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Research Article

ANTIBACTERIAL ACTIVITY OF THREE *STREPTOMYCES* SPECIES ISOLATED FROM SOILS OF SHIKARIPURA, KARNATAKA, INDIA

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Abstract

The present study was conducted to determine antibacterial efficacy of actinomycetes isolated from rhizosphere soils of Shikaripura, Karnataka, India. A total of 32 actinomycetes were recovered on Starch casein nitrate agar. Primary screening by cross streak method revealed marked antibacterial activity in case of three isolates viz., SRDP-S-03, SRDP-S-05 and SRDP-S-30. These isolates were grown in Starch casein nitrate broth and the culture filtrates were extracted using ethyl acetate. Secondary screening for antibacterial activity of ethyl acetate extract was performed by agar well diffusion assay. The extracts have shown marked inhibition of Gram positive bacteria when compared to Gram negative bacteria. Microscopic characteristics and other characteristics assisted identification of three actinomycete isolates as members of *Streptomyces*. Further studies on characterization of isolates and purification of bioactive compounds from ethyl acetate extracts are under progress.

Keywords: *Streptomyces*, Shikaripura, Cross streak, Agar well diffusion

INTRODUCTION

Actinomycetes, belonging to the order Actinomycetales, are among the microbial communities in the soil. These organisms play an important role in the degradation of complex biopolymers such as cellulose, lignin, chitin etc. They are responsible for producing the characteristic earthy odor of freshly turned soil. They are also a source of a variety of bioactive compounds having biotechnological applications. The species belonging to the genus *Streptomyces* represent the dominant actinomycetes population in the soil. The species of *Streptomyces* are well known for their ability to produce a variety of bioactive secondary metabolites such as antibiotics, immunomodulators, anticancer drugs, antiparasitic agents, antiviral agents, herbicides and insecticides. Among bioactive metabolites, about 75 % are produced by *Streptomyces*. Although a number of antibiotics have been produced from *Streptomyces*, the number still represents only a small fraction[1-5]. The present study was conducted with an aim of isolation of actinomycetes from soils of Shikaripura, Karnataka, India and to determine their antibacterial activity.

MATERIALS AND METHODS

Collection of Soil Sample

The rhizosphere soils (6 samples) were collected from a depth of 15 cm during the month of December 2012. The soils were collected in sterile plastic bags, brought to the laboratory and dried at 40°C under aseptic conditions[6].

Isolation of Actinomycetes

The soil samples were serially diluted and plated on Starch casein nitrate (SCN) agar (soluble starch, 10 g; potassium phosphate dibasic, 2 g; potassium nitrate, 2 g; sodium chloride, 2 g; casein, 0.3 g; $MgSO_4.7H_2O$, 0.05 g; $CaCO_3$, 0.02 g; $FeSO_4.7H_2O$, 0.01 g; agar, 15 g; distilled water 1000 ml) amended with antibiotic Fluconazole (in order to prevent fungal contamination). The plates were incubated aerobically at 30°C for up to 10 days. Colonies exhibiting typical morphology of actinomycetes were selected, sub cultured on SCN agar slants and maintained in refrigerator[6].

Primary Screening for Antibacterial Potential of the Actinomycete Isolates

We have employed Cross streak method to screen antibacterial efficacy of the actinomycetes recovered from soil samples. Here, the actinomycetes were streaked at the centre of the sterile SCN agar plates and the plates were incubated at 30°C for 5 days. Later, the test bacteria (*Staphylococcus aureus* NCIM-2079, *Bacillus cereus* NCIM-2016, *Escherichia coli* NCIM-2685, *Shigella flexneri* NCIM-4924, *Klebsiella pneumoniae* NCIM-2957 and *Vibrio cholerae* MTCC-3905) were inoculated perpendicular to the growth of the actinomycete isolates and the plates were further incubated for 24 h at 37°C. 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 actinomycete isolate was considered positive for production and secretion of antibacterial metabolite by the isolates[6]. Three isolates (designated as isolate SRDP-S-03, SRDP-S-05

and SRDP-S-30) showing marked inhibition of test bacteria were selected for further identification and for secondary screening for antibacterial activity.

Characteristics of the Isolates

Cultural Characteristics

The characteristics viz., aerial mycelium, substrate mycelium and diffusible pigment production of the isolates were studied on SCN agar plates.

Microscopic Characteristic

The characteristic spore arrangement in the isolates was studied by cover slip method. Here, thin blocks of SCN agar were cut, placed on sterile glass slides and inoculated with the isolates all over the agar block surface. A cover slip was then placed over each inoculated agar block and the slides were placed in a sterile moist chamber and incubated until good growth of the isolates was observed. The cover slips were removed from the agar blocks, mounted on a drop of dilute crystal violet stain taken on clean glass slides and observed under oil immersion objective in order to study the arrangement of spores[6].

Staining and Biochemical Characteristics

The isolates were subjected to Gram's and Acid-fast staining. Biochemical tests viz., starch hydrolysis, gelatin liquefaction, casein hydrolysis, catalyst test, citrate test, cellulose hydrolysis, nitrate reduction test, hydrogen sulfide (H_2S) production test and sugar fermentation test were performed[7,8].

Fermentation and Extraction

In order to obtain bioactive extracts, the spore suspensions of well grown cultures of isolates SRDP-S-03, SRDP-S-05 and SRDP-S-30 were inoculated into sterile SCN broth contained in Erlenmeyer flasks. The flasks were incubated aerobically at 30°C for 10 days. The content of the flasks were filtered through sterilized Whatman No. 1 filter paper under aseptic conditions[6]. The culture filtrates were subjected to centrifugation and the supernatants were used for extraction of bioactive metabolites. Equal volume (1:1) of culture filtrate and ethyl acetate were taken in a sterile separation funnel and agitated well for about 30 minutes. The solvent portion was separated and the aqueous portion was again extracted with ethyl acetate two more times. The solvent layers were pooled and evaporated to dryness at 40°C[9].

Secondary Screening for Antibacterial Activity

Agar well diffusion method[6] was employed to determine the efficacy of ethyl acetate extracts of isolates SRDP-S-03, SRDP-S-05 and SRDP-S-30 to inhibit the test bacteria viz., *S. aureus*, *B. cereus*, *E. coli*, *S. flexneri*, *K. pneumoniae* and *V. cholerae*. The test bacteria were grown overnight in Nutrient broth (HiMedia, Mumbai, India) and the broth cultures were inoculated on sterile Nutrient agar (HiMedia, Mumbai, India) plates using sterile cotton swabs. Wells of 6 mm diameter were punched in the inoculated plates using a sterile cork borer. 100 µl of ethyl acetate extracts (5 mg/ml of 10 % dimethyl sulfoxide [DMSO]), standard (Streptomycin, 1 mg/ml) and DMSO (10 %) were transferred into respectively labeled wells. The plates were incubated at 37°C for 24 h and the zone of inhibition formed around the wells was measured. The experiment was repeated twice and the average reading was noted.

RESULTS

A total of 32 actinomycetes isolates were recovered on SCN agar from the rhizosphere soils of Shikaripura. In primary screening for antibacterial activity, all the actinomycete isolates showed inhibition of at least one test bacteria. 18 out of 32 isolates displayed inhibition of all test bacteria. Three isolates, designated as SRDP-S-03, SRDP-S-05 and SRDP-S-30 caused marked inhibition of test bacteria when compared to other actinomycete isolates (Table 1). The isolates SRDP-S-03, SRDP-S-05 and SRDP-S-30 displaying marked antibacterial activity in primary screening were subjected to further identification up to genus level. Cultural, microscopic, staining and biochemical characteristics of isolates were studied and the results are shown in Table 2. Diffusible pigments were not produced by any of these three isolates. The spore arrangement was found to be retinaculum apertum (hook) in case of isolates SRDP-S-03 and SRDP-S-05 whereas SRDP-S-30 showed flexibilis type of spore arrangement. The isolates were Gram positive and nonacid-fast. The isolates were positive for amylase, cellulase, catalase, citrase and nitrate reductase. Fermentation of glucose with only acid production (no gas) was exhibited by all isolates. Proteolytic activity was observed in all isolates as revealed by either gelatin liquefaction (SRDP-S-03) or casein hydrolysis (SRDP-S-05 and SRDP-S-30). Based on these features, these isolates were identified as members belonging to the genus *Streptomyces*. Table 3 shows the result of antibacterial activity of ethyl acetate extracts of the three *Streptomyces* isolates. It was observed that the extracts of all three isolates were effective against all the test bacteria with zone of inhibition ranging from 1.1 cm to 2.0 cm. The extracts were more effective in inhibiting Gram positive bacteria than Gram negative bacteria as revealed by wider zones of inhibition formed around the wells. Among Gram positive and Gram negative bacteria, *B. cereus* and *V. cholerae* were highly susceptible to extracts respectively. Inhibition of test bacteria by standard antibiotic was higher than that of ethyl acetate extracts. DMSO did not cause inhibition of test bacteria.

DISCUSSION

The rhizosphere is a region of soil present in vicinity to plant roots and is a unique biological habitat inhabited by a diverse type of microflora comprising bacteria, fungi, protozoa and algae. The rhizosphere is influenced by secretions of the plant roots called root exudates. These secretions nutritionally favour the microbial community in rhizosphere as exudates are rich in organic compounds such as vitamins, organic acids, amino acids etc. Actinomycetes, in particular the members of the genus *Streptomyces*, are one of the important rhizosphere inhabitants and are known to have qualitative and quantitative influence on the plant health as they enhance plant growth and protect the plant roots from phytopathogenic fungi. Most soil actinomycetes are saprophytic. Among the various actinomycetes genera in soil, species of *Streptomyces* are best recognized and well-studied in terms of their number and the bioactive metabolites which they produce. The *Streptomyces* species are aerobic, filamentous, spore forming actinomycetes with DNA rich in GC content (69 – 73 %). They produce extensively branching substrate and aerial mycelia. They are considered as prolific producers of bioactive microbial metabolites and accounts for > 75 % of biologically active compounds.

Table 1: Inhibition of Test Bacteria by Actinomycetes Isolates in Cross Streak Method

Isolates	*E. coli*	*S. flexneri*	*K. pneumoniae*	*B. cereus*	*V. cholerae*	*S. aureus*
SRDP-S-01	+	2+	2+	+	+	2+
SRDP-S-02	+	-	2+	2+	3+	+
SRDP-S-03	4+	3+	3+	+	+	2+
SRDP-S-04	2+	+	+	-	-	+
SRDP-S-05	4+	2+	3+	3+	2+	2+
SRDP-S-06	2+	2+	+	+	+	+
SRDP-S-07	2+	+	+	+	+	-
SRDP-S-08	+	+	+	+	+	+
SRDP-S-09	+	+	+	+	+	+
SRDP-S-10	+	-	+	-	+	+
SRDP-S-11	-	-	+	-	-	+
SRDP-S-12	+	2+	+	+	2+	+
SRDP-S-13	+	-	3+	-	+	-
SRDP-S-14	2+	-	-	2+	3+	+
SRDP-S-15	+	2+	+	2+	+	2+
SRDP-S-16	+	+	+	+	+	+
SRDP-S-17	2+	2+	2+	+	+	+
SRDP-S-18	+	+	2+	+	2+	+
SRDP-S-19	+	2+	2+	+	2+	2+
SRDP-S-20	+	+	+	+	2+	2+
SRDP-S-21	+	+	+	+	+	2+
SRDP-S-22	2+	2+	2+	2+	+	2+
SRDP-S-23	+	+	+	+	+	-
SRDP-S-24	+	+	+	+	+	+
SRDP-S-25	+	+	+	+	+	+
SRDP-S-26	+	+	+	+	+	+
SRDP-S-27	2+	-	-	+	-	2+
SRDP-S-28	2+	-	-	+	+	+
SRDP-S-29	2+	-	-	2+	+	+
SRDP-S-30	2+	3+	3+	3+	3+	2+
SRDP-S-31	2+	-	-	3+	+	3+
SRDP-S-32	3+	-	-	+	3+	2+

'-'No inhibition; '+' inhibition; '2+' moderate inhibition; '3+ and 4+' marked inhibition

Table 2: Characteristics of Isolates SRDP-S-03, SRDP-S-05 and SRDP-S-30

Characteristics	SRDP-S-03	SRDP-S-05	SRDP-S-30
Aerial mycelium	Creamish	White	Light brown
Substrate mycelium	Yellow	Light brown	Light brown
Diffusible pigment	None	None	None
Spore arrangement	Retinaculum apertum (Hook)	Retinaculum apertum (Hook)	Flexibilis
Gram's staining	Gram positive	Gram positive	Gram positive
Acid-fast staining	Nonacid-fast	Nonacid-fast	Nonacid-fast
Starch hydrolysis	+	+	+
Gelatin liquefaction	+	-	-
Casein hydrolysis	-	+	+
Cellulose hydrolysis	+	+	+
Catalse test	+	+	+
Citrase test	+	+	+
Nitrate reduction test	+	+	+
H_2S production	-	-	-
Sugar fermentation (Glucose)	Acid, no gas	Acid, no gas	Acid, no gas

Table 3: Antibacterial Activity of Ethyl Acetate Extracts of Isolates SRDP-S-03, SRDP-S-05 and SRDP-S-30

Test bacteria	Zone of inhibition in cm			
	SRDP-S-03	SRDP-S-05	SRDP-S-30	Streptomycin
E. coli	1.3	1.5	1.5	3.1
V. cholerae	1.3	1.8	1.6	3.7
S. flexneri	1.2	1.6	1.6	3.4
K. pneumoniae	1.2	1.1	1.3	2.9
B. cereus	1.6	2.0	1.7	4.2
S. aureus	1.5	1.9	1.8	4.0

Even though a huge number of antibiotics have been isolated from *Streptomyces*, the number represents only a small fraction of the repertoire of bioactive compounds produced. Hence, isolation of new *Streptomyces* from natural resources and characterization of their secondary metabolites is valuable[10-14]. In the present study, we have isolated 32 actinomycetes from rhizosphere soils of Shikaripura by serial dilution followed by plating on SCN agar. SCN agar has been widely used for isolation of actinomycetes[6,14-19]. The isolates were screened preliminary for antibacterial activity by Cross streak method. This method detects antibiotic producing ability of actinomycetes and has been commonly employed by researchers to screen the potent antimicrobial activity of actinomycetes[6,17,18,20]. From the result of primary screening, three isolates viz., SRDP-S-03, SRDP-S-05 and SRDP-S-30 were selected and characterized up to genus level. Morphology plays an important role in distinguishing *Streptomyces* from other sporing actinomycetes. Features viz., vegetative mycelium, aerial mycelium baring chains of spores and the characteristic arrangement of spores and the spore ornamentation provides the distinct features for microscopic characterization. The spore arrangement provides the key diagnostic information[21,22]. Morphological characteristics together with cultural and biochemical characteristics assist in the identification of *Streptomyces*[6,17,18,23,24]. In the present study, the cultural and microscopic characteristics of the isolates SRDP-S-03, SRDP-S-05 and SRDP-S-30 were consistent with their classification as a member of the genus *Streptomyces*. Throughout history, infectious diseases caused by bacteria, fungi, protozoa, helminthes and viruses have threatened mankind and caused massive mortality and morbidity. Antimicrobial agents especially antibiotics play an important role in the prevention and control of infectious diseases. However, the selective pressure exerted by the use of antimicrobial drugs forms the major driving force behind the emergence and spread of drug-resistant pathogens. Resistance has been developed in pathogens after discovery of major classes of antimicrobial drugs. The development of resistance varies in time from as short as 1 year as in case of penicillin to more than 10 years in case of vancomycin[25]. This alarming and threatening situation stimulated search for new antimicrobial agents. It is well known that microorganisms are an inexhaustible source of natural compounds having several therapeutic applications. In the present study, the ethyl acetate extracts of three *Streptomyces* species exhibited inhibition of test bacteria. It has been observed in the present study that Gram positive bacteria were susceptible to higher extent to ethyl acetate extract of *Streptomyces* species when compared to Gram negative bacteria. Similar results were observed in earlier studies of Hassan *et al.*[26], Anansiriwattana *et al*.[27], Al-Hulu *et al*.[28], Kekuda *et al*.[6], Valli *et al*.[29], Manasa *et al*.[30] and Gunda and Charya[31]. The low susceptibility of Gram negative bacteria could be attributed to the presence of an outer membrane that possess hydrophilic polysaccharides chains and forms an additional barrier for the entry of extract as well as antibiotics into the cells[32,33].

CONCLUSION

In the present study, antagonistic actinomycetes were isolated from soils of Shikaripura, Karnataka, India. Three actinomycete isolates identified as species of the genus *Streptomyces* were found to be promising as producers of bioactive secondary metabolites. Further studies on molecular characterization of these isolates and purification of bioactive components from the solvent extracts of these isolates are under progress.

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