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Isolation and Screening of Actinomycetes Producing Antibacterial Substance

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ABSTRACT
Actinomycetes are potential source of many bioactive compounds which have diverse clinical effects and important applications in medicine for treating various human diseases and disorders. The present study was performed with an aim of isolating actinomycete strains with antimicrobial activities using the selective isolation media. Four different actinomycete strains were isolated from 10 soil samples which were collected from different locations of Salem mine area. These strains were selected on the basis of their morphology. These were characterized and screened for antibacterial activity against 5 bacterial pathogens. The antibiotic stability was observed against E.coli (100%), K.pneumoniae (80%), S.aureus (80%), S.mutans (50%), P.auroginosa (80%). 2 isolates (A2, A4) had high antimicrobial activity. The optimization was done with various carbon and nitrogen sources. There were produced in bulk and the maximum zone of inhibition were observed to be 29mm against S.aureus and P.auroginosa. The crude proteins were determined by SDS-PAGE, the band ranged 75kDa.The selected strains were amplified by PCR and were performed with gene specific primers. Totally 1400 bp fragments were produced. Thus the study reported that the unique soil actinomycetes serve as a good source with the potential antibacterial activity.
Keywords: *Actinomycetes, E.coli, K.pneumoniae, S.aureus, S.mutans, P.auroginosa, SDS-PAGE, PCR.*

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INTRODUCTION

Microbial diversity is a major frontier and future source for the biotechnology sector [1]. Microorganisms produced natural products that are a good source of antibiotics, including actinomycetes. Actinomycetes are gram-positive and slow-growing bacteria, distinguished by the development of aerial mycelium. They make mycelium from spores that anchor the substrate In the vegetative process, the substratum hyphae have diameter of around 0.5 to 1.0lm and lack cross-walls. Actino-mycetes have acquired prominence in recent years because of their antibiotic capacity. Actinomycetes are a group of microbes widely distributed across the world's natural ecosystems and are especially valuable for their organic cycling role. Actinomycetes have unique bioactive metabolites, including antibiotics, enzymes, and plant growth factors [3]. The development of multi-drug-resistant pathogens requires unique antimicrobial agents, at least 5000 known Streptomyces sp. (*Streptomyces avermitilis* and *Streptomyces verticillus*) were produced bioactive com-pounds.

Antibiotic resistance in bacterial isolates was recorded since the first use of antibacterial agents. Penicillin-resistant *Escherichia coli* were the first to be discovered in 1940 to possess penicillinases that inactivated the drug penicillin, followed by discovery of penicillin-resistant *Staphylococcus aureus* in 1944. In 2008, the NDM-1 gene, encoding novel beta-lactamase enzyme capable of hydrolyzing penicillins, cephalosporins and carbapenems was discovered in *Klebsiella pneumoniae*. Bacteria possessing the gene were found to be resistant for most of the tested antibacterial agents [4]. Several current antibiotics, particularly the aminoglycoside and macrolide group, originate from tropical bacteria of the actinomycete Streptomyces group [5]. Thus microbes also produce the currently poorly understood bacteriocins, which are broad spectrum peptides with efficacy against diverse gram positive bacteria. Therefore, we need to isolate and screen more and more *actinomycetes* from different sources in hope of finding new *actinomycetes* strains that can produce antibiotics that have not been discovered yet and active against drug resistant pathogens.

They belong to the order Actinomycetes (Superkingdom: Bacteria, Phylum: Firmicutes,Class: Actinobacteria, Subclass: Actinobacteridae) [6]. Bergey's manual divides actinomycetes in 8 diverse groups and comprise 63 genera [7]. Actinomycetes are large and diverse group and those originating from tropical locations have been shown to comprise novel species. The majority of actinomycetes are free living, saprophytic bacteria [8] found widely distributed in soil, water and colonizing plants and play an important role in the degradation of organic matter [9]. Several studies of the ecology of actinomycetes have shown that these microorganisms are widespread in nature and may occur in extreme environments. Thus, groups of acidophilic and alkaliphilic, psychrophilic andthermophile, halophile and haloalkaliphilic, and xerophilic actinomycetes have been described.

MATERIALS AND METHODS

COLLECTION OF SOIL SAMPLE

Soil samples were collected from the different places of Salem mine area. Samples were collected from 2 inch depth of the earth surface. They were collected in the sterile small plastic tubes and properly labelled with the date of collection. Ten soil samples were collected within a period of one month.

ISOLATION OF ACTINOMYCETES

Soil samples were air dried for 1-week prior isolation. This helps in decreasing the population of gram-negative bacteria. Soil suspension method [10] was used, where 1 g of the soil sample were taken and mix with 100 ml of sterile distilled water. The soil suspension was shaken vigorously under room temperature (25 ± 2^{o}C) on an orbital shaker at 200 rpm for 1 h. 200 ml of the soil suspension were pipetted and lawn onto Starch Casein Agar (SCA) (Soluble starch, 10.0 g; Casein hydrolysate, 0.3 g; KNO_3, 2.0 g NaCl, 2.0 g; K_2HPO_4, 2.0 g; $MgSO_4.7H_2O$, 0.05 g; $CaCO_3$, 0.02 g; $FeSO_4.7H_2O$, 0.01 g; Agar, 18.0 g; distilled water, 1000 ml; Nystatin, 100 µg/ml; ciprofloxin, 100µg/ml) at pH 7. A series of dilution of the suspension from 10^{-3} to 10^{-6} were done with duplicates. All the plates were incubated at 30^{o}C for 1 - 2 weeks. The identification of actinomycetes was done on the basis of morphology of spore chain, pigment production, colour of aerial mycelium, colour of substrate mycelium, consistency, gram's staining, and growth on actinomycetes media [11].

RESULTS AND DISCUSSIONS

ISOLATION OF ACTINOMYCETE

This study was performed with an aim of isolating actinomycete strains with antimicrobial activities using the selective isolation media. Four different actinomycete strains were isolated from 10 soil samples collected from different locations of Salem.All of these strains were collected by using Starch-casein-nitrate-agar media supplemented with nystatin (100μg/ml) to inhibit fungal growth and ciprofloxin (100μg/ml) to inhibit the bacterial growth. Number of colonies was found from each plate which was shown in (Fig.1). Colonies selected from each plate were 2 to 3 (Fig. 1) based on colony appearance which are shown in Fig. 1.Colonies having characteristic features such as powdery appearance with convex, concave or flat surface and colour ranging from white, gray to pinkish and yellowish were selected. Colonies observed at 3rd and 4nd day were eliminated because actinomycetes are considered as slow grower. Furthermore, bacterial configuration same as actinomycetes were accepted from gram staining. Thirty-five selected isolates were examined microscopically and identified by their morphological and culture characteristics. All isolates were inoculated in ISP2 agar media and store at 4^{0}C for further investigation.

Isolation of bacterial isolates from wound samples

Bacterial isolates were obtained from wound samples by selective media and biochemical test. Totally 20 isolates of 5 different bacterial isolates were obtained such as *E.coli, S.aureus, S.mutans, P.aeuroginosa* and *K.pneumoniae*. Among them highest occurrence was *S.aureus* and *S.mutans* (30%) second most *K.pneumoniae* (20%) and followed by *Pseudomonas* (15%) and *E.coli* (5%).

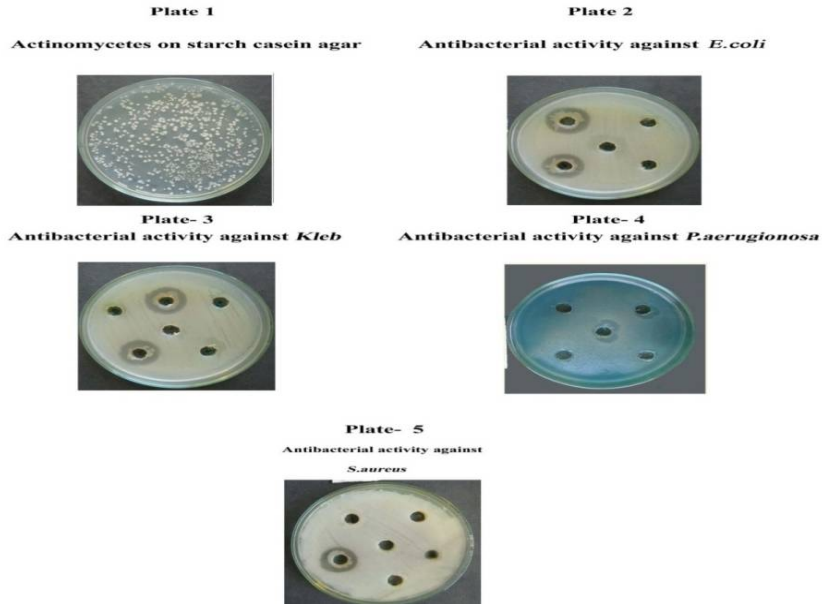

Fig. 1 Number of colonies found from each plate

Antibiotic stability of Bacterial isolates

All bacterial isolates were subjected to antibiotic stability with commercial antibiotics according to disk diffusion method were shown in Figure 2. In this study E.coli isolates were showed 100% resistance to 5 types of antibiotics such as Tertacycline, kanamycin, ciprofloxacin ,Nalidixic acid and cefatoxamine. In case of *K.pneumoniae* Erythromycin showed 100% resistance followed by Nalidixic acid and ciprofloxacin had 75%. Among the 4 isolates of *K.pneumoniae* 2 isolates had 80% of resistance against 5 types of antibiotics.Among the 5 types of bacterial isolates, highest percentage of biofilm produced by *E.coli* isolates (100%) followed by *S.aureus* and *P.auroginosa* (66.6%), *K.pneumoniae* (50%) and lowest from *S.mutans* (33.3%). In this study, among the 5 isolates highest strong biofilm formation observed in *E.coli* isolates followed by *S.aureus* and *P.auroginosa* which is shown in Figure 2.

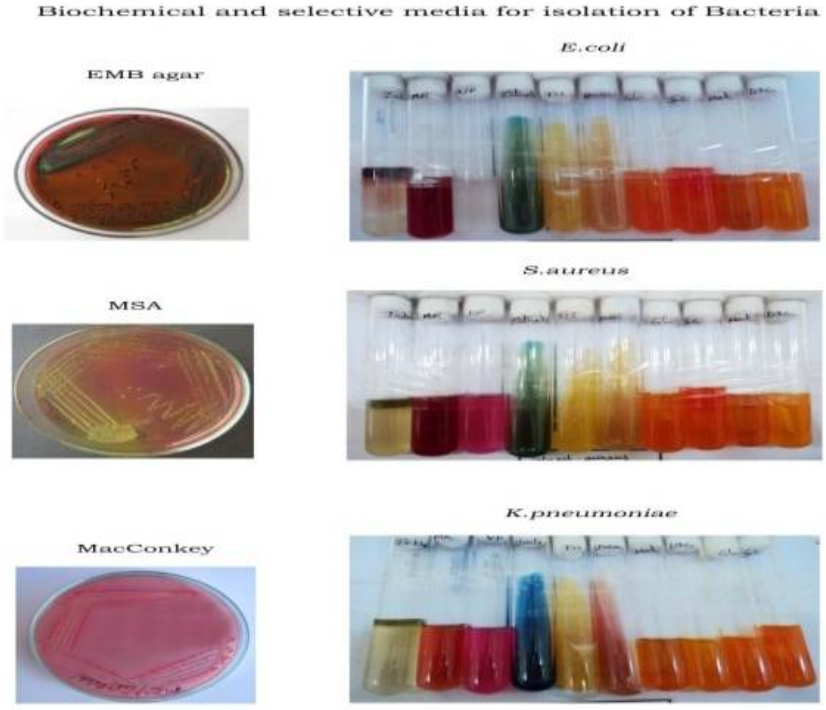

Fig. 2 Biofilm formation

ANTIBACTERIAL ACTIVITY OF ACTINOMYCES AGAINST WOUND ISOLATES

All isolates were subjected to antibacterial activity wound isolates such as *K.pneumoniae*, *S.mutans, E.coli*, *S.aureus* and *P.aeruginosa* were shown inFig.3Among the four isolates of actinomycetes 2 (71.4%) isolates had antimicrobial activity against all bacterial isolates. Out off 2 actinomycetes, 96% was active against *S.mutans* followed by 24% of actinomycetes against *K.pneumoniae,* 20% to *P.aeruginosa,* 16% to *Staphylococcus aureus* and 8% of isolates active against to *E.coli.* This is lowest percentage of result in our study that means our test pathogen of *E.coli* was highly resistance to actinomycetes. In this study most of the actinomycetes active against to gram positive bacterial pathogens compared to gram negative pathogens.

EFFECT OF CARBON SOURCES

The results indicated that the carbon sources obviously affected the synthesis of substances with antimicrobial activity producing A2 and A4 isolates.Both the strains were able to grow in all the tested carbon sources (Table 3). However maximum zone of inhibition was observed when cultures supplemented with maltose as a carbon source followed by sucroseand galactose. Cultures containing fructose did not shown any zone of inhibition. The utilization of maltose, sucrose and galactose for growth and production of antibiotic by A2 and A4 indicate the presence of an active uptake system for these substrates.

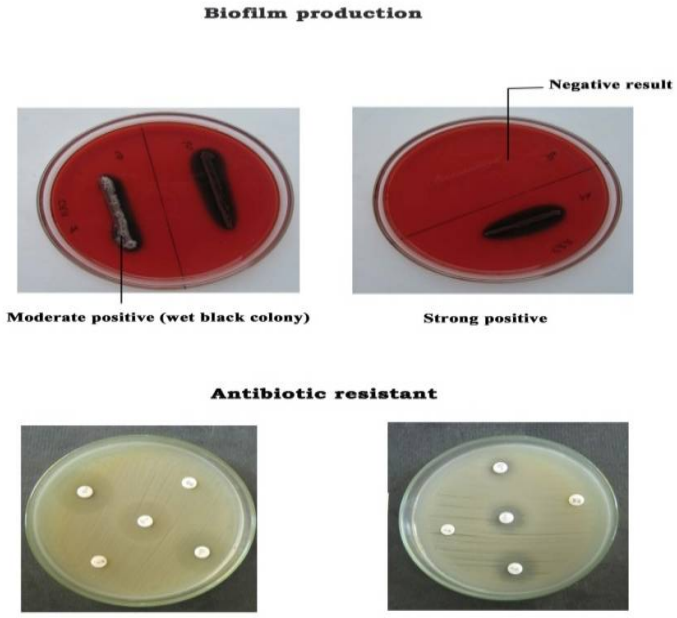

Fig. 3 Antibacterial activity wound isolates

Table 3. Effect of carbon source

S.no	Actinomycetes	Carbon sources Zone of inhibition in mm															
		Fructose				Sucrose				Galactose				Maltose			
		S.a	*K.p*	*P.a*	*S.m*	*S.a*	*K.p*	*P.a*	*S.m*	*S.a*	*K.p*	*P.a*	*S.m*	*S.a*	*K.p*	*P.a*	*S.m*
1.	A2	18	-	-	19	25	-	-	20	-	-	11	15	27	22	19	19
2.	A4	16	-	-	18	25	18	18	19	-	-	13	13	25	21	18	21

EFFECT OF NITROGEN SOURCES

Of all the tested nitrogen sources, A2 and A4 showed maximum growth and antibacterial activity when cultures are supplemented with peptone and followed by beef extract and lowest activity was when using Casein(results are not shown here). The results indicate that peptone and beef extract served as good nitrogen sources for both isolates of actinomycetes.

ISOLATION OF ACTINOMYCETES BY AMPLIFICATION OF 16SRRNA GENE

To evaluate the PCR carried out for 2 isolates from different types of sources were tested. WhenPCR was performed with gene specific primers, 1400 bp fragments were produced in 2 isolates. The PCR products

were visualized on ethidium bromide stained Agarose gel (Figure 4a).After staining with CBB stain the band was observed with white trans illuminator. The band ranged from 14.1kDa to above 97.4 kDa was observed (Figure 4b).

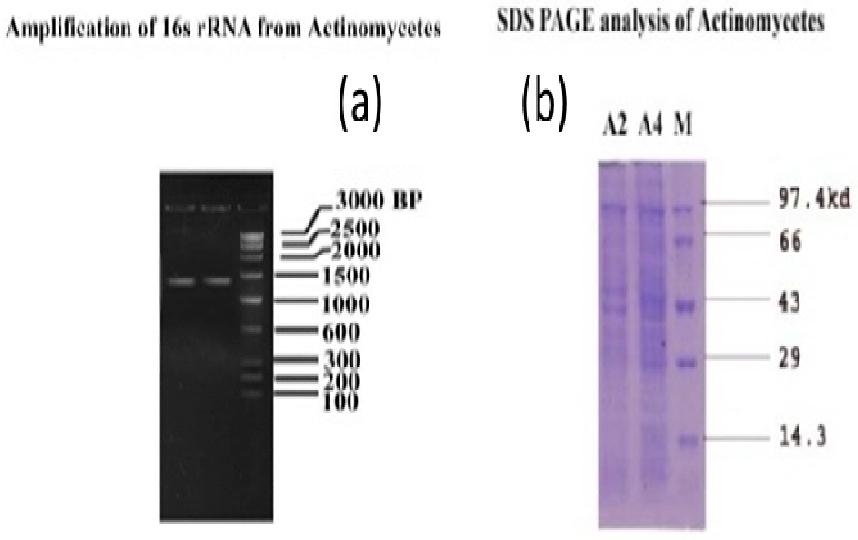

Fig. 4(a) Amplification of 16 s rRNA and (b) SDS PAGE analysis

In the present investigation actinomycetes were isolated from soil samples collected from different places in mine soil area, Salem, India, which are large and diverse, for the isolation of potent and broad-spectrum antibiotic producing actinomycetes. Totally 4 isolates of actinomycetes were observed from soil samples by using Starch-casein-nitrate agar media supplemented with nystatin (100µg/ml) to inhibit fungal growth and ciprofloxin (100µg/ml) to inhibit the bacterial growth. This media is very specific for isolation of actinomycetes, as only organisms (mostly actinomycetes) those are capable of degrading the polymers in the media are able to grow**.** Both primary and secondary screening methods were used to screen actinomycetes for antibacterial activity. The first screening was used to select the antibacterial isolates and determine the range of microorganism that was sensitive to the antibiotic. The secondary screening method was crucial to select the isolates for further studies. The result of the screening revealed that four isolates were against bacterial culture. But the best strain was found to be A2 and A4. Nutritional requirements of Streptomyces play an important role during metabolite synthesis process. Amongst various nutritional requirements, carbon source and nitrogen source are generally regarded as important factors of metabolism, and several examples of the production of metabolites in media with optimized contents of these components are also described in the literature.

CONCLUSION

In the present study, the actinomycetes isolated from soil samples of Salem, Tamil Nadu showed antibiotic activity against Gram positive and Gram-negative bacteria. These findings indicated that our produced substance might be the alternative antimicrobial substance as a tool for controlling human diseases. Further, attempts are being carried out to characterize the bioactive substances. To evaluate the PCR carried out for 2 isolates from different types of sources were tested. It is well known that when PCR was performed with gene specific primers, 1400 bp fragments were produced in 2 isolates. The PCR products were visualized on ethidium bromide-stained Agarose gel. After staining with CBB stain the band was observed with white transilluminator. The band ranged from 14.1 kDa to above 97.4 kDa were observed.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest

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