ORIGINAL ARTICLE

Stamford Journal of Microbiology, 2021.
ISSN: 2074-5346 (Print); 2408-8846 (Online)

Vol. 11, Issue 1, p. 11-13
DOI: https://doi.org/10.3329/sjm.v11i1.57145

Isolation and characterization of nitrogen-fixing bacteria from soil sample in Dhaka, Bangladesh

Farzana Yasmin Shomi, Md. Borhan Uddin and Tamanna Zerin*

Department of Microbiology, Stamford University Bangladesh, 51, Siddeswari Road, Dhaka-1217, Bangladesh

Received 30 July 2021/Accepted 02 September 2021

Biological nitrogen (N_2) fixation is very essential for limiting the growth of plants and agricultural crops. The present study was conducted to potentially isolate N_2-fixing bacteria from garden soil sample at Stamford University Bangladesh, Siddeswari, Dhaka. Here, we used culture-dependent method to perform this experiment. Firstly, we collected garden soil sample, diluted and inoculated in N_2-free Jensen's media by maintaining the aseptic procedure. We obtained 5 different colonies from soil samples. We cultured the isolates in N_2-free Jensen's media containing bromothymol blue (BMB) and also, in Yeast Extract Mannitol Agar (YEMA) media containing congo red to confirm nitrogen fixation capacity. We collected the colony characteristics of all the isolates. Only 1A isolate showed good growth after 24 h of incubation among all the isolates. We performed ammonification test with Nessler reagent to confirm N_2-fixing ability for our selected isolates. The 1A isolate was positive in ammonification test. Culture, microscopy and biochemical tests were performed to identify isolate 1A. This isolate was presumptively identified as *Azotobacter* sp. In the present study, *Azotobacter* sp. that was isolated from the soil sample was found to be a potential N_2-fixing bacterium. Isolate 1A can be used for N_2-fixation to boost production of crops.

Keywords: *Azotobacter* sp., N_2-fixation, N_2-free media, N_2-fixing bacteria, biofertilizer.

INTRODUCTION

Nitrogen is an essential element for the growth and development of plants and agriculture. Nitrogen is the most abundant element in the atmosphere but it is biochemically unavailable for plants and most microbes as they depend upon combined or fixed forms of nitrogen (1). There are four sources from where plants can obtain these forms of "combined" nitrogen as the addition of ammonia and/or nitrate fertilizer or manure to the soil, the release of these compounds during organic matter decomposition, the conversion of atmospheric nitrogen into the compounds by natural processes, such as lightning, and biological nitrogen fixation (2). So, nitrogen fixation is an important process by which the molecular nitrogen in the air is turned into ammonia, nitrite or related nitrogenous compounds in soil (2). Biological nitrogen fixation (BNF) is performed by a special group of prokaryotes including bacterial and archaeal domains and they fix about 50% of the total nitrogen per year (1). These prokaryotes include aquatic cyanobacteria, free-living soil bacteria as *Azotobacter*, bacteria like *Azospirillum* which form an associative relationship with plants, and bacteria that form symbioses with legumes and other plants as *Rhizobium* and *Bradyrhizobium* (3, 4). The remaining 50% is fixed industrially as nitrogen fertilizer by the Haber Bosch process which provides most of the necessary amount of nitrogen to cropping systems. The Haber Bosch process brought a revolution in agricultural production as it met up the immediate demands for nitrogen as a fertilizer early in the twentieth century (2). Nitrogenous fertilizer production is not only expensive but also has harmful consequences to the environment. The harmful consequences include nitrogen oxides emission, eutrophication of water, acidification of soil, and worldwide environmental problems such as the formation of coastal dead zones (1). So, BNF is both economically and ecologically beneficial to sustainable agricultural production (1). The dependence on chemical fertilizers in agriculture must be reduced and there is significant research interest on biological nitrogen fixation and prospects for increasing its application in agricultural settings. However, the process is solely confined to bacteria and archaea and it does not occur in nitrogenous eukaryotes. Symbiotic nitrogen fixation is part of a mutualistic relationship where plants and bacteria are equally benefitted by sharing a niche and fixed carbon to bacteria in exchange for fixed nitrogen and it is restricted mainly to legumes in agricultural systems but there are a number of microorganisms, including some diazotrophs that inhabit the rhizosphere of other crop plants, some of which have been shown to enhance plant growth (5). It is well documented that BNF mediated by nitrogenase enzymes is a process important to the biological activity of soil. Nitrogen-fixing free-living microorganisms have frequently been reported as plant growth promoters (6). Therefore, this study was conducted to isolate nitrogen-fixing bacteria from rhizospheric garden soil. Isolates were also characterized to determine their nitrogen-fixing ability time-dependently.

MATERIALS AND METHODS

Sample collection and processing. The sample was collected from the garden of Stamford University Bangladesh, Siddeswari, Dhaka on March, 2020. For soil collection, a sterile beaker, a marking pen, and a spatula were

**Corresponding Author*: *Mailing address. Dr Tamanna Zerin, Assistant Professor, Department of Microbiology, Stamford University Bangladesh, 51 Siddeswari Road, Dhaka-1217, Bangladesh; E-mail: tzerin1983@gmail.com.*

used. Sufficient amount of soil sample was collected from one point in the garden in a sterile beaker and tagged it. The sample was collected from the top 6 cm of the soil profile. This layer is the preferred zone of most of the microbial activities, where most of the bacterial population is concentrated.

Processing of soil sample. 1.0 g of soil sample was transferred in a sterile beaker containing 99 ml of autoclaved normal saline and homogenized. One ml of homogenized soil sample was transferred into 9 ml sterile normal saline and serial dilution was carried out from 10^{-1} to 10^{-8} dilutions.

Screening of nitrogen-fixing bacteria from soil. Serially diluted bacterial cultures (100 µl) were inoculated on Jensen's media, a nitrogen-free media and incubated at 37°C until growth was observed. Their colony morphology was noted and pure colonies were obtained by repeating subculture through streaking on Jensen's medium. Isolated colonies were streaked on Jensen's media containing Bromothymol Blue (BMB) and Yeast Extract Mannitol Agar (YEMA) media containing Congo red to determine nitrogen fixation capacity. The Petri plates were kept in the incubator at 37°C until the growth was observed. Followed by incubation, the colony characteristics were noted.

Determination of nitrogen-fixation capacity of isolated bacteria using Nessler's reagent . The isolates were grown in peptone water, shaken at 100 rpm, at 30°C for 3, 5, 7 and 9 days. Following incubation, the broth was centrifuged at 4,000 rpm for 20 minutes and the supernatant was reserved. After treatment with 0.5 ml of Nessler's reagent to 1 ml of supernatant, the sample develops color from yellowish to brown if ammonia is present there. The color intensity of the solution was found to correspond to the amount of ammonia present in the sample (7).

Morphological and biochemical characterization of nitrogen-fixing bacteria. The isolate that showed nitrogen-fixing capacity using Nessler's reagent was subjected to morphological identification through gram staining and biochemical characterization include oxidase, catalase, triple sugar iron (TSI), motility indole urea (MIU), citrate, methyl red (MR), voges-proskauer (VP), and starch hydrolysis tests (8).

RESULTS

Isolation and characterization of nitrogen-fixing bacteria. Five different microbial colonies were found on Jensen's media from soil samples. The isolates were marked as A1, A2, A3, A4 and A5. We further cultured them in Jensen's media containing BMB and YEMA containing Congo Red to get more evidence in nitrogen fixation. All those 5 isolates took more than 7 days to get visible colonies except isolate A1 that showed good growth after 48 h of incubation and changed media color from green to yellow in Jensen's BMB media. The five colony morphologies were observed and their characteristics are presented in Table 1. Isolate A1 was taken to continue for further work for its rapid growth and cultural characteristics as a potential nitrogen fixer. The growth on Jensen's BMB media of isolate A1 is shown in Figure 1.

Determination of nitrogen-fixing capacity of the selected isolate. Ammonification test was performed using Nessler reagent to determine N_2 fixing ability of A1 isolate. Time-dependent ammonia production potential was qualitatively measured from 0 to 9th day of incubation and showed in Table 2. With the time of incubation, the production of ammonia was increased till the 9th day.

The microscopic morphology and biochemical properties of isolate A1 from soil sample is presented in Tables 3 and 4, respectively. The isolate was presumably identified as *Azotobacter* sp.

DISCUSSION

Bangladesh is a developing country with a huge population that depends much on agriculture. So, crop development is essential for Bangladesh to meet up the huge demand of the population. Chemical fertilizers have good results on crop production but at the same time, it is not so cheap and it is polluting the whole environment causing harm to plants, animals, and humans. So, N_2-fixing microorganisms can be a

Table 1. Colony characteristics of isolated bacteria from garden soil.

Media	Morphology	A1	A2	A3	A4	A5
NA	color	watery	yellow, light zone	white	white	yellow
	size	large	very large	large	small	pinpoint
	form	convex	circular	wrinkled	undulate	convex
	elevation	raised	raised	flat	raised	raised
	Media color	unchanged	unchanged	unchanged	unchanged	unchanged
JENSEN+ BMB	color	watery	yellow	white	white	yellow
	size	small	very large	large	small	pinpoint
	form	convex	circular	wrinkled	undulate	convex
	elevation	raised	raised	flat	raised	raised
	Media color	yellow	unchanged	unchanged	unchanged	unchanged
YEMA +CR	color	Watery	yellowish pink	whitish pink	whitish pink	yellowish pink
	size	large	very large	large	small	pinpoint
	form	convex	circular	wrinkled	undulate	convex
	elevation	undulate	raised	flat	raised	raised
	Media color	unchanged	unchanged	unchanged	unchanged	unchanged

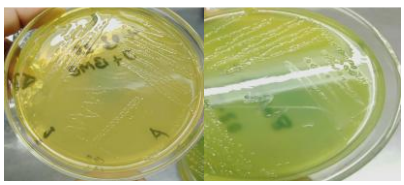


Figure 1. Colony morphology of isolate A1 from soil sample on Jensen's BMB agar media following 48 h of growth at 37°C.

Table 2. Qualitative ammonia production capacity of isolate A1.

Day	Ammonia production
0	-
3	+
5	+
7	++
9	++

Table 3. Microscopic observation of isolate A1.

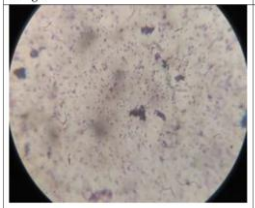
Image	Observation
	Oval or spherical, G -ve

Table 4. Biochemical characteristics of isolate A1.

Tests	**Results**
Oxidase	Positive
Catalase	Positive
TSI	Slant-yellow, Butt-yellow, H_2S-positive
MIU	Motile
Indole	Positive
Citrate	Negative
MR	Positive
VP	Negative
Starch hydrolysis	Positive
Presumptively Identified	***Azotobacter* sp.**

potential solution to all these problems due to their cost-effectiveness and eco-friendly role.

This study was focused to investigate and isolate nitrogen-fixing bacteria from soil sample which can be used as an alternative to chemical fertilizer. It is required to develop biofertilizers by determining its potent application in environment as well as cut huge costs in importing biofertilizers.

We presumptively confirmed our isolate as *Azotobacter* spp. based on all of our test results. *Azotobacter* sp. is a free N_2-fixing bacterium with proven capacity of biofertilizer for soil fertility and plant growth. It has plant promoting traits as nutrient use efficiency, protection against phytopathogens, phytohormone biosynthesis, etc. as well as high resistance to environmental stress due to cyst formation which may allow its use in various environmental conditions (9, 10). Previous studies reported that N_2-fixation in the aerobic condition is a promising criterion of the genus *Azotobacter* (11). *Azotobacter vinelandii* strain wild type isolates could excrete about 200 µM (12) and 260, 251 µM (13) concentrations of ammonia. Biofertilizers are carrier-based preparations containing mainly effective strains of microorganisms in sufficient numbers, which are useful for nitrogen fixation. Amongst the nutrients, nitrogen is the only nutrient, which plays a major role in the synthesis of chlorophyll, amino acids, and protein building blocks (14). Biofertilizers has substantial beneficial features in terms of their pollutant-free and in-expensive nature, in addition to its ability to utilize renewable resources along with the use of freely available solar energy, atmospheric nitrogen, and water. Moreover, they can provide vitamins and growth substances besides supplying N_2 to crops (14).

CONCLUSION

As a sustainable source of nitrogen, biological nitrogen fixation for plants may replace the need for industrial nitrogen production. This study revealed that our garden soil sample contained N_2-fixing bacterium which was identified as *Azotobacter* sp. based on morphological, physiological, and biochemical test results. This isolate can be used as a suitable candidate for the production of bio-fertilizer that helps to restore soil fertility for better crop response. *Azotobacter* sp. that was isolated from garden soil should be tested further in the field level to confirm their potential to be used as biofertilizer so that it can reduce the load of chemical fertilizer in crop production.

ACKNOWLEDGEMENTS

Authors are thankful to the Department of Microbiology, Stamford University Bangladesh for the technical supports provided during the study.

REFERENCES

1. Dahal B, NandaKafle G, Perkins L and Brözel VS. 2017. Diversity of free-Living nitrogen-fixing Streptomyces in soils of the badlands of South Dakota. Microbiol. Res. 195:31-39.
2. Alshibli NA. 2018. Diversity of Free – Living Nitrogen – Fixing Bacteria in Soil of Sioux Prairie of South Dakota. Electronic Theses and Dissertations. 2959. https://openprairie.sdstate.edu/etd/2959.
3. Smercina DN, Evans SE, Friesen ML and Tiemann LK. 2019. To fix or not to fix: Controls on free-living nitrogen fixation in the rhizosphere. Appl. Environ. Microbiol. 85:6.
4. Shridhar BS, Author C, Shrimant Shridhar B, Shridhar BS, Author C and Shrimant Shridhar B. 2012. Review: Nitrogen-fixing Microorganisms. Int. J. Microbiol. Res. 3(1):46-52.
5. Cummings S. 2005. The role and future potential of nitrogen-fixing bacteria to boost productivity in organic and low-input sustainable farming systems. Environ. Biotechnol. 1(1):1-10.
6. Sadik AS, Noof AE and Sonya HM. 2016. Isolation and Characterization Of Free-Nitrogen Fixer Bacterial Strains (*Azotobacter* Sp.) and Their Phages From Maize Rhizosphere Soil At TAIF. Pakistan J. Biotechnol. 13(1):31-37.
7. Alves Rodrigues A, Vinicius Forzani M, de Souza Soares R, Sibov ST and Gonçalves Vieira JD. 2016. Isolation and selection of plant growth-promoting bacteria associated with sugarcane. Pesqui. Agropecu. Trop. 46(2):149-158.
8. Harley JP and Prescott LM. 2002. Laboratory Exercises in Microbiology. 5th Edition, The McGraw-Hill Companies.
9. Aasfar A, Bargaz A, Yaakoubi K, Hilali A, Bennis I, Zeroual Y and Meftah Kadmiri I. 2021. Nitrogen Fixing *Azotobacter* Species as Potential Soil Biological Enhancers for Crop Nutrition and Yield Stability. Front. Microbiol. 12:628379.
10. Kasa P, Modugapalem H and Battini K. 2015. Isolation, screening, and molecular characterization of plant growth promoting rhizobacteria isolates of *Azotobacter* and *Trichoderma* and their beneficial activities. J Nat Sci Biol Med.6(2):360-363.
11. Brenner DJ, Noel RK and James TS. 2005. Bergeys manual Systematic Bacteriology. 2nd edition. Springer Science Business Media, Inc. New York. USA.
12. Bali A, Blanco G, Hill S and Kennedy C. 1992. Excretion of ammonium by a nifL mutant of *Azotobacter vinelandii* fixing nitrogen. Appl. Environ. Microbiol. 58:1711-1718.
13. Gordon JK and Jacobson MR. 1983. Isolation and characterization of *Azotobacter vinelandii* mutant strains with potential as bacterial fertilizer. Can. J. Microbiol. 29:973-978.
14. Alhia BMH. 2010. The Effect of *Azotobacter chrococcum* as Nitrogen biofertilizer on the growth and yield of *Cucumis Sativus*. December 2010. http://hdl.handle.net/20.500.12358/21551.