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RESEARCH ARTICLE

TAXONOMIC CHARACTERIZATION OF RARE ACTINOBACTERIA ISOLATED FROM MANGROVE ECOSYSTEM OF GILAKALADINDI, KRISHNA DISTRICT, ANDHRA PRADESH

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ABSTRACT

The present work is aimed to isolate, identify and characterize the rare actinobaterial strains VJSY-1 and VJSY-14 from the mangrove soils of Gilakaladindi, Krishna district of Andhra Pradesh. The soil samples were collected, pre treated with calcium carbonate and used for the isolation of rare actinobacterial strains. Identification of these strains was carried out by polyphasic taxonomical studies including morphological, cultural, physiological, biochemical characters along with 16S rRNA analysis. Phylogenetic tree was constructed using Molecular Evolutionary Genetic Analysis software (MEGA) version 6.0. Phylogenetic analysis by 16S rRNA sequencing revealed that the strains VJSY-1 and VJSY-14 are closely related to the genus *Nocardiopsis* and the bioactive metabolites produced by the strains inhibited Gram positive bacteria *Staphylococcus aureus* (MTCC 3160), *Bacillus megaterium* (NCIM 2187), *Bacillus subtilis*, Gram negative bacteria *Xanthomonas campestris* (MTCC 2286), *Pseudomonas aeruginosa* (ATCC 9027) and *Escherichia coli* (ATCC 35218), and fungi like *Aspergillus niger*, *Fusarium solani*, *F. oxysporum* and *Candida albicans* (MTCC 183). Attempts are being made to optimize the cultural conditions for enhanced production of bioactive metabolites by the strains as well as characterization of the bioactive compounds.

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INTRODUCTION

Mangrove actinobacteria remains a fundamental scientific challenge to unlock the biosynthetic capabilities that hold the greatest promise for the discovery of new agents from the mangrove environment. Mangrove environment is high moisture, high salinity and hypoxia tolerant ecosystem (Li *et al*., 2009) which breeds many kinds of novel microorganisms and plants that have been a rich source of bioactive natural products. Actinobacteria are the most fascinating microorganisms with their developmental lifecycle, including morphological and physiological differentiation and the rich repertoire of secondary metabolites constituting about 70-80% of bioactive secondary metabolites. Among the well-characterized pharmaceutically relevant microorganisms, actinobacteria remain major sources of novel, therapeutically relevant natural products. Bioactivity studies of secondary metabolites from mangrove actinobacteria have become a hot spot now adays. Recent reports of screening tests are not only on antimicrobial, antineoplastic (Hong *et al*., 2009) but also on production of commercially important enzymes such as protease, cellulase, amylase, esterase and L-asparaginase (Rajesh kumar *et al*., 2015; He *et al*., 2012). Compounds with unique structure and potential medicinal use have been obtained from mangrove actinomycetes in recent years as evidenced by the fact that the well-known compound salinosporamide-A produced by *Salinispora* is the first and most advanced anticancer drug to be processed for clinical trials (Feling *et al*., 2003). Xiamycins are Indolosesquiterpene compounds isolated from mangrove prokaryotes for the first time (Ding *et al*., 2010). The mangrove environment is a virtually untapped source of novel and diverse natural products and needs much more attention. Rare actinobacteria may result in increased chances of discovering novel structures but their genetics and physiology are not completely known. Knowledge about distribution of such unexploited groups of microorganisms must be augmented inorder to speed up their isolation process. Some genera of this group are *Actinomadura*, *Actinoplanes*, *Amycolatopsis*, *Actinokineospora, Acrocarpospora, Actinosynnema*, *Catenuloplanes*, *Cryptosporangium*, *Dactylosporangium*, *Kibdelosporangium*, *Kineosporia*, *Kutzneria*, *Microbiospora*, *Microtetraspora*, *Nocardiopsis, Nocardia*, *Nonomurea*, *Planomonospora*, *Planobispora*, *Pseudonocardia*, *Saccharomonospora*,

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Saccharopolyspora, *Saccharothrix, Streptosporangium*, *Spirilliplanes*, *Thermomonospora*, *Thermobifida* and *Virgosporangium* (Lazzarini *et al*., 2000). The genus *Nocardiopsis* was described by J. Meyer that comprises actinobacteria with fragmenting mycelium and currently comprises 18 species with validly published names (Meyer, 1976). In the last two decades several new species of *Nocardiopsis* have been described and many interesting substances, such as enzymes and antibiotics have been discovered including Dopsisamine, a novel antibiotic (Takahashi *et al*., 1986), Nocamycin, a new antineoplastic antibiotic (Brazhnikova *et al*., 1976), Lucentamycins, a cytotoxic peptide (Cho *et al*., 2007), 3-Trehaloseamine, a disaccharide antibiotic (Dolak *et al*., 1980), Apoptolidin, a new apoptosis inducer (Kim *et al*., 1997), Portmicin, a new antibiotic (Kusakabe *et al*., 1987) etc. In our continuous effort to explore the diversity of the potential bioactive rare actinobacteria from the mangrove ecosystem of Krishna district, we have isolated strains VJSY-1 and VJSY-14 with high antimicrobial activity from the 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-1 and VJSY-14 based on the polyphasic approach.

MATERIALS AND METHODS

Sampling and pretreatment of soil

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 (Krishna *et al*., 2014).

Selective isolation of rare actinobacteria

The pretreated soil samples were serially diluted in sterile distilled water and plated on selective media such as humic acid vitamin (HV) agar medium supplemented with 5% NaCl (Hayakawa, 2008). The medium was adjusted to pH 7.0 and 0.1 mL of diluted sample was spread over HV agar supplemented with 50µg/mL Cycloheximide and 50µg/mL Nalidixic acid to reduce the fungal and bacterial contamination respectively and incubated at 30±2^{o}C for two weeks. Actinobacterial colonies (Shirling and Gottlieb, 1966) were picked out, purified and preserved on YMD (yeast extract malt extract dextrose) agar slants at 4°C (Williams and Cross, 1971). The isolated actinobacterial strains were initially screened for antimicrobial activity with regard to their potential to generate bioactive compounds.

Identification of rare actinobacterial strains by polyphasic taxonomy

Among the 25 different actinobacterial strains tested for antimicrobial activity, two predominant rare actinobacterial strains, VJSY-1 and VJSY-14 found to be potent as they exhibited high antimicrobial activity. The rare actinobacterial strains were characterized by cultural, morphological, physiological, biochemical and molecular methods. The microscopic characterization was carried out by slide culture method (Kavitha *et al*., 2010) taking into account the nature of mycelium, color and spore arrangement (Pridham *et al*., 1980). 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 strains were grown on six 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 (Pridham and Lyons, 1980). Melanin pigment production was assessed by culturing the strain on tyrosine agar (ISP-7) medium (Williams and Cross, 1971). Hydrolysis of starch and nitrate reduction (Pridham and Gottlieb, 1948) and H_2S production were also tested (Gordon, 1966). Physiological characteristics such as the effect of pH (5-9), temperature (20-45 °C) and salinity on the growth of the strain analyzed. The ability of the strains to produce industrially important enzymes such as amylase, asparaginase, caseinase and cellulase were tested.

Molecular Identification of the potent strains VJSY-1 and VJSY-14

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 min followed by 30 cycles of amplification (Denaturation at 94 °C for 60 sec, annealing temperature of 55 °C 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 1µL 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).

Pair wise sequence alignment

The 16S rRNA gene sequence of the strains was aligned using BLAST against the gene library available for *Nocardiopsis* species in the GenBank. Pairwise evolutionary distances were computed by MEGA software version 6.0.

Multiple Sequence Alignment

The phylogenetic analysis was conducted using the Maximum Parsimony and Neighbor Joining methods using BLAST and CLUSTAL W. The closely related homologous strains were identified, retrieved and compared to the sequence of the isolated strain using CLUSTAL W available with the MEGA 6.0 Version (Tamura *et al*., 2011).

Nucleotide Sequence accession numbers

The 16S rRNA gene (rDNA) sequence of the strains VJSY-1 and VJSY-14 were registered in the GenBank database.

Determination of growth pattern

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 h 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.

Antimicrobial profile of VJSY-1 and VJSY-14

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 rare actinobacterial isolates VJSY-1 and VJSY-14 were inoculated and the fermentation was carried out at 30°C for 120 h 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 80µl of the crude extract and 80µl of negative control were poured in to separate wells. 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), *Bacillus subtilis* (ATCC 6633), *Bacillus megaterium* (NCIM 2187), *Xanthomonas campestris* (MTCC 2286), *Pseudomonas aeruginosa* and *Escherichia coli* (ATCC 9027).

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

RESULTS AND DISCUSSION

Among the 25 distinct Mangrove actinobacterial strains isolated, the predominant actinobacterial strains VJSY-1 and VJSY-14 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 *Nocardiopsis*. Morphological and micro morphological observation of the strains revealed the presence of fragmenting mycelium (Figs.1&2). The Strains did not produce any pigment on the media tested.

Identification of the strains VJSY-1 and VJSY-14

The cultural characteristics of the strains VJSY-1 and VJSY-14 are represented in the tables 1&2. Strain VJSY-1 exhibited luxurious growth on Yeast extract malt extract dextrose agar (YMD), good growth on Tryptone yeast extract agar (ISP-1), Peptone Yeast extract iron agar (ISP-6), Nutrient agar medium (NAM), Humic acid vitamin (HV) agar. The growth was moderate on Glycerol asparagine agar (ISP-5), Starch casein nitrate agar (SCN) while it was poor on Inorganic salts starch agar (ISP-4), Tyrosine agar (ISP-7). The color of aerial mycelium was white and the substrate mycelium was pale yellow. Strain VJSY-14 exhibited luxurious growth on YMD, Peptone Yeast extract iron agar (ISP-6), good growth on ISP-1, ISP-7 and HV agar. The growth was moderate on ISP-5 medium while it was poor on SCN agar. The color of aerial mycelium was white and the substrate mycelium was pale yellow. Soluble pigment production was not observed on the media tested.

Biochemical Characteristics

The biochemical characteristics of the strains are presented in table-3. The strain VJSY-1 had the ability to hydrolyze starch and exhibited positive response to indole production, catalase activity, urease activity, nitrate reduction, gelatin liquefaction but negative for Methyl red, Voges-Proskauer, citrate utilisation, H_2S production and casein hydrolysis. The strain VJSY-14 exhibits positive response to indole production, urease activity, catalase activity, nitrate reduction but negative for methyl red, Voges-Proskauer, citrate utilisation, H_2S production, casein hydrolysis and gelatin liquefaction. Both the strains could also produce enzymes like L-asparaginase and cellulase.

Physiological characteristics

Several physiological and biochemical tests were carried out as they are significant tools for identification of actinobacteria (Cowan, 1974). Growth of the strains VJSY-1 and VJSY-14 occurred in the pH range of 5-9 with optimum growth at pH 7. The temperature range for the growth of both the strains was 20-50^{o} C with the optimum growth at 30^{o} C. The strains VJSY-1 and VJSY-14 exhibited salt tolerance upto 9% with optimum growth at 4% and 5% NaCl respectively (Table 3).

Molecular Characterization

The 16S rDNA sequence data supported the assignment of the strain VJSY-1 and VJSY-14 to the genus *Nocardiopsis*. The partial 16S rDNA sequences of the strains were submitted to the GenBank database with accession numbers KP642767 and KP863922.

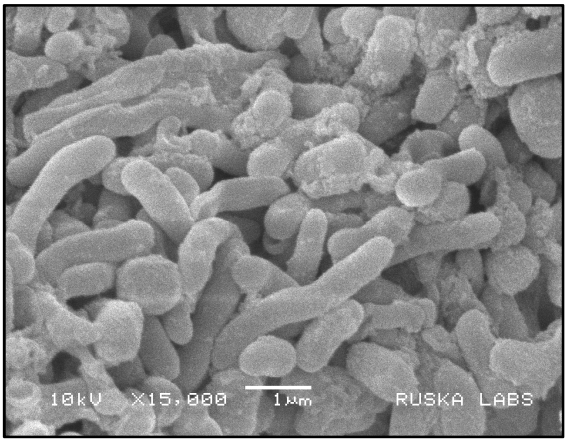


Fig. 1. Scanning electron microscopic photograph of *Nocardiopsis* sp.VJSY-1

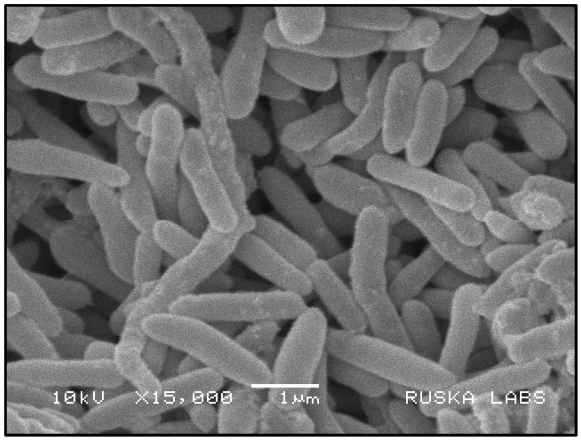


Fig. 2. Scanning electron microscopic photograph of *Nocardiopsis metallicus* VJSY-14

Table 1. Cultural characteristics of the strain VJSY-1

Name of the Medium	Growth	AM*	SM**	Pigmentation
Tryptone yeast-extract agar (ISP-1)	Good	White	Pale Yellow	NO
Yeast extract malt extract dextrose agar (ISP-2)	Luxurious	White	Pale Yellow	NO
Inorganic salts Starch Agar (ISP-4)	Poor	White	White	NO
Glycerol Asparagine agar (ISP-5)	Poor	White	Creamy white	NO
Peptone yeast extract iron agar(ISP-6)	Good	White	Pale brown	NO
Tyrosine agar (ISP-7)	Poor	White	Pale brown	NO
Starch casein nitrate agar	Moderate	White	Pale white	NO
Nutrient agar	Good	White	Pale Yellow	NO
Humic acid vitamin agar	Good	White	Hyaline	NO

*Aerial mycelium; **Substrate mycelium; '-' no growth.

Table 2. Cultural characteristics of the strain VJSY-14

Name of the Medium	Growth	AM*	SM**	Pigmentation
Tryptone yeast-extract agar (ISP-1)	Good	White	Pale Yellow	NO
Yeast extract malt extract dextrose agar (ISP-2)	Luxurious	White	Pale Yellow	NO
Inorganic salts Starch Agar (ISP-4)	-	-	-	-
Glycerol Asparagine agar (ISP-5)	Moderate	White	White	NO
Peptone yeast extract iron agar(ISP-6)	Luxurious	White	Grayish black	NO
Tyrosine agar (ISP-7)	Good	White	White	NO
Starch casein nitrate agar	Poor	White	Hyaline	NO
Nutrient agar	Poor	White	Pale Yellow	NO
Humic acid vitamin agar	Good	White	Hyaline	NO

*Aerial mycelium; **Substrate mycelium; '-' no growth.

Table 3. Morphological, biochemical and physiological characteristics of the strains

Character / Response	Response	
Morphological characters	VJSY-1	VJSY-14
Color of aerial mycelium	White	White
Color of substrate mycelium	Pale yellow	Pale yellow
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	20-50°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	7
NaCl tolerance	Up to 9%	Up to 9%
Optimum NaCl concentration	4%	5%
Asparaginase	+	+
Caseinase	-	-
Cellulase	+	+

VJSY-1 and VJSY-14, + = Positive; – = Negative.

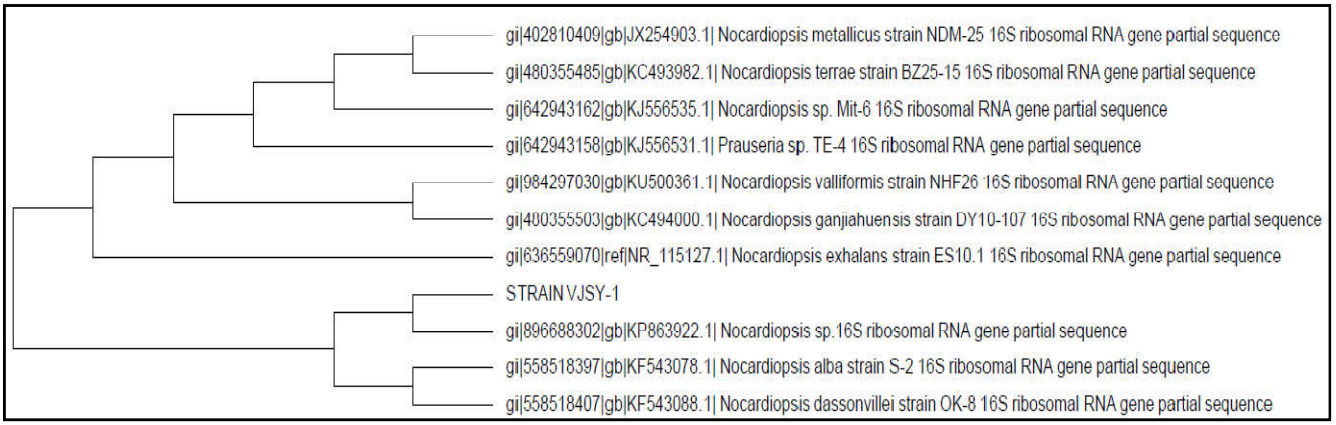


Fig. 3. Maximum parsimony tree based on partial 16S rRNA gene sequence showing relationship between strain VJSY-1 and related members of the genus *Nocardiopsis*

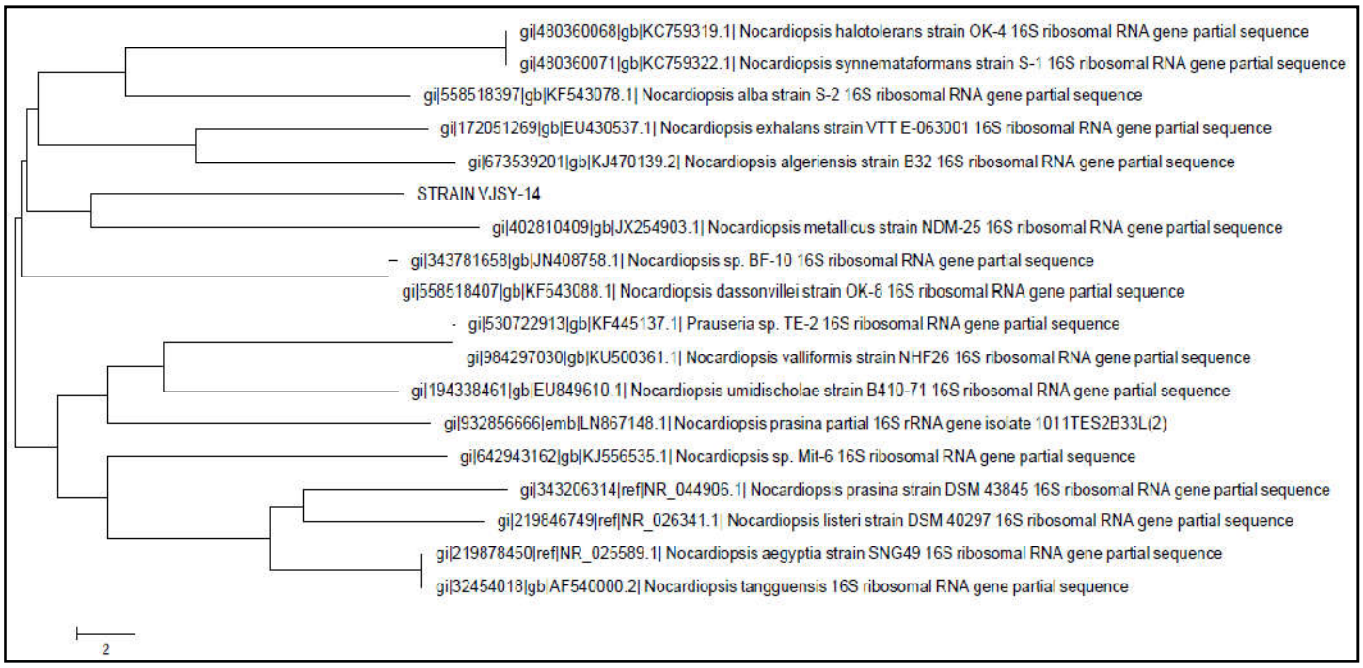
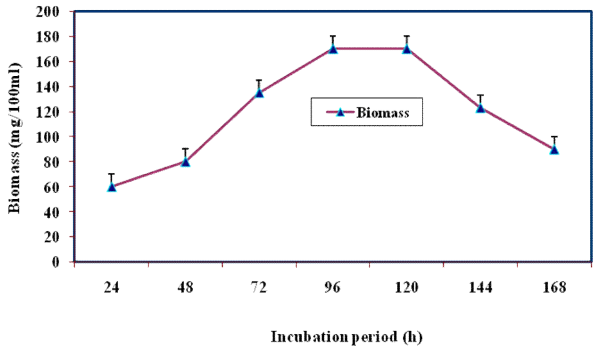


Fig. 4. Neighbor-Joining tree based on partial 16S rRNA gene sequence showing relationship between strain VJSY-14 and the related members of the genus *Nocardiopsis*



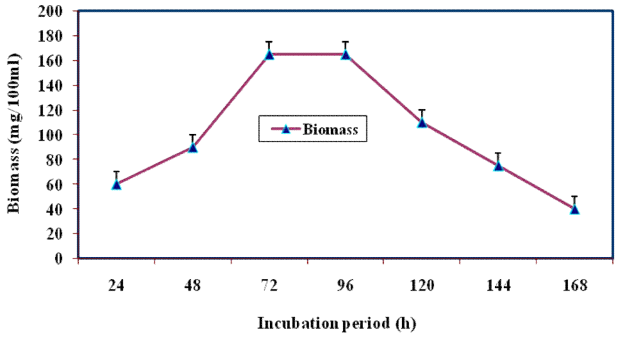


Fig. 5. Growth pattern of the strain *Nocardiopsis* sp. VJSY-1 **Fig. 6. Growth pattern of the strain *Nocardiopsis metallicus* VJSY-14**

Table 4. Antibacterial and antifungal activity of *Nocardiopsis* sp. VJSY-1 and *N. metallicus* VJSY-14

Test organism	Zone of inhibition (mm)		Positive control #
Bacteria	VJSY-1	VJSY-14	
Staphylococcus aureus	25	23	22
Bacillus megaterium	23	17	25
Bacillus subtilis	20	20	26
Xanthomonas campestris	25	17	22
Escherichia coli	25	21	20
Pseudomonas aeruginosa	30	20	22
Fungi			
Aspergillus niger	15	14	20
Fusarium solani	18	17	21
F. oxysporum	14	15	18
Candida albicans	30	23	28

#Positive control: Tetracycline against bacteria, Griseofulvin against yeast and Carbendazim against fungi.

The partial sequences were 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 6 Version. Based on the morphological, physiological, Biochemical and molecular characteristics by 16S rRNA sequencing, the strain VJSY-1 was identified as *Nocardiopsis* sp. and the strain VJSY-14 was identified as *Nocardiopsis metallicus*. The Phylogenetic trees were constructed using MEGA software Version 6.0. (Fig's.3&4).

Growth Pattern and antimicrobial profile of the strains Nocardiopsis sp. VJSY-1 and N. metallicus VJSY-14

The growth pattern of *Nocardiopsis* sp.VJSY-1 and *N. metallicus* VJSY-14 were studied on YMD broth. The stationary phase of the strain VJSY-1 extended from 96 h to 120 h of incubation and for the strain VJSY-14 is from 72 h to 96 h. The bioactive metabolites obtained from 5-day-old culture of VJSY-1 exhibited high antimicrobial activity against the test microorganisms (Fig. 5) while 4 day-old culture of VJSY-14 exhibited high antimicrobial activity (Fig. 6). Munaganti *et al*. (2015) and Naragani *et al.* (2014) reported that metabolites obtained from five day old culture of *Rhodococcus erythropolis* VL-RK_05 and *R. erythropolis* VLK-12 showed maximum antimicrobial activity (Munaganti *et al*., 2015; Krishna *et al*., 2014). The secondary metabolites obtained from four-day old culture of *Nocardia levis* MK-VL_113 showed high antimicrobial activity against the test microbes (Kavitha and Vijayalakshmi, 2009). The antimicrobial spectrum of the strains cultured on YMD broth for five days and four days were given in table 4. The metabolites extracted from the five days old culture broth of VJSY-1 showed maximum activity against *P. aeruginosa, C. albicans* followed by *S. aureus, X. campestris, E. coli, B. megaterium* and *B. subtilis* where as the metabolites from four day old culture broth of strain VJSY-14 showed maximum activity against *S. aureus, C. albicans* followed by *E. coli, B. subtilis, P. aeruginosa, B. megaterium* and *X. campestris.*

Conclusion

Based on the screening results, it is evident that mangrove habitats of Gilakaladindi of Andhra Pradesh, India, serve as a good source for the isolation of potent rare actinomycetes with broad spectrum antimicrobial activity. The extraction and purification of bioactive compounds produced by *Nocardiopsis* sp.VJSY-1 and *N. metallicus* VJSY-14 are in progress.

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