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Isolation, Identification and Molecular Characterization of Rare Actinomycetes from Mangrove Ecosystem of Nizampatnam

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ABSTRACT

Aims: Isolate and characterize the antimicrobial actinomycetes from sediments of Mangrove ecosystems of Nizampatnam located in the south coastal region of Andhra Pradesh, India.
Methodology and Results: The Mangrove soil samples were collected, pre-treated and plated on asparagine-glucose agar medium. Identification of the strain was carried out by employing the polyphasic taxonomical studies including the 16S rRNA sequence based analysis. Phylogenetic tree was constructed using the Molecular Evolutionary Genetic Analysis (MEGA) version 5. The potent bioactive metabolite strain was isolated and designated as VUK-10. Further polyphasic studies revealed that the Isolate VUK-10 belongs to the genera *Pseudonocardia.* Phylogenetic analysis of 16S rRNA sequencing studies revealed that the strain is closely related to *Pseudonocardia endophytica* and the bioactive metabolites produced by the isolate inhibited Gram positive, Gram negative and Fungi.
Conclusion, significance and impact of study: The isolation, characterization of the rare actinomycetes from the mangrove ecosystem will be useful for the discovery of the novel bioactive metabolites that are effective against wide range of pathogens.

Keywords: Pseudonocardia, Mangrove ecosystem, Pre-treatment, Bioactive metabolites, Polyphasic Taxonomy.

INTRODUCTION

Mangroves are the coastal wet land forests mainly found in the intertidal zone of estuaries, back waters, deltas, creeks, lagoons, marshes and also mud flats of the tropical and subtropical latitudes (Sahoo *et al*., 2009). The ecosystems where the mangrove plants grow are termed as “Mangrove Ecosystem” which occupies millions of hectors across the world coastal areas (Spalding *et al.,* 1997; Alongi, 2002). Mangrove marine ecosystems are largely uncaged source for screening and isolation of new microbes with rich potential to produce the important active secondary bioactive metabolites. The environment of the mangrove ecosystem is saline, and highly rich in an organic matter because of its various microbial enzymatic and metabolic activities (Kizhekkedathu and Parukuttyamma, 2005). The natural products remain to be the most important source of antibiotics (Bull and Starch, 2007). Marine derived compounds are more efficient in action against the pathogens that are resistance to the existing antibiotics (Donia and Hamman, 2003).

The risk undermining the health care system is because of the relentless and rapid spread of the multiple antibiotic resistant pathogens causing life threatening infection (Talbot *et al.,* 2006) and so the demand for new antibiotics continues to grow. Although, considerable progress has been made within the fields of chemical synthesis and engineered biosynthesis of antibacterial compounds, nature remains the richest and the most versatile source for new antibiotics (Baskaran *et al.,* 2011). Since actinomycetes have an important proven capacity to produce novel antibiotics (Bentley *et al.,* 2002), the practice in screening such organisms for the new bioactive compounds is continued (Bérdy, 2005). However, difficulty to discover the commercially potent secondary metabolites from well-known actinomycetes is becoming increasingly difficult due to the practice of wasteful screening that is leading to rediscovery of the known bioactive compounds (Kui *et al*., 2009). This stringent condition emphasizes the need to screen and isolate the undiscovered representatives of the unexplored actinomycetes taxa. It is a clear object that the mangrove ecosystem is a rich source of novel actinomycetes that have the capacity to produce interesting new bioactive compounds including antibiotics.

Screening for the microbial species is an important aspect as there is a remarkable source for the production of structurally diverse secondary metabolites that possess pharmaceutically relevant biological activities (Berdy, 2005). It has long been an observed fact that the search for the new secondary metabolites from microorganisms in general has been confounded because different strains belonging to the same species are capable of producing different secondary metabolites (Waksman and Bugie, 1943) but identical secondary metabolites are produced by taxonomically diverse strains (Larsen *et al*., 2005).

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Challenges raised questions against using the traditional taxonomic methods to differentiate among closely related actinomycetes contribute to the former. Sequence based approaches can now be addressed towards these challenges that resolute the taxonomic and opportunities to extract the relationship between the groups of related strains and the secondary metabolites they produce. In addition, these methods are the tools to probe the evolutionary history of the metabolic pathways and thus infer the root and action of the lateral gene transfer (LGT) responsible for the unrelated organisms to produce similar compounds (Paul *et al*.In view of the above discussion, the present study is taken up to isolate, screen and characterize the biologically diverse strains of actinomycetes from the mangrove sediment samples for bioactive secondary metabolites. In the systematic screening programme, an actinomycetes strain VUK-10 was isolated from a sediment sample taken from mangrove area on the south coast of India. Taxonomic characterization was carried out based on 16S rRNA sequence analysis in combination with morphological, physiological and biochemical data. The ability to produce antibacterial and antifungal compounds was also investigated.

MATERIALS AND METHODS

Sample Collection

Sediment samples were collected from different areas of the Nizampatnam mangrove ecosystem (Lat, 15° 54'0 N; Long 80° 40'0 E) situated along the south coast of Andhra Pradesh, India. The central portion of the 6-10 cm sediment sample was taken and transferred to a sterile bag and transported immediately to the laboratory.

Isolation

The samples thus collected were air-dried at room temperature. Actinomycetes were isolated by suspending pre-treated soil sample at 55 °C for 15 min in a suspension fluid containing osmoprotectant (i.e. quarter strength ringer's solution), serially diluted up to 10^{-5} and placed on asparagine–glucose agar medium (Smith, 1943) supplemented with nalidixic acid (25 µg/mL) and secnidazole (25 µg/mL) in order to retard the growth of bacteria and fungi respectively. After incubation of the plates at room temperature for two weeks, typical actinomycetes colonies selected on morphological basis (Shirling and gottlieb, 1996) were picked out, purified and preserved on medium at 4 °C (Williams and Cross, 1971). The isolated actinomycetes strains were then screened with regard to their potential to generate bioactive compounds (Atta *et al*., 2009).

Identification by Polyphasic Taxonomy

The potent actinomycete isolate was characterized by morphological, cultural, physiological, biochemical and molecular methods. Morphological methods consist of macroscopic and microscopic methods. The microscopic characterization was done by slide culture method (Williams and Cross, 1971). The mycelium structure, colour and arrangement of spores on the mycelium were observed through inverted microscope [400X, Olympus]. The cultural characteristics of the strain were studied on various ISP and non-ISP media (Shirling and Gottlieb, 1966). Melanin pigment production was determined by growth on tyrosine agar (ISP-7) medium (Pridham *et al.,* 1957). Hydrolysis of starch and nitrate productions was carried out according to the method of (Gordon, 1966). H_2S production was tested according to (Cowan, 1974). Liquefaction of gelatine was evaluated by the method of (Waksman, 1961). Catalase test was evaluated by the method of (Jones, 1949). Carbohydrate utilization was determined by growth on carbon utilization medium (ISP-9) (Pridham and Gottlieb, 1948) supplemented with 1 % carbon source. The cultural characteristics of the strain were studied on different ISP and non ISP media. Physiological characterization such as the effect of pH (5-9), temperature (20-60 °C) and salinity were analysed. In addition, the susceptibility of the strain to different antibiotics was determined by paper disc method (Cappuccino and Sherman, 2004).

Actinomycete strains were grown in 10 mL tryptone yeast extract broth (ISP 1) (Shirling and Gottlieb, 1966) with agitation at 30 °C for 18–24 h. Cells were harvested by centrifugation (7500 *g* for 2 min), washed once with 500 mL of 10 mM Tris-HCl/ 1 mM EDTA (TE) buffer (pH 7) and resuspended in 500 mL TE buffer (pH 7). The samples were heated in boiling water for 10 min, allowed to cool for 5 min and centrifuged (7500 *g* for 3 min). The supernatant (300 mL) was transferred to a clean tube and stored at 4 °C. If melanin or other pigments were produced during growth in ISP 1, cultures were grown in Middle brook 7H9 broth, as these pigments interfere with the PCR (Polymerase chain reaction).

Molecular Identification

The genomic DNA (Deoxyribo nucleic acid) used for the PCR was prepared from the single colonies grown on the Yeast extract malt extract dextrose agar (ISP-2) medium for 3 days. The total genomic DNA extracted from the potent strain (VUK-10) was isolated 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 Universal Primers. (Actino specific forward Primer -5'-GCCTAACACATGCAAGTCGA-3' and Actino Specific Reverse primer - 5'-CGTATTACCGCGGCTGCTGG-5') (Nilsson and Strom, 2002). 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 is 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

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(Eppendorf, Germany). Each reaction mixture contained 1 µL of DNA, 1 µL of 10 pmol 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 (Messner *et al.,* 1994). PCR product was analysed using 1 % agarose gel and the fragment was purified (Helini Pure Fast PCR clean up kit, Helini Bio molecules, India) according to the manufacturer 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 obtained sequences were analyzed for homology using BLASTN (Entrez Nucleotide database).

Nucleotide Sequence Accession Numbers

The 16S rRNA gene (rDNA) sequences of the strain VUK -10 are registered in the GenBank database with the accession number.

Extraction of Antimicrobial Metabolites

The inoculum was prepared by suspending spores from one week old culture (Yeast extract malt extract dextrose agar) in 10 mL of sterile saline. 5 mL of the homogenous spore suspension was used to inoculate 50 mL of asparagines-glucose broth (seed medium) in a 250 mL Erlenmeyer flask, and the culture was incubated at 30 °C for 48 h on a rotator shaker at 120 rpm. Seed culture at the rate of 10 % was transferred into 500 mL of starch - casein broth (Fermentation medium). The fermentation was carried out at 28±2 °C for 96 h under agitation at 120 rpm. Antimicrobial compound was purified from the filtrate by solvent extraction method. Ethyl acetate was added to the filtrate in the ratio of 1:1 (V/V) and shaken vigorously for 1 h for complete extraction. The ethyl acetate phase that contains bioactive metabolites was separated from the aqueous phase. It was evaporated to dryness in water bath and residue obtained was used to determine antimicrobial activity by agar well diffusion method (Cappuccino and Sherman, 2004) and effectiveness was measured by zone of inhibition.

RESULTS AND DISCUSSION

The present study was designed to investigate the mangrove ecosystem of Nizampatnam for novel actinomycetes and their antimicrobial properties. During our continuous search for novel antimicrobial metabolites of Nizampatnam mangrove region led to the isolation of morphologically distinct actinomycetes isolate VUK-10 on asparagine glucose agar media by employing soil dilution plate technique. The strain VUK -10 exhibited typical morphological characteristics of the genus *Pseudonocardia* (Warwick *et al*., 1994; Huang *et al*., 2002). Morphological and micro morphological observation of the strain revealed that aerial and vegetative hyphae were abundant, well developed and fragmented with rod shaped smooth surfaced spores. Soluble pigment production by the strain was not found on the culture media tested except melanin pigmentation on tyrosine (ISP-7) agar media.

Cultural Characteristics

The cultural characteristics of the strain are represented in Table 1. The strain VUK- 10 exhibited good growth on tryptone-yeast extract (ISP-1) agar, yeast extract-malt extract-dextrose (ISP-2) agar, oat-meal (ISP-3) agar, glycerol-asparagine (ISP-5) agar, czapek-dox agar and maltose-tryptone agar. The growth was moderate on starch-inorganic salts (ISP-4) agar, tyrosine agar (ISP-7) and starch casein agar while it was poor on nutrient agar. Creamy white aerial mycelium was found on ISP-1, ISP - 2, ISP-7, maltose tryptone and starch casein agar where as the aerial mycelium was cream colour in ISP-4, ISP-5 and creamy orange in czapek-dox agar. No aerial mycelium was formed on nutrient agar medium. The substrate mycelium ranges from brownish orange (on ISP-1 and ISP-2) to brown (ISP-3, ISP-4, ISP-5 and ISP-7) or light orange (czapek-dox agar, maltose tryptone agar and nutrient agar). Siva Kumar (2001) reported that the cultural characteristics could be used as markers by which an individual strain can be recognized.

Physiological Characteristics

The physiological tests are indispensable tools for classification and identification of actinomycetes and influencing the growth rate of actinomycetes (Kampfer *et al*., 1991; Hasegawa *et al*., 1978; Kim *et al.,* 1999 and Shimizu *et al*., 2000). Growth of the strain VUK-10 occurred in the pH range of 6-9 with optimum growth at pH 7. The temperature range for growth was 20-45 °C with the optimum temperature being 35 °C. The strain exhibited salt tolerance up to 10 % with optimum growth at 3 % NaCl; hence, the strain could be placed in intermediate salt tolerance group according to Tresner *et al. (*1968).

Biochemical Characterization

VUK-10 exhibited positive response to catalase production and citrate utilization but negative for urease, hydrogen sulphide production, nitrate reduction, starch hydrolysis, gelatine liquefaction, indole, methyl red and vogues-proskauer tests. The details of morphological, physiological and biochemical characteristics of the isolate are given in (Table 2).

Utilization of carbon sources by the strains could be used as an aid for species determination (Pridham and Gottlieb, 1948). The strain VUK-10 efficiently utilized the carbon sources such as D-glucose, lactose, maltose, sucrose,

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galactose, fructose, starch but not utilized xylose, arabinose and mannitol (Table 3). Good fellow and Orchard (1974) reported that the antibiotic sensitivity of some nocardioform bacteria as one of the valuable criteria for their taxonomic differentiation. Antibiotic susceptibility testing showed that the isolate was susceptible to streptomycin, gentamicin, rifampicin, chloramphenicol and oxytetracycline and resistance to nalidixic, acid and penicillin (Table 4).

Table 1: Cultural Characteristics of *Pseudonocardia* sp. VUK- 10

S No	Name of the Medium	*Pseudonocardia* sp. VUK-10
1	**Tryptone yeast-extract agar (ISP-1)**	
	Growth	Good
	Aerial mycelium	Creamy white
	Substrate mycelium	Brownish orange
	Pigmentation	Nil
2	**Yeast extract malt extract dextrose agar (ISP-2)**	
	Growth	Good
	Aerial mycelium	Creamy white
	Substrate mycelium	Brownish orange
	Pigmentation	Nil
3	**Oat-meal agar (ISP-3)**	Good
	Growth	White
	Aerial mycelium	Brown
	Substrate mycelium	Nil
	Pigmentation	
4	**Oat-meal agar (ISP-3)**	
	Growth	Good
	Aerial mycelium	White
	Substrate mycelium	Brown
	Pigmentation	Nil
5	**Glycerol asparagine agar (ISP-5)**	
	Growth	Good
	Aerial mycelium	Cream
	Substrate mycelium	Brown
	Pigmentation	Nil
6	**Tyrosine agar(ISP-7)**	
	Growth	Moderate
	Aerial mycelium	Creamy white
	Substrate mycelium	Brown
	Pigmentation	Melanin
7	Starch-casein agar	Moderate
	Growth	Creamy white
	Aerial mycelium	Light brown
	Substrate mycelium	Nil
	Pigmentation	
8	Czapek-dox agar	
	Growth	Good
	Aerial mycelium	Creamy orange
	Substrate mycelium	Light orange
	Pigmentation	Nil
9	Maltose tryptone agar	
	Growth	Good
	Aerial mycelium	Creamy white
	Substrate mycelium	Light orange
	Pigmentation	Nil
10	Nutrient agar	
	Growth	Good
	Aerial mycelium	Creamy white
	Substrate mycelium	Orange
	Pigmentation	Nil

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Table 2: Differential characteristics of strain VUK-10 and type strains of closely related *Pseudonocardia* species. Strain VUK-10 (Data from the study). *P. endophytica* YIM 56035 (Data obtained from study Hua *et al*., 2009). *P.kongjuensis* DSM 44525 (Data obtained from Hua *et al*., 2009). *P.ammonioxydans* H9 (data obtained from Liu *et al*., 2006). (+) - Positive or Present; (W) weakly positive; (-) – Negative; ND- No data available

CHARACTER		RESPONSE			
Morphological Characters					
S No	**Characteristics under study**	**VUK-10**	***P. endophytica* YIM 56035**	***P. kongjuensis* DSM 44525**	***P. ammonioxydans* H9**
1	Sporophore morphology	Fragmentation	Fragmentation	Fragmentation	Fragmentation
2	Color of aerial mycelium	Creamy white	White	White	White
3	Color of substrate mycelium	Brownish orange	Yellowish-brown	Yellowish-brown	Brown
Physiological Characters					
4	Gram reaction	+, aerobic	+, aerobic	+, aerobic	+, aerobic
5	Acid-fast reaction	-	ND	-	ND
6	Production of melanin pigment	+	-	-	-
7	Production of Diffusible pigment	-	-	-	-
8	Range of temperature for growth	25-50 ºC	15-37 ºC	4-37 ºC	10-40 ºC
9	Optimum temperature for growth	30 ºC	28 ºC	ND	ND
10	Range of pH for growth	5-9	6-8	ND	ND
11	Optimum ph for growth	7	ND	ND	ND
12	NaCl Tolerance	10 %	5 %	7 %	8 %
13	Optimum NaCl Concentration	3 %	1 %	ND	3.5 %
Biochemical Characters					
14	Catalase production	+	+	+	+
15	Urease production	-	-	+	+
16	Hydrogen sulfide production	-	-	+	+
17	Nitrate reduction	-	-	-	+
18	Starch hydrolysis	-	-	-	W
19	Gelatin liquefaction	-	-	-	W
20	Methyl red test	-	ND	ND	ND
21	Vogues-proskauer test	-	ND	ND	ND
22	Indole production	-	ND	ND	ND
23	Citrate utilization	+	ND	ND	ND
24	Casein hydrolysis	-	-	+	+

Molecular Characterization

Analysis of the 16s rDNA gene sequence of the strain vuk-10

The 16S rDNA sequence data supported the assignment of this isolate VUK-10 to the genus *Pseudonocardia and* species *endophytica* (*Pseudonocardia endophytica*). The 800 bp partial 16S rDNA sequence of the strain VUK10 was obtained and submitted to the GenBank database under an accession number JN087501. The partial sequence was aligned and compared with all the 16S rRNA gene sequence available in the GenBank database by using the multisequence advanced BLAST comparison tool that is available in the website of National Centre for Biotechnology Information. The highest 16S rRNA sequence similarity value of 98% was obtained for the *Pseudonocardia endophytica* 16s rRNA (GenBank accession no: DQ887489) (Frederic *et al*., 1999). The phylogenetic analysis of the 16S rRNA gene sequence was aligned using the CLUSTAL W programme from the MEGA 5 Version. Phylogenetic tree was constructed using Molecular Evolutionary Genetics Analysis (MEGA) software Version 5 using Maximum parsimony method (Saitou and Nei, 1987; Takahashi and Nei, 2000; Tamura *et al*., 2007, 2011). The topologies of the constructed tree were evaluated by bootstrap analysis with 1000 resamplings by Maximum parsimony tool. Sequence comparison of the strain VUK-10 with the corresponding

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Figure 1: Maximum Parsimony tree based on partial 16S rRNA gene sequence showing relationship between *Pseudonocardia* isolate VUK-10 and related members of the genus *Pseudonocardia.* 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.

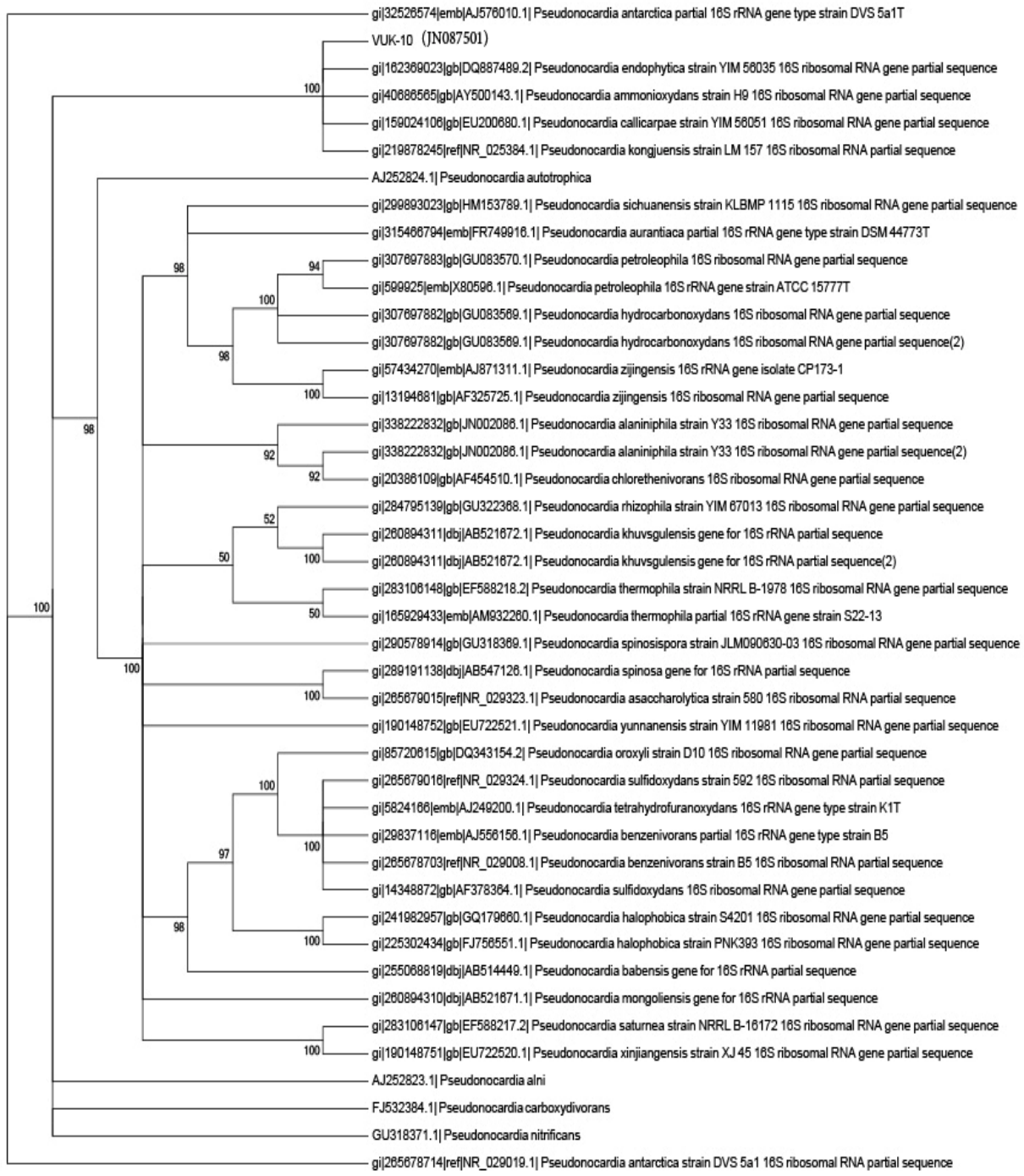

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Table 3: Utilization of the carbon sources. Excellent (+++), Moderate (++), Poor (-)

Utilization of Carbon Sources		
1	Lactose	++
2	Maltose	++
3	Xylose	-
4	Sucrose	++
5	Arabinose	-
6	D-Glucose	+++
7	Galactose	++
8	Fructose	++
9	Starch	+
10	Mannitol	-

Table 4: Antibiotic susceptibility testing of VUK-10

Susceptibility to Antibiotics: (µg/disc)		
1	Nalidixic acid (30)	R
2	Streptomycin (10)	S
3	Penicillin (10)	R
4	Chloramphenicol (30)	S
5	Rifampicin	S
6	Oxy tetracyclin	S

Table 5: Antimicrobial Activity of *Pseudonocardia* sp. VUK-10

S.No	Test organism	Diameter of Clear zone
1	*Staphylococcus aureus MTCC 3160*	13
2	*Streptococcus mutans MTCC 497*	15
3	*Bacillus subtilis ATCC 6633*	12
4	*E.coli ATCC 35218*	11
5	*Enterococcus faecalis MTCC 439*	13
6	*Pseudomonas aeruginosa ATCC 9027*	15
7	*Candida albicans ATCC 10231*	10
8	*Fusarium oxysporum MTCC 3075*	7
9	*Aspergillus niger*	9

sequences of the close representative strains of *Pseudonocardia* from the GenBank database showed that this strain formed a close distinct phyletic line with clade encompassed by *Pseudonocardia endophytica*, *Pseudonocardia ammonioxdans* and *Pseudonocardia kongjuensis* (fig. 1). Differential characteristics of strain VUK-10 and type strains of closely related *Pseudonocardia* sp. are represented in Table 2.

Based on the morphological, cultural, physiological, biochemical and molecular characteristics the strain has been included under the genus *Pseudonocardia* and deposited at NCBI genbank with an accession number JN087501.

Antimicrobial spectrum of *Pseudonocardia* species VUK-10 is represented in Table 5. The strain inhibited growth of Gram positive and Gram negative bacteria, yeast and fungi suggesting a broad spectrum nature of the active compound.

A perusal of literature indicates the bioactive metabolite profile of *Pseudonocardia* sp. includes antibacterial, antifungal, enzyme inhibitors, neuro protective and anti-*Helicobacter pylori* compounds. Rajendra *et al. (*2003) isolated a new phenazine derivative, phenazostatin D from *Pseudonocardia* sp.B6273, acts as a neuroprotective. Omura *et al.* (1979) reported antimicrobial activity of the antibiotics azureomycins A and B from *Pseudonocardia azurea* sp. Dekkar *et al.* (1998) extracted new quinolone compounds from *Pseudonocardia* sp. with selective and potent anti-*Helicobacter pylori* activity.

CONCLUSION

The present study is mainly involved in the isolation and identification of actinomycetes based on the cultural, morphological physiological, biochemical and molecular characteristics. Further studies on optimization, purification and elucidation of chemical structure of active compound are currently in progress. It is expected that the current attempt for the isolation and characterization of actinobacteria from Nizampatnam mangrove ecosystem will be useful for the identification of novel antibiotics effective against various pathogens.

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