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Research article

Isolation, Characterization and Molecular Identification of Nitrogen Fixing Bacteria from Rhizosphere of *Xylocarpus Moluccensis*

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ABSTRACT

The research was aimed to isolate, characterize, and molecularly identify nitrogen-fixing bacteria from the rhizosphere of *Xylocarpus moluccensis*, collected from the Sundarbans, Khulna, Bangladesh. This exploratory study focused on isolating bacteria capable of nitrogen fixation using Yeast Extract Mannitol Agar and Nitrogen-Free Burk's Media. In addition, an ammonification test was performed to identify ammonia-producing bacteria for further evaluation. Through the isolation process, ten nitrogen-fixing bacterial strains were identified, all of which demonstrated the ability to produce indole-3-acetic acid (IAA), a key secondary metabolite involved in plant growth promotion. Biochemical tests were conducted to presumptively identify the bacterial isolates, with colony morphology suggesting the presence of *Staphylococcus* spp. and *Kocuria* spp. in the rhizosphere soil samples. To assess the plant growth-promoting potential of these isolates, a pot experiment was conducted using maize seedlings. The results indicated that bacterial inoculation substantially stimulated seedling growth and development, suggesting their potential as biofertilizers. However, further research is necessary to evaluate their impact on seed germination and crop yield before large-scale application in agricultural settings. Overall, this study highlights the presence of nitrogen-fixing bacteria in the rhizosphere of *X. moluccensis*, with promising implications for sustainable agriculture. The findings suggest that these bacterial strains could contribute to enhancing soil fertility and plant growth, supporting the development of eco-friendly biofertilizers.

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Introduction

Nitrogen is an essential element for plant growth and agricultural productivity, yet most plants cannot directly utilize atmospheric nitrogen (N_2). Instead, they depend on bioavailable forms such as ammonia or nitrate. Biological nitrogen fixation (BNF), carried out by specialized prokaryotes including bacteria and archaea, converts atmospheric N_2 into plant-usable forms and contributes to approximately 50% of the global nitrogen supply (Zayed et al., 2023; Shomi et al., 2021).

BNF plays a particularly vital role in tropical agriculture, where it enhances both sustainability and productivity, especially in legume-based systems (Moura et al., 2020; Kass et al., 1997). Nitrogen-fixing bacteria that also produce plant growth-promoting compounds like indole-3-acetic acid (IAA), an important auxin involved in cell division and elongation, can function as dual-action biofertilizers (Kumar et al., 2022; Spaepen et al., 2007). Such bacteria offer an environmentally friendly alternative to synthetic fertilizers, with potential to improve crop growth and reduce agricultural costs.

While symbiotic nitrogen fixation has been extensively studied in legumes, free-living nitrogen-fixing bacteria- such as *Azotobacter*, *Beijerinckia*, and R*hodospirillum*—remain underexplored, especially in ecologically unique environments like the Sundarbans mangrove forest (Das et al., 2024; Vitousek et al., 2013). This region harbors distinct microbial communities with untapped agricultural potential.

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This study focuses on the isolation, characterization, and evaluation of nitrogen-fixing bacteria from the rhizosphere of *X. moluccensis* in the Sundarbans. In addition to their nitrogen-fixing capacity, these isolates were assessed for IAA production and their effect on maize seedling growth. The findings highlight their potential as biofertilizer candidates that could support sustainable farming in developing countries like Bangladesh (Bashan et al., 2014; Vessey, 2003).

Materials and Methods

Sample Collection and Isolation of Bacteria

Soil samples were aseptically collected from the rhizosphere (6 cm depth) of *X. moluccensis* in the Sundarbans using sterile tools and stored at 4°C in 50 ml conical tubes. Nitrogen-fixing bacteria were isolated using nutrient agar (NA), Yeast Extract Mannitol Agar (YEMA), and nitrogen-free Burk's medium. Cultures were incubated at 37°C: overnight for NA, 3 days for YEMA, and 10 days for Burk's medium. Growth on nitrogen-free media indicated nitrogen-fixing ability (Timofeeva et al., 2023).

Nitrogen Fixation Assays

Ammonia Production Test

Fresh cultures were incubated in peptone water at 28–30°C for 48–72 h. After adding Nessler's reagent, ammonia production was qualitatively assessed by color change (Kifle & Laing, 2016).

IAA Production Assay

Isolates were cultured in yeast malt dextrose (YMD) broth at 37°C for 5 days. After centrifugation, the supernatant was mixed with Salkowski's reagent and incubated in the dark for 30 min. IAA concentration was measured at 530 nm using a standard curve (Gang et al., 2019).

Biochemical Characterization

Colony morphology, cell size, motility, and Gram staining were observed following standard procedures (Roy et al., 2023). Nine biochemical tests (oxidase, catalase, citrate, urease, TSI, methyl red, indole, starch hydrolysis, and nitrate reduction) were performed in triplicate and compared with reference profiles for presumptive identification.

Molecular Identification

Genomic DNA was extracted using lysis buffer, lysozyme, RNase A, proteinase K, chloroform:isoamyl alcohol, and isopropanol. DNA quality was assessed by 1% agarose gel electrophoresis. The 16S rRNA gene was amplified using universal primers, and products were visualized under UV after ethidium bromide staining. Sequencing was conducted using the SeqStudio™ Genetic Analyzer (NIB, Bangladesh). Sequence data were analyzed using BLAST (NCBI), DNA Baser, and MEGA software to construct phylogenetic trees (Rimi et al., 2026; Tamura et al., 2021).

Pot Test

To evaluate plant growth-promoting effects, maize seeds were sown in sterilized soil with 10 ml of bacterial inoculum (prepared in nutrient broth and incubated for 3 days). Control plants received only tap water. After 15 days, root, shoot, and leaf lengths were measured. Inoculated plants showed enhanced growth and nutrient uptake compared to controls.

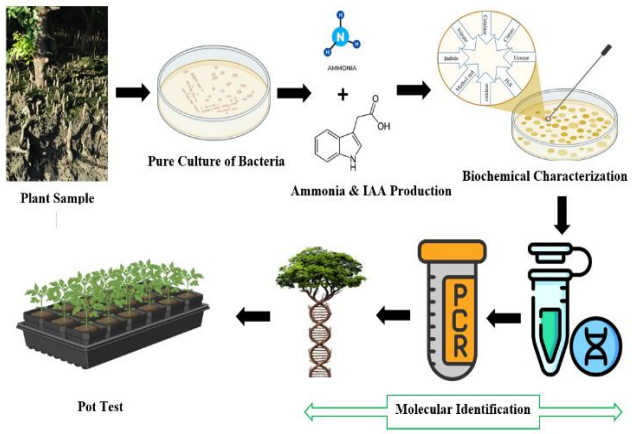

Figure 1: Schematic representation of the experimental methodology. The diagram illustrates the step-by-step procedures followed in the isolation, screening, and characterization of bacterial isolates, along with indole-3-acetic acid (IAA) production assay and data analysis.

Results

Isolation and Characterization of Bacteria

Ten bacterial strains (RP-1 to RP-10) were isolated from the rhizosphere of *X. moluccensis* in the Sundarbans using nutrient agar. All isolates demonstrated nitrogen-fixing ability on nitrogen-free media. The colony-forming unit (CFU) count was 6.1×10^3 CFU/mL at the second dilution. Morphological variations in size, color, and opacity were observed. All isolates grew on YEMA, while RP-8 failed to grow on Burk's medium, suggesting reduced viability or nitrogen-fixation capacity.

Ammonia Production

All isolates produced ammonia, indicated by a color change upon addition of Nessler's reagent. RP-2, RP-3, RP-6, and RP-10 showed the strongest reactions, while RP-1 and RP-8 showed weaker responses.

IAA Production

IAA concentrations ranged from 13.84 to 22.61 mg/mL (Figure 2). RP-5 exhibited the highest IAA production (22.61 mg/mL), while RP-4 had the lowest (13.84 mg/mL). Most isolates showed consistent IAA levels between 15–20 mg/mL, indicating potential plant growth-promoting capacity.

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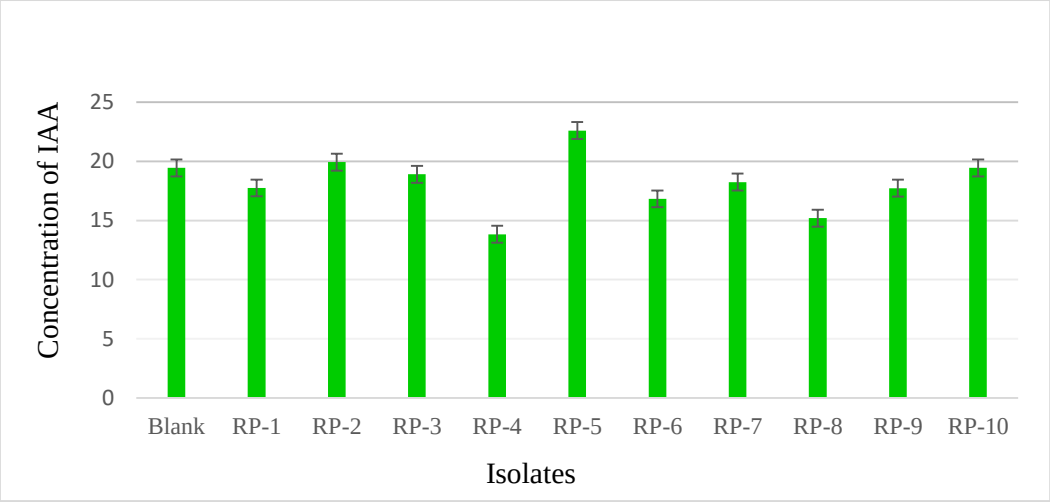

Figure 2: Indole-3-acetic acid (IAA) production by different bacterial isolates. The bar chart displays IAA levels across ten isolates (labeled 1 to 10) along with a blank control. IAA concentrations are presented on the vertical axis (0–25 µg/mL). Data represent the mean of triplicate measurements; error bars indicate standard deviation.

Biochemical Characterization

Colony morphology varied in pigmentation and form. Gram staining revealed four Gram-positive and six Gram-negative isolates. Biochemical profiling identified genera such as *Rossellomorea, Bacillus, Enterobacter, Kocuria, Micrococcus, and Nesterenkonia* (Table 1).

Table 1: Biochemical characteristics of the bacterial isolates to identify and characterize the bacterial isolates based on metabolic and enzymatic activity profiles.

Isolate	Indole	Catalase	Oxidase	Urease	Citrate	Glucose	Lactose	Sucrose	H_2S	Methyl	Nitrate	Starch	Species
RP-1	-	+	+	-	-	+	+	+	-	+	+	+	*Micrococcus sp.*
RP-2	-	+	+	-	+	+	+	+	-	+	+	+	*Rossellomorea oryzaecorticis*
RP-3	-	+	-	-	+	+	-	-	-	+	-	-	*Bacillus safensis*
RP-4	-	+	+	+	+	-	-	-	-	-	-	+	*Micrococcus sp.*
RP-5	-	+	-	-	+	+	+	+	-	-	-	+	*Rossellomorea aquimaris*
RP-6	-	+	+	-	-	+	+	+	-	-	+	+	*Bacillus halotolerans*
RP-7	-	+	-	-	+	-	-	-	-	+	-	+	*Enterobacter hormaechei*
RP-8	-	+	-	-	-	+	+	+	-	+	+	-	*Kocuria sp.*
RP-9	-	+	-	-	+	+	+	+	-	+	+	-	*Kocuria sp.*
RP-10	-	+	+	-	+	-	-	-	-	-	-	+	*Nesterenkonia sp.*

Plant Growth Promotion (Pot Test)

The growth-promoting impact of ten bacterial isolates (RP-1 to RP-10) on plant development was assessed by measuring leaf, shoot, and root lengths (Figure 3). All treated groups showed improved growth compared to the control. Notably, RP-7 exhibited the greatest overall plant height, reaching 31.97 cm—approximately 1.92-fold higher than the control—suggesting a strong growth-promoting effect. RP-6 showed the maximum leaf elongation (~30 cm), whereas RP-8 exhibited the lowest leaf growth (~15 cm). Shoot length varied between 5 and 10 cm, with RP-2 and RP-3 inducing the longest shoots. Root length was relatively stable among most isolates (15–20 cm), although RP-5 demonstrated the highest root elongation (~25 cm), indicating a balanced enhancement of all three growth parameters. These findings align with previous observations by Aasfar et al., (2024), supporting

the potential of these isolates as plant growth-promoting rhizobacteria (PGPR) (Laskar et al., 2024).

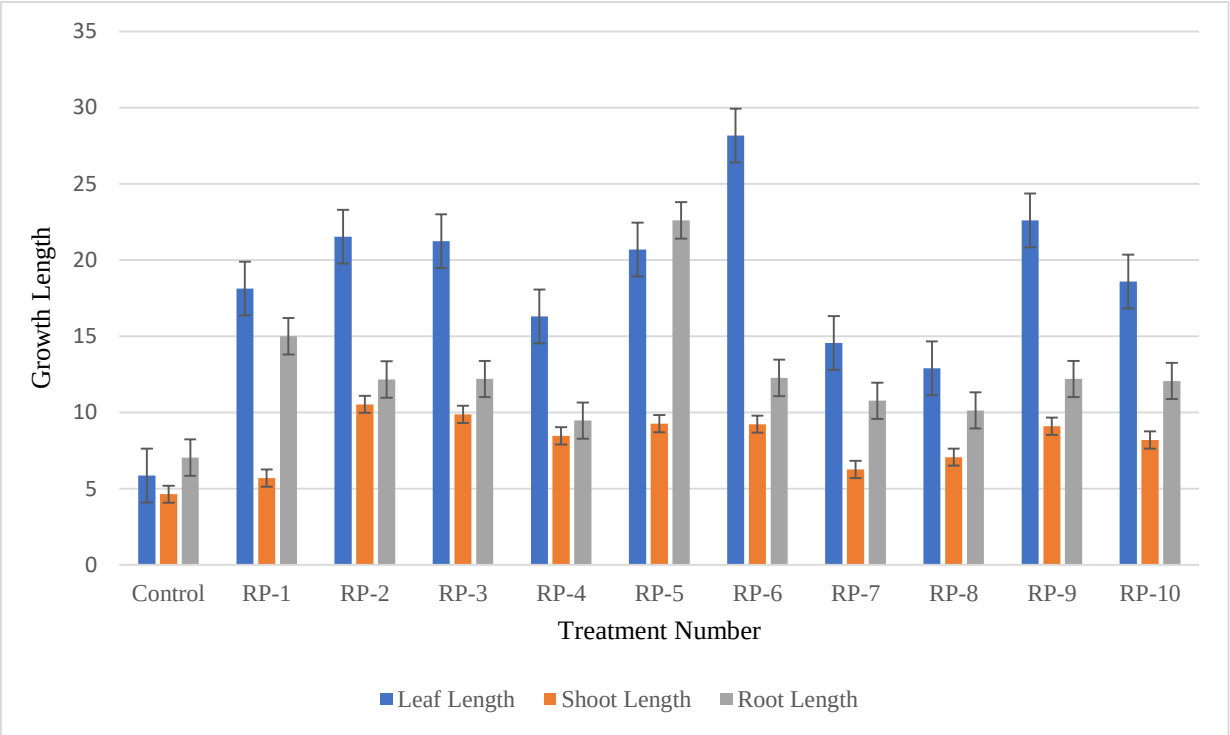

Figure 3: Effect of bacterial isolates on leaf, shoot, and root growth in treated plants. The bar chart presents growth measurements of leaves, shoots, and roots after treatment with ten bacterial isolates (RP-1 to RP-10) compared to an untreated control. Error bars represent standard deviation from triplicate measurements.

DNA Extraction and Sequencing

Agarose gel electrophoresis confirmed successful DNA extraction from all isolates, with visible bands of varying intensity (Figure 4).

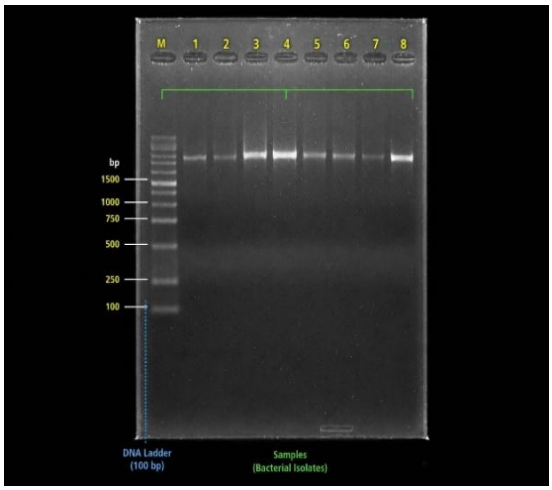

Figure 4: Agarose gel electrophoresis profile of genomic DNA extracted from bacterial isolates.

16S rRNA gene sequencing identified five isolates with 87.89% to 99.85% similarity to known species, including *Rossellomorea oryzaecorticis*, *Bacillus safensis*, *Rossellomorea aquimaris*, *Bacillus halotolerans*, and *Enterobacter hormaechei* (Table 2).

Phylogenetic Tree

The phylogenetic relationships among five bacterial strains were inferred using the Neighbor-Joining method. The tree reveals two major clades: Bacillus halotolerans grouped with B. safensis, and Rossellomorea oryzaecorticis clustered with R. aquimaris. Enterobacter hormaechei formed a distinct outgroup, indicating early divergence from the other taxa. Bootstrap values (based on 1,000 replications) are shown at branch points to indicate the reliability of clustering (Figure 5) (Martínez-Romero, E. (2003).

Table 2: Identification of bacterial isolates based on 16S rRNA gene sequencing. DNA sequencing results of selected bacterial isolates, including closest matched species, accession numbers, and percentage similarity to reference sequences in the NCBI database.

Internal Code	Name of Bacteria	Accession Number	Reference Number	Query Length	Similarity
RP-2	*Rossellomorea oryzaecorticis*	OQ331226.1	*Rossellomorea oryzaecorticis* KU933480.1	1391bp	87.89%
RP-3	*Bacillus safensis*	OQ331225.1	*Bacillus safensis* MK543013.1	1291bp	99.85%
RP-5	*Rossellomorea aquimaris*	OQ335971.1	*Rossellomorea aquimaris* KX033476.1	1290bp	94.87%
RP-6	*Bacillus halotolerans*	OQ335968.1	*Bacillus halotolerans* MK537363.1	1283bp	94.47%
RP-7	*Enterobacter hormaechei*	OQ335970.1	*Enterobacter hormaechei* PQ416037.1	1270bp	99.06%

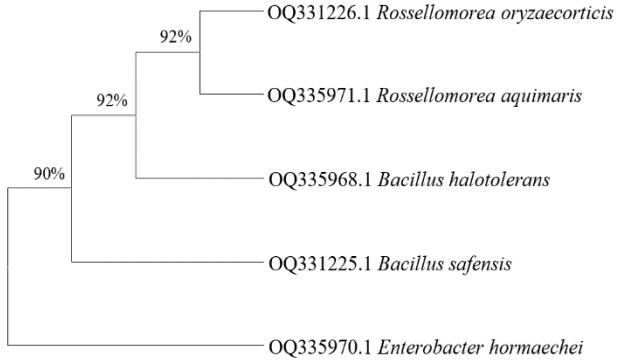

Figure 5: Phylogenetic tree based on 16S rRNA gene sequences of bacterial isolates.

Discussion

In this study, nitrogen-fixing bacteria were isolated from the rhizosphere of *X. moluccensis* collected from the Sundarbans, Khulna, Bangladesh, using specific nitrogen-fixing media. Ten bacterial isolates were selected based on their morphological characteristics and were confirmed to be nitrogen-fixing bacteria through DNA sequencing. The isolates were identified as *Rossellomorea oryzaecorticis* (RP-1), *Bacillus safensis* (RP-2), *Rossellomorea aquimaris* (RP-3), *Bacillus halotolerans* (RP-4), and *Enterobacter hormaechei* (RP-5), with the sequences deposited in the Gene Bank. The identification of these bacteria reflects previous studies emphasizing the diversity and significance of nitrogen-fixing microorganisms in the rhizosphere (Laskar et al., 2024; Santi et al., 2013).

These bacteria are recognized for their ability to produce Indole Acetic Acid (IAA), a crucial plant growth hormone, and showed plant growth-promoting activity in a short time (Shomi et al., 2021). The study revealed that the rhizospheric bacteria from Sundarbans soil are a rich source of IAA, with an average production of 19.997958 mg/ml at 530 nm wavelength, consistent with the findings of Ahmad et al. (2008) that highlight IAA production as a key function of rhizobacteria promoting plant growth. In addition, ammonia production was measured using Nessler's reagent, confirming the nitrogen-fixing capabilities of the isolates, aligning with similar studies on nitrogenase enzyme activity in soil bacteria (Nawaz et al., 2025).

The isolates demonstrated significant potential for promoting plant growth and improving soil fertility. Using these bacteria as biofertilizers could reduce the need for chemical fertilizers, offering a more sustainable solution for agriculture in developing regions. This is particularly important for countries where agriculture is a primary contributor to the national economy, as suggested by similar findings in sustainable agriculture practices. The molecular characterization of the isolates further established their role in plant growth enhancement, making these bacteria a valuable resource for biofertilizer development (Bashan et al., 2014).

However, a limitation of the study was the lack of field trials to test the efficacy of the isolates in real-world agricultural conditions. While biochemical tests like ammonia and IAA production confirmed the potential of these bacteria, testing their performance through pot trials on maize plants would have provided more comprehensive results (Kristek et al., 2020). Despite this, the selection process based on morphological traits, and the use of Burk's media and Yeast Extract Mannitol Agar (YEMA) media, ensured the reliability of the findings by minimizing contamination risks. Additionally, high-quality reagents and equipment further strengthened the accuracy of the experiments (Hamza et al., 2017).

The Sundarbans rhizosphere remains an underexplored region for nitrogen-fixing bacteria research. The practical application of these findings as biofertilizers in agriculture has the potential to positively impact society by promoting sustainable farming practices. Further studies on the host plants could deepen our understanding of their interactions with nitrogen-fixing bacteria and enhance agricultural practices based on these findings (Pallavi et al., 2023).

Conclusion

This study successfully isolated and characterized nitrogen-fixing bacteria from the rhizosphere of *X. moluccensis* in the Sundarbans, Khulna, Bangladesh. The isolates exhibited significant nitrogen-fixing capabilities and produced indole-3-acetic acid (IAA), which promotes plant growth. Pot tests confirmed their potential as biofertilizers by enhancing maize seedling development. These findings suggest that these bacteria could serve as sustainable alternatives to chemical fertilizers.

Future research should focus on field trials to evaluate the impact of these bacterial isolates on crop yields and seed germination. Additionally, exploring the molecular mechanisms underlying their nitrogen-fixing and growth-promoting activities could provide deeper insights into their application as biofertilizers. This study contributes to the growing body of knowledge on the use of beneficial microbes in sustainable agriculture, particularly in unique ecosystems like the Sundarbans.

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Competing Interest

The authors report that there are no competing interests to declare.

Credit Author Statement

Fatiha Islam Roshnee, Arifa Afrose Rimi, Kumar Shubhro, Nazmul Rahman Chowdhury, Shaikh Mufrad Rahaman Anim: Methodology, Investigation, Data Analysis, Writing- the first draft; Md. Morsaline Billah: Supervision, Conceptualization; Kazi Mohammed Didarul Islam, Md. Emdadul Islam: Methodology, Physical and Chemical Analyses; S. M. Mahbubur Rahman: Data Analysis; Review, Anti Islam: Writing – final draft, Data Analysis, review & editing.

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