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ANTAGONISTIC ACTIVITY OF *STREPTOMYCES* SPECIES MPPO-02 ISOLATED FROM RHIZOSPHERE SOIL OF MAHISHI, KARNATAKA, INDIA

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ABSTRACT

Western Ghats of India are one of the global biodiversity hotspots. The present study was conducted to isolate and determine antibacterial activity of actinomycetes from a Western Ghat soil of Mahishi village, Thirthahalli Taluk, Shivamogga district, Karnataka. Out of 5 actinomycetes, one isolate designated MPPO-02 displayed marked antibacterial activity in primary screening by Cross streak technique. The isolate was identified as a species of *Streptomyces* based on microscopic and biochemical characteristics. In Agar well diffusion assay, the culture filtrate of MPPO-02 showed inhibitory activity against a panel of 10 bacteria. Gram positive bacteria were more susceptible than Gram negative bacteria to culture filtrate. Further studies on molecular characterization of the isolate MPPO-02 and purification of bioactive principle from culture filtrate are under progress. Further screening of soils of Mahishi can be fruitful in isolation of bioactive actinomycetes.

Keywords: Rhizosphere, Western Ghats, Mahishi, *Streptomyces*, Cross streak, Agar well diffusion.

INTRODUCTION

The rhizosphere is the region of soil present in vicinity of plant roots. It is a unique biological niche and comprises of a variety of microflora. Rhizosphere is profoundly influenced by plant root exudates and the microbial community is nutritionally favoured by root exudates. Actinomycetes (order Actinomycetales) are among the important rhizosphere inhabitants. Most of these soil actinomycetes are saprophytic, they enhance the plant growth and protect the plants against attack by phytopathogens such as fungi, nematodes etc. Among various actinomycetes genera of soil, the genus *Streptomyces* is the best recognized and well studied. The species of *Streptomyces* are aerobic spore formers and possess DNA rich in GC content (69-73 %). *Streptomyces* species are filamentous and they form extensive branching substrate and aerial mycelia. They are considered as prolific producers of bioactive compounds as they produced around 75% of biologically active compounds[1-4]. Western Ghats of India represent one of the global biodiversity hotspots. Western Ghats covers an area of 1,80,000 km^2 (just under 6% of the land area of India) and harbor >30% of all plant, fish, herpeto-fauna, birds, and mammal species found in India. The mountain range of Western Ghats runs through five states *viz.*, Gujarat, Maharashtra, Goa, Karnataka and Kerala. The biodiversity of Western Ghats are protected and conserved by the establishment of biosphere reserves, national parks and wildlife sanctuaries. A number of globally threatened species of plants and animals are found in the Western Ghats[5-7]. Earlier studies revealed marked bioactivities of soil actinomycetes of Western Ghats of Karnataka isolated from places such as Thirthahalli, Agumbe, Kodachadri, Talakaveri, Dandeli and Kudremukh[8-20]. In this study, we report antibacterial activity of a *Streptomyces* species MPPO-02 isolated from a rhizosphere soil of village Mahishi, Karnataka, India.

MATERIALS AND METHODS

Collection of soil sample

Rhizosphere soil sample was collected at Mahishi, Thirthahalli Taluk, Shivamogga district, Karnataka during September 2011. The surface soil was removed and the soil sample was collected from a depth of 5cm in a sterile plastic pouch, brought to the laboratory and dried aseptically[20].

Isolation of actinomycetes

The dried soil sample was serially diluted up to 10^{-5} dilution in tubes containing sterile physiological saline. The dilutions *viz.*, 10^{-3}, 10^{-4} and 10^{-5} were spread on sterile Starch Casein Nitrate (SCN) Agar (soluble starch 10g; potassium phosphate dibasic 2g; potassium nitrate 2g; sodium chloride 2g; casein 0.3g; $MgSO_4.7H_2O$ 0.05g; $CaCO_3$ 0.02g; $FeSO_4.7H_2O$ 0.01g; agar 15g; distilled water 1000ml) supplemented antifungal antibiotic Fluconazole (Fluka-150, Cipla Ltd., Uttarakhand) in order to prevent fungal contamination. The plates were incubated at 28°C aerobically for 14 days. The typical actinomycete colonies were subcultured on sterile SCN agar slants[20]. Five actinomycete isolates were recovered.

Test bacteria

Three Gram positive bacteria *viz.*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Bacillus subtilis* and seven Gram negative bacteria *viz.*, *Shigella sonnei*, *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter aerogenes*, *Proteus mirabilis*, *Vibrio cholerae* and *Pseudomonas aeruginosa* were used to assess antibacterial activity of actinomycetes.

Primary screening for antibacterial activity of actinomycetes

To screen antibacterial activity of isolated actinomycetes, we employed Cross streak method. Here, the actinomycete isolates were inoculated at the centre of the sterile SCN agar plates and the plates were incubated at 30°C for 5 days. After incubation, the Nutrient broth (Peptone 5g; Beef extract 3g; Sodium chloride 5g; Distilled water 1000ml) cultures of test bacteria were streaked perpendicular to the growth of the actinomycete isolates. The plates were incubated at 37°C for 24 hours and the extent of growth inhibition of the test bacteria was observed. The absence of growth or a less dense growth of test bacteria near the growth of actinomycete isolate was considered positive for production and secretion of antibacterial metabolite[20]. Out of five isolates, one isolate designated as isolate MPPO-02 showed marked inhibition of test bacteria. The isolate MPPO-02 was selected for identification to genus level and to assess its antibacterial activity.

Characteristics of isolate MPPO-02

Cultural characteristics

The cultural characteristics *viz.*, colour of aerial and substrate mycelium and production of diffusible pigments were studied on SCN agar plates.

Microscopic characteristic

The characteristic spore arrangement was studied using cover slip method. Here, thin blocks of SCN agar were cut and kept on sterile glass slide. The well grown culture of isolate MPPO-02 was inoculated all over the surface of agar block using sterile inoculation loop. A cover slip was placed over the agar block, the slide was placed in a sterile moist chamber and incubated until good growth of the isolate was observed. Then the cover slip was removed carefully and placed on a drop of dilute crystal violet stain taken on a clean glass slide. The slide was observed under oil immersion objective and characteristic spore arrangement was observed[20].

Staining and biochemical characteristics

The isolate MPPO-02 was subjected to Staining techniques *viz.*, Gram's and Acid-fast staining and biochemical tests *viz.*, starch hydrolysis, gelatin liquefaction, casein hydrolysis, catalse test, citrate test, cellulose hydrolysis, nitrate reduction test, hydrogen sulfide (H_2S) production test and carbohydrate utilization tests were performed for the isolate[21-23].

Fermentation

A loopful well sporulated culture of isolate MPPO-02 was inoculated into Erlenmeyer flask containing 250ml Sterile SCN broth and the flask was incubated aerobically at 28°C for 10 days. After incubation, the content of the flask was aseptically filtered through sterilized Whatman No. 1 filter paper. The culture filtrate thus obtained was screened for antibacterial activity[20].

Antibacterial activity of culture filtrate of isolate MPPO-02

Agar well diffusion assay was performed to determine antibacterial effect of culture filtrate of isolate MPPO-02 against a panel of ten bacteria. The 24 hours old Nutrient broth cultures of test bacteria were swabbed on sterile Nutrient agar (Peptone 5g; Beef extract 3g; Sodium chloride 5g; Agar 20g; Distilled water 1000ml) plates using sterile cotton swabs and wells of 6mm diameter were punched in the inoculated plates using a sterile cork borer. 100µl of culture filtrate and standard (Streptomycin, 1mg/ml) were transferred into labelled wells. The plates were incubated at 37°C for 24 hours. The zone of inhibition formed around the wells was measured after incubation[20].

Statistical analysis

The experiment was performed in triplicates. The results are represented as Mean±Standard deviation (SD).

RESULTS

A total of 5 actinomycete isolates (designated MPPO-01 to MPPO-05) were recovered from a rhizosphere soil sample collected in Mahishi.

All isolates were found to exhibit antibacterial activity against one or more test bacteria in primary screening. Out of 5 isolates, one isolate designated MPPO-02 showed marked inhibition of test bacteria. Among bacteria, *B. subtilis* was shown to display high susceptibility. None of the isolates showed inhibitory activity against *P. mirabilis* (Table 1).

Table.1.Primary screening for antibacterial activity of isolate MPPO-02

Test bacteria	Extent of inhibition of test bacteria by isolates				
	MPPO-01	MPPO-02	MPPO-03	MPPO-04	MPPO-05
S. aureus	+	++	+	+	+
S. epidermidis	+	+	+	+	+
B. subtilis	+	+++	+++	++	++
S. sonneii	-	++	-	+	-
E. coli	-	+	+	+	+
K. pneumoniae	+	+	-	-	-
E. aerogenes	+	-	-	+	-
P. mirabilis	-	-	-	-	-
V. cholerae	+	++	+	-	+
P. aeruginosa	-	-	-	-	+

The cultural, microscopic, staining and biochemical characteristics of the isolate MPPO-02 is shown in Table 2 and Figure 1. The isolate grew well on SCN agar medium and produced grey colored aerial mycelium and violet colored substrate mycelium. Diffusible pigment production was not observed. The spore arrangement was found to be straight. The isolate was Gram positive and non acid-fast. The isolate showed hydrolysis of starch, cellulose and gelatin. The isolate showed positive result for lipase, oxidase, catalase, citrase and nitrate reductase. H_2S production was observed. The isolate was found to utilize glucose, fructose, galactose, maltose, lactose and mannitol. Based on the microscopic and other characteristics, the isolate MPPO-02 is assigned to the genus *Streptomyces*.

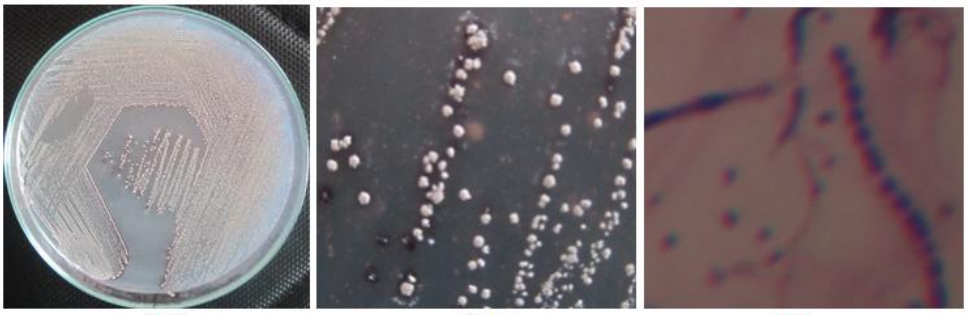

Fig.1.Culture [(a) and (b)] and spore arrangement [(c)] of isolate MPPO-02

Table.2.Characteristics of isolate MPPO-02

Characteristic/test	Isolate MPPO-02
Aerial mycelium	Grey
Substrate mycelium	Violet
Diffusible pigment	None
Spore arrangement	Rectus (straight)
Gram's staining	Gram positive
Acid-fast staining	Non acid-fast
Starch hydrolysis	+
Cellulose hydrolysis	+
Gelatin liquefaction	+
Casein hydrolysis	-
Lipase activity	+
Oxidase test	+
Catalse test	+
Nitrate reduction	+
Citrate utilization	+
Indole production	-
MR test	+
VP test	-
H_2S production	+
Utilization of carbon sources	Glucose + Fructose + Galactose + Maltose + Lactose + Mannitol +

The result of antibacterial activity of culture filtrate of isolate MPPO-02 is shown in Table 3. The crude culture filtrate was effective against all test bacteria but to a varied extent. *B. subtilis* and *S. sonnei* were inhibited to higher and least extent among Gram positive and Gram negative bacteria respectively. Overall, Gram positive bacteria have shown higher susceptibility than Gram negative bacteria. Standard antibiotic caused higher inhibition of test bacteria when compared to culture filtrate and the inhibitory activity was marked against Gram positive bacteria.

Table.3.Antibacterial activity of culture filtrate of isolate MPPO-02

Test bacteria	Zone of inhibition in cm	
	Culture filtrate	Standard
S. aureus	1.8±0.1	3.9±0.0
S. epidermidis	1.6±0.1	3.6±0.1
B. subtilis	2.4±0.2	3.9±0.0
S. sonneii	1.7±0.0	2.6±0.2
E. coli	1.0±0.2	2.7±0.1
K. pneumoniae	1.6±0.1	2.5±0.0
E. aerogenes	1.0±0.2	2.3±0.0
P. mirabilis	0.8±0.0	2.4±0.2
V. cholerae	1.6±0.1	2.3±0.2
P. aeruginosa	1.2±0.1	2.6±0.2

DISCUSSION

Microorganisms are attractive sources of biologically active compounds having pharmaceutical and agricultural significance. Actinomycetes are Gram positive eubacteria with high G+C content, found in terrestrial, fresh water and marine water and are responsible for the production of geosmin responsible for characteristic earthy odor. They have the ability to degrade a variety of compounds such as

pectin, lignocelluloses and many other complex polymers in soil and litter, and a range of xenobiotics. In addition to biodegradation role, these actinomycetes also produce lytic enzymes, antibiotics and other bioactive metabolites such as plant growth promoters, herbicides, insecticides, antitumor agents etc due to their metabolic diversity. These actinomycetes are biotechnologically and industrially valuable prokaryotes as they produce a large number of bioactive compounds with pharmaceutical and agricultural importance[2,4,24,25,26].

In the present study, we have recovered 5 actinomycete isolates (MPPO-01 to MPPO-05) from a Western Ghat rhizosphere soil sample collected at Mahishi. Out of 5 isolates, one isolate designated MPPO-02 displayed marked inhibitory activity against test bacteria in primary screening by cross streak technique. The isolate was characterized as a species of the genus *Streptomyces* on the basis of microscopic and biochemical characteristics. The members of *Streptomyces* can be differentiated from other sporing actinomycetes based on morphology. The vegetative mycelium, aerial mycelium baring chains of spores and the characteristics of spores themselves are used as diagnostic keys in the identification of *Streptomyces*. Cultural characteristics and characteristic spore arrangement along with biochemical characteristics assist classification of actinomycetes as members of the genus *Streptomyces*[14,20,27,28,29].

Throughout history, a continuous battle exists between humans and pathogenic microorganisms. Infectious diseases have been the major cause of morbidity and mortality in human population. The discovery of antibiotics from microbes is considered as a turning point and their subsequent use saved countless lives and revolutionized medicine field. However, the use of antibiotics has been accompanied by the rapid development of resistance in microbes. A wide range of biochemical and physiological mechanisms are involved in the development of resistance against antimicrobials. Moreover, the ability of pathogens to acquire and transmit resistance has made the situation even worst and the therapy more complicated. These drug resistant pathogens impose a substantial burden to human population. Therefore, search for new antimicrobials is a continuous process to keep pace with continually evolving pathogens[30-33]. Actinomycetes have shown to be promising sources of antimicrobials active even against drug resistant pathogens[34,35].

Western Ghats represents one of the biodiversity hotspots in the world. In particular, Western Ghats of Karntaka houses a large number of plants and animals including threatened species[7]. Antimicrobial activity of actinomycetes from Western Ghat soils of various parts of Karnataka such as Agumbe[12,14], Kudremukh[13], Talakaveri[19], Thirthahalli[20], Kodachadri[11] and Dandeli[17] has been reported. In the present study, we have observed inhibitory activity of culture filtrate of *Streptomyces* species MPPO-02 against pathogenic bacteria. The culture filtrate was found to inhibit Gram positive bacteria to higher extent when compared to Gram negative bacteria. Similar results (i.e., higher susceptibility of Gram positive bacteria) were observed in earlier studies of Kekuda *et al.*[12], Anansiriwattana *et al.*[36], Valli *et al.*[37], Manasa *et al.*[13], Kekuda *et al.*[20] and Junaid *et al.*[19]. In Gram negative bacteria, the presence of an outer membrane possessing hydrophilic polysaccharides chains forms an additional barrier for the entry of compounds into the cells. This might have been ascribed to the lower susceptibility of Gram negative bacteria[38,39].

CONCLUSION

The present study was successful in isolating antagonistic *Streptomyces* from Western Ghat soil of Mahishi, Karnataka, India. The crude culture filtrate of the isolate MPPO-02 exhibited inhibitory efficacy against Gram positive and Gram negative bacteria. The *Streptomyces* isolate can exploited for the development of therapeutic agents active against human pathogens. Studies are under progress to characterize the isolate by molecular studies and to purify bioactive components from culture filtrate and to determine their inhibitory activities. Further screening of soils of Western Ghats of Karnataka for antagonistic actinomycetes has to be carried out.

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CONFLICT OF INTEREST

Authors declare no conflict of interest

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