added, vortexed and centrifuged at 10000 rpm for 10 min. The solvent extracts were scanned from 190–800 nm using UV-visible spectrophotometer (Shimadzu UV–2450UV, Japan). **(b) Lysis in distilled water.** Response of cells to distilled water was studied as described by Mani et al. (2012a) using spectrophotometer (Shi-

madzu UV–2450UV, Japan). The mid log phase cells were harvested, suspended in distilled water, and absorbance was recorded at 600 nm after every 5 minutes for 30 minutes. The cells were also checked for their viability by spot inoculating the distilled water suspended cells on complex media and incubating the plates at 37°C for 10 days. **(c) Carbohydrate utilization.** The carbohydrate utilization for the various extremely halophilic isolates was studied. Stock of various carbohydrates (10% w/v) were made and sterilized separately at 121°C for 15 minutes and were added to test tubes containing Norberg and Hofstein (NH) medium pre-added with 0.0025% of phenol red as indicator and inverted Durham's tubes (Norberg and Hofstein, 1969). The test sugars used were Dextrose, Lactose, Sucrose, Arabinose, Maltose and Mannitol. Hundred µL of the log phase cultures were inoculated and the tubes were incubated at 37°C, 110 rpm for 15–20 days. Results were recorded as positive on appearance of turbidity in the medium and sugar fermentation was recorded by visual observation of change in the colour from red (neutral pH of 7.0) to yellow-orange (acidic pH below 6.0).

Antibiotic susceptibility analysis. Antibiotics such as penicillin, ampicillin, streptomycin and rifampicin were used for determining the susceptibility of the halophilic isolates. Media containing respective antibiotic (Stock 1 mg/mL or 1000 U/mL) was added to each flask such that the final antibiotics concentration was 10 µg/mL or 10 U/mL. About 10 µL of the log phase cultures were spot inoculated on the plates, followed by incubation at 37°C for 15–30 days and results were recorded as either growth (resistant) or no-growth (susceptible).

Molecular characterization. (a) Genomic DNA extraction. Genomic DNA was extracted from 1.5 mL of log phase halophilic isolates using Wizard Genomic DNA Purification Kit (Promega, WI, USA) according to manufacturer's instructions. **(b) PCR amplification.** The extracted genomic DNA was subjected to PCR for the amplification of 16S rRNA gene using universal archaea-specific primers—A109(F) AC(G/T)-GCTCAGTAACACGT and 1510(R) GGT-TACCTTGTTACGACTT (Mani et al., 2012a). The PCR was performed in a thermal cycler (Applied Biosystems, USA) using following conditions: Initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 53.5°C for 30 s and extension at 68°C for 1 min and final extension at 68°C for 10 min. Each PCR reaction mixture contained 10× *Taq* buffer, 2 mM $MgCl_2$, 10 mM of dNTPs (Sigma, India), 10 µM of each primer (IDT technologies, Singapore), 2 U *Taq* Polymerase and 10 ng template DNA. The PCR products were verified on a 1.5% agarose gel through submerged DNA electrophoresis and was visualised over a UV illuminator (Bio-Rad, USA) after staining with ethidium bromide. Intact amplified PCR products were eluted from the gel and purified with gel elution kit (Chromous Biotech Pvt. Ltd., India), according to manufacturer's instructions. **(c) Sequencing and phylogenetic analysis.** The purified PCR products were sequenced using an automated DNA sequencer employing dideoxy chain termination method (Applied Biosystems, USA). The taxonomic identities of the isolates and the calculation of pair-wise 16S rRNA gene sequence similarity against the type strains with validly published names were ascertained through EzTaxon server (Kim et al., 2012). Multiple sequence alignment was performed with Multiple Sequence Comparison by Log-Expectation (MUSCLE) and the phylogenetic tree was constructed using the neighbor-joining method of MEGA 5.0 (Tamura et al., 2011). Validity of the tree was established though bootstrapping analysis employing 1000 replicates and displaying the values for 100. The obtained 16S rRNA sequences were deposited in GenBank with the accession numbers MG008998 (Salgaonkar et al., 2019), MF045362–MF045365 and MF045367.

RESULTS AND DISCUSSION

Enumeration and isolation of halophilic microorganisms. The results of this study indicated that, the crystals salt obtained from the solar salterns of Vedaranyam, Tamil Nadu, India, harboured a rich diversity of halophilic microorganisms. The culturable halophilic microbial diversity, of the crystal salt was studied by surface spread plating on two media, NTYE and EHM both containing 25% (w/v) NaCl, along with other salts (Fig. 1). Growth was not observed up to 7 days of incubation. However, after 15 days of incubation, colonies with faint red pigmentation appeared along with few non-pigmented colonies. On further incubation of the plates up to 45 days, the faint pigmented colonies appeared brightly pigmented and outnumbered the non-pigmented ones (Fig. 2a). After 45 days of incubation, the CFU/g of halophilic pigmented/non-pigmented microbial counts were ~$1.1 \times 10^4/5.5 \times 10^3$ and ~$1.0 \times 10^4/4.0 \times 10^3$ on NTYE and EHM, respectively (Fig. 2b). Though the salt crystals represent an extreme hypersaline environment, a high viable counts were obtainedwhich could be incomparison with any other mesophilic environments. There are few reports on microbiological analysis on food grade salt crsytals, which indicated a similar CFU/g values. Henriet et al. (2014) studied the halophilic archaeal diversity in 14 food-grade salts produced by solar evaporation of hypersaline water and reported the viable counts of $\geq 10^5$ CFU/g. Cojoc et al. (2009) reported the halophilic archaeal count of ~3.0×10^3 CFU/g in subterranean salt crystals from salt mine Unirea, in Slanic Prahova. One possible explanation for an abundant halophilic microbial community in salt crystals might be, due to their low-level metabolism while simultaneously feeding on the carbon substrates released by the dead cells. Another source for

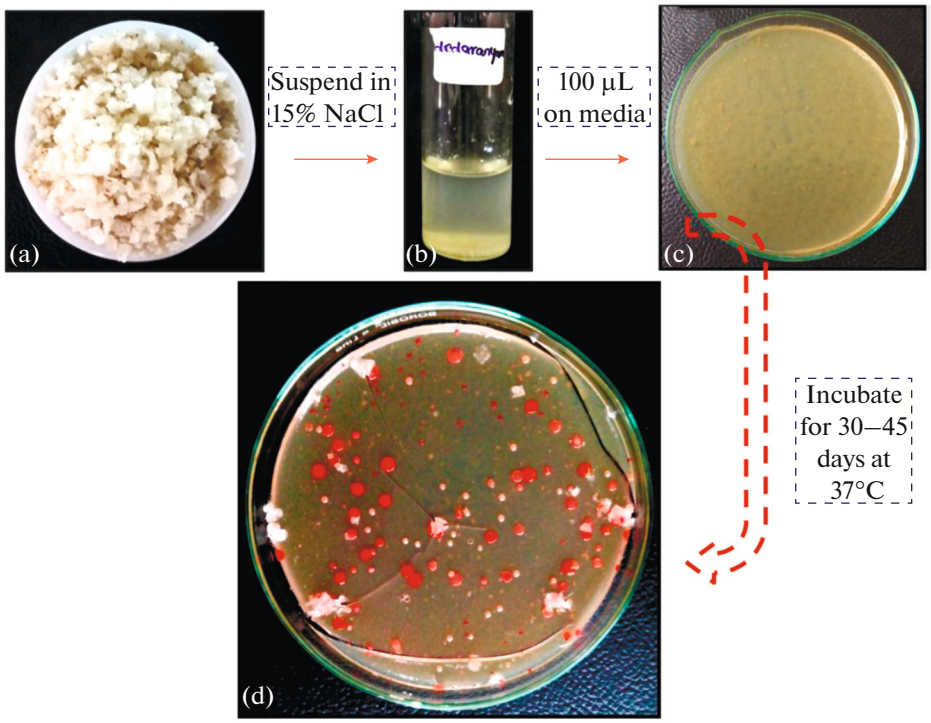

Fig. 1. Isolation of extremely halophilic microorganisms from (a) salt crystal sample of Vedaranyam, Tamil Nadu, India, (b) brine obtained after dissolving the salt crystals in 15% NaCl, (c) complex media agar plate without growth and (d) brightly pigmented colonies of halophilic microorganisms obtained after 30–45 days of incubation.

the carbon might be through the presence of glycerol, an osmoprotectant accumulated by *Dunaliella* (a halophilic algae) which might be released from the dead algal cells. It is a well known fact that *Dunaliella* coexists with halophilic archaea in the crystallizer pans of the solar salterns (Schubert et al., 2010).

Halophilic archaeathrive and flourish in high salt environments (15% NaCl upto saturation) (Oren, 2014). Since the isolates were obtained from salt crystals, and could grow on media containing 25% (w/v) NaCl, they were categorized as extreme halophiles. Among the isolates, six unique isolates were randomly selected based on the colony morphology and pigmentation. The colonies were purified by repetitive quadrant streaking method on the NTYE/EHM media and maintained on respective agar plates at 28°C (Figs. 3a, 3b). The purified isolates were named as GUVSC (Goa University Vedaranyam Salt Crystal) series, i.e., GUVSC1, GUVSC3, GUVSC5, GUVSC6, GUVSC8, GUVSC11.

Morphological characterization of the isolates. The isolates obtained exhibited varying colony morphology and pigmentation. After 30 days of incubation the colonies appeared ~1.0–3.0 mm in diameter, circular, smooth withshades of yellow-orange–red pigmentation (Fig. 3a). The cells stained gram-negative and appeared as single cocci or as pairs/tetrads or as irregular clusters. SEM analysis revealed cell morphology of the isolate GUVSC1, GUVSC6, GUVSC8 and GUVSC11, as coccus in pairs, tetrads or sarcina packets and GUVSC5 as irregular clusters, which is a typical morphology of the genus *Halococcus*family *Halococcaceae* (Mani et al., 2012a; Namwong et al., 2007). Interestingly cells of the isolate GUVSC3 appeared singly as involuted/cup shaped, a typical morphology of the genus *Halorubrum* of family *Halorubraceae* (Fig. 3d) (Kamekura and Dyall-Smith, 1995).

Chemotaxonomic characterization of the isolates. The intracellular pigment from the isolates was extracted using a ratio of solvents, $CHCl_3$: CH_3OH (2 : 1 v/v) and analysed using spectrophotometer (Salgaonkar et al., 2016). The UV–Visible spectra of the pigment scans gave a maximum absorption peak at ~492–500 nm, along with two shoulder peaks at ~472–496 nm and ~530–534 nm characteristic of C-50 bacterioruberin carotenoids from halophilic archaea (Fig. 3c). Apart from these main peaks, two more peaks were observed at 374 and 393 nm. Britton et al. (1995) described that spectrophotometric profile of bacterioruberin carotenoids and its derivatives, exhibiting the characteristic three fingered peaks at 467, 493, and 527 nm (nearly identical absorption maxima) and two cis peaks at 370 and 385 nm. Red-orange carotenoids such as 50-carbon compounds α-bacterioruberin (BR) and its derivatives mono-anhydrobacterioruberin (MABR) and bis-anhydrobacterioruberin (BABR) are signature pig-

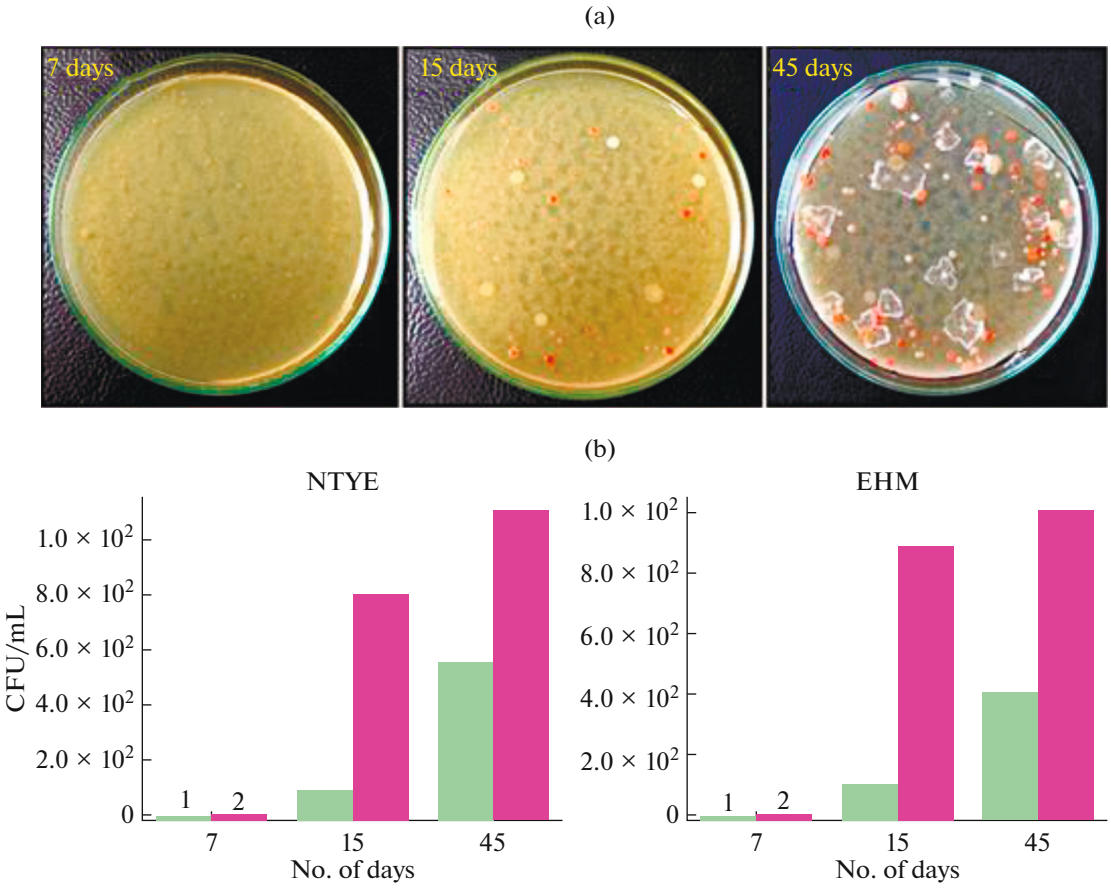

Fig. 2. (a) Colonies of halophilic microorganisms obtained from Vedaranyam salt crystals obtained on NTYE medium after incubation for various time intervals. (b) Comparison of non-pigmented (1) and pigmented (2) halophilic counts obtained on complex media NTYE and EHM.

ments synthesized by almost all members of the class *Halobacteria*, except the two species, *Natrialba asiatica* and *Halorhabdus tiamatea*. They are derived from the terpenoid biosynthetic pathway and play major functionas antioxidants and protects the organisms from lethal UV radiations.

The isolates GUVSC1, GUVSC5, GUVSC6, GUVSC8, grew on complex media even after suspending in distilled water, thereby indicating their rigid-cell wall and failure to lyse. However, GUVSC3 cells lysed immediately in distilled water and did not grow on media. Mani et al. (2012a) studied the culturable haloarchaeal diversity during two stages of salt production in Goa, India and reported the survival of members of genus *Halococcus*, in less saline environments. Utilization of various carbohydrates revealed that most of the isolates could utilise Dextrose, Arabinose and Maltose whereas Lactose, Sucrose and Mannitol was weakly consumed. Isolate GUVSC3 could utilize only dextrose and maltose (Table 1). Though halophilic archaea can be grouped, based on their requirement for salinity, their nutritional requirements and metabolic potential varies widely. While some isolates can metabolize all hexoses and pentoses, other isolates can metabolize only a narrow range of sugars. However, this limitation is overcome by the improved ability of these isolates to metabolize amino acids and osmoprotectant compound which are common in the hypersaline environments (Falb et al., 2008). Therefore, the pattern of sugar utilisation indicates a completely different metabolic potential of the isolates.

Antibiotic susceptibility test: The extremely halophilic isolates screened for their susceptibility to various antibiotics were found to be resistant to penicillin (10 IU/mL), ampicillin (10 μg/mL), and streptomycin (10 μg/mL). However, growth of the isolates was inhibited by Rifampicin (10 μg/mL). These results corroborated with the previous known data about the action of microbially synthesized antibiotics on halophilic archaea. Penicillin and ampicillin belong to β-lactam class antibiotics and prevent cross-linking process of the cell wall by interfering in the final transpeptidation process in bacteria. In general archaea are resistant to β-lactams, because they lack peptidoglycan and further they do not produce β-lactamase

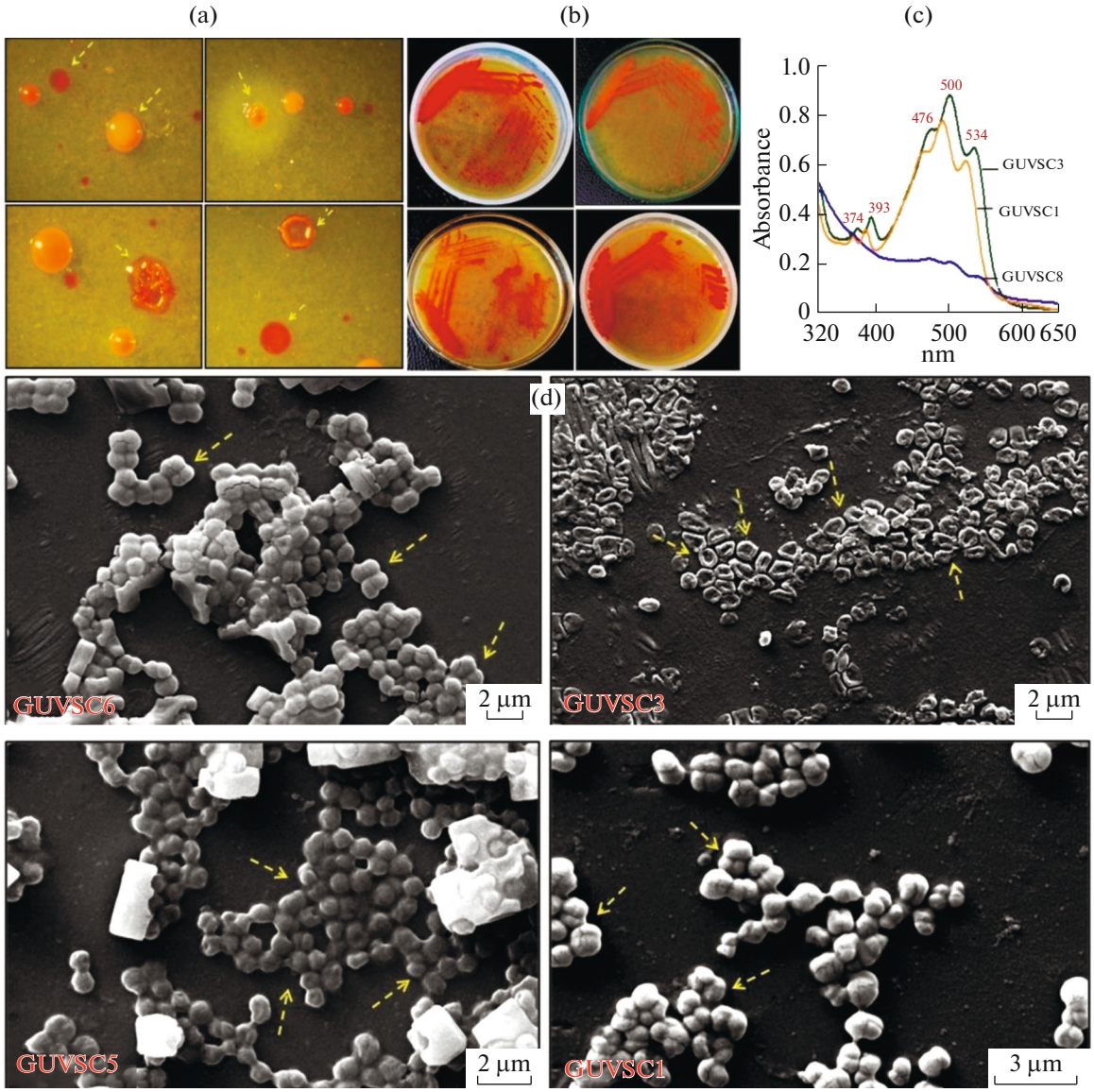

Fig. 3. (a) Various colony morphologies of brightly pigmented halophilic microorganisms obtained from Vedaranyam salt crystals on media containing 25% NaCl. (b) Some of the purified halophilic cultures. (c) Spectrophotometric scans of pigments obtained from various halophilic isolates. (d) Scanning electron micrographs depicting the cell morphology of some of the halophilic cultures.

enzyme. Streptomycin inhibits protein synthesis, by binding to the 70S ribosome of bacteria. It has no effect on archaeal growth because of the resemblance of archaeal rRNA structure to eukaroytes. The antibiotic Rifampicin is a bactericidal drug which acts by inhibiting the DNA-dependent RNA polymerase, thereby inhibiting the RNA synthesis in bacteria. Since, the archaeal RNA polymerase is structurally similar to bacterial RNA polymerase, archaea is susceptible towards rifampicin (Campbell et al., 2001).

Molecular characterization of the isolates. Small subunit rRNA (16S rRNA) gene sequence comparisons revealed that a representative strain, GUVSC1, GUVSC6, GUVSC8, GUVSC11 were affiliated to *Halococcus hamelinensis* 100A6^T (DQ017835), GUVSC5 to *Halococcus sediminicola* CBA1101^T (JX989265) and GUVSC3 to *Halorubrum sodomense* ATCC 33755^T (D13379) (levels of similarity of ≥ 95%). Figure 4 represents the tree representing the phylogenetic position of the halophilic archaeal isolates. The 16S rRNA sequence has been deposited in NCBI GenBank with accession number MF045362–65, MF045367 and MG008998.

Studies have demonstrated that, salt crystals obtained from various sea water acts as a reservoir of novel halophilic archaea (Table 2). Recently, many novel haloarchaeal isolates, such as *Halobaculum mag-*

Table 1. Characterization of the extremely halophilic isolates

Characteristic	GUVSC 1	GUVSC 3	GUVSC 5	GUVSC 6	GUVSC 8	GUVSC 11
Colony/cell characterization						
Pigmentation	Orange	Bright Red	Orange-red	Orange	Light orange	Orange
Colony size	1.5 mm	3 mm	1.5 mm	1 mm	2 mm	3 mm
Cell morphology	Cocci	Involuted/cup	Cocci	Cocci	Cocci	Cocci
Cell size (*D*)	~1.0 μm	~1.2 μm	~1.2 μm	~1.0 μm	~1.0 μm	~1.0 μm
Cell arrangement	Tetrad/groups	Single	Irregular clusters	Tetrad/groups	Tetrad/groups	Tetrad/groups
Elevation	Raised	Flat	Raised	Raised	Raised	Raised
Margin	Entire	Entire	Entire	Entire	Entire	Entire
Opacity	Opaque	Translucent	Opaque	Opaque	Opaque	Opaque
Consistency	Rough	Butyrous	Butyrous	Rough	Rough	Rough
Chemotaxonomic characterization						
Bacterioruberin	+	+	+	+	+	+
Lysis in D/W	NL	L	NL	NL	NL	NL
Carbohydrates						
Dextrose	+	++	+++	++	+++	+
Arabinose	+++	–	++	+	+++	+++
Lactose	+	–	+	+ +	+ + +	+ +
Sucrose	+		+	+ +	+ + +	+ +
Maltose	+ +	+	+ +	+ + +	+ + +	+
Mannitol	+	–	+	+	+ +	+
Antibiotics Susceptibility (10 U/mL or 10 μg/mL)						
Penicillin	R	R	R	R	R	R
Ampicillin	R	R	R	R	R	R
Streptomycin	R	R	R	R	R	R
Rifampicin	±	–	±	±	–	–
Molecular characterization						
16S rRNA gene analysis	*Hcc. hamelinensis*	*Hrr. sodomense*	*Hcc. sediminicola*	*Hcc. hamelinensis*	*Hcc. hamelinensis*	*Hcc. hamelinensis*

mm milli meter; μm micro meter; +++ very good growth; ++ good growth; + growth; ± negligible growth; *D* diameter; NL no lysis; L; lysis; D/W distilled water; R Resistant; S sensitive; *Hcc. Halococcus*; *Hrr. Halorubrum*.

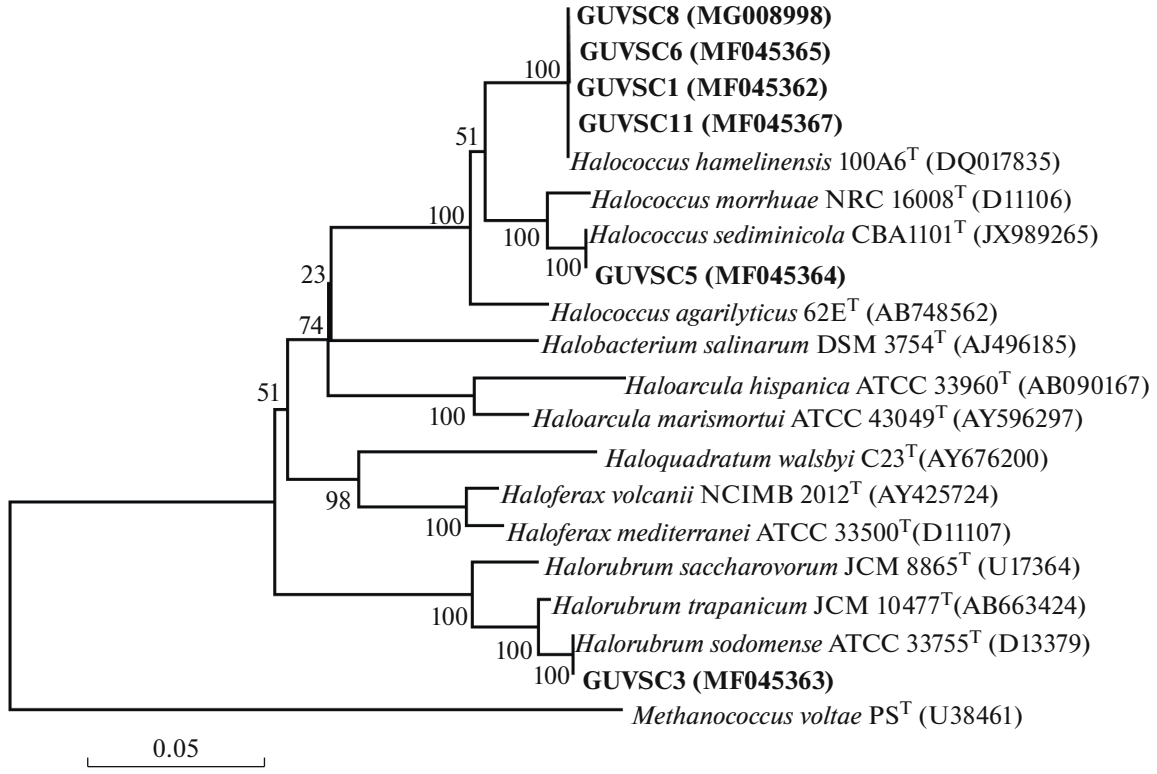

Fig. 4. Phylogenetic tree representing the position of the halophilic isolates, based on the partial 16S rRNA gene sequence, obtained by the neighbor-joining method and constructed with MEGA 5.0. The DDBJ GenBank sequence deposition number for the obtained cultures and the accession numbers for the reference strains are included in brackets. Percentage bootstrap values based on 1000 replications and displaying for 100 are indicated at nodes. *Methanococcusvoltae* PST (U38461) is represented as an out-group.

nesiiphilum MGY-184^T, *Salarchaeum japonicum* YSM-79^T, and *Natronoarchaeum mannanilyticum* YSM-123^T, have been reported from commercial salt obtained from seawater in Japan (Shimoshige et al., 2013; Shimane et al., 2010, 2011). Likewise, *Haloparvum alkalitolerans* MK62-1^T and *Natronoarchaeum philippinense* 294-194-5^T have been isolated from commercial sea salt of Philippines (Kondo et al., 2016; Shimane et al., 2013). *Halarchaeum acidiphilum* MH1-52-1^T (Minegishi et al., 2010) and *Halopiger thermotololerans* SR-441^T, SR-188, SR-412 have been isolated from commercial salt of Australia, China and Mexico (Minegishi et al., 2016). *Halostella salina* CBA1114^T, *Halapricum salinum* CBA1105^T (Song et al., 2016; Song et al., 2014) and *Halarchaeum salinum* MH1-34-1^T (Yamauchi et al., 2013) have been isolated from solar salts from Republic of Koreaand Indonesia, respectively.

In this study, we isolated six extremely halophilic archaeal isolates and identified them to be *Halococcus hamelinensis, Halococcus sediminicola* and *Halorubrum sodomense*. However, to the best of our knowledge, till date, there have been no study conducted on culturable halophilic diversity from commercial iodised crystal salt obtained from seawater of Bay of Bengal bodering Vedarenayam, Tamil Nadu, India. Neverthless, members of the genus *Halococcus* have been isolated from fermented fish sauce in Thailand (*Halococcus thailandensis*) (Namwong et al., 2007), crude sea-salt sample near Qingdao, China (*Halococcus qingdaonensis*) (Wang et al., 2007), stromatolites in Shark Bay, of Australia (Goh et al., 2006), dry rock salt (Permian alpine salt deposit) in an Austrian salt mine (*Halococcus dombrowskii* and *Halococcus salifodinae*) (Stan-Lotter et al., 2002), nostrils salt glands of the seabird (*Calonectris diomedea*) and feathers of Flamingoes (*Phoenicopterus* spp.) (Yim et al., 2015).

Additionally, Gibtan et al. (2017) explored microbial diversity of commercial salts from two countries, Ethiopia and Korea, using amplicon sequencing approach and reported that 91.58% archaea, belonged to the class *Halobacteria*, whereas the remaining were *Nanoarchaea, Methanobacteria,* and *Thermococci*, respectively. Members of genera, *Halorubrum, Halobacterium, Haloarcula, Halonotius, Natromonas, Halarchaeum, Halomicrobium,* and *Salarchaeum* were present in each salt. Study on the haloarchaeal diversity in 9 food-grade salts revealed, that *Halorubrum*, *Haloarcula* or *Halobacterium* to be the dominant genera (Henriet et al., 2014).

Table 2. Comparative list of reports on halophilic archaea isolated from commercial/rock/halite/crystal salt

Sr. No.	Haloarchaeal strains	Isolation medium	Source and place	Salt type	References
1.	*Halococcus hamelinensis, Hcc. sediminicola, Halorubrumsodomense*	EHM/NTYE	Vedaranyam, Tamil Nadu, India	CCS	Present study
2.	*Halopiger thermotololerans* SR-441^T, SR-188, SR-412	JCM no. 168	Australia, China, Mexico	CS	Minegishi et al., 2016
3.	*Haloparvum alkalitolerans* MK62-1^T	MH4.2	Philippines	CS	Kondo et al., 2016
4.	*Halostella salina* CBA1114^T	DSM no. 954	Republic of Korea	SS	Song, et al., 2016
5.	*Halapricum salinum* CBA1105^T	DSM no. 372	Gomso Bay, the Republic of Korea	NPSS	Song et al., 2014
6.	*Halobaculum magnesiiphilum* MGY-184^T	JCM 225	Japan	CSS	Shimoshige et al., 2013
7.	*Natronoarchaeum philippinense* 294-194-5^T	JCM no. 294	Philippines	CSS	Shimane et al., 2013
8.	*Halarchaeum salinum* MH1-34-1^T	MH1	Indonesia	CSS	Yamauchi et al., 2013
9.	*Salarchaeum japonicum* YSM-79^T	Medium A	Yonaguni Island, Okinawa, Japan	CS	Shimane, et al., 2011
10.	*Halarchaeum acidiphilum* MH1-52-1^T	MH1	Solar salt of Australia	CSS	Minegishi et al., 2010
11.	*Natronoarchaeum mannanilyticum* YSM-123^T	Medium A	Japanese seawater in Niigata prefecture	CS	Shimane et al., 2010

Hcc. Halococcus; EHM extremely halophilic media; NTYE NaCl tryptone yeast extract; JCM Japan Collection of Microorganisms; DSMZ Deutsche Sammlung von Mikroorganismen; CCS commercial crystal salt; CS commercial salt; CSS commercial sea salt; SS solar salt; NPSS non-purified solar salt.

Halococcus is known for resisting cell lysis owing to the tough cell wall and the very same property might provide the ability for the halococcal isolates to survive the prolonged entrapment in the salt crystals. Though halophilic archaeal 16S rRNA sequence has been detected in the human gut, majority of the sequences belonged to *Halorubrum* (Oxley et al., 2010). Another study involved in culturing the gut microbes, isolated halophilic archaea belonging to *Haloferax* (Lagier et al., 2016).However, our study indicates the dominance of *Halococcus* which can be unique to the salt crystals obtained from the salterns of India. This may be due to the transient operational nature of the solar salterns of India, which are operated only between the months of December and July owing to the monsoon rainfall, ceasing the salt production. This transient nature might favor growth of halophilic archaea belonging to *Halococcus* which can resist the fluctuations of salinity change (Mani et al., 2012a). Therefore, the dominance of *Halococcus*in the salt crystals might be unique to India.

India is the third largest country in salt production. However, there is limited research conducted on exploring the microbial diversity of the commercial salt. The salts contained surprisingly diverse microbial communities with various novel genus and strains. Recently halophilic archaea has been implicated in intestinal bowel diseases and as a part of the skin microbiota (Lurie-Weinberger and Gophna, 2015). The most possible entry route for halophilic archaea inside the human system might be through the intake of the salt crystals or food products processed with these salt crystals. Therefore, exploring the microbial diversity of the salt crystals might give us a clearer picture of the association of halophilic archaea with human health.

The present study describes the culturable halophilic archaeal diversity of the iodised crystal salt sample obtained from solar salterns of Vedaranyam, Tamil Nadu, India. Six haloarchaeal cultures were isolated by surface spread plating on two nutrient-rich complex media, NTYE and EHM both containing 25% (w/v) NaCl, along with other salts. The estimated number of grown colonies, pigmented/non-pigmented, per gram of crystal salt was ~$1.1 \times 10^4/5.5 \times 10^3$ and ~$1.0 \times 10^4/4.0 \times 10^3$ on NTYE and EHM media, respectively. The cells of the isolates were coccus in pair/tetrads/groups, involuted/cup shaped, producing C-50 bacterioruberin pigment. Based on the 16S rRNA gene sequencing, the isolates were assigned to two genera *Halococcus* and *Halorubrum*. To the best of

author's knowledge, this is the first study reporting the prevalence of *Halococcus* and *Halorubrum* in the commercial iodised crystal salt of Vedaranyam, Tamil Nadu, India. Studying halophilic microorganisms (bacteria and archaea) in commercial salt can further help us in understanding their role in food, employing salt as preservative and thereby, further uneviling their importance in human health.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

Author Contributions

B.B. Salgaonkar, conceived the idea and designed the experiments. R. Rodrigues performed the experiments and B.B. Salgaonkar analyzed the data and drafted the manuscript.

Statement on the Welfare of Animals

This article does not contain any studies involving animals performed by any of the authors.

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