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Halophilic and halotolerant actinomycetes from a marine saltern of Goa, India producing anti-bacterial metabolites

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Marine salterns are estuarine ecosystems in Goa, receiving inputs from riverine and marine waters. The Salinity fluctuates between 0 and 300 psu which makes it a conducive niche for salt tolerant and salt loving *Actinomycetales*. Halotolerant and halophilic *Actinomycetales* producing anti-bacterial metabolites were studied from crystallizer pond sediments of Ribandar saltern, Goa. Three media viz. Starch casein, R2A and Inorganic salt starch agar at four different salinities (35, 50, 75 and 100 psu) were used for isolation. R2A agar at 35 psu was the most preferred by hypersaline actinomycetes. The dominant group was halotolerant Streptomyces spp. others being rare actinomycetes viz. *Nocardiopsis*, *Micromonospora* and *Kocuria* spp. More than 50% of the isolates showed anti-bacterial activity against one or more of the fifteen human pathogens tested. Eight strains from 4 genera showed consistent anti-bacterial activity and studied in detail. Most halotolerant isolates grew from 0 to 75 psu, with optimum antibiotic production at 35 psu whereas halophiles grew at 20 to 100 psu with optimum antibiotic production at 35 psu. Four *Streptomyces* strains showed multiple inhibition against test organisms while four rare actinomycetes were specific in their inhibitory activity. This is the first report of a halophilic *Kocuria* sp., *Nocardiopsis* sp., and halotolerant *Micromonospora* sp. producing anti-bacterial compound(s) against *Staphylococcus aureus*, *Staphylococcus citreus*, and *Vibrio cholerae*, respectively. Sequential extraction with varying polarity of organic solvents showed that the extracts inhibited different test pathogens. These results suggest that halophilic and halotolerant actinomycetes from marine salterns are a potential source of anti-bacterial compounds.

[**Key words:** Hypersaline; Halophilic; Halotolerant actinomycetes; Antibiotic; Marine saltern]

Microbial communities in extreme environments have an immense potential as an untapped resource for the discovery of novel microbes of taxonomic significance. These organisms are a prolific source of several important biomolecules which are the results of their evolution and adaptation in terms of metabolic biochemistry such as enzymes, antibiotics, compatible solutes, etc. (1). Among the Domain *Prokaryotae*, *Actinomycetales* inhabit diverse ecological niches including extreme environments and undoubtedly this signifies their existence as psychrophilic, thermophilic, alkaliphilic, acidophilic and halophilic (2). Extremophilic actinomycetes are the most promising source for anti-microbial compounds (3) due to their unique and diverse community structure and various unexplored metabolic pathways existing in various genera even in the level of species variants. A review on actinomycetes of India have highlighted that extreme habitats viz. hot springs, deserts, marine salterns and deep sea oceanic floors need to be explored for novel bioactive compounds (4). In recent years there are few reports on their bioactive metabolites such as siderophores, indole acetic acid (5), antagonistic metabolites against dermatophytes (6) and anti-bacterial metabolites against clinical pathogens (7) from saline soil actinomycetes of the Indian subcontinent.

Marine salterns being coastal ecosystems are known to harbour halotolerant bacteria (which grow at less than 20 psu, i.e., 0.2 M NaCl), which perhaps gain entry from terrestrial ecosystems but have eventually adapted to hypersaline conditions with time. However autochthonous halophilic bacteria, (grow at 20–300 psu; 0.25 M–5.1 M NaCl) have an obligate requirement of salt for growth (8). These organisms hold a rich bioresource for the discovery of unique and novel microbes with a potential to produce new chemical entities of biotechnological importance. But till date there is no comprehensive study documenting the study of diversity of halophilic and halotolerant actinomycetes from marine salterns producing bioactive compounds. Ribandar saltern is situated along the Mandovi estuary, Goa. The salinity decreases as low as 5–10 psu during the monsoon seasons and reaches high values up to 350 psu during the non-monsoon or salt manufacturing season. In the salt crystallizer ponds, sea water gains entry during the high tide and is concentrated up to saturation levels by evaporation. These crystallizer ponds represent a unique marine hypersaline environment, with salinity levels from 10 to 350 psu; pH 6 to 9 and temperature 10°C to 42°C (9). Thus crystallizer ponds could be a potential source for known and rare actinomycetes genera with the capacity to produce novel secondary metabolites. In the present study our focus lies on exploring hypersaline

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actinomycetes for anti-bacterial metabolites from a marine saltern of Goa, India.

MATERIALS AND METHODS

Sample collection Four sediment samples were collected with the help of the sterile corers (0–5 cm) from four different crystallizer ponds of Ribandar saltern (15° 30.166 N and 73° 51.245 E), Goa, India during the peak salt manufacturing season (April–May 2012). Sediment cores were sealed right after collection and immediately brought to the laboratory for further processing. The physico–chemical parameters viz. pH and temperature were measured using a hand held multi-parameter device (Eutech Instruments, Singapore) whereas salinity with a hand held refractometer (S/MillE, Atago, Co. Ltd., Japan) during sample collection.

Isolation of halophilic and halotolerant actinomycetes Halotolerant and halophilic actinomycetes were isolated from sediment samples in three different media viz. Starch casein agar (soluble starch 10 g/L; casein powder 1 g/L; sea water 37 g/L; agar 15 g/L; pH 7.2 ± 2), R2A agar (casein acid hydrolysate 0.5 g/L, yeast extract 0.5 g/L, proteose peptone 0.5 g/L; dextrose 0.5 g/L; soluble starch 0.5 g/L; di-potassium phosphate 0.3 g/L; magnesium sulphate 0.024 g/L; sodium pyruvate 0.3 g/L; pH 7.2 ± 2) and Inorganic salt starch agar (soluble starch 10 g/L; di-potassium phosphate 1 g/L; magnesium sulphate 1 g/L; ammonium sulphate 2 g/L; calcium carbonate 2 g/L; ferrous sulphate 0.001 g/L; manganous chloride 0.001 g/L; agar 15 g/L; pH 7.2 ± 2) at 4 different salt concentrations, 32, 50, 75 and 100 psu. The isolation media used in the study was also supplemented with natural sea salt from salterns of Goa and was used to simulate the ecobiome. Sediment sample (10 g from the mixed core 0–5 cm) were added to 90 ml of sterile sea water (32 psu) in 250 ml conical flask and kept on the shaker at 120 rpm for 30 min to obtain a uniform suspension. Sediment slurry (10 ml) in 90 ml of salt pan water was used for enumeration. Further dilutions were made in sterile salt pan water (by adding 10 ml–90 ml of the salt pan water). Further the 10^{-1}, 10^{-2} and 10^{-3} dilution were plated (100 µl) on to each media plate supplemented with Nalidixic acid (25 µg/ml) and Nystatin (25 µg/ml) at the respective salinities. Plates were incubated at 28°C ± 2 for 4 weeks. Morphologically different actinomycetes colonies were picked and further subcultured onto their respective isolation media and salinities. The isolates were stored at 4°C on slants and as glycerol stocks at −20°C.

Identification Identification was carried up to genus level, based on morphological characterization and microscopic observation. Morphologically characteristics of the isolates were studied based on 7 International *Streptomyces* Project (ISP) media as described by Shirling and Gottlieb (10). Based on the spore production 70 isolates were initially divided into *Streptomycete* and non-*Streptomycete* (rare actinomycetes). *Streptomycete* isolates were differentiated based on the colour and appearance of the aerial and substrate mycelia and presence or absence of extracellular diffusible pigments. Cultures were grown on media plates with coverslips, placed at an angle of 45° in the agar to promote mycelial growth and sporulation. Mycelial filaments and spore structures on the coverslip were visualized using light microscopy (at 100×) and scanning electron microscopy (SEM-Hitachi TM 3000 Table Top Electron Microscope). The isolates were Gram stained. The isolates were grouped into genera based on the characteristic colony morphology, mycelial pattern and spore chain structure. Identification based on morphology of the rare actinomycetes genera was done as suggested by Al-Zarban et al. (11), Li et al. (12), Kim et al. (13), and Tanasupawat et al. (14).

Screening for the production of anti-bacterial compounds Seventy isolates were grown in their respective media broth used for isolation, and the activity of the anti-microbial compound was assessed with 15 different human pathogenic bacteria viz *Aeromonas hydrophila*, *Acinetobacter baumannii*, *Citrobacter freundii*, *Escherichia coli*, *Klebsiella* sp., *Morganella morganii*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Salmonella typhimurium*, *Shigella dysenteriae*, *Shigella boydii*, *Staphylococcus aureus*, *Staphylococcus citreus* and *Vibrio cholerae* using agar disc diffusion assay. The culture supernatant (10 µl) was loaded onto sterile Whatman no. 1 filter paper discs (5 mm), dried and then placed onto a seeded bacterial lawn grown on Nutrient agar plates. Zones of inhibition were measured after 24 h incubation at 37°C using Hi-Antibiotic ZoneScale (HiMedia). Assays were repeated in triplicates to confirm the consistent production of anti-bacterial metabolites along with media controls. The data were tabulated as mean ± standard deviation (SD).

Growth and antibiotic production at various salinities Eight selected isolates showing maximum production of anti-bacterial metabolite(s) were further studied for their salt requirement for growth and proliferation at 0 psu (no salt), 10, 20, 35, 50, 75 and 100 psu salt supplement in the broth medium. The isolates were spread plated onto their respective agar media and then allowed to grow for 2 weeks at 28°C ± 2. Spores were collected from agar plates by adding either 1 ml of sterile saline water of the respective salinity in the experimental broth medium (in case of non-sporulating cultures, vegetative cells were used). The spore and cell suspensions were collected in sterile eppendorf tubes and the spore or cell density was adjusted ~0.3 at OD_{590} with sterile saline water and 100 µl of this suspension was inoculated into 50 ml of the respective isolation media broths with respective salt concentrations. The flasks were kept in shaker incubator for 2 weeks (28°C ± 2). The cells and mycelia were pelleted down, lyophilized and the dry weight was calculated. The culture supernatant was checked for the production of anti-bacterial compounds against the inhibited pathogens by agar disc diffusion assay after a period of one week.

Identification of the eight selected actinomycetes by 16S rRNA gene sequencing Eight selected (both halotolerant and halophilic) actinomycetes strains which grew high salinities and consistently producing anti-bacterial compounds, were identified using 16S rRNA gene sequencing. Genomic DNA was isolated using Genomic DNA isolation kit (Chromous Biotech) and this DNA was further used for the PCR reaction for the amplification of 16S rRNA gene using bacterial universal primers for 16S rRNA gene; (i) forward primer: 5′-CCGAATTCGTCGACAAC AGAGTTTGATCCTGGCTCAG-3′ and reverse primer: 5′-CCCGGGATCCAAGCTTAC GGCTACCTTGTTACGACTT-3′ and (ii) forward primer 27F: AGAGTTTGATCCTGGCTC-CAG and reverse primer 1492R: TACGGTTACCTTGTT ACGACTT. The expected 1.5 kb length band was excised with the help of a sterile scalpel and gel elution was carried out using GE Healthcare, illustra GFX PCR DNA and Gel band purification kit. These eluted products were used as templates for the sequencing PCR using both the forward and reverse primer (mentioned above) separately using Applied Biosystem Kit. The amplified PCR products were sequenced using Applied Biosystem DNA analyser and sequences were screened using BioEdit software and aligned with Clustal W (15). These sequences have been submitted to the GenBank database under accession numbers KC166135.1, JQ424911, KC166136.1, JX235970.1, KF175508, KF208685, JX291105.1 and KC765077. The 16S rRNA gene sequence based phylogenetic tree was constructed using MEGA4 (16) software by Neighbour-joining method. *Bacillus subtilis* strain BCRC 10058 was used as an out group.

Sequential extraction of anti-bacterial compound(s) using various organic solvents From the eight potential antibiotic producing isolates, four from each genera were selected based on the highest activity and consistent production of anti-bacterial metabolite for further studies. Each isolate was scaled up for extraction of the anti-bacterial metabolite(s) with organic solvents. For scaling up, each culture was inoculated into 25 ml culture broth at 35 psu (being the optimum salt concentration for antibiotic production) and grown for 7 days in a shaker incubator at 140 rpm and 28°C ± 2°C temperature. This media was scaled up to 500 ml using the above 25 ml as an inoculum and incubated for 2 weeks. The culture broth was then centrifuged at 13,000 rpm for 5 min at 4°C. The culture supernatant was decanted and collected. Sequential extraction was carried out with six different organic solvents ranging from non-polar to polar solvents, (petroleum ether > hexane > diethyl ether > chloroform > ethyl acetate > butanol) in a volume ratio of 1:3 (culture supernatant: organic solvent). The organic layer was evaporated to dryness using a rotary vacuum evaporator (Equitron Roteva) at 45°C and the resultant residue was then dissolved in 1 ml of the respective organic solvent and then checked for its bioactivity against pathogens.

RESULTS

Halophilic and halotolerant actinomycetes The abundance of halophilic and halotolerant actinomycetes from the sediments of crystallizer pond at 4 different salinities and in 3 different isolation media is shown in Fig. 1. The actinomycetes numbers were high at lower salinities (35 psu and 50 psu), suggesting the abundance of halotolerant actinomycetes in this saltern. R2A agar was found to be a suitable medium for isolation of hypersaline actinomycetes, irrespective of whether the isolates were halotolerant or halophilic.

Characterization of the actinomycetes Based on their growth in ISP media and morphological observations, the 70

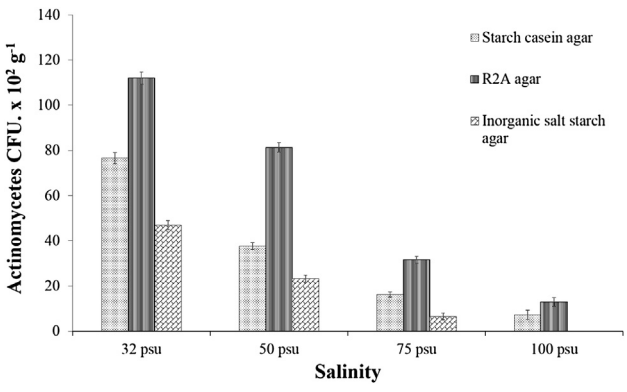

FIG. 1. Abundance of actinomycetes from saltern sediment in 3 different media.