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# Isolation Characterization and Optimization of Growth Conditions for Azotobacter spp.

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**Abstract**

**The role of genus *Azotobacter* in increasing the growth and health of plants is very important. The present study deals with the optimization of growth condition for *Azotobacter* species. Four colonies were isolated from soil and *Azotobacter* were found in them. The medium M2 allowed for the highest growth of *Azotobacter* (0.015mglml) and 0.3mg/ml. Optimum temperature ,pH and incubation period for growth of *Azotobacter* were 25 degree Celsius,9 and 48 hrs., respectively .By enhancing the growing medium with various carbon and nitrogen sources ,the growth speciality of *Azotobacter* was investigated. The most effective concentration for Azotobacter were 2.5% mannitol (as a C- source) and 2%$(NH_4)_2$ $SO_4$ (as an N-source) 24hrs old inoculum at a level of 1% was found best for the growth of *Azotobacter* .The bio fertilization activity was studied on maize plant in pot experiment and it was found that plant inoculated with *Azotobacter* gave better growth as compared to control plants.**

**Keywords: Azotobacter, Maize plant, pH**

## 1. Introduction

The microbes that are alive enhance the plant nutrient either by catalysing or by increasing the availability of nutrients in soil are termed as bio fertilizers. Currently used bio fertilizers include useful bacteria and fungi due to their successful colonization within the rhizosphere, root surface, or root interior. Even though they have the potential to improve soil fertility, organic fertilizers have yet to replace conventional chemical fertilizers in commercial agriculture.

The most generally used organic fertilizers include *Azotosppacter* Organic Fertilizer, Phosphorus Organic Fertilizer, LysoOrganic Fertilizer, and Trichoderma Organic Fertilizer. *Azotobacter* has been one of the most significant microorganisms widely used as a bio fertilizer (Pereira and Shetty, 1987). *Azotobacter* is a Gram-negative, pleomorphic bacterium. H. Various sizes and shapes. *Azotobacter* grows well in liquid Bark medium at pH 7-9, but does not grow below pH 6.

The Two scientist Hennequin and Blachere (1966) discovered small amounts of IAA in previous cultures of *Azotobacter* to which tryptophan was not added. *Azotobacter* uses auxins, cytokines, and GA-like substances, and these growth materials are the major drivers of tomato overgrowth (Azcorn and Barea, 1975). These hormonal substances, originating from the rhizosphere, influence the growth of closely related higher plants. Eklund (1970) showed that the presence of his *Azotobacter* chroococcum in the

tomato and cucumber rhizosphere correlated with increased germination and seedling growth. Puertas and Gonzales (1999) discovered that the dry weight of tomato plants were inoculated with *Azotobacter* chroococcum and grown on low-phosphate soil was mostly higher than in uninoculated plants. Plant hormones (auxins, cytokinins, gibberellins) stimulate root development.

Therefore the present study deals with the isolation characterization and optimization of growth conditions for *Azotobacter spp*

## 2. Materials and Methods

**2.1 Collection and Isolation serial dilution:** Soil samples were collected in April 2022 from different areas of Mundakkal, Kollam. The samples were taken from the rhizospheres of several crops (Tomato, Pink amaranths, cashew, and Banana) Four various locations had their soil samples taken.The soils were then brought to the laboratory in zip-lock plastic packets for the isolation purpose of *Azotobacter sp*. strains. The bacterial isolates were characterized using biochemical test such as Indole test, MVP test, Citrate test, Catalase and Oxidase test, Urease, TSI accordingly performed.

**2.2 Plating Method:** To prepare medium plates, approximately 12-15 ml of sterilized, cool and melted (45°C) medium was transferred aseptically into petriplates under laminar flow. After solidification of medium, one ml of suspension from $10^{-3}$, $10^{-4}$ dilutions of each rhizosphere soil sample was poured into each medium plate in double. The plates were incubated at 28±2°C in an incubator in inverted position for 7-10 days and growth of presumptive *Azotobacter* was observed on each during the entire 7 days incubation period.

**2.3Preparation of pure culture slants from the respective petriplates*:*** Following the exterior morphology identification of the bacterial colonies, a few well- developed colonies were chosen in order to isolate the pure culture of Azotobacter *sp*. strains.

**2.4 Morphological characterization of Azotobacter isolates*:*** Colony characteristics such as shape, size and appearance of each strain of *Azotobacter* were recorded as: Cell shape - Oval, rod (small/ medium/large), oval in chain, rod in chain shaped. Consistency- Based on light transmitted through the colony, these were categorized as transparent, translucent and opaque colonies (usually firm with little gum whereas the less dense colonies were often gummy, soft, glistening or dull). According to Cerney's (1993) normal staining approach, all Azotobacter isolates were tested by having them react with Gram stains . A clean microscopic slide's central region was evenly covered with a diluted bacterial cell suspension that had been taken with the use of an inoculating loop. The bacterial cells were fixed by heating it through passing repeatedly few seconds over a flame. They were stained with a drop of crystal violet solution (30 seconds), iodine (30 seconds) followed by ethyl alcohol, and safranin (1 to 1.5 minutes) solution. After each staining period, the cells were washed under a tap water jet and examined under a compound microscope using an oil immersion lens, and these bacterial cells were classified as Gram-negative (G-) or gram-positive (G+) or gram variable (G±) strains. The pigment of all colonies of *Azotobacter* isolates was noted after a week on an Ashby's Mannitol Agar medium.

***2.5* IAA production** Culture Media: LB (Luria-Bertani) broth/Nutrient Broth/YEM broth (Based on the growth of Bacteria Media may be customized). 0.1% tryptophan may or may not be added. If assumes that the bacteria may produce lower amount of IAA, in that case 0.1% tryptophan may be added with the media. Culture media (LB/Nutrient broth/YEM) inoculated with bacteria ± tryptophan (0.1%)Grow in Shaking incubator (28ºC, 100 rpm) for 48 hours (for obtaining log phase)Non inoculated broth culture was kept as control. Salkowski method was used to gauge IAA generation following incubation. Below is a picture of how to prepare a Salkowski reagent. Absorbance at 540 nm was plotted vs Concentration for the standard curve. We can now calculate the Concentration (g/mL) by plotting the absorbance of several bacterial strains

**2.6 Batch experiments*:*** .In 250 ml shake flasks containing culture medium that was made by dissolving agar, a batch experiment for the growth of Azotobacter was conducted. Peptone 0.005(mg/ml) Salt concentration: 0.005 mg/ml HM peptone/beef extract 0.0015 (mg/ml), yeast extract 0.0015 (mg/ml), and final pH (at 25°C) 7.4±0.2. The flasks were cotton plugged and autoclaved at 121 $^{0}$C (15 lb/inch2 pressure) for 15min. Then the medium was cooled down at room temperature and inoculated by adding 1ml of inoculum with the help of micropipette in laminar air flow chamber. The flasks were then incubated at 30ºC in a rotary shaker for 48hrs at 200rpm for growth of microorganisms.

**2.7 Selection of culture medium:** The growth of *Azotobacter* was identified using different culture media through batch fermentation process. M1, M3 medium are taken respectively. They are cotton plugged and autoclaved, cooled at room temperature and are inoculated by adding 1ml of the inoculum, with help of a micropipette in laminar air flow chamber.

**2.8 Effect of pH:** The effect of pH on different *Azotobacter* spp.was identified by varying the pH of the medium from 6.0 to 9.0. M3 medium is taken respectively.

**2.9 Effect of incubation period**: The effect of incubation period on *Azotobacter* spp. were studied by varying the incubation period 24-120 hrs.M3 medium was taken respectively.

**2.10 Effect of incubation temperature: The** effect of incubation temperature on *Azotobacter* spp. Were studied by varying the incubation temperature 25 – 40o C.M3 medium was taken respectively

**2.11 Screening of Carbon Sources: The** M3 medium was supplemented with 2.5% carbon source such as Sucrose and Mannitol.

**2.12 Screening of Nitrogen sources:** The M3 medium was supplemented with 2% of different nitrogen sources. Casein and Ammonium Sulphate is used as the nitrogen source in this experiment.

**2.13 Application of bio fertilizer*:*** A pot experiment in which seeds of maize injected with bacterial growth were sown in sterilized soil in clean pots allowed researchers to analyze the impact of bio fertilizer on plant growth. Seeds were watered and placed under sunlight. A control was also taken in which seeds were not inoculated with bacterial strains. After the growth of plants, the size of leaves, and roots were recorded in all experimental and control pots

## 3. Results and discussion

The results of the study entitled "Isolation characterization and optimization for growth conditions for *Azotobacter spp.*" are presented below

Table 3.1 Morphological and cultural characteristics of isolate Azotobacter strains (Colony characteristics one week after streaking)

| Sl.No | Type | Colony shape/margin | Color | Consistency | Gram staining | Catalase test |
|---|---|---|---|---|---|---|
| 1 | Sample 1 | Spherical, convex | White t light yellow | Slimy | Negative | Positive |
| 2 | Sample 2 | Slimy,circular,convex | Dull white | Viscous | Negative | Negative |
| 3 | Sample 3 | Circular, wrinkled | Creamy white | Gummy | Negative | Positive |
| 4 | Sample 4 | Poor growth | Yellowis white | Mucoid | Positive | Negative |
| 5 | Sample 5 | Slimy, circular convex | Clear transluce t | Mucoid | Positive | Negative |
| 6 | Sample 6 | Circular, contoured, convex | Yellowis | Dry | Positive | Positive |

**Figure 3.1** Growth on Luria betani medium

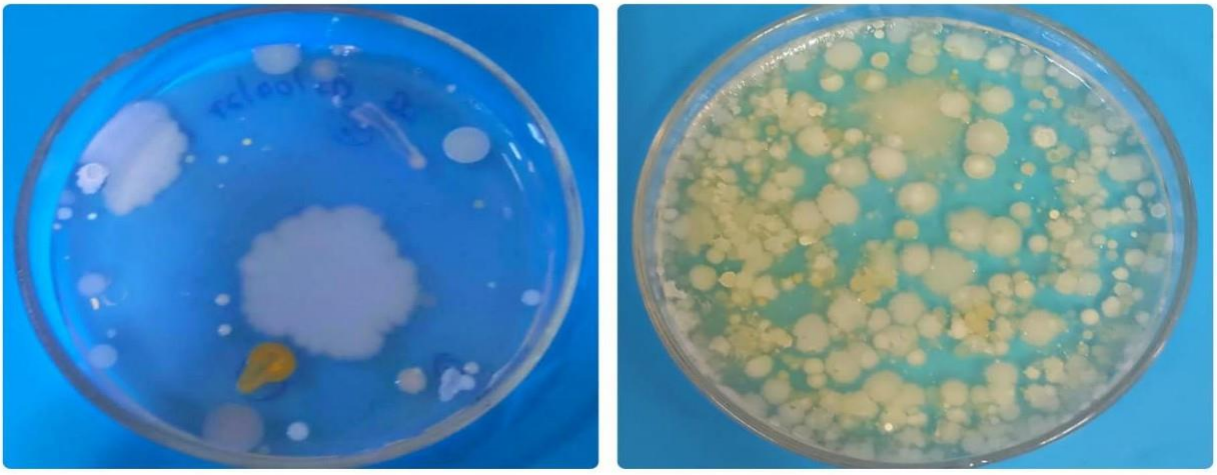


**Figure 3.2** Growth on Ashby's Mannitol Agar Medium

**Figure 3.3** Gram staining of the isolates

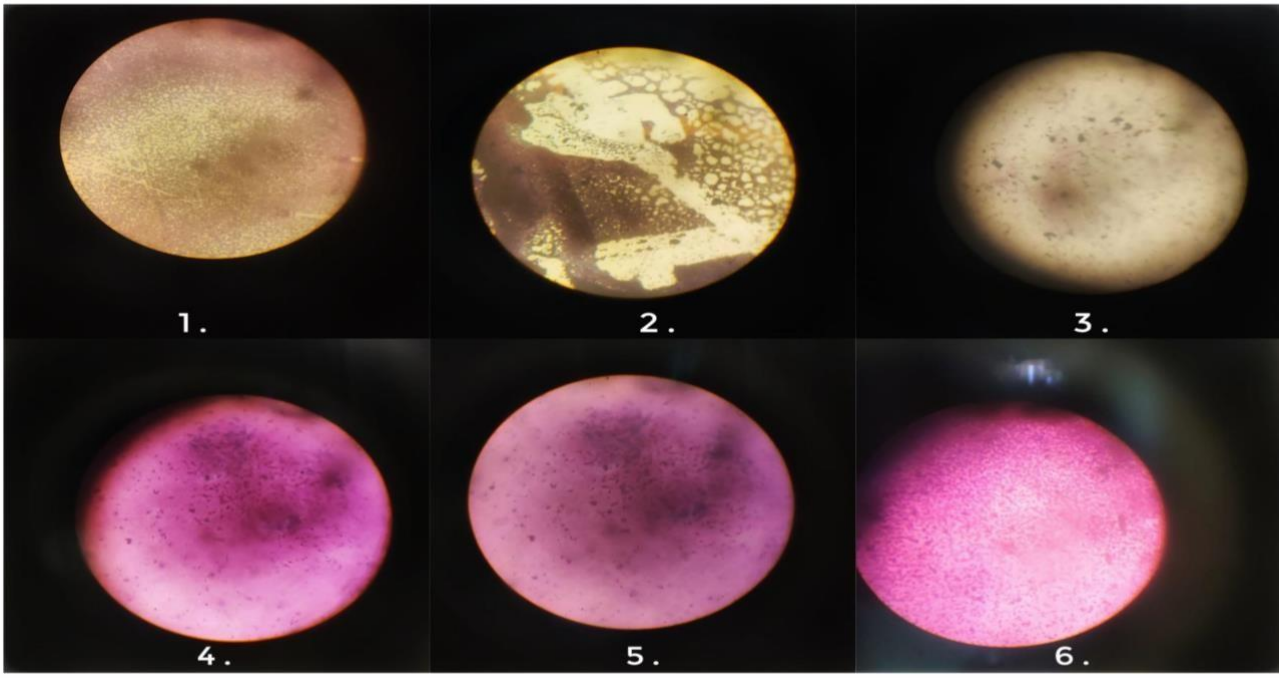



**Table 3.2** Discrimination of *Azotobacter* isolates characters

| Sl.No | Test Name | Sample 1 | Sample 2 | Sample 3 | Sample 4 | Sample 5 | Sample 6 |
|---|---|---|---|---|---|---|---|
| 1 | Motility | + | + | + | + | + | + |
| 2 | Citrate utilization test | - | - | - | - | - | - |
| 3 | Methyl Red test | + | + | + | - | + | + |
| 4 | Voges– | - | + | + | - | - | + |

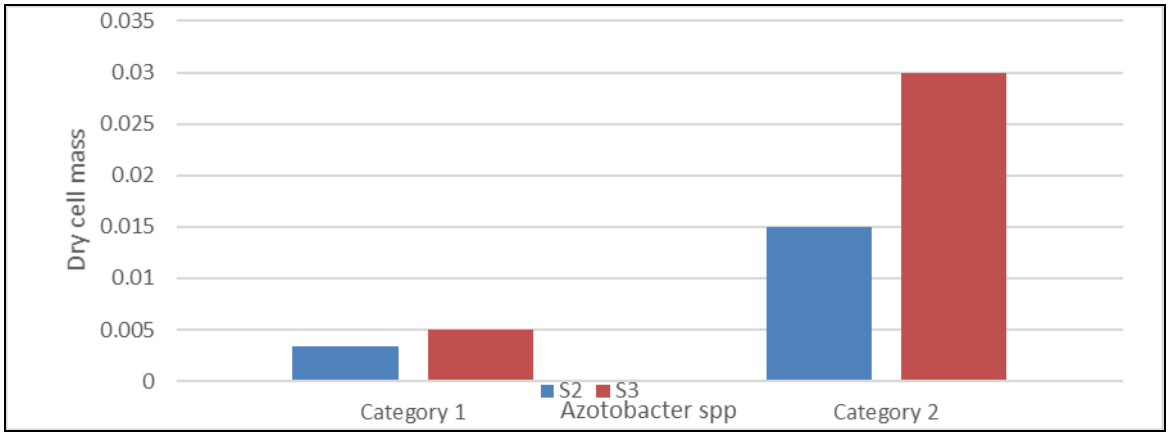
| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | Proskauer test | | | | | | |
| 5 | Starch hydrolysis test | + | + | - | + | - | - |
| 6 | Oxidase test | - | + | + | - | - | - |
| 7 | Extracellular polysaccharide production | + | + | + | + | + | + |

The six isolates were grown on Ashby's Mannitol Agar Medium, Luria betani medium (fig 3.1 and 3.2). Azotobacter isolates were pleomorphic. The variation in cell shape, colony size, and appearance was because of various factors such as the presence of disorganized, shapeless masses symptoms, giant spindle-shaped cells branched form and even spore formation in cultures of Azotobacter (Pleomorphism) may also be attributed to the composition of the medium and the stage of its growth in the medium as has been reported by Aquilantiet al. (2004). The colony characters such as colony margin, size, color, and consistency also differed among different isolates of Azotobacter (Table 3. 1). Three isolates were Gram -ve and three were Gram +ve. For Gram Staining test (Fig3.3), Five Azotobacter isolates were found catalase-positive one isolates were catalase-negative (Table 3.2). The isolates of Azotobacter were studied for their carbon source utilization and cyst forming behaviour to confirm and differentiate them into different species according to the Bergey's Manual of Systematic Bacteriology (Table 3.1). Three isolates showed Gram positive test and three were gram negative. Different Azotobacter isolates showed wide variability in consistency and colony color. Isolates were reported unevenness in color and flowing consistency mucoid, viscous, milky, glistening, gummy with dull to cream white, pale yellow, as described by John et al. (1994). Isolates were medium rods and cocci however, long; medium rods or cocci were observed in sample isolates

**Table 3.3** IMVIC TEST of Aztotobacter

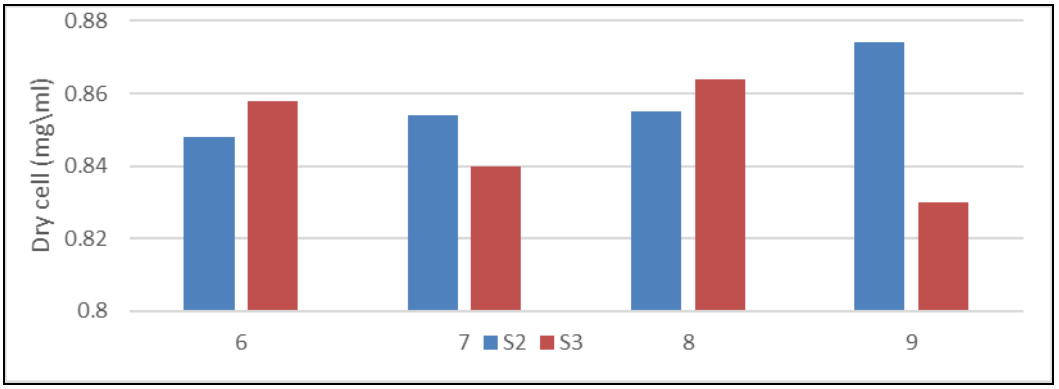
| **Sl. No** | **Test name** | **Sample 1** | **Sample 2** | **Sample 3** | **Sample 5** |
|---|---|---|---|---|---|
| 1 | Indole test | - | - | - | - |
| 2 | Urease test | - | - | - | - |
| 3 | Glucose fermentation test | + | + | + | + |
| 4 | Nitrate test | - | + | + | + |
| 5 | Casein test | - | + | - | - |
| 6 | IAA production | - | + | + | + |

**Figure 3.4** Screening of different media for growth of *Azotobacter spp*



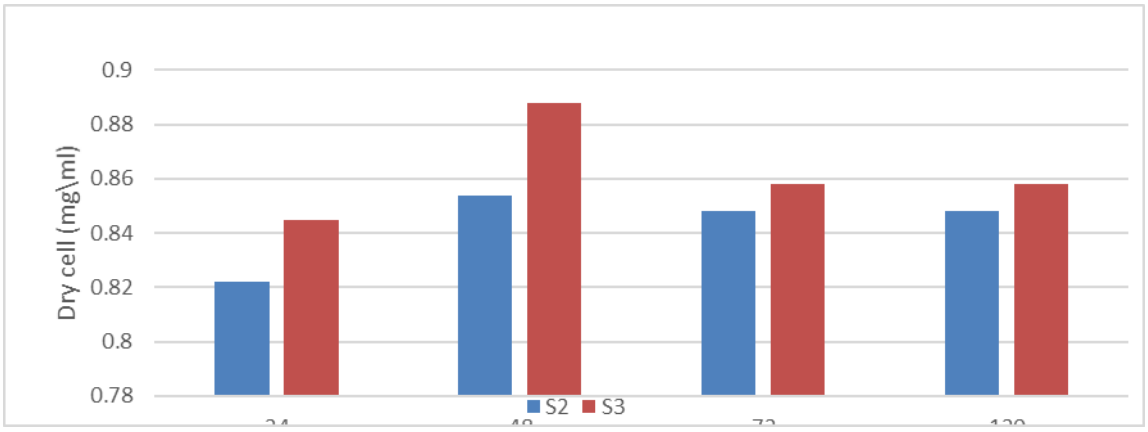

**Screening of Azotobacter spp. for maximum biomass production**: Data of Table 3. 3 show the screening of isolates for maximum growth on synthetic medium in shake flasks. Six different isolates of Azotobacter were screened and Sample 6 was found best strain showing highest dry cell biomass production i.e.0.007 mg/ml. Through batch fermentation, several culture media were used to study Azotobacter growth (Figure 3.4). Azotobacter IIB-3 grew to its maximum potential (0.015 mg/ml) in medium Luria betani. While Azotobacter vinelandii's growth was at its highest (0.3 mg/ml) on Luria betani medium

**Figure 3.5** Effect of initial pH of the culture medium on growth of *Azotobacter spp*.



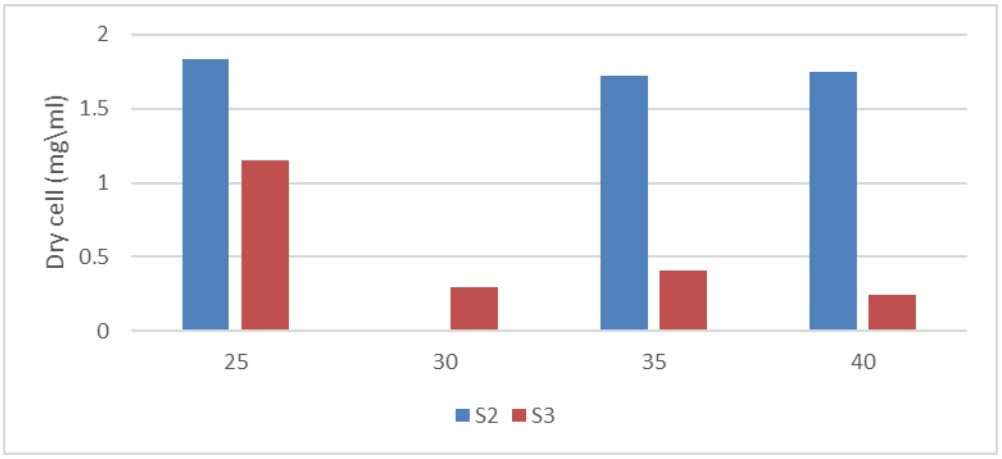

**Effect of pH:** Effect of pH on Azotobacter spp. were studied by varying the pH of the medium from 5.0 to 9.0 (Figure 3.5). Azotobacter sample 2 showed maximum growth i.e. 0.87mg/ml at pH 9.0. While Azotobacter sample 3 showed maximum growth of 0.86 mg/ml at pH 8.0.

**Figure 3.6** Effect of incubation period on the growth of Azotobacter spp.




**Effect of incubation period:** The effect of incubation period on Azotobacter spp. was studied by varying the incubation period 24-120 hrs. (Figure 3.6). Sample-2 showed maximum growth i.e. 0.85mg/ml after incubation period of 48 hrs. While sample 3 showed maximum growth of 0.88mg/ml in 48hrs incubation period. Incubation period is directly proportional to the growth of bacteria up to a certain extent and after that growth of bacteria start decreasing that can be attributed to the decrease in the supply of nutrients to microorganisms or may be accumulation of some toxic compounds in the broth.

**Figure 3.7** Effect of incubation temperature on the growth of Azotobacter spp




***Effect of incubation temperature :***The effect of incubation temperature on Azotobacter spp. were studied by varying the incubation temperature 25-40$^{o}$C for 48hrs (Figure 3.7). Sample 2 showed maximum growth i.e.1.83mg/ml after incubation at 25$^{o}$C. While sample 3 showed maximum growth of 1.15 mg/ml at 25$^{o}$C incubation temperature.

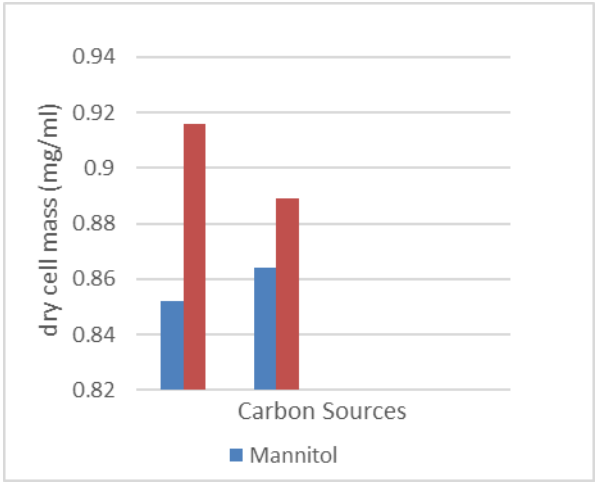



**Figure 3.8** Screening of different carbon Sources for enhanced growth of *Azotobacter spp*.

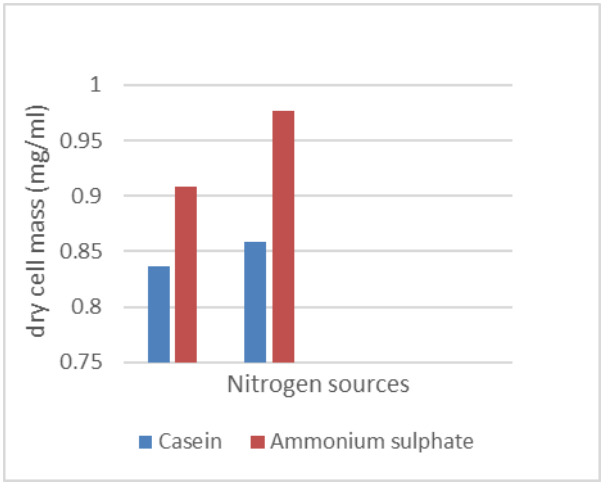



**Figure 3.9** Screening of different nitrogen sources on the growth of *Azotobacter spp*.

**Screening of Carbon Sources:**

Fermentation medium was supplemented with 2.5% of different carbon sources (Figure 3.8). Sample 2 showed maximum growth (0.86mg/ml) in the presence of mannitol as carbon source. While Sample 3 showed maximum growth of 0.92mg/ml in Sucrose medium.

**Screening of nitrogen sources:**

Fermentation medium was supplemented with 2% of different nitrogen sources (Figure 3.9). Sample 2 showed maximum growth (0.85mg/ml) in the presence of casein as nitrogen source. While sample 3 showed maximum growth of 0.96 mg/ml in medium containing casein as nitrogen source.

**Figure 3.10** – Effect of bio fertilizer on the growth of Maize Seedlings ( leaves size).

**Figure 3.11** Effect of bio fertilizer on the growth of Maize Seedling (Roots size)

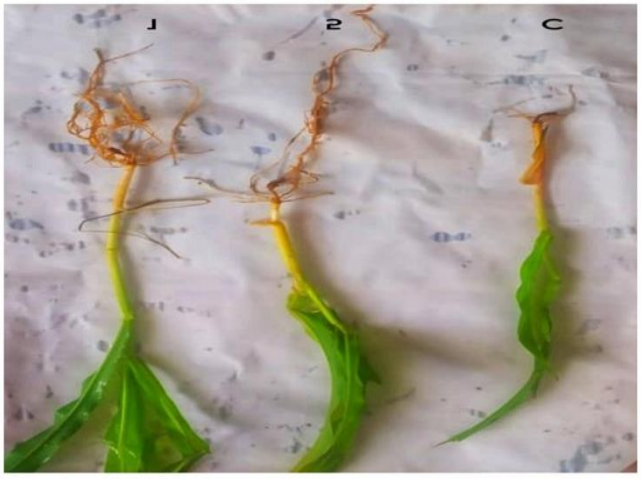



**Figure 3.12** Effect of application of *Azotobacter spp as bio fertilizer*

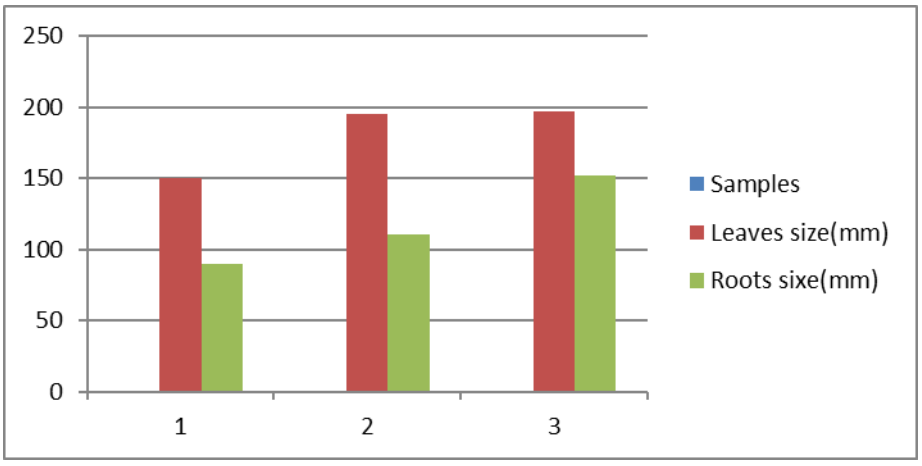



**Table 3.4** Effect of application of *Azotobacter spp as bio fertilizer*

| | **Samples** | **Leaves size(mm)** | **Roots size(mm)** |
|---|---|---|---|
| 1 | Control | 150mm | 90mm |
| 2 | Sample 2 | 195mm | 110mm |
| 3 | Sample 3 | 197mm | 152mm |

Application of Azotobacter biomass as bio fertilizer : The effects of bio fertilizers on the growth capacity of maize (Zeamayz) plants were investigated (Table 3.4). The experimental plants incubated with Azotobacter vinelandii showed the greatest growth of leaves (195mm) and a less extensive system of roots (110mm) (Sample 2). While the seeds treated with Azotobacter IIB-3 (Sample 3) displayed 197 mm-long leaves and 152 mm-long roots (fig 3.12).

Results showed that plants inoculated with bacterial strains gave maximum leaf growth which confirms that these contained the maximum nutrients available for their growth and minimum proliferation of roots because sufficient nutrients were present in the soil for uptake of roots due to which their penetration into

the soil was not so much high. It has been reported that inoculation of plants with Azotobacter increases their growth and yield as well.

## Conclusion

Azotobacter are isolated from the rhizospheric soil of different crops. From the above mentioned investigations, it was deduced that improving the cultural and nutritional conditions led to an improvement in Azotobacter species' growth during fermentation. However, quantitative understanding of the ecological factors that control the performance of biological N2fixation systems of the bacterium in crop fields is essential for promotion and successful adoption of the bio-fertilizer production technology

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## References

1 .Aquilanti L., Favilli F., Clementi F., "Comparison of Different Strategies for Isolation and Preliminary Identification of Azotobacter from Soil Samples", Soil Biology and Biochemistry, 2004, 36, 1475–1483.
2. Cerney G., "Method for the Distinction of Gram Negative and Gram Positive Bacteria", European Journal of Applied Microbiology, 1993, (3), 223–225.
3. John G.H., Noel R.K., Peter H.A.S., James T.S., Stanley T.W., "Aerobic/Microaerophilic, Motile, Helical/Vibroid Gram Negative Bacteria", Bergey's Manual of Determinative Bacteriology, 1994, , 39–45.
4. Gandora V., Gupta R.D., Bhardwaj K.K.R., "Abundance of Azotobacter in Great Soil Groups of North-West Himalayas", Journal of the Indian Society of Soil Science, 1998, 46 (3), 379–383.
5. Salhia B., "The Effect of Azotobacter chroococcum as Nitrogen Biofertilizer on the Growth and Yield of Cucumis sativus", Deanery of Higher Education Faculty of Science, Master of Biological Sciences, Botany: The Islamic University Gaza, 2013, ,
6. Tchan Y.T., New P.B., "Azotobacteraceae", Bergey's Manual of Systematic Bacteriology Volume 1, 1989, 220–229.
7. Chen J., "The Combined Use of Chemical and Organic Fertilizers and/or Biofertilizer for Crop Growth and Soil Fertility", International Workshop on Sustained Management of the Soil-Rhizosphere System for Efficient Crop Production and Fertilizer Use, Thailand, 2006, , 1–10.
8. 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.
9. Sariv Z., "Biogenic Levels of the Horizons of Calcerous Chernozem in Vojvodina", Contemporary Agriculture, 1969, 17, 819–825.
10. Sariv Z., "Biogenity of Limeless Chernozem in Vojvodina", Annals of Scientific Work at the Institute for Research in Agriculture Novi Sad, 1969, 7, 145–151.
11. Malek A.Y., Hosny I., Shawky B.T., "Nitrogen-Fixing Capacity of Azotobacter as Affected by the Type and Depth of Substrate", Zentralblatt für Bakteriologie Naturwissenschaften, 1979, 134 (5), 390–397.
12. Kalaigandhi V., Kannapiran E., Harimuraleedharan, et al., "Azotobacter Population in Rhizosphere

and Non-Rhizosphere Sediments of Tondi Coast", International Journal of Biological Technology, 2010, 1 (1), 63–65.
13. Sariv Z., Ragoviv B., "The Influence of the Maize on the Dynamic of Azotobacter in the Soil", Soil Plant, 1963, 13, 273–277.
14. Sariv Z., Ragoviv B., "The Effect of Some Plants on the Dynamics of Azotobacter in the Soil", Annals of Scientific Work at the Faculty of Agriculture Novi Sad, 1963, 7, 1–11.
15. Dong H., Li W., Eneji A.E., et al., "Nitrogen Rate and Plant Density Effects on Yield and Late-Season Leaf Senescence of Cotton Raised on a Saline Field", Field Crops Research, 2012, 126, 137144.
16. Monib M., Hosny I., Fayez M., "Effect of Azotobacter Inoculation on Plant Growth and Soil Nitrogen", Zentralblatt für Bakteriologie Naturwissenschaften, 1979, 134 (2), 140–148.
17. Rajaee S., Alikhani H.A., Raiesi F., "Effect of Plant Growth Promoting Potentials of Azotobacter chroococcum Native Strains on Growth, Yield and Uptake of Nutrients in Wheat", Agris Records, 2007, 11 (41), 285–297.
18 . Kizilog I.U., Bilen F.T., Ataplu N., "Effect of Inoculation Eight Azotobacter chroococcum and Nitrogen Fertilizer on Plant Growth of Corn (Zea mays) Carbohydrate and Protein Contents", Ziraat Fakultesi Dergisi Ataturk Universitesi, 2001, 32, 215–221.
19. Meshram S.U., Shende S.T., "Response of Maize to Azotobacter chroococcum", Plant and Soil, 1982, 69, 265–273.
20. Monib M., Malek A.E.Y., Hosny I., et al., "Seed Inoculation with Azotobacter chroococcum in Sand Cultures and Its Effect on Nitrogen Balance", Zentralblatt für Bakteriologie Naturwissenschaften, 1979, 134 (3), 243–248.
21. Beijerinck M.W., "Über Oligonitrophile Mikroben", Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten II Abt, 1901, 7, 561–582.
22. Bhardwaj D., Medici A., Gojon A., Lacombe B., Tuteja N., "A New Insight into Root Responses to External Cues", Plant Signal Behaviour, 2015, 10 (12), DOI: 10.1080/15592324.2015.1049791.
23. Chen J.H., Czajka D.R., Lion L.W., Shuler M.L., Ghiorse W.C., "Trace Metal Mobilization in Soil by Bacterial Polymers", Environmental Health Perspectives, 1995, 103 (1), 53–58.