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Isolation of indigenous actinomycetes and screening of their antibacterial and antifungal Activity

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Abstract : *The present study aimed at isolating indigenous strains of actinomycetes from soil sample collected in Patna region and screening of the isolates for their antibacterial and antifungal activity against seven pathogenic test bacterial and fungal genera. A total of sixteen strains were investigated for their morphological and cultural characteristics and antimicrobial activity. Upon initial identification all the isolated strains were found to belong to Genera Streptomyces. Out of sixteen strains, tested, only five showed antibacterial activity and four strains exhibited antifungal inhibition potential. One strain (Strain No. 14) exhibited inhibition potential against gram-positive, gram-negative bacteria and also against mold. However, strain no. 2, 13 and 15 exhibited activity only against the Gram negative strains i.e., Pseudomonas sp, Shigella sp and Serratia sp, while strain no. 9 only against Gram positive bacteria (both S. aureus and B. subtilis). Further investigations are needed to identify the strains to species level and also to check whether the metabolites responsible for these antimicrobial activity are active substances or not.*

Key Words : *Streptomyces, antimicrobial activity, antibiotic susceptibility, bioactive compound, characterization.*

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Introduction :

Actinomycetes have provided important bioactive compounds of high commercial value and continue to be routinely screened for new bioactive substances. These searches have been remarkably successful and approximately two-thirds of naturally occurring antibiotics, including many of medical importance, have been isolated from actinomycetes (Okami and Hotta 1988). Actinomycetes are abundant in terrestrial soils, a source of the majority of isolates shown to produce a number of bioactive compounds. The result of intensive screening program carried out over the

past several decades is that there is a growing problem of rediscovery of already known bioactive compounds (Nolan and Cross 1988).

Actinomycetes have the capability to synthesize many different biologically active secondary metabolites such as antibiotics, herbicides, pesticides, anti-parasitic compounds and enzymes like cellulase, xylanase, etc. (Ouhdouch et al 2001) Serious infections caused by bacteria that have become resistant to commonly used antibiotics has resulted in a major global healthcare problem which emphasizes the need for the development of other newer antimicrobial agents. Our knowledge of microbial diversity, antimicrobial and biochemical control ability of the microbes isolated from the soil is still inadequate, it is anticipated that the isolation, characterization and the study on actinomycetes can be useful in the discovery of antibiotics and novel species of actinomycetes.

The present study aimed to isolate indigenous strains of actinomycetes from soil sample of Patna region and for their antimicrobial (antibacterial and antifungal) properties against pathogenic bacterial and fungal genera.

Materials and Methods :

Soil sample collection and preparation : The soil samples were collected up to a depth of 15-20 cm from organic matter rich soil.. All samples were selected randomly from the PWC garden, compost soil and grass field of ICAR. The samples were placed in polyethylene bags, and stored in a refrigerator until pretreatment. Soil pretreatment was required for inhibiting or eliminating unwanted microorganisms by heating the samples at 50^{o}C in hot air oven for one hour. The samples were diluted till 10^{-6} dilutions for inoculation purposes.

Isolation of Actinomycetes : In this investigation, starch-casein agar (soluble starch 10 g, casein 0.3 g, K NO_3 2.0 g, NaCl 2.0g, K_2HPO_4 2.0g, $MgSO_4.7H_20$ 0.05g, $CaCo_3$ 0.02g $FeSO_4.7H_2O$ 0.01g, agar 20 g, distilled water 1000 ml) was used for isolating actinomycetes and pH of media used was set to 7.2. Actidione (50 µg/ml) were added into the medium as antifungal agent. Nutrient agar media and Potato Dextrose agar were used for routine culture of the bacteria and fungi under test respectively. The isolation plates of actinomycetes contaminated with bacteria and fungi were purified by repeated subculturing by streak-plate technique. A small portion of typical isolated colonies were streaked on starch casein agar media and incubated at 26°C for 2-7 days. Plates were checked for the growth of typical actinomycetes colonies upto 10 days.

Identification and morphological characterization of actinomycetes isolates : Identification and characterization of the pure strains were done according to International *Streptomyces* Project (Shirling and Gottlieb 1966). Morphological characters of isolates were observed by smears from colonies upto 10 days, stained by Gram's method as described by Hucker and Conn (1923). Colonies were identified on the basis of their colony morphology and color (Shirling and Gottlieb 1966). Color of aerial mycelium was determined from mature, sporulating aerial mycelia of the actinomycetes colonies on starch-casein agar media (Pridham 1964).

The utilization pattern of carbon sources by the strains was carried out according to the methods of Gottlieb (1961). The actinomycete strains were tested for their ability of tolerance to 45°C temperature, 7 and 10% NaCl concentration, production of H_2S, catalase and melanin pigments (Shirling and Gottlieb 1966). In addition, the sensitivity of the strains to different antibiotics was determined by disc diffusion method.

Screening of antimicrobial properties : The actinomycetes isolates were cultivated on Basal broth(yeast extract 10g, K_2HPO_4 1.0 g, $MgSO_4.7H_2O$ 0.5 g, $CaCl_2.2\ H_2O$ 0.04 g, $FeSO_4.7\ H_2O$ 0.05 g, $ZnSO_4.7\ H_2O$ 0.005g and distilled water 1000 ml) supplemented with yeast extract in a shaker incubator. The pH was adjusted to 7.5 and incubated at 27^0C and 160 rpm for 10 days. After 10 days, the basal broth was subjected to filtration using Whatman filter paper No.1 to remove the mycelial growth and the crude filtrate containing the antimicrobial bioactive compound was obtained which was used as source of antimicrobial substance to be tested against bacterial and fungal pathogens.

Test Organisms : Five bacterial and two fungal strains were used. All the strains used were pathogenic. Two Gram Positive bacteria : *Bacillus subtilis* and *Staphylococcus aureus*, and three Gram Negative bacteria *Serratia marcescens*, *Pseudomonas* sp., *Shigella* sp. were used. Fungal strains: One yeast *Candida albicans* and one mold *Aspergillus niger* were used. All the test organisms were obtained from Dept. of Industrial Microbiology, Patna Women's College, Patna.

Determination of Antibacterial activity and Antifungal activity : Determination of antibacterial activities of crude actinomycetes culture filtrate was performed by agar well-diffusion method (Cappuccino and Sherman 2004). Test bacterial and fungal strains were inoculated on Mueller Hinton (beef infusion 300g, acid hydrolysate of casein 17.5g, starch 1.5g, agar 20g, distilled water 1000ml) and Potato Dextrose agar medium respectively. Then small wells were cut from the agar surface with the help of sterile cork borer, approximately 100µl culture filtrates were added into each wells and plates were incubated at 37° and 26° C. Antimicrobial activity was measured by the determination of the size of the inhibition zone.

Results and Discussion :

Isolation plates developed various types of bacterial actinomycetes and fungal colonies (Table 1). All isolates grew on a range of agar media showing morphology typical of streptomycetes (Anderson and Wellington 2001). Colonies having characteristic features such as powdery appearance with convex, concave or flat surface and color ranging from white, grey to green and yellowish were selected. The color of the substrate mycelium and aerial spore mass was varied.Rizk et al (2007) reported grey and white color series of Actinomycetes as the dominant forms in the soil as compared to yellow, red, violet and green ones. Some isolates (No. 4, 7, 8, 10, 12 and 13) produced diffusible pigments on several agar media. Melani was produced on Tyrosine agar media (ISP 7) (Shirling and Gottlieb 1966) by isolate No. 4, 6 and 10.

Furthermore, bacterial colonies similar to that of actionmycetes were subjected to Gram's staining. Sixteen selected isolates were examined microscopically and identified by their morphological and culture characteristics. All the strains appeared Gram-positive with differing spore chain morphology ranging from spira, flexibilis and retinaculus – apertum, (Anderson and Wellington 2001; Williams et al 1989) as shown in the Table 2 . The utilization of carbohydrates, growth characteristics on 45°C temperature, 7% as well as 10% NaCl concentrations are summarised in Table 3. The Antibiotic Susceptibility Test showed varying results as showed in Table 4 below:

Table 1. Actinomycetes isolated from soil samples

Soil Sample	Dilution	No. of colonies	CFU
PWC Garden area	10^{-6}	3	3×10^7
PWC Garden area	10^{-7}	2	2×10^8
PWC Sports area	10^{-6}	2	3×10^7
PWC Sports area	10^{-7}	1	1×10^8
ICAR Fields	10^{-6}	5	5×10^7
ICAR Fields	10^{-7}	3	3×10^8

Table 2. Morphological characters of the isolated strains

Strain No.	Aerial Mass Colour	Substrate Mycelium colour	Diffusible Pigment Production	Gram Reaction	Spore Chain Morphology	Melanin Production
1	Cream	Off white	–	+ve	Rectus	–
2	Grey	Brown	–	+ve	Spira	–
3	Greenish Grey	Green	–	+ve	Rectus	–
4	Dark Grey	Brown	Red	+ve	Retinaculum-apertum	Brown
5	Grey	Brownish Grey	–	+ve	Flexibilis	–
6	Grey	Brown	–	+ve	Flexibilis	Orange red
7	Grey	Black	Brown	+ve	Spira	–
8	Dark Green	Violet	Violet	+ve	Rectus	–
9	Green	Light Brown	–	+ve	Flexibilis	–
10	Greenish Grey	Violet	Violet	+ve	Rectus	Brown
11	Grey	Light Green	–	+ve	Retinaculum-apertum	–
12	Green	Violet	Violet	+ve	Flexibilis	–
13	Yellow	Dark Yellow	Yellow	+ve	Rectus	–
14	Greenish Grey	Reddish Orange	Red	+ve	Spira	–
15	Off-white	Orangish Brown	Dark Yellow	+ve	Rectus	–
16	Greyish white	Greyish White	–	+ve	Flexibilis	–

Table 3. Observation for carbohydrate utilisation, for growth at 45^{0}C, growth with 7% and 10%(w/v) NaCl, catalase test, H_2S Production Test

Characteristics	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Carbohydrate utilization																
a) Glucose	–	++	+++	++	–	–	–	–	++	+	+	+	++	++	++	–
b) Sucrose	+	++	++	+++	++	++	++	++	+	+	++	+	++	++	++	–
c) Rhamnose	–	+	+	+++	++	–	–	+	+++	+	++	+	–	–	–	+
d) Mannitol	–	+	+	++	+	+	+	+	++	–	++	+	++	+	–	+
e) Xylose	+	++	++	++	++	+	+	++	++	+	+	+	–	+	+	–
f) Inositol	–	+	++	+++	+++	++	++	+	++	–	+	–	+	++	–	+
Growth at 45^{o}C	–	+	–	–	++	–	++	–	++	–	–	–	+	++	–	–
Growth with 7% NaCl	–	+	–	–	–	–	–	–	–	–	–	–	–	+	–	+
Growth with 10% NaCl	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–
Catalase Test	+	+	–	+	–	+	–	+	–	+	+	+	–	+	–	–
H_2S Prod^n Test	–	–	–	+	–	–	–	+	–	–	++	+	–	–	–	–

Interpretation:- No growth, + growth, ++ good growth, +++ very good growth

Table 4. Antibiotic susceptibility of the isolated strains (mm)

Isolate No.	Ampicillin	Erythromycin	Rifampicin	Tetracyclin	Oleandomycin
1	14	–	5	16	–
2	–	–	–	6	19
3	–	–	–	–	–
4	–	–	12	–	–
5	20	–	–	–	–
6	24	26	6	13	–
7	15	–	13	–	18
8	–	18	–	16	–
9	13	–	–	–	–
10	16	19	18	5	6
11	–	–	–	–	–
12	24	–	–	–	12
13	–	–	7	–	–
14	–	–	–	–	–
15	23	–	11	–	–
16	–	–	–	–	–

Carbohydrate utilization test plays prominent role in the taxonomic characterization of actinomycete strains (Pridham and Gottlieb 1948). Isolate No. 3, 4, 5 and 9 efficiently utilized the various carbon sources. Sucrose was utilized by all the isolates (except No. 16). Tolerance of the strains to NaCl concentration also serves as an important character for identification. Isolate No. 2, 14 and 16 could tolerate7% NaCl concentration while only isolate no. 14 could tolerate 10% NaCl concentration. Hence the isolates 2, 4 and 16 could be placed in intermediate salt tolerance group as suggested by Tresner et al (1968). Isolate no. 2, 5, 7, 9, 13 and 14 were able to tolerate high temperature of 45^{o}C.

Shirling and Gottlieb (1966) stated that the actinomycetes strains tested for resistance to different antibiotics could be useful as a taxonomic aid. Isolate no. 3, 14 and 16 were resistant to all the antibiotic used while isolate no. 10 was isolated by all of them, Erythromycin being the most effective.

The sixteen actinomycetal isolates were screened for their antibacterial activity against five species of two gram-positive and three gram-negative pathogenic genera and for their antifungal activity against a mold and a yeast as summerised in Table 5 and the Figures 1,2,3,and 4 below.

Table 5: Antimicrobial activity shown by the isolates of Actinomycetes (mm)

Isolate No.	*Pseudomonas sp*	*Serratia sp*	*Sheigella sp*	*S. aureus*	*B. subtilis*	*Candida albicans*	*Aspergillus niger*
1	–	–	–	–	–	–	–
2	34	17	18	–	–	–	–
3	–	–	–	–	–	15	–
4	–	–	–	–	–	–	16
5	–	–	–	–	–	–	–
6	–	–	–	–	–	–	–
7	–	–	–	–	–	–	–
8	–	–	–	–	–	–	–
9	–	–	–	15	17	–	–
10	–	–	–	–	–	–	–
11	–	–	–	–	–	23	–
12	24	–	–	–	–	–	–
13	–	19	–	–	–	–	–
14	47	15	18	–	28	–	18
15	–	–	–	–	–	–	–
16	–	16	–	–	10	–	–

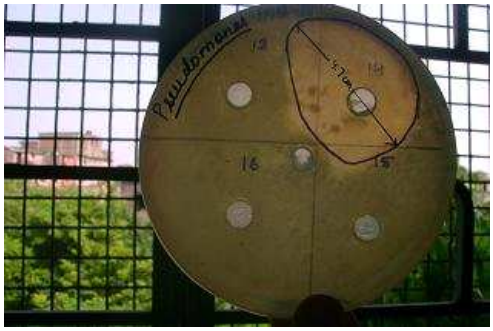

Fig 1. Zone of inhibition on *Pseudomonas sp.*

Fig 2. Zone of inhibition on *S.marcescens*

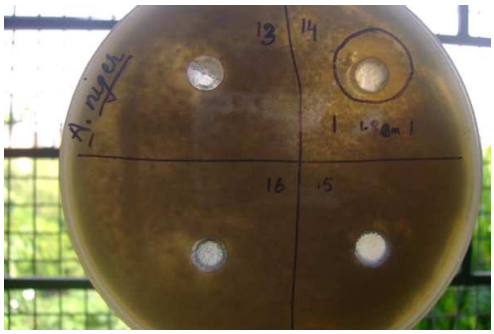

Fig 3. Zone of inhibition on *A. niger*

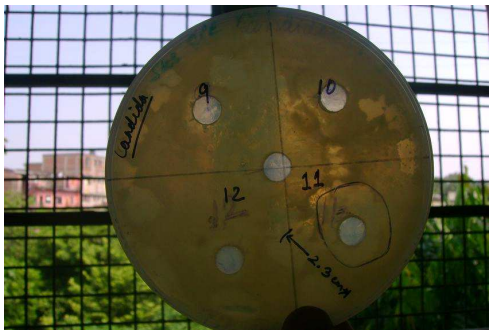

Fig 4. Zone of inhibition on *C. albicans*

Out of 16 of the investigated strains only five possess antibacterial activity against the test organisms. Three strains showed high inhibition potential against *Pseudomonas sp.* with 34, 24 and 47 mm sterile zone. The isolate no. 14 has shown the highest inhibition zone diameter of about 47mm against *Pseudomonas sp* among all the bacterial test organisms. It should be pointed out that only one of the studied strains (No. 9) suppressed the growth of *Staphylococcus aureus* and two strains were effective against *Shigella sp.* but the zone diameter was 25 mm. The lowest activity was exhibited against Gram-positive bacterium *S. aureus* (6%). The highest activity was shown against *Serratia sp*. (25%) as 4 isolates (No. 2, 13, 15 and 16) showed inhibition potential. Three isolates exhibit activity against *Pseudomonas sp*. and *Bacillus subtilis.* There was a very clear and distinct demarcation of activity against Gram positive and negative bacteria. Isolate No. 2, 13 and 15 exhibited activity against the three Gram negative strains i.e., *Pseudomonas sp, Shigella sp and Serratia sp*, and isolate no. 9 only against Gram positive bacterium (both *S. aureus* and *B. subtilis*). However, isolate no. 14 and 16 had showed activity against both Gram positive and Gram negative bacteria thus exhibiting a wide spectrum of antibacterial activity. The crude extracts collected from the culture filtrates of the 4 strains exhibited good antifungal activity, two (3,11) on *Candida albicans* and two (4,14) against *Aspergillus niger*, however none formed good zone of inhibition. Antifungal metabolites have been reported from the genus *Streptomyces* and a few from *Nocardia* (Kavitha et al 2010). Isolate no. 14 showed maximum inhibition potential as it is active against both Gram positive as well as Gram negative bacteria .*(B. subtilis and Pseudomonas sp, Shigella sp)* and also against mold (*A. niger*).

The search for novel metabolites especially from actinomycetes requires a large number of isolates (over thousands) in order to discover a novel compound of pharmaceutical interest. The search can be more promising if diverse actinomycetes are sampled and screened. This is based on the hypothesis that actinomycetes diversity may be influenced by the diversity of cultivated plant species as these bacteria grow profusely in the humus and leaf litter layer. Furthermore, different plants produce different type of secondary metabolites and some of these chemical compounds are toxic to soil microorganisms including actinomycetes. However, adaptation has in turn led the actinomycetes to produce their own secondary metabolites. Although the collection sites have mainly been limited to garden and field area, yet they possess many actinomycetes in the humus layer. The fact that actinomycetes number was lower on the soil surface than 11-15 cm deep down into the soil may be attributed to the favourable combination of suitable pH and water content.

A total of 16 different *Streptomyces* isolates were recovered from 3 soil samples studied. Initial morphological characterization using light microscope and biochemical tests showed that all the isolates belong to the genus *Streptomyces* spp. *Streptomyces* are Gram positive filamentous bacteria which belong to the order Actinomycetales (Madigan and Martinko 2005). Their antibiotic susceptibility results exhibited varied results with the isolate no. 6 and 10 showing sensitivity to four out of five antibiotics used and strain 3, 14 and 16 have showed resistance against all the antibiotics. Strain 4, 5, 9 and 13 showed sensitivity to three antibiotics. The highest zone of inhibition was showed by erythromycin against strain 6. Antibacterial activity was exhibited by 31% of the isolates. In this work, we have shown that a total of 16 different Streptomyces isolates associated with soil have the ability to produce antimicrobial compounds against microorganisms, especially multiple antibiotic resistant Gram positive and Gram negative bacteria.

The reason for different sensitivity between gram positive and gram negative bacteria could be ascribed to the morphological differences between these microorganisms, gram negative bacteria having an outer polysaccharide membrane carrying the structural lipopolysaccharide components. This makes the cell wall impermeable to lipophilic solutes, The gram positive should more susceptible having only an outer peptidoglycan layer which is not an effective permeability barrier (Scherrer and Gerhardt 1971). In contrast to the findings of various researchers where they observed that antagonistic reaction against the Gram positive bacteria were much higher than the Gram negative (Balagurunathan et al 1996; Basilio et al 2003; Oskay et al 2004; Sacramento et al 2004), in this research we found that the isolated actinomycetes were more inhibitory towards gram negative bacteria in comparison to gram positive bacteria and fungus tested. These strains produced either a broad-spectrum antimicrobial compound or several compounds with different activities. Further investigations are needed in order to determine whether the metabolites responsible for the antimicrobial activity are active substances or not. The isolates showed persistent antimicrobial activity and are likely to be potential candidates for discovery of novel secondary metabolites for bio-control and biotechnological application.

Conclusion :

The present work has resulted in selective isolation of 16 Streptomyces spp from soil of Patna region, exhibiting antibacterial and antifungal activity against some pathogenic bacteria and fungi on the basis of initial morphological and physiological characterization. Further investigations are needed for identification upto species level. The study shows that the isolated actinomycetes have potential as sources of new antibacterial compounds and may opens up a vista for further research opportunities towards characterizing the active compound(s) in the antibiotic metabolites. However, in depth analysis is required to produce more potent bioactive antifungal compounds from actinomycetes commonly available in the soil.

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