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Phenotypic and Genotypic Probing of Biofertilizing Halotolerant *Azosprillum* Spp. and *Bacillus* Spp.

[1, 2]*Santhiyagu Prakash,* [3]*Natarajan Kumari Ahila,* [2]*Sundaram Ravikumar and* [3]*Ethiraj Kannapiran*

[1]SRM-Research Institute, SRM University, Kattankulathur - 603 203, Tamilnadu, India
[2] Department of Oceanography and Coastal Area Studies, School of Marine Sciences, Thondi Campus, Alagappa University, Thondi - 623 409, Tamilnadu, India
[3]Directorate of Distance Education, Alagappa University, Karaikudi - 630 003, Tamilnadu, India

Abstract: The present study was conducted to study the divergence of phenotypic and genotypic characterization of six isolated Plant Growth Promoting Bacterial (PGPBs) strains such as four *Azosprillum* spp. (*A. lipofereum*, *A. amazonense*, *A. haloperense* and *A. brasilense*), nitrogen fixers and two *Bacillus* spp. (*B. megaterium* and *B. subtilis*), phosphate solubilisers from the mangrove environment. From the result, it was clear that the phenotypic and genotypic characteristics of the test bacterial species were significantly varied. The analysis of genotypic characterization by ARDRA profiles showed different polymorphic band patterns and which were interrelated by different statistical and cluster dendrogram analysis.

Key words: Biofertilizers · Halotolerant · *Azospirillum* spp. · *Bacillus* spp. · ARDRA profile

INTRODUCTION

Mangroves are nutrient deficient ecosystems, especially on nitrogen and phosphorus [1]. In spite of this, mangroves are one of the most productive ecosystems. This paradox can be explained by the fact that recycling by microbes in this environment keeps the scarce nutrients within the system [2, 3]. Among them, nitrogen (N_2) fixation by nitrogen fixing bacteria in the halotolerant environment facilitates the nitrogen for the mangrove flora and phosphate solubilizing bacteria (PSB) are the potential suppliers of soluble forms of phosphorus. These two groups would provide a great advantage of availability of limited nutrients to the mangrove plants. Also, they have Plant Growth Promoting Substances (PGPS) which enhances the growth of plants.

Nitrogen-fixing bacteria belonging to the members of genera *Azospirillum*, *Azotobacter*, *Rhizobium*, *Clostridium* and *Klebsiella* have been isolated from the sediments, rhizosphere and root surfaces of various mangrove species [4]. In particular, nitrogen fixation by *Azospirillum* spp. has been of interest for many years [5]. Most of the phosphate solubilizing bacteria (PSB) isolated from the mangrove environment belong to the genera, *Pseudomonas*, *Bacillus, Enterobacter*, *Rhizobium*, *Mesorhizobium, Burkholderia* and *Erwinia* genera [6, 7]. Moreover, the PGPS producing bacterial strains *Azospirillum* spp. and *Bacillus* spp. isolated from the mangrove rhizospheric and root samples of Manakudi estuary and it was inferred that the biofertilizing effect on significantly vigorous seed growing in the mangrove plants likes *Rhizophora* spp, *Avicennia officinalis* and also on economically important plant, *Jatropha curcas* seedlings [8, 9, 10]. With the following background, the present study was made to investigate the phenotypic and genotypic (16S rRNA- ARDRA profile) variations in plant growth promoting rhizobacterial (PGPR) strains *i.e. Azospirillum* spp. and *Bacillus* spp., isolated from mangrove environment. The relationships between the two genera; which are widely varied in their functions but serve as a plant growth promoting strains are common. Thus, considering the importance of biofertilizing microorganisms, in the present study, an attempt was undertaken to investigate the genotypic characteristics of the selected PGPS producing bacterial strains by using molecular tool with strong statistical assistance.

Corresponding Author: Santhiyagu Prakash, SRM-Research Institute, SRM University, Kattankulathur - 603 203, Tamilnadu, India.

MATERIALS AND METHODS

Selection of Potent Biofertilizing Halotolerant Strains: A total of 6 potent biofertilizing strains of two genus namely *Azospirillum* spp. and *Bacillus* spp. were collected from the Microbial Culture Collection, School of Marine Science, Department of Oceanography and Costal Area Studies, Thondi campus, Alagappa University, Tamil Nadu, India. Among them, 4 strains were nitrogen fixing *Azospirillum* spp. (*A. lipoferum, A. amazonense, A. halopreferens* and *A. brasilense*) and two were phosphate solublizing *Bacillus* spp. (*Bacillus megaterium* and *B. subtilis*).

Phenotypic and Genotypic Characterization of PGPBs
Phenotypic Characteristics: All the selected biofertilizing halotolerant bacterial strains were identified by various biochemical and physiological characteristics by adopting the method of Holt *et al*. [11].

Genotypic Characteristics
Genomic DNA Isolation: The total genomic DNA was isolated from the selected PGPS producing bacterial strains by the method followed by Ganesh Babu *et al*. [12]. In brief, the selected bacterial strains were inoculated in nutrient broth (Hi-media) prepared with filter sterilized seawater and incubated overnight at 28°C (150 rpm). Then, the individual bacterial culture was centrifuged at 10,000 rpm for 10 min and the resultant pellet was resuspended in 400 µl of Sucrose TE buffer. To each culture pellet, lysozyme was added at a final concentration of 8 mg/ml and it was incubated at 37°C for 1 h. Then, 100 µl of 0.5 M - EDTA (pH 8.0), 60 µl of 10% SDS and 3 µl of proteinase - K were added and incubated at 55 °C overnight. After incubation, 160 µl volume of phenol: chloroform (1:1) was added to the supernatant and centrifuged at 10,000 rpm for 10 min. Furthermore, the supernatant was extracted twice with phenol: chloroform: isoamylalcohol (25:24:1), once with chloroform: isoamylalcohol (24:1) and then ethanol precipitated. The ethanol precipitated genomic DNA was resuspended in TE buffer and stored at -20°C for long term storage.

16S rRNA PCR amplification: The eubacterial universal primers were used to amplify the 16S rRNA genes (forward primer - 5'AGAGTTTGATCCTGGCTCAG3' and reverse primer 5'ACGGCTACCTTG TTACGACTT3') [13]. PCR was performed with total reaction mixture of 50 µl, containing 10 µl genomic DNA as the template, each primer at a concentration of 0.5 µM, 1.5 mM $MgCl_2$ and dNTPs at a concentration of 50 µM, 1 U of Tag polymerase and buffer used as recommended by the manufacturer (MBI Fermentas). Thirty nine cycles with initial denaturation at 95°C for 4.5 min, 95 °C - 1 min, 55°C - 1 min, 72°C - 2 min and a final extension at 72 °C for 5 min were run in thermocycler (Eppendorf gradient thermocycler, 96). Finally, the amplified PCR products were checked in 1 % agarose gel electrophoresis.

Amplified Ribosomal DNA Restriction Analysis (ARDRA): For ARDRA profile analysis, the amplified PCR products (16S rRNA) of all the six selected strains were digested with *RsaI* restriction enzyme (MBI Fermentas). In this process, 25 µl reaction mixture containing 10 µl (~500 ng) of PCR product, 2.5 µl of 10X PCR buffer and 5 U of *RsaI* restriction enzyme were added and incubated at 37 °C for 3 h. Finally, the digested PCR amplicons of 16S rRNA were analyzed by submarine horizontal electrophoresis using 2% agarose gel containing (0.5 µl mL^{-1}) ethidium bromide. A 1 kD ladder (MBI - Fermentas) was used as a molecular marker and the ARDRA gel was documented by Alpha Imager Mini, Cell-Biosciences, at Center for Marine Science and Technology, Manonmanium Sundaranar University, Tamil Nadu, India.

Data Analysis: The molecular weight of the individual bands was determined by using Gelcompare (Applied bio - Maths) Software, from a standard curve based on the standard DNA marker of a 1 Kb. The data was analyzed by zero and one matrix constructed by (No of bands) medium / strong PCR- ARDRA fragments, where '1' for the presence of band and '0' for the absence of band. Low intensity bands were not considered. The ARDRA data matrix was subjected to multivariate analysis of Principal Component Analysis (PCA), one way - ANOSIM (Analysis of Similarity) and SIMPER (Similarity Percentage) analyzed by Bray - Curtis similarity index. The resulting similarity coefficient was used to appraise the relationship between the N_2 fixing bacterial strains (*Azospirillum* spp.) and phosphate solubilizing bacterial strains (*Bacillus* spp.) which are commonly a potent biofertilizing and halotolerant species. The Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method was employed to construct a dendrogram for the genetic distances obtained by the complement of Jaccard's co-efficient. The multivariate and cluster dendrogram was analyzed by using PAST - 2.15 software.

RESULTS AND DISCUSSION

The phenotypic characteristics of the selected six halotolerant and potent biofertilizing bacterial strains (*A. lipoferum*, *A. amazonense*, *A. haloperense*, *A. brasilense*, *B. megaterium* and *B. subtilis*) were confirmed by the conventional methods (Table 1) and our results are in comparison with other reports [14-17]. The marine microorganisms have the ability to tolerate high salt, nitrogen fixing property, enhancing mineral uptake and production of growth promoting substances [10]. The tested biofertilizing bacterial strains (*Azospirillum* spp. and *Bacillus* spp.) showed good growth in the media with NaCl concentration up to 5%, as they were isolated from the mangrove environment i.e. they are halotolerant. The optimum concentration varied from 1 to 2%. The results of the present study was in consonance with several authors who have reported that NaCl tolerant strains such as *Azotobacter* spp., *Azospirillum* spp. and *Bacillus* spp. etc. were isolated from the mangrove and salty estuary sediments [6, 7, 18, 19].

The PCR amplification result revealed the presence of ~1.5 Kb fragment of 16S rRNA in all test potent strains (Fig. 1A). Several authors have been used ARDRA profile for the identification and genetic variations of variety of *Azospirillum* spp. and *Bacillus* spp. with diverse restriction enzymes such *HinfI*, *HaeIII*, *RsaI*, *AluI*, *EcoRI*, *BamHI* and *PstI* [20-25]. As per their recorded results, the maximal no. of restricted bands were observed by using the *RsaI* restriction enzyme for the digestion of microbial PCR products. Therefore in the present study also, the PCR amplified products of test bacterial strains such as *A. lipoferum*, *A. amazonese*, *A. haloperferens*, *A. barasiliense*, *B. megaterium* and *B. subtilis* were digested by *RsaI* restriction enzyme and the results were clearly obtained to analyze the different polymorphic band patterns by *in silico* method (Fig. 1B). Similarly, Ishii *et al*. [21] reported that the genetic variations of five different bacterial strains such as *A. brasilense* JCM1224T; *Geobacillus kaustrophilus* JCM12893; *Stenotrophomonas nitritireducens* JCM13311; *Ochrobactrum anthropi* JCM21032T; and *Cupriavidus*

Table 1: Phenotypic Characteristic of Potent Bacterial Strains.

		Azospirillum spp.			*Bacillus* spp.		
S. No.	Different Tests	*A. lipofereum*	*A. amazonense*	*A. halopreferense*	*A. brasilense*	*B. megaterium*	*B. subtilis*
1	Morphology	R	R	R	SR	R	R
2	Gram's	-	-	-	-	+	+
3	Motility	+	+	+	+	+	+
4	Catalase	+	+	+	+	+	+
5	Oxidase	+	+	+	+	-	-
6	% of NaCl tolerance	1 - 4	1 - 5	1 - 5	1 - 4	1 - 4	1 – 4
7	% of optimum NaCl for growth	2	3	2	3	1.5	2.5
8	pH range	6 - 7.5	6 - 9	6 - 7.5	5.5 - 9	5 - 7	5.5 - 9
9	Optimum pH for growth	7	7.5	7	7.5	7	7.5
10	Biotin requirement	+	-	+	-	-	+
11	Indole	-	-	-	-	-	-
12	Methyl Red	-	-	-	+	-	-
13	VP	-	-	-	+	-	+
14	Citrate	+	-	-	+	+	+
15	H_2S	+	+	-	+	+	+
16	Urease	+	-	+	-	+	-
17	Nitrate	+	-	-	+	-	+
18	Phosphatase	+	+	-	+	+	+
19	Glucose	+	+	-	-	+	+
20	Fructose	+	+	-	-	+	+
21	Sucrose	-	+	-	-	+	+
22	Dextrose	±	±	±	-	±	-
22	Lactose	±	+	-	-	+	-
23	Maltose	+	+	±	+	+	+
24	Xylose	-	-	-	±	+	+
25	Mannitol	+	-	-	-	+	+
26	Glycerol	+	-	+	+	+	+

R: Rod; SR: Short Rod; NG: No Growth; A/A: Alkaline/ Acid; A: Acid; nd: not done; +: Positive;
- : Negative; ±: Doubtful.

Table 2: Number of Polymorphic and Monomorphic Band Patterns of ARDRA Profile in *Azospirillum* spp. and *Bacillus* spp.

S. No	Name of the Strains	No. of bands	Molecular weight (bp)*
1	*A. lipoferum*	2	968.33, 500.23
2	*A. amazonense*	3	976.87, 394.48, 300
3	*A. haloperferense*	3	498.00, 398.48, 309.38
4	*A. brasilense*	3	482.44, 389.24, 312.00
5	*B. megaterium*	2	486.62, 378.89
6	*B. subtilis*	3	492.37, 396.34, 300.38
Total No. of bands		16	

*Gel documented by the position of no. of bands on DNA finger printing on 2% agarose gel.

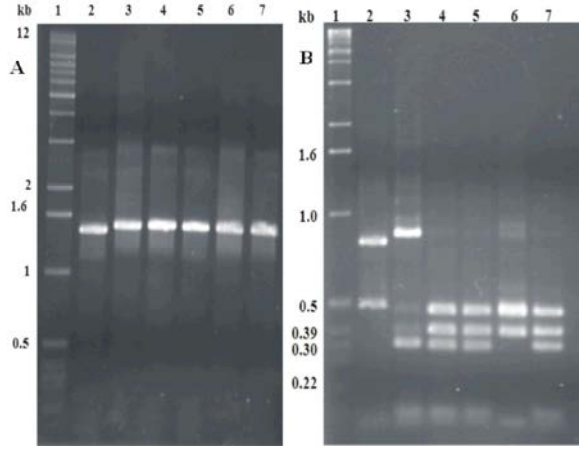
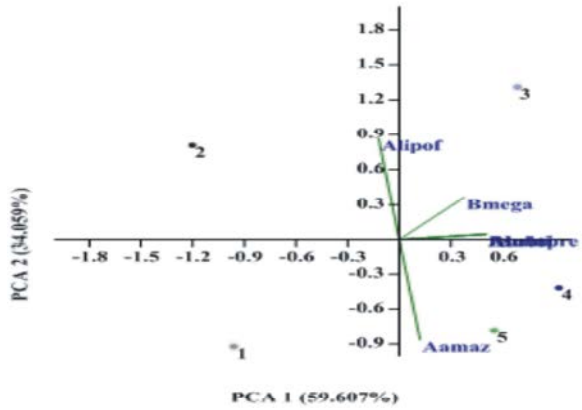


Fig. 1: 16S rRNA - Bands Patterns of the ARDRA Profile. A) Amplification of genomic DNA; B) Individual bands of test halotolerant bacterial strains on 2% agarose gel electrophoresis. M: 1 kb ladder (Marker); Lane 2: *A. lipoferum,* Lane 3: *A. amazonense,* Lane 4: *A. halopreferense,* Lane 5: *A. brasilense*, Lane 6: *B. megaterium* and Lane 7: *B. subtilis.*



The axes explain the data variation for the coordinates PCA1 (59.607%) and PCA2 (34.059%).

Fig. 2: Principal Component Analysis (PCA) of Halotolerant, *Azospirillum* spp. and *Bacillus* spp. by ARDRA Profile.

metallidurans JCM21315T on ADRDA profiles generated by the PCR products were digested with restriction endonuclease, *Afa I* (= *Rsa I*).

The molecular weight of ARDRA profile bands of each bacterial strains by using *RsaI* restriction enzyme was recorded and it ranged from 300.00 to 976.87 bp, followed by *A. lipoferum* (500.23 – 968.33 bp), *A. amazonense* (300.00 – 976.87 bp), *A. haloperference* (309.38 – 498.00 bp), *A. brasiliense* (312.00 – 482.44 bp), *B. megaterium* (378.89 – 486.62 bp) and *B. subtilis* (300.38 – 492.37 bp), respectively (Table 2). The ARDRA profile data of the selected bacterial communities (*Azospirillum* spp. and *Bacillus* spp.) analyzed by multivariate analysis of PCA, resulted 59.61%, 34.05% and 6.33% (1, 2 and 3 coordinates) variations among the tested bacterial strains. A separation of these bacterial communities was found along the coordinates 1 and 2 as shown by PCA (Fig. 2). The one way - ANOSIM calculated for the ARDRA profile bands inferred that the mean rank of within and between the bacterial groups of individual bands has no significant differences (ANOSIM; R= 0.8571; P_{same}= 0.1975). The similarity percentage (SIMPER) analysis based on the abundance estimates of individual bacterial strains of ARDRA profiles indicated the average dissimilarity between the bacterial strains which varied from 18.92% to 100% calculated by Bray- Curtis dissimilarity and it was a good differentiator between the tested bacterial communities.

The similarity index was calculated by Jaccard's similarity co-efficient and cluster dendrogram constructed by UPGMA. From the dendrogram of UPGMA, two main clusters were observed. The exclusive distant cluster I formed by *A. lipoferum* with other test bacterial strains, while cluster II was formed by other five strains (three *Azospirillum* spp. and two *Bacillus* spp.). The cluster II was subdivided into subcluster IIa with three strains (*A. haloperferense, A. brasiliense* and *B. subtilis*) and the cluster IIb was formed with single strain of *B. megaterium*. On the other hand, the ward's method of dendrogram was formed by three distinct clusters. The first cluster-I was formed with *A. lipoferum, A. amazonense*. Next to, the cluster - II was grouped with *A. haloperferense* and *A. brasiliense*. Finally, the cluster - III was formed by two *Bacillus* spp. (*B. subtilis* and *B. megaterium*) which showed an identical ARDRA profile. Thus, the dendrogram analysis revealed that the bacterial strains are highly distinct among the tested nitrogen fixers and phosphate solubilizers (Fig. 3). The nitrogen fixers and phosphate solubilisers are the two important families which play an important role in the

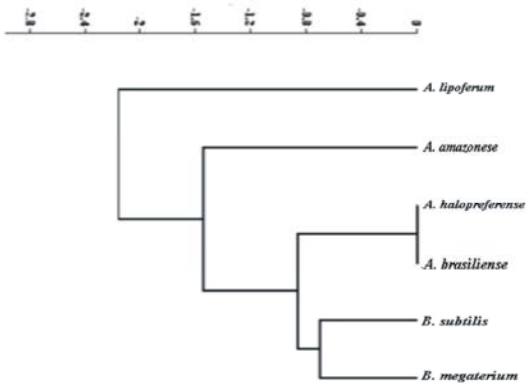


Fig. 3: Cluster Dendrogram Analysis of Halotolerant *Azospirillum* spp. and *Bacillus* spp. by UPGMA Method.

biogeochemical cycles. In the mangrove environment, these groups facilitate the mangrove flora by producing PGPS. In the present study, these two different families were phenotypically and genotypically characterized and proved that they are distinct by different methods. Further, the exact genetic variations of potential PGBS strains have to be characterized by molecular taxonomical studies.

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