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ORIGINAL RESEARCH ARTICLE

Isolation and Identification of Bioactive Compounds from *Bacillus Megaterium*e5 from the South East Coastal Region of India against Urinary Tract Infectious Pathogens

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ABSTRACT

Fifty-one bacterial colonies were isolated from water sample of South east coast region of India (Tamil Nadu). All these isolates were culture-purified and screened for antimicrobial activity against a battery of Urinary tr act inf ections (UTI) pathogens such as *Escherichia coli, Klebsiella* sp., *Pseudomonas aeruginosa, Proteus vulgaris, Enterococcus faecalis* and *Candida albicans*. These pathogens were procured by PSG Institute of Medical Science and Research, Coimbatore, collected from PSG hospital. Among these,31 % of the isolates (n=31) exhibited strong bactericidal properties against pathogens. One promising strain, designated as E5,with strong antimicrobial activity against most of the pathogens tested was selected for further studies. Morphological, biochemical properties and molecular characterization, 16S rRNA sequencing and BLAST analysis Synergy Scientific Center, Chennai, Tamil Nadu, indicated this s train as *Bacillus megaterium* (100% s imilarity with Gen Bank s equences). The s elected and identified *Bacillus megaterium* was grown and extracellular extract was taken, for GC-MS analysis.

Keywords: Antagonistic activity, *Bacillus megaterium*, Urinary t ract infections pathogens, Secondary metabolites and GC-MS analysis.

1. INTRODUCTION

Marine organisms are a rich source of structurally novel and biologically active metabolites. So far, many chemically uni que c ompounds of m arine origin with different bi ologically a ctivity h ave been i solated a nd a nu mber of t hem are unde r investigation are being de veloped as ne w pharmaceuticals [1]. Among the thr ee ma jor habitats of the biosphere, the marine realm which covers 70% of the ear th's s urface pr ovides t he largest inhabitable space for living organisms, the study of marine bacterial diversity is important in order t o unde rstand t he c ommunity s tructure a nd pattern of di stribution. T he s ea h arbors a n extensive popul ation of bacteria, va rying greatly in num bers a nd i n t he variety o f t heir a ctivities. Microorganisms a re w idely di stributed i n s ea water and on the ocean floor, where the influence chemical, physiochemical, geological, and biological conditions [2].

Urinary tract infections are serious health problem affecting m illions of pe ople e ach year. U rinary tract infections a ccount f or a bout 8.3 m illion doctor visits each year. O ne w oman i n f ive develops a UTI during her life time. *Escherichia coli, Klebsiella, Streptococcus pyogens, Streptococcus faecalis, Pseudomonas vulgaris* are the bacteria responsible for urinary tract infection. Commonly us ed antibiotics f or the tr eatment o f urinary tract i nfection are Clindamycin, Vancomycin, Bacitracin, Ampicillin, Chloramphenicol and E rythromycin. T hese antibiotics m ay r educe t he bur den but i t ha s its own s ide effects. T o ove rcome t he pr oblems associated with antibiotic treatment, people turned traditional me dicine like A yurveda, S iddha, Unani, Homeopathy and Herbal medicines. About 80% of world populations rely on herbal medicine for primary health care [3,4].

In this present, we reported the characterization of *Bacillus megaterium* E5 recently isolated from the south east coastal environment of T amil N adu (South I ndia). T he pr esent s tudy focuses on t he production of s econdary m etabolite f rom t his isolate w ith treatmendous antimicrobial pot ential. Our r esults pr ovide i nsights i nto t he w ide spectrum a ntimicrobial ability of the ide ntified

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Bacillus species from the Indian coastal environment.

2. MATERIALS AND METHODS

2.1. Isolation of organisms from coastal waters

Fifty-one bacterial isolates from coastal water samples at Rameswaram, Pamban, Dhanushkodi, Kilakarai, Uchapuli, Sethukarai, Erwadi and Devipatinam, Tamil Nadu, India was studied. Samples were collected in sterile plastic containers according to microbiological procedures and shifted to the laboratory for further analysis. All the bacterial colonies were isolated marine agar (High Media), purified and screened for antimicrobial activity against a battery of urinary tract pathogenic microorganisms [5].

2.2. Screening for marine bacteria for antagonistic activity

Primary and secondary screening: A total of 51 bacterial strains isolated (Marine Agar 2216, High Media) from the coastal water samples were screened for the production of antibacterial substance. In the primary screening, antimicrobial activity was assessed against the target pathogenic microorganisms [5]. From the pure cultures, marine bacterial strains were spotted on target organisms swabbed in Muller Hinton Agar (MHA) plates. Based on the zone of inhibition, each bacterial strain was selected for secondary screening and further analysis.

Secondary screening was done by agar well diffusion assay for testing the antagonistic activity of marine bacterial isolates. The isolated bacteria were inoculated in 300 ml of production media composed of marine broth with ferric phosphate for the production of secondary metabolites. Flasks were incubated on the rotary shaker at 220 rpm at 25ºC for 7 days. The broth was centrifuged at 5000 x g for 30 min to pellet the cells. The supernatant was collected and extracted thrice with ethyl acetate crude extract was concentrated by evaporation at 37°C. The residue obtained was dissolved in methanol. The crude extract was used for further antimicrobial activity screening. The wells were cut using a sterile cork of 6 mm diameter and 50 µl of supernatant was loaded into each well for assay of antagonistic activity by well diffusion assay against different pathogens [6]. The results were recorded after incubation at 37°C for 24 hrs.

2.3. Identification of antibiotic – producing strain

The isolated marine bacterial strain with antibacterial activity was identified to the species level by observing its morphology and biochemical reaction according to the methods described by Bergey's manual for systemic of bacteriology [7].

2.4. Genomic DNA Extraction

The selected colonies were inoculated in Artificial Sea water Nutrient broth and incubated overnight at 28°C. The culture was spun at 7000 rpm for 3 min. The pellet was resuspended in 400 µl of Sucrose TE. Lysozyme was added to a final concentration of 8 mg/ml and the solution was incubated for 1hr at 37°C. To the tube,100 µl of 0.5M EDTA (pH 8.0), 60 µl of 10% SDS and 3 µl of proteinase K were added and incubated at 55ºC overnight. Extracted with equal volume of phenol:chloroform (1:1), centrifuged (10000 rpm; 10 min) and the supernatant was transferred to a sterile tube. The supernatant was extracted twice with phenol:chloroform and once with chloroform: isoamylalcohol (24:1) and ethanol precipitated. The DNA pellet was resuspended in sterile distilled water and stored at4°C for immediate use and at 20°C for long-term storage [8].

2.5. PCR Amplification of 16S rRNA

Bacterial 16S rDNA was amplified from the extracted genomic DNA by using the following universal eubacterial 16S rRNA primers: forward primer 5'AGA GTT TGA TCC TGG CTC AG 3' and reverse primer 5' ACG GCT ACC TTG TTA CGA CTT 3'. Polymerase chain reaction was performed with a 50 -µl reaction mixture containing 2 µl (10 ng) of DNA as the template, each primer at a concentration of 0.5 µM, 1.5 mM $MgCl_2$, and each deoxynucleotide triphosphate at a concentration of 50 µM, as well as 1 U of Tagpolymerase and buffer used as recommended by the manufacturer (MBI Fermentas). After the initial denaturation for 4.5 min at 95°C, there were 40 cycles consisting of denaturation at 95°C for1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min and then a final extension step consisting of 5 min at 72°C; Master cycler Personal (Eppendorf, Germany) was used. The amplification of 16S rDNA was confirmed by running the amplification product in 1% Agarose gel electrophoresis in 1X TAE [8].

2.6. Sequence and Phylogenetic analysis

Sequence analysis was performed with sequences in the NCBI database using BLAST as well as with the sequences available with Ribosomal Database Project (RDP). These Sequences were aligned by using the Clustalw program and phylogenetic tree was constructed using software from the PHYLIP package [9].

2.7. Gas chromatography-mass spectrophotometer (GC-MS) analysis

Analysis of crude extract was conducted using an AB 45M F (Thermo, 8000 series И GC hyphenated with 5989 Mass Spectrometer). MS conditions were as follows: Detector mass spectrometer voltage 70eV and its source temperature was 300 ^{o}C. The injector temperature was 250 ^{o}C and the splitless mode 0.5µL injection. The oven was adjusted at 80°C for 1 min and initial time 1.5 min with 40 ^{o}C which ended by a final temperature of 300 ^{o}C and 4 min hold time where the total run time was 15 min. The components were identified by comparing their retention times with those of authentic samples, as well as by comparing their mass spectra with those of Wiley 275 Library. Quantitative data were obtained by the peak normalization technique using integrated FID response [10].

3. RESULTS

Overall, 51 strains of marine bacteria were isolated from eight places water samples of the south east coastal region of India, but only one-non pigmented bacteria was exhibited the capability of producing antibiotic compounds.

3.1. Antagonistic activities – primary and secondary screening

Out of bacterial isolates screened in the preliminary study, 6 (12%) were selected for secondary screening. These isolates were able to extract an inhibitory effect against at least one of the target organisms tested. Among the 6 isolates, a strain of *Bacillus megaterium* E5 revealed the efficiency of broads pectrum antimicrobial activity. Hence, the strain E5 isolated from the coastal region of Erwadi was selected for further studies (**Table 1**).

3.2. Identification of *Bacillus megaterium*

Morphological studies revealed that the isolate E5 was non-pigmented, circular and smooth colonies.The cells were Gram positive, citrate negative and urease positive. Biochemical reactions of the isolates with different biochemical are listed in (**Table 2**). On the basis biochemical diagnostic tests, the bacterium was identified as *Bacillus megaterium.*

3.3. PCR amplification and sequencing of 16S rRNA

The amplified product was run on a garose (1%) gel along with λDNA/EcoRI Hind III double digest as marker. The resulting band was observed using gel documentation system. The PCR of E5 was near 1413bp. Partial sequencing of 16S rRNA of the isolated data revealed 100% identity with *Bacillus megaterium* when the sequences were compared with related sequences available in data bases. Hence the strain E5 was identified as *Bacillus megaterium* based on 16S r RNA sequences analysis. The sequence was submitted to NCBI (Accession number JF416939) and deposited as type strain.

3.4. Phylogenetic analysis

The sequences were aligned using CLUSTAL W and a phylogenetic tree was constructed. The isolate (E5) showed (100%) similarity towards *Bacillus megaterium* phylogenetic tree constructed using PHYLIP version 3.68.

3.5. GC-MS Analysis of methanolic extract

GC-MS analyses of the extracts showed 7 major peaks and were identified from wiley library. The compounds molecular weight and name to the corresponded peaks were listed in the (**Table 3 & Fig 2**). From the mass spectrum, we conclude the compounds identified showed antimicrobial activity. The compound responsible for the inhibition of urinary tract organisms will be further purified in future.

4. DISCUSSION

In 1947, Rosenfeld and Zobell Rosenfeld *et al.,*[11] had carried out the first detailed study of antibiotic-producing marine bacteria [12]. Since then, there are several reports of antibiotic-producing marine bacteria showing the antagonistic effect against human pathogens, [13] as strains of pathogenic bacteria that recently emerged are unresponsive or multi drug resistant to the already discovered antibiotics that are in use.

The emergence of resistant strains to commonly used antibiotics. Among human microbial pathogens has necessitate the researches to discover the new antimicrobial agents that are produced in natural way. The oceans, which cover almost 70% of the earth‘s surface [6] contain a variety species, many of which have no terrestrial counterparts. So, marine bacteria as largely untapped source secondary metabolites.

In the present study, the isolates from the coastal region of Erwadi were identified as *Bacillus megaterium* (our laboratory reference – E5) and *Bacillus licheniformis* with haloalkaliphilic culture characteristics. *Bacillus* sp. has been described traditionally as aerobic saprophytic soil microorganisms. Many members of the *Bacillus* group continue to be dominant bacterial

workhorses i n m icrobial f ermentation for t he production of novel proteins [14].

The *Bacillus megaterium* E5 grew well in nutrient broth i n t he pH r ange 8 –9 a nd s howed s alt tolerance up t o 10 % (w/v), NaCl, i ndicating t hat the isolate is an obligate alkalophilic. Although 73 bacterial i solates w ere i dentified in an extensive survey o f m icrobial di versity a t ma rine s alterns near B havanagar, G ujarat, no *Bacillus* sp. ha s been documented [15]. In t he m arine environment, 90% of ba cteria are Gram-negative w ith different characteristics [16] and the Gram-negative cell wall is be tter a dapted f or survival i n the m arine environment. However, i n our s tudy t he *Bacillus* sp. reported were G ram-positive, i ndicating t hat their origin could be due to terrestrial run-off from rivers, as r eported w idely for m arine *Actinomycetes* [17].

In t he pr esent s tudy, t he c ulture s upernatant from *Bacillus megaterium* E5 showed potent and wide-spectrum a ntimicrobial activity. At a c ulture condition of 35 °C, the E5 isolate pr oduced supernatant showing antimicrobial activity against all the organisms t ested (both G ram-positive and Gram-negative ba cteria a nd f ungi). T he r esults revealed a hi gh degree of a ntimicrobial a ctivity (zone of inhibition m ore than or equal to 18 mm) towards *Escherichia coli, Klebsiella* sp., *Pseudomonas aeruginosa, Enterococcus faecalis* (Table-1). Several *Bacillus* isolates de monstrated antagonistic a ctivity against a br oad r ange of bacterial strains *Bacillus subtilis* isolate 259 (from broilers) pr esented the l argest ant ibacterial spectrum, exhibiting inhi bitory a ctivity against several Gram positive species such as *Clostridium perfringens, Enterococcus faecalis, Staphylococcus aureus* and different *Bacillus* species [18,19].

PCR a mplification pe rformed w ith uni versal bacterial 16S r RNA primer produced a f ragment of a pproximately 1500 bp. T he s equence of 16 S rRNA f rom E 5 strain was a ligned against the nearest B LAST sequences us ing th e mul tiple-alignment C ULSTAL W pr ogram. Phylogenitic analysis using 16S r RNA s equences a nd t he neighbor-joining method showed the E5 strain is a member of *Bacillus megaterium* close to *Bacillus cereus, Bacillus mucilaginosus, Bacillus gaemokensis* and *Bacillus massiliensis* (**Fig 1**).

The G C-MS a nalysis of *Bacillus megaterium* methanolic extract s howed that t he ex tract w as seven m ajor c ompounds. T hey were (1) Geranyl 3-methylbutanoat, (2) 1-Dodecanol, (3) Beta-H-Pregnane, (4) 9-Hexadecanoic aci d, (5) 9-Octadecenoic aci d, (6) 1,2-Diazabicyclo 2-2.2.octan-3-one, (7) L-Proline. T he bioactivity o f the compounds resulted from *Bacillus megaterium* methanolic crude extract was estimated and found to be pos itive a gainst ur inary t ract pa thogens t he inhibition zone w ere r anged f rom 13 t o 18 mm. Our da ta pr esented t he h ighest z one of i nhibition against *Enterococcus faecalis*. T his s train of *Bacillus megaterium* E5 with supernatant showing high de gree of thermal stability opens new insight into the future application of antibiotics.

Figure 1 : N eighbour-joining tree s howing t he p hylogenetic relationship o f E 5 t he members of t he g enus *Bacillus*. Booststrap values expressed as percentage of 100 replication

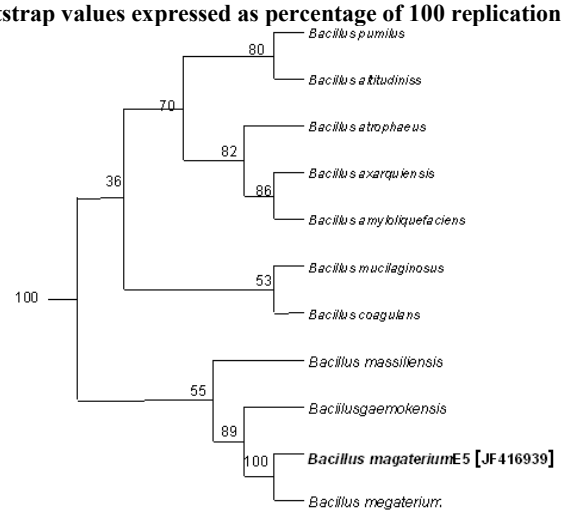

Figure 2: GC-MS Aanalysis of methanolic extract from marine bacteria

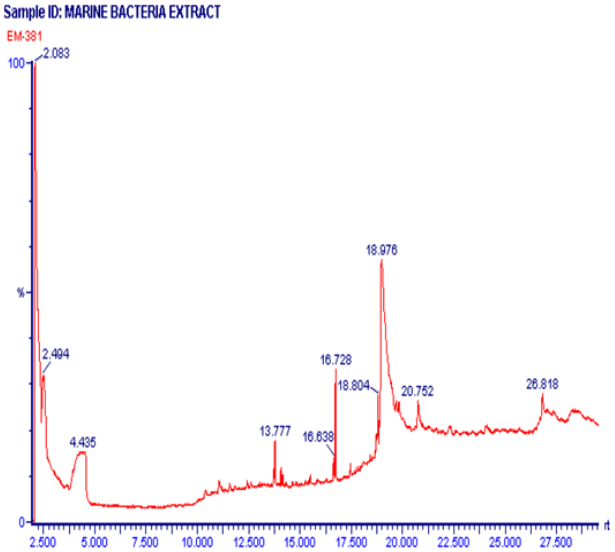

Table 1 : Antimicrobial a ctivity of p rimary a nd s econdary (methanolic ex tract) o f *Bacillus megaterium* (E5) a gainst Urinary tract (UTI) pathogens

	Zone of inhibition (mm)	
Target organisms	**Primary screening**	**Secondary screening (Methanolic crude extract)**
E.coli	11	13
Klebsiella sp.	12	15
Pseudomonas aeruginosa	10	12
Enterococcus faecalis	15	18

Table 2: Morphology and biochemical test for identification of (E5 strain)

S. No	Characteristics	Results
1	Colony morphology	Circular
2	Pigment	White
3	Gram's reaction	Positive
4	Indole test	Negative
5	Methyl red test	Positive
6	VogesProskauer test	Negative
7	Citrate utilization	Positive
8	Urea hydrolysis	Positive
9	Catalase test	Positive
10	Oxidase test	Positive
11	Nitrate test	Positive
12	Gelatin hydrolysis	Negative
13	Sucrose	Negative
14	Glucose	Negative
15	Lactose	Negative

Table 3: GC-MS Analysis of methanolic extract

Peaks	Compound Name	Molecular Formula	Molecular Weight	Activities
4.435	Geranyl 3-methylbutanoat	$C_{15}H_{26}O_2$	238	Antimicrobial activity
13.777	1-Dodecanol	$C_{12}H_{26}O$	186	Antibacterial
16.638	Beta-H-Pregnane	$C_{21}H_{36}$	288	Antibacterial
16.728	9-Hexadecanoic acid	$C_{17}H_{34}O_2$	270	Antimicrobial
18.804	9-Octadecenoic acid	$C_{19}H_{36}O_2$	296	Bronchitis, Rheumatalgia, Pneumonia, Lymphadenitis.
18.976	1,2-Diazabicyclo 2-2.2.octan-3-one	$C_6H_{10}ON_2$	126	Reduce blood microfilaria.
20.752	L-Proline	$C_5H_9O_2$	115	Antibacterial

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