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Characterization and Screening of N_2-fixing Microorganisms at Maturity Stage in Rhizosphere of Rice Grown in Brahmaputra Alluvium Soil of Bangladesh

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Studies on the existing non-symbiotic diazotrophic systems still are the most promising for better use of biological nitrogen fixation (BNF) in agriculture. The possibilities for the extension of nitrogen fixation to rice plants still speculative. The prospect of extension of N_2-fixation to other plants was originally formulated to simulate the possibilities for the biological use of atmospheric nitrogen in order to overcome the ecological and economical problems of nitrogenous fertilizers. In view of this, the present study was conducted for the characterization and identification of N_2-fixing bacterial strains at the maturity (110 days) stage in rhizosphere of rice (BR 10, *Oryza sativa* L.) grown in Brahmaputra Alluvium soil of Bangladesh. The soil is characterized as 'Inceptisol' order and 'Aquept' suborder. It was identified as 'Dhamrai series', had 'silt' texture, pH 6.0 and 6.8 C/N ratio. The present results of the microbial tests on the rice rhizosphere soil demonstrated that out of 401 isolates, only 94 were branded as nitrogen fixing organisms per gram of soil, which is about 23.4 % of the total isolates. Based on the selection criteria, four individual strains were selected for identification. Biochemical tests were conducted for proper identification. They were identified as *Closteridium spp., Klebsiella spp., Bacillus spp.* and *Azospirllum spp.*

Key words: *Azospirllum spp.*, *Bacillus spp.*, BNF, *Closteridium spp.*, Diazotrophs, *Klebsiella spp.* and *Oryza sativa* L.

1 NTRODUCTION

Nitrogen fertilizer rank first among the external inputs to maximize output in agriculture. Its use has been rising to increase crop yields. The continued and unabated use of nitrogen fertilizers would further accelerate depletion of non-renewable energy resources used in fertilizer production and contribute substantially to environmental pollution. Recently, all over the world, diminishing of input into agriculture without output decreases and more efficient use of resources have been demanded for sustaining environment and agricultural productivity.

Free living N_2 fixing bacteria of the genus *Azospirillum* are associated with roots of many tropical grasses and cereal crops and have beneficial effects on crop yield (Neyra and Dobereiner, 1977; Torres *et al.*, 2000 and Klipp *et al.*, 2004). Nitrogen fixation is the reduction of atmospheric molecular nitrogen to ammonia and other biologically essential nitrogen containing compounds. Nitrogen input through BNF can help to substitute for nitrogen fertilizer for obtaining better crop production.

The first nitrogen-fixing microbe as discovered was *Clostridium Pasteurium*, obtained by a famous microbiologist S. Winogradsky in Paris in 1893. 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). 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.

Sen (1992) suggested that the heterotrophic bacteria associated to the root system of rice could contribute efficiently to the nitrogen fixation. Yoshida (1972) in the Philippines, Riando and Dommerques (1971); 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

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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 axenic rice plantlets as a selective medium, a collection of 23 N_2 fixing bacterial strains was isolated from the rhizosphere of rice cultivated in the Brahmaputra alluvium soil tract of Bangladesh. The aim of the study was to enumeration, isolation and identification of nitrogen fixing organisms at the maturity stage of rice rhizosphere soil in order to diminish the use of chemical nitrogen fertilizers by production of biofertilizers.

2 MATERIALS AND METHODS

2.1 Soil Sampling

The topsoils at a depth of 0 to 15 cm were sampled during December from Dautia Bil at Dhamrai under the district of Dhaka at the harvesting time of rice (BR 10, *Oryza sativa* L.) at maturity (110 days after transplantation) stage. 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, this area is included in the Brahmaputra alluvium soil tract 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 (about 1.5 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 collected rhizosphere soil sample 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

Total numbers of bacteria were calculated 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

When discrete well-separated colonies developed on the surface of a nutrient medium plate culture, 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 by conventional of Cerney (1993) including morphological and physiological features. Gram staining was used for the study of the bacterial

Table 1 Selected morphological characteristics of the soil

Parameters	Description
1. Location	1. Mouga and Vill.: Dautia Bil; Union and P.S.: Dhamrai; Dist.: Dhaka.
2. Soil Series	2. Dhamrai series
3. Soil Tract	3. Brahmaputra Alluvium Soil
4. General Soil Type	4. Non Calcareous Grey Flood Plain Soil
5. FAO-UNESCO System	5. Areni Euteric Gleysols
6. USDA Siol Taxonomy	6. Subgroup: Typic Halaquepts; Greatgroug: Halaquepts; Suborder: Aquept; Order: Inceptisol.
7. Topography	7. Low land
8. Present land use	8. Mainly mixed Aus, Aman, Jute and Rabi crops
9. Soil Color	9. Grey

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

Physical properties	Values	Chemical properties	Values
Soil sampling depth	0-15 cm	pH	6.0
Maximum water holding		EC (1 : 5)	0.02 mS cm^{-1}
capacity	47.2 %	Organic carbon	1.02 %
Particle sizes:		Organic matter	1.75 %
Sand	16 %	C/N ratio	6.8
Silt	63 %	Total Nitrogen	0.15 %
Clay	21%	Available Nitrogen	20 mg kg^{-1}
Textural Class	Silt		

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

To detect the physiological activities of the selected strains 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 tests were conducted following 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 air-tight screw capped tubes, stored at 4 to 8°C. The working cultures were maintained on Trypticase Soyagar (TSA) slant in one dram air-tight 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. The plates were incubated overnights at 37±0.5°C to yield 10 g phase culture.

2.9 Media and Reagent

In this study, the RCV and complete nutrient media were used for the 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 (GA), MR-VP medium, Nitrate reduction medium (NR), Simmons citrate medium, TSA+0.6% yeast extract, starch agar, Tributyrin agar, milk agar and TSA agar.

Reagents 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 characterized as 'Inceptisol' order and 'Aquept' suborder on the basis of the USAD Soil Taxonomy, 1999. Among the seven soil tracts in Bangladesh, the studied soil was in the Brahmaputra Alluvium tract. It was identified as 'Dhamrai series' had 'silt' texture, pH 6.0 and 6.8 C/N ratio (Tables 1 and 2). The serial dilution agar plating technique was used for the enumeration of the existing total biomass and only nitrogen-fixing bacteria. Molten agar at 45°C was poured into a petridish. From the each dilution factor putting one ml suspension into a petridish. 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 found that 401 microorganisms was randomly counted from incubated plated of solid complete nutrient agar media and 94 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*

Table 3 Enumeration of total biomass and N-fixing bacteria

Dilution Factor	Total Number	No. of N-fixer	% of N-fixer
10^{-1}	100	25	
10^{-2}	80	21	
10^{-3}	78	19	
10^{-4}	48	15	23.4 %
10^{-5}	40	09	
10^{-6}	30	03	
10^{-7}	20	02	
10^{-8}	05	0	
Grant Total:	401	94	23.4 %

spp., *Pseudomonas spp.* and *Azospirillum.* Rennic and Vose (1983) used a 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 study showed that only 94 microorganisms of the total of 401 were nitrogen fixing organisms which was about 23.6% of the total isolates per gram of the rhizosphere soil (Table 3).

Watanabe and Baraqui (1979) suggested that nitrogen-fixing bacteria are present in greater number in the root of wetland rice. *Azospirillum spp.* were found in large numbers, which 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 showed that significantly much higher yields of grain and straw of rice by inoculation *Azospirillum* compared with control. The findings of the experiment have proved the above mentioned facts. The present results also have similarities with the ways of investigation and findings as reported by Mukhopadhyay et al. (1996) and Stoltzfus *et al.* (1997).

From the 94 nitrogen fixers (23.6%), 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; white, yellow, grey, off white in color, respectively. On the other hand, the colonies appearances on RCV petridish were nonpigment, off white, grey and grey to white in color; flat, smooth, circular, serrate in elevation; pinpoint and small in size, which are presented in table 4.

The strain-3 was identified as gram positive and the rest strains were gram negative rods and mostly true motile. All the strains were oxidase positive, indole positive, starch and lipid hydrolysis positive, produce H_2S in KIA media, catalase positive, methyl red positive (strain-2, strain-3) and catalase and methyl red negative (strain-1, Strain-4), Voges-Proskauer (acetone production) positive (strain-1, strain-4) and negative (strain-2, strain-3), reduced nitrite to nitrate (strain-2) and did not reduce nitrite to nitrate (strain-1, strain-3, strain-4), did not utilize citrate or liquefication. The results of biochemical and carbohydrate fermentation test are portrayed 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 *Closteridium spp.*, strain-2 is as *Klebsiella spp.*, strain-3 is as *Bacillus spp.* and strain-4 is as *Azospirillum spp.*

4 CONCLUSION

The present study concluded that a significant number of nitrogen fixing organisms (23.6% of total) exists in soil at the time of harvesting the rice (BR 10) at maturity (110 days after transplantation). Depending on the selection criteria, the four individual strains were microbiologically identified. Their biochemical tests were strictly similar to *Closteridium spp.* for strain-1, *Klebsiella spp.* for strain-2, *Bacillus spp.* for strain-3 and *Azospirillum spp.* for strain-4.

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	Flat	Smooth	Circular	Serrate
Pigmentation	White	Yellow	Grey	Off-white	Non-pigm.	Off-White	Grey	White-Grey

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

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

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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