

## TAXONOMIC CHARACTERIZATION, ANTIMICROBIAL ACTIVITY AND ANTIOXIDANT POTENTIAL OF RARE ACTINOBACTERIUM ISOLATED FROM MANGROVE ECOSYSTEM OF MACHILIPATNAM, ANDHRAPRADESH, INDIA

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### ABSTRACT

**Objective:** The objective of the present study is to isolate, characterize the potent actinomycete strain from mangrove habitats of South coast of Andhra Pradesh (Machilipatnam), India.

**Methods:** The samples collected from mangrove niches were pretreated with calcium carbonate, diluted, Plated on Starch casein agar, Humic acid vitamin agar and yeast extract malt extract dextrose (YMD) agar media to isolate actinomycetes. The three selective media were supplemented with clotrimazole (25 µg/mL) and streptomycin (25 µg/mL) to inhibit the growth of fungi and bacteria.

**Results:** A total of ten actinobacterial strains were isolated and designated as VLCH-1 to VLCH-10. All of them were screened and evaluated for their antimicrobial and antioxidant properties. Among them, one strain VLCH-6 was found to be potent as it exhibited high antimicrobial potential and it was identified as *Pseudonocardia parietis* VLCH-6 through polyphasic taxonomy (GenBank accession number: MZ951166). The growth profile of the strain was studied by culturing it in YMD broth. The culture broth extracted with ethyl acetate was tested for antimicrobial assay and found to be active against *Escherichia coli*, *Xanthomonas campestris* and *Aspergillus niger*. The antioxidant potential of the strain VLCH-6 was evaluated by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity. In the screening, the culture extract exhibited strong antioxidant activity against DPPH free radicals.

**Conclusion:** Over all, it was evident from the present study that mangrove habitats of Machilipatnam serve as a good source for potent actinomycetes possessing broad spectrum of antimicrobial and antioxidant metabolites.

**Keywords:** Mangrove actinomycete, *Pseudonocardia parietis* VLCH-6, Antimicrobial activity, Antioxidant potential, Bioactive compounds.

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### INTRODUCTION

Mangroves are the most productive ecosystem that occupies millions of hectares across the world [1]. It includes the coastal wet land forests mainly located in the intertidal zone of estuaries, deltas, back waters, lagoons, creeks, and also mud flats of the subtropical and tropical latitudes [2]. Microbes dwelling in mangrove sediments have been focused as a significant source and virtual potential resource for exploring novel bioactive compounds [3,4].

Actinomycetes are widely exploited group for the production of novel bioactive compounds and enzymes of commercial importance. The actinomycetes metabolites of mangrove sediments are endowed with novel chemical skeletons with strong biological activities [5,6]. Actinomycetes are free living, aerobic, Gram-positive bacteria, frequently filamentous and sporulating with a high GC content DNA (>70%) belonging to the order *Actinomycetales*. The filamentous actinomycetes providing precious bioactive compounds have been remarkably successful and approximately two thirds of known antibiotics have been purified from them. The metabolites produced by actinomycetes are known to possess antimalarial, antimicrobial, anti-fungal, neurotogenic, immunosuppressive, and anti-inflammatory activities [7,8].

Over all more than 10,000 bioactive secondary metabolites have been extracted from *Actinobacteria*, which constitute 45% of all proactive microbial metabolites. Members of the class *Actinobacteria* and especially family *Pseudonocardiaceae* have been recognized as newly exploited group for the production of rare bioactive

compounds. *Pseudonocardia* species are excellent producers of the antibiotics vancomycin, erythromycin, quinolone compounds and phenazostatin D [9-11].

In view of the significance of mangrove *Actinobacteria*, screening of actinomycetes from unique mangrove habitats will provide rare bioactive metabolites with antimicrobial and antioxidant properties.

### METHODS

#### Collection and processing of sediment samples

Soil samples were collected at a depth of 10 cm from mangrove habitats of Machilipatnam situated along the south coast of Andhra Pradesh, India. The samples were air-dried samples to control contaminant microbes and pretreated with calcium carbonate to enrich actinobacterial population (10:1w/v) [12,13].

#### Selective isolation of actinomycetes

The isolation of actinomycetes was carried out by serial dilution plate technique ( $10^{-1}$ - $10^{-4}$ ), by adding 1 g of soil sample in 100 ml of distilled water and spreaded on three selective media, yeast extract-malt extract-dextrose (YMD) agar, Starch casein agar and Humic acid vitamin Agar amended with 3% NaCl at pH 7.0. The media were also supplemented with streptomycin (25 µg/ml) and clotrimazole (25 µg/ml) to inhibit the bacterial and fungal contamination, respectively. The plates were incubated at  $30 \pm 2^\circ\text{C}$  for 3 weeks. The colonies showing the characteristics of actinomycetes (rough, chalky, powdery appearance with radiating growth, aerial filamentous and leathery texture) were observed. The predominant actinobacterial colonies were subcultured

on YMD agar slants for further preservation. These isolates were screened for their ability to generate bioactive compounds [14].

#### Screening of potent actinomycete strains for bioactive metabolites

Over all ten actinomycete strains were isolated and screened for bioactive metabolite production. From the seed medium, 10% of seed culture was inoculated into the YMD broth as production medium and incubated at room temperature for 9 days at 120 rpm. The flasks were harvested and biomass was separated from the broth. The obtained culture filtrate was extracted with ethyl acetate and this solvent extract was evaporated in water bath for residue and it was used to determine antimicrobial assay by agar well-diffusion method [15,16]. Among the ten isolates tested for bioactive compounds, the strain VLCH-6 was found potent compared to other strains.

#### Identification of potent strain VLCH-6 by polyphasic taxonomy (Morphological, cultural, physiological, and biochemical characteristics)

The actinobacterial strain was characterized by polyphasic taxonomic studies. The microscopic study was carried out by slide culture method to observe the nature of mycelium and spore arrangement. The morphological characteristics of 4-day old culture were assessed using scanning electron microscopy (SEM: Model- JOELJSM 5600, Japan) [17].

The strain was grown on five ISP media, three non-ISP media to observe the cultural characteristics such as color of both aerial and substrate mycelium, pigmentation and spore formation. Hydrolysis of starch, nitrate reduction and production of H<sub>2</sub>S was also tested [18,19]. Physiological characteristics such as the effect of pH (5-9), temperature (20–45°C), and salinity on the growth of strain were analyzed. The ability of the strain to produce industrially important enzymes such as amylase, asparaginase, glutaminase and cellulase was tested [20,21]. The susceptibility of the strain to different antibiotics was also decided by paper disc method.

#### Molecular identification of the potent strain VLCH-6

The genomic DNA used for the polymerase chain reaction was prepared from the colonies grown on YMD agar for 3 days. The chromosomal DNA of strain was isolated according to the manufacturer protocol (Pure Fast® Bacterial Genomic DNA purification kit, Helini Bio molecules, India). The 16S r DNA was amplified with the primers such as (518F 5'- CCAGCAGCCGCGTAATACG-3' and 800R 5'-TACCAGGTATCTAATCC-3'). The 1% agarose gel electrophoresis was used to separate the amplified DNA fragments and purified using Qiaquick gel extraction kit (Qiagen, Germany). The deduced 16s rDNA sequence was compared with the sequences in GenBank using the BLAST then aligned with the related *Pseudonocardia* species retrieved from GenBank databases. Phylogenetic and molecular evolutionary analyses were conducted using MEGA 6.0 [22]. The 16S rRNA gene sequences of the strain VLCH-6 were submitted in GenBank.

#### Growth pattern of the strain VLCH-6

To study the growth curve of the strain, it was inoculated into YMD broth (100 ml) and incubated at 30±2°C at 120 rpm for 8 days. The flasks were harvested at 24 h interval and growth of the strain 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 the crude extract was studied by agar well diffusion method [23].

#### Extraction of antimicrobial compounds and *in vitro* antimicrobial assay

Seed medium was prepared for VLCH-6 culture, from this 10% of seed culture was transferred to YMD broth (Fermentation medium) and incubated at 30±2°C for 8 days at 120 rpm. Bioactive compound was recovered from the filtrate using ethyl acetate (solvent extraction method) and residue obtained was tested for antimicrobial activity by agar well diffusion method. For this Nutrient agar for bacteria and Czapek-Dox agar for fungi were prepared. About 80 µl of the

crude extract was filled in separate wells and the standard antibiotic (Streptomycin) was as a positive control. Plates were incubated at room temperature for 24–48 h and the effectiveness was measured by zone of inhibition (mm) [24].

#### Test organisms

##### Bacteria

*Staphylococcus aureus* (MTCC 3160), *Streptococcus mutans* (MTCC 497), *Klebsiella pneumoniae* (ATCC 10031), *Bacillus megaterium* (NCIM 2187), *Xanthomonas campestris* (MTCC 2286), *Proteus vulgaris* (ATCC 6380), *Pseudomonas aeruginosa* (ATCC 9027) and *Escherichia coli* (ATCC 9027).

##### Fungi

*Aspergillus niger* (ATCC 189), *Candida albicans* (MTCC 183) and *Penicillium citrinum* (MTCC 6849).

#### *In vitro* antioxidant potential of the strain VLCH-6

##### 2,2-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay

The antioxidant capacity of the crude extract was evaluated by the DPPH free radical [25]. The strain solvent extract and ascorbic acid standard (100–500 µg/mL) were prepared in ethanol. 0.002% of DPPH in ethanol was used as the free radical. The 3 ml of DPPH solution was mixed with different concentrations (100–500 µg/mL) of VLCH-6 crude extract and standard separately. The flasks were incubated in dark conditions at room temperature for 30 min. The optical density was measured at 517 nm using ultraviolet visible spectrophotometer. The absorbance of DPPH control was also measured. All the experiments were made in triplicates and represented in mean±SD. The DPPH scavenging activity was expressed in percentage using the following formula.

$$\text{DPPH Scavenging activity (\%)} = [(A_o - A_e) / A_o] \times 100$$

Where A<sub>o</sub> is absorbance of the control; A<sub>e</sub> is absorbance of the sample/standard. The half-inhibitory concentration (IC<sub>50</sub>) values were calculated using regression analysis. IC<sub>50</sub> values signify the concentration of sample, which is appropriate in scavenging 50% of the DPPH free radicals.

## RESULTS AND DISCUSSION

Among the ten actinobacterial strains isolated from mangrove ecosystem of Machilipatnam, Andhra Pradesh, VLCH-6 was found to possess high antimicrobial and antioxidant activities. It exhibited typical morphological characteristics of genus *Pseudonocardia*. The morphology of 4 day-old culture was studied through SEM analysis. The color of aerial mycelium was creamy white and substrate mycelium was brownish orange (Fig. 1). The aerial and substrate mycelia are well developed, extensively branched and fragmented. The strain showed heavy sporulation with non-motile rod shaped spores having rough surface (Fig. 2).

The cultural characteristics of the strain are represented in Table 1. The strain exhibited good growth on tryptone yeast extract agar (ISP-1), YMD (ISP-2), inorganic salts starch agar (ISP-4) and glycerol asparagine agar (ISP-5) while it was moderate on starch casein salts agar (non-ISP) and tyrosine agar (ISP-7). The growth was poor on nutrient agar and no growth was found on Czapek-Dox agar. The color of aerial mycelium was creamy white and substrate mycelium was brownish orange on different media tested (ISP-1, ISP-2, ISP-4, ISP-5, ISP-7 and Starch-casein agar). No pigment production was observed on the media tested.

The physiological and biochemical characteristics were significant tools for the identification of *Actinobacteria*. Several tests were conducted for identifying strain VLCH-6 (Table 2). The strain exhibited positive response to catalase production, starch hydrolysis, citrate utilization, indole production, casein hydrolysis and gelatin liquefaction but negative to urease production, H<sub>2</sub>S production, nitrate reduction,



Fig. 1: The strain VLCH-6 cultured on yeast extract malt extract dextrose agar medium

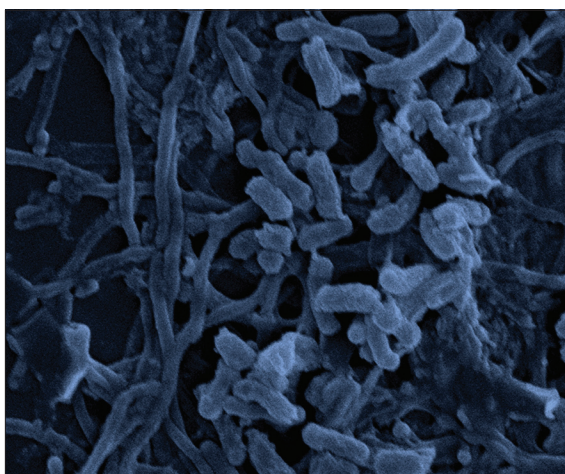


Fig. 2: Scanning Electron Microscopic photograph of VLCH-6

methyl red, and Voges-Proskauer test. The strain could also produce enzymes such as amylase, cellulose, L-asparaginase and glutaminase.

The strain VLCH-6 showed good growth at pH range between 5 and 9 with the optimum being 7 and range of temperature for growth was 25–37°C with the optimum being 35°C. Sodium chloride tolerance of the strain was also studied as the salt concentration has a profound effect on the production of antibiotics by microorganisms. The strain could grow well in the medium supplemented with 3% sodium chloride and showed tolerance up to 9%. The strain utilized a wide range of carbon sources. Maltose and D-glucose were supported excellent growth whereas arabinose and mannitol supported poor growth of the strain (Table 3). The strain was resistant to the majority of antibiotics tested and showed sensitivity to azithromycin, chloramphenicol, neomycin, and streptomycin (Table 4) [26].

The phylogenetic tree was constructed using MEGA software Version 6 employing maximum parsimony method and the strain VLCH-6 was identified as *Pseudonocardia parietis* VLCH-6 (Fig. 3). The partial 16S rRNA sequences of the strains were submitted to the GenBank database with accession number MZ951166.

The growth curve and antimicrobial profile of *P. parietis* VLCH-6 were studied at regular intervals up to 8 days in batch culture. The culture broth was harvested at 24 h intervals and dry weight of biomass was expressed in mg/100 mL. The stationary phase of the strain extended from 144 h to 168 h of incubation (Fig. 4). The culture filtrate was

Table 1: Cultural characteristics of the strain VLCH-6

Medium	Growth	A.M	S.M	Pigmentation
Tryptone yeast extract agar (ISP-1)	Good	Creamy white	Brownish orange	Nil
YMD agar (ISP-2)	Good	Creamy white	Brownish orange	Nil
Inorganic salts starch agar (ISP-4)	Good	Creamy white	Brownish orange	Nil
Glycerol asparagine agar (ISP-5)	Good	Creamy white	Pale orange	Nil
Tyrosine agar (ISP-7)	Moderate	Creamy white	Pale orange	Nil
Starch-casein agar	Moderate	Creamy white	Pale orange	Nil
Czapek-Dox agar	-	-	-	Nil
Nutrient agar	Poor	-	-	Nil

AM: Aerial mycelium, SM: Substrate mycelium. -: No growth, ISP: International Streptomyces Project, YMD: Yeast extract malt extract dextrose agar

Table 2: Morphological, physiological and biochemical characteristics of VLCH-6

Character	Response
Morphological characters	
Sporophore morphology	Fragmentation
Color of aerial mycelium	Creamy white
Color of substrate mycelium	Brownish orange
Physiological characters	
Grams reaction	Gram Positive, aerobic
Acid-fast reaction	-
Production of melanin pigment	+
Range of temperature for growth	25 – 37oC
Optimum temperature for growth	35oC
Range of pH for growth	5-9
Optimum pH for growth	7
NaCl tolerance	9%
Optimum NaCl Concentration	3%
Biochemical characters	
Catalase production	+
Urease production	-
H2S production test	-
Nitrate reduction	-
Starch hydrolysis	+
Gelatin liquefaction	+
Methyl red test	-
Voges-Proskauer test	-
Indole production	+
Citrate utilization	+
Casein hydrolysis	+
Enzymatic activity	
Amylase	+
Protease	-
Cellulase	+
Asparaginase	+
Glutaminase	+
Chitinase	-

extracted with ethyl acetate and evaporated to dryness in water bath [27]. The secondary metabolites obtained from 7-day-old culture showed high antimicrobial activity against *X. campestris*, *E. coli*, and *A. niger* (Fig. 5) (Table 5). Radical scavenging activities are very important to prevent the deleterious role of free radicals in cancer and immunological disorders. The DPPH produces violet/purple color in ethanol and fades to shades of yellow color due to the presence of antioxidants. The DPPH free-radical scavenging activity of different concentrations of standard ascorbic acid and VLCH- 6 crude extract is shown in Fig. 6. The results



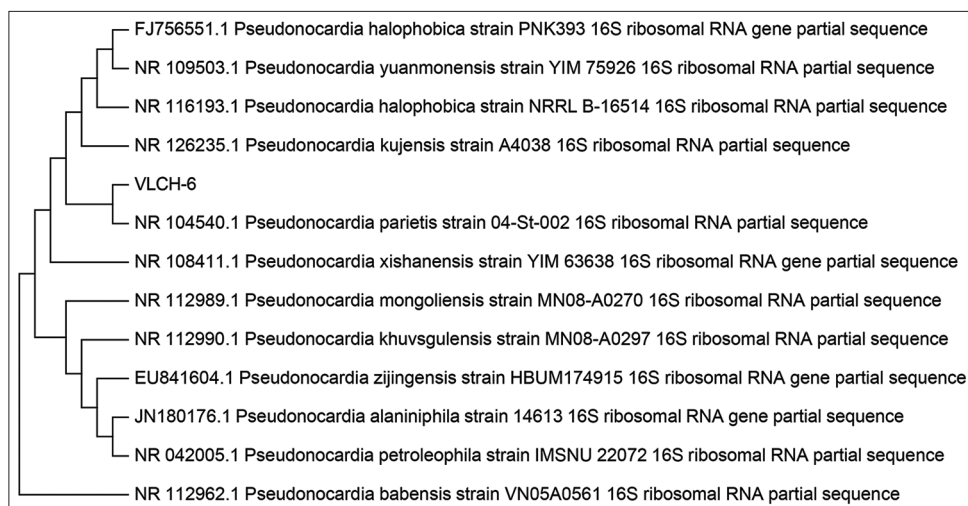


Fig. 3: Maximum parsimony tree based on partial 16S rRNA gene sequence showing relationship between strain VLCH-6 and related members of the genus *Pseudonocardia*

Table 3: Utilization of carbon sources by the strain VLCH-6

Carbon Sources	Response
D-Glucose	+++
Maltose	+++
Sucrose	++
Lactose	++
Arabinose	-
Fructose	++
Galactose	++
Starch	+
Mannitol	-

Excellent (+++), Moderate (++), Poor (-)

Table 4: Antibiotic susceptibility/resistance of VLCH-6 ( $\mu\text{g}/\text{disc}$ )

Antibiotics	Susceptibility/Resistance
Azithromycin (15)	S
Erythromycin (15)	R
Penicillin (10)	R
Chloramphenicol (10)	S
Neomycin (30)	S
Streptomycin (25)	S
Amoxyclav (30)	R
Amikacin (30)	R

Table 5: Antimicrobial assay of *Pseudonocardia parietis* VLCH-6

Test organism name	Zone of Inhibition (mm)
Bacteria	VLCH-6
<i>Staphylococcus aureus</i>	19.52 $\pm$ 0.38
<i>Escherichia coli</i>	20.21 $\pm$ 0.21
<i>Xanthomonas campestris</i>	21.21 $\pm$ 0.11
<i>Pseudomonas aeruginosa</i>	17.26 $\pm$ 0.12
<i>Bacillus megaterium</i>	19.45 $\pm$ 0.27
<i>Streptococcus mutans</i>	16.28 $\pm$ 0.18
<i>Klebsiella pneumoniae</i>	17.49 $\pm$ 0.16
Fungi	
<i>Candida albicans</i>	20.48 $\pm$ 0.15
<i>Aspergillus niger</i>	21.39 $\pm$ 0.26
<i>Penicillium citrinum</i>	09.26 $\pm$ 0.13

showed dose-dependent scavenging activity and it was expressed as  $IC_{50}$  ( $\mu\text{g}/\text{mL}$ ). The radical scavenging activity was found to be 51.16 $\pm$ 0.22, 59.68 $\pm$ 0.11, 66.58 $\pm$ 0.21, 77.85 $\pm$ 0.26 and 80.43 $\pm$ 0.29 (Fig. 6).

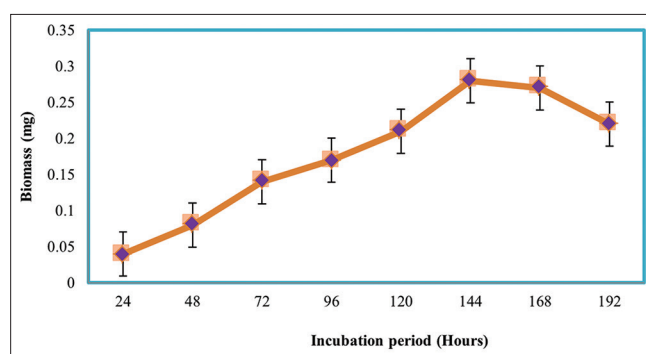


Fig. 4: Growth pattern of the strain *Pseudonocardia parietis* VLCH-6

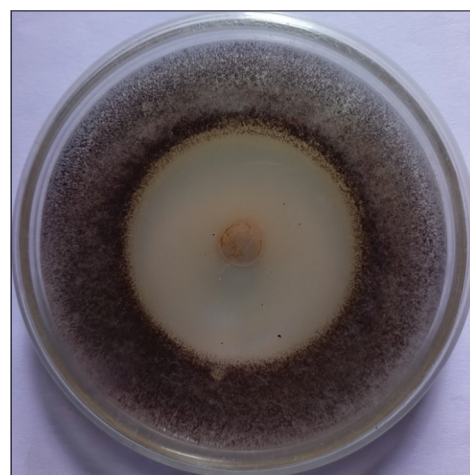


Fig. 5: Antimicrobial activity of culture crude extract against *Aspergillus niger*

#### Statistical analysis

Values are the means of three replicates  $\pm$  SD.

#### CONCLUSION

The objective of present study is isolation, screening and molecular identification of potent actinomycetes. Based on the screening results, it is evident that mangrove habitats of South coast of Andhra Pradesh

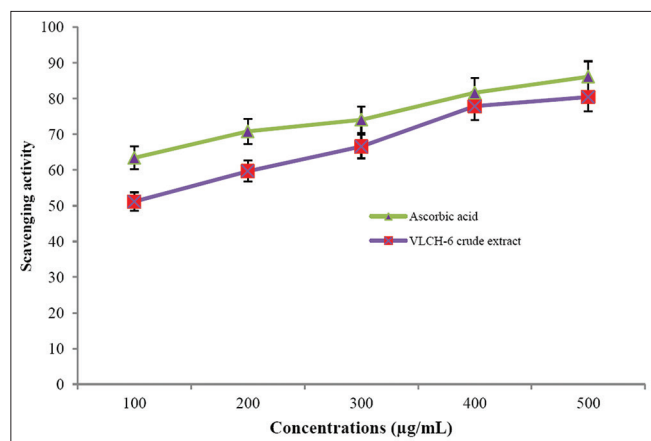


Fig. 6: Antioxidant assay of *Pseudonocardia parietis* VLCH-6

(Machilipatnam) serve as a good source for the isolation of potent actinomycetes with broad spectrum of antimicrobial and antioxidant potential. Further studies on purification of bioactive compounds are in progress.

To the best of our knowledge, this is the first report on taxonomic characterization, antimicrobial and antioxidant properties of *P. parietis* VLCH-6 isolated from mangrove habitats of Machilipatnam, South Coastal Andhra Pradesh, India.

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#### AUTHORS' CONTRIBUTION

Concept and design of the study, Data collection, Data Analysis, and manuscript writing were done by first and corresponding author.

#### CONFLICT OF INTERESTS

Declared None.

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