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Screening and Isolation of Plant Growth Promoting, Halotolerant Endophytic Bacteria from Mangrove Plant *Avicennia officinalis* L. at Coastal Region of Corangi Andhra Pradesh

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ABSTRACT

Background: Main objective of this study is to isolate and characterize halo tolerant plant growth promoting endophytic bacteria from leaves and pneumatophores of mangrove plant *Avicennia officinalis* L. Isolates were also screened for their ability to provide resistance to extreme salt tolerance and temperatures and to produce exo enzymatic activity like catalase, amylase, protease, phosphate solubilization and plant growth promoting hormone (Indole 3-acetic acid).

Methods: The present study was carried out during 2020-21 to 2021-22 at field and laboratory level. Eleven endophytic bacterial isolates were obtained from mangrove *Avicennia officinalis* L. grown at coastal region of Corangi Wildlife Sanctuary, East Godavari. All Isolates were enumerated, purified and preserved for further studies.

Result: Out of eleven isolates obtained bacteria "AOL2" isolated from leaves of *Avicennia officinalis* L. was found promising in production of exo enzymes, Indole 3-acetic acid (IAA) and phosphate solubilization parameters. Isolate AOL2 was a Gram's negative, motile, moderately halophilic, rod shaped diplobacilli which grows optimally at 32°C, pH 8.3 and had a salt tolerance of 10% NaCl. Microbial identification was carried out by morphological, biochemical and by 16S rRNA sequence. Based on nucleotide homology and phylogenetic analysis isolate AOL2 showed high degree of resemblance with *Salinicola salarius*. On the basis of the experimental findings, strain *Salinicola salarius* AOL2 had a capability in producing plant growth promoting substances, exo enzymes, phosphate solubilization ability and was first reported in endophytic bacteria isolated from *Avicennia officinalis* L. that can be studied further.

Key words: Catalytic activity, Endophytic bacteria, Mangrove, Plant growth promoters, *Salinicola*.

INTRODUCTION

Direct extraction of secondary metabolites from mangrove plants that have superior in antibacterial activity requires a large amount of biomass and this is potentially damage the mangrove community and their ecosystem. Endophytic bacteria that live in plant tissues can be used to obtain the bioactive compounds efficiently. Endophytic microbes are groups of organisms associated with various tissues and organs of several terrestrial and aquatic plants (Stone *et al.,* 2000). Some endophytic bacteria are known to produce secondary metabolites that are useful in pharmaceutical industry (Gouda, *et al.,* 2016). Endophytic organisms are those that live internally in apparently healthy and asymptomatic hosts. Endophytes appear to be ubiquitous; indeed, no study has yet shown the existence of a plant species without endophytes (Nisa *et al.,* 2015). Microorganisms from marine ecosystems contain useful enzymes, proteins, antibiotics and salt tolerant genes that have pharmacological significance (Thatoi *et al*., 2013). Endophytic bacteria had several potential applications in medicine and in other various sectors of biotechnology including agriculture. One of the potential applications of bacterial endophytes in agricultural is to enhance the growth of the agricultural crops.

Mangrove belong primarily to the families *Rhizophoraceae*, *Acanthaceae*, *Lythraceae*, *Combretaceae* and *Arecaceae*; that grow in dense thickets along tidal estuaries, in salt marshes and on muddy coasts. Mangrove ecosystem is a bridge between terrestrial and marine environment and harbors unique microbial diversity. Recently, among the marine microorganisms, marine derived bacteria have been recognized as good resources for new biologically active secondary metabolites including antitumor, antibacterial, antiviral, antifungal and enzyme inhibitor compounds. Bacterial diversity from these ecosystems has been studied worldwide for their unique biochemical processes. Various groups of bacteria are typically present in the mangrove ecosystem (Holguin *et al*., 2001) where they perform diverse activities including photosynthesis, nitrogen fixation and methanogenesis

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(Das *et al*.,2007). Bacterial communities can be found living freely in mangrove sediments (Roy *et al*., 2002) or as endophytes associated with the native flora (Garcias-Bonet *et al.,* 2012). Microorganisms from mangrove ecosystems contain useful enzymes, proteins, antibiotics and salt tolerant genes, all of which have biotechnological significance.

Avicennia officinalis L. found sporadically on the banks of rivers and rarely found near the sea. It prefers clay soil and usually found inland. *Avicennia officinalis* L. is a species of mangrove also known as Indian mangrove.

One of the bacterial isolates in this study belongs to genus *Salinicola*, a member of the family Halomonadaceae, was originally described with *Salinicola salarius* as the type species. The genus comprises six recognized species with validly published names: *S. socius, S. halophilus, S. salarius, S. rhizosphaerae, S. peritrichatus* and *S. acroporae*. Salinicola strains have been isolated from different environments including seawater, salt mines, a solar saltern, the rhizosphere of mangrove and deep-sea sediment. *Salinicola* strains are described as Gram-stain-negative, aerobic, rod-shaped, motile and moderately halophilic. *Salinicola* strain isolated from the leaves of mangroves, *Avicennia officinalis* L. was studied in this experiment.

MATERIALS AND METHODS

This present study was conducted during 2020-21 and 2021-22 at Department of Botany andhra University, Visakhapatnam, Andhra Pradesh (Fig 1).

Sample collection

Samples were collected from leaves and pneumatophores of mangrove plant *Avicennia officinalis* located at mangrove zone of Corangi Wildlife Sanctuary, East Godavari district within latitude of 16°49′53.0″N and longitude of 82°20′12.0″E (16.831389, 82.336667). The collected samples were sealed in sterile container before sending to the Research Laboratory (Fig 5).

Surface sterilization and Sterility check

The sample was first washed with sterile water, blotted dry with filter paper dipped in 70% ethanol and kept for about 1 min. The sample was again washed in sterile water dipped in 4% Sodium hypochlorite solution and kept for about 5 minutes. The whole process was repeated twice.

For sterility check, the shoots were rolled on nutrient agar plates as well as 0.1 ml aliquot from the final wash was inoculated to 10 ml nutrient broth (NB) (Gyaneshwar *et al.,* 2001). Samples were discarded if any growth was detected in the sterility check.

Isolation of endophytic bacteria from leaves and pneumatophores of mangrove plant *Avicennia officinalis* L.

After surface sterilization of the explants, 1g of each sample was grounded into paste and diluted by serial dilution technique. The highest diluted solution (4^{th}) were spread (100 µl) on Zobell Marine Agar plates (Composition-Zobell-Marine Broth 2216 Himedia @ 5.5% + Agar agar @ 1.5%) and incubated. After incubation individual colony of a different type were observed isolated and purified by quaternary streaking method on Zobell Marine Agar plates with a code assigned. Pure cultures obtained were subjected to further studies. They were also named according to their host plant followed by part (leaf or pneumatophore) they are collected and finally by numbering in serial number (Table 2).

Preservation of endophytic bacterial isolates

All the isolates were streak on 3% sodium chloride (NaCl) nutrient agar plates and stored with 20% (w/v) glycerol and

Fig 1: Sample collection site (Coringa Wildlife Sanctuary, East Godavari).

stored at -20°C. Viability of the cultures was retrieved by periodic sub-culturing into new media at monthly intervals (Fig 2).

Phenotypic characterization of the endophytic isolates

Eleven isolates were inoculated on 3% NaCl Nutrient agar and the colony morphology was observed as per guidelines given by Hawksworth *et al*. (1983). The purified cultures, at log phase were observed microscopically for the cell morphological characters as per the method outlined by Aneja, (2006).

Growth at different NaCl concentration

The young cultures were streaked on Nutrient agar plates with different salt percentages *i.e.* 8% NaCl, 10% NaCl and 15% NaCl. The streaked plated were incubated at 32°C and observations on growth was made for 24hrs and 48 hrs (Fig 4).

Biochemical characterization of endophytic bacterial isolates

12 hours old cultures were subjected to catalase test, oxidase and KOH string test as per the methods outlined by Aneja (2006). Indole, Methyl Red, Voges Proskauer and Citrate Utilization Tests was conducted as per the method summarized by Seeley (1962).

Property studies of endophytic isolates

The isolated bacteria were subjected to different property studies like Protease activity (Gelatin hydrolysis), Amylase Production (Starch hydrolysis), IAA production, Phosphate solubilizing and Sideophore production ability (Fig 3).

Gelatin and starch hydrolysis

Gelatin and Starch hydrolysis test was conducted by growing the organism on 1% Gelatin Nutrient Agar and 1% Starch Nutrient agar plates in that order. After 48 hours of incubation the plates were flooded with saturated solution of Ammonium

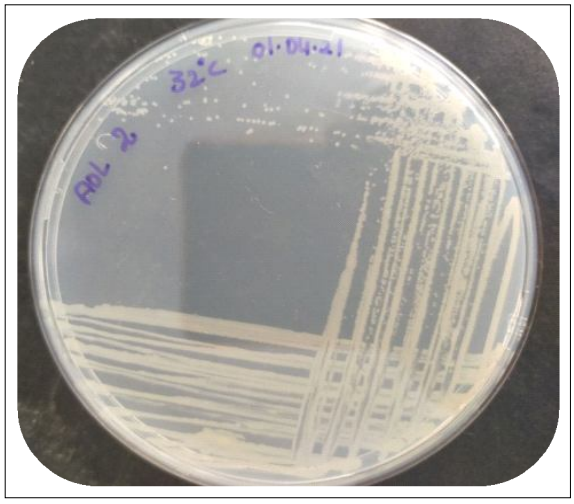

Fig 2: AOL2 Colonies isolated from *Avicennia officinalis* L.

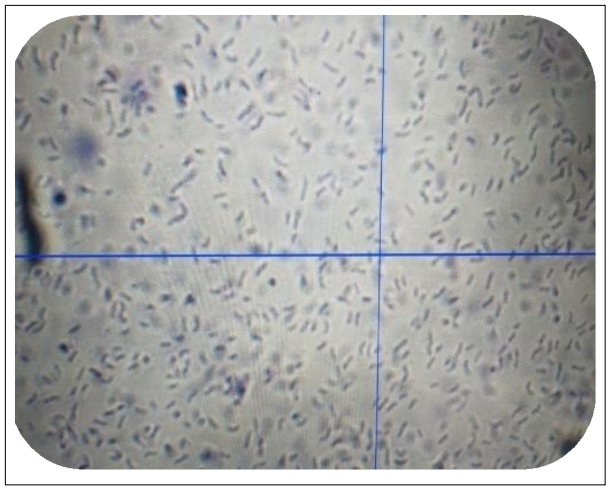

Fig 3: Endophytic bacteria under 100X.

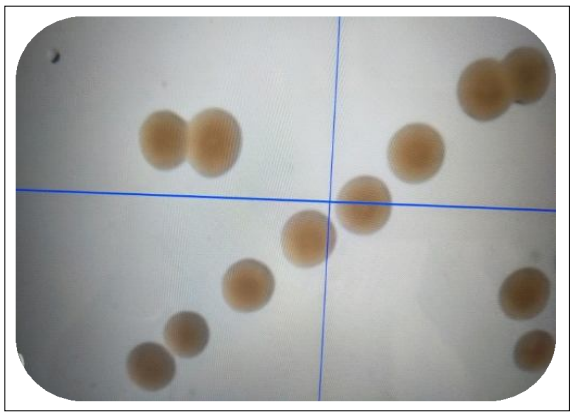

Fig 4: Colony morphology of strain AOL2.

Fig 5: *Avicennia officinalis* L. leaves and pneumatophores.

Chloride (5 g/10 ml) and diluted Iodine solution for Gelatin and Starch utilization respectively.

IAA production and phosphate solubilizing ability

Bacteria isolated from plant rhizosphere produce various phytohormones in the form of secondary metabolites, the most common of which is Indole-3-acetic acid (IAA). IAA production was screened for the presence of Indole using Salkowski reagent and colour developed was measured spectrophoto metrically at 536 nm using UV Spectrophotometer.

Phosphate solubilization ability was carried out by Pikovskaya's broth and the presence of Phosphate was tested by adding 750 µl of phosphate reagent and colour development was measured spectrophotometrically at 680nm by using UV Spectrophotometer (Fig 5).

Microbial identification using 16S rRNA gene based molecular method

DNA was isolated from the culture. Its quality was evaluated on 1.0% agarose gel, a single band of high-molecular weight DNA has been observed. Fragment of 16S rRNA gene was amplified by 16S rRNA-F and 16S rRNA-R primers. A single discrete PCR amplicon band of 1500 bp was observed when resolved on agarose gel. The PCR amplicon was purified to remove contaminants. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with 16S rRNA-F and 16S rRNA-R primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Consensus sequence of 16S rRNA gene was generated from forward and reverse sequence data using aligner software. The 16S rRNA gene sequence was used to carry out BLAST with the 'nr' database of NCBI GenBank database. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix and phylogenetic tree was constructed using MEGA 10. 7.

RESULTS AND DISCUSSION

Enumeration of endophytic bacterial population in leaves and pneumatophores of mangrove plant *Avicennia officinalis* L.

The endophytic bacterial population in leaves (3.5×10^5 cfu/g) and pneumatophores (4.2×10^5 cfu/g) of mangrove plant *Avicennia officinalis* were isolated. Three isolates from leaves and eight isolates from pneumotaphores of *Avicennia officinalis* were obtained and purified by Streak plate technique.

Among 11 isolates, which belongs to the genus Bacillus, Exiguobacterium, Salinicola, Pseudomonas, Enterobacter and Vibrio, Strain AOL2 (*Aveicenia Officinalis* Leaf strain-2) is of particular interest due to its salt tolerance at 10-15% NaCl and also showed distinct morphology and phenotypic characters and was selected for further studies (Fig 2 to 5) (Table 1).

Colony and cell morphology

The colonies of AOL2 strain on agar medium were medium, round in shape, yellow in colour, convex in elevation. The cells when viewed microscopically are single rods and motile. Gram's staining was carried out and the cells were gram negative in appearance. When grow in nutrient broth cells showed aerobic in nature and are non-endospore forming.

Growth at saline conditions

AOL2 grows well at temperature 25-30°C with 3% NaCl nutrient agar, pH ranges 05-10 and salt tolerance up to 15% NaCl nutrient agar. Similar findings were presented by Ali *et al.* (2017) in their study with *Bacillus pumilus* AM11 and *Exiguobacterium* sp. AM25 showed significantly higher growth in saline media.

Biochemical characteristic of endophytic bacterial isolates

Biochemical studies were carried out by the isolate AOL2 and presented in Table 2. The results showed that the AOL2 isolate were KOH positive with stringy appearance. Catalase positive when 3% H_2O_2 was added and oxidase negative when para-aminodimethyl aniline oxalate solution were added. IMViC test showed Indole positive with red colour when Kovac's reagent was added, Methyl red positive with the production of red colour, Voges-Proskeaur test negative with no colour and Citrate utilization positive with production of blue colour due to pH change in the medium. AOL2 showed positive result with Gelatin (0.5 cm) and Starch (0.8 cm) hydrolysis (Table 2).

Indole-3-acetic acid (IAA) production

IAA production was screened for the presence of Indole compounds by colorimetric assay using Salkowski reagent. Strain AOL2 was cultured and inoculated in 3% Nutrient broth with 0.5% Tryptophan. After 48 hrs incubation the culture were subjected to centrifugation and the supernatant was mixed with 1.5 ml of Salkowski's reagent. The colour intensities were measured by using UV Spectrophotometer at 536 nm. The OD values were compared with standard graph of IAA and 7.5 µg/ml of IAA production was recorded with strain AOL2 (Table 2). Similar results were obtained by Hardoim *et al.* (2015).

Phosphate solubilizing ability

AOL2 culture were inoculated in 4 ml of 3% pikovskaya's broth in ria vials and kept for 48 hrs incubation as mentioned in material and methods. After incubation 750 µl of phosphate reagent to the 4ml of the sample and blue colour intensities were measured by using UV Spectrophotometer at 680 nm wavelength. 3.5 ppm was recorded with strain AOL2. Similar results were also reported Rylo Sona Janarthine *et al.* (2011) in their study isolated Endophytic bacteria from the surface sterilized pneumatophores of

Table 1: Coding of endophytic bacterial isolates.

Plant	Plant part	Isolates*
Avicennia officinalis L.	Leaf (03 isolates)	AOL1, AOL2, AOL3
	Pneumatophore (08 isolates)	AOP1, AOP2, AOP3, AOP4, AOP5, AOP6, AOP7, AOP8

*A- Aviceniia, O- Officinalis, L- Leaf, P- Pneumatophores.

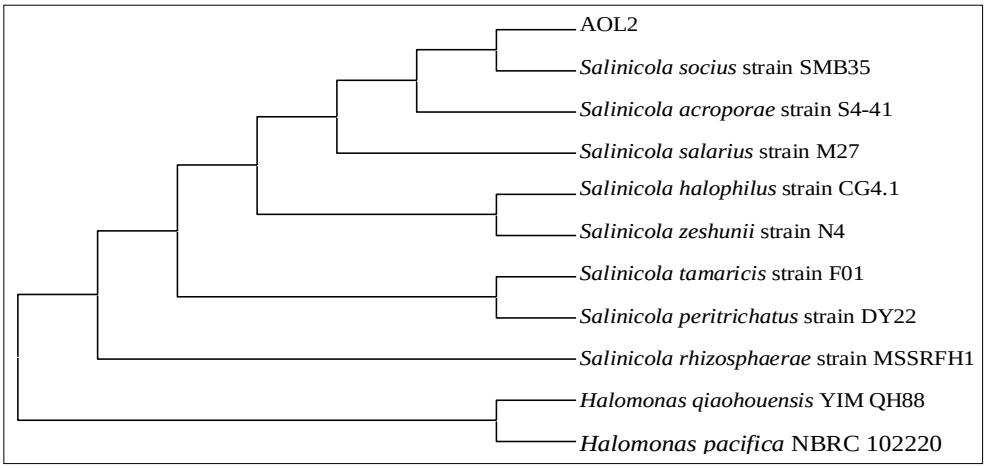

Fig 6: Phylogenetic tree based on nucleotide sequence of the 16S rRNA sequence of AOL2 and other related type strains.

Table 2: Phenotypic studies of endophytic bacteria isolates strain AOL2.

Parameter	Results
Morphological observation	
Gram's reaction	-ve
Cell shape	Rod
Endospore forming ability	-
Motility	+
Biochemical studies	
KOH	+
Catalase	+
Oxidase	-
Indole production	+
Methyl red	+
Voges-proskauer test	-
Citrate utilization	+
Enzymatic assay	
Starch hydrolysis (Amylase production)	0.5 cm
Gelatin hydrolysis (protease production)	0.8 cm
Phosphate solubilization	3.5 ppm
IAA production	7.5 µg/ml

Avicennia marina. They identified isolates as GU930357 (*Bacillus* sp.), GU930358 (*Enterobacter* sp.) and GU930359 (*S. aquimarina*). *Bacillus* sp., fixed nitrogen, *S. aquimarina* produced siderophore and all the three strains solubilized phosphate molecule. Same findings were also obtained by El-Tarabily and Youssef, (2010) conducted a study on mangrove *Avicennia* marina rhizosphere identified 129 bacterial strains with the ability to solubilize rock phosphate, with *Oceanobacillus picturae* able to mobilize 97% of this mineral. Richardson (2009) in their study stated that Phosphate solubilization by microorganisms has an important function in supplying phosphorus (P) to plants with the potential to be used as inoculants. Kim *et al.* (1997) also reported that the ability of microorganisms to solubilize phosphate correlates with the ability to produce organic acids and/or extracellular polysaccharide.

Microbial identification using 16S rRNA gene based molecular method

Isolated DNA from the culture was evaluated on 1.0% agarose gel and single band of high molecular weight DNA was observed. Fragment of 16S rRNA gene was amplified by 16SrRNA-F-5′-TCTTCGGACTTCGCGCTATC-3′ and 16SrRNA-F-5′-CAGACCAGCTACGGATCGTC-3′ primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. A comparison using 16S rRNA gene sequences from the databases revealed that the 16S rRNA gene sequence of the type strain of AOL2 displayed high levels of similarity to those of *Salinicola salarius.* The percentage of 16SrRNA sequence similarity between strain AOL2 and *Salinicola salarius* was 99.19%. The phylogenetic tree reconstructed using the neighbour-joining algorithm showed that the strain AOL2 and *Salinicola salarius* clustered together and constituted a separate group from the other closely related species (Fig 6).

CONCLUSION

Interaction between plants and endophytic bacteria is very crucial in the development and evolution of both organisms. Endophytes contributes nutrients, resistance to the plants from extreme biotic and abiotic stress. Endophytes produce several active secondary metabolites that are useful in many applications. Much research is still needed to understand their relationship. Present study revealed the interaction between endophytic bacteria and mangrove plants. Isolated strain *Salinicola salarius* AOL2 has an ability in producing plant growth promoting phytohormones, bioenzymes, phosphate solubilization and was first reported endophytic bacteria isolated from *Avicennia officinalis* and had importance in further research studies.

Conflict of interest: None.

Authors' contribution

All authors listed have made a substantial direct, personal and intellectual contribution to this research work and all

authors edited, proofread and approved for publication of this manuscript.

Data availability

All datasets generated or analyzed during this study are included in this manuscript.

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