

STUDIES ON BIOACTIVE POTENTIAL OF *STREPTOMYCES* SPP. (KX710212). ISOLATED FROM ENVIRONMENTAL POLLUTED SAMPLE, RANIPET, VELLORE, INDIA

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ABSTRACT

Objective: Natural products from bacterial origin played and still play a valuable role in drug discovery and provide diverse health-promoting compounds that help to cure or alleviate serious diseases.

Methods: A total of 4 *actinobacterial* strains were isolated from 2 different environmental polluted soil samples collected from Ranipet, Vellore, Tamil Nadu, India. Of 15 isolates screened for antibacterial property, 4 isolates (PS1, PS2, and PS3) were found potent and characterized phenotypically and genotypically.

Results: The 16S rDNA gene sequences of potent strain showed 97% similarity with *Streptomyces* spp. and termed as *Streptomyces* sp. The bioactivity was revealed by antibacterial and antioxidant activity.

Conclusion: Hence, these findings provide scientific evidence to validate the pharmacological use and have the potential contribution in the discovery of new lead that play a key role in the field of therapeutics.

Keywords: Actinomycetes, Bioactive compounds, Antibacterial property.

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INTRODUCTION

Microorganisms and especially bacteria are being examined as a source of novel antibiotics that are active against antibiotic resistant human pathogens [1,2]. It was rapidly found that *actinomycetes* were most frequently positive; in particular, nearly 50% of *Streptomyces* strains isolated were active, mainly against Gram-positive bacteria. This widely applied approach was very fruitful by the early 1960s, and members of all the main families of clinically useful antibiotics were discovered. With the exception of the penicillins, cephalosporins, and a few minor products, all were produced by *actinomycetes*. Their collective spectra of action covered, practically all the important bacterial pathogens. The Gram-positive, soil-dwelling bacteria of the order *Actinomycetales*, *Streptomyces* exhibit an unusual, developmentally complex life cycle [3]. Moreover, the *Streptomyces* cell differentiation is typically accompanied by the production of secondary metabolites and is regulated by complex interrelated mechanisms [4]. To assess the potential of bioactive compounds isolated from new *actinomycetes* from environmental contaminated soil and to understand its mechanism underlying for its potency.

METHODS**Samples collection and isolation**

Soil samples were collected from environmental polluted area of Ranipet, Vellore, Tamil Nadu. The soil were collected and were transported to laboratory and stored at 4°C to isolate the microorganisms. Serially diluted soil were plated on starch casein agar incubated at room temperature for about 4 days.

Physiological, biochemical, and cultural characteristics

The morphological and biochemical studies of *actinomycetes* were carried out using International Streptomyces Project. Growth on the media was compared with growth of the control and was recorded as negative or positive [5].

16S ribosomal RNA amplification

The 16S ribosomal RNA was amplified using the polymerase chain reaction (PCR) method with Taq DNA polymerase. PCR amplification was detected by agarose gel electrophoresis and was visualized by ultraviolet fluorescence after ethidium bromide staining. The sequence was compared for similarity with the reference species of bacteria contained in genomic database banks, using the NCBI BLAST available at <http://www.ncbi.nlm.nih.gov/> [6].

Phylogenetic analysis

Phylogenetic and molecular evolutionary analyses were conducted using software included in MEGA version 3.0 package. The evolutionary history was inferred using the neighbor-joining method. The 16S rRNA sequence of the strains ERI-1, ERI-3, ERI-4, and ERI-26 were aligned using the Clustal W program against corresponding nucleotide sequences of representatives of the genus *Streptomyces* retrieved from GenBank. Phylogenetic tree was inferred by the neighbor-joining method. The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed.

Fermentation medium

Fermentations were carried out for the potent isolate PS5 in production medium. At 200 rpm, 30°C for 5 days, different culture conditions such as temperatures (20, 25, 30, 37, and 45°C), pH (6.0, 6.5, 7.0, 7.5, 8.0, and 8.5), and incubation hours (24, 48, 72, 96, 120, and 144) were studied to standardize the antibiotic production.

Liquid-liquid extraction

The 6 days grown culture broth was collected and filtered through Whatman No. 1 filter paper. The total culture filtrate 100 ml was extracted using Ethyl acetate. The solvents were removed using simple distillation and vacuum rotary evaporator at 40°C. The extracts were stored at 4°C until further use. Water extract was lyophilized and the concentrated metabolites were stored at 4°C until further use.

Structure elucidation

Characterization and identification of the compound and the chemical bonds were carried out using Fourier transform-infrared (FT-IR). The FT-IR spectra of the purified compound were recorded using Thermo Nicolet, Avatar 370 spectrometer over the 4000–400 cm^{-1} range at the resolution of 4 cm^{-1} .

Antibacterial assay

The antibacterial activity was determined by agar well diffusion assay. Various concentrations of the crude extracts. The following test organisms were used for antibacterial studies. The selected microorganisms (*Salmonella typhi* [MTCC 441], *Staphylococcus aureus* [ATCC 25923]), (*Escherichia coli* [ATCC 25922], *Pseudomonas aeruginosa* [ATCC 27853]) were inoculated in 3 ml of Mueller-Hinton Broth and incubated at 37°C for 24 hrs. After incubation period, the culture was diluted.

Free radical scavenging activity

The antioxidant activity was determined by 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) scavenging assay. Various concentrations (0.1, 0.5, 1.0, 3.0, and 5.0 mg/mL) of crude extracts were taken in separate tubes. Ascorbic acid was used as reference compound (0.1, 0.5, 1.0, 3.0, and 5.0 mg/mL). A freshly prepared solution of 0.002% DPPH in methanol was added to each tube containing different concentrations of extracts (2 mL). The samples were incubated in dark place at 37°C for 20 minutes and read at 515 nm. Percentage inhibition of radical scavenging activity was calculated.

Cell viability assay

The 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, MTT assay, was incorporated to assess the viability of the cells 5×10^3 were seeded per well into 96 well plates. 100 μL of the PS4 pure compound in triplicates with different concentrations ranging from 250, 500, 750, and 1000 $\mu\text{g}/\text{mL}$ was tested. 5 $\mu\text{g}/\text{mL}$ of doxorubicin was used as internal positive control and 100 μL of Dulbecco's modified Eagle medium was used as negative control and wells without any cells were considered as blank.

RESULTS

Screening for potent actinobacterial strains

In this study, the pharmaceutically useful *actinomycetes* were isolated from 2 different locations were selected at Ranipet, Vellore district, Tamil Nadu, India (Fig. 1). The samples were processed and isolated were characterized on starch casein agar. A number of 4 promising *Streptomyces* strains were identified, of which 1 strain was identified as novel.

The percentage frequency of the most common genera was determined as *Streptomyces* with 93% occurrence soil samples tested. All the four potent *actinomycetes* isolates, which were evaluated for their antibacterial activity by cross streak assay, revealed 6 isolates with maximum activity (Fig. 2).

Further, the cross streak assay revealed the potent bioactive strains by the inhibiting pathogens. The isolate showed activity against *Bacillus cereus* (2 ± 0.05 mm), *S. aureus* (3 ± 0.05 mm), *P. aeruginosa* (2 ± 0.05 mm), and *E. coli* (1 ± 0.05). Preliminary assay confirmed as the best isolate and it was further considered as the study organism. The cultures characteristics of all the potent four isolates were found aerobic, mesophilic, and grew readily on most of the nutrient media, which further showed round colonies with powdery texture. The morphological characterization of the strain was done based on the color of aerial and substrate mycelia which was identified with smooth surface. The strain was observed by the presence of spiral spores with smooth surface with gray-colored aerial mycelium. The frequent production of spreading substrate mycelia was observed, and the colonies were found darkened during sporulation with spirals. The colonies with powdery appearance had a characteristic feature with smooth surface. The ornamentation of spore surface of was observed under light microscopy and scanning electron microscope analysis (0.7 micron) (Figs. 3 and 4).

Molecular characterization

16S rDNA sequence of all the six potent isolates was submitted to GenBank. Clustering analysis for the six strains was found taxonomically very close to *Streptomyces*. Agarose electrophoresis revealed that



Fig. 1: Soil samples collected from Ranipet, Vellore district, Tamil Nadu, India

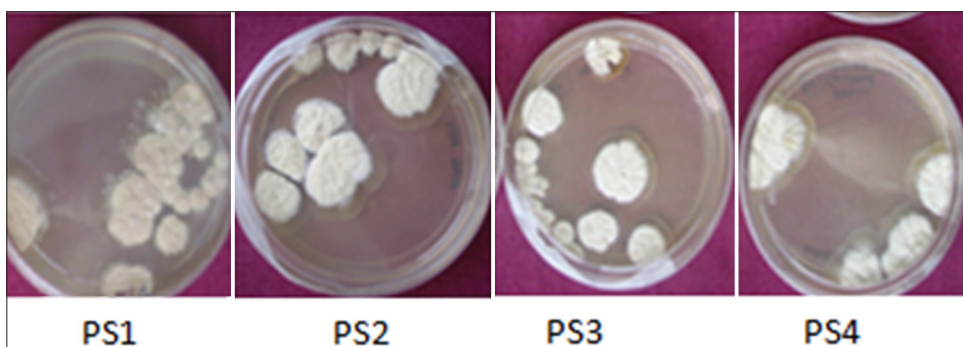


Fig. 2: Colony morphology of four potent *actinomycetes* isolated from soil samples

the size of 16S ribosomal RNA was around >1.2 Kb. Identification and phylogenetic affiliation of the new strain was determined and found similar to the family Streptomycetaceae belonging to genus *Streptomyces*. The 16S rDNA gene sequences of strain PS4 showed 97%

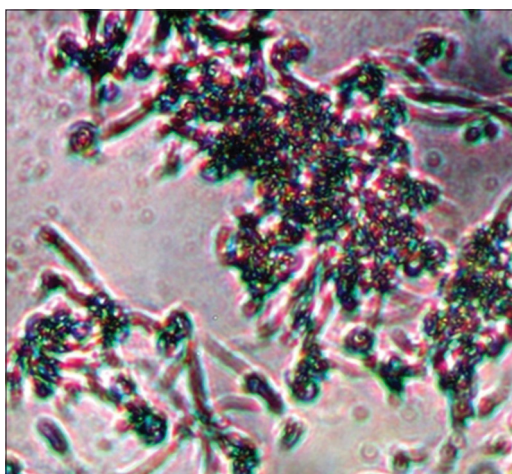


Fig. 3: Microscopic view of potent actinomycete isolates (Magnification, ×100)

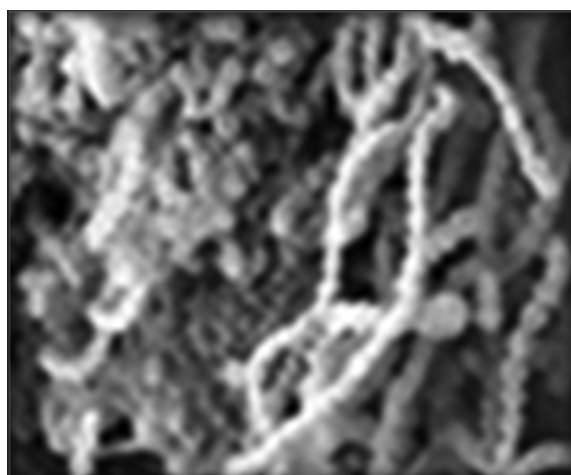


Fig. 4: Scanning electron microscope analysis of potent actinomycete isolates

similarity with *Streptomyces species*, the most closely related species, further it was taxonomically assigned using the BLAST program of NCBI and the phylogenetic tree was constructed based on GeneBee software. The guanine-cytosine content of the strain was found to be 72.2%. The unique combination of genotypic and phenotypic characterization allowed the parallel relatedness to its closest genotypic neighbors (Fig. 5). Hence, they represent a species of the genus *Streptomyces*, for which the name *Streptomyces* sp. PS4 is proposed, submitted to the GenBank database under the accession number.

Extraction of secondary metabolites from six potent isolates

The extraction of secondary metabolites was carried out for the potent isolate by liquid-liquid extraction methods. The bioactive metabolites from the shake flask cultures containing secondary metabolites were separated based on the enhancement of solvent specificity. In spite of using various solvent systems, maximum yield of metabolite and biomass was obtained from ethyl acetate extract. Different fermentation media were used as the base to determine the optimal nutritional conditions for the production of antimicrobial compounds. The influence of pH on the production of antimicrobial compounds revealed the maximum production with antibacterial activity for the strain *Streptomyces* sp. Among the different pH used, pH 7.0 exhibited good growth and antimicrobial activity against tested bacteria. Maximum cell growth was observed at pH 7.0 and pH 7.5 compared to other pH. The effect of growth and antimicrobial metabolite production of strain *Streptomyces* sp. PS4 was analyzed at different temperatures. The temperature 27°C was found to be optimum for antimicrobial metabolite production and exhibited activity against all tested bacteria at different time intervals such as 24, 48, 72, and 96 hrs (Fig. 6). Ethyl acetate extract of the isolate *Streptomyces* sp. showed activity against bacterial pathogens. Alteration of sources resulted in a change of biological activity caused by variation in synthesis rate of bioactive compounds. The maximum activity was found utilizing starch as carbon source and beef extract as the nitrogen sources. The cultural conditions for the production of bioactive metabolites were found at pH 7.2 and temperature 27°C. The influence of rpm on the bioactive metabolite production was found higher at 200 rpm.

Analysis

The IR spectrum showed respective functional groups, which authenticates the characteristic compound. The IR spectrum of the compound showed α , β unsaturated carboxylic acid (Fig. 6).

Bioactivity

The antibacterial activity of the crude extracts from *Streptomyces* sp. exhibited maximum activity *P. aeruginosa* and *S. aureus* (23 mm±0.05) activity compared to the standard drug chloramphenicol (Fig. 7).

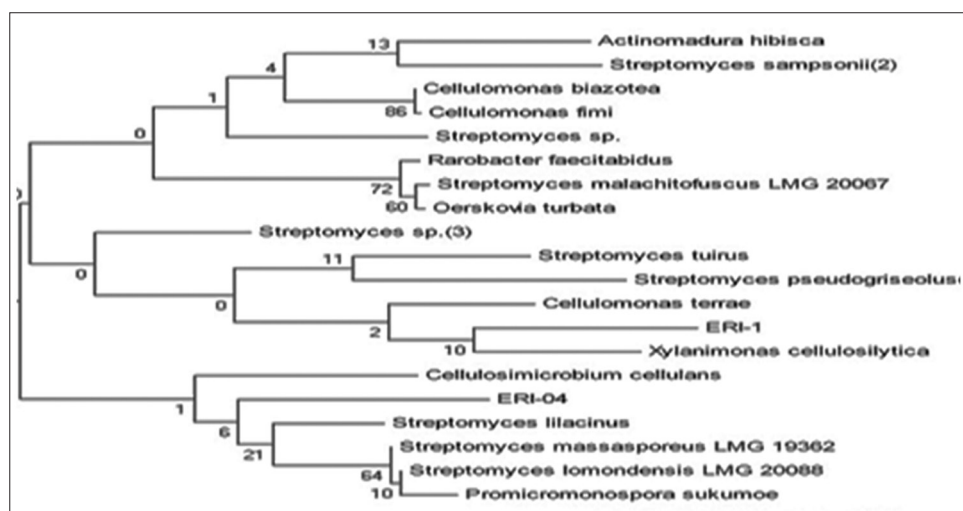


Fig. 5: Phylogenetic tree based on 16S rDNA gene sequences of *Streptomyces* sp

Antioxidant activity of potent isolate *Streptomyces*

The antioxidant potential of the crude extract tested at (0.1, 0.5, 1.0, 3.0, and 5.0 mg/mL) concentrations exhibited strong reducing power activity with $70 \pm 0.15\%$ percent inhibition with the IC_{50} of 0.95 mg/mL (Fig. 8).

DISCUSSION

In the present study, *Streptomyces* strain was found to exhibit potential bioactivity. 518 *Streptomyces* strains and checked for their antibacterial and antifungal activities. It was found that 47.01% showed activity against *S. aureus* and 30% showed activity against *E. coli*. The majority of the isolates (46.43%) showed combined antibacterial and antifungal activity and 25% showed only antibacterial activity. 280 *actinomycetes* were isolated from Kanyakumari district [5]. They showed good antibacterial and antifungal activity. Among the isolates, ERI-1, ERI-3, and ERI-26 showed significant activity against both Gram-positive and Gram-negative bacteria. These isolates were also exhibited good antifungal activity.

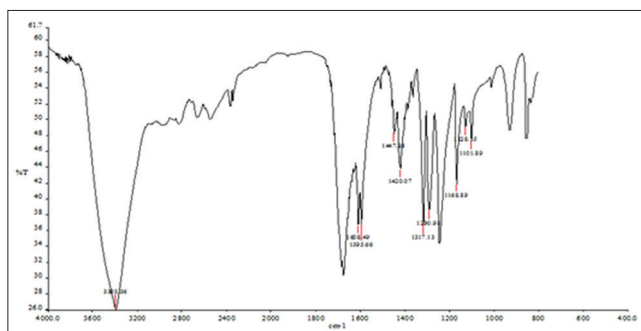


Fig. 6: Fourier transform infrared profile pattern of 3-benzylhexahydropyrrolo[1,2-A]pyrazine-1,4-dione

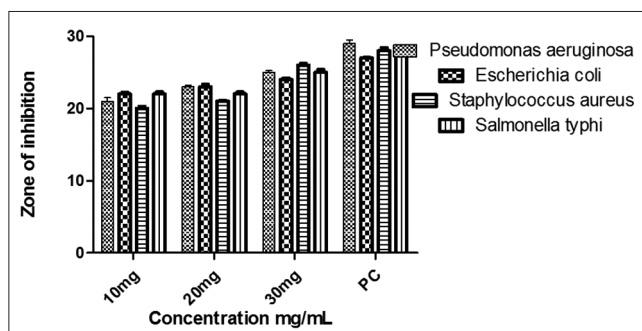


Fig. 7: Antibacterial activity of six potent strains against pathogens

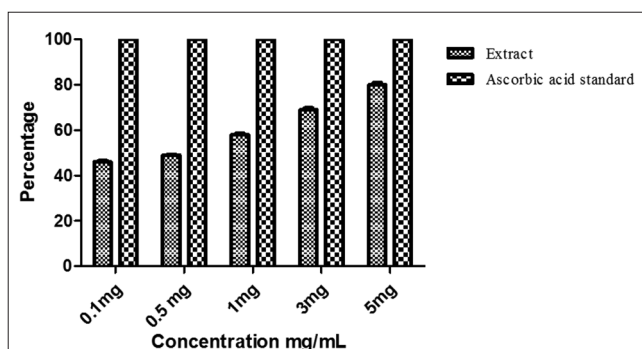


Fig. 8: Antioxidant activity

The compound from *Streptomyces*, p-hydroxybenzoic acid exhibited good activity against fungi. *Trichophyton rubrum* MTCC 296 and *E. floccosum* 73/01 showed MIC of 150 μ g/ml. *Trichophyton simii* 110/02, *Curvularia lunata*, *Aspergillus niger*, *Botrytis cinerea*, *T. rubrum* 57/01, *Scopulariopsis* sp., and *Candida albicans* exhibited minimum inhibitory concentration of 250 μ g/ml. Di-(2-ethyl hexyl)-phthalate from chloroform extract of *actinomycetes* and exhibited good activity against both Gram-positive and Gram-negative organisms at 128-256 μ g ml. Quinone-anthracycline from *Streptosporangium* isolated from an Algerian soil, which exhibited activity against both Gram-positive and Gram-negative bacteria. Broad spectrum, an active compound isolated from *Streptomyces* sp. isolate number 419, showed good activity against wide range of Gram-positive and Gram-negative *E. coli*, *Klebsiella pneumoniae*, *Micrococcus luteus*, *Proteus vulgaris*, *Providencia rettgeri*, *Proteus mirabilis*, *P. morgani*, *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, and *Pseudomonas syringae* pv. *syringae*. Similarly, cytotoxicity studies and the apoptosis have been reported [7]. This study also provides an excellent basis for the investigation for a broad range of antimicrobial and anticancer activities [8-11]. With the above significant features, the strain could be recommended for its use in medicinal and agricultural sectors, an extensive knowledge on the behavior of natural compounds can be gained for the benefit of health [8-11].

CONCLUSION

The aim of this study was to explore the bioactive potential of *Streptomyces* sp. isolated from soil and understand the bioactive properties of the crude and the pure compounds. Hence, high mortality rates, serious side effects, deficiencies of the available chemotherapeutics, and high costs during treatment clearly underscore the need to develop new anticancer agents. With the above significant features, the strain could be recommended for its use in medicinal and agricultural sectors, an extensive knowledge on the behavior of natural compounds can be gained for the benefit of health.

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