RESEARCH ARTICLE

ISOLATION, AND CHARACTERIZATION OF THERMOSTABLE *AZOTOBACTER* FOR BIO FERTILIZER PRODUCTION

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DOI: https://doi.org/10.5281/zenodo.17442168

Abstract:

Azotobacter species, as free-living nitrogen-fixing bacteria in the soil, are important for soil fertility and crop productivity because they convert atmospheric nitrogen to plant-useable forms and produce other plant growth-promoting compounds. The focus of this study was to isolate, identify, and characterize Israeli strains of *Azotobacter* that are thermostable and are from compost enriched soils. Organisms were isolated using enrichment, followed by serial dilution on Ashby's Mannitol Agar, and underwent morphological, biochemical, and physiological characterization. The strains that were identified as thermostable were characterized for nitrogen fixation, tolerance to salinity, and biofertilizer potential. Seed germination assays demonstrated significant improvement in root and shoot growth of treated wheat and mung bean seedlings as compared to their controls. The results in this study demonstrate that thermostable *Azotobacter* species have the potential to be novel biofertilizers as they can be used as an environmentally safe alternative.

Keywords: Thermostable Azotobacter; Nitrogen Fixation; Biofertilizer; Sustainable Agriculture.

Introduction:

Nitrogen is a crucial macronutrient for plant development and has a basic role in amino acid, nucleic acid, and chlorophyll formation. Deficiency of nitrogen found in the soil is one of the most pervasive limiting factors of crop yield and fertility in many areas of the world. To combat nitrogen deficiency of soil, farmers use synthetic fertilizers containing readily available nitrogen for the plant. However, nitrogen fertilizers have excessive and indiscriminate applications potentially leading to various environmental issues, including: acidification of soil; degradation of soil structure; groundwater contamination with nitrate; and increased emissions of greenhouse gases, including nitrous oxide [1]. These limits and issues fueled the development of sustainable systems for soil fertility management such as the use of biofertilizers.

Biofertilizers are formulations composed of live microorganisms that enhance nutrient availability and support plant growth via natural mechanisms. Nitrogen-fixing bacteria, especially those belonging to the genus Azotobacter, are very important biofertilizers. Azotobacter species are free-living, aerobic, heterotrophic bacteria that can fix atmospheric nitrogen into forms that are bioavailable to plants, and do not form any symbiotic associations with plants [2,3]. These nitrogen-fixing bacteria can be utilized for a wide variety of crop species, such as cereals, legumes, and vegetables. The genus Azotobacter was first described by Beijerinck in 1901, which contains Gram-negative, rod-shaped, cyst-forming bacteria that produce extracellular polysaccharides. These exopolysaccharides not only impart a characteristic slimy, mucoid colony morphology, but also help to protect the cells from desiccation, UV radiation, and other environmental stresses [4,7,8].

Aside from nitrogen fixation, Azotobacter possesses a variety of plant growth-promoting traits. It secretes phytohormones like auxins, gibberellins, and cytokinins which increase plant development through promoting root elongation and cellular division. Some strains can solubilize phosphate and make it available for plants while producing siderophores to chelate iron, making a more available micronutrient within the rhizosphere [6,8–11,14]. With these many advantages, Azotobacter is an excellent candidate for biofertilizer production, especially in low-input agriculture and sustainable agricultural systems.

Thermostable and halotolerant Azotobacter strains are worth researching and testing, particularly for agriculture in warmer/hotter temperatures or saline soil types, such as semi-arid or tropical climates. High soil temperatures can reduce the viability and activity of microbes and, as a result, the potential to have biofertilizer strains be effective. Thermostable strains have been shown to withstand high soil temperatures and continue to fix nitrogen [9,15]. Halotolerant strains can survive and function in saline soils that otherwise create limitations to normal agricultural practices [19,24,25]. Previous studies have confirmed that inoculation of crops with Azotobacter promotes root growth, nutrient uptake, seedling vigor, and overall productivity in crops like wheat, mung bean, or legumes [16,20,21,26].

Although these attributes are promising, we have very little overall research regarding the isolation, characterization, and biofertilizer value of thermostable *Azotobacter* strains. Much of the literature has described general characterization at best, or laboratory-based nitrogen fixation trials, with

significantly less regard for stress tolerance, or promotion of plant growth under controlled germination conditions. This study aims to address this gap in research by isolating thermostable *Azotobacter* strains from compost soils. After characterizing morphologically, biochemically, and physiologically, their tolerance to salinity and high temperature will be evaluated, and the potential use as biofertilizers is to be assessed through seed germination and seedling growth trials. By finding strains that have both stress tolerance and the ability to be metabolically active, this study hopes to address a direction towards sustainable alternatives to chemically derived fertilizers, and provide biofertilizer options to improve soil fertility and crop output under challenging agro-climatic conditions.

Materials and Methods:

Sample Collection

Soil samples enriched with compost were collected aseptically from the Dogarshelki village area, near Nanded, Maharashtra, during the winter of 2025, using sterile spatulas and putting the soil samples in sterile zip-lock bags for immediate transport to the laboratory [10].

Isolation of Thermostable *Azotobacter*

To isolate thermostable *Azotobacter* strains, 100 mL of Ashby's mannitol broth was prepared and enriched with 1% (1 g in 100 mL) of soil sample. The inoculated broth was then incubated at 40 °C for 72 hours to allow the growth of thermotolerant cultured bacteria. After the incubation period, turbidity in the broth was seen signifying increased growth and proliferation of heterotrophic bacteria. Serial dilution of the enriched *Azotobacter* culture was performed to reduce the density of cells, with dilutions done from 10^{-1} to 10^{-10}. Aliquots (100 µL) from the selected dilutions (10^{-2} to 10^{-10}) were plated onto Ashby's mannitol agar plates and incubated under the same conditions to isolate individual *Azotobacter* colonies for further assessment and characterization of important morphological, biochemical, and functional properties.

Morphological and Biochemical Characterization

Examination of the isolates at the microscopic level comprised standard Gram staining and motility tests to determine the specific cellular morphology and motility. Biochemical characterization was conducted following standard protocols [11-13] utilizing various assays to assess the metabolic abilities. The isolates were assessed for catalase and amylase utilization as well as starch hydrolysis, which assessed the isolates ability to produce extracellular enzymes. Carbohydrate metabolism was assessed through sugar utilization tests involving glucose, sucrose, mannitol and maltose. One of the denominations of IMViC (indole, methyl red, Voges-Proskauer, and citrate) tests and assays (nitrate reduction) were assessed to study metabolic versatility. All together, these analyses presented a comprehensive profile of the physiological and biochemical characterization of the isolates that are a metabolically active free-living nitrogen-fixing bacteria for application in soil and plant applications.

Salt Tolerance Test

The salt tolerance level of the *Azotobacter* isolates was tested using Ashby's medium supplemented with varying concentrations (0.5%, 1%, 2%, 3%, 4%, and 5%) of NaCl. The growth of the isolate was determined visually and by measuring the cultue optical density at 600nm (OD600) after

72 hours [13,19]. This test provides evidence of the isolates ability to grow and remain metabolically active in salt, and the significance of this is helpful in their use in soils prone to stress.

Nitrogen Fixation Assay

The nitrogen-fixing ability of the isolates was measured using the acetylene reduction assay (ARA) to measure nitrogenase activity in micromoles C_2H_4/hour/mL of culture [5]. This assay demonstrated the functional ability of the isolates to convert atmospheric nitrogen into bioavailable forms to support soil fertility and plant productivity.

Seed Germination Assay

The germination of seeds and their promotion of early growth performance were tested with wheat (*Triticum aestivum*) and mung bean (*Vigna radiata*) seeds that were surface sterilized with 0.1% $HgCl_2$ for 2 minutes and rinsed thoroughly, followed by soaking in an *Azotobacter* suspension (10^8 CFU/mL) for 30 minutes. Control seeds were soaked in sterile distilled water instead of an *Azotobacter* suspension, and the germination of each seed type was done on moist filter progressive paper in Petri plates at 25±2°C and recorded for seed germination percentage, root and shoot length and vigor index after 7 days [14–16,20,21]. This assay was designed to determine the effects of *Azotobacter* inoculation on seedling growth in a controlled setting.

Percent increment in length

Increment in the length of root and shoot of the seeds was calculate using the following formula

$$\text{Percent Increment (\%)} = \frac{\text{Length (Treated)} - \text{Length (Control)}}{\text{Length (Control)}} \times 100$$

Where:

- **Length (Treated)** = Root or shoot length of seeds under treatment.
- **Length (Control)** = Root or shoot length of seeds without treatment.

Statistical Analysis

Data for all experiment application were analyzed using one-way analysis of variance (ANOVA). Differences between treatments were considered statistically significant at $p < 0.05$ to confirm reliability and reproducibility of effects, if they were observed.

Results and Discussion:

Morphological and Biochemical Characterization

After 72 h incubation at 40 °C, the *Azotobacter* colonies were large, slimy, and mucoid, with a glistening surface. Microscopic examination showed the cells were Gram-negative, rod-shaped, and motile, which is in accordance with previous reports on *A. chroococcum* and *A. vinelandii* [2,7]. The colony morphology is characteristic of polysaccharide-producing strains that usually provide protective barriers against desiccation and other environmental stressors [5].

Biochemical characterization confirmed positive catalase and amylase activity, which indicates aerobic metabolism and production of extracellular enzymes. All the isolates were able to utilize glucose, sucrose, and mannitol, demonstrating metabolic versatility that allows them to colonize a variety of niches in soils (Table 1). However, all isolates tested negative for both the methyl red (MR) and nitrate reduction tests, which is consistent with earlier work that has shown limited fermentative

pathways in *Azotobacter* [6,8]. The results observed thus far confirm the isolates' metabolic flexibility, which allows them to survive and perform ecological functions in soils with abundant but variable nutrient availability [9,12].

Salt Tolerance and Thermostability

All isolates showed halotolerance, growing well with NaCl concentrations as high as 5%. Growth was also observed with concentrations above 5%, demonstrating that the isolates will tolerate salinity and can grow in saline, semi-arid and alkaline soils [15,19,24]. Halotolerance of all isolates will broaden their applications as a biofertilizer for marginal lands, where high salinities limit crop yields.

All isolates also showed thermotolerance, where good growth was observed at 40 °C. Thermotolerance is particularly relevant for use in tropical and subtropical agricultural systems where high soil temperatures naturally reduce the viability of many beneficial microorganisms. Furthermore, similar findings were presented in the study by Mishra *et al.* [25] which indicated thermotolerant *Azotobacter* strains could survive under field conditions during periods of extreme temperatures and would therefore benefit plant growth hormones during the summer months.

Nitrogen Fixation Potential

The acetylene reduction assay (ARA) confirmed nitro-genase activity across all isolates, with Isolate 2 showing the most rapid rates of total reduction. This confirms that these organisms can convert atmospheric nitrogen into ammonia, thus increasing the fertility of the soil with no addition of chemical fertilizers. According to previous work, *Azotobacter spp.* not only fix nitrogen but also contribute organic matter to the soil as extracellular polysaccharides, thereby improving soil structure [5,9,15]. The differences in nitrogenase activity among isolates indicate the potential for selection of higher-performing strains for future use in biofertilizers.

Seed Germination and Plant Growth Promotion

Seed germination tests indicated that Azotobacter inoculation positively impacted root and shoot growth when compared to non-inoculated control seeds (Figure 2, Tables 2). The root length of mung beans had a 35% increase and the vigor index improved by almost 35% compared to controls. Similarly, the vigor index of wheat seedlings improved by 20%. We also noted similar effects on seedling growth of pigeon pea, moth beans, and chick peas at 48-72 hours of germination (Table 3). The increased seedling growth is likely due to multiple interactions, including biological nitrogen fixation providing bioavailable nitrogen to growing seedlings, production of phytohormones including indole-3-acetic acid and gibberellins, that promote root and shoot elongation [6,14], and improving iron uptake with siderophores [22]. Furthermore, Azotobacter could produce ammonia and hydrogen cyanide, with the possibility of reducing soil pathogens, all factors improving seedling vigor. The current results are in agreement with other studies, indicating that Azotobacter improved seed germination, improved root and shoot growth, and improved the overall health of seeds and seedlings of several cereals and legumes [3,4,7].

This study reveals that the Azotobacter strains obtained from the isolates shared morphological and biochemical traits similar to other earlier descriptions of free-living diazotrophs [2,7,8]. Also, the strains halotolerance and thermotolerance imply that these strains could be used in agricultural soils at

risk of salinity and high temperatures [15,19,25]. Moreover, azotobacters improved seed germination and vigor index, which resides with other studies stating the ability of Azotobacter to not only fix nitrogen but provide a multiple of plant growth-promoting substances [5,9,15,16,19,26].

These results are in line with a prospective use of *Azotobacter* biofertilizer in sustainably managed agriculture systems. Compared to chemical fertilizers, *Azotobacter* biofertilizer provides ecological and advantageous long-term outcomes while improving soil fertility, increasing plant growth, and decreasing farmers' input rates.

Table 1: Biochemical characteristics of isolated *Azotobacter* strains

Test	Isolate 1	Isolate 2	Isolate 3
Gram Staining	-	-	-
Motility	+	+	+
Catalase	+	+	+
Amylase	+	+	+
Glucose Utilization	+	+	+
Sucrose Utilization	+	+	+
Mannitol Utilization	+	+	+
MR	-	-	-
Nitrate Reduction	-	-	-

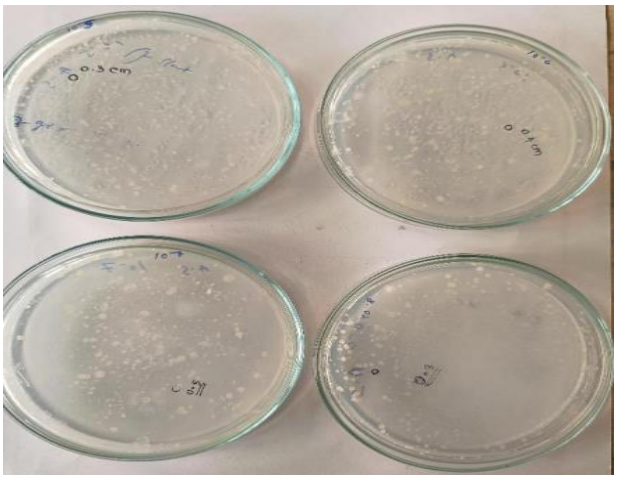

Figure 1: Colony morphology of *Azotobacter* on Ashby's Mannitol Agar (72 h at 40°C)

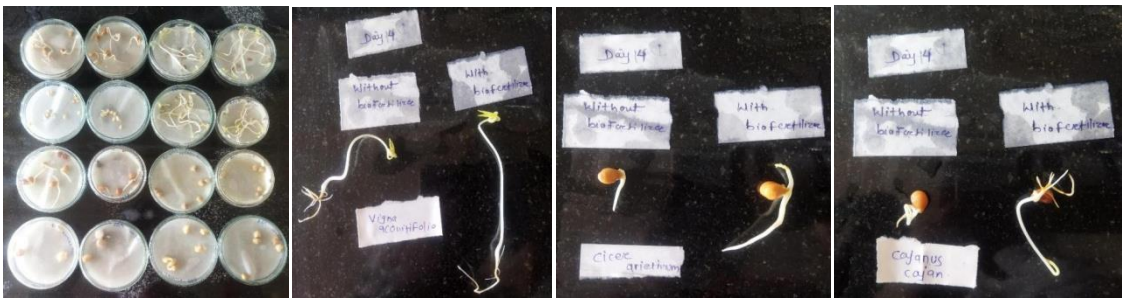

Figure 2: Seed germination process observed after 72 hours

Table 2: Effect of *Azotobacter* inoculation on seed germination and seedling growth

Crop	Treatment	Germination (%)	Root Length (cm)	Shoot Length (cm)	Vigor Index
Wheat	Control	85	4.2	5.1	789
Wheat	Azotobacter	95	5.1	6.3	947
Mung Bean	Control	80	3.8	4.6	704
Mung Bean	Azotobacter	92	5.2	6.4	949

Table 3: Seed germination assay after 48 hours and 72 hours

Seed Type	Root (Control, cm)	Shoot (Control, cm)	Root (Treated, cm)	Shoot (Treated, cm)	% Growth Root	% Growth Shoot
Brown Chickpea (*Cicer arietinum*) (48h)	0.7	0	3.8	1.7	442%	–
Pigeon Pea (*Cajanus cajan*)(48h)	1.6	0.5	3.7	2.5	131%	400%
Moth Beans (*Vigna aconitifolia*)(48h)	5.6	0.8	7.8	0.9	39%	12.50%
Mung Beans (*Vigna radiata*)(48h)	5.4	0.5	8.6	1.5	59%	200%
White Chickpea (*Cicer arietinum*)(48h)	1	0	2	0	100%	–
Brown Chickpea(*Cicer arietinum*) (72 h)	2.5	0.5	6	2.6	140%	420%
Pigeon Pea Pea (*Cajanus cajan*) (72 h)	3.5	2.3	6.8	3	94%	30%
Moth Beans (*Vigna aconitifolia*) (72 h)	7	1.3	10	4	43%	208%
Mung Beans(*Vigna radiata*) (72 h)	6.5	1.5	11	4	69%	166.70%
White Chickpea (*Cicer arietinum*) (72 h)	2	0	3.5	0.5	75%	–

Conclusion:

The study has successfully isolated and characterized multiple strains of Azotobacter from local soils that exhibited significant traits that could be applied as biofertilizers. Results of morpholoical and biochemical characterization showed that the isolates were Gram-negative, motile and had substantial metabolic versatility, utilizing different forms of sugars and producing several extracellular enzymes such as catalase and amylase.

With respect to tolerance capabilities, all isolates displayed halotolerance and thermotolerance characteristics and suggested that they would survive and be active in agriculture soils that are saline, warm, or semi-arid. Nitrogenase activity suggests that they can fix atmospheric nitrogen. Seed germination assays of the isolates showed significant improvements in overall root and shoot growth and vigor index of the tested crops, especially in the case of mung beans and moth beans.

In summary, these Azotobacter isolates confer multi-plant growth-promoting traits (including nitrogen fixation, phytohormone production, and nutrient availability) - further demonstrating their promise as a sustainable biofertilizer. The application of the Azotobacter isolates as defined through this research promotes soil fertility, crop productivity, and reduces the need for reliance of chemical fertilizers to achieve eco-friendly practices.

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