

Isolation, 16S rRNA-based Taxonomic Profiling and Potential Roles of Endophytic Bacteria Colonizing *Dalbergia reniformis* (Koroch)

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Abstract

Endophytic bacteria represent a functionally diverse group of plant-associated microorganisms with significant potential as bioinoculants for sustainable agriculture. This study investigated the diversity and plant growth-promoting (PGP) attributes of endophytic bacteria colonising root, stem, and leaf tissues of *Dalbergia reniformis* from Ratargul Swamp Forest, Bangladesh's only freshwater swamp forest. Of 34 bacterial colonies isolated, 18 morphologically distinct isolates were characterised through 16S rRNA gene sequencing, revealing ten genera: *Bacillus*, *Fictibacillus*, *Priestia*, *Pseudomonas*, *Enterobacter*, *Stenotrophomonas*, *Serratia*, *Pantoea*, *Klebsiella*, and *Leclercia*, with *Pantoea* being the most frequently recovered genus. Screening across 13 PGP traits demonstrated that all isolates produced ammonia, siderophores, and gibberellic acid. Zinc and phosphate solubilization were recorded in 94.4% and 66.7% of isolates, with solubilization indices reaching 5.80 and 3.66, respectively. Organic acid production and phytase activity were detected in 88.89% of isolates, lipolytic activity in 83.33%, proteolytic activity in 55.56%, HCN production in 50%, IAA production in 38.89%, cellulolytic activity in 44.44%, and oxalate oxidase activity in 27.78% of test isolates. These findings highlight the functional richness of the endophytic community in this ecologically unique habitat, with promising implications for biofertilizer and biocontrol applications.

1. Introduction

Plant growth-promoting (PGP) microbes are beneficial microorganisms, including bacteria, fungi, and actinomycetes, that colonize various plant compartments. They enhance plant growth through nutrient acquisition, phytohormone production, stress alleviation, and biocontrol of phytopathogens [1]. These microbes play a crucial role in sustainable agriculture by improving plant health, yield, and resilience, particularly in nutrient-poor or contaminated soils [2–6]. Some reported PGP-associated genera include *Bacillus subtilis*, *Pseudomonas fluorescens*, *Azospirillum brasilense*, and *Rhizobium leguminosarum*, alongside species from genera such as *Azotobacter*, *Mycobacterium*, *Serratia*, *Xanthomonas*, *Proteus*, and *Clostridium*. PGP fungi encompass *Trichoderma harzianum*, *Glomus intraradices*, and *Piriformospora indica*, while actinomycetes like *Streptomyces lydicus*, *Actinoplanes missouriensis*, and *Micromonospora* spp. also contribute significantly.

Endophytes are a subset of PGP microbes that reside within plant tissues without causing apparent harm, forming symbiotic relationships that have coevolved over millions of years [7]. These microorganisms, primarily bacteria and fungi, produce bioactive compounds with applications in medicine, agriculture, industry, and environmental management [8–10]. Endophytes enhance plant resistance to stressors like drought, salinity, and pests by modulating phytohormones, strengthening root systems, and activating defense mechanisms [11]. They also facilitate nutrient cycling, including nitrogen fixation and phosphorus solubilization, reducing reliance on synthetic fertilizers [12]. Challenges include culturing difficulties and understanding interactions within microbial communities, yet their potential, particularly in bioremediation of pollutants such as heavy metals and organics, remains promising [13–15].

Dalbergia reniformis (Roxb.) Prain (Fabaceae), commonly known as Korocho in Bangladesh, is a small to medium-sized deciduous tree native to Bangladesh, India, and Myanmar [16]. It is characterized by compound imparipinnate leaves, axillary panicle flowers, and reniform pods, and exhibits remarkable tolerance of waterlogging, remaining submerged for up to six months during the monsoon season [17]. In Bangladesh, it is found primarily in the haor wetlands of the Sylhet Division and is the dominant tree species of the Ratargul Swamp Forest, the country's only freshwater swamp forest, spanning 3,325 acres along the Gowain River, Sylhet [18]. This ecosystem supports high plant and animal biodiversity but faces increasing degradation from anthropogenic pressures [19]. Despite the ecological significance of *D. reniformis* and its dominance in this unique habitat, its endophytic microbiome remains entirely unexplored, representing a clear gap in the literature.

This study combines assessments of 18 bacterial isolates from *D. reniformis* tissues, evaluating PGP traits (production of indole acetic acid, gibberellic acid, ammonia, siderophore, HCN, organic acids, phytase, oxalate oxidase, lipase, cellulase, protease, along with solubilization of phosphate and zinc) and taxonomic affiliations via 16S rRNA gene sequencing, to explore their potential as biofertilizers and biocontrol agents for sustainable agriculture.

2. Results

2.1 Isolation and morphological characterization

Surface sterilization of root, stem, and leaf tissues of *Dalbergia reniformis* yielded 34 bacterial colonies, from which 18 morphologically distinct isolates (EB_01-EB_18) were selected for further study (Fig. 1). The absence of growth on plates inoculated with the final rinse water confirmed the efficacy of the sterilization procedure. The isolates exhibited considerable variation in colony morphology, including differences in color, texture, margin, and elevation (Table S1), with six isolates recovered from each of the three tissue types.

2.2 Biochemical characterization

Biochemical profiling revealed diverse phenotypic profiles across all 18 isolates (Table S2). Gram-positive strains, several of which were endospore-forming, were predominant among root-derived isolates and were consistent with affiliation to the Firmicutes. The remaining isolates were Gram-negative and non-spore-forming. Variation was observed in catalase and oxidase activities, carbohydrate fermentation patterns, citrate utilization, gelatin hydrolysis, amylase production, motility, and H₂S production. The overall biochemical profiles were broadly consistent with the taxonomic assignments derived from 16S rRNA gene sequencing (Fig. 2).

2.3 Molecular identification and phylogenetic analysis

PCR amplification of genomic DNA using universal primers 27F and 1492R produced amplicons of approximately 1465 bp for all 18 isolates, confirmed by agarose gel electrophoresis. Subsequent Sanger sequencing yielded readable sequences ranging from 819 to 1409 bp across isolates. BLASTn analysis

resolved the isolates into ten genera, with *Pantoea* being the most frequently recovered, represented by five isolates showing sequence similarity to four recognized species (Table S3). The neighbor-joining phylogenetic tree constructed in MEGA v11 with 1000 bootstrap replicates, using *Mycobacterium kansasii* (ATCC 12478) as the outgroup, resolved the isolates into two principal lineages corresponding to Firmicutes and Gammaproteobacteria, consistent with the BLASTn-based assignments (Fig. 2).

2.4. Plant growth-promoting (PGP) attributes

2.4.1. Qualitative PGP traits

All 18 isolates produced ammonia and siderophores (100%), confirming universal nitrogen mobilization and iron-chelation capacity across the community (Figs. 3a, 3b). Both organic acid production and phytase activity were detected in 16 isolates (88.89%). Lipolytic activity was observed in 15 isolates (83.33%; Fig. 4b), proteolytic activity in ten (55.56%; Fig. 4f), and HCN production in nine isolates (50%), of which four were classified as strong producers (+++) and two as moderate producers (++) (Fig. 3c). IAA production was recorded in seven isolates (38.89%; Fig. 4a), cellulolytic activity in eight (44.44%; Fig. 4e), and oxalate oxidase activity in five isolates (27.78%; Fig. 3f). Complete qualitative trait data for each isolate are presented in Table 1.

Table 1

Overview of the plant growth-promoting attributes of the 18 endophytic bacterial isolates from *Dalbergia reniformis*. (Qualitative traits are shown as presented as mean $\pm$ SD (n = 3). Different letters within the same column indicate significant differences according to one-way ANOVA followed by Fisher's LSD post hoc test (p < 0.05).

Origin	Isolates	Plant Growth Promoting Activity Tests									
		Ammonia production	Siderophore production	Oxalate oxidase production	Lipase production	Phytase production	Organic acids production	HCN production	Indole acetic acid (IAA) production	Gibberellin production ($\mu\text{g/mL}$)	Phosphate solubility index
ROOT	EB_01	Positive	Positive	Negative	+++	+++	+++	+++	+	59.65 $\pm$ 1.42 ^{de}	3.20 $\pm$ 0.18
	EB_02	Positive	Positive	Negative	-	+	++	-	-	46.40 $\pm$ 2.68 ^g	3.07 $\pm$ 0.30
	EB_03	Positive	Positive	Positive	+	++	+	+++	-	65.00 $\pm$ 0.68 ^{bc}	-
	EB_04	Positive	Positive	Positive	-	+	+	-	-	47.89 $\pm$ 2.52 ^g	1.84 $\pm$ 0.04
	EB_05	Positive	Positive	Negative	++	+++	++	+	-	64.78 $\pm$ 2.48 ^{bc}	-
	EB_06	Positive	Positive	Negative	++	-	-	-	+++	54.10 $\pm$ 1.08 ^{ef}	2.76 $\pm$ 0.11
STEM	EB_07	Positive	Positive	Positive	++	+	+	-	-	54.74 $\pm$ 5.12 ^{ef}	-
	EB_08	Positive	Positive	Negative	++	-	+++	-	+	54.52 $\pm$ 3.52 ^{ef}	2.67 $\pm$ 0.24
	EB_09	Positive	Positive	Negative	+++	+++	+	++	-	75.47 $\pm$ 3.72 ^a	2.57 $\pm$ 0.39
	EB_10	Positive	Positive	Negative	-	+++	-	-	++	53.24 $\pm$ 3.22 ^f	2.48 $\pm$ 0.05
	EB_11	Positive	Positive	Negative	++	++	++	+	-	68.83 $\pm$ 4.18 ^b	3.30 $\pm$ 0.29
	EB_12	Positive	Positive	Negative	+++	+++	++	++	+++	61.58 $\pm$ 3.98 ^{cd}	3.66 $\pm$ 0.15
LEAF	EB_13	Positive	Positive	Negative	+++	+++	+++	+	-	77.40 $\pm$ 1.92 ^a	2.17 $\pm$ 0.05
	EB_14	Positive	Positive	Negative	+++	+++	++	+++	+++	65.43 $\pm$ 3.68 ^{bc}	1.48 $\pm$ 0.04
	EB_15	Positive	Positive	Positive	++	+	+++	-	-	53.67 $\pm$ 0.58 ^f	-
	EB_16	Positive	Positive	Negative	+++	+++	+	+++	+++	60.51 $\pm$ 5.48 ^d	2.31 $\pm$ 0.05
	EB_17	Positive	Positive	Positive	-	++	+	-	-	52.17 $\pm$ 2.42 ^f	-
	EB_18	Positive	Positive	Negative	++	++	+	-	-	64.78 $\pm$ 5.12 ^{bc}	-

2.4.2 Quantitative PGP traits

One-way ANOVA followed by Fisher's LSD post hoc test (p < 0.05) revealed significant differences among isolates for all five quantitatively assessed traits: phosphate solubilization index (SI), zinc SI, cellulolytic index, proteolytic index, and gibberellic acid (GA) concentration. LSD groupings are presented in Table 1 and Fig. 5.

Phosphate solubilization was recorded in 12 isolates (66.67%), with SIs ranging from 1.48 $\pm$ 0.04 (EB_14) to 3.66 $\pm$ 0.15 (EB_12) (Fig. 4c, Fig. 5d). Isolates EB_01 (3.20 $\pm$ 0.18), EB_11 (3.30 $\pm$ 0.29), and EB_12 (3.66 $\pm$ 0.15) formed the highest-performing LSD group (group a), while EB_02 (3.07 $\pm$ 0.30), EB_08 (2.67 $\pm$ 0.24), EB_06 (2.76 $\pm$ 0.11), and EB_09 (2.57 $\pm$ 0.39) formed LSD group b. Zinc solubilization was detected in 17 isolates (94.44%), with SIs ranging from 1.28 $\pm$ 0.05 (EB_18) to 5.80 $\pm$ 0.31 (EB_12) (Fig. 4d, Fig. 5c). EB_12 was the sole member of LSD group a and was significantly higher than all other isolates. EB_18 (1.28 $\pm$ 0.05) and EB_05 occupied the lowest LSD group (group h).

Cellulolytic activity was detected in eight isolates, with indices ranging from 5.33 ± 0.58 (EB_07) to 9.47 ± 0.76 (EB_15) (Fig. 4e, Fig. 5a). EB_15 formed the highest LSD group (group a), followed by EB_17 (8.25 ± 0.41 , group b), EB_02 (7.37 ± 0.59 , group b), EB_11 (6.90 ± 1.47 , group c), EB_04 (7.06 ± 0.99 , group c), and EB_18 (6.98 ± 0.56 , group c), with EB_06 (6.12 ± 0.28) and EB_07 (5.33 ± 0.58) in the lowest groups. Proteolytic activity was recorded in ten isolates, with indices ranging from 1.12 ± 0.08 (EB_15) to 3.68 ± 0.73 (EB_04) (Fig. 4f, Fig. 5b). EB_04 formed the highest LSD group (group a), followed by EB_08 (3.11 ± 0.17 , group b) and EB_01 (2.21 ± 0.11 , group c), with EB_15 and EB_03 (1.41 ± 0.03) occupying the lowest groups.

All 18 isolates produced gibberellic acid (GA), with concentrations ranging from 46.40 ± 2.68 $\mu\text{g/mL}$ (EB_02) to 77.40 ± 1.92 $\mu\text{g/mL}$ (EB_13) (Fig. 5e, Table 1). EB_13 (77.40 ± 1.92) and EB_09 (75.47 ± 3.72) formed the highest LSD group (group a), followed by EB_11 (68.83 ± 4.18 , group b). Mid-range producers included EB_03 (65.00 ± 0.68), EB_05 (64.78 ± 2.48), EB_18 (64.78 ± 5.12 , group bc), and EB_14 (65.43 ± 3.68 , group bc). The lowest GA concentrations were recorded for EB_02 (46.40 ± 2.68) and EB_04 (47.89 ± 2.52), both in LSD group g.

3. Discussion

The recovery of endophytic bacteria spanning ten genera from root, stem, and leaf tissues of *D. reniformis* is consistent with the broad phylogenetic diversity reported for endophytic communities of tropical legumes [12, 20]. The co-occurrence of Firmicutes and Gammaproteobacteria representatives has similarly been observed in other leguminous hosts grown under environmentally variable conditions [14]. *Pantoea* was the most frequently recovered genus, with five isolates showing sequence similarity to four recognized species, supporting the reports of its prevalence as an endophyte of tropical plants [21]. Species-level identification based solely on 16S rRNA gene sequencing carries inherent limitations due to the low interspecies resolution of this marker within certain genera, and further genomic characterization could provide more precise assignments.

All 18 isolates produced ammonia, siderophores, and gibberellic acid, indicating that nitrogen mobilization, iron acquisition, and phytohormone production are widespread functional traits within this endophytic community. Siderophore-producing bacteria have been shown to limit iron availability to competing phytopathogens, contributing to biocontrol in addition to plant nutrition [22]. Phosphate and zinc solubilization were recorded in 66.67% and 94.44% of isolates, respectively, with solubilization indices within the range reported for endophytes of other leguminous hosts [23]. In waterlogged soils, nutrient availability is closely linked to redox dynamics, and the high frequency of solubilization traits observed here may reflect an adaptation to the periodically anaerobic conditions of Ratargul Swamp Forest [12]. Phytase activity, detected in 88.89% of isolates, contributes to the mineralization of organic phosphorus in soil, supplementing inorganic phosphate solubilization [24]. HCN production in 50% of isolates and IAA production in 38.89% further broaden the functional profile of this community, with established roles in pathogen suppression and root development [25].

Among the isolates, EB_01, EB_11, EB_12, and EB_16 demonstrated strong performance across multiple PGP traits simultaneously. EB_12 recorded the highest solubilization indices for both phosphate and zinc, combined with IAA, HCN, phytase, and organic acid production. EB_01 showed comparably higher phosphate and zinc solubilization alongside strong HCN, organic acid, and phytase activities. EB_11 is the second-highest gibberellic acid producer alongside high phosphate SI, while EB_16 expressed the highest count of active PGP traits (12 out of 13) with strong zinc solubilization, IAA, HCN, lipase, and phytase activities. These isolates could be potential bioinoculant candidates, as their combined functional capacity may provide more consistent plant growth benefits across varying environmental conditions [23, 25]. The broad distribution of PGP capacity across diverse taxa and tissue types, without apparent tissue-specific or phylogenetic clustering, suggests functional redundancy as a characteristic of this endophytic community, likely shaped by the fluctuating abiotic conditions of the swamp habitat.

While this study provides a solid foundation for the PGP potential of *D. reniformis* endophytes in Ratargul Swamp forest, several limitations should be acknowledged. The work was based on culturable isolates, which represent only a fraction of the total endophytic community. Future studies employing culture-independent metagenomic approaches would provide a more comprehensive view of microbial diversity [26]. Moreover, the promising isolates identified here require further validation through greenhouse and field trials to confirm their efficacy in promoting plant growth, nutrient uptake, and stress tolerance under varying natural conditions.

4. Materials and Methods

4.1 Plant sampling and bacterial endophyte isolation

Plant samples were collected from the Ratargul Swamp Forest, geographically located at ($25^{\circ}00.025'\text{N}$ and $91^{\circ}58.180'\text{E}$) on the southeast bank of the Gowain River, Sylhet, Bangladesh. Healthy roots, stems, and leaves of mature *Dalbergia reniformis* (Koroch) were collected in sterile zip bags and stored at 4°C . Samples were taken from five different plots, mixed, and the isolation procedure was carried out within 24 hours. The collected plant samples were washed with distilled water, air-dried, and surface sterilized by sequential treatment with 70% ethanol (3–5 min), 2% NaOCl (3 min), and 0.1% HgCl_2 (30 s), followed by 70% ethanol (30 s) and triple rinsing with sterile distilled water. The final rinse water was used as a control to verify sterilization [27].

For isolation, Luria-Broth (LB) agar media supplemented with nystatin (30 $\mu\text{g/mL}$) was used [28]. Surface-sterilized roots, stems, and leaves were cut into small pieces (1–3 mm), placed onto the agar plates, and sealed with parafilm, followed by incubation at 37°C for 1–7 days to allow the growth of endophytic bacteria, and were checked every 24 hours for counting [29]. Morphologically distinct colonies from the margins of the inoculated sample parts were repeatedly streaked on fresh NA to obtain pure cultures, which were preserved at 4°C on NA slants [30].

The plant was formally identified as *Dalbergia reniformis* (Roxb.) Prain by Rabeya Sultana, Assistant Professor, Department of Forestry and Environmental Science, Shahjalal University of Science and Technology, Sylhet, based on standard taxonomic keys and relevant literature. A voucher herbarium specimen has been deposited in the Herbarium of the Dendrology and Species Silviculture Laboratory, Department of Forestry and Environmental Science, Shahjalal University of Science and Technology, Sylhet, Bangladesh (Deposit Number: SUST/FES/2022/08), where it is available for examination by researchers upon

request. Collection of plant material was conducted in accordance with the institutional guidelines of the Department of Forestry and Environmental Science and the Department of Genetic Engineering and Biotechnology, SUST. Permission for field sampling was granted by the Forest Department of Bangladesh. All sampling procedures complied with relevant national and international guidelines. This study did not involve any endangered or protected species. The authors confirm compliance with the IUCN Policy Statement on Research Involving Species at Risk of Extinction and the Convention on the Trade in Endangered Species of Wild Fauna and Flora (CITES). *Dalbergia reniformis* is classified as Least Concern on the IUCN Red List [16].

4.2 Biochemical analysis

Once the pure cultures were obtained, several morphological and biochemical tests were conducted for each isolate, viz., Gram staining, Catalase, Oxidase, Endospore staining, Carbohydrate fermentation (dextrose, lactose, sucrose, and mannitol), Amylase, Gelatin hydrolysis, Simmons citrate, KOH, Motility, and H₂S production test to obtain a phenotypic profile that could be compared with the molecular identification. The tests were carried out according to the protocols described in *Bergey's Manual of Systematic Bacteriology* [31].

4.3 Molecular identification of endophytic bacterial isolates

Molecular identification was conducted based on the bacterial 16S rRNA gene sequence analysis. The genomic DNA of the bacterial isolates was extracted using the FAVORGEN FavorPrep™ Tissue Genomic DNA Purification Kits (Catalog No. FATGK 001, Lot No. CC927123324) according to the supplier's manual. The quality and quantity of the extracted bacterial genomic DNA were checked using a nanodrop spectrophotometer (Model: ND2000, Origin: Thermo Scientific, USA). PCR amplification of the genomic DNA was performed employing universal bacterial primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTACGACTT-3'), which amplify approximately 1465 bp of the 16S rRNA region. The PCR reactions were prepared using a 15 µL mixture containing 7.5 µL of GoTaq™ G2 Hot Start Master Mix (Promega, USA), 1.5 µL of genomic DNA, 1 µL of each primer, and 4 µL of nuclease-free water. Amplifications were performed in a thermocycler (Gene Atlas, Model: G2, Astec, Japan) under the following conditions: initial denaturation at 95°C for 2 min; 35 cycles of 95°C for 30 s, 50°C for 45 s, and 72°C for 90 s; followed by a final extension at 72°C for 10 min. The amplified products were then analyzed on 1.2% agarose gel electrophoresis to confirm successful amplification and were sequenced at Apical Scientific Sdn. Bhd., Selangor, Malaysia.

The obtained sequences were edited and assembled into consensus sequences using BioEdit version 7.2.5 software. The bacterial sequences in this study have been deposited in GenBank under accession numbers from PZ256760 to PZ256777. The sequences were compared against the NCBI database using the BLASTn tool to determine their closest phylogenetic affiliations. Multiple sequence alignment and phylogenetic tree construction were performed using the MEGA version 11.0.13 software, employing the neighbor-joining method with 1000 bootstrap replications to assess the robustness of the tree topology [32, 33].

4.4 Screening of Plant Growth-Promoting Attributes

4.4.1 Ammonia production

To assess ammonia-producing ability, bacterial strains were inoculated into 10 mL peptone water and incubated at 28 ± 2°C for 10 days. Upon addition of approximately 1 mL of Nessler's reagent to the culture broth, the development of a yellow to brown color indicated ammonia production [34].

4.4.2 Siderophore production

Siderophore production was qualitatively detected by using King's B agar medium supplemented with Chrome Azurol S (CAS), Fe³⁺ solution, and hexadecyltrimethylammonium bromide (HDTMA) as an indicator. The CAS solution was prepared by mixing CAS (60.5 mg in 50 mL distilled H₂O) with 10 mL of iron (III) solution (1 mM FeCl₃·6H₂O, 10 mM HCl). The HDTMA solution was prepared by dissolving 72.9 mg of HDTMA in 40 mL of distilled H₂O. The prepared CAS dye solution was added to King's B medium with a ratio of 1:15. The fresh bacterial culture was spotted on a King's B agar plate and incubated at 28 ± 2°C for 72 h. The appearance of an orange halo around the endophytic bacterial growth indicated a positive result for siderophore production [34].

4.4.3 HCN production

The HCN-producing ability of the test isolates was evaluated on King's B agar medium supplemented with glycine (4 g L⁻¹), using a non-inoculated plate as a control [35]. A Whatman No.1 filter paper dipped in picric acid solution (0.5% picric acid in 2% sodium carbonate) was affixed to the Petri dish lid and sealed with parafilm, followed by incubation at 30°C for 7 days. HCN production was indicated by a color change of the filter paper from yellow to light brown (+), brown (++), or brick red (+++), while no color change was recorded as a negative (-) reaction [36].

4.4.4 Organic acid production

The bacterial isolates were inoculated in minimal salt medium (MN9 broth) for a week using methyl red as a pH indicator. Production of organic acids by the bacterial isolates causes the pH of the medium to drop. Upon the addition of methyl red, the appearance of a pink color confirmed organic acid production [37].

4.4.5 Phytase production

The ability of the bacterial isolates to solubilize phytate was determined by inoculation on a phytate-specific medium containing 1.5% glucose, 0.5% (NH₄)₂SO₄, 0.05% KCl, 0.01% MgSO₄·7H₂O, 0.01% NaCl, 0.2% CaCl₂·2H₂O, 0.5% sodium phytate, 1.2% agar (pH 6.5). Phytase-producing isolates were identified by the clear halo zone around them due to the hydrolysis of sodium phytate [38].

4.4.6 Oxalate oxidase production

The oxalate-oxidizing ability of the bacterial isolates was screened on basal mineral media containing (g L^{-1}) KH_2PO_4 – 3, Na_2HPO_4 – 6, NaCl – 5, NH_4Cl – 2, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ – 0.1, Agar – 15, and calcium oxalate – 5, pH 7 [39]. As calcium oxalate was used as the sole carbon source in the medium, bacterial growth with transparent areas around the bacterial colonies due to solubilization of calcium oxalate indicated oxalate-oxidizing ability [40].

4.4.7 Phosphate solubilization

Bacterial isolates were inoculated into NBRIP medium (pH 7.0) containing 1% glucose, 0.5% $\text{Ca}_3(\text{PO}_4)_2$, 0.01% $(\text{NH}_4)_2\text{SO}_4$, 0.02% KCl, 0.025% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.5% Agar, followed by incubation at 37°C for 5–7 days. Positive results were observed as clear zones around the bacterial colonies [41]. The solubilization index (SI) was calculated as (halo + colony diameter)/colony diameter [42].

Solubilization Index (SI) = (Halo diameter + Colony diameter) / Colony diameter

4.4.8 Zinc solubilization

Zinc solubilization ability of the bacterial isolates was assessed on Bunt and Rovira's agar medium (1% glucose, 0.1% $(\text{NH}_4)_2\text{SO}_4$, 0.02% KCl, 0.01% K_2HPO_4 , 0.02% MgSO_4 , 1.5% agar, and 0.1% ZnO or ZnCO_3 ; pH 7) [43] by incubation at 37°C for 10 days. Isolates forming clear halos were considered zinc-solubilizing [44].

4.4.9 Indole acetic acid (IAA) production

To detect IAA production, bacterial cultures in Luria-Bertani (LB) broth supplemented with tryptophan (0.5 g L^{-1}) were incubated at $28 \pm 2^\circ\text{C}$ for 4 days with shaking. After incubation, 1 mL of culture was mixed with 4 mL of Salkowski reagent and incubated in the dark for 20 minutes. The development of a pinkish-red color indicated indole-3-acetic acid (IAA) production [45].

4.4.10 Gibberellic acid (GA) production

Gibberellin production by the bacterial isolates was estimated using the 2,4-DNPH method [46]. Bacterial isolates were suspended in sterile nutrient broth for 10 days at 28°C. GA in the culture supernatant was collected using the ethyl acetate pooling method [47]. A 0.2 mL aliquot of the extract was mixed with 1 mL of DNPH, incubated at 100°C for 5 min, cooled, and treated with 5 mL of 10% KOH until a red wine color developed. The mixture was diluted with sterile distilled water (1:2), and absorbance was measured at 430 nm using a UV-VIS spectrophotometer. Standard GA solutions (0.8 mg mL^{-1}) were prepared in absolute alcohol and analyzed similarly to obtain a standard curve. The absorbance values for each isolate were compared to the standard curve to determine the amount of GA produced by each isolate.

4.4.11 Cellulase production

Extracellular cellulolytic activity was screened by spotting bacterial isolates onto CMC agar medium (0.2% $(\text{NH}_4)_2\text{SO}_4$, 0.1% K_2HPO_4 , and 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05% KCl, 0.001% FeSO_4 , 0.1% Carboxymethylcellulose (CMC), 1.4% agar, pH 7) described by [48] with slight modifications and incubating at 37°C for 48–72 hours. Cellulase production was confirmed by the formation of clear halo zones around colonies after flooding the plates with Gram's iodine solution (which stains non-hydrolyzed cellulose black-bluish).

4.4.12 Protease production

Proteolytic activity of the bacterial isolates was assessed on skim milk agar medium containing 0.5% casein, 0.25% yeast extract, 0.1% glucose, 2.8% skim milk powder, and 1.4% agar, while the media pH was adjusted to 7.4 [49]. After incubation at 37°C for 24–48 hrs., the positive isolates were identified by the formation of clear zones around the bacterial colonies due to the proteolysis of skim milk [50].

4.4.13 Lipase production

Lipolytic ability was screened on Tween agar media containing (g L^{-1}): peptone, 10; calcium chloride, 0.1; NaCl, 5; agar, 15; and 10 mL of sterile Tween 20 [51]. After inoculation, the cultures were incubated at 30°C for 2 days. Formation of opaque zones around colonies indicated lipase activity [52].

4.5 Statistical analysis

All quantitative assays were performed in triplicate and expressed as mean $\pm$ SD. Data were analyzed using one-way ANOVA, followed by Fisher's LSD test at $p < 0.05$ for multiple comparisons. Qualitative and semi-quantitative traits were presented as percentages or frequency distributions without inferential analysis. Statistical analyses were conducted using SPSS v26.

Declarations

Competing interests:

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author Contribution

R.S. conceptualized the study, designed the experiments, and drafted the initial manuscript. A.A.A. conducted the laboratory work, collected and analyzed the data, interpreted the results, and contributed to manuscript writing and editing. M.F.A. conducted the laboratory work, collected and analyzed the data, interpreted the results, and contributed to manuscript writing. M.M.R.S. and M.T.I. conducted the laboratory work, collected and analyzed the data, and interpreted the results. M.N.H., N.I., and S.H.P. interpreted the results and critically revised the manuscript. H.H. conceptualized the study, acquired funding, provided resources, supervised the research, and reviewed and finalized the manuscript. All authors have read and approved the final version of the manuscript.

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Data Availability

The nucleotide sequences generated in this study have been deposited in the NCBI GenBank database under accession numbers PZ256760–PZ256777. All other experimental data supporting the findings of this study are available from the corresponding author upon reasonable request.

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Figures

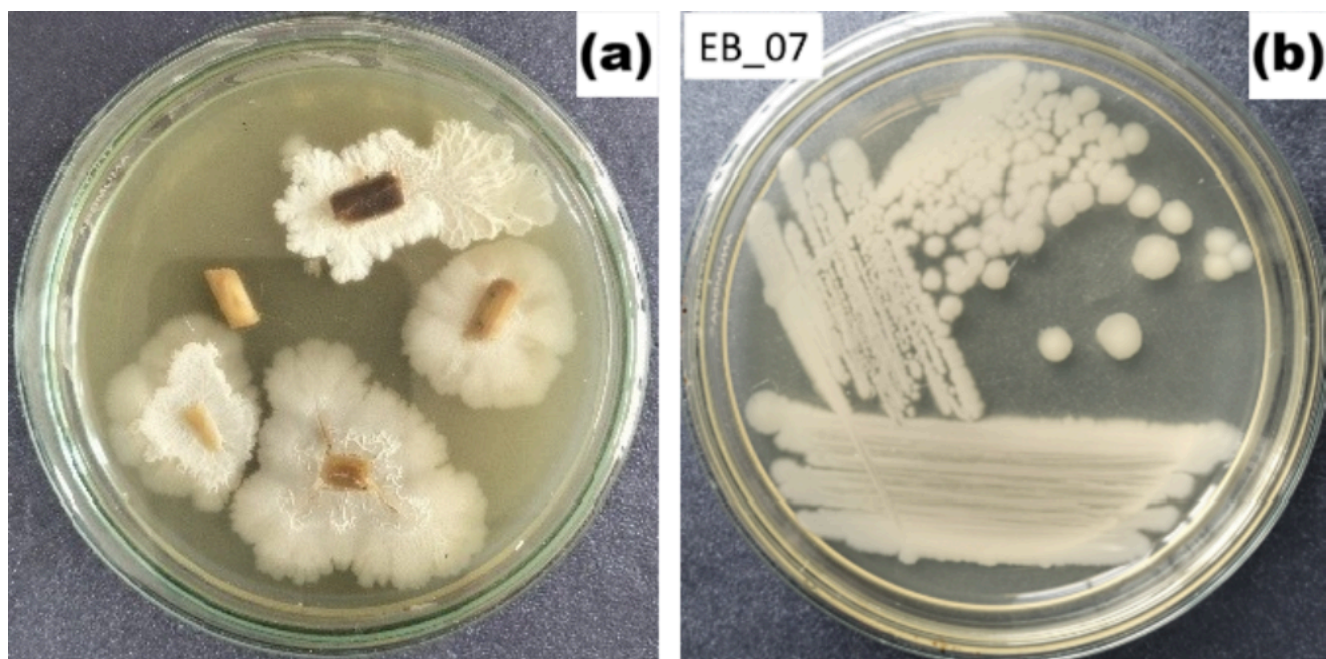


Figure 1

Isolation and pure culture of endophytic bacteria from *Dalbergia reniformis* . **(a)**Bacterial colonies emerging from stem tissue on LB agar; **(b)**pure culture of isolate EB_07 on nutrient agar.

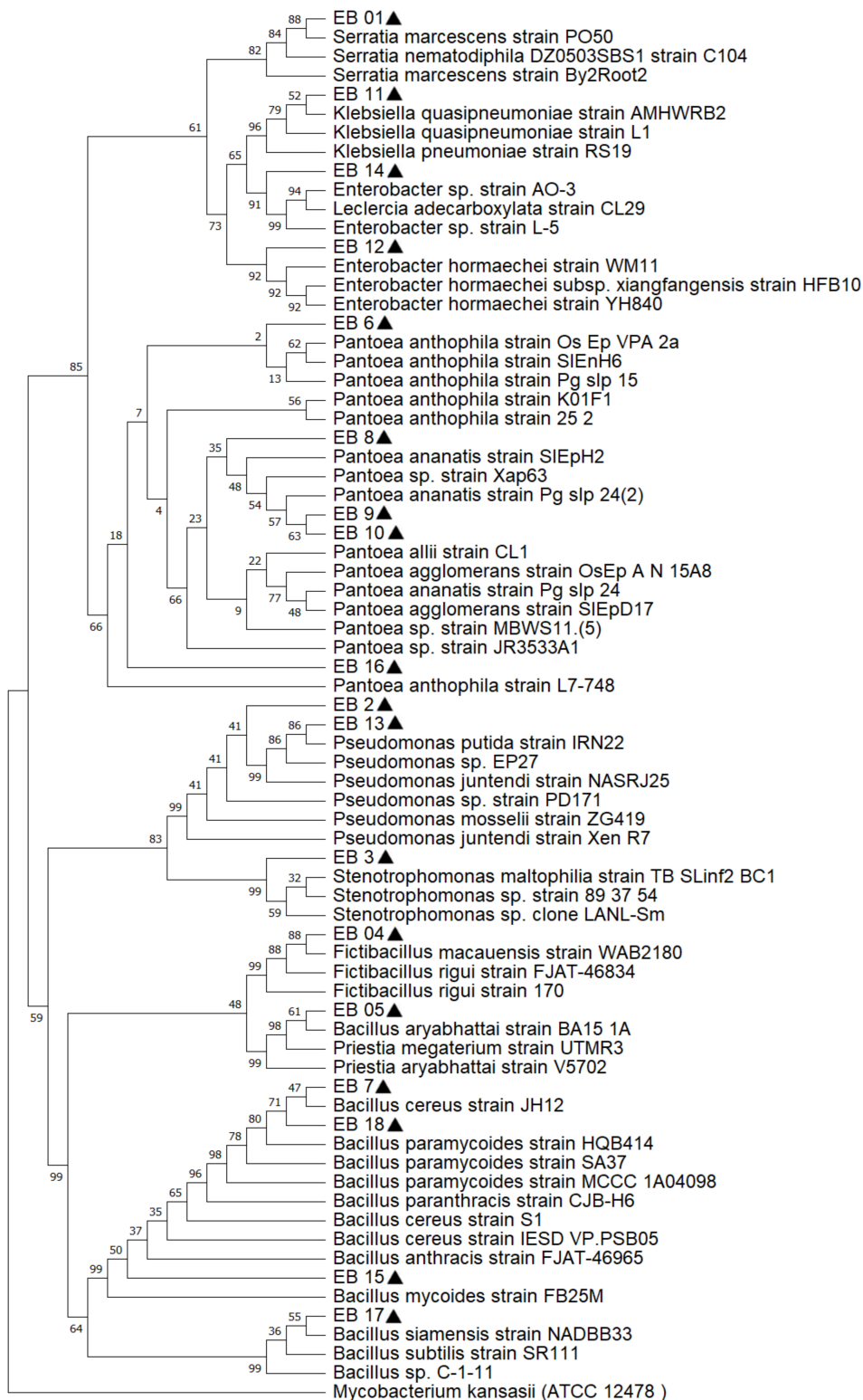


Figure 2

Phylogenetic analysis of 16S rRNA sequences of the endophytic bacterial isolates.

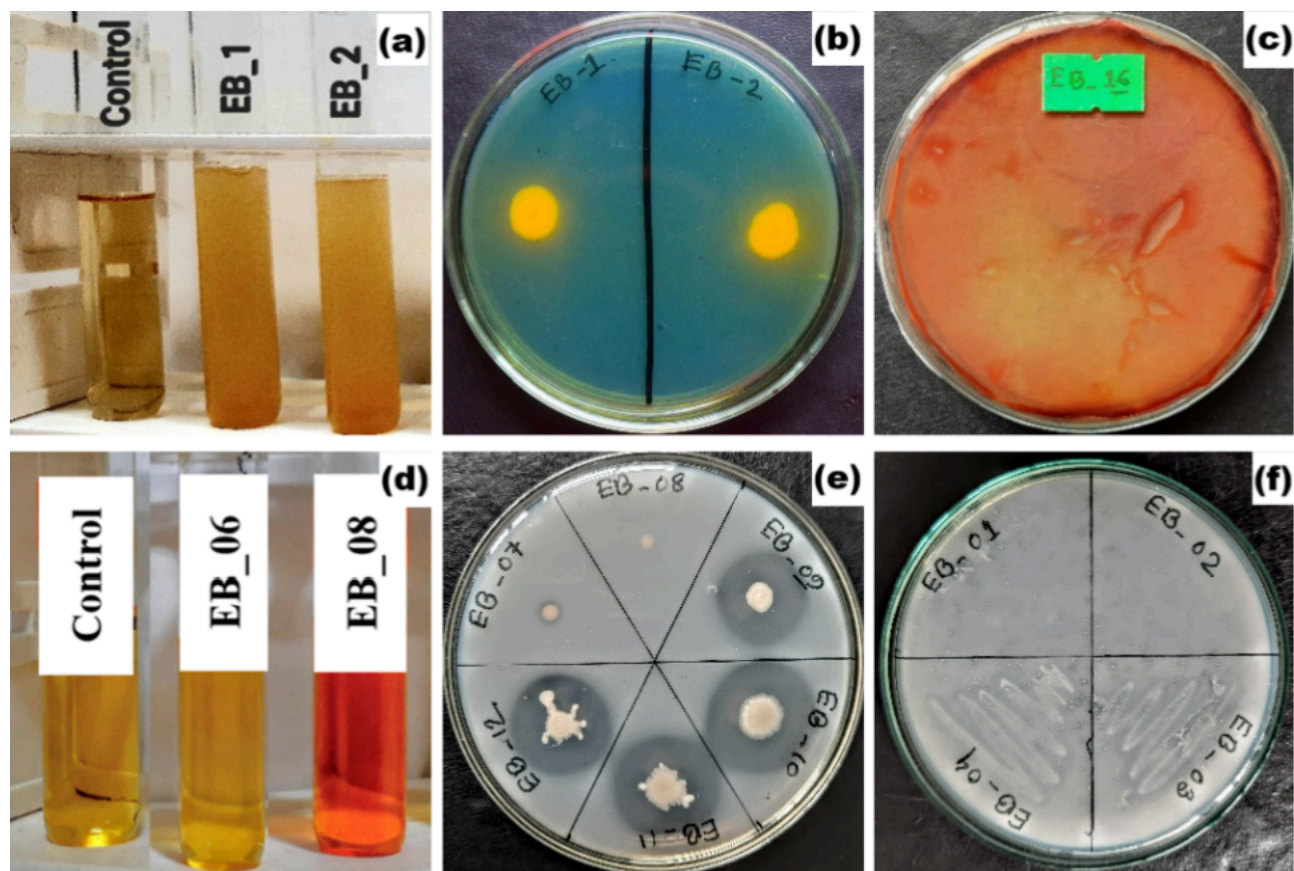


Figure 3
Results of PGP tests across selected bacterial isolates. (a) Ammonia produced by EB_01 and EB_02 compared to control; (b) Siderophore produced by EB_01 and EB_02; (c) HCN produced by EB_16; (d) EB_08, a strong organic acid producer compared to control, whereas EB_06 provided negative results; (e) Phytate solubilization shown by EB_09, EB_10, EB_11, EB_12, and EB_07 (minimal activity) whereas EB_08 showed no enzymatic activity; (f) Oxalate oxidase activity demonstrated by EB_03 and EB_04.

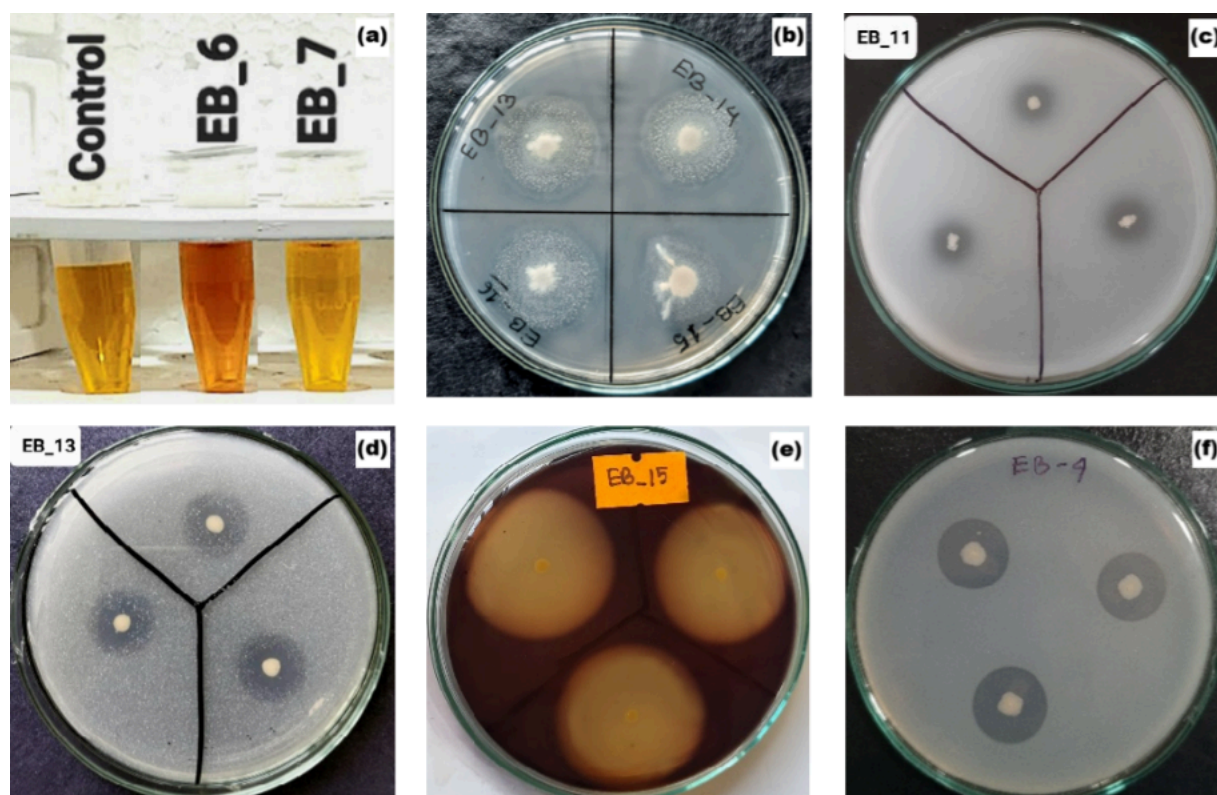


Figure 4

Results of PGP tests across selected bacterial isolates. (a) IAA produced by EB_06, whereas EB_07 is a non-producer compared to the control; (b) Lipolytic activity identified in EB_13, EB_14, EB_15, and EB_16 by Ca-salt deposition around bacterial colonies; (c) Phosphate solubilization ability of EB_11; (d) Zn solubilization shown by EB_13; (e) Cellulolytic ability of EB_15; (f) Proteolysis of milk casein by EB_04.

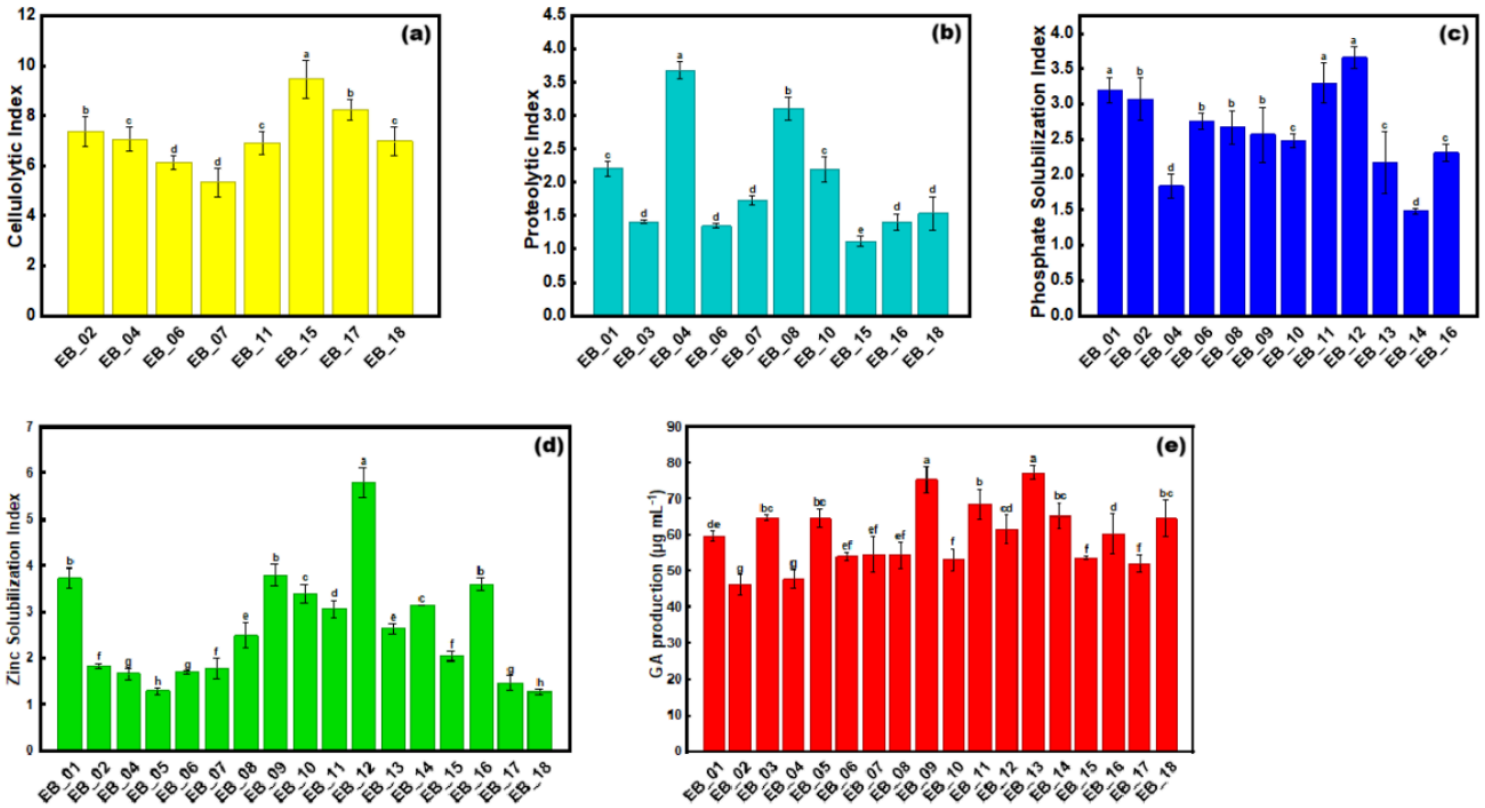


Figure 5

PGP activities of the bacterial isolates tested in this study. (a) Cellulolytic activity exhibited by eight of the bacterial isolates; (b) Proteolytic activity exhibited by ten of the bacterial isolates; (c) Zinc solubilization indices of the bacterial isolates; (d) Phosphate solubilization indices of the bacterial isolates; (e) Amount ($\mu\text{g L}^{-1}$) of gibberellic acid (GA) produced by each bacterial isolate.

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