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# *IN VITRO* ANTAGONISTIC ACTIVITY OF SOIL *STREPTOMYCES COLLINUS* DPR20 AGAINST BACTERIAL PATHOGENS

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**ABSTRACT**

Actinomycetes are one of the most important groups that produce useful secondary metabolites. They play a great role in pharmaceutical and industrial uses. The search for antibiotic producing soil actinomycetes to inhibit the growth of pathogenic microorganisms has become widespread due to the need for newer antibiotics. The present work was aimed to isolate soil actinomycetes from pinus tree rhizosphere from Doddabetta, Western Ghats, Tamil Nadu, India. Thirty one actinomycetes were isolated based on heterogeneity and stability in subculturing; they were screened against 5 Gram positive and 7 Gram negative bacteria in an *in vitro* antagonism assay. In the preliminary screening, out of 31 isolates, 12.09% showed good antagonistic activity; 25.08% showed moderate activity; 19.35% showed weak activity and 41.93% showed no activity against the tested bacteria. Among the isolates tested, DPR20 showed good antibacterial activity against both Gram positive and Gram negative bacteria. The antibacterial crude secondary metabolites were extracted using ethyl acetate from micromonospora medium (M3) in which DPR20 was grown for twelve days at 30ºC. The lowest MIC value of ethyl acetate extract against *Bacillus subtilis* and *Staphylococcus epidermis* was 31.25µg/ml. The active isolate was identified as *Streptomyces collinus* DPR20 based on morphology, physiology, scanning electron microscopy and 16S rRNA gene sequence analysis.

**Keywords:** Antibacterial activity, *Streptomyces collinus* DPR20, Minimum inhibitory concentration (MIC)

## INTRODUCTION

Currently many of the pathogens implicated in infectious diseases are rapidly developing resistance to available antibiotics **(Singer *et al.*, 2003)** making treatment of these infections very difficult **(Bhavnani *et al.*, 2000)**; hence there is a need to look for alternative sources for novel bioactive compounds with strong activity against these pathogens. Microbial natural products have been the source of most of the antibiotics in current use for the treatment of various bacterial infectious **(Jiang *et al.*, 2013).** During the past decades, the actinomycetes have provided many important bioactive compounds of high commercial value. Actinomycetes, which are prolific producers of antibiotics and important suppliers to the pharmaceutical industry, can produce a wide variety of secondary metabolites **(Baltz, 2008).** Investigation on the biosynthesis of antibiotics involves optimization of culture media for their production that can be achieved by a systematic study of the suitability of physiological parameters and supplementary nutrition. Cultural parameters and medium constituents are important factors influencing the production of antimicrobial agent **(Iwai and Omura, 1982; Singh *et al.*, 2009).** To maximize the production of antibiotics by any producer strain, it is necessary to optimize nutrient and environmental conditions. Therefore, isolation of antibiotic producing *Streptomyces* from natural sources and characterization of their secondary metabolites are valuable endeavors. Soil, in particular, is an intensely exploited ecological niche, the inhabitants of which produce many useful biologically active natural products, including clinically important antibiotics. The species belonging to the genus *Streptomyces* are Gram-positive, aerobic microorganisms with high DNA G+C contents, which constitutes 50% of the total population of soil actinomycetes (**Miyadoh, 1993**) and 75-80% of the commercially and medicinally useful antibiotics **(Watve *et al.*, 2001; Mellouli *et al.*, 2003).** The aim of the present study was to isolate and evaluate the potential antibacterial activity of soil derived actinomycetes isolated from soil samples collected from pinus tree rhizosphere from Doddabetta, Western Ghats, Tamil Nadu, India. This area is poorly studied and represents diverse and largely unscreened ecosystem.

## METHODOLOGY

### Sample collection

The soil sample was collected near the root zone of pinus tree at 5-7m depth using sterile spatula in sterile zip lock polypropylene bags from Doddabetta, Western Ghats, Tamil Nadu, India (Latitude: 11º40'116"N, Longitude: 76º35'32.28''E Elevation ft 217). The soil samples were transported aseptically to the laboratory and stored at -20°C until use **(Govindarajan *et al.*, 2014).**

### Processing and isolation of soil actinomycetes

The soil sample was sieved through a 2mm siever to get rid of large debris and the sieved soil was used for the isolation of actinomycetes. Tenfold serial dilution of soil samples was made using distilled water and overlaid on the surface of starch casein agar (SCA) which contained casein powder-1.0g; starch-10g; sea water-37L; agar-18g; pH-7.2 ± 0.20 and amended with 20mg/L of the fungicide actidione and 100mg/L antibiotic Nalidixic acid to isolate actinomycetes. The plates were incubated at 28°C for 21 days **(Saadoun *et al.*, 2002)**.

### Selection of actinomycetes

The growth of actinomycetes colonies especially streptomycetes was carefully observed for the typical morphology as per Bergey's manual and as per the proceedings of the International *Streptomyces* project published in the International Journal of systemic bacteriology by **Breed *et al.*, (1957), Shirling and Gottlieb (1966), Kersters and Vancanneyt, (2005).** The pure colonies were

transferred to ISP-2 medium and incubated at 28±2ºC for 7-14 days until powdery, pigmented and leathery colonies were observed on the plates. Pure isolates were continually subcultured in fresh ISP-2 by repeated streak plate technique and stored in 20% glycerol as stock for further analysis **(Saravana Kumar *et al.*, 2012).**

**Microbial organisms**

Bacterial pathogens used in tests were obtained from the Institute of Microbial Technology (IMTECH), Chandigarh, India-160036. They were: Gram positive bacteria: *Staphylococcus aureus* MTCC 96, *Micrococcus luteus* MTCC 106, *Bacillus subtilis* MTCC 441, *Staphylococcus epidermidis* MTTC 3615 and Methicillin resistant *Staphylococcus aureus* (MRSA); Gram negative bacteria: *Klebsiella pneumoniae* MTCC 109, *Enterobacter aerogenes* MTCC 111, *Vibrio parahaemolyticus* MTCC 451, *Yersinia enterocolitica* MTCC 840, *Salmonella typhimurium* MTCC 1251, *Shigella flexneri* MTCC 1457 and *Proteus vulgaris* MTCC 1771. Prior to the experiment, bacterial inoculums were prepared by growing cells in Mueller Hinton broth (MHB) (Hi-media, Mumbai, India) at 37°C for 24 h in order to reach the exponential phase; then the cell suspension was diluted with peptone water to obtain $1.0 \times 10^8$ CFU/mL (0.5 McFarland standard) **(Mahomoodally *et al.*, 2015).**

**Preliminary antibacterial activity of the DPR isolates**

In our pilot scale screening, a total of 31 actinomycetes was isolated and designated as DPR1 to DPR31. The antibacterial activities of the pure actinomycetes isolates were performed by using cross streak method **(Oskay 2009)**. Modified nutrient glucose agar (MNGA) plates were prepared and the test actinomycetes cultures were inoculated in a single streak down the middle of a plate and incubated at 28°C for 4-7 days for the production of antibiotics **(Al-Dhabi *et al.* 2016).** After incubation the plates were inoculated with the test organisms by a single streak at 90° angles to the actinomycetes strains and incubated at 37°C overnight. Antagonism was observed by the inhibition of test organism **(Ramesh *et al.*, 2009).**

**Culture characterization of DPR20**

**Morphological characterization**

The active strain DPR20 was characterized morphologically by following the method of **Lechevalier, (1989), Shirling and Gottlieb, (1966).** Colony morphology including growth, color and soluble pigments which are highly characteristic and useful in the classification of actinomycetes were observed by growing the isolate in different media such as ISP-1, 2, 4, 5, 7 (International *Streptomyces* project), Agar, MHA- Mueller Hinton Agar, SCA- Starch casein Agar, SDA- Sabouraud dextrose agar, SKM- Skim milk agar and YPG- Yeast peptone glucose agar and incubating for 7 - 14 days at 28°C ± 2°C (**Saravana Kumar *et al.* 2014)**.

**Physiological characterization**

**Effect of temperature on the growth**

The effect of temperature on the growth of DPR20 was determined using ISP-4 medium. A loopful of DPR20 was streaked separately on the medium and incubated at different temperatures viz., 25°C, 27°C, 30°C, 33°C, 35°C and 37°C. After 7-14 days of incubation, the plates were observed for the growth of actinomycetes **(Sanghvi *et al.*, 2014).**

**Effect of pH on the growth**

The effect of pH on the growth of DPR20 was determined using modified nutrient glucose agar (MNGA); it contained glucose- 10g, peptone- 5g, beef- 3g, yeast- 3g, NaCl-3g, agar- 18g. Acidic range (pH 3-5) was prepared with acetate buffer; neutral range (pH 6-7) was prepared with phosphate buffer and alkali range (pH 8-12.5) was prepared with Tris-buffer. A loopful of active isolate DPR20 was streaked separately in the above medium with different pH and the plates were incubated at 28°C ± 2°C. After 7-14 days of incubation, the plates were observed for the growth of actinomycetes **(Balachandran *et al.*, 2014).**

**NaCl tolerance test**

DPR20 was grown in 20 mL of different concentrations of NaCl (1, 3, 5, 7, and 9%) amended MNG agar media in petri plates at 28°C±2°C. After 7-14 days of incubation, the growth of active isolate DPR20 was visually observed to determine the tolerance of selected actinomycetes to NaCl. The MNGA medium without amendment of NaCl served as control **(Saravana Kumar *et al.*, 2017).**

**Biochemical characterization**

Biochemical test was done by following the manufacturer's procedure (KIT no: KB003; HiMedia, Mumbai, India). 100 µL of inoculum was poured into the plate and incubated at 28°C ± 2°C for 24 hr. The growth of actinomycetes on a given carbon source was compared with both positive and negative controls.

**Scanning electron microscope observation**

Morphological features of spores and mycelia were observed by scanning electron microscope (Hitachi S-4800 model SEM, Japan) after growth in ISP-2. The preparation of samples for scanning electron microscopy was done as described previously **(Praveen Kumar *et al.*, 2015).**

**Optimization of antibacterial metabolite production**

To determine the optimal nutritional and cultural conditions for growth and antibacterial metabolites production, the spore suspension of active isolate DPR20 was prepared from the freshly grown culture on ISP-2 plates for seven days and inoculated in 100mL of the following media: APM- Antibiotic production medium, FEM- Fermentation medium, GLM- Glucose yeast extract malt medium, MNG- Modified nutrient glucose medium, M3 media- Micromonospora medium, M6 medium and YPG- Yeast peptone glucose medium and incubated at 150 rpm at 30ºC for twelve days **(Saravana Kumar *et al.*, 2014, 2017)**. To study the time course of antibacterial metabolites production, the active strain DPR20 was maintained in the production medium at 150 rpm at 30ºC. After every 24 h, the fermented broth was tested for antibacterial activity for up to twelve days using agar well diffusion methods of **Neha and Vibhuti (2013).** Each experiment was done in triplicate and mean values of inhibition zones were calculated.

**Large-scale submerged fermentation and extraction**

Antibacterial production was studied in a submerged culture containing the optimized production medium. Well grown spores of DPR20 were transferred into 250-ml Erlenmeyer flasks containing 50mL of the seed medium. Inoculation was prepared with a 10% culture grown on a rotary shaker (150 rpm) at 30°C for 24 h; this was transferred to 500mL production medium in 1L Erlenmeyer flasks and grown under the same conditions for 12 days. Five liters of fermented broth were centrifuged at 8,000 g for 5 min and the extracellular metabolites were extracted through manual shaking twice with equal volume of ethyl acetate (1:1) in a separating funnel. The respective organic phases were collected and were dried over $Na_2SO_4$ (anhydrous). The organic solvent was evaporated to dryness in a vacuum evaporator at 55°C to yield 3.3 g reddish brown crude fraction **(Salamoni *et al.*, 2010, Crevelin *et al.*, 2013).**

**Isolation and purification of genomic DNA**

DPR20 was grown in modified nutrient glucose agar (MNGA) at 30°C for 7 days for obtaining full growth. A loopful of spores was inoculated in the modified nutrient glucose broth and incubated for 2-5 days on a shaker incubator at 200 rpm at 30°C to form a pellet of vegetative cells **(Supong *et al.*, 2016)**. Total genomic DNA from DPR20 was extracted and purified using Hipura *Streptomyces* DNA spin kit-MB 527-20pr from Hi-media, according to the manufacturer's protocol.

**16s rRNA and sequence determination**

PCR-mediated amplification of the 16S rRNA gene was carried out using the bacterial universal primers 27F (5' AGT TTG ATC CTG GCT CAG 3') and 1492R (5' ACG GCT ACC TTG TTA CGA CTT 3') in Thermal Cycler (ep gradient Eppendorf) as described by **Barakate *et al.*, (2002)**. The cyclic conditions were as follow: initial denaturation at 94ºC for 3min, 35cycles of 94ºC for 1min, 54ºC for 1min, and 72ºC for 2mins, and final extension of 10 min and held at 4ºC. The PCR products were confirmed by 1.5% agarose gel electrophoresis.

**Database searching and phylogenetic analysis**

Amplified PCR product was sequenced and nucleotide sequence was matched using BLAST program with the reference strains contained in the genomic database banks, using the NCBI BLAST (Blast'n') tool

(http://www.ncbi.nlm.nih.gov/BLAST). Molecular phylogeny and pair wise evolutionary distances were analyzed using 16 rRNA gene sequence together with other sequence homologues retrieved from GenBank. The phylogenetic tree was constructed using UPGMA method (Bjerga, et al., 2014) and the stability of relationships was assessed by bootstrap analysis based on resamplings of 1000 times using a software MEGA version 6.0. (Tamura et al., 2007).

**Restriction sites analysis of DPR20**

The restriction sites of the DNA of DPR20 were analyzed using NEB cutter online tool version 2.0 (nc2.neb.com/NEBcutter2/; (**Vincze *et al.*, 2003).**

**Antibacterial assay**

The antibacterial activities of filtrate and metabolic extract were assayed using the standard Kirby-Bauer well and disc diffusion methods; they were estimated via comparison with streptomycin, a standard commercial antibiotic **(Manimaran *et al.*, 2015).** By means of a sterile cork borer, wells were punctured in plates containing Mueller Hinton agar previously seeded with one of the test Gram positive or Gram negative bacterium. 100 µL of fermented broth of each medium was added in each well. About 2.5 mg of the DPR20 crude extract was impregnated on sterile disc and dried under sterile conditions at room temperature. The loaded discs were placed on the surface of the medium and left for 30 min at room temperature for compound diffusion and the plates were incubated overnight at 37$^{o}$C. The area of no growth around the well was recorded as zone of inhibition. Negative control was prepared using the respective solvents (DMSO) **(Barakate *et al.*, 2002)**. Diameters of the zones of inhibition were measured using a zone scale from Hi-media and expressed in millimeters. The assays were performed in triplicate.

**Determination of minimal inhibitory concentration**

The minimum inhibitory concentrations (MIC) of the ethyl acetate extract against selected bacterial pathogens were determined by following the method of Clinical and Laboratory Standards Institute **(Andrews *et al.*, 2001)**. The ethyl acetate extract (1000µg/mL) was serially two fold diluted to obtain the following concentrations of 1000, 500, 250, 125, 62.5, 31.3, 15.6µg/mL. The growth medium employed was Muller Hinton Broth. The organisms were added to 96 well micro titter plate containing 0.1mL broth. The 3µL of log phase culture was introduced into respective wells and the final inoculum size was 1x10$^{5}$cfu/mL. The plates were incubated at 37$^{o}$C overnight. Streptomycin was used as positive control. Negative (water) and solvent controls (DMSO) were also included **(Zgoda *et al.*, 2001)**. 5µL of the test broth was introduced into plain Mueller Hinton agar for bacteria to observe the viability of the organism. MIC was determined as the lowest concentration which inhibited complete growth.

## RESULTS AND DISCUSSION

**Isolation, selection, and preliminary screening of actinomycetes**

Thirty one *Actinomyces* were isolated from pinus tree rhizosphere soil from Doddabetta, Western Ghats, Tamil Nadu, India which exhibited the morphological features of *Streptomyces* bacterial community such as dimorphic mycelial forms as aerial and substrate mycelium, spore production, non-motile spores and earthy odor (**Andayani *et al.*, 2015, Malviya *et al.*, 2014).** The isolates were purified on ISP-2 slants and they were screened for antibacterial activity against selected human bacterial pathogens. In preliminary antibacterial screening 4 isolates (12.09%) showed good antibacterial activity; 8 isolates (25.08%) showed moderate antibacterial activity; 6 isolates (19.35%) showed weak antibacterial activity and 13 isolates (41.93%) showed no antibacterial activity against the tested bacterial pathogens (Table 1). Among these, the isolate DPR20 was found to produce broad spectrum and significant antibacterial activity against the screened Gram positive and Gram negative bacteria. Similarly, **Sanjivkumar *et al.* (2016)** and **Sanghvi *et al.* (2014)** studied the potential of many soil *Streptomyces* species to suppress or reduce bacterial pathogens.

**Identification of the active isolate DPR20: morphological, biochemical, physiological and molecular characteristics**

The active isolate DPR20 was identified by morphological, biochemical and physiological characteristics, SEM features and 16S rRNA amplification. DPR20 grown on various media at 30ºC for 14 days was observed to be 3-8 mm in diameter, circular and smooth. DPR20 grew well in ISP-1, 2, 7, Sabouraud dextrose agar and yeast peptone glucose agar. Moderate growth was observed in skim milk agar; weak growth was observed in ISP- 4, 5, plain agar and Muller Hinton agar; it had a white to grey aerial mycelium and dull to dark yellow vegetative mycelium. No soluble pigment was produced in all media used (Table: 2; Fig. 1a). It was found that DPR20 was Gram positive filamentous bacterium forming long or short chains of oval shaped abundant spores spirally arranged in the aerial mycelia (Fig. 1b). Based on the morphological characteristics, Gram staining and SEM (Fig. 1c, 1d), DPR20 was tentatively attached to the genus *Streptomyces* species. Table 3 shows the physiological properties of DPR20. Optimal growth of DPR20 was observed at 30°C and at pH 7 and in the presence of NaCl in the range of 1-7% (very good growth). The isolate was able to grow at 11% NaCl (moderate to good growth) at 37°C (maximum temperature growth). The strain was able to hydrolyze cellulose, melibiose, glucose and lactose; our results are in agreement with previous reports of **Nandhagopal *et al.* (2017), Balachandran *et al.* (2014)** and **Arasu *et al.* (2008).**

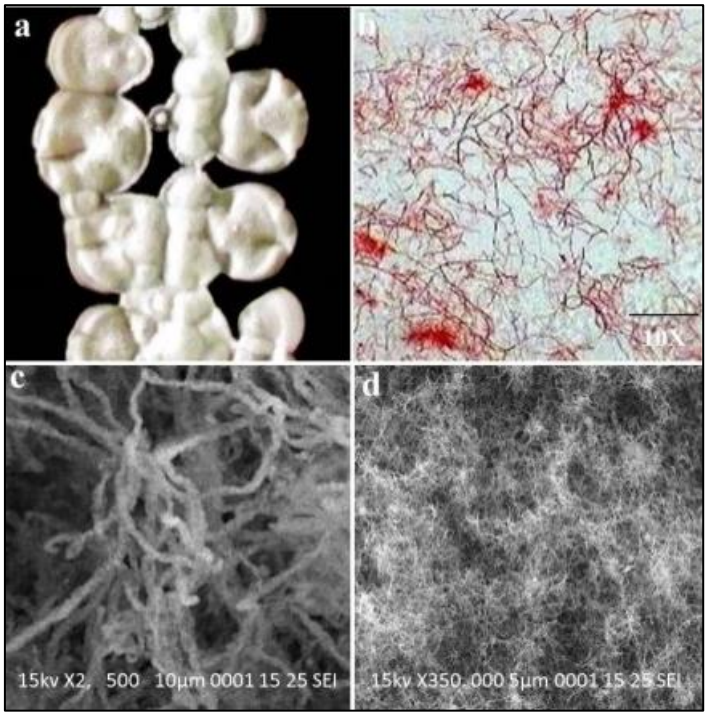


**Figure 1** Cultural characterization of Streptomyces collinus DPR20, (2a) Macroscopic image in AIA, (2b) Light microscopic image under 10X, Scanning electron microscopy images of spore chain (SEM) (2c) under 10µM, (2d) under 5µM

In order to provide further support for the identification features mentioned above 16S rRNA gene was amplified from the genomic DNA of the active isolate DPR20 and sequenced. Modern *Streptomyces* identification systems are based on 16S rRNA sequence data, which have provided invaluable information about streptomycetes systematics; they have been used to identify several newly isolated *Streptomyces* **(Lee *et al.*, 2005, Kim *et al.*, 2006).** The partial 16S rRNA gene sequence (811base pair) obtained was subjected to NCBI BLAST search program; it showed that the sequence had high similarity with the other taxonomically identified *Streptomyces collinus* strain 3MA2 (KC119170.1 (100%) and (99%) *Streptomyces collinus* strain BG4 (KF766107.1) with total scores and E values of 1467, 1476 and 0 respectively. The active strain DPR20 showed maximum sequence homology with *Streptomyces* species from NCBI database and evolutionary history was inferred. The phylogenic tree also suggested that the isolate (DPR20) was clustered within the lineages of the Streptomycetaceae with the clade containing the type strains including *Streptomyces collinus* BG4 (KF766107.1), which formed the sub-clade and clustered with *Streptomyces albogriseolus* (NR_042760.1), *Streptomyces fradiae* (AB184063.2), *Streptomyces lividans* (KT362142.1), *Streptomyces rubrogriseus* (KX431235.1), *Streptomyces tendae* (KC794689.1), *Streptomyces tritolerans* (NR_043745.1), *Streptomyces violaceorubidus* (NR_042309.1); *Actinomycetales bacterium* (JQ924135.1) belongs Actinomycetaceae with high bootstrap values in the range of 40-82% (Fig. 2).Our results are in agreement with the previous report of **Nandhagopal *et al.*, (2017).** This species seems to be free living in soil as well as in association with the marine sponge *Echinodictyum gorgonoides*. The present result also supports that the endosymbiotic *S. collinus* ICN1 possessed good antibacterial activity against multidrug resistant *S. aureus*. Based on the

above mentioned morphological, biochemical and physiological characteristics, SEM features and 16S rRNA analysis, we strongly conclude that the 16S rRNA gene partial sequence of the isolate DPR20 has been deposited in the GenBank database under the accession number KF766110. The strain DPR20 is *Streptomyces collinus* DPR20.

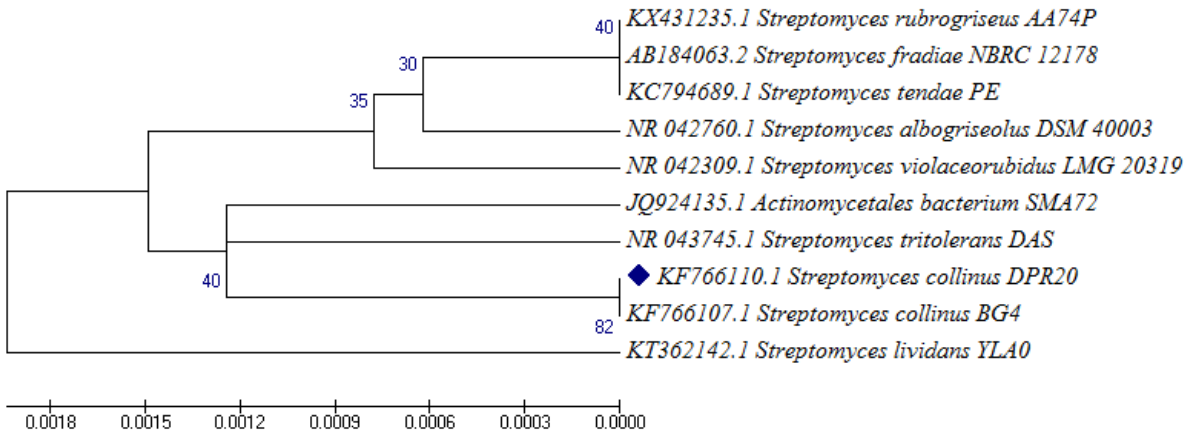


**Figure 2** Phylogenetic tree of *Streptomyces collinus* DPR20 (Kf766110) showing relatedness to other *Streptomyces* species constructed using UPGMA method with the aid of MEGA 6.0

**Table 1** Preliminary screening of actinomycetes isolates using cross streak method

| Isolates | 96 | 106 | 441 | 3615 | MRSA | 109 | 111 | 451 | 840 | 1251 | 1457 | 1771 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| DPR-1 | - | - | - | - | - | - | - | - | - | - | - | - |
| DPR-2 | - | - | - | - | - | - | - | - | - | - | - | - |
| DPR-3 | +++ | ++ | +++ | +++ | ++ | +++ | +++ | +++ | +++ | +++ | ++ | - |
| DPR-4 | - | ++ | +++ | - | + | - | ++ | +++ | - | - | - | - |
| DPR-5 | - | - | - | - | - | - | - | - | - | - | - | - |
| DPR-6 | + | ++ | ++ | - | + | + | ++ | +++ | ++ | - | - | - |
| DPR-7 | +++ | +++ | - | - | +++ | +++ | +++ | +++ | - | - | +++ | +++ |
| DPR-8 | - | +++ | ++ | - | ++ | + | + | ++ | ++ | ++ | - | - |
| DPR-9 | - | + | +++ | - | +++ | - | + | - | - | +++ | - | - |
| DPR-10 | ++ | +++ | ++ | - | ++ | ++ | ++ | ++ | ++ | +++ | - | - |
| DPR-11 | - | - | - | - | - | - | - | - | - | - | - | - |
| DPR-12 | - | - | +++ | - | ++ | + | + | - | - | +++ | - | - |
| DPR-13 | - | + | +++ | + | ++ | + | + | - | - | +++ | - | - |
| DPR-14 | - | - | - | - | - | - | - | - | - | - | - | - |
| DPR-15 | + | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | - | ++ |
| DPR-16 | - | - | - | - | - | - | - | - | - | - | - | - |
| DPR-17 | - | - | + | - | - | - | - | - | - | - | - | - |
| DPR-18 | - | + | - | - | +++ | +++ | ++ | - | - | - | - | - |
| DPR-19 | - | - | - | - | - | - | - | - | - | - | - | - |
| DPR-20 | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ |
| DPR-21 | - | - | - | - | - | - | - | - | - | - | - | - |
| DPR-22 | - | - | - | - | - | - | - | - | - | - | - | - |
| DPR-23 | ++ | +++ | +++ | - | +++ | ++ | +++ | +++ | +++ | +++ | - | - |
| DPR-24 | - | - | - | - | - | - | - | - | - | - | - | - |
| DPR-25 | - | - | - | - | - | - | - | - | - | - | - | - |
| DPR-26 | + | + | - | - | + | - | - | - | - | - | - | - |
| DPR-27 | - | - | +++ | - | - | - | - | - | - | +++ | - | - |
| DPR-28 | - | - | - | - | - | - | - | - | - | - | - | - |
| DPR-29 | + | + | - | - | - | - | - | - | + | - | - | - |
| DPR-30 | - | - | - | + | - | + | - | - | - | - | - | - |
| DPR-31 | - | - | - | - | - | - | - | - | - | - | - | - |

+++ Good activity; ++ Moderate activity; + Weak activity; - no activity

Gram positive bacteria: *Staphylococcus aureus* MTCC 96, *Micrococcus luteus* MTCC 106, *Bacillus subtilis* MTCC 441, *Staphylococcus epidermis* MTTC 3615 and Methicillin resistant *staphylococcus aureus* (MRSA); Gram negative bacteria: *Klebsiella pneumoniae* MTCC 109, *Enterobacter aerogenes* MTCC 111, *Vibrio parahaemolyticus* MTCC 451, *Yersinia enterocolitica* MTCC 840, *Salmonella typhimurium* MTCC 1251, *Shigella flexneri* MTCC 1457, *Proteus vulgaris* MTCC 1771

**Table 2** Morphological features of *Streptomyces collinus* DPR20 in different media

| Medium | Aerial mycelium | Substrate mycelium | Reverse side | Growth |
|---|---|---|---|---|
| ISP-1 | - | Dull Yellow | Dull Yellow | +++ |
| ISP-2 | White | Yellow | Greyish Yellow | +++ |
| ISP-4 | - | Yellow | Yellow | + |
| ISP-5 | - | Pale Orange | White | + |
| ISP-7 | White | White | White | +++ |
| SCA | - | - | - | - |
| SDA | Black | Black | Black | +++ |
| AGAR | White | White | White | + |
| MHA | - | Yellow | Yellow | + |
| SKM | White | White | White | ++ |
| YPG | Grey | Grey | White | +++ |

+++ Good growth; ++ Moderate growth; + Weak growth; - no growth
ISP- International *Streptomyces* project, SCA- Starch casein Agar, SDA- Sabouraud dextrose agar, MHA- Mueller Hinton Agar, SKM- Skim milk agar and YPG- Yeast peptone glucose agar

**Table 3** Biochemical and physiological characterization and sugar analysis of *Streptomyces collinus* DPR20

| S. No | Test | Results | Indication |
|---|---|---|---|
| 1. | Gram staining | Positive | Purple |
| 2. | Shape and growth | filamentous aerial growth | Good Growth |
| 3. | Production of diffusible pigment | - | - |
| 4. | Range of temperature for growth | 25°C to 37°C | Good Growth |
| 5. | Optimum temperature | 30°C | Good Growth |
| 6. | Range of pH for growth | 7 to 9 | Good Growth |
| 7. | Optimum pH | 7 | Good Growth |
| 8. | Growth in the presence of NaCl | 1 to 7% | Good Growth |
| 9. | ONPG | +++ | Yellow |
| 10. | Lysine utilization | +++ | Purple |
| 11. | Ornithine utilization | +++ | purple |
| 12. | Urease | +++ | Pinkish red |
| 13. | Phenylalanine deamination | - | Colorless |
| 14. | Nitrate reduction | +++ | Pinkish red |
| 15. | $H_2S$ production | - | Orange yellow |
| 16. | Citrate utilization | +++ | Blue |
| 17. | Voges Proskauer's | - | Yellow |
| 18. | Methyl red | - | Colorless |
| 19. | Indole | - | Colorless |
| 20. | Malonate utilization | - | Light green |
| 21. | Esculin hydrolysis | +++ | Black |
| 22. | Oxidase | + | Purple |
| 23. | Cellulose | ++ | Yellow |
| 24. | Melibiose | ++ | Yellow |
| 25. | Glucose | +++ | Yellow |
| 26. | Lactose | +++ | Yellow |
| 27. | Saccharose | - | Red |
| 28. | Raffinose | - | Red |
| 29. | Trehalose | - | Red |
| 30. | Arabinose | - | Red |
| 31. | Xylose | - | Red |
| 32. | Adonitol | - | Red |
| 33. | Rhamnose | - | Red |

+++ Good activity; ++ Moderate activity; + Weak activity; - no activity; ONPG- ortho-Nitrophenyl-β-galactoside

**Restriction sites analysis of *Streptomyces collinus* strain DPR20**

Figure3 shows the restriction sites of 16S rRNA gene linear view of *S. collinus* DPR20. It predicted the restriction sites for various commercial and NEB restriction enzymes such as BstXI, Bst BI, StyI, EcoNI, HaeII, EcoRI and BstAP. Type II restriction enzymes are among the most valuable tools available to researchers in molecular biology. These enzymes recognize short DNA sequences (4-8 nucleotides) and cleave at, or close to, their recognition sites **(Pingoud *et al.*, 2001, Roberts *et al.*, 1993)**. Also the restriction site analysis showed the GC and AT content to be 60% and 40% respectively **(Telugu *et al.*, 2014**).

**Optimization of media and time course for antibiotic production**

Optimization for production of antibacterial metabolites was carried out on seven different types of media in a batch culture for 12 days. Preliminary screening of nutrient substrates showed that *Streptomyces collinus* DPR20 supported good growth in modified nutrient glucose medium, fermentation medium, Glucose yeast extract malt medium and yeast peptone glucose medium; moderate growth

was observed in Micromonospora medium (M3 medium), M6 medium and antibiotic production medium. Among the media screened M3 medium and modified nutrient glucose medium were found favorable for the production of antibacterial metabolites. However, maximum growth was obtained in fermentation medium, Glucose yeast extract malt medium and yeast peptone glucose medium; antibiotic production medium did not produce antibiotic under similar conditions. These results indicated that the production of biomass and antibiotics was found to be dependent on the composition of the medium. The results of time course experiment revealed that the antibacterial production by *Streptomyces collinus* DPR20 strain was growth dependent.

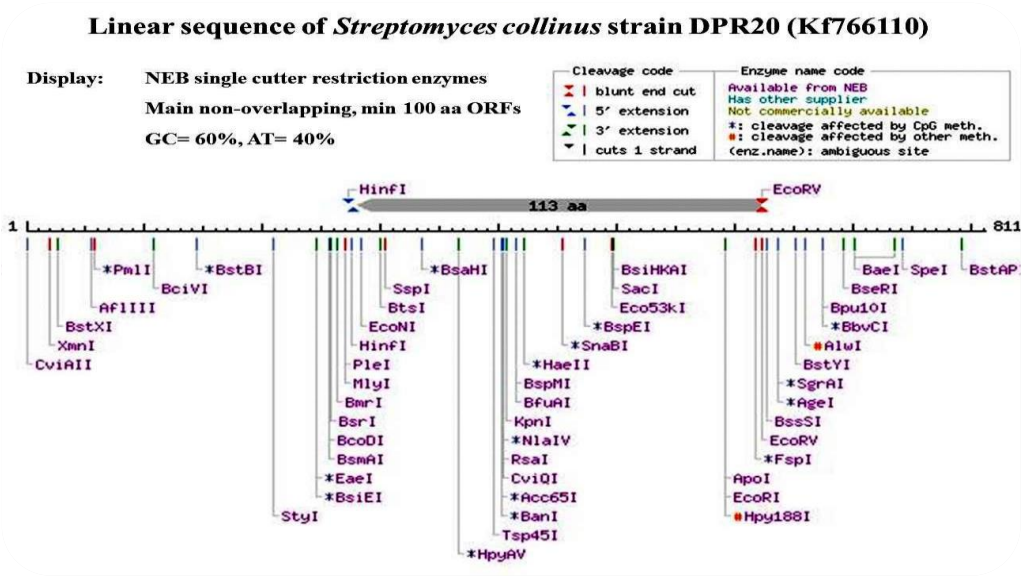


**Figure 3** Restriction sites on the 16s rRNA sequence of *Streptomyces collinus* DPR20

The study of culture conditions on antibacterial metabolite production indicated that the highest biological activities were obtained when Micromonospora medium was used as a base and the level of antibiotic yield increased gradually with increase in the incubation period up to 12 days; beyond this period, the antibiotic production began to diminish gradually; the diameters of zone of inhibition ranged from 16 to 25 mm against the tested pathogens (Fig.4b). Hence the incubation period was maintained up to 12 days for further antibacterial metabolite production**.** The time course study revealed that the production of antibacterials depended on the growth rate of the isolate. This is in accordance with the previous reports of **Gogoi *et al.* (2008)** and **Atta, (2015).**

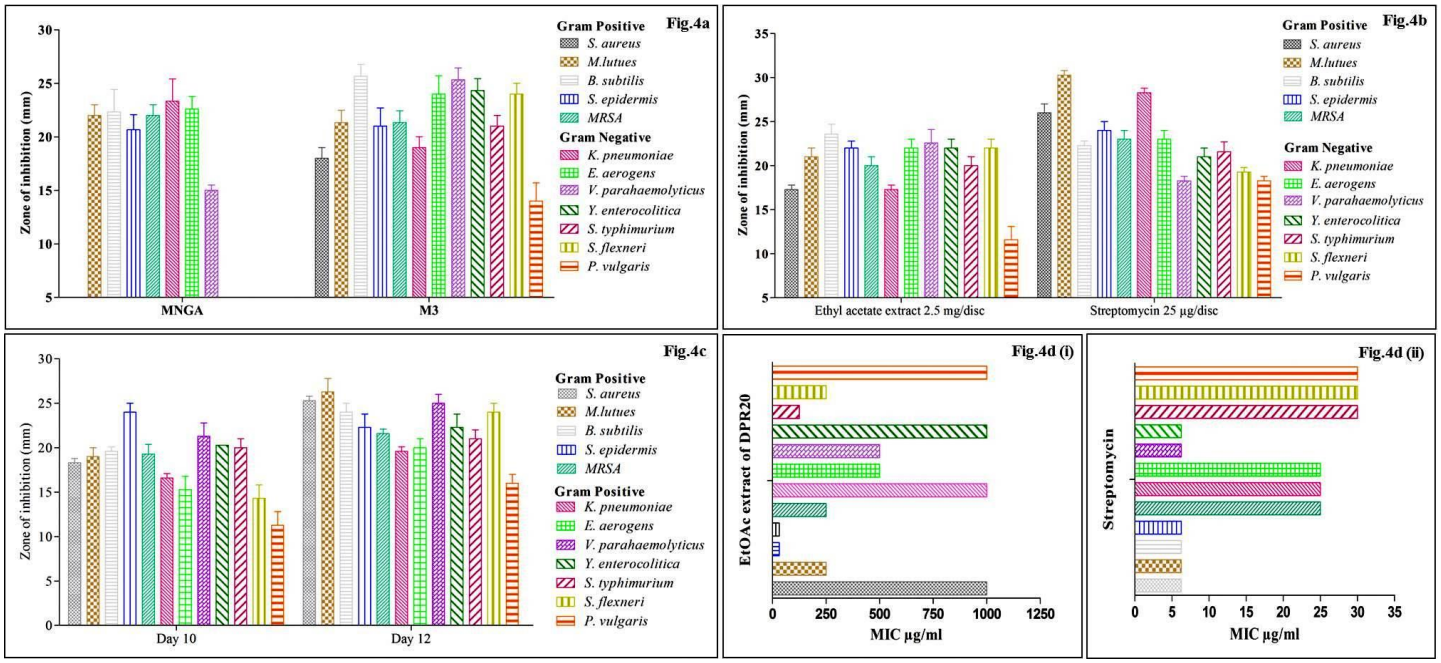


**Figure: 4** Antibacterial activity of *Streptomyces collinus* DPR20

**(4a)** Antibacterial activity of *Streptomyces collinus* DPR20 in different culture media by using Kirby-Bauer well diffusion method**; (4b)** Day optimization for antibacterial activity of *Streptomyces collinus* DPR20 in fermentation media by using Kirby-Bauer method**; (4c)** Antibacterial activity of crude ethyl acetate extract of *Streptomyces collinus* DPR20 using disc diffusion method**; (4d)** Minimum inhibitory concentrations of 4d (i) ethyl acetate extract of *Streptomyces collinus* DPR20 and 4d (ii) streptomycin

**Mass production, extraction and antibacterial activity**

To prove the ability of DPR20 to produce maximum antibacterial antibiotic, the culture was mass produced in M3 medium with glucose as carbon source and extracted twice with ethyl acetate to give a reddish brown residue. At 2.5mg/disc concentration, ethyl acetate extract of *S. collinus* DPR20 showed good activity against *B. subtilis* (23.6 ± 1.1 mm), *S. epidermis*, *E. aerogenes*, *Y. enterocolitica*, *V. parahaemolyticus*, *S. flexneri* (22 ± 0.5 mm), *M. luteus* (21±1), MRSA, *S. typhimurium* (20 ± 1 mm), *S. aureus*, *K. pneumoniae* (17.3 ± 0.5 mm), *P. vulgaris* (11.6 ± 1.5 mm) (Fig.4c). Overall the ethyl acetate extract showed effective antibacterial activity against the tested pathogens. Extraction using ethyl acetate additionally brought out the antibacterial activity and thus validated its capability as an ideal solvent for extraction of compounds from culture supernatants. These results are in agreement with **Huining *et al.* (2016),** who reported that the ethyl acetate extract from *Streptomyces* sp. P294 showed board spectrum activity against the tested bacterial pathogens. Moreover **Shu *et al.* (2007), Thakur *et al.* (2009)** and **Radhakrishnan *et al.* (2010)** had also reported that the ethyl acetate extract showed similar antibacterial activity.

**Minimum inhibitory concentration of antibiotic metabolite**

Screening for antibacterial activity provided the required preliminary observation to select the crude ethyl acetate extract for further chemical and pharmaceutical investigation. The ethyl acetate extract of DPR20 showed broad spectrum activity against Gram positive and Gram negative bacteria with the MIC values in the range of 31.25µg/mL - 1000µg/mL (Fig 4). The ethyl acetate extract showed significant activity against *B. subtilis*, *S. epidermis* (31.25µg/mL), *S. typhimurium* (125µg/mL), *M. luteus*, MRSA, *S. flexneri* (250µg/mL), *E. aerogenes*, *V. parahaemolyticus* (500µg/mL), *S. aureus*, *K. pneumoniae*, *Y. enterocolitica* and *P. vulgaris* (1000µg/mL) (Fig.4d (i). The results were compared with the standard drug streptomycin (Fig.4d (ii). Our findings are in agreement with the reports of **Inagaki *et al.* (1998), Sekiguchi *et al.* (2007)** and **Atta *et al.* (2009).**

**CONCLUSION**

We isolated of 31 naturally occurring soil actinomycetes from pinus tree rhizosphere. Among them, *Streptomyces collinus* DPR20 was selected for its antibacterial activity against Gram-positive and Gram-negative bacteria. Cultural characteristic studies and analysis of the nucleotide sequence of active isolate DPR20, strongly suggested that this isolate was *Streptomyces collinus* DPR20. M3 medium with glucose as a carbon source was found to be good base for fermentation and the production of antibacterial metabolites. Our results strongly support that *Streptomyces collinus* DPR20 can be utilized to produce compounds having broad spectrum antibacterial activity.

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