Jahangirnagar University J. Biol. Sci. **3**(1): 47-53, 2014 (June)

Nitrogen fixing efficiency and physiological characteristics of *Azospirillum* isolates from the paddy fields of North Bengal

Md. Mozammel Hossain, Salina Akter*, Md. Mahmudul Hasan, Ashraful Hasan, Kazi Rasel Uddin, Afroza Parvin, Iffat Jahan, Nazibur Rahman and S. M. Badier Rahman

Department of Biochemistry and Molecular Biology, Jahangirnagar University, Savar, Dhaka-1342, Bangladesh

Abstract

The microbial activity of ten selected *Azospirillum* isolates was measured in terms of the amount of CO_2 evolved by the isolates after incubation for 5 days which ranged from 6.88 to19.25 mg. Nitrogen fixing efficiency all of the isolates was determined by microkjeldhal method and the nitrogen fixing efficiency ranged from 10.03 to 13.11 mg N/g substrate. The growth of *Azospirillum* isolates was significant affected by different physiological factors such as pH, temperature and salinity. Most of the isolates showed optimum growth at pH 6.8, temperature 37°C and in the absence of NaCl. *Azospirillum* has the potential to be used as a substitute and or supplement of N-fertilizers. Further research is needed to estimate N-supplement potentials of biological nitrogen fixation (BNF) systems at the farm level.

Key words: *Azospirillum*, isolates, north Bengal, pH, temperature, salinity, biofertilizer.

INTRODUCTION

Biological approaches are usually less expensive, harmless and in the reach of all the countries. The utilization of biological nitrogen fixation (BNF) technology can also decrease the use of urea-N, prevent the depletion of soil organic matter and reduce environmental pollution to a considerable extent. *Azospirillum* a plant growth prtomoting bacteria is being used as biofertilizer in several countries of the world. It is a soil bacterium capable of producing associative symbiosis in the roots of various plants including grain crops including rice. *Azospirillum* promotes plant growth by fixing atmospheric nitrogen and by some other ways like production of growth promoting substances and influencing root development, causing increased uptake of nutrients from the land, and inhibiting pathogenic fungi and bacteria in the rhizosphere. *Azospirillum* inoculation increases percentage of rice seed germination (treated 50% : untreated 20%) (Kannan & Ponmurugan, 2010; Ravikumar *et al*., 2002). Inoculation of plants with *Azospirillum* has been found to cause significant increases in growth and yield which is equivalent to that is attainable by application of 15-20 kg N/ha. A yield increase in rice due to inoculation of *Azospirillum* is reported to be in the 5–60% range (Kumar & Balasubramanian, 1986). The aims and objectives of the present study were to determine microbial activity, nitrogen fixing efficiency and the effect of different physiological factors such as pH, temperature, salinity on the growth of different *Azospirillum* isolates.

* Author to whom all correspondences should be made.

MATERIALS AND METHODS

Isolates collection: Ten *Azospirillum* isolates (Table 1), isolated from rhizosphere soils (M-6 and M-9) and roots of rice(M-1, M-2, M-3, M-4, M-5, M-7, M-8 and M-10) (*Oryza sativa*) growing on rice fields of particular locations of three different districts of North Bengal- Bogra, Naowgoan and Dinajpur were selected for inoculation. These isolates were identified by different biochemical tests.

Table 1. Screening of *Azospirillum* isolates

Selected isolates	Identified species	Location	Selected isolates	Identified species	Location
M-1	*Azospirillum brasinense*	Shahjahnpur, Bogra	M-6	*Azospirillum lipoferum*	Naowgaon Sadar, Naowgaon
M-2	*Azospirillum brasinense*	Shahjahnpur, Bogra	M-7	*Azospirillum lipoferum*	Adamdighi, Bogra
M-3	*Azospirillum lipoferum*	Adamdighi, Bogra	M-8	*Azospirillum lipoferum*	Dinajpur Sadar, Dinajpur
M-4	*Azospirillum lipoferum*	Dinajpur Sadar, Dinajpur	M-9	*Azospirillum halopraeferens*	Dinajpur Sadar, Dinajpur
M-5	*Azospirillum brasinense*	Naowgaon Sadar, Naowgaon	M-10	*Azospirillum lipoferum*	Dinajpur Sadar, Dinajpur

Determination of microbial activity: Microbial activity of the selected isolates was determined by measuring the amount of CO_2 evolved (mg) by the culture. CO_2 evolution was estimated according to the method described by Pramer & Schmidt (1964).

Determination of nitrogen-fixing efficiency of the *Azospirillum* isolates: Nitrogen fixation was determined in terms of the quantity of nitrogen (mg N/g substrate) gained in the 72 hours old cultures of each isolates developed in 25 ml semi-solid nitrogen free malate medium (without bromothymol rblue). Total nitrogen content in culture was estimated by microkjeldahl method.

Effect of pH on the activity of the *Azospirillum* isolates: Semi-solid Nfb medium was prepared and pH of the medium was adjusted to 5.0, 6.0, 6.8, 8.0 and 9.0 respectively just prior to adding agar. Effect of different pH on the activity of selected isolates was determined by CO_2 evolution method (Pramer & Schmidt, 1964).

Effect of temperature on the activity of the *Azospirillum* isolates: To find out the maximum, minimum and optimum temperature, each isolate was allowed to grow in Nfb semi-solid medium at temperature 30°C, 37°C, 40°C, 45°C respectively for 5 days. Then the activity of each isolate was estimated by CO_2 evolution method (Pramer & Schmidt, 1964).

Effect of salinity on the growth of the *Azospirillum* isolates: To determine the effect of salinity on the growth of the *Azospirillum* isolates tubes of liquid malate medium with no

Bromothymol blue but containing 1.0% NH_4Cl was used. Tubes of the medium containing various concentrations of NaCl (1.0%, 1.5%, 2.0%, 2.5%, 3.0%, 3.5%, and 4.0%) were inoculated with the selected isolates and incubated for 48 hours. The growth level was measured by spectromertically at 620 nm (Usha & Kanimozhi, 2011).

RESULTS AND DISCUSSION

The activities of the selected isolates of *Azospirillum* under N_2-fixing condition have been studied. Carbon dioxide is one of the principle metabolic products of microorganisms and CO_2 evolution during microbial growth has frequently been used as a measure of microbial activity (Waksman & Starkey, 1957). Activity of selected isolates of *Azospirillum* measured in terms of quantity of CO_2 (mg) evolved after 5 days varied significantly (Table 2). Amounts of CO_2 evolved by the selected isolates ranged from 6.88 to 19.25 mg. M-6 and M-8 evolved more CO_2 than the other selected isolates. On the basis of activities the selected isolates could be arranged as M-6, M-8>M-1>M-9>M-7>M-4>M-10>M-2>M-5>M-3.

Table 2. Microbial activity of *Azospirillum* isolates in N_2-fixing condition at 37°C and pH 6.8 after 5 days

Selected isolate	Evolved CO_2 (mg)
M-1	16.5
M-2	8.56
M-3	6.88
M-4	14.44
M-5	8.43
M-6	19.25
M-7	15.75
M-8	19.25
M-9	15.81
M-10	13.75

Azospirillum readily utilize organic acids like malate, succinate, pyruvate, and lactate for its growth (Tarrand *el al*., 1978). In this study malate was used as sole carbon source for the determination of nitrogen fixing potential of the selected isolates. Values equivalent to the highest efficiencies of nitrogen fixation first reported by Dobereiner and Day (1976). 115 mg N per g lactate has not been reported in other studies. About *in vitro* nitrogen fixation, Okon *et al.*, (1977) reported values of 20 to 24, Nelson & Knowles (1978) 4.7 to 28 and Lakshmi *el al.*, (1977) 12 to 36 mg N per g substrate. Lakshmi & Dhala (1984) reported that some aquatic isolates of *Azospirillum* has nitrogen fixing potential ranging from 3.08 to 11.9 mg N/100 ml culture. In the present investigation, the selected isolates were found to fix nitrogen ranging from 10.03 to 13.11 mg N per g substrate (malate) (Table 3). As per their nitrogen fixing capability the selected isolates could be arranged as M-10>M-7>M-6>M-1>M-4>M-9>M-8>M-3>M-2>M-5. Khan & Hossain (1990) found that nitrogen fixation by ten isolates of *Azospirillum* ranged from 2.9 to 7.3 mg N/50 ml culture. Khan & Akond (1996) however reported lower values, the amount of nitrogen

fixed by their isolates ranged from 448 to 658 µg N/25 ml culture. Khan *et al*. (2001) reported that the N_2-fixing potentials of *Azospirillum* isolated from wheat fields of Dhaka ranged from 15.12 to 22.16 mg N/g substrate. Khan *et al.* (2001) also reported that some thermophilic isolates of *Azospirillum* isolated from Bangladesh could fix nitrogen well at 55°C, and the values ranged from 10.08 to 28.00 mg N/g substrate.

Table 3. Nitrogen fixing efficiency of the selected *Azospirillum* isolates

Selected isolate	mg N/g substrate
M-1	12.00
M-2	10.11
M-3	10.20
M-4	11.33
M-5	10.03
M-6	12.02
M-7	12.15
M-8	10.21
M-9	11.29
M-10	13.11

In this study, activities of all the selected isolates were observed optimum at pH 6.8 but least at pH 5.0 and 9.0 (Table 4). At pH 6.8 both M-6 and M-8 showed the highest activities evolving 19.25 mg CO_2, whereas M-3 demonstrated its lowest activity evolving 6.88 mg CO_2 after 5 days incubation. Tilak *et al.* (1988) found that N_2-fixation for 3 isolates of *Azospirillum* ranged for 6.5 to 8.5 mg at their optimum pH values.

Table 4. Effect of pH on the activity of *Azospirillum* isolates in N_2-fixing condition at 37°C after 5 days

Isolate	Evolved CO_2 (mg) at different pH				
	5	6	6.8	8	9
M-1	3.44	8.95	16.5	8.94	7.18
M-2	3.44	5.26	8.56	8.25	2.06
M-3	6.19	6.42	6.88	6.19	3.43
M-4	6.19	10.12	14.44	13.75	6.89
M-5	3.44	5.22	8.43	7.56	7.56
M-6	4.81	15.33	19.25	12.38	5.5
M-7	4.81	13.12	15.75	15.13	12.38
M-8	5.84	9.63	19.25	8.94	4.81
M-9	4.81	10.11	15.81	6.89	4.81
M-10	4.13	8.52	13.75	8.25	5.5

In this study, the activity of the selected isolates was found to be optimum at 37°C, except M-9 the optimum temperature of which was 40°C (Table 5). At 37°C the values of activity ranged from 19.25 mg CO_2 in M-6 and M-8 to 6.88 mg CO_2 in M-3 after incubation for 5 days. The activities were low at 30°C and 45°C. The results indicated that activity decreases with the increase of temperature above 40°C. Khan *et al.* (2001) found that some thermophilic isolates of *Azospirillum* exhibited higher growth and N_2-

fixation at 55°C than at 35°C. The high temperature requirements of these organisms are of great ecological importance as in temperate regions soil temperatures seldom reach 28°C for any significant period. In the tropics, however, optimal temperatures for nitrogenase activity of this system occur during the main growing season almost daily for most of the days. Nitrogen fixation by a tropical *Azospirillum* isolate was highly reduced when the isolate was transferred from 36°C to 17°C (Day & Dobereiner, 1976). It shows that *Azospirillum* are highly adaptive to their native environment.

Table 5. Effect of temperature on the activity of *Azospirillum* isolates in N_2-fixing condition at pH 6.8 after 5 days

Isolate	Evolved CO_2 (mg) at different temperature			
	30°C	37°C	40°C	45°C
M-1	5.26	16.5	10.3	2.09
M-2	7.38	8.56	6.23	3.15
M-3	5.56	6.88	5.25	2.09
M-4	9.5	14.44	10.19	5.26
M-5	6.22	8.43	6.45	4.21
M-6	7.38	19.25	15.47	3.15
M-7	9.5	15.75	13.42	3.15
M-8	8.44	19.25	16.82	6.32
M-9	7.38	15.81	17.4	2.09
M-10	7.91	13.75	9.63	3.15

In this study, growth of the selected isolates of *Azospirillum* was highly affected by NaCl, and growth in all isolates, except isolate M-8 and M-9 gradually declined with increasing concentrations of NaCl in the medium (Table 6). Growth of isolate M-1, M-2 and M-5 was absent at all concentration of NaCl. Growth was almost completely inhibited at 4% NaCl in all isolates except M-8 and M-9 at which little growth was observed for M-8 and highest growth was observed for M-9. The growth of M-9 gradually increased with the increasing of NaCl concentration. Low concentrations of NaCl produce an accelerating effect on the growth of bacteria (Salle, 1967). High concentrations of NaCl are generally inhibitory. Maximum count of E. coli was found at a concentration of 0.2 M (1.17%) (Salle, 1967). In addition to affecting osmotic pressure, high salt concentrations tend to denature proteins and obligate halophiles possess specialized enzymes that are in their active configuration only at high salt concentrations (Atlas & Bartha, 1981). In the present investigation none of the isolates, except M-9 preferred saline condition for proper growth. Similar results were reported by Khan & Akond (1996). They found that N_2-fixation by 5 isolates of *A. brasilense* gradually decreased with the increase of the concentrations of NaCl in the medium. Only isolate M-9 preferred saline condition as reported by Rahman *et al.* (2007).

Table 6. Growth of the selected *Azospirillum* isolates at different concentration of NaCl

Isolate	Growth in term of O.D. at 620 nm						
	Different concentration of NaCl (%)						
	1	1.5	2	2.5	3	3.5	4
M-1	0.000	0.000	0.000	0.000	0.000	0.000	0.000
M-2	0.000	0.000	0.000	0.000	0.000	0.000	0.000
M-3	0.063	0.042	0.029	0.010	0.000	0.000	0.000
M-4	0.119	0.095	0.038	0.037	0.037	0.000	0.000
M-5	0.000	0.000	0.000	0.000	0.0000	0.000	0.000
M-6	0.022	0.016	0.007	0.007	0.003	0.000	0.000
M-7	0.077	0.062	0.026	0.026	0.023	0.010	0.000
M-8	0.040	0.040	0.038	0.036	0.036	0.031	0.028
M-9	0.007	0.009	0.015	0.013	0.010	0.026	0.039
M-10	0.074	0.056	0.043	0.033	0.029	0.010	0.000

It has been observed in this study that Azospirillum can fix significant amount of atmospheric nitrogen that may have profound effect on agriculture. It has also been demonstrated that the growth and nitrogen fixation of *Azospirillum* are affected by pH, temperatures, salinity. Like other countries, *Azospirillum* has the potential to be used as a substitute and/ or supplement of N-fertilizer. But prior going to a large-scale extension of biological nitrogen fixation (BNF) systems at the farm level, further research is needed to determine their N supplement potentials.

REFERENCES

Atlas, R. M. and Bartha, R. 1981. Microbial Ecology: Fundamentals and application. Addison-Wesley Publishing Company, Inc. London.

Dobereiner, J. and Day, J. M. 1976. Associative symbiosis in tropical grasses: Characterization of microorganisms and dinitrogen fixing sites. pp. 518-538. *In:* W. E. Newton and C. J. Nyman (ed.). Proceedings of the first international symposium on nitrogen fixation. Vol. **2**. Wahington State University Press, Pullman.

Kannan, T. and Ponmurugan, P. 2010. Response of paddy (*Oryza sativa* L.) varieties to *Azospirillum brasilense* inoculation. *J. Phytol*. **2**(6): 08–13.

Khan, Z. U. M. and Hossain, M. M. 1990. Distribution and nitrogen fixing potential of A*zospirillum* in rice field ecosystem [in Bangladesh]. *Bangladesh J. Bot.* **19**(2): 161-166.

Khan, Z. U. M., Akond, M. A. and Mubassara, S. 2001. Population of heterotrophic bacteria and *Azospirillum* in wheat field soil and the nitrogen fixing potential of *Azospirillum* of the same soil. *Bangladesh J. Life Sci*. **13**(1 & 2): 1-5.

Kumar, K. and Balasubramanian, B. 1986. Field response of rice to *Azospirillum* biofertilizer. Current Research. **15**: 74–76.

Lakshmi, V. and Dhala, S. A. 1984. Occurrence and characteristics of *Azospirillum brasilense* from aquatic sources. *Indian J. Microbiol*. **24**(1): 1-6.

Lakshmi, V., Rao, A. S., Vijayalakshmi, K., Lakshmi-Kumari, M., Tilak, K. V. B. R. and Subba Rao, N. S. 1977. Establishment and survival of *Spirillum lipoferum*. Proc. Indian Acad. Sci. Sect. B. **86**: 397-404.

Nelson, L. M. and Knowles, R. 1978. Effect of oxygen and nitrate on nitrogen fixation and denitrification by *Azospirillum brasilense* grown in continuous culture. *Can. J. Microbiol*. **24**(11): 1395-1403.

Okon, Y., Albrecht, S. L. and Burris, R. H. 1977. Methods for growing *Spirillum lipoferum* and for counting it in pure culture and in association with plants. Appl. Environ. Microbiol. **33**: 85-87.

Pramer, D. and Schmidt, E. L. 1964. Experimental soil microbiology. Burgess Publishing Company. pp. 70-71.

Rahman, M. M., Hoque, S. and Khan, Z. U. M. 2007. Nitrogen fixation and respiratory activity of *Azospirillum* spp. isolated from saline habitat of Bangladesh. *Bangladesh J. Life Sci*. **19**: 55-60.

Ravikumer, S., Ramanthan, G., Suba, N. and Jeyaseeli, L. 2002. Quantification of hlophilic *Azospirillum* from mangroves. *Indian J. of Marine Sci*. **31**(2): 157-160.

Salle, A. J. 1967. Fundamental principles of bacteriology. 6th edition, TATA McGraw-Hill Publishing Co. Ltd. Bombay, New Delhi.

Usha, D. K. and Kanimozhi ,K., 2011. Isolation and Characterization of Saline Tolerant *Azospirillum* Isolates from Paddy of Thanjavur District. Adv. Appl. Sci. Res. **2**(3): 239-245.

Waksman, S. A. and Strakey, R. L. 1957. Microbiological analysis of soil as an index of fertility. Soil Sci. **17**: 141.