Isolation and identification of *Rhizobium* from non-saline coastal soils of Bangladesh and preparation of mother culture

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**Abstract:** Nitrogen is the essential mineral macro nutrients which are required for the maximum magnification and yield of agriculturally paramount crops. Microbial inoculants may supplement and abbreviate the dependency on synthetic costly N-fertilizers in reverence of crop yield. The categorical objectives of the research works are to isolate and identify the *Rhizobium* from culled soils. The ability of soil microorganisms to fix atmospheric nitrogen is an important trait in promoting plant growth and increasing crop yield. The study was conducted for the isolation and identification of nitrogen fixing bacteria from saline and non-saline soils of different locations of tidal floodplain region of Bangladesh. Six *Rhizobium* strains were isolated and purified. The isolates were preliminary identified on the basis of their morphological and biochemical characteristics. Based on the results, it can be concluded that the isolates possess great potential to be developed as biofertilizers to enhance soil fertility and plant growth. However, their performance under green house and field conditions should be assessed afore being recommended for biofertilizer production and their applications.

**Keywords:** biofertilizer; non-saline soils; *Rhizobium*

1. **Introduction**

Nitrogen is an essential nutrient for plant growth and development. Plants usually depend upon combined, or fixed, forms of nitrogen, such as ammonia and nitrate because it is unavailable in its most prevalent form as atmospheric nitrogen. Much of this nitrogen is provided to cropping systems in the form of industrially produced nitrogen fertilizers. Use of these fertilizers has led to worldwide ecological problems as well as affects the human health (Vitousek, 1997). Biological nitrogen fixation (BNF) is the cheapest and environment friendly procedure in which nitrogen fixing microorganisms, interacting with leguminous plants, fix aerobic nitrogen into soil (Franche et al., 2009).

*Rhizobium* is the most well-known species of a group of bacteria that acts as the primary symbiotic fixer of nitrogen. These bacteria can infect the roots of leguminous plants, leading to the formation of lumps or nodules where the nitrogen fixation takes place. The bacterium’s enzyme system supplies a constant source of reduced nitrogen to the host plant and the plant furnishes nutrients and energy for the activities of the bacterium. *Rhizobium* bacteria stimulate the growth of leguminous plants and they are able to fix atmospheric nitrogen into soil by interacting symbiotically with leguminous plants, using the nitrogenase enzyme complex (Kiers et al., 2003).

*Rhizobium* is the soil microorganisms that can survive in the soil or forms a symbiotic association with the host legume. The most convenient method of obtaining *Rhizobium* from nature is by isolation from root nodules. In contrary to popular belief, many of the bacteriods in nodule are viable. It is impractical to isolate rhizobia...
directly from the soil because of their fastidious growth. The primary objective of the proposed research is to develop a cheap organic nitrogen fertilizer that could supplement synthetic nitrogen fertilizer.

2. Materials and Methods
2.1. Sampling site
Selected sampling sites were Charfashion upazilla of Bhola district under AEZ 18 (Young Meghna Estuarine Floodplain) and Dumki upazilla of Patuakhali district under AEZ 13 (Ganges Tidal Floodplain) of Bangladesh.

2.2. Collection of soil samples
For isolation of rhizobia, soil samples were collected from selected areas. Ten surface soil samples were collected from each location. For the convenience of discussion, the 6 soil samples are referred to as soil-1, soil-2, soil-3, soil-4, soil-5 and soil-6 and these soil samples were collected from Aslampur, Ginnagor, Osmangonj of Charfashion upazila in Bhola district and Srirampur, PSTU Farm, Jamla of Dumki upazila in Patuakhali, respectively.

2.3. Preparation of the soil sample
Some portions of collected soil sample were kept in refrigerator at 4°C for isolation of bacteria. The rest portions of soil samples were then air dried ground to pass through a 2 mm sieve and then mixed to from a composite sample. Then these composite samples were kept in clean and sterilized bottles for physical and chemical analysis.

2.4. Experimental site
The laboratory experiment was conducted at the Department of Soil Science and Central Laboratory, Patuakhali Science and Technology University, Dumki, Patuakhali during July 2014 to June 2015.

2.5. Soil analysis
The initial soil samples were analyzed for physical and chemical characteristics. The physical characteristics includes textural class and the chemical properties include soil pH, electrical conductivity, organic matter, total N, Exchangeable K, available P and S content. Results of this analysis have been presented in Table 1.

| Properties of soils | Soil-1 | Soil-2 | Soil-3 | Soil-4 | Soil-5 | Soil-6 |
|---------------------|--------|--------|--------|--------|--------|--------|
| %Sand               | 19.2   | 19.5   | 19.3   | 21.7   | 20.9   | 21.5   |
| %Silt               | 67     | 66     | 68     | 70.75  | 70.86  | 70.92  |
| %Clay               | 13.8   | 13.9   | 13.6   | 7.55   | 7.75   | 7.68   |
| pH (H$_2$O)         | 7.65   | 7.88   | 7.75   | 6.56   | 5.98   | 6.43   |
| EC                  | 0.67   | 0.80   | 0.73   | 0.12   | 0.20   | 0.17   |
| %OC                 | 1.070  | 1.069  | 1.081  | 1.091  | 1.079  | 1.086  |
| % OM                | 1.845  | 1.762  | 1.812  | 1.880  | 1.743  | 1.810  |
| %N                  | 0.047  | 0.042  | 0.062  | 0.014  | 0.020  | 0.019  |
| P(ppm)              | 8.73   | 8.67   | 8.59   | 10.65  | 10.76  | 10.63  |
| S(ppm)              | 32.77  | 32.67  | 32.82  | 13.52  | 13.35  | 12.98  |
| K(meq/100g)         | 0.989  | 0.974  | 0.871  | 1.209  | 1.214  | 1.192  |

2.6. Culture media
Yeast mannitol agar media were used for culture of *Rhizobium*.

2.7. Method of isolation
Enrichment culture technique (in liquid medium) was used for isolation of bacteria.

2.8. Composition of yeast extract mannitol agar
Mannitol - 10.0 g, K$_2$HPO$_4$ - 0.5 g, MgSO$_4$, 7H$_2$O - 0.2 g, NaCl - 0.1 g, Yeast extract - 0.5 g, Distilled water - 1 liter and Agar- 15 g. The medium was prepared and was autoclaved at 121°C and 15 psi for 20 minutes. In the meantime, all accessories like Petridis and pipette (1 ml) was also sterilized by autoclave.
2.9. Colony isolation
The growth of *Rhizobium* was streaking on medium and incubated until pure growth was obtained. Finally, pure *Rhizobium* was cultured on slant medium as mother culture and stored in refrigerator. Then different biochemical test is to be done and new mother culture was done after 3-4 months. The colonies showing clear zones around them developed within 48 hours were transferred to agar slants of Yeast mannitol agar medium and allowed to grow at 30°±2°C for three days. The cultures were then repeatedly plated in the same agar medium till pure strains were obtained and finally 20 bacterial cultures were maintained in the Yeast mannitol agar medium.

2.10. Estimation of bacterial population
The viable cells were calculated by the following formula stated by Somasegaran and Hobben (Somasegaran and Hobben, 1985).
\[
\text{Number of cells/ml (CFU/ml)} = \left(\frac{\text{Number of colonies} \times \text{(Dilution factor)}}{\text{(Volume per drop)}}\right)
\]

2.11. Purification of isolates
Six isolates of each *Rhizobium* were taken from respective cultured media and streaked on respective prepared plate’s media. The streaked plates were incubated at 28°C for 2-4 days. Repeated streaking was done until purification.

2.12. Identification of *Rhizobium* isolates
The isolates of *Rhizobium* obtained from soils were described according to their growth characteristics on solid and liquid Yeast Mannitol Agar media. Some morphological characters such as the shape, size, color, texture of colonies and ability to alter pH and some biochemical characters such as carbohydrate utilization and fermentation, gelatin and starch hydrolysis, Congo red dye absorption.

2.13. Preparation and preservation of mother culture
Purified isolates of *Rhizobium* were transferred into Yeast Mannitol Agar media and preserved for further study.

3. Results and Discussion

3.1. Estimation of Rhizobia
Bacterial populations of collected soils were determined and presented in Table 2. The results show the highest populations of *Rhizobium* $2.8 \times 10^6$ were found in soil no. 1 (Aslampur, Charfashion, Bhola) and the lowest populations of *Rhizobium* $2.2 \times 10^6$ were found in soil no. 2 (Jamla, Dumki, Patuakhali).

| Location | Rhizobium (CFUg$^{-1}$) |
|----------|-------------------------|
| 1. Aslampur, Charfashion, Bhola | $2.8 \times 10^6$ |
| 2. Ginnagor, Charfashion, Bhola | $2.6 \times 10^6$ |
| 3. Usmangonj, Charfashion, Bhola | $2.7 \times 10^6$ |
| 4. Srirampur, Dumki, Patuakhali | $2.3 \times 10^6$ |
| 5. PSTU Farm, Dumki, Patuakhali | $2.5 \times 10^6$ |
| 6. Jamla, Dumki, Patuakhali | $2.2 \times 10^6$ |

3.2. Isolation of *Rhizobium* from saline soils of coastal region
Six *Rhizobium* isolates were obtained from Non-saline soil of coastal region. They were designated as R1, R2, R3, R4, R5 and R6 respectively (Table 3).

| Soil No. | Isolate name | Location |
|----------|--------------|----------|
| 1.       | R1           | Aslampur, Charfashion, Bhola |
| 2.       | R2           | Ginnagor, Charfashion, Bhola |
| 3.       | R3           | Usmangonj, Charfashion, Bhola |
| 4.       | R4           | Srirampur, Dumki, Patuakhali |
| 5.       | R5           | PSTU Farm, Dumki, Patuakhali |
| 6.       | R6           | Jamla, Dumki, Patuakhali |
3.3. Characterization of the isolates
Results of isolation as well as morphological and biochemical characteristics of isolates are presented below.

3.3.1. Morphological characteristics
Morphological characteristics of the isolates i.e. colony morphology have been presented in Table 4 and Table 5. The colony characteristics of isolates did not vary widely. All the isolates were found round shape, medium flat elevation, whitish colour, smooth surfaces, odour less, viscous consistency, opaque opacity with entire margin on Congo red yeast extract mannitol agar (CRYFMA) plates. All the isolates were found medium, small, large in size.

3.3.2. Microscopic tests
3.3.2.1. Simple staining (shape of cells)
The shape of the cells of rhizobia isolates are presented in Table 5. All the isolates were found short rod in shape. Vincent stated that *Rhizobium* was rod/ short rod shaped (Vincent *et al.*, 1980).

3.3.2.2. Motility test
All the 6 isolates under study were found motile in nature. Vincent stated that *Rhizobium* was generally motile (Vincent *et al.*, 1980).

3.3.2.3. Gram reaction test
All the 6 isolates have shown gram negative in reaction. Vincent stated that *Rhizobium* was gram negative (Vincent *et al.*, 1980).

Table 4. Colony characteristics of *Rhizobium* isolates on Yeast Mannitol Agar media.

| Isolate | Shape  | Elevation     | Odor   | Margin | Surface | Opacity | Colour  | Consistency |
|---------|--------|---------------|--------|--------|---------|---------|---------|-------------|
| R1      | Round  | Medium flat   | Odor less | Entire | Smooth  | Opaque  | Whitish | Viscous     |
| R2      | Round  | Medium flat   | Odor less | Entire | Smooth  | Opaque  | Whitish | Viscous     |
| R3      | Round  | Medium flat   | Odor less | Entire | Smooth  | Opaque  | Whitish | Viscous     |
| R4      | Round  | Medium flat   | Odor less | Entire | Smooth  | Opaque  | Whitish | Viscous     |
| R5      | Round  | Medium flat   | Odor less | Entire | Smooth  | Opaque  | Whitish | Viscous     |
| R6      | Round  | Medium flat   | Odor less | Entire | Smooth  | Opaque  | Whitish | Viscous     |

Table 5. Morphological (microscopic) characteristics of *Rhizobium* isolates.

| Isolate | Shape    | Gram reaction | Motility |
|---------|----------|---------------|----------|
| R1      | Short rod | Gram negative | Motile   |
| R2      | Short rod | Gram negative | Motile   |
| R3      | Short rod | Gram negative | Motile   |
| R4      | Short rod | Gram negative | Motile   |
| R5      | Short rod | Gram negative | Motile   |
| R6      | Short rod | Gram negative | Motile   |

3.3.3. Biochemical tests
Results of biochemical tests are presented below-

3.3.3.1. Congo red absorption test
From the Table 6 it was presented observed that all the bacterial isolates did not absorb Congo red at young stage but absorbed slightly when cultures became old. The isolates absorbed counter stain. Vincent *et al.* (1980) stated that *Rhizobium* was gram negative, rod shaped and generally motile. The isolates produce circular, low convex to convex, mucous and opaque white. The isolates were observed to lack the ability to absorb Congo red from yeast extract mannitol agar medium containing this dye. Similar result was observed by Barbar (Barbar *et al.*, 1983).
3.3.3.2. BTB test
All the bacterial isolates produced acid on BTB-YEMA plates. The results are presented in Table 7. The growth of the all fast growers develops yellow color that results acidic in nature.

3.3.3.3. Hofer’s alkaline broth test
Among the six isolates none had grown on Hofer’s alkaline broth (Table 7).

Table 7. Biochemical observation of different rhizobial isolates.

| Isolate | BTB test | Hoffer’s alkaline test |
|---------|----------|------------------------|
|         | Growth   | Observation | Result | Growth |
| R1      | Fast growth | Yellow colour | Acidic | No growth |
| R2      | Fast growth | Yellow colour | Acidic | No growth |
| R3      | Fast growth | Yellow colour | Acidic | No growth |
| R4      | Fast growth | Yellow colour | Acidic | No growth |
| R5      | Fast growth | Yellow colour | Acidic | No growth |
| R6      | Fast growth | Yellow colour | Acidic | No growth |

3.3.3.4. Growth on Different pH
The growth responses of the Rhizobium isolates were investigated in the YEMA medium having 5 levels of pH. The pH levels 4.0, 5.0, 6.0, 7.0 were created adding HCl solution and 8.0 adding NaOH as required. Results in the Table 8 show that all the isolates viz., R1, R2, R3, R4, R5 and R6 were heavy growers at pH 6.0 and 7.0. At pH 8.0 all isolates viz., R1, R2, R3, R4, R5 and R6 were found medium growth. But at pH 4 and 5 isolates were found minimum to medium growth. At pH 5.0 most of the isolates were found medium growth except R3. Kucuk found that rhizobia grew on pH levels 5 and 9 (Kucuk et al., 2006). Similarly Shraddha Bhatt also found that rhizobia were grown in YEM medium with pH values of 4, 5, 7 and 9 (Shraddha Bhatt et al., 2013).

Table 8. Effect of different pH on Rhizobium isolates in Yeast Mannitol Agar media.

| Isolate | Different pH |
|---------|--------------|
|         | 4 | 5 | 6 | 7 | 8 |
| R1      | - | + | ++ | ++ | + |
| R2      | + | + | ++ | ++ | + |
| R3      | - | - | ++ | ++ | + |
| R4      | - | + | ++ | ++ | + |
| R5      | + | + | ++ | ++ | + |
| R6      | + | + | ++ | ++ | + |

++ = Heavy growth, + = Medium growth, - = Minimum growth

3.3.3.5. Carbohydrate utilization
Results of carbohydrate utilization by the isolates are presented in Table 9. The sign of carbohydrate utilization was observed from the growth and fermentation characteristics of the isolates in a given carbohydrate medium and the variation in growth was identified by measuring the optical density of the media. It was observed that the isolates R1 and R5 showed heavy growth in mannitol and sucrose. R2, R3, R4 and R6 showed minimum growth in mannitol and sucrose. It was also observed that all the isolates showed minimum growth in glucose and produced gas in carbohydrate media used. Chowdhury and Knan (1968) and Podder (1977) working with
chickpea isolates also recorded similar results. Graham reported that most of the rhizobial strains utilized glucose, xylose and arabinose but lactose and sucrose were utilized by a few slow growing rhizobia (Graham et al., 1964).

Table 9. Carbohydrate utilization and fermentation by the strains.

| Strain | Sucrose | Glucose | Mannitol |
|--------|---------|---------|----------|
| R1     | ++      | +       | ++       |
| R2     | +       | +       | +        |
| R3     | +       | +       | +        |
| R4     | +       | +       | +        |
| R5     | ++      | +       | ++       |
| R6     | +       | +       | +        |

++ = Heavy growth, + = Minimum growth

3.3.3.6. Gelatin hydrolysis
Results in Table 10 show that R4, R5 and R6 isolates had the capacity to hydrolyse gelatin. On the other hand, R1, R2 and R3 gave negative result for gelatin hydrolysis. But Podder reported that none of the isolates from chickpea used in his study could hydrolyse gelatin (Podder et al., 1977).

3.3.3.7. Strach hydrolysis
R1, R2, R3, R4 and R6 of the isolates gave positive results for starch hydrolysis (Table 10). R5 of the isolates gave negative results for starch hydrolysis (Table 10). Halos developed around the bacterial colonies. Podder also noted that the isolates from chickpea failed to cause hydrolysis of starch (Podder et al., 1977).

3.3.3.8. Catalase test
Results in Table 10 show that all of the test isolates gave positive results for catalase test. All of the isolates produced bubbles within a few seconds.

Table 10. Gelatin hydrolysis, starch hydrolysis and catalase test.

| Isolates | Gelatin hydrolysis | Starch hydrolysis | Catalase test |
|----------|--------------------|-------------------|--------------|
| R1       | (-)                | (+)               | +            |
| R2       | (-)                | (+)               | +            |
| R3       | (-)                | (+)               | +            |
| R4       | (+)                | (+)               | +            |
| R5       | (+)                | (-)               | +            |
| R6       | (+)                | (+)               | +            |

(+)= hydrolytic, (-)= nonhydrolytic and + = positive result

3.3.4. Growth at different temperature conditions
All the isolates showed good growth at temperature 28°C and 32°C (Table 11). Most of the isolates grew weakly (poor growth) at 14°C except R3, R5 and R6 isolates. At 22°C most isolates exhibited medium growth while three isolates (R3, R5 and R6) recorded poor growth. All the isolates grew at 38°C. Only three isolates (R3, R5 and R6) showed medium growth at 38°C while rest three showed poor growth. At 45°C most isolates exhibited no growth while two isolates (R5 and R6) recorded very poor growth (Table 11).

Table 11. Growth of Rhizobium isolates in different temperature conditions.

| Isolate | Growth in different temperature condition |
|---------|------------------------------------------|
|         | 14°C | 22°C | 28°C | 32°C | 38°C | 45°C |
| R1      | ++   | +    | ++   | ++   | +    | -    |
| R2      | ++   | +    | ++   | ++   | +    | -    |
| R3      | -    | +    | ++   | ++   | +    | -    |
| R4      | +    | +    | ++   | ++   | +    | -    |
| R5      | -    | +    | ++   | ++   | +    | +    |
| R6      | -    | +    | ++   | ++   | +    | +    |

- = No growth, + = Very poor growth, ++ = Poor growth, +++ = Medium growth, ++++ = Good growth
4. Conclusions
It is concluded from the present study that the utilization of as bioinoculants may increase the fix atmospheric nitrogen in soil. It avails to minimize the nitrogen fertilizer application, minimize environmental pollution and promotes sustainable agriculture. The study ventilated that *Rhizobium* isolated from rice rhizosphere could be utilized for sustainable rice crop production system in Bangladesh. Their performance under green house and field conditions should be assessed afore being recommended for biofertilizer production and commercial applications.

Conflict of interest
None to declare.

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