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ORIGINAL ARTICLE

Antimicrobial Activity of Actinomycetes Isolated From Coal Mine Soils of Godavari Belt Region, A.P, India

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ABSTRACT

Actinomycetes are best known for their ability to produce antibiotics and are gram positive bacteria which comprise a group of branching unicellular microorganisms. Among actinomycetes, the streptomycetes are the dominant. The non-streptomycetes are called rare actinomycetes, comprising approximately 100 genera. The potentiality of a particular antibiotic for important therapeutic usefulness in the treatment of one or more infectious diseases depends upon its action on the causative agents of the disease and its lack of toxicity of the affected animals. The screening programs for new actinomycetes and for their antibiotics are still proceeding at a very rapid pace. There is a need for the development of new antibiotics to overcome the problems associated with the existing antibiotics. Antibiotic resistance was approximately recorded in 50% of the strains with broad or narrow range. All the strains under study showed negative activity to two gram negative bacteria strains, Enterobacter aerogens and Klebsiella pneumonia. MMKK5 strain found to be highly active against test microorganisms like Bacillus subtilis, Bacillus megaterium and Bacillus stearothermophilus and was moderately active against Bacillus cereus and staphylococcus aureus, Micrococus luteus and E. coli showed slightly active 'inhibition while Enterobacter aerogenes, Proteus vulgaris and Klebsiella pneumonia showed passive activity.

Keywords: *Actinomycetes, Coal mine soil, MMKK5 strain*

INTRODUCTION

Microbial natural products still appear as the most promising source of the future antibiotics that society is expecting [1]. Antibiotics are produced by bacteria, fungi, actinomycetes, algae, lichens and green plants. Since the isolation of actinomycin and streptomycin the actinomycetes have received tremendous attention [2]. Members of streptomyces are a rich source of bioactive compounds, notably antibiotics, enzymes, enzyme inhibitors and pharmacologically active agents and about 75% of the known commercially and medically useful antibiotics are produced by streptomyces [3]. Beijerinck[4] and Eriko [5] established that actinomycetes occur in great abundance in the soil and have a great role in the management of microbial stability with the production of antibiotic substances.

Actinomycetes live in soil and decompose organic matter such as cellulose, hemi-cellulose, pectin, chitin etc. In the drug discovery, these microorganisms are widely recognized for their ability to produce secondary metabolites which commercially viable antibiotic activity. *Streptomyces* antibiotics group was first recognized as a common and important group of soil actinomycetes [6]. *Streptomyces rochei* was first recognized as an important group of soil bacteria [7]. Streptomycin, the first treatment for tuberculosis, was derived from the largest genus of these bacteria, *Streptomyces.* Erythromycin and tetracycline are two other common medicines derived originally from these microorganisms. Streptomycetes synthesize about two-thirds of the currently known antibiotically active substances [8].

The economic importance of these antibiotics has led to increased interest in the physiological and genetic aspects of antibiotic biosynthesis by actinomycetes [9-10]. Actinomycetes, especially *Streptomyces,* are of particular industrial importance in antibiotics production and account for more than 70% of the world's naturally occurring antibiotics. Vancomycin therapy of staphylococcal infections has been associated with a slow and inadequate response in many instances [11]. Effective treatment of the infections caused by these organisms is yet to be established. Thus, the need

for the discovery and development of new and effective antibiotics is a priority. Presently, there is little documentation is available on the *Streptomyces* sp. for their potential to produce antimicrobial compounds. Wang *et al.,* [12] isolated glycopeptide, antitumor, antibiotic, zorbamycin from *Streptomyces* sp. and macrolide antibiotic producing streptomycete strains by the regeneration of protoplasts. Synthesis of new antibiotic monomycin by *Actinomyces circulatus* var *monomycini* was a remarkable discovery in the antibiotic field [13].

The potentiality of a particular antibiotic for important therapeutic usefulness in the treatment of one or more infectious diseases depends upon its action on the causative agents of the disease and its lack of toxicity of the affected animals. Meevootisom and Nomi, [14] isolated antibiotic producing *Streptomyces* from the soil. Porter (15) reported prevalence and distribution of antibiotic producing actinomycetes. Waksman and Lechevalier (16) read guide to the classification and identification of the actinomycetes and their antibiotics. William *et al.* [17] has revealed the use of antibiotics for selection isolation an enumeration of actinomycetes in soil.

A survey of the Antibiotic Literature Database (ABL) from the Bioresearch Italia Database provided more than twenty-three thousand microbial products possessing biological activity, i.e., antifungal, antibacterial, antiviral, antitumor, cytotoxic and immunosuppressive. Among these majorities are from the fungal kingdom followed by strains belonging to the genus Streptomyces (32%) Common actinomycetes produce 15.1% of the microbial products reported in the database and rare actinomycetes produce about 10.6%. Out of more than 8000 antimicrobial products described in the ABL database, 45.6% are produced by stretomycetes. The discovery of streptomycin as an amino glycoside antibiotic from streptomyces, a large number of antibiotics and major therapeutic agents such as chloramphenicol, tetracyclines, macrolides and β-lactam cephancyin group have been obtained from many strains of Streptomyces and Streptoverticillium [18].

Microbes from extreme environments have attracted considerable attention in recent years. This is primarily due to the secret that they hold about the molecular evolution of life and stability of the macromolecules. The extremophilic actinomycetes are relatively less explored group. Antibiotic production was favoured with addition of variety of nitrogen sources, like ammonium sulphate, ammonium nitrate and urea. Streptomyces kananmyceticus also yielded maximum antibiotic production with ammonium sulphate [19]. In Thailand, the production of a novel antibiotic, streptothricin and the antimicrobial activity of streptomyces strains were elaborated and recorded [20]. Rinehart et al. [21] and Omura et al. [22] reported the comparison of the spectral data of geldanamycin a known compound which was obtained from the culture broth of Streptomyces hygroscopicus. It was evaporated to dryness in water bath a1 80 - 90 ^{o}C and the residue obtained was weighed. Thus obtained compound was used to determine antimicrobial activity, minimum inhibitory concentration and to perform bioautography.

Different soils all over the world had been exploited in search of bioactive actinomycetes to discover new antibiotics. Abdelghani, [23] investigated the unexplored regions of the world with the aim of isolating organisms for the production of antibiotics, whose, potential was neglected throughout the history. In the present investigation an attempt was made to understand the diversity of actinomycetes in the coalmine soils of Godavari belt region, A.P, India. Apart from that the actinomycetes isolated from the coal mine soils were screened for the antimicrobial activity against certain commonly occurring bacteria (eg. *E. coli*) and (eg. *P. aeruginosa*) pathogenic bacteria. In the present investigation, an attempt was made to isolate and assess the antimicrobial activity of actinomycetes from coal mine soils of Godavari belt region, A.P, INDIA.

MATERIALS AND METHODS

Soil sample collection

Soil samples were collected from different coal mine soils of Godavari belt soils *viz* Mandamarri Kalyani Khani (MMKK), Godavarikhani (GDK), Kottagudem (KGM), Sattupally Opencast (SPOL-OC), Bhupalapally (BPA). Each collection was made from 10-15 cm depth of the soil. These were air-dried for 1 week, crushed and sieved. The sieved soils were then used for actinomycete isolation.

Isolation and culture condition

For each collected sample, 1g of the soil were suspended in 100 ml of physiological water (NaCl 8.5 g/1) then incubated in an orbital shaker incubator at 28 ^{o}C with shaking at 200 rpm for 30 min. Mixtures were allowed to settle, and serial dilutions up to 10^{-5} were prepared using sterile physiological water and agitated with the vortex at maximum speed. An aliquot of 0.1 ml of each dilution from 10^{-2} to 10^{-5} was taken and spread evenly over the surface of actinomycetes isolation agar and starch casein agar medium. Rifampicin (2.5 mg/ml) and amphotericin B (75 mg/ml) was added to the both media to inhibit bacterial and fungal contamination, respectively. Plates were incubated at 28

and 37 °C, and monitored after 48, 72, and 96 h. Repeated streaking on starch casein agar plates led to purify bacterial colonies that showed actinomycetes like appearance. The isolated strains are preserved at 4 °C during two months and maintained for longer period by serial subculture.

Test organisms

For testing the antibiotic activity of the investigated strains the following test microorganisms were used. *Bacillus subtilis* ATCC6633, *Bacillis cereus* ATCCI1778, *Bacillus megaterium* ATCC9885, *Staphylococcus aureus* AT CC297 37, *Micrococus luteus* ATCC2I70, *Bacillus stearothermophilus* ATCC2328, *E.coli* ATCC2343, *Enterobacter aerogenes* ATCC 13048, *Proteus vulgaris* ATCC2027, *Klebsiella pneumoniae*. The diameter of the zones of complete inhibition was measured in millimeter (mm).

Antimicrobial activity

Eight actinomycetes strains isolated from coalmine soils were used in the screening and the actinomycetes isolates were cultured on glycerol-broth, ISP-2 and ISP-3 at 28 °C the antimicrobial activity was determined by agar well diffusion method [24]. The partially purified extracts obtained by the evaporation of the ethyl acetate was dissolved in 1ml 0.2 M phosphate buffer (pH 7.0) and 100 µl of it was loaded into well borer and inoculated into petriplates and were incubated at 37 °C for l8-24 h and examined for six days

Composition of the media used

1) Glycerol Asparagine Broth

Glycerol	-	10.0 g
Asparagine	-	1.0 g
K_2HPO_4	-	1.0 g
Distilled Water	-	1000 ml

2) Starch – Casein Agar Medium

Starch	-	10.0 g
Casein	-	0.3 g
Potassium Nitrate		2.0 g
NaCl	-	2.0 g
K_2HPO_4	-	2.0 g
$CaCo_3$	-	0.02 g
$FeSo_4 7H_20$	-	0.01 g
Distilled Water	-	1000 ml

The media were solidified with 1.5 % agar, and the pH of media was adjusted to 4.0, 5.5, 7.0, 8.5, and 10.0 with 1 or 2 M NaOH or HCl before the autoclaving at 121 °C for 15 min. The pH 11.5 was adjusted from pH 10.0 after the autoclaving.

Table -1 Antimicrobial activity of the four strains of actinomycetes in glycerol broth (A) and Starch-casein broth (B).

Strains	A- Days of incubation			B-Days of incubation		
	7	14	21	7	14	21
MMKK5	++	+++	++++	+	++	++++
GDK	+	++	+++	+	+	++
KGM	+	++	+++	++	++	++
SPL-OC	-	-	-	-	-	-

+ = Weak growth; ++ = Moderate growth;
+++ = Good growth; ++++ = Excellent growth

RESULTS AND DISCUSSION

Antimicroial activity of the four strains of actinomycetes in glycerol broth (A) and Starch-casein broth (B) is shown in Table-01, it reveals that all the four strains shoed minimum growth on the 7th day and moderate growth on 14th day but showed maximum growth on 21st day except SPL-OC. Antibiotic resistance was approximately recorded in 50% of the strains with broad or narrow range (Table-02). The strains exhibited sensitivity to a number of antibiotics like penicillin, tetracycline, ampicillin, streptomycin, streptothricin, actinomycin A, D, ciprofloxin, MMKK5 showed resistance to HIV drug, Lanostad N-30, while, Actinomycin C does not showed any resistance against the strains like MMKK5, GDK, SPL-OC and MMKK5, KGM showed high range of antibiotic resistance showed less resistance but 70% of the antibiotics showed complete absence.

Table – 2 : Antibiotic resistance of eight strains of actinomycetes

Antibiotics	MMKK5	GDK	KGM	SPOL-OC	BPA	GDK-OC	MMK2	BP
Pencillin	+	+	+	-	+	-	+	+
Tetracycline	+	+	+	-	+	-	+	+
Ampicillin	+	+	+	-	+	-	+	+
Steptomycin	+	+	+	-	+	-	+	+
Streptothricin	+	+	+	-	+	-	+	-
Actinomycin A	+	-	+	-	+	-	+	-
Actinomycin D	+	+	+	-	+	-	+	-

+ = Positive; - = Negative

The antibacterial activity of eight strains of actinomycetes against six gram-positive and four gram-negative bacteria was studied and reported (Table-3). The diameter of .the bacterial clear zone was measured and the minimum inhibition concentration of the actinomycetes extracts was calculated. The inhibitory effect was divided into three groups, i.e., negative, group one, two and three. The negative inhibition shows passive nature with $\leq$ l0mm, group shows one 11-20mm with slightly active, group two shows 2l-33 mm with moderately active while group three shows $\geq$ 33 mm with highly active inhibition.

All the strains under study (Table-3) showed negative activity to two gram negative bacteria strains, *Enterobacter aerogens* and *Klebsiella pneumoniae* MMKK5 strain found to be highly active against test micioorganisms like *Bacillus subtilis megaterium* and *Bacillus* and *Bacillus stearothermaphilvs* and was moderately active against *Bacillus cereus* and *staphylococcus aureus*: *Micrococus luteus* and *E. coli* showed slightly active 'inhibition while *Enterbacter aerogenes*, *Proteus vulgaris* and *Klebsiella pneumonia* showed passive activity. GDK strain was moderately active against *B. subtilis, B. megaterium* and *B. sterothermophilis*. KGM was moderately active to *B, subtilis* and *S. aureus*; all other organisms were slightly active or negative. SPL-OC and GDK-OC were negative to all the bacteria under study. BPA showed its moderate activity with *B. subitilis* and *M. luteus* and all other test bacteria were slightly active or negative. MMKK2 showed its high inhibitory activity to *B. cereus* and *B. megaterium*. While showed moderate inhibition with *B. subitilis, S. aereus* and *B.sterothermophilus* BP showed moderately active inhibition to only *B. subitilis* while all other bacteria were passive or with slight activity.

Table – 3: Antimicrobial activity of eight strains against Gram positive and Gram negative bacteria

Test Bacteria	**Diameter of the inhibition zone (in mm) Gram – positive bacteria**							
	MMKK5	GDK	KGM	SPOL-OC	BPA	GDK-OC	MMK2	BP
Bacillus subtilis	3	2	1	--	2	--	2	2
Bacillus cereus	+	1	1	--	-	--	3	1
Bacillus megaterium	3	2	1	--	1	--	2	1
Staphylococcus aureus	2	1	2	--	1	--	2	1
Gram-negative bacteria								
Esherichia coli	1	1	1	--	1	--	1	1
Enterobacter aerogenes	-	1	-	--	1	--	1	-

The inhibitory effect of the strains was divided into five groups according to their size of the group -- = No zone of inhibition; -= Passive $\leq$ 10mm; Group 1 = 11-20, slightly active: Group 2 = 21 = 33mm, moderately active; Group 3=> 34mm Highly active.

Similar work was reported by Waksman [25], Williams *et al.*[17] and recorded the *Streptomyces* flora of soil samples, collected from different locations and screened for their potentials as a source of antibiotics. All the isolates were tested for their ability to produce inhibitory substances against several test microorganisms. The test microorganisms included gram positive bacteria, gram negative bacteria and yeast. A total of 15 different Streptomycetes isolates were shown to have a very potent in vitro antimicrobial activity against the test organisms, and all these belong to the *Streptomyces* spp. The active strains belong to both *Streptomyces* and rare species in moderately high proportion. This rather low estimate of the proportion of active strains may be due to the method of preliminary screening used. Although most of the actinomycetes isolates could inhibit only gram-positive bacteria, some of them were rare actinomycetes from which novel antimiorobial substances are expected. *Actinamadura*, *Micromonospora, Microbispora* and *Streptomyces* especially the strains produced antimicrobial substances inhibiting both gram-positive and gram-negative bacteria [26]. Actinomycetes were isolated from different soil samples discarding isolates with identical characteristics showed antibacterial activity and antimicrobial spectrum of the culture filtrate was studies [27].

Similar findings were reported by Gopinath and Singara Charya [28] reveals that the antibiotic production of actinomyctes isolated from different etiological conditions were surveyed it was evident that the antimicrobial activity of the eight strains of actinomycetes grown on two different media glycerol-broth and starch-casein broth for 7, 14 and 21 days.Out of thirty seven, 2l isolates were subjected to submerged to submerged culture and 12 (57%) isolates were found to exhibit antimicrobial activity while the other 9(43%) isolates did not exhibit any activity in broth culture. The diameter of zone of inhibition of the 12 showed antimicrobial activity of the active actinomycete isolates by standard well diffusion method. But the emergence and dissemination of antibacterial resistance is well documented as a serious problem worldwide [29]. The emergence of bacterial resistance threatens to return us to the era before the development of antibiotics [30]. The perspective of rapid emergence of drug resistance among bacterial pathogens shows that the potencies of prevalent antibiotics are decreasing steadily, leading to reduce useful period of drugs. This situation compounds the need for the investigation of new, safe and effective antimicrobial for replacement with invalidated antimicrobials (or) use in antibiotic rotation programs [31].

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