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Bioprospecting of Halotolerant *Bacillus subtilis*: A Study depicting its Potential Antimicrobial Activity against Clinically Important Pathogens

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Abstract

Objectives: The current study focuses on characterization and isolation of bioactive compounds from a marine halophilic bacterium and testing its antimicrobial potentiality against clinically important pathogens like *Proteus mirabilis, Salmonella typhi A, Salmonella typhi B, Pseudomonas aeruginosa* and *Klebsiella pneumoniae.* The study also portraits presence of possible bioactive compounds responsible for such antimicrobial activity through Gas Chromatography-Mass Spectrometry (GC-MS). **Methodology:** Surface water samples were collected from Kovalam beach at Thiruvananthapuram, Kerala state, a South Indian coast of Indian peninsula and were screened for potential bacteria. Morphological, biochemical and molecular profiling were performed to identify and isolate the bacterium of interest. Antibiogram studies were conducted to understand pathogenic trends in the isolate. Culture supernatant of the test bacterium was screened against clinically important pathogens. Further, crude extract of the culture supernatant was subject to GC-MS analysis. **Findings:** Morphological, biochemical and molecular profiling of the isolate revealed the organism to be *Bacillus subtilis.* The culture supernatant had significant activity against clinically important pathogens. GC-MS analysis revealed possible antimicrobial compound to be (Pyrrolo [1, 2-a] pyrazine-1, 4-dione). **Novelty:** The study has significant importance as it depicts antimicrobial activity of halophiles against potent clinical pathogens.

Keywords: Antibiogram Profiling, Bioactive Compounds, Gas Chromatography-Mass Spectrometry (GC-MS), Marine Halophiles, Molecular Profiling

1. Introduction

Over the past twenty years there has been an unprecedented rise in emergence of multidrug resistant bacterial strains in the field of medicine, making it laborious in the treatment process[1]. Anthropogenic emissions of hospital / industrial wastes into aquatic sources, spraying of pesticides in the farmland for pest control and exploitation of antibiotics in therapies could have triggered rising trends in antibiotic resistance among bacteria. It is estimated that at least 10 million people will die every year owing to Antimicrobial Resistance (AMR) until a global solution is mounted[2]. It is alarming to witness common environmental pathogens like *Acinetobacter baumannii* and *Klebsiella pneumonia*

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are turning to Multidrug Resistant (MDR) and are currently untreatable with commonly used antibiotics[3]. Horizontal gene transfer of resistant plasmids from the clinical pathogens has led to the emergence of MDR in environmental and clinical isolates. It would have taken place through transfer of plasmids, intergrons, genomic islands, insertion elements, mobile genetic elements or through bacteriophages[4]. Some resistant genes are also found cardinal in the chromosomes of many environmental bacteria.

Colistin has been considered to be the last resort antibiotic in medicine for treating MDR bacteria like *Escherichia coli* which exhibited resistance to almost all drugs types[5]. With recent discovery of resistance to colistin through MCR-1 genes, a mobile DNA which can be transferred among bacteria is raising questions on our situation to post antibiotic era[6]. According to the reports released by World Health Organization, the number of currently available antibiotics is insufficient to meet the rising issue of antibiotic resistance[7]. It is understood that, to cope the modern-day crisis of antibiotic resistance, there is an urgent need of finding novel antibiotic molecules with significant drug efficacy at regular interval.

About 70% of the earth is covered with sea and remains as an unexplored source of different beneficial microorganisms capable of producing bioactive molecules. Halophiles are extremophilic organisms that thrive in environments with very high concentrations of salt that is five times greater than that of salt concentration of the ocean[8]. Halophilic bacteria have been explored industrially and biotechnologically for its potentiality to produce different bioactive compounds with antitumor, anticancer, cytotoxic and antimicrobial activities[9]. The present study focuses on isolation of halophilic bacteria from marine sediments of Kovalam beach of Thiruvananthapuram, Kerala state. Through our research, we further portraits screening of a particular bacterium capable of inhibiting growth of clinically important pathogens. Through GC-MS analysis we further predict the chemistry of possible bioactive compound responsible for its antimicrobial activity and also lay footprints for scientist to explore further in characterization of this bioactive compound in future.

2. Materials and Methods

2.1 Sample Collection

Water sample were collected from Kovalam beach at Thiruvananthapuram (8.4004° N, 76.9787° E), Kerala on 12th of December 2018. The temperature of the location was between 27°C-30°C at the time of sampling. Surface water sample were collected in one-liter sterile plastic container, well-sealed and transported to the Dr. Thacharodi's Laboratories, Puducherry, India in ice cold condition at 4°C for further processing [Figure 1].

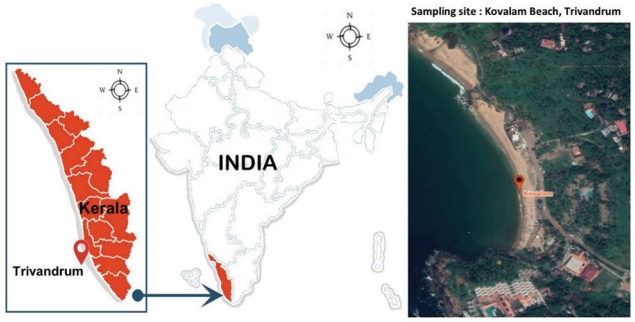

Figure 1. Sample isolation.

2.2 Isolation of Halophilic Bacteria

The water samples were taken into sterile hood and standard serial dilution procedures were carried out. Plating were done in triplicates on Luria-Bertani Agar (HiMedia Laboratories, India) and Zobell Marine Agar (HiMedia Laboratories, India) and incubated overnight at 37°C for 24 hrs.

2.3 Characterization of the Isolates

After incubation, a particular colony with most prominent zone of clearance with communal bacteria was picked up. This zone of clearance producing bacterium was then analyzed for its macroscopic characterization based on the colony morphology, pigmentation, colony elevation and size. Microscopic characterization was also performed using Gram staining. Conventional biochemical screening methods were performed which includes MR-VP tests, cytochrome oxidase activity analysis, mannitol motility test, triple sugar iron tests, nitrate reduction tests and urea reductase tests. Utilization of carbohydrates and fermentation of amino acids were tested using sugars like D-Glucose, Sucrose, Lactose, Galactose, D-Fructose,

Xylose, Dextrose, Arabinose, Raffinose and Mannitol and Amino acid like Cysteine, Tyrosine, Histidine and Arginine[10].

2.4 Molecular Characterization of the Strain

Molecular characterization of the isolate was performed using 16S rRNA sequencing. Universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3')[11] were used for characterization of the isolate. DNA extracts from cells and the 16S rRNA sequence was determined by fluorescent dye terminator method using the sequencing kit (ABI Prism Big dye terminator cycle sequencing ready reaction kit v.3.1). Products were run on an ABI13730XL capillary DNA sequencer (ABI Prism 310 genetic analyzer, Korea). The aligned sequences were computed using ClustalW software and sequence homologies were determined using BLASTn search to create an evolutionary distance matrix.

2.5 Scanning Electron Microscope

The bacterial cells were immobilized on a coverslip for 1 hr. at 24°C using 2.5% of glutaraldehyde in 0.1 M sodium cacodylate buffer of pH 7.2. Serial concentrations of ethanol were used as a dehydrating agent to wash the cover slip. Dehydrated bacterial cells were analyzed after gold sputtering under scanning electron microscope (JSM-7900F Schottky Field Emission SEM).

2.6 Antibiogram Profiling

Antibiogram profiling was performed for the bacterial isolate using Kirby-Bauer disc diffusion method. The test was done on Muller Hinton agar (HiMedia Laboratories, Mumbai, India) plate by impregnating with different antibiotic discs[12]. The antibiotic discs used in the study includes 30 mcg of Ceftriaxone (CTR), 300 mcg of Cefpodoxime (CPD), 300 mcg of Polymixin (PB), 30 mcg of Gentamicin (GEN), 300 mcg of Nitrofuranton (NIT), 30 mcg of Nalidixic acid (NA), 30 mcg of Chloremphenicol (C), 30 mcg of Ceftazidime (CAZ), 10 mcg of Ampicillin (AMP), 30 mcg of Tetracyclin (TE), 10 mcg of Norfloxacin (NX), 15 mcg of Erythromycin (E), 10 mcg of Penicillin G and 30 mcg of Vancomycin (VA) (HiMedia Antibiotic sensitivity disc). The experiment was performed by using overnight bacterial cultures of 0.1 OD with an incubation time of 24 hrs at 37°C. The results were interpreted using Clinical Laboratory Standard Institute guidelines (CLSI 2010)[13].

2.7 Antimicrobial Assay against Clinical Pathogens

The test organism was inoculated in 30 ml Luria-Bertani broth and was maintained on a rotary shaker at 220 rpm for 24 hrs at 37°C. The incubated culture was then taken and centrifuged at 10,000 rpm for 5 mins at 4°C. The supernatant was transferred into new falcon tubes and filter sterilized with 0.2 µm filter[14]. *Proteus mirabilis, Salmonella typhi A, Salmonella typhi B, Pseudomonas aeruginosa* and *Klebsiella pneumonia* were tested with this supernatant for its antimicrobial activity. The assay was performed in 96 well plates with 100 µL log phase culture of all the isolates and 100 µL of LB broth as control. The test wells had 100 µL of the log phase cultures of different isolates and 100 µL supernatant of the test organism incubated at 37°C on a rotary shaker at 100 rpm for 12 hrs. After Incubation, the cell density in wells were determined using plate Reader (Bio-Tek) at optical density of 595 nm.

2.8 Crude Extract Preparation

The test isolate was inoculated in Luria-Bertani broth (1 liter) (HiMedia Laboratories, Mumbai, India) supplemented with 2% NaCl and incubated at 37°C for 4 days on a rotary shaker at 180 rpm. Then the broth was filtered using Whatman No 1 filter paper and the filtrate was blended with equal volume of Chloroform (1:1). The mixture was transferred into a separation flask, agitated vigorously for 5 hrs and kept static overnight. The chloroform organic phase was separated and was brought to dryness by evaporation using rotary-vacuum evaporator (BioRad, USA)[15].

2.9 Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

The crude extract of cell free supernatant of the test isolate was studied using GC-MS (GCMATE II GC-MS, Agilent Technologies 6890N Network GC system for GC) to identify the potential antibacterial compound[16]. Fused silica column (HP5) of 50 m x 0.25 mm L.D was used for separation. Analysis conditions were set at 100°C for 25 min, column temperature maintained at 200°C for 5 min, followed by injection temperature of 220°C and helium

as carrier gas with split ratio of 5:4. A run time for 40 minutes was set and the samples were injected in a split less injector at 300°C. GC coupled with MS was used to identify the compounds of interest by matching the hits with the referral compounds available.

3. Results and Discussion

3.1 Sample Isolation and Characterization

Mixed colonies of both gram negative and gram-positive bacteria were obtained from marine water samples. Colony with most prominent zone of clearance (inhibition) against communal bacteria in the petri plates were picked up. Morphological characterization of the isolate (JAAK-P) revealed them to be Gram positive rods with irregular pinpoint colonies identified under the genus Bacillus. SEM imaging revealed them to be approximately 0.5 µm in thickness when observed under 35,000X (magnification) [Figure 2].

Gram positive rods with pinpoint colonies are common salient features of Bacillus sp[17]. Genotypic characterization using 16S sequencing and comparative analysis using Blast tool of NCBI revealed them as close relatives of *Bacillus subtilis* (NCBI accession no: MN049469)

Figure 2. SEM image of the Isolate visualized at X35000.

3.2 Biochemical Characterization and Amino Acid Breakdown

Biochemical profiling for assimilation of sugars as a sole carbon source revealed the ability of isolate to ferment all sugars used in the studies except arabinose. The results have shown concomitance with previous studies[18]. The isolate JAAK-P were found incapable of either gas production or utilizing Indole broth. Amino acid decarboxylase test also revealed their inability in hydrolyzing amino acids used in the study. Our results showing, *Bacillus subtilis* capable of hydrolyzing gelatin is previously reported using rat skins gelatin[19].The complete profiling of the isolates has been explained in Table 1.

Table 1. Characterization of the isolate

S.No	Biochemical analysis	Results
1	Indole	
2	MR	
3	VP	
4	Citrate	
5	Catalase	
6	Urease	
7	Motility	
8	Gas Production	
9	Gelatinase	
	Carbohydrate fermentation test	
10	Glucose	
11	Sucrose	
12	Fructose	
13	Galactose	
14	Lactose	
15	Maltose	
16	Xylose	
17	Raffinose	
18	Arabinose	
19	Cellobiose	
	Amino acid fermentation	
20	Arginine	
21	Cystine	
22	Histidine	
23	Tyrosine	
	Positive results	
	Negative results	

3.3 Antibiogram Profiling

The Antibiogram profiling of JAAK-P revealed that they are resistant towards Vancomycin, Penicillin G and Polymyxin B. They also showed intermediate resistance towards Ampicillin, Ceftazidime and Nalidixic acid which

makes them to be resistant to three classes of Antibiotics falling under multidrug resistant category. Polymyxin are last resorts of antibiotics and resistance to polymyxin are rare. Identification of polymyxin synthetase genes in Bacillus subtilis could be possible results of such resistance[20]. The complete antibiogram profiling of JAAK-P has been explained in Table 2.

3.4 Antagonistic Activity of Culture

Table 2. Antibiogram profiling of JAAK-P

S. No	Antibiotics	Results
1	Vancomycin	
2	Penicillin G	
3	Ampicillin	
4	Erythromycin	
5	Gentamicin	
6	Polymyxin B	
7	Cefpodoxin	
8	Cefpodoxime	
9	Norfloxacin	
10	Tetracycline	
11	Ceftazidime	
12	Chloramphenicol	
13	Nalidixic acid	
14	Nitrofurantoin	
	Sensitive	
	Intermediate	
	Resistance	

Supernatant against Clinical Pathogens

The overnight culture supernatant which was tested for its antimicrobial activity against important clinical strains of *Proteus mirabilis, Salmonella typhi A, Salmonella typhi B, Pseudomonas aeruginosa* and *Klebsiella pneumonia* had significant results. Significant results of inhibition were observed in both 12 and 24 hrs study [Figure 3].

This significant result further prompted us in analyzing possible secondary metabolite in culture supernatant using Gas Chromatography-Mass Spectrometry.

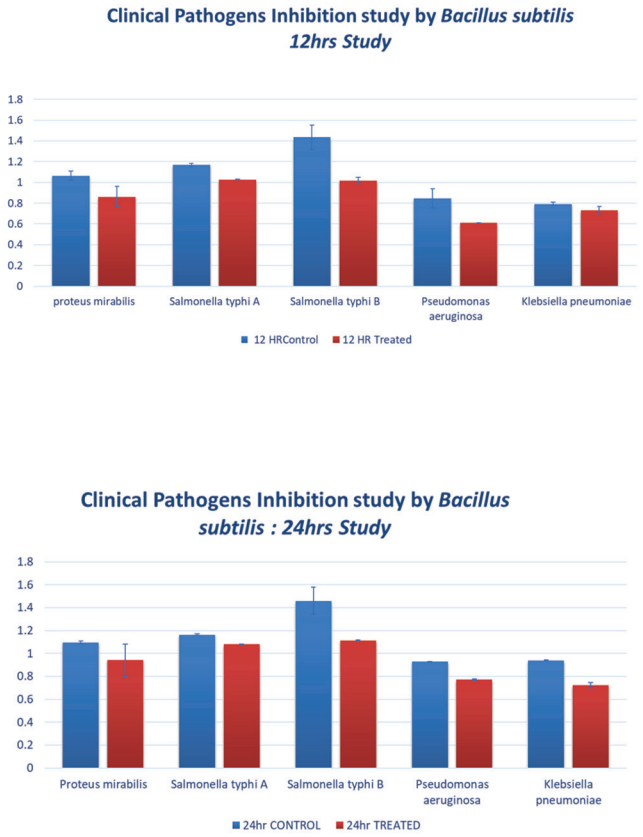

Figure 3. Antagonistic activity of culture supernatant of JAAK-P.

3.5 GC-MS Analysis of the Crude Extract

GC-MS analysis of the crude extracts indicates that isolates had a majority of peaks obtained from 19.13 min to 29.06 min of Retention Time (RT) which is being displayed in the chromatograms of crude extract [Figure 4].

NIST library analysis reviled that majority of compound were derivatives of aliphatic and alkane hydrocarbons. Of which, (Pyrrolo[1, 2-a] pyrazine-1, 4-dione, hexahydro-3-(2-methylpropyl) eluted at 26.70 min of retention time is our field of interest as it has been reported to possesses antimicrobial activities against clinical pathogens.

4. Conclusion

Pyrrolo pyrazine compound has been previously reported to possess antimicrobial activity against clinical pathogens and certain environmental strains[21]. Purified pyrrole [1, 2-a] pyrazine-1, 4-dione, hexahydro-3-(2-methylpropyl) from sponge associated marine bacterium

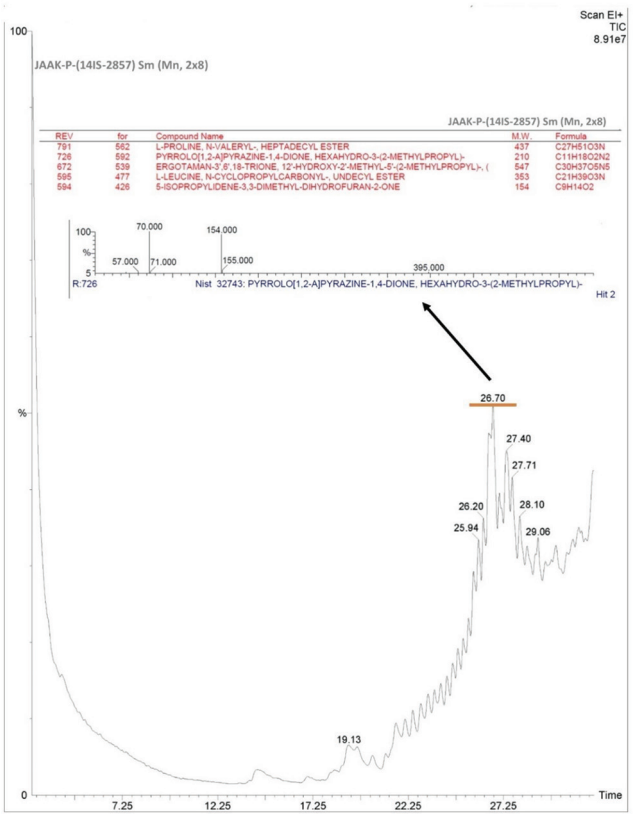

Figure 4. GC-MS analysis of crude extract from JAAK-P.

has also depicted biofilm disruption activity in *Loktanella honkongensis* and *Vibrio halioticoli*[22]. It has also been reported that chloroform extracts of pyrrolo [1,2-a] pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl) extracted from Antarctic endophytic fungus is capable of inhibiting growth of *E. coli* with a MIC of 26.9 µg/mL, *P. aeruginosa and Enterococcus faecalis*, with a MIC of 107 µg/mL[23]. The study thus portraits potentiality of the isolated halophilic bacterium to produce the compound pyrrolo [1, 2-a] pyrazine-1, 4-dione, hexahydro-3-(2-methylpropyl) and its biological significances that could be exploited in the field of medicine in developing novel antibiotics.

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