Saudi Journal of Biological Sciences (2018) 25, 1411–1419

King Saud University

Saudi Journal of Biological Sciences

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ORIGINAL ARTICLE

The heterotrophic eubacterial and archaeal co-inhabitants of the halophilic *Dunaliella salina* in solar salterns fed by Bay of Bengal along south eastern coast of India

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Received 23 April 2015; revised 29 October 2015; accepted 30 October 2015
Available online 6 November 2015

KEYWORDS

Bacterioruberin;
Dunaliella salina;
Group 2 *Halomonas* sp.;
Halobacterium sp.;
rRNA;
Solar salterns

Abstract Halophilic microbes are studied to understand the metabolic pathways adopted by organisms in such extreme environment and for their biotechnological exploitation. In thallosohaline environments worldwide, the autotrophic alga *Dunaliella salina* Teodoresco is omnipresent, but it is being recently realised that the heterotrophic components vary in different regions. The unexplored eastern coastline of India abutted by Bay of Bengal was investigated for the heterotrophic halophilic microbes in this region. The waters in the salterns – replicas of natural hyper-saline water bodies of that region, were collected at four sites along 650 km of the coastal belt. In cultures set up from these waters, green and pink colonies were observed. The green colonies were found to be those of *D. salina* while the pink colonies were of heterotrophs. To identify the heterotrophic microbes, light microscopy, 16S rRNA typing and pigment profiling through spectrophotometry and HPLC were done. The cells in pink colonies were rod shaped. 16S rRNA typing of cells in these colonies detected the presence of *Halomonas* sp. – a eubacterium. The pigment profile of cells in pink cultures matched that of the archaea – *Halobacterium*; bacterioruberin derivatives were found. Thus, it was concluded that *Halomonas* and *Halobacterium* spp. are among the co-inhabitant heterotrophs of *D. salina*. Cultures of *D. salina* established from these salterns showed the typical three

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Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

http://dx.doi.org/10.1016/j.sjbs.2015.10.019

colours seen in the ponds of different sub-plots of salterns. They were green until 30 days, turning dark orange by 60 days and pink when 90 day old. In the 90 day old cultures, innumerable rod shaped cells were found. These cells were similar to the cells of the waters from the ponds of pink sub-plots of salterns and the pink colonies established from saltern waters in the laboratory. In the old (90 days) laboratory cultures of *D. salina*, the glycerol and proteins released from degenerating cells and the increase in salt concentration to super saturation levels due to evaporation of water in the medium led to the gregarious appearance of the heterotrophs – the co-inhabitants in natural environment.

1. Introduction

The colourful hyper-saline water bodies constitute gorgeous arenas along sea coasts worldwide. In some seasons, they are coloured pink/dark red/orange due to the presence of dense communities of carotenoid rich prokaryotes, archaea and the β carotene rich unicellular alga *Dunaliella salina* (Elevi Bardavid et al., 2008). The extremophilic (halophilic) inhabitants of these aquatic systems are intriguing and the subject of study for diverse objectives. Because of their remarkable resilience, they are investigated for the genes and proteins that bestow this property and as models for studying mechanisms of survival away from our planet (Das Sarma, 2006). They are sought by multibillion dollar industries – the most spectacular being β carotene production from *D. salina*. The bacterial and archaean inhabitants of hyper-saline environments have a biotechnological potential not only for their special carotenes but also as source of enzymes, compatible solutes (eg. ectoine), biosurfactants and exopolysaccharides among other products (Haba et al., 2011). The halophilic prokaryotes can also serve as very convenient tools for production of recombinant proteins (Haba et al., 2011). Stringent aseptic conditions are not required for culture of these resilient organisms – the salt concentration at which they grow eliminates the possibility of contamination with undesirable microbes. Contamination is a serious problem with normal microbial cultures requiring expensive measures to prevent it. In comparison to the other groups of extremophilic microorganisms such as the thermophiles and alkaliphiles, halophiles of all three domains have been relatively less exploited in biotechnological processes, with notable exceptions of β carotene from *D. salina*, bacteriorhodopsin from *Halobacterium*, and ectoine from *Halomonas* (Oren, 2010).

Based on the limited metabolic diversity observed in halophilic microbes, it was for a long time assumed that the hyper-saline environments worldwide have a simple ecosystem with very similar microbial inhabitants (Oren, 2002). However, the studies in the last decade have indicated that while the microalga *D. salina* is ubiquitous in these environments, the members of halophilic heterotrophs are not similar in different geographic regions (Oren, 2002). Many new bacterial and archaean members have been discovered, the dominant members of these groups being distinct in different regions. The microbial ecology of these special environmental expanses has not been as exhaustively investigated as the conventional marine and terrestrial ecosystems (Oren, 2001).

Solar salterns artificially constructed along sea coasts with sub-plots for incremental evaporation of sea water for harvest of common salt (NaCl), are replicas of the natural salt water bodies. They offer an opportunity to explore the microbial diversity in different geographic regions. Just as was the earlier belief on microbial diversity of natural salt water bodies, it was held that "despite minor variations attributable to differences in availability of nutrients, the general microbial properties of crystallizer brines in salterns worldwide are markedly similar" (Elevi Bardavid et al., 2008). From their studies Elevi Bardavid et al. (2008) stated that the most abundant heterotrophs in salterns are *Haloquadratum* and *Salinibacter* species. They discounted the contention of Borowitzka (1981) that *Halococcus* and *Halobacterium* are the dominant heterotrophs in salterns. Further, it has been indicated that *Halobacterium* is present in very few numbers in salterns and it can be obtained in culture only through special enrichment procedures (Oren and Litchfield, 1999). A recent investigation of salterns along the south western coast of India (Goa) revealed the presence of entirely different genera of heterotrophs (Mani et al., 2012). The dilute waters of the salterns in the pre-harvest stage were found to harbour exclusively bright orange red *Halococcus salifodinae* while in the dark red waters at salt harvest stage, along with *H. salifodinae*, *Haloarcula* sp. and *Haloferax* sp. were found abundant (Mani et al., 2012). Another study in the west coast of India (Mumbai) reported a wide diversity in the archaea spp. in the saltern sediments consisting of *Haloarcula* spp. *Haladaptatus* spp. and members of methanosarcinales (Ahmad et al., 2008).

The microbial components of hyper-saline ecosystems along the east coast of Indian subcontinent in the tropical belt have not been investigated. We examined the heterotrophic microbes associated with *D. salina* in these unexplored salterns fed by the waters of Bay of Bengal. Salterns are the simplest salt water bodies to investigate the relationship between the primary producer *Dunaliella* and heterotrophic microbes (Elevi Bardavid et al., 2008).

2. Materials and methods

2.1. Sample collection

Water samples were collected from evaporation saltpans to establish cultures of heterotrophs and the carotenogenic *Dunaliella* species – *D. salina*. Samples were collected from a 650 km belt along the south eastern coast of India in the state of Andhra Pradesh starting from Visakhapatnam to Nellore districts in four regions: Bheemunipatnam, Parawada, Kakinada and Iskapalli (Fig. 1). The water in the collection sites were in various shades of green, orange and pink in the ponds in different sub-plots of salterns (Fig. 2A–C). The water in different sub-plots have different salt concentrations ranging

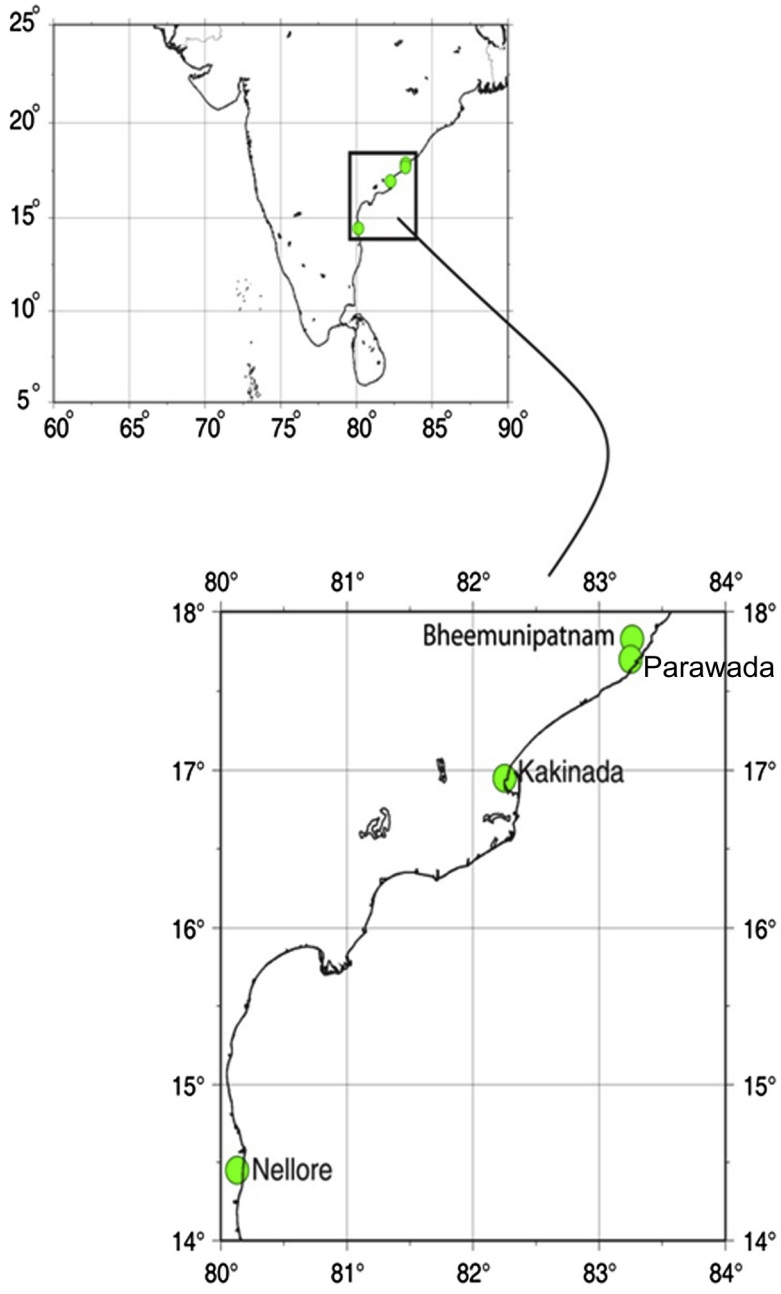

Figure 1 Location of the salterns along south eastern coast of India from which samples were investigated.

from dilute to super-saturated. The diverse colours of the sub-plots in the salterns are due to the characteristic microbial community in each of them – being composed of the types that thrive in the prevailing salt concentration (Elevi Bardavid et al., 2008). Brine samples were collected from orange sub-plots with a saturated concentration (~26%) of salt. *D. salina* is predominant in the orange sub-plots (the orange colour being due to the β carotene in *D. salina*). We chose the orange sub-plots as we wanted to examine the heterotrophic microbes associated with *D. salina* in the salterns.

2.2. Sampling procedure

Spatial and temporal sampling was done. In each collection area, one saltern field was randomly selected from among the several distributed in a 1 km radius. In each saltern field, one orange sub-plot (with easy access for collection) was chosen. The sub-plots were of ~900 m^2. The sub-plot was divided approximately into nine 30 m^2 units and one sample (10 ml) was collected from each of them. In the borders of the sub-plot with crystallising salt, a spatula full of salt with brown patches was collected and dissolved in the water from the saltern. Samples were collected from the same saltern in two consecutive years.

2.3. Culture establishment

The water samples were filtered through 20 μ nylon liquid filter mesh and 30 ml of the filtrate was inoculated into 100 ml of NORO culture medium with 12% NaCl (Takagi and

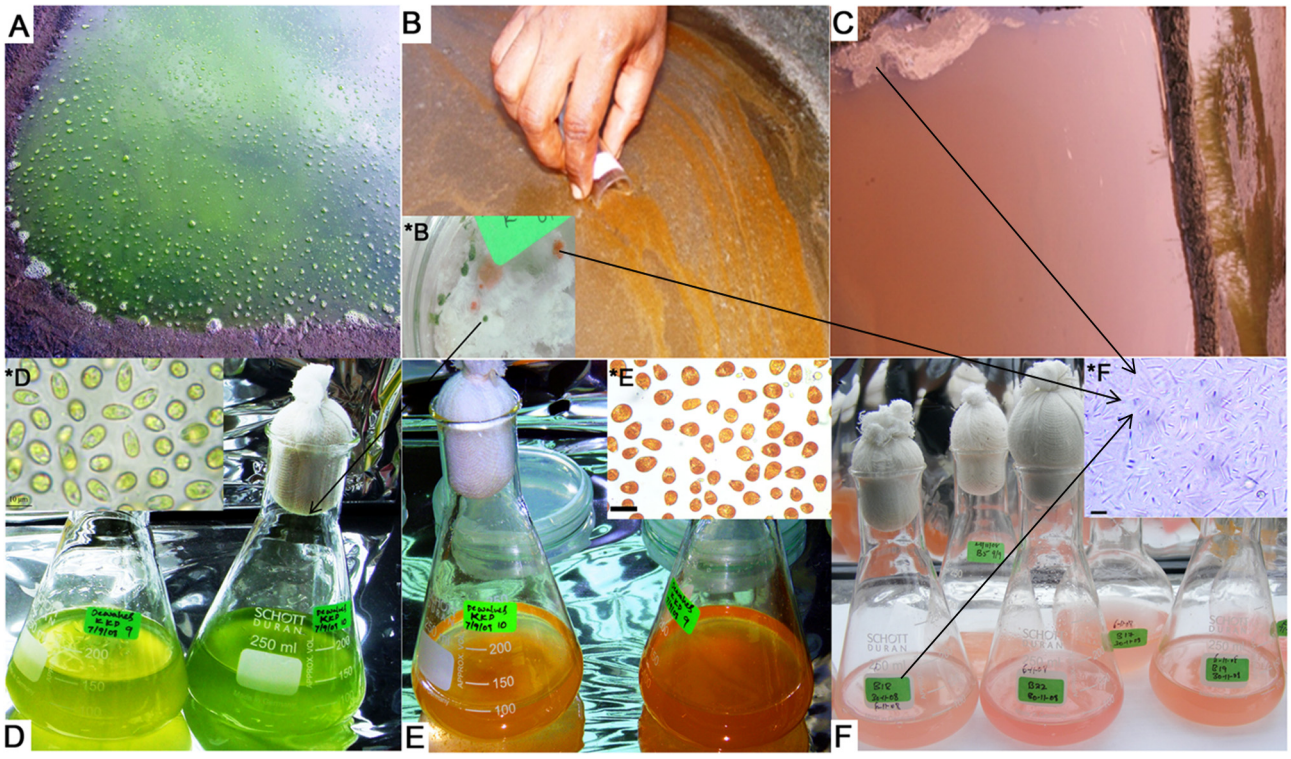

Figure 2 Water in solar salterns and *Dunaliella salina* cultures. Similarity between nature and laboratory. (A–C) Colours of the water in different sub-plots of the evaporation salt pans at increasing salt concentration from A to C. Water samples for establishing *D. salina* cultures were collected from B. (B*) Green and pink cultures that developed from the water samples plated on NORO medium. (D–F) *Dunaliella salina* cultures at different stages. (D) Up to 30 days. (E) By 60th day. (F) 90 day old. (D*, E* and F*) The cells in the corresponding culture. (D* and E*) Green and orange *D. salina* cells respectively. (F*) Rod shaped cells of the heterotroph (seen in pink waters of salterns, ~90 day old *D. salina* cultures and pink colonies developed from the ponds with orange coloured water in Petri dish (B*) and the liquid culture developed from them). The bar represents 10 μm.

Yoshida, 2006) in 250 ml Erlenmeyer flasks. The flasks were kept in a culture room maintained at 26 ± 1 °C, 100 μmol photons m^2 s^{-1} light on a 12/12 h light/dark cycle. After 2 weeks, 1 ml of culture from such flasks was spread on the surface of solidified NORO medium (with 1.5% agar) in Petri dishes. The Petri dishes were incubated in the same conditions as described above for 8 weeks. By this time the salt was found to crystallise in the medium and single cell colonies developed in them. Both green and pink colonies appeared in cultures set up from all the collected samples (Fig. 2B*). When viewed under the microscope, the green colonies showed *Dunaliella* cells (2D*) while the pink colonies showed rod shaped cells (Fig. 2F*). Similar rod shaped cells were also observed in water from the pink ponds in sub-plots of salterns (2F). These cells represent the predominant heterotrophic components associated with *Dunaliella* in the salterns. The green colonies in the Petri dishes turned dark orange when the cultures were left for 14 weeks. Thus it was inferred that the cultures were of carotenogenic *Dunaliella* sp. – *D. salina*.

2.3.1. Establishment of cultures of heterotrophic microbe

Culture of heterotrophic microbial cells in the pink colonies was established in a modified Johnson's medium (J/l) (Borowitzka and Borowitzka, 1988) with 1% (W/V) maltose, 2% yeast and 17.5% NaCl. The cultures were maintained under conditions described above. From each collection site, five cultures established from five pink colonies from five different samples totalling 20 were selected for analysis.

The cultures grew well by 25 days. The cells were observed under the microscope and their length was measured with Progress Capturer® 2.8 software provided with the Lx 400 trinocular research microscope with iVu 3000 LCD camera module (JENOPTIK I Optical Systems Germany).

2.3.2. Establishment of cultures of Dunaliella

The cells from green colonies were picked and single cell cultures of carotenogenic *Dunaliella* were developed from them in De Walne's medium (Orset and Young, 1999) using standard procedures (Andersen, 2005). For eliminating any probability of bacterial contamination, 2000 ppm penicillin treatment was given for one day (Spencer, 1952). The cyanobacterial contaminants were purged by treatment with 2000 ppm of streptomycin sulphate for 30 min under 150 μmol photons m^{-2} s^{-1} (Guillard, 2005). Possible diatom contamination was eradicated by treating the samples with 5 mg l^{-1} of germanium dioxide for 3 days (Lewin, 1966). The cultures were washed thrice thoroughly to remove the antibiotic and then transferred to culture medium. To ensure that there was no bacterial contamination, verification was done by spreading an aliquot from these cultures on TYG agar and inoculating in TYG broth (0.5% tryptone [Difco], 0.25% yeast extract [Difco] and 0.1% glucose) and incubating

for 3 weeks at 26 °C (Ferris and Hirsch, 1991). The *Dunaliella* cultures were also grown in conditions described above. The carotenogenic nature of *Dunaliella* isolates and their growth kinetics were studied and cultures of 16 highly carotenogenic isolates of *D. salina* were established (Suman, 2012). The cultures were maintained in the laboratory through sub culture.

2.4. Identification of heterotrophic microbe isolated from salterns

2.4.1. 16S rRNA typing

The most commonly used primers specific for 16S rRNA of eubacteria (Lane, 1991) – 16F27F forward primer (5′- GAGTTTGATCCTGGCTCAG-**3**′) matching nucleotides from 8th to 27th position of 16S rRNA and reverse primer (5′-ACGGCTACCT-TGTTACGACTT-3′) were used. An aliquot of pelleted cells from a 1.5 ml culture of each of the 20 pink cultures was used for DNA extraction. DNA was extracted as described by Okamoto et al. (2004). The size of the amplified product was examined through electrophoresis on a 1.5% agarose gel. The amplified product from one of the cultures (B22) was sequenced. For this, the PCR product was purified and its entire length was sequenced as described by Shivaji et al. (2000). Along with the primers used for amplification of 16S rRNA, forward primers – TAACACATGCAAGTCGAACG (50–70); CTACGGGAGGC-AGCAGTGGG (341–361); CAG CAGCCGCGGTAATAC (518–536); AAACTCAAAG-GAA TTGACGG (908–928) and CATGGCTGTCGTCAGCTCGT (1053–1073) and reverse primers – GTATTACCGCG GCTGCTG (536–518) and ACGAGCTGACGACA-GCC ATG (1073–1053) were used to sequence the entire (~1.5 Kb) length of 16S rRNA gene. The numbers given in parentheses refer to the nucleotide positions in the 16S rDNA of *Escherichia coli* to which the primers match. The sequence was deposited in GenBank. Similarity of this sequence was assessed through BLAST (Altschul et al., 1990).

2.4.2. Phylogenetic analysis

Sequences that showed more than 90% similarity (in BLAST) with the sequence obtained in our study were chosen for phylogenetic analysis. The rRNA sequence from the red rod shaped bacterium *Salinibacter ruber* reported to be a dominant eubacterial component in salterns of Spain and Israel (Elevi Bardavid et al., 2008) was included to serve as an outgroup. The data set consisted of 21 nucleotide sequences. MEGA (ver. 5) (Tamura et al., 2011) was used for phylogenetic analysis. The sequences were aligned with the alignment tool of MEGA 5. Codon positions included were 1st + 2nd + 3rd + noncoding. All positions containing gaps and missing data were eliminated. There were a total of 1232 positions in the final dataset. Both distance based (neighbour joining) and character based (maximum parsimony, maximum likelihood and minimum evolution) phylogenetic trees were constructed to examine the conformity of clades. For neighbour joining, maximum likelihood and minimum evolution trees, evolutionary distances were estimated using Tamura-Nei method (Tamura and Nei, 1993). The maximum parsimony tree was obtained using Close-Neighbour-Interchange algorithm (Nei and Kumar, 2000) with search level 1 in which initial trees were obtained with random addition of sequences (10 replicates). Branch lengths of the tree were calculated using average pathway method (Nei and Kumar, 2000).

2.4.3. Pigment analysis

Analysis of the pigments in the microbe in the pink cultures was done initially through spectrophotometry. For one of the cultures – B22 (used in 16S rRNA gene sequencing), a comprehensive qualitative analysis of the pigments was done through HPLC. Pigments were extracted from a 2 ml aliquot of the culture. Cells were pelletised through centrifugation at 12,000 rpm for 10 min. To the pellet, 5 ml ice cold 90% (v/v) acetone was added and vortexed for 5 min and left overnight in a refrigerator at 4 °C. The sample tubes were covered with an aluminium foil to prevent exposure to light. This treatment was found sufficient for complete extraction of pigments. The samples were centrifuged for 10 min at 12,000 rpm. The supernatant was used for spectrophotometric and HPLC analysis. Absorption spectrum was obtained with a dual beam spectrophotometer (Shimadzu UV 1800, Kyoto, Japan) using one cm light path. HPLC analysis was done in an Agilent 1200 HPLC system (Santa Clara, CA) equipped with Chemstation software. Pigments were detected with the diode array detector at 450 nm wavelength with a 20 nm bandwidth. An injector programme was optimised to deliver sample extract and buffer composed of 28 mM aqueous tetra butyl ammonium acetate (TBAA) (AR Grade, Fluka, USA) at pH 6.5 and methanol (GC Assay 99.7% pure, Merck) in 90:10 ratio. The method proposed by Van Heukelem and Thomas (2001) was adopted. A volume of 400 µl was injected. The oven temperature was set to 60 °C and solvent flow rate was 1.1 ml/min. A ZORBAX eclipse XDB-C8, 4.6/150 mm (diameter by length) PN: 963967-906 column was used. Binary gradient elution as described by Chakraborty et al. (2010) was used. Carotenoids and xanthophylls were detected and quantified at 445 nm.

3. Results and discussion

The bacterial cells in all the twenty pink cultures established showed uniform morphology. They were rod shaped (Fig. 2F*) and measured 10–12 µ in length at 150× magnification.

An ~1.5 kb amplification product was obtained in PCR with 16S rRNA gene primers in all the 20 cultures of the heterotrophic microbe. A sequence read of 1.49 kb was obtained from the PCR amplified product of the B 22 culture. The sequence was deposited in GenBank with the accession number JN613434. In BLAST analysis, the sequence showed 98% similarity with *Halomonas variabilis* Fendrich, *Halomonas janggokensis* Kim et al., *Halomonas venusta* Baumann et al., and *H. subterranea* Xu et al., – all of which belong to group 2 members of the genus *Halomonas* of Halomonadaceae (Haba et al., 2011).

The topology was similar of the all the four phylogenetic trees obtained from the 16S rRNA nucleotide sequences – neighbour joining, maximum parsimony, maximum likelihood and minimum evolution. The maximum likelihood tree is represented in Fig. 3. *Salinibacter ruber* which was used as the outgroup separated out as a distinct branch (Fig. 3). *H. variabilis* members grouped into two clusters (Fig.3). One of these clusters consisted exclusively of *H. variabilis* (five members) while the other had three *H. variabilis* sequences grouped along with *H. janggokensis* and *H. venusta* (Fig. 3). A similar phylogenetic

relation between *H. variabilis* and *H. venusta* as evident in this analysis was also reported in the 16S rRNA analysis by Lim et al. (2004) and Lee et al. (2005). The bacterial sequence from our study (JN613434) diverged as a separate branch in this cluster (Fig. 3). Thus the bacterial sequence from our study showed affiliation to a distinct group with members of the *H. variabilis*, *H. janggokensis* and *H. venusta* species.

The spectrophotometric profile of the pigment extract from all the 20 strains from pink cultur was identical: absorption maxima were observed at 371, 389, 471, 495 and 528 nm (three fingered with two *cis* maxima) (Fig. 4A). This is typical of bacterioruberin derivatives (Gochnauer et al., 1972; Oren, 1983). In HPLC of the B22 culture, 12 peaks were observed (Fig. 4B) of which four (4–7) were major (Fig. 4B). The absorption spectra of these four peaks (4–7) matched the peaks of HPLC reported for *Halobacterium* spp. (Yachai, 2009) which were described as C 50 derivatives of bacterioruberin (Britton, 1995). Bacterioruberin (a carotene with a high free radical scavenging ability just as β carotene (Oren, 2010)) and its derivatives are the dominant pigments of the rod shaped archaea – *Halobacterium*. The HPLC profile in our study however had additional unidentified carotenoids

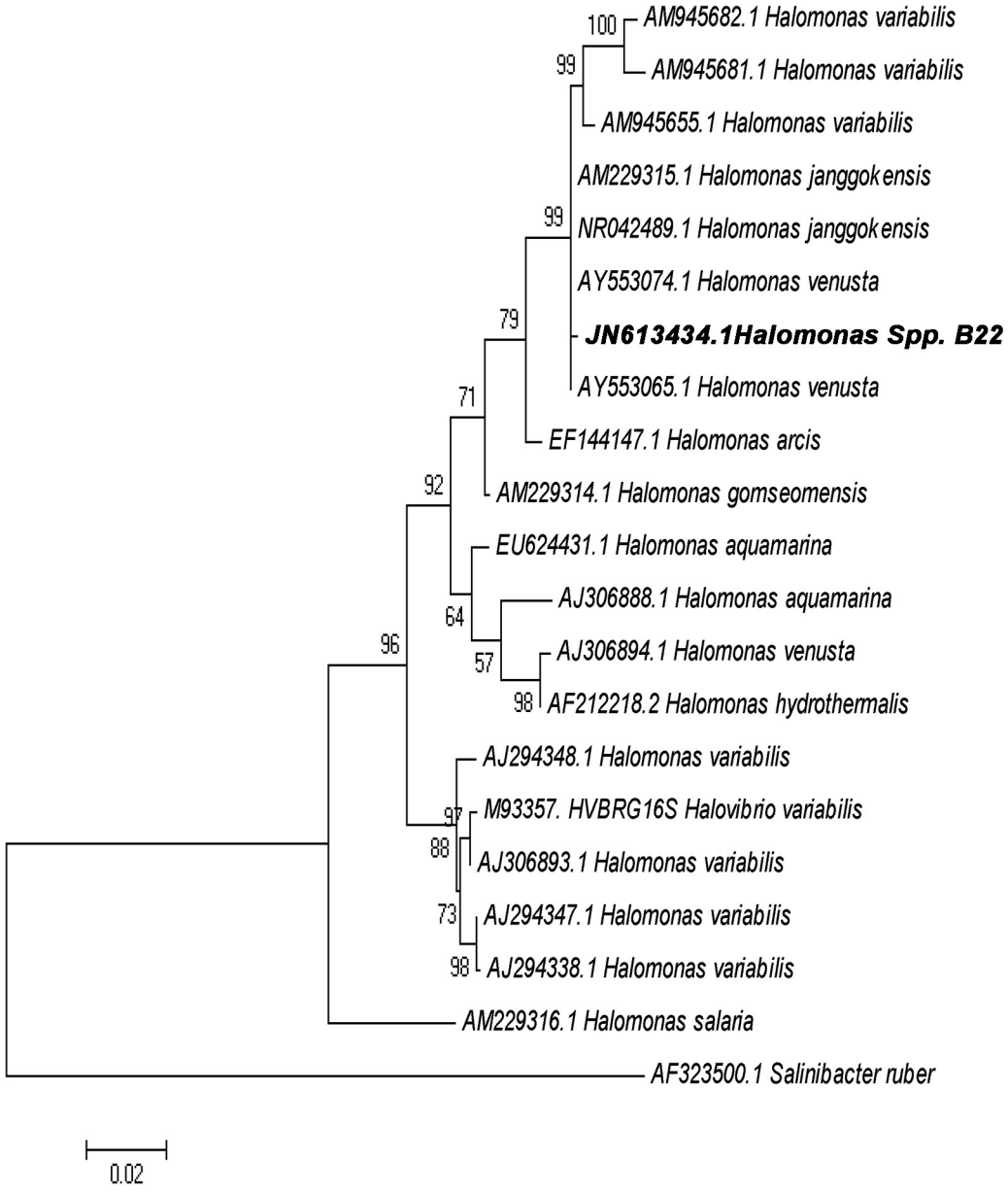

Figure 3 Maximum likelihood tree constructed based on the Tamura-Nei model using MEGA5 from aligned 16S rRNA sequences of the species of *Halomonas*, a halophilic eubacterium that showed more than 90% similarity with the B22 of our study. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for heuristic search were obtained automatically as follows. When the number of common sites was < 100 or less than one fourth of the total number of sites, the maximum parsimony method was used; otherwise BIONJ method with MCL distance matrix was used. The tree is drawn to scale, with branch lengths measured as the number of substitutions per site.

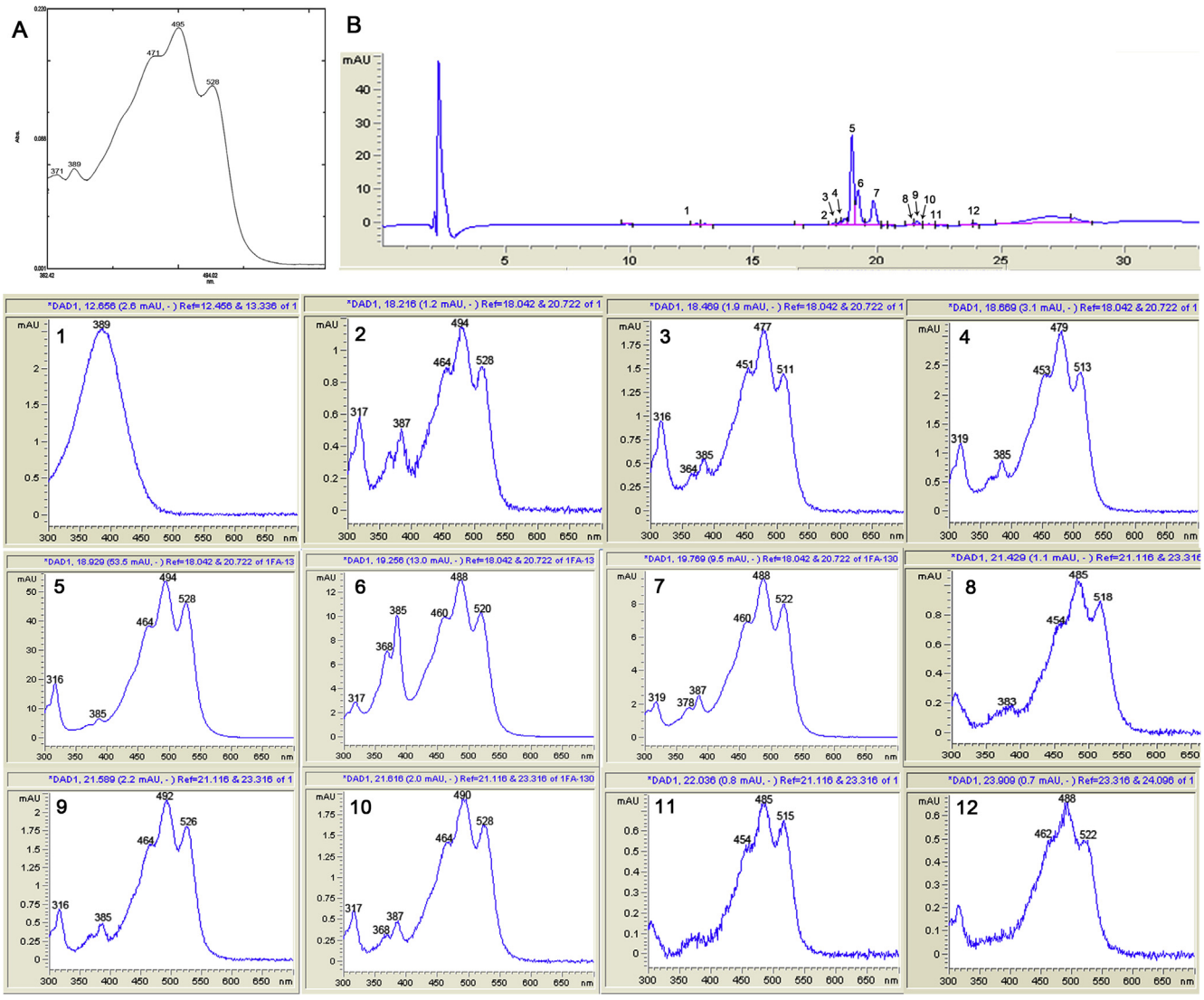

Figure 4 Absorption spectrum of acetone (90%) extract of cells pelletised from culture of heterotrophic cells established from solar salterns. (A) UV absorption spectrum – the maximum absorption peaks at 371, 389, 471, 495, 528 and 663 nm correspond to bacterioruberin derivatives. (B) HPLC chromatogram using a ZORBAX eclipse XDB-C8, 4.6 × 150 mm (diameter by length) column. (1–12) are absorption spectra of the peaks 1–12 in the HPLC chromatogram. Peaks 3–7 represent bacterioruberin derivatives.

corresponding to the HPLC peaks 1, 2, 3 and 8–12 (Fig. 4) not reported in the study of *Halobacterium* species by Yachai (2009). The HPLC column and conditions we used are different from those used by Yachai (2009).

In the heterotrophic microbial cultures established from the water samples of salterns, while the 16S rRNA analysis revealed the presence of *Halomonas* sp. (a eubacterium), the pigment profile matched that of *Halobacterium* (an archaea). Species of *Halomonas* are cream/yellow or orange (Mnif et al., 2009) while those of *Halobacterium* are pink. Thus it is inferred that the eubacterium *Halomonas* sp., and an archaea *Halobacterium* sp. are among the microbial components in the saltern samples collected along the south eastern Indian coast. Both these microbes are rod shaped. The cells in the pink cultures appeared rod shaped of a nearly uniform size; two morphologically discrete types (corresponding to the two heterotrophic species) could not be made out. The laboratory cultures of the heterotrophs are pink in colour. The planktonic populations, which bestow a pink to brightly red colour to the brines is reported to be due to the α-bacterioruberin and other 50-carbon bacterioruberin derivatives present in archaea of the family Halobacteriaceae (Oren and Dubinsky, 1994). Therefore it is inferred that the *Halobacterium* like archaea with bacterioruberin is the predominant component in the pink cultures in our study. Similarly, *Halobacterium* has been reported as the predominant archaea in the northern arm of Great Salt Lake, Utah, the Dead Sea and a few other natural salt-saturated lakes and crystalliser ponds of multi-pond solar salterns (Borowitzka, 1981). But Oren et al. (1996) reported that *Halobacterium* is not a dominant component of the heterotrophic community in saltern brines and is present in low numbers. Further, it was reported that *Halobacterium* can be

recovered from salterns only by a selective enrichment procedure (Oren and Litchfield, 1999).

In the pink heterotrophic microbial cultures in our study, *Halomonas* should have been present in very few numbers and may not have been distinguishable from the dominant *Halobacterium* cells with the resolving power of ordinary light microscope that was used for observations. Though very few in number, *Halomonas* was detectable through PCR of 16S rRNA gene because of the amplification power of the technique.

In all 16 carotenogenic *Dunaliella* isolates, liquid cultures were green for ~30 days (Fig. 2D) and thereafter started turning orange to dark orange in 60 days (Fig. 2E). When left for another month, the cultures turned pink (Fig. 2F) and salt crystals were observed at the bottom of the flasks. The cells from the green and orange culture conformed to the morphology of *D. salina* being biflagellate and green (Fig. 2D*) and dark orange (Fig. 2E*) respectively. Microscopic examination of the old cultures of *D. salina* that turned pink showed innumerable rod shaped cells (Fig. 2F*) similar to the cells observed in cultures developed from pink colonies arising from saltern waters. The pink colour of these cultures was similar to the pink brine in the sub-plots of evaporation saltpans where salt was getting crystallised (Fig. 2C and F). The water in pink ponds in salterns also had similar rod shaped cells. No cells with typical morphology of *D. salina* could be observed in old *D. salina* cultures that turned pink. When 30 ml of the 90 day old *D. salina* culture that turned pink was transferred to 100 ml of fresh De Walne's medium and incubated in the laboratory conditions described above, green cells of *D. salina* appeared after ~21 days. When these cells were transferred to fresh culture medium, *D. salina* cultures could be established. The pink cultures must have had a few *D. salina* cells in a form that does not conform to their normal morphological state.

The different colours of ponds in the sub-plots of solar salterns at varying salt concentrations are thus replicated in the laboratory cultures of *D. salina*. Laboratory cultures of *D. salina* turned from green to orange and finally pink over time as the salt concentration increased in the medium (Fig. 2D–F). These colours were similar to the colours observed in the different sub-plots of salterns at different salt concentrations (Fig. 2A–C). Though the cultures of *D. salina* were established from single cell colonies and given antibiotic treatment, halophilic bacteria and archaea were found developing when the cultures were very old (~90 days) and most of the *D. salina* cells were dead. Salt was found to crystallise in these culture flasks. The *Halomonas* and *Halobacterium* spp. in these salterns are thus resistant to the antibiotics (penicillin and streptomycin sulphate) used for treating the brine samples before setting up cultures. Members of Halobacteriaceae have been reported to be insensitive to bacteria specific antibiotics (Bonelo et al., 1984). *Halomonas* is a Gram negative bacterium. Gram negative bacteria are reported to be generally resistant to antibiotics due to the special membrane under their capsule (Kaplan, 2000). β lactamase mediated resistance to antibiotics has been reported in *Halomonas hydrothermalis* (Rameshpathy et al., 2012). Being very tiny, they might have been included in the cultures along with the *D. salina* cell in cultures being established from single cells. They remain dormant in the *D. salina* cultures in mineral medium at low (12.5%) salt concentration. With the passage of time, there is slow evaporation of water in the cultures leading to an increase in salt concentration to a level of super saturation – salt crystals were observed in the very old *D. salina* laboratory cultures that turned pink. At this salt concentration, most of the carotenogenic *D. salina* cells are lysed. Glycerol is released from these cells. With the availability of carbon source and the increased salt concentration in the medium, the latent heterotrophs in the culture multiplied and became predominant. Members of Halobacteriaceae are known to readily utilise glycerol (Oren and Gurevich, 1994). The halophilic heterotrophs were thus found to replace *D. salina* in old laboratory cultures reflecting the cycle in nature. These heterotrophic organisms constitute unintended components of *D. salina* cultures. All the *Dunaliella salina* cultures established in our laboratory painstakingly as single cell cultures still did not turn out to be axenic. The tiny co-inhabitant heterotrophic halophilic prokaryote and archaea got transferred along with single *D. salina* cells into the cultures. The fact that all the ~60 strains of *D. salina* established from the saltern waters turned out to show the presence of these heterotrophs in very old cultures indicates that it is very difficult if not impossible to obtain axenic cultures of *D. salina*. The heterotrophic microbial components of the *D. salina* cultures are latent and not in noticeable counts in *D. salina* cultures during the log phase and in the stationary phase (with β carotene accumulated in cells) at which time the cells are harvested.

The observation of Borowitzka (1981) regarding predominance of *Halobacterium* in salterns is thus valid in some salterns of the world. The salterns along the south eastern Indian coast were also found to be dominated by *Halobacterium* sp. The opinion of Oren et al. (1996) that *Halobacterium*, in spite of its high salt requirement and tolerance, is not at all successful in colonising the saltern environment is not true in all locations. Special enrichment procedures found to be required for culture of *Halobacterium* spp. (Oren and Litchfield, 1999) may be relevant only with waters of salterns where it is not a dominant member. *Halobacterium* could be readily cultured from the saltern samples in our study. Along with *Halobacterium* sp., the eubacterial species of *Halomonas* was also detected (through 16S rRNA typing) in these salterns. In the study site of Borowitzka (1981) along with *Halobacterium* which was predominant, the eubacterium – *Halococcus* sp. was reported. The heterotrophic microbial population of salterns worldwide is thus indeed not similar as noted by Elevi Bardavid et al. (2008) and is site specific.

Among the halophiles, only one representative from each of the three domains – *Halomonas* of eubacteria, *Halobacterium* of archaea and *D. salina* of eukaryotes, is commercially exploited for biotechnological purposes (Oren, 2010). All three are found in salterns along the eastern coast of India. If required for biotechnological purposes, *Halomonas* and *Halobacterium* can be separated from mixed cultures established from saltern waters using suitable antibiotics (Elevi Bardavid et al., 2008).

Acknowledgements

We thank Department of Science and Technology, New Delhi, India (DST/IS-STAC/CO2SR-32/07) and Ministry of Earth Sciences, New Delhi, India (MoES/11-MRDF/1/20/P08) for financial support. We thank Mr. T Acharyya and Dr. V.V.S. S. Sarma, NIO Regional Centre, Visakhapatnam, India for their help in HPLC work.

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