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Exploration and Biochemical Profiling of Native *Azotobacter* and *Azospirillum* Isolates from Chhattisgarh and Uttar Pradesh Soils

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Abstract
This study focused on isolating and characterizing native plant growth-promoting rhizobacteria (PGPR) from soils of Chhattisgarh and Uttar Pradesh, emphasizing *Azotobacter* and *Azospirillum* species. A total of nine isolates were obtained through standard serial dilution and streak plating on Jensen's and Okon's media. Comprehensive morphological and biochemical analyses, including Gram staining, catalase activity, starch hydrolysis, citrate utilization, and methyl red test, were conducted for each isolate. Observed variations in colony characteristics and biochemical reactions revealed notable diversity among the isolates. These findings highlight the potential of locally adapted PGPR strains as candidates for sustainable agricultural practices and biofertilizer development.

Keywords: *Azotobacter*, *Azospirillum*, PGPR, Isolation, Morphological Characterization, Biochemical Tests, Jensen's Medium, Okon's Medium

Introduction
Spinach (*Spinacia oleracea*), a major leafy vegetable of the *Amaranthaceae* family, is widely cultivated in India due to its high nutritional value and adaptability to diverse agro-climatic conditions (Hanif *et al*., 2006; Kisan *et al.*, 2015; Tewani *et al.*, 2016) [2, 3, 4]. In states like Chhattisgarh and Uttar Pradesh, favorable soil and water availability support its cultivation, making it an important crop for local farmers (Ministry of Agriculture and Farmers Welfare, India). Spinach leaves are rich in vitamins A, C, and K, minerals like iron and magnesium, and bioactive compounds such as flavonoids and phenolics, which contribute to human health.

However, spinach productivity can be affected by nutrient deficiencies, poor soil health, and environmental stresses. Modern agriculture often relies heavily on chemical fertilizers, which may disrupt beneficial soil microorganisms, reduce soil fertility, and increase input costs for marginal farmers (Itelima *et al*., 2018) [9]. Biofertilizers containing PGPR offer an eco-friendly alternative by improving nutrient availability, enhancing plant growth, and maintaining soil microbial balance.

Among PGPR, *Azotobacter* and *Azospirillum* are free-living nitrogen-fixing bacteria that support crop growth by fixing atmospheric nitrogen, producing phytohormones like indole-3-acetic acid, and improving nutrient and water uptake. They also reduce soil-borne diseases and mitigate abiotic stresses such as salinity and drought (Bashan & De-Bashan, 2005; Dobbelaere *et al.*, 2003; Rashid *et al.*, 2016) [1, 5, 14].

The natural genetic and phenotypic diversity of native rhizobacterial populations provides opportunities to select superior strains for biofertilizer development (Raaijmakers & Weller, 2001; Hartmann & Amarger, 1991) [10, 11]. Isolation and characterization of locally adapted strains are therefore crucial to develop effective, environmentally safe biofertilizers tailored to specific agro-climatic regions (Zhou *et al.*, 2020) [6]. Such an approach enhances crop productivity while reducing dependency on chemical fertilizers, supporting sustainable agricultural practices.

Plant growth-promoting rhizobacteria employ direct mechanisms such as nutrient solubilization and phytohormone production, and indirect mechanisms like biocontrol of pathogens and enhancement of soil microbial health (Garcia-Fraile *et al.*, 2015; Gouda *et al.*,

[7]. Their use in crops like spinach can increase yield by 10-40% under controlled and field conditions (Kawalekar, 2013) [12]. Solid and carrier-based formulations using lignite, humus, peat, or wheat bran improve microbial shelf-life and ease of application (Bhattacharjee & Dey, 2014) [13].

Scope of the Present Study

The present paper focuses exclusively on the isolation and biochemical characterization of *Azotobacter* and *Azospirillum* obtained from the spinach rhizosphere. The detailed evaluation of their inoculation effects on spinach growth parameters, including plant height, biomass, chlorophyll content, and NPK accumulation, will be reported in a subsequent research paper (hereafter referred to as "the second paper").

2. Experimental Site

The present study was conducted during the Master's research period (2024-2025) in the Microbiology Laboratory of the Department of Microbiology, College of Agriculture, Indira Gandhi Krishi Vishwavidyalaya (IGKV), Raipur, Chhattisgarh. All experimental work from handling soil samples to isolating and characterizing bacterial cultures was carried out in a well-maintained laboratory environment, ensuring aseptic conditions and reliable observations throughout the research.

3. Materials and Methods

3.1 Sample Collection

Soil samples were collected from the rhizosphere of spinach plants across Chhattisgarh and Uttar Pradesh, including Dharsiwa, Surajpur, Balrampur, Nardaha (Raipur), Sarguja, Sakti, Achanakmar Biosphere Reserve, and research fields of Indira Gandhi Krishi Vishwavidyalaya, Raipur. Samples were taken at 0-15 cm depth using sterile soil augers to ensure collection of microorganisms closely associated with roots. Samples were immediately transferred to sterilized polythene bags, labeled, and stored at 4 °C to minimize microbial changes prior to analysis.

3.2 Culture Media

Jensen's medium was used for *Azotobacter*, and Okon's medium for *Azospirillum*. Media were prepared using standard protocols, sterilized at 121 °C for 15-20 minutes, and poured under aseptic conditions. Media were routinely checked for contamination.

3.3 Isolation of PGPR (*Azotobacter* & *Azospirillum*)

One gram of soil was suspended in 9 mL of sterile distilled water to prepare a stock solution, followed by serial dilutions up to 10^{-9}. From suitable dilutions, 0.1 mL was spread on Jensen's or Okon's medium and incubated at 28-30°C for 24-48 hours. Well-separated colonies were selected and purified by repeated streaking to obtain single, uncontaminated cultures, which were maintained on slants at 4 °C for further analysis.

3.4 Colony Morphology

Colony size, shape, margin, elevation, surface texture, and pigmentation were observed for each isolate to provide preliminary identification cues.

3.5 Gram Staining

Smears were prepared on sterilized slides, air-dried, and heat-fixed. Sequential staining with crystal violet, Gram's iodine, 95% ethanol, and safranin was performed. Microscopic examination provided cell shape, arrangement, and Gram reaction.

3.6 Biochemical Characterization

Catalase Test: Fresh colonies were tested with 3% hydrogen peroxide. Rapid bubbling indicated positive catalase activity.

Starch Hydrolysis Test: Isolates were streaked on starch agar. Iodine application revealed clear zones for positive starch hydrolysis.

Methyl Red Test: Cultures in MR broth were incubated and tested with MR indicator. Red color indicated positive acid production.

Citrate Utilization Test: Cultures were streaked on Simmons citrate agar. Color change from green to blue indicated citrate utilization.

4. Results and Discussion

4.1 Isolation of PGPR

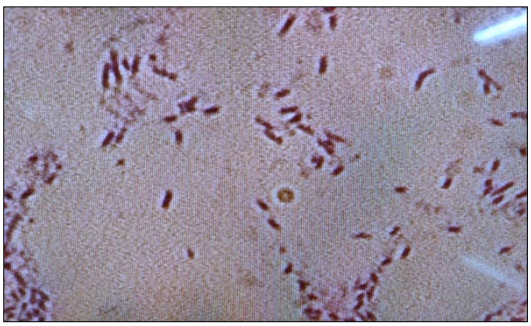
Nine bacterial isolates (*Azotobacter* and *Azospirillum*) were successfully obtained from spinach rhizosphere soils and maintained as pure cultures.

4.2 Colony Morphology and Gram Staining

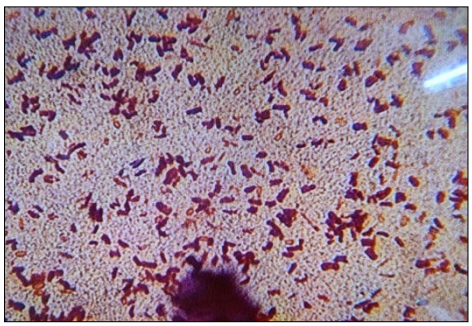
***Azotobacter*:** Gram-negative, spherical or short rod-shaped cells; off-white, circular, convex colonies with smooth surfaces and entire margins.

***Azospirillum*:** Gram-negative, slightly spiral cells; dark bluish, circular, convex colonies with smooth surfaces and entire margins.

Representative plate images (Plate 4 - 4.5) illustrate these distinctions.



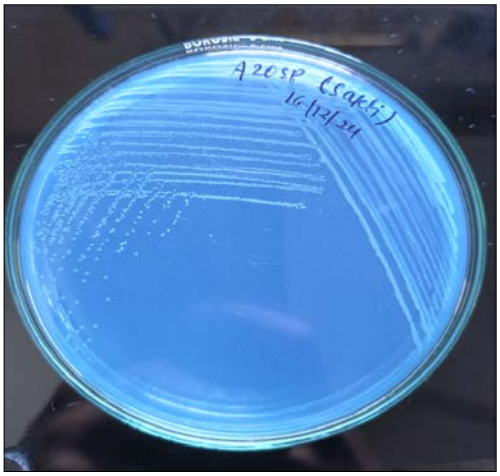
Gram stain of *Azotobacter*



Gram stain of *Azospirillum*

Plate 4: Gram staining of *Azotobacter* & *Azospirillum* isolates.

Azospirillum isolates in slants.



Azotobacter isolates in slants

Plate 4.1: Colony morphology of *Azotobacter* & *Azospirillum* isolates.

4.3 Biochemical Characteristics

Parameter	**Isolates of *Azotobacter***								
	Azoto-1	***Azoto-2***	***Azoto-3***	***Azoto-4***	***Azoto-5***	***Azoto-6***	***Azoto-7***	***Azoto-8***	***Azoto-9***
Citrate utilization	_	_	_	+	_	_	_	+	_
Catalase test	+	_	_	+	+	+	_	+	+
Starch hydrolysis	+	+	+	_	+	+	+	+	+
Methyl red test	_	_	+	_	_	_	_	_	+
Isolates of *Azospirillum*									
	Azosp-1	***Azosp-2***	***Azosp-3***	***Azosp-4***	***Azosp-5***	***Azosp-6***	***Azosp-7***	***Azosp 8***	***Azosp-9***
Citrate utilization	+	+	_	_	_	+	+	+	+
Catalase test	+	+	+	_	_	+	+	+	_
Starch hydrolysis	_	_	_	_	+	_	_	+	_
Methyl red test	+	_	_	_	_	_	_	_	_

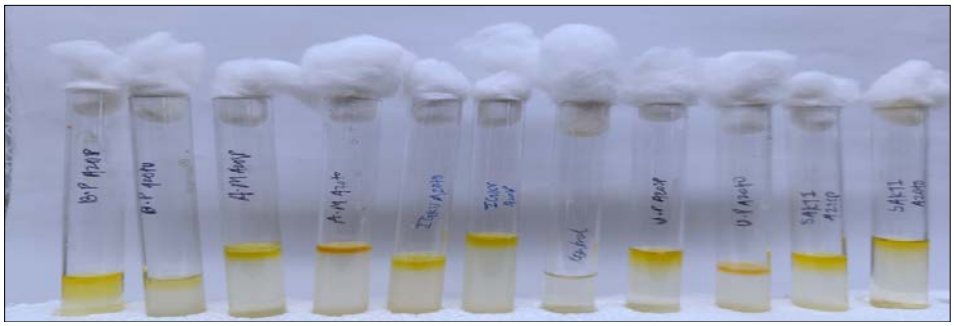
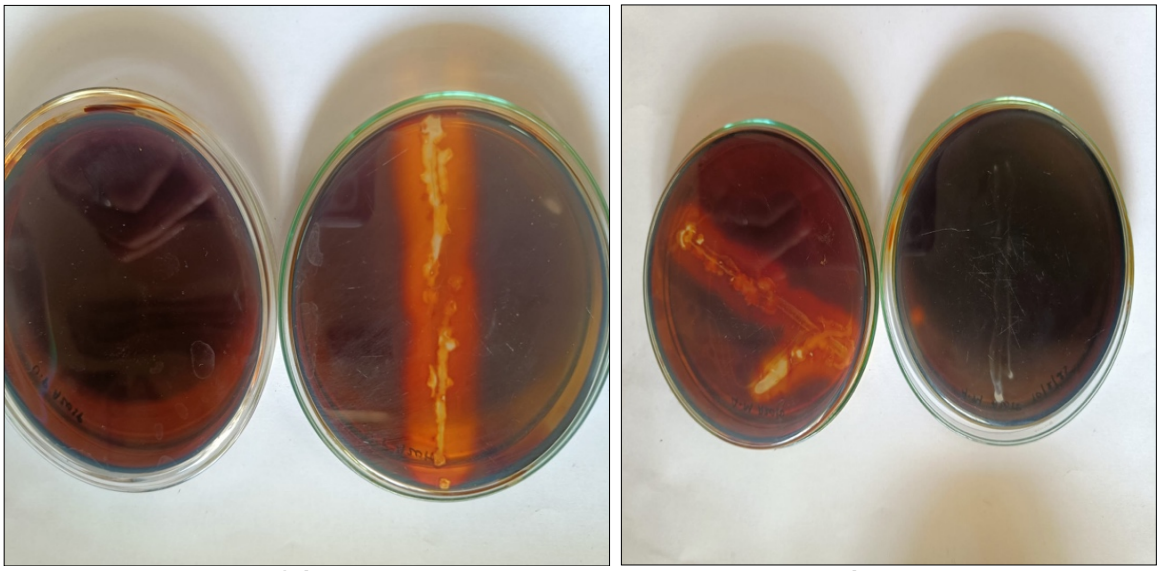


Plate 4.2: Methyl Red Test

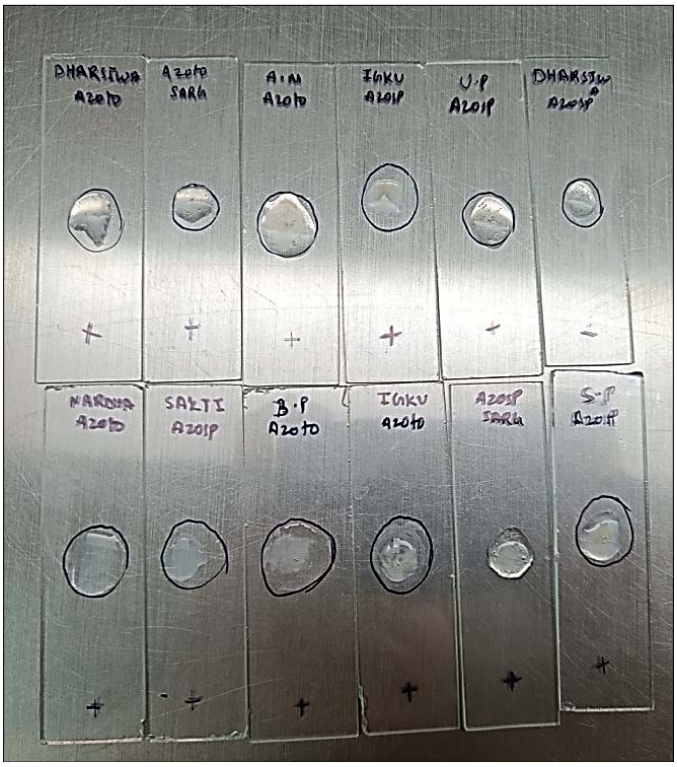


T3 Azoto- **T5 Azosp-**

Plate 4.3: Catalase test

T9 Azoto-9 T9 Azosp-9

Plate 4.4: Citrate utilization test



T3 Azoto-3 T3 Azosp-3

T1 Azosp-1 T1 Azosp-1

T3 Azoto-3 T5 Azosp-5

Plate 4.5: Starch hydrolysis test

4.4 Comparative Analysis and PGPR Potential

Morphological and biochemical differences were observed between the two genera, while functional similarities were noted within each genus. Biochemical tests confirmed oxidative stress tolerance (catalase) and ability to use citrate as carbon source. Minor differences in starch hydrolysis and uniformly negative methyl red reactions suggest stable metabolic profiles.

These native strains demonstrated potential as plant growth-promoting rhizobacteria suitable for biofertilizer development.

4.5 Summary of Findings

All nine isolates were Gram-negative, showed genus-specific colony morphology, positive catalase and citrate activity, and minor variability in starch hydrolysis. Negative methyl red results confirmed the absence of strong acid production. The isolates possess characteristics associated with effective PGPR.

5. Conclusion

Nine native strains of *Azotobacter* and *Azospirillum* were successfully isolated and characterized from spinach rhizosphere soils of Chhattisgarh and Uttar Pradesh. Morphological and Gram staining traits differentiated the two genera, while biochemical profiling revealed positive catalase and citrate activity and minor variability in starch hydrolysis. Negative methyl red reactions confirmed metabolic stability.

These isolates demonstrated key plant growth-promoting traits, indicating their suitability for biofertilizer development in sustainable crop production. Further studies on field inoculation and spinach growth performance will be addressed in the next phase.

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