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Research Article

Antibacterial Activity of Actinomycetes Collected From an Extreme Environment and Coal Mines

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ABSTRACT

Actinomycetes are saprophytic bacteria that secrete important hydrolytic enzymes, antibiotics and medicinally important secondary metabolites. The objective of this study is to study the antibacterial activity of actinomycetes collected from an extreme environment and coal mines. Soil sample were collected from five important coal field regions of Singareni Colleries Company Limited (SCCL), Telangana State. A total of 106 actinomycetes were isolated by perpendicular streak method against gram-positive test bacteria i.e. *Bacillus subtilis, Bacillus cereus, Bacillus megaterium, Staphylococcus aureus, Micrococcus luteus, Bacillus stearothermophilus* and gram-negative test bacteria i.e. *Escherichia coli, Enterobacter aerogenes, Proteus vulgaris, Klebsiella pneumoniae* species. The isolated actinomycetes were identified using different staining techniques, various biochemical methods and molecular approaches. Antibacterial compound was recovered from the filtrate grown with different actinomycetes strains by solvent extraction method. Eight actinomycetes strains isolated from coalmine soils were used in the screening and the actinomycetes isolates were cultured on glycerol-broth, starch-casein broth, ISP-2 and ISP-3 at 28^{O}C. The antimicrobial activity was determined by agar well diffusion method. It was observed that two isolates were active against only gram-negative bacteria and sixteen against gram-positive bacteria and twenty six against gram-positive and gram-negative bacteria. Finally sixteen isolates were selected for further study on the basis of a) broad spectrum activity and b) layer zone of inhibition in comparison to others.

Keywords: Actinomycetes, antibiotics, coal mines, SCCL.

1. Introduction

The actinomycetes are noteworthy as antibiotic producers, making three quarters of all known products; the Streptomyces are especially prolific and can produce a great many antibiotics and other class of biologically active secondary metabolites. Between 1988 and 1992 more than hundred different new molecules from actinomycetes were discovered. Approximately 75% of these originated from Streptomyces genus (Sanglier et al., 1993; Sacromento et al., 2004). Therefore, and because of their ability to secrete valuable proteins, Streptomyces have been considered as an alternative host organism for producing recombinant proteins (Dela Cruz et al., 1992). Induction of mutation and selection to improve the producitivity of cultures has been strongly established for over fifty years and is still recognized as a valuable tool for many antibiotics (Venkateshwarlu et al., 2000). The mutant strains can be achieved by inducing genetic variation in the natural strain with increasing productivity. An actinomycetes strain (Streptomyces sp.) isolated from Kuwait tropical desert (Hashem and Diab, 1973), was chosen due its marked stability and tested for its double capacity to produce antimicrobial agents active against some pathogenic with special focus on the cytotoxic activity against brain human tumor cell line. The possibility of producing mutants from the parent strain has been explored and direct cytotoxic activity against brain cancer cell lines was also investigated. The molecular weight and the structure of the isolated proteo-polysaccharides was also investigated of these mutant forms.

The antibiotics produced by actinomycetes represent a great variety of chemical compounds. These compounds differ greatly in their physical properties, including solubility in water and in organic solvents; in their chemical composition; in their antimicrobial activities both in vivo and in vitro; and in their toxicity to animals (Swapna Gurrapu et al, 2017). These differences are frequently not only qualitative but also quantitative in nature. Hence, the differences emphasized in these keys are often not sufficiently sharp to make such separation distinct. Frequently, a compound placed in one position in the keys could just as easily have been placed in another on the basis of certain minor differences. The

difficulties involved in the classification of these antibiotics become particularly sharp when one realizes that many of them have not been crystallized.

The first true antibiotic from a culture of an actinomyces may, therefore, be said to have been isolated in 1940. It was designated as actinomycin, Waksman and Woodruff (1940). The first such antibiotic which possessed chemotherapeutic possibilities was isolated in 1942 and was designated as streptothricin (Waksman and Woodruff, 1942); it was a result of an extensive screening program on actinomycetes for the production of antibiotics (Waksman et al., 1946). The first actinomyces-produced antibiotic that found application as a chemotherapeutic agent was streptomycin (Schatz et al., 1944; Gurrapu S et al, 2017).

The present research work on antibacterial activity of actinomycetes from an extreme environment, coal mines was studied. A total of 106 actinomycetes were isolated by perpendicular streak method against gram-positive test bacteria i.e. Bacillus subtilis ATCC6633, Bacillus cereus ATCC11778, Bacillus megaterium ATCC9885, Staphylococcus aureus ATCC29737, Micrococcus luteus ATCC2170, Bacillus stearothermophilus ATCC 2328 and gram-negative test bacteria i.e. Escherichia coli ATCC 2343, Enterobacter aerogenes, ATCC 13048, Proteus vulgaris, ATCC 2027, Klebsiella pneumoniae species. It was observed that two isolates were active against only gram-negative bacteria and sixteen against gram-positive bacteria and twenty six against gram-positive and gram-negative bacteria. Finally sixteen isolates were selected for further study on the basis of a) broad spectrum activity and b) layer zone of inhibition in comparison to others.

2. Materials and Methods

2.1 Isolation of Soil Samples

Soils is the habitat for a variety of organisms, including bacteria, viruses, fungi, actinomycetes, protozoa, insects, nematodes, worms and many other animals. The microbial population in soils can be very high. In surface soil the bacterial population can approach 108 to 109 cells per gram of dry weight. The gram-positive bacteria, which show varied degree of branching and mycelial development, are an important and less studied part of the microbial community. They include the coryneforms, the nocordioforms, and the true filamentous bacteria or actinomycetes. Further, the examination of soil of extreme environments for isolation of actinomycetes are very less. Hence, in the present study an attempt was made to isolate the actinomycetes in coal field soils.

Soil sample were collected from five important coal field regions of Singareni Colleries Company Limited (SCCL), Andhra Pradesh, i.e., Mandamarri (Plate 1 and 7) and Bellampally (Plate 5) (Adilabad district), Godavarikhani (Plate 2 and 6) (Karimnagar district), Bhupalpally (Plate 8) (Warangal district), Kothagudem (Plate 3) and Sattupally (Plate 4) (Khammam district).

In the process of sample collection one inch of superfial layer was dug and collected soil was kept in clean polythene bags and carried to the laboratory for isolation of actinomycetes. The standard soil dilution technique was procedure were adopted for the separation of actinomycete colonies from the soil. Generally, actinomycetes from the soil were isolated by pour-plate technique on Glycerol-aspargine agar, Starch-casein agar and Inorganic salt-starch agar media after serial dilutions in the distilled water. Dry colonies of actinomycetes were selected and isolated. Thus, isolated colonies were preserved in Glycerol based agar media and stored at 20oC. The actinomycetes of which antimicrobial activity should be determined were revived by streaking on starch-casein agar and incubated at 28^{0}C for 7 days.

2.2 Serial dilution technique:

Microorganisms were isolated from the soil samples collected from coal mines and make serial dilutions as 10-1 (1/10), 10 2 (1/100), 10-3 (1/1000), 10-4 (1/10,000), 10-5(1/1, 00,000). One ml portion of the dilutions were inoculated into Glycerol-aspergine, Starch-casein and inorganic salt starch agar media by pour-plate method and usually 10-4 and 10-5 were used for enumeration and separation of actinomycete colonies.

2.3 Selective media:

A selective media is the one that contains one or more agents that inhibit the growth of a certain microorganisms and there by encourage or select the microorganism of interest and allow it to grow. Selective media are very important in primary isolation of a specific type of microorganism from soil samples containing different types of microorganisms of interest and allow it to grow. They enhance isolation by suppressing the unwanted background organisms and favouring growth of the desired ones.

2.4 Antimicrobial activity:

Eight actinomycetes strains isolated from coalmine soils were used in the screening and the actinomycetes isolates were cultured on glycerol-broth, starch-casein broth, ISP-2 and ISP-3 at 28oC. The antimicrobial activity was determined by agar well diffusion method (Yilmaz et al., 2006). The partially purified extracts obtained by the evaporation of the ethyl acetate was dissolved in 1 ml 0.2 M phosphate buffer (pH 7.0) and 100 □l of it was loaded into well boarer and inoculated into petriplates and were incubated at 37oC for 18-24 hrs and examined. For testing the antibiotic activity of the investigated strains the following test microorganisms were used. Bacillus subtilis ATCC6633, Bacillus cereus ATCC11778, Bacillus megaterium ATCC9885, Staphylococcus aureus ATCC29737, Micrococcus luteus ATCC2170, Bacillus stearothermophilus ATCC 2328, Escherichia coli ATCC 2343, Enterobacter aerogenes, ATCC 13048, Proteus vulgaris, ATCC 2027, Klebsiella pneumoniae. The diameter of the zones of complete inhibition was measured in mm sterile zone

3. Results and Discussion

In spite of many significant advances in antibacterial therapy, the widespread use and misuse of antibiotics have caused the emergence of bacterial resistance to antibiotics. The development of new compounds to deal with resistant bacteria has become one of the most important areas of antibacterial research today. Although the antibiotic mechanism was not scientifically understood until the 20th century, the principal of using organic compounds to fight infection has been used to fight many infectious diseases. The first observation of what would now be called an antibiotic effect was made in the 19th century by the French Chemist Louis Pasteur. In addition to the

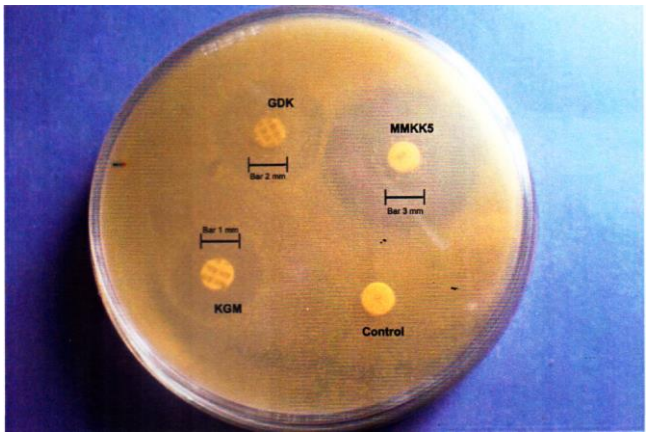

Plate-1. Photograph showing the antimicrobial activity of three strains of actinomycetes against *Bacilus subtils* ATCC 6633

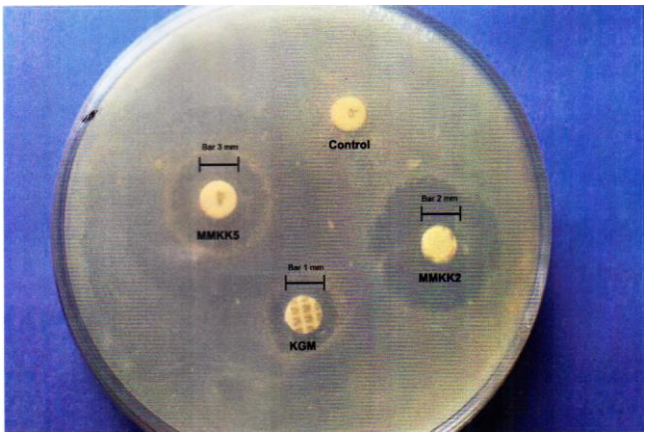

Plate-2. Photograph showing the antimicrobial activity of three strains of actinomycetes against *Bacillus stearothermophilus* ATCC2328

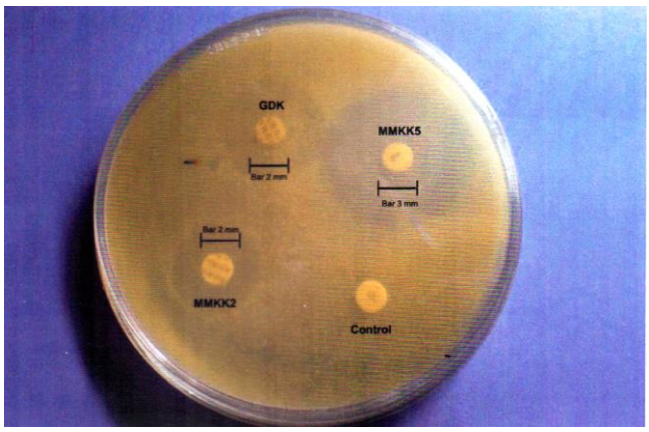

Plate-3. Photograph showing the antimicrobial activity of three strains of actinomycetes against *Bacillus megaterium* ATCC 9885

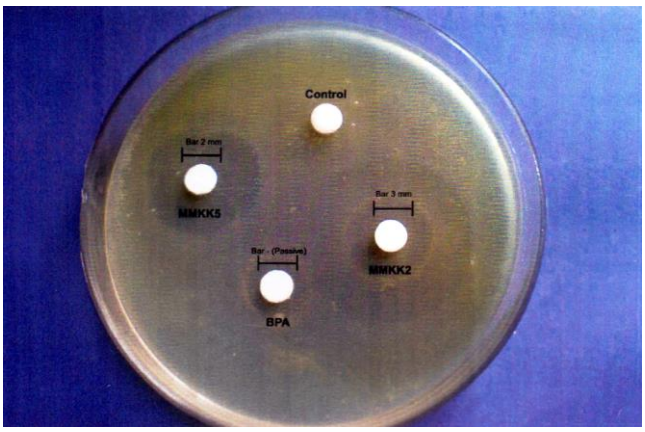

Plate-4. Photograph showing the antimicrobial activity of three strains of actinomycetes against *Bacillus cereus* ATCC 11778

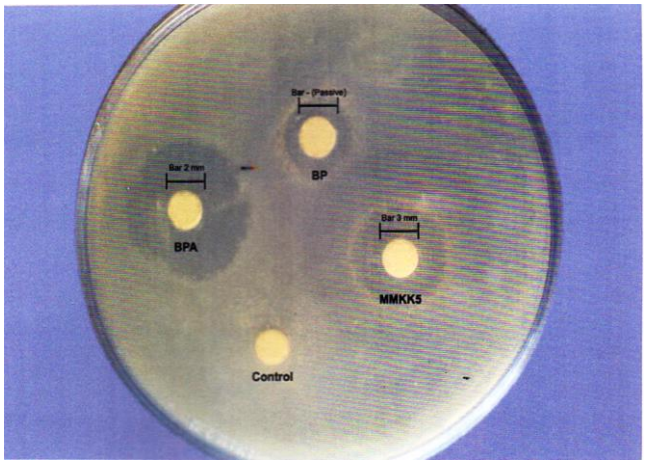

Plate-5. Photograph showing the antimicrobial activity of three strains of actinomycetes against *Micrococcus luteus* ATCC 2170

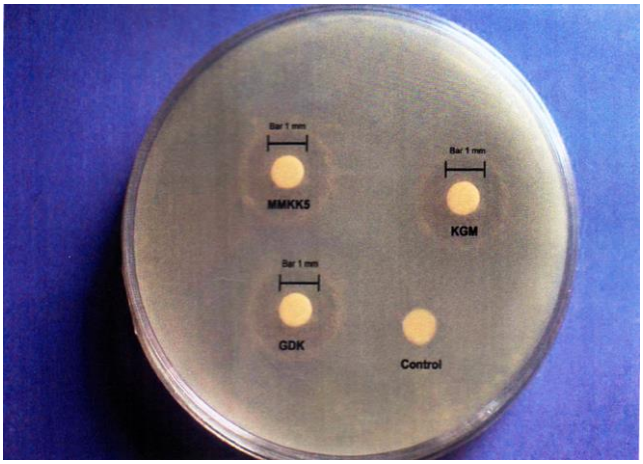

Plate-6. Photograph showing the antimicrobial activity of three strains of Actinomycetes against *Escherichia coli* ATCC 2343

development of new and effective antibacterial agents against multi drug resistant gram – Positive bacteria, recently attention has focused on the treatment of tuberculosis. Minimum inhibitory concentration values of the synthesized compound were determined and evaluated as per, National Committee for Clinical Laboratory Standards (2002).

Table–1. Antimicrobial activity of the eight 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	-	-	-	-	-	-
BPA	+	++	++	+	++	++
GDK-OC	-	-	-	-	-	-
MM-KK2	++	+++	+++	++	+++	+++
BP	++	++	+++	+	++	+++

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

Table–2. Antibiotic resistance of eight strains of actinomycetes

Antibiotics	MMKK5	GDK	KGM	SPOL-OC	BPA	GDK-OC	MMK2	BP
Penicillin	+	+	+	-	+	-	+	+
Tetracycline	+	+	+	-	+	-	+	+
Ampicillin	+	+	+	-	+	-	+	+
Streptomycin	+	+	+	-	+	-	+	+
Streptothricin	+	+	+	-	+	-	+	-
Actinomycin A	+	-	+	-	+	-	+	-
Actinomycin D	+	+	+	-	+	-	+	-
Ciprofloxin	+	+	+	-	-	-	+	-
Norfloxin	-	-	+	-	-	-	+	-
Actinomycin C	-	-	+	-	+	-	+	-
Lamostad – N- 30	+	-	+	-	-	-	-	-
Zidovudine	-	-	-	-	-	-	-	-

+ = Positive; - = Negative

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

Test bacteria	Diameter of the inhibition zone (in mm)							
	MMKK5	GDK	KGM	SPL- OC	BPA	GDK-OC	MMKK2	BP
Gram-positive bacteria								
Bacillus subtilis	3	2	1	--	2	--	2	2
Bacillus cereus	2	1	1	--	--	--	3	1
Bacillus megaterium	3	2	1	--	1	--	2	1
Staphylococcus aureus	2	1	2	--	1	--	2	1
Micrococcus luteus	3	1	1	--	2	--	1	--
Bacillus stearothermophilus	3	2	1	--	1	--	2	1
Gram-negative bacteria								
Escherichia	1	1	1	--	1	--	1	1
Enterobacter aerogenes	--	1	--	--	1	--	1	--
Proteus vulgaris	--	--	--	--	--	--	--	--
Klebsiella pneumoniae	--	--	--	--	--	--	--	--

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

Based on the criteria mentioned by different scientists the antibiotic production of actinomycetes isolated from different ecological conditions were surveyed and presented in plates (1-6) and tables (1-3). From the plates and tables 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. The MMKK5 strain showed moderate, good and excellent growth in glycerol broth during 7, 14 and 21 days of incubation while in starch – casein broth weak growth observed in 7 days incubation but finally at twenty one days showed excellent growth. GDK, KGM, MMKK2, BP showed moderate to good while the SPL – OC and GDK – OC showed no growth at all up to 21 days of incubation. MMKK2, BP, KGM showed good growth in both the media in 21 days of incubation.

Antibiotic resistance was approximately recorded in 50% of the strains Both board or narrow range (Table–2). 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, BP, SPL – OC and GDK – OC. MMKK2, MMKK5, KGM and BPA showed high range of antibiotic resistance. BP showed less resistance but 70% of the antibiotic showed complete absence.

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 < 10mm, group shows one 11 – 20 mm with slightly active, group two shows 21 – 33 mm with moderately active while group three shows > mm with highly active inhibition.

All the strains under study (Table–3) showed negative activity to twogram negative bacteria strains, *Enterobacter areogenes* and *klebsiella pneumonia*. MMKK5 strain found to be highly active test microorganisms like *Bacillus*, *Bacillus megaterium* and *bacillus* and *Bacillus Stearothermophilvs* and was moderately active against Bacillus cereus and Staphylococcus aureus (plates 1-3). *Micrococcus luteus* and *E. coli vulgaris* and *Klebsiella pneumonia* showed passive activity. GDK strains was moderately active against *B. subtilis. B. megaterium and B. sterothermophiles.* 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 bacterial 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 moderate inhibition with *B. subtilis, S. aureus* and *B. sterothermopilus*. BP showed moderately active inhibition to only *B. subtilis*, while all other bacteria were passive or with slight activity.

Waksman (1961), Shirling and gottileb (1966), Nonomura (1974), Willams et al. (1983), Cross (1989), Goodfellow(1989), Lechevalir (1989) and Locci, (1989) recorded the Streptomyces flora of soil samples, collected from 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 species. 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.

Competing interests

The authors have declared that no competing interests exist.

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