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Study of Antimicrobial activities of Actinomycetes Obtained From River Cauvery (Karnataka) Terrestrial Soil and River Sediments

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ABSTRACT

This study screened antagonistic actinomycetes isolated from terrestrial soil and river sediment isolates of Cauvery basin, Karnataka, India, for antimicrobial activity. A total of 54 actinomycetes isolates were obtained from the various terrestrial soil and river sediments samples collected and were tested for antagonistic activity against E.coli (NCIM-2563), S.aureus (NCIM-2492), C.utilis (NCIM-3055) and A.niger (NCIM-1222). Results indicated that 14 out of 54 isolates were active against at least one of the test microorganism, 13 isolates were active against at least one of the test bacteria and 4 were active against one of the test fungi. It was noted that the terrestrial site was the richest source of the antibiotic – producing actinomycetes where approximately 40% of isolates were antibacterial and 4% were antifungal. Selected bioactive isolates were chosen for further screened against other strains of the test microorganisms during secondary screening. Resulting mean diameter of inhibition zones revealed isolates SII-45, SIV-05 and RI-12 are the most potent of all remaining isolates with a minimum inhibitory microbial concentrations of 20% (MIC) for SII-45 and 15%(MIC) for SIV-05 and RI-12. Cultural and morphological characterization classified them under the genus Streptomyces. It can be recommended therefore terrestrial soil and river sediment samples from Cauvery basin, Karnataka, India, be further investigated for antibiotic producing actinomycetes. The number of actinomycetes isolated with persistent activity suggests Cauvery basin of Karnataka state may be India's potential source of novel antibiotics.

Keywords: Antimicrobial, Actinomycetes, Cauvery basin, Minimum Inhibitory Concentration.

INTRODUCTION

Actinomycetes are considered to be the most widely distributed group of microorganisms in nature which primarily inhabit the soil[1]. Many of them are prolific producers of various kinds of bioactive

compounds. They provide over two-third of the naturally occurring antibiotics discovered and continued to be a major source of novel and useful compounds.

Most of actinomycetes are known to have the capacity to synthesize bioactive secondary metabolites which include enzymes, herbicides, pesticides and antibiotics. Almost 80% of the world's antibiotics are known to come from actinomycetes, mostly from the genera Streptomyces and Macromonospora[2].

Actinomycetes consist of an extensive and diverse group of gram-positive, aerobic and mycelia bacteria that play an important ecological role in soil cycles. Current efforts are focused on the development and massive application of selective methods for the isolation of members of the genus actinomycetes and related genera.

According to the World Health Organization, over prescription and improper use of antibiotics has led to the development of resistance by many pathogens. Clinically-important bacteria, such as *Staphylococcus aureus*, are becoming resistant to commonly used antibiotics. At present, new resistant strains are being emerged more quickly, while the rate of discovery of new antibiotics is slowing down. Because of this, many scientists have focused on screening programs of microorganisms, primarily of actinomycetes, for the production of antibiotics[1].

The present work reports isolation, characterization and screening of potent actinomycetes.

MATERIALS AND METHODS

Sample collection and processing : Soil samples and sediments were obtained from twelve different locations each throughout a river site, a terrestrial site in Cauvery basin, Mysore and Madikeri district, Karnataka, India. From different locations, seven soil samples and five river sediment samples of 5g each were collected from 10-15cm below the surface.

All the samples were placed in small pre-labeled sterile plastic bags which were tightly sealed. The temperature and pH of the soil were determined. Sediment samples were air dried for one week. For isolation studies serial dilutions were made up to 10^{-6}.

Isolation of actinomycetes from terrestrial soil and river sediments: About 0.1 ml of each dilution was surface plated onto Yeast Malt Extract Agar (YMA) supplemented with cyclohexamide (50 µg ml^{-1}) and tetracycline (20 µg ml^{-1}). These were incubated for one week at room temperature. After incubation, actinomycetes isolates were distinguished from other microbial colonies by characteristics such as tough, leathery colonies which are partially submerged into the agar[3]. Each isolate was the coded based on the sample of origin. Terrestrial isolates were given the code Si#, where Si which stands for terrestrial isolate and the # for the number of the isolate. River isolates were given the code Ri#, respectively. Pure cultures were inoculated in 10ml of yeast malt extract broth (YMB) and incubated at room temperature for 24 to 48 hours in Rotary shaker (200 rpm) prior to screening.

Antibacterial screening of actinomycetes isolates : Two stages of antibacterial screening were done. All isolates were subjected to primary screening. While secondary screening was only performed for the upper 50% of the total actinomycetes isolates tested that showed inhibition during the primary screening.

Preliminary antimicrobial screening : Actinomycetes isolates were screened for antimicrobial property using the Agar well method on Muller-Hinton Agar plates. Pure cultures of the test bacteria (*Escherichia coli, Staphylococcus aureus, Candida utilis and Aspergillus niger*) were obtained in nutrient broth from the national collection of industrial microorganism, national chemical laboratory, Pune, India.

Suspensions of test organisms were adjusted to 10^8 cfu ml^{-1} (Mac Farland standard 0.5) for use in the primary and secondary screening. The YMB tubes containing the actinomycetes isolates were centrifuged for 15minutes at 7000 rpm to separate the pellet and the supernatant. Each resulting supernatant was decanted into a sterilized test tube. Inoculation of 100ml of the test microorganism in culture broth on MHA plate was done by spread-plating. The antimicrobial activity was determined by the Agar well method[2], distinguishing the pellet from the supernatant. YMB was used as the negative control. 50 µl of each were then loaded into their respective wells. The plates were sealed and incubated at 37^{o}C for 18-24 hours, and then observed. The diameters of the zones of complete inhibition were measured in millimeter to the nearest hundredths using calibrated vernier calipers. Four millimeters were subtracted from the obtained diameter of complete inhibition. The isolates exhibiting zones of inhibition against any test bacteria were chosen. Triplicates of each were made, and the diameters of inhibition zones were again measured by the millimeter using the calibrated Vernier calipers.

Secondary antimicrobial screening : The most potent 3 isolates were noted for each test microorganism. Based on the mean diameter of inhibition zones, they were subjected to secondary screening of antimicrobial assay using clinical and pathogenic strains of bacteria and fungi which they inhibited.

Determination of Minimum Inhibitory Concentration (MIC) : The MIC of the actinomycetes isolate having the largest inhibition zone measured based from the secondary screening was determined by broth dilution test using varying concentrations of the actinomycetes suspension (0, 5, 10, 15, 20, 25, 60, 75, 100%) prepared using YMB.

Characterization of the Most Potent Actinomycetes isolate : The most potent actinomycetes isolate was characterized by macroscopic, microscopic and morphological methods. The macroscopic method was done by colony characterization on YMA. Color of colony and presence of pigmentation was recorded. The microscopic characterization was done by cover slip culture method observed after 3 days. The presence or absence of aerial and substrate mycelium, spore formation, fragmentation of the vegetative or substrate mycelium were observed.

RESULTS AND DISCUSSION

Isolation of actinomycetes from terrestrial soil and river sediments: Terrestrial soil samples were obtained from seven locations and river sediments from five locations in each of the two sites that are terrestrial and river.

In each area, temperature and pH were obtained to determine appropriate culture conditions. Temperature of sediments ranged from 22-32^{o}C, while the pH varied from 7.5 to 9.2. These two readings for the substrates were within ranges that are said to be optimum for actinomycetes growth and survival[4].

The two sediment types of the sampling area yielded 54 actinomycetes isolates. A total of 30 actinomycetes are isolated from terrestrial sites, while 24 were recovered from the river sites[5] showed that actinomycetes were less common in river sediments relative to terrestrial soils. Another study by Good fellow and Haynes[6] suggested that actinomycetes represent only a small component of the total bacterial population in river sediments. It was observed that most of the isolates were obtained from terrestrial origin. Terrestrial soils have the main reservoir of actinomycetes[7]; they comprise the large part of the microbial population of the soil[8].

Antimicrobial screening of actinomycetes isolates

Preliminary antibacterial screening : Thirteen of the 54 actinomycetes isolates showed antagonistic property in at least one of the two test bacteria. Meanwhile, four (three from the terrestrial site and one from the river site) inhibited at least one of the test fungus. Based on the findings, about 40% of the 30 terrestrial isolates and 4% of 24 river isolates were antibacterial whereas only two actinomycetes isolates were found active against C.utilis. Five actinomycetes isolates were found active against *E. coli* and twelve against *S. aureus*. Three replicates were made for the 13 actinomycetes isolates active against the test bacterial microorganisms. Their mean diameter of inhibition zones were computed and recorded. Four millimeters, the diameter of the well, was subtracted from the obtained mean diameter. From the five actinomycetes isolates which inhibited *E.coli*, only one (SII-45) was chosen to advance to the secondary screening. Three of the five isolates showed activity against *E.coli*, with both the supernatant and pellet. The other two, namely, SIII-42, SII-24, had an inhibitory effect only with the use of pellet (Figure 1).

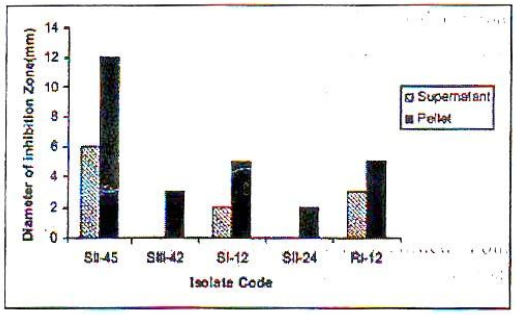

Fig.1: Activity of Actinomycetes isolates against *E.coli.*

Both supernatant and pellets of nine out of the 10 isolates were found to inhibit the growth of *S.aureus*. Isolates SIII-26 has recorded inhibitions for the pellets only. Results also revealed that pellets were more active for most isolates (Figure 2).

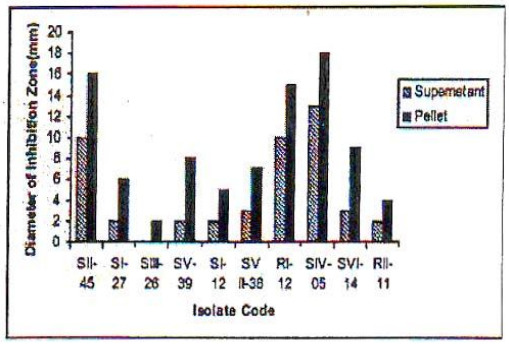

Fig.2: Activity of Actinomycetes isolates against *Staphylococcus aureus*

Only the pellet of SVI-14 was active on *C.utilis* which also gave the greatest mean diameter of inhibition zones (Figure 3).

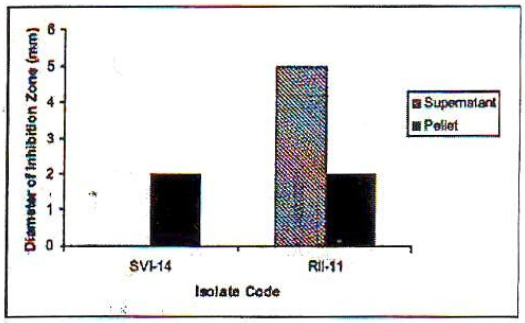

Fig.3: Activity of Actinomycetes isolates Against *C.utilis*

Likewise, only two isolates were active against *Aspergillus niger* with isolate SIV-32 & SV-18 having the greatest diameter of inhibition zone for both pellets and supernatant (Figure 4).

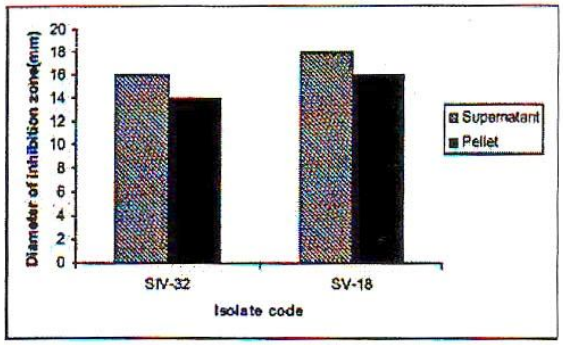

Fig.4: Activity of Actinomycetes isolates against *A.niger*

Results of the primary screening suggested that the actinomycetes found in Cauvery basin exhibited antimicrobial activity against the two test bacteria, namely *E.coli* and *S.aureus*. Most except one active actinomycetes were isolated from terrestrial sediments which suggest that this proportion of antibiotic producers and where they were found, were influenced by the chemical soil characteristics of the area since production of secondary metabolites by actinomycetes is directly affected by its environment. He demonstrated in his study that 78% of Actinomodura isolates obtained from reportedly rich mangrove soils exhibited antimicrobial activity, while only 14% were active from poor, actinomycetes -rich tropical soils[9].

Results further revealed that Cauvery basin isolates were found to be more active against gram-positive bacteria (*S.aureus*) than gram-negative bacteria (*E.coli*) which collaborated with previous observations [1,2,10] likewise observed that activities against gram-negative bacteria were less frequent than against gram-positive bacteria. Results also showed that most of the active isolates were more active against bacteria than fungi. This agreed with the findings of the study done by Nedialkova and Naidenova[11]. The study showed that isolated active actinomycetes strains Antarctica with a broader spectrum of antibacterial activity and only limited antifungal activity against various strains of yeasts and phytopathogenic fungi.

Although, various studies have also shown the above mentioned pattern of antimicrobial activity, this is not always the case[12]. Comparison of antibacterial and antifungal activities of isolated actinomycetes strains has not been the focus of majority of the studies dealing with antimicrobials since most fungi require longer generation time compared to bacteria. Many antibacterial agents inhibit steps important for the formation of peptidoglycan, a very important cell wall component. On the other hand, most antifungal compounds target either the formation or the function of Ergosterol, an important component of the fungal cell membrane[13]. However, this dominance of actinomycetes strains with antibacterial activity in Cauvery basin, Karnataka might be a reflection of the soil ecology. Secondary metabolites (antibiotics)

are produced by some actinomycetes in order to overcome competition with other soil microorganisms by killing or destroying them. From this, we could infer that the sediments from which the actinomycetes isolates were obtained have a rich bacterial population which competes with them. As a means of adaptation, they produce secondary metabolites which are harmful to these bacteria.

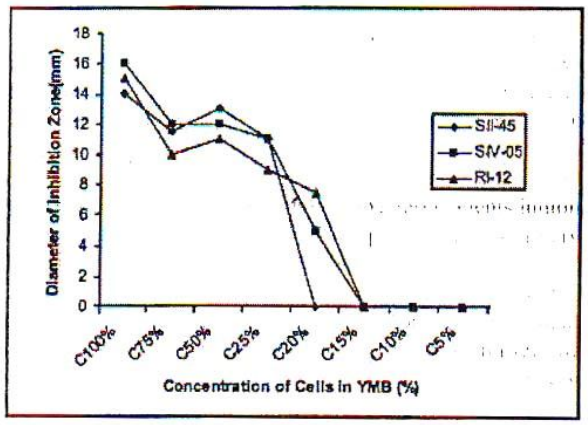

Fig.5: Minimum Inhibitory Concentration (MIC) of the different concentration of SIV-32 supernatant.

Secondary antimicrobial screening : Out of the 13 actinomycetes isolates subjected to the preliminary antibacterial screening, only five were subjected to secondary antibacterial screening to verify their consistent activity on other strains of the same test microorganism. It can be noticed in (Table 1) that there were varied observed activities as compared to the primary screening. No isolate was found to inhibit the clinical strain of *E. coli* and only one isolate was found to be active against the clinical strain of *S.aureus*. Isolate SVI-14 supernatant, compared to its pellet, showed an improved activity against *S.aureus* relative to the primary screening results but still exhibited larger inhibition zone than its supernatant. Screening of the active actinomycetes isolates against clinical stains of *E.coli* and *S.aureus* showed considerable changes in their antimicrobial activities. Those isolates active against non pathogenic *E.coli*, except for SVI-14 which was active against non pathogenic *S.aureus*, exhibited no activity against the respective clinical strains of test bacteria which may be attributable to the fact that these clinical strains of bacteria may possess characteristics that differ or are absent from the non-pathogenic strains. Pathogenic strains of *E.coli* may possess specific virulence determinants (toxins and adhesion, etc.) encoded by monocistronic genes, plasmids, or pathogenicity islands as well as plasmids that code for drug resistance, which may partially account for the ineffectiveness of most of the antibiotics produced by the isolate[14]. Pathogenicity of *S.aureus*, on the other hand, is mainly caused by several virulence factors, one of which is its inherent and acquired resistance to antimicrobial agents[15].

Table 1. Mean diameters of inhibition zones (mm) of test microorganisms by bioactive actinomycetes isolates in primary (1^o) and secondary (2^o) screening

Isolate code	Mean diameter of zones of inhibition (mm)							
	Escherichia coli				*Staphylococcus aureus*			
	Supernatant		Pellet		Supernatant		Pellet	
	1^0	2^0	1^0	2^0	1^0	2^0	1^0	2^0
SII-45	6	0	12	0	10	0	16	0
RI-12	3	0	5	0	10	0	15	0

SIV-05 SVI-14 RII-11	0 0 0	0 0 0	0 0 0	0 0 0	13 3 2	0 5 0	18 9 4	0 7 0

Only *A.niger* AN-20 was used as the test fungus for the secondary screening since primary screening revealed that no isolate were found to be active against the yeast test organism. Results showed that both isolate SIV-32 and SV-18 that inhibited *A. niger* showed activity in the secondary screening. Improved activity was shown by the SV-18 supernatant against a Cauvery basin strain of *A.niger*. Isolate SVI-32 had the largest mean diameter of inhibition zones (Table 2).

Table 2. Mean diameters of zones of inhibition (mm) of A.niger by bioactive actinomycetes isolates in primary (1^0) and secondary (2^0) screening

Isolate code	Mean diameter of zones of inhibition (mm)			
	Supernatant		Pellet	
	1^0	2^0	1^0	2^0
SIV-32	16	12	14	17
SV-18	18	11	16	15

Bacteria and fungi usually share a common substrate. Spatial proximity has allowed for the emergence of synergistic or antagonistic interactions between them, probably as a result of competition. Filamentous fungi, including *Aspergillus niger*, are very proficient at secreting native proteins. Some of these enzymes have the ability to counteract another organism, i.e., actinomycetes, and their activity. Different strains of *A.niger* produce different types and amounts of enzymes. It could then be inferred that different strains of the fungus, when exposed to a certain antifungal actinomycetes isolate, may vary in their responses. That is, they may either remain unaffected or be inhibited. In this study, *A.niger* differs in certain ways from the Cauvery basin strain. Using another strain of *A. Niger* was necessary to test the consistency of the activity of both isolates SIV-32 and SV-18 on the specific species of mold. In another observation, in all active isolates obtained from the terrestrial site, inhibition zones appear around the wells containing either pellet or supernatant. Moreover, among all the active terrestrial isolates, the measurements of inhibition zones around wells containing the pellet were larger than those around wells containing the supernatant. Nkanga [16] stated that the antimicrobial activity of freshly isolated actinomycetes in liquid culture is low because of their initially low production of antibiotics. In order to detect an inhibition, the antibiotic produced must be concentrated. The actinomycetes isolates were grown in 10 ml YMB which may have been diluted in the broth. Hence, they would have been more diluted in the supernatant than in the pellet, and consequently cause a larger inhibition zone in the latter.

Surprisingly, a larger inhibition zone of *S.aureus* was observed in the supernatant of RII-11 as compared to its pellet. This may be due to the nature of the antibiotic produced by this isolate. According to Waksman[17], pigments play various roles in the growth and survival of actinomycetes. One such role is for defense against foreign cells, they being referred to as antibiotic pigments. Production of more than one antimicrobial compound has been proven in several studies. They mentioned that in addition to the calcium-dependent antibiotic (CDA), Streptomyces coelicolor produces three other known antibiotics, namely, actinorhodin, methylonomycin and undecylprodigiosin, and one novel polyketide, mutactin. In this study, intracellularly and extracellularly produced antibiotics were accounted through the use of pellet and supernatant, respectively.

Determination of the Minimum Inhibitory Concentration (MIC) : Isolate SII-45, SIV-05 & RI-12 were chosen to be subjected for minimum inhibitory concentration (MIC) assay since it exhibited the larger zone of inhibition after the two screenings. Data{Fig.5) shows that the MIC of isolate SII-45, SIV-05, RI-12 falls between 15-20%. It can be inferred then that to exhibit antimicrobial activity against

S.aureus, a microbial suspension having at least 20% actinomycetes cells is needed. Pandey[2] mentioned about the MIC is not a constant for a given agent, since it is influenced by a number of factors. These factors include the nature of the test organism used, the inoculums size, and the composition of the culture medium, the incubation time, and aeration. Hence, to obtain more accurate results for MIC than what have been reported in this study, it would be better if the bioactive compound will be isolated by extraction techniques in future studies.

Characterization of the most potent actinomycetes isolate : Both macroscopic and microscopic methods were employed to describe SIV-05, SII-45 and RI-12(Table.3). According to Waksman [18], such color and form (Table 5) are exhibited by both colonies of Streptomyces. However the color of the growth and the form of the colony could not serve as basis for finding out the genus to which actinomycetes isolate belongs to. Hence, its morphological characters must serve as the primary basis of characterization. One distinguishing morphological character isolates SIV-05, SII-45 and RI-12 commonly possess abundant reddish brown to reddish pink aerial mycelium[19]. A well developed substrate mycelium partly penetrates the medium and the formation of smooth, straight or spiny and hairy spores with occasional hooks at the sporophore tips are the two characteristics that may well qualify it as a species of Streptomyces [20-23]. The formation of dark brown pigment that dissolves into the medium has also been mentioned by Waksman [18] to be a possible characteristics of the genus although not exclusive. After growing form broth isolates SIV-05, SII-45 & RI-12 were placed on yeast extract malt extract agar and was seen to exhibit yellowish brown, brown pale yellow colonies respectively.

Table 3. Cultural and morphological characteristics of SIV-05, SII-45 & RI-12

Character	Observations		
	SIV-05	S II-45	RI-12
Temperature	28^0C	28^0C	29^0C
pH	7.0	7.0	7.2
Colony color	Brown	Yellow brown	Pale brown
Nature of colony	Round, convex colonies and spreading edges	Round, flat to convex compact	Round, convex colonies with concentric circles & powdery spreading edges
Pigmentation in medium	Present (pale brown)	Absent	
		Present (dark pink)	Present (pale yellow)
Aerial mycelium	Abundant (reddish brown)		Present (bluish grey)
Substrate mycelium	Present	Present	
			Present (bluish grey)
Sporophores	Long and straight with occasional hooks	Straight to waves & occasionally open as hooks	Present
			Short coiled spirals.

However subsequent transfer of isolates shows brownish yellow colonies. The form of colonies were described to be round, flat too convex with concentric circled colonies with powdery and spreading edges. It was also observed that very pale brownish pigmentation was produced on the medium. Also both well defined reddish brown to reddish pink aerial mycelium and well developed substrate mycelium were observed on the medium. 28^0 Studies made supporting the anti tumor antibiotic activity of streptomyces have further strengthened the claim that isolated SIV-05, SII-45 & RI-12 does belongs to said genus.

SIV-05 is nearer to *S.fulvicimus* but differed from the reference culture in the following respects sporophore morphology, color of substrate mycelium and its fragmentation cell wall composition, tyrosinase reaction, starch hydrolysis, nitrate reduction. In view of this large number of significant differences it is proper to consider that our isolate SIV-05 is a new species and it is designated as variant of *S.fulvicimus.*

SII-45 is closer to *S.fumicarius* but differed in following aspects: sporophore morphology, inability to hydrolysis of gelatin, nitrate reduction, absence of antifungal activity. In view of these significant differences it is proper to consider out isolate as a new species and is designated as *S. fumicarius*.

Few differences between our isolate RI-12 and the reference culture *S.xanthocidicus* could be noticed like sporophore morphology, absence of antifungal activity, sodium chloride tolerance. In view of these minor differences, it is proposed to consider our isolate RI-12 as a variant of *S.xanthocidicus.*

CONCLUSION

Based on the screening results, it has been shown that terrestrial soil of Cauvery basin, Karnataka state, India posses' antibiotic producing actinomycetes and may be tapped as one of the Indians potential source of novel antibiotics.

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