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# Identification and Enumeration of Bacterial Species From Rice Rhizosphere to Determine the Ratio of Nitrogen Fixers on Agricultural Aspect

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***Abstract:*** *Soil sample was collected from agricultural land of Chuadanga district, Bangladesh for Microbial Characterization and Analysis of $N_2$-Fixing bacterial species. The process was initiated with rice rhizosphere during seedling (after 30 days) of rice (BR 28, Oryza sativa L.). N-fertilizers mixing ration at that soil was 60kgha-1yr.-1. The soil was usually cultivated by Aus, Aman and Boro crops, as well as Rabi crops in winter. 1500 grams of crushed wet soil samples were collected. Drying process was initiated under 65°C for four days. 1227 grams samples were found after drying process which was then separated into 4 equal parts for post processing. Total 188 isolates were found in which only 65 were nitrogen fixer, in a ratio of 34.58%. Isolated nitrogen fixer were observed physiologically and biochemically and tested with a variety of enzymatic reaction, four species were found based on study criteria- Enterobacter spp., for strain-1, Klebsiella spp. for strain-2, Bacillus spp. for strain- 3 and Azospirillum spp. for strain-4.*

***Keywords:*** *Enterobacter spp., Klebsiella spp., Bacillus spp., Azospirllum spp.,Rhizosphere, Bangladesh*

## I. Introduction

Nitrogen fixation is a process in which nitrogen ($N_2$) in the atmosphere is converted into ammonium ($NH_4$+) or nitrogen dioxide ($NO_2$) (Postgate1998).Nitrogen is very abundant in nature which causes a higher reduction in agriculture productivity in rural portion worldwide. About 386 x 1016 kg nitrogen contains in the Earth's atmosphere (Stevensen, 1986).

Oxygen is highly reactive with nitrogenase enzyme complex. Organisms have various methods to overcome the problem. Azotobacter species also produce copious amounts of extracellular polysaccharide. Diffusion rate of oxygen into the cell can be limited by these bacteria by balancing water into polysaccharide slime layer.

In the symbiotic nitrogen-fixing organisms such as Rhizobium, the root nodules can contain oxygen-scavenging molecules such as leghaemoglobin, which shows as a pink color when the active nitrogen-fixing nodules of legume roots are cut open.

Some of the cyanobacteria have yet another mechanism for protecting nitrogenase: nitrogen fixation occurs in special cells (heterocysts) which possess only photosystem I whereas the other cells have both photosystem I and photosystem II.

In 1901, after successfully studying a specialized group of prokaryotes Beijerinck discovered Biological nitrogen fixation (BNF). These organisms utilize the enzyme nitrogenase to catalyze the conversion of atmospheric nitrogen ($N_2$) to ammonia ($NH_3$). (Postgate 1982).Plants can readily assimilate $NH_3$ to produce the aforementioned nitrogenous biomolecules. Rice is the staple food of over half the world's population.

Non-symbiotic (or "free-living") nitrogen-fixing bacteria may reside in the rhizosphere just outside the roots of certain plants (including many grasses), and similarly "fix" nitrogen gas in the nutrient-rich plant rhizosphere.

## II. Materials And Methods

### 2.1 Sample Collection

Soil sample was collected from agricultural land of Chuadanga district, Bangladesh for Microbial Characterization and Analysis of $N_2$-Fixingbacterial species. The process was initiated with rice rhizosphere during seedling (after 30 days) of rice (BR 28, *Oryza sativa L.*). N-fertilizers mixing ration at that soil was $60kgha^{-1}yr.^{-1}$. The soil was usually cultivated by Aus, Aman and Boro crops, as well as Rabi crops in winter.

### 2.2 Raw Materials Processing

1500 grams of crushed wet soil samples were collected. Drying process was initiated under 65°C for four days. 1227 grams samples were found after drying process which was then separated into 4 equal parts for post processing.

**2.3 Serial Dilution**

10gm of sample was dissolved with vortex in 90ml of saline water and labeled as $10^{-1}$. Others test tubes were labeled as $10^{-2}$, $10^{-3}$, $10^{-4}$, $10^{-5}$, $10^{-6}$, and $10^{-7}$. 1mL of sample from $10^{-1}$ dilution was transferred into the $10^{-2}$ test tube with 9ml saline solution by using a sterile 1mL pipette. Each bottle contains 90mL of sterile water. This first bottle now has a 1:100 dilution. 1mL of sample from $10^{-2}$ test tube was transferred into the $10^{-3}$ test tube by using a new sterile 1mL pipette and shacked with vortex. Then 1mL of sample from $10^{-3}$ test tube was transferred into the $10^{-4}$ test tube by using a new sterile 1mL pipette and shacked with vortex. This process was continued for test tube labeled as $10^{-5}$, $10^{-6}$, and $10^{-7}$. Then 1ml sample from each tube was used for pour plate method with RCV and incubated the plates at 30˚C for 48 hours.

**Table 1:** Characterization of Soil

| Parameters | Details |
|---|---|
| **Location** | Village: Hajrahati, Thana: Chuadanga Sadar, Post: Chuadanga-7200, District: Dhaka, Country: Bangladesh |
| **General Soil Type** | Calcareous Dark Grey Floodplain soils |
| **FAO-UNESCO System** | Calcaric Cambisols and Gleysols |
| **AEZ** | AEZ-1 (High Ganges River Flood Plain) |
| **Topography** | High Land |
| **Present land use** | Aus, Aman, Boro and Rabi crops |
| **Soil pH** | 5.2 |
| **Organic Matter** | Low |
| **CES** | Medium |
| **Soil Color** | Dark Grey |
| **Soil sampling depth** | 0-15 cm |
| **Maximum water holding capacity** | 49% |
| **Texture** | Silt |
| **Potassium** | Medium |
| **Zinc** | Low |
| **Nitrogen** | Low |

**2.4 Pure Culture Isolation**

The procedures for the isolation and identification were based on the Nordic Committee methods. Bacteria were isolated on RCV media slant and left in incubator at $37^{O}$C for 24 hours. After growth in all slant, bacterial culture was observed physically and biochemically.

**2.5 Microscopic Examination**

Glass slide corner was labeled with a wax pencil of the respective bacteria. An inoculating loop was used to apply a small amount of bacteria to one end of a clean microscope slide 1 to 2 loops of nigrosin was added, to the bacteria and mixed thoroughly. The mixture was spreaded over the slide using a second slide. The second was hold at a 45°angle so that the bacteria-nigrosin solution. The smear was allowed to air dry and then observed under microscope. (Prescott H. 2002)

**2.6 Gram Staining**

Heat-fixed smears were prepared from the sample. The slides were placed on the staining rack. Flood The smears was flooded with crystal violet and rested for 30 seconds 4. Rinsed with water for 5 seconds. Then smear was covered with Gram's iodine mordant and rested for 1 minute. Rinsed with water for 5 seconds. Next the smear was decolorized with 95% ethanol for 15 to 30 seconds.

The decolorizer was added drop by drop until the crystal violet fails to wash from the slide. Rinsed with water for 5 seconds. The smear was counter-stained with safranin for about 60 to 80 sec. Rinsed with water for 5 seconds. Smear was blot dried with bibulous paper. Smear was examined under oil immersion. Organisms were stained blue to purple.

**2.7 Catalase Test**

A clear slide was taken and 1 drop of hydrogen peroxide was added. A single colony was taken and added on slide. Then wait for 2-3 minutes. (Prescott 2002)

**2.8 Oxidase Test**

A disk was taken. 2-3 drops of reagent (tetra-methyl-p-phenyl-diaminedihydro chloride) was added on the disk. A loop full of bacteria colony was added on the disk. (Prescott 2002)

**2.9 Citrate Utilization Test**

5ml of Simmons citrate media was taken in to sterile test tube. A loop full of bacteria was inoculated in to the tube. Then incubate at 37°c for 24 hours. (Prescott 2002)

**2.10 Indole Test**

A loop full of overnight growth bacteria was inoculated in to the TSB. Incubate for at least 48 hours. Kovac´s reagent was added to the media. (Prescott 2002)

**2.11 Methylene-Red Test**

Using sterile technique, inoculate each experimental organism into its appropriately labeled tube of medium by means of a loop inoculation. The last tube was served as a control and all culture were incubated for 24 to 48 hours at 37ºC. (Prescott 2002)

**2.12 Vogues Proskauer Test**

A loop full of organisms was inoculated in to the MR-VP medium in the tube. Barrits A (alpha-napthol)+ Barrits B (potassium-hydroxide) was added to the medium. Keep it 20 to 30 minutes. (Prescott 2002)

**2.13 Gelation Liquification and Carbohydrate Fermentation**

Gelation liquification and carbohydrate fermentation methods were initiated as demonstrated by Collee and Miles (1989).

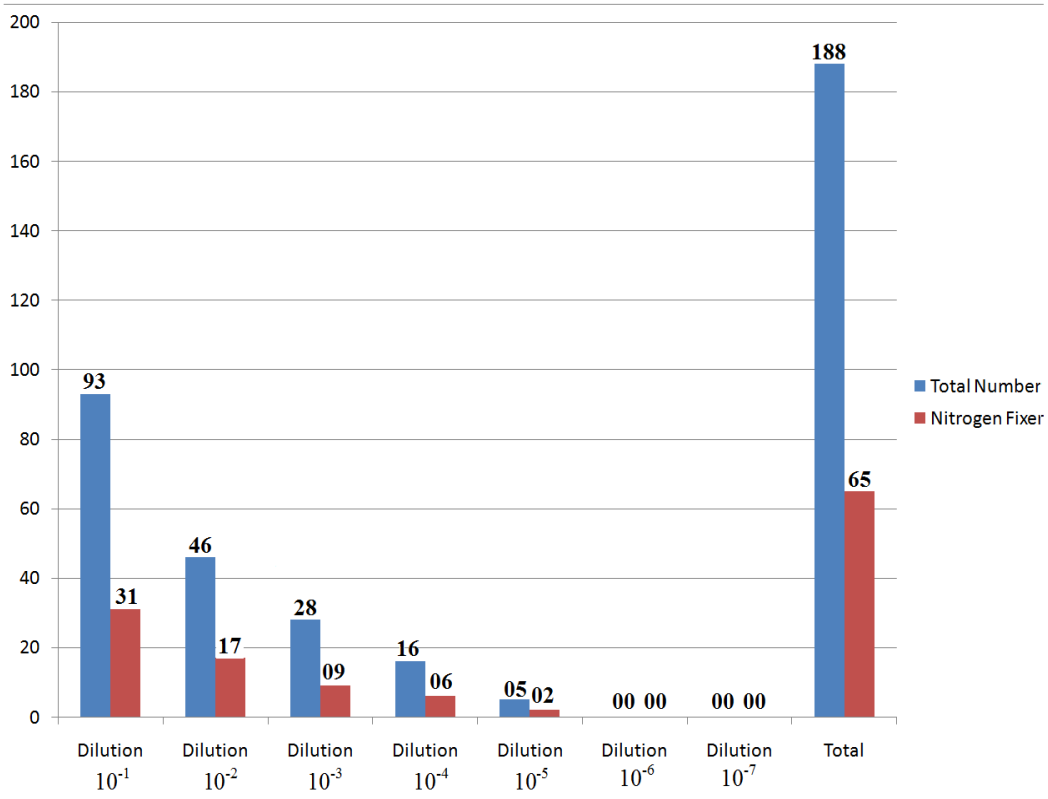
**2.14 Enzymatic Reaction**

Enzymatic Reactions were initiated as demonstrated by Collee and Miles (1989).

## III. Results

Total 188 isolates were found in which only 65 were nitrogen fixer, in a ratio of 34.58%.

**Chart 1:** Enumeration of Bacteria




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.*

Among the 65 nitrogen fixers (34.58 %), 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.

**Table 2:** Physiological Identification

| Tests | Results | | | |
|---|---|---|---|---|
| | **Strain 1** | **Strain 2** | **Strain 3** | **Strain 4** |
| **Gram Stain** | -ve | -ve | +ve | -ve |
| **Shape** | Rod Shaped | Rod Shaped | Rod Shaped | Rod Shaped |
| **Spor formation** | Non-Spor Forming | Non-Spor Forming | Spor Forming | Non-Spor Forming |

**Table 3:** Biochemical Identification

| Tests | Results | | | |
|---|---|---|---|---|
| | **Strain 1** | **Strain 2** | **Strain 3** | **Strain 4** |
| **Indole production** | + | + | + | + |
| **Methyl Red reaction** | + | + | + | + |
| **Voges-Proskauer reaction** | + | - | - | + |
| **Citrate Utilization** | - | - | - | - |
| **Urease activity** | + | + | + | - |
| **Catalase** | + | - | + | + |
| **Oxidase** | + | + | + | + |
| **Gelatin Liquefication** | - | - | - | - |

**Table 4:** Enzymatic Reaction

| Tests | Results | | | |
|---|---|---|---|---|
| | **Strain 1** | **Strain 2** | **Strain 3** | **Strain 4** |
| **Acetoin Production** | - | + | - | - |
| **Esculin Hydrolysis** | + | + | + | - |
| **Lipase** | - | - | - | + |
| **Lysine** | - | + | - | + |
| **ONPG (β-galactosidase)** | - | + | - | - |
| **Ornithine Decarboxylase** | - | - | - | + |
| **Phenylalanine Deaminase** | - | - | - | - |

**Table 5:** Carbohydrate Fermentation

| Tests | Results | | | |
|---|---|---|---|---|
| | **Strain 1** | **Strain 2** | **Strain 3** | **Strain 4** |
| **Adonitol** | - | + | - | + |
| **Arabinose** | - | + | + | + |
| **Glucose** | + | + | + | + |
| **Lactose** | + | + | + | + |
| **Maltose** | + | - | + | + |
| **Mannitol** | + | + | + | + |
| **Mannose** | + | + | + | + |
| **Raffinose** | + | + | + | + |
| **Sorbitol** | + | + | + | - |
| **Sucrose** | + | + | + | - |

## IV. Discussion

In this study 65 microbes were identified as $N_2$-fixing out of 188 isolates which is about 34.58% of total from rice rhizosphere during seedling (after 30 days) of rice (BR 28, *Oryza sativa L.*).

After physiological identification, biochemical identification and carbohydrate fermentation, four species were found based on study criteria- *Enterobacter spp.*, for strain-1, *Klebsiella spp.* for strain-2, *Bacillus spp.* for strain- 3 and *Azospirillum spp.* for strain-4.

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