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Isolation and Identification of Azotobacter from Crop Rhizosphere Soils of Ahilyanagar District

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Abstract: Soil microorganisms contribute significantly to nutrient cycling and the maintenance of soil fertility in agricultural ecosystems. Among beneficial microorganisms, species belonging to the genus *Azotobacter* are recognized for their capacity to fix atmospheric nitrogen and stimulate plant growth through multiple biochemical activities. The present investigation was conducted to isolate and characterize *Azotobacter* species from rhizospheric soils collected from major crop fields in Ahilyanagar district, Maharashtra, India. A total of twenty soil samples were obtained from the root zones of crops such as sugarcane, onion, pigeon pea, maize and wheat.The collected soil samples were examined to determine their physicochemical characteristics, specifically pH, moisture level, and capacity to retain water, following established analytical protocols. Bacterial isolates were obtained through serial dilution and plating techniques and subsequently screened on Ashby's mannitol agar for the selective recovery of *Azotobacter*. Colony morphology, Gram staining, and biochemical assays including catalase activity, starch hydrolysis, and nitrate reduction were used for characterization of isolates. Twenty bacterial strains were recovered from the soil samples and designated as ANAZO-1 to ANAZO-20. Among these isolates, fourteen strains showed characteristics consistent with *Azotobacter*, while the remaining isolates displayed different phenotypic features. The analyzed soil samples exhibited pH values ranging from 5.6 to 6.7, indicating slightly acidic to near-neutral conditions. Moisture content ranged from 29.25% to 49.37%, while water-holding capacity varied between 31.90% and 51.25%. The occurrence of *Azotobacter* species in the rhizosphere suggests their potential contribution to soil fertility and crop productivity. These isolates may serve as promising candidates for future development of biofertilizer formulations aimed at sustainable agricultural practices.

Keywords: Azotobacter, *Rhizospheric Soil, Biofertilizer, Nitrogen fixation, Soil Microbiology.*

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I. INTRODUCTION

Soil represents a complex ecological system that supports a wide variety of microorganisms responsible for essential biochemical processes. These microorganisms participate in nutrient transformation, organic matter decomposition, and maintenance of soil health, thereby influencing overall agricultural productivity. The microbial population present in soil plays a crucial role in sustaining plant growth through several biological interactions with plant roots. Certain beneficial soil bacteria known as plant growth-promoting rhizobacteria (PGPR) have received significant research interest for their capacity to support and stimulate plant growth.. These bacteria promote plant growth through mechanisms such as atmospheric nitrogen fixation, solubilization of mineral nutrients, production of phytohormones, and synthesis of siderophores that facilitate nutrient uptake by plants.

Azotobacter represents a group of free-living aerobic bacteria widely distributed in productive soils and recognized for their capacity to fix atmospheric nitrogen. These microorganisms can fix atmospheric nitrogen and convert it into usable forms independently, without establishing symbiotic associations with plants. Due to this capability, by fixing atmospheric nitrogen, *Azotobacter* helps maintain soil fertility and positively influences crop yield. In addition to nitrogen fixation, species of this genus are known to synthesize several biologically active substances including vitamins, amino acids, and plant growth regulators such as indole-3-acetic acid and gibberellins.

The abundance and activity of *Azotobacter* in soil depend on environmental factors such as soil pH, organic matter content, moisture level, and nutrient availability. Soils with adequate organic matter and near-neutral pH

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conditions generally favor the growth of these bacteria. Their presence in the rhizosphere can enhance plant growth by improving nutrient availability and stimulating root development.The soil region adjacent to plant roots, known as the rhizosphere, functions as an important site for biological interactions between plants and soil microbes. Root exudates released by plants supply organic compounds that support microbial growth, leading to higher microbial density compared to non-rhizospheric soil.

Ahilyanagar district in Maharashtra is an important agricultural region where crops such as wheat, maize, onion, sugarcane, and pigeon pea are widely cultivated. Despite the agricultural importance of this region, limited information is available regarding the distribution of beneficial rhizospheric microorganisms such as *Azotobacter*. Therefore, the present study was undertaken to isolate and characterize *Azotobacter* species from rhizospheric soils associated with major crops cultivated in this district. Investigating the occurrence of these bacterial populations could support the advancement of eco-friendly agricultural systems based on biofertilizer applications.

II. MATERIALS AND METHODS

➢ *Soil Sample Collection*

Rhizospheric soil samples were collected from twenty agricultural locations within Ahilyanagar district of Maharashtra, India. Samples were obtained from the root zones of commonly cultivated crops including onion (*Allium cepa*), sugarcane (*Saccharum officinarum*), wheat (*Triticum aestivum*), pigeon pea (*Cajanus cajan*) and maize (*Zea mays*). Approximately 100 g of soil was collected from a depth ranging between 1 and 15 cm using sterilized sampling tools.All samples were immediately stored in sterilized polyethylene bags and subsequently taken to the laboratory for further investigation.

➢ *Physicochemical Analysis of Soil*

The collected soil samples were subjected to physicochemical analysis to determine parameters such as pH, moisture content, and water-holding capacity. Soil pH was measured using a calibrated digital pH meter in a soil-water suspension. To determine moisture levels, soil samples were dried in a hot air oven maintained at 105 °C until no further change in weight was observed.. Water-holding capacity was estimated by measuring the amount of water retained by soil under controlled laboratory conditions.

➢ *Isolation of Bacteria*

Bacterial isolation from soil samples was carried out using the serial dilution and spread plate method. To prepare the initial suspension, 1 g of rhizospheric soil was homogenized in 9 ml of sterile distilled water (The serial dilution is carried out for 7 time). Serial dilutions were prepared and aliquots of diluted samples were spread onto Ashby's mannitol agar plates. The plates were incubated at a temperature of 26 ± 2°C for period of 72 hours.

Individual colonies appearing on the Ashby's mannitol agar plates were selected and repeatedly subcultured to obtain pure bacterial cultures. For selective isolation of *Azotobacter*, purified isolates were streaked onto Ashby's mannitol agar medium, which lacks combined nitrogen and favors the growth of nitrogen-fixing bacteria belonging to this genus.

➢ *Morphological Characterization*

Colony morphology of bacterial isolates was observed after incubation on agar media. Characteristics such as colony color, shape, size, texture, elevation, and margin were recorded. The morphology of bacterial cells and their Gram nature were assessed through microscopic observation after Gram staining.

➢ *Biochemical Characterization*

Biochemical tests were performed to confirm the identity of the bacterial isolates. The assays included catalase activity, starch hydrolysis, and nitrate reduction tests. The obtained results were compared with reported characteristics of Azotobacter species.

- *Catalase Test*

Catalase activity was determined using the hydrogen peroxide method. Catalase activity was determined by adding a drop of 3% hydrogen peroxide to bacterial cells placed on a clean glass slide, where rapid bubble formation signified a positive result.

- *Starch Hydrolysis Test*

To determine amylolytic activity, bacterial isolates were cultured on starch agar plates with soluble starch and incubated at around 30 °C for 72 hours. The plates were then flooded with iodine solution to reveal zones of starch hydrolysis.

- *Nitrate Reduction Test*

Nitrate reduction capability of the isolates was evaluated by inoculating bacterial cultures into nitrogen-free basal medium containing 0.2% potassium nitrate (KNO_3). The inoculated tubes were incubated at 30°C for three days. Following incubation, detection of nitrite resulting from nitrate reduction was carried out by applying Griess–Ilosvay reagent to the culture medium. The nitrate reduction test was regarded as positive when the medium showed a red coloration.

Table 1 Physicochemical Properties of Soil Samples

Sr. No.	Location	Soil. Color	pH	Water Holding capacity in %	Moisture %	Botanical name of the crop (Vernacular Name)
1	Ukkadgaon, tq. Kopargaon dist. Ahilyanagar	Dark Brown	5.9	38.00	33.52	*Triticum aestivum (*Wheat*)*

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2	Kopargaon, tq. Kopargaon dist. Ahilyanagar	Black	6.4	36.28	33.25	*Triticum aestivum (*Wheat*)*
3	Ladgaon, tq. Shrirampur dist. Ahilyanagar	Brown	6.0	31.90	30.35	*Zea Mays (Maize)*
4	Galeem, tq. Shrirampur dist. Ahilyanagar	Black	6.1	32.46	31.42	*Triticum aestivum (*Wheat*)*
5	Mamadapur tq. Rahata dist. Ahilyanagar	Dark Brown	6.2	48.52	45.37	*Zea Mays (Maize)*
6	Khandala, tq. Rahata dist. Ahilyanagar	Black	6.3	32.35	34.82	*Saccharum officinarum (Sugarcane)*
7	Kesapur, tq. Rahuri dist. Ahilyanagar	Brown	5.6	50.18	47.33	*Triticum aestivum (*Wheat*)*
8	Rahuri, tq. Rahuri dist. Ahilyanagar	Brown	6.3	48.56	44.82	*Triticum aestivum (*Wheat*)*
9	Belpimpalgaon, tq. Nevasa dist. Ahilyanagar	Blackish Brown	6.1	36.48	32.72	*Triticum aestivum (*Wheat*)*
10	Ghogargaon, tq. Nevasa dist. Ahilyanagar	Black	6.4	42.25	30.82	*Triticum aestivum (*Wheat*)*
11	Sangmaner, tq. Sangamner dist. Ahilyanagar	Black	5.9	37.35	33.65	*Triticum aestivum (*Wheat*)*
12	Rajapur, tq. Sangamner dist. Ahilyanagar	Black	6.0	40.25	42.80	*Zea Mays (Maize)*
13	Nandgaon, tq. Ahilyanagar dist. Ahilyanagar	Black	6.2	36.55	30.52	*Zea Mays Maize)*
14	Ahilyanagar, dist. Ahilyanagar	Light Brown	6.1	34.85	30.92	*Allium cepa (Onion)*
15	Pimpaldari, tq. Akole dist. Ahilyanagar	Dark Brown	6.3	51.25	47.62	*Allium cepa (Onion)*
16	Belapur, tq. Akole dist. Ahilyanagar	Black	6.1	49.21	46.37	*Cajanus cajan* (tur)
17	Pathardi, tq. Pathardi dist. Ahilyanagar	Dark Gray	5.9	32.27	29.25	*Triticum aestivum (*Wheat*)*
18	Sakegaon, tq. Pathardi dist. Ahilyanagar	Gray	6.3	36.52	34.37	*Triticum aestivum (*Wheat*)*
19	Pokhari, tq. Parner dist. Ahilyanagar	Dark Brown	6.7	48.37	46.76	*Triticum aestivum (*Wheat*)*
20	Gajadipur, tq. Parner dist. Ahilyanagar	Black	6.4	50.26	49.37	*Triticum aestivum (*Wheat*)*

Table 2 Morphological Characteristics of Bacterial Strains Collected from *Rhizosphere.*

Strain	**Cell Morphology**			**Colony Morphology**				
	Gram's Staining	**Cell Size**	**Cell Shape**	**Color**	**Shape**	**Size**	**Appearance**	**Margins**
ANAZO-1	-ve	1.02 to 2.03µm	Rod	Dull White	Spherical	Medium	Slimy	Smooth
ANAZO 2	-ve	1.05 to 1.60µm	Large Rod	Creamy White	Oval	Large	Opaque	Smooth
ANAZO 3	+ve	3.05µm	Rod	Creamy yellowish	Irregular	Medium	Opaque	Smooth
ANAZO 4	-ve	1.30 to 1.70µm	Large Rod	Dull White	Spherical	Medium to Large	Slimy	Smooth
ANAZO 5	-ve	1.07 to 1.55µm	Large Rod	White	Oval	Medium	Opaque	Smooth
ANAZO 6	-ve	1.11 to 1.90µm	Rod	Creamy White	Spherical	Large	Slimy	Smooth
ANAZO 7	+ve	1.15µm	Rod	Creamy White	Circular	Medium	Opaque	Rough
ANAZO 8	-ve	1.07 to 1.60µm	Large Rod	White	Spherical	Small	Slimy	Smooth
ANAZO 9	+ve	1.10 to 1.80 µm	Large Rod	Milky White	Spherical	Small	Opaque	Rough
ANAZO 10	-ve	1.08 to	Rod	White	Spherical	Medium	Opaque	Smooth

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		2.0µm						
ANAZO 11	+ ve	4µm	Rod	Cream to pale orange	Circular	Large	Opaque	Rough
ANAZO 12	-ve	1.10 to 1.80 µm	Rod	White	Spherical	Small	Slimy	Smooth
ANAZO 13	-ve	1.20 to 1.70µm	Large Rod	Dull White	Spherical	Medium to Large	Slimy	Smooth
ANAZO 14	-ve	1.10 to 1.60 µm	Rod	White	Spherical	Small	Slimy	Smooth
ANAZO 15	-ve	1.30 to 1.90µm	Large Rod	Dull White	Spherical	Medium to Large	Slimy	Smooth
ANAZO 16	+ ve	4µm	Rod	Light brown	Circular	Large	Opaque	Rough
ANAZO 17	-ve	1.10 to 1.90 µm	Rod	White	Spherical	Small	Slimy	Smooth
ANAZO 18	-ve	1.40 to 1.90µm	Large Rod	Dull White	Spherical	Medium to Large	Slimy	Smooth
ANAZO 19	-ve	1.10 to 1.90 µm	Rod	White	Irregular	Small	Slimy	Smooth
ANAZO 20	-ve	1.40 to 1.90µm	Large Rod	Dull White	Spherical	Medium to Large	Slimy	Smooth

Fig 1 Azotobacter Colonies on Ashby Mannitol Agar and Gram Staining Results

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III. RESULTS AND DISCUSSION

Considerable variation in physicochemical parameters was observed among soil samples collected from various farming locations within Ahilyanagar district. Soil pH values ranged from 5.2 to 6.9, indicating that the soils were slightly acidic to nearly neutral. This pH environment is conducive to the proliferation of beneficial soil microbes, including bacteria capable of fixing atmospheric nitrogen. Previous studies have reported that *Azotobacter* species grow optimally in soils with near-neutral pH and sufficient organic matter content.

Moisture content of the analyzed soil samples showed variation between 29.25% and 49.37%. Soil moisture plays a critical role in regulating microbial metabolic activity because it influences nutrient solubility and microbial mobility within the soil matrix. Higher moisture levels generally promote microbial growth by maintaining adequate hydration and facilitating biochemical reactions necessary for microbial metabolism.

The water-holding capacity of the soil samples ranged from 31.90% to 51.25%, suggesting moderate ability of the soils to retain water. Soil texture and organic matter content significantly influence the water-holding capacity, which in turn affects microbial survival and root–microbe interactions in the rhizosphere. Soils with higher water-retention ability typically provide a more stable environment for microbial colonization and activity.

A total of twenty bacterial isolates were obtained from the rhizospheric soil samples using the serial dilution and spread plate technique. These isolates were purified and designated as ANAZO-1 to ANAZO-20. Cultivation on Ashby's mannitol agar revealed that a number of isolates formed colonies showing features typical of *Azotobacter* species.The colonies appeared large, circular, smooth, and slightly raised, with a mucoid texture, which is commonly attributed to the production of extracellular polysaccharides.Microscopic examination through Gram staining revealed that the majority of the isolates were Gram-negative rods, which is consistent with the morphological characteristics reported for members of the genus *Azotobacter*. These bacteria are known to possess relatively large cells and exhibit active motility under favorable growth conditions.

Biochemical analysis further supported the identification of the isolates. Catalase activity was detected in most of the selected isolates, indicating that they can degrade hydrogen peroxide into water and oxygen. This feature is typical of aerobic bacteria that use this enzyme system to defend against oxidative stress generated during metabolism.In the starch hydrolysis test, several isolates demonstrated the ability to degrade starch, which was evident from the formation of clear zones surrounding the bacterial colonies after iodine treatment. This observation indicates the presence of extracellular enzymes capable of breaking down complex carbohydrates into simpler forms that can be utilized for microbial growth.Similarly, results obtained from the nitrate reduction test suggested that some isolates were capable of reducing nitrate compounds, indicating their involvement in nitrogen transformation processes within the soil ecosystem. Such metabolic versatility contributes to nutrient cycling and enhances soil fertility.

Based on morphological and biochemical characteristics, fourteen isolates were found to display features consistent with Azotobacter species, while the remaining isolates exhibited characteristics that differed from those typically associated with this genus. The identification of Azotobacter in rhizosphere soils emphasizes their beneficial role in enhancing nitrogen availability and supporting crop growth.

The occurrence of Azotobacter species in soils associated with crops such as wheat, maize, onion, and sugarcane suggests that these bacteria may contribute to the maintenance of soil fertility in agricultural ecosystems of the Ahilyanagar region. The dual ability of these microorganisms to perform nitrogen fixation and generate plant growth–promoting metabolites highlights their potential application in eco-friendly biofertilizer development.

- *Key Observations*

The isolated bacterial colonies predominantly exhibited large, circular, smooth, and mucoid morphology, which is characteristic of Azotobacter species. Microscopic examination revealed that most of the isolates were Gram-negative rod-shaped bacteria. Biochemical characterization demonstrated that the majority of isolates were catalase positive, indicating their ability to decompose hydrogen peroxide and tolerate oxidative stress. Several isolates showed positive results for starch hydrolysis, suggesting the production of extracellular enzymes capable of degrading complex carbohydrates. Additionally, nitrate reduction activity was observed in some isolates, indicating their involvement in nitrogen transformation processes within the soil environment.

IV. CONCLUSION

The investigation provided insight into the occurrence of beneficial nitrogen-fixing bacteria in the rhizospheric soils of major agricultural crops cultivated in the Ahilyanagar region of Maharashtra. Examination of soil characteristics revealed environmental conditions that support microbial activity and the establishment of plant-associated bacteria. Isolation and laboratory characterization of microbial cultures demonstrated the presence of several strains exhibiting features typical of *Azotobacter*. These microorganisms are known for their ecological role in improving nitrogen availability in soil systems and supporting plant growth.

The recovery of such bacterial populations from different crop rhizospheres indicates that local soils harbor naturally occurring microbial resources that may contribute to maintaining soil productivity. Exploring these indigenous

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strains can be valuable for the development of environmentally friendly agricultural inputs. Future investigations focusing on molecular identification and evaluation of additional plant growth-promoting properties may further clarify the potential application of these isolates in sustainable farming systems.

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