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Isolation, identification and characterization of efficient free-living nitrogen-fixing bacteria from rice rhizosphere ecosystem

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Abstract

Biological nitrogen fixation (BNF) is a natural bacterial process of utmost importance due to its potential to supply alternative source of plant available nitrogen for the crops to enable sustainable and economical production. The aim of the present study is to isolate efficient nitrogen fixing bacteria with a potential to be developed into bio-fertilizer for supplying nitrogen and for improving growth and yield of rice. With this background, rice (*Oryza sativa L.*) rhizosphere soil samples were collected from different locations and 197 nitrogen-fixing bacterial strains were isolated. Among these, eight promising bacterial strains that showed highest nitrogen fixing ability based on Acetylene Reduction Assay **(**ARA) were isolated and identified as *Stenotrophomonas sp.*, *Paenibacillus sp.* (isolate 1), *Ochrobactrum sp.*, *Paenibacillus sp.* (isolate 2), *Burkholderia cepacia* (isolate 1), *Burkholderia cepacia*, *Xanthomonas sacchari* and *Rhizobium sp.* The reduction rates of the eight potential isolates indicating the nitrogen-fixing ability ranged from 115 to 490 nm of C_2H_4 produced h^{-1} mg^{-1} protein. These bacterial isolates were further characterized for their morphological features and for their plant growth-promoting (PGP) activity like indole acetic acid (IAA) production and insoluble mineral (P, K, Zn) solubilization. All isolates exhibited IAA production ranging from 11.90 to 47.40 µg/ml while two isolates possessed phosphate and zinc solubilization activity.

Keywords: Nitrogen-fixing bacteria, IAA, ARA, Phosphate-solubilizing bacteria, rice ecosystem

Introduction

Microbes are considered as engineers of soils, playing a vital role in maintaining soil fertility by enriching the availability of nitrates, phosphorous and other nutrients (Rajendhran and Gunasekaran, 2008). Rice (*Oryza sativa)* is a primary food source for half of the world's population. Rice cultivated in different ecologies supports a wide diversity of soil microbes and soil fauna in aqua terrestrial ecosystems. Biologically fixed nitrogen is a potential alternative source of nitrogen for sustainable cereal crops production (Rogers and Oldroyd, 2014). Diazotrophic free-living bacteria is known to contribute 20 kilograms per hectare per year to the long-term nitrogen needs of cereals rotation cropping system (30-50% of the total needs) (Vadakattu and Paterson, 2006). Diazotrophs are found among most of the bacterial groups like alphaproteobacteria, gammaproteobacteria, firmicutes, beta proteobacteria and cyanobacteria, but are not the most abundant (dominant) bacteria in plant rhizospheres, so there is scope for increasing nitrogen-fixation by favouring their populations in rice rhizosphere. Many rhizospheric bacteria also contribute to plant growth promotion by producing plant hormones. Indole acetic acid (IAA) producing bacteria promotes the growth and root architecture of the host plant and is also thought to be a trait required for better rhizosphere colonization and competence (De Salamone *et al.,* 2005). IAA from plant growth-promoting rhizobacteria (PGPR) can loosen cell walls of the plant, which leads to enhancing level of root exudation that provides nutrients for the growth and development of bacteria in rhizosphere (Chi *et al.*, 2005).

Phosphate-solubilizing bacteria (PSB) enhance phosphorus availability to plant which leads to improved root architecture, uptake of mineral nutrients and water from the soil (Rodriguez *et al*., 2006). Phosphate solubilizing microorganism (PSM) includes a diverse group of microorganisms including bacteria, fungi and

actinomycetes with plant growth-promoting abilities like, biological nitrogen fixation, phytohormones production, biocontrol activities etc. and bacteria hold foremost position as PSM than fungi and actinomycetes with a population of 1-50% among total soil microbial populations (Alam *et al.,* 2002). Plant growth-promoting, zinc solubilizing rhizobacteria have also been found to enhance the plant growth and development by colonizing the rhizosphere and making zinc available to the plants (Rodriguez *et al*., 2006). The aim of the present study was to isolate, identify and characterise nitrogen-fixing bacteria from different rice ecosystems.

Materials and methods

Soil samples: Rice rhizosphere soil samples were collected from farmers fields in Ranga Reddy, Vikarabad (Telangana) and Mandya districts (Karnataka) where rice was grown under irrigated and aerobic ecologies. Soil samples were collected at the rice reproductive stage (10-15 cm depth) and immediately stored in sterile polythene bags at 4°C until further analysis.

Isolation of nitrogen-fixing bacteria: Ten grams of rhizosphere soil samples were suspended in Erlenmeyer flask containing 90 ml of sterile distilled water and mixed thoroughly in an incubated shaker (120 rpm) at 28 °C for 1 hr. Appropriate serial dilutions were prepared and 0.1 ml (10^{-3} to 10^{-6} dilutions) aliquots were plated on N (Nitrogen) free Rennie's media (Atlas, 2004). The petri plates were incubated at 28 ± 2 ºC in an incubator for 3-7 days. Bacterial isolates showing visually differential growth were picked up and purified on the same media. The purified bacterial cultures were maintained on N-free Rennie's slants and stored on 50% glycerol stock at 4 ºC and -80 ºC. Sub culturing of purified bacterial cultures were carried out as and when required.

Acetylene Reduction Assay (ARA): Nitrogenase activity *i.e*. acetylene reduction assay of isolated and purified culture was determined by using Gas Chromatography (GC) method (Hardy *et al.,* 1971). Purified cultures were inoculated on Rennie's slant and incubated at 28 ± 2 ºC for 3 days in an incubator. Acetylene gas (10% V/V) was injected in the tube and incubated for 24 hrs. Appropriate uninoculated controls were maintained. After incubation, 1 ml air sample was removed from the tubes and injected into Gas chromatograph for analysis. The ethylene produced by reduction of acetylene was assayed using a Gas chromatograph (Thermo Scientific, GC1110 model) with FID detector having Porapak N column using N_2 as a carrier gas. After completion of ARA, to determine protein content, cells were suspended in 2 ml of 0.2 N NaOH, incubated for 10 min at 65 ºC, followed by neutralization using 2 ml of 0.2 N HCl. Protein concentration in resultant mixture was determined by using Bradford method (Bradford, 1976). ARA was expressed in terms of hmoles of ethylene produced per mg protein per hr. The observations were recorded in three replications per treatment.

Genomic DNA Extraction: Eight cultures with high ARA activity were selected and grown in 5 ml of N- free Rennin's broth at 28 ± 2 ºC for 24 hrs. Log phase cultures were used for isolation of total genomic DNA using CTAB /NaCl method (William *et al.,* 2012). Precipitated DNA was pelleted down by centrifuging at 8000 rpm for 10 min, washed with 70% ethanol, air-dried, dissolved in the TE buffer and stored at 4 °C for further use.

Amplification of 16S rDNA and identification of isolates: The 16S rRNA gene from the bacterial genomic DNA was amplified using FGPS6 (forward primer) 5'GGAGAGTTAGATCTTGGCTCAG3' and FGPS1509 (reverse primer) 5'AAGGAGGGGATCCAGCCGCA3 (Normand *et al.,* 1992). In a reaction mixture, 50 ng of template DNA, primer (10 pmol each), dNTP (200 µM each) and 5 U Taq DNA polymerase (Takara) were used. The final volume of the reaction mixture was adjusted to 50 µl. Amplification was carried out under standard conditions (initial denaturation at 95 ºC for 5 min, 30 cycles of denaturation at 95 ºC for 1 min, 30 cycles of annealing at 60 ºC for 45 sec, extension at 72 ºC for 1 min and a final extension at 72 ºC for 7 min). Amplified PCR product was resolved on 1.2% of agarose gel in 1xTAE buffer. The amplified product was purified by using PCR cleanup kit (Favorgen) according to manufacturer instructions. Amplified 16S rRNA gene of the selected isolates was sequenced by Sanger sequencing (Eurofins, India). The sequences were compared using BLAST search tool with already known and identified microbial databases of National Center for Biotechnology Information (NCBI) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) for identification of the isolates.

Indole acetic acid (IAA) production: Indole Acetic Acid (IAA) production ability of the identified cultures was

carried out by using the Hartmann method by growing cultures in Tryptic soya broth amended filter sterilized tryptophan (100 µg/ml broth) for 7 days at 28°C (Hartmann *et al.*, 1983). After incubation, an aliquot of the culture supernatant was mixed with equal volumes of Salkowski reagent solution and incubated for 30 minutes to allow the colour to develop before taking reading absorbance at 530 nm using Spectrophotometer (model Shimadzu, UV-1800). The IAA production by the cultures was expressed as µg IAA produced/ ml. Appropriate uninoculated controls were maintained. Three replications for each treatment were maintained and the experiment repeated twice.

Phosphorous-solubilization: The ability of the isolates to solubilize inorganic phosphate was determined by spotting 5 µl (10^8 CFU/ ml) of fresh bacterial culture on Pikovskaya media (Pikovskaya, 1948). Plates were observed for the formation of transparent halos around each bacterial colony and formation of a clear zone around the colony indicates solubilization.

Zinc solubilisation: Cultures were inoculated as spot on respective zinc medium plates for studying the zinc solubilization (Sharma *et al.*, 2012). These plates were covered with aluminium foil and incubated in dark at 28 °C for 14 days and further observed for the zinc solubilization which appears as a halo around the colony.

Potassium solubilisation: A loopful of culture of each bacteria were inoculated into 25 ml Aleksandrov medium and incubated at 28 ±2 °C for 10 days. The growth suspension was centrifuged at 8,000 rpm for 10 min to separate the supernatant from the cell growth and insoluble potassium. One ml of the supernatant was diluted to 50 ml with distilled water, mixed thoroughly and the resultant solution was used to determine K content with atomic absorption spectrometer. (Alexander, 1985).

Results and Discussion

In the present investigation, a total of 197 nitrogen-fixing bacterial cultures were isolated from the rice fields. Among the 197 isolates, eight potential bacterial cultures were selected and further processed for molecular identification (16S rRNA gene sequencing), morphological and plant growth-promoting activities.

Acetylene reduction by the promising isolates

Nitrogenase, the enzyme that is responsible for biological nitrogen fixing, reduces N_2 to NH_3. It also has the capacity of reducing acetylene to ethylene. So, measuring the quantity of ethylene that is produced in the medium provides a way of estimating nitrogenase activity. Acetylene Reducing Assay (ARA) was carried out to examine the nitrogen-fixing ability of isolated cultures through gas chromatographic (GC) analysis. Out of 197 cultures, eight cultures showed a high ARA activity ranging from 115 to 490 nmoles ethylene/h/mg protein (Table 1).

Table 1: Nitrogenase (ARA) activity of promising isolates

S. No.	Selected isolates	Acetylene reduction assay (ARA) ηM of C_2H_4 produced h^{-1} mg^{-1} protein
1	IIRR A3	193
2	IIRR C4	314
3	IIRR E3	149
4	IIRR F4	490
5	IIRR J3	140
6	IIRR J4	115
7	IIRR M4	123
8	IIRR N	410

Identification of selected isolates

The molecular characterization of eight promising isolates (based on higher ARA activity) was carried out using PCR based 16S rRNA gene amplification and Sanger sequencing. After PCR based amplification of 16S rRNA gene (1,450 bp) and Sanger sequencing a BLASTN search of the amplified 16S rRNA gene sequence database was performed by the BLAST search tool and identified based on the similarity to the closest type strain affiliated to each of the isolated strains. The 16S rRNA gene nucleotide sequences of IIRR E3 isolate was showed the high similarity (100%) to the 16S rRNA gene sequences of strain *Ochrobactrum sp*. JF313266.1, while the other cultures *viz.,* IIRR A3 (92%), IIRR C4 (82%), IIRR J3 (97.49), IIRR F4 (96.57%), IIRR J4 (97.98%), IIRR M4 (90.65%) and IIRR N (95.7%) were showed the similarity to the 16S rRNA gene nucleotide sequences with *Stenotrophomonas sp., Paenibacillus sp., Burkholderia cepacia, Paenibacillus sp.* and *Burkholderia cepacia,* respectively (Table 2). Based on the identification, highest ARA activity among the promising isolates was recorded in *Rhizobium sp.* (490 nmoles ethylene/h/mg protein) and lowest activity was seen in *Burkholderia cepacia* (115 nmoles ethylene/h/mg protein). All eight promising nitrogen-fixing cultures were stored at 4 °C and -20 °C in glycerol stock for further studies.

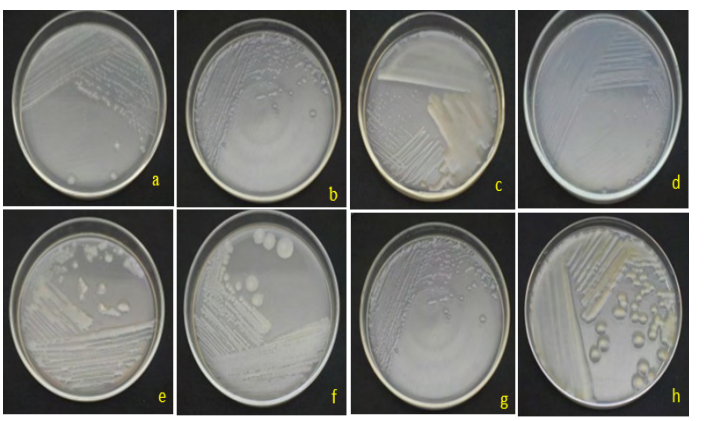
Table 2: Promising N-fixing bacteria identified from different rice ecosystems

S. No.	Selected isolates	Identified promising N-fixing bacteria
1	IIRR A3	*Stenotrophomonas sp.*
2	IIRR C4	*Paenibacillus sp.* (isolate 1)
3	IIRR E3	*Ochrobactrum sp.*
4	IIRR F4	*Paenibacillus sp.* (isolate 2)
5	IIRR J3	*Burkholderia cepacia* (isolate 1)
6	IIRR J4	*Burkholderia cepacia* (isolate 2)
7	IIRR M4	*Xanthomonas sacchari*
8	IIRR N	*Rhizobium sp.*

Our results are similar to earlier reports on the identification of nitrogen-fixing bacteria in various crops. Majeed *et al.* (2015) demonstrated that diazotrophic strains of *Stenotrophomonas sp.* exhibited multiple plant growth promotion activities and colonized the wheat rhizosphere as well as in the root interiors. They also identified five *Paenibacillus sp.* exhibiting IAA production and nitrogenase activities (*Paenibacillus sp.* SZ-10, *Paenibacillus sp.* JS-4, *Paenibacillus sp.* SZ-14, *Paenibacillus sp.* SZ-15 and *Paenibacillus sp.* BJ-4), which significantly enhanced the growth and dry weight of wheat (Liu *et al.* 2019). Also, *Paenibacillus polymyxa* M1 isolated from root tissues of wheat was reported to promote plant growth (Yao *et al.*, 2008; Niu *et al.*, 2011). In relation with our results, *Ochrobactrum sp.* has been earlier reported in some legumes showed the complete symbiotic relationship and also establishing the biological nitrogen fixation with host plant (Trujillo *et al.*, 2005, Meng *et al.*, 2014). Dobereiner (1997) identified microaerophilic bacteria *viz. Burkholderia sp.* that colonize shoots, leaves and roots of rice, wheat and maize leading to enhanced growth.

Morphological characterization

The identified eight bacterial cultures were characterized morphologically based on their size (which varied from very small to small, moderate and large) and apparent differences in slime production. Among these, four bacterial cultures (*Stenotrophomonas sp.*, *Paenibacillus sp.* isolate 1, *Xanthomonas sacchari* and *Rhizobium sp.*) were fast growers and the other four cultures (*Ochrobactrum sp.*, *Paenibacillus sp.* isolate 2, *Burkholderia cepacia.* isolate 1 and *Burkholderia cepacia.* isolate 2) showed slow growth on N-free medium. Pigmentation of colonies was less and varied from non-pigmentation, to creamy yellow (*Xanthomonas sacchari*) pigmentation. (Figure 1, Table 3*)*.



a- *Stenotrophomonas* sp, b- *Paenibacillus* sp, c- *Burkholderia cepacia*, d- *Burkholderia cepacia*, e- *Onchrobactrum* sp, f- *Rhizobium* sp, g- *Paenibacillus* sp, h- *Xanthomonas sacchari*

Figure 1: Colony morphology of identified eight promising nitrogen-fixing bacteria

Table 3: Morphological characterization of identified nitrogen-fixing bacteria

S. No.	Selected isolates	Growth	Size	Slime Production	Pigmentation
1	*Stenotrophomonas sp.*	Active	Very small	++	Creamy - Non-pigmentation
2	*Paenibacillus sp.* (isolate 1)	Active	Small	++	Creamy - Non-pigmentation
3	*Ochrobactrum sp.*	Slow	Small	+	Creamy- Yellow Pigmentation
4	*Paenibacillus sp.* (isolate 2)	Slow	Moderate	++	Non-pigmentation
5	*Burkholderia cepacia* (isolate 1)	Slow	Large	+	Creamy Non-pigmentation
6	*Burkholderia cepacia* (isolate 2)	Slow	Large	++	Cream - Non-pigmentation
7	*Xanthomonas sacchari*	Active	Moderate	++++	Cream - Non-pigmentation
8	*Rhizobium sp.*	Active	Large	+++	Yellow - Pigmentation

Media: NA - Nutrient Agar (NA) and Rennie, ++++ More slime production, + Less slime production

Characterization for Indole acetic acid (IAA) production and Mineral solubilization

The eight isolates were further tested for their ability to produce IAA and to solubilize insoluble potassium, phosphorous and zinc solubilization. All eight isolates grown in the minimal medium supplemented with tryptophan exhibited considerable production of IAA with amounts ranging from 11.90 to 47.40 µg/ml. Among these, *Orthobacterium sp*. showed the greatest IAA production (47.40 µg/ml) and *Burkholderia cepacia* had lower IAA production of 11.90 µg/ml. (Table 4). In addition to the nitrogen-fixing ability of isolates, the two isolates *viz. Ochrobacterum sp*. and *Xanthomonas sacchari* displayed solubilization of phosphate and zinc. None of the isolates demonstrated potassium solubilizing activity (Table 4).

Table 4: IAA production and mineral solubilizing capacity of promising isolates

S. No.	Selected isolates	Indole acetic acid (IAA) assay	Insoluble minerals solubilization assay		
		(µg/ml)	Potassium	Phosphorous	Zinc
1	*Stenotrophomonas sp.*	28.70	-	-	-
2	*Paenibacillus sp.* (isolate 1)	15.60	-	-	-
3	*Ochrobactrum sp.*	47.40	-	+	+
4	*Paenibacillus sp.* (isolate 2)	30.75	-	-	-
5	*Burkholderia cepacia* (isolate 1)	14.80	-	-	-
6	*Burkholderia cepacia* (isolate 2)	11.90	-	-	-
7	*Xanthomonas sacchari*	38.50	-	+	+
8	*Rhizobium sp.*	19.91	-	-	-

+ (Positive) - Showing solubilization, - (Negative) - Not showing solubilization

Leinhos (1994) reported bacterial biosynthesis of IAA in many rhizobacteria and it has been assumed that approximately 80% of rhizospheric bacteria can secrete IAA. However, Joseph *et al.* (2007) reported that the ability of bacteria to produce IAA in the rhizosphere depends on the availability of precursors and uptake of microbial IAA by the plant. In the present study, all the eight promising N-fixing bacteria are capable of producing the IAA similar to earlier studied reports (Reetha *et al*., 2014, Oteino *et al*., 2015). The capacity to produce IAA is widespread among soil plant growth-promoting bacteria such as *Azospirillum sp., Enterobacter cloacae* and *Klebsiella sp.* (Spaepen *et al*., 2007). Furthermore, IAA production has been reported in *P. stutzeri* strain A15 group of bacteria (Mehnaz *et al*., 2009) wherein the strain A15 was re-isolated from inoculated rice roots (Lalucat *et al*., 2006). Also, *Pseudomonas sp*. have been reported to enhance the growth of onion crop (Reetha *et al*., 2014, Oteino *et al*., 2015). *Pseudomonas fluorescens* has shown indole acetic acid (IAA) production resulting in enhanced growth of onion as well as phosphate solubilization (Reetha *et al*., 2014, Oteino *et al*., 2015).

Phosphate solubilizing bacteria (PSB) provides available form of phosphorus (P) to the plants and is hence a viable substitute to chemical phosphatic fertilizers (Khan *et al*., 2009). Our results are similar to the previous reports on phosphate solubilizing bacterial genera *viz. Azotobacter, Bacillus, Burkholderia, Erwinia, Enterobacter, Pseudomonas* and *Rhizobium* (Bhattacharyya and Jha, 2012). Current results in relation to phosphate solubilization also correlate with earlier studies on plant growth-promoting abilities of *Enterobacter* species (Ahemad and Khan, 2010, Khalifa *et al*., 2016). *Enterobacter asburiae* has shown phosphate solubilization, IAA and ammonia as well as siderophore production (Ahemad and Khan, 2010). Similarly, *Rhizobium sp.* and *E. cloacae* have been found to possess phosphate solubilization and ability for phytohormone production like acetoin, which leads to significant growth and development of the pea (*Pisum sativum*) (Khalifa *et al*., 2016).

Our results on zinc solubilization are correlated with the earlier reports on identified zinc solubilizing bacterial genera belonging to *Pseudomonas, Bacillus, Enterobacter, Xanthomonas, Stenotrophomonas* and *Acinetobacter* (Saravanan *et al.*, 2003, Gandhi and Muralidharan 2016; Sunithakumari *et al.*, 2016). Zinc solubilizing ability has been proven for *Bacillus sp*. and *Pseudomonas sp*. which was observed as production of visible halo zones on the zinc oxide-amended media (Saravanan *et al.*, 2003).

In summary, from our investigation, we have isolated and identified eight promising N-fixing bacteria *viz. Stenotrophomonas sp.* (IIRR A3), *Paenibacillus sp.* (isolate 1) (IIRR C4), *Burkholderia cepacian.* (isolate 1) (IIRR J3), *Burkholderia cepacian* (isolate 2) (IIRR J4), *Ochrobactrum sp.* (IIRR E3), *Paenibacillus sp.* (isolate 2) (IIRR F4), *Xanthomonas sacchari* (IIRR M4) and *Rhizobium sp*. (IIRR N) with additional plant growth promoting traits with a potential for enhancing rice plant growth.

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