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Research Article

Molecular characterization of *Nocardiopsis* species from Didwana dry salt lake of Rajasthan, India

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Abstract
The genus *Nocardiopsis* is well known to produce secondary metabolites especially antibacterial bioactive compound. Isolation and characterization of bioactive compounds producing novel isolates from unusual habitats are crucial. The present study was aimed to explore Didwana dry salt lake of Rajasthan state in India for the isolation and characterization of actinomycetes. The isolated actinomycetes isolates were characterized based on culture characteristics, biochemical tests and 16S rRNA gene sequencing. The 16S rRNA gene sequence analysis revealed that all the five isolates inhabiting soil of the said dry salt lake of Didwana, Rajasthan belonged to four species of *Nocardiopsis* viz., *N. synnemataformans, N. potens, N. prasina* and *N. dassonvillei* subsp. *albirubida.* The molecular identification based on 16S rRNA gene sequences was found accurate and robust. The phylogram generated through multiple sequence alignment of all the test isolates of *Nocardiopsis* revealed that the isolates aroused from a single branch and validated monophyletic association. The present study is the first report of exploring *Nocardiopsis* isolates from the dry salt lake. These characterized *Nocardiopsis* isolates isolated from Didwana dry salt lake habitat are novel stains and can be of significance in the detection and utilization of novel bioactive compounds.

Keywords: Actinomycetes, Molecular characterization, *Nocardiopsis species*, 16S rRNA gene

INTRODUCTION

Actinomycetes are gram-positive filamentous bacteria and are distributed across habitats, including extreme environments. As compared to other Eubacteria, they have a large linear genome with higher Guanine and Cytosine (GC) contents and form an extensively branched mycelium fragmenting into non-motile spore chains (Kampfer, 2006). They are the acknowledged source of bioactive chemical compounds having medicinal and pharmaceutical significance. Berdy (2005) reported that out of the known bioactive microbial metabolites, 45% (~10,000) compounds had been isolated from a various group of actinobacterial species. Out of which, 34% (~7600) compounds were from *Streptomyces* and 11% (~2500) compounds were from the rare actinobacteria.

Nocardiopsis is one of the most important genera of Actinomycetes group having 62 species (https://lpsn.dsmz.de/genus/nocardiopsis) ubiquitously distributed in the environment. *Nocardiopsis* species are aerobic, Gram-positive, non-acid-fast, catalase-positive with nocardioform substrate mycelia and their aerial mycelia bear long chains of spores. They are halophilic

or halotolerant in nature and have higher GC contents in their genomic DNA. Their cell walls contain meso 2,6 -diaminopalmelic acid (Kroppenstedt and Evtushenko, 2006). Phospholipid and menaquinones composition are used to distinguish species of *Nocardiopsis* (Tulskaya *et al.*, 2014). Members of *Nocardiopsis* has distinctive genetic make-up accumulation of compatible solutes, surfactants and extremozymes allow them to survive under adverse conditions such as hot desert, marine, salterns, mine tailings, hypersaline and alkaline regions (Bennur *et al.*, 2014). *Nocardiopsis* are known to produce various bioactive compounds such as antibiotics, anticancer substances, tumour inducers, immune-modulators and novel extracellular enzymes (Bennur *et al.*, 2014).

Saline soils of the arid zone are reported to have novel and rare isolates of Actinomycetes *(*Binayke *et al.,* 2018). It has been found that these rare isolates produce novel bioactive secondary metabolites compounds having a broad range of pharmacological applications (Harwani, 2013). Although various studies have been reported on exploration and identification of Actinomycetes isolates from Rajasthan (Kumar, 2018; Begani *et al*, 2019) still, review of the literature revealed no comprehensive study on the site of dry salt lake inhabiting *Nocardiopsis*.

Therefore, the present study was undertaken to isolate and molecularly identify the promising isolates of *Nocardiopsis* based on culture characteristics, biochemical tests and 16S rRNA gene diversity.

MATERIALS AND METHODS

Study area

The dry salt lake of Didwana at Nagaur district of Rajasthan was selected for the exploration of *Nocardiopsis* isolates. The lake is located in the NE-SW direction (27°23'46" N latitude and 74°33'57" E longitude) expanded over an area of 10 km^2. This lake is mostly dry except few patches of shallow water and is characterized by centripetal drainage without outflow as alkaline brine (Roy *et al*., 2006).

Collection of soil sample

The soil samples were collected aseptically from a depth of 5 cm from the randomly selected locations of dry salt lake, Didwana in airtight plastic bags. The samples were brought to the laboratory and were stored in the refrigerator at 4°C.

Selective isolation of Actinomycetes isolates

Air-dried soil samples were kept in an oven at 50°C for 24 hours. One gram of soil was suspended in 100 ml of 0.9% saline solution to prepare stock culture and 0.5 ml of this stock culture was used for serial dilution up to 10^{-6} as suggested by Seong *et al.* (2001). An amount of 0.5 ml from this dilution was spread on to Actinobacteria Isolation Agar (AIA) medium supplemented with 0.5M NaCl. Nalidixic acid (20 µg/ml) and Nystatin (25 µg/ml) were also added in media to avoid fungal and bacterial contamination. Inoculated petriplates were incubated at 37°C for 10-15 days. The suspected actinobacterial isolates were purified and sub-cultured on Starch Casein Agar (SCA) culture medium for characterization.

Molecular marker based identification

Genomic DNA isolation, PCR amplification and 16S rRNA gene sequencing

Extractions and amplification of genomic DNA of isolated isolates were carried out using protocol from our laboratory (Kumar *et al.,* 2021). The 16S rDNA sequences were amplified using universal forward primer EUB-1 (5' AGA GTT TGA TCC TGG CTC A 3') and reverse primer EUB-2 (5' GCT CGT TGC GGG ACT TAT CC 3').The purified PCR products were sequenced using Big Dye termination method in ABI prism DNA sequencer.

BLAST search and phylogeny analysis

16S rDNA sequences were aligned and subjected to Basic Local Alignment Search Tool (BLAST) search for their similarity analysis. The 16SrDNA gene sequences were submitted to the GenBank database of NCBI and gene accession numbers were obtained. The phylogenetic tree was constructed using MEGA-7 software (Kumar *et al.*, 2016b). The evolutionary history was inferred using the Neighbor-Joining method developed by Saitou and Nei (1987).

Morphological and biochemical characterization

Identified isolates of *Nocardiopsis* were characterized by their phenotypic and biochemical characters using standard methods (Shirling and Gottlieb, 1966; Gordon *et al.*, 1974). The morphological and cultural characteristics of identified isolates were observed by analyzing their growth and colour of aerial and substrate mycelia on AIA and SCA media. Both media were supplemented with 0.5M NaCl and incubated at 37°C. The microscopic features of purified isolates were observed by light microscopy using the cover-slip culture technique (Arifuzzaman *et al.*, 2010) and compared with Bergay's manual of Determinative Bacteriology (Holt *et al.*, 1994). The biochemical characteristics viz., methyl red and Vogues-Proskauer test, degradation of starch, casein, and urea, production of indole and catalase, nitrate reduction and citrate utilization were tested by method described by Cappuccino and Sherman (2004). Utilization of carbohydrates viz., glucose, fructose, rhamnose, mannose, xylose, arabinose, cellobiose, lactose, sucrose and raffinose as carbon source were determined on International Streptomyces Project (ISP)

medium (Shirling and Gottlieb, 1966).

RESULTS AND DISCUSSION

A total 18 actinobacterial colonies were isolated on AIA media using serial dilution method. The BLASTn search of 16S rRNA gene sequences of isolates at the National Center for Biotechnology Information (NCBI) web revealed that five isolates belonged to the genus *Nocardiopsis* (Table 1). The BLAST result further resolved that out of five isolates, two isolates (DW-1 and DW-13) showed more of the 99% similarity with *Nocardiopsis synnemataformans* and another three, i.e. DW-5, DW-9 and DW-11 had affinities with different species viz., *N. potens, N. prasina* and *N. dassonvillei* subsp. *albirubida,* respectively, with more than 98% similarity. The GC values of 16S rDNA sequences of these isolates were ranged from 58.31-60.15%. 16S rRNA gene sequences of these isolates were submitted to GenBank, NCBI to get accession numbers (MT669267, MT669269, MT669270, MT669272 and MT669273).

The morphological and cultural characteristics of the five *Nocardiopsis* isolates are presented in Table 2. Phenotypic observation and growth pattern of identified isolates were observed on both AIA and SCA media (Fig. 1). The colour of the aerial and substrate mycelia with growth response of all five isolates varied significantly from white, off white, yellow and grey on both selected media. Tiwari *et al.* (2015) reported the diversity of Actinomycetes isolates by using culture characteristics, including aerial and substrate mycelia, growth patterns, and pigments from the great Indian Thar Desert. Kumar *et al.* (2016a) used morphological features and biochemical test for the characterization of S*treptomyces* species in semi-arid soils of Western Rajasthan.

The biochemical and physiological characteristics of all the five isolates are presented in Table 3. Methyl red test and Voges-Proskauer test were performed to differentiate the Actinomycetes isolates on the basis of fermentation of glucose into mixed acids and digestion of glucose into acetyl methyl carbinol, respectively. All the five identified isolates exhibited gram-positive cell wall as distinctive features of Actinomycetes isolates. Out of five isolates, three isolates (DW-9, DW-11 and DW1-3) were positive to methyl red, while three isolates (DW-5, DW-9 and DW-11) were positive to Vogues Proskauer test. Indole test was used to determine the ability of Actinomycetes to split amino acid tryptophan to indole and pyruvic acid using of enzyme tryphophanase. Only one isolate (DW-5) was found positive to indole production test. All the five isolates

Table 1. Molecular characterization of *Nocardiopsis* isolates.

Isolates	Nucleotide	GC (%)	Reference sequence	Similarity (%)	Isolates designation	GenBank accession
DW-1	1389	58.31	NR_112742	99.78	*N. synnemataformans* DW-1	MT669267
DW-5	1413	60.15	NR_116914	99.86	*N. potens* DW-5	MT669269
DW-9	1419	58.35	NR_044906	99.30	*N. prasina* DW-9	MT669270
DW-11	1433	59.24	NR_112743	98.95	*N. dassonvillei* subsp. *albirubida* DW-11	MT669272
DW- 13	1394	58.46	NR_112742	99.86	*N. synnemataformans* DW-13	MT669273

Table 2. Culture characteristics and growth responses of *Nocardiopsis* isolates.

Isolates	AIA media			SCA media		
	Colour of aerial mycelia	Colour of substrate mycelia	Growth response	Colour of aerial mycelia	Colour of substrate mycelia	Growth response
N. synnemataformans DW-1	White	Off white	Good	Off white	Light yellow	Good
N. potens DW-5	Yellow	Grey	Poor	Light yellow	Grey	Poor
N. prasina DW-9	White	White	Poor	White	Off white	Good
N. dassonvillei subsp. *albirubida* DW-11	Grey	Off white	Good	Grey	Off white	Good
N. synnemataformans DW-13	Off white	Off white	Good	Off white	Light yellow	Good

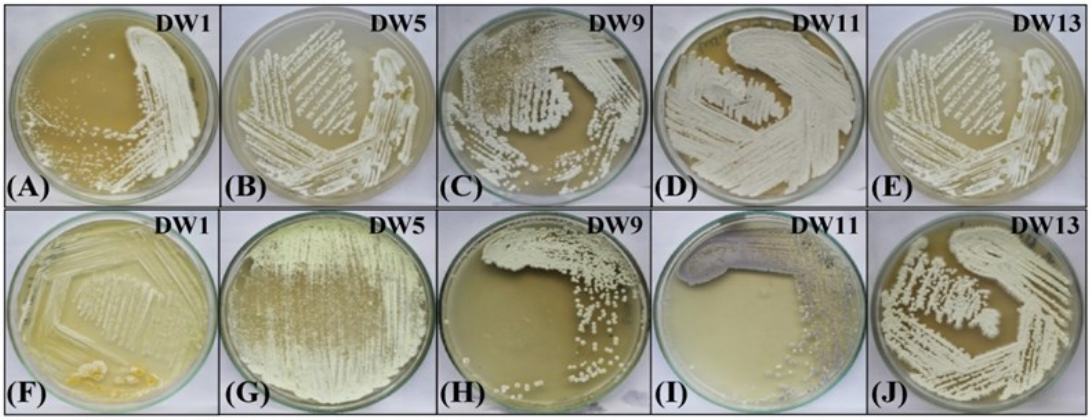

Fig. 1. *Growth characteristic of Nocardiopsis isolates on AIA (A to E) and SCA (F to J) media.*

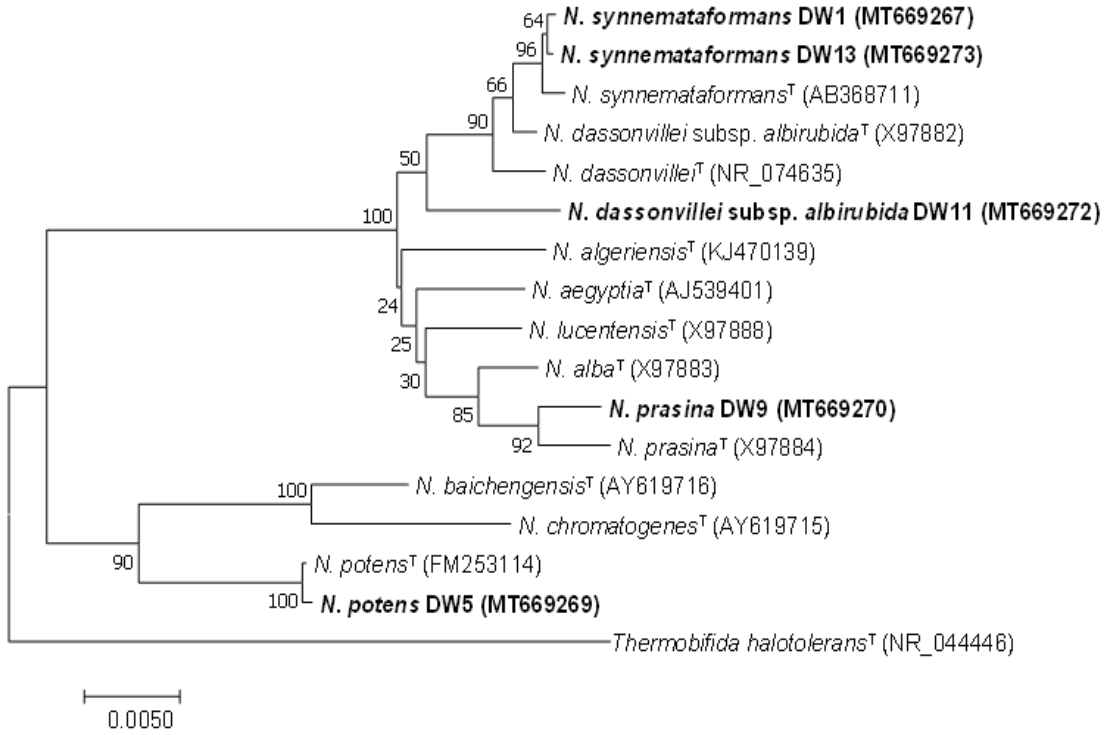

Fig. 2. *Neighbor-Joining phylogenetic tree of Nocardiopsis isolates using 16S rRNA gene sequences. Tree was constructed with 1000 bootstraps. GenBank accession numbers are in parenthesis.*

(DW-1, DW-5, DW-9, DW-11 and DW-13) hydrolyzed starch and casein as they utilized AIA and SCA media for their growth that revealed they produced amylase and protease enzymes. Four isolates (DW-1, DW-5, DW-9 and DW-13) produced urease and citrate enzyme that hydrolyzed urea and citrate salt and converted into end product ammonia, pyruvic acid and CO_2, respectively. These four isolates also exhibited catalase enzyme activity as they neutralize toxic forms of oxygen (H_2O_2) metabolites. Three isolates viz., DW-1, DW-5and DW-13 exhibited nitrate reductase activity as they hydrolyzed nitrate (NO_3^-) to nitrite (NO_2^-). The carbon utilization ability of all the isolates varied significantly exhibiting their ability to ferment different carbohydrates. Kumar *et al.* (2016a) studied biochemical parameters of *Streptomyces* isolates isolated from western Rajasthan and advocated that biochemical traits are strain-specific rather than species traits. The phylogenetic tree of characterized *Nocardiopsis* isolates using the multiple sequence alignment with high bootstrap values indicated a close phyletic line with distinct clades. It delineated all the five isolates from each other along with closeness of phylogenetic relations (Fig.2).The *Nocardiopsis* isolates delineated into two main clusters. Cluster I included only one isolate *N. potens* DW-5 while cluster II included four isolates N. *synnemataformans* DW-1, N. *synnemataformans* DW-

Table 3. Biochemical and physiological characteristics of *Nocardiopsis* isolates.

Characteristics		Isolates				
		***N.synnemataformans* DW-1**	***N.potens* DW-5**	***N.prasina* DW-9**	***N. dassonvillei*subsp. *albirubida* DW-11**	***N.synnemataformans* DW-13**
Biochemical test	Methyl Red	-	-	+	+	+
	Voges-Proskauer	-	+	+	+	-
	Indole Production	-	+	-	-	-
	Citrate Utilization	+	+	+	-	+
	Nitrate reductase	+	+	-	-	+
	amylase	+	+	+	+	+
	Protease	+	+	+	+	+
	Urease	+	+	+	-	+
	Catalase	+	+	+	-	+
Carbon source utilization test	Glucose	+	+	+	+	+
	Fructose	-	+	+	+	+
	Rhamnose	+	-	-	+	+
	Mannose	+	+	+	-	+
	Xylose	+	-	-	+	+
	Arabidose	-	-	+	+	-
	Cellobiose	+	+	+	+	+
	Lactose	-	+	-	-	-
	Sucrose	-	+	-	+	-
	Raffinose	-	-	-	+	-

(+ = positive response, -=negative response)

13, *N. dassonvillei* subsp. *albirubida* DW-11 *and N. prasina* DW-9, indicating the presence of genetic variability within the genus. This may be attributed to the single nucleotide polymorphisms (SNPs) in 16S gene region during the process of evolution by insertion, deletion or substitution (López-Pérez *et al.* 2014; Santos-Beneit, 2018).

Conclusion

The present study is the first report of exploring *Nocardiopsis* species from the dry salt lake of Didwana, Rajasthan, which resulted in identifying four *Nocardiopsis* species with five distinct isolates viz. *N.synnemataformans, N. potens, N. prasina* and *N. dassonvillei* subsp. *albirubida*. These promising *Nocardiopsis* isolates isolated from unusual habitat can be sources for novel bioactive compounds for industrial and therapeutic use.

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Conflict of interest

The authors declare that they have no conflict of interest.

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