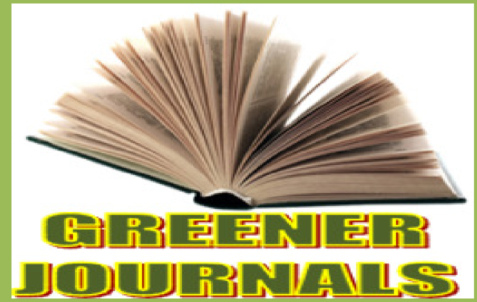
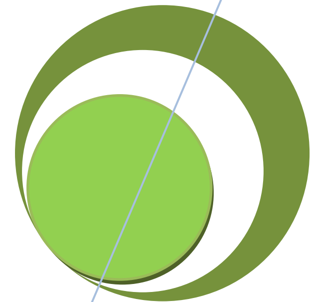


Greener Journal of Biological Sciences

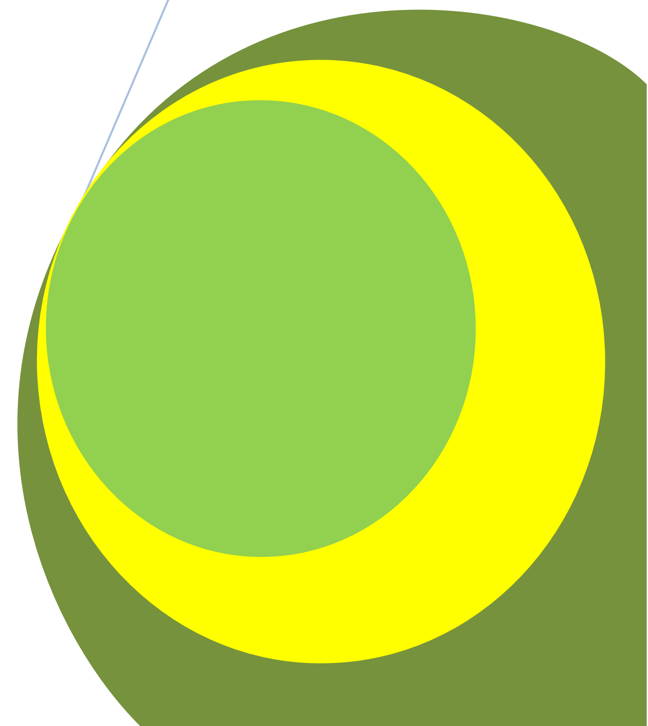
ISSN: 2276-7762



Isolation and Characterization of Salinity Tolerant Azotobacter sp

By

Md. Shamim Akhter
Sheikh Julfikar Hossain
SK. Amir Hossain
Rakesh Kumar Datta



Research Article

Isolation and Characterization of Salinity Tolerant *Azotobacter* sp

Md. Shamim Akhter[1*], Sheikh Julfikar Hossain[2], SK. Amir Hossain[3], Rakesh Kumar Datta[4]

[1]Ph D Research Fellow, Brain Protein Malfunction Lab., Dept. of Medicine, University of Santiago De Compostela, Spain
[1,3]Biotechnology and Genetic Engineering Discipline, Khulna University, Khulna, Bangladesh

[1]*Corresponding author's Email: shamim11_akhter@yahoo.com

ABSTRACT

The research work was conducted to isolate and characterize salinity tolerant *Azotobacter* sp. to be use as biofertilizer. In this study, soil samples were collected from different salinity affected areas, namely Pankhali Bazar of Dakop Upazilla, Khulna, Bangladesh and Dargapur Village, Assasuni Upazilla, Satkhira, Bangladesh. Thirty- five colonies were isolated from pore-plate containing Ashby's medium. Out of thirty -five isolates, fifteen were finally selected for detail study. All the isolates were rod-shaped, gram negative and non spore-forming. Biochemical tests indicated that they were obligate aerobes, catalase, oxidase and starch hydrolysis positive. Most of them were motile except four isolate nos. 02, 05, 09 and 13. Five isolates of *Azotobacter* were found which could tolerate 6% NaCl concentration whereas only two isolates of *Azotobacter* were found which could stand for at 10% NaCl concentration, which may become a promising source for further study regarding salinity tolerant *Azotobacter* sp. Finally, nitrogen fixation potentiality of the selected isolates was estimated ranging from 04.95 to 10.55 mg N/g substrate.

Keywords: salinity tolerant, *azotobacter*, biofertilizer, nitrogen fixation

INTRODUCTION

Due to the increased population in the world, it has become important for us to increase the crop productivity by using various fertilizers, insecticides and pesticides. The soil has been affected badly due to tremendous uses of these chemical fertilizers because of the depletion in the essential minerals of the soil [1]. Therefore, use of biofertilizers in place of chemical fertilizers is getting importance today.

The total area of Bangladesh is 147, 570 km^2. The coastal area covers about 20% of the country and over 30% of the net cultivable area. It extends inside up to 150 km from the coast. Out of 2.85 million hectares of the coastal and offshore areas about 0.83 million hectares are arable lands, which cover over 30% of the total cultivable lands of Bangladesh [2]. A part of the coastal area, the Sundarbans, is a reserve natural mangrove forest covering about 4,500 km^2. The remaining part of the coastal area is used in agriculture. The cultivable areas in coastal districts are affected with varying degrees of soil salinity [3].

Observations in the recent past indicated that due to increasing degree of salinity of some areas and expansion of salt affected area as a cause of further intrusion of saline water, normal crop production becomes more restricted [4]. According to salinity survey findings and salinity monitoring information, about 1.02 million ha (about 70%) of the cultivated lands are affected by varying degrees of soil salinity. about 0.282, 0.297, 0.191, 0.450 and 0.087 million hectares of lands are affected by very slight, slight, moderate strong and very strong salinity, respectively. Salinity in the country received very little attention in the past. Increased pressure of growing population demands more food. Thus, it has become increasingly important to explore the possibilities of increasing the potential of these (saline) lands for increased production of crops [2].

The study to find out efficient strains of nitrogen-fixing bacteria to develop biofertilizer for crops like wheat, rice, etc. is very essential. Biological nitrogen fixation is an inexpensive source of nitrogen for higher yields in non-leguminous crop, *e.g.*, rice and wheat farming systems [5]. *Azotobacter* is the genus consists of the members which are aerobic, free-living, nitrogen-fixing bacteria that are found throughout the world [6,7,8]. This organism was first isolated and described by Beijerinck in 19011 [9]. Besides nitrogen fixation, *Azotobacter* has been found to

synthesize growth promoting substances and antibiotics [10]. These bacteria have one of the most highly active cytochrome oxidases known [11,12], as well as notably active superoxide dismutase and catalase systems [13]**.** By virtue of these attributes, *Azotobacter* can play nutritional and stimulatory roles and can benefit the plants with its manifold actions. Furthermore, *Azotobacter* inoculation has been found to increase the growth and yield of a wide variety of cereals, pulses, vegetable crops, fruit crops and cash crops [14]. It is indeed interesting to note that *Azotobacter* is among the first organisms to develop in a newly formed soil, and the number of this organism in soil runs parallel with its fertility [15]. The occurrence and distribution of *Azotobacter* have been studied in the soils of different parts of the world but in Bangladesh get little attention.

Among the free-living nitrogen-fixing bacteria, those belonging to genus *Azotobacter* play a remarkable role, being broadly dispersed in different environments, such as soil, water and sediments [16]. In fact, field trials have demonstrated that under certain environmental conditions, inoculation with *Azotobacter* has beneficial effects on plant yields [17,18, 19] due to the increase of fixed nitrogen content in soil [20, 21,22] and to the microbial secretion of stimulating hormones, like gibberellins, auxins and cytokinins [23,24]. Several authors have shown the beneficial effects of *Azotobacter chroococcum* on vegetative growth and yields of maize [25,26] as well as the positive effect of inoculation with this bacterium on wheat [27]. Recently, it has been also shown that strains of *Azotobacter* could be usefully employed both in aquaculture systems [28] and in vermicompost production [29], due to their ability of fixing nitrogen and solubilizing phosphates. An additional reason which justifies the interest on these micro-organisms is that the species *Azotobacter vinelandii* and *A. chroococcum* produce exopolysaccharides with high potential value related to their wide range of commercial applications [30].

Many workers have reported about salt-tolerant strains of *Azotobacter* [31]. The efficiency of N_2-fixing efficiency of several *Azotobacter chroococcum* isolates was generally better in nonsaline and slightly saline strains than in saline strains observed by Mahmoud *et al*. [32]. A strain of Azotobacter which showed maximal N_2 fixation at 30%o NaCl, with good fixation still observed at 10 to 40% was isolated by Blinkov [33]. Several studies have been done on the isolation, distribution and abundance of *Azotobacter* in the soils of Jahangirnargar University Campus, Bangladesh [34,35]. However, no work has been done on strains isolated from saline affected regions of Bangladesh. Therefore, the present study is designed to achieve the following objectives:

- Isolation, characterization and determination of potentiality of salinity tolerant *Azotobacter* spp for nitrogen fixation.

MATERIALS AND METHODS

Collection of Samples

Soil samples were collected from the different salinity affected areas, namely Pankhali Bazar of Dakop Upazilla of Khulna district, Bangladesh and Dargapur Village of Assasuni Upazilla of Satkhira, Bangladesh in sterilized polythene bags. Then samples were used to investigate on salinity tolerant *Azotobacter* sp. The tests were conducted at the Environmental Biotechnology Laboratory of Biotechnology and Genetic Engineering Discipline and Soil Chemistry Laboratory of Soil Science Discipline of Khulna University, Khulna, Bangladesh.

Sampling Procedure

For the purpose of soil sample collection, sampling kit consisting of several sterile polythene bags, a marking pen, spatula, alcohol, knives, etc. were taken. Sufficient amount of soils were collected from each site; kept in a polythene bag and tagged. Soil samples were collected from top 4 cm of the soil profile, as this is where most of microbial activities take place. At each time of collection, hands were sterilized with alcohol (95%) and the bags were partially filled with soil; then the bags were properly tied and labeled. Special care was always taken to avoid contamination as far as practicable**.**

Preservation of the sample

After collection, the samples were brought to the laboratory carefully and preserved in the refrigerators for immediate use; however for long- term usage, samples were stored at 4ºC**.**

Determination of soil pH

25 gm (field moist) was taken in a clean dry 150 ml beaker and 50ml distilled water was added. The contents were thoroughly stirred with vortex machine. pH of the suspension was measured with a digital pH meter.

Medium Preparation

To prepare one liter of Ashby culture medium, the following steps were followed**.** According to media composition, the reagents (Manitol, K_2HPO_4, $MgSO_4 . 7H_2O$, NACL, K_2SO_4, $CACO_3$) weighted by electronic balance. One thousand ml distilled water was measured by volumetric flask and taken in a conical flask. The reagents (except agar) were mixed with the distilled water. After mixing reagents, pH was adjusted by adding HCl or NaOH solution, if required. After pH adjustment, agar was mixed into the solution. After mixing the agar, the medium was autoclaved by autoclave machine. Finally, the medium was poured in sterile Petri Plates.

Isolation

Ten gm collected soil sample was added to 90 ml of sterile distilled water in a sterile conical flask (250 ml), shaked well by vortex machine allowing to stand for 30 minutes. From the top of the suspension, 1 ml of sample suspension was then transferred to sterile 9 ml distilled water containing McCarty bottle, shaked well by hand and allowed to stand for 30 minutes. In this way, samples were diluted up to 10^5 dilution fraction. One ml of sample suspension (from 10^3 to 10^5 fraction) was taken in a sterilized Petri Plates containing approximately 15-20 ml melted (45ºC), Ashby's medium and then incubated at 28±2ºC temperature for about 2 – 3 days. After incubation, the individual colony was appeared on the medium. The number of *Azotobacter* per gram of soil was then calculated. The well spaced colonies on these plates were transferred into nutrient agar slants.

Purification

The isolates were purified through streak plate technique. Media used for the purpose were Ashby's medium and/or nutrient agar medium.

Preservation

The purified isolates were then transferred to the slants of nutrient agar media. The one -dram vial containing purified isolate, nutrient broth medium and sterilized glycerol was kept in the polyethylene bags, properly tied and preserved as stock culture.

Simple staining

For this purpose, 5% aqueous solution of basic stains as crystal violate was used.

Gram staining

For gram staining the fixed smear was treated with ammonium oxalate, crystal violates solution for 1 min, this was gently rinsed off and iodine solution applied for 1 min. This in term was drained off _ ethyl alcohol (95%) was then applied 10-12 drops to decolorize the strain. Finally safranine was used as a counter stain for 10 seconds. Then slide was gently rinsed off with water and blotted off. The result was recorded as gram positive and gram negative.

Spore staining

For spore staining 15-24 hours old culture were used. The fixed smear was flooded with 5% aqueous solution of malachite green and heated for about 10-15 minutes. Boiling was strictly avoided. The necessary malachite green was added from time to time. The excess die was then washed off and 5% aqueous solution of Mercuroehrome was then applied as a counter stain 1 and ½ minutes. The slide was washed dried and examined. Morphology of the spore and sporangia were studied under 100x using immersion oil. The results were recorded.

Motility test

For the determining the mortality of the selected bacteria by cultural method "motility medium" was used. The tubes containing the medium were inoculated by stabbing with straight wire. After incubation, motile organisms usually disperse through the semi solid, soft-agar medium and the growth of non- motile organism was confined to the stab. A Chemical 2, 3, 5-triphhenyltetrazoliun chloride was used with the medium which was reduced to a red color by growing bacteria contribute easier observation of the growth of bacteria.

Deep glucose agar test

The method was derived by Hall (1929) with relation to free oxygen. Organisms are classified as strict aerobes, facultative anaerobes, microaerophiles and strict anaerobes. By this method a rough grouping of the selected organisms, in regard to their oxygen requirements were made. Inoculate the tubes that contain deep glucose agar medium and incubate. After incubating at 37ºc for 2-5 days, observation was made to find out whether (A) the organisms grew on the surface and in the upper layer of the medium (strict aerobes), or (B) the organism grew just a lv millimeter below the surface (microaerophiles), or (C) the organisms trough out the medium (facultative anaerobes), or (d) the organism grew deeper in the medium (strict anaerobes).

Catalase test

To determine the ability of some microbes to degrade hydrogen peroxide by producing the enzyme catalase. Inoculate each experimental isolate into its appropriately labeled tube by means of a streak inoculation, then incubate three or four drops of 3% hydrogen peroxide flow over the entire surface of each slant culture. Then each culture examined for the presence or absence of bubbling or foaming, if bubble positive.

Nitrate reduction test

To determine the ability of microbes to reduce nitrates to nitrites.
First producing color change indicate nitrates were reduced.

Oxidase test

Medium: agar plate

A single-line streak inoculation of each test organism on the agar surface of its appropriate section of the plate (a) then incubates.
Reagent: p-aminodimethylaniline oxalate (light pink in color)
(black color positive, no color change negative)

Hydrolysis of starch

Organisms capable of hydrolyzing starch to maltose possess the enzyme amylase. By this test, the presence or absence of this enzyme in the organisms was ascertained. For this test, starch agar plates were incubated by streak method and the plates were incubated at 37ºc for 24 hours. After growth, iodine solution was added to the plates. Development of the blue color indicated that starch had not been hydrolyzed. Complete hydrolysis and partial hydrolysis of starch were indicated by the development of clear white or brownish white color respectively.

Salt tolerance

Nutrient agar slant containing different concentration of NaCl (viz. 0%, 2%, 4%, 6% and 10%) were inoculated and incubated at 28 ºC for 48 hours. The growth of isolates at different concentrations of NaCl was then compared with the control.

Determination of nitrogen fixing capacity:

Potentiality for nitrogen fixation of *Azotobacter* isolates was determined in terms of the quantity of nitrogen accumulated in five days old culture of each isolate developed in fifty ml LG broth medium. Nitrogen in culture was estimated by Kjeldahl method.

RESULTS AND DISCUSSION

In the present study, the P^H was in the range of 7.21 to 4.48 (Table No. 01). The difference in P^H value is most probably due to collection of samples from different locations. Soil samples were examined, in addition, for the calculation of total number of bacteria (Table No. 01). The highest number of bacteria was found as 7×10^5/gm soil in soil sample no. 01 whereas the lowest number of bacteria was found as 1×10^5/gm soil in soil sample nos. 06, 08 and 11. It was reported that *Azotobacter* sp. counts in soil samples in Bangladesh were very much lower in a soil of P^H 5.7 than in soils of P^H 6.8, 6.9 and 7.3[36]. However, from the bacteria that grown on nitrogen-free Ashby's medium, well-spaced colonies were isolated. Then pure cultures were prepared. Hereafter, depending on cultural characteristics and salt tolerant, fifteen isolates were finally selected for detail studies.

The isolates were microscopically studied. Simple staining, Gram staining, Spore staining were performed (Table No. 02). All the isolates were rod shaped, Gram negative and non-spore former. Biochemical tests needed for characterization of the isolates were performed (Table No. 03). Deep Glucose Agar test indicated that all the isolates were obligate aerobes. The isolate nos. 2, 5, 9 and 13 were found non-motile. All the isolates were catalase and oxidase positive.

Table 01: P^H, number of cfu of *Azotobacter* in different soil samples.

Sample No.	P^H	Number of cfu ($\times10^5$/gm soil)
01	7.11	7
02	6.61	2
03	5.73	2
04	5.59	5
05	5.48	2
06	6.90	1
07	4.48	2
08	6.37	1
09	5.31	5
10	4.95	2
11	6.77	1
12	7.19	3
13	5.91	5
14	6.11	3
15	7.21	4

Salinity test was done for obtaining salinity tolerant *Azotobacter* isolates (Table No. 04). Saline tolerant capability is one of the most important attribute of *Azotobacter* for salinity affected regions like Khulna, Satkhira in Bangladesh. If it is possible to use saline tolerant *Azotobacter* as biofertilizer in this region, the production of crops will certainly increase. All the isolates showed growth in 0%, 2% and 4% NaCl concentration but isolates No. 1, 2, 4, 5, 6, 8, 9, and 12 showed poor growth. Out of which, isolates No. 1, 2, 4, 5, 6, 8, 10, 12, 13 and 14 showed no growth at 6%

NaCl. No growth was found from isolate No. 1, 2, 3, 4, 5, 6, 8, 9, 10, 11, 12, 13 and 14 at 10% salt concentration. In this experiment, isolate nos. 3, 7, 9, 11 and 15 were salt resistant *Azotobacter* at 6% NaCl concentration where as only isolate nos. 7 and 15 were more salt resistant *Azotobacter* as they showed growth at 10% salt concentration. The result co-relates the findings of Islam M. Z. et al (2008) [36]. According to Bergey's Manual of Systematic Bacteriology, *A. chroococuum, A. vinelandii* and *A. armeniacus* can tolerate more than 1% NaCl [36]. It was reported that *Azotobacter* sp. could tolerate up to 1.5% salt concentration [37]. Besides, many workers have reported about salt tolerant *Azotobacter* sp [31---35].

Out of the *Azotobacter* isolates, isolate no. 06 showed the maximum potentiality to fix nitrogen (10.55 mg N/g substrate), whereas isolate no. 06 showed the least potentiality to fix nitrogen (04.95 mg N/g substrate). Kizilkya R. reported the range of nitrogen fixation by *Azotobacter* from 3.50 to 29.35 µg N/ml for Ashby medium culture [38]. The potentiality of nitrogen fixation was also reported within the range of 0.69 to 1.18 mg N/25 ml culture [35]. Another findings reported the capacity of nitrogen fixation by *Azotobacter* isolates as 10.00 mg N/g substrate [39].

Table 02: Morphological Characterization of selected isolates

Morphological Tests	Isolate no.														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Simple staining	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod
Gram staining	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Spore staining	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 03: Biochemical Characterization of selected isolates

Biochemical tests	Isolate no.														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Motility test	+	-	+	+	-	+	+	+	-	+	+	+	-	+	+
Deep Glucose Agar test	Growth at the top of the tube (Obligate aerobe)														
Catalase test	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Nitrate reduction test	-	-	+	+	-	+	+	+	+	-	+	+	-	+	+
Oxidase test	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Hydrolysis of starch	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Table 04: Growth of *Azotobacter* isolates in different salt (NaCl) concentration and their nitrogen fixing capacity

Isolate No.	0% salt	2% salt	4% salt	6% salt	10% salt	Nitrogen fixing capacity(mg N/g substrate)
01	+++	++	+	_	_	7.15
02	++	++	+	_	_	5.07
03	++	++	++	+	_	7.00
04	++	+	+	_	_	5.21
05	+++	++	+	_	_	7.13
06	++	++	+	_	_	10.55
07	+++	++	++	++	+	9.87
08	++	++	+	_	_	6.32
09	+++	+++	+	+	_	4.95
10	++	++	++	_	_	5.60
11	+++	++	++	+	_	8.05
12	++	++	+	_	_	5.45
13	++	++	++	_	_	6.41
14	++	++	++	_	_	5.32
15	++++	++	++	++	+	6.59

'+' sign indicates degree of growth. '_' sign indicates no growth.

CONCLUSION

The significance of the study is the acknowledgement of salt tolerant *Azotobacter* sp. in Khulna and Satkhira regions affected by salinity in Bangladesh. The use of these salt tolerant *Azotobacter* sp. may offer the opportunity to use as biofertilizer in the concerned areas, especially for their average standard potentiality of nitrogen fixing in the laboratory environment. However, the successful exploitation of these isolates with proper biotechnology as eco-friendly biofertilizer replacing chemical fertilizers will be definitely beneficial, especially in salinity affected areas of Bangladesh. Therefore more advance research is required for a deeper understanding about this salt tolerant *Azotobacter* sp. to further improve biofertilization process. In many cases, geochemical influences of the microbes

are potentially great. The ability to quantify and more importantly, to predict the rates & extents of these processes for biofertilization, bioremediation, etc remains a challenge for the future.

ACKNOWLEDGEMENTS: This work is supported by grants from the University Grants Commission (UGC) of Bangladesh.

REFERENCES

Boraste A, Vamsi KK, Jhadav A, Khairnar Y, Gupta N, Trivedi S, Patil P, Gupta G, Gupta M, Mujapara AK, Joshi B (2009). Biofertilizers: A novel tool for agriculture. International Journal of Microbiology Research, 1(2): 23-31.

Haque SA (2006). Salinity problems and crop production in coastal regions of Bangladesh. *Pak. J. Bot.*, 38(5): 1359-1365.

Petersen L and Shireen S (2001). Soil and water salinity in the coastal area of Bangladesh. SRDI.

Rahman MM and Ahsan M (2001). Salinity constraints and agricultural productivity in coastal saline area of Bangladesh, Soil Resources in Bangladesh: Assessment and Utilization.

Akond MA, Sanzida M and Motiur RM (2007). Distribution and abundance of *Azotobacter* in wheat fields of Bangladesh. Bangladesh J Microbiol. 24(2): 151-153.

Becking JH (1981). The family *Azotobacteraceae,* p. 795-817. *In* M. P. Stan-, H. Stolp, H. G. Triiper, A. Balows, and H. G. Schlegel (ed.), The prokayotes. A handbook on habitats, isolation and identification of bacteria, vol. 1. Springer-Verlag, Berlin.

Tchan YT and New PB (1984). Genus I. *Azotobacter* Beijerinck 1901, 567AL, p. 220-229. *In* N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1. The Williams & Wilkins Co., Baltimore.

Thompson JP and Skerman VBD (1979). *Azotobacteraceae:* the taxonomy and ecology of the aerobic nitrogen-fixing bacteria. Academic Press, Inc., New York.

Subba Rao NS (1982). Biofertilizers in Agriculture, pp 77-91. Oxford & IBH Publishing Co Pvt Ltd, New Delhi.

Robson RL and Postgate JR (1980). Oxygen and hydrogen in biological nitrogen fixation. Annu. Rev. Microbiol. 34:183-207.

Jurtshuk PJr, Mueller TJ, McQuitty DN and Riley WH (1978). The cytochrome oxidase reaction in *Azotobacter vinelandii* and other bacteria, p. 99-121. *In* H. Degn, D. Lloyd, and G. C. Hill (ed.), Functions of alternative terminal oxidases. Proceedings of the 1lth Federated European Biochemical Societies, Copenhagen. Pergamon Press, Oxford.

Jurtshuk PJr, Mueller TJ and Wong TY (1981). Isolation and purification of the cytochrome oxidase of *Azotobacter vinefundii.* Biochim. Biophys. Acta. 637:374382.

Jurtshuk PJr, Liu JK and Moore ERB (1984). Comparative cytochrome oxidase and superoxide dismutase analyses on strains of *Azotobacter vinefandii* and other related free-living nitrogen-fixing bacteria. Appl. Environ. Microbiol. 47:1185-1187.

Bhandari SC & Somani LL (1990). *Azotobacter*. In Biofertilizers (Somani LL ed), pp 355-361. Scientific Publishers, Jodhpur.

Bhat JV (1961). Bacterization experiment with *Azotobacter*. In *Radioisotopes, Fertilizers and Cow Dung Gas Plant* (Raghvan D, Kachroo P & Doraiswami S eds), p 317. ICAR, New Delhi.

Palleroni NJ (1984). Gram negative aerobic rods and cocci. In: Krieg, N.R., (Ed.), Bergey's Manual of Systematic Bacteriology, Williams and Wilkins, Baltimore, pp. 140–199.

Jackson RM, Brown ME, Burlingham SK (1964). Similar effects on tomato plants of Azotobacter inoculation and application of gibberellins. Nature. 203:851–852.

Rovira AD (19650. Interactions between plant roots and soil microorganisms. Annal Review of Microbiology 19:241–266.

Denarie´ J, Blanchere H (1966). Inoculation de graines de vegetaux cultives a` l'aide de souches bacteriennes. Annales de l' Institut Pasteur Suppl. 3:57–74.

Gouri PSVM, Jagasnnatathan R (1995). Biotechnology in organicfarming. Biotechnology Review 5:34–47.

Maltseva NN, Nadkernichnaya EV, Kanivets NA (1995). Associations of nitrogen-fixing bacteria with winter rye, Proceedings of the 10th International Congress on Nitrogen Fixation, St Petersburg, Russia, No. 614.

Mrkovacˇki N, Mezei S, Kovacˇev L (1996). Effect of Azotobacter inoculation on dry matter mass and nitrogen content in the hybrid varieties of sugar beet. A Periodical of Scientific Research on Field and Vegetable Crops 25:107–113.

Mishustin EN, Shilnikova VK (1971). Biological fixation of atmospheric nitrogen, Macmillan, London.

Martinez T, Moreno MY, De la Rubia J, Gonzalez-Lopez T (1989). Root exudates of Zea mays and production of auxins, gibberellins and cytokinins by Azotobacter chroococcum. Plant and Soil. 110:149–152.

Mishra OR, Tomar US, Sharama RA, Rajput AM (1995). Response of maize to chemicals and biofertilizers. Crop Research. 9:233–237.

Pandey A, Sharma E, Palni L (1998). Influence of bacterial inoculation on maize in upland farming systems of the sikkim Himalaya. Soil Biology and Biochemistry 3:379–384.

Elshanshoury AR (1995). Interaction of Azotobacter chroococcum, Azospirillum brasilense and Streptomyces mutabilis, in relation to their effect on wheat development. Journal of Agronomy and Crop Science. 175:119–127.

Garg SK, Bhatnagar A, Kalla A, Narula N (2001). In vitro nitrogen fixation, phosphate solubilization, survival and nutrient release by *Azotobacter* strains in an aquatic system. Bioresearch Technology. 80:101–109.

Kumar V, Singh KP (2001). Enriching vermicompost by nitrogen fixing and phosphate solubilizing bacteria. Bioresource Technology. 76:173–175.

De la Vega, Cejudo MG, Paneque FJ (1991). Production of exocellular polysaccharide by Azotobacter chroococcum. Applied Biochemistry and Biotechnology. 30:273–284.

Babak NM (1966). The effect of different salts on Azotobacter chroococcum and Azotobacter galophilum Mikrobiologiya. 35:137-141.

Mahmoud SA, El-Sawy ZM, Ishac YZ and El-Safty MM (1978). The effects of salinity and alkalinity on the distribution and capacity of N2-fixation by Azotobacter in Egyptian soils. Ecol. Bull. NFR (Naturvetensk. Forskningsradet). 26:99-109.

Blinkov GN (1963). Salt-tolerant Azotobacter. Mikrobiologiya. 31:715-717.

Shilpi RY, Akhond MA and Khan ZUM (1993). Distribution and abundance of Azotobacter in the soils of Jahangirnagar University Campus. Bangladesh J. Life Sci., 5: 19-24.

Shilpi RY, Akhond MA and Khan ZUM (1998). Growth and nitrogen-fixing potential of Azotobacter isolated from Jahangirnagar University Campus. Bangladesh J. Life Sci. 10:1-5.

Islam M. Z., D.I. Sharif and Hossain M. A. (2008). A Comparative Study of Azotobacter spp. From Different Soil Samples. J. Soil. Nature. 2(3):16-19.

Tippannavar, C M, Rddy, M M V, Rajashekara, E.(1989). Tolerance of *Azotobacter chroococcum* strains to different salt concentrations and P^H levels. Farming Systems, 5:1 & 2, 24-48.

Kizilkya R. 2009. Nitrogen fixation capacity of Azotobacter spp. Strains isolated from soils in different ecosystems and relationship between them and the microbiological properties of soils. J. Environ. Biol. 30(1) : 73---82.

Hamdi YA. 1982. Application of nitrogen-fixing systems in soil improvement and management. FAO Soil Bull. 49 : 45---82.