Studies on Isolation and Characterization of *Actinomycetes* from Soil for Potential of Antibacterial Activity.

Dinesh Kasbe[1], Milind Kharat[2]

[1]Department of Microbiology, Indira Gandhi (Sr.) College, CIDCO, Nanded. (M.S.)
[2]Arts, Science and Commers College, Ambad Dist. Jalna (M.S.)

Abstract: The purpose of this study was to screen soil samples from diverse crop areas in Maharashtra's Nanded District for actinomycetes with powerful antibacterial chemicals. In total, 49 actinomycetes were isolated from six soil samples. In the initial screening, 21 isolates shown antibacterial activity against the test pathogens. To establish antibacterial potential, all isolates were submitted to additional agar well screening. Only four actinomycetal isolates out of 21 demonstrated the highest zone of inhibition against both gramme positive and gramme negative organisms. One actinomycetal isolate with the highest antibacterial activity was chosen and identified as belonging to the genus Streptomyces. The effect of carbon and nitrogen sources on antibiotic synthesis was investigated using this isolate. Maltose and potassium nitrate were shown to be the best carbon and nitrogen sources for antibiotic synthesis in the study.

Key Words: Antibiotics, Streptomyces, Screening, Antibacterial.

1. INTRODUCTION

Actinomycetes are a large and diversified group of Gram-positive, aerobic, mycelial bacteria with a high G+C nucleotide content (> 55%) that perform an important ecological function in the soil cycle. The name actinomycetes comes from the first discovered anaerobic species, *Actinomycetes bovis*, which causes "actinomycosis," or "ray-fungus illness" in cattle. They were once thought to be an intermediate group between bacteria and fungi, but they are now classified as prokaryotic microorganisms. The majority of actinomycetes are free-living, saprophytic bacteria that are found in soil and water. Actinomycetes have been found as a significant soil population. Actinomycetes can produce a wide range of secondary metabolites, including antibiotics. Among prokaryotes, actinomycetes have a high commercial and biotechnological value. They are responsible for the majority of the bioactive secondary metabolites found. Actinomycetal taxa that are rare or unique have been a prominent focus in the hunt for medicinal medicines. Actinomycetes, particularly Streptomyces, have been responsible for the isolation of approximately 61% of all bioactive microbial metabolites.

Thousands of antibiotics are known now, with the majority of them produced by actinomycetes, particularly the species Streptomyces. Because of their almost limitless ability to create secondary metabolites, including antibiotics with diverse chemical structures and biological activity, actinomycetes have taken a major role in the pharmaceutical business. Streptomyces is the greatest antibiotic-producing genus found so far in the microbial world. For more than two decades, the number of antibiotic compounds reported from the genus's species expanded practically rapidly.

The purpose of this research is to isolate *actinomycetes* from farm soil samples in the Maharashtra district of Nanded, to evaluate their antibacterial activity against test microorganisms, identify potential isolates, and select suitable carbon and nitrogen sources for antibiotic synthesis.

2. MATERIALS AND METHODS:

I. Collection of soil sample:

The farm soil samples were collected in newly purchased polythene bags (swab led with cotton dipped in 70% alcohol) and transported to the laboratory to avoid contamination. They were kept at temperatures ranging from 6 to 10 ^{0}C until they were used.

II. Isolation of Actinomycetes:

Dilutions of soil samples in sterile water (1/10 w/v) were prepared. Each diluted soil sample got a thermal shock of 70 ^{0}C for five minutes, and 5 ml of soil sample was inoculated in a 250 ml conical flask containing 50 ml of enrichment medium (Starch-2.0g, Yeast extract-0.8g, Peptone-0.4g, Distilled water-1L, pH-7.2). The antifungal drug griseofulvin was added to the medium at a concentration of 50 g/ml. Temperature shock depresses related gram-negative bacteria, while antibiotics in the medium destroy fungi that cause problems during isolation. The flasks were incubated at 30 ^{0}C for 10 days. The enriched cultures were isolated by using the streak plate method on starch nitrate agar.

Composition:

Starch	-	20.0g,
KNO_3	-	1.0g,
K_2HPO4	-	0.5g,
$MgSO_4.7H_2O$	-	0.5g,
NaCl	-	0.5g,
$FeSO_4.7H_2O$	-	0.01g,
Agar	-	20.0g,

Distilled water - 1L,
pH - 7.2

The medium was mixed with the antifungal drug griseofulvin at a concentration of 50µg/ml. These plates were then incubated at 30°C for 10 days.

III. Study of characteristics of isolates:

After incubation, dried, leathery actinomycetes colonies were observed. The colour of aerial mycelium and the colour of vegetative mycelium were examined using the "colour and Streptomyces" and ISCC-NBS colour charts. Cover slip cultures of actinomycetal isolates were produced, and morphological characteristics were examined. Pure colonies were sub cultured onto the appropriate media slants and stored at 40 ^{0}C for later investigation.

IV. Selection and culturing of bacteria for antibacterial:

Bacterial cultures were obtained from NCIM Pune. From these cultures, master cultures were prepared, and every 3-4 weeks, subculturing was performed for each experiment a 24 hour actively growing culture was used.

Bacillus subtilis (NCIM 2195)
Staphylococcus aureus (NCIM 2602)
Proteus vulgaris (NCIM 2027)
Pseudomonas aeruginosa (NCIM 2945)
Escherichia coli (NCIM 2685).

V. Primary screening of actinomycetes for antimicrobial activity:

The cross-streak technique was used to test the antagonistic activity of 49 isolates. *Bacillus subtilis* NCIM 2195, *Staphylococcus aureus* NCIM 2602, *Proteus vulgaris* NCIM 2027, *Escherichia coli* NCIM 2685, and *Pseudomonas aeruginosa* NCIM 2945 were selected as test organisms. Secondary screening was performed on actinomycetes that shown high activity.

VI. Secondary screening:

Those isolates that shown significant antibacterial activity in primary screening were employed in secondary screening. 5 ml of sterile starch nitrate broth were placed in 25 ml conical flasks. Aseptically, 2.5% actinomycetes inoculums were added to the broth. For 10 days, the flasks were shaken at 30 ^{0}C. After incubation, supernatant was recovered by aseptic centrifugation at 4000 rpm for 20 minutes, and a portion of it was utilised to assess antimicrobial activity against test organisms using the agar well method. One actinomycetal isolate with the highest antibacterial activity was chosen based on the zone of inhibition against test organisms and characterised according to Shirling and Gottlieb 1966; Holt 1974. Morphological, cultural, and biological characteristics were investigated. Morphology of actinomycetal isolate was further studied by scanning electron microscopy (SEM). Cultural characteristics were studied on ISP4 medium. Utilization of different sugars and biochemical test were performed according to Bergey‟s Manual of Determinative Bacteriology.

VII. Effect of carbon and nitrogen source on antibiotic production:

To investigate the effect of carbon and nitrogen sources on antibiotic synthesis, starch, glucose, glycerol, sucrose, maltose, xylose, lactose, and arabinose sugars were utilised. Shirling and Gottlieb's carbon utilisation medium was created in a 250 ml Erlenmeyer flask,

Composition

$(NH4)_2SO_4$ - 2.64g
KH_2PO4 - 2.38g
K_2HPO_4 - 5.65g
$MgSO_4.7H_2O$ - 1.0g
Trace salt solution - 1ml
Carbon source - 10g
Distilled water - 1L
pH - 7.2

In an Erlenmeyer flask, 100 ml of each sugar medium was taken. The broth was sterilised before being inoculated with 2.5% actinomycetes inoculums. For 10 days, all flasks were incubated on a rotating shaker at 30 ^{0}C. After incubation, supernatant was collected by aseptic centrifugation at 4000 rpm for 20 minutes, and a portion of it was utilised to assess antimicrobial activity against test organisms using the agar well method (*P. vulgaris S. aureus, P. aeruginosa B. subtilis, E. coli).* The zone of inhibition against test organisms was used to monitor antibiotic synthesis. To investigate the influence of a single nitrogen source on antibiotic synthesis, various nitrogen sources were tested, which including ammonium sulphates, ammonium chloride, KNO_3, $NaNO_3$, asparagine, casein, soybean meal, and yeast extract. The previous experiment was repeated, but this time 1% maltose was utilised as the carbon source and 2% nitrogen was used as the nitrogen source.

3. RESULTS AND DISCUSSION:

Initially, 49 actinomycetes were isolated from 6 farming soil samples. In primary screening using the cross-streak method, out of these 21 isolates shown significant antibacterial activity against test pathogens.

Table -1 Antimicrobial activity of potent actinomycetes against test organisms

Sr. No.	Isolates	Zone of inhibition (mm) against test organisms				
		Bs	Sa	Pv	Ec	Pa
1	S1	16	18	15	18	17
2	S4	15	15	15	12	14
3	S9	16	20	24	19	24
4	S13	16	15	13	16	20

(Bs= Bacillus subtilis, Sa=Staphylococcus aureus Pv= Proteus vulgaris , Ec=Escherichia coli
Pa= Pseudomonas aeruginosa)

To validate antimicrobial activity, 21 isolates that inhibited test organisms in primary screening were submitted to secondary screening using the agar well method. 04 actinomycetal isolates (S1, S4, S9, S13) were found to have significant antibacterial activity against both gram positive and gram-negative pathogens in both primary and secondary screening procedures. Finally, one of the most potent actinomycetal isolates, S9, was chosen for further investigation based on its largest zone of inhibition.

Table-2 Cultural characteristics of isolates.

Sr. No.	Cultural Characteristics	Streptomyces Spp.
1	Colony morphology	4-8 mm. diameter, circular , rough, convex, opaque, velvety, white to light brown.
2	Aerial mycelium Colour	White
3	Vegetative mycelium Colour	Yellow
4	Diffusible pigment	None
5	Nature of sporulating aerial mycelium & spore	Long & spiral chains of spores on aerial mycelium. Spores are circular with smooth surface

The morphological, cultural, physiological, and biochemical properties of the selected potent actinomycetal isolate S9 were investigated. Actinomycetal isolate S9 colonies were 4-8 mm in diameter, round, rough, convex, opaque, leathery, and white on ISP4 medium. Actinomycetal isolate included both vegetative and aerial mycelium, and lengthy and spiral chains of spores on aerial mycelium were detected in coverslip culture and SEM pictures. The spores were round and had a smooth surface. SEM examination of Actinomycetal isolate S9 confirmed it to be a Streptomyces species. Many researchers employed SEM to identify actinomycetes.

Table-3. Physiological Characteristics of actinomycetal isolate.

Sr. No.	Sugars	Result
1	Maltose	+++
2	Xylose	+
3	Glucose	++
4	Arabinose	+
5	Mannitol	+
6	Lactose	+
7	Sucrose	+
8	Raffinose	--
9	Mannose	+

(+++ Very good, ++ Moderate, + Poor, + Positive Test, - Negative Test)

The ability of actinomycetal isolate S9 to use various sugars was studied. It could use D-glucose, maltose, xylose, arabinose, sucrose, mannitol, and lactose. Catalase, oxidase, gelatinase, caseinase, cellulase, lecithinase, and amylase enzymes were produced by Actinomycetal isolate.

Table-4 Biochemical Characteristics of actinomycetal isolate.

Sr. No.	Biochemical Characteristics	Result
1	Indole	-ve
2	Methyl red	+ve
3	V.P	-ve
4	Citrate	-ve
5	NO_2 reduction	-ve
6	H_2S production	+ve
7	Catalase	+ve
8	Oxidase	+ve
9	Gelatinase	+ve
10	Casienase	+ve

11	Cellulase	+ve
12	Lecithinase	+ve
13	Amylase	+ve

(+++ Very good, ++ Moderate , + Poor , + Positive Test , - Negative Test)

Actinomycetal isolate shown positive methyl red and H_2S generation. Thus, it was observed that actinomycetal isolate S9 is biochemically diverse and has a high capacity for biodegrading a wide range of organic molecules in soil. Actinomycetal isolate S9 was recognised as a species of Streptomyces spp. based on morphological, cultural, physiological, and biochemical characteristics. Li Hua et al. (1996) examined 4200 Yunnan soil samples. He observed that the genus Streptomyces appears to be the most important in ecological function it represents up to 90% of all soil actinomycetes in Yunnan. Oskay et al. (2004) identified and examined antibacterial activity of 80 distinct actinomycetes strains obtained from farming soil samples against phytopathogenic bacteria *Agrobacterium tumefaciens*, *Erwniaamylovora*, and *Psedomonas viridiflora.* When the effect of carbon source on antibiotic synthesis in Streptomyces S9 was tested, it was discovered that when medium containing maltose as a carbon source, Streptomyces S9 gave the largest zone of inhibition, i.e. 24, 20, 24 mm against *P. vulgaris, S. aureus*, and *P. aeruginosa.* Streptomyces S9 discovered maltose to be the best carbon source when compared to starch, glucose, glycerol, sucrose, xylose, lactose, and arabinose. According to Narayana et al. (2001), *Streptomyces albiaqflavus* produces the most antibiotics when maltose is present as a carbon source. When inorganic carbon source KNO_3 was utilised as a nitrogen source in the medium, the maximal zone of inhibition formed by Streptomyces S9 was 18, 19, 16 mm for *P. vulgaris, S. aureus, and P. aeruginosa*, respectively. Thus, inorganic KNO_3 was discovered to be the optimal nitrogen source for Streptomyces S9 antibiotic synthesis. According to Bulchandani and Parvateesam (2007), ammonium nitrate is the best substrate for Streptomyces antibiotic synthesis. Aruna et al. investigated the optimal conditions for antibiotic synthesis in Streptomyces spp. They discovered that yeast extract and KNO_3 were the most effective for antibiotic synthesis. Vorar laid Rabah et al. (2007) discovered a new actinomycetes strain called RAF10 from Egyptian soil. It proved effective against both gram positive and gram-negative bacteria, yeast, and filamentous fungi.

4. CONCLUSION:

According to the primary and secondary screening results, farming soil samples from Nanded, Maharashtra, contain antibiotic producing actinomycetes and could be used as a source of new antibiotics.

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