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Isolation and molecular characterization of microorganisms producing novel antibiotics from soil sample

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ABSTRACT

Antibiotics are one of the most important commercially exploited secondary metabolites produced by the bacteria and employed in a wide range. Most of the antibiotic producers used today are the soil microbes. Fungal strains and Streptomyces members are extensively used in industrial antibiotic production. Bacteria are easy to isolate, culture, maintain and to improve their strains. Although thousands of antibiotics have been isolated from soil microbes, these represent only a small part of the repertoire of bioactive compounds so far produced. Majority of the Actinomycetes in soil that are potential drug sources remain uncultivable, and therefore inaccessible for novel antibiotic discovery. Hence there is need to rediscover new drugs active against these drug resistance pathogens. The main objective of the present study was isolation, purification, and characterization of soil isolates having antimicrobial activity against Bacillus strains. Soil samples were serially diluted and plated on isolation agar media. Potential colonies were screened, purified, and stored in glycerol stock. Isolates were morphologically and biochemically characterized. Genomic DNA was extracted from the identified isolate, and analyzed using 16s rRNA sequencing. The sequence analysis revealed of the strain to be Streptomyces, Bordetella and Achromobacter. The culture isolate was grown in the production medium and then isolated with the antibiotic compound. The compound isolated was tested for the antibacterial activity using the tube method and Well plate method. The compound showed high potential of antibacterial activity and the activity is dose dependent.

Key words: 16s rRNA sequencing, Soil microorganisms, PCR, Genomic DNA

INTRODUCTION

With the increasing number of drug-resistant pathogens, particularly the acquired multi-drug resistant strains, serious public health problems have arisen throughout the world. Therefore, the need for antimicrobial discovery and better treatments of these infections, particularly in hospitals where antibiotic resistance is immediately life threatening, is becoming a rapidly growing concern [1]. The study of different environments throughout the world has yielded a lot of antimicrobial agents that are of great value for the treatment of many infectious diseases.

Antibiotics are one of the most important commercially exploited secondary metabolites produced by the bacteria and employed in a wide range. Most of the antibiotic producers used today are the soil microbes. Fungal strains and Streptomyces members are extensively used in industrial antibiotic production [2]. Bacteria are easy to isolate, culture, maintain and to improve their strains. Microbes are Omni present and exist in a competitive environment.

Bacillus species being the predominant soil bacteria because of their resistant endospore formation and production of vital antibiotics like bacitracin etc. are always found inhibiting the growth of the other organisms [3].

The microbial products of secondary metabolism carry an important role in human health, providing roadmaps for the biosynthesis of many synthetic and semi-synthetic drugs [4,5]. In nature, microbial bioactive products are present as mycotoxins or bacteriocins derived from filamentous and non-filamentous bacteria and fungi [6]. The soil-based actinomycetes have been the source of countless drugs, from streptomycin and actinomycin, to erythromycin and vancomycin. Natural soil harbors over 109 microorganisms/ gram and provides an ideal reservoir for bioactive microbiota, which springs virtually all clinical antibiotics used today. Today nearly 500 antibiotics are found each year and over 80% of antibiotics in clinical use are obtained from soil isolates [7,8]. These bioactive microorganisms are most abundantly present at the top few inches of the soil, in soil containing straw and agricultural products [9,10]. Studies have also suggested that soil from areas containing residential-derived materials, such as human fecal matter, contains 10-20 times more antibiotic resistant strains than recreational or industrial soils [4]. Consequently and in addition to the elevated use of therapeutic drugs in urban or residential environments, residential soils contain exceptionally high bioactivity and are abundant reservoirs of antibiotic-resistant microorganisms [11,12,13].

Plasmid-mediated antibiotic producing genes can be acquired environmentally by transposition from one microorganism to another. The phenomenon of intracellular plasmid acquisition enables antibiotic-producing bacteria to co-exist with antibiotic-resistant strains through the synthesis of novel bioactive compounds, which allows them to combat their continuously evolving antibiotic resistant counterparts [14,15]. This sophisticated co evolutionary adaptation is perpetual within microbial-rich soil and represents a great reservoir for novel, natural (non-synthesized) antibiotics [16].

The successful advancement of non-synthetic and synthetic antibiotic therapeutic applications therefore requires constant identification and characterization of natural antibiotic-producing microorganisms [17, 18, 19]. After the identification of super-bugs (bacteria able to resist to different antibiotics), the research about new and more effective antibiotics became more and more relevant. The two antimicrobial peptides of Bacillus subtilis strain were isolated from a rhizosphere soil sample. It grew optimally up to 14% NaCl and produced antimicrobial peptide within 24 h of growth. Bacteriosins are antimicrobial peptides that are produced by bacteria as a defense mechanism in complex environments [20]. Identification and characterization of novel bacteriocinsin novel strains of bacteria is one of the important fields in bacteriology. Bacteriocins are ribosomal synthesized antimicrobial peptides and have drawn attention in recent years due to their potential therapeutic applications in treating bacteria, including multiple drug resistant bacteria [21,22].

In the recent years, there are many studies about the biological activities of the secondary metabolites isolated from the marine microorganisms, and these studies mainly focus on the antibacterial, cytotoxic, antioxidation, and antiviral, immunosuppressant activities [23]. In the screening program for bioactive principles from marine microorganisms, it is essential to study the biological activities of the marine microorganisms producing the bioactive secondary metabolites [24].

In this study, we investigated the evaluation of antimicrobial agent produced by a bacterium isolated from the soil samples collected from various locations in Bangalore, India by establishing the molecular and phylogenetic identity of the organism by 16S rDNA and DNA sequence analysis. The results of these analyses have indicated that the isolated organism belonged to *Streptomyces spp* and other soil bacteria like *Bordetella* and *Achromobacter*. The DNA sequence and subsequent BLAST analysis indicated a high similarity of the obtained sequence corresponding to *Streptomyces spp*. Solvent extraction of antimicrobial substance with chloroform and subsequent activity testing by agar diffusion and agar dilution methods indicated results positive for the antimicrobial activity of compound.

MATERIALS AND METHODS

Isolation and Culture of soil microorganisms:

Soil samples were collected from various locations. The soil samples were weighed and approximately 1gm of each soil sample was dissolved in 10ml of distilled water. The samples were serially diluted 10 fold and 100µl of each soil sample was plated on nutrient agar plates and were incubated at 65°C. These bacteria were further streaked on

fresh nutrient agar plates to procure isolated colonies. The isolated colonies of putative soil bacteria were sub-cultured in nutrient broth for DNA extraction.

Isolation of genomic DNA from bacteria:
Total genomic DNA from the bacteria was isolated by N- Cetyl- N, N, N-trimethylammonium bromide (CTAB) method. Total genomic DNA from the bacteria was isolated by N- Cetyl- N, N, Ntrimethyl-ammonium bromide (CTAB) method described elsewhere (Wilson, K. 2001). In brief, the culture was centrifuged at 10000 rpm at 4°C and lysed with 675µl extraction buffer (100mM Tris HCl, 100mM EDTA, 1.4M NaCl, 1% CTAB and Proteinase K - 0.03µg/µl). The suspension was incubated at about 370C for 30 minutes. To the mixture 75µl of 20% SDS was added and incubated at 65°C for 2 hours. The suspension was then centrifuged and the supernatant was extracted with equal volumes of Chloroform and Isoamyl alcohol (24:1). The aqueous phase obtained after centrifugation was then extracted with 0.6 volumes of isopropyl alcohol. The mixture was allowed to stand undisturbed at RT for 1hour. The suspension was then centrifuged again and the DNA was pelleted with 500µl of 70% ethanol. The DNA collected was then quantified using UV spectrophotomer (Vivaspec Biophotometer, Germany).

PCR amplification:
The bst polymerase gene was amplified by PCR using purified genomic DNA as a template. Oligonucleotide primers were synthesized to amplify the intact region of *bst* gene. The forward primer for bst, 5′ AGAGTTTGATCCTGGCTCAG 3′ and the reverse primer, 5′ ACGCTTACCTTGTTACGACTT 3′, were purchased from Eurofins, Bangalore. These primers correspond to the gene bst gene and thus the final PCR product was 1400bp. The PCR mixture consisted of 10x reaction buffer with MgCl2 (1.5mM), 2µL of dNTP mix (2.5mM), 2µL each of forward and reverse primers (10picomoles/µl each primer), 0.3µL of Taq DNA polymerase (5 U/µL), and 50ng/ µL of template DNA in a total volume of 20µL.

The PCR was performed with the following cycling profile: initial denaturation at 94°C for 2 min, followed by 30 cycles of 50s denaturation at 94°C, annealing at 51°C for 30s, and extension at 72°C for 1min. The time for the final extension step was increased to 6 min. The PCR products amplified were then qualitatively analysed on 1% agarose gel. The PCR product was recovered using the QIA quick gel extraction kit, and the amplified product was then purified and used for cloning purpose.

Sequencing:
The DNA eluted from agarose gel was sequenced using ABI PRISM Big Dye Terminators v1.1 cycle sequencing kit (Applied Biosystems Foster city, CA, USA) according to the manufacturer's instruction employing T7 or M13 primers. The comparison of the nucleotide sequences of the unique fragment with the sequences available in the GenBank database was carried out using the NCBI BLAST program (http//www.ncbi.nlm.nih.gov/blast).

Extraction of the putative antibiotic compound:
To extract the antimicrobial agent, culture filtrate was mixed with 10% (v/v) of organic solvents such as chloroform, vigorously shaken and the mixture was allowed to separate. The lower phase (organic phase) was separated from the exhausted supernatant (upper phase). The solvent phase was concentrated by drying the samples at 37°C until no solvent was left. Then, two phases were tested for their antimicrobial activity by performing a 10-fold serial dilution of the putative organic compound and subsequently the Minimum Inhibitory Concentration (MIC) was analyzed by testing 10µl of the 10-fold serial dilutions by the agar disk diffusion method. In an additional assay, 200µL of overnight grown pathogenic cultures were taken.

RESULTS AND DISCUSSION

Isolation of bacteria from soil:
4 isolated colonies were obtained from the culture plates. The DNA quality was checked with spectrophotometer for purity. The pure DNA was resolved on 1% agarose gel.

16S rDNA PCR analysis of the soil bacterial isolates:
16S rDNA oligonucleotides primers were designed for the identification of the soil bacterium using Primer3 software. The primers were validated *in silico* and subsequently in wet lab. The primers could yield an amplicon of the expected size to produce ~1500 bp amplicon which shown in the figure 1.

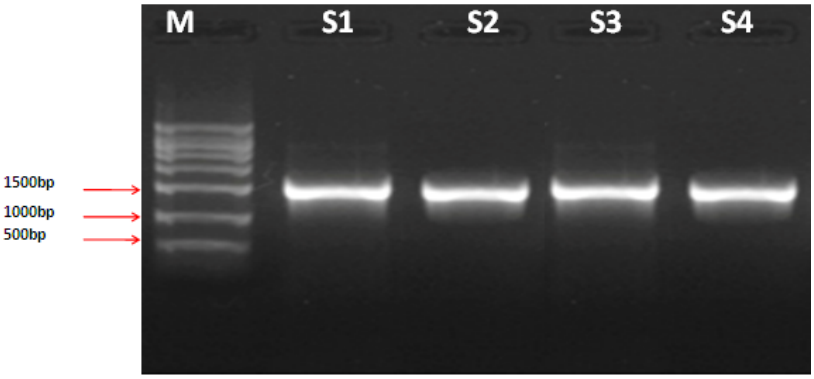

Figure 1: PCR amplification of bacterial genomic DNA with 16S primers. S1, S2, S3, S4: samples. M: DNA marker

Sequencing of the bacterial isolates:
The 16S PCR product of the bacterial isolates from were further characterized by DNA sequence analysis. The DNA sequencing was performed at Eurofins, Bangalore. A sequence read of ~1500 bp DNA sequence was obtained.The sequence data is shown below**.** The DNAsequence obtained was further used to investigate the identity of the bacterial isolate. The identified sequence showed 100% similarity with the gene cluster sequences of Streptomyces species available from GenBank database. The BLAST hits had a significant e-value of 0.0.

Multiple Sequence Alignment:
The sequence identified by DNA sequencing was further characterized by multiple sequence alignment with the highly similar sequences found in BLAST search. The alignment results indicate a significant alignment of the putative DNA sequences (identified in the current study) to the 16s rRNA regions of *Streptomyces, Bordetella and Achromobacter*. The members of *Streptomyces* spp. Produce antibiotics.

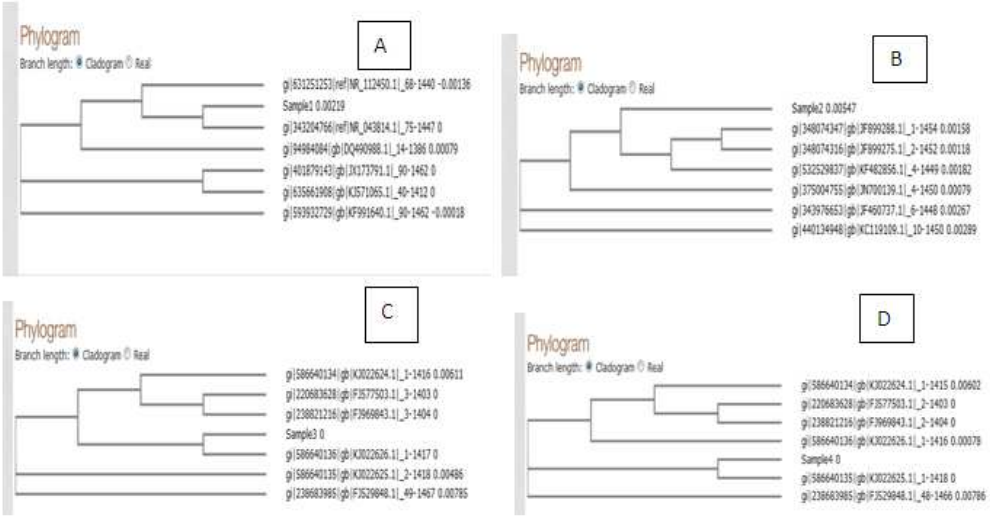

Figure 2: Phylogenetic analysis of the samples. A: Sample 1; B: Sample 2; C: Sample 3; D: Sample 4

Agar Diffusion Method:
In order to disclose the effective factors on their antibacterial activity, all the four isolates were screened for their antibacterial activity.

Samples	Inhibition zone diameter (mm)
Sample -1	4
Sample -2	10
Sample -3	3
Sample -4	10

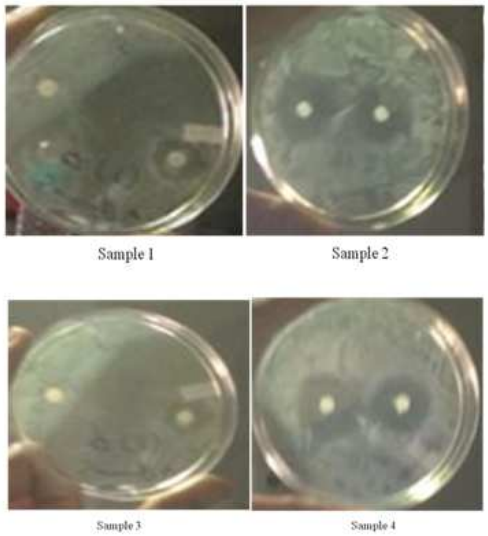

Figure 3: Left: Disc diffusion method. Zone of inhibition diameter against isolated isolates from the soil. Right: Diameter of the inhibition zones measured in mm

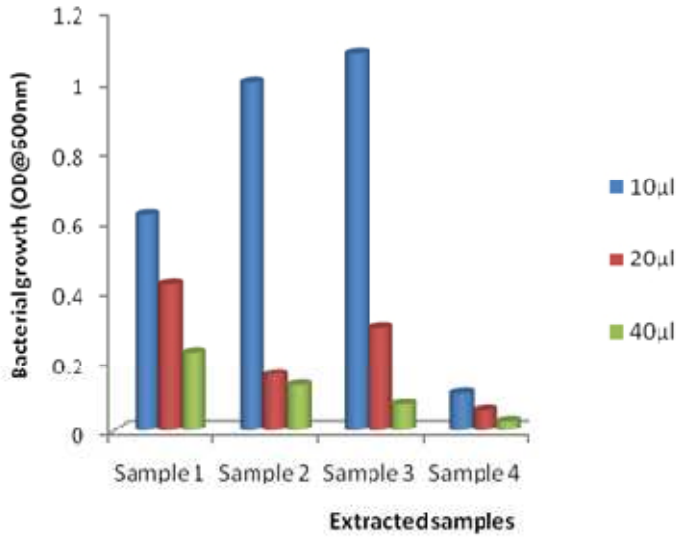

Figure 4: Graph depicting bacterial growth inhibition with varying amounts of the antimicrobial compound

To these cultures, 10, 20 and 40 µL of the putative antibiotic compound was added separately to each bacterial test cultures. The results are indicated in the graph below. Bacterial growth was inhibited at higher volumes of the putative antimicrobial compound (20 and 40µL) whereas lesser growth inhibition is observed with 10µL of the antimicrobial compound. The inhibition was found to be dose dependent. Isolated antimicrobial compound exhibited a lowest MIC of 2µg/ml indicating excellent antibacterial effect.

CONCLUSION

Today, increase in the number of drug-resistant pathogens, particularly the acquired multi-drug resistant strains, cause serious public health problem throughout the world. Therefore, the need for antimicrobial discovery and better treatments of these infections, particularly in hospitals where antibiotic resistance is immediately life threatening, is becoming a rapidly growing concern. The study of different environments throughout the world has yielded a lot of antimicrobial agents that are of great value for the treatment of many infectious diseases. Among many methods available for evaluation of antimicrobial activity, the methods described below have been used for determining the antimicrobial activity of compounds produced by microorganisms. Agar and broth dilution methods are used as quantitative methods, suitable for microorganisms with variable growth rate and for anaerobic, micro-aerophilic microorganisms. The results are expressed as MIC, which is the lowest concentration of an antimicrobial that prevents growth of a microorganism after a specific incubation period. In this test, an antimicrobial is serially diluted and a single concentration added to a culture tube or plate added with nonselective broth or melted agar medium, which is then inoculated with test organisms and incubated. The MIC is defined as the lowest concentration at which no growth occurs (absence of turbidity) in a medium following incubation.

In this work, we investigated the evaluation of antimicrobial agent produced by a bacterium isolated from the soil samples collected from various locations in Bangalore, India by establishing the molecular and phylogenetic identity of the organism by 16S rDNA and DNA sequence analysis. The results of these analyses have indicated that the isolated organism belonged to Streptomyces spp and other soil bacteria like Bordetella and Achromobacter. The DNA sequence and subsequent BLAST analysis indicated a high similarity of the obtained sequence corresponding to *Streptomyces spp*. Solvent extraction of antimicrobial substance with chloroform and subsequent activity testing by agar diffusion and agar dilution methods indicated results positive for the antimicrobial activity of compound. The growth inhibition was found to be dose dependent.

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