Archives of Agriculture and Environmental Science 10(1): 10-15 (2025)
https://doi.org/10.26832/24566632.2025.100102

e-ISSN: 2456-6632

This content is available online at AESA

Archives of Agriculture and Environmental Science

Journal homepage: journals.aesacademy.org/index.php/aaes

ORIGINAL RESEARCH ARTICLE

Isolation and identification of Azotobacter from saline and non-saline soils of Bangladesh

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ARTICLE HISTORY

Received: 08 December 2024
Revised received: 28 January 2025
Accepted: 10 February 2025

Keywords

Azotobacter
Biofertilizer
Nitrogen fixing bacteria

ABSTRACT

Nitrogen is the most important mineral nutrient required for the plants growth and development. Microbial inoculants have the potential to augment and reduce reliance on expensive chemical fertilizers specially Urea, while maintaining crop productivity. Urea fertilizer not only expensive but also destroy our environment by nitrate pollution. Some microbes have ability to fix atmospheric nitrogen in soil symbiotically in legume crops. However, there are a few reports on non-symbiotic nitrogen fixers for non- leguminous crops. The present study aimed to isolate efficient non symbiotic or free living *Azotobacter* spp. that could be utilized as potential bio inoculants for resolving the nitrogen deficiency in soil for better growth and development of non-leguminous crops as well as industrial benefits. Nine *Azotobacter* isolates from saline and non-saline soils of south coastal zones of Bangladesh cultured on Ashby's agar plates at 28°C for 1 week. The colonies on the medium were picked up and used for the investigations. Based on morphological and biochemical identifications the isolates were confirmed as *Azotobacter* spp., isolates showed higher nitrogen fixing abilities (6.95 to 9.89 mg N/g) were selected. Among them, isolate NFA3 performed the best (9.89 mg N/g) regardless of all the tests. These isolates could survive neutral to slightly saline soils and higher temperature. Therefore, isolate NFA3 was considered to be the candidate for industrial usage for the development of nitrogenous biofertilizer.

Citation of this article: Uddin, M. R., Islam, M. K., Tasmin, M. F., Hossin, M. S., & Hoque, M. F. (2025). Isolation and identification of Azotobacter from saline and non-saline soils of Bangladesh. *Archives of Agriculture and Environmental Science*, *10*(1), 10-15, https://dx.doi.org/10.26832/24566632.2025.100102

INTRODUCTION

Nitrogen is a crucial ingredient for the growth and enhancement of plants. As atmospheric nitrogen, the most common form of nitrogen, is unavailable to plants, they typically depend on merged, or fixed, forms of nitrogen, such as ammonia and nitrate (Dahal *et al*., 2017). Plants can get these types of "united" nitrogen from four different sources: addition ammonia and/or nitrate manure or fertilizer to the soil, discharging these compounds during the breakdown of organic matter, converting atmospheric nitrogen into these compounds through natural processes like biological nitrogen fixation (BNF) and levin (Alshibli *et al*., 2018). Nitrogen fertilizers made in factories supply a large portion of this nitrogen to cropping schemes. The use of these fertilizers has impacted human health and caused global problems with the environment (Vitousek, 1997). One of the most significant biological processes in nature is biological nitrogen fixation (BNF). The application of BNF technology can furthermore significantly reduce environmental pollution, stop the loss of soil organic matter and minimize the need for Urea-N (Bhardwaj *et al*., 2014). Through their interactions with leguminous plants, nitrogen-fixing microorganisms fix aerobic nitrogen into soil through a procedure known as biological nitrogen fixation (BNF), which is both economical and environmentally

friendly (Franche *et al.*, 2009). As the principal symbiotic nitrogen fixer, *Azotobacter* is the utmost well-known species among this group of bacteria. Leguminous plants are susceptible to bacterial root infections, which can result in the development of lumps or nodules where nitrogen fixation occurs. The plant provides nutrients and energy for the bacterium's activities, while the bacterium's enzyme system provides the host plant with a steady supply of reduced nitrogen. A genus of bacteria known as *Azotobacter* is typically oval or spherical, motile, forms thick-walled cysts, and can produce abundant quantities of capsular slime. These are free-living, aerobic soil microorganisms that are crucial to the nitrogen cycle in nature. They fix nitrogen in the soil by binding atmospheric nitrogen, which is unavailable to plants, and releasing it as ammonium ions. These bacteria consume nitrogen gas from the atmosphere to synthesize proteins in their cells. After *Azotobacter* cells die, this cell protein becomes mineralized in the soil, which increases the amount of nitrogen available to agricultural plants. *Azotobacter* stimulates rhizosphere microorganisms, produces phytopathogenic inhibitors, and biosynthesis physiologically active chemicals, all of which have positive effects on crop growth and yield (Chen, 2006; Lenart, 2012). Nutritional absorption modification, which in turn increases biological nitrogen fixation. In case of chemical fertilizers, they have an adverse effect on soil health and environment (Nagananda *et al.*, 2010). To improve soil fertility in such a scenario, microbial inoculants like *Azotobacter* is the best option.

Microbial products are thought to be a key element of integrated nutrient management since they are safer, renewable and cost-effective compare to the chemical fertilizers. Many research works already have done on *Rhizobium* inoculant development. It provides nitrogen only for leguminous crops, but a few research has been done on non-leguminous crops specially rice. So, the research aimed to develop a *Azotobacter* inoculants for the fulfilment of nitrogen requirement for non-leguminous crop specially in rice.

MATERIALS AND METHODS

Sampling site

Three upazillas in Bangladesh were chosen for the sampling: Dumki in Patuakhali district, Dumuria in Khulna district under AEZ 13 (Ganges Tidal Floodplain) and Charfashion in Bhola district under AEZ 18 (Young Meghna Estuarine Floodplain).

Collection of soil samples

Soil samples were taken from certain locations for the purpose to isolate *Azotobacter*. Nine composite surface soil samples were taken at a predetermined area. The soil samples were taken from Aslampur, Ginnagor, Osmangonj of Charfashionupazilla in Bhola district; Srirampur, PSTU Farm, Jamla of Dumki upazila in Patuakhali district; and Rangpur, Rudroghor, Gutudia of Dumuria upazilla in Khulna district. For the purposes, the nine soils are referred to as soil -1, soil-2, soil-3, soil-4, soil-5, soil-6, soil-7, soil -8, and soil-9.

Preparation of the soil sample: A portion of the soil sample that was taken was refrigerated at 4°C for the purpose of isolating bacteria. After the remaining soil sample portions were air dried, they were mixed and passed through a 2 mm sieve to form a composite sample. Following that, these composite samples were stored for chemical and physical analysis in sterile, clean bottles.

Soil analysis: Physical and chemical properties were examined in the initial soil samples. Textural class is one of the physical qualities, whereas soil pH, EC, organic carbon, organic matter, percent of total N, available P, S content, and exchangeable K are among the chemical features. The analysis's findings are shown in Table 1.

Culture media: *Azotobacter* cultures were conducted using Ashby's media.

Method of isolation: Bacteria were isolated using the enrichment culture technique (in liquid media).

Table 1. Physico-chemical properties of collected soils.

Attributes of soils	Soil-1	Soil-2	Soil-3	Soil-4	Soil-5	Soil-6	Soil-7	Soil-8	Soil-9
Sand (%)	19.2	19.5	19.3	21.7	20.9	21.5	29.2	28.9	29.15
Silt (%)	67	66	68	70.75	70.86	70.92	60.75	60.82	60.56
Clay (%)	13.8	13.9	13.6	7.55	7.75	7.68	10.05	10.12	10.21
pH (H_2O)	7.65	7.88	7.75	6.56	5.98	6.43	7.77	7.67	7.65
EC	0.67	0.80	0.73	0.12	0.20	0.17	0.411	0.423	0.416
OC (%)	1.070	1.069	1.081	1.091	1.079	1.086	1.195	1.21	1.179
OM (%)	1.845	1.762	1.812	1.880	1.743	1.810	2.06	2.08	2.02
N (%)	0.047	0.042	0.062	0.014	0.020	0.019	0.017	0.018	0.015
P (ppm)	8.73	8.67	8.59	10.65	10.76	10.63	10.01	10.09	10.10
S (ppm)	32.77	32.69	32.82	13.52	13.35	12.98	99.33	99.45	99.57
K (meq/100g)	0.988	0.975	0.873	1.208	1.215	1.193	1.136	1.207	1.263

Medium preparation: The following procedures were used to prepare one liter of Ashby's culture media. The reagents (Manitol, K_2HPO_4, $MgSO_4$, $7H_2O$, NaCl, K_2SO_4, $CaCO_3$) were weighted by electronic balance based on the composition of the media. A conical flask was filled with one thousand milliliters of distilled water, which was measured using a volumetric flask. The reagents (except agar) were mixed with the distilled water. The pH was adjusted by adding HCl or NaOH solution as necessary after the ingredients were mixed. After the pH was adjusted, the liquid was combined with agar. The agar was mixed and then an autoclave machine was used to autoclave the medium. At last, the medium was transferred into sterile Petri dishes.

Isolation, population count, purification and preservation of *Azotobacter* isolates

Isolation was done in the Central Laboratory and Soil Science Laboratory of Patuakhali Science and Technology University under Class-II biosafety cabinet to avoid the contamination of any infectious disease. Sterile 50 ml disposable dilution tube containing 5g of collected soil sample and sterile distilled water was filled up to the mark. Suspension was made by vortex mixture and made dilution 10^{-1} to 10^{-5}. Transferred 1 ml of the sample suspension on Ashby's agar plate. Incubation was done at 28±2°C for two to three days. The following formula was used to count the *Azotobacter* population (Somasegaran and Hobben's 1985). After being purified through repeated streaking, the isolates were kept as mother cultures in nutrient agar slants.

$$\text{No.of cells/ml (CFU/ml)} = [(\text{Number of colonies}) \times (\text{Dilution factor})\} \div (\text{Volume per drop})]$$

Determination of nitrogen fixing capacity

The amount of nitrogen that accumulated in each isolates were studied using five-day-old culture, which was grown in fifty milliliters of broth medium to assess the nitrogen fixing potential of isolates. The amount of nitrogen in the culture medium determined by The Kjeldahl method.

RESULTS AND DISCUSSION

Estimation of *Azotobacter* population

According to the findings, soil no. 7 had the highest numbers of *Azotobacter* (2.6×10^6) which was collected from saline zone and the lowest *Azotobacter* populations (1.4×10^6) have been found in soil no. 6 (Table 2) which was collected from non-saline zone of Bangladesh. It might be due to the presence higher pH and higher organic matter content in soil no. 7. We know, organic matter plays a vital role on the growth and proliferation of microorganisms.

Isolation of Azotobacter from saline and non-saline soils of coastal region

From coastal region soil, nine *Azotobacter* isolates were obtained. They were given the designations NFA1, NFA2, NFA3, NFA4, NFA5, NFA6, NFA7, NFA8, and NFA9 (Table 3).

Characterization of the isolates: Isolates were characterized based on their morphological and biochemical characteristics.

Table 2. List of isolates from saline and non-saline soil areas.

Soil No.	Isolate name	Location	Azotobacter (CFU/g)
1	NFA1	Achlampur, Charfassion, Bhola	2.0×10^6
2	NFA2	Zinnagor, Charfassion, Bhola	1.9×10^6
3	NFA3	Osmangonj, Charfassion, Bhola	1.7×10^6
4	NFA4	Srerampur, Dumki, Patuakhali	1.5×10^6
5	NFA5	PSTU Agriculture Farm, Dumki, Patuakhali	1.6×10^6
6	NFA6	Zamla, Dumki, Patuakhali	1.4×10^6
7	NFA7	Rangpur, Dumuria, Khulna	2.6×10^6
8	NFA8	Rudroghor, Dumuria, Khulna	2.2×10^6
9	NFA9	Gutudia, Dumuria, Khulna	2.3×10^6

Table 3. Colony characteristics of *Azotobacter* isolates on Ashby's media.

Isolate	Shape	Elevation	Odor	Margin	Surface	Gram reaction	Motility	Consistency
NFA1	Circular	Medium flat	Odor less	Entire	Smooth	Gram negative	Motile	Viscous
NFA2	Short rod	Medium flat	Odor less	Entire	Smooth	Gram negative	Motile	Viscous
NFA3	Short rod	Medium flat	Odor less	Entire	Smooth	Gram negative	Motile	Viscous
NFA4	Short rod	Medium flat	Odor less	Entire	Smooth	Gram negative	Motile	Viscous
NFA5	Short rod	Medium flat	Odor less	Entire	Smooth	Gram negative	Motile	Viscous
NFA6	Short rod	Medium flat	Odor less	Entire	Smooth	Gram negative	Motile	Viscous
NFA7	Round	Medium flat	Odor less	Entire	Smooth	Gram negative	Motile	Viscous
NFA8	Circular	Medium flat	Odor less	Entire	Smooth	Gram negative	Motile	Viscous
NFA9	Short rod	Medium flat	Odor less	Entire	Smooth	Gram negative	Motile	Viscous

Morphological characteristics

The morphological traits of the isolates are presented in table 3. There was little variation in the colony features of the isolates. Every isolate that was found on agar plates had a round, short rod and circular form, medium flat elevation, whitish and transparent color, smooth surfaces, no odor and a viscous consistency over the entire edge. All isolates, except for NFA1, NFA7, and NFA8, were found to be short rods or rod-shaped. Under assessment, it was found that each of the nine isolates was motile and gram negative. The same findings were found by Vincent *et al.* (1980).

Growth on different pH

Five different pH values of Ashby's medium were used to test the growth responses of the *Azotobacter* isolates. HCl solution was added to create pH levels 4.0, 5.0, 6.0 and 7.0; NaOH was added to achieve pH 8.0 when needed. All the isolates were heavy growers at pH 6.0 and 7.0 (Table 4). All the isolates showed medium growth at pH 8.0. However, isolates showed minimal to medium growth at pH 4 and 5 which is similar to the findings of Kucuk *et al.* (2006) and Shraddha *et al.* (2013).

Starch hydrolysis and catalase test

All the isolates, namely NFA1, NFA2, NFA3, NFA4, NFA5, NFA6, NFA7, NFA8, and NFA9, give positive results in starch hydrolysis and catalase tests (Table 4).

Growth at different temperature conditions

At 28°C and 32°C temperature, every isolate showed good growth (Table 4). At 14°C temperature, almost all the isolates developed slowly. At 22°C most isolates exhibited medium growth were found except NFA6 and NFA8. All the isolates exhibited good growth at 38°C except NFA1, NFA2, NFA4 and NFA9. All the isolates exhibited no growth at 45°C except NFA6, NFA7 and NFA8. Amarger *et al.* (1994) reported that freeze dried *R. meliloti* from old cultures survive better during storage at 30°C than did freeze dried bacteria from young cultures. Deschodt & Strijdom (1976) observed that *Azotobacter meliloti* survived well at a temperature of 33°C to 37°C but *R. trifotii*, *R. japonicum* and a cowpea *Rhizobium* survived less well at 37°C.

Table 4. Effects of varying pH on *Azotobacter* isolates in Ashby's media.

Isolate	Different pH					Development under varying temperature conditions (°C)						SH	CT
	4	5	6	7	8	14	22	28	32	38	45		
NFA1	-	+	++	++	+	+	+ +	+ + +	+ + +	+ +	-	(+)	(+)
NFA2	+	+	++	++	+	-	+ +	+ + +	+ + +	+ +	-	(+)	(+)
NFA3	-	-	++	++	+	-	+ +	+ + +	+ + +	+ ++	-	(+)	(+)
NFA4	-	+	++	++	+	+	+ +	+ + +	+ + +	+ +	-	(+)	(+)
NFA5	+	+	++	++	+	-	+ +	+ + +	+ + +	+ + +	-	(+)	(+)
NFA6	+	+	++	++	+	-	+	+ + +	+ + +	+ + +	+	(+)	(+)
NFA7	-	-	++	++	+	+	+ +	+ + +	+ + +	+ + +	+	(+)	(+)
NFA8	+	+	++	++	+	-	+	+ + +	+ + +	+ ++	+	(+)	(+)
NFA9	-	+	++	++	+	+	+ +	+ + +	+ + +	+ +	-	(+)	(+)

pH: ++ = Heavy growth, + = Medium growth, - = Minimum growth; Temperature: - = No growth, + = Poor growth, + + = Medium growth, + + + = Good growth; SH =Strach hydrolysis, (+) = positive result; CT = Catalase test, (+) = positive result.

Table 5. Nitrogen fixing capacity of *Azotobacter* isolates.

Isolate	Nitrogen fixing capacity (mg N/g substrate)
NFA1	7.56 c
NFA2	6.98 d
NFA3	9.89 a
NFA4	6.95 d
NFA5	8.45 b
NFA6	6.98 d
NFA7	7.65 c
NFA8	7.86 c
NFA9	7.43 c
CV (%)	0.5491
SE (±)	0.0347
Level of Significance (0.1%)	***

NFC = Nitrogen fixing capacity; Similar letter(s) in a column is not statistically different at 0.1% level by DMRT; ***= significant at 0.1% level of probability; CV= Co-efficient of variation; SE= Standard error of means.

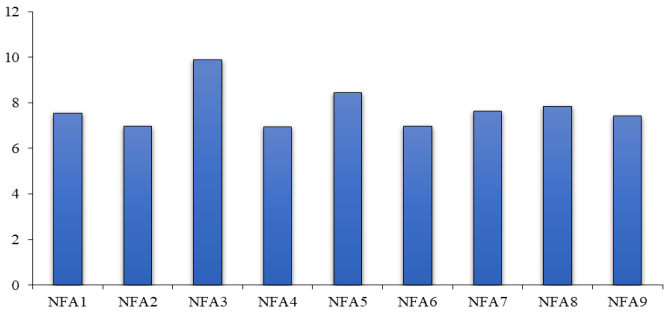

Figure 1. *Nitrogen fixing capacity of different isolates.*

Nitrogen fixing capacity

The Kjeldhal method was used to assess the nitrogen fixing capability. The effectiveness of *Azotobacter sp.* in fixing nitrogen was shown by the results. The nitrogen-fixing capacity of *Azotobacter sp.* ranged from 6.95 to 9.89 mg N/g (Table 5). The nitrogen fixing ability of NFA3 isolate was the highest at 9.89 mg N/g, while NFA4 isolate had the lowest at 6.95 mg N/g (Figure 1). The activity of the nitrogenase enzyme is one of the criteria that determines *Azotobacter* isolates' capacity to fix nitrogen (Sant'Anna *et al.*, 2011). According to Hossain *et al.* (2014), *Azospirillum sp.* had nitrogen fixing efficiencies ranging from 10.03 to 13.11 mg N/g.

Conclusion

The current investigation has led to the conclusion that using as bio inoculants may boost soil's capability to fix atmospheric nitrogen. All the isolates exhibited good growth at 38°C except NFA1, NFA2, NFA4 and NFA9. All the isolates exhibited no growth at 45°C except NFA6, NFA7 and NFA8. According to the study, Bangladesh may utilize *Azotobacter* that was isolated from the crop's rhizosphere to produce growing rice in a sustainable manner. It decreases the amount of chemical nitrogen fertilizer applied, minimizes pollution to the environment, and promotes sustainable agriculture. Further, it is advisable to evaluate their effectiveness in both greenhouse and field settings before recommending them for use in the production of biofertilizer and commercial applications.

ACKNOWLEDGEMENTS

Authors are thankful to the Department of Soil Science, Patuakhali Science and Technology University, Dumki, Patuakhali-8602, Bangladesh for the technical supports provided during the study.

DECLARATIONS

Author contribution statement

Conceptualization: M.R.U. and M.K.I.; Methodology: M.R.U.; Software and validation: M.R.U. and M.F.T.; Formal analysis and investigation: M.R.U.; Resources: M.R.U. and M.F.T.; Data curation: M.R.U. and M.K.I.; Writing—original draft preparation: M.R.U.; Writing—review and editing: M.F.T., M.S.H., M.K.I. and M.F.H.; Visualization: M.R.U. and M.K.I.; Supervision: M.R.U.; Project administration: M.R.U.; Funding acquisition: M.R.U. and M.F.T. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest: The authors declare no conflict of interest.

Ethics approval: This study did not involve any animal or human participant and thus ethical approval was not applicable.

Consent for publication: All co-authors gave their consent to publish.

Data availability: The data that support the findings of this study are available on request from the corresponding author.

Supplementary data: No supplementary data is available for this paper.

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