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ORIGINAL ARTICLE

Investigation of the Antimicrobial Activity of Some Soil Actinomycetes

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ABSTRACT
Soil samples from various farming soils of Beed District of Maharashtra were screened as a source of actinomycetes with potent antimicrobial compounds. In all 08 soil samples 53 actinomycetes were isolated. In primary screening by cross streak method 26 actinomycetal isolates showed antibacterial activity against test organisms. To confirm antibacterial potential all 26 actinomycetal isolates were subjected for secondary screening by agar well method. Out of 26 actinomycetes only 05 actinomycetal isolates showing maximum zone of inhibition against both Gram positive and Gram negative organisms. Finally one potent antibacterial actinomycetal isolate was selected for detailed characterization and was identified as belonging to genus Streptomyces. This isolate was subjected to studies to establish the effect of carbon and nitrogen sources on antibiotic production. The study established that maltose and potassium nitrate were the most suitable carbon and nitrogen sources for antibiotic production.
***Key Words**: Actinomycetes, Soil, Antibacterial, Streptomyces*

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INTRODUCTION

Actinomycetes comprise an extensive and diverse group of Gram positive, aerobic, mycelial bacteria with high G+C nucleotide content (> 55 %) and play an important ecological role in the soil cycle. The name of the group actinomycetes is derived from the first described anaerobic species *Actinomycetes bovis* that causes 'actinomycosis" the "ray-fungus disease" of cattle. They were originally considered to be intermediate group between bacteria and fungi but are now recognized as prokaryotic microorganisms. [1]

The majority of actinomycetes are free living, saprophytic bacteria found widely distributed in soil, water and colonizing plants.Actinomycets population has been identified as one of the major group of soil population. Actinomycetes have ability to form wide variety of secondary metabolites including antibiotic. Actinomycetes are economically and biotechnologically most valuable prokaryotes. They are responsible for the production of about half of the discovered bioactive secondary metabolites [2]. Rare and novel actinomycetal taxa have become a major focus in the search for pharmaceutical agents [3]. About 61% of all bioactive microbial metabolites isolated from actinomycetes especially from *Streptomyces.*[4] Moreover, the majority of antibiotics in use today were discovered in the 1950.1000 antibiotics are known today and most of them are produced by actinomycetes especially the genus *Streptomyces.* [5]

The actinomycetes have occupied a prominent position in the pharmaceutical industry for their seemingly unlimited capacity to produce secondary metabolites including antibiotics with divers chemical structure and biological activities. [6] *Streptomyces* is the largest antibiotic producing genus in the microbial world discovered so far. The number of antibiotic compounds reported from the species of the genus per year increased almost exponentially for about two decades. A recent report shows that this group of microorganisms still remains an important source of antibiotics [7].

The present investigation deals with isolation of actinomycetes from farm soil samples of Beed district of Maharashtra, evaluation of their antibacterial activity against test microorganisms and identification of promising isolate and selection of suitable carbon and nitrogen source for antibiotic production.

MATERIALS AND METHODS

The farm soil samples were collected in freshly purchased polythene bags (swabbed with cotton dipped in 70% alcohol and solarised) were brought to the laboratories preventing any contamination on the way. They were stored at temperature between 6^0C to 10^0C until further use.

Isolation of actinomycetes

Enrichment: Dilutions of soil samples in sterile water (1/10 w/v) were made. A temperature shock 70^0C for five minutes were given to each diluted soil samples and 5ml of soil sample were inoculated in 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 medium was supplemented with antifungal antibiotic griseofulvin at 50 µg/ml concentration.[8] The temperature shock depress associated gram negative bacteria and added antibiotic in medium kill fungi which create problem during isolation. The flasks were incubated at 30^0C for 10 days. [9]

Isolation: Actinomycetes from enrichment medium were isolated by streak plate method using starch nitrate agar (Starch-20.0g, KNO3-1.0g, K_2HPO_4-0.5g, $MgSO_4.7H_2O$-0.5g, Nacl-0.5g, $FeSO_4.7H_2O$-0.01g,Agar-20.0g,Distilled water-1L, pH 7.2). The medium was supplemented with antifungal antibiotic griseofulvin at 50µg/ml concentration. Then these plates were incubated at 30^0C for 10 days.

Study of characteristics of isolates: After incubation dry, leathery colonies of actinomycetes were studied. Color of aerial mycelium, color of vegetative mycelium was studied using "color and Streptomyces 'and ISCC-NBS color charts. [10, 11] The cover slip cultures of actinomycetal isolates were prepared and morphological characters were studied. [12] Pure colonies were subculture on to the respective media slants and were stored at 4^0C for further study.

Primary screening of actinomycetes for antimicrobial activity: Antagonistic activity of 53 isolates were tested by using the cross streak method .[13, 14, 15, 16] The test organisms used were *Bacillus subtilis* NCIM 2195, *Staphylococcus aureus* NCIM 2602, *Proteus vulgaris* NCIM2027, *Eschrishia coli* NCIM2685, *Pseudomonas aeruginosa* NCIM 2945.Those actinomycetes which showed prominent activity were selected for secondary screening.

Secondary screening: In primary screening those isolates showed prominent antibacterial activity were used for secondary screening. Sterile 5 ml of starch nitrate broth were taken in 25ml of conical flasks. 2.5% inoculums of actinomycetes were added in the broth aseptically. The flasks were incubated on shaker 30^0C for 10 days. After incubation supernatant was obtained by aseptic centrifugation 4000 rpm for 20 minutes, a part of which was used for testing antimicrobial activity by agar well method against test organisms.[17,18] One actinomycetal isolate showing highest antibacterial activity was selected on the basis of zone of inhibition against test organisms and was subjected to characterization following standard procedures of Shirling and Gottlieb1966 ; Holt1974.[19,20] The morphological, cultural and biochemical characters were studied . Morphology of actinomycetal isolate was further studied by scanning electron microscopy (SEM). [21, 22] Cultural characters were studied on ISP4 medium. Utilization of different sugars and biochemical test were performed according to Bergey's Manual of Determinative Bacteriology.

Effect of carbon and nitrogen source on antibiotic production: To study the effect of carbon source on antibiotic production starch, glucose, glycerol, sucrose, maltose, xylose, lactose and arabinose sugars were used. The carbon utilization medium Shirling and Gottlieb was prepared10gl^{-1} concentration of carbon source ($(NH4)_2SO_4$ -2.64g , KH_2PO_4 - 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).100 ml of each sugar medium was taken in 250ml capacity Erlenmeyer flask. The broth was sterilize and inoculated with 2.5% inoculums of actinomycetes. All flasks were incubated on rotary shaker 30^0C for 10 days. After incubation supernatant was obtained by aseptic centrifugation 4000 rpm for 20 minutes, a part of which was used for testing antimicrobial activity by agar well method against test organisms (*P. vulgaris S aureus, P. aeruginosa*). Antibiotic production was checked by measuring zone of inhibition against test organisms.

To study the effect of single nitrogen source on antibiotic production different nitrogen sources viz. Ammonium sulphates, ammonium chloride, KNO3, NaNO3, aspargine, casein, soybean meal, yeast extract were used. The above mentioned experiment was repeated except that1% maltose was used as carbon source and 2% of nitrogen source was used.

RESULTS AND DISCUSSION

Initially 53 actinomycetes were isolated from 8 farming soil samples. Out of these 26 isolates showed prominent antibacterial activity against test organisms in primary screening by using cross streak method (plate-1). To confirm antimicrobial activity of 26 isolates giving inhibition of test organisms in primary screening were again subjected to secondary screening by agar well method (plate-2). It was observed that 05 actinomycetal isolates (BD3, BD5,BD8 ,BD11.BD17) were showing prominent antimicrobial activity in both primary and secondary screening methods against Gram positive and Gram negative organisms (Table-1).Finally one potent actinomycetal isolate BD8 was selected for further study on the basis of maximum zone of inhibition .

The morphological, cultural, physiological and biochemical properties of the selected potent actinomycetal isolate BD8 was studied. 4-8 mm diameter, circular, rough, convex, opaque, leathery, white colonies of actinomycetal isolate BD8 were observed on ISP4 medium (Table-2, Plate-3). It was found that vegetative and aerial mycelium was present in actinomycetal isolate and long and spiral chains of spores on aerial mycelium were observed in cover slip culture and SEM images. Spores were circular and having smooth surface (Plate-4). Actinomycetal isolate BD8 was confirmed as *Streptomyces* species on the basis of morphology by SEM analysis. Many researchers used SEM for identification of actinomycetes.

The ability of actinomycetal isolate BD8 to utilize different sugars was tested .It was able to utilize D-glucose, maltose, xylose, rhamnose, arabinose, sucrose, mannitol, lactose. Actinomycetal isolate BD8 was able to produce catalase, oxidase, gelatinase, caseinase, cellulase lecithinase and amylase enzymes .Methyl red and H_2S production positive (Table- 3). Thus it was found that actinomycetal isolate BD8 is biochemically versatile and it has good potential in biodegradation of varieties of organic compounds in soil. On the basis of morphological, cultural, physiological and biochemical characters the actinomycetal isolate BD8 was identified as species belonging to the gnus *Streptomyces spp.*

Li Huo *et al*. [23] studied 4200 soil samples from Yunnan. 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 Yunan. Oskay *et al*.[16] isolated 80 different actinomycetes strain from farming soil samples and studied antibacterial activity against phytopathogenic bacteria *Agrobacterium tumefaciens, Erwnia amylovora* and *Psedomonas viridiflora.*

Effect of carbon source on antibiotic production in a *Streptomyces* BD8 was studied it was observed that when medium containing maltose as carbon source, *Streptomyces* BD8 was giving maximum zone of inhibition i.e. 22, 25, 20 mm against, *P. vulgaris, S. aureus, P . aeruginosa* respectively .Thus maltose was found best carbon source by *Streptomyces* BD8 as compare to starch, glucose, glycerol, sucrose, xylose, lactose and arabinose (Fig-1).

Table -1 Antimicrobial activity of potent soil actinomycetes against test organisms

		Zone of inhibition (mm)against test organisms									
		Primary Screening					Secondary Screening				
Sr.No	actinomycetes isolates	*Bs*	*Sa*	*Pv*	*Ec*	*pa*	*Bs*	*Sa*	*Pv*	*Ec*	*pa*
1	BD3	17	18	14	13	17	18	19	15	14	18
2	BD5	16	16	16	13	16	15	18	17	16	14
3	BD8	26	22	20	20	24	28	24	22	21	24
4	BD11	18	17	19	15	21	18	18	19	15	21
5	BD17	17	18	14	14	19	18	19	15	14	19

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

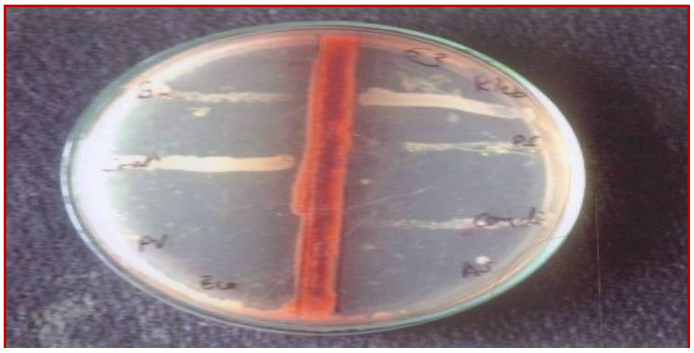


Plate-1 Antibacterial activity of actinomycetal isolate against test organisms (cross streak method)

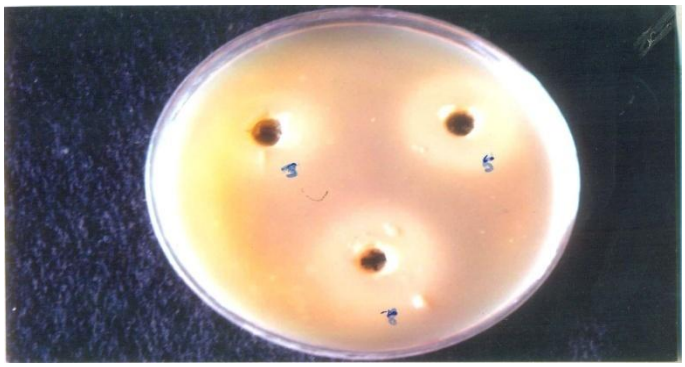


Plate -2 Antibacterial activity of actinomycetal isolate against *P. aeruginosa (*agar well method)

Table -2. Cultural Characteristics of actinomycetes isolate *Streptomyces* BD8

S.N	Cultural characters	*Streptomyces* BD8
1	Colony morphology	4-8 mm.diameter,circular,rough, convex, opaque, velvety, white to light brown
2	Aerial mycelium color	White
3	Vegetative mycelium color	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

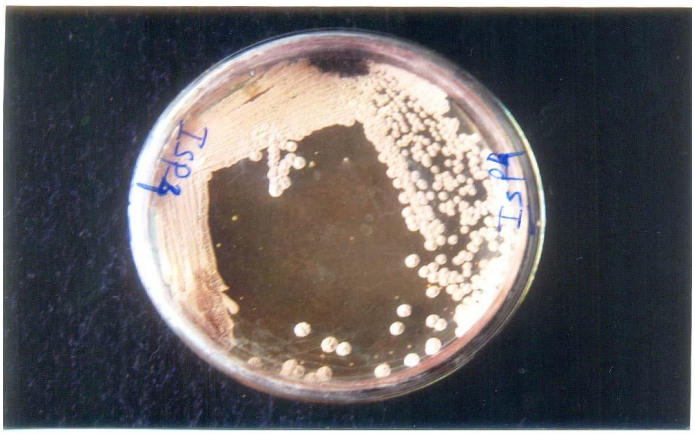


Plate -3Colony morphology of *Streptomyces* BD8 on ISP4 medium

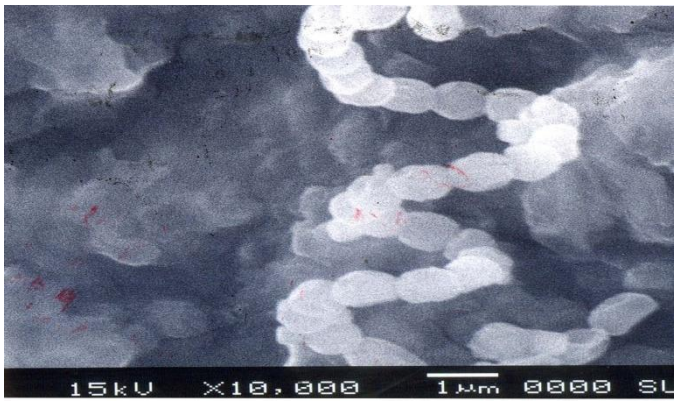


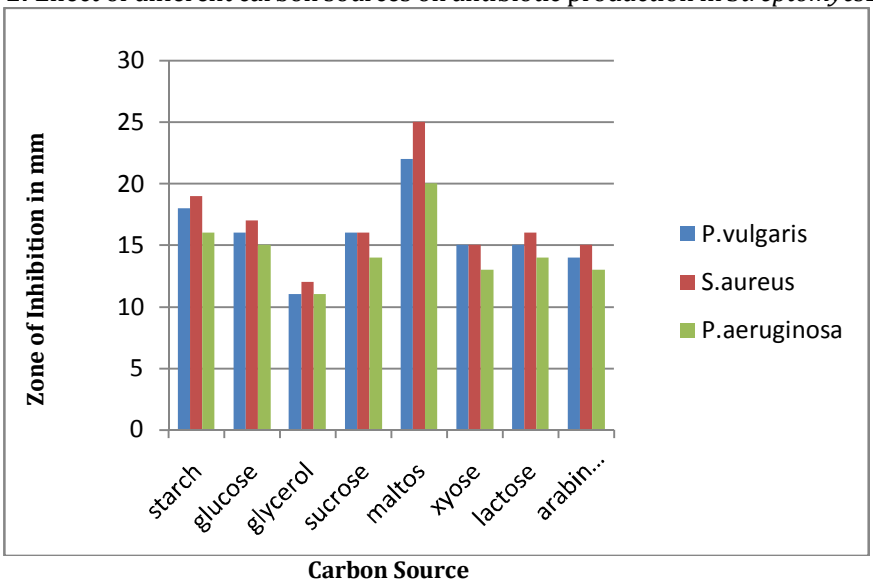
Plate -4 Spore chain morphology of *Streptomyces* BD8 (SEM)

Table 3. Physiological and Biochemical Characters of actinomycetal isolate *Streptomysces- BD8*

Biochemical Tests			Utilization of Sugars		
Sr .No.		**Results**	Sr. No.	Sugar	Result
1	Indol	**-ve**	**1**	Maltose	**+++**
2	Methyl Red	**+ve**	**2**	Xylose	**++**
3	V P	**--ve**	**3**	Glucose	**+++**
4	Citrate	**--ve**	**4**	Raffinose	**----**
5	NO_2 Reduction	**--ve**	**5**	Rhamnose	**+**
6	H_2S Production	**+ve**	**6**	Arabinose	**++**
7	Catalase	**+ve**	**7**	Sucrose	**++**
8	Oxidase	**+ve**	**8**	Mannitol	**++**
9	Gelatinase	**+ve**	**9**	Lactose	**++**
10	Caseinase	**+ve**			
11	Cellulase	**+ve**			
12	Lcithinase	**+v**			
13	Amylase	**+ve**			

+++ - Very good, ++ Moderate , + Poor , - No growth; + = Positive Test , - = Negative Test

Fig-1: Effect of different carbon sources on antibiotic production in *Streptomycs*BD8



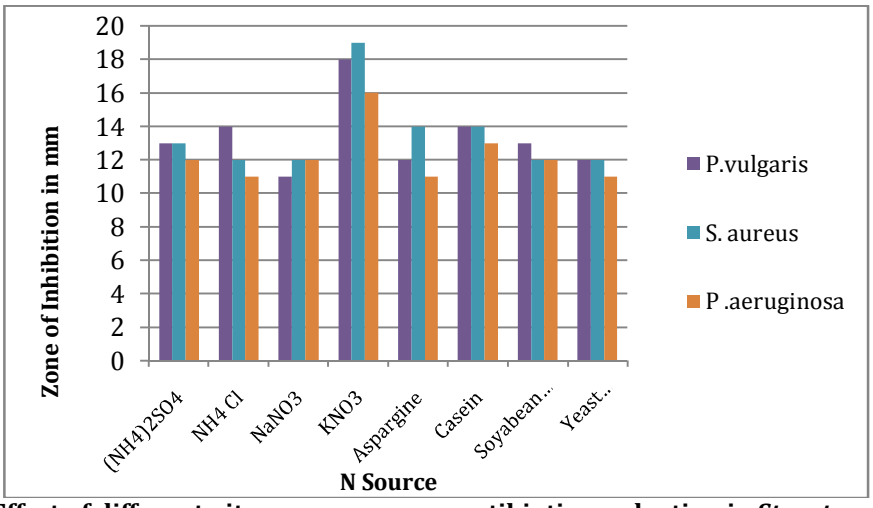


Fig-2: Effect of different nitrogen sources on antibiotic production in *Streptomycs*BD8

Narayana *et al.* [24] stated that *Streptomyces albiaqflavus* was giving maximum antibiotic production in presence of maltose as carbon source.

When inorganic carbon source KNO_3 was used as nitrogen source in medium the maximum zone of inhibition developed by antibiotic compound produced by *Streptomyces* BD8 i e 18, 19, 16 mm again *P. vulgaris, S. aureus, p. aeruginosa* respectively. Thus inorganic KNO_3 was found the best nitrogen source for antibiotic production in *Streptomyces* BD8 (Fig-2).

Bulchandani and Parvateesam [25] have reported that ammonium nitrate was most suitable for antibiotic production by *Streptomyces* . Aruna *et al*. studied optimum condition required for antibiotic production in *Streptomyces spp.* They reported that yeast extract and KNO_3 was best for antibiotic production [26]. Vorar laid Rabah *et al.* [27] found a novel actinomycetes strain designated RAF 10 isolated form Egyptian soil. It was active against Gram positive and Gram negative bacteria, yeast and filamentous fungi.

CONCLUSION

Based on the screening results, it has been shows that farming soil samples of Beed, Maharashtra possess antibiotic producing actinomycetes and may be tapped as one of the potential source of novel antibiotics.

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