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ACTINOBACTERIA FROM MANGROVE HABITATS: DIVERSITY AND ANTIMICROBIAL ACTIVITY

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ABSTRACT
The mangrove ecosystem is considered as the natural reservoir of resources likely to provide innovative applications. It facilitates the growth of diverse microorganisms. The present study was carried out to assess seasonal enumeration and antimicrobial activity of actinobacteria from two mangrove ecosystems namely Nizampatnam and Gilakaladindi located along the south coast of Andhra Pradesh, India. The samples collected from these locations were pretreated with calcium carbonate and plated on three different selective media. High actinobacterial count was found on the starch-casein agar medium, when compared to asparagine glucose agar and glycerol-asparagine agar media. The incidence of actinobacterial population in two mangrove ecosystems exhibited seasonal variation. The high count of actinobacteria in two locations was observed in January while the count was minimum in July. Out of 55 strains screened for antimicrobial activity, seven were found effective against test bacteria. Among the seven actinobacterial strains, two highly potent strains were identified by morphological, cultural, physiological, biochemical characteristics and 16S rRNA analysis. The strains were identified as *Nocardiopsis alba* VLK-A and *Streptomyces albiaxialis* VLK-10.

KEYWORDS: Mangrove ecosystem; Actinobacteria; Diversity.

1. INTRODUCTION
The mangrove ecosystem is unexplored source for actinobcteria with the potential to produce biologically active metabolites. It facilitates the isolation and identification of new and potential actinobacteria that can produce many novel compounds with antibacterial, anti-tumour, antifungal, antiviral and antiparasitic properties. The exploration of the novel strains for the new antimicrobial compounds continues to be the most important research programme around the world.[1] Extensive exploration, identification and screening are suggested for the search of new leads for microbial drugs.[2] Mangroves are unique intertidal ecosystems of the tropics and hold up genetically diverse groups of microorganisms.[3] The distribution and biological individuality of mangrove actinobacteria are expected to be different from the terrestrial ones.[4] They are Gram positive, free living, saprophytic bacteria widely distributed in different habitats, frequently filamentous and sporulating with DNA rich in G+C (55-75%). They are the potential source for many bioactive secondary metabolites. More than 10,000 biologically active molecules have been isolated from actinobacteria.[2] Rare actinobacterial strains such as *Nocardiopsis* spp [5&6] and *Saccharomonospora oceani*[7] were reported from mangrove soils of Gilakaladindi. The mangrove ecosystems of Nizampatnam and Gilakaladindi regions of Andhra Pradesh are not yet much explored for microbial diversity and microbial compounds. Hence the present work was designed to study the diversity, distribution and seasonal variation of actinobacterial population of the mangrove ecosystems of Nizampatnam and Gilakaladindi regions located along the south coast of Andhra Pradesh, India.

2. MATERIALS AND METHODS
2.1 Sample collection
Mangrove sediment samples were collected at bimonthly intervals over a period of one year (March 2014 to January 2015) from Nizampatnam as well as Gilakaladindi mangrove ecosystems located along the south coast of Andhra Pradesh, India. Samples were collected from 6-10cm depth and transported to the laboratory in sterile bags and air-dried at room temperature.

2.2 Isolation of actinobacteria
The air-dried samples were subjected to $CaCO_3$ pre-treatment to enrich the actinobacterial population as well as to reduce the unnecessary microbial population.[8] The treated soil samples suspended in sterile distilled water were homogenized and 0.1ml of serially diluted sample (10^{-3} to 10^{-4}dilution) was spread over the surface of different media such as starch-casein agar[9], asparagine-

glucose agar[10] and glycerol-asparagine agar[11] containing 3% NaCl supplemented with nalidixic acid (25µg/ml) and secnidazole (25µg/ml). After incubation for a week at 30°C, distinct colonies were selected and maintained by sub culturing on yeast extract, malt extract and dextrose (YMD) agar slants at 4°C.

2.3 Extraction of secondary metabolites

YMD broth was used as a production medium for the extraction of crude secondary metabolites. All the actinobacterial isolates were inoculated and incubated at 30°C in a rotatory shaker (120 rpm) for primary screening. The crude culture filtrate obtained from a five day old culture was extracted with equal volumes of different solvents viz. chloroform, ethyl acetate and acetone separately to extract the antimicrobial compounds. The solvent extracts were evaporated to dryness in a water bath at 40°C and the compound obtained from each solvent was tested for its activity against the test microorganisms by agar well diffusion method.[12]

2.4 Antimicrobial activity against test organisms

The antimicrobial metabolites produced by the strains were tested against test bacteria such as *Staphylococcus aureus* (MTCC 3160), *Escherichia coli* (ATCC 35218), *Bacilus subtilis* (ATCC 6633), *Pseudomonas aeruginosa* (ATCC 9027), *Proteus vulgaris* (MTCC 7299) and *Xanthomonas campestris* (MTCC 2286) and fungi like *Candida albicans* (ATCC 10231) by agar-diffusion assay.

2.5 Identification of the actinobacteria strains

Morphological, biochemical and physiological characteristics together with genomic (16S rRNA gene sequencing) analysis of the strains were carried out to identify the strains.[13]

2.6 Molecular identification

The Molecular identification of the potential strains was carried out by amplifying the bacterial genome. DNA samples were extracted using a Insta Genetm Matrix (BIO-RAD.) The primers 27F 5' (AGA GTT TGA TCM TGG CTC AG) 3' and 1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) 3' were used for the PCR. The PCR reaction was performed with 20 ng of genomic DNA as the template in a 30 $\mu\ell$ reaction mixture using EF-Taq (SolGent, Korea) as follows: activation of Taq polymerase at 95°C for 2 minutes, 35 cycles at 95°C for 1 minute 55°C and 72°C for 1minutes each were performed, finishing with a 10-minute step at 72°C. The amplification products were purified with a multi screen filter plate (Millipore Corp., Bedford, MA, USA). Sequencing reaction was performed using a PRISM Big Dye Terminator v3.1 Cycle sequencing Kit. The DNA samples containing the extension products were added to Hi-Di formamide (Applied Biosystems, Foster City, CA). The mixture was incubated at 95°C for 5 min, followed by 5 min on ice and then analyzed by ABI Prism 3730XL DNA analyzer (Applied Biosystems, Foster City, CA). The deduced 16s rRNA sequence was compared with the sequences in Gene Bank using the Basic Local Alignment Search Tool (BLAST) against the gene library available for potential actinobacterial strains in the NCBI and the GenBank. The phylogenetic tree was constructed using the Maximum Parsimony method. The closely related homologous strains were identified, retrieved and compared to the sequence of the isolated strain using CLUSTAL W available with the MEGA-6 Version.[14]

3. RESULTS AND DISCUSSION

The mangrove ecosystem of south coastal Andhra Pradesh (Nizampatnam and Gilakaldindi) was selected for studying the diversity of actinobacteria and their antimicrobial properties. The number of actinobacterial strains isolated from Nizampatnam and Gilakaladindi was 24 and 31 respectively in which the Gilakaladindi mangrove sediment supported high counts when compared to Nizampatnam mangrove sediments

The incidence of actinobacteria in two mangrove ecosystems exhibited seasonal variation. The highest number of actinobacteria in two regions was observed in January while the counts were minimum in July. Usha kiranmayi *et al.*[4] reported that the highest number of actinobacteria was observed in February while the counts were least in December.

High counts of actinobacteria were observed in Thondi in the month of July and January.[15] Low incidence of actinobacteria was recorded in cooler climates.[16] The actinobacterial population in two mangrove ecosystems exhibited seasonal variation with high counts in winter.

The enumeration of actinobacteria from soil samples collected from two mangrove ecosystems is presented in Table 1. High actinobacterial count was found on the Starch-casein agar medium (Fig.1), when compared to asparagine glucose agar and glycerol-asparagine agar media. Anindita *et al.*[17] reported that maximum count of actinobacteria was observed on starch casein agar medium.

Table 1: Seasonal incidence of actinobacteria from samples collected from Nizampatnam and Gilakaladindi

Location	Media used for isolation	Actinobacterial count at different months					
		March 2014	May 2014	July 2014	September 2014	November 2014	January 2015
Nizampatnam	Starch-casein agar medium	25 x10^4	37 x10^4	13 x10^4	23 x10^4	12 x10^4	54 x10^4

	Asparagine glucose agar	10 x10^4	32 x10^4	14 x10^4	16 x10^4	14 x10^4	37 x10^4
	Glycerol-asparagine agar	23 x10^4	26 x10^4	17 x10^4	12 x10^4	11 x10^4	22 x10^4
Gilakaladindi	Starch-casein agar medium	23 x10^4	21 x10^4	10 x10^4	16 x10^4	12 x10^4	47 x10^4
	Asparagine glucose agar	26 x10^4	24 x10^4	12 x10^4	16 x10^4	14 x10^4	35 x10^4
	Glycerol-asparagine agar	10 x10^4	10 x10^4	12 x10^4	10 x10^4	12 x10^4	23x10^4

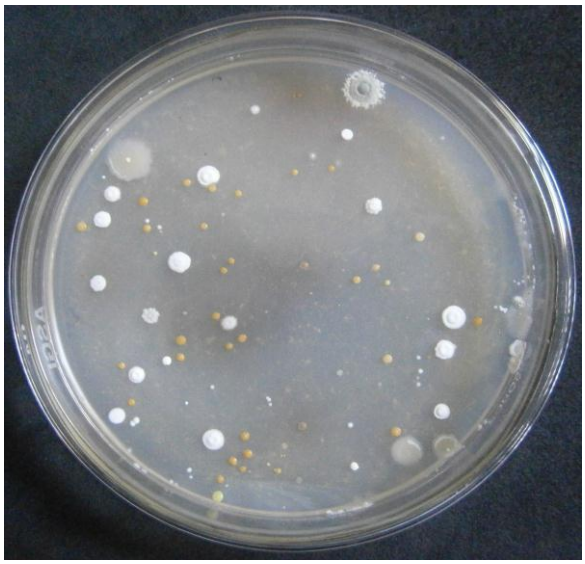

Fig 1: Actinobacterial colonies on starch-casein agar plate

The antimicrobial activity of the strains was evaluated by using three different solvents such as ethyl acetate, chloroform and acetone. Among the solvents used, ethyl acetate extract exhibited high antimicrobial activity (Tables 2 and 3), whereas the other solvent extracts showed moderate to minimum activity against the microbes tested (Figs. 2 and 3). Among the 55 isolates, 28 were found to be active against the test microorganisms. Overall, two (VLK-A and VLK-10) were found to be potential, hence attempts were made to identify them.

The cultural characteristics of the potential strains VLK-A and VLK-10 grown on nine culture media are presented in tables 4 and 5. The strain VLK-A exhibited good growth on ISP-2 ISP-4, ISP-6 out of nine culture media tested. The growth was moderate on ISP-1, ISP-5, ISP-7, asparagine glucose agar and nutrient agar media while it was poor on ISP-3. The color of aerial mycelium varied from white to grayish when cultured on different media, while the substrate mycelium was brown.

Table 2: Screening of Nizampatnam mangrove actinobacteria for Antimicrobial activity

S. NO	ISOLATE CODE	Antimicrobial activity of Ethyl acetate extracts represented as zone of inhibition (mm)						
		Sa	**Bs**	**Pa**	**Pv**	**Xc**	**Ec**	**Ca**
1	VLK-4	12	12	13	11	14	12	11
2	VLK-7	14	13	12	12	14	11	10
3	VLK-8	12	10	9	9	10	10	14
4	**VLK-10**	**19**	**14**	**15**	**17**	**16**	**18**	**17**
5	VLK-12	19	15	16	16	15	16	12
6	VLK-15	17	15	13	9	14	14	13
7	VLK-17	15	14	12	10	12	12	12
8	VLK-18	15	15	14	10	13	13	12
9	VLK-19	13	14	12	13	13	15	13
10	VLK-21	18	15	13	8	9	11	15
11	VLK-24	19	16	17	16	16	17	12
12	VLK-27	14	12	12	9	9	12	10
13	VLK-28	20	17	16	14	14	12	12
14	VLK-29	10	9	7	9	8	12	11
15	VLK-31	18	16	14	13	17	15	12

Sa-*Staphylococcus aureus*, **Bs**- *Bacilus subtilis*, **Pa**- *Pseudomonas aeruginosa*, **Pv**- *Proteus vulgaris*, **Xc**-*Xanthomonas campestris*, **Ec**- *Escherichia coli* and **Ca**- *Candida albicans*

Table 3: Screening of Gilakaladindi mangrove actinobacteria for Antimicrobial activity

S. NO	ISOLATE CODE	Antimicrobial activity of Ethyl acetate extracts represented as zone of inhibition (mm)						
		Sa	**Bs**	**Pa**	**Pv**	**Xc**	**Ec**	**Ca**
1	**VLK-A**	**20**	**16**	**14**	**16**	**14**	**19**	**19**
2	VLK-B	19	16	13	15	14	17	17
3	VLK-C	17	15	10	9	10	15	15
4	VLK-D	17	15	9	8	8	16	16
5	VLK-F	17	14	12	11	12	18	15
6	VLK-H	17	12	10	9	14	14	11
7	VLK-I	15	12	11	10	12	10	10
8	VLK-K	16	13	9	9	10	12	12
9	VLK-N	18	15	14	13	13	15	13
10	VLK-O	9	8	8	8	9	13	12
11	VLK-R	16	12	10	10	10	14	10
12	VLK-S	15	10	9	9	9	12	10
13	VLK-T	12	8	8	7	8	12	10

Sa-*Staphylococcus aureus*, **Bs**- *Bacilus subtilis*, **Pa**- *Pseudomonas aeruginosa*, **Pv**- *Proteus vulgaris*, **Xc**-*Xanthomonas campestris*, **Ec**- *Escherichia coli* and **Ca**- *Candida albicans*

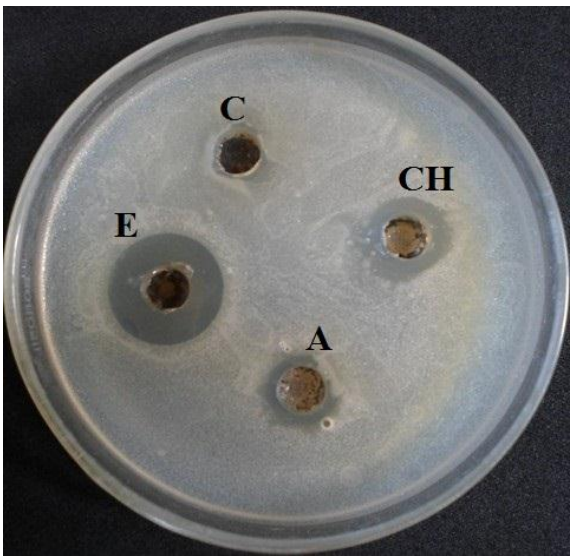

Fig: 2. Antibacterial activity of the strain VLK-A against *Staphylococcus aureus*
E= Ethyl acetate extract, CH= Chloroform extract, A= Acetone extract and C= control (Ethyl acetate)

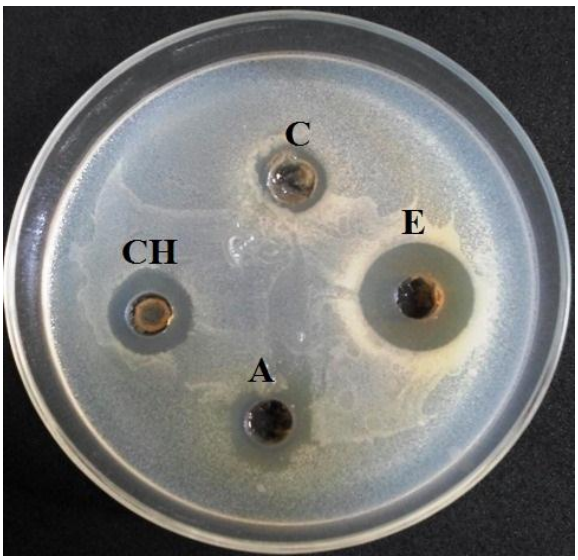

Fig.3. Antifungal activity of the strain VLK-10 against *Candida albicans*
E= Ethyl acetate extract, CH= Chloroform extract, A= Acetone extract and C= control (Ethyl acetate)

Table 4: Cultural characteristics of the strain VLK-A

S.No	Medium	Growth	Aerial mycelium	Substrate mycelium	Pigmentation
1	Tryptone Yeast extract agar(ISP-1)	Moderate	White to Grayish	Brown	Nil
2	Yeast extract Malt extract dextrose agar(ISP-2)	Good	White to Grayish	Brown	Nil
3	Oat-meal agar(ISP-3)	Poor	Nil	Nil	Nil
4	Inorganic salts starch agar(ISP-4)	Good	white to Grayish	Brown	Nil
5	Glycerol asparagine agar (ISP-5)	Moderate	white to Grayish	Brown	Nil
6	Starch-casein agar(ISP-6)	Good	white to Grayish	Brown	Nil
7	Tyrosine agar (ISP-7)	Moderate	white to Grayish	Brown	Nil
8	Asparagine glucose agar	Moderate	white to Grayish	Brown	Nil
9	Nutrient agar	Moderate	white to Grayish	Brown	Nil

Table 5: Cultural characteristics of the strain VLK-10

S.No	Medium	Growth	Aerial mycelium	Substrate mycelium	Pigmentation
1	Tryptone Yeast extract agar(ISP-1)	Moderate	White	Pale yellow	Nil
2	Yeast extract Malt extract dextrose agar(ISP-2)	Good	White	Pale yellow	Nil
3	Oat-meal agar(ISP-3)	Poor	Nil	Nil	Nil
4	Inorganic salts starch agar(ISP-4)	Good	White	Pale yellow	Nil
5	Glycerol asparagine agar (ISP-5)	Good	White	Pale yellow	Nil
6	Starch-casein agar(ISP-6)	Good	White	Pale yellow	Nil
7	Tyrosine agar (ISP-7)	Moderate	White	Pale yellow	Nil
8	Asparagine glucose agar	Moderate	White	Pale yellow	Nil
9	Nutrient agar	Good	White	Pale yellow	Nil

The strain VLK-10 exhibited good growth on ISP-2, ISP-4, ISP-5, ISP-6 and nutrient agar media out of nine culture media tested. The growth was moderate on ISP-1, ISP-7 and asparagine glucose agar while it was poor on ISP-3. The color of aerial mycelium was white when cultured on different media, while the substrate mycelium was pale yellow.

3.1 Morphological characteristics of the strains

The strain VLK-A exhibited typical morphological characteristics of *Nocardiopsis.* Micromorphology of the strain was examined by cover slip culture method as well as by Scaning electron microscope. The aerial mycelium is unbranched, white in color with thin substrate mycelium. The spores are smooth and rectangular in shape (Fig.4).

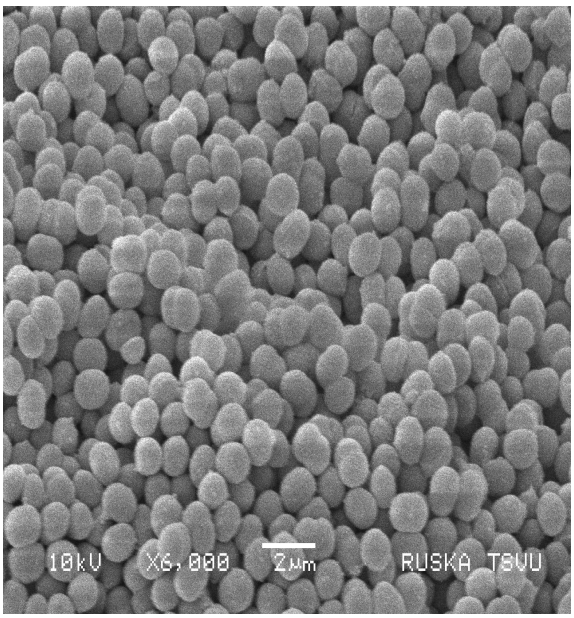

Fig: 4. Scanning electron microscopic photograph of the strain VLK-A

The strain VLK-10 has extensively branched aerial mycelium and bear short chains of spores (fig.5). As its sporophore morphology is of rectus-flexibilis type, the strain is placed in the rectus-flexibilis group of *Streptomyces.*[18]

3.2 Physiological and biochemical characteristics of the strains

Physiological and biochemical characteristics of the strains VLK-A and VLK-10 are recorded in table 6. Kampfer *et al.* [19] suggested the physiological tests as indispensible tools for classification and identification of actinobacteria. Both the strains had the ability to hydrolyze starch and exhibited positive response to citrate utilization, Urease production and catalase production but negative for indole, methyl red, Vogues-Proskauer and nitrate reduction tests and also hydrogen sulphide production. The utilization of starch revealed the ability of the strain to produce extracellular amylase. Positive reaction with catalase revealed its potential to withstand the stress generated by reactive oxygen species.

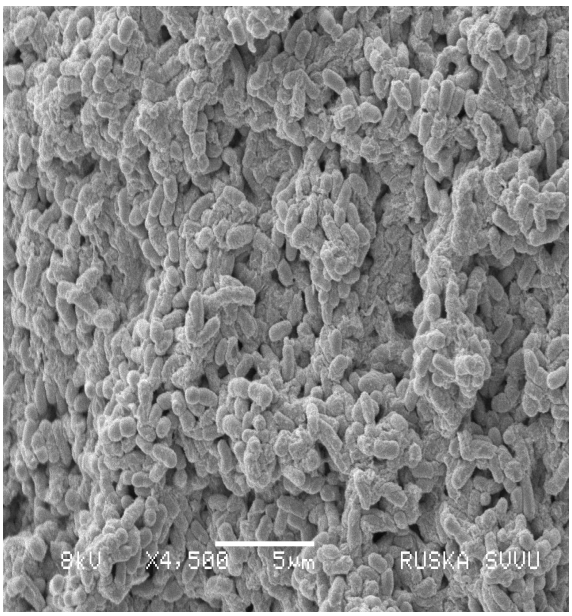

Fig: 5. Scanning electron microscopic photograph of the strain VLK-10

Table 6: Morphological, Physiological and biochemical characteristics of strains VLK-A and VLK-10

Character	Response	
	VLK-A	VLK-10
Morphological characters		
Color of aerial mycelium	White to grayish	white
Color of substrate mycelium	Brown	Pale yellow
Physiological characters		
Gram's reaction	+	+
Production of melanin pigment	-	-
Biochemical characters		
Catalase production	+	+
Urease production	+	+
Hydrogen sulphide production	-	-
Nitrate reduction	-	-
Starch hydrolysis	+	+
Gelatin liquefaction	-	-
Methyl red test	-	-
Voges proskauer test	-	-
Indole production	-	-
Citrate utilization	+	+

Gene sequences of 16S rRNA of VLK-A and VLK-10 were blasted against nucleotide database of the NCBI. The library search reported matching strains and the sequences were aligned with the set of published sequence on the basis of the conserved primary sequence and also by nucleotide BLAST similarity search analysis. Based on the 16S rRNA gene sequences, the strain VLK-A was identified as *Nocardiopsis alba* and the strain VLK-10 as *Streptomyces albiaxialis* (Figs. 6 & 7).

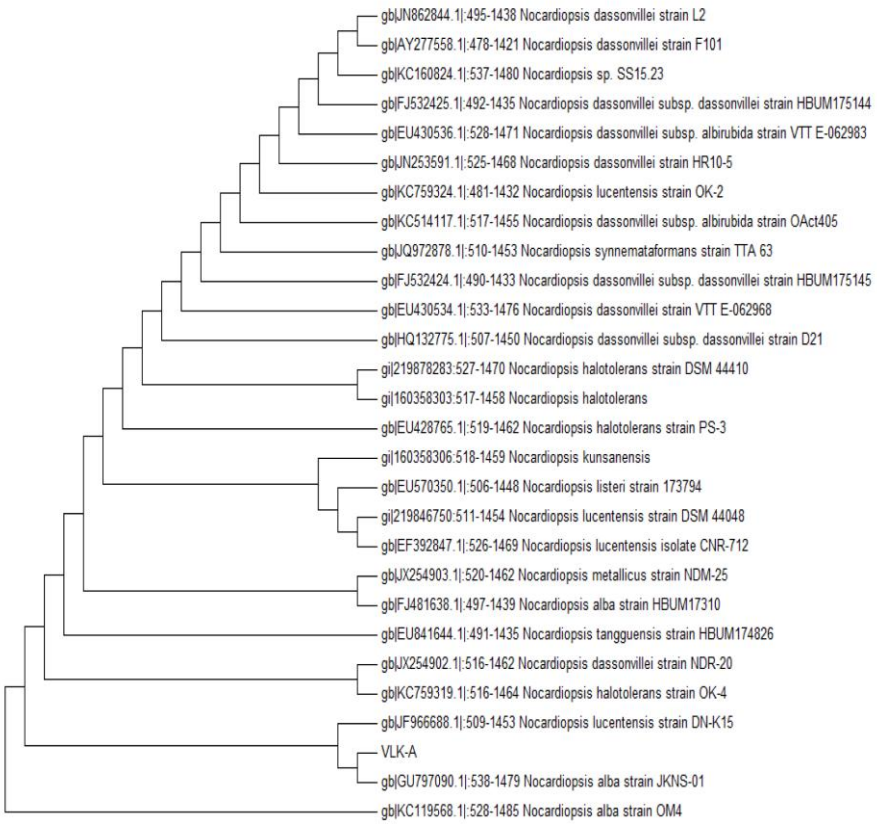

Fig.6 Maximum Parsimony tree based on partial 16S rRNA gene sequence showing relationship between the strain VLK-A and related members of the genus *Nocardiopsis.* The numbers at the nodes indicate the level of bootstrap support based on Maximum parsimony analysis of 1000 resampled datasets; only values above 50% are given.

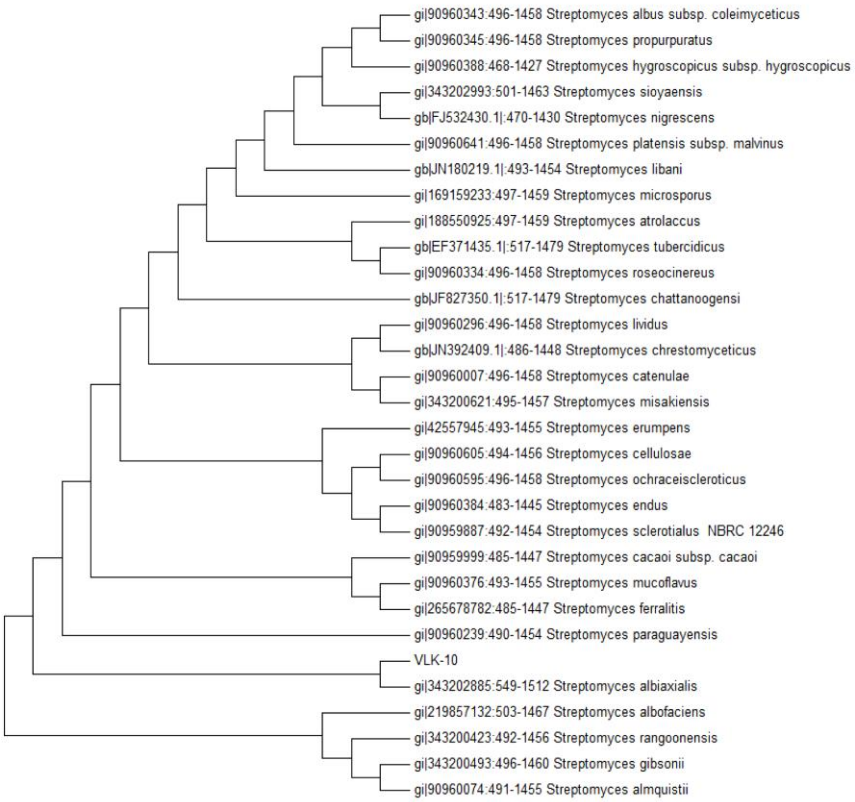

Fig.7 Maximum Parsimony tree based on partial 16S rRNA gene sequence showing relationship between isolate VLK-10 and related members of the genus *Streptomyces.* The numbers at the nodes indicate the level of bootstrap support based on Maximum parsimony analysis of 1000 resampled datasets; only values above 50% are given.

The 16S rRNA sequences were deposited in the GenBank database of NCBI under the accession numbers of the strains **KP170479** (VLK-A) and **KP170480** (VLK-10). Further studies regarding the optimization of nutritional and physiological parameters, extraction, purification and structural elucidation of bioactive compounds are in progress.

4. CONCLUSION

Actinobacterial population in two mangrove ecosystems exhibited seasonal variation with high counts in winter season. Two potent actinobacteria namely *Nocardiopsis alba* VLK-A and *Streptomyces albiaxialis* VLK-10 were reported from these locations. It is evident from the study that marine habitats of South coast of Andhra Pradesh, India serve as a good source of potent actinobacteria with broad spectrum antimicrobial activity.

5. ACKNOWLEDGEMENTS

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6. REFERENCE

1. Mustafa O. Effects of some environmental conditions on biomass and antimicrobial metabolite production by Streptomyces Sp., KGG32. Inter J Agri Biol., 2011; 3: 317-324.
2. Solecka J, Zajko J, Postek M, Rajnisz A. Biologically active secondary metabolites from actinomycetes Cent Eur J Biol., 2012; 7: 373-390.
3. Surajit D, Lyla PS, Ajmal Khan. Marine microbial diversity and ecology: Importance and future perspectives. Curr Scien., 2006; 90: 1325-1335.
4. Usha Kiranmayi M, Muvva V, Sudhakar P. Exploration of Actinobacteria from Mangrove Ecosystems of Nizampatnam and Coringa for Antimicrobial Compounds and Industrial Enzymes. British Biotechnol J., 2014; 4: 173-184.
5. Prasada Rao Naradala, Vijayalakshmi Muvva, Ushakiranmayi Mangamuri and Kavi Kishor PB Exploration of potent actinobacterium Nocardiopsis halotolerans VJPR-2 isolated from mangrove habitats Asian J Pharm Clin Res., 2016; 9: 113-117.
6. Saba Yasmeen, Vijayalakshmi Muvva and Rajesh Kumar Munaganti. Taxonomic characterization of rare actinobacteria isolated from mangrove ecosystem of golakaladindi Krishna district Andhra Pradesh. International journal of current research, 2016; 8: 31060-31066.
7. Manideepa Iindupalli Vijayalakshmi Muvva, Rajesh Kumar Munaganti usha kiranmayi mangamuri.

Saccharomonospora oceani vjds-3, a potent actinobacterial strain from mangrove ecosystem, Asian j pharm clin Res., 2016; 9: 370-373.
8. Krishna Naragani, Rajesh kumar Munaganti, Chandra Kala Sirigiri, Vijayalakshmi Muvva. Antimicrobial Potential of Streptomyces violaceoruber VLK-4 Isolated from South Coast of Andhra Pradesh, India. Int. J. Pharm. Sci. Rev. Res., 2014; 25: 125-129.
9. Wellington EMH, Cross, T. Taxonomy of antibiotic producing actinomycetes and new approaches to their selective isolation. In Bushell M.E. editor. Progress in industrial Microbiology. Elsevier, Amsterdam, 1983.
10. Smith CE. Coccidioidomycosis. Med Clinics. N. America., 1943; 27: 790-807.
11. Waksman SA. The Actinomycetes classification, identification and description of genera and species. The Williams and Wilkins company, Baltimore, 1961.
12. Cappuccino JG, Sherman N. Microbiology, laboratory manual. 8th Ed Pearson education, Inc., New Delhi. 2004.
13. Shirling EB, Gottlieb D. Methods for characterization of Streptomyces sp. Int J Syst Bacteriol., 1966; 16: 313-340.
14. Tamura K, Stecher G, Peterson D, Filipski A and Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Molecular Biology and Evolution, 2013; 30: 2725-2729.
15. Ravi kumar S, Suganthi P. Biodiversity of actinomycetes along the south east coast of Tamilnadu, India. World Appli Scin J., 2011; 13: 119-124.
16. Xu LH, Qi-Renli Cheng-Lin Jiang. Diversity of soil actinomycetes in Yunnan, China. Appl Environ Microbiol, 1996; 62: 244-248.
17. Anindita M, Subhas Chandra S, Mukherjee J. Distribution of actinomycetes, their antagonistic behavior and the physico-chemical characteristics of the world's largest tidal mangrove forest. Appl Microbiol Biotechnol., 2008; 80: 685-695.
18. Pridham TG and Gottlieb D. The utilization of carbon compounds by some Actinomycetales as an aid for species determination. J. Bacteriol., 1948; 56: 107-114.
19. Kampfer P, Kroppenstedt RM, Dott W.A numerical classification of the genera Streptomyces and Streptoverticillium using miniaturized physiological tests. J. Gen. Microbiol., 1991; 137: 1831-1891.