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Enumeration, Isolation and Identification of Nitrogen-Fixing Bacterial Strains at Seedling Stage in Rhizosphere of Rice Grown in Non-Calcareous Grey Flood Plain Soil of Bangladesh

Md. Harunor Rashid KHAN*, Md. MOHIUDDIN** and M. RAHMAN*

Non-symbiotic diazotrophic systems for biological nitrogen fixation (BNF) in agriculture are most promising but the possibility for the extension of nitrogen fixation by rice is still speculative. Accordingly, the present study was conducted for the Enumeration, isolation and identification of nitrogen fixing bacterial strains at seedling stage (30 days after seed sowing) in rhizosphere of rice (BR 10, *Oryza sativa* L.) grown in Non-Calcareous Grey Flood Plain soil of Bangladesh. The soil is classified as 'Inceptisol' order and 'Aquept' suborder. It was identified as 'Dhamrai series', had 'silt' texture, pH 7.1 and 5.5 C/N ratio. The present results of the microbial tests on the rice rhizosphere soil evinced that out of 263 isolates, only 91 were branded as nitrogen fixing organisms per gram of soil, which was about 34.6 % of the total isolates. As per selection criteria, four individual strains were considered for identification. Biochemical tests were conducted for proper identification and the selected strains were identified as *Enterobacter spp., Klebsiella spp., Bacillus spp.* and *Azospirllum spp.*

Keywords: *Azospirllum spp., Bacillus spp., diazotrophs, Enterobacter Spp., Klebsiella spp.* and *Oryza sativa*.

1 INTRODUCTION

The source of soil nitrogen is the atmosphere where nitrogen gas occupies about 79% of the total atmospheric gases. Living organisms that are present in the soil have profound effect on transformation, which provides food and fiber for an expanding world population. Although nitrogen is very abundant in nature, it often limits plant productivity because atmospheric nitrogen is only available to a very range of organisms symbiotically associates with higher plants and non-symbiotically. About 386 x 10^{16} kg nitrogen contains in the Earth's atmosphere (Stevensen, 1986). He stated that nitrogen returned to the earth every year through microbiologically is of the order of 139 x 10^{9} kg of which about 65% (89 x 10^{9} kg) contributed by nodulated legumes. Biological fixation of the atmospheric nitrogen can be estimated at about 175 million metric tons per year or about 70% of all nitrogen fixed on the Earth per year, the remaining is by some micro-organisms, autotrophs or heterotrophs 'free' fixers. According to Ishizuka (1992), total world biological nitrogen fixation is 17.2 x 10^{7} tons per year. This figure is three times of those of industrially or other ways of nitrogen fixation. The world population increased day by day but the expansion of land is limited. Moreover, almost half of the world's population is consuming rice (*Oryza sativa* L.) as the primary food grain, making it the most important food crop currently produced (Cottyn, et al. 2001; Klipp, et al. 2004). Hence for the higher yield of rice for over population, people of the world use expensive nitrogenous fertilizers. These are used to fulfill the nitrogen demand of rice that can be overcome partially by using biofertilizers when they are scientifically applied. Biofertilizer is important in crop farming systems because it is an inexpensive source of nitrogen for the higher yields of crops. This process diminishes the need for expensive chemical fertilizer. Thus the extensive use of biofertilizers would provide economic benefits to farmers, improve the socio-economic condition of people and preserve natural resources.

Now days, scientists of the world in the field of agriculture are very much concerned about the fixation of atmospheric nitrogen associated with rice. In Bangladesh rice is the main staple cereal crop. It is the basic nutrition of the inhabitants of this country. Rice covers 80% of the total cropped areas and is the main source of cash income for farmers.

Sen (1992) suggested that the heterotrophic bacteria associated to the root system of rice could contribute efficiently to the nitrogen fixation. Youshida (1972) in the Philippines and Balandreau and Dommerques (1978) in the Ivory Coast demonstrated that BNF supplies a part of the nitrogen necessary for the growth of rice. Statistically significant grain yield increases due to *Azospirillum* inoculation have been reported from India and Israel (Dobereiner, 1981 and 1988). But most of the cases, these trials have been done using bacterial strains from international collections. Using an axenic rice

*Department of Soil, Water and Environment, University of Dhaka, Dhaka 1000, Bangladesh. Present address: Department of Environmental Management Engineering, Faculty of Environmental Science and Technology, Okayama University.
**Department of Soil Science, Govt. Barisal College, Barisal, Bangladesh.

plant lets as a selective medium, a collection of 23 N_2 fixing bacterial strains were isolated from the rhizosphere of rice cultivated in the Brahmaputra alluviam soil tract of Bangladesh. The aim of the present research was to characterize and identify nitrogen-fixing organisms at the seedling stage of rice rhizosphere soil in order to reduce the use of chemical nitrogen fertilizers by the production of biofertilizer.

2 MATERIALS AND METHODS

2.1 Soil Sampling

Soil at a depth of 0 to 15 cm was sampled during October from Dautia Bil at Dhamrai under the district of Dhaka at the seedling stage (30 days after seed sowing) of rice (BR 10, *Oryza sativa* L.). Six samples of rhizosphere soil were collected from an area of 100 m^2. According to 1981 reviewed Reconnaissance Soil Survey report of Dhaka district, the studied site is identified as Non-Calcareous Grey Flood Plain soil as per the general soil type of Bangladesh and the collected soil is representing Dhamrai series. The soil responded well when Aus and Boro-aman were cultivated. The field was used to receive N-fertilizers at the rate of 60 kg $ha^{-1}yr.^{-1}$. In the winter season Rabi crops are cultivated. The soil was deeply flooded (about1.5 to 2.5 m) during rainy season. Selected morphological and physico-chemical properties of the studied soil are presented in **Tables 1 and 2**, respectively.

2.2 Dilution of Soil Samples

The sampled rhizosphere soil was mixed thoroughly to make a composite soil. Then 10 gm of sub-soil sample diluted to 100 ml that considered being 10^{-1} dilution factor. Transferring of 1 ml of 10^{-1} dilution to 9 ml sterilized water with the help of a sterilized pipettes yielded 10^{-2} dilution. In this way, a series of up to 10^{-8} dilution was prepared under aseptic condition. Screw cap test tubes and glass petridishes were used to culture microorganisms. Sterility is the hallmark for successful works in the microbiological studies were also kept in mind throughout the study.

2.3 Bacterial counts

The calculation for the total numbers of bacteria was done by plating soil dilutions on nutrient agar and total numbers of N_2 fixing bacteria were counted by plating soil dilutions on nitrogen free medium –RCV media. One ml of the suspension from each (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}) was taken and poured into the nutrient agar media and the nitrogen free media on petridish separately. Then incubated the plates at 30˚C for 48 hours. The total count of the microorganisms was obtained by multiplying the number of cells per plate by the dilution factor, which was the reciprocal of the dilution.

2.4 Isolation of pure culture

Discrete well-developed and separated colonies on the surface of a nutrient medium plate culture were each picked up with a sterile niddle and transferred separately in RCV medium slant. Each of these new slant cultures represents the growth of a single bacterial species. The colonies, which are different in appearances and characters were picked and purified.

2.5 Preparation for microscopic examination

The strains were studied following the methods of Cerney (1993) including morphological and physiological features. Gram staining was used for the study of the bacterial morphology. Bacteria were grown on nutrient slants for overnight incubation at 30±0.5˚C. A portion of the bacterial culture was taken out by a sterile loop and was suspended in sterile normal saline. The suspension was sufficiently diluted. A drop of suspension was taken on the slide and was spread evenly covering an area of about 15-20 mm diameter. The slide was then kept in a safe place to air-dry. The smear was fixed by rapidly passing the dry slide; smear uppermost three times through the flame of a Bunsen burner. After passing the slide through the flame, it should be possible to lay the slide on the back of the hand without the hand feeling uncomfortably hot. The slide was allowed to cool before staining.

2.6 Identification of gram stain

A dried fixed smear was covered with oxalate crystal violet reagent for sixty seconds. The strain was then rapidly washed off with clean water. All the water was then tipped off and the smear was covered with Lugol's iodine for sixty seconds. The iodine was then washed off with clean water. The smear was then decolorized rapidly for 10 seconds with acetone alcohol and was washed immediately with clear water. Finally, the smear was covered with safranine for 30 seconds. The slide was then washed thoroughly in water and blotted dry. The smear was examined under microscope by using high power (5 x 1000) immersion oil objective. The gram-negative organisms were stained pink and the gram positives were dark violet in color.

2.7 Physiological studies of the selected strains

The physiological activities of the selected strains were tested through oxidase, catalase motility indole urease (MIU), methyl red (MR), aceton production (Voges-Proskauer), nitrate reduction, citrate utilization, hydrogen sulfide (H_2S) production, gelation liquification and carbohydrate fermentation methods as demonstrated by Collee and Miles (1989).

2.8 Maintenance of culture

Stock cultures were maintained in soft agar (0.7% agar in nutrient broth, Difco Lab, Detroit) stab on one-dram airtight screw capped tubes, stored at 4 to 8˚C. The working cultures were maintained on Trypticase Soyagar (TSA) slant in one dram airtight screw capped (150 x 16 mm) test tubes and stored at 4 to 8˚C. Isolated colonies were then streaked on TSA slant in 5 mm screw capped test tubes and incubated overnight at 37±0.5˚C. The TSA slant cultures were stored at 4 to 8˚C and were used as working culture. For routine culture or routine use, culture was transferred from TSA slant on TSA plate.

Table 1 Selected morphological characteristics of the soil used in the experiment.

Parameters	Description
1. Location	1. Mouga and Vill.: Dautia Bil; Union and P.S.: Dhamrai; Dist.: Dhaka.
2. Soil Series	2. Dhamrai series
3. General Soil Type	3. Non-Calcareous Grey Flood Plain Soil
4. FAO-UNESCO System	4. Areni Euteric Gleysols
5. USDA Siol Taxonomy	5. Subgroup: Typic Halaquepts; Greatgroug: Halaquepts; Suborder: Aquept; Order: Inceptisol.
6. Topography	6. Low land
7. Present land use	7. Mainly mixed Aus, Aman, Jute and Rabi crops
8. Soil Color	8. Grey

Table 2 Selected physical and chemical properties of the soil used in the experiment.

Physical properties	Values	Chemical properties	Values
Soil sampling depth	0-15 cm	pH	7.1
Maximum water holding capacity	49 %	EC (1 : 5)	0.02 mS cm^{-1}
Particle sizes:		Organic carbon	10.5 g kg^{-1}
Sand	15 %	Organic matter	18.1 g kg^{-1}
Silt	59 %	C/N ratio	5.5
Clay	26%	Total Nitrogen	1.9 g kg^{-1}
Textural Class	Silt	Available Nitrogen	25 mg kg^{-1}

The plates were incubated overnights at 37±0.5˚C to yield 10 g phase culture.

2.9 Media and Reagent

The RCV and complete nutrient media were used for the present study in order to enumeration, isolation and identification of bacterial strains.

The RCV media: A carbon and nitrogen free media, which was adjusted for the culture of Rhodopseudomonas capsulate. The composition of this media is **Elements solution** ($ZnSO_4$ $7H_2O$ 430 mg, $MnSO_4$ H_2O 1.30 g, Na_2MoO_4 $2H_2O$ 750 mg, H_3BO_3 2.80 g, $CuSO_4$ 26 mg, $CoSO_4$ $2H_2O$ 70 mg and distilled water 1 L), **Super salt solution** (Elements solution 20 ml, EDTA 400 mg, $MgSO_4$ $7H_2O$ 2.00 g, $FeSO_4$ $7H_2O$ 440 mg, $CaCl_2$ $2H_2O$ 200 mg and distilled water 1 L), and **Phosphate buffer** (KH_2PO_4 40 mg, K_2HPO_4 60 mg and distilled water 1 L). The super salt solution and phosphate buffer were sterilized separately and then they were carefully mixed after cooling in order to avoid flocculation. Carbon source (yeast extract 0.10 g L^{-1}, Malic acid 3.5 g L^{-1}) was added in the super salt solution and adjusted to pH 6.8 with 1*N* KOH. The final solution media was a mixture of super salt solution 50 ml, phosphate buffer 15 ml and distilled water 1 L. The complete media for the development of bacterial strain were Luria Bertani Broth (LB), Nutrient agar (NA), Nutrient Broth (NB), Gelation agar (AG), MR-VP medium, Nitrate reduction medium (NR), Simmons citrate medium, TSA+0.6% yeast extract, starch agar, Tributyrin agar, milk agar and TSA agar.

The chemicals were Kovac's reagent, mercuric chloride solution, methyl red indicator, NR test reagent, Phosphate buffer saline, VP reagent, ammonium oxalate crystal violet solution, Lugol's iodine solution and safranine solution.

3 RESULTS AND DISCUSSION

The studied soil was classified as 'Inceptisol' order and 'Aquept' suborder on the basis of the USAD Soil Taxonomy, 1999. The general soil type of the studied area was Non-Calcareous Grey Flood Plain Soil. It was identified as 'Dhamrai series', had 'silt' texture, pH 7.1 and 5.5 C/N ratio (**Tables 1 and 2**). The enumeration of the existing total biomass and only nitrogen-fixing bacteria were done through serial dilution agar plating technique. Molten agar at 45˚C was poured into a petridish. One ml of the suspension was putted onto a petridish from the each fold of dilution. Then the plate was gently rotated in a circular motion to achieve uniform distribution. This procedure was repeated for all dilutions to be plated. Dilutions were plated in duplicate for greater accuracy, incubated overnight before colonies develop and counted by naked eye. The total counts of the suspension are obtained by multiplying the number of cells per plate by the dilution factor, which is the reciprocal of the dilution. It was observed that 263 microorganisms was randomly counted from incubated plated of solid complete nutrient agar media and 91 organisms were picked up in the same manner from the N-free medium (RCV) plates and the results are presented in **Table 3**.

The present results have close conformity with findings of Torres, et al. (2000). They obtained *Azotobacter Chroococcum*, *Azotobacter vinelandii*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* strains from rhizosphere of rice cultivated in the Tolima region, Colombia S.A. Thomas-Bauzon and Balandreau (1982) isolated N-fixing bacteria with a frequency of 65%, 32 of the many isolates were *Klebsiella spp.*, *Enterobacter spp.*, *Pseudomonas spp.* and *Azospirillum.*

Table 3 Enumeration of total biomass and N-Fixing bacteria.

Dilution Factor	Total Number	No. of N-Fixer	% of N-Fixer
10^{-1}	120	30	34.6 %
10^{-2}	58	21	
10^{-3}	42	18	
10^{-4}	29	14	
10^{-5}	08	06	
10^{-6}	03	02	
10^{-7}	02	0	
10^{-8}	01	0	
Grant Total:	263	91	34.6 %

Rennic and Vose (1983) used single nitrogen free medium for isolating nitrogen-fixing bacteria and showed that at the higher dilutions 75% of the isolates exhibited acetylene reduction. They also showed that *Erwinia herbicola* comprised 50% of the total population, which 13 *Polymixa* and *K. Pneumoniae* accounted for 33% and 17% exist in the rhizosphere of rice, respectively. But the present results showed that only 91 microorganisms of the total of 263 were nitrogen fixing organisms which was about 34.6% of the total isolates per gram of the rhizosphere soil (**Table 3**). Watanabe and Baraqui (1979) revealed that nitrogen-fixing bacteria are present in greater number in the root of wetland rice. *Azospirillum spp.* were found in large numbers were associated with rice. Yoshida (1972) also reported that biological nitrogen fixation in Philippine rice fields were ranged from 2.30 to 33.3 kg ha^{-1}. Sanoria and Maurya (1982) also reported that significantly much higher yields of grain and straw of rice by inoculation *Azospirillum* compared with control. The present results have proved the above-mentioned facts and also have similarities with the ways of investigation and findings as reported by Mukhopadhyay, et al. (1996) and Stoltzfus, et al. (1997).

Among the 91 nitrogen fixers (34.6%), only 4 types of strains (strain-1, strain-2, strain-3 and strain-4) were selected for the identification on the basis of their colonies appearances on NA media and on RCV media. The appearances of the colonies of strains (strain-1, strain-2, strain-3 and strain-4) on the nutrient agar (NA) plate were circular, flat, raised and convex in elevation; small and pinpoint in size; non-pigment, yellow, grey, off white in color, respectively. On the other hand, the colonies appearances on RCV petridish were white, off white, grey and grey to white in color; circular, flat, raised, serrate in elevation; small and pinpoint in size, which are presented in **Table 4.**

All the selected strains were identified as gram-negative rods and mostly true motile. All the strains were oxidase positive, indole positive, starch and lipid hydrolysis positive, but did not produce H_2S in KIA media, catalase positive except strain-2, methyl red positive (strain-2, strain-3) and methyl red negative (strain-1, Strain-4), Voges-Proskauer (acetone production) positive (strain-1, strain-4) and negative (strain-2, strain-3), only strain-2 was found to be reduced nitrite to nitrate, did not utilize citrate or liquefication. The results obtained from the biochemical and carbohydrate fermentation tests are stated in **Tables 5 and 6**, respectively. According to Bargey's Manual of Systemic Bacteriology, the above (**Tables 5 and 6**) biochemical and carbohydrate fermentation tests indicated that the characters represented by the strain-1 is similar to *Enterobacter spp.*, strain-2 is as *Klebsiella spp.*, strain-3 is as *Bacillus spp.* and strain-4 is as *Azospirillum spp*.

4 CONCLUSIONS

The 34.6% of the total isolates was identified as nitrogen fixing microorganisms during the seedling (30 days after seed sowing) stage of rice (BR 10) rhizosphere soil. Based on the selection criteria, the four individual strains were microbiologically identified. Their biochemical tests were strictly similar to *Enterobacter spp.*, for strain-1, *Klebsiella spp.* for strain-2, *Bacillus spp.* for strain-3 and *Azospirillum spp.* for strain-4. They were anaerobic in nature.

Table 4 Colony appearance on NA media and RCV media.

Features	#NA media				*RCV media			
	Appearances of the strains				Appearances of the strains			
	Strain-1	Strain-2	Strain-3	Strain-4	Strain-1	Strain-2	Strain-3	Strain-4
Size	Small	Pinpoint	Small	Pinpoint	Small	Pinpoint	Small	Pinpoint
Elevation	Circular	Flat	Raised	Convex	Circular	Flat	Raised	Serrate
Pigmentation	Non. pig.	Yellow	Grey	Off-white	White	Off-White	Grey	White-Grey

#NA = Nutrient agar; *RCV = A carbon and nitrogen free media which was adjusted for the culture of Rhodopseudomonous capsulate.

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Table 5 Selected biochemical tests of unknown strains after 48 hours of incubation at 37±0.5ºC.

Name of the Tests	Response of strain			
	Strain-1	Strain-2	Strain-3	Strain-4
Gram strain	-	-	-	-
H_2S production	-	-	-	-
NO_3 production	-	+	-	-
Indole production	+	+	+	+
Methyl Red reaction	-	+	+	-
Voges-Proskauer reaction	+	-	-	+
Citrate Utilization	-	-	-	-
Urease activity	+	+	+	-
Catalase	+	-	+	+
Oxidase	+	+	+	+
Gelatin liquefication	-	-	-	-
Starch hydrolysis	+	+	+	+
Liquid hydrolysis	+	+	+	+
Motility	++	++	++	++

Table 6 Selected carbohydrate fermentation tests for the studied strains.

Carbohydrate tests	Response of strain			
	Strain-1	Strain-2	Strain-3	Strain-4
Glucose	+	+	+	+
Lactose	+	+	+	+
Sucrose	+	+	+	-
Maltose	+	-	+	-
Mannose	+	+	-	+
Mannitol	-	-	+	-
Inositol	-	+	-	+

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