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Bacteria from Salt Pans: A Potential Resource of Antibacterial Metabolites

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Abstract

Marine salt pans are important ecological niches which inhabit halobacteria. These bacteria tolerate and thrive in salt concentrations ranging from 0.5 to more than 5 M in which only very few other organisms are able to survive. Bacteria from marine salt pans of varying salinities of 220 to 395 psu were isolated during the peak salt harvesting season and screened to evaluate their antibiotic producing potential. In this report, a total of 119 bacteria were screened on 12 different solid media supplemented with either natural salt or sea water or distilled water to check their substrate utilization and salinity requirement. Based on their morphological variations, 94 isolates were further screened for their antagonistic properties, against 20 different clinical pathogens. Thirty one isolates were found to produce antibacterial compounds of which, 21 showed bactericidal action and one was bacteriostatic while 9 isolates exhibited both bacteriostatic and bactericidal activity. Eleven isolates were broad spectrum antibiotic producers. This study provides information regarding the applied value of potential halotolerant and halophilic isolates as pharmaceutically important microorganisms.

Key Words: Bioactivity, Hypersaline, Secondary metabolites, Salt pans

Introduction

Marine salt pans in Goa are thalassohaline, multi-pond systems which are interconnected; allowing a discontinuous salinity gradient. These ponds have a continuous inflow of sea water which is evaporated for the commercial production of sodium chloride i.e. natural salt.

Microbes from salt pans are yet to be fully explored as potential producers of antimicrobial agents. However, few reports are available on the antimicrobial potential of microorganisms in Indian salterns. Dhanasekaran *et al.* (1) have reported antibacterial potential of salt pan actinomycetes from Tamil Nadu, India. Kamat and Kerkar (2) have carried out studies on a marine salt pan bacterium, producing a broad spectrum antibiotic, from Goa, India.

In the present study our goal was to focus on antibiotic producing potential of salt pan bacteria from two marine salterns namely Batim and Ribandar from North Goa. The bacterial isolates have been screened against twenty human pathogens to establish their inhibitory activity. Additionally, studies have been carried out on the substrate utilization and salinity requirement of the isolates. Because of the growing interest in the study of secondary metabolites from marine environments these hypersaline ecosystems could be highly promising habitats for the discovery of microorganisms capable of producing novel and useful bioactive compounds for the future development of medicine, agriculture and biotechnology industry.

Materials and Methods

Sample collection and isolation of marine bacteria:

Sampling was restricted to the crystallizer ponds from the salt pans of Batim and Ribandar, Goa, India during the pre-monsoon i.e. the peak salt manufacturing season of 2007 and 2008. Water samples (four) from overlying salt pans, were collected in sterile disposable bottles, chilled immediately on ice and transported to the laboratory for processing within a 24h period. The salt pan water salinity, temperature and pH were measured at the site using S/Mill-E Hand-held refractometer (ATAGO, Japan) and pHTestr 30 (Eutech Instruments, Thermo Scientific, USA) respectively. Sediment samples (four) were collected using a 10 cm corer. Core samples were sealed in sterile plastic bags and transported at 4°C in an ice box. For the isolation of hypersaline bacteria in brief, serial dilutions were carried out with sterile salt pan water and 0.2 ml of the sample was spread plated, in duplicates, on International *Streptomyces* Project (ISP) media 1 to 7 (ISP 1: Tryptone Yeast extract agar, ISP 2: Yeast Malt agar, ISP 3: Oat meal agar, ISP 4: Inorganic salt Starch agar, ISP 5: Glycerol Asparagine agar base, ISP 6: Peptone Yeast extract Iron agar, ISP 7: Tyrosine agar base) (3) and Nutrient Agar (NA) +5% crude NaCl. All the media used were prepared in filtered sea water except NA+5% crude NaCl which was prepared in distilled water. The plates were incubated at 37 °C for 72h. The strains isolated were purified, sub cultured and

stored at 4 °C. All the media and media components used for the experiments were procured from Hi Media, Mumbai, India unless otherwise specified.

Growth and tolerance to salinity:

All the isolates were spotted on 12 different agar media to assess their growth and their tolerance to salinity. The media used for the study included NA (may also be referred as NA D/W i.e. NA prepared using distilled water), NA+10% crude NaCl, NA prepared using sea water (NA S/W) and Kuster's agar prepared in sea water as well as the 8 media used for isolation as mentioned above. The isolates were streaked on respective agar plates and incubated at 37 °C for 24-48h.

Antibacterial activity:

Twenty clinical pathogens namely *Acinetobacter baumanii* (AB), *Aeromonas hydrophila* (AH), *Citrobacter diversus* (CD), *Citrobacter freundii* (CF), *Escherichia coli ATCC 25922* (ECATCC), *Klebsiella pneumoniae* (KP), *Morganella morganii* (MM), *Proteus mirabilis* (PM), *Pseudomonas ATCC 27855* (PATCC), *Pseudomonas spp. (Pigmented)* (PP), *Salmonella paratyphi A* (SPA), *Salmonella typhi* (ST), *Salmonella typhimurium* (STM), *Shigella boydii* (SB), *Shigella flexneri* (SF), *Vibrio cholerae* (VC), *Methicillin Resistant Staphylococcus aureus* (MRSA), *Methicillin Sensitive Staphylococcus aureus* (MSSA), *Staphylococcus ATCC 25923* (SATCC), *Staphylococcus citreus* (SC) were obtained from Goa Medical College & Hospital, Goa, India to be used as test organisms against isolates. Preliminary screening was performed on 94 isolates by the cross streak method. The isolates were streaked as a ribbon on NA plates, in duplicates, and incubated at 37 °C for 48 h. After incubation, the test pathogens were streaked perpendicular to the ribbon and the plates were incubated again for 16-24 h at 37 °C. The observations were recorded as mm of inhibition.

Secondary screening by Kirby-Bauer disc diffusion method:

Six isolates namely 6, 7, 12, 17, TSK 32 and TSK 71 with most promising antimicrobial potential were tested. The isolates were grown in 50 mL Nutrient broth (NB) at 120 rpm, room temperature (28 ± 2 °C) for 3 days. After every 24 h, 1 mL of each culture was centrifuged at 8000 rpm. Sterile discs (Whatman filter no. 1) 6mm each, were impregnated with 10 µL of the filter sterilised culture supernatant and allowed to air dry under aseptic conditions. The test pathogen *Staphylococcus citreus* was suspended in 0.85% sterile saline (6.25 x10^7 cells per mL), vortexed and spread plated on NA. The impregnated discs were then placed on the culture lawn along with positive control (Streptomycin 10µg) and incubated at 37 ºC for 24 hours. The zones of inhibition were recorded in mm. Assays were carried out in triplicates.

Characterization of the active isolates:

The six active isolates were tested for utilization of 35 carbohydrates using KB009 HiCarbohydrate™ Kit (Part A, B and C) from Hi Media, Mumbai, India. The bacterial suspension grown in ISP-1 media was inoculated into KB009 strips and incubated at 37 °C. Results were recorded after 24 h. Presence of endospores was confirmed using Schaeffer-Fulton's staining method.

DNA was isolated from the active isolates and was PCR amplified using universal bacterial primers for 16S rDNA. The PCR amplified product was separated electrophoretically on 1% agarose gel. Approximately 1.5kb fragment was gel purified and sequenced.

Antimicrobial susceptibility test for active isolates:

Active isolates were grown for 16-18 hours and 0.1 mL suspension was spread on Muller Hinton agar plates. The antibiotic discs Cephalexin (30 µg), Tetracycline (30 µg), Cefuroxime (30 µg), Lincomycin (10 µg), Methicillin (5 µg), Gentamicin (10 µg) from Hi Media, India were placed on the lawn of the respective culture and incubated overnight at 37 °C. The test was performed as per the CLSI methodology provided by the HiMedia Laboratories Pvt. Ltd., Mumbai, India (4). The zones of inhibition were recorded.

Results and Discussion

The bioactive molecules currently available in the market have been obtained after decades of intensive screening and research. The past successes make discovering new bioactive metabolites from microbial sources much more difficult, since thousands of compounds are described in literature (5, 6). Newer niches are frantically being explored to screen potential producers of novel bioactive molecules (7).

Marine actinomycetes viz. *Streptomyces* and *Micromonospora* strains have been reported to produce antibiotics like enterocin and ikarugamycin which have previously been isolated from terrestrial strains whereas abyssomicins, a novel family of antibiotics, produced by marine *Verrucosispora* strains (8), marinomycins by *Marinispora* sp., Salinosporamide A (NPI-0052) by *Salinispora tropica* and many other novel metabolites produced by marine actinomycetes have been reported (9). In India, a halophilic *Actinopolyspora* species AH1, with antibacterial activity, was isolated from the sediments of Alibag coast of Maharashtra. The strain exhibited antagonistic activity against Gram positive bacteria and some fungi. (10).

Salt pans are an extreme environment, which inhabit organisms that survive at very high salinities, high temperatures and withstand severe solar radiations. To survive in such extreme environment, these organisms are known to produce secondary metabolites. These metabolites can sometimes be of great importance due to their bioactive potential. Hence organisms isolated from these environments could serve as a source for the discovery of novel secondary metabolites.

Sample collection and isolation of marine bacteria:

We have explored two salt pans in north Goa namely Batim and Ribandar to investigate the antibiotic producing potential of these bacteria. The sampling parameters are recorded in Table 1. Hundred and nineteen bacterial isolates which include bacteria (94%) and actinobacteria (6%) were isolated on eight different media. The number of isolates obtained on various media are shown in Table 2. Maximum number of isolates (33) were obtained on ISP 6 media. Nearly equal number of isolates i.e. 60 from Ribandar and 59 from Batim were obtained from both the sampling sites.

Table 1. Sampling parameters for collections during pre-monsoon season of 2007 and 2008.

Physical parameters during sampling	Sampling site 1 (May 2007)	Sampling site 2 (May 2007)	Sampling site 1 (May 2008)	Sampling site 2 (May 2008)
Place	Ribandar	Batim	Ribandar	Batim
GPS data	N 15° 29' 58.1" E 073° 50' 49.2"	N 15° 27' 27.6" E 073° 52' 58.0"	N 15° 30' 8.1" E 073° 51' 19.6"	N 15° 27' 28.6" E 073° 52' 50.6"
Temperature	45°C	38°C	45.3°C	45°C
Salinity (psu)	320	220	310	395
pH	7.09	7.09	6.63	6.43

Table 2. Isolates obtained on various media.

Media of isolation	No. of isolates May 2007	No. of isolates May 2008	Total no. of isolates
ISP No. 1	0	18	18
ISP No. 2	11	16	27
ISP No. 3	5	5	10
ISP No. 4	2	3	5
ISP No. 5	0	1	1
ISP No. 6	9	24	33
ISP No. 7	5	3	8
NA + 5% NaCl	5	12	17
Total	37	82	119

With salinities at or near sodium chloride saturation hypersaline environment of salt pans supports only limited microbiota. These NaCl saturated salt pans allow the growth of moderately halophilic and halotolerant bacteria (11). Manikandan and Kannan (11) assessed the diversity of cultivable microorganism from three solar salterns along the shoreline of Bay of Bengal in Tamil Nadu, India. They used three different media to isolate halophiles. Vijayakumar *et al.* (12) have reported 19 actinomycetes isolates in the salt pans of Vedaranyam, Thondi and Tuticorin in Palk Strait region of Bay of Bengal, India. Dhanasekaran *et al.* (3) have isolated 9 *Streptomyces* isolates from salt pan soil (1 from Porto Novo and 3 from Thaikkalthurai, Tamil Nadu, India).

Growth and tolerance to salinity:

Qualitative analysis of growth on various media indicated that 97% isolates showed dense growth (matt or confluent growth where colonies are merged and uncountable) on ISP 2 media followed by ISP 1 (87%). Most of the isolates (79%) did not require salt for growth, and only 21% isolates did not show growth in the absence of crude NaCl. Nearly 50% of the isolates could tolerate up to 10 % crude NaCl while 61% could tolerate up to only 5% crude NaCl. The results are shown in Figure 1. There are numerous reports which support that most actinomycetes isolated from marine sources are of terrestrial origin and reside in the marine ecosystem as spores or resting propagules (13). Thus some of the salt tolerant bacteria are likely to have a terrestrial origin, entering the salt pans from the surrounding ecosystems.

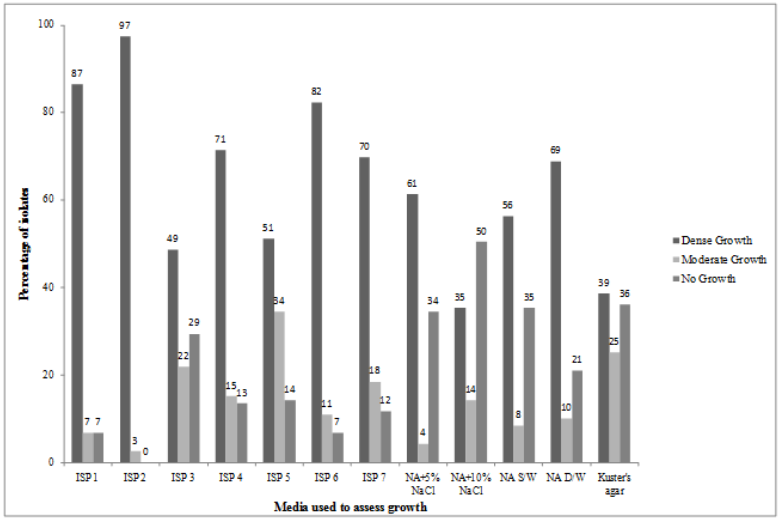

Figure 1. Growth of isolates on various media and salt tolerance study.

Antibacterial activity

Ninety four isolates, based on morphological differences, were screened for antibacterial activity. Numerous screening methods to determine antimicrobial activity of natural products have been discussed in the literature (14, 15). We used simple cross streak method as described above. When no growth of pathogen was seen it was considered to be bactericidal action whereas when few colonies (compared with the control) were seen it was considered to be bacteriostatic activity. Only 31 isolates were found to be potentially active. Of these, 21 showed bactericidal action [Isolate codes: 1, 2, 3, 5, 6, 7, 8, 9, 10, 12, 13, 16, 17, 18, 21, 22, TSK 7, TSK 11, TSK 28, TSK 38 and TSK 40] and one was bacteriostatic [Isolate code: TSK 31] while nine were bacteriostatic as well as bacteriocidal [Isolate codes: TSK 10, TSK 19, TSK 24, TSK 32, TSK 33, TSK 43, TSK 44, TSK 45 and TSK 71]. Figure 2 shows preliminary screening by cross streak method for isolate no. 6. Out of the 31 actives, two isolates [Isolate codes: TSK 38 and TSK 40] inhibited Gram positive pathogen SC; eighteen [Isolate codes: 1, 2, 3, 5, 8, 10, 13, 16, 18, 21, 22, TSK 7, TSK 10, TSK 11, TSK 24, TSK 28, TSK 31 and TSK 33] inhibited Gram negative pathogens viz. AB, CF, PP, SPA, ST, SB, SF & VC whereas eleven [Isolate codes: 6, 7, 9, 12, 17, TSK 19, TSK 32, TSK 43, TSK 44, TSK 45 and TSK 71] were broad spectrum antibiotic producers. The profiles of most significant activity are shown in Figure 3.

Secondary screening by Kirby-Bauer disc diffusion method:

Secondary screening was performed on the six most promising isolates, namely 6, 7, 12, 17, TSK 32 and TSK 71. During primary screening a maximum inhibitory zone was observed with SC and hence chosen for secondary screening. Amongst the six cultures tested for activity against SC, TSK 71 had the maximum activity ($p<0.05$). Also there were no significant differences ($p>0.05$), over time (three days tested), in the bactericidal action of TSK 71 on SC. Hence, as shown in Table 3, the maximum zone of inhibition was exhibited by TSK 71 consistently for 3 days.

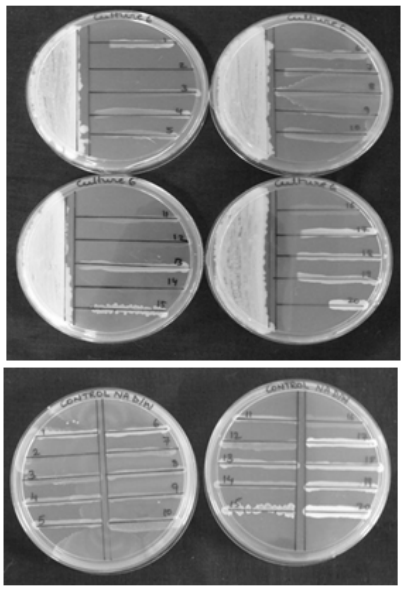

Figure 2. Preliminary screening by cross streak method. Top four plates show preliminary screening by cross streak method for isolate no. 6 and bottom two plates show growth of pathogens on NA (control). 1: *Acinetobacter baumanii*; 2: *Aeromonas hydrophila;* 3: *Citrobacter diversus;* 4: *Citrobacter freundii;* 5: *Escherichia coli ATCC 25922 ;* 6: *Klebsiella pneumoniae;* 7: *Morganella morganii;* 8: *Proteus mirabilis;* 9: *Pseudomonas ATCC 27855;* 10: *Pseudomonas spp. (Pigmented);* 11: *Salmonella paratyphi A;* 12: *Salmonella typhi;* 13: *Salmonella typhimurium;* 14: *Shigella boydii;* 15: *Shigella flexneri;* 16: *Vibrio cholerae;* 17: *Methicillin Resistant Staphylococcus aureus;* 18: *Methicillin Sensitive Staphylococcus aureus;* 19: *Staphylococcus ATCC 25923;* 20: *Staphylococcus citreus*.

Table 3. Zone of inhibition in mm against *Staphylococcus citreus*.

Culture no.	Zone of inhibition in mm		
	Day 1	Day 2	Day 3
6	11	17	15
7	0	13	10
12	9	11	10
17	11	12	11
TSK 32	14	17	16
TSK 71	15	17	17

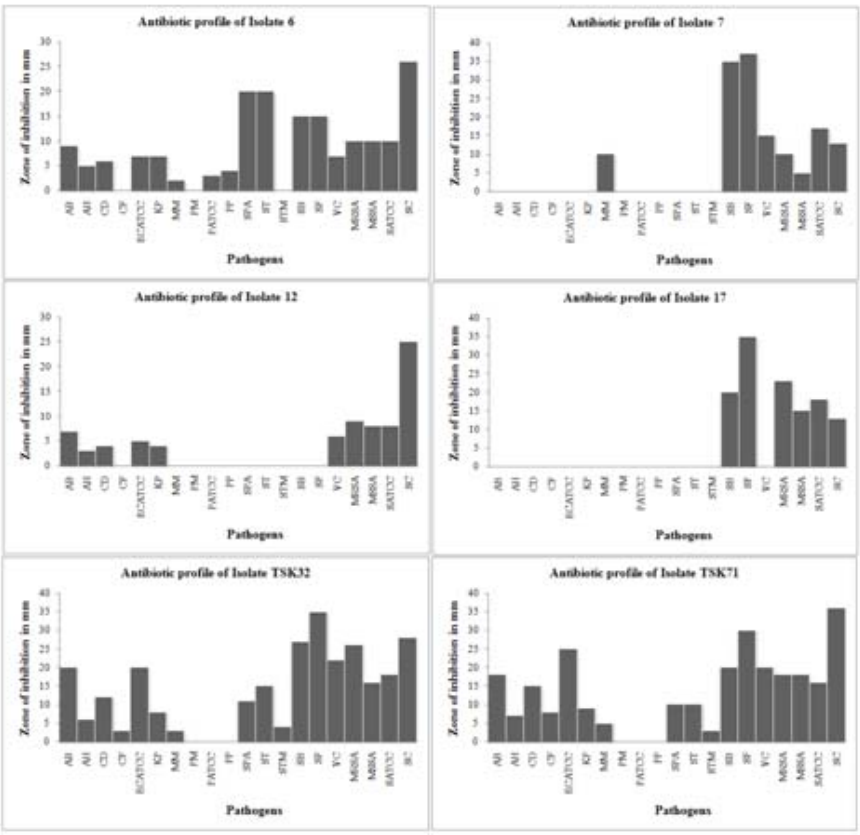

Figure 3. Antibiotic profiles of active isolates showing most significant bactericidal activity. A: Profile of Isolate 6; B: Profile of Isolate 7; C: Profile of Isolate 12; D: Profile of Isolate 17; E: Profile of Isolate TSK32; F: Profile of Isolate TSK71. AB: *Acinetobacter baumanii;* AH: *Aeromonas hydrophila;* CD: *Citrobacter diversus;* CF: *Citrobacter freundii;* ECATCC: *Escherichia coli ATCC 25922;* KP: *Klebsiella pneumonia;* MM: *Morganella morganii;* PM: *Proteus mirabilis;* PATCC: *Pseudomonas ATCC 27855;* PP: *Pseudomonas spp. (Pigmented);* SPA: *Salmonella paratyphi A;* ST*: Salmonella typhi;* STM: *Salmonella typhimurium;* SB: *Shigella boydii;* SF: *Shigella flexneri;* VC: *Vibrio cholerae;* MRSA: *Methicillin Resistant Staphylococcus aureus ;* MSSA: *Methicillin Sensitive Staphylococcus aureus;* SATCC: *Staphylococcus ATCC 25923;* SC*: Staphylococcus citreus*.

Suthindhiran and Kannabiran (16) have reported antimicrobial activity of *Saccharopolyspora salina* VITSDK4 isolated from a salt pan marine soil sample collected at the Marakkanam coast of the Bay of Bengal, India. One hundred and sixteen strains were isolated on screening for bioactive marine actinobacteria, from which only 7 isolates exhibited broad spectrum activity. *Saccharopolyspora salina* VITSDK4 was profoundly antagonistic with fungal and Gram positive pathogens. Gokulkrishnan et al. (17) have reported antimicrobial activity of marine bacteria isolated from the Mangalore coast, along the west coast of India. Out of 38 isolates only 21 showed activity during primary screening against test organisms. Of which 3 were active against Gram negative, 10 against Gram positive and 12 against both Gram positive and Gram negative organisms. Kamat and Kerkar (2, 18) have reported a halotolerant *Acinetobacter* sp. from salt pans of Ribandar, Goa producing antibacterial compound. Halocins have been reported to be produced by extremely halophilic bacteria. Some of the halocins characterized and studied include halocin H4 from *Haloferax mediterranei* R4 (19), halocin H6 from *Haloferax gibbonsii* (20) and halocin S8 from an uncharacterized extremely halophilic rod –strain S8 (21). Dhanasekaran *et al.* (1) have reported three actinomycetes, from salt pan regions of Cuddalore and Parangipettai, Tamil Nadu, India, showing promising antibacterial activity against eight test organisms. Dhanasekaran *et al.* (22) have also reported six broad spectrum antibacterial *Streptomyces* out of nine (66.7%) from salt pan soil. Four out of nine (44.44%) strains also showed extra- and intra cellular antifungal activity.

Characterization of the active isolates:

As shown in Table 4, the isolates TSK 32 and TSK 71 could utilize six carbohydrates and the carbohydrate utilization pattern of TSK 32 resembled that of TSK 71. The colony morphology on various media and salinity tolerance pattern of both the isolates was also similar. Depending on their substrate utilization and morphology TSK 32 and TSK71 were found to be similar. The isolate number 6 and 12 could utilize eighteen carbohydrates while isolate 17 could utilize ten carbohydrates. The carbohydrate utilization pattern of isolate 7 was stringent. The isolate metabolised mannose, glycerol, salicin and inositol. All the isolates showed the presence of endospores.

Table 4. Biochemical characterization of active isolates.

Sr.No.	Test	6	7	12	17	TSK 32	TSK 71
1	Lactose	-	-	-	-	-	-
2	Xylose	-	-	-	-	-	-
3	Maltose	+	-	+	-	-	-
4	Fructose	+	-	+	+	+/-	+/-
5	Dextrose	+	-	+	-	+	+
6	Galactose	+	-	-	-	-	-
7	Raffinose	-	-	-	-	-	-
8	Trehalose	+	-	+	-	-	-
9	Melibiose	-	-	+	-	-	-
10	Sucrose	+	-	+	+	+/-	+/-
11	L-Arabinose	+	-	+	+	+/-	+/-
12	Mannose	+	+	+	+	+	+
13	Inulin	+	-	-	+	-	-
14	Sodium gluconate	-	-	+	-	-	-
15	Glycerol	+	+	+	+	+	+
16	Salicin	+	+	+	-	-	-
17	Glucosamine	+	-	-	-	-	-
18	Dulcitol	-	-	-	-	-	-
19	Inositol	+	+	+	-	-	-
20	Sorbitol	+	-	+	+	-	-
21	Mannitol	+	-	+	+	-	-
22	Adonitol	-	-	-	-	-	-
23	A Methyl-D-glucoside	-	-	-	-	-	-
24	Ribose	+	-	+	+	-	-
25	Rhamnose	-	-	-	-	-	-
26	Cellobiose	+	-	+	-	-	-
27	Melezitose	-	-	-	-	-	-
28	A Methyl-D-mannoside	-	-	-	-	-	-
29	Xylitol	-	-	-	-	-	-
30	ONPG	-	-	+	-	+	+
31	Esculin	-	-	-	-	+	+
32	D-Arabinose	+	-	+	+	+/-	+/-
33	Citrate	-	-	-	-	+	+
34	Malonate	-	-	-	-	-	-
35	Sorbose	-	-	-	-	-	-
36	Gram character	Positive	Positive	Positive	Positive	Positive	Positive

Based on 16S rDNA sequence and biochemical characterization we assign isolate number 6, 12, 17 and TSK 71 to the genus *Bacillus* (GenBank accession numbers JF430795, JF411054, JF411055 and JF411056 respectively) and isolate number 7 to *Virgibacillus* (GenBank accession number JF411053). *Bacillus* spp. are also marine sediment inhabitants. Nowlan *et. al.* (23) have reported *Bacillus okhensis* sp. nov., a halotolerant and alkalitolerant bacterium from a natural salt pan near Okha, Gujarat, India.

Bacillus okhensis (23) isolated from salt pan in Gujarat utilized dextrose, mannose, L-arabinose, galactose, ribose, xylose and rhamnose but not glycerol, mannitol, and sucrose. Our four *Bacillus* isolates viz. 6, 12, TSK 32 and TSK 71 could utilize dextrose, mannose, glycerol and sucrose but not xylose and rhamnose. Isolate 17 showed a similar pattern but could not utilize dextrose. Only isolate 6 could utilize galactose while isolate number 6, 12 and 17 could utilize ribose and mannitol.

Antimicrobial susceptibility test was carried out for all the active isolates and results were recorded as sensitive or resistant to the antibiotic concentration tested. Our *Bacillus* isolates (6, 12 and TSK 71) were sensitive to methicillin, cefuroxime, cephalexin and also gentamicin and tetracycline but resistant to lincomycin as shown in Table 5. Isolate 17 showed a similar sensitivity pattern but was sensitive to lincomycin as well. Nowlan *et al.* (23) reported that *Bacillus okhensis* was sensitive to lincomycin, methicillin, cefuroxime and cephalexin but resistant to gentamicin and tetracycline.

Table 5. Antibiotic sensitivity test.

Name of the antibiotic	Concentration (µg)	6	7	12	17	TSK 71
Cephalexin	30	S	S	S	S	S
Tetracycline	30	S	S	S	S	S
Cefuroxime	30	S	S	I	S	S
Lincomycin	10	R	S	R	S	R
Methicillin	5	S	S	I	S	S
Gentamicin	10	S	S	S	S	S

I: Intermediate; R: Resistant; S: Sensitive

To conclude, our results indicate that salt pan bacteria from Batim and Ribandar have an interesting antibiotic producing profile and hence can be looked upon as potential resource of antibacterial metabolites. Very few reports are available on the antimicrobial potential of the cultures isolated from Indian salt pans. This study encourages exploration of other salt pans in the discovery of potential halotolerant and halophilic isolates as pharmaceutically important microorganisms with a possibility of being "novel antibiotic" producers.

Acknowledgements

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