

## **A Report on Antimicrobial Activity of Actinomycete, Streptomyces S12c Species**

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### **Abstract**

The focus of this study was to isolate and to identify the actinomycetes having antimicrobial activity. A total of 145 actinomycetes were recovered from soil samples collected from different areas in Tamilnadu, India. All these isolates were screened for their antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia* and *Candida albicans*. On primary screening for antimicrobial activities, out of 145 isolates of actinomycetes, only 22 isolates showed the activity against test organisms. On Secondary screening of 22 isolates, the strain S12c with good antimicrobial activity was selected for secondary metabolite fermentation. The active metabolite was extracted using ethyl acetate (1:1; v/v) at pH 7.0. The separation of the active ingredient and its purification was performed using column chromatography technique. The partially purified antibiotic was subjected to partial characterizations viz., thermal stability, pH stability, effect of detergents, effect of enzymes, and effect of ergosterol. The minimum inhibition concentrations “MICs” of the purified antibiotic were also determined. Thus the antibiotic produced by *Streptomyces* species S12c was found to exhibit potent activity against *Staphylococcus aureus* and *Candida albicans*. Various studies done and the results obtained in the present investigation indicated that *Streptomyces* species S12c produced an effective and stable antifungal and antibacterial antibiotic.

**Keywords:** Soil samples, Actinomycetes, Antimicrobial activity, Screening, Antibiotics,

## Introduction

Search for new antibiotics effective against multi-drug resistant pathogenic bacteria is presently an important area of antibiotic research. Natural products having novel structures have been observed to possess useful biological activities. Soil is a natural reservoir for microorganisms and their antimicrobial products (Dancer 2004). Among microorganisms, actinomycetes are one of the most attractive sources of antibiotics and are biologically active substances of high commercial value and from which, *Streptomyces* species has been the most fruitful source of all types of bioactive metabolites that have important applications in human medicines as anti-viral and anti-cancer compounds and in agriculture as herbicides, insecticides and parasitic compounds (Watve et al, 2001). *Streptomyces* species are considered exceptionally well endowed for “chemical warfare”, presumably allowing them to eliminate bacterial and fungal competitors in soil ecosystems. For more than two decades, clinicians and public health officials have faced hospital acquired methicillin-resistant *S. aureus* (MRSA), which also bears resistance too many antibiotics. During much of this time, vancomycin has been the therapeutic answer to MRSA, but that paradigm has changed. Vancomycin-resistant strains have emerged clinically (Bozdogan *et al.*, 2003; Chang *et al.*, 2003). Effective treatment of the infections caused by these organisms is yet to be established. So the present study aims at isolation and screening of biologically diverse strains of actinomycetes from soil for the production of novel antibiotics.

## Materials and Methods

**Isolation of Actinomycetes:** 30 different soil samples were collected from various places in and around the areas of Palakkad and Coimbatore. The collected sample were weighed as 1 gram and mixed with sterile 100 ml distilled water. The mixture was serially diluted up to 10<sup>-8</sup> dilution. From each dilution 0.1 ml was taken and inoculated onto sterile starch casein nitrate agar (SCN) medium (Ph-7.0) using L-rod method. The inoculated plates were incubated at 27<sup>0</sup>C for 4 days. Individual colonies with characteristics of actinomycetes morphology were isolated and pure cultures of respective isolates were maintained on SCN plates and preserved at 4<sup>0</sup>C.

**Test microorganisms:** The following test microorganisms procured from microbial Type Culture Collection and Gene bank (IMTECH, Chandigarh, India) were used during the investigation: *Staphylococcus aureus* (MTCC 96), *Escherichia coli* (MTCC 739), *Pseudomonas aeruginosa* (MTCC 2453), *Klebsiella pneumonia* (MTCC 4030) and *Candida albicans* (MTCC 227). The fungi were grown at 28<sup>0</sup>C on potato dextrose agar medium and the bacterial cultures were grown at 37<sup>0</sup>c on Nutrient agar medium. All the cultures were stored at 4<sup>0</sup>C and sub-cultured as needed.

The following works deals with the partial characterization of the antibiotic from actinomycete isolate, S12c selected on the basis of strong antimicrobial activity against target organisms by Agar well diffusion method.

**Extraction of the antibiotic using different solvents:** The submerged fermentation of the actinomycete isolates was terminated on the 5<sup>th</sup> day and the broth was centrifuged at 10,000 rpm for 20 minutes to separate the mycelial biomass. Different solvents used were n-hexane, chloroform, n-butanol, ethanol and ethyl acetate to determine the ideal solvent for extraction of the antibiotic from the culture supernatant. The solvent was added to the supernatant in 1:1 proportion. Solvent – supernatant mixture was agitated for 45 minutes with homogenizer. The solvent was separated from the broth by separating funnel. The solvent was evaporated by subjecting the sample to rotating flash evaporator at 40<sup>o</sup> (50rpm) under vacuum. The substance obtained was dissolved in ethyl acetate and concentrated, following which the crude antibiotic powder was obtained. The crude antibiotic was collected and dried in vacuum oven at 40<sup>o</sup>c overnight. All extracts were assayed for their antibacterial and antifungal activity using respective solvents as control by well plate method. The residue (crude antibiotic) obtained was subjected to further purification by chromatography.

**Purification of the antibiotic:** The crude antibiotic powder derived after ethyl acetate extraction and ethanol treatment was subjected to column chromatography using silica gel (3 x 25 cm) as stationary phase. The crude antibiotic powder was dissolved in 5ml of ethyl acetate. The column was developed with solvent mixture chloroform: methanol 95:5 (v/v). Fifteen fractions were collected at an interval of 10 minutes and were subjected to antimicrobial assay.

**Thermal stability of the antibiotic from S12c:** To determine the effect of temperature on the stability of the antibiotic, screw cap ampoules, each with 100µg/ml of the partially purified antibiotic in water were kept at temperatures 30, 40, 50, 60, 70 and 80<sup>o</sup> C for one hour in water bath and also subjected to autoclaving temperature (121<sup>o</sup> C for 15 minutes). After heat treatment at various temperatures the antibiotic solutions were cooled to room temperature and the residual antibacterial and antifungal activity were determined against the target test organisms.

**Effect of pH on activity and stability of the antibiotic:** To determine the effect of pH on the antibiotic, 100µg of the antibiotic was mixed with 1ml of 0.1 N phosphate buffer of varied pH (5.7-8.0) in various tubes, and incubated for one hour at 30<sup>o</sup> C. After incubation the antibacterial and antifungal activity in each tube was determined against the target test organisms.

**Effect of enzymes on the activity of the antibiotic:** The sensitivity of the antibiotic to denaturation by enzymes proteinase K and lysozyme was tested. Both the enzymes were dissolved in distilled water at the concentration of 1 mg/ml. One hundred µl of the antibiotic solution (100µg/ml) was mixed with 100µl enzyme and incubated at 30<sup>o</sup> for 3 hours. The antibiotic solution without any enzymes served as control (Munimbazi and Bullerman, 1998). After incubation the residual antibacterial and antifungal activity were determined against the target test organisms.

**Effect of detergents on the activity of the antibiotic:** Susceptibility of the antibiotic to denaturation by various detergents, viz Tween 20, Tween 40, Tween 80, Sodium dodecyl sulphate (SDS) and was determined by mixing the detergents with the antibiotic and incubating them at 30<sup>0</sup> C for 6 hours. Detergents were dissolved in distilled water at concentration of 0.01g/ml. One hundred µl of the antibiotic solution (100µg/ml) was mixed with 100 µl detergent and incubated as mentioned above. Detergents added to distilled water were used as controls to check the effect of detergents themselves on the target cultures.

**Effect of ergosterol on the antifungal activity of the antibiotic:** To determine the effect of the antibiotic on the ergosterol present in the fungal cell membrane, ergosterol was used as the reversal agent to test for its ability to reverse the inhibition of *Candida albicans* caused by the antibiotic. Muller – Hinton agar plates with 0.5% ergosterol was prepared along with a control without ergosterol. About 0.1 ml of the antibiotic (100 µg/ml) was added to the well and checked for antimicrobial activity.

**Determination of the minimum inhibitory concentration(MIC):** The minimum inhibitory concentration and minimum bactericidal and fungicidal concentration values of the antibiotic were determined by broth tube dilution procedure using two-fold dilution in Nutrient and Sabouraud Dextrose broth respectively at 28<sup>0</sup> C (Baron *et al.*, 1994).

## Results

**Colony Isolation:** Out of 30 soil samples total 145 actinomycetes colonies were selected. This selection was done based on morphological characterization of bacterial colonies. The gram stain of selected isolates showed a filamentous structure of actinomycetes. On primary screening process only 22 isolates showed activity against test organisms and on secondary screening 9 isolates were found to exhibit antimicrobial activity. The isolate S12c showed effective antimicrobial activity against target organisms. According to antimicrobial activity and spectrum of broadness the isolate S12c alone was subjected to further study.

**Extraction and purification of the antibiotic:** Different solvents like n-hexane, chloroform, n-butanol, ethyl acetate and ethanol were tested for the extraction of the antibiotic. In the case of *Streptomyces* species S12c, the antibiotic yield was observed in the residue extracted using ethyl acetate. (Figure-1) Fraction 13 obtained by column chromatography was found to exhibit antimicrobial activity against the target test organisms.

**Thermal stability of the antibiotic:** The antibiotic was stable at different temperatures ranging from 30<sup>0</sup>C to 60<sup>0</sup>C but it lost its antibacterial as well as antifungal activity completely after autoclaving at 121<sup>0</sup>C for 15 minutes. This indicates that the antibiotic produced by the isolate S12c is very heat stable.

**pH stability of the antibiotic:** The antibiotic was stable within the pH range of 5.8 to 8.0 as tested against *S. aureus* (MTCC 96) and *C. albicans* (MTCC 227) after incubating the antibiotic at pH values in the range of 5.8 to 8.0, whereas the activity decreased slightly at pH 6.4 and below. Thus the antibiotic is active over a wide range of pH values.

**Effect of detergents on the activity of antibiotic:** The antibiotic alone showed 20mm to 24mm inhibition zone against *S. aureus* (MTCC 96) and *C. albicans* (MTCC 227) respectively. The inhibition zone diameter obtained with mixture of antibiotic and detergents ranged between 14mm to 18mm against *S. aureus* (MTCC 96) and ranged between 15mm to 19mm against *C. albicans* (MTCC 227).

**Effect of enzymes on the activity of the antibiotic:** The antibiotic alone showed 22mm and 23mm inhibition zone diameter against *S.aureus* (MTCC 96) and *C. albicans* (MTCC 227) respectively. The inhibition zone diameter obtained with mixture of antibiotic and enzymes ranged from 15mm to 17mm against *S. aureus* (MTCC 96) and ranged from 16mm to 19mm against *C. albicans* (MTCC 227).

**Effect of ergosterol on the antifungal activity of the antibiotic:** The control plate without ergosterol showed an inhibition zone diameter of 20mm, whereas the plate containing the reversal agent, ergosterol showed a slightly reduced inhibition zone diameter which was 18mm.

**Determination of Minimum inhibitory concentration (MIC):** MIC was determined by broth tube dilution procedure using two-fold dilutions of the antibiotic in Sabouraud Dextrose broth and Nutrient broth for *C. albicans* (MTCC 227) and *S. aureus* (MTCC 96) respectively. These results indicate that the antibiotic has antibacterial as well as antifungal activity, but at particular concentration it is bacteriostatic and fungistatic only (Table-2).

## Discussion

The actinomycetes are known to be rich sources of novel antibiotics. They are the most economic producers of pharmacologically potential compounds with antibiotic and antitumour properties. In the present study the actinomycetes with potential activity against bacteria and fungi was isolated from Coimbatore. In the course of systemic screening for actinomycetes 145 strains were isolated and only 9 isolates showed broad spectrum of activity. One promising isolate S22c with strong antibacterial and antifungal activity was selected for further studies.

The antimicrobial efficacy of the isolates was tested by using five different solvents. The maximum inhibitory zone observed in ethyl acetate extract. Similarly Franco and Coutinho 1991 reported that antifungal antibiotics are extracted using ethyl acetate. The antibiotic produced by S12c was very heat stable and active over a wide range of pH. The antibiotic was kept at various temperatures (30 °c to 80 °c) for 1 hour and was found to be stable at different temperature but lost its activity at 121 °c

for 15 minutes. Similar observations were reported by Augustine *et al.*, 2005 on antifungal metabolites produced by *Streptomyces albidoflavus* PU23. The antibiotic was stable within the pH range of 5.2 to 8.0. Most isolates showed a difference of concentration between inhibitory and cidal values indicating that although the antibiotic has bactericidal and fungicidal but at particular concentration it is fungistatic only. There was a significant loss of antimicrobial activity of the antibiotic after treatment with detergents and enzymes. The effect of detergents and enzymes on the antifungal antibiotic produced by *Streptomyces albidoflavus* PU23 was studied by Augustine *et al.*, (2005). They found that the activity of the antifungal antibiotic was retained after the enzymatic treatment.

The antibiotic from *Streptomyces* species S12c probably binds to the ergosterol present in the fungal cell membrane resulting in the leakage of intracellular material and eventually death of the cell. A similar study was carried out by Augustine *et al.*, (2005) on *Streptomyces albidoflavus* PU23. They noticed a reduced antifungal activity in the presence of added ergosterol. Further research is needed to determine the chemical structure of the antibiotic compound. With the seemingly exponential emergence of microorganisms becoming resistant to the clinically available antibiotics already marketed; the need for discovering novel drugs is real. Further study on the bioactive metabolite produced by isolate S12c is under progress.

Optical density values at 520 nm indicating the MIC values of the partially purified antibiotic from *Streptomyces* species S12c.

Concentration of the antibiotic ( $\mu\text{g/ml}$ )	Test organism	
	<i>Staphylococcus aureus</i> (MTCC 96)	<i>Candida albicans</i> (MTCC 227)
100	0.08	0.23
200	0.07	0.21
300	0.06	0.20
400	0.06	0.20
500	0.05	0.13
600	0.05	0.11
*Positive control	0.03	0.14
**Negative control	0.16	0.23

\*Medium containing 600  $\mu\text{l}$  of 0.1% of a known antibiotic and inoculated with the test organism.

\*\*Medium without any antibiotic and inoculated with the test organism.

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