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Study of antifungal potential of some actinomycetes isolated from soil

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Abstract : *The present investigation was aimed to study the antifungal activities of actinomycetes, isolated locally from soil samples collected from five different regions of Patna. On the basis of powdery texture of the colonies having musty smell and gram positive filamentous micro morphology, twenty isolates presumed to be actinomycetes strains were selected. Among these, five isolates, possessing antifungal potential and designated as 2,3,5,11, and 16; were selected on the basis of primary screening by cross plug method against Penicillium sp., Aspergillus niger, Curvularia sp., Coccidiodes sp., Aspergillus flavus, and Candida albicans obtained from the Department of Industrial Microbiology of Patna Women's College. The antifungal activities of the selected actinomycetes strains were assayed by the Agar well diffusion method and the zone of inhibition were measured in mm. The results revealed that the zone of inhibition (in diameter) was maximum by strain 2 against Aspergillus niger (26 mm), followed by strain 3 against Coccidioides sp. (24 mm), strain 5 against Curvularia sp. (23 mm), strain 11 against Curvularia sp. (18 mm) and strain 16 against Penicillium sp. (21 mm), respectively.*

Key words: *actinomycetes strains, antifungal activity, fungal strains.*

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Introduction :

Actinomycetes are gram positive filamentous bacteria and are originally considered as an intermediate group between bacteria and fungi. The group comprises a ubiquitous order of bacteria which exhibit wide physiological and morphological diversity. Actinomycetes may be found in rhizosphere region of medicinal plant and are known to have ability to produce new and novel inhibitory compound (Khamna *et al.*, 2009). Actinomycetes inhabiting soil particularly *Streptomyces* sp. enhances soil fertility and has antagonistic activity against wide range of soil borne plant pathogens (Aghighi *et al.,* 2004; Anitha and Rebeeth, 2009). The antagonistic activity of *Streptomyces* sp. to fungal pathogen is usually related to the production of antifungal compounds (Taechowisan *et al.,* 2005). Antagonistic microorganisms usually compete with the pathogen for nutrients, inhibit pathogen multiplication by secreting antibiotics or toxins or reduce pathogen population through hyper parasitism (Svetlana *et al.,* 2010). Approximately 70% of antibiotics are known to be obtained from actinomycetes, in which 75% are known to be used in medicine and 60% in agriculture (Miyadoh, Tanaka and Mura, 1993). Attention has been paid as actinomycetes can protect root by inhibiting the development of potential fungal pathogen by producing antifungal compound (Goodfellow and William, 1983). Certain *Streptomyces* strains are known to have strong antifungal activity against *A.niger* and *C.albicans* (Laidi *et al.*, 2007; Oskay, 2009). Conventional practice to reduce the population of *A.niger* and *C.albicans* has been the use of chemical fungicides, which have adverse environmental effect causing health hazards to human and other non targeted organisms, including beneficial life forms (Dahiya *et al* ., 2006). Recent reports show that this group of microorganism is still an important source of antibiotics (Watve *et al.*, 2001) and continues to be screened for new bioactive compounds (Hayakawa et al., 1996; Sajid *et al.,* 2008). During present study antifungal activity of isolated strains has been described.

Materials and Methods:

Media used :

For isolation and preservation of actinomycetes: Starch Casein Agar (Soluble starch 10g, Casein 0.3g, KNO_3 2.0g, NaCl 2.0g, K_2HPO_4 2.0g, $MgSO_4.7H_2O$ 0.05g, $CaCO_3$ 0.02g, $FeSO_4.7H_2O$ 0.01g, Agar 15g, Distilled water 1 litre, pH 7.3)

For preservation of fungi (obtained from the Department of Industrial Microbiology of Patna Women's College): Potato Dextrose Agar (Peeled potato 200g, Dextrose 20g, Agar 15g, Distilled water 1 litre, pH 5.6)

For biochemical and physiological characterization: Methyl red and Voges-Proskaur broth (Peptone 7.0g, Potassium phosphate 5.0g, Dextrose 5.0g, Distilled water 1 litre, pH 6.9), Simmon's Citrate Agar (Ammonium dihydrogen phosphate 1.0g, Dipotassium hydrogen phosphate 1.0g, Sodium chloride 5.0g, Sodium citrate 2.0g, Magnesium sulphate 0.2g, Bromothymol blue 0.08g, Agar 15g, Distilled water 1 litre, pH 6.9) Nutrient Agar (Peptone 5.0g, Beef extract 3.0g, Sodium chloride 5.0g, Agar 15g, Distilled water 1 litre, pH 7.0), SIM (Sulfide Indole Motility) Agar (Peptone 30g, Beef extract 3.0g, Ferrous ammonium sulphate 0.2g, Sodium thiosulphate 0.025g, Agar 3.0g, Distilled water 1 litre, pH 7.3) and Trypticase soya Agar (Trypticase 15g, Phytone 5.0g, Sodium chloride 5.0g, Agar 15g, Distilled water 1 litre, pH 7.3).

For Screening of antagonistic activity: Czapek-Dox Agar medium (Sucrose 30g, Sodium nitrate 3.0g, Magnesium sulfate 0.5g, Potassium

chloride 0.5g, Iron (III)sulfate 0.01g, Dipotassium hydrogen phosphate 1.0g; Agar 13g Distilled water 1 litre, pH 6.8)

Collection of soil samples : The soil samples were collected from a depth of 3-4 inches below the upper layer of the soil, from five different locations of Patna that included the garden near pond of Patna women's college campus, agricultural field ICAR, rhizospheric region of medicinal plant of Anisabad locality, compost soil from ICAR campus and bank of Ganges near Danapur. The samples were air dried in the laboratory for 4 days to reduce the population of other bacterial cells. The physico-chemical characteristics of the soil samples regarding temperature, pH and moisture were recorded.

Isolation and preservation of actinomycetes strains : The soil samples were serially diluted in 0.85% normal saline and plated on Starch Casein Agar plates with five fold dilutions under aseptic condition (Aneja, 2003). Different colonies were obtained among which 20 colonies, considered as actinomycetes strains on the basis of powdery texture of the colonies, were selected for the present study and were preserved on slants of Starch Casein Agar at 4^{0}C with periodic sub culturing.

Screening of antifungal activities of the selected strains : The actinomycetes strains were screened for their antifungal activities against six fungal strains namely *Aspergillus flavus*, *Candida albicans, Penicillium* sp., *Aspergillus niger, Curvularia* sp., *Coccidioides* sp. These strains are being maintained in the Department of Industrial Microbiology of Patna Women's College. The cross plug method described by Crawford et al.(1993) was used for screening. Using sterile technique, a single line streak inoculation of each of the selected strains of actinomycetes was made on the surface of the Czapek-Dox medium plates in triplicates and incubated at 26^{o}C for 6 days. After incubation the cultures of six fungal strains were placed one on each side of the central strip of actinomycetes culture with the help of a sterilized cork borer, incubated at 26^{0}C for 48 hours and observed for zone of inhibition.

Bioassay of antifungal potential of screened actinomycetes strains : The antifungal activities of the isolated actinomycetes strains were further assayed by Agar well diffusion method (Ismet, 2004) and zone of inhibition formed was measured in millimeter. The fungal strains were plated on Czapek-Dox medium plates by spread plate technique and incubated at 26^{0}C for 48 hours. Wells of about 8mm diameter were made in the plates with the help of sterilized cork borer. Then the discs of freshly grown actinomycetes strains were cut with sterilized cork borer and were placed in the wells with help of sterile forcep and the plates were incubated at 26^{0}C for 48 hours. The zone of inhibition formed was measured to the nearest millimeter.

Characterization of the actinomycetes : The actinomycetes strains with potential antifungal activities were characterized on the basis of macro and micro morphology of the colonies. Macro-morphological characterization of the selected actinomycetes strains was done on the basis of aerial mass colour, substrate mycelium colour and diffusible pigment production. The micro-morphological characterization was done with the help of Gram's reaction and spore chain morphology. The biochemical behaviour of the strains that included Indole, Methyl red, Voges-Proskauer, Citrate utilization, Hydrogen sulphide production and Catalase tests were studied according to International *Streptomyces* Project (Shirling and Gottlieb, 1966).

Results:

Characteristics of soil samples: The temperature of the soil samples was recorded at the site of collection and the pH and the moisture of the samples were recorded in the laboratory.The data obtained has been shown in Table-1.

Table 1: Physico-chemical characteristics of soil Samples

Soil Sample	Temperature (^{0}C)	pH	Moisture (gm)
Near the pond (PWC)	25	5.8	0.875
Agriculture field (ICAR)	27	6.8	0.931
Rhizospheric region of medicinal plant (Anisabad)	24	6	0.997
Compost soil (ICAR)	29	6.5	1.231
Near Ganges (Danapur)	16	7	0.892

Isolation and preservation of actinomycetes strains: A total of 20 colonies of actinomycetes with the characteristic powdery texture and musty smell were obtained from five soil samples collected from different regions of Patna. The percentage of the isolates obtained from each of the soil samples has been shown in the pie chart (Fig.1).

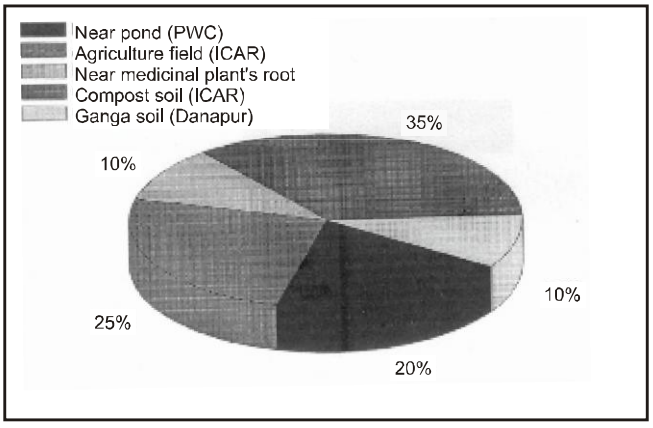
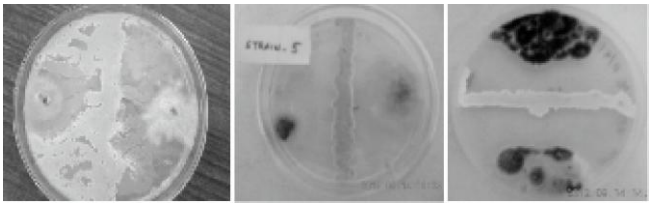


Fig. 1: Percentage of strains of actinomycetes isolated from different soil samples

Screening for antagonistic activity of actinomycetes isolates: All the 20 actinomycetes isolates were screened for their antagonistic activitities against selected fungal strains by cross plug method (Crawford et al., 1993). Among them, 5 most effective strains designated as 2, 3, 5, 11 and 16 were selected (Fig. 2). Two fungal strains *Candida albicans* and *Aspergillus flavus* against which the actinomycetes strains were least effective were eliminated from further testing.



(A) (B) (C)

Fig.2: (A) negative (B) partial (C) positive antagonistic activity of actinomycetes isolates.

Bioassay of antifungal potential of screened actinomycetes strains: The results of bioassay of antifungal potential of the selected actinomycetes strains carried out by Agar Well Diffusion method (Ismet, 2004) has been shown in Table 2. The growth of *Aspergillus niger* inhibited by all the screened actinomycetes strains has been shown in Fig. 3.

Table 2: Antifungal potential of actinomycetes isolates

FUNGUS	ACTINO-MYCETES	INHIBITON ZONE (diameter in mm)
Coccidioides sp.	2	17
	3	24
	5	15
	11	13
	16	16
Curvularia sp.	2	18
	3	20
	5	23
	11	18
	16	19
Penicillium sp.	2	20
	3	14
	5	13
	11	10
	16	21
Aspergillus niger	2	26
	3	15
	5	15
	11	12
	16	19

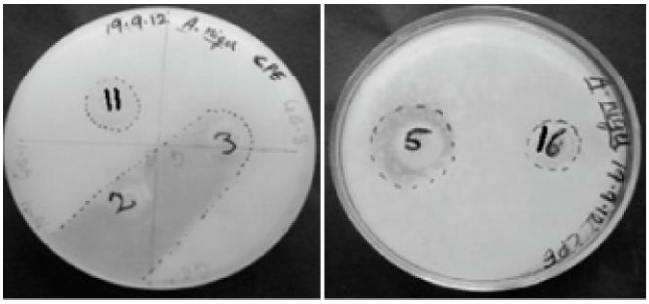


Fig. 3: Bioassay of antifungal potential of screened actinomycetes strain no. 2, 3, 11, 5 and 16 against *Aspergillus niger*

Characterization of the actinomycetes: The results of macro and micro morphology of the isolated strains are listed in Table 3.

Table 3: Morphological characterization of the isolated strains

Strain No.	Aerial mass colour	Substrate mycelium colour	Diffusible pigment production	Gram Reaction	Spore Chain Morph-ology
2	Dark Grey	Brownish Grey	Red	Positive	Flexible
3	Cream	Off white	–	Positive	Spira
5	Grey	Brown	–	Positive	Rectus
11	Grey	Green	–	Positive	Flexible
16	Grayish white	Grayish White	–	Positive	Rectus

The results of biochemical tests of selected strains that included Hydrogen sulphide production test, Indole production test, Methyl-red test, Voges-Proskauer test, Citrate Utilization test, Hydrogen sulphide production and Catalase test are shown in Table 4.

Table 4: Biochemical characterization of the selected strains

Biochemical Test	Strain 2	Strain 3	Strain 5	Strain 11	Strain 16
Hydrogen Sulphide Production	–	+	+	–	–
Indole Production	+	–	+	–	–
Methyl-red	–	+	+	–	–
Voges-Proskauer	–	–	–	–	–
Citrate Utilization	+	–	–	+	–
Catalase Test	+	+	+	+	+

Discussion:

A total of 20 different actinomycetes isolates were obtained from 5 soil samples collected from different regions of Patna. On the basis of morphological (Table 3) and biochemical characterization (Table 4) all the 20 isolates were found to be Gram positive filamentous bacteria which seems to belong to the order Actinomycetales. For species Identification, besides morphological and physiological properties, various other biochemical properties such as cell wall chemo type, whole cell sugar pattern, peptidoglycan type, phospholipids type and G+C% of DNA should be determined. However, further confirmation by 16S rRNA analysis and other molecular characterization is still needed.

The screening of antagonistic activitities of the isolated actinomycetes strains showed that strain no. 2, 3, 5, 11 and 16 were effective against all the test fungi. Among them, three strains namely 2, 3 and 5 showed high inhibition potential against *Aspergillus niger, Coccidiodes* sp. and *Curvularia* sp., with 26mm, 23mm and 24 mm of sterile zone, respectively. The strain no.2 has shown the highest zone of inhibition of diameter measuring 26mm against *Aspergillus niger*. The lowest activity was exhibited against *Pencillium* sp. by strain no. 11 (Table 2). Similar results have been found by Gebreel et al., (2008) and Kavitha et al., (2010). Khamna (2009) also reported that the crude extract of antifungal compounds was active against *R. stolonifer*, *A. flavus*, *F. oxysporum* and *Alternaria*. Lim et al. (2000) selected 32 Actinomycetes isolates, which showed the inhibitory activity against mycelial growth of plant pathogenic fungi like *Alternaria mali*, *Colletotrichum gloeosporides*, *F.oxysporum*, *cucumerinum*, *Magnaporthe grisea*, *Phytophthora capsici,* and *Rhizoctonia solani*. Although exact mechanisms by which actinomycetes operate to reduce disease incidence is not elucidated, one possibility is that

this strain exerted a direct inhibitory effect on hyphal growth and structure of fungal pathogens (Loqman et al., 2009). Streptomycetes have the ability to produce iron chelating compounds and siderophores that starve pathogens for iron (Tokala et al., 2002).

Further investigations are needed in order to determine whether the metabolites obtained from the actinomycetes strains in the present study are commercially usable or not.

Conclusion:

The antifungal potential of actinomycetes strains against some pathogenic fungi was previously reported from different locations. Studies on actinomycetes are very limited and have been mentioned incidentally on the microbial community of soil habitats.

The present study thus shows that the test actinomycetes are potential sources of antifungal compounds. On review of result described above it can be concluded that the soil samples of Patna are rich source of actinomycetes which produce metabolites inhibitory to fungi and may be helpful in new drug discovery.

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