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Original Article

STREPTOMYCES CELLULOSAE VJDS-1, A PROMISING SOURCE FOR POTENTIAL BIOACTIVE COMPOUNDS

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ABSTRACT

Objectives: The aim of the present study was to isolate, identify and analyze the phylogenetic characteristics of the potent actinobacterial strain VJDS-1 with antagonistic activities isolated from Mangrove ecosystems of Nizampatnam, Guntur Dist, A.P., India.

Methods: Soil samples collected were pre treated with calcium carbonate and used for isolation of potent actinobacterial strain designated as VJDS-1. Identification of the strain was carried out by studying the micro morphological, cultural, biochemical and physiological methods. The Phylogenetic study of the strain was carried out by employing 16S rDNA sequence based analysis. Phylogenetic tree was constructed using the MEGA (Molecular Evolutionary Genetic Analysis) software version 6.

Results: The potent actinobacterial strain was identified as *Streptomyces cellulosae* VJDS-1 and the bioactive metabolites produced by the strain inhibited Gram positive bacteria (*Staphylococcus aureus, Bacillus megaterium*), Gram negative bacteria (*Xanthomonas campestris, Proteus vulgaris*, *Pseudomonas aeruginosa and Escherichia coli*) and fungi (*Aspergillus niger, Botrytis cinerea, Fusarium solani F. oxysporum and Candida albicans*).

Conclusion: The results of the experiment showed that the crude ethyl acetate extract of *Streptomyces cellulosae* VJDS-1 showed significant antimicrobial potential hence it can be used for isolation of compounds with pharmaceutical importance.

Keywords: Mangrove ecosystems, Phylogenetic study, *Streptomyces cellulosae*, Bioactive compounds.

INTRODUCTION

Marine bacteria produce unique and novel secondary metabolites with interesting biological activities. Among different types of marine bacteria, actinomycetes play an extensive role in the pharmaceutical and medical industry for their capacity to produce secondary metabolites with diverse chemical structures and biological activities. Thousands of bioactive compounds have been isolated and characterized, many of which have been developed into drugs for treatment of wide range of diseases in human, veterinary and agriculture sectors [1]. The salinity, low temperature, pressure and stress conditions in marine environments provoke a set of different metabolic pathways and defense systems compared to their terrestrial counterparts, ensuring the potential ability to produce novel antibiotics.

The genus *Streptomyces* consists of 640 species with validly published names [2]. Since the discovery of streptomycin, various pharmaceutically important drugs have been discovered from the genus *Streptomyces* [3-6] and exploration of the natural environment with the aim of discovering novel species of this genus is important. In addition, characterization of the physiological and genotypic features of members of this genus will broaden the understanding of the behavior of these organisms in various ecosystems.

The complex metabolic machinery of *Streptomycetes* can produce an infinite variety of secondary metabolites, including dark-brown to black melanoid pigments that play important ecological roles in the environment [7, 8]. The competitive superiority of some *Streptomyces* species in the rhizosphere protects plants against root pathogens [9, 10], which could be important in the early stages of plant establishment [11].

As part of our ongoing screening of different habitats of our near marine ecosystems of Guntur district resulted in the isolation of a potent strain VJDS-1 with a broad spectrum activity against different Gram positive and Gram negative bacteria as well as fungi. An attempt was made in the present study to identify the strain based on the polyphasic taxonomic approach along with its antimicrobial profile.

MATERIALS AND METHODS

Sample collection

Soil samples were randomly collected from mangrove ecosystem of Nizampatnam, Guntur district, south coast of Andhra Pradesh, India. They were brought to the laboratory in sterilized containers and air dried for 2-5 days at room temperature (30±2 °C). The air dried soil samples were pre treated with $CaCO_3$ (10:1w/w) and incubated at 30 °C for four days [12].

Isolation

Dilution plate technique was employed for isolation of actinobacteria by employing humic acid vitamin (HV) agar medium [13]. The medium was adjusted to pH 7.0 and 0.1 mL of diluted soil sample spread over HV agar supplemented with 25 µg/ml Nystatin and 25 µg/ml Streptomycin to reduce the fungal and bacterial contamination respectively and incubated at 30±2°C for 7 days. Actinobacterial colonies [14] were picked out, purified and preserved on YMD (yeast extract malt extract dextrose) agar slants at 4 °C [15]. The actinobacterial strains were then screened for their potential to generate bioactive compounds [16]. Among the 20 isolates tested for biological activity, one isolate designated as VJDS-1 was found to be potent as it exhibited high antimicrobial activity.

Identification of the potent strain VJDS-1 by polyphasic approach

Morphological, Cultural, Physiological and biochemical characteristics of the strain

The potent actinobacterial strain was characterized by cultural, morphological, physiological, biochemical and molecular methods. The microscopic characterization was carried out by slide culture method [17] taking into account the nature of mycelium, color and spore arrangement [18]. The morphological characteristics were assessed using scanning electron microscopy (SEM: Model-JOELJSM 5600, Japan) of 4-day old culture grown on yeast extract malt extract dextrose agar (YMD) medium at various magnifications. The strain was grown on seven International Streptomyces Project (ISP) media and four non-ISP media to observe the cultural characteristics such

as color of aerial mycelium, substrate mycelium, pigment production and spore formation [19]. Melanin pigment production was assessed by culturing the strain on tyrosine agar (ISP-7) medium [20]. Hydrolysis of starch and nitrate reduction [21] and H_2S production were also tested [22]. Physiological characteristics such as the effect of pH (5-9), temperature (20-45 °C) and salinity on the growth of the strain analyzed. The susceptibility of the strain to different antibiotics was also determined by paper disc method [23].

Molecular identification

The genomic DNA used for the PCR (Polymerase Chain Reaction) was prepared from the colonies grown on YMD agar for 3 days. The total genomic DNA extracted from the strain was isolated by employing the DNA purification Kit (Pure Fast® Bacterial Genomic DNA purification kit, Helini Bio molecules, India) according to the manufacturer protocol. The 16S rRNA gene fragment was amplified using Actino specific forward Primer-5'-GCCTAACACATGCAAGTCGA-3' and actino specific reverse primer-5'-CGTATTACCGCGGCTGCTGG-3'. Conditions of the PCR were standardized with initial denaturation at 94 ℃ for 3 min followed by 30 cycles of amplification (Denaturation at 94 °C for 60 sec, annealing temperature of 55 ℃ for 60 sec and extension at 72 °C for 60 sec) and an addition of 5 min at 72 °C as final extension. The amplification reactions were carried with a total volume of 50 µl in a Gradient PCR (Eppendorf, Germany). Each reaction mixture contained 1 µl of DNA, 1 µl of 10 p mol forward 16S Actino specific primer (5´-AAATGGAGGAAGGTGGGGAT-`3), 1 µl of 10 pmol reverse 16S Actino specific primer (5´-AGGAGGTGATCCAACCGCA-`3), 25 µl of Master Mix and 22 µl of molecular grade nuclease free water. The separation was carried out at 90 Volts for 40 min in TAE buffer with 5 µl of Ethidium bromide. PCR product was analyzed using 1 % agarose gel and the fragment was purified (Helini Pure Fast PCR clean up kit, Helini Bio molecules, India) as per the manufacturer's instructions. The bands were analyzed under UV light and documented using Gel Doc. The direct sequencing of PCR products was performed by dideoxy chain termination method using 3100-Avant Genetic Analyzer (Applied Bio systems, USA). The sequences thus obtained were analyzed for homology using BLASTN (Entrez Nucleotide database). The deduced 16s rDNA sequence was compared with the sequences in GenBank (http://www. ncbi. nlm. nih. gov/) using the Basic Local Alignment Search Tool (BLAST) then aligned with the related reference sequences retrieved from GenBank databases using the Clustal *W* method. Phylogenetic and molecular evolutionary analyses were conducted using MEGA (Melecular Evolutionary Genetic Analysis) version 6.0 [24].

Nucleotide sequence accession numbers

The 16S rRNA gene (rDNA) sequence of the strain VJDS-1 is registered in the Gen Bank database.

Growth pattern of the strain VJDS-1

To determine the growth pattern, the strain was inoculated into 250 ml flasks containing 100 ml YMD broth and incubated at 30±2 ℃ on a rotary shaker at 180 rpm. The flasks were harvested at 24 h interval and the growth of the strain was determined by taking the dry weight of biomass. The culture filtrates obtained after separating the biomass were extracted with ethyl acetate and antimicrobial activity of crude extract was determined by agar well diffusion method.

Extraction of metabolites and antimicrobial assay

The antimicrobial activity of the strain was determined by agar well diffusion assay. The homogenous culture suspension prepared by suspending three day old culture in sterile saline was used to inoculate YMD broth (seed medium) and the culture was incubated at 30 °C for 48 h on a rotator shaker at 180 rpm. Seed culture at the rate of 10 % was transferred to YMD broth (Fermentation medium). The fermentation was carried out at 30±2 ℃ for 120 h under agitation at 180 rpm. Antimicrobial compound was recovered from the filtrate by solvent extraction method. Ethyl acetate was added to the filtrate (1:1) and shaken vigorously. The ethyl acetate extract was evaporated to dryness in the water bath and the residue thus obtained was used to determine antimicrobial activity. Ethyl acetate itself was used as negative control. 80µl of the crude extract and 80µl of negative control were poured in to separate wells. The standard antibiotic disc was placed on the agar surface as positive control. For each bacterial strain, controls were maintained utilizing pure solvent. Plates were incubated at 37 °C for 48 h and inhibition zones (in mm) were measured after 24-48 h. Experiment was carried out in triplicates for each test organism and the mean values were computed.

Test organisms

Bacteria: *Staphylococcus aureus* (MTCC 3160), *Lactobacillus casei, Bacillus megaterium* (NCIM 2187), *Xanthomonas campestris* (MTCC 2286), *Proteus vulgaris* (ATCC 6380), *Pseudomonas aeruginosa* (ATCC 9027), *Salmonella entericus and Escherichia coli* (ATCC 9027).

Fungi: *Aspergillus niger, Botrytis cinerea, Fusarium solani F. oxysporum* and *Candida albicans* (MTCC 183).

RESULTS AND DISCUSSION

A total of 20 actinobacterial strains were isolated from the mangrove ecosystem of Nizampatnam designated as VJDS-1 to VJDS-20. Among the 20 strains, one actinobacterial strain VJDS-1 was found to be potent and exhibited strong antimicrobial activity against Gram positive and Gram negative bacteria. The strain VJDS-1 exhibited typical morphological characteristics of the genus ***Streptomyces***. Morphological and micro morphological observation of the strain was studied by SEM and revealed that the strain exhibited heavy sporulation with spiral arrangement and the surface of the spore was rough (fig. 1).

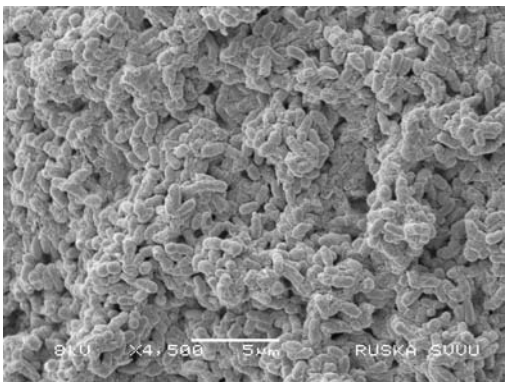

Fig. 1: Scanning electron microscopic photograph of *Streptomyces cellulosae* VJDS-1

Cultural characteristics

The cultural characteristics of the strain are represented in table 1. The strain VJDS-1 exhibited good growth on Tryptone yeast extract agar (ISP-1), YMD agar (ISP-2), Glycerol asparagine agar (ISP-5) and Glucose tryptone agar (Non ISP). The growth was moderate on Nutrient agar (Non ISP) and Starch casein salts agar (Non ISP). The colour of aerial mycelium was white and substrate mycelium was pale brown on the different media tested. Soluble pigment production was not observed on the media tested.

Biochemical and physiological characteristics of the strain VJDS-1

The physiological tests are significant tools for classification and identification of Actinobacteria and influencing the growth rate of Actinobacteria [20-22]. Details of biochemical and physiological characteristics of the strain are given in table 2. The strain VJDS-1 exhibited the positive response.

To catalase production, H_2S production and citrate utilization. Growth of the strain VJDS-1 occurred in the pH range of 5-10 with optimum growth at pH 7 and the range of temperature for growth, was 25-55 °C with the optimum being 30 °C. Sodium chloride tolerance of the strain was also studied as the salt concentration has a profound effect on the production of antibiotics from

microorganisms. The strain could grow well in the medium supplemented with 3% sodium chloride and shown tolerance upto 9%. Though the strain utilized a wide range of carbon sources, the growth was good when supplemented with glucose. The strain was resistant to the majority of antibiotics tested and showed sensitivity to gentamicin, kanamycin and ciprofloxacin.

Table 1: Cultural characteristics of the strain VJDS-1

Name of the medium	Growth	AM*	SM**	Pigmentation
Tryptone yeast-extract agar (ISP-1)	Good	White	Pale Brown	No
Yeast extract malt extract dextrose agar (ISP-2)	Good	White	Pale Brown	No
Oat-meal agar (ISP-3)	Poor	White	Pale Brown	No
Inorganic salts Starch Agar (ISP-4)	Poor	White	Pale Brown	No
Glycerol Asparagine agar (ISP-5)	Good	White	Pale Brown	No
Tyrosine agar (ISP-7)	Poor	White	Pale Brown	No
Czapek-Dox agar	Poor	White	Pale Brown	No
Nutrient agar	Moderate	White	Pale Brown	No
Bennets agar	Poor	White	Pale Brown	No
Starch casein salts agar	Moderate	White	Pale Brown	No
Glucose tryptone agar	Good	White	Pale brown	No

*Aerial mycelium **Substrate mycelium

Table 2: Morphological, biochemical and physiological characteristics of the strain VJDS-1

Character	Response
Morphological characters	
Sporophore morphology	Rectiflexible
Color of aerial mycelium	White
Color of substrate mycelium	Pale yellow to brown
Biochemical characters	
Catalase production	-
Urease production	-
Hydrogensulfide production test	+
Nitrate reduction	-
Starch hydrolysis	+
Gelatin liquefaction	-
Methyl red test	-
Voges proskauer test	-
Indole production	-
Citrate utilization	+
Physiological characters	
Gram reaction	+
Production of melanin pigment	-
Range of temperature for growth	25-55 °C
Optimum temperature for growth	30 °C
Range of pH for growth	5-10
Optimum pH for growth	7
NaCl tolerance	Up to 9%
Utilization of carbon sources (w/v)*	
Lactose	-
Maltose	-
Raffinose	-
Sucrose	+
Arabinose	+
D-Glucose	+++
Galactose	+
Fructose	++
Starch	++
Mannitol	+
Cellulose	+
Antibiotic sensitivity	
Gentamicin (10µg)	S
Kanamycin (10µg)	S
Penicillin (10µg)	R
Co-trimoxazole (25µg)	R
Ciprofloxacin (10µg)	S
Erythromycin (10 µg	R
Lincomycin (10µg)	R
Cefuraxime (30µg)	R

* Growth of the strain measured as dry weight of the mycelium '+++'-good growth; '++'-moderate growth; '+'-weak growth; '-'indicates negative/no growth; S–Sensitive; R–Resistant; P–Positive; N–Negative.

Molecular characterization

Analysis of the 16S rDNA gene sequence of the strain VJDS-1

The 16S rDNA sequence data supported the assignment of the strain VJDS-1 to the genus *Streptomyces* and species *cellulosae.* The partial 16S rDNA sequence of the strain VJDS-1 was submitted to the GenBank database under an accession number KP170478. The partial sequence was aligned and compared with all the 16S rDNA gene sequence available in the GenBank database by using the multi sequence advanced BLAST comparison tool.

The phylogenetic analysis of the 16S rRNA gene sequence was aligned using the CLUSTAL W programme from the MEGA 5 Version. Phylogenetic tree (Fig-2) was constructed using MEGA software Version 5 using Neighbor-joining method. The topology of the constructed tree was evaluated by bootstrap analysis with 1000 re samplings by Maximum parsimony tool.

Fig. 2: Neighbor-Joining tree based on partial 16S rRNA gene sequence showing relationship between *Streptomyces* strain VJDS-1 and related members of the genus *Streptomyces*

Growth Pattern and antimicrobial profile of *Streptomyces cellulosae* VJDS-1

The growth pattern of *Streptomyces cellulosae was* studied on YMD broth. The stationary phase of the strain extended from 120 hr to 144 h of incubation. The bioactive metabolites obtained from 5-day-old culture exhibited high antimicrobial activity against the test microorganisms (fig. 3). Naragani *et al.* (2014) reported that metabolites obtained from five day old culture of *Rhodococcus erythropolis* VLK-12 showed maximum antimicrobial activity [25]. Narayana *et al.* (2004) showed that *Streptomyces* sp. isolated from virgin soil elaborated maximum antimicrobial metabolites production after 120 h. Narayana *et al.* (2007) stated that *Streptomyces albidoflavus* elaborated maximum antimicrobial metabolites production after 120 h.

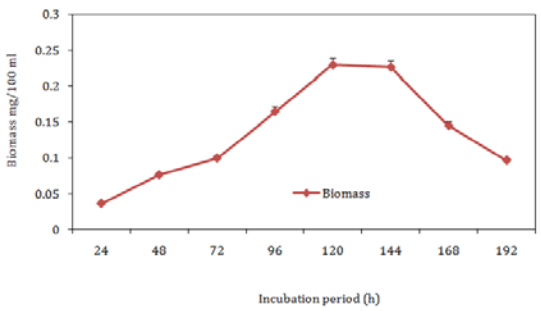

Fig. 3: Growth pattern of the strain *Streptomyces cellulosae* VJDS-1

The secondary metabolites obtained from four-day old culture of *Nocardia levis* MK-VL_113 [26], *Streptomyces tendae* TK-VL_333 [27], *S. cheonanensis* VUK-A [28] showed high antimicrobial activity against the test microbes. The antimicrobial spectrum of the strain cultured on YMD broth for five days was shown in table 3.

The metabolites extracted from the five day old culture broth showed maximum activity against *Proteus vulgaris*, *Bacillus megaterium* and *Xanthomonas campestris*. In case of fungi *Candida albicans* showed maximum sensitivity when compared to the other fungi tested.

Table 3: Antibacterial and antifungal activity of *Streptomyces cellulosae* VJDS-1

Test organism	Zone of inhibition (mm)	Positive control #
Bacteria:		
Staphylococcus aureus	20±0.15	22±0.05
Lactobacillus casei	20±0.08	28±0.10
Xanthomonas campestris	22±0.12	25±0.12
Bacillus megaterium	24±0.14	25±0.08
Escherichia coli	16±0.09	20±0.05
Pseudomonas aeruginosa	18±0.11	22±0.15
Salmonella entericus	10±0.14	15±0.12
Proteus vulgaris	26±0.11	28±0.08
Fungi:		
Aspergillus niger	16±0.05	20±0.05
Botrytis cinerea	15±0.08	28±0.08
Fusarium solani	16±0.12	21±0.05
F. oxysporum	15±0.12	18±0.12
Candida albicans	27±0.05	30±0.05

#Positive control: Tetracycline against bacteria, Griseofulvin against yeast and Carbendazim against fungi, Values are mean±S. E. M (n = 3), Mean percent inhibition on bacteria: 84.3%, Mean percent inhibition on fungi: 76.0%.

CONCLUSION

The present investigation highlights the antimicrobial potential of *Streptomyces cellulosae* VJDS-1. The results state important evidence of use of crude ethyl acetate extract for isolation of antimicrobial compounds. Further study on optimization, purification and chemical characterization of bioactive compounds of the strain are in progress.

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CONFLICT OF INTERESTS

Declared None

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