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Studies on isolation, biochemical characterization and nitrogen fixing ability of *Azotobacter sp*. isolated from agricultural soils.

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Abstract

The objective of the work was to isolate and characterize *Azotobacter* sp. from agricultural soils. Further estimation of ability of nitrogen fixation was also determined . *Azotobacter* population was isolated from 10 soil samples collected from agricultural fields. From the population 7 isolates were obtained and they were biochemically characterized. All the isolates were gram –ve and most isolated showed +ve results for the tests. The estimation of N_2 fixation was carried out by micro kjeldahl method. On estimation, the N_2 fixation ranged from 6.58 mg N/ml to 14.86 mg N/ml. Isolates Azo_4 and Azo_5 showed maximum of 14.86 mg N/ml and 13.25mg N/ml respectively. The minimum ability was recorded in Azo_2 (6.58 mg N/ml). It was also reported that the efficiency of nitrogen fixation decreases with increase in N content in soil.

Key words: nitrogen fixation, *Azotobacter*, biochemical charectrization and Kjeldahl method.

1. Introduction

In recent years, farming communities generally depends on synthetic fertilizers rather than the application of bio fertilizers which boosts organic matter in our soils. Nitrogen (N_2) is found in the gaseous forms in the atmospheric air, plants and animals do not get to use of it in this form for their metabolism (Dobereiner, 1997). Relatively loss of N_2 will be higher because of their gaseous nature microbially through mineralization-nitrification-denitrification in addition to leaching, volatilization, soil erosion, seepage and other naturally occurring processes. Simultaneously the drastic increase in the demand of nitrogen for plants can be fixed by synthetic fertilizers where yearly usage accounts for $17.2x10^7$ tons of synthetic nitrogen. Besides being quite expensive and high cost of production, synthetic fertilizers affect adversely on soil health and microbial population.

A. chroococcum is the first aerobic free-living nitrogen fixer (Beijerinck, 1901). *Azotobacter* sp. are non-symbiotic heterotrophic bacteria capable of fixing an average 20kg N/ha/year (Kizilkaya ., 2009). These *Azotobacter* sp. utilize atmospheric nitrogen for their cell protein synthesis and then mineralized in soil after their death.Thereafter nitrogen is available to plants. *Azotobacter* sp. is sensitive to acidic pH, higher salt concentration, and temperature (Tchan *et a*.,1989). Being economic and environmental friendly, biofertilizers can be used in crop production for better yield (Nagananda *et al*.,2010). In such situation, biofertilizers can be

the alternative source for improving soil fertility and other beneficial effects on plants where abundance will increase organic matter, pH, temperature, soil moisture.

Nitrogen is the important source for larger number of leaves growth wherein have influence in chlorophyll. Therefore, adequate amount nitrogen supply is needed for high yield potential in crops. The atmosphere comprises of ≈78% nitrogen as an inert form. Above every hectare of ground there are ≈80000 tons of this unavailable nitrogen. In order to convert into available form it needs to be fixed through either the industrial process or through Biological Nitrogen Fixation (BNF). Without these nitrogen-fixers, life on this planet may be difficult (Jnawali *et al.*,2015). Bacterization helps to improve plant growth and to increase soil nitrogen through nitrogen fixation by utilizing carbon for its metabolism(Monib et al., 2015). We duly made investigations in the *Azotobacter* population in agricultural soils, and their are confirmed by biochemical tests and then nitrogen fixing ability by micro kjeldahl method.

2. Materials and Methods

2.1 Collection of soil sample

Soil samples were collected from 10 agricultural fields in Krishnagiri district of Tamil Nadu. Five samples were taken at a depth of 5 to 10 cm from each field and pooled together as one sample. The samples were packed and bought to the laboratory for the further work. Later the pH of all the 10 samples were recorded.

2.2 Isolation of *Azotobacter*

Totally 10soil samples were used to isolate *Azotobacter*. From the collected soil sample 1g was taken and serially diluted upto 10^{-6} dilutions in sterile water. All the glasswares used for this work was completely sterilized under hot air oven at 160° C for 2 hrs. Isolation was done on Ashby's medium consisting of mannitol 20.0g, dipotassium phosphate 0.20g, $MgSO_4$ 0.20g, NaCl 0.20g, K_2SO_4 0.10g, $CaCO_3$ 5.00g. agar 15.00g, pH 7.4±0.2 for 1000 ml distilled water. The medium was prepared and sterilized. Isolation was done as per the procedures of pour plate method. 1ml of sample from 10^{-6} dilution was inoculated in the medium and was allowed to solidify. Three replications were maintained for each. The plates were incubated at 30° C for 48 hrs. Each well developed *Azotobacter* colonies with different morphology were purified and assumed as distinct isolates.

2.3 Morphological and biochemical characterization

Gram staining

This test helps in differentiating the bacteria into G+ve and G-ve . A thin smear of bacteria was prepared on a clean slide and air dried. Then heat fixed. The smear was then covered with crystal violet and allowed it for 20 sec and rinsed with water. Then it was covered with gram's iodine

for 1 min and washed with decolouriser . Then covered with safranin for 20 sec and washed in water and observed under microscope. The bacteria stained with pink or red is G-ve and bacteria with purple or violet is G+ve .

Catalase test

This is used to test the ability of organisms to produce catalase enzyme. For this about 4 to 5 ml of 3% hydrogen peroxide was taken in a test tube, a loop full of bacteria was taken and placed into the solution and it was observed for immediate bubble formation. This is taken as +ve for the test.

Urease test

It is used to test the ability of organism to split urea by enzyme urease. Urea agar slants were prepared and the test organisms were streaked and incubated for 37ºC for 48 hrs. Appearance of pink colour indicates +ve and no colour change as –ve.

Citrate utilization test

Simmons citrate agar slants were prepared and test organisms were streaked on the slants. The slants was incubated for 24 hrs at 37ºC. Bacterial growth and colour change to blue indicates +ve and no colour change indicates –ve.

Carbohydrate fermentation test

It is used to test the ability of microorganisms to ferment the given carbohydrate and produce organic acids. Test broth was prepared with peptone, sodium chloride, phenol red. Fructose, glucose, maltose and mannitol was added as carbohydrate source in separate tubes. Then it was inoculated with the culture and incubated at 37ºC for 18 hrs. The medium changes to yellow indicates +ve result and no colour change indicates –ve result .

MR and VP test

MR-VP broth was prepared and culture was inoculated in separate tubes and left for incubation at 37ºC for 3 days. For MR test, 5 drops of MR reagent was added. Appearance of red colur indicates +ve result. For VP test, 6 drops of 5% α napthol and 2 drops of 40% KOH was added and mixed. Formation of pink red colour at surface indicates +ve result .

Oxidase test

It is to determine the ability of the organisms to produce the cytochrome oxidase enzyme. The oxidase disc was taken and rubbed with the test organism. The development of deep purple colour within 10 sec indicates +ve and absence of colour indicates –ve.

2.4 Estimation of N_2 fixing ability of *Azotobacter* isolates

The efficiency of N_2 fixation of *Azotobacter* isolates weas made in Ashby broth containing mannitol source. All the seven isolates were inoculated separately in Ashby broth in respective conical flasks. The flask was incubated at 37º C for 10 days. The total amount of N_2 fixation was determined by micro kjeldahl method (Bergerson, 1980).

Sample preparation and digestion

After the incubation of isolates for 10 days, the broth containing isolates were digested in 250ml micro kjeldahl flask by adding the digestion salt mixture (50:10:01 ratio of K_2SO_4, $CuSO_4$ and Metallic Selenium) and 3ml of conc. H_2SO_4 and digested in a sand bath. After digestion, 100ml of distilled water was added and cooled. The appearance of apple green colour indicates the complete digestion of the sample and move for further distillation and back titration.

Distillation

The digested samples were taken in micro kjeldahl distillation apparatus. For the quick estimation, 10ml of 40% NaOH was added into distillation flask. In a conical flask, 10ml of 4% boric acid reagent and 3-4 drops of mixed indicator (prepared by dissolving 0.5g bromo cresol green & 0.1g methyl red in 100ml 95% Ethanol) were added. The flask was connected to the condenser and the tip of the condenser outlet is dipped into the flask containing boric acid. The entire system is regulated by water flow for heat control.

Titration and calculation

The flask containing solution, boric acid and mixed indicator containing the 'distilled off' NH_3 was titrated against standard HCL until the blue colour disappears. Excess addition of 1ml HCL will give pink colour as end point to stop titration. The percentage of N_2 fixation is estimated by the formula

$$\% \text{ of } N_2 \text{ in sample} = \frac{\text{Sample titer} - \text{Blank titer}}{\text{Vol. of sample in ml.}} \times \text{Normality of HCL} \times 14 \times 100$$

3. Result and discussion

3.1 Morphology and biochemical identification

The pH of all the soil samples ranged from 6.25 to 7.44 and has been reported optimal for the growth of *Azotobacter* sp. (Balandreau, 1986). From the 10 soil samples, 7 different isolates *viz*

Azo $_1$ to Azo $_7$ were obtained. These 7 isolates were purified by streaking and preserved for the further work. The colony morphology on Ashby's medium and various biochemical test like gram staining, catalase test, pigment production, Urease test, citrate utilization test, carbohydrate fermentation (fructose, glucose, sucrose, lactose and mannitol) test, oxidase test and MR VP test were conducted for identification. Out of the 7 isolates some showed +ve for the tests (fig.1) and the results were tabulated (table.1) Our biochemical test results were almost similar with Yusminah Hala *et al*., (2019), who isolated and characterized *Azotobacter* from neem rhizosphere. Further all the seven isolates were studied for their nitrogen fixing ability.

Table.1 Results of biochemical test for *Azotobacter* isolates.

Isolates	Azo_1	Azo_2	Azo_3	Azo_4	Azo_5	Azo_6	Azo_7
Gram staining	–	–	–	–	–	–	–
Catalase test	+	+	–	+	+	+	+
Pigment production	+	+	+	+	+	+	+
Urease test	+	+	+	+	+	+	+
Citrate utilization test	+	–	+	+	+	–	–
Lactose	+	+	+	+	+	+	+
Glucose	–	–	–	–	–	–	–
Fructose	+	–	–	+	–	+	+
Mannitol	–	–	–	–	–	–	–
Oxidase test	+	+	+	+	+	+	+
MR test	+	+	+	+	+	+	+
VP test	+	+	+	+	+	+	+

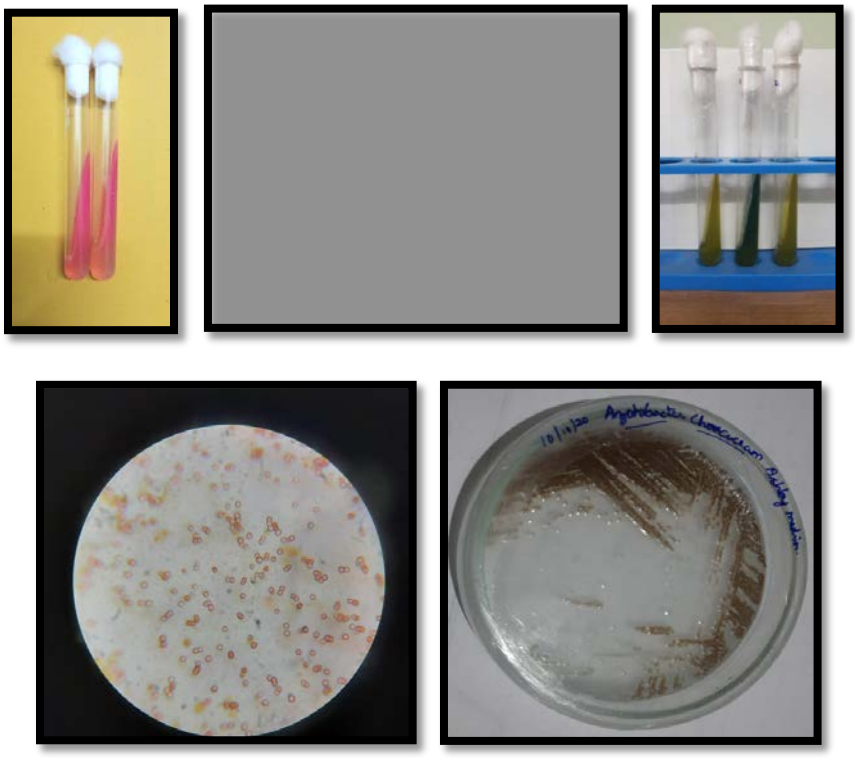

Fig.1 Results of urease test, carbohydrate fermentation test, citrate utilization test, gram staining and colony of Azo_4 isolate.

3.2 Estimation of N_2 fixing ability of *Azotobacter* isolates

All the seven isolates tested were able to fix atmospheric nitrogen. The N_2 fixation ranged from 6.58 to 14.86 mg N/ml (fig.2). Among them, the maximum N_2 fixation ability of 14.86mg N/ml was recorded from *Azotobacter* isolate Azo_4 and next 13.25 mg N/ml from Azo_5. Then 12.98, 9.57,12.46 and 13.22 mg N/ml was recorded in Azo_1, Azo_3, Azo_6 and Azo_7 respectively. The Azo_2 isolate were found to have the minimum ability of nitrogen fixation (6.58 mg N/ml).

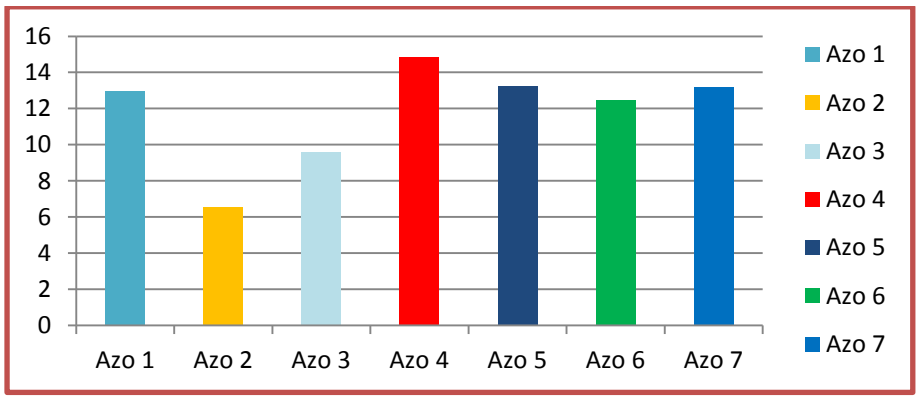

Fig.2 Ability of the *Azotobacter* isolates in N_2 fixation

The population of *Azotobacter* sp. in soil has a beneficial effect on plant growth but the abundance of these bacteria may be related or dependent on the factors like organic matter content present in the soil, pH of the soil, soil temperature, soil moisture and also other microbiological properties. *Azotobacter* are much more abundant in the rhizosphere of plants than in the sourrounding soil and that this abundance depends on the crop species (Sariv *et al.*, 1963). Research results showed that all the *A. chroococcum* strains had positive effect on the yield and N concentration of wheat (Kizilkaya, 2008). Jnawali *et al*., 2015, reported that the population of *Azotobacter* may suffer due to high amount of nitrates and the acidic environment created because of chemical fertilizers. So, it is said that the efficiency of the strains get reduced with increase in nitrogen content present in the soil. In order to maintain soil and plant health, these beneficial microorganisms play a vital role. Hence further more works may be carried out in identifying efficient strains of *Azotobacter* sp. and can be use in mass multiplication for biofertilizer production.

Reference

1. Balandreau J. (1986). Ecological factors and adaptive processes in N2 fixing bacterial populations of the plant environment. Plant Soil. 90, 73-80.

2. Beijereinck MW. Ueber Oligonitophile Mikroben, Zentralblattfir Bakteriologie, Parasitenkunde, Infektionskrankheiten and Hygiene. Abteilung II. 1901;7:561-582.

3. Bergerson, F. J., 1980. Measurement of nitrogen fixation by direct means, In: F.J. Bergerson (ed.), Methods of evaluating biological nitrogen fixation. John Wiley and Sons, Inc., New York., pp: 65-110.

4. Dobereiner, J., 1997 Soil. Biol. Biochem., 29:771-774

5. Jnawali A D , Ojha R B, Marahatta s. Role of Azotobacter in soil fertility and Sustainability - a review. Adv Plant Agric Res. 2015; 2(6) : 250-253.

6. Kizilkaya R. 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*. 2009;30(1):73–82.

7. Kizilkaya R. Yeild response and nitrogen concentrations of spring wheat (*Tritricum aestivum*) inoculated with *Azotobacter chroococcum* strains. Ecological Engineering. 2008; 33(2) : 150-156.

8. Monib M, Hosny I, Fayez M. Effect of *Azotobacter* inoculation on plant growth and soil nitrogen. *Zentralbl Bakteriol Naturwiss*. 1979;134(2):140– 148.

9. Nagananda G S, Das A, Bhattachrya S, et al. *In vitro* studies on the Effects of Biofertilizers (*Azotobacter* and *Rhizobium*) on Seed Germination and Development of *Trigonella foenum–graecum* L. using a Novel Glass Marble containing Liquid Medium. *International Journal of Botany*. 2010;6(4):394–403.

10. Sariv Z, Ragoviv B. The influence of the maize on the dynamic of *Azotobacter* in the soil. Soil Plant, 1963. 13 : 273-277.

11. Tchan YT, New PB. Azotobacteraceae. In:Holt JG, Williams, et al. editors. Bergey's Manual of Systematic Bacteriology Volume 1. Baltimore, USA; 1989. p. 220-229

12. Yusminah Hala and Alimuddin Ali (2019). Isolation and characterization of Azotobacter from neem rhizosphere. J. Phys : conf. ser. 1244012019.