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Antimicrobial Activity of Streptomyces halstedii, an Endophytic Streptomyces and its Charecterization Studies

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ABSTRACT

This study was designed to isolate and evaluate the antimicrobial potentials of endophytic Streptomyces from Catharanthus roseus, Phyllanthus niruri L. and Lantana camara L. While primary screening was performed by agar over lay method, secondary screening was done by well diffusion assay against various Gram positive and Gram negative bacterial strains. Upon primary and secondary screening one best antagonistic isolate was selected and secondary metabolite production was carried out by shake flask fermentation process in glucose–malt yeast extract (GMYE) broth and subjected to solvent extraction. The antimicrobial spectrum of each extract of the selected Streptomyces sp was studied using well diffusion and broth dilution method. The results show that methanolic extracts shows better antimicrobial activity against the other extracts. The methanolic extracts shows activity against three test strains E. coli, S. aureus and B. subtilis, followed by ethyl acetate and chloroform. No inhibitory activity against K. pneumoniae was observed for all the three extracts. The isolate was characterized based on its morphological, biochemical, cultural characteristics and identified as Streptomyces halstedii.

Keywords: *Antimicrobial activity, Endophytic Streptomyces, MIC.*

INTRODUCTION

Over the last few decades, products from natural resources have continued to play a major role in the drug discovery and development of therapeutics products. According to Newman and Cragg, around 50% of new drugs launched worldwide between 1981 and 2006 were of natural products and their derivatives [1]. Due to their rich source of bioactive metabolites, microorganisms were considered to be a greater interest next to plants. Research confirms that, more than 22,000 active compounds of biological origin have been produced from different forms of microbes by the end of the year, 2002. Almost half of them were contributed by actinobacteria, especially the genus *Streptomyces* considered as potential antibiotic producers compared to other microbes accounting for approximately 10,000 such products [2]. These actinobacteria have made an exceptional contribution to the health and well-being of people throughout the world [3].

Actinomycetes are the best producers of various antibiotics and secondary metabolites such as anti-inflammatory agents, antitumor agents, etc. [4]. Among actinomycetes *Streptomyces*, the Gram positive filamentous bacteria produce approximately 75% of commercially and medically useful antibiotics. About 60% of antibiotics produced by these ubiquitous organisms are used in agriculture. The major group of antibiotics produced by *Streptomyces* comprises anthracyclins, aminoglycosides, nucleosides, glycopeptides, macrolides, b-lactams, peptides, tetracyclines and polyethers [5, 6].

Bacterial endophytes are a diverse group of symbionts found virtually in every plant on earth. These organisms must have entered the host from soil or phyllosphere through wounds or openings [7]. The first endophytic actinobacteria to be identified and studied were the *Frankia* sp. These nitrogen fixing actinobacteria resemble legume-rhizobia in their evolution, structure and function in view of their symbiotic relationship with angiosperms [8].

The role of actinobacteria in soil and plant is very significant because of its ability to produce large number of secondary metabolites. Many of these metabolites posses antibacterial activity. They are the producers of about two-thirds of all the known antibiotics. While the genus *Streptomyces* alone produces nearly 80% of the actinobacterial antibiotics, the genus *Micromonospora* produces one-tenth as many as that of *Streptomyces*. Apart from being antibacterial the secondary metabolites are antifungal agents that degrade cell walls and inhibit the synthesis of mannan and β-glucan enzymes, anti-parasitic agents and insecticidal agents. The actinobacteria produces more than 60% of secondary metabolites produced by microorganisms and *Streptomyces* over 80% of it. Actinobacteria are able producers of plant growth regulatory compounds a few which are used commercially as herbicides. Apart from the above said secondary metabolites they are also the producers of compounds that functions as enzyme inhibitors, immunomodulators and antihypertensives [9].

The present study was aimed to isolate a potential endophytic *Streptomyces* sp for the production of antibacterial compounds effective against various human pathogens.

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MATERIALS AND METHODS

Chemicals:

The chemicals and reagents used in the present were of AR grade obtained were procured from Himedia Laboratories, India and Merck Specialties Pvt. Ltd. (Mumbai, India).

Sample collection:

Healthy plant leaves of three different plants, *Catharanthus roseus, Phyllanthus niruri* L. and *Lantana camara* L. were collected and washed with running water. Leaves were collected, labelled, placed in plastic bags, and stored inside a cooler until processed. The leaves were cut into segments (5×5 mm) aseptically and were subjected to surface treatment procedures to eliminate surface contaminating microbial communities.

Isolation of endophytic *Streptomyces* spp:

The leaf samples were washed with 70% alcohol to eliminate surface microbes followed by exposure to 95% ethanol and immersion in 0.9% NaOCl and allowed for drying. The leaves were aseptically removed and the tissues lying beneath were excised and placed onto the agar surface of sterile starch casein agar with following composition (g L^{-1}): soluble starch 10.0, casein 0.3g; KNO_3 2.0; NaCl 2.0, $MgSO_4.7H_2O$ 0.05, $CaCO_3$ 0.02, $FeSO_4.H_2O$ 0.01,

K_2HPO_4 2.0, agar 18.0, pH 7.0 (SCA) containing cyclohexamide (50 mg L^{-1}) at 25 °C. The actinomycetes, yielding an earthy odor in culture, seemed as likely candidates as potential streptomycetes were isolated and maintained in SCA slant till further studies [10].

Screening of *Streptomyces* spp for Antibacterial activity:

Primary screening: Agar overlay method:

Antimicrobial property of the *Streptomyces* was screened by agar overlay method [11] where the spores of isolated *Streptomyces* were streaked on Petri plate containing 15 mL of starch casein agar and incubated for five days at 28 ± 2 °C. Then, 10 mL of sterile soft nutrient agar (0.75 %) medium was mixed with 0.1 mL test bacteria (*E. coli* ATCC 8739 and *S. aureus* ATCC 29736) separately and overlaid with five days old growth of actinobacterial isolates and further incubated for 24 h at 37 °C. The *Streptomyces* producing antimicrobial compounds were selected based on the presence of inhibition zone in the plates.

Well diffusion method:

The positive isolates from the primary screening test was further subjected for antibacterial property against the pathogenic bacteria (*E. coli* and *S. aureus*) using well diffusion method. The selected positive isolates were inoculated into 100 mL of starch casein liquid medium and incubated at 28 °C for 7 days at 200 rpm in shaker incubator. After incubation, the cell free culture filtrates was collected by centrifuging the broth for 8,000 rpm for 10 minutes and tested for their antibacterial activity against pathogenic test strains. Briefly, 24 h old nutrient broth cultures of test bacteria were swabbed uniformly on solidified sterile nutrient agar plates using sterile cotton swab. Then, wells of 4 mm diameter were bored in the inoculated plates with the help of sterile cork borer and each extract were added and the plates were incubated at 37 °C for 24 h in upright position and the zone of inhibition formed around the well was measured. The *Streptomyces* extracts which shows maximum clearance zone was selected and used for further study.

Cultivation and extraction of metabolites:

The Streptomycete strain showing maximum antibacterial activity was transferred aseptically into 100 mL Erlenmeyer flasks, containing 50 mL glucose–malt yeast extract (GMYE) broth and incubated for 7 days at 25°C. One per cent (v/v) inoculum of each vegetative culture was then transferred into the same type of flasks with 100 mL glucose–malt yeast extract (GMYE) broth. These flasks were incubated for 7 days at 25°C with agitation (120 rpm) [12].

After incubation the broths were filtered through Whatmann No.1 filter paper and the filtrate was centrifuged for 8000 rpm for 10 minutes which was then transferred aseptically into a conical flask and stored at 4°C for further assay. To the culture filtrate, equal volume of various solvents (viz., chloroform, ethyl acetate and methanol) was added separately and centrifuged at 8000 rpm for 10 min at 4°C to extract the antimicrobial compound which was dried, stored at 4°C for further analysis.

Antimicrobial studies of solvent extracts:

The antimicrobial spectrum of each extracts of the selected *Streptomyces* spp was studied using well diffusion and broth dilution method. The compound obtained from each solvent was tested for their activity against the test pathogens, *E. coli* ATCC 8739, *S. aureus* ATCC 29736, *K. pneumoniae* ATCC 10031 and *B. subtilis* ATCC 6633 by well diffusion method.

Briefly, 24 h old nutrient broth (HiMedia, Mumbai) cultures of test bacteria were swabbed uniformly on solidified sterile nutrient agar (HiMedia, Mumbai) plates using sterile cotton swab. Then, wells of 6mm diameter were bored in the inoculated plates with the help of sterile cork borer and each extract (10mg/ml of sterile water) and standard (Streptomycin, 1mg/ml of sterile water) were added separately into respectively labeled wells. The inoculated plates were incubated at 37°C for 24 h in upright position and the zone of inhibition formed around the well was measured. The experiment was carried in triplicates to get average reading. The MIC (minimum inhibitory concentration) of active strain results were noted based on zone of inhibition.

MIC of the antibacterial agent produced by *Streptomyces* spp isolate was determined by the broth two-fold dilution method. The crude extract was serially diluted in Mueller Hinton broth (Difco, USA). Different concentrations of the pure extracts (µg/ml) were prepared from the stock solution. The tubes were incubated aerobically at 37°C for 24 hrs for growing of bacteria. After incubation, the tube with lower concentration of extract shows no growth was taken as the MIC value for the respective organism [13].

Identification of *Streptomyces* spp SES 18:

Purified isolates of actinomycetes were identified using PIBwin (probabilistic identification of bacteria) software. This software is based on various cultural, morphological and biochemical characteristics. A probabilistic identification matrix for *Streptomyces* is based on 50 characteristics like spore chain morphology, pigmentation, antibiotics, antibiotic sensitivity, growth tolerances and nutritional requirements [14].

Isolated strain was identified based on the morphological, physiological and biological characterization [15].

Physiological and Biological characteristics:

Media used were those recommended by Shirling and Gottlieb [16] in the International *Streptomyces* Project (ISP) and by Waksman [17]. Mycelium was observed after incubation at 28°C for two weeks. Colors were determined according to Prauser [18]. Temperature range for growth was determined on inorganic salts starch agar medium (ISP 4) using a temperature gradient incubator.

RESULTS AND DISCUSSION

The present study was aimed to exploit the healthy plants for the isolation of endophytic *Streptomyces* spp with antimicrobial potential. Actinomycetes are known well for their source of secondary metabolites. Recent studies also confirm that among the 10,000 antibiotics reported; more than half are produced by streptomycetes. Several antimicrobial substances were isolated and characterized from different actinomycetes which include anthracyclines, aminoglycosides, macrolides, betalactams, glycopeptides, peptides, nucleosides, polyenes, polyester, polypeptides, actinomycins and tetracyclines [19]. Most of the antibiotic productions are extracellular in nature which are normally secreted in culture media and have been used as drugs, herbicides, anticancer agents, immunoregulators and antiparasitic drugs [20-22].

In the present study, healthy plant leaves of *C. roseus, P. niruri and L. camara* were processed for endophytic isolation of *Streptomyces* spp. A total of 21 isolates (7 from *C. roseus,* 6 from *P. niruri* and 8 from *L. camara*) were obtained and all the isolates were inoculated into the starch casein agar slants for further study. Among the 21 isolates 3 isolates gave positive results when screened for antibacterial activity against both the pathogens, *S. aureus* and *E. coli* using over agar lay method, further those 3 isolates was subjected to secondary screening. The secondary screening antimicrobial evaluation for the selected strains was performed by well diffusion method. Among the culture filtrates of three selected strains, one showed clearance zone against the two test strains, *E. coli* and *S. aureus* and was chosen as potential strain and used for further study.

The major advantage of actinomycetes include, their major role in various ecological processes in different habitats such as plant and animal degradation in soils, hydrocarbons degradation in the polluted soil, controlling of soil nature by nitrogen fixation and production of several secondary metabolites [23]. Mostly, actinomycetes grow and colonize the soil which makes easy to isolate them from internal cortical tissues of the roots where they grow [24], however the special features of these endophytic population is connected with their physiology. As a consequence, several streptomycetes exhibit similar physiological characteristics which are also considered discriminating in the numerical taxonomy of the genus. This aspect confirms that endophytic microorganisms have peculiar characteristics which favour their growth inside plant tissues [25, 26].

The shake flask fermentation process for the extraction of antimicrobial metabolites was carried out for 7 days at 25°C using glucose–malt yeast extract (GMYE) broth. After incubation, cell free extracts were collected by filtration and centrifugation. The clear filtrates containing the active metabolite were extracted using equal volume of various solvents (viz., chloroform, ethyl acetate and methanol) and which was further analyzed for antimicrobial evaluation against test strains. The various extracts of the selected *Streptomyces* spp. was studied for antimicrobial activity using well diffusion and broth dilution method for the test pathogens, *E. coli* ATCC 8739, *S. aureus* ATCC 29736, *K. pneumoniae ATCC 10031* and *B. subtilis ATCC 6633*. The results of the antimicrobial spectrum of the selected strains against the pathogens are given in the table 1. The results show that methanolic extracts shows better

antimicrobial activity against the other extracts. The methanolic extracts shows activity against three test strains *E. coli, S. aureus* and *B. subtilis*, followed by ethyl acetate and chloroform. There is no inhibitory activity against *K. pneumoniae* was observed for all the tested three extracts. Based on the results, the methanolic extracts of the *Streptomyces* spp. was evaluated for minimum inhibitory concentration against the three test strains *E. coli, S. aureus* and *B. subtilis*. The minimum inhibitory concentration of the methanolic extract was found to be 12.5 µg/ml against *E. coli* and 6.25 µg/ml against *S. aureus* and *B. subtilis* (table 2).

Prashith Kekuda and co-workers investigated the biological properties of crude extract developed from soil *Streptomyces* species isolated from Agumbe, Karnataka, India. They screened the antimicrobial activity using both primary and secondary screening method by Cross streak method and agar diffusion method, respectively [27]. Maximum activity was found to be against Gram positive bacteria compared to the Gram negative pathogens; similarly, *Candida albicans* showed greater sensitivity compared to *Cryptococcus neoformans*. Rajput and their co-workers studied the antimicrobial property seven *Streptomyces* strains isolated from subterranean cave against different pathogens. They also found that greater antibacterial activity resulted against *E. coli* when compared with *S. aureus* and *P. aeruginosa* [28]. In a different study, Arasu and co-workers isolated an actinomycetes isolate ERI-26 from Nilgiri forest soil of Western Ghats and investigated its antimicrobial activity against different pathogenic bacteria. They also found that maximum antibacterial activity was determined against *B. subtilis, S. aureus, S. epidermidis* and *Enterococcus faecalis*. Further, MIC of the methanol fractions of ERI-26 was also performed using microdilution technique showing potential activity against *S. epidermidis* (375 µg/ml) [29]. Afifi and co-workers, produced an aminoglycoside antibiotic, Hygromycin B which has an potential antibacterial and antifungal activity by directly inhibiting protein synthesis. They have isolated potential *Streptomyces* sp. AZ151 from soil and identified using both conventional and molecular sequencing method to confirm as *Streptomyces crystallines* [30].

Cao et al. isolated enophytic *Streptomyces* strains from the surface of the tomato roots and investigated their potential antimicrobial activity against various pathogens [31]. Similarly, Gangwar et al. isolated 40 endophytic actinomycetes from root, stem and leaf tissues of three medicinal plants, namely *Aloe vera, Mentha arvensis* and *Ocimum sanctum.* They also reported that maximum isolates was recovered from roots (7 %) compared to the stems (17.5% and leaves (12.5%); interestingly *Streptomyces* sp alone contributes about 60% of the total isolates [32]. Ghadin and co-workers isolated *Streptomyces* (SUK 06) from *Thottea grandiflora* and investigated the antimicrobial property of ethyl acetate extracts against various pathogens. They also found that maximum activity was observed against MRSA ATCC 700699 with inhibition zones of 37 mm, followed by *Bacillus cereus* (22 mm) and *Pseudomonas aeruginosa* ATCC 27853 (20 mm). In addition they also studied the antifungal activity and the results confirmed that maximum inhibition percentage occurred against *Fusarium solani* with 62%; followed by *Aspergillus fumigatus* with 44% and *Phytophthora erythroseptica* and *Geothrichum candidum* showing 23% [10].

The spore-bearing hyphae of the strain were Rectus-Flexibilis (RF), spore mass was grey or white, spore surface was smooth; substrate mycelium was light brown without any diffusible pigment. The isolate was Gram positive and non motile. The isolate was found to hydrolyse chitin and gelatin but not caesin. Rhamnose and inositol were utilized but sucrose and maltose were not. Hydrogen sulfide was not produced. The isolate produced brown pigment. The cultural characteristics of the selected isolate SES18 are shown in table 3. The isolate *Streptomyces* spp. grew well on starch casein medium. Particularly, the colonies were convex and some part of the aerial mycelia and spore chains could be observed around the colony margin. Aerial mycelia were initially white, becoming grey colour. Melanin and other soluble pigments were not produced. The physiological properties were also shown in table 3. There was growth at 45°C and pH 8.0.

Good growth was observed at 45°C, pH 8.0 and NaCl concentration of 8% which could be intended to be moderate halophilic actinomycete. No melanoid pigments were produced. It showed positive results for utilization of carbon sources such as rhamnose and inositol. However, carbon sources D-fructose, L-arabinose, sucrose, mannitol, raffinose, xylose, and galactose could not be utilized. It showed resistance to ampicillin, penicillin and streptomycin. The morphological, physiological and biochemical characterization based on ISP [16] and Bergey's manual of Systematic Bacteriology and ISP strongly suggested that the strain belonged to the genus *Streptomyces* [33]. The isolate SES18 was identified using PIBWin which provides probabilistic identification of unknown isolates against identification matrices of known strains. The results were confirmed on the basis of the highest ID score of the organism. The isolate SES 18 showed PIBWin ID score of 0.99 and hence has been identified as *Streptomyces halstedii*.

Shetty and co-workers have isolated actinomycetes strain from marine soil sediments of Visakhapatnam sea coast, Bay of Bengal. Among the ten strains isolated, *Streptomyces parvulus* RSPSN2 produced antibacterial compound, a polypeptide antibiotic (Actinomycin D) which was purified and characterized by thin layer chromatography (TLC), column chromatography (CC), UV-visible, Fourier transform infrared spectroscopy (FTIR) and Nuclear magnetic resonance (NMR) techniques [34]. Taechowisan and co-workers investigated the antimicrobial potential of endophytic *Streptomyces* sp. against *Colletotrichum musae* and *Fusarium oxysporum*. They also reported that out of 330 isolates, 212 were recovered from roots, 97 from leaves and 21 isolates were from stem with a prevalence ratio of 3.9, 1.7 and 0.3%, respectively. They have identified the endophytic actinomycetes based on their microscopic and macroscopic morphology, amino acid composition of the cell extract and found that, *Streptomyces* sp. (n = 277) were predominant followed by Microbispora sp. (n = 14), Nocardia sp. (n = 8) and Micromonospora sp. (n = 4) [35].

Similar studies were also reported by various researchers who investigated the antimicrobial properties of *Streptomyces* spp. metabolites against various Gram positive and Gram negative pathogens [36 – 40].

Table No. 1: Antimicrobial activity various extracts of *Streptomyces* isolate against bacterial pathogens

Extracts	Pathogenic bacterial strains			
	***E. coli* ATCC 8739**	***S. aureus* ATCC 29736**	***K. pneumoniae ATCC 10031***	***B. subtilis ATCC 6633***
	Inhibition zone (mm)			
Chloroform	5 mm	7 mm	-	6 mm
Methanol	10 mm	16 mm	-	13 mm
Ethyl acetate	6 mm	6 mm	-	6 mm
Streptomycin	11 mm	14 mm	-	13 mm

Table No. 2: Effect of the Methanolic extract of *Streptomyces* sp on bacterial pathogens

Strains	MIC (mg/ml)
***E. coli* ATCC 8739**	12.5
***S. aureus* ATCC 29736**	6.25
B. subtilis ATCC 6633	6.25

Table No. 3: Morphological, biochemical and physiological characteristics of the *Streptomyces* isolate

Characteristic feature	Result
Morphology	
Gram staining	Gram positive

Motility	-
Spore chain	Rectus flexible
Spore mass	Grey
Pigmentation of substrate mycelium	+
Test for Diffusible pigment	-
Test for Melanin production	-
Test for H_2S production	+
Test for Nitrate reduction	+
Growth	
At 45°C	+
NaCl tolerance	8%
Test for Gelatin hydrolysis	+
Test for Pectin hydrolysis	-
Test for Chitin hydrolysis	+
Utilization of carbon sources	
L – arabinose	-
D – fructose	-
Sucrose	-
Ramnose	+
Mannitol	-
D – xylose	-
Raffinose	-
M – inositol	+
Galactose	-
Antibiotic resistance	
Ampicillin	+
Chloramphenicol	-
Nalidixic acid,	-
Nystatin	-
Rifampicin	-
Penicillin	+
Streptomycin	+

CONCLUSION

Many endophytic streptomycetes isolated from plants are precious resources which possess remarkable antimicrobial activity. The obtained results show the potential use of endophytic forms as antibacterial agents against both Gram positive and Gram negative forms. Thus these endophytic forms can be exploited as an alternative source for the production of bioactive compounds that cannot be easily synthesized by chemical methods. Further understanding and analysis of the compound will enhance the possibility of its use as potential antimicrobial agent.

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