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Plant growth promotion activity of endophytic bacteria isolated from *Acalypha indica* L., Euphorbiaceae

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ABSTRACT

Medicinal plants serve as natural reservoirs for various types of beneficial microorganisms. Among these, endophytic bacteria act as a promising source of bioactive compounds with significant applications in multiple fields, including medicine, agriculture, and industry. This research aims to isolate and characterise endophytic bacteria inhabiting *Acalypha indica* L. and evaluate their potential in plant growth promotion and enzyme production. Based on 16S rRNA gene sequencing analysis, the seven morphologically distinct bacterial isolates exhibited close sequence similarity to *Brucella anthropi*, *Priestia megaterium*, *Staphylococcus epidermidis*, *Microbacterium paludicola*, *Agrobacterium tumefaciens*, *Enterobacter cloacae*, and *Nocardioides zeae*. Among these, *B. anthropi* demonstrated superior plant growth-promoting potential, as evidenced by its higher production of indole-3-acetic acid and ability to enhance the growth of *Vigna radiata* seedlings. *Priestia megaterium* exhibited notable potassium-solubilising activity, whereas *S. epidermidis* showed efficient phosphate solubilization. All seven isolates showed aminocyclopropane-1-carboxylic acid deaminase activity and ammonia production, indicating their collective potential in plant growth promotion. Enzymatic profiling revealed that *S. epidermidis* exhibited lipase activity, while *E. cloacae* demonstrated cellulase and amylase production. Although the isolation of endophytic bacteria is well documented, the specific functional attributes of these isolates from this host plant have not been previously reported. Therefore, this study provides a foundational screening of promising endophytic bacteria with potential applications in sustainable agriculture and biotechnology.

1. INTRODUCTION

Endophytes are microorganisms that live inside plants without causing any disease and complete their life cycle either partially or completely within the host tissue [1–3]. Endophytes and plants have evolved together, forming a mutually beneficial relationship in which plants provide nutrients to endophytes, while endophytes help maintain plant health [4,5]. They enhance plant growth and health by absorbing nutrients more efficiently, increasing the availability of essential elements, and improving nitrogen fixation. Additionally, endophytes can promote plant development, strengthen their stress resistance, and protect them from pests and diseases [6].

Fungal and bacterial endophytes are valuable in various industries due to their ability to produce a diverse range of metabolites with potential applications. These metabolites can be utilised as antitumor, antiviral, and antimicrobial agents, plant growth promoters, bio-control agents, stress tolerance enhancers, and immunosuppressants. Endophytes also produce compounds with antibiotic, anti-diabetic, and antioxidant properties [7]. Recent research has highlighted their potential in the synthesis of innovative nanomaterials and their significance in agricultural practices [8,9]. Plant cell modification is necessary for the entry of bacteria and their spread inside the plant. This is accomplished through the secretion of cell wall-degrading enzymes, such as xylanases, endoglucanases, pectinases, and cellulases [10,11]. Endophytic microbial enzymes have a significant role in biotechnology across various sectors, including medical treatments, agricultural and pharmaceutical research, detergent and textile industries, food processing, and molecular biology. Endophytic microbes synthesize a wide range of enzymes, including chitinases, cellulases, β-1,3-glucanases, pectinases, glucanases, and proteases. These enzymes degrade the cell walls of phytopathogens and/or inhibit spore germination. Through these mechanisms, endophytic microbes suppress pathogen growth, thereby enhancing host plant protection against biotic stress [12].

The plant *Acalypha indica* L., commonly known as "Kuppaimeni," in Malayalam, belongs to the Euphorbiaceae family [13]. It is a common weed plant found in India, Sri Lanka, Thailand, and Pakistan, and has significant medicinal value. Extracts from its leaves, roots, and stems are used to treat various ailments, including eye infections, respiratory problems, rheumatism, skin conditions, and diabetes [14]. India has a

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diverse array of medicinal plants, and traditional knowledge regarding their phytochemical properties has been effectively utilised to develop pharmaceutical and nutraceutical products, thereby improving human health status [15]. The present study reports the isolation and identification of endophytic bacteria from *A. indica* and their ability to promote plant growth and produce various enzymes.

2. MATERIAL AND METHODS

2.1 Collection of Plant Material

Healthy asymptomatic plants of A. indica were collected from the district of Ernakulam (10°07′00″N 76°21′00″E), Kerala State, India. The plant was taxonomically identified by the Department of Botany, Botanical Survey of India, Coimbatore. A voucher specimen of the plant was deposited at the Madras herbarium with specimen number MH 178294. Samples were taken to the laboratory in a sealed plastic bag and kept for further studies.

2.2. Confocal Microscopy

Surface-sterilised tissues (stems, leaves, and roots) of *A. indica* were sectioned using a sterile, sharp razor blade to obtain extra-thin hand sections (approximately 60–80 µm thick). The sections were immediately processed to preserve cellular integrity and prevent microbial displacement. For fixation, the sections were immersed in 4% paraformaldehyde (PFA) prepared in phosphate-buffered saline (PBS) for 1 hour at room temperature. Following fixation, the sections were washed three times with PBS to remove residual fixative. To facilitate the penetration of staining dyes through the plant cell wall and membranes, the sections were permeabilised with 0.1% TritonX-100 for 10 minutes, and rinsed with PBS.

The sections were first stained with 4′,6-diamidino-2-phenylindole (DAPI) (30 nM) by adding 100 µL of the staining solution and incubating for 15–20 minutes. Excess stain was removed by washing with PBS. Subsequently, the sections were stained with propidium iodide (PI) (1 µg/ml) for 30 minutes, followed by PBS washes to remove excess dye. The stained sections were kept in the dark at room temperature until observation. Finally, the sections were examined using a Leica SP8 WLL confocal microscope (Germany). The system was controlled using LAS X software with the following laser parameters: DAPI excitation at 405 nm with emission detection at 415–450 nm, and PI excitation at 561 nm with emission detection at 575–630 nm.

2.3. Culture-Dependent Isolation

The whole plants of *A. indica* were thoroughly rinsed under running tap water to remove soil and dust. They were then washed with a mild detergent solution and rinsed twice with double-distilled water. Further surface sterilisation procedure was carried out under a Laminar Air Flow (LAF) chamber. Plant samples were initially rinsed in 70% ethanol for 20–30 seconds, followed by sterilisation in 2.5% sodium hypochlorite for 5 minutes. Then they were washed five to six times with sterile double-distilled water. Surface-disinfected whole plant was macerated using a mortar and pestle. It was serially diluted up to 10-6 by mixing 1 ml of the suspension with 9 ml of sterile distilled water in each tube. 0.5 ml was taken from each tube and plated on Luria Bertani(LB) agar (HiMedia Laboratories Pvt. Ltd.) and nutrient agar media (HiMedia Laboratories Pvt., Ltd.). It was incubated at 28°C for 24–48 hours to ensure the maximum number of bacterial colonies. A portion of the water used for the final wash was plated as a control. No growth on the control plate indicates no contamination.

Out of the two media used, bacterial growth was higher in the LB media. Therefore, for further isolation, LB agar was used. Morphologically unique colonies observed on LB agar plates after incubation were isolated individually by repeated streaking. These isolated bacterial strains were grown on LB agar slants and then stored at 4°C for later use [17].

2.4. Morphological and Biochemical Characterization

Morphological characters, such as colony and cell characters, were studied. Cell character, such as Gram's staining [18] and endospore staining [19], was carried out. Biochemical tests such as catalase test, Voges Proskauer test, methyl red test, citrate test, mannitol motility test, urease test, indole test, Kligler iron agar test, phenylalanine deaminase, and nitrate reduction test were conducted using the guidelines outlined in Bergey's Manual of Determinative Bacteriology [20].

2.5. Enzymatic Assay

For the catalase test, a drop of 3% hydrogen peroxide (H_2O_2) was applied to the slide. A colony of bacterial isolate from LB plates was then placed on the glass slide. Immediate formation of bubbles after the addition of bacterial isolates onto the slide indicates a positive result for the presence of catalase enzyme [21]. For detecting the presence of amylase enzyme, Starch agar with 0.2% soluble starch and 1.5% agar was used. Bacterial isolates were streaked on this agar plate and incubated for 18–24 hours at 30ºC. A clear zone formation around the colony after the addition of iodine gives a positive result for amylase production. The enzymatic index was calculated by dividing the average diameter of the halo zone by the average diameter of the colony growth [22,23]. For determining the cellulase activity, bacterial isolates were cultured on LB media with 0.5% yeast extract and 1% carboxymethylcellulose (CMC). The plates were incubated for 48 hours, and 10 ml of 1% Congo red dye was applied, followed by washing with 5 M NaCl. The presence of clear yellowish zones around their colonies shows a positive result for the test [23]. LB culture media supplemented with 1% (v/v) of sterilised Tween 20 was used for testing the lipase activity. The formation of clear zones around bacterial growth confirmed the presence of lipase enzyme [24]. Pectin (0.5%) containing agar medium is used for culturing bacterial isolates for detecting the presence of pectin. Incubated for 3–5 days at room temperature. The formation of a clear zone around the bacterial colony by the drop-wise addition of 1% cetyltrimethylammonium bromide (CTAB) gives a positive result for pectin. Enzymatic index was also calculated for all isolates [25].

$$\text{Enzymatic index} = \frac{\text{Colony diameter (cm)+Halozone diameter (cm)}}{\text{Colony diameter (cm)}}$$

2.6. IAA Production

The production of indole-3-acetic acid (IAA) by the isolates was determined [23]. Bacterial cultures were grown in Luria–Bertani (LB) broth supplemented with 1% tryptophan at 28°C ± 2°C for 72 hours. Then centrifuged at 10,000 rpm for 20 minutes, and 2 ml of the supernatant was collected. Two drops of orthophosphoric acid and 4 ml of Salkowski reagent (0.5 M FeCl3 in 35% perchloric acid) were added to the supernatant. Tubes were then incubated for 25 min in the dark at room temperature. Formation of pink colour indicates the presence of IAA production. The quantitative estimation was done by taking absorbance at 530 nm using spectrophotometry and compared with a standard curve prepared using various concentrations of analytical grade IAA.

2.7. ACC Deaminase Production

ACC deaminase production was assessed by following the protocol described by [26] using sterile minimal Dworkin and Foster (DF) salts medium. The bacterial isolates were grown on DF salts medium plates at 28°C for 3 days. The presence of growth on the plates indicates positive for ACC deaminase production, and the DF salts medium without ACC was used as the negative control.

2.8. Phosphate Solubilisation

Phosphate solubilization was determined using NBRIP medium. This medium consists of glucose (10 g/l), tricalcium phosphate ($Ca_3(PO_4)2$, 5 g/l), magnesium chloride hexahydrate ($MgCl_2.6H_2O$, 5 g/l), magnesium sulphate heptahydrate ($MgSO_4.7H_2O$, 0.25 g/l), potassium chloride (KCl, 0.2 g/l), and ammonium sulphate ($(NH_4)2SO_4$, 0.1 g/l). Cultures were incubated at 28°C in a shaking incubator at 120 rpm for 6 days. Absorbance was measured at 430 nm using a spectrophotometer (Shimadzu UV-1800). The amount of phosphate solubilized was then determined by comparing the absorbance values to a standard curve generated using known concentrations of KH2PO4 [27].

2.9. Ammonia Production

Bacterial cultures were inoculated into the test tube containing 5 ml of peptone water and incubated at 28°C ± 2°C for a period of 48 hours. After incubation, 0.5 ml of Nessler's reagent was added to each tube. Formation of brown to yellow colour indicates the presence of ammonia production [28].

2.10. HCN Production

Luria–Bertani (LB) medium was supplemented with 4.4 g/l glycine, and the bacterial culture was streaked on this plate. A Whatman No. 1 filter paper was saturated with an alkaline picrate solution (2% sodium carbonate, 5% picric acid) and then placed on the inner side of the upper lid of the petri plate. Plates were then covered with parafilm and incubated for 4 days at 28°C ± 2°C. Colour change of the filter paper from yellow to reddish brown indicates production of HCN [29].

2.11. Siderophore Production

Isolates were first cultured in King's A medium and then transferred to King's B medium and incubated at 30°C for 6 days. After centrifugation, an equal volume of 2% $FeCl_3$ solution was added to 1 ml of the supernatant. The formation of a reddish-brown or orange colour indicated the siderophore production [30].

2.12. Potassium Solubilization

The test was carried out on Aleksandrov agar (5 g $C_6H_{12}O_6$, 0.5 g $MgSO_4$ $7H_2O$, 0.1 g $CaCO_3$, 0.006 g $FeCl_3$, 2 g $Ca_3(PO_4)_2$, 3 g $KAlSi_3O$ (K-feldspar), and 20 g agar in 1 L of distilled water at pH ± 7, using the spot test method. Bacterial isolates were inoculated on Aleksandrov agar medium at room temperature for 3 days. Diameter of the clear zone around the bacterial colonies was measured [30].

Potassium Solubilization index (PSI) = the diameter of the clearance zone(D)/the diameter of the colony growth (d).

2.13. Identification of Endophytic Bacteria

16SrRNA sequencing was done based on a modified procedure [31]. Total genomic DNA was isolated from a 24-hour bacterial culture using the Nucleospin® Tissue kit (Macherey-Nagel). Polymerase chain reaction (PCR) was used to amplify the partial 16S rRNA gene (v3–v4 region) of the bacterial DNA using the universal primers 27F (AGAGTTTGATCMTGGCTCAG) and 1492R (CGGTTACCTTGTTACGACTT). Amplification was performed using a thermal cycler (BIORAD, USA). The reaction was carried out in 20 µl containing 10 µl Takara -EmeraldAmp GT PCR Master Mix, 2 µl forward primer (10pmol/ µl), 2 µl reverse primer (10pmol/ µl), and 2µl template DNA,and the volume was made up to 20 µl with nuclease-free water (4µl). Thermocycling conditions consist of an initial denaturation step at 95°C for 5 minutes, followed by denaturation at 95°C for 30 seconds for 35 cycles, annealing for 30 seconds at 53°C, and extension for 1 minute at 72°C. A final extension step was performed for 1 minute at 72°C. Phylogenetic analysis based on 16S rRNA gene sequences was performed using the neighbour-joining method in MEGA version 12 to determine the evolutionary relationships among the bacterial strains [32].

2.14. Influence of Endophytic Bacteria on the Growth of *Vigna radiata*

Vigna radiata was selected as a model system due to its rapid germination and high sensitivity to growth-promoting factors. An overnight bacterial culture was centrifuged at 8000 rpm for 10 minutes to obtain the cell pellet. The supernatant was carefully discarded, and the pellet was resuspended in sterile 0.85% NaCl solution. The suspension was centrifuged again under the same conditions, and the washing step was repeated two to three times to ensure complete removal of residual culture medium. Finally, the washed bacterial cells were resuspended in sterile saline solution. Seeds of *Vigna radiata* were surface sterilized using 70% ethanol for 30 seconds, followed by 1% sodium hypochlorite treatment for 1–2 minutes. The seeds were then rinsed 3–4 times with sterile distilled water to remove any traces of sterilizing agents. The sterilized seeds were subsequently soaked in the bacterial suspension (OD_{600} = 1.0) for 30–60 minutes.

Vigna radiata seeds were then sown in sterilised soil and grown under controlled conditions (14-hour light/10-hour darkness, temperature–27°C ± 2°C). Each treatment consisted of three replicates, with three seeds per replicate. After growing for 15 days, plant tissues were harvested, and root length, shoot length, fresh weight, dry weight, and chlorophyll content were measured. Fresh weight was recorded immediately, while dry weight was determined after oven-drying at 60°C for 48 hours [33]. Chlorophyll content (a and b) was quantified using the method described by Akhtar et al. [34]. Approximately 0.5 g of fresh leaf tissue was accurately weighed and homogenized in 10 ml of 80% (v/v) acetone using a pre-chilled mortar and pestle. The homogenate was filtered through Whatman No. 1 filter paper to remove cellular debris. To compensate for any solvent loss during extraction and filtration, the final volume of the filtrate was adjusted to 10 mL with 80% acetone using a volumetric flask. The absorbance (A) of the extract was measured at 645 nm, 663 nm, and 470 nm using a UV–Visible spectrophotometer (Model 5520XR, Wescor Inc., Logan, UT, USA). All measurements were carried out using a 1 cm pathlength quartz cuvette, and the instrument was calibrated with 80% acetone as the blank prior to analysis.

2.15. Statistical Analysis

The data was analysed statistically utilizing R Studio version 4.5.2. Normality and homogeneity of variance were first tested using Shapiro-Wilk and Levene's tests, respectively. Subsequently, a one-way ANOVA was performed to identify statistically significant differences, followed by a post hoc Tukey's HSD test for determining the specific differing groups at a *p*-value less than 0.05. Relevant

graphical representations were done using the ggplot2 package in R studio [35,36].

3. RESULTS

Confocal laser scanning microscopy shows the presence of bacterial colonies within the thin sections of the stem and root, visualised as bright blue spots in Figure 1. Mostly, bacteria enter the plants from the soil through the roots, and hence, the highest bacterial diversity is seen in the roots compared to the stem.

Sterilised plant was homogenised and plated on LB media. Morphologically similar and different colonies were obtained as in Figure 2. Individual colonies were numbered, isolated, and subcultured for further analyses. Based on easily observable morphological characteristics, 23 bacterial isolates were selected from a total of 60 colonies obtained.Cell characters such as Gram staining, motility, and endospore staining were studied. All the bacterial isolates showed negative for endospore staining with one exception. Both Gram-positive and Gram-negative bacteria were obtained. The majority of isolates exhibited a rod-shaped morphology, with a few coccoid bacteria. Most of the bacterial colonies studied were circular and opaque in nature. Orange, white, yellow, and cream-coloured bacteria were isolated as shown in Table 1.

Biochemical tests such as catalase, citrate, indole, MR, VP, urease, phenylalanine deaminase, nitrate reduction, mannitol motility, and Kligler iron agar were done. Almost all bacterial isolates are positive for urease, with a few exceptions, as shown in Table 2.

All 23 isolates were screened for cellulase, pectinase, lipase, and amylase production, as shown in Table 3. Of the 23 isolates, 17 produced cellulase. Production of cellulase was significantly higher in the Ac17 isolate. Pectinase activity was observed in 8, and lipase production was observed in 9 out of 23 isolates. Lipase production was higher in Ac10. Isolate Ac1 and Ac17 exhibited increased production of amylase (Table 3).

Plant growth-promoting traits, including indole-3-acetic acid (IAA) production, 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity, siderophore production, potassium solubilisation, phosphate solubilisation, HCN and ammonia production, were studied in 23 isolates. A highly promising result was obtained for IAA production, with Ac3 and Ac5 showing higher IAA production. Potassium solubilisation was most pronounced in isolate Ac5, whereas phosphate solubilisation was most evident in isolate Ac10. The majority of isolates showed ACC deaminase activity, while all isolates exhibited ammonia production (Table 4, Fig. 4). DF salt medium lacking ACC supplementation did not support the growth of any isolate.

Seven bacterial strains were selected for further studies based on their plant growth promotion activity, such as Ac3, Ac5, Ac10, Ac12, Ac13, Ac17, and Ac22 and were renamed as A1, A2, A3, A4, A5, A6, and A7, respectively. For molecular identification, these isolates were subjected

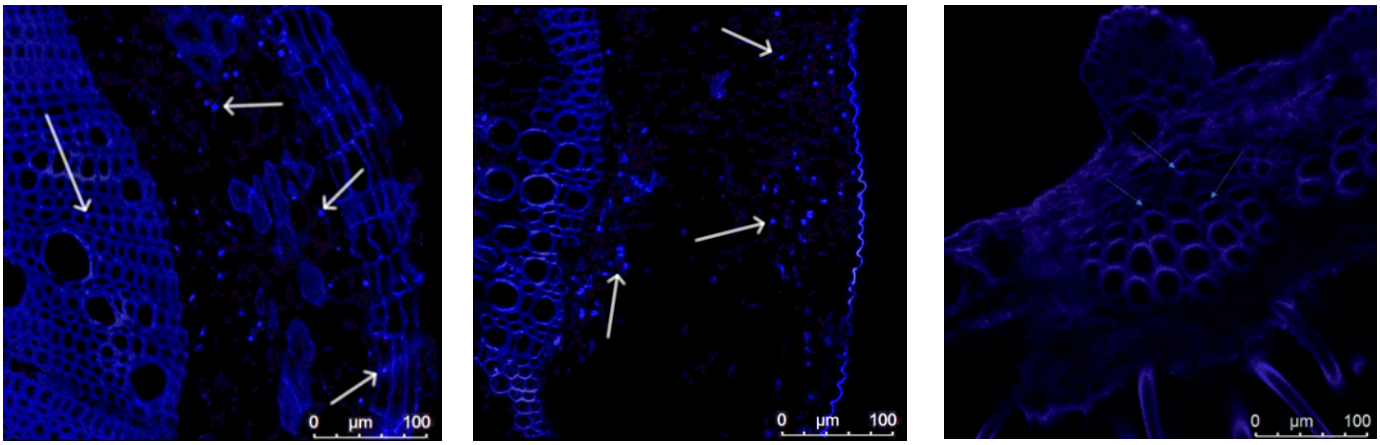

Figure 1. Confocal laser scanning microscopic image of root (a), stem (b) and leaf (c).

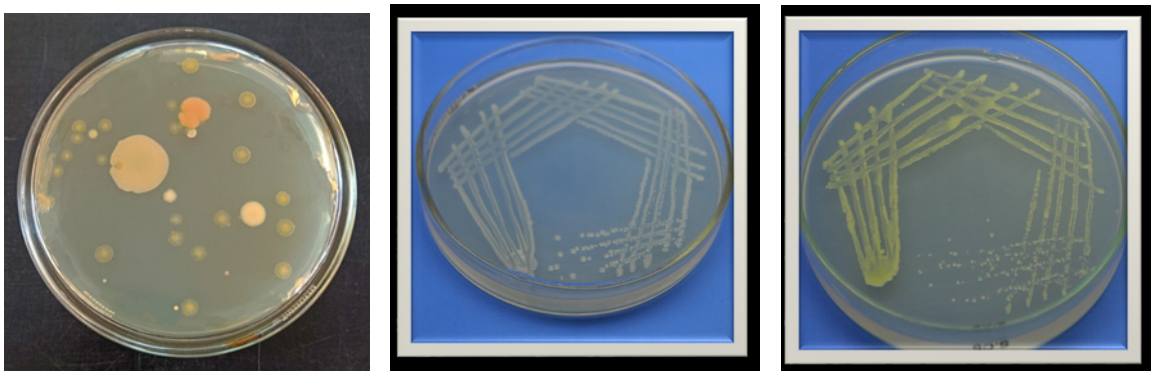

Figure 2. Different bacterial colonies, streak plates of two different bacterial culture.

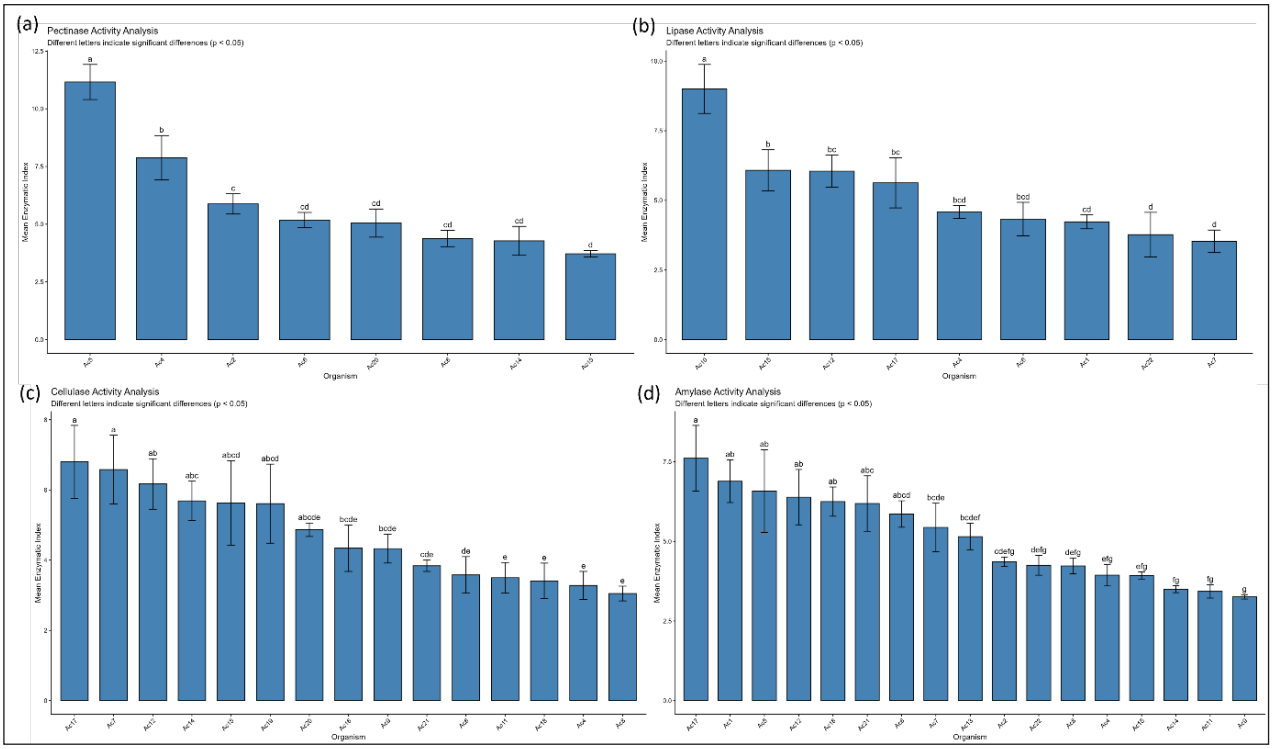

Figure 3. (a) Functional diversity in pectinase production across different samples. One-way ANOVA: $F(7,16) = 53.77$, $p < 0.001$. (b) Analysis of lipase across different samples. One-way ANOVA: $F(8,18) = 20.79$, $p < 0.001$. (c) Comparative analysis of cellulase across different samples. One-way ANOVA: $F(14,30) = 10.27$, $p < 0.001$. (d) Comparative analysis of amylase across different samples. One-way ANOVA: $F(16,34) = 15.38$, $p < 0.001$. Means followed by different letters (a–e) indicate significant differences between samples according to Tukey's HSD post-hoc test ($p < 0.05$). Error bars represent mean ± SD ($n = 3$).

Table 1. Cell and colony characters of endophytic bacteria isolated from A. indica. (Ac- represents the bacterial species isolated from A. indica. + indicates presence and – indicates absence).

Endophytes	Shape	Colour	Elevation	Edge	Opacity	Consistency	Texture	Gram staining and shape	Endospore	Motility
Ac1	Circular	Orange	Umbonate	Entire	Opaque	Creamy	Rough	+ Rod	–	+
Ac2	Circular	White	Raised	Entire	Opaque	Sticky	Smooth	+ Rod	–	+
Ac3	Circular	Cream	Raised	Entire	Opaque	Creamy	Smooth	– Rod	–	+
Ac4	Circular	Yellow	Raised	Entire	Opaque	Creamy	Smooth	+ Rod	–	+
Ac5	Wavy	Off white	Raised	Wavy	Opaque	Creamy	Rough	+ Rod	+	–
Ac6	Circular	White	Raised	Entire	Opaque	Creamy	Smooth	+ Rod	–	+
Ac7	Wavy	White	Umbonate	Wavy	Opaque	Sticky	Rough	– Coccus	–	+
Ac8	Circular	Yellow	Raised	Entire	Opaque	Creamy	Smooth	+ Rod	–	+
Ac9	Wavy	Cream	Raised	Wavy	Opaque	Creamy	Smooth	– Rod	–	+
Ac10	Circular	Greyish white	Raised	Entire	Translucent	Creamy	Smooth	+ Coccus	–	–
Ac11	Wavy	White	Umbonate	Wavy	Opaque	Sticky	Rough	– Rod	–	+
Ac12	Circular	Yellow	Flat	Entire	Translucent	Sticky	Smooth	+ short rod	–	–
Ac13	wavy	Off white	Umbonate	Entire	Translucent	Creamy	Smooth	– Rod	–	+
Ac14	Circular	Orange	Raised	Entire	Transparent	creamy	Rough	+ Rod	–	+
Ac15	Wavy	White	Umbonate	Wavy	Transparent	Creamy	Smooth	+ Coccus	–	–
Ac16	Wavy	Off white	Umbonate	Wavy	Opaque	Sticky	Rough	+ Rod	–	+
Ac17	Circular	Creamy	Umbonate	Entire	Opaque	Creamy	Smooth	– Rod	–	+
Ac18	Wavy	Light yellow	Raised	Wavy	Opaque	Sticky	Rough	+ Rod	–	+

Continued

Endophytes	Shape	Colour	Elevation	Edge	Opacity	Consistency	Texture	Gram staining and shape	Endospore	Motility
Ac19	Circular	Yellow	Raised	Entire	Opaque	Sticky	Rough	+ Rod	–	+
Ac20	Circular	Orange	Umbonate	Entire	Opaque	Creamy	Smooth	– Coccus	–	–
Ac21	Wavy	White	Raised	Wavy	Opaque	Sticky	Rough	+ Coccus	–	+
Ac22	Circular	White	Raised	Entire	Transparent	Sticky	Rough	+ Rod	–	+
Ac23	Circular	Milky white	Raised	Entire	Opaque	Creamy	Smooth	+ Rod	–	–

Table 2. Biochemical analysis of each bacterial isolate (+ indicates presence, ++ indicates greater degree of presence and – indicates absence).

	Catalase test	Citrate test	Indole test	MR	VP	Urease	Phenyl alanine deaminase	Nitrate reduction	Mannitol motility test	Kligler Iron Agar test
Ac1	+	–	–	–	–	+	–	–	–	Glucose only
Ac2	+	–	–	+	+	–	–	+	+	glucose and lactose
Ac3	+	+	–	–	–	+	+	+	–	Glucose only
Ac4	+	+	–	–	–	+	–	+	–	Glucose only
Ac5	+	+	–	–	–	+	++	–	–	Glucose only
Ac6	–	–	+	–	–	+	–	–	–	Glucose only
Ac7	+	–	–	–	–	++	–	–	–	Glucose only
Ac8	+	–	–	–	–	+	–	–	–	Glucose only
Ac9	+	+	–	–	–	++	–	–	–	Glucose only
Ac10	+	–	–	+	+	+	–	+	–	glucose and lactose
Ac11	+	+	–	+	+	+	–	+	+	glucose and lactose
Ac12	+	–	–	–	–	–	+	–	–	Glucose only
Ac13	–	+	–	–	–	–	+	+	–	Glucose only
Ac14	+	–	–	–	–	+	+	–	–	Glucose only
Ac15	+	–	–	–	–	++	–	+	–	Glucose only
Ac16	+	–	–	–	–	+	–	–	–	Glucose only
Ac17	+	+	–	+	+	+	–	+	+	glucose only + Gas
Ac18	+	–	–	–	–	+	–	+	–	Glucose only
Ac19	+	+	+	–	–	+	–	–	–	Glucose only
Ac20	+	+	–	+	+	+	–	+	–	glucose and lactose
Ac21	+	–	–	–	–	+	+	–	–	Glucose only
Ac22	+	–	–	–	–	+	–	+	–	Glucose only
Ac23	+	+	–	+	+	++	–	+	+	glucose only + gas

Table 3. Screening of extracellular enzyme production (cellulase, pectinase, lipase, and amylase) by isolated bacterial strains, showing qualitative enzyme activity and the corresponding enzymatic index calculated from hydrolysis zone and colony diameter. (+ indicates presence, ++ indicates greater degree of presence, and – indicates absence).

Organism	Cellulase		Pectinase		Lipase		Amylase	
	Presence	Enzymatic Index	Presence	Enzymatic index	Presence	Enzymatic index	Presence	Enzymatic index
Ac1	–	–	–	–	+	4.23 ± 0.25	++	6.89 ± 0.67
Ac2	–	–	+	5.88 ± 0.44	–	–	+	4.36 ± 0.15
Ac3	–	–	–	–	–	–	–	–
Ac4	+	3.28 ± 0.40	+	7.88 ± 0.96	+	4.58 ± 0.23	+	3.94 ± 0.34
Ac5	–	–	+	11.16 ± 0.76	–	–	+	6.58 ± 1.5
Ac6	+	3.58 ± 0.52	+	5.18 ± 0.35	+	4.33 ± 0.6	+	5.86 ± 0.42

Continued

Organism	Cellulase		Pectinase		Lipase		Amylase	
	Presence	Enzymatic Index	Presence	Enzymatic index	Presence	Enzymatic index	Presence	Enzymatic index
Ac7	+	6.58 ± 0.98	–	–	+	3.53 ± 0.41	+	5.44 ± 0.76
Ac8	+	3.05 ± 0.21	+	4.38 ± 0.37	–	–	+	4.23 ± 0.25
Ac9	+	4.33 ± 0.41	–	–	–	–	+	3.26 ± 0.07
Ac10	–	–	–	–	++	9 ± 0.88	–	–
Ac11	+	3.5 ± 0.44	–	–	–	–	+	3.43 ± 0.21
Ac12	+	6.17 ± 0.72	–	–	+	6.05 ± 0.58	+	6.39 ± 0.87
Ac13	–	–	–	–	–	–	+	5.15 ± 0.42
Ac14	+	5.69 ± 0.56	+	4.28 ± 0.63	–	–	+	3.5 ± 0.11
Ac15	+	5.63 ± 1.21	+	3.72 ± 0.14	+	6.08 ± 0.74	+	3.93 ± 0.11
Ac16	+	4.34 ± 0.64	–	–	–	–	–	–
Ac17	++	6.8 ± 0.97	–	–	+	5.63 ± 0.90	++	7.61 ± 0.79
Ac18	+	3.41 ± 0.51	–	–	–	–	+	6.25 ± 0.46
Ac19	+	5.61 ± 1.13	–	–	–	–	–	–
Ac20	+	4.8 ± 0.2	+	5.05 ± 0.61	–	–	–	–
Ac21	+	3.84 ± 0.16	–	–	–	–	+	6.19 ± 0.88
Ac22	–	–	–	–	+	3.77 ± 0.8	+	4.25 ± 0.3
Ac23	–	–	–	–	–	–	–	–

Table 4. Evaluation of plant growth–promoting traits of isolated bacterial strains, including indole-3-acetic acid (IAA) production, phosphate and potassium solubilization, siderophore production, hydrogen cyanide (HCN), ACC deaminase activity, and ammonia production.

Endophyte	IAA (µg/ml)	Phosphate (µg/ml)	Potassium		Siderophore	HCN	ACC deaminase	Ammonia
				PSI				
Ac1	6.20 ± 0.33	7.18 ± 0.83	+	3.61 ± 0.35	–	+	+	+
Ac2	7.53 ± 0.89	13.89 ± 0.64	–	–	–	+	++	+
Ac3	28.06 ± 0.71	22.15 ± 0.61	–	–	+	–	+	+
Ac4	21.56 ± 0.67	19.53 ± 0.95	–	–	+	–	–	+
Ac5	24.68 ± 0.04	19.93 ± 0.62	+	6.61 ± 0.79	–	–	++	+
Ac6	18.84 ± 0.25	12.38 ± 0.67	+	5.08 ± 0.8	+	–	++	+
AC7	7.49 ± 0.8	7.6 ± 0.62	–	–	+	–	+	+
Ac8	18.95 ± 0.61	2.94 ± 0.84	–	–	–	+	–	+
Ac9	6 ± 0.35	19.78 ± 0.23	+	2.25 ± 0.12	–	+	–	+
Ac10	23.71 ± 0.82	25.24 ± 0.32	–	–	+	–	++	+
Ac11	5.66 ± 0.31	12.47 ± 0.64	+	2.82 ± 0.31	+	+	+	+
Ac12	23.85 ± 0.68	21.89 ± 0.54	–	–	+	–	+	+
Ac13	23.45 ± 0.47	21.66 ± 0.42	+	4.89 ± 0.67	–	–	++	+
Ac14	21.33 ± 0.86	20.47 ± 0.2	–	–	–	–	++	+
Ac15	18.09 ± 3.06	16.96 ± 0.66	–	–	–	–	+	+
Ac16	21 ± 0.53	5.11 ± 0.27	–	–	+	–	+	+
Ac17	23.36 ± 0.79	21.38 ± 0.78	+	4.35 ± 0.49	+	–	++	+
Ac18	18.63 ± 0.18	5.16 ± 0.34	–	–	+	+	–	+
Ac19	15.23 ± 0.36	19.84 ± 0.58	+	4.05 ± 0.58	+	+	+	+
Ac20	17.93 ± 0.2	7.91 ± 0.29	–	–	–	–	++	+
Ac21	16.59 ± 0.82	14.29 ± 0.49	–	–	–	+	–	+
Ac22	22.96 ± 0.45	22.09 ± 0.5	–	–	–	–	++	+
Ac23	7.46 ± 0.47	2.94 ± 0.32	–	–	–	+	+	+

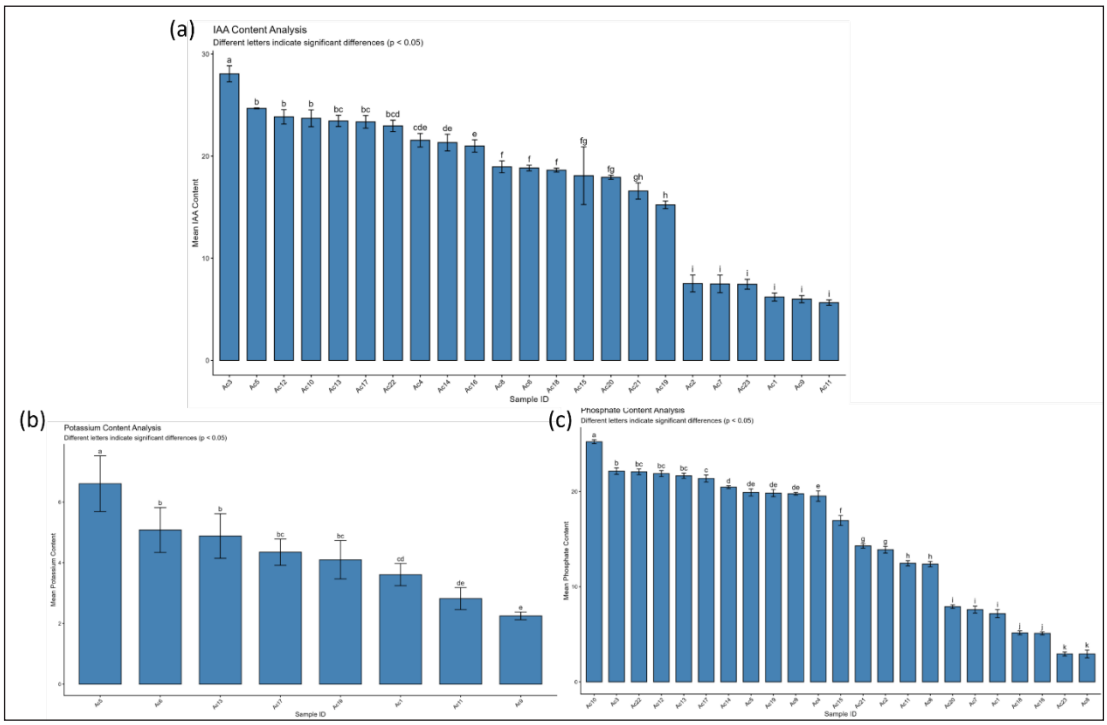

Figure 4. (a) Quantitative profiling of IAA across twenty-three distinct samples. One-way ANOVA: F (22,92) = 369.9, p < 0.001. (b) Comparative analysis of potassium absorption across different samples. One-way ANOVA: F (7,32) = 26.86, p < 0.001. (c) Comparative analysis of phosphate absorption across different samples. One-way ANOVA: F (22,92) = 2469, p < 0.001. Means followed by different letters (a–e) indicate significant differences between samples according to Tukey's HSD post-hoc test (p < 0.05). Error bars represent mean ± SD (n = 5).

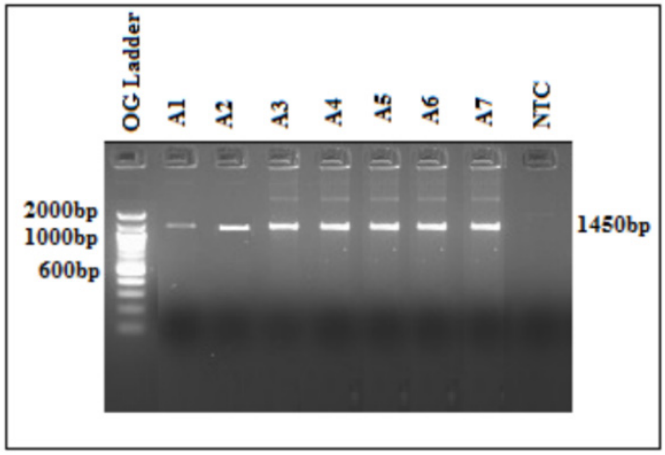

Figure 5. Agarose gel electrophoresis image of the bacterial isolates amplified with 16S F & R / 27F & 1492R primers.

to 16S rRNA gene sequencing. These were then verified by 2% agarose gel electrophoresis (Fig. 5). Bacterial isolates A1, A2, A3, A4, A5, A6, and A7 were closely related to the identified taxa. We present these identifications as the most probable matches based on the NCBI database as *Brucella anthropi, Priestia megaterium, Staphylococcus epidermidis, Microbacterium paludicola, Agrobacterium tumefaciens, Enterobacter cloacae*, and *Nocardioides zeae,* respectively. The sequences were deposited in GenBank, and accession numbers were obtained (Table 5, Fig. 6).

The plant growth-promoting potential of the isolates was evaluated by analysing their impact on seeds of Vigna radiata (Fig. 7). Compared to the control (seeds grown without bacterial inoculation), isolates show higher plant growth activity. Isolate A1 exhibited an increase in root (7cm) and shoot length (16.5cm) compared to the control. While isolate A3 showed the lowest growth (root length 5cm and shoot length 12.8cm) among the tested isolates, it still exhibited enhanced growth compared to the control group (Fig. 8). Total chlorophyll content was calculated as shown in Table 6.

Table 5. Endophytic bacterial species identified using 16S rRNA gene sequencing, along with their corresponding query coverage and GenBank accession numbers.

No.	Organism	Strain	% identity	Query coverage	Accession number
A1	*B. anthropi*	DP5 16S	100	100 %	OR886245
A2	*P. megaterium*	E71CS3	100	99 %	OR886609
A3	*S. epidermidis*	TWSL 18	99.85	100 %	OR886931
A4	*M. paludicola*	EB385	100	100 %	OR888720
A5	*A. tumefaciens*	NAC54	100	100%	OR892550
A6	*E. cloacae*	HNXY160623	98.44	100 %	OR888723
A7	*N. zeae*	SCSIO 43764	96.89	100 %	OR888758

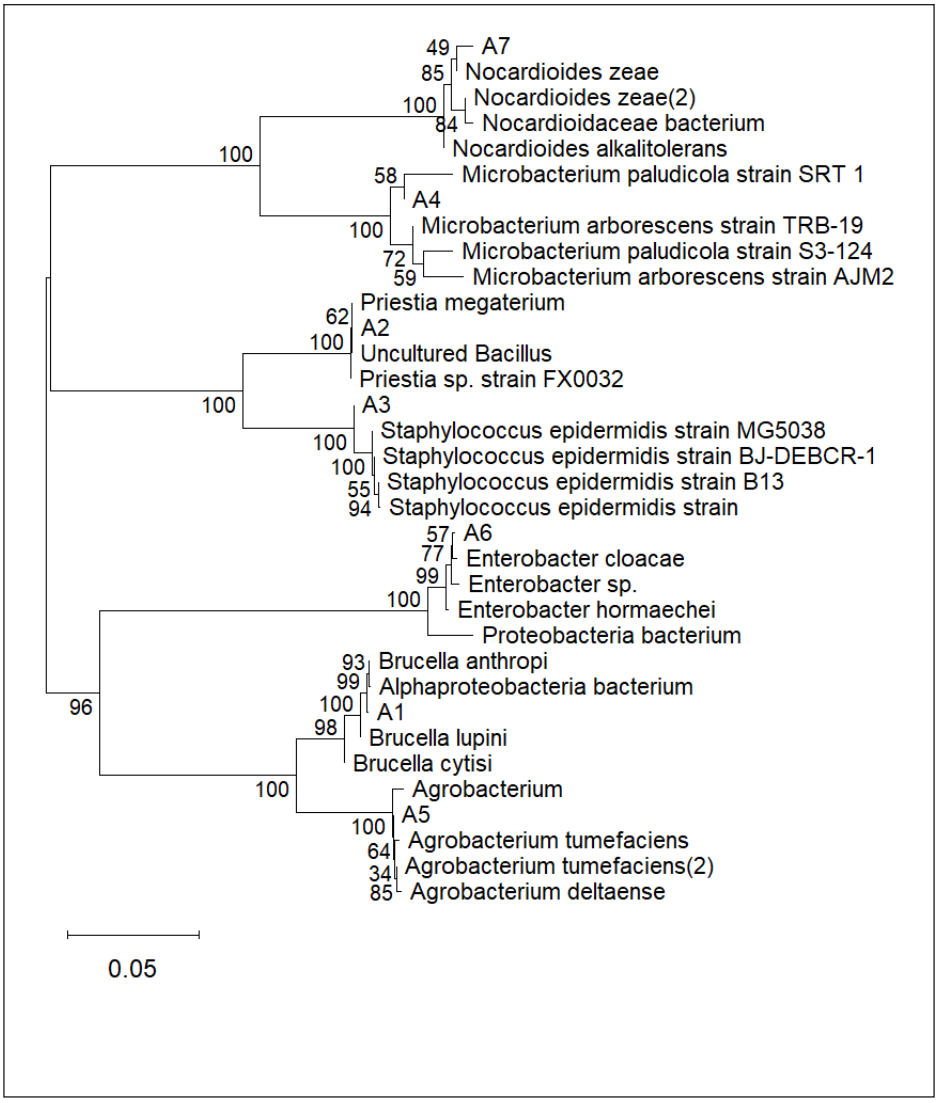

Figure 6. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences illustrating the evolutionary relationships among bacterial strains, constructed using MEGA version 12. The scale bar indicates 0.05 nucleotide substitutions per site, and bootstrap support values (1,000 replicates) are shown at the branch nodes.

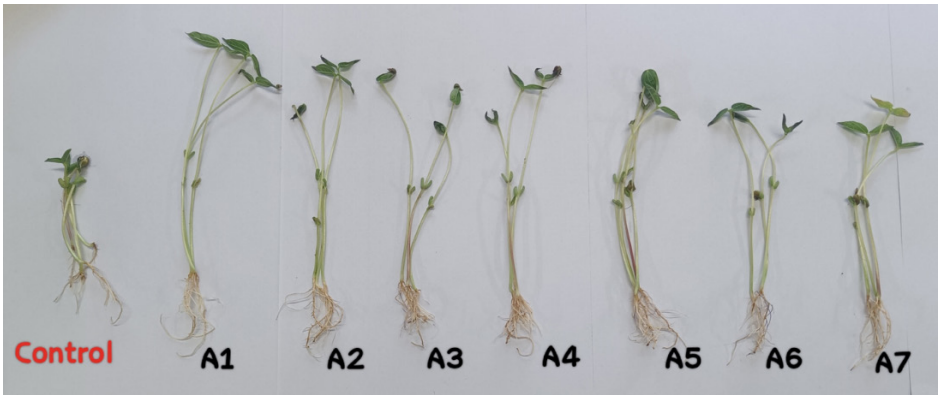

Figure 7. Comparative growth response of *Vigna radiata* seedlings following inoculation with different bacterial isolates, and overall enhanced growth in treated plants relative to the uninoculated control.

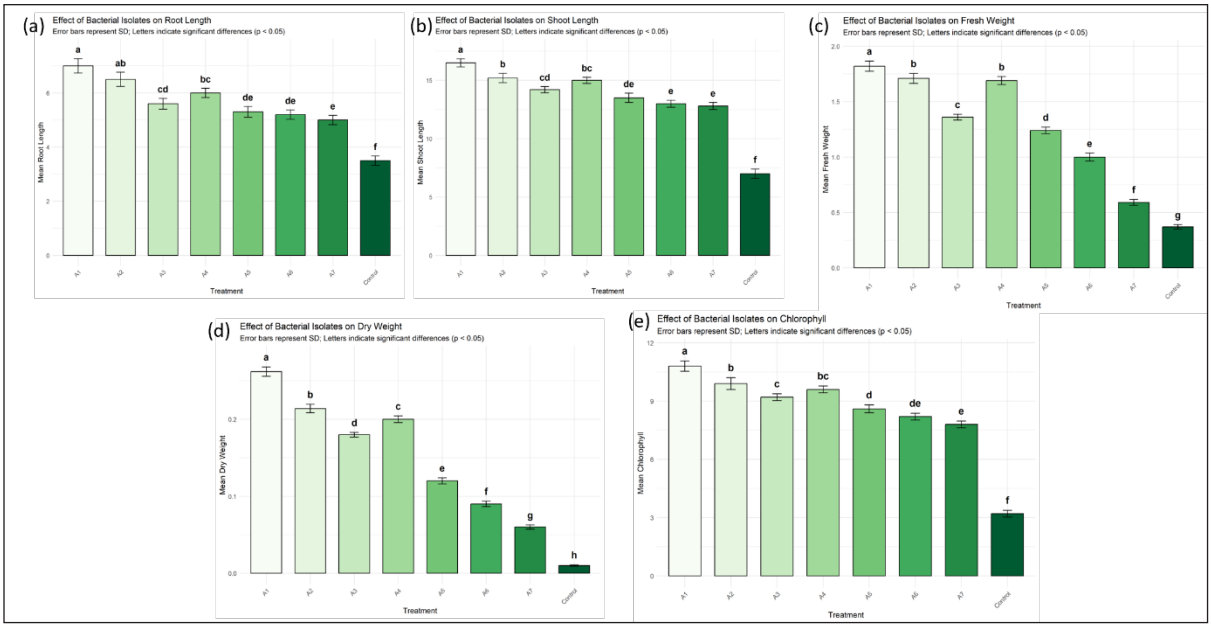

Figure 8. (a) Effect of treatments on Root Length (cm). One-way ANOVA: F (7,16) = 79.55, $p < 0.001$. (b) Impact of treatments on Shoot Length (cm). One-way ANOVA: F (7,16) = 214.4, $p < 0.001$. (c) Variation in Fresh Weight (g) across treatment groups. One-way ANOVA: F (7,16) = 725, $p < 0.001$. (d) Treatment-wise comparison of Dry Weight (g). One-way ANOVA: F (7,16) = 1333, $p < 0.001$. Total Chlorophyll content (mg/g) under various treatment. One-way ANOVA: F (7,16) = 368, $p < 0.001$. Means followed by different letters (a, b, c, etc.) indicate significant differences between groups based on Tukey's HSD post-hoc test. Error bars represent mean ± SD ($n = 3$).

Table 6. Growth and physiological parameters of *Vigna radiata* seedlings inoculated with different bacterial isolates, showing variations in root length, shoot length, fresh and dry biomass, and chlorophyll content in comparison with the untreated control.

Sl No	Root length (cm)	Shoot length (cm)	Fresh weight (g)	Dry weight (g)	Chlorophyll content (mg/g)
Control	3.5 ± 0.21	7 ± 0.37	0.37 ± 0.03	0.01 ± 0.0012	3.2 ± 0.18
A1	7 ± 0.29	16.5 ± 0.42	1.82 ± 0.05	0.262 ± 0.0064	10.8 ± 0.31
A2	6.5 ± 0.24	15.2 ± 0.35	1.71 ± 0.04	0.214 ± 0.0051	9.9 ± 0.27
A3	5.6 ± 0.18	14.2 ± 0.28	1.36 ± 0.03	0.18 ± 0.0032	9.2 ± 0.19
A4	6 ± 0.20	15 ± 0.31	1.69 ± 0.04	0.2 ± 0.0043	9.6 ± 0.22
A5	5.3 ± 0.23	13.5 ± 0.36	1.24 ± 0.03	0.12 ± 0.0041	8.6 ± 0.24
A6	5.2 ± 0.19	13 ± 0.33	1 ± 0.04	0.09 ± 0.0035	8.2 ± 0.21
A7	5 ± 0.17	12.8 ± 0.29	0.59 ± 0.03	0.06 ± 0.0028	7.8 ± 0.20

4. DISCUSSION

Thomas and Sekhar [37] reported visualisation of bacterial colony in the apical leaf sheath of banana using confocal laser scanning microscopy. The bacterial colony was localised in the periplasmic space between the cell wall and plasma membrane. Abundant bacterial colonisation in cytoplasmic and periplasmic areas localised using confocal microscopy was studied by Thomas and Reddy [38]. Similar observations were made in our study; bacterial colonies were abundant in the root and stem of *A. indica*. The majority of the isolates in this study were Gram-positive, except for a few Gram-negative bacteria. Our results are in line with the findings of Kiros et al. [39], who reported that the majority of bacterial isolates obtained from *Gloriosa superba* were Gram-positive. Endophytes from *Tinospora cordifolia* studied by Duhan et al. [40] did not produce endospores, but in our results, among the 23 isolates, one of the bacterial isolates showed positive for endospore formation.

Extracellular enzymes, produced by microbes, function outside the cell and play important roles in various biological and environmental processes. Endophytic bacteria and fungi produce a variety of extracellular enzymes, including xylanases, hemicellulases, phytases, proteases, asparaginase, cellulases, pectinases, tyrosinase, gelatinase, chitinase, and amylases [17]. Dogan and Taskin [41] reported that bacterial endophytes isolated from Poaceae are involved in the production of lipases, proteases, amylases, cellulases, pectinases, and xylanases. Bacterial isolates obtained from *A. indica* in this study exhibited the ability to produce different enzymes, including amylase, cellulase, lipase, pectinase, catalase, phenylalanine deaminase, urease, and nitrate reductase. Application of the enzyme cellulase to crops has positive impacts on plant growth, including improved seed germination and enhanced plant protection [42]. Hassan et al. [43] studied the potential for sustainable agriculture by utilising endophytic fungi, *Penicillium chrysogenum* and *Penicillium crustosum*, which possess the ability

to produce a range of extracellular enzymes, including amylase, pectinase, xylanase, cellulase, and carboxymethyl cellulase.

Yaish et al., [44] reported that some of the endophytic bacteria isolated from seedling roots of *Phoenix dactylifera* (date palm) can produce ACC deaminase, IAA, and ammonia and can solubilise phosphate and potassium. To our knowledge, this study reports the first isolation and identification of these bacterial strains as endophytes with the particular functional analysis from *A. indica*. *Brucella anthropi* reported from *A. indica* has the ability to produce IAA, ACC deaminase, ammonia, and siderophore. Our findings align with the results of Lee et al. [45], who previously reported that *B. anthropi* shows plant growth-promoting activities. Indole-3-acetic acid (IAA) is a plant hormone known to promote plant growth and thereby increase plant biomass [46]. At low concentrations, IAA primarily stimulates primary root elongation, while higher concentrations promote the formation of both lateral and adventitious roots [47]. Meng et al. [48] reported that the secretion of Ochrobactrum anthropi (synonym of B. anthropi) has a high concentration of IAA, and it produces siderophore and chelates iron.

Endophytic bacteria isolate A2, which was identified as *P. megaterium* in our study, was previously reported as *Bacillus megaterium* [49]. This bacterium was initially named due to its large size, nearly 100 times larger than *E.coli* [49]. *Bacillus megaterium* was developed and utilised in industry for more than 50 years due to its ability to produce various enzymes such as amylases, proteases, glucose dehydrogenase, penicillin amidase, vitamin B12, and a few antibiotics [49–51]. The antagonistic activity exhibited by the endophytic bacteria *P. megaterium* isolated from chickpea root suggests their possible application in integrated approaches for managing Fusarium wilt of chickpea [52]. Our study reports that *B. megaterium* shows higher IAA production and improved growth of *Vigna radiata*, which is in line with reports of many genes involved in the biosynthesis of IAA and improved growth of *Arabidopsis* seedling by *B. megaterium* isolated from *Retama monosperma* [53].

Staphylococcus epidermidis and *M. paludicola* reported in our study have an endophytic report isolated from *Commiphora gileadensis* [54] and from tomato, showing plant growth promotion activities [55], respectively. *Staphylococcus epidermidis* isolated from *A. indica* shows plant growth promotion activities such as IAA production, phosphate solubilization, ACC deaminase production, Siderophore production, and ammonia production. It is also involved in the production of lipase. Different strains are reported to be involved in phosphate solubilisation [56], IAA production [57], and lipase production [58]. *Nocardioides zeae* reported in the present study has an endophytic report isolated from *Zea mays* [59] *Agrobacterium* species responsible for crown gall disease are recognised as soil-borne phytopathogens. But *A. tumefaciens* is found to be isolated from *A. indica,* and previous studies of members of this genus isolated as endophytes from the root nodules of legume species are also reported [60,61]. *Enterobacter cloacae* reported in our study produces cellulase, amylase, IAA, siderophore, ammonia and has phosphate and potassium solubilisation ability. *E. cloacae* have previous reports as an endophyte showing multiple plant growth-promoting activities like IAA, siderophore, and ammonia production and can solubilise phosphate and potassium [62]. Cellulase enzyme was produced by *E. cloacae* isolated from bovine rumen fluid waste [63].

5. CONCLUSION

Endophytic bacteria possess the capacity to produce a wide range of bioactive metabolites with significant potential for promoting plant health. Recent studies have explored these microorganisms as promising sources of biofertilizers and biopesticides. In the present study, bacterial endophytes were isolated and identified from *A. indica*, and their roles in plant growth promotion and enzyme production were evaluated. Among the isolates, B. anthropi (OR886245) demonstrated promising in vitro plant growth-promoting traits, indicating its potential for further development as a biofertilizer, pending detailed investigation. Endophytes exhibiting enhanced plant growth-promoting activity represent a sustainable alternative to conventional agrochemicals and contribute to environmentally friendly agricultural practices. The increasing reliance on chemical fertilizers has raised serious concerns regarding environmental sustainability and soil health. Additionally, endophytic bacteria capable of enzyme production hold considerable promise for diverse industrial applications, including those in the textile, paper, and food industries.

6. CONFLICT OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

7. AUTHORS' CONTRIBUTION

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

8. FUNDING

There is no funding to report.

9. ETHICS APPROVAL

This study does not involve experiments on animals or human subjects.

10. DATA AVAILABILITY

All the data is available with the authors and shall be provided upon request.

11. PUBLISHER'S NOTE

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12. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declare that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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