Secondary Metabolites of *Streptomyces* and Their Antibacterial, Antifungal Properties

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Abstract **- *Actinomycetes* are abundantly present in soil and they produce a variety of antimicrobial compounds that can be used as chemotherapeutic agent in order to limit the infection. In present study, *Actinomycetes* were isolated from soil and vermicompost samples collected from the Guntur district, Andhra Pradesh, India. Total 93 isolates were isolated from soil and vermicompost samples. Isolates were identified by colony characterizations, microscopy and biochemical tests. Out of the 93 isolates six isolates are showing six *Streptomyces* sp ESS-4 (*Streptomyces fradiae*), DVS-11 (*Streptomyces olivoviridis*), KZSS-6 (*Streptomyces koyangensis*), NVS-10 (*Streptomyces flavofuscus*), TSS-3 (*Streptomyces griseorubens*), and TVS-2 (*Streptomyces lunaelactis*) were previously reported to have antagonistic activity in fungal pathogens, Plant Growth Promoting traits, Greenhouse and field conditions in black gram, antibacterial activity, antibiotic resistances, and optimization of pH, salinity, temperature, carbon sources, and heavy metals. Microorganisms, extracts of botanicals and secondary metabolites of microorganisms are commonly used as bio-pesticides. Primarily these isolates were screened for intercellular metabolites production by conventional methods. *Actinomycetes* will be screened against fungal pathogens. Culture filtrates of the most promising *Actinomycetes* will be evaluated for their effectivity. Compounds from the filtrate will be identified and isolated by using various chromatographic techniques.**

Index Terms **- *Actinomycete*, Antibacterial activity, Antibiotic resistances, Antifungal activity.**

1.INTRODUCTION

Biological control is one of the better ways to control plant pathogens and pests of important crops as it causes less damage to the environment. Some of the bicontrol agents include the use of whole microorganisms, extracts of botanicals and secondary metabolites (antibiotics) of microorganisms. It is in practice to use various microorganisms like *Trichoderma sp, Pseudomonas sp*, and arbuscular mycorrhizae fungi to control various fungal pathogens and nematodes of plants in the form of seed coatings and soil treatment.

Actinomycetes have been identified as one of the major groups of the soil population, which may vary with the environment of ground type Kuster HJ 1968. *Actinomycetes* are Gram positive bacteria and are an important source of antibiotics Valli et al., 2012. They have high G+C content 55-77% (Lo et al., 2002; Ningthoujam et al., 2009; Lam et al., 2006 and Ndonde et al., 2000). They belong to order *Actinomycetales* and are composed of around eighty genera Stackebrandt et al., 1997 and Goodfellow et al., 1983. *Actinomycetes* are economically and biotechnologically feasible prokaryotes. They produce a variety of bioactive compounds that can be used to treat infections, they include antitumor, antifungal, antibacterial agents Bizuye et al., 2013 and enzymes Jeyadharshan et al., 2013. Among all genus, *Streptomyces* are the best secondary metabolites producers (Valli et al., 2012). First antibiotic, streptomycin has been isolated from *Streptomyces* in 1945 by A. Waksman Atta et al., 2010.

Streptomyces are filamentous bacteria with a complex life cycle. Aerial growth coincides with the production of many secondary metabolites; *Streptomyces* is widely recognized as industrially relevant microorganism for their ability to produce different types of novel secondary metabolites including many clinically important antibiotics Hopwood DA 2007.

Streptomyces species produce about 75% of commercially and medically useful antibiotics Baltz

RH 2006. They have provided more than half of the naturally occurring antibiotics discovered to date and continue to screen for useful compounds Miyadoh S 1993. Still, microbial natural products remain the most promising source of novel antibiotics although new approaches are required to improve the efficiency of the discovery process Thumar JT et al., 2010.

Because of the increasing resistance of microorganisms towards discovered antibiotics there is a need to explore the potential of novel strains of *Actinomycetes* for their new secondary metabolites and to study its role in the field of antibiotics. Thus, we collected soil samples from different sites viz; from forest, cultivated river side and open waste fields for *Actinomycetes* isolation and screened for their antimicrobial activity. Inhibition of the test pathogens in this study proved their antimicrobial potency of isolates and has opened the way for further investigations regarding the novelty of the metabolites and isolated *Actinomycetes* strain. Thus, the focus of this study is to isolate *Actinomycetes* from soil and vermicompost and purify their bioactive compounds to inhibit bacteria related to antibiotic resistance nature. Out of 93 Actinomycetes, against 14 human bacterial pathogens. Studies conducted by Castillo et al. (2002) and Compant et al. (2005) demonstrated that Streptomyces sp and S. griseoviridis produced antibiotic, which can inhibit the growth of Fusarium and Phomopsis.Streptomyces sp produces multiple antibiotics to suppress diverse microbial organisms, and also enhance the plant growth (Pal & Gardener 2006). Streptomyces sp. produces antibiotics that help in the control of bacterial diseases

2. MATERIALS AND METHODS

2.1 Extraction and purification of active *Streptomyces* species

The *Streptomyces* sp will be grown in antibiotic production medium at room temperature (28±2ºC) for 10 days. The cultures were harvested and filtered through cheesecloth to separate the mycelial biomass. The culture broths were centrifuged at 12000×g for 15 min and the supernatants were collected. The cell-free supernatants and mycelial biomass were used separately for the extraction of bioactive principles. Extraction of extracellular metabolites was done by adding ethyl acetate to the cell-free supernatant in the ratio of 1:1 (v/v). The solvent layer (upper) was collected and concentrated using a rotary evaporator. The mycelial biomasses were used for the extraction of intracellular bioactive metabolites. Acidified acetone (Acetone: HCl at 99:1 ratio) was added to the mycelial biomass in the ratio of 2:1 (v/w) and kept under shaking at 200 rpm in a rotary shaker. After 12 h, the acetone extracts were separated by vacuum filtration and concentrated using a rotary evaporator. Similarly, bacteria were grown in antibiotic production medium at room temperature (28±2ºC) for 2 days and extracted similarly described as above.

2.2 Purification by using TLC

The compound will be purified by commercially available TLC (Silica gel sheets) using various solvents such as methanol, chloroform, acetic acid, n-butanol, n-hexane and water in different proportions. Crude compound will be dissolved in 200 micro litres of ethyl acetate with the help of capillary tube and chromatogram will be run. After drying, chromatogram will be kept in the iodine chamber to visualize the separated compound as clear spots.

2.3 Antibiotic production

On the basis of zone inhibitory size produced in *Actinomycetes* crude extract was subjected for antibiotic activity by agar well diffusion method. Briefly, *Actinomycetes* was selected and grown in a Starch Casein Broth (200ml) incubated at 37°C for 7 days. Thenmozhi M, Kannabiran K 2010. To concentrate the antimicrobial metabolite, equal volumes of ethyl acetate (200 mL) was added in culture for 1 hour in thermostat water bath shaker at 37°C Selvameenal L et al., 2009. Then the ethyl acetate containing active metabolite was separated from the solid residue with Whatman No. 1 filter paper. The crude extracts obtained from each isolates was dissolved in ethyl acetate (76 mg/mL) and used as stock concentration for determination of antibiotic activity against test antibiotics. The potential isolates were tested for their antibiotic susceptibility and resistance activity against 11 different antibiotics such as Amoxicillin, Ampicillin, Cefpodoxime, Chloramphenicol, Ciprofloxacin, Neomycin, Novobiocin, Penicillin, Rifampicin, Tetracycline, Vancomycin. Muller Hinton Agar media was prepared, autoclaved at 121°C for 15 minutes at 15 lbs pressure, cooled and poured into petri plates. After solidification, 3 days old *Actinomycetes* culture was

swabbed and followed by placing the antibiotic disc aseptically (Benveniste, & Davies 1973). The plates were incubated at 28 ± 2°C for four to five days. The results were noted as the zone of inhibition in mm.

2.4Antibacterial activity

The inoculums were separately prepared by the cultivated Actinobacterial isolate on starch casein nitrate agar at 30°C for 7 days. Then, the liquid media (50 ml media/250 ml Erlenmeyer flask) was incubated with 2 disks (6 mm in diameter) of Actinobacterial inoculum and incubated at 30°C in a rotary shaker at 250 rpm (revolution per minutes) for 7 days. After that, the culture broth was centrifuged at 8000 rpm at 4°C for 20 minutes. The aliquot supernatant was filtered using 0.45 µm membrane filter (millipore). The filtrate of Actinobacterial isolate was tested as antibacterial metabolite Karthick L et al.,2010.

The antibacterial activity was determined, pathogenic bacteria were grown overnight in nutrient broth at 37°C for 24 hrs (O.D = 0.5; McFarland 1×108 CFU/ml). The cultures of test organisms were streaked on Mueller-Hinton agar medium separately. Wells (6 mm in diameter) were prepared in each seeded agar plate, and each well was filled with 60 µl of the aliquot supernatant containing the active metabolites Anansiriwattana W et al., 2006. The plates were kept at 4°C for 24 hrs for the diffusion of the metabolites. Then, the plates were incubated at 37°C for 24 hrs. After the incubation period, the diameter of inhibition zones was measured. Each test was in triplicate, and the activities were shown as the mean of the diameter of the inhibition zone Shahrokhi S et al.,2005; Kavanagh F 1972, and Panwar D, Vipin SK 2012.

2.5 Screening of *Actinomycetes* for antifungal activity

Antifungal activities of *Actinomycetes* were tested by agar plug method. The isolated strains were seeded over the entire surface of ISP 2 medium Petri dishes. As soon as the organism developed, agar discs were cut out by the cork borer (6 mm diameter) and transferred to the surface of Sabouraud dextrose agar plates seeded with the test organisms i.e., *Aspergillus flavus, Aspergillus fumigatus, Candida albicans* and *Penicillium.* The Petri dishes were then kept in incubator at 27°C to allow the growth of test organisms.

3. RESULTS

The optimal conditions for antibiotic production were 30°C in shaker incubator (250 rpm) for 7 days using basal fermentation medium composed of (g/100 ml distilled water): 2.5% glucose, 0.5% soybean, 0.5% NaCl, 2% K2HPO4, 0.05% MgSO4, and 0.01% CaCO3 at pH 7. The purpose of this study was to evaluate antimicrobial activity of Actinomycetes. Due to emergence of multidrug resistant human pathogens there is a need to discover new antibiotics which are effective against these pathogens.

Six isolates were screened against human bacterial pathogens. The percentage of activity to each pathogen is listed below: *Bacillus subtilis*, *Micrococcus luteus*, *Staphylococcus aureus*, *Streptococcus mutans*. Gram negative bacteria *Klebsiella pneumonia*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Saccharomyces cerevisiae* ESS-4 shows five fungal strains except for Klebsiella pneumonia, DVS-11 shows six strains, KZSS-6 shows maximum growth of four bacterial strains except for Streptococcus mutans, Pseudomonas aeruginosa, NVS-10 shows six strains, TVS-2 shows four strains except for Micrococcus luteus, Saccharomyces cerevisiae, TSS-3 shows five strains except Micrococcus luteus (Table 01 and Figure 01). The six *Streptomyces sp* were able to resist all 11 antibiotics. The zone of inhibition is ranged from 14mm-20mm. DVS-11 showed maximum resistance to Amoxiclav, Ampicillin, Ciprofloxacin, and Vancomycin, ESS-4 showed maximum resistance to Cefpodoxime and Tetracycline, TVS-2 showed maximum resistance to Chloramphenicol and Rifampicin, NVS-10 showed maximum resistance to Neomycin and Penicillin and KZSS-6 showed maximum resistance to Novobiocin. The results are represented in the Figure 02 and Table 02.

ESS-4 shows five fungal strains except *Candida krusei*, DVS-11 shows four strains except *Candida krusei*, *Aspergillus flavus*, KZSS-6 shows maximum growth of six fungal strains, NVS-10 shows five strains except *Trichoderma viride*, TVS-2 shows four strains except *Candida albicans*, *Aspergillus niger*, TSS-3 shows five strains except *Candida albicans* the results are represent in Table 03 and figure 03.

4. DISCUSSION

Vijayakumar *et al*. (2012) reported 68 morphologically different *Actinomycetes* and tested

antimicrobial activity. Among the strains tested, VPTSA18 showed strong antimicrobial activity. Siva Kumar *et al*. (2011) reported 78 *Actinomycetes* and carried out the antibacterial activity by cross-streak technique.

Deshmukh (2012) studied the antibacterial activity of *Actinomycetes* against human bacterial pathogens by using the streak method. Study conducted by Wadetwar & Patil (2013) showed the antimicrobial potential of *Actinomycetes* against *Bacillus subtilis, B. cereus, Escherichia coli.* and *Staphylococcus aureus.*

Jaganmohan et al. (2013) studied the antimicrobial potential in plants, such as Blight of pear, apple, and peach (Stockwell et al., 2012).

Atta et al. (2010 b) also revealed that growth medium amended with pH 7.5 showed good results in metabolite production of *Streptomyces ramulosus* AZ-SH-29. Saha *et al.* (2010) reported that *Streptomyces* sp. MNK-7 showed the production of antibacterial and bioactive metabolites at 35°C.

Studies performed by Askar et al. (2011) revealed that *Streptomyces spororaveus* RDS28 showed good growth at 31°C, pH 7.5, and glucose as carbon sources. El-refai et al. (2011) reported starch as the carbon source for the production of metabolites in the *Actinomycetes*.

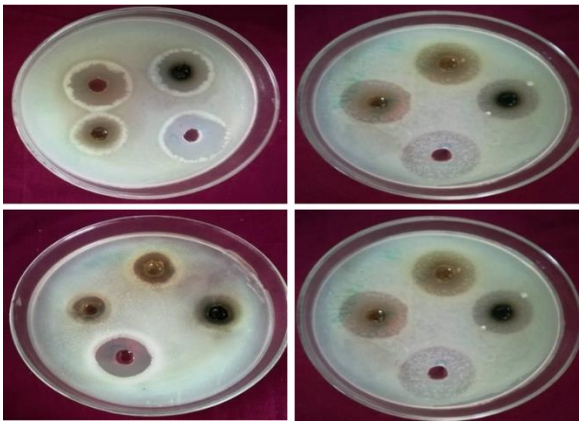
(Mohseni et al., 2013), to overcome this problem we can use the potential of *Actinomycetes*, that are able to produce bioactive compounds and an important source of secondary metabolites (Suthindhiran et al., 2009).

Iznaga et al., 2004 reported *Actinomycetes* from soils rich in minerals are promising producers of antifungal compounds. The high number of polyene macrolide producer strains demonstrate the effective and powerful antifungal activity of the compounds belonging to this family and their important protective effect to the *Actinomycetes'* life

5. CONCLUSION

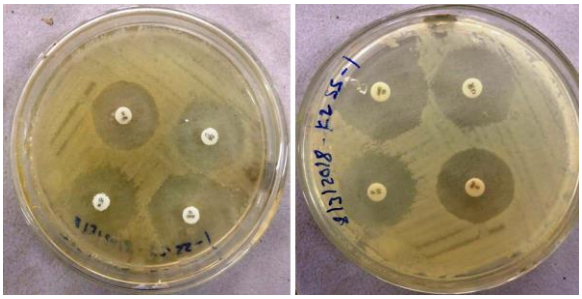
The present research highlights the importance of soil *Actinomycetes* which are quite active in producing antagonistic metabolites. Six *Streptomyces* sp., were showing antimicrobial activity against the microbial pathogenic bacteria its shows 40-50% activity. Our finding on effective zone of inhibition size (8-18mm) on secondary screening of isolates against test fungal pathogens is comparable with most of the previous similar studies against different test pathogens. Antibiotic assay was performed, the zone of inhibition of the six *Streptomyces* are 10mm to 30mm. Further, larger numbers of samples would have produced more significant results. Future research will be carried out in determining the molecular nature of this bioactive metabolite, produced from soil *Actinomycetes*.

Figure 01 Antibacterial activity for six selected *Streptomyces* species



These images represented show the antibacterial activity with six *streptomyces* crud extract.

Figure 02 Antibiotic assays for six *Streptomyces* species

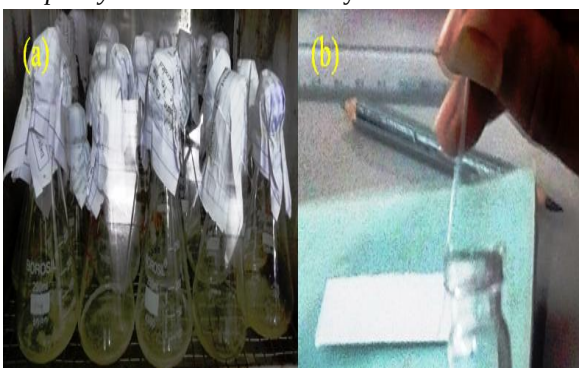


These images are representing the antibiotic assay for six promising *Streptomyces* species.

Figure 03 Antifungal activities for promising isolates

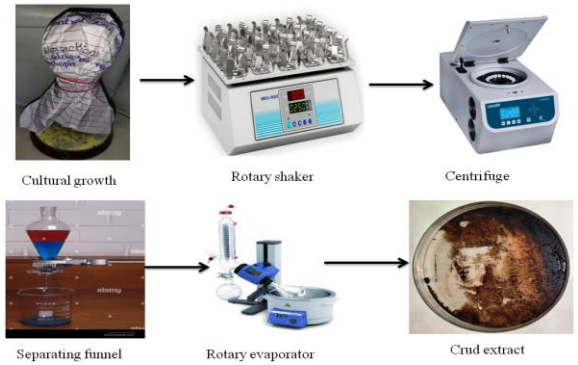
This image is represent in the antifungal activity for *streptomyces* species

Figure 04 Extraction and purification of active *Streptomyces* and Purification by TLC



(a) Growth of *Streptomyces* species (b) Purification of TLC by *Streptomyces koyangensis.*

Figure 05 Process of secondary metabolites production



Semitic diagram is representing the process of secondary metabolites production for *Streptomyces* species.

Table 01 Antibacterial activity against the gram positive and negative bacteria

Isolated cultures	*Bacillus subtilis*	*Micrococcus luteus*	*Staphylococcus aureus*	*Streptococcus mutans*	*Klebsiella pneumonia*	*Proteus vulgaris*	*Pseudomonas aeruginosa*	*Saccharomyces cerevisiae*
ESS-4	++	+++	++	+	-	++	+++	++
DVS-11	++	+++	++	+++	+++	++	+	+++
KZSS-6	+++	++	+++	-	++	++	-	++
NVS-10	+	+++	++	+++	++	++	+++	++
TVS-2	+++	-	++	+++	++	++	++	-
TSS-3	++	-	++	+	++	++	++	+++

Based upon growth the scale was given as follow; (- no growth, + slight growth, ++moderate growth, and +++ good growth)

Table 02 Antibiotic activity for six *Streptomyces* species and zone of inhibition

Isolates	AM	AMP	CF	CP	CIP	NE	NB	P	RF	TR	VC
TVS-2	18	16	15	20	16.33	18	19	15	20	16	16
KZSS-6	15	17	15	14	17	17	20	15	14	17	17
ESS-4	18	16	17	15	17	18	13	15	15	19	15
NVS-10	15	17	16	18	16	18	17	18	19	16	18
DVS-11	18	17	17	15	17	16	18	17	16	16	18
TSS-3	14	16	18	19	15	15	18	19	18	16	19

Am= Amoxyclav, AMP= Ampicillin, CF= Cefpodoxime, CP= Chloraphnicol, CIP= Ciproflaxacin, NE= Neomycin, NB= Novobiocin, P= Penicillin, RF= Rifampicin, TR= Tetracycline, VC= Vencomycin

Table 03 Antimicrobial susceptibility of antagonistic *Actinomycetes* against test organisms

Isolated cultures	*Penicillium*	*Candida albicans*	*Candida krusei*	*Aspergillus niger*	*Aspergillus flavus*	*Trichoderma viride*
ESS-4	12	08	00	16	10	10
DVS-11	12	10	00	14	00	12
KZSS-6	10	12	14	16	18	10
NVS-10	12	14	16	14	18	00
TVS-2	10	00	14	00	14	16
TSS-3	14	00	14	12	14	16

*Isolates showing maximum inhibition against all test organisms, #isolate showing highest zone of inhibition against test pathogen.

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