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Extreme Halophilic Archaea *Halobacterium* sp. SP1 in Bioremediation of Hypersaline Water Polluted with Metal, Metalloid, Organometal and Aromatic Compound

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Abstract: Extremely halophilic rod shaped, motile prokaryotic microorganism was isolated from sediment of Ribandar salt pan, Goa, India on NTYE agar (25% crude salt) with 500 units/mL penicillin and was designated as SP1. By performing antibiotic sensitivity test, sodium taurocholate sensitivity test, cell lysis in distilled water, glycerol diether lipid moieties in membrane, spectrophotometric analysis of carotenoid pigment and biochemical tests, the isolates SP1 was confirmed to be extreme haloarchaea *Halobacterium* sp. Extremely haloarchaeal isolate, *Halobacterium* sp. SP1 could resist 50µM $HgCl_2$ in NGSM broth with 0.1mM MIC (minimum inhibitory concentration). The isolate showed the potential of reducing soluble, toxic selenite into less toxic, insoluble elemental selenium (Se^0) which is indicated by change in colony color from red to bright orange. The isolate showed selenite reduction to elemental selenium up to 10 mM with MIC 11 mM. *Halobacterium* sp. SP1 could degrade up to 1.5 mM sodium benzoate as sole source of carbon on NSM broth (25 % crude salt) and showed 2 mM MIC. *Halobacterium* sp. SP1 can also capable of utilizing up to 1 mM tributyltin chloride (TBTCl) having 2 mM MIC on NTYE (25% crude salt) agar. Haloarchaea *Halobacterium* sp. SP1 is multi extremophilic archaea i.e. resistant/degrading multiple pollutants (Hg, Se, sodium benzoate and TBTCl) in hypersaline conditions and is of great importance for bioremediation of hypersaline environments contaminated with multiple pollutants. This is first report of haloarchea *Halobacterium* sp. resistant to multiple pollutants such as metal, metalloid, organometal and aromatic compound in hypersaline environment.

Key words: Extreme haloarchaea, salt pan, pollutants, bioremediation.

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Introduction

Marine pollution alters the physical, chemical and biological properties of water and sediments due to contaminants. Pollution causes deterioration of the natural quality and affects health and existence of all forms of marine life [5, 14]. Marine environments can be contaminated with pollutants such as oil spills, heavy metals (Hg, Cd, Pb), metalloids (As, Te, Se), organometals (tributylin, tetraethyl lead, methyl mercury), Xenobiotic compounds (Polychlorinated biphenyls, polychlorinated poly aromatic compounds), organic wastes (hotels, domestic) and medical wastes, which have deleterious effects on marine biota and need to be removed [1, 5, 14,18]. Oceans have average salinities ranging from 32-35 psu and are the largest saline environment. Hypersaline environments like saltpans are formed as a result of evaporation of sea water and are inhabited by salt loving halophiles (archaea and bacteria) [12, 21]. Hypersaline environments are not polluted with single contaminant but simultaneously polluted with metals, metalloids and aromatic pollutants from different sources. Metal, Metalloid, aromatic compounds and organometal contamination in hypersaline environment like salt pan is a major concern due to their harmful effects on flora and fauna. Mercury poisoning causes severe and strange neurological disorder due to consumption of marine fish and shellfish from mercury-contaminated marine waters and was discovered in Minamata Bay, Japan, in 1956 and therefore called Minamata disease [14]. Discharge of selenium polluted industrial effluent in marine waters results in biomagnification of selenium in fishes and thus, ultimately reaching to human and higher concentration of selenium/selenite (>400µg/mL) in body causes selenosis [18]. Sodium benzoate finds its application in preservatives in soft drinks, fruit products, pickles and sauces but, Sinha and D'Souza, 2010, reported liver cell damaging potential of aromatic pollutant sodium benzoate. TBTCl is commonly used in marine antifouling paints, biocides in agriculture, PVC stabilizers, preservatives for wood and leather industry [19]. Report exists on contamination of marine environment with TBTCl, leading to immunological, neurological, renal, reproductive, carcinogenic, and teratogenic effects on marine life and humans [11]. Bioremediation of polluted saltpans and other hypersaline environments with conventional microorganisms is not possible due to high salinities of these environments which may disrupt their osmotic balance and denature metabolic enzymes [9]. Therefore, there is pressing need to isolate extremely halophilic microorganisms for bioremediation of polluted hypersaline environments with metal, metalloids and aromatic pollutants.

The archaeal phylum Euryarchaeota includes mainly methanogens and haloarchaea. Haloarchaea generally require salt concentrations in excess of 1.5 M (or about 10%) to grow, and optimal growth usually occurs at much higher concentrations, typically 20–25% (3.4 M-4.3 M). However, haloarchaea can grow up to saturation (37% salt or 6.3 M) [12, 21]. Haloarchaea thrive in places such as the Great Salt Lake Utah, Owens lake in California, evaporation salt pans and the dead sea - places that provide an inhospitable environment to most life forms [15]. There are very few reports of isolation and application of haloarchea for bioremediation. Fairley *et al.* 2002, isolated novel haloarchaea *Haloarcula* sp. strain D1 capable of metabolising 4-hydroxybenzoic acid aerobically [10]. *Haloferex mediterranei* strain M-11 which was able to utilize oil as sole souse of carbon was isolated by Zvyagintseva *et al.* 1995 from Kalamkass oil field [22]. Bonfa *et al.* 2011

showed ability of haloarchaea (*Haloferex* strains) to grow on mixture of benzoic acid, p-hydroxy benzoic acid, and salicylic acid in media containing 20% crude salt [4]. *Halobacterium piscisalsi*, *Halorubrum ezzemoulense*, *Halobacterium salinarium*, *Haloarcula hispanica*, *Haloferax* sp., *Halorubrum* sp. and *Haloarcula* sp. isolated by Erdogmus *et al.* 2013 were capable of degrading aromatic hydrocarbons (namely, *p*-hydroxybenzoic acid, naphthalene, phenanthrene and pyrene) as sole carbon source in hypersaline environment [9]. Haloarcheon *Halococcus salifodinae* BK3 was able to tolerate up to 5.5 mM Na_2SeO_3 using NADH-dependent nitrate reductase mechanism [20]. Al-Mailem *et al.* 2011, reported mercury resistance and volatilization by oil utilizing haloarchaea (*Halobacterium*, *Haloferex* and *Halococcus*) under hypersaline conditions [2].

Salt making is practiced for many years in Ribandar region of Goa, which is a costal state of India [12]. Saltpans are flooded by Mandovi estuarine water during high tides and due to evaporation of estuarine waters in salt pans salinities gradually increases and reaches saturation (hypersaline environment), at this time crude salt crystals precipitate out. There are reports that Mandovi estuary is highly polluted with metals, metalloids, aromatic compounds and organometals due to anthropogenic activities [1, 7, 13, 18]. More than 50% of Goan population consumes crude salt formed at the salt pans in Goa. This practise may prove very detrimental for their health if the salt comes from the salt pans located in the Mandovi estuary that encounters frequent pollution. Therefore, main objective of this study is to isolate extremely halophilic archaea (haloarchaea) which can resist/degrade to multiple pollutants such as mercury, selenite, Tributyltin (organometal) and sodium benzoate and can be used in bioremediation of saltpans polluted with these pollutants.

Materials and Methods

1) Isolation of extremely halophilic archaea from sediment sample of Ribandar salt pan.

Sediment sample was collected from salt pan at Ribandar-Goa in January 2017. Serial dilutions of sediment in 20% crude salt solution up to 10^{-4} were prepared and 0.1 mL from 10^{-4} dilution were plated out on NTYE media containing 25% crude salt and 500 units/mL penicillin. The plates were incubated at 37^{o}C for 10-15 days.

2) Determination of growth characteristics

Single red colored colony appeared on 10th day on NTYE medium containing 25% crude salt was selected and designated as SP1. Isolate SP1 was tested for salt requirement, antibiotic resistance, lysis in distilled water and response to sodium taurocholate.

i. Salt requirement for growth

Isolate SP1was cultured on NTYE media containing 1%, 10% and 25% of crude salt and plates were incubated at 37^{o}C and were checked for the requirement of optimum salt concentration by observing growth pattern after 7 days.

ii. Response to antibiotics

Isolate SP1 was streaked on NTYE media (25% crude salt) containing choloramphenicol 100 µg/ml and plates were incubated at 37°C and the response of the isolates was recorded after 7 days. Control plate was also maintained with NTYE media (25% crude salt) without chloramphenicol.

Antibiotic susceptibility test of isolate SP1 was performed following Kirby–Bauer disc diffusion method [3] using NTYE agar plates (20% crude salt) and antibiotic disc. HiMedia Hexa G-plus disc containing Ciproflaxin (10µg/disc), Gentamyin (10 µg/disc), linezolid (30µg/disc), Streptomycin (300µg/disc) and Vancomycin (30 µg/disc) was used.

iii. Response of isolate SP1 to sodium taurocholate (bile salt)

Isolates SP1 was streaked on NTYE media (25% crude salt) containing 4% sodium taurocholate (bile salt). The plates were incubated at 37°C for 7 days. NTYE media (25% crude salt) without 4% sodium taurocholate (bile salt) was maintained as control.

iv. Analysis of SP1 cell lysis in distilled water

Extremely halophilic archaeal cells tend to lyse when exposed to distilled water due to osmotic imbalance [12]. Therefore, in order to investigate whether SP1 is haloarchaea or not, isolate SP1 was grown in NTYE media (25% crude salt) at 37ºC for 7 days and then centrifuge at 8000 rpm for 10 minutes. Pellet obtained was suspended in distilled water for 30 min and centrifuged at 8000 rpm for 10 minutes and observed under phase contrast microscope. Also, cells treated with distilled water were inoculated on NTYE agar plate (25% crude salt) and incubated at 37ºC for 7 days and growth was monitored.

3) Identification of isolate SP1

i. Detection of glycerol diether lipid moieties

100 mL NTYE medium (25% crude salts) was inoculated with SP1 and was incubated for 7 days at 37°C with constant shaking at 150 rpm. Culture broth was then centrifuged at 8000 rpm for 10 mins. Supernatant was discarded and 3 mL methanol + 3 mL toluene + 0.1 mL conc. H_2SO_4 was added to the pellet. The suspension was mixed well and was kept in boiling water bath at 50°C for 12 hrs. The suspension was then taken into separating funnel and 1.5 mL of hexane was added and mixed well. This was allowed to separate in dark for 4 hours. Upper hexane layer was collected, concentrated and then spotted on TLC plate. The solvent system included petroleum ether: diethyl ether (85:15). After solvent run the plate was sprayed with 10% dodecamolybdo phosphoric acid in absolute ethanol and heated for 15 min at 100ºC. Retardation factor (Rf) of the blue spot obtained was calculated [17].

ii. Pigment analysis

NTYE (25% crude salt) media (50 mL) was inoculated with isolate SP1 and incubated at 37°C for 7 days with constant shaking at 150 rpm, illuminated with light. Broth was centrifuged at 37°C at 8000 rpm for 10 min and acetone (10 mL) was added to the cell pellet and was sonicated for 10

min (at a pulse rate of 0.5 second). The resultant suspension was again centrifuged at 10,000 rpm for 10 minutes and supernatant was analyzed by taking absorption spectrum in the range 190 - 600 nm using UV- visible spectrophotometer (Shimadzu, Model–UV 2450, Japan) with acetone as blank [12].

iii. Biochemical tests

Biochemical tests such as sugar fermentation, motility were conducted in NTYE media containing 20% salt and were incubated at 37^{o}C for 7 days. The isolate was tested for catalase, indole, nitrate reductase, oxidase and amylase (starch utilization) production.

iv. Scanning electron microscopy

Isolate SP1 grown in NTYE (20% salt) at 37ºC for 7 days was pelleted at 10000 rpm and 100 µL of this suspension was mounted on glass coverslips, air dried and desalted with 2% acetic acid. SP1 cells were fixed with 2% glutaraldehyde, overnight. The coverslips were then exposed to a series of increasing acetone concentrations (10%, 30%, 50%, 70%, 90%), each for 15 min and finally with 100% acetone for 15 min. These dehydrated samples were air dried and mounted onto metal stubs. The sample was then coated with gold for SEM analysis (Zeiss EVO18). SEM analysis was carried out within 2 days for morphological identification.

4) Studies on mercury and selenite resistance

Mercury and selenite resistance potential of isolate SP1 was studied in Minimal media (NGSM) with 25% crude salt [6]. Growth of *Halobacterium* strain SP1 in minimal media (NGSM) broth amended with $HgCl_2$ concentrations of 30 µM, 50 µM, 0.1 mM and 0.2 mM was studied. Control flask without $HgCl_2$ was maintained for all experiments. All flasks were incubated at 37ºC for 7 days. The growth was recorded after 7 days. Similarly, growth of *Halobacterium* strain SP1 in minimal media (NGSM) plates amended with Na_2SeO_3 concentrations 1 mM, 2 mM, 5mM, 10 mM, 11 mM and 12 mM was studied. Stock solutions of $HgCl_2$ (0.5 M) and Na_2SeO_3 (0.5 M) was prepared in double distilled water and filtered through 0.2 µ nitrocellulose filter paper and stored at 4ºC until used.

5) Sodium benzoate and Tributylin chloride degradation studies

Sodium benzoate and Tributylin chloride (TBTCl) degrading potential of isolate SP1 was studied in minimal media without glucose (NSM) with 25% crude salt. Growth of *Halobacterium* strain SP1 in minimal media (NSM) broth amended with sodium benzoate concentrations of 0.1 mM, 0.5 mM, 1 mM, 1.5mM and 2 mM was studied. Control flask with glucose as carbon source (without sodium benzoate) was maintained for all experiments. All the flasks were incubated at 37ºC for 7 days. Similarly, growth of *Halobacterium* strain SP1 on NTYE plates (25% crude salt) amended with different TBTCl concentrations 0.1 mM, 0.5 mM, 1mM, 1.5 mM, 2 mM was studied. Stock solutions of sodium benzoate (0.5 M) was prepared in double distilled water and filtered through 0.2 µm nitrocellulose filter paper and stored at 4ºC till use. TBTCl stock (3.7M) was procured from Sigma Aldrich and stored at room temperature (RT).

Results and Discussion:

1) Isolation of extremely halophilic archaea from sediment sample of Ribandar salt pan.

After incubation of NTYE plates containing 25% crude salt solution and 500 units/mL of penicillin for 10 days at 37^0C, showed red colored colonies (Online resource 1). Single red colony was selected and was purified by streaking on NTYE media containing 25% crude salt (Fig.1) and maintained on NTYE slants (25% crude salt).

2) Determination of growth characteristics

i. Salt requirement for growth

After observing the NTYE plates with 1%, 10% and 25% salt concentrations, SP1 showed best growth at 25% crude salt concentration. SP1 did not show growth on NYTE media containing 1% crude salt but there was good growth on NTYE media having 10% crude salt. This indicates that the isolate SP1 is extremely halophilic and it requires 25% crude salt concentration for their optimum growth (Online resource 2).

ii. Response to antibiotics

The isolates SP1 was not inhibited by 500 units/mL penicillin (Fig.1) and 100 µg/mL chloramphenicol (Fig. 2) which were incorporated into NTYE media (25% crude salt). Also the isolate SP1 showed resistance to a wide range of antibiotics such as Ciproflaxin (5 µg/disc), Gentamyin (10 µg/disc), linezolid (30 µg/disc), Penicillin G (10 µg/disc), Streptomycin (10µg/disc) and Vancomycin (30 µg/disc) (Online resource 3).Therefore, isolates SP1 may be haloarchaea because archaea are insensitive to antibiotics which inhibit bacteria [8, 12] since archaea differ from bacteria by cell wall, replication, transcription and translation.

iii. Response of the isolates to sodium taurocholate (bile salt)

After observing NTYE plates (25% crude salt) containing 4% sodium taurocholate on seventh day, there was no growth of isolate SP1. This indicates that sodium taurocholate inhibited the growth of SP1 (Online resource 4). Elevi and Oren, 2008, reported that haloarchaea are inhibited by sodium taurocholate but halobacteria (*Salinobacter*) are not inhibited by sodium taurocholate [8]. The above result is suggestive of isolate SP1 being a haloarchaea.

iv. Analysis of SP1 cell lysis in distilled water

SP1 cells showed lysis when treated with distilled water and after centrifugation colored supernatant obtained suggests the extraction of pigment in the water phase. Also after observing under phase contrast microscope cell lysis was confirmed and there was no growth of the isolate when re-streaked on NTYE plates (25% crude salt) even after 7 days of incubation. This result clearly suggests isolate SP1 as extremely halophilic archaea [12].

3) Identification of extremely halophilic isolate SP1

i. Detection of glycerol diether lipid moieties

Fig 1: Growth of isolate SP1 on NTYE media with 25% crude salt

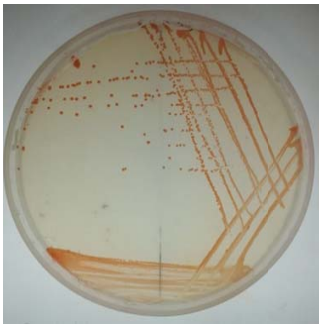


Fig 2: Growth of isolate SP1 on NTYE media with 25% crude salt and 100 µg/mL chloramphenicol

Fig 3: SEM image of isolate SP1

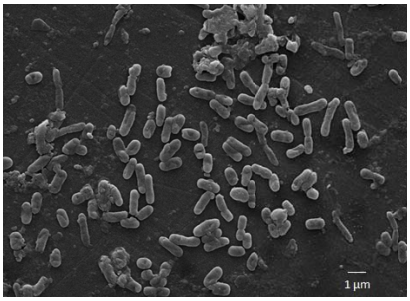


Fig 4: Growth of *Halobacterium* sp. SP1 on NGSM plates with 10 mM sodium selenite

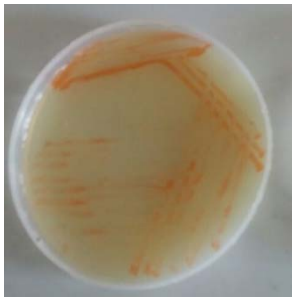
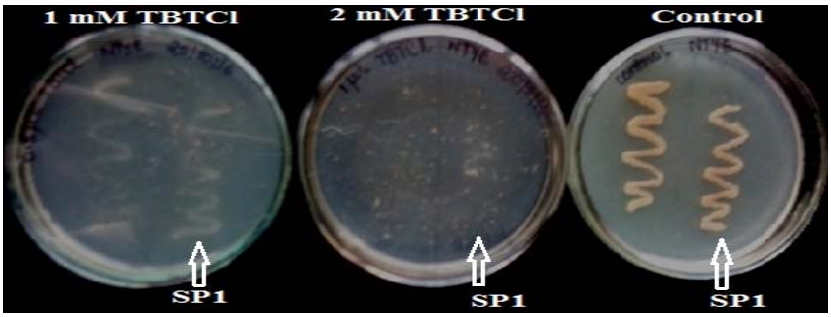


Fig :5 Growth of *Halobacterium* sp. SP1 on NTYE media (25% crude salt) with (a)1mM TBTCl (b) 2mM TBTCl and (c) 0 mM TBTCl (Control).



On performing thin layer chromatography SP1showed distinct dark blue spot at 4.3 cm and solvent front was at 18.3 cm. The Rf was calculated to be 0.23, that matched with glycerol diether moieties which is a characteristic of haloarchaeal membrane. The Rf matched with the Genus *Halobacterium* [17].

ii. Pigment analysis

Culture SP1 produced bright red pigment after 7 days of incubation in NTYE media (Online resource 5). Spectroscopic analysis of acetone extract of pigment showed 3 peaks with absorption maxima at 470nm, 500nm and 585nm, respectively, which are the characteristics of carotenoid pigment of haloarchaea [12]. These peaks correspond to bacterioruberin pigments which are typical pigments of haloarchaea, that protects haloarchaea from strong sun light and UV rays in solar saltern and therefore proves that SP1 is haloarchaea.

iii. Biochemical tests

Isolate SP1 was negative for all sugar fermentation (glucose, sucrose, fructose, mannitol, galactose and lactose). Isolate SP1 was motile and positive for catalase, indole, nitrate reductase, oxidase, and amylase production. These biochemical results were comparable to that of haloarchaea, *Halobacterium* sp. [16].

iv. Scanning electron microscopy

Scanning electron microscopy confirmed that isolate SP1 was rod shaped (Fig. 3). This result is in concurrence with the earlier report by Pathak and Sardar, 2012, which revealed that genus *Halobacterium* is rod shape, motile archaea [16].

Therefore, based on SEM analysis, antibiotic resistance test, salt requirement, sodium taurocholate sensitivity test, lysis in distilled water, glycerol diether lipid moieties in membrane, spectrophotometric analysis of carotenoid pigment and biochemical tests, the isolate SP1 was confirmed to be extreme haloarchaea belonging to *Halobacterium* sp.

4) Studies on mercury and selenite resistance

Halobacterium sp. SP1 was seen to resist 50µM $HgCl_2$ on NGSM broth with 0.1mM MIC *Halobacterium* sp. SP1 could also reduce up to 10 mM toxic selenite into less toxic and insoluble elemental selenium (Se^0) which was indicated by change in colony color from red to bright orange on NGSM plate (Fig. 4). The MIC of selenite for the haloarchaeal isolate was found to be 11 mM. *Halobacterium* sp. SP1, therefore have potential to resist both metal and metalloid in NGSM media with 25% crude salt.

Online resource 1: Red color colonies on NTYE media plates containing 25% crude salt and 500 units/mL penicillin after incubating 10 days at 37°C

Online resource 2: Growth of *Halobacterium* sp. SP1 on NYTE media containing 1%, 10% and 25% crude salt.

Online Resource 3: Antibiotic sensitivity study of *Halobacterium* sp. SP1 on NTYE media (20% crude salt) using disc diffusion method

Online resource 4: NTYE media (25% Crude Salt) having 4% sodium taurocholate

Online resource 5: Red coloured pigment produced by isolate SP1 in NTYE broth (25% crude salt) at 37^{o}C after 7 days.

5) Sodium benzoate and Tributyl tin chloride degradation studies

Halobacterium sp. SP1 could degrade up to 1.5 mM sodium benzoate as sole source of carbon on NSM broth, showing 2 mM MIC. *Halobacterium* sp. SP1 was also capable of utilizing up to 1 mM TBTCl with MIC 2 mM in NTYE plates having 25% crude salt (Fig. 5). *Halobacterium* sp. SP1 have potential to degrade both aromatic compound sodium benzoate and organometal TBTCl in NSM (without glucose) and NTYE media with 25% crude salt, respectively.

Chemical pollutants such as metals, metalloids, organometals and aromatic compounds may persist in hypersaline environment if not removed or undergone microbial degradation. These pollutants are deleterious to the marine biota either directly or via biomagnifications [5, 7, 11], that may even result in irreversible damage of marine ecosystem. In order to minimize the hazardous effects caused by mercury, selenite, sodium benzoate and tributylin in marine environment, bioremediation strategy can be practiced since bioremediation is eco-friendly and cost-effective method of environmental clean-up. Extremely haloarchaea *Halobacterium* sp. SP1

isolated from Ribandar salt pan have potential to resist/degrade mercury, selenite, sodium benzoate and tributyltin in hypersaline conditions and therefore can be used for bioremediation of hypersaline environmental sites contaminated with mixture of pollutants. There are many reports of marine organisms resisting/degrading mercury, selenite, sodium benzoate and tributylin [7, 11, 14, 18] but these bacteria may not tolerate hypersaline conditions therefore may prove inefficient to bioremediate marine sites contaminated with these pollutants. Also, microorganism resistant/degrading only one or two pollutants may not be effective in bioremediation of environmental sites polluted with a number of pollutants. Haloarchaea *Halobacterium* sp. SP1 which is multi extremophilic i.e. resistant to multiple pollutants and also able to grow in hypersaline environment would be of great importance to bioremediate hypersaline environments contaminated with multiple pollutants. The application of this haloarchaeal isolate could be focused to treat the estuarine polluted waters, prior to its use in saltpans for crude salt preparation, which would directly result in the healthier crude salt supply for Goan population.

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Conflict of interest: Nil

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