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Screening and characterization of putative antibiotic compound using PCR

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ABSTRACT

Although thousands of antibiotics have been isolated from Streptomyces, these represent only a small part of the repertoire of bioactive compounds so far produced. Therefore, isolation of new Streptomyces from natural resources and characterization of their secondary metabolites is a valuable endeavor. 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 actinomycetes from soil samples, having antimicrobial activity against Bacillus strains. Soil samples were taken from the bank of the Ulsoor lake, Bangalore, India. These samples were serially diluted and plated on actinomycete 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 carpaticus. 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.

Keywords: Actinomycetes, *Streptomyces caprpaticus*, Agar well diffusion method, antimicrobial activity.

INTRODUCTION

Soil microorganisms provide an excellent resource for the isolation and identification of therapeutically important products. Among them, actinomycetales are an important group[1] The order actinomycetales is composed of approximately 80 genera, nearly all from terrestrial soils, where they live primarily as saprophytes, water and colonizing plants showing marked chemical and morphological diversity, but from a distinct evolutionary line[2,3]

The actinomycetes are Gram positive bacteria having high G+C (>55%) content in their DNA. Actinomycetes were originally considered to be an intermediate group between bacteria and fungi but now are recognized as prokaryotic organisms [4]. The majority of actinomycetes are free living, saprophytic bacteria found widely distributed in soil, water and colonizing plants [5]. The actinomycetes are noteworthy as antibiotic producers, making three quarters of all known products; the *Streptomyces* are especially prolific and can produce a great many antibiotics and other class of biologically active secondary metabolites [6,7]. They cover around 80% of total antibiotic product, with other genera trailing numerically; Micromospora is the runner up with less than one-tenth as many as *Streptomyces* [8]. If we include secondary metabolites with biological activities other than antimicrobial, actinomycetes are still out in front, over 60%; Streptomyces spp. accounting for 80% of these [9].

Streptomyces, as the most important genus of Actinomycetes, are the most abundant soil microorganisms under a wide variety of conditions. Actinomycetes strains are characterized by the production of important extracellular bioactive compounds and majority of those strains belong to species within the genus Streptomyces which produce two–thirds of the clinically important antibiotics [10]. This genus was confirmed to be promising bacteria against several pathogens and is well known for their potential to produce a large number of inhibitory metabolites used in industry and pharmacy [11, 12]. Historically a large percentage of the world's antibiotics have been found in actinomycetes. Actinobacteria are commonly known as producers of antibiotics and other metabolites [13]. In fact, 70-80% of the commercially available secondary metabolites have been isolated and characterized from several species of actinomycetes [14]. These secondary metabolites represent an opulent source of biologically active compounds such as antibiotics, agrochemicals, enzyme, immunosuppressants, antiparasitics and anticancer agents [15]. However, over the last decade, the search for antibiotics and other metabolites in actinomycetes and other microorganisms has become less popular due to the diminishing success of discovering novel strains [15, 16]. As a result, screening for potential chemicals increased but did not yield much success, which is not surprising since Actinobacteria have evolved their capabilities over a billion years [17].

Research in finding newer antibiotics and increasing productivity of such agents has been a very important activity. This is because some important drugs are expensive and/or have side effect to the host, some microbes have no successful antibiotics and others are developing multidrug resistance [18]. These situations require more attention to find solutions by searching and producing new and effective antibiotics from microbes like actinomycetes. All these studies searching for discovering new antibiotics, in order to synthesize new drug that is able to kill different type of microorganisms.

The present study was undertaken to isolate actinomycetes from the soil samples along the bank of Ulsoor lake, Bangalore and to assess their anti-bacterial properties. Along side the isolates were analysed using the 16s rRNA sequencing and the antibiotic gene was amplified suing the specified primers.

MATERIALS AND METHODS

Isolation and Culture of soil microorganisms: Soil samples were collected from the sediments of the Ulsoor lake, Bangalore. The samples were collected in sterile containers from the site and transported to the lab. The soil samples were weighed and approximately 1 gram of each soil sample was dissolved in 10 ml 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.

Genomic DNA isolation: Total genomic DNA from the bacteria was isolated by N- Cetyl- N, N, N-trimethyl-ammonium bromide (CTAB) method. Total genomic DNA from the bacteria was isolated by N- Cetyl- N, N, N-trimethyl- 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 37^0C 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).

16rDNA Sequencing: The purified genomic DNA was later used for sequencing 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.

PCR amplification of the putative antibiotic producing gene: Oligonucletide primers were designed to amplify a region in the putative gene cluster *Streptomyces*. For this purpose, a web based software, Primer 3 plus was utilized. A 940bp region has been targeted and the DNA sequence was amplified by PCR as shown in the figure below. The amplified Sequence was sub-cloned into the pTZ57R/T cloning vector (Fermentas, USA). The sequence was later confirmed for the gene characterization using the ClustalW.

The oligonucleotide primers designed are listed below:

Oligonucleotide	Sequences (5'- 3')	GC %	Tm Value	Length	Product Size
FP	ACAGCATCCTGTCCATCCA	52	60^0C	19	940 bp
RP	GAGCAGCAGGTCGTTGATCT	55	60 ^{0}C	20	

Extraction of the putative antibiotic compound:
All the components of nutrient broth are weighed and added to distilled and sterilized. Sterilized broth is inoculated with test culture under aseptic conditions and is incubated in an orbital shaker at 37^0C over night.

The pure colonies isolated were inoculated into 200ml of the nutrient broth. The cultures were incubated at about 30^0C for over night. The culture was used for the extraction of the antimicrobial 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 an antimicrobial activity assay. To isolate an oil-soluble antibiotic such as penicillin, a solvent extraction method is used. In this method, the broth is treated with organic solvents such as chloroform, which can specifically dissolve the antibiotic.

Antimicrobial activity assay: Antimicrobial activity of an antibiotic can be expressed by determining the antibiotic activity quantitatively. It is done by using the known sensitivity of a test organism towards a particular antibiotic. In the assay, 200µL of overnight grown bacterial cultures were taken. To these cultures, 10, 20, 40, 60 and 80µL of the putative antibiotic compound was added separately to each bacterial test cultures. The experiment was repeated four times to confirm the activity.

Well Method of Antibiotic Assay: Nutrient agar medium is poured into petri plates under aseptic conditions with the selected bacterial species (Bacillus sp) for inhibition. And five wells were cut for increasing concentrations of the crude extract. About 20-40µl of the crude extract was added to the wells in the increasing order of their concentration. The plates were the incubated overnight at 37^0C for 24 hours.

RESULTS AND DISCUSSION

Genomic DNA isolation and quantification: The bacteria were cultured in the Nutrient broth media and genomic DNA was isolated by CTAB method. The isolated DNA was analyzed by Agarose (1% w/v) gel electrophoresis (Fig.1). The quantity and quality of DNA was analyzed by UV visible spectrophotometer and the data was shown in the table 1. The A260/280 ratio was recorded, showing the purity profile of the isolated DNA.

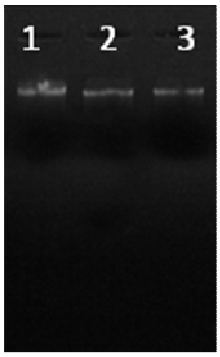

Fig. 1. Genomic DNA isolated from soil bacteria

16S rDNA PCR analysis of the soil bacterial isolates
16S rDNA oligonucleotide primers were designed for the identification of the soil bacterium using Primer3 software. The predicted 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 2.

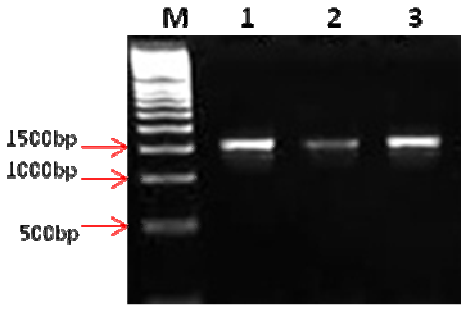

Fig. 2. PCR amplification of bacterial genomic DNA with 16S rDNA primers

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 DNA sequence obtained was further used to investigate the identity of the bacterial isolate. A **B**asic **L**ocal **A**lignment **S**earch **T**ool, BLAST (version 2.7) analysis was performed to establish the quality and accuracy of DNA sequencing results. The sequences were compared to their corresponding GenBank sequences. The identified sequence showed 100% similiarity with the gene sequences of *Streptomyces carpaticus* which were available from GenBank database. The BLAST hits had a significant e-value (as shown in the figure below).

5’AGAGGCGAACGGGTGAGTACCACGTGGGCAATCTGCCCTGCACTCTGGGATAAGCCCGGGAAACTG
GGTCTAATACCGGATACGACACTCCGAGGCATCTCGGGGTGTGGAAAGTTCCGGCGGTGCAGGATGA
GCCCGCGGCCTATCAGCTTGTTGGTGGGGTAATGGCCTACCAAGGCGACGACGGGTAGCCGGCCTGAG
AGGGTGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATA
TTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAAC
CTCTTTCAGCAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCACCGGCTAACTACGTGCCA
GCAGCCGCGGTAATACGTAGGGTGCGAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCG
GTTTGTCGCGTCGATTGTGAAAGCCCGGGGCTTAACCCTGGGTCTGCAGTCGATACGGGCAGGCTAGA
GTTCGGCAGGGGAGACTGGAATTCCTGGTGAAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGG
TGGCGAAGGCGGGTCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATT
AGATACCCTGGTAGTCCACGCCGTAAACGGTGGGCACTAGGTGTGGGCAACATTCCACGTTGTCCGTG
CCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTAGGGCCGCAAGGCTAAAACTCAAAGGAATTG
ACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGG
CTTGACATACACCGGAAACGGCCAGAGATGGTCGCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGC
TGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCTGTGTTGC
CAGCGAACTCTTCGGACTGCCGGGGACTCACGGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGG
ACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAG
CTGCGATGCCGTGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCG
ACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCATTGCTGCGGTGAATACGTTCCCGGGCCT
TGTACACACCGCCCGTCACGTCATGAAAGTCGGTAACACCCGAAGCCGGTGGCCTAACCCCTTGTGGG
AGGGAGCTGTCGAAGGTGGGCCTGGCG3’

Primer design and cloning of the putative antibiotic producing gene:
Oligonucletide primers were designed to amplify a region in the putative gene cluster *Streptomyces*. For this purpose, a web based software, Primer 3 plus was utilized. A 940bp region has been targeted and the DNA sequence was amplified by PCR as shown in the figure below. The amplified Sequence was sub-cloned into the pTZ57R/T cloning vector (Fermentas, USA). Upon further query of the identified sequence *in silico*, it was revealed that the sequence is a part of a gene cluster responsible for antibiotic production.

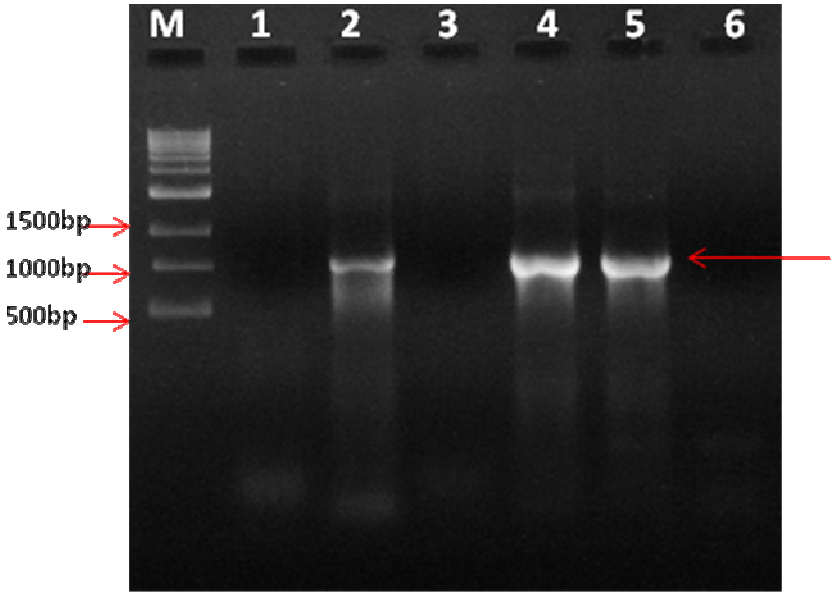

Figure 4: PCR amplification of the putative antibiotic gene. The amplified gene was 940bp. Lane M: Standard; Lane 2, 4,5: Amplified product; Lane 1: Negative control

Multiple Sequence alignment: Multiple sequence alignment was performed with CLUSTAL alignment software (http://www.ebi.ac.uk/Tools/msa/clustalo/). The gene sequences which were exhibiting high similarity to the identified sequence in BLAST analysis were selected for sequence alignment in order to establish the molecular identity. The results of the alignment showed significant sequence match with the corresponding highly similar *Streptomyces* sp. as shown below.

CLUSTAL 2.1 multiple sequence alignment:

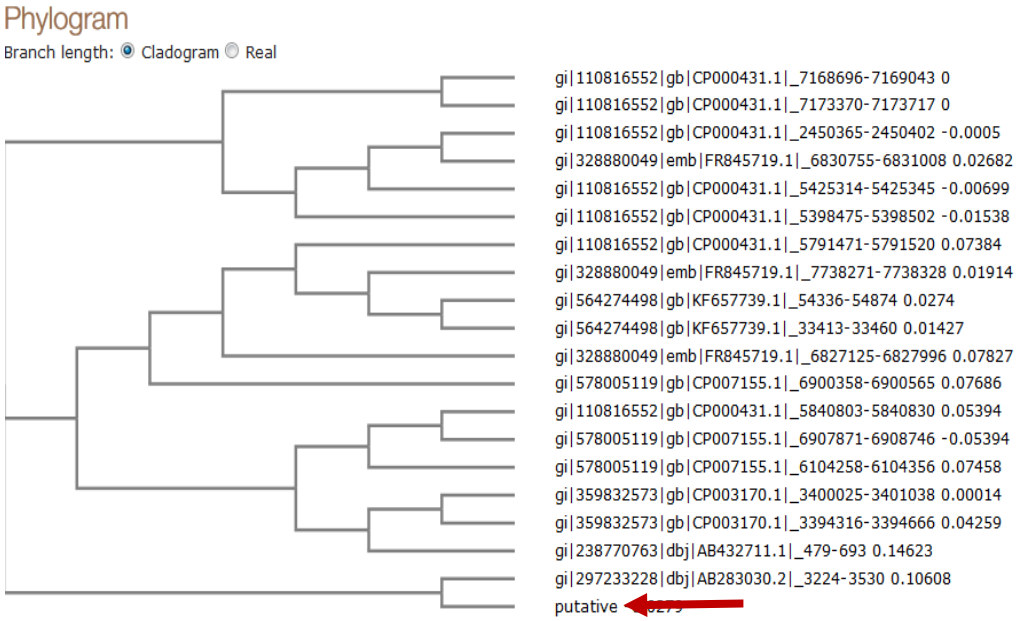

Figure 5: Phylogenetic tree displaying the relationship of the identified gene with the corresponding genes

Antimicrobial activity assay

Antimicrobial activity of an antibiotic can be expressed by determining the antibiotic activity quantitatively. It is done by using the known sensitivity of a test organism towards a particular antibiotic. In the assay, 200µL of overnight grown bacterial cultures were taken. To these cultures, 10, 20, 40, 60 and 80µL of the putative antibiotic

compound was added separately to each bacterial test cultures. The results are indicated in the graph below (Fig 6). Bacterial growth was inhibited at higher volumes of the putative antimicrobial compound (20, 40, 60 and 80µL) whereas lesser growth inhibition is observed with 10µL of the antimicrobial compound as shown in figure 6, 7. The growth inhibition was found to be dose dependent in the case of activity.

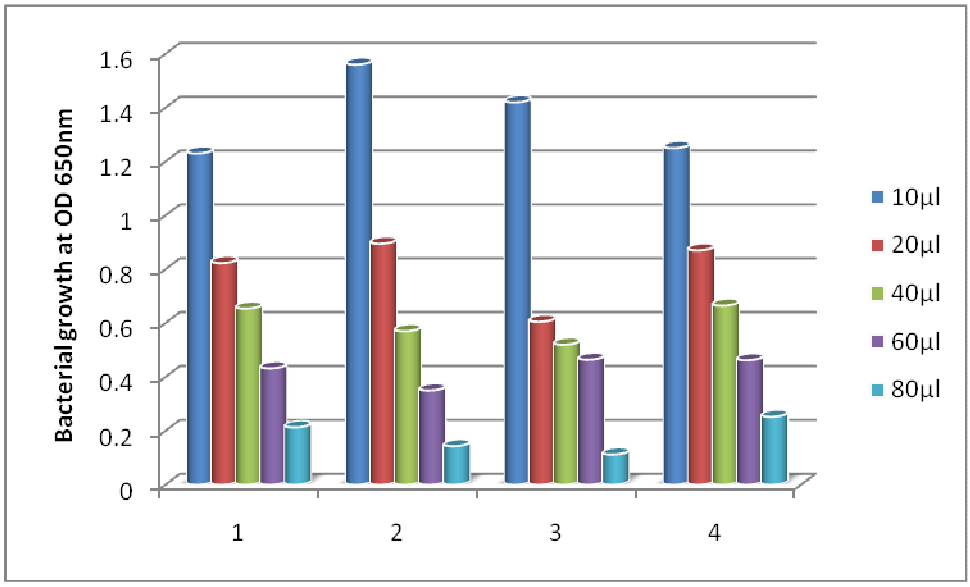

Figure 6: Graph depicting the antimicrobial activity of the extracted compound. The activity was shown to be dose dependent. All the results are average of triplicates. The experiment was done four times

Bacterial growth was inhibited at higher volumes of the putative antimicrobial compound (20, 40, 60 and 80µL) whereas lesser growth inhibition is observed with 10µL of the antimicrobial compound as shown in figure 6 & 7.

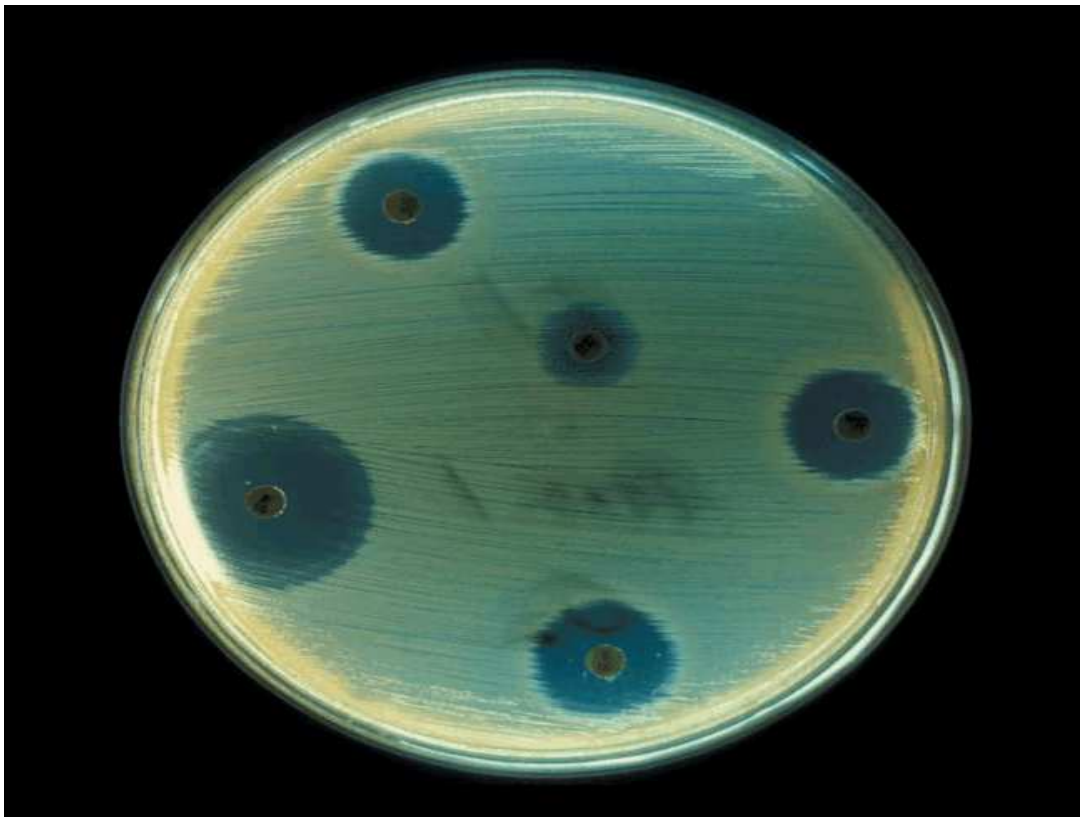

Figure7: Well plate method of antimicrobial assay

DISCUSSION

Actinomycetes are the most widely distributed group of microorganisms in nature which primarily inhabit the soil. They have provided many important bioactive compounds of high commercial value and continue to be routinely screened for new bioactive compounds. Almost 80% of the world's antibiotics are known to come from actinomycetes, mostly from the genera *Streptomyces* and *Micromonospora.*

The primers designed yielded an amplicon of the expected size of about ~1500bp. The sequences were compared to their corresponding GenBank sequences. The identified sequence showed 100% similiarity with the gene sequences of *Streptomyces carpaticus* which were available from GenBank database. Oligonucletide primers designed to amplify a region in the putative gene cluster *Streptomyces*. For this purpose, a web based software, Primer 3 plus was utilized. A 940bp region has been targeted and the DNA sequence was amplified by PCR as shown in the figure below. The amplified Sequence on BLAST analysis revealed that the sequence is a part of a gene cluster responsible for antibiotic production. The isolated compound was found to show high level of antibacterial activity in both tube method and well plate method. Bacterial growth was inhibited at higher volumes of the putative antimicrobial compound (20, 40, 60 and 80µL) whereas lesser growth inhibition is observed with 10µL of the antimicrobial compound as shown in figure 6, 7. The growth inhibition was found to be dose dependent in the case of activity.

CONCLUSION

Streptomyces is the largest genus of Actinobacteria and the type genus of the family Streptomycetaceae. Over 500 species of Streptomyces bacteria have been described. As with the other *Actinobacteria*, *Streptomycetes* are Gram-positive, and have genomes with high GC content. Found predominantly in soil and decaying vegetation, most of the *Streptomycetes* produce spores, and are noted for their distinct "earthy" odor that results from production of a volatile metabolite, geosmin. *Streptomycetes* are the most abundant source of antibiotics. Typically, each species produces several antibiotics, with the profile being species specific. *Streptomyces coelicolor*, the model species, produces at least five different antibiotics. The regulation of antibiotic biosynthesis in *S. coelicolor* and other, nonmodel streptomycetes of each antibiotic is specified by a large gene cluster, usually including regulatory genes (cluster-situated regulators [CSRs]). In the present study we isolated different actinomycetes species and one of the efficient strain was identified based on 16S rDNA sequencing. The identified *Streptomyces carpaticus* was efficiently inhibiting most of the pathogens tested. The sequences flanking the identified sequence in this work need to be amplified and cloned into an expression vector to achieve the expression of the antibiotic compound. Further we plan to characterized and analyze the compounds with antimicrobial activity.

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