Exploring Anti-microbials of Actinomycetes isolated from rhizosphere soil

[1]Sasikala C,[2]Hemapriya R, [3]Tobias Noel A, [4]Deebini G, [5]Thirisha Y

Department of Microbiology
Dr. N.G.P. Arts and Science College
Coimbatore, Tamil Nadu, India.

Abstract- **Actinomycetes have provided a wealth of bioactive secondary metabolites with interesting activities such as antimicrobial, antiviral and anticancer. At present, there is a need to find out novel antimicrobial-producing strains as the pre-existing drugs have failed due to the development of resistance among the microorganisms. The present study is also a little contribution towards this need. The isolates which showed broad-spectrum activity against the test microorganisms can be considered as candidates in regards of searching for potential antimicrobial compounds. The objective of this study was to isolate, identify, and screen the potential antimicrobial-producing actinomycetes from soil. The isolates were identified on the basis of morphological study, different sugar utilization, protein utilization, and hydrolysis tests. They were also characterized on the basis of temperature and pH. Primary screening for antimicrobial activity was carried out against several test organisms. These results highlighted the scope for further characterization of the metabolite and could be a candidate in the generation of new antimicrobial agents.**

Key Words- **Actinomycetes, antimicrobial, Soil, Isolation, Characterization, Exploring, Rhizosphere.**

INTRODUCTION

Actinomycetes are Gram-positive filamentous bacteria with fungal morphology (Bhatti *et al.*, 2017). They are widely distributed in nature, particularly in soil. Actinomycetes are widespread in nature and may occur in extreme environments. Microorganisms found in extreme environments have attracted a great deal of attention, due to the production by such microorganisms of various natural compounds and their specialized mechanisms for adaption to extreme environments (Bawazir and Shantaram, 2018). Among the various extremophiles, halophilic microorganisms have developed several strategies to survive and to function in hypersaline ecosystems, such as salterns, salt mines and other hypersaline environments. Actinomycetes are known as the most biotechnologically valuable prokaryotic microorganisms. They are well known as a source of antibiotics and bioactive molecules. Most of their bioactive molecules have been hown to have antibacterial (streptomycin, tetracycline, and chloramphenicol), antifungal (nystatin), antiviral (tunicamycin) and antiparasitic properties (Manandhar and Sharma, 2017). Currently, over 5000 antibiotics have been screened from Gram- positive, Gram-negative bacteria as well as fungi. However, only 100 of these antibiotics have been developed to clinical applications (Rai *et al.*, 2016). Narendra Kumar *et al.*, 2010 isolated 117 antibiotic producing actinomycetes from non-agricultural wasteland alkaline soils and compost rich garden soils in which most of the isolates inhibits Gram negative bacterial growth. Four potential antibacterial actinomycetes were isolated from the aquatic environment. Valli *et al.* (2012) isolated 21 potential actinomycetes from marine environment and reported that all the isolates were promising against at least one tested organism. Kalyani *et al*., 2012 isolated 20 species from marine soil samples in which three showed significant antimicrobial activity against *Staphylococcus aureus* and *Escherichia coli* (*E. coli*). The present study aimed to evaluate the anti-microbial activity of the actinomycetes which is isolated from the rhizosphere soil (*Arachis hypogea*). Natural product antimicrobials have been used for centuries by native peoples all around the world but it was not until the late 1800s that people began to search for single compounds that could be used to kill disease-causing bacteria (Kavitha *et al*., 2016). It was concluded that actinomycetes isolates from different soil samples nearby medicinal plant area of Sarastro region are a good source to produce many useful antimicrobial compounds and other metabolites. (Ram Darshit *et al*., 2018). Though an oversized listing of antibiotics is well-known to be commercially accessible, the quest for the foremost capability one continues to be on, and this work could provide some ability facts at the antibiotic manufacturing and also the control of microbial strains. (Anshika *et al*., 2021). Among the microbial products, the fermentation product of Streptomyces is the most abundant source of antibiotics and various industrial compounds. The previous study describes a simple, accurate, reproducible and precise UV Spectrophotometric method for the estimation of AZI (pH 6.8 Phosphate buffer). The findings of the study shows the diminishing effort in the identification of proficient antibacterial especially from a new source and increase in bacterial resistance towards antibiotics has created an apprehensive scenario to control bacterial infection. The study reveals the present investigation deals with the extracellular metabolites from the soil bacterium against the devastating vector, *Aedes aegypti*.

I. MATERIALS AND METHODS

Collection of samples

Sample of soil was collected from a small village near Annur (Mondipalayam) as rhizosphere soil (Figure 1). The samples were taken up to the depth of 10 cm after removing approximately 3 cm of the soil surface. Temperature at time of soil collection is 27°C. About 5 g of this soil was taken and it was stored in sterile poly bags, sealed tightly for further identification. The test organisms (*Klebsiella pneumonia, Bacillus subtilis, Staphylococcus aureus, Escherichia coli*) were obtained from Kovai Medical Centre and Hospital, Coimbatore,India.

Figure 1 Collection of soil sample

Culture media

The culture media used for the isolation of actinomycetes from soil sample is Actinomycetes isolation agar (Hi-Media). The cultural media used for testing antimicrobial activity was Mueller-Hinton agar. The culture media was prepared and sterilized following the manufacturer's instructions.

Isolation of the Micro-organism

9 test tubes were taken; 9 ml of distilled water was poured in each test tube. The tubes were subject to sterilization in an autoclave, 121°C for 15 minutes. 1gram of soil sample was taken and diluted in 99ml of distilled water and serially diluted in each test tube. Actinomycetes isolation agar medium was prepared. The medium along with glass Petri dishes was sterilized in an autoclave at 121°C for 15-20 minutes. After sterilization, the media was slowly cooled to 50°C. 20 ml of the media was poured into each of these Petri dishes and allowed to solidify the media. Crowded plate technique was used to isolate the antibiotic producing colonies. 0.1 ml of suspension 10^{-2} to 10^{-4} was used to spread on agar medium aseptically using L-shaped glass rod. For each sample, three plates were used and incubated at 37°C for 48 hrs. After the period of incubation, the clear zone of inhibitions around the microorganism was observed. The microorganism colony responsible for the clear zone was isolated.

Preservation of soil isolates:

The pure cultures were obtained from selected colonies by repeated sub culturing on Actinomycetes isolation agar plates by the method of streaking. Streaking was done using a sterile nichrome loop inside a laminar air flow. The plates were kept for incubation at 37°C for 24 hrs. And preserved them by keeping into the refrigerator at 4°C and maintained for a longer period by serial sub culturing.

In-vitro screening of isolates:

The potential isolates selected from the in-vitro screening were characterized by morphological, biochemical, and physiological methods.

Morphological characterization:

The morphological characters of the selected isolates were studied by inoculating into sterile Actinomycetes isolation agar plate. The media was sterilized and poured into sterile Petri dishes. After solidification, selected isolates were streaked in plates aseptically and incubated at 30°C for 5 days. Morphological characters, such as colony characteristics were observed.

Microscopical characterization:

The Gram stain was used to determine positive and negative strain of bacteria with the help of oil immersion microscope at magnification of ×100. The motility test is performed by preparing wet mount of isolates by Hanging drop method and viewed at ×100 magnification under phase contrast microscope.

Biochemical characterization:

After preliminary studies, isolates were used for biochemical studies. The various biochemical tests methyl red test, Voges-Proskauer test, citrate utilization test, urease test, triple sugar iron test and starch hydrolysis test were performed for the identification of the potent isolates and incubated at 37°C±0.2 for about 24hrs.

Screening for anti-microbial activity:

These isolates were used in the secondary screening. In 50 ml conical flask 5 ml of inoculum medium was taken, and inoculums of actinomycetes about 2.5 % were added into media (broth) in aseptic condition. After this procedure flask is incubated at temperature 37°C for 7 to 9 d of duration. By using the centrifugation method at 4000 rpm for 30 min, supernatant was obtained. This supernatant was used in agar well diffusion method for secondary screening against various pathogens in test. For the evaluation of antimicrobial activity well diffusion method is the preferred method of choice. The procedure of this method is similar to the disk diffusion method. First microbes are inoculated on all surface of agar by spreading volume of inoculums of the surface of media. Then after prepared media 6to 8 mm diameter hole is made in aseptic conditions by using cork borer which was previously sterile take 20 to 100 µl of the volume of an extract of the sample or antimicrobial agent is added with specific concentrations into the hole.

Extraction of antimicrobial metabolites:
For further study actinomycetes isolates were incubated in 500 ml of agar medium under aseptic conditions. Then these flasks incubated for 8 d at 30 °C. Then the broth culture was filtered using Whatman no.1 filter paper. In ratio 1:1 of volume-by-volume chloroform added infiltrates. Extraction was done under shaking conditions. Antibiotic-containing chloroform part was separated. Using separating funnel aqueous part was separated. And it is subjected to rotary evaporator in the temperature of 40 °C which is used to evaporate and make concentrate this antibiotic containing chloroform extract part. Methanol was used to purification purpose. Usinga well diffusion method antibacterial activity of this compound was checked.
Anti-microbial activity from crude extract:
The soil isolates for antimicrobial activity screening against the pathogenic test organisms by Agar well diffusion method. In Agar well diffusion method, the test microorganisms were inoculated on Mueller-Hinton Agar plates using sterilized cotton swabs. Wells bored by sterileborer, and 100 µl of each crude extracts were poured into wells. Plates were incubated at 37°Cfor 24 hrs.

III RESULTS & DISCUSSION
Serial Dilution:

1gram of soil is mixed in 100ml of distilled water and is serially diluted in test tube to reduce the microbial count from the collected sample.
Spread Plate:
The diluted liquid specimen containing one or more microorganism is spread over suitable agar media in which each of the viable microorganisms is multiplied and formed separate colonies (Fig.2). The serial dilution is performed in order to reduce the total number of microbial counts in theculture plate. This reduced the group size and the concentration that are more applicable.

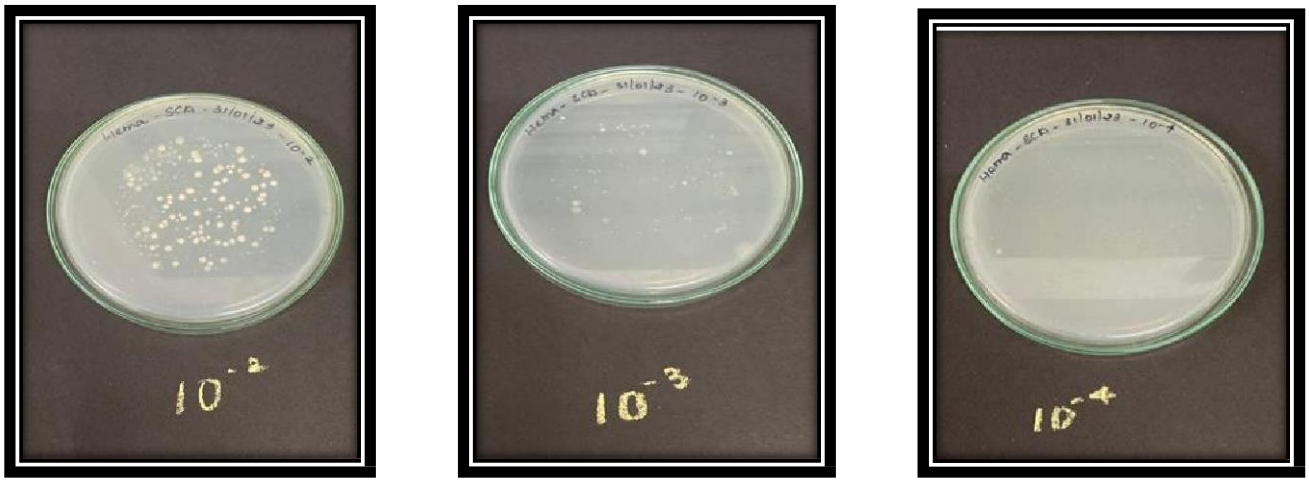

Figure 2 Spread plate techniques

Subculture of the isolates:

The isolated colonies after the incubation period of 5-6 days were macroscopically observed for white powdery and earthy smell was observed on the sub cultured plate with isolated colonies. The quadrant streaking when performed with single inoculum with four streaking withouteach turn to red hot doesn't give isolated colonies.

Gram's staining:
The isolate was found Gram positive and showed violet colour rod on the slide under microscopic view of 100x with oil immersion. Gram-positive bacteria have higher peptidoglycan content and when stained with a primary dye and fixed by a mordant, bacteria retained the primary stain by resisting decolorization, that doesn't get decolorized by a decolorizer.
Broth culture:

The isolated colonies were sub cultured in broth medium for further biochemical analysis.
Biochemical Analysis:

Biochemical test such as Indole, Methyl Reed, Voges Proskauer, Citrate, Triple sugar ion test and Starch hydrolysis was done to identify the genus of the organism and the results are tabulated (Fig.3 & Fig.4).

Figure 3 IMVIC & TSI

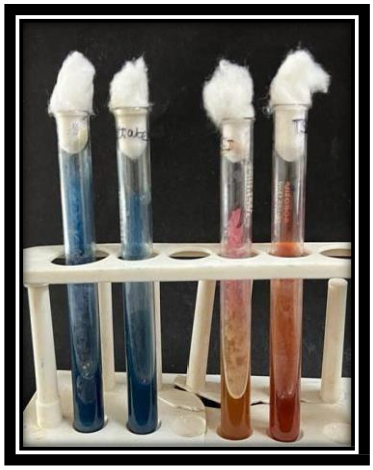

Figure 4 Starch Hydrolysis

Table 1 Biochemical Analysis

Test	**Result**
Gram's Staining	Positive
Indole	Positive
Methyl Red	Positive
Voges- Proskauer	Positive
Triple sugar ion test	Alkaline slant, acid bud
Fermentation of citrate	Negative
Strach Hydrolysis	Positive

Broth Culture for production of secondary metabolites:

After the culture identification the actinomycetes were sub cultured in actinomycetes broth medium and is incubated for 5-6 days at room temperature for the production of secondary metabolite from the isolates. After incubation mat like culture growth is seen on the top of the broth medium.

Extraction of intracellular metabolites from broth to solvent:

The broth culture is mixed vigorously and filtered and the infiltrate is mixed with the solvent (Chloroform) in 1:1 ratio and is kept in bench top shaker for overnight.

Separation of the solvent containing metabolites:

The broth culture with solvent is placed in separating funnel for about 1hr, for formation of a clear Layer which contains

secondary metabolites.

Crude extract from the secondary metabolites:

The extracted solvent is set to rotary evaporator at 40°C for about 15mins and the solvent is evaporated and the solvent which contained the secondary metabolites is separated as crude extract.

Centrifugation:

The broth culture is set in centrifugation for 4000rpm for 15mins for the separation of the supernatantfrom the pellet.

UV- Spectroscopy:

The crude extract and the supernatant were set in UV-Spectroscopy and the peaks of absorbance was between the range of 190nm-400nm. The peaks obtained for the crude extract was well defined than the supernatant, and this shows the presence of antibiotic. The peak obtained by ranges between 200nm-250nm. Which shows lower absorption when compared to the results obtained in this study.

Table 2 UV-Spectroscopy values for crude extract

Wave Length	**Absorbance for crude extract**		
200-300nm	0.102	2.793	1.879
300-400nm	1.662	1.566	0.421
400-500nm	0.416	0.408	0.128
500-600nm	0.158	0.189	0.09
600-700nm	0.116	0.039	0.031

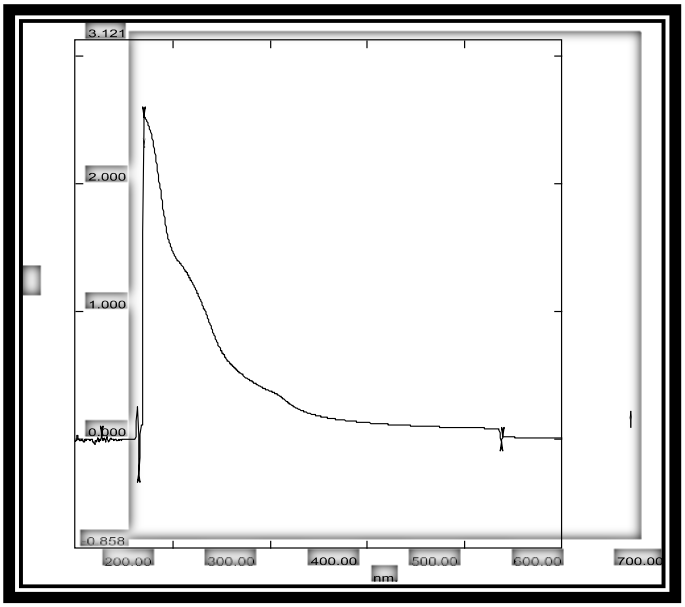

Figure 5 Absorbance peak obtained for crude extract

Table 3 UV-Spectroscopy values for Supernatant

Wave Length	**Absorbance for Supernatant**		
200-300nm	0.018	2.529	1.642
300-400nm	1.459	1.346	0.376
400-500nm	0.371	0.365	0.128
500-600nm	0.128	0.157	0.09
600-700nm	0.08	0.04	0.006

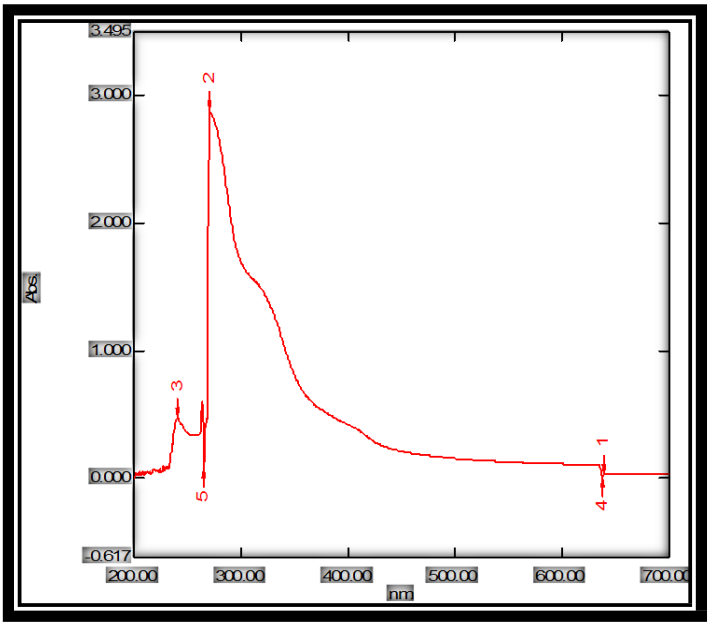

Figure 6 Absorbance peak obtained for Supernatant

Anti-Microbial Activity using collected secondary metabolite* (Fig.7)*:

Table 4 Comparative zone of inhibition on MHA medium

S.no	**Test organisms**	**Zone of inhibition(mm)**
1	*Escherichia coli*	24
2	*Staphylococcus aureus*	26
3	*Klebsiella pneumonia*	18
4	*Bacillus subtilis*	No zone

Table 5 Comparative zone of inhibition on MHA medium

S.no	**Test organisms**	**Zone of inhibition(mm)**
1	*Escherichia coli*	21
2	*Staphylococcus aureus*	26
3	*Bacillus subtilis*	28
4	*Proteus vulgaris*	19

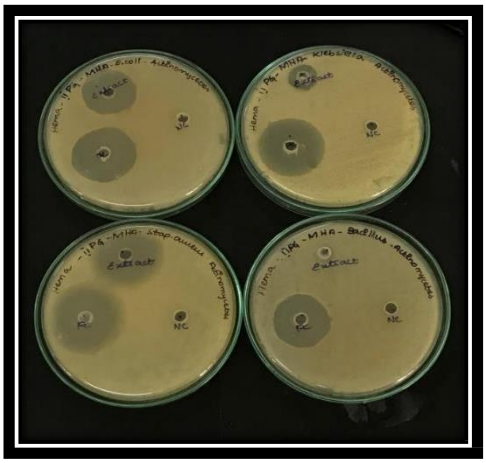

Figure 7 Zone of inhibition on MHA medium

IV. CONCLUSION:

The study concluded that actinomycetes from rhizosphere soils may produce a useful compound which has antimicrobial properties and also there is a chance to develop other industrially important metabolites from them. The isolated culture was found most potent, and further in future, there is a chance to produce new and use a full compound which may cause the death of the microbes which has been got resistance.

V. ACKNOWLEDGEMENT:

The authors are grateful to DST-FIST Scheme, DBT Star Scheme, Management and Principle of Dr. N. G. P. Arts and Science College (Autonomous) for their extended support for this work.

REFERENCES:

1. S, Bhatti, S. Haq, and R. A. Bhat, "Actinomycetes benefaction role in soil and plant health," Microbial Pathogenesis, vol. 111, pp. 458–467, 2017
2. A.L.T, Kalyani, Ramya Sravani KM, Annapurana J. Isolation and characterization of antibiotic producing actinomycetes from marine soils samples. *Int J Curr Pharmaceu Res.* 2012;4(2):109–112.
3. Kavitha. R, Dhamodharan. N, Dhivya. C. (2017). Screening, isolation and antibacterial activity of antibiotic producing bacteria obtained from saprophytic soil samples. Asian Journal of Pharmaceutical and clinical Research.
4. Kumar N, Singh RK, Mishra SK, Singh AK, Pachouri UC. Isolation and screening of soil actinomycetes as source of antibiotics active against bacteria. *Int J Microbiol Res.* 2010;2(2):12–16.
5. M. A. Bawazir and M. Shantaram, "Ecology and distribution of actinomycetes in nature—a review," International Journal of Current Research, vol. 10, no. 7, pp. 71664–71668, 2018.
6. M. Rai, N. Bhattrai, N. Dhungel, and P. K. Mandal, "Isolation of antibiotic producing actinomycetes from soil of Kathmandu valley and assessment of their antimicrobial activities," International Journal of Microbiology and Allied Sciences, vol. 2, no. 4, pp. 22–26, 2016.
7. Ram Darshit, Dr. Devang Pandya. (2018). Screening and Characteristic study of antimicrobial actinomycetes from nearby soil of medical plants. Innovare Academic Sciences.
8. S. Manandhar and S. Sharma, Practical Approach to Microbiology, pp. 273–276, National Book Centre, Kathmandu, Nepal, 3rd edition, 2017.
9. Sharma, I. Soni and J. Tyagi, "Isolation of actinomycetes from different soil sample and check their antimicrobial activity," (2021).. Journal of Biological Science.
10. Valli S, Suvathi Sugasini S, Aysha OS, Nirmala P, Vinot Kumar P, Reena A. Antimicrobial potential of actinomycetes species isolated from marine environment. *Asian Pacific J Trop Biomed.* 2012; 9:416–473.