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Diversity and EPS Production Potential of Halotolerant Bacteria from Veraval and Dwarka

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Abstract: *The microbial EPS are used in several biotechnological applications viz., cosmetics, textiles, pharmaceuticals, agricultural, paints and petroleum industries. Due to their extensive range of applications and also their bioactive roles there is increased interest for unusual and novel EPS. Less exploited saline and hypersaline environment may harbor microorganisms capable of producing unusual EPS with biotechnological interest. There are no reports of EPS production by halophilic and halotolerant bacteria from Gujarat. Therefore, the main aim of the study was to isolate halophilic and halotolerant bacteria, study their diversity and test their potential for EPS production. Four saline soil samples were collected, three from Veraval and one from Dwarka. The halophilic bacteria were enriched in media supplemented with 5% glucose containing increasing salt concentration from 5% to 35% NaCl. After enrichment organisms were isolated on solidified media containing salt concentration mentioned. Increasing salt concentration adversely affected growth. A total of 73 morphologically distinct isolates were studied. Less diverse colonies were obtained at low as well as extremely high salt concentration. The incidence of gram negative isolates decreased with increasing salt concentration. Organisms capable of growing at 35% NaCl concentration have been isolated. Out of 73 isolates, 23 isolates showing mucoid appearance were tested for EPS production which ranged from 0.2* gl^{-1} *to 10.60*gl^{-1}.

Key words: *Dwarka, Exopolysaccharide, Halotolerant, NaCl, Veraval*

I. Introduction

Bacterial growth is often accompanied by the production of exopolysaccharides (EPS), which have number of important ecological and physiological functions [1]. The microbial exopolysaccharides (EPS) are polymers that consist principally of carbohydrates and are excreted by some bacteria outside of their cell walls. Their composition and structure is varied: they may be either homo-or heteropolysaccharides; may also contain a number of different organic and inorganic constituents [2, 3]. Ecological and physiological functions assigned to microbial exopolysaccharides, include biofilm formation, self-protection against antimicrobial compounds, antibodies and bacteriophages and allowing the adherence to other bacteria or inert surfaces [4, 5]. The microbial EPS are used in several biotechnological applications counting food, textile, pharmaceutical, agricultural, paint and petroleum industries where emulsifying [6], viscosifying, suspending and chelating agents are required [4, 7, 8]. The advantages of microbial polysaccharides over plant or marine macroalgal polymers are their novel functionality, easily reproducible chemical and physical properties and stable cost and supply [2, 9]. Due to their bioactive role and their extensive range of applications increasing attention is being paid to production of these biomolecules. A new approach to encountering EPS with novel properties might entail investigating different environments such as less exploited saline and hypersaline habitats.

Saline and hypersaline environments are found in wide variety of aquatic and terrestrial ecosystems. They are inhabited by halotolerant as well as halophilic microorganisms [10, 11]. Halophiles are the microorganisms requiring salt for their growth. Non-halophilic microorganisms, able to grow in the absence as well as in the presence of salt are designated halotolerant. According to most widely used definition, that of Kushner (1978) [12], one can distinguish between

- **Slight halophiles** (many marine organisms; seawater containing about 3% w/v NaCl)
- **Moderate halophiles** (optimal growth at 3-5% w/v NaCl)
- **Borderline extreme halophiles** (requirement of atleast 12%w/v NaCl) and
- **Extreme halophiles** (optimal growth at 25% w/v NaCl) [13, 14].

There are several categories of halotolerant microbes:

- **Non-tolerant**, those which tolerate only a small concentration of salt (about 1% w/v)
- **Slightly tolerant**, tolerating upto 6-8%
- **Moderately tolerant**, upto 18-20%
- **Extremely tolerant**, those microbes that grow over the whole range of salt concentrations from zero up to saturation [15].

Saline habitats have not been studied intensively [16] and therefore very little information is available regarding diversity of halophilic microorganisms [17]. Understanding diversity within saline environmental context is very necessary in order to study the survivability and adaptation of halophiles at different levels of tolerance [17]. Hypersaline conditions are prevailing on Mars and thus the studies of microbial diversity in terrestrial saline environments may also have implications for the possibility of extinct and/or extant life on Mars [18, 19, 20]. During the last two decades, there have been many attempts to study diversity, isolate and characterize halophilic organisms so as to provide a firm systematics base for halophilic bacteria and archaea. The most widely studied ecosystems are the Great Salt Lake (Utah, USA), the Dead Sea (Israel), the alkaline brines of Wadi Natrun (Egypt), and Lake Magadi (Kenya) [10]. These habitats have one or more harsh environmental conditions such as high salinity, high temperature, low oxygen availability, high nutrient availability, high light intensity, and extremely alkalinity [17]. Major saline sites studied in India are Sambhar salt lake, Rajasthan, coastal regions of Gujarat, Tamil Nadu, Maharashtra, Andhra Pradesh, Orissa and West Bengal [21]. It can be hypothesized that a relatively large diversity of strains, species, or both exists within the bacterial floras, a condition that requires experimental scrutiny rather than the examination of gross morphology used for identifying unicellular and multicellular eukaryotes [22]. Such environments may harbor unusual halophiles and halotolerant microorganisms of biotechnological interest. There are number of reports on EPS production by moderately halophilic species e.g., *Halomonas eurihalina*, *Halomonas maura*, *Halomonas ventosae*, *Halomonas anticariensis*, *Alteromonas hispanice*, *Idiomarina rambicola* and *Idiomarina fontisalpitosi*. Polymers produced by these bacteria show potential interest as viscosifying, jellying, emulsifying and metal binding compounds. Sulfated EPS also provide interesting applications for pharmaceutical industry as antitumoral [23], antiviral [24, 25, 26] and anticoagulant [3, 27] properties.

II. Research Objectives

The reports regarding the study of diversity of halotolerant and halophilic bacteria from Gujarat are scanty. Thus the main aims of the study were : 1) To study culturable diversity of halotolerant and halophilic bacteria from the saline soil samples of Veraval and Dwarka. 2) To isolate halotolerant and halophilic bacteria from the saline soil samples and test their potential for EPS production.

III. Materials and Methods

1.1 Saline soil sample collection:

Saline soil samples were collected from four different sites:

A. Veraval coast
B. Somnath beach
C. Dwarka beach
D. Fish drying unit in Veraval

Samples were collected in November, 2010. The saline soil samples were collected in sterile plastic bags and were transported to laboratory within 24 hours of collection and refrigerated till further analysis.

3.2 Enrichment and isolation:

1-2 gram weight of each soil sample was mixed with the liquid media for enrichment of halotolerant bacteria. Because no one media is known to support the growth of all the halotolerant and halophilic bacteria, the general-purpose, complete media was used for both enrichments and direct plate streaking. This liquid media was supplemented with 5% glucose containing 5%, 7.5%, 10%, 15%, 20%, 25%, 30% and 35% NaCl. Incubation was done at 30±0.2ºC at static conditions. Growth intensity was visually graded. Enriched organisms were transferred on solidified media containing 5% glucose and respective concentration of salt. Morphologically distinct colonies were purified by streaking to obtain pure cultures. Isolated pure cultures were differentiated by colony morphology, size and pigment production pattern and gram staining.

3.3 Screening for EPS production in liquid medium:

Colonies showing mucoid appearance were selected for further screening in liquid medium.

3.3.1 EPS production:

Actively growing cultures were inoculated in liquid media with 5% glucose and 7.5% salt concentration. These were grown for 5 days (on shaker) in 250 ml Erlenmeyer flasks, each containing 100ml of the media.

3.3.2 EPS extraction:

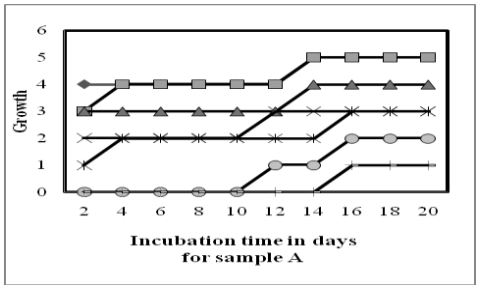
Broth samples after incubation were centrifuged (5000g for 30 min) to pellet out cells. EPS was separated from the supernatant by adding three volumes of chilled ethanol (15ml broth + 45ml alcohol). Precipitated EPS was filtered onto preweighed Whattman no. 1 filter paper and dried to constant weight. Amount of EPS in culture broth was calculated in terms of dry weight [1, 28].

IV. Results and Discussions

1.2 Effect of increasing salt concentration on enrichment of halotolerant bacteria

At low salt concentration (5%) intense growth was observed in all samples within 24 hours. Increasing salt concentration delayed growth as well as decreased intensity of growth in all samples. At high salt concentration (15% and 20%) moderate to scanty growth was observed after 2 days but the intensity of growth was less as compared to low salt concentration. At very high salt concentration (25% & 30%) and also at extremely high salt concentration (35%) near to saturation scanty growth was observed after prolonged incubation; indicating adverse effect of very high salt concentration on enrichment and growth of organisms. In the study of microbial diversity of marine salterns of Bhavnagar also total microbial count as well as diversity was found to decrease with increasing NaCl concentration [25]. Sample D showed considerable variation as compared to other samples. A notable difference was observed for sample D where growth was observed at all salt concentration within 24 hours (except at 25%, 30% and 35%). The sample D had been collected from fish drying unit where salt is used for drying and preservation of fish. The microbial flora in this soil would have been exposed and adapted to high salt concentration, therefore rapid growth was observed within 24 hours.

Graph-1, 2, 3, 4: Effect of increasing salt concentration on enrichment of halotolerant organisms from sample A, B, C and D, respectively



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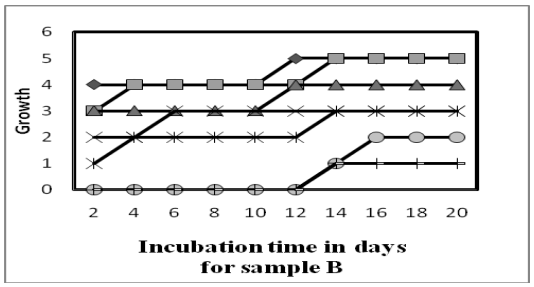

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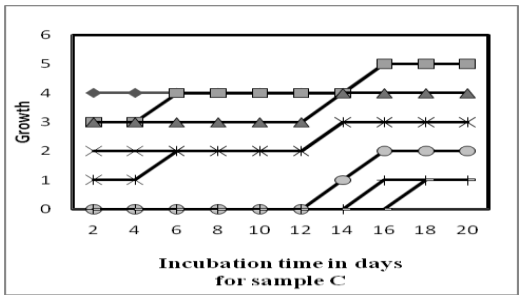
Key: 5 highly intense growth, 4 intense growth, 3 good growth, 2 moderate growth, 1 scanty growth, 0 no growth.



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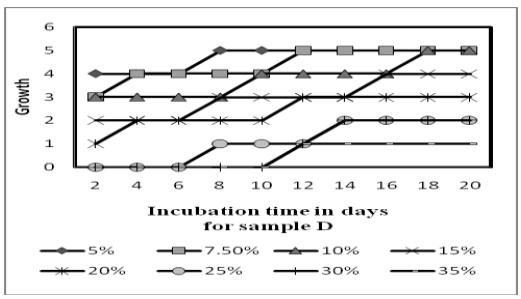

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4.2 Morphological observations

A total of 73 isolates were obtained from all the samples at different salt concentrations. Under the incubation condition of 30±0.2ºC the colonies had size ranging from pin-point to 3mm. Maximum number of isolates were obtained from sample D and least isolates were obtained from sample A. At low salt concentration from 5% to 7.5% varieties ranged from 3-5 while at high salt concentration from 20% to 35% till saturation no diversity was observed; only one isolate was obtained. Majority of isolates were colourless to white. Only 22 isolates out of 73 were pigmented. Pigmentation pattern could separate sample from different sites. Yellow pigmented colonies were obtained from sample A and B both of which were collected from Veraval beach. Amongst yellow pigmented colonies various shades of yellow viz., light yellow, lemon yellow and golden yellow were obtained. Other pigmented colonies were either pink-peach or orange and were obtained only from sample D. Sample D was collected from fish drying unit in Veraval and was expected to have different microbial flora. Red coloured colonies were obtained only from sample C that too only above 20% salt concentration. Another interesting finding was that in sample A, B and D the pigmented colonies were observed at salt concentration till 15% while in sample C colonies were obtained above 20% till saturation [29].

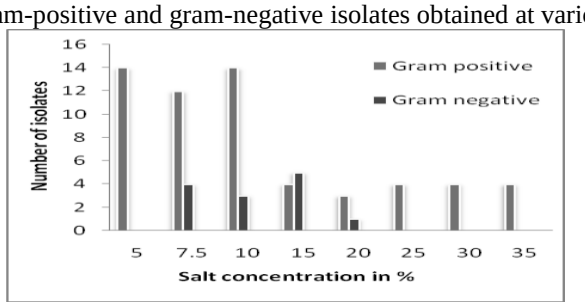
Table-1: Gross morphological diversity of isolates obtained from all the samples.

Sample	Salt Conc. (in %)	Varieties of isolates	Gram nature			Pigment			Consistency		Opacity	
			Gram +ve Bacilli	Gram +ve Coccii	Gram -ve Short-rods	W	Y	P/R	M	N-M	O	T
A	5	3	2	1	0	2	1	0	2	1	1	2
	7.5	3	2	0	1	2	1	0	1	2	3	0
	10	3	0	3	0	3	0	0	1	2	2	1
	15	2	1	1	0	1	1	0	1	1	1	1
	20	1	1	0	0	0	1	0	1	0	1	0
	25	1	1	0	0	1	0	0	1	0	0	1
	30	1	1	0	0	1	0	0	1	0	0	1
	35	1	1	0	0	1	0	0	1	0	0	1
B	5	3	2	1	0	2	1	0	0	3	3	0
	7.5	5	2	1	2	4	1	0	3	2	3	2
	10	5	3	0	2	4	1	0	0	5	2	3
	15	2	0	0	2	2	0	0	1	1	1	1
	20	1	0	0	1	1	0	0	1	0	0	1
	25	1	1	0	0	1	0	0	1	0	1	0
	30	1	1	0	0	1	0	0	1	0	1	0
	35	1	1	0	0	1	0	0	1	0	1	0
C	5	4	2	2	0	3	1	0	1	3	1	2
	7.5	3	1	2	0	2	1	0	1	2	3	0
	10	4	1	2	1	3	1	0	1	3	2	2
	15	2	0	1	1	2	0	0	0	2	0	2
	20	1	0	1	0	0	0	1	0	1	0	1
	25	1	1	0	0	0	0	1	0	1	0	1
	30	1	1	0	0	0	0	1	0	1	0	1
	35	1	1	0	0	0	0	1	0	1	0	1
D	5	5	4	1	0	1	3	1	1	4	5	0
	7.5	5	3	1	1	4	0	1	1	4	5	0
	10	5	3	2	0	3	0	2	1	4	5	0
	15	3	1	0	2	3	0	0	0	3	2	1
	20	1	1	0	0	1	0	0	0	1	1	0
	25	1	1	0	0	1	0	0	0	1	1	0
	30	1	1	0	0	1	0	0	0	1	1	0
	35	1	1	0	0	1	0	0	0	1	1	0

Key: W-White, Y-Yellow , P/R-Peach/Red, M-Mucoid, N-M-Non-mucoid, O-Opaque, T-Translucent

4.3 Gram nature

Graph-5: Comparision of gram-positive and gram-negative isolates obtained at various salt concentrations



Majority of the isolates studied were gram positive. Only 13 isolates were found to be gram negative. At low salt concentration of 5% as well as at very high salt concentration of 25% to 35% no gram negative organisms were recorded. Gram negative isolates were obtained only at 7.5%, 10% and 15% NaCl. Maximum numbers of gram negative isolates were obtained at 15% NaCl. Amongst gram positive isolates gram positive coccii were more predominant as compared to gram positive bacilli. Out of 60 gram positive isolates obtained 19 were gram positive bacilli and 41 were gram positive coccii. From all the samples at very high salt concentration above 25% all the isolates were found to be gram positive cocci. In a study of hypersaline soil of Alcinate similar results have been reported where media containing 10% yielded mostly gram positive rods whereas gram negative rods dominated between 10% and 20% and gram positive cocci developed above 20% [29].

1.4 Screening for EPS

From the 73 isolates obtained, 23 isolates showed mucoid and viscous consistency and appeared to be EPS producers. However, morphologically different 16 isolates were screened to test their EPS production potential in liquid medium. EPS yield ranged from as low as $0.27gl^{-1}$ to as high as $10.6gl^{-1}$. From the isolates tested five isolates gave EPS yield less than $1gl^{-1}$, five isolates gave yield less than $5.0gl^{-1}$ and three isolates gave EPS yield above $5.0gl^{-1}$. Best EPS yield of $10.6gl^{-1}$ was obtained from isolate $10A_2$. Isolates obtained from media with very high salt concentration gave no growth this may be because of their extremely halophilic nature and inability to grow at low salt concentration as EPS production was tested at 7.5% NaCl. The isolate may be halophilic archaea which lyse below 10% NaCl. However, we are further characterizing these isolates. Best halotolerant EPS producers were obtained from sample A and B. Isolates $10A_2$, $7.5B_1$ and $7.5C_3$ were found to be best EPS producers with $10.6gl^{-1}$, $8.07gl^{-1}$ and $5.2gl^{-1}$ EPS yield respectively. The reported EPS yield for various moderately halophilic bacteria like *Halomonas anticariensis* [28], *Halomonas ventosaev* [28], *Bacillus licheniformis* [29] is $0.5gl^{-1}$, $0.29\ gl^{-1}$ and $0.165gl^{-1}$. However, the maximum EPS yield is reported by *Halomonas eurihalina* is $1.6gl^{-1}$[1] which is 6.6 times less than isolate, $10A_2$. Other halophilic organisms belonging to α-Protobacteria are also known to produce good EPS. One of these includes *Salipiger mucosus* [2] which is capable of producing $1.35\ gl^{-1}$ EPS under optimized conditions. So this also accounts to the yield much less than that produced by most of our isolates. Other halophilic organisms known to produce EPS include cyanobacterium, *Aphanocopsa halophytica* [30] and archaeon, *Haloferax mediterranei* [31] with EPS yield of $0.80gl^{-1}$ and $3.0gl^{-1,}$ respectively. Thus, the range of EPS yield by most of the halotolerant organisms is from $0.16gl^{-1}$ to $3.0gl^{-1}$ whereas the range of EPS yield by our isolates was from $0.27gl^{-1}$ to $10.60gl^{-1}$.

Table 2: EPS production by selected isolates

SR. NO.	ISOLATE	GRAM NATURE	EPS(gl^{-1})
1.	5A1	Gram +ve Cocci	4.733
2.	5D2	Gram +ve Bacilli	3.200
3.	5D3	Gram +ve Cocci	0.266
4.	7.5A1	Gram +ve Cocci	0.733
5.	7.5B1	Gram +ve Cocci	8.066
6.	7.5B3	Gram –ve Short rods	0.266
7.	7.5C3	Gram +ve Cocci	5.200
8.	10A1	Gram +ve Bacilli	2.733
9.	10A2	Gram +ve Bacilli	10.600
10.	10C1	Gram +ve Bacilli	2.000
11.	15A2	Gram +ve Bacilli	2.733
12.	15B1	Gram –ve Short rods	1.000
13.	15B2	Gram –ve Short rods	1.933
14.	20A1	Gram +ve Cocci	-
15.	25A1	Gram +ve Cocci	-
16.	35A1	Gram +ve Cocci	-

V. Conclusions

Increasing salt concentration adversely affected growth as indicated by delayed growth as well as decreased intensity of growth at high salt concentration. Less diverse colonies were observed at low concentration as well as at extremely high salt concentration. At low salt concentration serial dilution followed by plating would have allowed isolation of diverse and rare organisms. Ability to grow at extremely high salt concentration is restricted to few organisms. These organisms would have been selected during enrichment process resulting in less diverse colonies at extremely high salt concentration. The data provides considerable information about diverse morphological varieties of isolates obtained at varying salt concentration. The interesting findings of study are dominance of gram positive isolates at low and extremely high salt concentration, complete absence of gram negative isolates low and extremely high salt concentration and the distinct pigmentation pattern of isolates from different samples. There are number of reports on isolation of extremely halophilic organisms from marine salterns but few from saline soils. The interesting finding of study is the isolation of microorganisms which are capable of growth at salt concentration of 35% i.e., near saturation and ten times more salt concentration from their natural habitats. Three isolates obtained gave promising EPS yield. The maximum amount of EPS was produced by isolate $10A_2$ which was 6.6 times more than reported. Further work will be centered on optimization of nutritional and environmental variables for each EPS producing isolate in order to obtain maximum production, together with the best functional properties of polymers. Both EPS yield and its chemical composition may be influenced by several parameters such as carbon source, limiting nutrients and aeration and incubation temperature. EPS produced by these halotolerant bacteria

can be exploited for bioremediation and MEOR where these polymers act as excellent emulsifiers and mobility controllers. So the search for halophiles with such useful applications will in turn deepen our understanding of the functioning of hypersaline ecosystems also.

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