Asian J. Med. Biol. Res. 2020, 6 (2), 222-228; doi: 10.3329/ajmbr.v6i2.48053

Asian Journal of Medical and Biological Research
ISSN 2411-4472 (Print) 2412-5571 (Online)
www.ebupress.com/journal/ajmbr

Article

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

Md. Rafiq Uddin[1*], Mosa. Farjana Tasmin[1], Md. Shahin Hossin[1], Md. Mainul Islam Rashad[2], Md. Fazlul Hoque[1] and Md. Kabirul Islam[1]

[1]Department of Soil Science, Patuakhali Science and Technology University, Dumki, Patuakhali-8602, Bangladesh
[2]Department of Genetics and Plant Breeding, Patuakhali Science and Technology University, Dumki, Patuakhali-8602, Bangladesh

[*]Corresponding author: Md. Rafiq Uddin, Department of Soil Science, Faculty of Agriculture, Patuakhali Science and Technology University, Dumki, Patuakhali-8602, Bangladesh. E-mail: rafiq_ssc@pstu.ac.bd

Received: 30 April 2020/Accepted: 30 May 2020/ Published: 30 June 2020

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 micro-organisms, 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. *Rhizibium* 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 4^0C 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.

Table 1. Physical and chemical characteristics of collected soils.

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_2O)	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	32.69	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_2HPO_4- 0.5 g, $MgSO_4$, $7H_2O$ - 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 121^0C 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 $30^0 \pm 2^0$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).

Number of cells/ml (CFU/ml) = [((Number of colonies) × (Dilution factor)} ÷ (Volume per drop)].

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 28^0C 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* $2.8*10^6$ were found in soil no. 1 (Aslampur, Charfashion, Bhola) and the lowest populations of *Rhizobium* $2.2*10^6$ were found in soil no. 2 (Jamla, Dumki, Patuakhali).

Table 2. Bacterial population of *Rhizobium* from collected soil sample.

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

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).

Table 3. List of isolates from saline and non saline soil areas.

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 *el al.,* 1983).

Table 6. Congo red absorption of different rhizobial isolates.

Isolate	Congo red absorption	
	Young culture	**Old culture**
R1	Not absorbed	Weakly absorbed
R2	Not absorbed	Weakly absorbed
R3	Not absorbed	Weakly absorbed
R4	Not absorbed	Weakly absorbed
R5	Not absorbed	Weakly absorbed
R6	Not absorbed	Weakly absorbed

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 the 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 28^0C and 32^0C (Table 11). Most of the isolates grew weakly (poor growth) at 14^0C except R3, R5 and R6 isolates. At 22^0C most isolates exhibited medium growth while three isolates (R3, R5 and R6) recorded poor growth. All the isolates grew at 38^0C. Only three isolates (R3, R5 and R6) showed medium growth at 38^0C while rest three showed poor growth. At 45^0C 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					
	14^0 C	22^0C	28^0 C	32^0 C	38^0 C	45^0 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.

References

Barbara EK and AL Thomas, 1983. Congo red absorption by *Rhizobium leguminosarum*. Appl. Environ. Microbiol., 45: 340-342.

Chowdhury MS and AKM Khan, 1968. Cultural characteristics of certain native strains of root nodule bacteria of East Pakistan. J. Soil Sci., 4: 21-29.

Franche C, K Lindstrom and C Elmerich, 2009. Nitrogen fixing bacteria associated with leguminous and non-leguminous plants. Plan Soil, 321: 35-59.

Graham PH, 1964. Diagnostic features in the characterisation of the root nodule bacteria of legumes. Plant and Soil, 20: 383-396.

Kiers ET, RA Rousseau, SA West and RF Denison, 2003. Host sanctions and the legume–rhizobium mutualism. Nature, 425: 79-81.

Kucuk C, M Kivanc and E Kinac, 2006. Characterization of *Rhizobium* sp. Isolated from Bean. Turk. J. Biol., 30: 127-132.

Podder AK and AKM Habibullah, 1982. Performance of some isolated native rhizobial strains on the production and nodulation of chickpea. Bangladesh J. Soil Sci., 18: 30-35.

Shraddha Bhatt DrRV, H Vyas, N Shelat and J Sneha, 2013. Isolation and Identification of Root Nodule Bacteria of Mung Bean (*Vigna radiate* L.) for Biofertilizer Production. International Journal of Research in Pure and Applied Microbiology, 3: 127-133.

Somasegaran P and HJ Hobben, 1985. Methods in legume-Rhizobium technology. NifTAL project and MIRCEN, Department of Agronomy and Soil science, University of Hawaii.

Vincent JM, PS Nutman and FA Skinner, 1980. Some General Techniques and Procedures - Identification and Classification of Rhizobium. Research for development seminar on "Nitrogen Fixation by Legumes for Tropical Agriculture" held in Canberra. Australia, during Nov.-Dec. 1980.

Vitousek PM, 1997. Human alteration of the global nitrogen cycle: sources and consequences. Ecol. Appl., 7: 737-750.