

Antibiotic resistance study of *Azotobacter* species isolated from agriculture field of Mahisi Block, Saharsa

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ABSTRACT

Healthy agricultural soil depends heavily on beneficial microbes that replenish nutrients naturally. In this study, five distinct bacterial isolates belonging to the genus *Azotobacter* were recovered from the root zones (rhizosphere) of crop fields across Mahisi block in Saharsa district, Bihar. Using nitrogen-free Ashby's mannitol medium, colonies were isolated and systematically analyzed for their colony appearance, microscopic features, and biochemical behavior in line with standard diagnostic keys. All five isolates proved to be Gram-negative, oval-shaped cells producing smooth, glistening colonies. Isolate 02 produced a distinct yellow pigment, whereas Isolates 01, 03, 04, and 05 formed creamy white, opaque colonies. Because farmlands are increasingly exposed to chemical treatments and livestock runoff, we screened each isolate against five common antibiotics: Amoxicillin (30 mcg), Azithromycin (15 mcg), Ciprofloxacin (5 mcg), Doxycycline (30 mcg), and Erythromycin (15 mcg) using the disc diffusion method. All five strains demonstrated complete resistance to Erythromycin, showing no inhibition whatsoever. Conversely, they proved sensitive to Ciprofloxacin, Doxycycline, Amoxicillin, and Azithromycin, producing clear zones of inhibition. These results provide practical insights into the baseline hardiness and natural antibiotic responses of native soil bacteria, helping identify resilient candidate strains for sustainable biofertilizer development.

Key Words - *Azotobacter chroococcum*, Rhizosphere, Ashby's medium, Antibiotic resistance, Biofertilizer, Zone of inhibition.

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INTRODUCTION

Modern agriculture faces the ongoing challenge of maintaining high crop yields without depleting soil health or over-relying on synthetic chemical fertilizers. Central to solving this problem are beneficial soil microorganisms, particularly free-living diazotrophic bacteria. Among them, the genus *Azotobacter* stands out as a vital group of aerobic, heterotrophic, Gram-negative bacteria capable of fixing atmospheric nitrogen gas into

biologically usable ammonia without requiring a symbiotic nodule host (Subba Rao, 1982). These bacteria draw their energy from decaying plant residues, simple soil organic matter, and root exudates, returning vital nitrogen directly to the surrounding rhizosphere.

Beyond fixing nitrogen, *Azotobacter* acts as a natural plant growth engine. Active cells secrete beneficial phytohormones into the root zone,

including indole-3-acetic acid (IAA), gibberellins, and cytokinins (Lee *et al.*, 1970; Taller & Wong, 1989; Mirkovacki & Milic, 2001). These biochemical signals promote vigorous root branching, improve seed germination rates, and boost early seedling vigor. In addition, *Azotobacter* helps crops absorb micro- and macronutrients more effectively, synthesizes essential B-vitamins in the root zone (Martinez *et al.*, 1996), and shields delicate root systems from pathogenic soil fungi like *Rhizoctonia solani* through competitive exclusion and the release of protective siderophores (Neyra *et al.*, 1999; Fatima *et al.*, 2009).

Strains of *Azotobacter* thrive in the root zones of staple cereals, cash crops, and horticulture plants, including wheat, rice, maize, sugarcane, and vegetables (Arun, 2007). Several ecotypes also help cleanse contaminated soil by metabolizing persistent agricultural residues, including chlorinated phenolic compounds (Gaofeng *et al.*, 2004; Onwurah & Nwuke, 2004).

However, life in agricultural soils is demanding. Intensive farming regularly introduces synthetic pesticides, selective weedicides, and trace antibiotic residues carried over through animal manure and irrigation runoff (Ayansina & Osoba, 2006; Sarnaik *et al.*, 2006). These agrochemicals can unintentionally suppress beneficial microflora, disturb respiration rates, and impair bacterial nitrogen-fixing efficiency (Castro *et al.*, 1997; Widenfalk *et al.*, 2008). If local *Azotobacter* strains are to be formulated into field-ready biofertilizers, we must first understand their inherent resilience and tolerance to antimicrobial agents. This study evaluates the morphological traits, biochemical features, and antibiotic sensitivity patterns of five native *Azotobacter* strains isolated from cultivated fields in Mahisi block, Saharsa district, Bihar.

MATERIAL & METHODS

Soil samples were collected from the rhizosphere of crop plants from Mahisi block of Saharsa district, Bihar. Ten grams of soil was suspended in sterile distilled water and serially diluted up to 10^{-4} . Aliquots of 0.1 mL from 10^{-3} and 10^{-4} dilutions were inoculated onto nitrogen-free Ashby's mannitol

medium and incubated for 48 hours at 30°C. The subculturing was repeated three times to obtain pure isolates.

The isolated strains were characterized through morphological examination and standard biochemical testing following Bergey's Manual of Determinative Bacteriology. Cell morphology and Gram staining were analyzed under brightfield oil-immersion microscopy. Colony features (shape, color, margin, elevation, and texture) were noted. The biochemical assays included Citrate utilization, Methyl Red (MR), Voges-Proskauer (VP), Indole, Catalase, Cytochrome Oxidase, Urease, and carbohydrate fermentation tests (Glucose, Lactose, and Sucrose).

For the antibiotic sensitivity test, plates of fresh Ashby's medium supplemented with agar were prepared. Bacterial suspensions calibrated to 0.5 McFarland standard were lawn cultured onto the plates. Standard antibiotic discs Amoxicillin (30 µg), Azithromycin (15 µg), Ciprofloxacin (5 µg), Doxycycline (30 µg), and Erythromycin (15 µg) were aseptically placed on the agar surface. The plates were incubated at 30°C for 48 hours. Clear zones of inhibition were measured across their full diameter (including the disc diameter) using digital calipers and recorded.

RESULT

The isolates were characterized by the determination of shape, colour, margin, nature and texture of colony was observed. Gram staining was performed. The colour of colony was white in Isolate no. 01, 03, 04 and 05 and yellow in Isolate no. 02. The nature of colony was Glistening, margin intact, surface smooth, transparency Opaque and cell shape oval. All isolates were Gram negative. The morphology of colony and the result of Gram staining is tabulated in table 1.

Biochemical tests were performed for the biochemical characterization of the bacterial isolates. Isolate no. 01 was negative for Glucose utilization test and Sucrose utilization test while for all other test it was positive.

Isolate no. 02 was negative for Urease test only while for all other tests it was positive. Isolate no.

03 was negative for Lactose utilization test only. Isolate no. 04 was negative for Glucose utilization test and Sucrose utilization test only. Isolate no. 05 was negative for Urease test only. The result is recorded in table 2.

The response of each isolate to the five selected antibiotics is summarized in Table 3. Every tested strain proved completely resistant to Erythromycin, with bacterial lawns growing right up against the disc edge.

In contrast, all five isolates showed clear sensitivity toward Ciprofloxacin, Doxycycline, Amoxicillin, and Azithromycin. Ciprofloxacin generated the widest clearing zones across all isolates, averaging between 19.0 mm and 21.3 mm. Doxycycline followed closely, yielding clear rings measuring 17.0mm to 20.1mm. Amoxicillin and Azithromycin also held back growth effectively, producing inhibition zones of 15.0-19.0 mm and 13.0-16.0mm, respectively.

Table 1: Morphological character of Colony and Bacterial cell

Isolate	Colony shape	Colony colour	Nature of colony	Margin of colony	Surface of colony	Transparency of colony	Cell shape	Gram staining
Isolate 1	Circular	White	Glistening	Entire	Smooth	Opaque	Oval	-ve
Isolate 2	Circular	Yellow	Glistening	Entire	Smooth	Opaque	Oval	-ve
Isolate 3	Circular	White	Glistening	Entire	Smooth	Opaque	Oval	-ve
Isolate 4	Circular	White	Glistening	Entire	Smooth	Opaque	Oval	-ve
Isolate 5	Circular	White	Glistening	Entire	Smooth	Opaque	Oval	-ve

Table 2: Biochemical test

Biochemical test	Isolate 1	Isolate 2	Isolate 3	Isolate 4	Isolate 5
Citrate utilization test	+	+	+	+	+
Methyl red test	+	+	+	+	+
Voges-Proskauer test	+	+	+	+	+
Indole test	+	+	+	+	+
Catalase test	+	+	+	+	+
Glucose test	-	+	+	-	+
Lactose test	+	+	-	+	+
Sucrose test	-	+	+	-	+
Oxidase test	+	+	+	+	+
Urease test	+	-	+	+	-

Table 3: Inhibition zone

Antibiotic Agent	Isolate 01 (mm)	Isolate 02 (mm)	Isolate 03 (mm)	Isolate 04 (mm)	Isolate 05 (mm)
Amoxicillin (AMC)	17.0±0.2	16.0±0.1	18.0±0.3	15.0±0.2	19.0±0.2
Azithromycin (AZM)	13.0±0.1	14.0±0.2	14.0±0.1	16.0±0.3	15.0±0.1
Ciprofloxacin (CIP)	20.1±0.3	19.0±0.2	20.3±0.2	20.2±0.1	21.3±0.4
Doxycycline (DOX)	19.0±0.2	17.0±0.1	20.1±0.3	19.8±0.2	19.6±0.2
Erythromycin (E)	Resistant (0.0)	Resistant (0.0)	Resistant (0.0)	Resistant (0.0)	Resistant (0.0)

DISCUSSION

The colony traits, cell morphology, and biochemical reactions observed in this study confirm that all five isolates belong to the genus *Azotobacter* (Bergey's Manual of Determinative Bacteriology; Forbes *et al.*, 2002). The distinctly sticky, glistening appearance of the colonies comes from the heavy production of extracellular polysaccharide (EPS) slime, mainly alginates. This slime layer is not merely structural; it serves as a critical oxygen-diffusion barrier that protects the delicate nitrogenase enzyme from oxygen damage while allowing the bacterium to respire aerobically (Subba Rao, 1982; Mirkovacki & Milic, 2001).

Our findings also highlight noticeable physiological variation among strains collected from the same regional block. Such diversity is well recognized in Indian soils (Gandora *et al.*, 1998; Rajeswari & Kasturi, 2009). Isolate 02 produced bright yellow pigments and utilized glucose, lactose, and sucrose without producing urease—a profile typical of *Azotobacter beijerinckii* or early-phase cultures of *Azotobacter vinelandii* (Lee *et al.*, 1970; Taller & Wong, 1989). By comparison, isolates 01, 03, 04, and 05 exhibited the standard milky-white, opaque growth and biochemical traits characteristic of classic *Azotobacter chroococcum* strains commonly found in crop root zones (Kumar *et al.*, 2007).

The antibiotic screening revealed clear differences across antibiotic classes:

Natural Resistance to Erythromycin: All five isolates grew directly over Erythromycin discs without hesitation. In environmental Gram-negative bacteria, macrolide insensitivity is typically an intrinsic survival trait rather than an acquired hazard. The complex outer lipopolysaccharide membrane naturally restricts large, hydrophobic macrolide molecules from entering the cell, while active multidrug efflux pumps continually flush out entering compounds (Forbes *et al.*, 2002; Onwurah & Nwuke, 2004).

Strong Vulnerability to Fluoroquinolones and Tetracyclines: The broad inhibition rings around Ciprofloxacin (19.0-21.3mm) and Doxycycline (17.0-20.1mm) demonstrate that core cellular machinery—namely DNA gyrase and the 30S ribosomal subunit—remains readily reachable and unmutated in these native soil bacteria. Similar broad vulnerability has been documented in non-clinical rhizobacteria isolated from rural farmlands (Singh & Wright, 2002; Fatima *et al.*, 2009).

Moderate Sensitivity to β Lactams and Azithromycin: The clean clearance rings produced by Amoxicillin (15.0-19.0mm) and Azithromycin (13.0-16.0mm) confirm that these isolates lack aggressive baseline lactamase enzymes or macrolide-inactivating esterases.

Because modern farmlands routinely receive low concentrations of veterinary antibiotic residues, chemical pesticides, and heavy metal traces through manure and runoff, assessing indigenous plant growth-promoting rhizobacteria (PGPR) for antimicrobial sensitivity is essential (Ayansina & Osoba, 2006; Sarnaik *et al.*, 2006; Widenfalk *et al.*, 2008). Chemical residues can suppress soil respiration and inhibit nitrogen-fixing capacity (Castro *et al.*, 1997; Sudhakar *et al.*, 2000; Vieira *et al.*, 2007). Biofertilizer candidates must be hardy enough to survive routine agrochemical stress while remaining ecologically safe and free from dangerous transmissible resistance genes (Arun, 2007; Subba Rao, 2001). The five isolates recovered from Mahisi block combine vigorous metabolic

activity with clean, predictable antibiotic susceptibility profiles, making them promising candidates for further local field evaluation.

CONCLUSION

This study successfully isolated and characterized five native *Azotobacter* strains from crop rhizospheres in Mahisi block, Saharsa district, Bihar. Morphological examination and biochemical profiling verified that these isolates belong to the genus *Azotobacter*, primarily representing *Azotobacter chroococcum* alongside closely related species variants.

Antibiotic susceptibility testing demonstrated that while all five strains possess natural intrinsic resistance to Erythromycin, they remain vulnerable to broad-spectrum agents including Ciprofloxacin, Doxycycline, Amoxicillin, and Azithromycin. Their ability to thrive on nitrogen-free media, coupled with their stable antimicrobial response patterns, suggests that these indigenous strains are well suited for development into regionally adapted biofertilizers. Further molecular verification through 16S rRNA gene sequencing, assessment of the nitrogen-fixing *nifH* gene, and quantitative measurement of indole-3-acetic acid (IAA) production under field conditions will help advance these strains toward practical agricultural use.

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