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Assessment of the Plant Growth-Promoting Potential and Biochemical Traits of Foliar Endophytic Bacteria from *Hibiscus rosa-sinensis*

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ABSTRACT

Endophytic microorganisms constitute an integral component of the plant microbiome, colonizing internal tissues asymptomatically and significantly influencing host physiology, growth and stress resilience. The present investigation was designed to isolate, characterize, and taxonomically identify endophytic bacterial isolates inhabiting the foliar tissues of leaves of *Hibiscus rosa-sinensis*. The obtained isolates from leaf were systematically characterized based on phenotypic and molecular attributes. The isolates were evaluated for their plant growth–promoting potential through the assessment of multiple PGPR-associated traits and were concurrently screened for the production of extracellular hydrolytic enzymes. Among the obtained endophytic isolates, four prominent bacterial isolates HE1, HE2, HE3 and HE4 exhibited distinct plant growth–promoting activities. Enzymatic profiling revealed that four isolates expressed substantial hydrolytic capabilities, particularly amylolytic activity, and indicative of their proficiency in the degradation of complex polysaccharide substrates. Positive catalase and oxidase reactions observed among the isolates reflect their metabolic adaptability and capacity to withstand oxidative stress conditions within the plant microenvironment. All the four distinct isolates were subjected for detailed molecular identification via 16S rRNA gene sequencing and identified as *Bacillus toyonensis*, *Pantoea dispersa*, *Cytobacillus firmus*, and *Neobacillus drentensis* respectively. These all four identified strains of endophytes are reported first time together from *Hibiscus rosa-sinensis*. Collectively, the findings elucidate the structural diversity and functional competence of endophytic bacterial populations associated with *Hibiscus rosa-sinensis*, emphasizing their prospective roles in nutrient turnover, modulation of host metabolic processes, enhancement of plant growth, and augmentation of tolerance to biotic and abiotic stresses.

1. Introduction

Endophytic microorganisms are a diverse group of bacteria and fungi that reside asymptomatically within the internal tissues of plants. These endophytes establish mutualistic associations with their host plants and contribute significantly to plant growth, yield enhancement, and protection against phytopathogens. Endophytic bacteria and fungi benefit their hosts by producing a wide range of bioactive secondary metabolites that have potential applications in medicine, agriculture, and industry (Ryan et al., 2008). In addition to growth promotion, endophytes have been reported to play an important role in environmental sustainability by enhancing phytoremediation processes and contributing to soil fertility through mechanisms such as phosphate solubilization and biological nitrogen fixation. Owing to these functional attributes, there is increasing interest in exploring endophytic microorganisms for biotechnological applications, including sustainable agriculture and biomass production (Panigrahi et al., 2018).

Hibiscus rosa-sinensis, an important ornamental and medicinal plant of the family Malvaceae, is widely cultivated in tropical and subtropical regions and is known for its antioxidant, antimicrobial, and anti-inflammatory properties. However, limited information is available on the diversity and plant growth–promoting potential of its associated endophytic microorganisms (Jain et al., 2017; Panigrahi et al., 2018).

Plant growth–promoting (PGP) endophytes enhance host plant performance through both direct and indirect mechanisms. These mechanisms include nitrogen fixation, phosphate solubilization, siderophore production, ammonia production, synthesis of phytohormones such as indole-3-acetic acid (IAA), and modulation of plant defence responses. Additionally, endophytic microorganisms help plants tolerate biotic and abiotic stresses by producing antioxidant enzymes, antimicrobial compounds, and stress-responsive signalling molecules, thereby improving overall plant fitness (Manasa et al., 2017).

The present study focused to explore and identify the endophytic microorganisms associated with *Hibiscus rosa-sinensis* and to evaluate their plant growth–promoting traits, with a view toward their potential application in sustainable agriculture.

2. Materials and Methods

Sample Collection & Isolation of Endophytic Bacteria

Healthy leaves of *Hibiscus rosa-sinensis* were collected from the botanical garden of Shri R. L. T. College of Science, Akola (MS). Surface-sterilized plant leaves were aseptically macerated using a sterile mortar and pestle with sterile distilled water to obtain a homogenized suspension. The resulting extract was serially diluted (10^{-1} to 10^{-5}), and 0.1 mL aliquots from each dilution were inoculated on nutrient agar and incubated at 28 ± 2°C for 24-48 h. After incubation, morphologically distinct bacterial colonies were selected and repeatedly streaked on fresh nutrient agar plates to obtain pure cultures (Panigrahi et al., 2018).

Phenotypic and Biochemical Characterization of Endophytic Bacterial Isolates

The isolated endophytic bacterial cultures were phenotypically characterized based on their growth and cultural characteristics on nutrient agar, including colony morphology and Gram reaction, following standard microbiological procedures. Biochemical and physiological characterization was performed using conventional assays, including indole, methyl red, Voges–Proskauer, citrate utilization, carbohydrate fermentation, and hydrogen sulfide production on triple sugar iron (TSI) agar. (Jain et al., 2017; Manasa et al., 2017).

Enzyme Test

The bacterial isolates were screened for amylase, catalase, oxidase, and urease activities using standard methods. Clear zones on starch agar after iodine flooding, bubble formation with hydrogen peroxide, rapid colour change on oxidase discs, and a pink colour on Christensen's urea agar indicated positive enzyme reactions. (Panigrahi et al., 2018 & Manasa et al., 2017).

Plant Growth–Promoting Activity Test

All selected endophytic bacterial isolates were qualitatively screened for plant growth–promoting traits, including indole-3-acetic acid (IAA) production, hydrogen cyanide (HCN) production, ammonia production, nitrogen fixation and phosphate solubilization, following standard protocols. IAA production was assessed by culturing isolates in 1% peptone water supplemented with 1% tryptophan at 28 ± 2 °C for 24 h, followed by reaction of the culture supernatant with orthophosphoric acid and Salkowski's reagent, where development of a pink coloration and absorbance at 540 nm indicated IAA synthesis. HCN production was evaluated on glycine-amended nutrient agar using picric acid–impregnated filter paper, with orange to red colour development indicating positive results after incubation. Phosphate solubilization was determined on Pikovskaya's agar by the formation of a clear halo zone around colonies, and the solubilization index was calculated based on the ratio of the total halo zone diameter and colony diameter to colony diameter. All bacterial endophytes were also screened for atmospheric nitrogen fixation by using Jensen's medium and Ammonia production by Nessler's reagent.

Molecular identification via 16S rRNA Sequencing of endophytic isolates

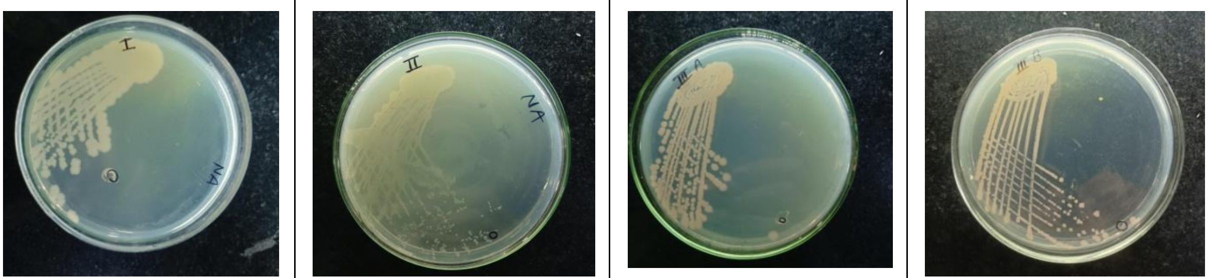
To confirm the taxonomic identity of the isolated endophytes i.e., HE1, HE2, HE3 and HE4, molecular characterization was conducted using 16S rRNA gene sequencing by Sanger's method. Genomic DNA was extracted from a pure culture of the isolates. The 16S rRNA gene was amplified using universal primers (e.g., 27F and 1492R) via Polymerase Chain Reaction (PCR). The resulting amplicons were purified and sequenced. The sequence data were then analysed using the Basic Local Alignment Search Tool (BLAST) through the NCBI database to determine phylogenetic relatedness, identifying isolates HE1, HE2, HE3 and HE4.

3. Result and Discussion

3.1 Isolation and Characterization of Endophytic Bacteria

Total 20 endophytic bacteria were isolated from leaves of *Hibiscus rosa-sinensis* by culturable method on nutrient agar medium. Out of these 20 isolates 4 were selected for further study named as HE1, HE2, HE3, and HE4 respectively. Four bacterial isolates (HE1 to HE4) from *Hibiscus rosa-sinensis* showed distinct colony and microscopic features as shown in Table 1. Among those, HE1 and HE3 were Gram-positive, rod-shaped isolates with opaque colonies, while HE2 was Gram-negative short rods with small, pale, watery colonies, and HE4 formed flat, opaque colonies with endospore-forming Gram-positive rods (Refer Fig. 1).

Fig. 1: Colony morphology on nutrient agar and Gram-stained microscopic appearance of endophytic bacterial isolate



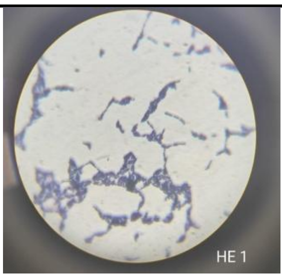
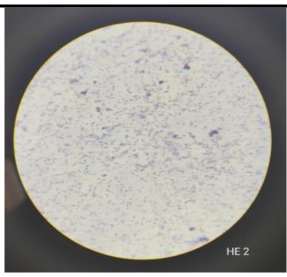
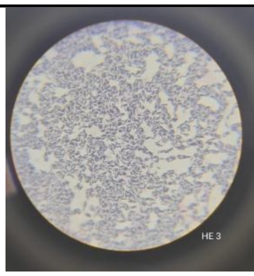
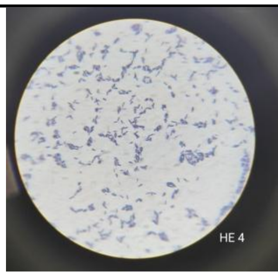
			
Isolate HE 1	Isolate HE 2	Isolate HE 3	Isolate HE 4

Table 1. Gram Staining and Morphological Characterization on Nutrient Agar Plate

Parameters	**HE1**	**HE2**	**HE3**	**HE4**
Colony Size	4 mm	1 mm	2 mm	2 mm
Shape	Circular	Circular	Circular	Circular
Colour	White	Pale White	Orange	Orange-Pink
Margin	Irregular	Even	Even	Irregular
Elevation	Flat	Flat	Slightly Elevated	Flat
Opacity	Opaque	Translucent	Opaque	Opaque
Gram reaction	Gram-positive rods	Gram-negative rods	Gram-positive rods	Gram-positive rods
Motility	Motile	Motile	Non-motile	Non-motile
Endospore	Present	Absent	Present	Present

3.2 Biochemical Characteristics of selected isolates

Biochemical analysis of the bacterial isolates obtained from *Hibiscus* spp. showed variation in carbohydrate fermentation and enzymatic activities as mentioned in Table 2. Most isolates utilized glucose, while lactose, mannitol, sucrose, ribose, xylose, and maltose utilization differed among the isolates, with variable acid and gas production. The MR and VP tests showed differential fermentation pathways among the isolates. Citrate utilization was positive in some isolates, indicating their ability to use citrate as a sole carbon source. TSI test reactions supported the carbohydrate fermentation patterns observed with H_2S gas production. The majority of isolates were catalase positive, whereas oxidase activity varied among the isolates.

Table 2. Biochemical Test Results for endophytes bacterial isolates

Isolate No.	Glucose		Lactose		Mannitol		Sucrose		Ribose		Xylose		Maltose		Indole	MR	VP	Citrate	TSI
	A	G	A	G	A	G	A	G	A	G	A	G	A	G					
HE1	+	-	-	-	-	-	+	-	+	-	+	-	+	-	-	+	+	-	+
HE2	+	-	-	-	+	-	+	-	+	-	+	-	-	-	-	-	+	+	+
HE3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
HE4	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	+	-

Where, A for Acid and G for Gas

3.3 Hydrolysing Enzyme Activities

All the endophytic bacterial isolates were subjected to evaluate the production of hydrolytic enzymes like catalase, amylase, and oxidase. All the 4 bacterial isolates were positive for catalase as well as oxidase activity. Among the 4 bacterial isolates, 3 isolates namely (HE1, HE3 and HE4) positive for production of amylase enzyme as shown in Fig. 2.

Fig. 2: Amylase activity of entophytic bacterial isolates showing a clear zone on starch agar

3.4 Determination of plant growth promoting traits

All four endophytic isolates (HE 1, HE 2, HE 3 and HE 4) were evaluated for plant growth–promoting traits, including indole acetic acid (IAA) production, phosphate solubilization, hydrogen cyanide (HCN) production, ammonia production and nitrogen fixation ability. Qualitative screening showed that all isolates were positive for IAA, HCN production (Fig. 3), ammonia production and nitrogen fixation indicating their potential role in plant growth promotion and biocontrol activity, while none of the isolates exhibited phosphate solubilization on pikovskaya's agar. All the isolates showed positive results for ammonia production with slight change in colour of precipitation formed as shown in Fig. 4. Ammonia production helps plants by supplying nitrogen needed for growth and metabolism.

Fig. 3 HCN Production by bacterial endophytic isolates

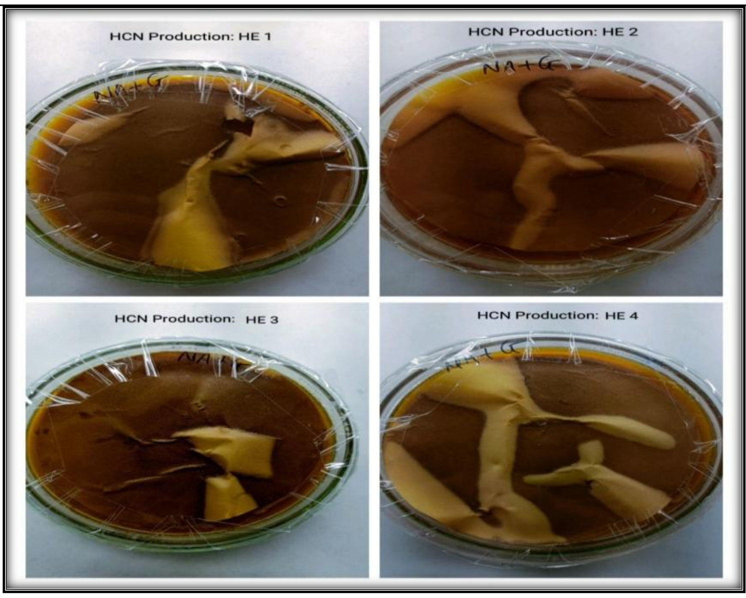


Fig. 4 Ammonia production by bacterial endophytes

3.5 Endophytic isolates sequencing data

After sequencing and BLAST analysis using NCBI database, the isolated strains HE1, HE2, HE3 and HE4 were found to be as *Bacillus toyonensis*, *Pantoea dispersa*, *Cytobacillus firmus*, and *Neobacillus drentensis* respectively as mentioned in Table 3.

Table 3. Endophytic bacterial isolates sequencing data

Sr. No.	Isolate Code	Scientific Name	Percentage identity	NCBI Accession Number
1.	HE1	*Bacillus toyonensis*	97.16%	PX909695
2.	HE2	*Pantoea dispersa*	98.88%	PX909698
3.	HE3	*Cytobacillus firmus*	99.70%	PX909701
4.	HE4	*Neobacillus drentensis* (formerly *Bacillus drentensis*)	99.90%	PX910700

Fig. 4 Genomic DNA of samples compared to 1kb ladder

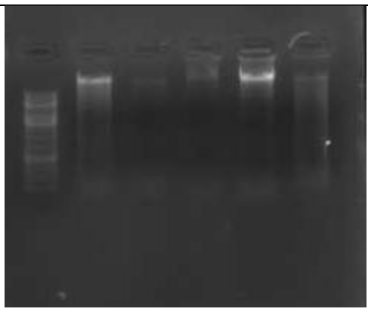


Fig. 5 16s gene of 1400bp, compared to 1kb ladder

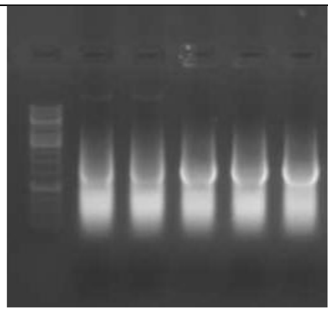


Fig. 6 Phylogenetic tree for *Bacillus toyonensis*

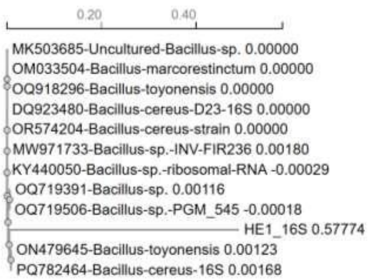


Fig. 7 Phylogenetic tree for *Pantoea dispersa*

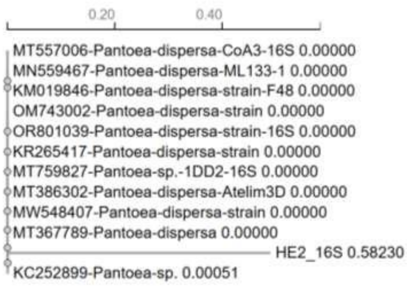
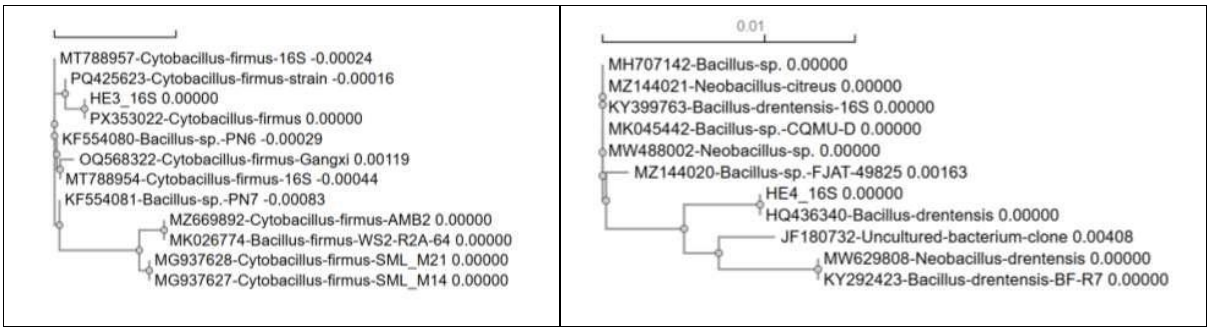


Fig. 8 Phylogenetic tree for *Cytobacillus firmus*

Fig. 9 Phylogenetic tree for *Neobacillus drentensis (formerly Bacillus drentensis)*



4. Discussion

The present study demonstrated that *Hibiscus rosa-sinensis* harbours diverse endophytic bacteria with multiple plant growth–promoting traits. All the four distinct isolates were subjected for detailed molecular identification via 16S rRNA gene sequencing and identified as *Bacillus toyonensis*, *Pantoea dispersa*, *Cytobacillus firmus*, and *Neobacillus drentensis* respectively. These all four identified strains of endophytes are reported first time together from *Hibiscus rosa-sinensis*.

Endophytes have shown to promote plant growth by producing phytohormone like IAA, which increase the root size and nutrient absorption. The production of IAA by all isolates suggests their potential role in enhancing root development and plant growth. Additionally, the positive nitrogen fixation ability exhibited by the isolate indicates their role in improving nitrogen availability to the host plant. The positive ammonia production by the isolates supports their contribution to plant nutrition and growth. HCN production reflects their possible role in biocontrol against phytopathogens.

The hydrolytic enzyme secreted by the bacteria play an important role in suppression of pathogens which aids to the defence mechanism of the host plant (Buchenauer, 1998). In the present study maximum number of isolates were found positive for catalase, oxidase and amylase activities indicating their capabilities to protect the host from pathogen attack. These findings are in agreement with earlier reports on endophytic bacteria from medicinal and ornamental plants, highlighting their ecological and agricultural significance.

5. Conclusion

The study concludes that endophytic bacteria isolated from *Hibiscus rosa-sinensis* possess significant plant growth–

promoting attributes. Nitrogen fixation by the isolates indicates their role in enhancing nitrogen availability to the host plant, thereby improving plant nutrition and growth. The combined abilities of IAA production, ammonia production, nitrogen fixation and HCN production suggest their potential application as bioinoculants in sustainable agriculture. Molecular identification and field-level evaluation will contribute to our existing knowledge regarding plant and microbiome interaction.

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