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DIVERSITY ASSESSMENT AND EPS PRODUCTION POTENTIAL OF CULTIVABLE BACTERIA FROM THE SAMPLES OF COASTAL SITE OF ALANG

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ABSTRACT

In this study diversity of cultivable bacteria and their exopolysaccharide production potential were investigated from the water and sediment samples of coastal site of ALang. The studied ten samples represented diversity in pH, conductivity, salinity and TDS, in the range of 7.25 to 8.92, 9.4 to 76.8 mS, 3570 to 35100 mg/L and 15120 to 53100 mg/L respectively. Total 141 bacteria were isolated from the collected ten samples, from which 105 were gram positive and 36 were gram negative in nature. Amongst the isolates 33% were able to produce variety of pigments. The diversity indices, including Shannon-Wiener index (H'), Richness, and Evenness Indices based on the metabolic characteristics of the organisms were calculated. The isolates were characterized morphologically and biochemically. Identification of 41 isolates was confirmed by 16S rRNA gene sequencing. They represented 22 genera and various species of these genera. The bacterial isolates were able to grow in the range of 3-25% NaCl concentrations. When 141 cultures were grown in liquid medium, the viscosity of the medium ranged between $9.20x10^{-6}$ to $2.09x10^{-4}$ m.pa.s^{-1}. The EPS yield in terms of dry weight also ranged from 0.76 to 10.7g/L.

Keywords: Alang, coastal, diversity indices, exopolysaccharide, phylogeny, pigment

INTRODUCTION

India has the coastline of 7516 kilometers, out of this Gujarat state contributes about 21% (~1600 kilometers) of the coastline **(Venkatraman, 2008; Parikh *et al*., 2012)**. The coastal region of the Bhavnagar is a center for varied economic activities like salt production, mining, fishing, ship building and breaking. Alang is the largest ship breaking yard in the world and is a census town of Bhavnagar district. Its economical importance with regards to its salt production value and ship breaking process makes it a valuable site for the microbiological studies. Studied saline sites of India are Sambhar salt lake, Rajasthan (**Sahay *et al*., 2012)**, Coastal regions of Gujarat, **(Dave and Desai, 2006)**, Little Rann of Kutchh, Gujarat **(Thomas *et al*., 2012)**, Tamilnadu, Andhra Pradesh **(Kumari *et al*., 2013)**, Maharashtra **(Deshmukh *et al*., 2011)**, Orissa **(Bal *et al*., 2009)**, and West Bengal **(Das *et al*., 2012)**. Some of the coastlines studied worldwide are the Great Salt Lake (Utah, USA), the Dead Sea (Israel), the alkaline brines of Wadi Natrun (Egypt), and Lake Magadi (Kenya) **(Nanjani and Soni, 2012)**. These habitats represent extreme conditions of high salinity, high pH, low oxygen conditions and different range of temperature values and are inhabited by halotolerant as well as halophilic microorganisms **(Moreno *et al*., 2013)**. Members of the domain bacteria are of special interest to scientists, as they play an important role in saline as well as hypersaline environments and have the potential to produce compounds of industrial interest, one of these compounds is the exopolysaccharide (EPS) **(Antoin *et al*., 2000; Hedi *et al*., 2014)**. Novel EPS with better characteristics can be developed from the isolates of such ecosystem than those of the existing one **(Bejar *et al*., 1998)**. EPS produced by microorganisms has a number of applications in pharma, food, petroleum and other industries **(Sutherland, 1990)**. Because of their unique properties they can be used as coagulants, thickening agents, binder, emulsifiers, stabilizers, lubricants and gelling agents **(Sutherland, 1997)**. EPS have been reported to increase the viscosity of solution at low pH values, as a good surface active agent for heavy metal remediation and provides gluing properties in soil aggregation **(Kalpan *et al*., 1987; Nisha *et al*., 2007)**. The saline site of Alang, Bhavnagar, is less explored_ in the bacterial diversity study and particularly for the EPS production from such organisms, thus the purpose was to analyze the physico-chemical properties of the collected samples, elucidation of the culturable diversity of marine bacteria isolated from the Alang coastline and their potential to produce EPS.

MATERIALS AND METHODS

Sampling site and sample collection

Ten different samples comprising five sea water (sample 1-5) and five sediments (sample 6-10) were collected for the study from the different sites (about 50m distance between two points) of the coastal region of Alang, Bhavnagar, having area of 160 x10 m (latitude 21°36' N, longitude 72°18'E). The surface water samples were collected directly into sterile bottles while the sediments (0.5-1.0m depths from water surface) were collected in sterile plastic bags.

Physico-chemical analysis of the sediment and water samples

From the collected water samples, pH and conductivity were measured directly using a portable multimeter analyzer (Eutech, Singapore). Where as for the collected sediment samples, 10% w/v sediments were suspended in distilled water for all the analysis. Parameters like total solids (TS) and total dissolved solids (TDS) were determined using gravimetry method. Soluble chloride estimation was done by titration with $AgNO_3$, Ca^{2+} and Mg^{2+} were measured using EDTA. Standard analytical procedures were followed for the determination of all the parameters **(APHA, 1995)**.

Isolation and characterization of marine bacteria

Collected samples were serially diluted and spreaded on Zobell Marine Agar (ZMA), Zobell Marine Sea Water Agar (ZMSA), R2A Agar, Artificial Sea Water Agar (ASWA) with 0.5 g/L peptone and 1.0 g/L glucose, Nutrient Agar (NA) with five different concentrations of sodium chloride (3, 5, 10, 15 and 20% w/v) and Alang Sea Water Medium (ASWM) containing 0.5 g/L peptone and 1.0 g/L glucose in sea water. All the media constituents used in the study were acquired from Hi Media, India. All the plates were incubated at 30±2 °C for 48 -72h to grow bacteria. The viable bacterial count was determined in terms of Colony Forming Unit (CFU) and from apparently different colonies, organisms were purified and sub cultured in the respective medium **(Bianchi *et al*., 1992)**. Isolates were preliminary identified on the basis of morphological and biochemical observations according to the methods described in the Bergey's manual of systematic bacteriology **(Brenner *et al*., 2005; Vos *et al*., 2009)**.

Salt tolerance study

Salt tolerance study of all the bacterial isolates was carried out using nutrient broth with increasing NaCl concentrations (0-30% w/v NaCl). In each tube containing 5.0 mL of the growth medium, 100 µL of activated bacterial culture (OD_{620}=1.0) was inoculated. Growth was measured at 620 nm after 24 h, 48 h and 72 h. Based on the salt tolerance potential all the isolates were categorized as non-halophiles, halotolerant, slight and moderate halophiles as described in The Prokaryotes **(Oren, 2006)**.

Diversity indices and statistical analysis

Based on the phenotypic characteristics of the organisms, Shannon Weiner diversity index (H'), Richness ($R_{margalef}$, $R_{menhinik}$) and Evenness (E_{Pielou}) were calculated by the standard formula **(Derry *et al*., 1998; Dave *et al*., 2002)**. The data of biochemical tests were applied to Principal Component Analysis (PCA) and Hierarchial Cluster Analysis (HCA) using the statistical package (IBM SPSS Statistics, version 20, 2011) for windows **(Soni *et al*., 2002)**.

Molecular identification by 16S rRNA gene sequencing and phylogenetic analysis

The taxonomic identity of the selected bacterial isolates was confirmed by 16S rRNA gene sequencing along with the biochemical tests. The 16S rRNA sequences of the bacteria were submitted to the GenBank and accession numbers were obtained. All sequences were aligned with a multiple sequence alignment and phylogenetic tree of the isolates was constructed by the neighbor joining analysis using Kimura's two-parameter model using MEGA 6.0 **(Kimura, 1980; Saitou and Nei, 1987)**.

Screening of EPS producers

Bacterial isolates that produced high mucoid colonies were selected for further screening and their EPS production ability was checked using EPS broth medium **(Atlas, 1993)**.

EPS production and extraction

The growth of various isolates was harvested in sterilized normal saline, cell count was performed spectrophotometrically at 600 nm and 10% (v/v) inoculum consisting $1x10^8$ cells/mL was inoculated into 250 mL capacity Erlenmeyer flask containing 100 mL EPS broth medium consisting of (g/L): casein hydrolysate, 15; sodium acetate, 12, K_2HPO_4, 10; yeast extract, 5; L-–Cystine, 0.5; NaOH, 30 and sucrose, 50. The flasks were incubated on orbital shaker (Newtronics, India) rotating at 150 rpm at 30±2 °C. At different time intervals the viscosity of the medium was studied. Extraction of EPS was carried out using chilled acetone in 1:3 ratio **(Watanabe *et al*., 1999)**, and was kept at 4 °C for overnight. Precipitated EPS was separated by centrifugation at 10,000 g and wet weight of EPS was recorded **(Ashok *et al*., 2011; Razack *et al*., 2013)**. The separated EPS was dried at 65 °C in oven to get the constant dry weight.

RESULTS AND DISCUSSION

Physico-chemical characterization of samples

The physico-chemical characteristics of the samples are represented in Table 1. The pH of the samples ranged from 7.25 to 8.92 obviously due to the presence of dissolved salts in marine water. The temperature at the time of sample collection was 34±4 °C. The conductivity of the samples ranged from 9.4 mS to 76.8 mS. The sediment samples showed salinity ranging from 3570-18400 mg/L, whereas, water samples showed the salinity ranging from 21950-35100 mg/L. The chloride (Cl^-) concentration ranged from 2170-10250 mg/L and 12053-22688 mg/L for sediment samples and water samples respectively. NaCl concentration of sediment samples ranged from 2095.5-16912.5 mg/L and that of water samples ranged from 866.25-2179.55 mg/L. Hardness of the samples in terms of Ca^{2+} and Mg^{2+} was found in the range from 78-758 mg/L and 320-1200 mg/L respectively. Samples had TDS values between 15120-53100 mg/L. The overall composition of the samples differed depending on the site of sampling. Physico-chemical analysis of the liquid samples showed minor pH variations as compared to solid samples. However, conductivity, TS, salinity, Cl^-, Mg^{2+}, Ca^{2+} content of water sample showed variation of 1.73, 1.72, 1.80, 1.88, 1.51, 1.66 fold respectively. Where as conductivity, TS, salinity, Cl^-, Mg^{2+}, Ca^{2+} content of sediment sample showed variation of 3.98, 2.41, 4.74, 4.72, 5.48, 1.62 fold respectively, which were analyzed by preparing the 10% samples. However, the results presented here are of one particular day and hence the variation was due to the different sites that too within 160x10m are and not due to the season. Sediment systems were more diverse as compared to water samples collected from the same place. As per the reported results of the coastal region of Alang and South Saurashtra coastal stretch of Gujarat, pH ranges from 7.9 to 8.39 **(Bhadeja and Kundu, 2011)**.

Table 1 Physico-chemical analysis of the samples

Samples	pH	Conductivity (mS)	TS (mg/L)	TDS (mg/L)	Salinity (mg/L)	Cl^- (mg/L)	Ca^{2+} (mg/L)	Mg^{2+} (mg/L)	NaCl (mg/L)
1	7.35	58.92	44450	44350	30240	21979	608	880	36265.35
2	7.25	69.55	48814	48750	35100	22688	588	1050	37435.2
3	7.45	76.8	53232	53100	19775	12053	758	1200	19887.45
4	7.56	48.39	37592	37520	27360	14889	488	850	24566.85
5	7.25	44.15	30896	30800	21950	15598	456	790	25736.7
6	8.62	12.06	30400	29800	3570	2170	318	480	2095.5
7	8.92	9.4	26350	25900	4090	3210	119	369	5296.5
8	8.75	11.8	37250	36500	6390	4180	428	520	6732
9	8.33	37.5	29050	28500	18400	10250	268	460	16912.5
10	8.01	14.19	15420	15120	8370	5250	78	320	8662.5

1-5 water and 6-10 sediment samples

Morphological and physiological characterization of isolates

Initially sample number 4, 6 and 8 were studied on all the 10 culture media described above to select the most suitable medium for the study of all the samples. Detail of the bacterial viable count of these samples is given in Table 2a. Amongst all the media used, the highest bacterial count was obtained on ZMA, and Nutrient agar with 3% NaCl concentrations, respectively, hence further isolation study from all the samples was carried out with ZMA and NA (both with 3% NaCl) and the CFU of bacteria on ZMA in comparison with NA is listed in Table 2b. The highest CFU/mL of culturable bacteria was found as $134x10^6$ in the sample number 8 on ZMA; whereas, the lowest CFU/mL of culturable bacteria was found as $0.5x10^6$ in the sample number 5 on NA . ZMA proved to be the medium of choice for the isolation of the organisms from such saline habitat. As can be seen from the results some of the bacteria grew even in the presence of 20% NaCl concentration in NA(Table 2a), indicated the presence of helophilic or helotolerant organisms.

Table 2a Total bacterial count of three samples on all media studied

Sample	Total viable count ($x10^6$ CFU/ml)									
	Medium									
	ZMA	ZMSA	R2A	ASWA	ASWM	NA (3%)	NA (5%)	NA (10%)	NA (15%)	NA (20%)
4	2.2	0.68	0.021	0.024	0.028	0.8	0.74	2*	0.012*	0.01*
6	84	62	0.044	0.05	0.044	24	0.88	4.7*	0.03*	0.024*
8	134	40	0.052	0.062	0.088	32	1.2	7.0*	0.021*	0.026*

*= _$x10^3$ CFU/mL

Table 2b The viable count of bacteria on ZMA in comparison with NA.

Medium	Total viable count ($x10^6$ CFU/mL)									
	Sample number									
	1	2	3	4	5	6	7	8	9	10
ZMA	6.8	3.1	4.4	2.2	1.2	84	40	134	12	7
NA	3.2	2.5	1.1	0.8	0.5	24	13	32	40	20

Morphologically different 141 bacterial isolates were isolated from the collected samples. The colony size of the isolates ranged from 0.4 to 2.8 mm. Out of 141 isolates, 105 were gram positive and 32 isolates were gram negative and 4 were actinomycetes. In case of gram positive isolates, cocci were large in numbers as compared to gram positive rods. Out of 105 gram positive isolates, 55% were cocci, 41% were gram positive rods. Amongst 141 isolates, 23% of the isolates were able to produce varieties of pigments while the rest of them gave nonpigmented colonies. Pigmented colonies showed white, creamish white, yellow, red, orange, pink and bluish green pigments. Yellow pigmented colonies showed variation in shades like golden yellow, lemon yellow and light yellow. Comparison of pigmented and non-pigmented colony from all the samples studied is shown in Fig.1a. Sample 8 showed the highest percent of pigmented isolates and sample 10 showed the lowest percent of pigmented isolates. Light yellow pigments were the highest in number where as bluish green was the lowest. Gram positive cocci produced variety of pigments as compared to gram positive bacilli and gram negative rods (Fig. 1b).

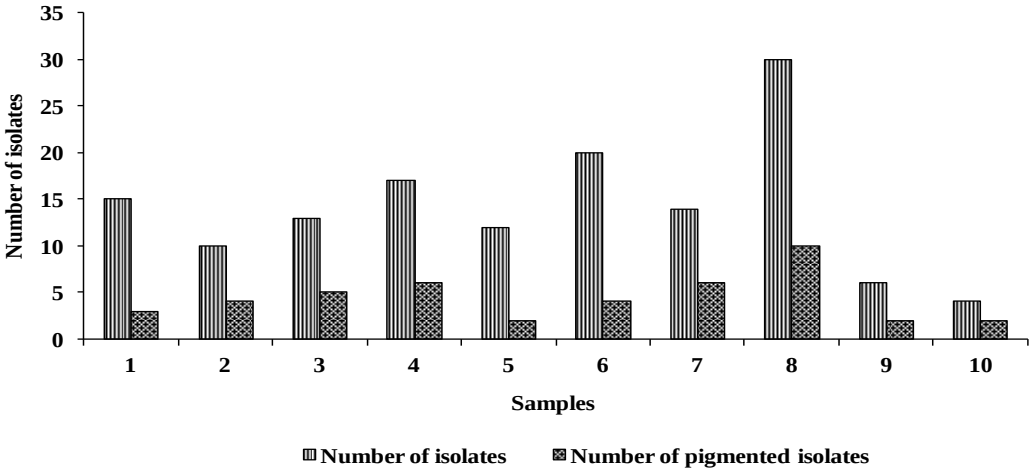

Figure 1a Comparison of pigmented bacteria with the total number of isolates in different samples

Amongst 141 bacterial isolates, 132 isolates were able to grow up to 5% of NaCl concentration, 105 isolates up to 10%, 27 isolates up to 15% and only 5 isolates showed growth up to 20% of salt concentration. From these five, only two were able to grow up to 25% of NaCl concentration. None of them showed any growth at 30% w/v of salt concentration. As the salt concentration was increased the growth of the organisms was found to be decreased into the medium. Organisms showed intense growth at low salt concentrations (3-5%) within 24-48 h, moderate growth at 10% of NaCl concentration and scanty growth at high NaCl concentration (15-25%) after 2-3 d of incubation period. There are some reports on the diversity study of marine salterns near Bhavnagar and from the coastal region of Dwarka-Veraval. According to that the microbial diversity and growth of the microorganisms found to decrease with higher salt concentration **(Dave and Desai, 2006; Nanjani and Soni, 2012)**. Gram positive bacteria were more in numbers between 10-15% of NaCl concentrations; whereas, gram negative isolates showed dominance between 20-25% of NaCl concentrations. The comparable results are also obtained in a study of isolation of halotolerant and halophilic bacteria **(Purohit *et al.*, 2015)**.

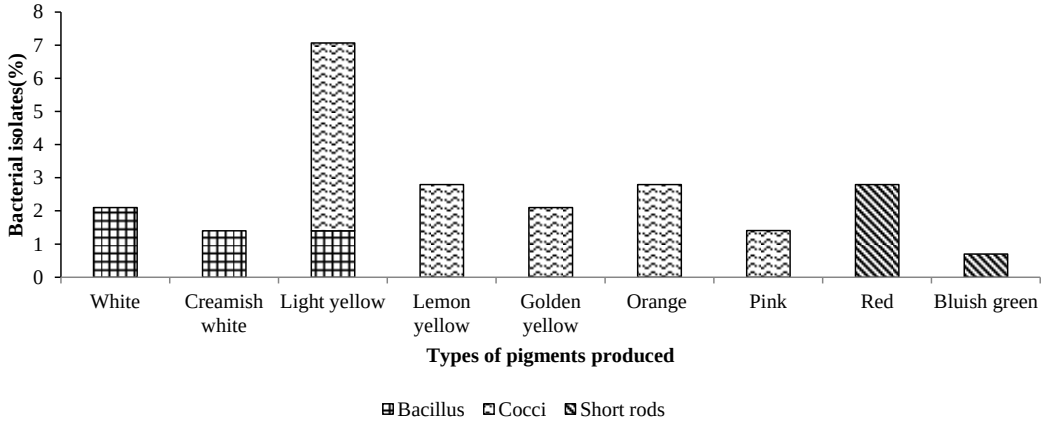

Figure 1b Dominance of the group of bacteria, in particular pigment production

Diversity indices

The results of various diversity indices are presented in Table 3. As can be seen for all the samples, Shannon Weiner index (H') varied from 1.043 to 2.86. Sample 3, 6 and 8 had H'values above 2.5. Sample 1, 2 and 4 had H' values above 2, forming another group. The rest of the samples showing H' index values above 1 were grouped separately. Evenness values ranged from 0.752 to 1.209. Richness, $R_{margalef}$ ranged from 0.333 to 1.140 and $R_{menhinik}$ ranged from 0.0422 to 0.0848. Based on all the four indices, it can be concluded that the sample number 10 had overall low diversity where as sample 8 showed high diversity, high richness and high evenness. Moreover, it represented the most diverse bacterial communities capable of growing with a wide range of salt concentrations and producing variety of pigments could be the reason for its high diversity value. The lowest dissolved solids, Ca^{2+} and Mg^{2+} content could be responsible for low diversity and evenness of the organisms in sample 10. However, there are some samples with high diversity and richness, but low evenness and vise versa. **Haque *et al.*, (2004)** have reported that the samples, which shows similar diversity values may incorporate low evenness and the high richness or consist of high evenness and low richness. In some cases the sample shows all three parameters high or low simultaneously. Therefore, one should consider the diversity, richness and evenness collectively during diversity studies.

Table 3 Diversity indices for bacterial populations from various samples based on their physiological profile

Sample no.	Shannon Diversity (H')	Richness		Evenness
		$R_{Margalef}$	$R_{Menhinick}$	E_{Pielou}
1	2.47	1.11	0.0848	0.994
2	2.15	0.723	0.0632	1.034
3	2.51	0.696	0.0527	1.209
4	2.09	0.844	0.0789	0.952
5	1.54	0.418	0.0422	0.96
6	2.73	0.87	0.0567	1.189
7	1.41	0.549	0.0632	0.787
8	2.86	1.140	0.0675	1.11
9	1.55	0.537	0.0572	0.866
10	1.043	0.333	0.0447	0.752

Principal component and Hierarchical Cluster Analysis

PCA based on different biochemical tests was able to group the 39 bacterial isolates on the basis of the similarities and differences in their metabolic behaviour. Results are shown in the Fig. 2a and 2b. Total 7 principal components were extracted. PC1 and PC2 explained 18.84% and 14.19% diversity, respectively, of the total variance present in the original data. Bacterial isolates formed different groups in PCA, among these groups isolates E2 and E3, SR31 and SR 103, SR 49, SR52 and KD, SR 24, K5 and K8, SR 40 and SR 47 were closely placed because of their similar metabolic behaviour. The Hierarchical Cluster Analysis represents the similar observations as PCA. It shows the least distance between the isolates explained above. Isolate SR 104 placed distinctly from the rest of the isolates in PCA. It could be due to its distinct metabolic properties . Isolates also showed a wide diversity With respect to fermentation of 21 sugars studied(Results are not shown).

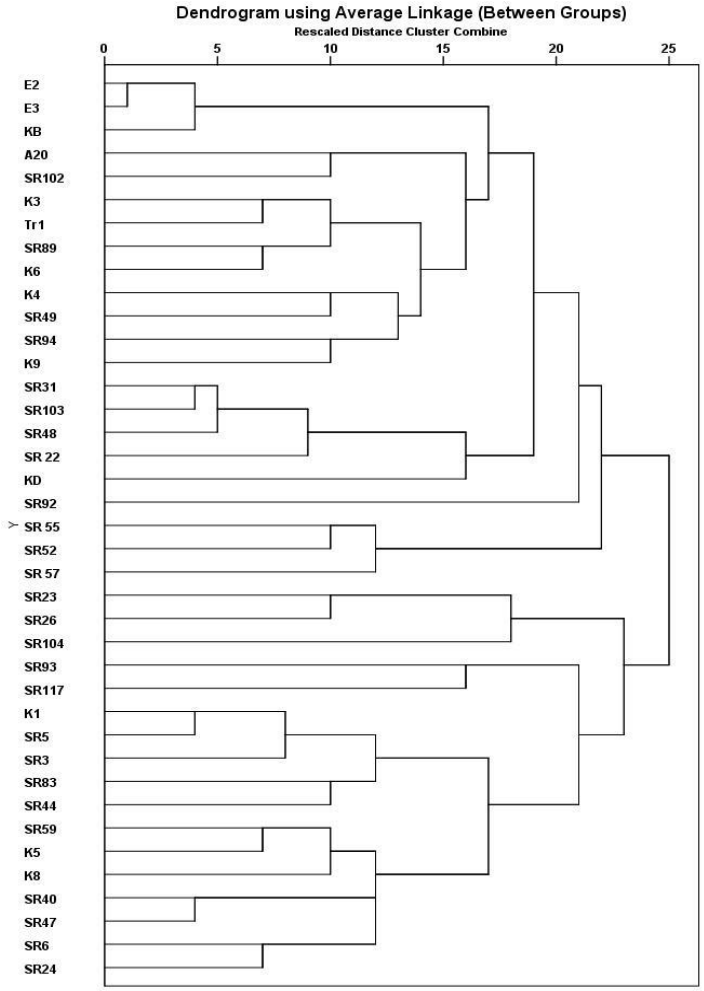

Figure 2a The Dendogram showing relationship amongst bacterial isolates using Hierarchical cluster analysis of biochemical test data

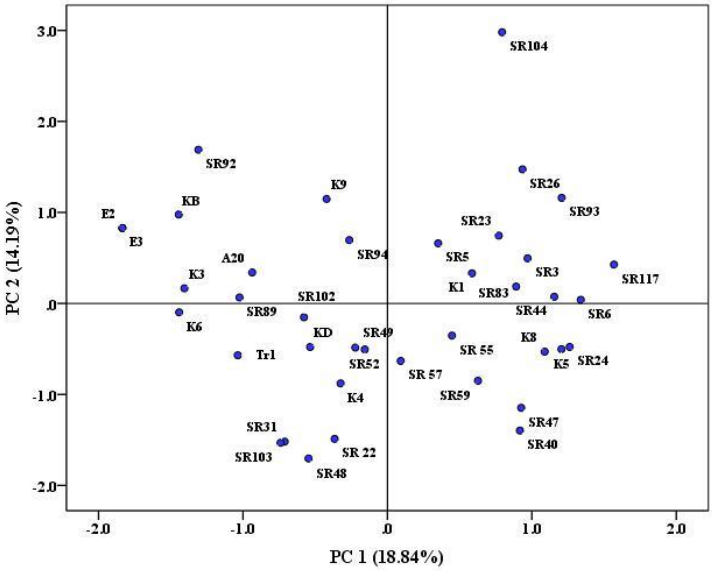

Figure 2b PCA of biochemical tests of 39 isolates at their optimal incubation time

Phylogenetic analysis of the isolates

Based on the results of 16S rRNA partial gene analysis selected isolates represented 22 genera and various species of these genera. Thus, it is obvious to have morphological, metabolical and EPS production diversity. Phylogenetic analysis of the isolates showed that most of them belonged to the phylum Firmicutes, followed by gamma-Proteobacteria. Gram positive bacteria of genera *Bacillus, Exiguobacterium, Micrococcus, Arthrobacter, Kocurea, Planococcus, Cellulosimicrobium, Actinotalea* and some of the Gram negative isolates related to genera *Pseudomonas, Enterobacter, Pontibacter, Sinorhizobium, Mesorhizobium* and *Dyadobacter* were cultured from the Alang ecosystem. Gamma-Proteobacteria included isolates of *Halomonas* and *Marinobacter* sp. The studied samples were from the same plot at the Alang coastal line, but they are from 10 different sites, which could be the reason of great diversity observed in the bacterial isolates. Members of the genus *Bacillus, Exiguobacterium, Micrococcus, Arthrobacter, Planococcus, Cellulosimicrobium, Pseudomonas* and *Enterobacter* have also been isolated from other saline environments, including Krishna Godavari basin (Bay of Bengal), Lonar Lake (Maharashtra) and coastal area of Tamilnadu **(Jaynath *et al.*, 2002; Kanekar *et al.*, 2007; Devi *et al.*, 2011)**. Based on the 16S rRNA gene analysis, evolutionary relationship between the cultivable microbes, isolated from the coastal region of Alang, Bhavnagar was studied using the neighbor- joining method (Fig. 3). The accession numbers are given in parentheses. Only bootstrap values are shown at nodes (based on 500 bootstrap resampling). The scale bar represents 1% divergence. Total 41 different microbial species were identified. The evolutionary distance is depicted by the length of the horizontal line. The branch point along the length of the horizontal line is the point of divergence of two microbial species. The outer, *Paenibacillus polymyxa* represents a distinct group with minimum evolution in relationship with the rest of the isolates. On the other hand, the significant divergence (2 to 3 step divergence) is observed among other microbes, including halotolerant *Halomonas*, chemoorganotrophs *Salinicola salaries*, aerobes *Pseudomonas*, anaerobes and facultative anaerobes *Enterobacter*. The community also contains some unique plant pathogens like *Xanthomonas campestris*. On the basis of this diversity study, it can be concluded that the selected site is highly enriched with the various types of microbes having divergent metabolic potential in spite of ship breaking activity at the site.

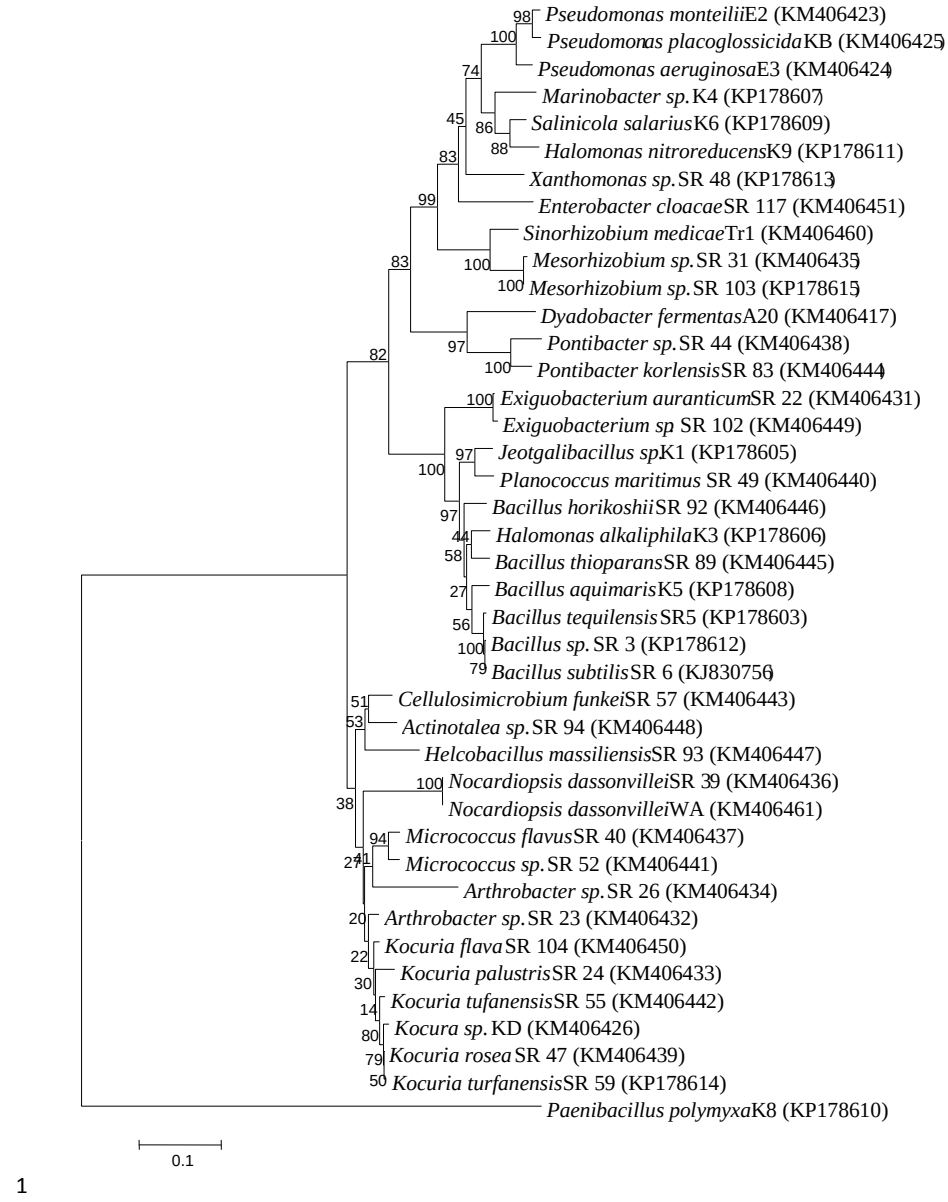

Figure 3 Neighbor joining phylogenetic tree of the 16S rRNA partial gene sequences of bacteria isolated from the coastal region of Alang, Bhavnagar

Rheological properties and EPS production study

Change in viscosity of the media was measured, in which EPS producers were grown and studied organisms showed quite a broad variation in viscosity. Out of 141 isolates 88 isolates showed variation in viscosity between $9.20x10^{-6}$ to $1.6x10^{-5}$ m.pa.s^{-1}, 28 isolates showed it between $1.7x10^{-5}$ to $2.6x10^{-5}$ m.pa.s^{-1} and for remaining 25 isolates it was $2.7x10^{-5}$ to $2.09x10^{-4}$ m.pa.s^{-1} when cultivated in EPS broth medium. Isolate SR5, SR6, SR40, SR48, SR55, SR83, SR117, Tr1 and K9 were found to be high EPS producers. In this study EPS production was observed in the range from 0.76 g/L to 10.7 g/L. The observed variation was obviously due to the diverse variety of the species as well as genus. EPS production yield by *Halomonas nitroreducens* was 0.76 g/L, *Sinorhizobium medicae* was 3.44 g/L, *Enterobacter cloacae* was 4.2 g/L, *Xanthonas* sp. was 6.48 g/L, *Kocurea turfenensis* was 7.1 g/L, *Micrococcus flavus* was 8.0 g/L, *Bacillus tequilensis* was 8.8 g/L, *Pontibacter korlensis* was 9.8 g/L, and *Bacillus subtilis* was 10.7 g/L. Reported range of EPS production from halotolerant organism is 0.16 g/L to 3.0 g/L. *Halomonas ventosa*, *Bacillus licheniformis*, *Halomonas anticariensis* are moderate halophiles showing EPS yield 0.29 g/L, 0.165 g/L and 0.5 g/L respectively **(Bejar *et al*., 2006; Maugeri *et al*., 2002)**. As halotolerant organisms survive in extreme conditions, they may be used for production of biopolymers, halophilic enzymes, compatible solutes and in the bioremediation process **(Ventosa *et al*., 1998; Margesin and Schinner, 2001; Mellado and Ventosa, 2003)**.

CONCLUSIONS

The site of Alang could be a good source of culturable bacterial diversity as different halotolerant and moderate halophilic bacteria were isolated from it. To the best of the authors' knowledge, the presence of the organisms, namely *Pontibacter korlensis*, *Kocurea tirfenensis*, *Actinotalea*, *Sinorhizobium* sp., *Cellulosimicrobium funkei*, *Helcobacillus massiliensis* and *Dyadobacter fermentas* from the coastal area of Alang, Bhavnagar is the first report. The significant finding was that many of these isolates grew in the presence of as high as 3-10% NaCl concentrations and some grew even in 20-25% NaCl, more over they produce a significant amount of EPS. EPS production by *Kocurea turfenensis* (SR55) and *Pontibacter korlensis* (SR 83) might be the new finding of this study. EPS produced by these organisms can be explored for various economical uses as it may have distinct properties with biotechnological applications.

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