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EXOPOLYSACCHARIDE PRODUCING HALOPHILIC MICROORGANISMS FROM WEST COAST OF MAHARASHTRA, INDIA

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ABSTRACT

Halophilic microorganisms are routinely isolated from saline environments like solar salterns, salt lakes, salt pans and low saline environments like ocean water, sand and soil in the vicinity of salt pans. They produce exopolysaccharides (EPS) to protect the cells from high concentration of salt. EPS are carbohydrate polymers produced extracellularly by microorganisms. In the present studies, halophilic microorganisms have been isolated from saline environments from West Coast of Maharashtra (MS), India and explored for production of exopolysaccharide (EPS). Out of 43 isolates obtained, 6 isolates showing potential in production of EPS were selected for the studies. Among the 6 isolates, 4 isolates were identified as *Halomonas smyrnensis*, and other two were as *Halomonas denitrificans* and as *Haloferax chudinovii*. The isolates produced EPS in a range of 2-10.65 g/l the maximum being produced by *Halomonas smyrnensis*. This is presumably the first report of production of EPS by *Halomonas denitrificans* and *Haloferax chudinovii*, and also the exploration of the coastal regions of Sindhudurga district, Maharashtra, India for EPS producing halophilic microorganisms.

KEYWORDS: **Exopolysaccharides, Halophiles, West Coast of India, *Halomonas smyrnensis, Haloferax chudinovii, Halomonas denitrificans.***

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INTRODUCTION

Halophiles are salt loving microorganisms, which thrive in hypersaline environment. They are classified based on salt requirement as halotolerant (no requirement of salt but can tolerate salt up to 15%), slight halophiles (2-5%), moderate halophile (5-15%) and extreme halophile (20-30%)[1]. Halophilic microorganisms have been isolated from many ecosystems like Great salt lake Utah, USA, Dead Sea and alkaline brines of Wadi Natrun and lake Magadi, Kenya[2,3]. The work on halophiles is reviewed by DasSarma and DasSarma[4] and Kanekar et al[5]. Exopolysaccharides (EPS) are high molecular weight carbohydrate polymers widely produced by a variety of microorganisms exterior to the cell surface as capsule or loosely associated as slime. EPS are involved in cell adhesion, dehydration, protection of cell from freezing, biofilm production[6,7], increase pathogenicity and virulence and storage of reserve carbon sources. Some of the EPS producing microorganisms are *Xanthomonas campestris, Klebsiella pnuemonie, Leuconostoc mesenteroides, Haemophilus influenza,* etc. Well studied microbial EPS include Xanthan, Dextran, Hyaluronic Acid (HA), etc. The microbial EPS are having unique rheological properties due to their pseudoplastic behavior and capacity of forming viscous solutions at low concentrations. Microbial EPS has different biotechnological applications such as gelling agents, biosurfactants, emulsifiers, viscofiers[8,9,10], biosorbants[11,12] and biologically active antimicrobials, anticancer agents and antioxidants[13,14,15]. Due to numerous applications in industrial sector, EPS are currently getting a global attention. Generally microbial EPS exhibit more diversity in composition and structure as compared to plant EPS. Since some of the microorganisms producing EPS are pathogenic and yield is low in case of lactic acid bacteria, other microorganisms are being explored globally. Extremophilic microorganisms which thrive in harsh environment are being looked upon as a source of EPS. Halophiles produce EPS to protect them from high concentration of salt. EPS production from halophilic bacteria from extreme marine habitat and their bioactivities have been reviewed by Poli et al[8]. Kanekar et al[16] have reviewed work on production of exopolysaccharides from halophilic microorganisms. Sulphated EPS from novel *Halomonas stenophila* strain B100 studied by Ruiz-Ruiz Carmen etal[17]. Kulkarni et al[18] have studied production of polyhydroxy-alkanoate (PHA) using moderately haloalkaliphilic microorganism *Halomonas campisalis*. In India, halophiles from saline environments of coastal regions in Gujarat, Karnataka, Tamilnadu and Goa have been studied. Compared to these areas, coast of Western Maharashtra has not been explored so far for halophilic microorganisms. The present paper reports isolation of halophilic bacteria and archaea from coastal regions of Western Maharashtra, India and their exploration for production of EPS.

MATERIAL AND METHODS

Media components were purchased from Hi Media laboratory, India. Laboratory grade ethanol was used for EPS extraction. Halophilic microorganisms have been isolated from sea water and sand from coastal regions of Maharashtra and soil sample in the vicinity of salt pans along the coast of Sindhudurg district of Western Maharashtra and studied for production of EPS.

Sample collection

Samples were collected from different places in Sindhudurg district of Western Maharashtra, India viz. from Malvan (16.0565°N, 73.4688°E), Deobaug, Chivla (15.8501°N, 73.6323°E) and Shiroda (15.87°N 73.63°E) (Fig 1). Water samples were collected in gamma ray sterilized Tarson's plastic containers and sand samples were collected in clean plastic bags with the help of clean spatula. Details of samples collected are described in Table 1. The temperature of the samples was recorded at site collection and pH in the laboratory using pH meter. Glycerol stocks were prepared from samples collected and all original samples were stored at -20°C. Chemical analysis of sea water and sand samples was conducted as per the standard methods described by Greenberg et al[19]. Samples were analyzed for sodium, chlorides and magnesium content. The results of chemical analysis of the samples collected showed that sodium, chloride and magnesium content of sea water were 0.71%, 2.76% and 0.07%, respectively, which is comparable to that of any ocean water while sodium, chloride and magnesium content of the sand were 0.29%, 6.91% and 0.06%, respectively. The samples represent low saline environment.

Isolation of halophilic microorganisms by enrichment method

The enrichment technique was used for isolation of halophilic microorganisms and nutrient medium containing high concentration of salt was used. Medium and growth conditions were; medium; Sehgal and Gibbon's (SG) medium[20], temperature, 37°C, agitation: 120 rpm and incubation period, one week. For water sample, 5 ml of water inoculated in 20 ml of SG medium in 100 ml Erlenmeyer flask. The salt concentration was 15%, 20% and 25%. For sand sample, 10 g of sand inoculated in 40 ml of SG medium containing 15% of NaCl in 250 ml flask. Isolation of haloarchaea from Shiroda salt pan soil sample was carried out by inoculating 10 g of soil in 40 ml SG medium containing 20% NaCl in 250 ml flask. After incubation of one week, enrichment cultures were then streaked on respective salt containing SG plates and incubated at 37°C. Well isolated colonies were then observed for morphological characteristics. Pink red colonies of haloarchaea were observed and Gram staining performed as described by Dussault[21].

Morphological and biochemical characterization

Morphological characterization of the selected isolates was done by Gram staining method. Motility of the selected isolates was checked by hanging drop method. Biochemical characterization included catalase test which is performed by addition of 1% (v/v) hydrogen peroxide to few drops of culture broth on slide. Presence of effervescence confirmed positive catalase activity. Oxidase test was carried out by placing microbial colony on Whatman filter paper No.1 followed by addition of oxidase reagent (Hi-media). Change in colony color to purplish blue confirmed positive oxidase test. Glucose

fermentation for acid and gas was checked in SG containing 15% NaCl, 1% glucose and Andrade's indicator (1% v/v). Salt tolerance study of isolates was carried out by checking growth of the isolates on SG with and without salt (2% and 5%). Based on results obtained, isolates were grouped in to halotolerant, slightly halophilic, moderately halophilic and borderline extreme halophilic. 16S rRNA gene sequencing of selected isolates was carried out. 16SrRNA gene sequences of selected cultures were deposited in NCBI GenBank and accession numbers were obtained.

16S rRNA gene sequencing

The bacterial cultures were identified using 16S rRNA gene sequencing method as described by Sharma et al[22]. The genomic DNA was isolated using a DNA isolation kit (Invitrogen) and the 16S rRNA gene sequence was amplified using the ARC20F: TTCCGGTTGATCCYGCCRG and ARC958R: YCCGGCGTTGAMTCCAATT archaeal primers and 27F (AGAGTTTGATCMTGGCTCAG) and 1492R (GGTTACCTTGTTACGACTT) eubacterial primers[23]. The DNA sequences covered with these primers were around 700 bps for archaea and 1200 bps for bacteria. The PCR product was then purified using a Rapid Tip kit from Diffinity Genomics and DNA sequencing was carried out using an ABI PRISM Big Dye Terminator v3.1 Cycle Sequencing kit on a 3730× l Genetic Analyzer (Applied Biosystems). The sequence data were edited with the Seqman DNA Star software. The taxonomic identity of the 16S rRNA gene sequence of strains and its percentage similarity with other taxa was calculated using the 'Identify' option of the EzTaxon-e server29. The 16S rRNA gene sequences of all halophilic microorganisms were deposited in NCBI GenBank to obtain accession numbers. Phylogenetic tree was constructed based on 16S rRNA gene sequencing by neighbor joining method (Fig.2). Evolutionary analyses were conducted in MEGA 4 software.

Screening of EPS producers

All the isolates showing no growth on SG without salt were selected and streak inoculated on SG containing varying concentrations of salt and 3% glucose for screening of EPS producers. The isolates showing glistening growth were further selected for EPS production in broth.

EPS production, isolation and purification

Production of EPS by selected isolates was checked in SG broth with 3% glucose. Selected isolates were grown in SG medium at 37°C for one week in shaker incubator (~120 rpm). The culture broth was centrifuged at 5000 rpm for 30 min to obtain cell free supernatant, which was treated with 20% trichloroacetic acid (TCA) to remove protein fraction. To the supernatant, two volumes of chilled ethanol were added to precipitate EPS. Amount of EPS obtained by filtration was calculated gravimetrically on the dry weight basis.

RESULTS

The total number of isolates obtained from all samples was 43. Out of 43 isolates, 21 were found to be halotolerant, 19 slightly halophilic, 2 moderately halophilic and 1 borderline extreme halophilic microorganisms. All the isolates showing no growth on SG without salt were selected and streak inoculated on SG amended with varying concentrations of salt and 3% glucose. Total 6 isolates showed glistening growth and hence further selected for EPS production. The results of morphological and biochemical characterization and their EPS yield are presented in Table 2. Out of 6 isolates, 5 belonged to bacterial genus *Halomonas,* while one isolate to haloarchaeal genus *Haloferax*. Identification of isolates using 16S rRNA gene sequencing is described in Table 3. In present study, halophilic microorganisms have been isolated from the habitats viz. Malvan, Deobaug, Chivla and Shiroda. The samples collected in the form of ocean water, sand and soil sample. The six isolates were identified as *Halomonas denitrificans, Halomanas smyrnensis* and *Haloferax chudinovii*. EPS yield was calculated gravimetrically. It has been observed that isolate with SeqID3 produced EPS 10.6 g/l which was maximum as compared to other isolates. This isolate was isolated from oceanic water of Deobaug MS, India and identified as *Halomonas smyrnensis.* Isolate with SeqID1 was isolated from rock pit sea water sample from rock garden of Malvan, MS,India and identified as *Halomonas denitrificans* and yielded 2.0 g/l of EPS. The isolate with SeqID2 was isolated from ocean water of Chivla, MS, India, identified as *Halomonas smyrnensis* and observed yield of EPS was 7.0 g/l. The isolate with SeqID4 from ocean water of Deobaug, MS, India, was identified as *Halomonas smyrnensis* and its EPS yield was 7.1 g/l. The isolate with SeqID5 from ocean water of Deobaug, MS, India, was identified as *Halomonas smyrnensis* and its EPS yield was 4.9 g/l. The isolate with SeqID6 from soil near salt pan of Shiroda, MS, India, was identified as a haloarchaeon *Haloferax chudinovii*. Its EPS yield was 3.0 g/l. In present investigation, it has been observed that halophilic bacteria are from low saline environment such as ocean water, sand and rock pit sea water. *Halomonas smyrnensis* is found to be major EPS producer with yield of 10.6 g/l. Exploration of the sites Malvan, Chivla, Deobaug and Shiroda from West coast of Maharashtra, for isolation of EPS producing halophilic microorganisms appears to be the first report.

Table 1
Sites of samples collection

Sample code	Sample name	Abbreviation	pH	Temperature (°C)
SVD 1	Deobaug beach water-6 feet deep	DBW	7.0	26
SVD 2	Deobaug beach sand	DBS	--	--
SVD 3	Deobaug beach water-3 feet deep near rock	DBW	6.8	26
SVD 4	Chivla beach water	CBW	7.0	2
SVD 5	Chivla beach rock scraping	CBRS	6.8	26

SVD 6	Shiroda soil sample near solar salt pan	Sh.5	--	--
RG 2.2	Rock garden, Malvan, rock pit sea water	RG 2.2	6.8	26

Table 2
Characterization of isolates and their EPS yield

Isolate code	Gram nature	Motility	Catalase	Oxidase	Glucose fermentation	Salt tolerance	EPS yield (g/l)
RG2.2 (RG2.10% rock garden-rock pit sea water)	Gram negative rods	Motile	Positive	Positive	Negative	Slightly halophilic	2.0
TE.SVD4.20%1 (Chivla beach water)	Gram negative rods	Motile	Positive	Positive	Negative	Slightly halophilic	7.0
TE.SVD1.20%1 (Deobaugbeach water-6ft)	Gram negative cocobacilli	Motile	Positive	Positive	Negative	Moderately halophilic	10.6
TE.SVD1.20%2 (Deobaug beach water-6ft)	Gram negative rods	Motile	Positive	Positive	Negative	Slightly halophilic	7.1
TE.SVD3.20%1 (Deobaug beach water-3ft)	Gram negative cocobacili	Motile	Positive	Positive	Weakly positive	Slightly halophilic	4.9
Sh5.15%+3% glucose.FE2 (Shiroda salt pan soil)	Gram negative cocobacili	Motile	Positive	Positive	Weakly positive	Borderline extreme halophilic	3.0

Table 3
Identification of cultures using 16S rRNA gene sequencing

Isolate sequence ID	Source	Accession number	Sequence length in base pairs	Closest sequence	% similarity
Seq ID 1	Malvan rock garden-rock pit sea water	KX057988	1365	*Halomonas denitrificans* M29(T)	98.02
Seq ID 2	Chivla beach water	KX057989	1179	*Halomonas smyrnensis* AAD6(T)	99.92
Seq ID 3	Deobaug beach water-6ft	KX057990	1252	*Halomonas smyrnensis* AAD6(T)	100
Seq ID 4	Deobaug beach water-6ft	KX057991	1121	*Halomonas smyrnensis* AAD6(T)	99.91
Seq ID 5	Deobaug beach water-3ft	KX057992	1387	*Halomonas smyrnensis* AAD6(T)	99.93
Seq ID 6	Shiroda salt pan soil	KX057993	775	*Haloferax chudinovii* RS75(T)	99.74

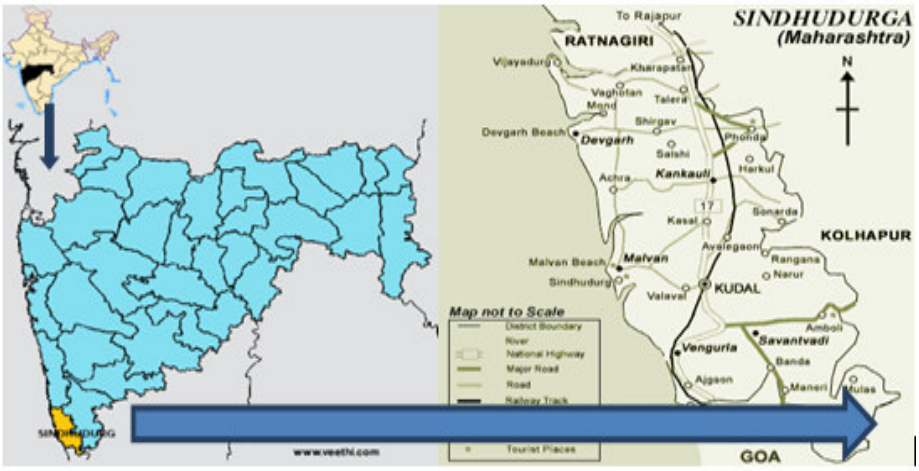

Figure 1
Geographic location of sample collection sites in India

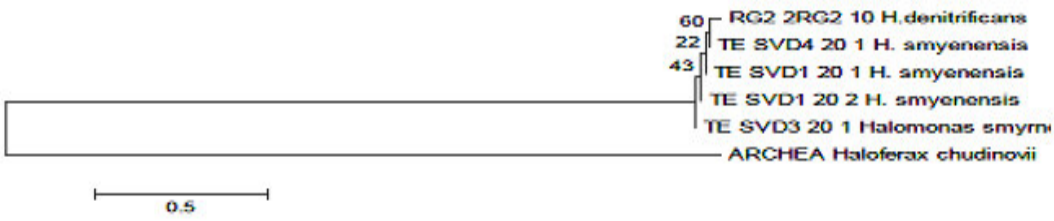

Figure 2
Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences

DISCUSSION

In the present studies, 4 strains of *Halomonas smyrnensis* were obtained from Deobaug sea water and Chivla beach water from the coastal regions of Western Maharashtra, India. *Halomonas smyrnensis* sp. nov. was first described by Poli et al[24] from Camalti Saltern area, a wildlife reserve in Sessali, Izmir, Aegeon region of Turkey. The organism *Halomonas* sp. was reported to produce exopolysaccharide[25]. The type strain of *Halomonas smyrnensis* was isolated from saltern area i.e. high saline environment while the present strains of *Halomonas smyrnensis* are isolated from sea water i.e. low saline environment of West Coast of Maharashtra, India. *Halomonas denitrificans* sp. nov. was isolated from saline water in Anmyeondo, Korea by Kim et al[26]. In the present studies *Halomonas denitrificans* was isolated from rock pit sea water of Rock garden, Malvan, West Coast of MS, India. The type strain has not been reported for production of EPS while the present strain of *Halomonas denitrificans* produces EPS. A halophilic archaeon, *Haloferax chudinovii* sp. nov. reported by Saralov et al[27] from Permian Potassium salt deposits in Solikamsk, Russia. In present investigation, the haloarchaeon was isolated from saline soil near by the solar salt pan at Shiroda, West Coast of Maharashtra, India. The present strain was found to produce EPS. Thus although the moderately halophilic bacteria *Halomonas smyrnensis* and *Halomonas denitrificans* were reported earlier from saline environments, the present isolates are from low saline environment. The haloarchaeon *Haloferax chudinovii* was isolated from soil in vicinity of solar salt pan. The type strain of *Haloferax chudinovii* has not been reported for EPS production. Thus novelty of present investigation lies in the isolation of EPS producing halophilic microorganisms from low saline environments from West Coast of Maharashtra, India, the unexplored sites so far. This is presumably the first report of isolation of EPS producing strains of *Halomonas denitrificans* and *Haloferax chudinovii* from West Coast of Maharashtra, India.

CONCLUSION

Total 43 isolates were obtained from different samples collected from West Coast of Maharashtra. Out of these 43 isolates, 6 isolates were found as potential EPS producers which were then identified as *Halomonas smyrnensis, Halomonas denitrificans* and *Haloferax chudinovii.* EPS production was observed in the range of 2-10.65 g/l and maximum yield was obtained with *Halomonas smyrnensis.*

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CONFLICT OF INTEREST

Conflict of interest declared none.

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