ORIGINAL ARTICLE

Stamford Journal of Microbiology, 2020.
ISSN: 2074-5346 (Print); 2408-8846 (Online)

Vol. 10, Issue 1, p. 12-15
DOI: https://doi.org/10.3329/sjm.v10i1.50726

Isolation of *Bacillus* spp. from rhizosphere of garden soil: their potential role in amylase production and nitrogen cycle

Tamanna Zerin*

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

Received 28 July 2020/Accepted 02 September 2020

Rhizobacteria influence plant growth by producing various substances like enzymes and play role in nitrogen cycle. Microbes are the most important sources of enzymes because of their stability and reduced price in production. In our present investigation, soil samples were collected from rhizosphere of garden soil and a total of eight isolates of *Bacillus* spp. were presumptively identified by morphological, cultural and biochemical analysis with the conventional technique. The isolates were named as A1, A3, A4, A5, B2, C1, D1 and D2. Amylase enzyme assay was performed by 3, 5-dinitrosalicylic acid method. The highest enzyme activity was observed with the isolate A4 (2.71 U/ml) followed by isolates D2 (2.54 U/ml) and D1 (2.52 U/ml). The highest ammonification was found to be positive with the isolates A4 and B2 followed by isolate D1. Denitrification potential was found to be highest with isolates A4 and A5. No isolates were found to fix atmospheric nitrogen in Jensen's media after two weeks of incubation. The isolates have a great potential of amylase production and they can be used in different industries as well as in starch rich waste degradation. Involvement of these bacteria in nitrogen cycle may help promote growth of plants.

Keywords: Amylase, Nitrogen fixation, *Bacillus*, Denitrification, Ammonification.

INTRODUCTION

The steadily increasing global population is limiting the arable land to cope with the increasing demand for food supply (1). Overuse of chemical fertilizers and pesticides, introduction of genetically modified crops with higher productivity and disease-resistance mechanism are often introduced to make up such loss. All these activities have prolonged detrimental effect on soil fertility, animal food chain and human health. Controversial application of genetic manipulation of plants may have unknown effect on them (1). Therefore, many agrochemicals are already banned or restricted due to their detrimental effect on the environment, which directly or indirectly affects animal and human health. Sometimes, not only the active components but also the disclosed additives used in agrochemical formulations, become the reason of a terrible impact on the environment (2). Thus, the use of environment-friendly and sustainable new biotechnological products such as, microbial biofertilizers (plant growth promoting rhizobacteria, PGPRs) is progressively increasing (3). Commercial biofertilizer 'Alicit' developed from *Bacillus subtilis* was found to improve crop yield by 40%. Other commercial *Bacillus* spp. based products used for increasing crop production are Kodiak (*B. subtilis* GB03), Quantam-400 (*B. subtilis* GB03), Rhizovital (*B. amyloliquefaciens* FZB42), Serenade (*B. subtilis* QST713) and YIB (*Bacillus* spp.) (4, 5, 6). Commercial formulations of *Bacillus* spp. have become major choice to improve crop yield due to their valuable metabolite production that improves plant yield and control plant pathogen, heat and desiccation resistant spore formation that improved the viability in commercial formulation (7, 8, 9, 10). Therefore, extensive study on *Bacillus* spp. pertaining to its plant growth promoting properties with an emphasis on plant pathogen controlling activities and their possible implication in crop ecosystem in Bangladesh will be valuable. This study will help primarily explore the scope of *Bacillus* spp. which are isolated from rhizosphere of garden soil for amylase production and their role in the nitrogen cycle.

MATERIALS AND METHODS

Sample collection and processing. Soil samples were collected in a sterile beaker using a sterile spatula at a depth of 2-3 cm from a garden in Ramna Park, Dhaka, Bangladesh. One gram of soil sample was dispensed into 99 ml of sterile distilled water and homogenized. One ml of homogenized soil sample was transferred into 9 ml sterile distilled water and serial dilution was carried out up to 10^{-8} dilution.

Isolation and screening of amylolytic bacteria. Serially diluted bacterial cultures (100 µl) were spread on nutrient agar media and incubated at 37°C for 24 h. Their colony morphology of isolated colonies were noted and streaked on starch agar media containing starch as sole carbon source for starch hydrolysis test to detect their amylolytic activity. The plates were incubated at 37°C for 24-48 h. Following incubation, plates were flooded with Gram's iodine solution (Gram's iodine- 250 mg iodine crystals added to 2.5 g potassium iodide solution and 125 ml of water) to identify zone of clearance around the colony. Deep blue color around the growth indicates negative result that is no amylolytic activity where zone of clearance was produced by amylase producers. The pure cultures showing clear zones were subcultured at regular interval and maintained on to nutrient agar slants at 4°C.

Identification of amylase producing bacteria. Isolated amylase producing bacteria were presumptively identified by Gram staining, cultural and biochemical characteristics. The biochemical tests included methyl red, voges-proskauer, citrate utilization, indole production, H_2S production, motility, gelatine hydrolysis, sugar hydrolysis, oxidase, catalase and carbohydrate fermentation. Mannitol egg yolk polymyxin (MYP) media (Egg yolk emulsion and Polymyxin B sulphate were added in MYP agar base) was

***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.*

Table 1. Microscopic observation of isolated *Bacillus* spp.

Microscopic observation	**A1**	**A3**	**A4**	**A5**	**B2**	**C1**	**D1**	**D2**
Shape	Short rod	Short rod	Long rod	Short rod	Short rod	Long rod	Long rod	Short rod
Arrangement	Single	Single	Single	Single	Single	Single	Single	Single
Gram reaction	Gram positive	Gram positive	Gram positive	Gram positive	Gram positive	Gram positive	Gram positive	Gram positive

Table 2. Colony characteristics of the *Bacillus* spp. on nutrient agar media.

Characteristics	**A1**	**A3**	**A4**	**A5**	**B2**	**C1**	**D1**	**D2**
Size	Medium	Small	Large	Medium	Medium	Large	Large	Small
Shape	Irregular	Circular	Irregular	Irregular	Irregular	Irregular	Irregular	Circular
Margin	Undulate	Entire	Undulate	Undulate	Undulate	Entire	Undulate	Entire
Elevation	Umbonate	Flat	Raised	Raised	Flat	Raised	Raised	Flat
Texture	Gummy	Smooth	Smooth	Dry	Dry	Dry	Smooth	Smooth
Pigmentation	Off white	Creamy white	White	Off white	Off white	Off white	White	Off white

Table 3. Biochemical characteristics of the *Bacillus* isolates.

Biochemical tests	**Media**	**Observation**							
		A1	**A3**	**A4**	**A5**	**B2**	**C1**	**D1**	**D2**
Starch hydrolysis	Starch agar plate	+	+	+	+	+	+	+	+
Lecithinase	MYP media	Y/No Halo	Y/No Halo	P/ Halo	Y/No Halo	Y/No Halo	P/Halo	P/ Halo	Y/ No Halo
Methyl red test	GPB broth	+	+	+	+	+	+	+	+
Voges-proskauer test	GPB broth	-	-	-	-	-	-	-	-
Indole production	1% peptone	-	-	-	-	-	-	-	-
H_2S production	2% peptone	-	-	-	-	-	-	-	-
Sugar hydrolysis	Triple sugar iron agar slant (Slant/Butt)	K/A	K/A	A/A	K/A	K/A	A/A	A/A	A/A
Oxidase	Nutrient agar	+	-	+	+	+	+	+	+
Catalase	Nutrient agar	+	+	+	+	+	+	+	+

Note: P, Y, K and A stand for pink, yellow, alkaline and acidic, respectively.

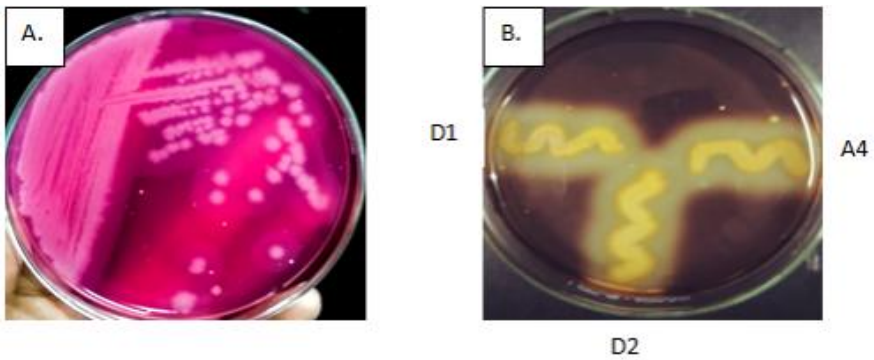

Figure 1. The representative picture for growth of isolates in MYP media and Starch agar media is presented. The growth of isolate A4 in MYP media is clearly visible with the zone for hydrolysis of lecithin (A). The clear zone of starch hydrolysis is visible after growth of isolates A4, D2 and D1 on Starch agar media (B).

used to selectively isolate only *Bacillus cereus* which was purchased as dehydrated media from Oxoid Ltd (Basingstoke, UK), prepared according to the manufacturer's instructions. Only those isolates that are presumptively isolated as *Bacillus* spp. were further analyzed by different experiments.

Crude enzyme preparation and extraction. To extract amylase enzyme, a loopful of pure culture was inoculated in production media containing starch (10 g/l), peptone (5 g/l), $(NH_4)_2SO_4$ (2 g/l), KH_2PO_4 (1 g/l), K_2HPO_4 (2 g/l), $MgCl_2$ (0.01 g/l) at pH 7 and incubated at 37°C in a shaking water bath for 24 h. Following incubation, 10 ml of 24 h old culture was centrifuged at 5,000 rpm for 15 min. Cells were discarded and supernatant was decanted for crude enzyme that was used for optimization of assay condition for amylase activity.

Amylase assay. Amylase activity was assayed by employing 3, 5 dinitrosalicylic acid (DNS) method as described previously with few modifications (11). In brief, 1% starch solution was prepared freshly by dissolving 1 g of soluble starch in 100 ml of 0.02 M sodium phosphate buffer (pH, 7). To prepare assay condition, 1 ml of 1% starch solution and 0.5 ml of crude enzyme extract was incubated at 50°C for 30 min. The assay was stopped by adding 3 ml of DNS reagent and heated the solution in a boiling water bath for 10 min. Then, with running tap water the solution was cooled. The solution volume was brought up to 10 ml by adding distilled H_2O and the absorbance was recorded using spectrophotometer. A blank was always prepared without the enzyme. Enzyme activity was measured by preparing a standard graph with known concentrations of standard (glucose) and plotted. Here, one unit (U/ml) of amylase activity is defined as the amount of amylase required to catalyze 1 µmol of reducing sugar (glucose) from starch per minute under the assay condition.

Production of ammonia. Isolates with the ability to release simple ammonia from complex proteins are considered as potential for plant growth. To detect ammonia excretion, freshly grown bacterial cultures were inoculated in 5 ml sterilized peptone water containing test tubes and incubated at 37°C for 48 h. Following incubation, 1 ml of Nessler's reagent was added to peptone water. The tubes were thoroughly shaken and centrifuged at 12,000 rpm for 15 min. The supernatants were taken to measure absorbance at 450 nm in a spectrophotometer (12).

Denitrification. Denitrification is a step where nitrates are reduced to nitrites, ammonia, nitrous oxide and finally to elemental nitrogen in the form of nitrogen gas. To detect nitrification, freshly grown bacterial isolates were inoculated in nitrate broth containing Durham tube. Following 48 h of incubation at 37°C, the test cultures were examined for the presence or absence of the air bubble in the Durham tube (12).

Nitrogen fixation capability. To detect whether the isolates can fix atmospheric nitrogen, freshly grown bacterial cultures were streaked in nitrogen-free Jensen's media (sucrose 10 g, dipotassium hydrogen phosphate 1 g, magnesium sulphate 0.5 g, sodium chloride 0.5 g, ferrous sulfate 0.1 g, sodium molybdate 0.005 g, agar 20 g, for 1 liter, pH 7.0-7.2) (13). The media is devoid of any nitrogen source. The media were incubated at 37°C and checked each day for any growth till 15 days.

RESULTS

Isolation and characterization of *Bacillus* spp.

Among a good number of bacterial isolates, only 8 bacterial isolates namely A1, A3, A4, A5, B2, C1, D1 and D2 were presumptively found to belong to the genus *Bacillus* following characterization by morphological (Table 1), cultural (Table 2) and biochemical tests (Table 3). A representative picture for the growth of isolates on MYP agar and Starch agar media are presented in Figure 1.

Comparative amylolytic activity of the *Bacilllus* isolates.

The highest amylolytic activity was observed with the isolate A4 and that was 2.71 U/ml where other isolates, D2, D1, A3, A1, A5, C1 and B2 showed lower amylolytic activity from 2.54 to 2.06 U/ml (Figure 2).

Role of *Bacillus* isolates in N_2 cycle.

Ammonification extent was measured by taking absorbance at 450 nm. The highest ammonification was observed with the two isolates namely A4 and B2. Isolate D1 also showed good potentiality in ammonification but the latter was gradually declined subsequently with the isolates A5, C1, D2, A3 and A1 (Figure 3). Denitrification potential also observed with those isolates by forming gas in Durham tube. Denitrification ability was found the highest between isolates A4 and A5, moderate among isolates B2, C1, D1, and D2 isolates, and the lowest between isolates A1 and A3. Whereas, no nitrogen fixation potential

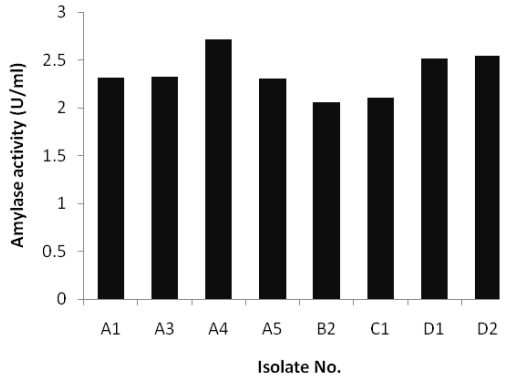

Figure 2. The amylolytic activity of the *Bacillus* isolates.

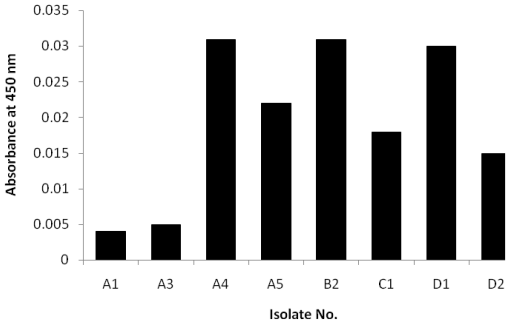

Figure 3. The capability of ammonia release from peptone broth by the isolates.

was observed among any of the isolates as no growth was found following 15 days of their inoculation in Jensen media (Table 4).

DISCUSSION

The eco-friendly indigenous microbes in the soil are becoming extinct due to the regular increasing usage of agrochemicals to keep the yield same. We have isolated eight *Bacillus* isolates from a garden soil at a depth of 2-3 cm to isolate rhizobacteria. All the isolates were presumptively distinguished as *Bacillus* sp. We observed great potential of amylase activity in all the isolates that is over 2 U/ml. The highest amylolytic activity was observed in A4 isolate (2.71 U/ml). However, *Bacillus* spp. are common sources of both α and β amylases (14, 15, 16). Those enzymes have great importance in various industries as food, textile, paper, detergent as well as they might contribute to starchy waste degradation that can be used as a raw material for high value enzyme production or can be used as biofertilizer (17, 18, 19). However, all the isolates showed capability of converting peptone water to ammonia but the highest yield was observed with the isolates A4 and B2. Ammonification potential is necessary for plant growth promotion. However we also tried to detect whether the isolates can perform denitrification or

Table 4: The strength of denitrification and N_2 fixation abilities by the isolates.

Properties	A1	A3	A4	A5	B2	C1	D1	D2
Denitrification	+	+	+++	+++	++	++	++	++
N_2 fixation	-	-	-	-	-	-	-	-

Note: -, negative; +, weak; ++, medium; +++, strong reaction.

nitrogen fixation. All the isolates were capable of releasing gas in the environment from nitrate or nitrite form but that was not firmly detected as nitrogen gas. Previous studies also showed the contribution of *Bacillus* spp. in ammonification and denitrification processes (20, 21). From our experiments, none of the *Bacillus* isolates were capable of growing in Jensen media that is devoid of nitrogen source. Conversely, some studies showed nitrogen fixation capabilities of some *Bacillus* spp. (22, 23, 24, 25). The picture for plant growth promotion characteristics by the isolates is incomplete due to the lack of sufficient relevant experiments. As *Bacillus* species are used in various countries as biofertilizer, therefore, detailed studies are necessary to come up with our own indigenous microbe for biofertilizer purpose. After proper investigation, with exact dosage, we may establish biofertilizer use in our country, as chemical fertilizers are reducing soil fertility and are very expensive as well.

CONCLUSION

Day by day, the use of increasing dosage of chemicals in lands reduces its productivity, making the land unfertile; losing the aroma/flavor and taste of vegetables, fruits, and crops; polluting the environment, and imposing a threat to human and animal health as well. Although there are very few researches going on and very few commercialized biofertilizer and biopesticides are available in Bangladesh, even in some cases, the quality is questionable. More research should be done on exploring new potential environmentally friendly microbe that can improve crop yield along with retaining soil fertility and will be easier to prepare in commercial formulations. In addition, optimum use of such microorganisms needs to be studied in order to achieve best outcome with optimal combinations and environmental conditions.

ACKNOWLEDGEMENTS

Author wants to thank the Department of Microbiology, Stamford University Bangladesh for the technical supports provided during the study.

REFERENCES

1. Borriss R. 2011. Use of Plant-Associated *Bacillus* Strains as Biofertilizers and Biocontrol Agents in Agriculture. In: Maheshwari D. (eds) Bacteria in Agrobiology: Plant Growth Responses. Springer, Berlin, Heidelberg.
2. Mann R and Bidwell J. 1999. The toxicity of glyphosate and several glyphosate formulations to four species of southwestern Australian frogs. Arch. Environ. Contam. Toxicol. 36:193.
3. Lugtenberg B and Kamilova F. 2009. Plant-growth promoting rhizobacteria. Annu. Rev. Microbiol. 63:541-556.
4. Brannen P and Kenney D. 1997. Kodiak®-A successful biological control product for suppression of soil borne plant pathogen of cotton. J. Ind. Microbiol. Biotechnol. 19:169-171.
5. Ngugi H, Dedej S, Delaplane K, Savelle A and Scherm H. 2005. Effect of flower-applied Serenade biofungicide (*Bacillus subtilis*) on pollination related variables in rabbit eye blueberry. Biol. Control. 33:32-38.
6. Cawoy H, Bettiol W, Fickers P and Ongena M. 2011. *Bacillus*-based biological control of plant diseases, in pesticides in the Modern World-Pesticides Use and Management, ed Stoytcheva (InTech Academic Press), 273-302.
7. Haas D and Defago G. 2005. Biological control of soil-borne pathogens by fluorescent pseudomonads. Nat. Rev. Microbiol. 3:307-319.
8. Emmert EAB and Handelsman J. 1999. Biocontrol of plant disease: a (Gram-) positive perspective. FEMS. Microbiol. Lett. 171:1-9.
9. Kloepper JW, Ryu C-M and Zhang S. 2004. Induced systemic resistance and promotion of plant growth by *Bacillus* spp. Phytopathology. 94:1259-1266.
10. McSpadden Gardener BB and Fravel DR. 2002. Biological control of plant pathogens: research, commercialization, and application in the USA. Plant Health Progress. doi;10.1094/PHP-2002-0510-01-RV.
11. Bernfeld P. 1955. Amylase α and β. Meth. Enzymology. 1:149-158.
12. Cappuccino JC and Sherman N. 1992. In: Microbiology: A Laboratory Manual, New York, pp. 125-179.
13. Jensen HL. 1955. Non-symbiotic nitrogen fixation In: Soil nitrogen (eds.) Barthdlomew, W.V. and Clark, F. E. American Soc. Agron. Inc. Madison, 10:436-480.
14. Divakaran D, Chandran A and Pratap Chandran R. 2011. Comparative study on production of α-Amylase from *Bacillus licheniformis* strains. Braz. J. Microbiol. 42:1397-1404.
15. Raplong H, Odeleye P, Chrinius H, Idoko M, Adudu J and Odeke E. 2014. Production of Alpha Amylase by *Bacillus cereus* in Submerged Fermentation. Aceh. Int. J. Sci. Technol. 3:124-130.
16. Niziołek S. 1997. beta-Amylase production by some *Bacillus cereus*, *Bacillus megaterium* and *Bacillus polymyxa* [correction of polymaxa] strains. Acta. Microbiol. Pol. 46:357–362.
17. de Souza PM and de Oliveira Magalhães P. 2010. Application of microbial α-amylase in industry - A review. Braz. J. Microbiol. 41:850-861.
18. Sayeki M, Kitagawa T, Matsumoto M, Nishiyama A, Miyoshi K, Mochizuki M et al. 2001. Chemical composition and energy value of dried meal from food waste as feedstuff in swine and cattle. Anim. Sci. J. 72:34-40.
19. Lin LL, Chyau CC and Hsu WH. 1998. Production and properties of a raw starch-degrading amylase from the thermophilic and alkalophilic *Bacillus* sp. TS-23. Biotechnol. Appl. Biochem. 28:61-68.
20. Hui C, Wei R, Jiang H, Zhao Y and Xu L. 2019. Characterization of the ammonification, the relevant protease production and activity in a high-efficiency ammonifier *Bacillus amyloliquefaciens* DT. Int. Biodeter. Biodegr. 142:11-17.
21. Kim JK, Park KJ, Cho KS, Nam SW, Park TJ and Bajpai R. 2005. Aerobic nitrification–denitrification by heterotrophic *Bacillus* strains. Bioresour. Technol. 96:1897-1906.
22. Moore A and Becking J. 1963. Nitrogen Fixation by *Bacillus* Strains isolated from Nigerian Soils. Nature. 198:915-916.
23. Abdel Wahab AM. 1975. Nitrogen fixation by *Bacillus* strains isolated from the rhizosphere of *Ammophila arenaria*. Plant Soil. 42:703-708.
24. Abdel Wahab AM and El-Sharouny HM. 1979. Nitrogen-fixing bacillus species from Egyptian soils: Acetylene reduction and cultural conditions. Plant Soil. 51:187-196.
25. Hiremath G. 2012. Isolation and characterization of nitrogen fixing *Bacillus subtilis* strain AS-4 from agricultural soil. Int. J. Recent Sci. Res. 3:762-765.