Studies on Enzyme Profile and N_2 Fixing Ability of *Azotobacter sp.* Isolated from Rhizospheric Soil

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***ABSTRACT*: In the present study a total of 16 different rhizospheric soil samples were collected from rhizospheric area of plants. Further the isolates were identify on the basis of cultural and biochemical characteristics. Enzyme profile of all the isolates were studied starch and urea hydrolysis as well as oxidase, cellulase and catalase were found to be positive. The study on effect of environmental parameter were studied and it was observed that the Azotobacter grows best 8.0 and 9.0 pH and it requires the temperature of 28°C and 2% salinity tolerant ability was observed. *Azotobacter sp*. also shows hydrogen cyanide (HCN) and ammonia production ability. N_2 fixing capacity was studied by Kjeldahl Method. Highest N_2 fixing ability was shown by AZ_5 isolated is 0.0068 mg/ml.**

***Keywords*: Azotobacter, Rhizosphere, N_2 Fixation, Enzyme Profile, Envoirnmental Factor, Hyrogen cyanide and Ammonia.**

INTRODUCTION:

Nitrogen is commonly considered as one of the foremost restrictive nutrients in plant growth andmajor reserve of nitrogen in the biosphere being available in the form of atmospheric nitrogen (molecular nitrogen) which actually cannot be utilized by the plant. The plant nitrogen are made available to the plants when nitrogen fixing bacteria fixed atmospheric nitrogen and convert them into ammonia (NH_3).

Azotobacter sp. are gram –ve, free living aerobic soil dwelling, oval or spherical bacteria that form thick walled cysts. There are around six species in the genus Azotobacter some of which are motile by means of peritrichous flagella, other are not. These bacteria utilized atmospheric nitrogen gas for their cell protein synthesis. This cell protein is then mineralized in soil after the death of Azotobacter cells thereby contributing towards the nitrogen availability of crop plants. Azotobacter tends to be sensitive to acid pH values, high temperature and salt.

Azotobacter naturally fixed atmospheric nitrogen in the rhizosphere. *Azotobactre sp.* populations, their activities and microbiological properties of soil contain microbial Biomass C, basal soil respiration and enzymes activities (Dehydrogenase, Catalase, Glucosidase, Urease, Phosphatase and sulphatase). Hydrogen Cyanide (HCN) was higher traits of Azotobacter (77.00%).

MATERIAL AND METHODS:

A total of 16 soil samples were collected from different location in sterile plastic bags from the depth of 10-15cm and use to isolate nitrogen fixing *Azotobacter sp*. Yeast Malt Dextrose Broth is used for enrichment for soil microorganism. Further identification of the obtained isolates was done on the basis of Bergey's Mannual Determinative Bacteriology (Holt J. G; 2001). Further the isolates were Screened for tolerance of envoirnmental parameter are also done as well as enzyme profile of 8 different enzymes were studied.

Amount of nitrogen fix by Azotobacter isolates was estimated by Micro Kjeldhal Method of Bregersen 1980. The isolates were grown in Lowenstein Jensen Media (LJ) supplied with L- glutamic acid. The nitrogen fixed invitro was calculated by formula:

$$\text{Nitrogen content (mg)} = \frac{(\text{Sample titer} - \text{Blank titer}) \times \text{Normality of Standard}}{\text{Sample wt. (gram)}} \times 14.007$$

Ammonia Production:

Ammonia production test was done within peptone water. The culture is inoculated in 10 ml of peptone water and incubate at 28 ± 2°C for 48 to 72 hours then add Nessler's reagent (0.5 ml) after incubation brown to yellow color indicates positive test for ammonia production (Mohd Musheer Altaf and Abdul Malik, 2019).

HCN Production:

The Hydrogen Cyanide production done with test tube containing Lowenstein Jensen media supplemented with 4.4 g/L glycine was inoculate with tested isolates using micropipettes and these tubes are incubate for 2 to 4 days at 28 ± 2°C. Each inoculated tube was overlaid with Whatman's paper NO. 2 that was impregnated with 0.5% picric acid and 2% sodium carbonate. Paper were saturated with the reagent the change in color from yellow to orange-brown on the filter paper is an indicator to the production of cyanide (Abdel-Hamid, Marwa S. et al., 2010).

Results and Discussion:

Plant Growth Promoting Rhizobacteria (PGPB) are considered to promote plant growth directly or indirectly. PGPB can exhibit a variety of characteristics responsible for influencing plant growth. The common traits include the production of plant

growth regulators (Auxins, Gibberellin, Ethylene, etc.), siderophores, HCN and antibiotics. In this study, an effort will be made to isolate several bacterial strains and investigated to know their skill for N_2 fixing ability by Kjelhaldal method.

Enzyme ProfileStudy

Table no. 1: Enzyme Profile of obtained *Azotobacter spp.*

Isolates	Gelatinase	Amalyase	Urease	Lipase	Protease	Oxidase	Cellulase	Catalase
AZ_1	+++	-	+	-	-	+	+	+
AZ_2	++	+	+	-	-	+	+	+
AZ_3	-	+	+	-	-	+	+	++
AZ_4	++	+	+	-	-	+	-	+++
AZ_5	-	-	+	-	-	+	+	+
AZ_6	-	-	++	-	-	+	-	+
AZ_7	+++	+	+	-	-	-	+	-
AZ_8	-	+	+	-	+	+	-	+
AZ_9	-	-	+	-	-	-	+	+
AZ_{10}	+++	-	++	+	-	-	+	++
AZ_{11}	++	-	+	-	-	-	+	+
AZ_{12}	-	-	+	-	-	+	+	+
AZ_{13}	-	-	+	-	-	+	-	+
AZ_{14}	-	-	+	-	+	+	+	-
AZ_{15}	-	+	+	-	-	-	+	-
AZ_{16}	-	+	+	-	-	+	+	+

Study of Environmental Parameter:

Table No. 2: Screening of *Azotobacter* isolates for pH, Temperature and Salt Tolerance

Isolates	AZ_1	AZ_2	AZ_3	AZ_4	AZ_5	AZ_6	AZ_7	AZ_8	AZ_9	AZ_{10}	AZ_{11}	AZ_{12}	$AZ_{`12}$	AZ_{14}	AZ_{15}	AZ_{16}
pH tolerance																
pH 5.0	+	+	-	-	-	-	+	-	+	-	-	-	-	-	+	-
pH 7.0	-	-	-	-	-	-	+	-	+	-	-	-	-	+	-	-
pH 8.0	+	+	+	+	+	-	+	-	+	-	+	-	+	+	+	+
pH 9.0	+	+	+	+	+	-	+	-	-	-	-	-	-	+	+	-
Temperature Tolerance																
2°C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5°C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
28°C	-	+	-	-	+	+	+	-	+	+	+	-	+	-	+	-
37°C	-	-	-	-	-	-	+	-	+	-	-	-	+	-	-	-
45°C	-	-	-	-	-	+	-	-	-	-	-	-	+	-	+	-

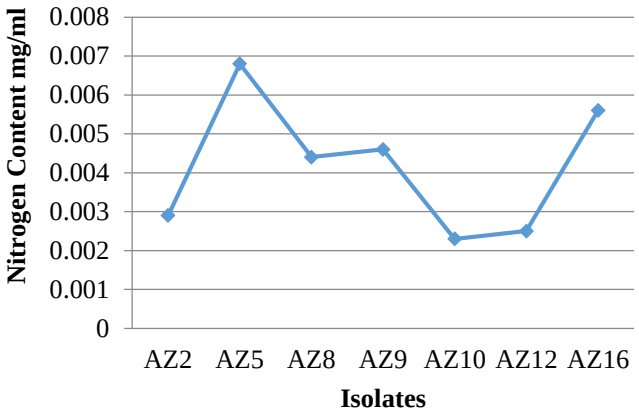
Salt tolerance																
0%	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2%	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5%	-	+	-	-	-	+	-	-	-	-	-	-	+	-	-	+
10%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Nitrogen Fixing Ability

Table no. 8: Nitrogen fixing capacity of selected *Azotobacter* isolates.

Isolates	Nitrogen content mg/ml on Johnson's broth
AZ_2	0.0029
AZ_5	0.0068
AZ_8	0.0044
AZ_9	0.0046
AZ_{10}	0.0023
AZ_{12}	0.0025
AZ_{16}	0.0056

Fig. No. 1: Nitrogen fixing capacity of selected Azotobacter isolates.



Production of HCN and Ammonia

Table no. 9: Production of HCN and Ammonia by *Azotobacter spp.*

Isolates	HCN	Ammonia
AZ_1	+	+
AZ_2	+	+
AZ_3	+	+
AZ_4	+	-
AZ_5	+	+
AZ_6	+	+
AZ_7	+	+
AZ_8	+	+
AZ_9	+	-
AZ_{10}	+	-
AZ_{11}	+	+
AZ_{12}	+	+
AZ_{13}	+	+
AZ_{14}	+	+
AZ_{15}	-	+
AZ_{16}	-	+

Nitrogen estimation by Kjeldhal Method

A total of 16 different rhizospheric soil samples were collected from various places and from rhizospheric area of different plants. Out of 16 soil samples 16 *Azotobacter spp*. were isolated on selective media i.e. Azotobacter Isolation Agar. Further the isolates were identified on the basis of morphological, cultural and biochemical characteristics and confirmation of the isolates was done on the basis of Bergey's Manual of Determinative Bacteriology (Holt J. G., 2001). Results were represented in Table No. 1 and 2.

The morphology and cultural characteristic of *Azotobacter spp.* was found to be Gram –ve. Most of isolates showing motility while AZ_6, AZ_9, AZ_{13} shows no motility. Size of colony is about 2-4 mm and the shape is short and long rod, bacilli. The colony characters of these isolates are round, entire, smooth, convex. Occurrence of colony on media are slimy, gummy, sticky, spread, mucoid and viscous having colour off white, yellowish white or dull white (Table No. 2). Abdel-Hamid, Marwa S. *et al*., 2010, shows the similar morphological and cultural characteristics. Most isolates presented whitish (cream color), smooth, irregular, shining, 3-8mm diameter colonies; nevertheless, colonies with transparent, glistening, shining, 2-5 mm diameter also appeared. Three cell type morphologies were identified: Gram-negative bacilli short and long; Gram-negative bacilli short and small; similar characters of *Azotobacter species* was reported by Jiménez, D.J. *et al.*,(2011).

Biochemical characteristics of *Azotobacter spp.* were shown in Table No. 3. Most of isolates ferment glucose and mannitol with the production of acid and gas. Whereas very few isolates were found to be lactose fermenter such as AZ_1, AZ_2, AZ_4, AZ_7, AZ_{10} and AZ_{11}.

According to Magda M. Aly (2011) Methyl red and Vogus Proskauer tests were positive. In our study it was observed that the VP tests show positive result whereas Methyl red and Indole test was found to be negative.

Enzyme Profile of all the obtained isolate was studied. Total 8 different enzymes such as Gelatinase, Amalyase, Urease, Lipase, Protease, Oxidase, Cellulase and Catalase were studied. Strong positive gelatinase activity was shown by AZ_1, AZ_7, AZ_{10} followed by AZ_2, AZ_4, AZ_{11}. Rest of the isolates shows negative gelatin liquification.

Starch hydrolysis were carried out by AZ_2, AZ3, AZ_4, AZ_7& AZ_8. All the isolates shows urea hydrolysis whereas, only AZ_{10} shows lipase production. Almost all Azotobacter shows oxidase, cellulase and catalase test positive whereas, only two isolates AZ_8 & AZ_{14} were found to be protease producer (Table no.4).

According to Jiménez, D.J. *et al.*,(2011), gram-negative bacilli, and catalase and oxidase negative tests, suggesting that they can be aerobic FNFB that do not belong to the genus *Azotobacter*. Almost all isolate shows catalase and oxidase positive except AZ_7 and AZ_{15} are negative for oxidase and catalase test. 100% positive result for oxidase and catalase in Mohd MusheerAltaf and Abdul Malik, (2019). Magda M. Aly (2011) during the study *Azotobacter spp.* shows indole, citrate, catalase and oxidase.

All the 16 isolates were screened for pH tolerance. From the study it was revealed that maximum pH tolerance was observed at pH 8.0 followed by pH 9.0. Indicating that *Azotobacter spp*. growth was favoured at alkaline pH whereas, very less growth was observed at acidic and neutral pH.Our results are not correlated with the results of Naveen Kumar Nag (2015), the pH tolerant capability is one of the most important attribute of Azotobacter for pH affected regions. All the isolates showed growth in pH 7.

During the study it was observed that maximum growth of *Azotobacter spp.* was observed at 28°C. Whereas no growth was found at 2 and 5°C. Similar finding were obtained from Naveen Kumar Nag (2015) reporting that the highest density of*Azotobacter* isolates was observed at 28°C. Similarly moderate growth was observed at 37° and 45°C. Whereas M. Z. Islam *et al*.,(2008), observed maximum growth both at 30ºC and 40ºC and no isolate survived at 50ºC.

The salinity tolerant ability of *Azotobacter* isolates were found at0% of NaCl and 2% of NaCl concentration. According to Naveen Kumar Nag (2011) few of the isolates shows tolerance to NaCl at 5% whereas no activity was observed at 10%.

The HCN production of this 16 isolates shows positive result only the isolate no. AZ_{15} and AZ_{16} are dose not produce HCN according to the Abdel-Hamid, Marwa S. *et al*.,(2010), the visual inspection of the tested plates revealed that *A. chrococcum* isolates and the reference strain have a cyanogenic potential changing due to the color of indicator paper. Asper the reports from Kurnal Modi (2017), HCN is produced by many rhizobacteria and is postulated to play a role in biological control of pathogensSuraj Singh (2013), observed the of nitrogen fixation ability even though the amount were in small quantity, is visible. According to study our isolate no. AZ_5 shows 0.0068 mg/ml nitrogen fixing ability which is very less in amount. It is observed that *Azotobacter chrococcum* is known for its nitrogen fixing potentiality with the cereal crops. Nitrogen fixing potentiality of *A. chroococcum* is affected by the various environmental and chemical factors in Raghvendra P. Narayan, (2011). The N_2 fixing capacity of *Azotobacter* strains also differed from strain to strain. According toSandeep Upadhyay *et al.*,(2015) the fixation of N_2 depends upon the activation of nitrogenase enzyme, which may vary from strain to strain.

The study reveals that the HCN and Ammonia production ability of all the isolates except AZ3, AZ9, AZ10, AZ_{15}, AZ_{16} respectively.

The Nitrogen fixing efficancy of each strain was determined in terms of the amount of total nitrogen fixed. The N_2 fixation depends upon the activation of nitrogenase enzyme which may vary from strain to strain.

The amount of N_2 fixed by different *Azotobacter spp.* was ranged from 0.0023 upto 0.0068 mg/ml on Johnson's Broth. Highest N_2 fixing ability was shown by isolate AZ_5 i.e. 0.0068 mg/ml whereas, very less N_2 fixing ability was shown by isolate AZ_{10}.

The wide variations in nitrogen fixing capacity of different strains of Azotobacter have also been reported by Sanoria and Sundara Rao (1975), Mahmoud et al. (1978) and Tippannavar and Reddy (1987). These isolation results have close conformity with findings of Torres, et al. (2000). They obtained *Azotobacter chrococcum, A. vinelandii*, *Pseudomonas aeruginosa* and *Klebsiella pneumonia* strains from rhizosphere of rice cultivated in the Tolima region.

CONCLUSION

From the complete study it was conclude that Azotobacter is having potential to produce Ammonia and Cyanide. The isolate AZ_5 shows the production of 0.0068 mg/ml of N_2 fixation.

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