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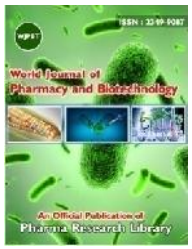

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Isolation and identification of bioactive potential actinomycetes from soil samples of Waddepalli village

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A B S T R A C T

The present study concludes that the bioactive antibiotic produced by the actinomycete *Streptomyces* It's also a potential antibiotic for the treatment of diseases caused by drug resistant bacterial pathogens It showed broad spectrum of antimicrobial activity as it inhibited both Gram positive and Gram negative bacteria and also some fungi. Therefore, this isolate proves to be a promising isolate which can be further studied for its applications in producing important pharmaceutical compounds and also as a biocontrol agent against pathogenic fungi. Further works on optimization of this isolate's antagonistic activity, purification of the important bioactive compounds from NMR studies are underway.

Keywords: Actinomycetes, Bioactive compounds, Anti microbial activity, NMR.

A R T I C L E I N F O

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1. Introduction

Actinomycetes perform significant biogeochemical roles in terrestrial soils and are highly valued for their unparalleled ability to produce biologically active secondary metabolites. Totally 22,500 bioactive secondary metabolites

have been reported, out of which 16,500 compounds show antibiotic activities. Out of the 22,500 total bioactive secondary metabolites, 10,100 (45%) are reported to be produced by actinomycetes in which 7630 from streptomycetes and 2470 from rare actinomycetes. A search of recent literature revealed that at least 4607 patents have been issued on actinomycete related products and processes (Baltz 2007, El-Tarabily 2006). For discovering novel bioactive compounds of biotechnological interest is important. Identification and study of soil microorganisms with unique physiological traits. Several factors such as: choice of screening source, pretreatment, selective medium, culture condition and recognition of actinomycetes colonies on a primary isolation plate are used for identification of unique actinomycetes (Bhaskaran 2011). Majority of streptomycetes and other actinomycetes members produce a diverse array of antibiotics including aminoglycosides, glycopeptides, β-lactams, macrolides, nucleosides, peptides, polyenes andtetracyclines (Berdy, 2005). As a result of the increasing prevalence of antibiotic-resistant pathogens and the pharmacological limitations of antibiotics, there is anexigency for new antimicrobial substances (Sahin and Unger, 2003). In view to the significance of Soil ecosystem which provide a rich source of novel actinomycetes, the present study is aimed for bioprospecting of actinomycetes from the marine sediments of Bay of Bengal, with special focus on the production of bioactive compounds by the isolates

2. Materials and methods

Collection of soil sample:

Nine different soil samples were collected in sterile plastic bags from different regions in Waddepally village area surroundings of Warangal district, Telangana State, India. All samples were transported to Microbiology Laboratory, labeled and stored in refrigerator for further investigation.

Isolation and culture condition:

One gm of the soil samples were weighed and dissolved in 10ml of distilled water and further serially diluted upto10-6 dilution, 0.1 ml of each dilution were spreaded on starch casein agar medium Table 3.1 uniformly and incubated for 6-7 days at room temperature (Narendra Kumar, Ravi Kant Singh 2010)**.** The emerging colonies of pigmented and non pigmented actinomycetes were sub cultured and maintained for future use.

Screening for Bioactive Compound Producing *Actinomycetes*

Primary screening:

The isolated strains were screened for the production of bioactive compounds. The isolated actinomycetes strains were inoculated in starch casein agar medium. The inoculated flask was kept for incubation at room temperature for a period of 6 days on rotary shaker (150 rpm) at 28°C. After incubation the broth was filtered and the filtrate was used to test antimicrobial activity. The overnight cultures like *Klebsiella pnuemoniae, Proteus vulgaris, Escherichia coli, Micrococcus luteus, B. subtilis, B. cereus, B. stearothermophilus and B.megaterium* were used as test organisms. The test organisms were spread uniformly on agar plate and wells were bored on the agar surface; then the wells were filled with the culture filtrate. Then plates were incubated at 37°C for 24 to 48 hrs.

Fermentation and extraction of crude extracts:

Based on the zone of inhibition in primary screening, isolates that have potential antimicrobial activity were selected for fermentation and extraction, and then the crude extracts were assessed following agar well diffusion methods (Thenmozhi 2010).

Antimicrobial activity:

The wells (6 mm diameter) were cut using a sterile cork borer on Muller Hinton agar (MHA, India). One day old culture of *S. aureus E. coli, S. typhi* ATCC9289, *K. pneumonia* and 48 h young culture of *Candia albicans* were swabbed with sterilized cotton swab on the surface of prepared Muller Hinton agar for bacteria and potato dextrose agar for fungi. Sixty micro litres of dissolved crude extract was loaded into each well and left for 30 min until the metabolite was diffused. Then the plates were incubated for 24 h at 37 °C for bacteria and 48 h at 28 °C for fungi. After incubation, the zone of inhibitions were measured and recorded.

Morphological and Taxonomical Identification of Isolated Strain: Based on the results of screening, one potential strain was selected for further investigations. The potential isolates were observed for aerial spore, mycelia color, spore chain morphology, and other microscopic characters. The taxonomic identification of actinomycetes sp. was based on Nonomura's key and Bergey's Manual ((Nonomura, 1974 and Buchanan 1974).

Molecular identification of bacteria

The molecular identification of 16S rRNA was performed at Xlearies Lab Pvt. Ltd India. DNA was extracted from the culture and its quality was evaluated by agarose (1.2%) gel electrophoresis. A fragment of the 16S r DNA gene was amplified by PCR and purified. Forward and reverse DNA sequencing reactions were carried out with primers 8F and 1492R (Aoust 1974) using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Standard nucleotide BLAST searches using the identified 16S rRNA sequence as a query were performed against NCBI's 16S ribosomal RNA sequences (Bacteria and Archaea) database. Additional searches were conducted against NCBI genomes, whole genome shotgun contigs, and the non-redundant nucleotide collection databases also available on NCBI Gene bank (www.ncbi.nlm.nih.gov). Sequences were aligned using the online version of MAFFT (Katoh 2002; Katoh 2008).The multiple sequence alignment was trimmed and manually adjusted where necessary. A phylogenetic tree displaying the evolutionary relationships between sequences was obtained with the maximum likelihood method implemented in RAxML (Stamatakis 2006). Node support was estimated with 1000 rapid bootstrap replicates

3. Result and Discussion

Antibiotics are the most important bioactive compounds for the treatment of infectious diseases. But now, because of the emergencies of multi-drug resistant pathogens, there are basic challenges for effective treatment of infectious

diseases. Thus, due to the burden for high frequency of multidrug resistant pathogens in the world, there has been increasing interest for searching effective antibiotics from soil actinomycetes in diversified ecological niches (Abo-Shadi 2010). Actinomycetes are one of the most efficient groups of secondary metabolite producers and are very important from an industrial point of view. Among its various genera, Streptomyces, *Saccharopolyspora, Amycolatopsis, Micromonospora* and Actinoplanes are the major producers of commercially important biomolecules. Several species have been isolated and screened from the soil in the past decades.

Consequently the chance of isolating a novel actinomycete strain from a terrestrial habitat, which would produce new biologically active metabolites, has reduced. The most relevant reason for discovering novel secondary metabolites is to circumvent the problem of resistant pathogens, which are no longer susceptible to the currently used drugs. Existence of actinomycetes has been reported in the hitherto untapped marine ecosystem. Marine actinomycetes are efficient producers of new secondary metabolites that show a range of biological activities including antibacterial, antifungal, anticancer, and insecticidal and enzyme inhibition. Actinomycetes have been proven as a potential source of bioactive compounds and richest source of secondary metabolites. (Suthindhiran2009). Bioactive compounds from marine actinomycetes possess distinct chemical structures that may form the basis for synthesis of new drugs that could be used to combat resistant pathogens. In the present study the bacterial strains collected and tested for antibiotic production. Among the 23 actinomycetes isolated from different regions of waddapelly village single strain showed antibacterial activities against at least one of the tested bacteria. In antagonistic activity method, results revealed that the isolates, **RNK018** exhibited broad spectrum activities against tested bacteria. After fermentation the antibiotic was extracted by solvent extraction method. 100 ml of supernatant was taken in three separating funnel and extracted with 100 ml of dichloromethane, ethyl acetate and n-butanol separately, each with three times. The organic solvent extracts were evaporated to dryness, dissolved in sterile water and subjected to antimi-crobial studies (Williams et al., 1971). The crude antimicrobial compound was stable even above 60°C when kept for 30 min, and after treatment with proteinase K enzyme (5 mg/ml) for 1hr. (Yucel and Yamac 2010). Determination of antibacterial activity by agar well diffusion assay showed that ethanolic extract of actinomycetes RNK018 exhibited the antibacterial effect against different bacterial pathogens was evaluated for its antibacterial activity using the agar well diffusion method with streptomycin as reference standard. The antibacterial studies revealed that the ethonolic leaf extract has antibacterial activity against both gram positive and negative bacteria. The standard streptomycin showed the all most same level of inhibition against *Escherichia coli*. The antimicrobial activity was observed against Escherichia coli (20 mm), followed by *Klebsiella pnuemoniae* (13 mm). The lowest activity levels were observed against *Proteus vulgaris* and *B.megaterium* (6 mm each).The antibacterial activity reveals that the produced extract has a broad spectrum activity against gram positive and gram negative bacteria. (Table 1).

Table 1: Antibacterial activity of *actinomycetes*

S.NO	Test Organisms	Extract	Standard (Streptomycin)
1	*Klebsiella pnuemoniae*	13 mm	20mm
2	*Proteus vulgaris*	06 mm	10 mm
3	*Escherichia coli*	20 mm	06mm
4	*Micrococcus luteus*	08 mm	09 mm
5	*B. subtilis*	01 mm	18 mm
6	*B. cereus*	11 mm	22 mm
7	*B.stearothermophilus*	15 mm	16 mm
8	*B.megaterium*	06 mm	12 mm

The morphological characteristics of the selected isolates revealed that the growth of the isolates was excellent in starch casein agar. The isolates of RNK018 showed excellent growth in starch casein agar. Showing the aerial mycelium with white in color and margin with filamentous. Physiological and biochemical characteristics result indicates that all isolates showed in the table.1

Table 2: Characteristic Morphological

Characteristic Morphological	RNK018
Aerial mycelium color	White
Substrate mycelium color	White
Colony diameter(mm)	3
Colony margin	Filamentous
Colony elevation	Flat
Spore chain	Spiral
Spore surface	Warty
Biochemical	
Indole production	-
Methyl red	-
Voges proskaur	-
Citrate utilization	+
H2S production	-
Nitrate reduction	-
Melanin production	+
Starch hydrolysis	+
Gelatin hydrolysis	+
Lipid hydrolysis	-
Casein hydrolysis	+
Carbon source utilization	
Starch	+
Dextrose	+
Fructose	+
Maltose	+
Nitrogen source Utilization	
D-Alanine	+
L-Arginine	+
L-Phenylalanine	+
L-Tyrosine	+

The identification of species and phylogenies has been made easier by molecular techniques in which primers that can target specifically the 16SrRNA sequence of the actinomycetes are used which makes identification fast and accurate (Isik 2014, Jeffrey 2008). The taxonomic position of the isolated marine strain was determined with molecular methods. A single discrete PCR amplicon band of 1500 bp was observed by agarose gel electrophoresis (Fig.1). A consensus sequence of *Streptomyces* SRL9 1507 bp length for the 16S r DNA gene was generated from forward and reverse sequence data using Auto Assembler software, version 2.1. Blast searches against NCBI Gene bank did not retrieve a perfect match (100% identity) to our query, indicating that this strain may represent a new taxonomic entity. With the closest match, *Streptomyces sp* it shared 99% identity between the 16S sequences. The phylogenetic tree gives a more detailed picture about the taxonomic position within the *Streptomyces* sp and confirmed that the identified organism is *Streptomyces sp*

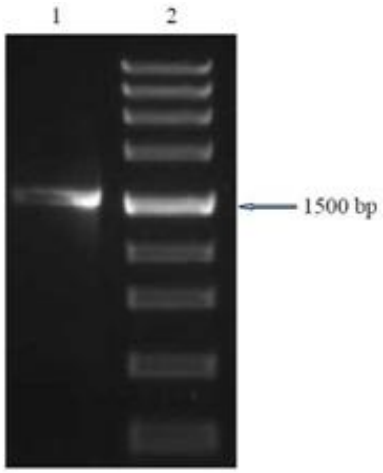

Figure 1: The PCR amplicon of 16S r RNA gene isolated from *Streptomyces* RNK018

4. Conclusion

The present study concludes that the bioactive antibiotic produced by the actinomycete *Streptomyces* It's also a potential antibiotic for the treatment of diseases caused by drug resistant bacterial pathogens It showed broad spectrum of antimicrobial activity as it inhibited both Gram positive and Gram negative bacteria and also some fungi. Therefore, this isolate proves to be a promising isolate which can be further studied for its applications in producing important pharmaceutical compounds and also as a biocontrol agent against pathogenic fungi. Further work on optimization of this isolate's antagonistic activity, purification of the important bioactive compounds from NMR studies are underway.

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