# MORPHOLOGICAL AND BIOCHEMICAL CHARACTERIZATION OF *AZOTOBACTER* ISOLATED FROM DIFFERENT RHIZOSPHERIC SOILS OF KATHMANDU VALLEY

**Kiran Bista***, **Parbati Adhikari**, **Shree Prasad Vista**, and **Rishi Ram Kattel**

*National Soil Science Research Centre (NSSRC), Nepal Agricultural Research Council (NARC), Khumaltar, Lalitpur, Nepal*

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*Corresponding author:
kkbista22@gmail.com

**ABSTRACT**

*Biological nitrogen fixation by free-living diazotrophic bacteria, particularly Azotobacter spp., offers a sustainable way to replenish soil nitrogen. This study aimed to isolate and characterize Azotobacter from rhizospheric soils of ten crop species collected across Kathmandu Valley, and examine the influence of soil pH on bacterial colony count and Azotobacter prevalence. The study was conducted at the Microbiology Laboratory of NSSRC, NARC, Khumaltar, Lalitpur over four months using a Completely Randomized Design (CRD) with 20 treatments established by subjecting two dilutions ($10^{-3}$ and $10^{-5}$) to ten soil samples, each replicated three times. A pure Azotobacter strain served as a reference. Bacteria were isolated on Jensen's nitrogen-free agar and identified through colony morphology, Gram staining, and biochemical tests. Out of 33 isolates obtained from 60 experimental units, Azotobacter was confirmed in 7 treatments (35%). Other $N_2$-fixing bacteria identified included Azospirillum (25%) and Clostridium (20%), while 20% of isolates remained unidentified. Soil pH showed a strong positive correlation with bacterial colony count ($r = 0.887$, $p \leq 0.001$), while Azotobacter occurrence showed a positive but non-significant relationship with pH (coefficient = 0.801, $p = 0.114$). Morphological and biochemical characterization confirmed the presence of Azotobacter in rhizospheric soils of five crop species, providing baseline information on native Azotobacter isolates in Kathmandu valley.*

## 1. INTRODUCTION

N is one of the most important nutrients limiting crop production, and its supply in agroecosystems is commonly maintained through the application of chemical fertilizers. However, excessive use of nitrogen fertilizers contributes to greenhouse gas emissions, soil acidification, and water pollution (Kumar, 2019; Tripathi et al., 2020). Biological nitrogen fixation by diazotrophic bacteria provides a sustainable alternative by converting atmospheric nitrogen into plant-available forms (Basalingappa, 2018; Jaweria & Qayum, 2020). Among these bacteria, Azotobacter spp. are free-living nitrogen-fixing microorganisms that contribute to soil fertility and plant growth.

*Azotobacter* spp. are free-living, aerobic, Gram-negative diazotrophic bacteria commonly found in neutral to alkaline soils (Aquilanti et al., 2004; Nongthombam et al., 2021; Shankar & Maitra, 2021). Besides fixing atmospheric nitrogen, they produce plant growth-promoting substances such as indole acetic acid, gibberellins, cytokinins, vitamins, and antifungal compounds, and also

help in phosphate solubilization and soil aggregation (Elbaz et al., 2010). These attributes make *Azotobacter* a versatile common bioinoculant for many crops. Its application as a biofertilizer can enhance soil health, enhance plant growth, and increase crop yield while reducing the environmental impact of chemical fertilizers (Jnawali et al., 2015; Gurikar et al., 2016).

The rhizosphere is the soil zone around plant roots where microbial activity is high due to root exudates that support diverse microorganisms, including PGPR (Hinsinger & Marschner, 2006; Ling et al., 2022). Previous studies have reported that the occurrence and abundance of *Azotobacter* are influenced by several environmental factors, particularly soil pH, moisture, temperature, and nutrient availability (Barnes et al., 2007; Chen et al., 2018; Sumbul et al., 2020; Aasfar et al., 2021). Although the association between soil pH and *Azotobacter* occurrence has been reported in different regions, information on the distribution and characteristics of native *Azotobacter* populations in the rhizospheres of crops grown in Kathmandu valley remains limited. Furthermore, little information is available regarding how soil pH relates to bacterial colony abundance and *Azotobacter* prevalence in these agricultural soils.

Kathmandu valley is an important agricultural region with diverse cropping systems, making it a suitable area for exploring native nitrogen-fixing bacterial populations. Characterization of indigenous *Azotobacter* isolates is important for understanding their occurrence and potential utilization in sustainable agriculture. Therefore, this study was conducted to isolate and characterize bacterial strains from rhizospheric soils of different crops, identify *Azotobacter* using morphological and biochemical characteristics with a reference strain, and assess the relationship between soil pH, bacterial colony count, and *Azotobacter* prevalence.

## 2. MATERIALS AND METHODS

### *2.1 Experimental location*

Rhizospheric soil samples were collected from districts of Kathmandu valley based on commonly cultivated crops. Only fields that had been continuously cultivated with the same crop for at least three years were included in the study to ensure a well-established crop rhizosphere. Laboratory isolation, culture, and characterization work was conducted at the Microbiology Laboratory of the National Soil Science Research Centre (NSSRC), National Agriculture Research Council (NARC), Khumaltar, Lalitpur.

### *2.2 Collection of soil samples*

Composite rhizospheric soil samples were collected from the uppermost 15 cm of soil from ten different crop species grown at ten locations across the three districts of Kathmandu valley: Kathmandu, Bhaktapur, and Lalitpur following strict aseptic technique. Approximately 100 g of composite soil per sample was collected in sterilized plastic bags, stored in an ice box, and processed promptly.

### *2.3 Experimental design and treatments*

The experiment was conducted using Completely Randomized Design subjecting soil samples to two serial dilutions ($10^{-3}$ and $10^{-5}$), yielding twenty experimental treatments, each replicated three times (Table 1). A known pure culture of *Azotobacter* obtained from the NSSRC microbiology laboratory was included as a reference check to confirm bacterial identification by comparison of morphological and biochemical characteristics.

**Table 1.** Experimental treatments used in the study.

| Soil Sample Treatment | Code | Soil Sample Treatment | Code |
|---|---|---|---|
| Asparagus soil @$10^{-3}$ dilution | T1 | Brassica soil @$10^{-3}$ dilution | T11 |
| Asparagus soil @$10^{-5}$ dilution | T2 | Brassica soil @$10^{-5}$ dilution | T12 |
| Colocasia soil @$10^{-3}$ dilution | T3 | Avena soil @$10^{-3}$ dilution | T13 |
| Colocasia soil @$10^{-5}$ dilution | T4 | Avena soil @$10^{-5}$ dilution | T14 |
| Cucumis soil @$10^{-3}$ dilution | T5 | Solanum soil @$10^{-3}$ dilution | T15 |
| Cucumis soil @$10^{-5}$ dilution | T6 | Solanum soil @$10^{-5}$ dilution | T16 |
| Phaseolus soil @$10^{-3}$ dilution | T7 | Raphanus soil @$10^{-3}$ dilution | T17 |
| Phaseolus soil @$10^{-5}$ dilution | T8 | Raphanus soil @$10^{-5}$ dilution | T18 |
| Zea soil @$10^{-3}$ dilution | T9 | Triticum soil @$10^{-3}$ dilution | T19 |
| Zea soil @$10^{-5}$ dilution | T10 | Triticum soil @$10^{-5}$ dilution | T20 |
| Azotobacter pure culture (Check) | — | | |

### *2.4 pH measurement*

The pH of each soil sample was measured using a glass electrode pH meter. The instrument was calibrated using buffer solutions of pH 4.0 and pH 7.0. A 1:2 soil-to-water suspension was prepared (20 g soil in 40 mL distilled water), stirred thoroughly, and allowed to settle for 30 minutes before measurement. The electrode was rinsed with distilled water between readings.

### *2.5 Preparation of Jensen's selective medium*

Jensen's agar medium, a nitrogen-free selective medium was used for the isolation and culture of $N_2$-fixing bacteria. Jensen's agar powder (39.1 g) was dissolved in 1000 ml of distilled water in a conical flask, heated to near boiling with continuous stirring in a water bath, and sterilized by autoclaving at 121°C, 15 psi for 30 minutes. The sterilized medium was poured into sterile Petri plates (approximately 2/3 full) inside a laminar airflow cabinet to avoid contamination and allowed to solidify before use.

### *2.6 Serial dilution and isolation*

One gram of each soil sample was transferred to a sterile test tube and serially diluted from $10^{-1}$ to $10^{-5}$ in sterile distilled water (1 mL into 9 mL blanks). Bacterial isolation was performed by the spread plate method. 0.1 mL aliquots of the $10^{-3}$ and $10^{-5}$ dilutions were inoculated onto Jensen's agar plates then well distributed using a sterilized bent glass rod. These dilutions were chosen to obtain countable, well-separated colonies for accurate enumeration and isolation of bacteria. Plates were incubated at 25–30°C for 48–72 hours.

After incubation, colony counting was performed on primary plates. Well-grown, water-droplet-like colonies (characteristic of *Azotobacter*) were selected for sub-culturing. Contaminated plates were discarded; uncontaminated replicates were processed further. Selected colonies were sub-cultured on fresh Jensen's medium using quadrant streak technique and incubated again at 25–30°C for 48–72 hours. The sub-culturing process was repeated two to three times to obtain pure cultures for downstream analyses (Hala & Ali, 2019).

### *2.7 Morphological characterization*

*2.7.1 Macroscopic examination.* Pure cultures were examined visually for colony morphology, including colour, shape, texture, transparency, surface features, and margin characteristics.

*2.7.2 Microscopic examination.* Gram staining was performed on all pure isolates. A loopful of each culture was smeared on a glass slide, heat-fixed, and stained using the standard four-step Gram protocol. Slides were examined under a compound microscope at

4×, 10×, 40×, and 100× magnification (oil immersion) to determine cell shape and Gram reaction.

### *2.8 Biochemical characterization*

All isolates were subjected to five biochemical tests. (i) **Nitrogen fixation:** growth on Jensen's nitrogen-free medium confirmed the ability to fix atmospheric $N_2$. (ii) **Catalase test:** 4–5 drops of hydrogen peroxide ($H_2O_2$) were placed in a test tube; positive results were indicated by bubble formation on addition of bacterial culture. (iii) **Oxidase test:** bacterial culture was rubbed onto an oxidase disk; appearance of deep purple or blue colour within 5–10 seconds indicated a positive result. (iv) **Simmons citrate utilisation test:** culture was inoculated onto Simmons citrate agar (SCA) slants, incubated at 37°C for 24–48 hours; a colour change from green to blue indicated citrate utilisation. (v) **Urease test:** culture was inoculated onto Christensen's urea agar slants, incubated at 37°C for 24–48 hours; colour change from yellow to pink indicated urease activity. (vi) **Indole production test:** culture was inoculated into SIM broth and incubated at 37°C for 24–48 hours; 1–2 drops of Kovac's reagent were added and a red/pink layer indicated positive indole production. Results were interpreted in comparison to the reference check, following Upadhyay et al. (2015) and Hala & Ali (2019).

### *2.9 Data analysis*

All data were recorded in Microsoft Excel, cleaned, and analyzed using R-Studio software. One-way analysis of variance (ANOVA) was performed to assess differences in mean colony count across treatments, with the least significant difference (LSD) test at the 5% level of significance. Pearson's correlation coefficient was calculated to evaluate the relationship between soil pH and bacterial colony count.

## 3. RESULT AND DISCUSSION

A total of 33 bacterial isolates were obtained from 60 experimental units (20 treatments × 3 replicates). Isolation via serial dilution and culture on Jensen's selective nitrogen-free agar yielded well-grown colonies after 48–72 hours of incubation at 28°C. The mother culture of Azotobacter from NSSRC was also cultured concurrently for comparative reference.

Colony counts varied significantly across treatments (ANOVA: $p \leq 0.001$). Higher mean colony counts were recorded in T6 (Cucumis soil @ $10^{-5}$; 34.67 CFU), followed by T17 (Raphanus soil @ $10^{-3}$; 32.67 CFU) and T4 (Colocasia soil @ $10^{-5}$; 31.00 CFU). The lowest colony counts were observed in T15 (Solanum soil @ $10^{-3}$; 16.33 CFU), T11 (Brassica soil @ $10^{-3}$; 18.00 CFU), and T19 (Triticum soil @ $10^{-3}$; 18.33 CFU). The LSD value at the 5% significance level was 0.0456, indicating that treatment differences exceeding this threshold are statistically significant. Overall, the highest bacterial populations were recorded in the rhizosphere of Cucumber (Cucumis sativus), Taro (Colocasia esculenta), and Radish (Raphanus sativus), while significantly lower populations were associated with Mustard (Brassica juncea), Oats (Avena sativa), and Wheat (Triticum aestivum).

The significant variation in mean colony counts across treatments (ANOVA $p = 0.000763$) indicates that crop species, soil physiochemical properties, and level of dilution influence bacterial population density. Treatments T6 (Cucumis @ $10^{-5}$) and T17 (Raphanus @ $10^{-3}$) showed markedly higher colony counts compared to T15 (Solanum) and T11 (Brassica), indicating that root exudate composition and rhizosphere pH of different crop species differentially support bacterial growth. These findings align with reports that soil factors such as pH, Mn, Zn levels, temperature, moisture, carbon, and nitrogen sources significantly influence the growth and diversity of Azotobacter (Chen et al., 2018; Aasfar et al., 2021).

Macroscopic and microscopic examinations of all 33 isolates are presented in **Table 2**. Colony morphology across all treatments

was consistent with Azotobacter-like characteristics, displaying round colonies with smooth surfaces and entire margins. Colony colour ranged from creamy to yellow, and cells were predominantly rod-shaped, with two treatments (T9 and T20) showing coccus cell shapes. All isolates were opaque except T10, which was translucent with a rough surface.

**Table 2.** Morphological characterization of bacterial isolates across all treatments

| Treatment | Cell Shape | Colony Shape | Colony Colour | Transparency | Surface | Margin |
|---|---|---|---|---|---|---|
| Check* | Rod | Round | Yellow | Opaque | Smooth | Entire |
| T1 | Rod | Round | Yellow | Opaque | Smooth | Entire |
| T2 | Rod | Round | Yellow | Opaque | Smooth | Entire |
| T3 | Rod | Round | Creamy | Opaque | Smooth | Entire |
| T4 | Rod | Round | Creamy | Opaque | Smooth | Entire |
| T5 | Rod | Round | Yellow | Opaque | Smooth | Entire |
| T6 | Rod | Round | Yellow | Opaque | Smooth | Entire |
| T7 | Rod | Round | Yellow | Opaque | Smooth | Entire |
| T8 | Rod | Round | Yellow | Opaque | Smooth | Entire |
| T9 | Coccus | Round | Yellow | Opaque | Smooth | Entire |
| T10 | Rod | Round | Yellow | Translucent | Unsmooth | Entire |
| T11 | Rod | Round | Creamy | Opaque | Smooth | Entire |
| T12 | Rod | Round | Creamy | Opaque | Smooth | Entire |
| T13 | Rod | Round | Creamy | Opaque | Smooth | Entire |
| T14 | Rod | Round | Creamy | Opaque | Smooth | Entire |
| T15 | Rod | Round | Yellow | Opaque | Smooth | Entire |
| T16 | Rod | Round | Creamy | Opaque | Smooth | Entire |
| T17 | Rod | Round | Creamy | Opaque | Smooth | Entire |
| T18 | Rod | Round | Yellow | Opaque | Smooth | Entire |
| T19 | Rod | Round | Creamy | Opaque | Smooth | Entire |
| T20 | Coccus | Round | Yellow | Opaque | Smooth | Entire |

*Check = Known Azotobacter strain from NSSRC.

Morphological characterization showed that the majority of isolates displayed Azotobacter-like colony characteristics: round colonies, smooth surfaces, entire margins, and creamy to yellow pigmentation, consistent with previously published descriptions (Gangavane & MJ, 2018; Upadhyay et al., 2015). However, morphological characteristics alone were insufficient to definitively identify Azotobacter, making biochemical characterization essential for confirmation.

Results of biochemical testing for all treatments are presented in **Table 3**. Comparing isolate profiles against the reference check (Gram-negative, oxidase-positive, catalase-positive, citrate-positive, urease-positive, indole-negative), Azotobacter was confirmed in seven treatments: T1, T2, T5, T6, T11, T18, and T19. This represents 35% of the total treatments. Azospirillum (oxidase-negative, catalase-positive, Gram-negative) was identified in T7, T8, T10, T12, and T17 (25%). Clostridium (oxidase-negative, catalase-positive, Gram-positive) was identified in T9, T13, T14, and T15 (20%). The remaining four treatments — T3, T4, T16, and T20 — showed variable results inconsistent with any single genus and remained unidentified (20%), indicating a heterogeneous microbial community requiring further molecular analysis.

**Table 3.** Biochemical characterization of bacterial isolates across all treatments

| Treatment | Gram Stain | Oxidase | Catalase | Simmons Citrate | Urease | Indole | Identification |
|---|---|---|---|---|---|---|---|
| Check* | − | + | + | + | + | − | ***Azotobacter*** |
| T1 | − | + | + | + | + | − | ***Azotobacter*** |
| T2 | − | + | + | + | + | − | ***Azotobacter*** |
| T3 | − | − | − | + | + | − | Unidentified |
| T4 | − | − | − | + | + | − | Unidentified |
| T5 | − | + | + | + | + | − | ***Azotobacter*** |
| T6 | − | + | + | + | + | − | ***Azotobacter*** |
| T7 | − | − | + | + | + | − | *Azospirillum* |
| T8 | − | − | + | + | + | − | *Azospirillum* |
| T9 | + | − | + | + | + | − | *Clostridium* |
| T10 | − | − | + | + | − | − | *Azospirillum* |
| T11 | − | + | + | + | + | − | ***Azotobacter*** |
| T12 | − | − | + | + | + | − | *Azospirillum* |
| T13 | + | − | + | + | + | − | *Clostridium* |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| T14 | + | − | + | + | + | − | *Clostridium* |
| T15 | + | − | + | + | − | − | *Clostridium* |
| T16 | + | + | + | + | + | − | Unidentified |
| T17 | − | − | + | + | + | − | *Azospirillum* |
| T18 | − | + | + | + | + | − | ***Azotobacter*** |
| T19 | − | + | + | + | + | − | ***Azotobacter*** |
| T20 | − | + | + | + | + | + | Unidentified |

**Check = Known Azotobacter strain from NSSRC. +, positive; −, negative.*

The biochemical characterization of isolates against the reference check confirmed Azotobacter in 35% of treatments. According to Bergey's Manual of Systematic Bacteriology, Azotobacter is typically oxidase-positive, catalase-positive, citrate-positive, urease-positive, Gram-negative, and indole-negative (Upadhyay et al., 2015; Gangavane & MJ, 2018), a profile matched precisely by T1, T2, T5, T6, T11, T18, and T19. Azotobacter was confirmed in the rhizospheres of five crops: Asparagus, Cucumber, Maize, Radish, and Wheat.

The identification of Azospirillum (25%) and Clostridium (20%) alongside Azotobacter indicates the presence of a broader $N_2$-fixing microbiome in Kathmandu valley soils. Azospirillum is a known diazotrophic PGPR associated with grasses and cereals, while Clostridium performs anaerobic nitrogen fixation (Timofeeva et al., 2023). The remaining 20% of isolates with variable biochemical profiles may represent mixed cultures or taxa requiring molecular characterization for confirmation.

The colony count did not show appreciable variability between the two dilution levels ($10^{-3}$ and $10^{-5}$), which is consistent with the literature: Azotobacter tends to form colonies at similar rates across a range of dilutions when selective nitrogen-free media are employed (Malek & Ishac, 1968). This finding lends confidence to the representativeness of results across dilutions.

Pearson correlation analysis revealed a strong positive relationship between soil pH and bacterial colony count ($r = 0.8874$, $p = 1.82 \times 10^{-7}$) (Figure 1). This highly significant result indicates that soil pH is an important determinant of bacterial population density in the rhizosphere under study: as soil pH increased, bacterial colony counts also increased.

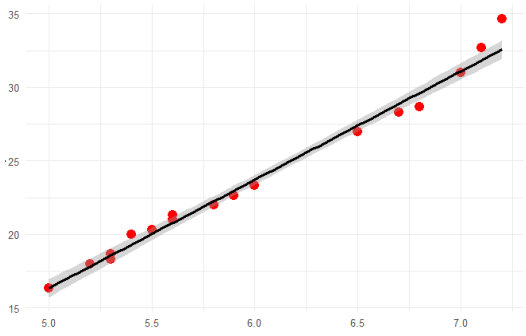


Figure 1. Scatter plot showing the correlation between colony count and pH

The strong positive Pearson correlation ($r = 0.887$) between soil pH and bacterial colony count is a notable finding and aligns with the

well-documented preference of Azotobacter for neutral to slightly alkaline conditions (pH > 6.5) (Barnes et al., 2007; Kumar et al., 2025). Soil pH influences microbial growth by affecting nutrient availability, enzyme activity, and cellular functions. Neutral to slightly alkaline conditions generally favour bacterial growth, whereas acidic conditions can reduce microbial activity and limit the survival of aerobic $N_2$-fixing bacteria such as Azotobacter. A highly significant p-value ($1.82 \times 10^{-7}$) confirms this relationship is not due to chance. Similar positive associations between soil pH and Azotobacter abundance have been reported in rice and other crop rhizospheres by Chen et al. (2018) and Aasfar et al. (2021).

The present study successfully isolated and characterized $N_2$-fixing bacteria, including Azotobacter, from the rhizospheric soils of diverse crop species cultivated across the Kathmandu Valley. A total of 33 isolates were obtained, representing a diversity of $N_2$-fixing taxa. The significant variation in mean colony counts across treatments indicates that crop species, soil physicochemical properties, and rhizosphere conditions may influence bacterial population density.

The results highlight the abundant presence of $N_2$-fixing bacteria, particularly Azotobacter, in the diverse rhizospheric soils of the Kathmandu Valley. These indigenous strains represent a valuable resource for developing locally adapted biofertilizer formulations. The use of Azotobacter-based biofertilizers provides a cost-effective and environmentally safe alternative to chemical nitrogen fertilizers, contributing to improved soil health and sustainable farming systems in Nepal (Jnawali et al., 2015; Sumbul et al., 2020).

## 4. CONCLUSION

This study demonstrated that $N_2$-fixing bacteria including *Azotobacter*, *Azospirillum*, and *Clostridium* are widely distributed in the rhizospheric soils of diverse crop species in the Kathmandu valley. *Azotobacter* was confirmed in 35% of isolates, representing five crop rhizospheres, using a combination of morphological and biochemical characterization. A robust positive correlation between soil pH and bacterial population density was established. These findings highlight the viability of *Azotobacter* strains as biofertilizers and underline the importance of soil pH management for effective nitrogen fixation in agricultural systems. Naturally occurring Azotobacter offers a cost effective, and eco-friendly pathway to improving soil fertility, reducing chemical fertilizer dependency, and supporting long-term agricultural productivity in Nepal.

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