

EXPLORATION OF POTENT ACTINOBACTERIUM *NOCARDIOPSIS HALOTOLERANS* VJPR-2 ISOLATED FROM MANGROVE HABITATSPRASADA RAO NARADALA¹, VIJAYALAKSHMI MUVVA^{1*}, USHAKIRANMAYI MANGAMURI¹, KAVI KISHOR PB²¹Department of Botany and Microbiology, Acharya Nagarjuna University, Guntur - 522 510, Andhra Pradesh, India. ²Department of Genetics, Osmania University, Hyderabad - 500 007, Telangana, India. Email: profmvl@gmail.com

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ABSTRACT

Objectives: This study was aimed at isolation and identification of potent bioactive metabolite producing actinobacterial strain VJPR-2 isolated from the mangrove ecosystem of Nizampatnam, Andhra Pradesh, India.

Methods: Soil sediments collected were subjected to pre-treatment with CaCO₃, and actinobacterial strains were isolated using selective media. The screening of the isolated strains was carried out and the potent bioactive metabolite producing strain was designated as VJPR-2. An identification of the strain was carried out by employing polyphasic approach including morphological, cultural, physiological, biochemical, and phylogenetic analysis of 16S rRNA gene sequence. Antimicrobial potency of the isolate was tested against bacterial and fungal pathogens.

Results: The strain VJPR-2 was identified as *Nocardiopsis halotolerans* by morphological, cultural, physiological, and biochemical studies along with 16S rRNA gene sequence analysis. The rRNA sequence was deposited in the NCBI GenBank with the accession number KP313613. The strain exhibited antimicrobial activities against Gram-positive as well as Gram-negative bacteria and fungi.

Conclusion: Actinobacterium strain *N. halotolerans* VJPR-2 having good antimicrobial potential was identified from the 16 strains isolated from the sediment samples of Nizampatnam mangrove ecosystem using CaCO₃ based approach. The present study reveals the isolation, identification and biological evaluation of the bioactive metabolites produced by strain VJPR-2.

Keywords: Mangrove ecosystem, *Nocardiopsis halotolerans* VJPR-2, Polyphasic approach, Bioactive metabolites.

INTRODUCTION

The mangrove ecosystems are often considered as the potential storehouse of actinobacteria [1]. Actinobacteria are considered as terrigenous bacteria due to their existence in extreme environments [2]. Actinobacterial strains dwell in marine environments, mangrove habitats, coastal environments, and even deep sea sediments [3,4]. Due to the constant changes of mangrove environmental factors such as tidal gradient and salinity, mangrove microbial communities exhibit metabolic pathway adaptations for the production of chemically diverse novel secondary metabolites. A some of the novel actinobacteria such as *Asanoa iriomotensis* [5], *Nonomuraea maheshkhaliensis* [6], and *Streptomyces xiamenensis* [7] were discovered from poorly explored mangrove environment.

Among the mangrove bacterial communities, actinobacteria play an important role due to their ability to produce novel, commercially diverse bioactive compounds [8]. These bacteria are well-recognized sources for various bioactive molecules such as lignocelluloses, hemicelluloses [9], immunomodulators [10], anti-infective, and anticellular agents [11]. They also play an extensive role in the pharmaceutical and medical industry.

Decrease in the rate of novel antibiotics and increase in the multi-drug resistant pathogens revealed the need to search for new antimicrobials. The exploration of the rare actinobacterial strains from mangrove sediments that exhibit potent antimicrobial activity with chemically diverse molecules against different human and plant pathogens [12] may be one of the answers to counter the current antibiotic resistant problem. Most of the researchers show great interest to explore the microbes dwelling the unexplored habitats which are a great direction toward the isolation of rare microbes for the production of potent antibiotics [13,14]. This study is aimed at isolation and identification of novel actinobacteria from the mangrove sediments of Nizampatnam,

Andhra Pradesh with potent biochemical activity against opportunistic and pathogenic bacteria and fungi.

METHODS**Collection and processing of sediment samples**

The sediment samples were collected at random from different areas of the Nizampatnam mangrove ecosystem spread across the coast of Andhra Pradesh, India. The collected samples were sealed in sterile bags and transported to the laboratory. They were air dried and pre-treated with calcium carbonate to enrich actinobacterial population.

Isolation of actinomycetes

The isolation of actinomycetes was carried out by suspending 1 g of pretreated soil sample in 100 ml of sterile distilled water and serially diluting it up to 10⁻⁴. The diluted sample was spread onto three selective media, International *Streptomyces* Project (ISP-2) (YMD agar), HV Agar and starch casein agar supplemented with nalidixic acid (25 µg/ml) and secnidazole (25 µg/ml) [15,16] to inhibit the growth of bacteria and fungi, respectively. The plates were incubated at 30°C for 10 days, and the actinobacterial colonies were selected based on the morphology and maintained at 4°C on ISP-2 slants.

Identification of the potent strain VJPR-2 by polyphasic approach

Cultural properties of the strain VJPR-2 were studied on ISP and non-ISP media [17]. The micro-morphology of the strain was studied by employing slide culture method [18] and examined using compound microscope (model Motic-BA410). In addition, the scanning electron micrograph of the isolate was presented.

Melanin pigment production of the strain was determined by growth on tyrosine agar media (ISP-7) [17]. Utilization of the carbohydrates was tested in minimal medium containing different carbon sources

(D-glucose, starch, lactose, maltose, sucrose, mannose, galactose, fructose, cellulose and xylose) at 1% concentration [19]. The strain was also examined for its ability to produce catalase, hydrolysis of starch, indole, methyl red, Voges-Proskauer, citrate utilization test, and urease production. Physiological characterization such as effect of pH, temperature, and salinity tolerance (up to 8%) was analyzed. In addition, the antibiotic sensitivity of the isolate to different antibiotics (gentamicin, amoxycylav, erythromycin, streptomycin, neomycin, azithromycin, cephalexin, and amikacin) was determined by paper disc method [20].

Molecular characterization

DNA isolation, amplification and sequencing of the 16S rRNA gene

The genomic DNA for the study was extracted from strain VJPR-2 grown in YMD broth at 30°C for 120 hrs by employing bacterial genomic DNA isolation kit RKT 41/42 (Chromous Biotech Pvt. Ltd., Bengaluru, India) according to the manufacturer instructions [21]. The 16S rRNA sequence amplification was carried out using a universal forward primer (5' CAGGCCTAACACATGCAAGTC3') and reverse primer (3'GGGCGGWTGTGTACAAGGC 5'). The polymerase chain reaction (PCR) condition was programmed with an initial denaturation for 3 minutes at 94°C followed by 30 cycles of amplification (1 minute denaturation at 94°C, 1 minute annealing at 57°C and 1 minute extension at 74°C) and a final extension of 5 minutes at 74°C. A total volume of reaction mixture contained 25 µl (1.2 µl genomic DNA, 1.0 µl of 10 pmol forward primer 5'-CAGGCCTAA CATGCAAGTC-3', 1.0 µl of 10 pmol reverse primer 5'-GGGCGGWTGTGTACAAGGC-3', 5.0 µl of 30 mM dNTP's, 2.5 µl of 10X PCR buffer and 0.2 µl of Taq polymerase (1 U) (Chromous Biotech Pvt. Ltd., Bengaluru, India) and 19.2 µl of molecular grade nuclease free water). The amplification was determined on 1% agarose gel (with ethidium bromide) electrophoresis and documented with Gel Doc (UV-Tech London) [21]. Amplified product was purified with gel purification kit (Invitrogen) as per the manufacturer protocol.

Analysis of sequenced data

The sequence was analyzed using molecular evolutionary genetics analysis software (MEGA, Version 6.0) [22] and aligned together with reference sequences obtained from the GenBank, EMBL, and DDBJ.

Nucleotide sequence accession number

The 16S rRNA gene (rRNA) sequences of the strain VJPR-2 was registered in the GenBank database.

Growth pattern of the strain VJPR-2

The growth pattern of the strain was studied at regular intervals up to 7 days. The strain VJPR-2 was cultured for 1 week and then transferred aseptically into the seed medium (malt extract 10 g, yeast extract 4 g, dextrose 4 g, sodium chloride 50 g, sea water 1000 ml and pH 7) and incubated at 30°C for 48 hrs at 120 rpm in a rotator shaker. 10% of the 48 hrs seed culture was inoculated into the production medium of the same composition. Fermentation was carried out at 30°C for 7 days under agitation at 120 rpm. The culture broth was harvested at every 24 hrs intervals and biomass was expressed. The culture filtrate thus collected was extracted with ethyl acetate and evaporated to dryness with the help of rotavap. The bioactive metabolite thus obtained was used for antimicrobial screening.

Determination of antimicrobial screening

The antimicrobial activity of the crude extract was assessed by measuring the diameter of inhibition zone against Gram-positive bacteria - *Staphylococcus aureus* MTCC 3160, *Bacillus megaterium* NCIM 2187, *Bacillus subtilis* ATCC 6633; Gram-negative bacteria - *Escherichia coli* ATCC 9027, *Xanthomonas campestris* MTCC 2286, *Pseudomonas aeruginosa* ATCC 9027, and fungi *Candida albicans* MTCC 183, *Aspergillus niger*, *Aspergillus flavus*, *Penicillium expansum*, and *Fusarium oxysporum* by agar well diffusion assay [20].

RESULTS AND DISCUSSION

A total of 16 actinobacterial strains were isolated from the mangrove ecosystem of Nizampatnam by employing soil dilution plate technique. All the strains were screened for antimicrobial potential. Out of 16 strains, six strains (VJPR-1, VJPR-2, VJPR-6, VJPR-7, VJPR-9 and VJPR-15) possessed antimicrobial activity, of which the strain designated as VJPR-2 exhibited strong antimicrobial activity against a wide range of pathogens. The strain VJPR-2 showed typical morphological characteristics of the genus *Nocardioopsis* that exhibited heavy sporulation with the fragmented mycelium and rough surfaced spores (Figs. 1 and 2) [23].

Cultural characteristics of the strain VJPR-2

Cultural characteristics of the strain VJPR-2 was studied on different media and abundant growth was observed on tryptone yeast extract agar (ISP-1), yeast extract malt extract dextrose agar (ISP-2), oat-meal agar (ISP-3), inorganic salts starch agar (ISP-4), glycerol asparagine agar (ISP-5), and starch casein salts agar (non-ISP). Moderate growth was observed on nutrient agar (non-ISP), Bennett's agar (non-ISP), while growth was poor on tyrosine agar (ISP-7) and glucose tryptone agar (non-ISP). The color of aerial mycelium was white, substrate mycelium was yellow, and soluble pigment production by the strain was not found on the culture media including melanoid pigmentation on ISP-7 [17]. Cultural characteristics of the strain are recorded in Table 1.

Biochemical and physiological characteristics of the strain VJPR-2

The temperature range for the growth of the strain VJPR-2 was 28-30°C with optimum growth at pH 7.0. The strain exhibited salt tolerance up to 8%, with optimum growth documented at 3% NaCl. In the carbohydrate

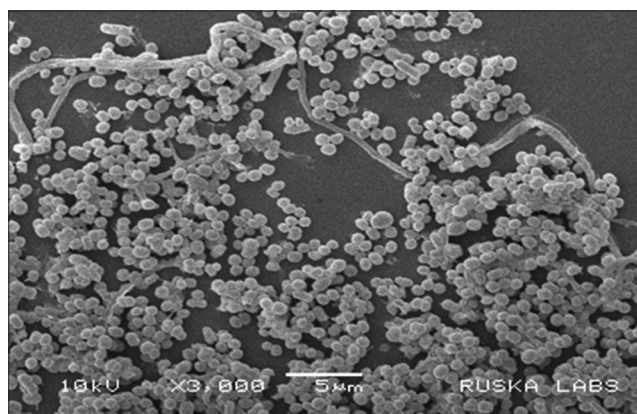


Fig. 1: Scanning electron microscopy photograph of *Nocardioopsis halotolerans* VJPR-2



Fig. 2: Micro-morphology of *Nocardioopsis halotolerans* VJPR-2

Table 1: Cultural characteristics of the strain VJPR-2

Name of the medium	Growth	Aerial mycelium	Substrate mycelium	Pigmentation
Tryptone yeast-extract agar (ISP-1)	Good	White	Yellow	No
Yeast extract malt extract dextrose agar (ISP-2)	Good	White	Yellow	No
Oat-meal agar (ISP-3)	Good	White	Yellow	No
Inorganic salts starch agar (ISP-4)	Good	White	Yellow	No
Glycerol asparagine agar (ISP-5)	Good	White	Yellow	No
Tyrosine agar (ISP-7)	Poor	White	Yellow	No
Nutrient agar	Moderate	White	Yellow	No
Bennett's agar	Moderate	White	Yellow	No
Starch casein salts agar	Good	White	Yellow	No
Glucose tryptone agar	Poor	White	Yellow	No

ISP: International *Streptomyces* Project

Table 2: Morphological characteristics of the strain VJPR-2

Character	Response
Sporophore morphology	Fragmented mycelium
Color of aerial mycelium	White
Color of substrate mycelium	Yellow

Table 3: Biochemical characteristics of the strain VJPR-2

Character	Response
Catalase production	-
Urease production	+
Indole production	+
Methyl red test	-
Voges proskauer test	-
Citrate utilization test	+
Strach hydrolysis	+

+: Positive, -: Negative

Table 4: Physiological characteristics of the strain VJPR-2

Character	Response
Gram reaction	+
Acid fast reaction	-
Production of melanin pigment	-
Optimum temperature for growth	28-30°C
Optimum pH for growth	7.0
NaCl tolerance up to	8%

+: Positive, -: Negative

Table 5: Utilization of carbon sources of the strain VJPR-2

Character	Response
D-glucose	+++
Strach	++
Lactose	+
Maltose	+
Sucrose	++
Mannose	++
Galactose	++
Fructose	+
Cellulose	+
Xylose	+

+++ : Good growth, ++ : Moderate growth, + : Weak growth

assimilation test, carbon sources such as glucose, starch, sucrose, mannose, and galactose were utilized by the strain efficiently. The strain exhibited a positive response to urease production, starch hydrolysis, and citrate utilization. It showed resistance to different antibiotics such as amoxyclav, erythromycin, streptomycin, neomycin, and cephalixin. Morphological, physiological, biochemical characteristics and antibiotic sensitivity of the strain are recorded in Tables 2-6.

Table 6: Antibiotic Sensitivity of the strain VJPR-2

Antibiotic (µg)	Response
Gentamicin (30)	S
Amoxyclav (30)	R
Erythromycin (15)	R
Streptomycin (10)	R
Neomycin (30)	R
Azithromycin (15)	S
Cephalexin (30)	R
Amikacin (30)	S

S: Sensitive, R: Resistant

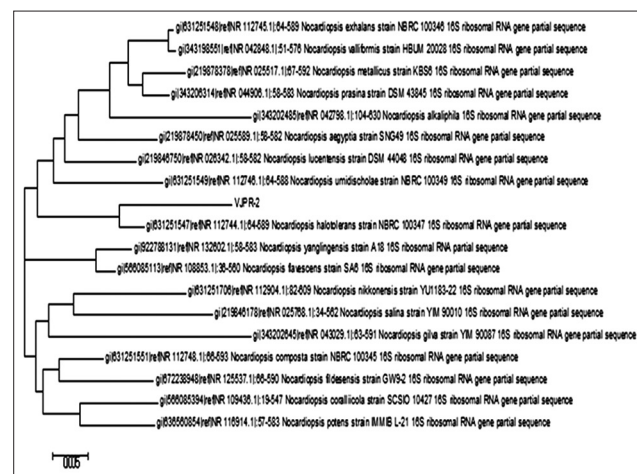


Fig. 3: Neighbor-Joining tree based on partial 16S rRNA gene sequence showing relationship between *Nocardiosis* strain VJPR-2 and related members of the genus *Nocardiosis*

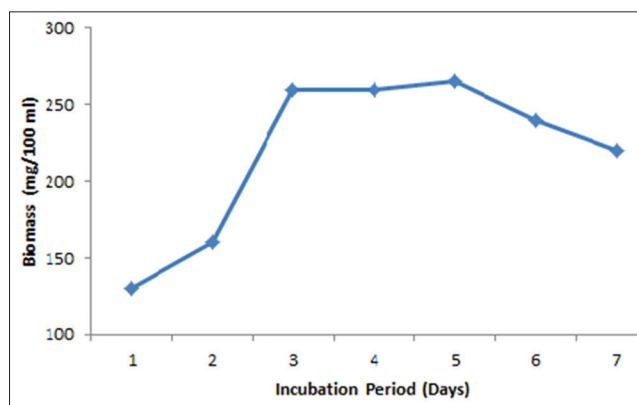


Fig. 4: Growth Pattern and antimicrobial profile of *Nocardiosis halotolerans* VJPR-2

Table 7: Antibacterial and antifungal activity *N. halotolerans* VJPR-2

Test organisms	Zone of inhibition (mm)	#Standard drug
Bacteria		
<i>B. megaterium</i> NCIM 2187	20±0.019	24±0.06
<i>E. coli</i> ATCC 9027	22±0.016	25±0.05
<i>X. campestris</i> MTCC 2286	21±0.015	24±0.07
<i>P. aeruginosa</i> ATCC 9027	19±0.17	22±0.10
<i>S. aureus</i> MTCC 3160	20±0.07	23±0.09
<i>B. subtilis</i> ATCC 6633	22±0.016	26±0.15
Fungi		
<i>C. albicans</i> MTCC 183	21±0.05	26±0.07
<i>A. niger</i>	17±0.08	21±0.04
<i>A. flavus</i>	18±0.04	22±0.08
<i>P. expansum</i>	16±0.07	19±0.10
<i>F. oxysporum</i>	20±0.10	24±0.06

Values are mean±SD (n=3), #Standard drug: Streptomycin against bacteria, cycloheximide against yeast, flucanazole against fungi.

N. halotolerans: *Nocardioopsis halotolerans*, *B. megaterium*: *Bacillus megaterium*, *E. coli*: *Escherichia coli*, *X. campestris*: *Xanthomonas campestris*, *P. aeruginosa*: *Pseudomonas aeruginosa*, *S. aureus*: *Staphylococcus aureus*, *B. subtilis*: *Bacillus subtilis*, *C. albicans*: *Candida albicans*, *A. niger*: *Aspergillus niger*, *A. flavus*: *Aspergillus flavus*, *P. expansum*: *Penicillium expansum*, *F. oxysporum*: *Fusarium oxysporum*

Molecular characterization

Analysis of the 16S rRNA gene sequence of the strain VJPR-2

The 16S rRNA gene sequence data supported the assignment of the strain VJPR-2 to the genus *Nocardioopsis* and species *halotolerans*. The sequenced 16S rRNA sequence of the strain VJPR-2 was submitted to the GenBank database with the accession number KP313613. The partial sequence was aligned and compared with all the 16S rRNA gene sequence available in the GenBank by BLAST online tool. The phylogenetic analysis of the 16S rRNA gene sequence was aligned using the CLUSTAL W program [24] from the MEGA 6.0 Version. Phylogenetic tree (Fig. 2) was constructed using MEGA Software Version 6.0 using neighbor-joining method [25]. The constructed tree topology was evaluated by 100 resampling bootstrap analysis by neighbor-joining tool.

Growth pattern and antimicrobial profile of *nocardioopsis halotolerans* VJPR-2

The growth pattern of the strain VJPR-2 was studied using the production medium ISP-2. The secondary metabolites obtained from 5-day old culture exhibited good antimicrobial activity against the test microorganisms (Fig. 3). Of all the bacteria tested, *E. coli* and *B. subtilis* were highly sensitive to the metabolites produced by the strain. Among the fungi tested, *C. albicans* followed by *Fusarium oxysporum* were sensitive to the metabolites elaborated by the strain (Table 7). Naragani et al. (2014) reported that metabolites obtained from 5-day old culture of *Rhodococcus erythropolis* VLK-12 showed maximum antimicrobial activity [26]. The metabolites collected from 4-day old culture of *Nocardia levis* and *Pseudonocardia* sp. also exhibited good antimicrobial activity [27,28].

CONCLUSION

In the course of our screening for bioactive compounds from mangrove actinomycetes, we found that *N. halotolerans* VJPR-2 was found to be active against pathogenic and opportunistic bacteria and fungi. The growth pattern and anti-microbial profile of the strain against a wide range of pathogens were also studied. Further study on optimization, purification and chemical characterization of bioactive compounds of the strain is in progress.

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