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Research Article

ISOLATION AND CHARACTERIZATION OF BIOACTIVE *STREPTOMYCES* FROM MANGROVE ECOSYSTEM OF MACHILIPATNAM, KRISHNA DISTRICT, ANDHRA PRADESH

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ABSTRACT

Objective: The aim of the present study was to isolate, identify, and analyze the taxonomic characteristics of the potent actinobacterial strains VJSY-2 and VJSY-3 isolated from mangrove soils of Gilakaladindi, Machilipatnam, Krishna District of Andhra Pradesh.

Methods: Soil samples pretreated with calcium carbonate were used for isolation of potent actinobacterial strains. Identification of the strains was carried out by studying the cultural, morphological, biochemical, and physiological characteristics. The phylogenetic study of the strains was carried out by employing 16S r DNA sequence-based analysis. Phylogenetic tree was constructed using the molecular evolutionary genetic analysis software version 6.

Results: Based on the polyphasic taxonomic studies, the potent strains belong to *Streptomyces* genus. The bioactive metabolites produced by the strains were active against Gram-positive bacteria (*Staphylococcus aureus, Bacillus subtilis,* and *Bacillus megaterium*), Gram-negative bacteria (*Xanthomonas campestris, Pseudomonas aeruginosa*, and *Escherichia coli*), and fungi (*Aspergillus niger, Fusarium solani, Fusarium oxysporum*, and *Candida albicans*).

Conclusion: The results of the experiment showed that the crude ethyl acetate extract of the strains VJSY-2 and VJSY-3 showed significant antimicrobial potential; hence, they can be used for isolation of compounds with pharmaceutical importance.

Keywords: Mangrove *Actinobacteria*, *Streptomyces,* Bioactive metabolites.

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INTRODUCTION

Microorganisms are capable of carrying out a tremendous variety of reactions with the ability to have adapted to a range of environments allowing them to be transplanted from nature to the laboratory. They can be grown on inexpensive carbon and nitrogen sources to produce valuable compounds [1]. Secondary metabolites of microbial origin are extremely important to our health and nutrition and have a tremendous economic importance due to their biological activity. Screening of microbial natural products continues to represent an important route to the discovery of novel chemicals, for development of new therapeutic agents and for evaluation of the potential of lesser-known or new bacterial taxa [2].

Streptomyces is a well-explored genus of Gram-positive bacteria in the phylum *Actinobacteria*. These prokaryotes present a strikingly similar lifestyle to that of filamentous fungi and, as do fungi, most *Streptomycetes* live as saprophytes in the soil [3]. Currently, it is reported that there are more than 2,400 different secondary metabolites produced by *Streptomyces* spp. The genus *Streptomyces* was first described by Waksman and Henrici, in 1943, and abundant with more than 668 species as reported recently (http://www.bacterio.net/streptomycesa.html). Since the discovery of streptomycin from *Streptomyces griseus* and various other pharmaceutically important drugs, studies have shown that over 74% of current antibiotics were derived from genus *Streptomyces* [4]. Given the importance of the *Streptomyces* as a source of pharmaceuticals, exploration of the natural environment with the aim of discovering novel species in this genus is important. In addition, characterization of the physiological and genotypic features of members of this genus will broaden understanding of the behavior of these organisms in various ecosystems. As per the accumulated knowledge and statistical data, the bioactive potency of the *Streptomyces* species should not be underestimated and their capacity to produce promising new compounds as well. Since long time they have been producing, the majority of the chemotherapeutically applied antibiotics.

Streptomyces spp. is distributed in a variety of habitats such as soil, freshwater, and marine environments particularly abundant in the soil and rhizosphere which can be distinguished by physiological and morphological characteristics, chemical composition of cell walls, type of peptidoglycan, phospholipids, fatty acids chains, percentage of GC content, 16S rRNA analysis, and DNA-DNA hybridization [5]. In terms of number and variety of identified species, *Streptomyces* represents one of the largest taxonomic items of recognized *Actinomycetes* [6]. The search for the pharmacological targets as well as the synthesis of modified natural compounds will keep on moving in the process of drug discovery. In connection with the excellent track record of *Streptomyces* with antimicrobial activity, an attempt was made in the present study to explore the diversity as well as the antimicrobial activity of the *Streptomyces* from the mangrove soils of Gilakaladindi, Machilipatnam, Krishna District of Andhra Pradesh. In the present study, an attempt was made to reveal the taxonomic characteristics of the strains VJSY-2 and VJSY-3 based on the polyphasic approach. It is the first report of diversity studies of Streptomyces from this region and an attempt was made to reveal the taxonomic characteristics of the potent bioactive strains VJSY-2 and VJSY-3 based on the polyphasic approach.

METHODS

Sample collection

Soil samples were randomly collected from the mangrove habitats of Gilakaladindi, Machilipatnam, Krishna District of Andhra Pradesh. Samples were collected from 20 cm depth and brought to the laboratory in sterilized containers and air dried at room temperature. The air-dried soil sample was pretreated with calcium carbonate (10:1w/w) and incubated at 37°C for four days [7].

Isolation of *Actinobacteria*

Dilution plate technique was employed for isolation of *Actinobacteria* on humic acid vitamin (HV) agar medium supplemented with 5% NaCl [8]. The medium was adjusted to pH 7.0 and 0.1 mL of diluted soil sample spread over HV agar supplemented with 50 µg/mL cycloheximide and 50 µg/mL nalidixic acid to reduce the fungal and bacterial contamination, respectively, and incubated at 30±2°C for 7 days. Actinobacterial colonies [9] were picked out, purified, and

preserved on yeast extract malt extract dextrose (YMD) agar slants at 4°C [10]. The actinobacterial strains were then screened for their potential to generate bioactive compounds [11]. Among the 25 different actinobacterial strains tested for antimicrobial activity, two predominant actinobacterial strains - VJSY-2 and VJSY-3 found to be potent as they exhibited high antimicrobial activity.

Identification of the potent strains by polyphasic approach

Morphological, cultural, physiological, and biochemical characteristics of the strains

The potent actinobacterial strains were characterized by cultural, morphological, physiological, biochemical, and molecular methods. The microscopic characterization was carried out by slide culture method [12] taking into account the nature of mycelium, color, and spore arrangement [13]. The morphological characteristics were assessed using scanning electron microscopy (SEM: Model - JOELJSM 5600, Japan) of 4-day-old culture grown on YMD agar medium at various magnifications. The strains were grown on seven International Streptomyces Project (ISP) media and three non-ISP media to observe the cultural characteristics such as color of aerial mycelium, substrate mycelium, pigment production, and spore formation [14]. Melanin pigment production was assessed by culturing the strains on tyrosine agar (ISP-7) medium [10]. Hydrolysis of starch and nitrate reduction [15] and H_2S production were also tested [16]. Physiological characteristics such as the effect of pH (5-9), temperature (20-45°C), and salinity on the growth of the strains were analyzed. The ability of the strains to produce industrially important enzymes such as amylase, asparaginase, caseinase, and cellulase was tested.

Molecular identification

The total genomic DNA extracted from the strains 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°C for 3 minutes followed by 30 cycles of amplification (Denaturation at 94°C for 60 seconds, annealing temperature of 55°C for 60 seconds, and extension at 72°C for 60 seconds) and an addition of 5 minutes 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 p mol 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 V for 40 minutes 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 Biosystems, 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 NCBI GenBank databases using the Clustal W method. Phylogenetic and molecular evolutionary analyses were conducted using molecular evolutionary genetic analysis (MEGA) version 6.0 [17].

Nucleotide sequence accession numbers

The 16S rRNA gene (rDNA) sequence of the strains VJSY-2 and VJSY-3 were registered in the GenBank database.

Growth pattern determination

For determination of growth pattern, the strains were inoculated into 250 ml flasks containing 100 ml YMD broth and incubated at 30±2°C on a rotary shaker at 180 rpm. The flasks were harvested at 24 hrs interval, and the growth of the strains 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 [18].

Antimicrobial profile of VJSY-2 and VJSY-3

The antimicrobial activity of the strains was determined by agar well diffusion assay. YMD broth was used as a production medium for the extraction of crude secondary metabolites. The selected potent actinobacterial isolates VJSY-2 and VJSY-3 were inoculated, and the fermentation was carried out at 30°C for 120 hrs under agitation at 180 rpm. Bioactive compounds were 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 water bath and the residue thus obtained was used to determine antimicrobial activity.

Ethyl acetate itself was used as negative control. About 80 µl of the crude extract and 80 µl of negative control were poured into separate wells. For each bacterial strain, controls were maintained utilizing pure solvent. Plates were incubated at 37°C for 48 hrs, and inhibition zones (in mm) were measured after 24-48 hrs. The experiment was carried out in triplicates for each test organism, and the mean values were computed.

Test organisms

Bacteria

Staphylococcus aureus (MTCC 3160), *Bacillus subtilis* (ATCC 6633), *Bacillus megaterium* (NCIM 2187), *Xanthomonas campestris* (MTCC 2286), *Pseudomonas aeruginosa*, and *Escherichia coli* (ATCC 9027).

Fungi

Aspergillus niger, Fusarium solani, Fusarium oxysporum, and *Candida albicans* (MTCC 183).

RESULTS AND DISCUSSION

Among the 25 distinct Mangrove actinobacterial strains isolated, the predominant actinobacterial strains VJSY-2 and VJSY-3 were found to be potent and exhibited strong antimicrobial activity against Gram-positive, Gram-negative bacteria, and fungi. Both the strains exhibited typical morphological characteristics of the genus *Streptomyces.* Morphological and micro morphological characteristics of the strain were studied by SEM (Figs. 1 and 2). The strains did not produce any pigment on the media tested.

Identification of the strains VJSY-2 and VJSY-3

The cultural characteristics of the strains VJSY-2 and VJSY-3 are presented in Tables 1 and 2. Strain VJSY-2 exhibited excellent growth

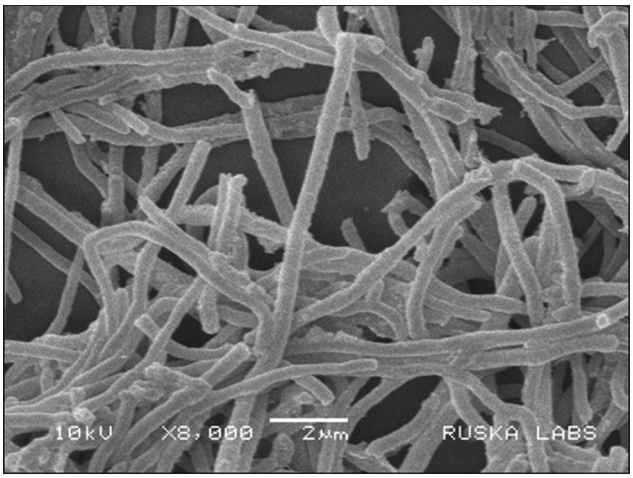

Fig. 1: Scanning electron microscopic photograph of *Streptomyces* sp. VJSY-2

on YMD and nutrient agar medium (NAM), good growth on tryptone yeast extract agar (ISP-1), and tyrosine agar (ISP-7), whereas the growth was moderate on oat meal agar (ISP-3), inorganic salts starch agar (ISP-4), glycerol asparagine agar (ISP-5) and starch casein nitrate agar. Sabouraud's dextrose agar did not support its growth. The color of aerial mycelium was white and the substrate mycelium was pale yellow. Strain VJSY-3 exhibited luxurious growth on ISP-1and ISP-2, good growth on ISP-4, ISP-5, ISP-7, and NAM. The growth was moderate on ISP-3, whereas no growth observed on ISP-6, Starch Casein Nitrate Agar medium and Sabouraud's dextrose agar. The color of aerial mycelium was gray, and the substrate mycelium was black. Black soluble pigment was produced by the strain VJSY-3.

Biochemical characteristics

The biochemical characteristics of the strains are presented in Table 3. The strain VJSY-2 had the ability to hydrolyze starch and exhibited a positive response to indole production, catalase activity, urease activity, citrate utilization, and gelatin liquefaction but negative for methyl red, Voges-Proskauer, and H_2S production. The strain VJSY-3 exhibited a positive response to catalase activity, nitrate reduction, starch hydrolysis, gelatin liquefaction, and indole production but negative for urease, methyl red, Voges-Proskauer, citrate utilization, and H_2S production. Both the strains could also produce amylase, L-asparaginase, and cellulase.

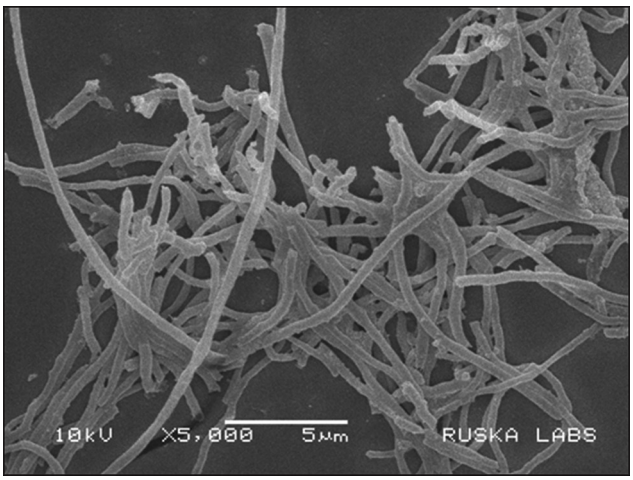

Fig. 2: Scanning electron microscopic photograph of *Streptomyces carpaticus* VJSY-3

Physiological characteristics

Growth of the strains VJSY-2 and VJSY-3 occurred in the pH range of 5-9 with with optimum being pH 7. The temperature range for the growth of the strains was 25-50°C and 20-50°C for VJSY-2 and VJSY-3, respectively, with the optimum growth at 30°C. The strains VJSY-2 and VJSY-3 exhibited salt tolerance up to 10% with optimum growth at 5% and 4% NaCl, respectively (Table 3).

Molecular characterization of the strains

The 16S rDNA sequence data supported the assignment of the strain VJSY-2 and VJSY-3 to the genus *Streptomyces*. The partial 16S rDNA sequences of the strains were submitted to the GenBank database with accession numbers KP863921 and KP863920. The sequences were aligned and compared with all the 16S rDNA gene sequence available in the GenBank database using the multisequence advanced BLAST comparison tool. The phylogenetic analysis of the 16S rRNA gene sequence was aligned using the CLUSTAL W program from the MEGA 6V. Based on the morphological, physiological, biochemical, and molecular characteristics by 16S r DNA sequencing, the strain VJSY-2 was identified as *Streptomyces* sp., and the strain VJSY-3 was identified as *Streptomyces carpaticus*. The phylogenetic trees were constructed using MEGA software version 6.0 (Figs. 3 and 4).

Growth pattern and antimicrobial profile of the strains *Streptomyces* sp. VJSY-2 and *Streptomyces carpaticus* VJSY-3

The growth pattern of *Streptomyces* sp. VJSY-2 and *Streptomyces carpaticus* VJSY-3 were studied on YMD broth. The stationary phase of the strain VJSY-2 and VJSY-3 extended from 96 hrs to 120 hrs (Figs. 5 and 6). The bioactive metabolites obtained from 5-day-old culture

Table 1: Cultural characteristics of the strain VJSY-2

Name of the medium	Growth	AM*	SM**	Pigmentation
Tryptone yeast extract agar (ISP-1)	Good	White	Pale yellow	Nil
YMD agar (ISP-2)	Excellent	White	Pale yellow	Nil
Oat meal agar (ISP-3)	Moderate	White	White	Nil
Inorganic salts starch agar (ISP-4)	Moderate	White	Pale yellow	Nil
Glycerol asparagine agar (ISP-5)	Moderate	White	Pale yellow	Nil
Peptone yeast extract iron agar (ISP-6)	-	-	-	-
Tyrosine agar (ISP-7)	Good	White	Pale yellow	Nil
Starch casein nitrate agar	Moderate	Pale white	Pale white	Nil
Nutrient agar	Excellent	White	Pale yellow	Nil
Sabouraud's dextrose agar	-	-	-	-

*Aerial mycelium; **Substrate mycelium; '-' no growth, ISP: International streptomyces project, YMD: Yeast extract malt extract dextrose

Table 2: Cultural characteristics of the strain VJSY-3

Name of the medium	Growth	AM*	SM**	Pigmentation
Tryptone yeast extract agar (ISP-1)	Excellent	Gray	Reddish brown	Black
YMD agar (ISP-2)	Excellent	Gray	Black	Black
Oat meal agar (ISP-3)	Moderate	Whitish gray	Gray	Nil
Inorganic salts starch agar (ISP-4)	Good	White	Pale yellow	Nil
Glycerol asparagine agar (ISP-5)	Good	Whitish gray	Greenish black	Nil
Peptone yeast extract iron agar (ISP-6)	-	-	-	-
Tyrosine agar (ISP-7)	Good	White	Reddish	Nil
Starch casein nitrate agar	-	-	-	-
Nutrient agar	Good	Grayish white	Greenish black	Nil
Sabouraud's dextrose agar	-	-	-	-

*Aerial mycelium; **Substrate mycelium, - no growth, ISP: International streptomyces project, YMD: Yeast extract malt extract dextrose

Table: 3 Morphological, biochemical, and physiological characteristics of the strains VJSY-2 and VJSY-3

Character response	Response	
Morphological characters	VJSY-2	VJSY-3
Color of aerial mycelium	White	Gray
Color of substrate mycelium	Pale yellow	Black
Biochemical and physiological characters		
Catalase production	+	+
Urease production	+	-
Hydrogen sulfide production	-	-
Nitrate reduction	-	+
Starch hydrolysis	+	+
Gelatin liquefaction	+	+
Methyl red test	-	-
Voges-proskauer test	-	-
Indole production	+	+
Citrate utilization	+	-
Gram reaction	+	+
Production of melanin pigment	-	+
Range of temperature for growth	25-50°C	20-50°C
Optimum temperature for growth	30°C	30°C
Range of pH for growth	5-9	5-9
Optimum pH for growth	7.0	7.0
NaCl tolerance	Up to 10%	Up to 10%
Optimum NaCl concentration	5%	4%
Asparaginase	+	+
Caseinase	-	-
Cellulase	+	+
Amylase	+	+

+: Positive, –: Negative

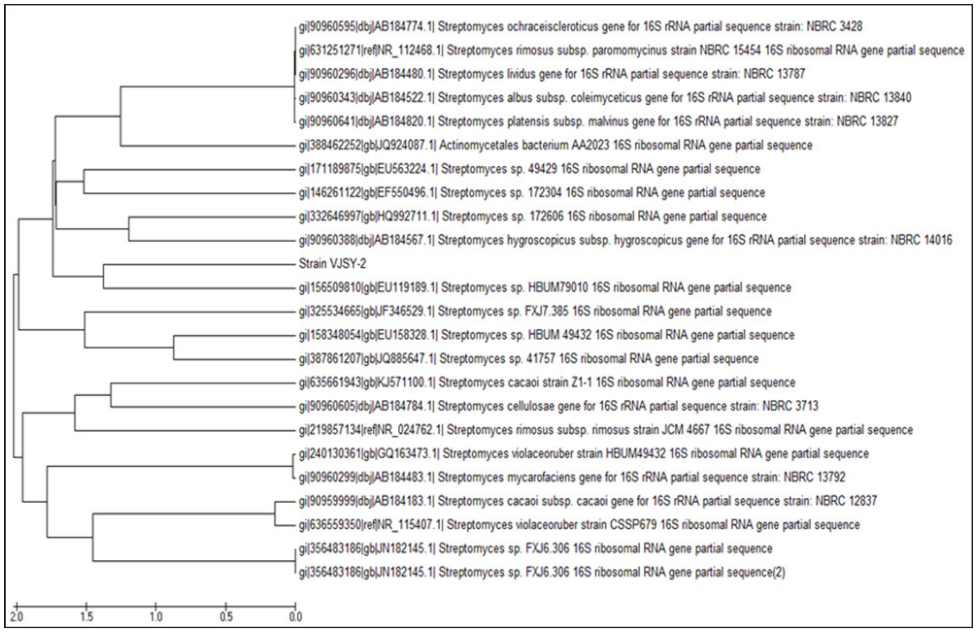

Fig. 3: Maximum parsimony tree based on partial 16S rRNA gene sequence showing relationship between strain VJSY-2 and related members of the genus *Streptomyces*

of both the strains (VJSY-2 and VJSY-3) exhibited high antimicrobial activity against the test microorganisms. The antimicrobial spectrum of the strains cultured on YMD broth for 5 days was given in Table 4. Secondary metabolites extracted from 5-day-old cultures of *Streptomyces* sp. CDRIL-312 [19] and *Streptomyces* spp. [20] exhibited good antifungal activity. 5-day-old culture of *Streptomyces clavuligerus* was reported to produce good yield of Clavulanic acid [21], whereas 6-day-old culture of *Streptomyces* sp. 201 exhibited good antimicrobial activity [22].

Naragani *et al*. (2014) reported that 5-day-old culture extracts of *Streptomyces violaceoruber* VLK-4 and *S. cheonanensis* VUK-A evidenced a high antimicrobial activity against the test microbes [23,24]. The metabolites extracted from 5-day-old culture of VJSY-2 showed maximum activity against *B. megaterium, S. aureus, P. aeruginosa,* and *C. albicans* followed by *E. coli*, *B. subtilis,* and *X. campestris,* whereas the metabolites from 5-day-old culture of the strain VJSY-3 showed maximum activity against *B. megaterium, S. aureus, P. aeruginosa, C. albicans* followed by *X. campestris, B. subtilis and E. coli.*

CONCLUSION

The present study revealed the taxonomic features of the two *Streptomyces* strains VJSY-2 and VJSY-3 and their spectrum of action

Table 4: Antibacterial and antifungal activity of *Streptomyces* sp. VJSY-2 and *Streptomyces carpaticus* VJSY-3

Test organism	Zone of inhibition (mm)		Positive control[#]
Bacteria	VJSY-2	VJSY-3	
S. aureus	22±0.12	27±0.06	28±0.08
B. megaterium	24±0.08	28±0.08	32±0.06
B. subtilis	18±0.10	21±0.04	28±0.04
X. campestris	17±0.04	22±0.05	22±0.07
E. coli	19±0.08	20±0.09	21±0.10
P. aeruginosa	22±0.11	25±0.11	28±0.12
Fungi			
A. niger	15±0.09	16±0.02	20±0.06
F. solani	17±0.04	18±0.03	21±0.08
F. oxysporum	14±0.11	15±0.08	18±0.01
C. albicans	22±0.10	24±0.06	28±0.02

[#]Positive control: Tetracycline against bacteria, griseofulvin against yeast and carbendazim against fungi. *S. aureus: Staphylococcus aureus, B. megaterium: Bacillus megaterium, B. subtilis: Bacillus subtilis, X. campestris: Xanthomonas campestris, E. coli: Escherichia coli, P. aeruginosa: Pseudomonas aeruginosa, A. niger: Aspergillus niger, F. solani: Fusarium solani, C. albicans: Candida albicans, Values are mean±S.E.M (n = 3)*

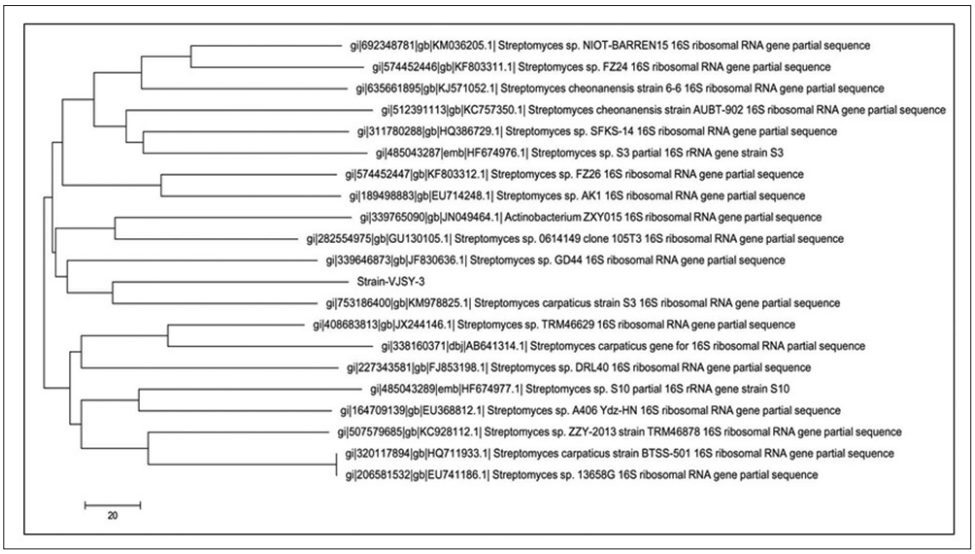

Fig. 4: Neighbor-joining tree based on partial 16S rRNA gene sequence showing relationship between strain VJSY-3 and the related members of the genus *Streptomyces*

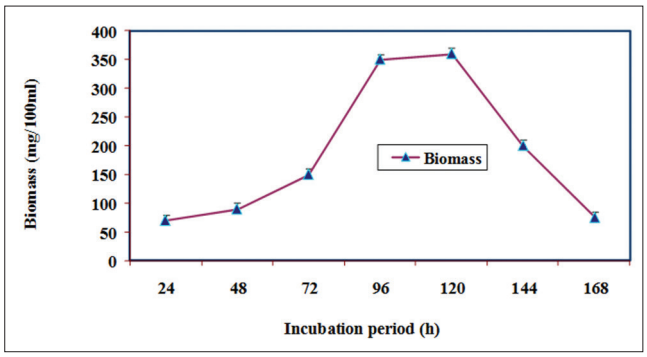

Fig. 5: Growth pattern of the strain *Streptomyces* sp. VJSY-2

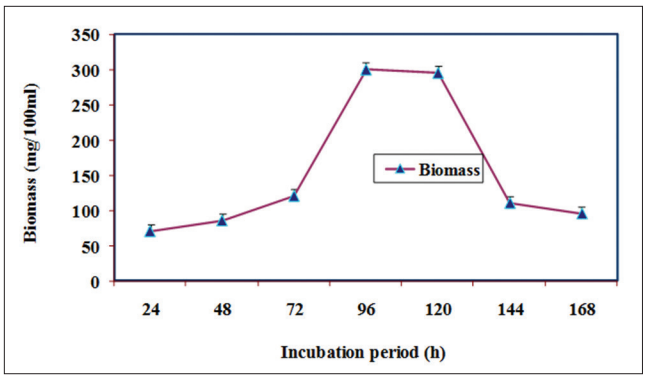

Fig. 6: Growth pattern of the strain *Streptomyces carpaticus* VJSY-3

against Gram-positive and Gram-negative bacteria along with yeast and filamentous fungi. It is evident from the present study that mangrove habitats of Gilakaladindi of Andhra Pradesh, India, serve as a good source for the isolation of potent actinomycetes with broad spectrum antimicrobial activity. Further studies on optimization of the cultural conditions for improved bioactive metabolite production are in progress.

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