Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2016, 8(7):463-469

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Diversity of Actinobacteria in Gilakaladindi Mangrove Ecosystem of Andhra Pradesh, India

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ABSTRACT

The mangrove ecosystem of Gilakaladindi, Andhra Pradesh, India was selected for studying the diversity of Streptomycetes spp. and their antimicrobial properties. The soil samples pretreated with $CaCO_3$ were plated on three selective media viz. ISP-2, ISP-4 and HV agar to isolate Streptomycetes spp. Among the 27 strains isolated, 13 possessed antimicrobial activity, of which 7 isolates were active against the test micro-organisms (Gram positive bacteria - Bacillus megaterium (NCIM 2187), Staphylococcus aureus (MTCC 3160); Gram negative bacteria - Escherichia coli (ATCC 9027), Xanthomonas campestris (MTCC 2286) and fungi - Candida albicans (MTCC 183), Penicillium expansum, Fusarium oxysporum). The bioactive strains were further analyzed by polyphasic taxonomy and identified as Streptomycetes harbinensis VJPR-1, S. plicatus VJPR-3, S. coelicolor VJPR-5, S. sodiphilus VJPR-6, S. nogalater VJPR-7, S. enissocaesilis VJPR-8 and S. xiamensis VJPR-24. The strains have been deposited in NCBI genbank with accession numbers. This is the first report on the diversity studies of Streptomycetes spp. from Gilakaladindi mangrove ecosystem.

Keywords: Mangrove habitats, *Streptomycetes* spp., Phylogenetic analysis, Secondary metabolites.

INTRODUCTION

Actinomycetes are most economically and biotechnologically ubiquitous group of microbes that are considered to be valuable prokaryotes. Several ecologically significant properties of actinomycetes were reported, which made the screening source expand into uncommon environments. Many actinobacterial species especially *Streptomycetes* spp. have been reported as producers of chemically diversified bioactive natural products [1, 2, 3, 4].

Streptomycetes are regarded as the best source of commercial interest due to their ability to produce novel metabolites. They are considered cosmopolitan in their distribution due to their ability to produce abundant spores that are readily dispersed [5]. These filamentous bacteria are readily adapted to marine environment and can readily break down complex biological polymers [6]. The genus *Streptomycetes* alone accounts for 80% of the actinobacterial bioactive metabolites reported till date. The natural genetic capacity of these wonderful actinomycetes makes them remain unchallenged in the microbial world [7].

In recent years the discovery of commercially significant novel chemical skeletons from mangrove actinobacteia has been increased. The mangrove ecosystem is saline and highly rich in organic matter and uncaged source for screening and isolation of potential bioactive metabolites [8]. The microbes in mangrove habitats not only produce primary and secondary metabolites but also involved in important ecological role in soil organic matter

decomposition and mineralization [9]. Studies on biodiversity of actinobacteria from mangrove ecosystem are important for the biotechnological exploitation [10].

In the present study, an attempt has been made to isolate diverse *Streptomycetes* spp. with antimicrobial potential from the mangrove habitats of Gilakaladindi, Andhra Pradesh and study their taxonomic characteristics.

EXPERIMENTAL SECTION

Collection and processing of sediment samples

Sediment samples were collected from Gilakaladindi mangrove ecosystem situated along the south east coast of Andhra Pradesh, India. Samples were collected from 6-10 cm depth and transported to laboratory in sterile bags and air-dried at room temperature. The air-dried samples were subjected to calcium-carbonate pre-treatment to enrich the actinobacterial population as well as to reduce the unwanted contaminants like fungi and bacteria [11, 12].

Isolation of mangrove actinobacteria

The pretreated sediment sample (1g) was suspended in 100 ml of sterile distilled water. Serial dilutions were prepared and 100 µl of 10^{-4} dilution obtained from the $CaCO_3$ pretreatment were spread onto the surface of three selective media, ISP-2, ISP-4 and HV Agar. These media were supplemented with streptomycin (25µg/ml) and fluconazole (30µg/ml) to retard the growth of bacteria and fungi respectively [13].The inoculated plates were incubated at 28±2°C for 15 days. After incubation, actinobacterial colonies were sub-cultured and further maintained on ISP-2 agar slants.

Identification of the potent strains by polyphasic approach

The cultural characteristics of the strain were studied on different ISP and non- ISP media. Biochemical characteristics of the potent actinobacterial strains along with physiological characterization such as effect of pH, temperature and salinity were also evaluated [14]. The ability of the strains to produce melanin pigment was examined by growing them on ISP-7 (Tyrosine agar) culture medium.

Molecular characterization and phylogenetic analysis

16S rRNA sequencing of the isolates

Actinobacterial isolates were cultured in ISP-2 broth and incubated for 10 days at 28±2°C at 120 rpm. The cell mass was filtered and used for genomic DNA isolation. DNA extraction was carried out using genomic DNA isolation kit (Chromus Biotech Pvt. Ltd., Bangalore, India). Specific sequence amplification was performed using universal 16S rRNA forward primer (5'AGAGTTTGATCCTGGCTCA–3') and 16S rRNA reverse primer (5'ACGGCTACCTTGTTACGACT–3'). The PCR products were purified and sequenced at Chromus Biotech Pvt. Ltd., Bangalore, Karnataka, India, for obtaining partial sequence [15].

Phylogenetic analysis of isolates

The sequences were analyzed with reference sequences obtained from the GenBank, EMBL and DDBJ using Molecular Evolutionary Genetics Analysis software (MEGA, 6.05) [16] Phylogenetic trees were constructed using the software MEGA Version 6.05 based on the Maximum parsimony method.

Nucleotide sequence accession numbers

The 16S r RNA gene sequences of the strains VJPR-1, VJPR-3, VJPR-5, VJPR-6, VJPR-7, VJPR-8 and VJPR-24 are submitted to Gen Bank database.

Extraction of potent secondary metabolites

The potent actinobacterial isolates VJPR-1, VJPR-3, VJPR-5, VJPR-6, VJPR-7, VJPR-8 and VJPR-24 identified from the primary screening were inoculated separately in yeast extract- malt extract dextrose medium (YMD) (malt extract 1 g, yeast extract 0.4 g, dextrose 0.4 g, sodium chloride 3 g, sea water 100 ml and pH 7.0) and incubated at 28±2ºC for 120 h in a rotatory shaker (120 rpm). After incubation for 120h, the culture filtrate collected was extracted with equal volume of ethyl acetate by solvent extraction method. The ethyl acetate was evaporated to dryness in rotavap, and the metabolite obtained was used to determine antimicrobial activity by agar well diffusion method [17]**.**

Growth pattern

To study the growth pattern, the strains VJPR-1, VJPR-3, VJPR-5, VJPR-6, VJPR-7, VJPR-8 and VJPR-24 were inoculated into 100ml YMD broth and incubated at 28±2ºC in a rotary shaker (120 rpm). The fermentation process

was allowed to run for seven days for each strain. The flasks were harvested at every 24h interval, and the growth of the strains was determined by taking dry weight of the biomass. The culture filtrates harvested at regular intervals were extracted with ethyl acetate to determine antimicrobial activity.

Test Microorganisms

The antimicrobial activity of the crude extract of actinobacterial strains was determined by measuring the diameter of inhibition zone against Gram positive bacteria - *Bacillus megaterium* (NCIM 2187), *Staphylococcus aureus* (MTCC 3160); Gram negative bacteria - *Escherichia coli* (ATCC 9027), *Xanthomonas campestris* (MTCC 2286) and fungi *Candida albicans* (MTCC 183), *Penicillium expansum, Fusarium oxysporum* by agar well diffusion assay [17].

RESULTS AND DISCUSSION

The mangrove ecosystem of Gilakaladindi was selected for studying the diversity of *Streptomycetes* spp. and their antimicrobial compounds. A total of 27 *Streptomycetes* strains were isolated from the mangrove ecosystem designated as VJPR-1 to VJPR-27. All the 27 isolates were identified up to generic level based on the morphological features and the isolates were assigned to *Streptomycetes* spp. Out of 27 strains, 13 possessed antimicrobial activity, of which 7 isolates VJPR-1, VJPR-3, VJPR-5, VJPR-6, VJPR-7, VJPR-8 and VJPR-24 exhibited strong antimicrobial activity against Gram positive and Gram negative bacteria as well as fungi. These potent strains were further evaluated for cultural, physiological and biochemical characteristics along with the phylogenetic analysis of the 16S rRNA.

The cultural characteristics of the strains are represented in Table-1. They could be used as markers by which an individual strain can be recognized [18]. The strains exhibited luxuriant growth on YMD agar medium. The strains VJPR-1, 3, 5, 6 & 7 exhibited grayish white aerial mycelium and dark yellow to grey substrate mycelium, while the aerial mycelium of VJPR- 8 and 24 was grey colored with substrate mycelium ranging from yellow to dark brown. The strains VJPR-1, VJPR-7 and VJPR-24 produced melanin pigment on ISP-7 (tyrosine agar) culture medium.

Table 1: Cultural characteristics of the *Streptomycetes harbinensis* VJPR-1, *S. plicatus* VJPR-3, *S. coelicolor* VJPR-5, *S. sodiphilus* VJPR-6, *S. nogalater* VJPR-7, *S. enissocaesilis* VJPR-8 and *S. xiamensis* VJPR-24 on YMD agar medium

S.No.	Isolate	Colour of aerial Mycelium	Colour of substrate mycelium	Pigment production
1.	VJPR-1	Greyish white	Dark yellow	Yes
2.	VJPR-3	Greyish white	Dark yellow	Nil
3.	VJPR-5	Greyish white	Grey	Nil
4.	VJPR-6	Greyish white	Dark yellow	Nil
5.	VJPR-7	Greyish white	Grey	Yes
6.	VJPR-8	Grey	Yellow	Nil
7.	VJPR-24	Grey	Dark brown	Yes

The physiological and biochemical characteristics of the strains are recorded in Table-2. The physiological tests are indispensable tools for classification and identification of Actinomycetes [19]. All the strains exhibited optimum growth at pH 7.0 and temperature 28±2°C. Tolerance of the strains to NaCl also serves as an important character for species identification. The strains could grow well in the medium supplemented with 3% NaCl. All the strains exhibited positive response to citrate utilization.

Table 2: Biochemical and physiological characteristics of *Streptomycetes harbinensis* VJPR-1, *S. plicatus* VJPR-3, *S.coelicolor* VJPR-5, *S. sodiphilus* VJPR-6, *S. nogalater* VJPR-7, *S. enissocaesilis* VJPR-8 and *S. xiamensis* VJPR-24

Characteristics	VJPR-1	VJPR-3	VJPR-5	VJPR-6	VJPR-7	VJPR-8	VJPR-24
Indole production	–	–	–	–	–	–	–
Methyl red test	–	–	–	–	–	–	–
Voges proskauer test	–	–	–	–	–	–	–
Citrate utilization test	+	+	+	+	+	+	+
Gram's reaction	+	+	+	+	+	+	+
Acid fast reaction	–	–	–	–	–	–	–
Temperature for growth	28±2°C	28±2°C	28±2°C	28±2°C	28±2°C	28±2°C	28±2°C
pH for growth	7.0	7.0	7.0	7.0	7.0	7.0	7.0
NaCl range for growth	3%	3%	3%	3%	3%	3%	3%

'– 'indicates Negative; '+'indicates Positive.

The 16S rRNA sequence data supported the assignment of the isolates VJPR-1 to *Streptomycetes harbinensis*, VJPR-3 to *Streptomycetes plicatus*, VJPR-5 to *Streptomycetes coelicolor*, VJPR-6 to *Streptomycetes sodiphilus*,

VJPR-7 to *Streptomycetes nogalater*, VJPR-8 to *Streptomycetes enissocaesilis* and VJPR-24 to *Streptomycetes xiamensis.* The partial 16S rRNA sequences of all the strains were submitted to the Genbank database under accession numbers - *Streptomycetes harbinensis* VJPR-1 (KP313611), *S. plicatus* VJPR-3 (KP313619), *S. coelicolor* VJPR-5 (KP318084), *S. sodiphilus* VJPR-6 (KP313617), *S. nogalater* VJPR-7 (KP313616), *S. enissocaesilis* VJPR-8 (KP313618) and *S. xiamensis* (KP313615). The partial sequence was aligned and compared with all the 16S rRNA gene sequence available in the Gen bank database using the multisequence advanced BLAST comparison tool. The phylogenetic analysis of the 16S rRNA gene sequence was aligned using the CLUSTAL W programme from the MEGA 6.05 version [16]. Phylogenetic trees were constructed using MEGA 6.05 version and maximum parsimony analyzing tool (figs.1to7).

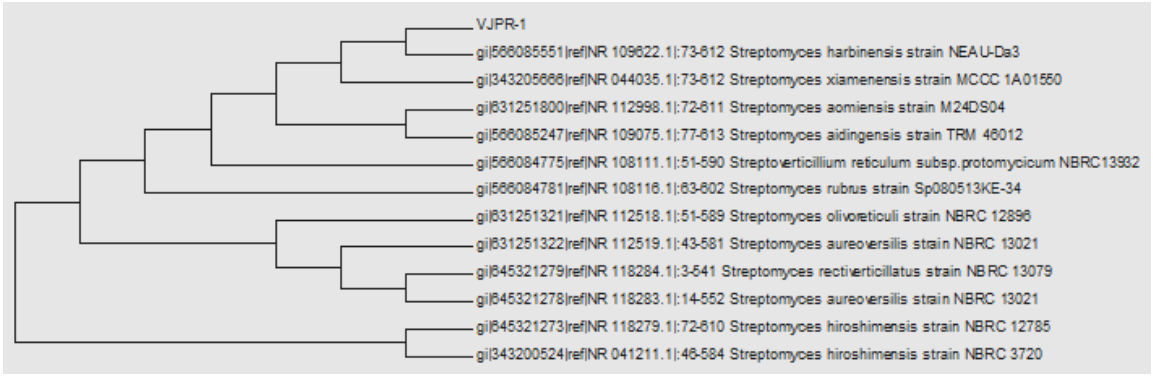

Fig. 1: Maximum parsimony tree based on partial 16S rRNA gene sequence showing relationship between *Streptomycetes* strain VJPR-1 and related members of the genus *Streptomycetes*

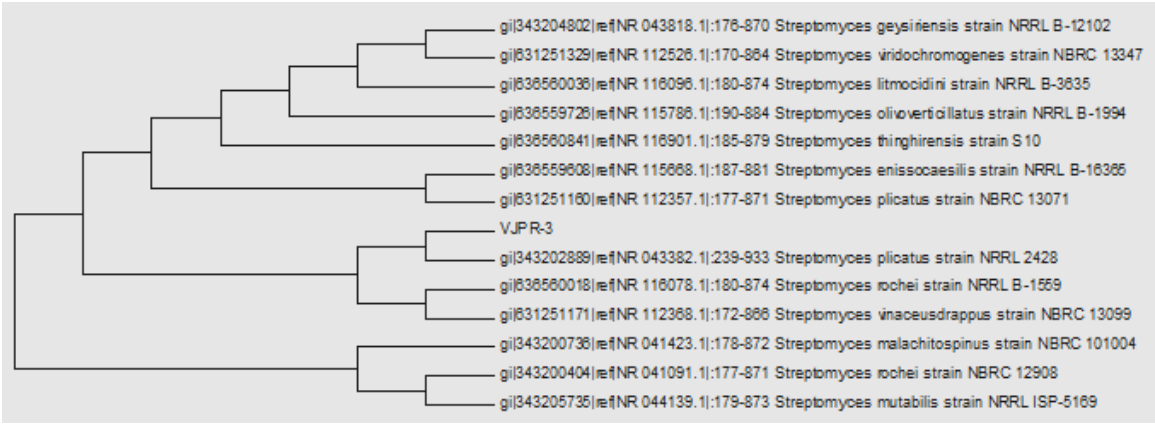

Fig. 2: Maximum parsimony tree based on partial 16S rRNA gene sequence showing relationship between *Streptomycetes* strain VJPR-3 and related members of the genus *Streptomycetes*

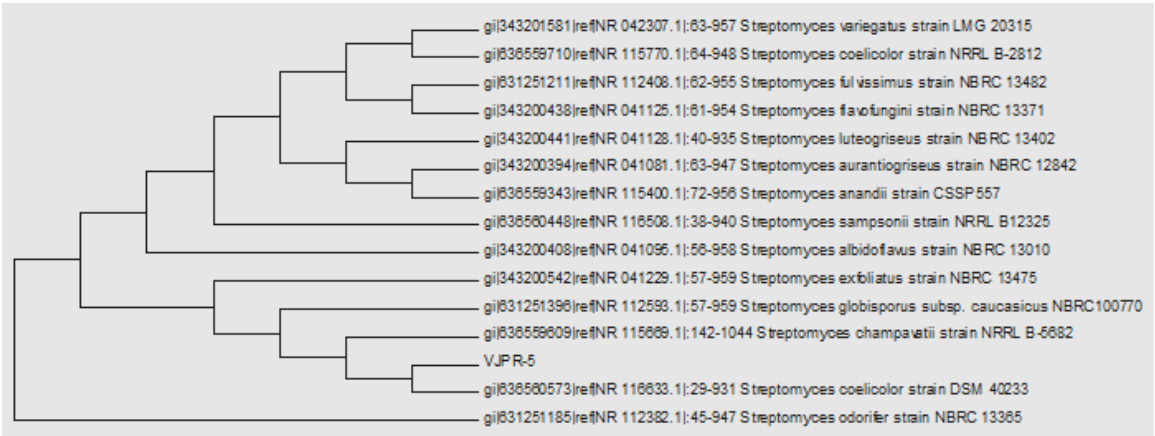

Fig. 3: Maximum parsimony tree based on partial 16S rRNA gene sequence showing relationship between *Streptomycetes* strain VJPR-5 and related members of the genus *Streptomycetes*

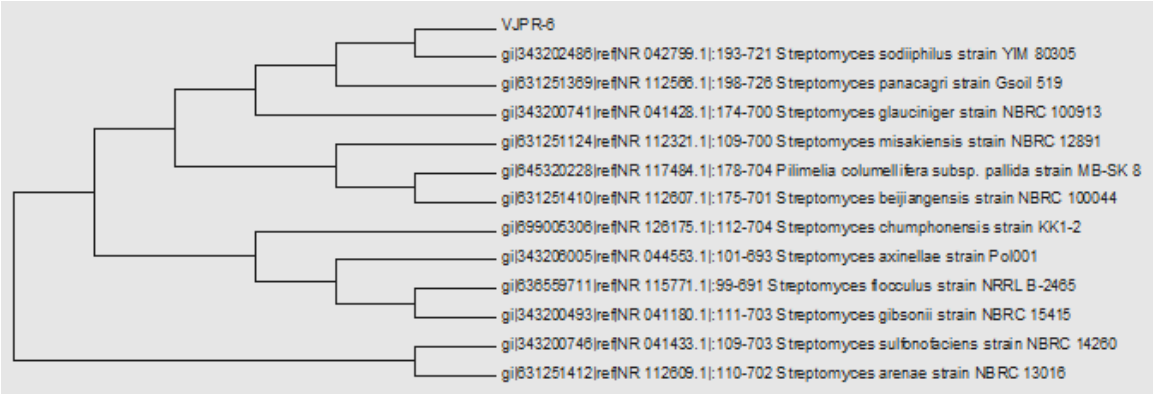

Fig. 4: Maximum parsimony tree based on partial 16S rRNA gene sequence showing relationship between *Streptomycetes* strain VJPR-6 and related members of the genus *Streptomycetes*

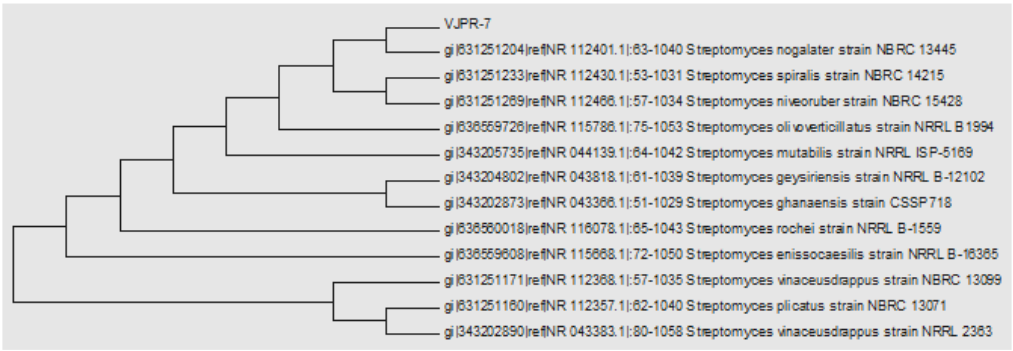

Fig. 5: Maximum parsimony tree based on partial 16S rRNA gene sequence showing relationship between *Streptomycetes* strain VJPR-7 and related members of the genus *Streptomycetes*

Fig. 6: Maximum parsimony tree based on partial 16S rRNA gene sequence showing relationship between *Streptomycetes* strain VJPR-8 and related members of the genus *Streptomycetes*

The growth pattern and antimicrobial profile of the strains were studied at regular intervals up to seven days. The secondary metabolites of the strains obtained from five day old cultures showed high antimicrobial activity against test microorganisms. Of all the strains tested, VJPR-1 and 3 showed significant antimicrobial activity compared to other strains (Table 3). The high antimicrobial activity of extracts obtained from five day old culture is in agreement with the earlier reports [20, 21]. Metabolites collected from four day old culture of *Nocardia levis* and *Pseudonocardia* sp. also showed good antimicrobial activity [22, 23] (Table 3).

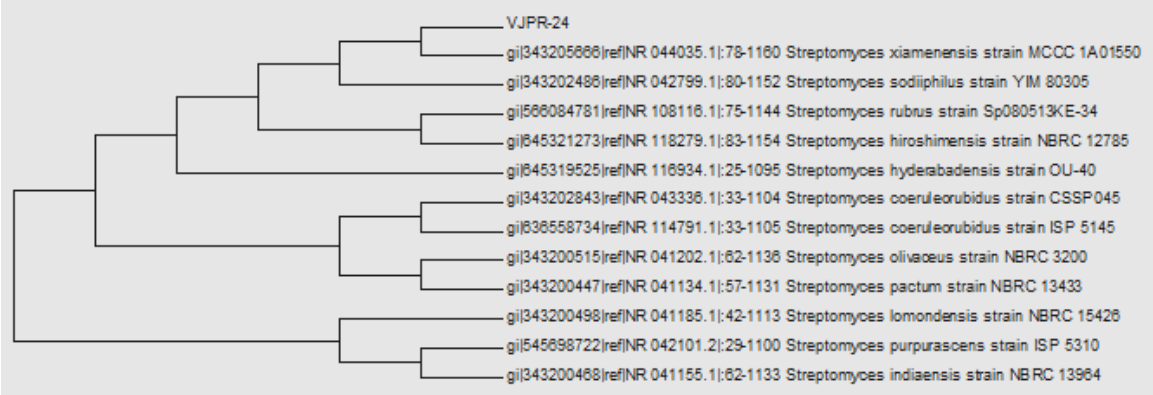

Fig. 7: Maximum parsimony tree based on partial 16S rRNA gene sequence showing relationship between *Streptomycetes* strain VJPR-24 and related members of the genus *Streptomycetes*

Table 3: Antibacterial and antifungal activity of *Streptomyces harbinensis* VJPR-1, *S. plicatus* VJPR-3, *S.coelicolor* VJPR-5, *S.sodiphilus* VJPR-6, *S.nogalater* VJPR-7, and *S. enissocaesilis* VJPR-8 and *S.xiamensis* VJPR-24.

Diameter of inhibition zone (mm)								
S.No.	Isolate	Bm	Ca	Ec	Sa	Fo	Pe	Xc
1.	VJPR-1	18	14	16	15	14	17	13
2.	VJPR-3	16	12	14	13	12	15	11
3.	VJPR-5	12	10	9	10	8	12	8
4.	VJPR-6	11	9	8	10	7	9	10
5.	VJPR-7	9	12	12	7	8	10	8
6.	VJPR-8	8	10	11	8	13	11	11
7.	VJPR-24	9	9	12	8	10	13	12

Bm -Bacillus megaterium NCIM (2187); *Ca - Candida albicans* MTCC (183); Ec -*Escherichia coli* ATCC (9027);
Sa - Staphylococcus aureus MTCC (3160); Fo –*Fusarium oxysporum* ; *Pe- Penicillium expansum*; Xc- *Xanthomonas campestris* MTCC (2286).

CONCLUSION

Diverse *Streptomycetes spp*. were isolated from Gilakaladindi mangrove habitats of Andhra Pradesh by combining pre-treatment technique with suitable culture media supplemented with antibacterial and antifungal antibiotics. The potent isolates were characterized up to species level by polyphasic taxonomy. The strains isolated were capable of synthesizing good antimicrobial metabolites. Hence further studies for purification and characterization of bioactive compounds produced by the strains are in progress. This is the first report on the diversity studies of *Streptomycetes* spp. from Gilakaladindi mangrove ecosystem of Andhra Pradesh, India.

Acknowledgement
This work was accomplished with the help of UGC, Research Fellowship in Sciences for Meritorious Students (RFSMS), Govt.of India, New Delhi.

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