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Phylogenetic Analysis and Antioxidant Activity of the Endophytic Bacterial Isolates from *Kalanchoe pinnata*

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Abstract

Endophytic bacteria are vital members of the plant microbiome, and their phylogenetic diversity has been extensively documented using next-generation sequencing. They are also essential for initiating different biological effects and regulating the synthesis of secondary metabolites with important therapeutic qualities. Plant tissues harbor endophytic bacteria that generate bioactive chemicals that enhance plant growth, increase resistance to infections, and promote plant tolerance to stress. The aromatic plant Kalanchoe pinnata possess unique chemical elements, including essential oils such as lipids, alkaloids, triterpenes, steroids, and flavonoids, which impart it with great therapeutic potential and medical significance. The present investigation separated endophytic bacteria from Kalanchoe pinnata. Three endophytic bacterial isolates, HMA1, HMA2, and HMA3, were isolated after surface sterilization. These isolates were characterized using biochemical parameters and 16S rRNA sequencing. Phylogenetic analysis and 16S rRNA sequencing data indicated that the bacterial isolates were members of the Bacillus genera. A more comprehensive understanding of the microbiome of this plant could highlight the importance of studying endophytes and their potential applications in agriculture, medicine, and environmental biotechnology

Keywords: *Kalanchoe pinnata, steroids, triterpenes, flavonoids, BLAST, RNA Sequencing, PCR*

1. Introduction

Endophytes are indispensable components of plant microbiomes. These harmless microorganisms are crucial for promoting plant growth and protecting plants from diseases (Aswani et al., 2020). Without endophytes, plants are more vulnerable to various diseases, leading to their decline and, ultimately, death. Therefore, it is vital to understand the significance of these microorganisms and take steps to maintain their presence in plant microbiomes. The ability of microorganisms to establish mutualistic relationships with plants is a key aspect of modern agriculture and plant science research (Adeleke et al., 2021). Such relationships are characterized by interactions that effectively enhance nutrient uptake, improve tolerance to environmental stress, and ultimately increase biomass production (Sharma et al., 2018; Tosi et al., 2021). The capacity of microorganisms to establish advantageous relationships with plants presents a viable strategy to maximize agricultural output and lessen the effects of biotic and abiotic stressors on crop yields (Hasanuzzaman et al., 2019). These characteristics make bacterial endophytes a promising candidate for sustainable agricultural practices and plant biotechnology research (Etesami & Beattie, 2017). This has led to increased interest in studying their potential benefits.

Endophytic bacteria and rhizobacterium are plant growth-promoting bacteria (PGPB) owing to their PGP mechanisms (del Carmen Orozco-Mosqueda et al., 2020; Tian et al., 2023). Endophytes are microorganisms that are found in almost all plant species (Fadiji & Babalola, 2020). The plant microbiome is currently the subject of much scientific interest because of its potential to protect plants from harsh environmental conditions, enhance nutrient use efficiency, increase nutrient uptake, and promote growth.

The *Kalanchoe* genus comprises over 125 succulent species that reproduce asexually through somatic cells(Lee, 2023). It has been discovered that various species of *Kalanchoe* possess anti-inflammatory, antioxidant, antibacterial, and wound-healing properties (Fernandes et al., 2019). As a result, these plants can be used in dermatological products that are available on the market. For instance, *Kalanchoe pinnata* has been found to accelerate wound healing(Suprapto et al. in 2015 & T. Sharma et al., 2023). The study revealed that two other extracts, namely petroleum ether extract and alcoholic extract obtained from the leaves, have been proven to enhance the healing process of incision wounds. Furthermore, histological analysis has demonstrated that *Kalanchoe pinnata* positively affects collagen matrix remodeling, repair, and subsequent epithelization of wounds (Araújo et al., 2023). The Gram-positive endophytic bacteria *Bacillus cereus*, *Bacillus paranthracis*, and *Bacillus thuringiensis* were isolated from the leaves of *Kalanchoe pinnata*. Moreover, previous research studies showed that a new type of *Streptomyces species*, named BJSG4, was discovered in *Kalanchoe pinnata* (Nirmala et al., 2023; Sankarganesh & Joseph, 2019). Gas chromatography-mass spectrometry confirmed the presence of 3-hydroxy-1-(4-13-(4-(3-hydroxy-3-phenylacryloyl) phenyl) tridecyl-phenyl)-3-phenylprop-2-en-1-one in the endophyte BJSG4. The compound was purified and exhibited in vitro activity against the malaria parasite Plasmodium falciparum3D7 with a log IC50 value of 3.47 nm. A statistical correlation coefficient (R2) of 0.9 confirmed the highest positive in vitro interaction between the compound and malarial parasite (Sankarganesh & Joseph, 2019). The aim of the present study was to isolate and characterize the endophytic bacteria from *Kalanchoe pinnata*.

2. Materials and methods

2.1 Collection of plant materials

The stems, roots, and leaves of *Kalanchoe pinnata* were used in the identification and isolation of endophytic bacteria. The healthy roots, stems, and leaves of *Kalanchoe pinnata* were meticulously harvested from both cultivated and wild plants in Bahadurgarh as shown in **Fig.1** . These carefully selected samples were transported to the laboratory for further experimental purposes. As part of the pre-treatment procedure, the leaves stems and roots were thoroughly washed with tap water for approximately 15 min to remove any clinging soil particles and a large percentage of the microbial epiphytes (Anjum & Chandra, 2015).

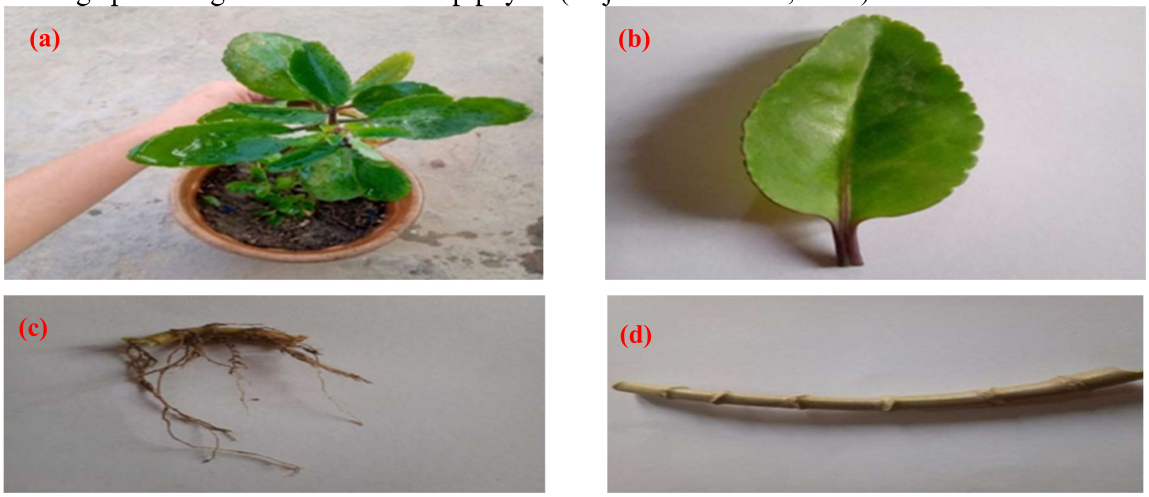


Fig. 1: *Kalanchoe pinnata* and excised plant parts (a) plant, (b) leaf, (c) root, and (d) stem.

2.2 Surface sterilization

The first crucial step in isolating endophytic bacteria is to eliminate surface microbes. This is achieved by treating plant tissues with sodium hypochlorite (NaOCl). Each sample was subjected to the following procedure: one minute of washing in 70% ethanol, two to three minutes of soaking in a sodium hypochlorite solution, thirty seconds of 70% alcohol washing, two rinses in distilled water, and three to five sterile rinses. Without endangering the host tissue or endophytic microorganisms, the sterilizing agent must eradicate surface bacteria. Samples were imprinted onto Luria Bertaini medium, aliquots of water from the final rinse were cultured on nutrient media, and surface-sterilized explants were dipped into LB broth to verify the sterility of the plant tissue. The isolated bacteria were regarded as endophytes, and surface sterilization was judged successful if no microbial growth was observed on the control media. If no microbial growth occurs on the control media, surface sterilization is deemed successful, and the isolated bacteria are considered endophytes (Maela, 2020; Damavandi et al., 2023).

2.3 Isolation of endophytic bacteria

The surface of the stems was removed using a sterile scalpel in a laminar airflow cabinet after the surface-sterilized plant material has been completely dried. After being cleaned and disinfected, the root, stem, and leaf were sliced into pieces that are 1-2 mm^2 and placed on plates containing Luria Bertani (LB) medium as shown in **Fig.2**. The plates were monitored daily while incubating for 3–4 days at 28°C (Easa et al., 2018). To improve the recovery of bacterial endophytes, plant tissue plates were incubated at 28°C and sealed with parafilm tape (Basumatary et al., 2021). The growth of the bacteria was observed up to 48 h. To obtain pure bacterial isolates, several bacterial colonies were chosen based on their shape and streaked to obtain pure colonies. After subculturing each of the chosen isolates on LB medium plates, the purified endophytes were stored at 4°C until the time of DNA extraction (Maela, 2020; Damavandi et al., 2023).

2a **2b** **2c**

Fig. 2: Thin sections of different plant parts: (a) stem, (b) root, and, (c) leaf, transferred to the plates containing Luria- Bertani (LB) media after surface sterilization of plant parts

2.4 Identification of Endophytic bacteria

The endophytic bacteria were identified using two methods: biochemical tests and 16S rRNA sequencing. In biochemical identification, isolates were characterized based on biochemical properties like methyl red test, citrate utilization test, phenylalanine deaminase test, sugar utilization test, Voges- Proskauer test, catalase test, and indole production test. All biochemical tests were performed according to the methodology reported by Anjum & Chandra, (2015) and Bariya et al. (2022) with some modifications.

2.5 16S rRNA sequencing

16S rRNA sequencing begins with DNA extraction followed by PCR amplification of the 16S rDNA gene. After the amplified products were sequenced, the resulting sequence was compared using the BLAST program with the sequence in the database. The total DNA from the microbial community present in our sample, whether it's environmental, clinical, or from another source was extracted and the extracted DNA was subjected to polymerase chain reaction (PCR) to amplify the 16S rRNA gene. It help in taxonomic identification because it is present in all bacteria and archaea but varies enough to provide species-level resolution. The amplified 16S rRNA gene products were sequenced using methods such as Sanger sequencing or high-throughput sequencing platforms (like Illumina). This generates a large amount of sequence data that reflects the microbial diversity present in the original sample. The resulting sequences were compared against a reference database using BLAST (Basic Local Alignment Search Tool). This allows for identification of the organisms present in the sample by matching the obtained sequences to known sequences in the database. After matching the sequences, further analysis was conducted to interpret the microbial composition, diversity, and potential functions of the identified organisms (Sunkar et al., 2018).

3. Results and discussion

3.1 Isolation of bacterial endophytes

The stem was cut into thin sections in the current research and transferred to plates containing LB media on October 29, 2023. On November 5, three bacterial endophytes were separated from the stem of *Kalanchoe pinnata* on the Luria-Bertani (LB) medium (S. K. Sharma et al., 2018). The streak-plate technique was used to isolate a pure culture of the bacteria from clusters of bacterial colonies. The quadrant method was used to isolate single colonies, and for 2–3 days, the plates were incubated.

Fig.3 Bacterial isolates obtained from the stem of *Kalanchoe pinnata* i.e. are labelled 3 (HMA1),4 (HMA2), and 5 (HMA3)

The results of this study demonstrated that the bacterial isolates were present on the plates containing LB media (**Fig. 3),** and further labelled HMA1, HMA2, and HMA3. According to earlier research, *Bacillus thuringiensis*, *Bacillus paranthus*, *Staphylococcus xylosus*, and *Bacillus cereus* have all been isolated from *Kalanchoe pinnata* leaves (Renugadevi et al., 2022; Renugadevi et al., 2024). Similarly, several authors have reported the effective isolation of *Streptomyces* sp. BJSG4 with possible anti-malarial properties from the stem of *Kalanchoe pinnata*.These findings have direct implications for the agricultural and biotechnological sectors, offering practical solutions for root growth promotion and disease control, thereby contributing to the sustainable development of these industries (Sankarganesh & Joseph, 2019). Streaking of the bacteria was carried out twice to obtain pure colonies, as shown in **Fig. 4.**

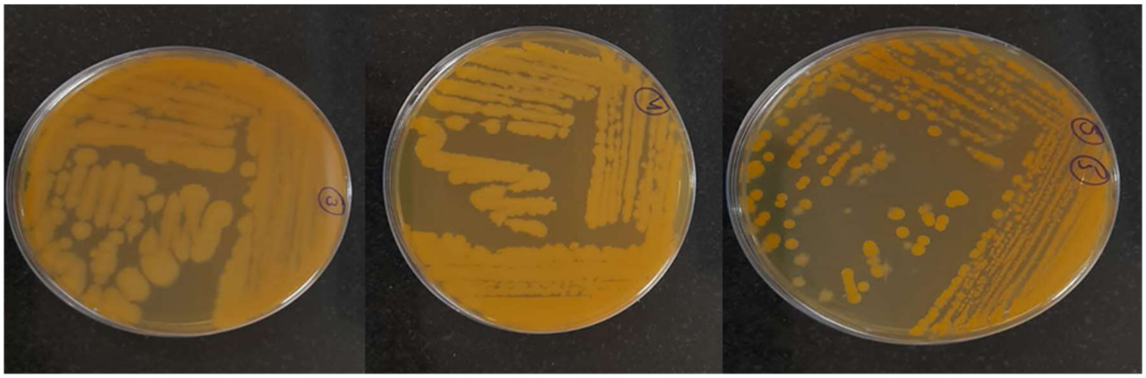
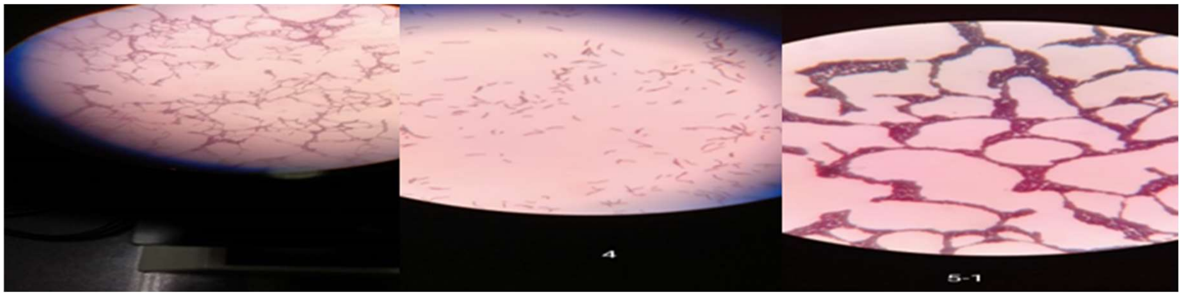

a b c

Fig. 4: Results of re-streaking (a) HMA1 (b) HMA2, and (c) HMA3

3.2 Preliminary identification of endophytic bacteria

This research identified three endophytic bacteria from the stem of *Kalanchoe pinnata*, which are named HMA1, HMA2, and HMA3. Morphological characterization showed that the endophytic bacterial isolates exhibited diverse colony shapes, elevations, and margins. Gram staining was performed to identify the bacterial isolates, which were gram-positive **(Fig. 5).** Furthermore, the growth of the bacterial isolates was assessed using EMB and MacConkey media. The observations indicate that the bacteria were gram-positive because there was no growth on the EMB or MacConkey agar media (**Table 1**).



a b c

Fig. 5: Results of Gram staining (a) HMA1 (b) HMA2, and (c) HMA3

Table 1: Gram staining and observations of EMB and MacConkey agar

Isolate	Gram	Shape	Margin	Elevation	EMB	MacConkey
HMA1	Positive	Rod	Irregular	No	No growth	No growth
HMA2	Positive	Rod	Irregular	No	Colorless	No growth
HMA3	Positive	Rod	Smooth	No	Colorless	No growth

Furthermore, all bacterial isolates were thoroughly characterized biochemically and physiologically using a variety of tests, including the methyl red test, citrate utilization, indole synthesis, phenylalanine deaminase, catalase, and sugar utilization tests (Panigrahi et al., 2020). The results unequivocally demonstrated that all three bacterial isolates were positive for indole production (**Table 2**).

Table 2: Biochemical analysis of endophytic bacterial isolates

Preliminary tests	**Bacterial isolates**		
Biochemical tests	HMA1	HMA2	HMA3
Methyl red	- - -	- - -	- - -
Citrate test	- - -	- - -	- - -
Indole production	+ + +	+ + +	+ + +
Catalase	- - -	- - -	- - -
Phenylalanine deaminase	- - -	- - -	- - -
Sugar utilization test	- - -	- - -	- - -

Where, + ++ sign indicated the colour change and the results to be positive for the tests performed in triplicates, and --- sign indicated no change in the colour and the results to be negative for the tests carried out in triplicates.

3.4. Identification of bacterial isolates using 16S rRNA sequencing

Agarose gel electrophoresis was used to separate and visualize genomic DNA from the endophytic bacterial isolates as shown in **Fig.6** .Through partial 16S rRNA region sequencing and BLAST analysis using the NCBI database, the present study was able to identify the genera and species of three endophytic bacterial isolates. Three bacterial isolates, namely *Bacillus kochii* strain Uyi_40, *Bacillus cereus* strain J6, and *Bacillus sp*. (in: firmicutes) strain HSH3, were identified after the bacterial isolates were sequenced. **Table 3** presents the identity of the isolated and NCBI sequences. The nucleotide sequences of this study were uploaded to GenBank and were given accession numbers PP765168, PP767370, and PP767371.

According to Janda and Abbot (2007), the 16S rRNA gene sequence is a useful informatics tool that can be used to identify isolates that do not fit any known biochemical profile, both generally and in species-wise. Thus, *Bacillus* emerged as the most represented genus among our isolates based on 16S rRNA sequencing results. The rod-shaped, spore-forming, gram-positive genus *Bacillus* is resistant to harsh environmental conditions. Previous studies have shown that endophytic bacteria of the genus *Bacillus* are present in sunflower, strawberries (Hirsch et al., 2024), *Panaxnoto ginseng* (Sun et al., 2018), *and Lonicera japonica* (Herman et al., 2020). In the context of *K. pinnata*, these *Bacillus* isolates may play a significant role, thereby opening up new avenues of research and development in the fields of plant biology and agriculture.

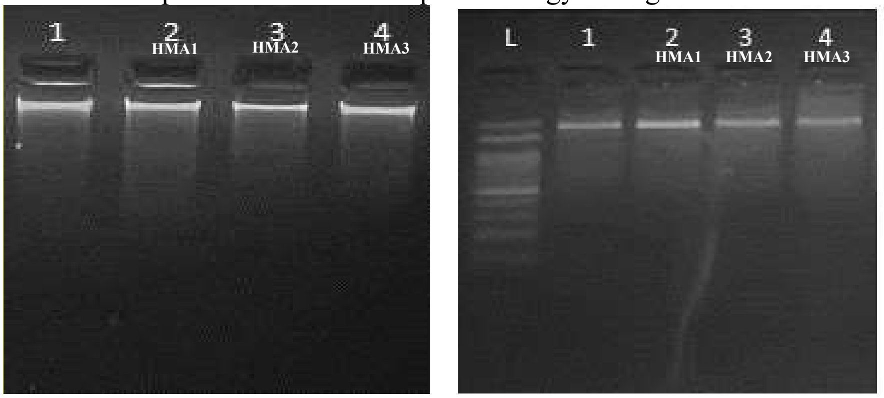


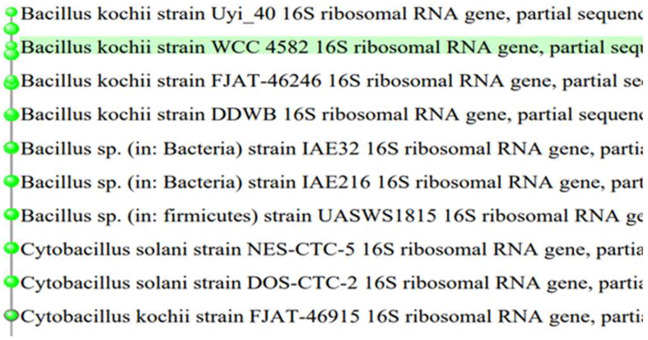
Fig.6 Agarose gel electrophoresis of genomic DNA from endophytic bacterial isolates

Table 3. Endophytic bacterial isolates identified and the percentage of similarities found in the NCBI database

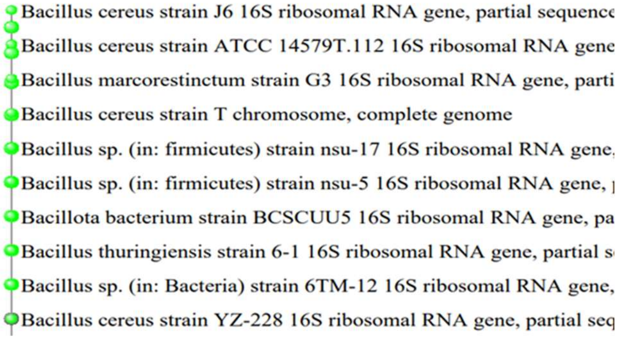
Endophytic bacterial isolates	Bacterial genera/species with the highest resemblance	Accession no. having maximum similarity	% similarity
HMA1	*Bacillus kochii* strain Uyi_40	MT507233.1	99.92%
HMA2	*Bacillus cereus* strain J6	PP414204.1	100%
HMA3	*Bacillus sp.* (firmicutes) HSH3	OR964086.1	99.41%

3.6. Phylogenetic tree of the 16 S rRNA region and BLAST data of bacteria isolated from the stem of *Kalanchoe pinnata*

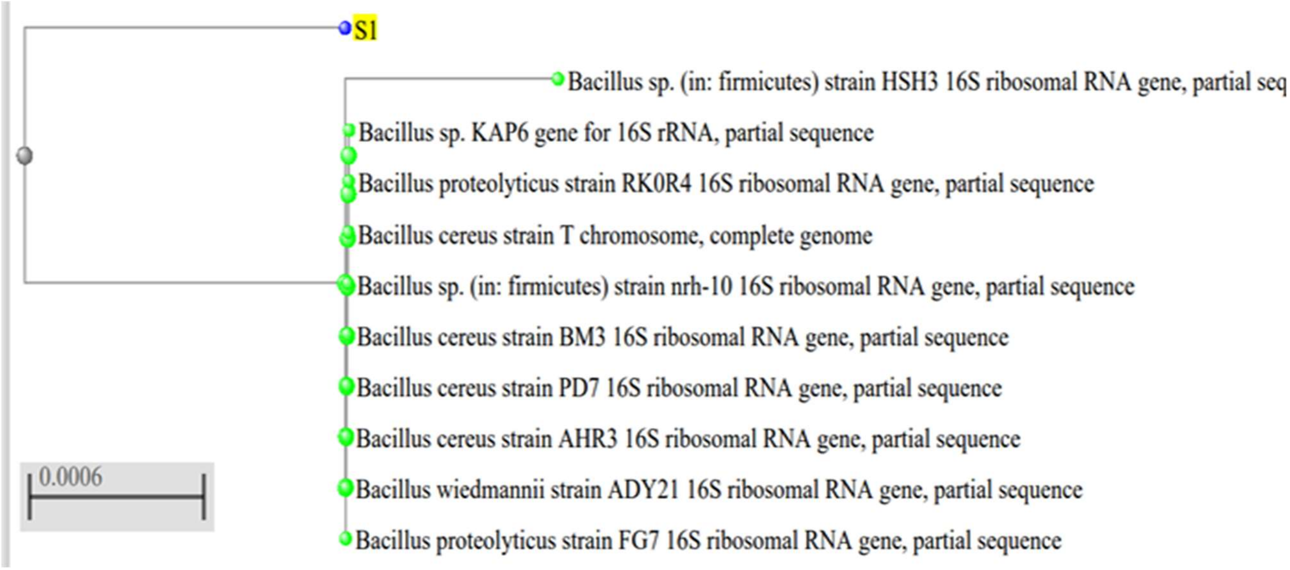
The amplified products were sequenced, and the resulting sequence was matched using the BLAST program with the sequence in the database. The experiment was successful for all three bacterial isolates i.e. HMA1, HMA2, and HMA3. The results inferred that isolate HMA1 showed that the bacteria belong to *Bacillus* sp. and HMA1 showed 99.92% resemblance to *Bacillus kochii* strain Uyi_40, as shown in (**Table 4)**, the isolate HMA2 showed 100% resemblance to *Bacillus cereus* strain J6, as shown in (**Table 5)** and the isolate HMA3 showed 99.41% resemblance to *Bacillus* sp. (in firmicutes) strain HSH3 as depicted in (**Table 6).** Additionally, a phylogenetic tree (**Fig 7)** with the matching sequence also confirmed that HMA1, HMA2, and HMA3 were closely associated with other *Bacillus* strains.



7 (a)



7 (b)



7 (c)

Fig. 7: Phylogenetic relationship between the isolate and its matching sequence for HMA1 (8a), HMA2 (8b) and HMA3 (8c)

Table 4: BLAST results of HMA1

BLAST DATA

Sl. No.	Organism Name	Accession No.	% Match
1	Bacillus kochii strain Uyi_40 16S ribosomal RNA gene, partial sequence	MT507233.1	99.92
2	Bacillus kochii strain WCC 4582 16S ribosomal RNA gene, partial sequence	MN075515.1	99.92
3	Bacillus kochii strain FJAT-46246 16S ribosomal RNA gene, partial sequence	MK859993.1	99.92
4	Bacillus kochii strain DDWB 16S ribosomal RNA gene, partial sequence	MK537364.1	99.92
5	Bacillus sp. (in: Bacteria) strain IAE32 16S ribosomal RNA gene, partial sequence	MK414976.1	99.92
6	Bacillus sp. (in: Bacteria) strain IAE216 16S ribosomal RNA gene, partial sequence	MK414919.1	99.92
7	Bacillus sp. (in: firmicutes) strain UASWS1815 16S ribosomal RNA gene, partial sequence	MH671860.1	99.92
8	Cytobacillus solani strain NES-CTC-5 16S ribosomal RNA gene, partial sequence	MF079294.1	99.92
9	Cytobacillus solani strain DOS-CTC-2 16S ribosomal RNA gene, partial sequence	MF076225.1	99.92
10	Cytobacillus kochii strain FJAT-46915 16S ribosomal RNA gene, partial sequence	MG651527.1	99.92

Table 5: BLAST results for HMA2

BLAST DATA

Sl. No.	Organism Name	Accession No.	% Match
1	Bacillus cereus strain J6 16S ribosomal RNA gene, partial sequence	PP414204.1	100
2	Bacillus cereus strain ATCC 14579T.112 16S ribosomal RNA gene, partial sequence	PP178677.1	100
3	Bacillus marcorestinctum strain G3 16S ribosomal RNA gene, partial sequence	PP177327.1	100
4	Bacillus cereus strain T chromosome, complete genome	CP130491.1	100
5	Bacillus sp. (in: firmicutes) strain nsu-17 16S ribosomal RNA gene, partial sequence	OR392899.1	100
6	Bacillus sp. (in: firmicutes) strain nsu-5 16S ribosomal RNA gene, partial sequence	OR392887.1	100
7	Bacillota bacterium strain BCSCUU5 16S ribosomal RNA gene, partial sequence	OR367221.1	100
8	Bacillus thuringiensis strain 6-1 16S ribosomal RNA gene, partial sequence	OR365308.1	100
9	Bacillus sp. (in: Bacteria) strain 6TM-12 16S ribosomal RNA gene, partial sequence	OP696627.1	100
10	Bacillus cereus strain YZ-228 16S ribosomal RNA gene, partial sequence	OK474777.3	100

Table 6: BLAST results for HMA3

BLAST DATA

Sl. No.	Organism Name	Accession No.	% Match
1	Bacillus sp. (in: firmicutes) strain HSH3 16S ribosomal RNA gene, partial sequence	OR964086.1	99.41
2	Bacillus sp. KAP6 gene for 16S rRNA, partial sequence	AB638891.1	99.41
3	Bacillus proteolyticus strain FG7 16S ribosomal RNA gene, partial sequence	MN833036.1	99.34
4	Bacillus wiedmannii strain ADY21 16S ribosomal RNA gene, partial sequence	MH084807.1	99.34
5	Bacillus cereus strain BM3 16S ribosomal RNA gene, partial sequence	KY773608.1	99.34
6	Bacillus cereus strain PD7 16S ribosomal RNA gene, partial sequence	KY773589.1	99.34
7	Bacillus cereus strain AHR3 16S ribosomal RNA gene, partial sequence	KT456542.1	99.34
8	Bacillus proteolyticus strain RK0R4 16S ribosomal RNA gene, partial sequence	PP177545.1	99.34
9	Bacillus cereus strain T chromosome, complete genome	CP130491.1	99.34
10	Bacillus sp. (in: firmicutes) strain nrh-10 16S ribosomal RNA gene, partial sequence	OR392955.1	99.34

3.7. Antioxidant activity of bacterial samples

Bacterial isolate HMA3, isolated from *Kalanchoe pinnata* showed the best antioxidant activity giving the % inhibition as 19.433 ± 0.68. Data revealed that bacterial isolate HMA2 gave the value of % inhibition as 16.659 ± 0.42, and bacterial isolate HMA1 showed the percentage as 14.144 ± 0.23 **(Table 7)**.

Table 7: Antioxidant activity of bacterial isolates

Samples	Antioxidant activity (%) inhibition
HMA1	14.144±0.23
HMA2	16.659±0.42
HMA3	19.433±0.68

Conclusion

The current investigation focused on separating endophytic bacteria from *Kalanchoe pinnata* and performing phylogenetic analysis of the isolated bacteria. The *Bacillus* species of bacteria that were isolated from the stem of *Kalanchoe pinnata* provided distinct advantages over other biocontrol bacteria due to their ease of growth, storage, and potential application as spores on plant seeds or in inoculants. It also exerts a protective effect against various microbial pathogens, potentially enhancing plant growth. These *Bacillus* isolates hold immense potential for agricultural applications, spurring new avenues of research and development and offering practical solutions for the farming and biotechnological sectors.

With an increasing understanding of endophytic bacteria, it may be possible to exploit their unique abilities to generate bioactive chemicals on their own or along with plants. Applying helpful microorganisms in the form of biofertilizers in combination increases crop benefits, making them a competitive option for organic farming. Endophytic bacteria may be a valuable source of novel antibiotics in the future. Therefore, the current research explores underutilized endophytic bacteria and conducted the phylogenetic analysis.

Conflicts of interest: Authors declared no conflict of interest.

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Author contribution: Himani- Conceptualization, Methodology, Data analysis, Writing–Original Draft, Writing – Review & Editing; Ishu Khangwal: Writing – Review & Editing, Conceptualization, Validation, Supervision, Nishant Kumar : Supervision, Review & Editing; Himanshu : Writing- Review & Editing.

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