

## ISOLATION OF ANTIBACTERIAL COMPOUND FROM MARINE SOIL *ACTINOMYCETES*

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### Abstract

The antibacterial study revealed that the isolated marine soil *actinomycetes* by crowded plate method and was identified by MIDI Advance technology (gas chromatographic) method and Thus, the microbes were identified according to their fatty acid sequences. The *actinomycetes* species were identified as *Streptomyces rimosus*, *Streptomyces fradiae*, and *Streptomyces griseoflavus* and the antibacterial assay was carried out by well diffusion method. Out of these only *Streptomyces rimosus* having high antimicrobial potential against Gram positive as well as Gram negative bacteria. In Gram positive *Streptomyces aureus* exhibits higher sensitivity followed by *Bacillus subtilis* where as gram negative bacteria *E.Coli* was extremely sensitive to the metabolites followed by *Pseudomonas aeruginosa*.

**Keywords:** *Actinomycetes*, Marine soil, Antibacterial assay

### 1. Introduction

*Actinomycetes* are aerobic, gram positive bacteria that form branching filaments or hyphae and asexual spores and frequently filamentous and sporulating with DNA rich in G+C from 57-75%. The name *Actinomycetes* are derived from Greek word “aktis” means a ray and “mykes” means fungus. Immediately after the first shower the earth’s smell of the musty odour is due to the presence of *Actinomycetes*. The *Streptomyces* metabolites known as the ‘Geosmin’ is responsible for the earthy odour. However volatile product secreted by *Streptomyces* may also be responsible for the characteristic smell.<sup>1</sup> After the revolution in the ‘golden era’, in forties and early fifties, when almost all groups of important antibacterial antibiotic (Tetracycline, Cephalosporines, Amino glycosides, Macrolides) were discovered, the success story had continued. It seemed in the fifties and sixties that the main problems of chemotherapy had been solved. Antibiotic discovered in this period were mainly isolated from *Streptomyces* species representing some 70-80 % of the all isolated compounds. During this period the discovery of antitumour, antiviral and non antibiotic-enzyme inhibitory-metabolites had just started. In the next period, between the seventies and nineties the efficiency of research had decreased. In this period, besides the leading role of *actinomycetes* products (65-70%), the considerable increase of the discovery of “rare *Actinomycetes*” products (up to 30%) were also noticeable. The chemical

structures of almost all discovered compounds had been elucidated.<sup>2</sup>

### 2. Materials and Methods

**2.1 Collection of soil sample:** Soil samples were collected from three locations, Chilka lake, Puri beach and Bhuvneshwar. In the month of May the soil samples were taken from the 20 cm depth after removing approximately 3 cm of upper soil surface. The samples were placed in the polythene bags, closed tightly and stored in a refrigerator. The processed samples were given proper identification code (ID).<sup>8</sup>

**2.2 Isolation of the *Actinomycetes* organism:** Collected soil samples were treated with 2% calcium carbonate and incubated at 37<sup>0</sup> C for 15 min. Isolation of the *Actinomycetes* was carried out by the serial dilution method and followed by the crowded plate technique.<sup>3</sup>

**2.3 Primary screening of the Isolates:** Few colonies were selected which showed the antimicrobial activity and grown by streaking horizontally on the nutrient agar medium plate and incubated for 24 hours at 28<sup>0</sup> C. After incubation test organisms were streaked perpendicular on it with sterile inoculating loop and then incubated for 24 hours at 28<sup>0</sup> C. The zones of inhibition near to the test organism were observed.<sup>4,5,6</sup>

**2.4 Microbial Identification of the Isolates:** Microbes are identified by modern technique MIDI (Gas Chromatographic) methods<sup>7</sup>

**2.5 Fermentation of Isolates:** Isolates no. 3, 9, and 11 were taken from seeded medium and inoculated and grown in the submerged culture

medium in 500 ml Erlenmeyer flask separately. Flask contained 100 ml of the fermentation medium (NaCl 0.8 gm,  $\text{NH}_4\text{Cl}$  1.0 gm, KCl 0.1 gm,  $\text{KH}_2\text{PO}_4$  0.1 gm,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.2 gm,  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  0.04 gm, Glucose 2.0 gm, Yeast Extract 3.0 gm and Distilled Water 1.0 L, pH 7.3) Flask were kept on mechanical rotary shaker at 105 RPM and incubated at  $28^\circ\text{C}$  for 124 Hours. In between fermentation process samples were taken and antibacterial activity checked by disc plate method. After the incubation the fermented broth was taken and centrifuged at 8000 RPM for 5 min and was filtered the broth. Filtrates of each isolates were collected in separate flask.<sup>8,9</sup>

**2.6 Chemical extraction:** Antibacterial compound was recovered from the filtrate by solvent extraction with equal volume of methanol and shaken vigorously for 2 hour and filter. Then again compounds were recovered by extraction with ethyl acetate. Ethyl acetate was added to the filtrate in the ratio 1:1(v/v) and shaken vigorously for 1 hour for complete extraction. The ethyl acetate phase that contains an antibiotic was separated out from the aqueous phase with the help of separating funnel. It was evaporated to dryness in a water bath at  $80^\circ\text{C}$  and Residue was collected and weighed.<sup>10,11,12</sup>

**2.7 Thin layer chromatography of Extracts:** The extracts showed promising antibacterial activities were placed as spots in the two TLC plates (silica gel 1mm thickness) exactly 0.5 cm from the bottom of the plates. The chromatogram was developed in two plates by using solvent Chloroform: Methanol (4:1). The spots in the chromatogram were visualized in the Iodine vapor chamber and the  $R_f$  values were calculated in the first plate. The second plate was used for Bioautography.<sup>13</sup>

**2.8 Bioautography:** Muller Hinton agar media was prepared and sterilized. The Muller Hinton agar was inoculated with test organism *Staphylococcus aureus*, *Bacillus subtilis*, and *E.coli*. Inoculated Muller Hinton agar media was poured over the developed TLC chromatogram. The TLC Plates were incubated overnight at  $37^\circ\text{C}$ . Inhibition zones were noted.

**2.9 Antibacterial activity of extracted crude Antibiotic**

**i. Well (Cup) diffusion method:** Antibacterial activity was determined by cup diffusion method on MHA medium. The sterile medium (20 ml) was poured into 9 cm petriplates. The medium was allowed to cool in a sterile condition and plates were then inoculated with

cultures of test bacteria. Agar cup of 5 mm diameter were made in the plates with the help of sterile borers. The desired different concentrations of the extracts were prepared by first reconstituting in methanol then diluting in sterile distilled water. A 100  $\mu\text{l}$  volume of each dilution was introduced in triplicate wells into MHA plates already seeded with the standardized inoculums of the test bacterial cells. All test plates were incubated at  $37^\circ\text{C}$  for 24 h. The least concentration of each extract showing a clear zone of inhibition was taken as the MIC. Negative controls were prepared using the same solvent employed to dissolve the extracts. Oxytetracyclin was used as positive reference to determine the sensitivity of each bacterial species tested.<sup>4,5,6</sup>

**ii. Determination of Minimum Inhibitory Concentration:** Minimum Inhibitory Concentration (MIC) is the lowest concentration of an antimicrobial agent that inhibits the growth of the test micro-organism. Moreover this method is also used to compare the activity of an unknown crude antibiotic with known antibiotic.<sup>14</sup>

### 3. Result and Discussion

**3.1 Primary screening for isolated marine Actinomycetes :** Primary screening of the isolated *Actinomycetes* were carried out against the test organism *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*. Out of 12 isolates only 3 isolates were shown the antibacterial activity in Table 1.

**3.2 Microbial Identification of Isolated marine soil Actinomycetes by MIDI (GC):** The identification of isolates by midi was carried out and the results were tabulated in table 2.

From the result of MIDI it was found that Isolate No.3, 9, and 11 were identified as *Streptomyces* species and found to be *Streptomyces rimosus*, *Streptomyces fradiae*, and *Streptomyces griseoflavus* respectively.

**3.3 Thin layer chromatography of Extracts:** The extracts were separated by TLC and observed on Iodine vapor and UV spectrophotometer and the  $R_f$  values were tabulated in Table 3.

**3.4 Antibacterial activity of extracted crude antibiotic**

Antibacterial activity was carried out by well diffusion method, zone of inhibition were observed and tabulated in Table 4.

**3.5 Determination of Minimum Inhibitory Concentration (MIC):** Minimum Inhibitory

Concentration of extracted crude antibiotics was determined by serial dilution method against the test organisms and values were tabulated in Table no. 5.

**3.6 Spectral Analysis:** The extracted crude antibiotics were run in the UV and FTIR the values were noted in table no. 6.

**Table No. 1. Primary screening against test organism (Cross streak method)**

Sr. No.	Strains	<i>Staphylococcus aureus</i>	<i>Bacillus subtilis</i>	<i>Escherichia coli</i>
1	Isolate 03	+	+	+
2	Isolate 09	NI	+	+
3	Isolate 11	+	+	NI

Note: NI - No Inhibition.

**Table No. 2. Microbial Identification of isolated soil *Actinomycetes* by MIDI (GC)**

Sr. No.	Strain	Sim Index	Organism found
01	Isolate no. 3	0.523	<i>Streptomyces rimosus</i>
02	Isolate no. 9	0.511	<i>Streptomyces fradiae</i>
03	Isolate no. 11	0.433	<i>Streptomyces griseoflavus</i>

**Table No.3. Thin layer chromatography of Extracts**

Sr. No.	Extracts ID	Solvent System	No. of Spots	R <sub>f</sub> value
01	Isolate. No. 03	Chloroform: Methanol (4:1)	Single	0.88
02	Isolate. No. 09	Chloroform: Methanol (4:1)	Single	0.85
03	Isolate. No. 11	Chloroform: Methanol (4:1)	Single	0.82

R<sub>f</sub>– Refractive Index

From the above table, it is evident that all the extracts have single spot against Chloroform: Methanol (4:1) solvent system. The R<sub>f</sub> values fall between 0.82 and 0.88. From the study it is evident that the isolated extract may be a single compound.

**Table No. 4. Antibacterial activity of extracted crude Antibiotic by well diffusion method zone of inhibition (Diameter in mm)**

Sr. No.	Isolate strains	Extracts ID	<i>S. aureus</i>	<i>B. subtilis</i>	<i>E.coli</i>
1	Isolate 03	03	15	18	20
2	Isolate 09	09	NI	18	20
3	Isolate 11	11	12	14	NI
4	Std.	Oxytetracycline	20	24	18
5	Blank	Ethyl acetate	NI	NI	NI

From the above table, it was evident that Isolate 3 had more potent activity against *Staphylococcus aureus*, Isolate 3 and 9 had more potent activity against *Bacillus subtilis*, and *Escherichia coli*.

**Table No. 5. MIC of extracted crude antibiotics against the test organisms**

Sr. No.	Isolate strains	Extracts ID	<i>S. aureus</i>	<i>B. subtilis</i>	<i>E.coli</i>
1	Isolate 03	03	500	1000	500
2	Isolate 09	09	----	1000	500
3	Isolate 11	11	1000	500	

**Table No. 6: UV Analysis of Extracts**

Sr. no.	Extracts ID	Wavelength (λ max)	Peak Absorbance
01	Isolate. No. 03	268.0	0.380
02	Isolate. No. 09	273.5	0.669
03	Isolate. No. 11	277.0	0.232

From the above table, it was evident that the extracts showed an absorbance at the UV range. All the extracts exhibited absorbance maximum between 268 nm and 277 nm when scanned between 200 to 400 nm confirming that the isolated extract was having single compound.

#### 4. Summary and Conclusion

The collected soil samples from the different locations were brought to the laboratory under aseptic condition and used for isolation of antibacterial compounds. Serial dilutions of sample were made and isolation of the antibiotic

producer *Actinomycetes* bacteria was done by crowded plate method. From these 12 *Actinomycetes* showed zone of inhibition around the colonies were selected for primary screening. It was done by cross streak method by using 3 standard test organism *Staphylococcus aureus*,

*Bacillus subtilis*, and *Escherichia coli*. It was found that only 3 isolates (3,9,11) showed the antibacterial and then chosen for secondary screening by well diffusion method and further antibacterial study.

These isolates were periodically subcultured and for further study. The microbial identification of isolates were done by MIDI using Gas chromatography, it was found that three isolates was *Streptomyces* species and named as *Streptomyces rimosus*, *Streptomyces fradiae*, *Streptomyces griseoflavus* for isolate 3, 9, and 11 respectively. For these extracted Antibiotics, Further TLC studies of extracts were carried out by using Chloroform: Methanol (4:1) solvent system these extracts showed single spot when plates were visualized by iodine and UV chamber, this confirmed that extracts contained only single compound.

The extracts were subjected to bioautography, which showed inhibitory zone around the area of spot. The antibacterial activity was carried out by well diffusion method, it was evident that crude antibiotic of isolate 3 showed activity against *Staphylococcus aureus*. *Bacillus subtilis*, *Escherichia coli*, isolate 9 showed activities against *Bacillus subtilis*, *Escherichia coli* and isolate 11 showed activities against *Staphylococcus aureus* and *Bacillus subtilis*. MIC studies were carried out to found that the exact concentration of drug required to inhibiting the growth of test organisms. Extracts were further studied for their spectral analysis by UV and FTIR Spectroscopic analysis. The UV spectrum of all crude antibiotics showed  $\lambda$  max between 268 to 277 nm. FTIR showed the antibacterial groups but the graphs didn't match with the previous antibiotic produced by *Streptomyces rimosus* *Streptomyces fradiae* and *Streptomyces griseoflavus*. Hence the produced antibiotics might be newer antibiotics. To conclude, all the isolated Actinomycetes had good antibacterial activity except few had some inhibitory effect against test organisms. Among these isolate 3 had got more potent activity as compared to isolate 9 and 11. The identification and elucidation of the structure of these antibiotics required  $C^{13}$  NMR, MS spectral analysis and can be carried out in future studies.

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