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ISOLATION AND CHARACTERIZATION OF BIOACTIVE ACTINOMYCETES FROM SOIL IN AND AROUND NAGPUR

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ABSTRACT: Fifteen soil samples were collected from different locations near Nagpur i.e. from Nagpur, Bhandara and Chandrapur. Air drying and pre-treatment of soil samples with $CaCo_3$ (1%) were found to be most effective for isolation of actinomycetes. Five different selective media were used for the isolation of actinomycetes and the best growth was observed in yeast malt extract agar medium with 1 mg/ml cycloheximide as antifungal agent and by using double layer agar technique for isolation. Significant differences were found between the counts of actinomycetes on different media. Total 78 actinomycetes species were isolated. More number of actinomycetes were isolated from Nagpur region (32 x 10 cfu/g) as compared to Bhandara (28 x 10 cfu/g) and Chandrapur (18 x 10 cfu/g) regions. Out of 78 isolates 23 isolates showed positive results in primary screening. Maximum isolates showed activity against *B. subtilis, B. cereus, S. aureus and E. coli.* Few isolates showed activity against *P. vulgaris, P. aeruginosa, C. albicans and A. niger.* No activity was seen against *Penicillium sp.* From 23 isolates 20 isolates showed good results in secondary screening. Finally one isolate was selected for further identification based on its broad spectrum activity and larger zone of inhibition compared to others. Phenotypic identification and biochemical and physiological characteristics of the isolated species were performed at MTCC Chandigarh and was identified as *Streptomyces violaceorubidus* and the strain was designated as RC 1796. These investigations clearly indicate that Nagpur and nearby area is a potent source for the isolation of bioactive actinomycetes useful for production of antimicrobial substances.

INTRODUCTION: Actinomycetes are Gram positive bacteria which possess many important and interesting features [1]. They are widely distributed in soil, water and colonizing plants. They are one of the major groups of soil population which may vary with the soil type [2]. The isolation of Actinomycetes from mixed microflora present in soil is difficult because of their slow growth compared to other bacteria [1, 2, 3]. Actinomycetes possess extraordinary biosynthetic capability of producing antibiotics. They are responsible for the production of about half of the discovered bioactive secondary metabolites notably antibiotics [4, 5], antitumour agents [6] enzymes [7, 8] and immunosuppressive agents [9]. It is because of these unique biosynthetic properties that actinomycetes are of industrial interest for several decades. Hence efforts are done on successful isolation of novel actinomycetes from terrestrial sources for drug screening programs in the past few years.

Recently, it is seen that the rate of discovery of new compounds from terrestrial actinomycetes has decreased, whereas the rate of re-isolation of known compounds has increased [10]. Thus, it is important that the new group of actinomycetes from unexplored or underexploited habitats be pursued as source of novel antibiotics. The study is carried out to isolate the antibiotic producing actinomycetes from Nagpur region because the geographic variation in different parts of the world leads to variation in soil type which leads to variation in type of actinomycetes sp. isolated.

MATERIALS AND METHODS:

Soil sampling and processing: Fifteen soil samples were collected from different locations like agricultural field, lakes, rivers, canals etc. from Nagpur and nearby region i.e. from Nagpur, Bhandara and Chandrapur region. Every sample was a mixture of soil collected from 5 to 6 holes whose depth was around 10 to 30 cm. The surface layer of soil was removed and the central portion was collected in sterile plastic bags with the help of polyvinyl corer (previously sterilised with alcohol). Soil samples were air dried in laminar airflow (HICON) for 24 hrs. at room temperature and was stored at 4°C until processed [11].

Calcium Carbonate soil treatment: All the soil samples were pre-treated with $CaCo_3$ to reduce the number of vegetative bacterial cells, while allowing many actinomycetes spores cells to survive [12]. Air dried soil sample (10 g) was taken in a mortar, was triturated with 1% $CaCo_3$ and was incubated at 30°C for 2 days in a closed sterile petridish. A high relative humidity was maintained in the petridish by water saturated filter paper [13]. To study the effect of $CaCo_3$ treatment soil samples without $CaCo_3$ were used as control [11].

Isolation of Actinomycetes: One gram of each air dried soil sample and the sample containing $CaCo_3$ were suspended in 100 ml of sterile distilled water and the flasks were rotary shaken at 150 rpm for 30 min. From this suspension dilutions were prepared as 1:10, 1:100, 1:1000 and 1:10000 in distilled water [14]. Each diluted suspension was placed on different types of media by spread plate technique, double layer agar technique [15] and by overlaying on the medium 0.22 to 0.45 µm pore cellulose ester membrane filter and inoculating the sample on the filter for isolation [3]. The different media used for isolation were starch casein agar (SCA), yeast malt extract agar (YMEA), nutrient agar (NA), maltose yeast extract agar (MYE), and kenknight and munaier's (KM) medium [16]. 1 ml of antifungal antibiotic cycloheximide prepared in distilled water (1mg/ml) and sterilized by autoclaving for 15 min at 121°C was mixed in each media. The plates were incubated at 28°C for 6 to 7 days. Typical actinomycetes colonies (rough chalky) were selected and further isolated by streak plate technique. Single isolated colonies were transferred on slants and stored at 4°C until further use [15].

Screening of actinomycetes for antibacterial activity: Two steps were involved in screening: primary screening and secondary screening.

Primary screening: The antibacterial activities of the isolates were determined on YMEA media by perpendicular streak method [17]. Isolated actinomycetes were cross streaked as a single line on solidified YMEA media in a petridish and were incubated at 28°C for seven days. The test organisms were then cross streaked perpendicular to the original streak of isolates as shown in **Figure 1**. The different test microorganisms used were *S aureus* (NCIM 2079), *B. subtilis* (NCIM 2063), *P. areuginosa* (NCIM 2200), *E. coli* (NCIM 2065), *P. vulgaris* (NCIM 2813), *C. albicans* (NCIM 3471), *A. niger* (NCIM 512) *and Penicillium sp.* (NCIM 1108) which were procured from National Collection of industrial microorganism (NCIM) at National Chemical Laboratory, Pune. The plates were incubated at 37°C for 24 to 48 hrs. for bacteria and at 25°C for 48 to 72 hrs. for fungi. The zone of inhibition was measured for each microorganism. Control plates of the same medium without actinomycetes growth were also simultaneously streaked with test organism to study their normal growth.

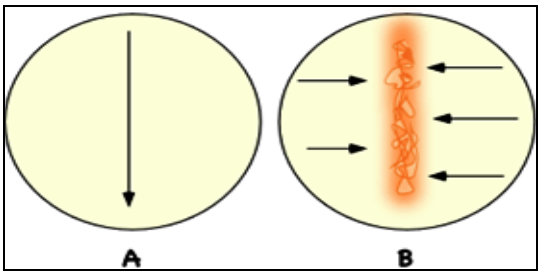

FIGURE1: STREAKING PATTERN FOR SCREENING BY PERPENDICULAR STREAK METHOD (A) THE PLACEMENT OF THE ACTINOMYCETES COLONIES (B) THE PLACEMENT OF THE TEST CULTURES [18].

Secondary screening: The isolates showing positive results in primary screening were subjected to the secondary screening procedures. Promising cultures were inoculated in test tubes containing 10 ml of sterile media such as yeast malt extract broth, nutrient broth, starch casein broth, and maltose yeast extract broth by submerged fermentation technique. The inoculated test tubes were kept for incubation on rotary incubator shaker (Biotechnich India) at 28°C and 150 rpm for 7 days. The fermentation broth was centrifuged (Remi, RM12C, India) at 10,000 rpm and 4°C. The supernatant was analysed for antimicrobial activity by agar diffusion method using the same test microorganisms used in primary screening [19].

Characterization of actinomycetes: The most potent actinomycete identified from secondary screening was characterized by morphological and biochemical methods [20]. Spore chain structure was studied by scanning electron microscopy (SEM, JSM 6360 Jeol, Japan).

RESULTS AND DISCUSSION:

Effect of calcium carbonate soil treatment on isolation of actinomycetes: Collected soil samples were analysed for presence of actinomycetes by $CaCo_3$ treatment procedure. The total number of actinomycetes colonies observed on five different media in each $CaCo_3$ treated soil samples were counted and total was represented as average number of culturable actinomycetes colonies which is demonstrated in **Table 1**. The same was done with the control samples (without $CaCo_3$ treatment). **Figure 2** summarizes that in the control samples comparatively lower numbers of actinomycetes were observed as compared to the $CaCo_3$ treated sample as shown in. This proves that pre-treatment of soil samples with $CaCo_3$ (1%) increased the number of actinomycetes observed on the isolation plates.

TABLE 1: AVERAGE OF CULTURABLE ACTINO-MYCETES COUNT FROM ALL $CaCo_3$ TREATED AND WITHOUT $CaCo_3$ TREATED SOIL SAMPLES ON DIFFERENT TYPES OF MEDIA

Type of Media	Average of culturable actinomycetes count from all soil samples ($x10^6$ cfu/g)	
	$CaCo_3$ treated sample	Without $CaCo_3$ treatment
SCA	4.1	2.8
YMEA	6.2	4.4
NA	1.8	0.7
MYE	3.2	1.9
KM	2.2	1.3

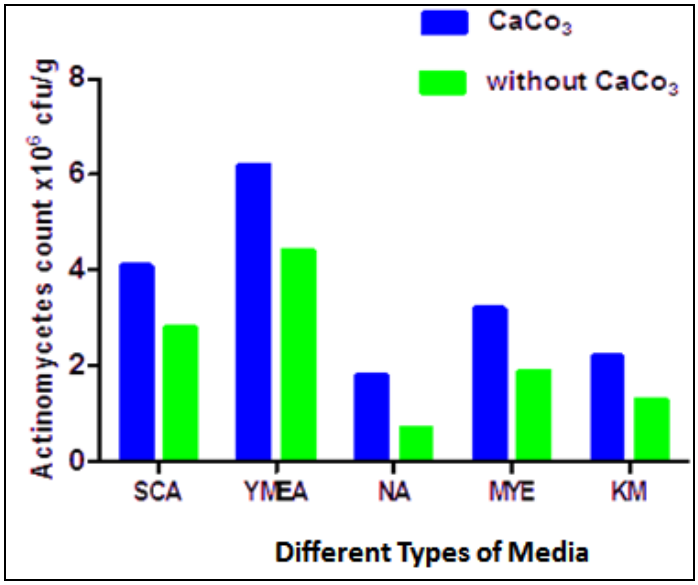

FIGURE 2: COMPARISON OF AVERAGE OF CULTURABLE ACTINOMYCETES COUNT FROM $CaCo_3$ TREATED AND WITHOUT $CaCo_3$ TREATED SOIL SAMPLES ON DIFFERENT TYPES OF MEDIA

Isolation of Actinomycetes on different media: The number of isolated colonies on each medium was counted and it was observed that amongst the five different media YMEA medium showed good growth and more number of actinomycetes compared to other media. YMEA was the best media for isolation of actinomycetes from soil. SCA was the second most effective media. MYE was the third, KM was the fourth and NA was the least effective media for isolation which is summarized in Table 1 and Figure 2. The growth of other bacterial and fungal contamination inhibited the growth of actinomycetes on isolation medium. The addition of cycloheximide (1mg/ml) in each medium reduced the fungal contamination, because of which isolation of actinomycetes was easy. **Figure 3** shows the isolation of the colony showing typical characteristic growth of actinomycetes (rough and chalky). Further, purification of single isolated colony was done by streak plate technique as shown in **Figure 4**.

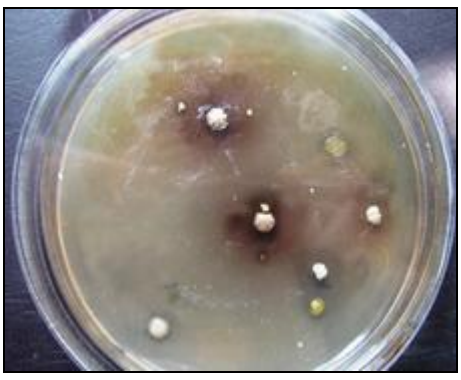

FIG. 3: ISOLATED COLONIES OF ACTINOMYCETES

FIG. 4: PURIFICATION OF ISOLATED COLONIES BY STREAK PLATE METHOD

Isolation of actinomycetes by various methods: The best method for isolation of actinomycetes was found to be double layer agar technique because only the thin top layer of the medium was inoculated with sample. Hence surface colonies predominated and identification and isolation of colonies was facilitated.

Antimicrobial activity of isolated actinomycetes: Total 78 actinomycetes were isolated. Primary screening was done for antibiotic producing activity by perpendicular streak method. The zone of inhibition near perpendicular streaking of actinomycetes indicated antibiotic producing activity of actinomycetes as depicted in **Figure 5**.

FIG. 5: PRIMARY SCREENING FOR ANTIBIOTIC PRODUCING ACTIVITY OF ISOLATED ACTINO-MYCETES BY PERPENDICULAR STREAK METHOD

Twenty three actinomycetes showed antibiotic producing activity in primary screening. These were subjected to secondary screening and were analysed by cup plate method. Twenty isolates showed antibiotic producing activity in secondary screening which is summarized in **Figure 6**.

From the results of primary and secondary screening we can say that more no. of actinomycetes were active against gram positive bacteria (*Bacillus subtilis, Bacillus cereus and Staphylococcus aureus)* than those against gram negative bacteria (*Escherichia coli, Proteus vulgaris and Pseudomonas aeruginosa).*

The reason behind this may be due to the presence of outer polysaccharide membrane carrying the structural lipopolysaccharide components in gram negative bacteria. This makes the cell wall impermeable to lipophilic solutes.

The gram positive bacteria are more susceptible because it carries only outer peptidoglycan layer which is not an effective permeability barrier[21]. Out of 20 isolates studied for secondary screening 100% isolates were showing positive results against *B. subtilis* and *B. cereus*. 75% isolates were active against *S. aureus*, 55% against *E. coli*, 40% against *P. vulgaris*, 35% against *C. albicans*, 25% against *A. niger*, 5% against *P. aeruginosa* and none against *Penicillium sp*.

From these isolates, actinomycetes sp. A2 was having the best antibacterial activity showing maximum zone of inhibition against most of the micro-organisms as shown in **Table 2** and hence it was further selected for identification.

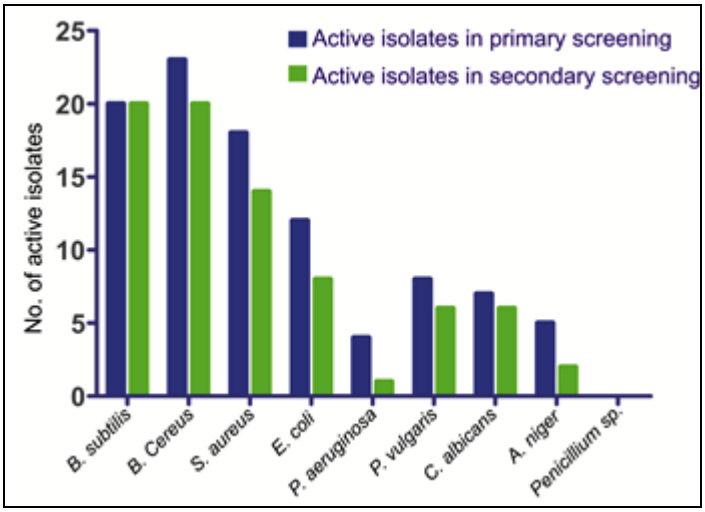

FIG. 6: NUMBER OF ISOLATES SHOWING ANTIMICROBIAL ACTIVITY IN PRIMARY AND SECONDARY SCREENING

TABLE 2: ANTIMICROBIAL ACTIVITIES OF ISOLATED ACTINOMYCETES IN SECONDARY SCREENING

Actinomycete Sp.	Test organisms and diameter of zone of inhibition (mm)								
	B. subtilis	***B. cereus***	***S.aureus***	***E.coli***	***P. vulgaris***	***P. aeruginosa***	***C. albicons***	***A. niger***	***Penicillium Sp.***
A1	10	15	10	--	--	--	07	--	--
A2	20	22	18	15	12	--	10	07	--
A3	12	10	09	08	06	--	--	--	--
A4	07	08	--	--	--	--	--	--	--
A5	15	13	09	07	--	--	--	--	--
A6	16	14	10	08	--	--	08	--	--
A7	13	08	--	08	--	--	--	08	--
A8	10	09	--	--	07	--	--	--	--
A9	10	09	--	--	--	--	--	--	--
A10	12	12	09	--	--	06	--	--	--
A11	13	14	12	--	10	--	08	07	--
A12	17	16	10	09	--	--	--	--	--
A13	14	12	10	--	--	--	--	--	--
A14	12	10	18	--	--	--	10	08	--
A15	09	08	--	--	--	--	--	--	--
A16	12	10	08	07	07	--	09	--	--
A17	10	10	07	08	06	--	---	--	--
A18	13	11	08	09	07	--	--	--	--
A19	09	10	08	08	07	--	10	08	--
A20	14	14	10	09	--	--	--	--	--

Morphological, physiological and biochemical characteristics of isolate: Biochemical and physiological characteristics of actinomycete sp. A2 were performed at MTCC Chandigarh. Based on the results the isolated species was identified as *Streptomyces violaceorubidus* and the strain was designated as RC 1796. The tests performed and the results are depicted in **Table 3**. Scanning electron microscope studies showed spore chain structure of actinomycetes sp. A2 as shown in **Figure 7**.

TABLE 3 : BIOCHEMICAL AND PHYSIOLOGICAL TESTS OF ACTINOMYCETES SP. A2

Sr. No.	Tests	Result
01	Gram Staining	+
02	Endospore staining	-
03	Motility	-
04	Growth at 15^0C	-
05	Growth at 25^0C	+
06	Growth at 37^0C	+
07	Growth at 42^0C	-
08	Growth at pH 5.2	+
09	Growth at pH 8.0	+
10	Growth at pH 9.0	+
11	Growth at pH 10.0	+
12	Growth on NaCl 2%	+
13	Growth on NaCl 5%	-
14	Growth on NaCl 7%	-
15	Growth on NaCl 10%	-
16	Starch hydrolysis	-
17	Casein hydrolysis	-
18	Citrate Utilization	-
19	Gelatin liquefaction	+
20	H2S production	+
21	MR	-
22	VP	-
23	Nitrate reduction	+
24	Indole	-
25	Catalase	+
26	Oxidase	+
27	Urea	-
28	Acid production from	-
29	Galactose	-
30	Glucose	+
31	Mannitol	+
32	Raffinose	-
33	Salicin	+
34	Xylose	-
35	Sucrose	-
36	Rhamnose	-
37	Meso-inositol	-
38	Fructose	-

FIG. 7: SPORE CHAIN STRUCTURE OF ISOLATED ACTINOMYCETES SP. A2 OBSERVED UNDER SCANNING ELECTRON MICROGRAPH

CONCLUSION: Hence it was concluded that the objective of the proposed project was achieved. Actinomycetes were isolated from different locations in and around Nagpur. The isolated actinomycetes species having antimicrobial activity was identified from MTCC Chandigarh as *Streptomyces violacerorubidus* and the strain was designated as RC 1796. This investigation clearly indicate that Nagpur and nearby area is a potent source for the isolation of bioactive actinomycetes.

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