



RESEARCH ARTICLE

Biocontrol potential of stem endophytic bacteria against blast disease (*Magnaporthe grisea*) in pearl millet

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Abstract

Pearl millet (*Pennisetum glaucum* (L.) R. Br.) is a cereal crop in Asia and Africa, with India being a significant centre for its diversification. Pearl millet faces numerous challenges, including various fungal diseases that significantly impact yields. Among these, the blast disease caused by the fungus *Magnaporthe grisea* has become a major concern for both forage and grain production in India. The characteristic symptoms include greyish, water-soaked lesions, leading to chlorosis and reduced yields. A promising approach is the use of indigenous bacterial endophytes with biocontrol capabilities. These endophytes produce phytohormones along with secondary metabolites, which increase the tolerance of the plant level against various stresses and pathogens. Therefore, this investigation was conducted to evaluate the effectiveness of indigenous bacterial endophytes. Field evaluation during kharif 2021 and 2022 demonstrated that seed treatment combined with foliar spray of PMSEB 3 (T4) recorded the lowest disease incidence at 60 DAS (56.94 % and 56.93 % respectively) compared to higher incidence in other treatments. It also improved germination up to 71.4 % and 69.33 % in the respective years. The morphological and biochemical traits of PMSEB 3 exhibited distinctive colony morphology, fast growth and rod-shaped cells. It showed metabolic flexibility, with the ability to perform mixed acid fermentation, utilise citrate as a carbon source and hydrolysed starch. These traits make PMSEB 3 a valuable resource for biocontrol of pearl millet blast disease. The study highlights the significance of utilising indigenous bacterial endophytes as a sustainable and eco-friendly approach for managing pearl millet blast disease.

Keywords: endophytic bacteria; *Magnaporthe grisea*; IDM strategies; phytohormone; disease severity; disease incidence

Introduction

Pearl millet (*Pennisetum glaucum* (L.) R. Br.) is an anciently cultivated cereal crop in Asia and Africa. India is a significant centre for pearl millet diversification, making it the world's 6th most important cereal crop, following maize, rice, wheat, barley and sorghum (1). In India, it ranks as the 4th most widely grown food crop, trailing only rice, wheat and maize. India holds the distinction of being the world's largest producer of pearl millet, encompassing an area of 7.653 million hectare, with an average production of 9.62 million tons with productivity rate of 1086 kg/ha during 2021–2022 season.

However, the crop also faces substantial challenges in the form of various diseases, which lead to reduced yields and ultimately economic losses for farmers and the nation (2). Among these diseases, fungal pathogens pose significant threats, including downy mildew or green ear disease (*Sclerospora graminicola*), rust (*Puccinia substriata* var. *indica*), smut (*Tolyposporium penicillariae*) and sugary disease or ergot of bajra (*Claviceps fusiformis*).

Additionally, leaf spot diseases caused by pathogens like *Pyricularia grisea*, *Bipolaris setariae*, *Cercospora penniseti*, *Curvularia penniseti* and *Drechslera dematioidea* further exacerbate the problem, leading to foliage damage and reduced yields. Among all of these, the blast disease caused by the ascomycete fungus *Magnaporthe grisea* ((anamorph: *Pyricularia grisea* (Cook) Sacc.). Earlier, it was considered insignificant, when recorded for the first time in Uganda in 1913, but now it has emerged as a serious threat, affecting both forage and grain production of pearl millet in India (3).

The first recorded instance of this disease was in Uganda in 1933 and it was long considered a minor issue (4). However, it has recently become a severe threat to pearl millet grain and fodder production. The disease manifests as greyish water-soaked lesions on the foliage, which gradually expand and become necrotic. This results in extensive chlorosis and premature drying of the leaves (5). The lesions are often surrounded by a chlorotic halo and in a shape of small, roundish, elliptical, diamond-shaped to elongated, measuring between 1–2 mm to 20 mm. Severe infections on plants lead to either no grain production or the

development of only a few shrivelled grains in blasted florets. The pathogen can cause both quantitative and qualitative damage that can result in losses of up to 100% (6).

Within the context of integrated plant disease management (IDM), researchers have explored the utilisation of indigenous bacterial endophytes with biocontrol capabilities as an environmentally benign and ecologically efficient technique (7). Bacterial endophytes have the potential to benefit host plants directly by promoting their growth through processes such as the production of phytohormones like indole acetic acid (IAA), cytokinin and the availability of assimilable nitrogen through biological nitrogen fixation (BNF). These endophytes also produce secondary metabolites such as siderophores, antibiotics, hydrogen cyanide (HCN) and enzymes like 1-Aminocyclopropane-1-carboxylate (ACC) deaminase, cellulase and protease. These compounds play a crucial role in enhancing tolerance to both biotic and abiotic stresses through indirect mechanisms (8, 9). Consequently, this strengthens the plant's immune system and protects it from infection by plant pathogens (10).

In this context, the present investigation was undertaken with the objective of isolating, characterising and evaluating stem endophytic bacteria of pearl millet for their antagonistic potential against *Magnaporthe grisea*, the causal agent of blast disease. The study also aims to assess the biocontrol efficacy and plant growth-promoting attributes of selected endophytic isolates under *in vitro* and *in vivo* conditions.

Materials and Methods

Collection, isolation and maintenance of endophytic bacteria from pearl millet

All tools, glassware and phyta jars were autoclaved for 60 min at 121 °C and 15 psi pressure in order to prepare them for tissue culture. Sterile double-distilled water was combined with the components of the MS medium and 1N HCl or 1N NaOH was used to bring the pH down to 5.6. BAP (6-Benzyl amino purine 1 to 2 mL/L), NAA (naphthalene acetic acid 0-0.4 mL/L) and 0-1 mg/l IBA were plant preservative combinations used to create half- and full-strength MS medium with sucrose (3 % w/v weight/volume) and varying PGR ratios. The media solidifying agent is 0.8 % agar media. The MS culture (8) medium is sterile at 121 °C and at 15 psi pressure for 15 min. Plant growth regulators were incorporated into sterile medium at temperature of 50–60 °C. The PGRs were filtered using a 22 µm syringe filter before being introduced to the medium. The media were dispensed into phytajars, exposed to UV- light overnight and then autoclaved autoclaved again at 121 °C for 15 min.

Evaluation of the biocontrol activity of endophytic bacteria of pearl millet

Isolated bacterial colonies were evaluated for biocontrol activity under *in vitro* conditions.

Siderophore production

Siderophore production was detected by CAS (Chrome azurol S) assay (11).

Solutions: Chrome azurol S (CAS) agar medium

Dye solution (Solution A): 10 mL of iron (III) solution containing 1 mM FeCl₃·6H₂O in 10 mM HCl were combined with 50 mL of dye

solution containing 60.5 mg CAS produced in distilled water. A solution comprising 40 mL of distilled water and 72.9 mg of hexadecyl trimethyl ammonium bromide (HDTMA) was gradually mixed with the mixture. 10 mL of this solution were taken for every 100 mL and the resulting dark blue liquid was autoclaved for 10 min at a pressure of 15 pounds.

Solution B: A basal medium containing deferrated 1M sucrose (3 mL); deferrated 1M CaCl₂ (0.4 mL); deferrated 1M MgSO₄·7H₂O (0.8 mL); 2 % K₂HPO₄ (10 mL), 10 % NaCl (2 mL); 5 % Na₂MoO₄ (0.1 mL); Pipes buffer (30.42 g) and agar agar (15 g) in 800 mL of distilled water was prepared. After adding 50 % (w/w) NaOH to the medium to bring its pH down to 6.8, it was autoclaved for 20 min at 15 pounds of pressure.

Once the mixture had cooled to 50 °C, 30 mL of a 10 % mannitol solution was added as a carbon source. The dye solution (solution A) was then gently mixed in to prevent air bubbles from forming. Sterilised petri dishes were filled with the molten media. Each log phase developed culture's 5 µL of inoculant were spotted on siderophore plates and incubated for 3–4 days at 28 ± 2 °C. The decolourisation of the blue-coloured ferric dye complex, which produced yellow halo zones surrounding the colonies, showed the presence of an iron chelator (siderophore).

ACC utilisation

The minimal medium supplemented with 2 mM ACC was prepared (12). On ACC-amended minimal medium plates, a loopful of a 48 hr-old culture of bacterial endophytes was seen. After 5 days of incubation at 28 ± 20 °C, growth on ACC and medium plates supplemented with ammonium sulphate was measured. The bacterial isolates that grew well on medium plates supplemented with ACC demonstrated a high efficiency of using ACC as a source of nitrogen and these isolates were chosen for more research. Ammonium sulphate-containing minimal medium plates were utilised as control plates to compare the development of various bacterial isolates.

Hydrogen cyanide production

Inoculum of different bacterial endophytes was prepared by inoculation of 48 hr old culture from nutrient agar slants to freshly prepared Kings B broth. After 72 hr of incubation at 28 ± 2 °C, cyanide generation was found utilising picrate/Na₂CO₃ paper glued to the underside of the test tube. Weak, moderate, or great cyanogenic potential was indicated by a shift in hue from yellow to brown, brown, reddish brown (13).

Indole-3-acetic acid (IAA) production

Indole-3-acetic acid production in different isolates of endophytes were detected by inoculating bacterial suspension in 10 mL Luria Bertanni broth containing tryptophan 0.01 % (L-Trp) separately and incubated at 28 ± 2 °C for 3–6 days (14). Appearance of pink colour confirmed the production of IAA. By adding 2 mL of Salkowski's reagent (1 mL of 0.5 mL FeCl₃ in 50 mL of 35 % HClO₄) to 1 mL of culture supernatant and uninoculated broth using Salkowski's reagent as a reference, the quantity of IAA (µg/mL-1) was quantitatively quantified. After by 20 min, the absorbance of pink colour was measured spectroscopically at 535 nm using an ELICO UV-VIS spectrophotometer. The IAA content was then determined using a standard curve.

Ammonia production

The ability of bacterial isolates to produce ammonia in peptone

water was evaluated. 10 mL of peptone water were added to each tube containing freshly developed cultures and the tubes were then incubated at 30 °C for 4 days. Each tube received 0.5 mL of Nessler's reagent. Small amounts of ammonia were indicated by the development of a light yellow hue, whilst increased ammonia production was indicated by a deep yellow to brownish tint.

Cellulolytic activity

Pearl millet root bacterial endophytes were screened for cellulolytic activity using carboxymethylcellulose (CMC) agar plates (15). On CMC agar plates, freshly grown bacterial cultures were spot-inoculated, incubated at 28 ± 2 °C for 48 hr and then flooded with a 0.1 % aqueous solution of congo red for 15–20 min before the plate surface was cleaned with 1 M NaCl. The clear zone surrounding the colony showed the generation of cellulase.

Zn solubilisation

Zn solubilisation in endophytic bacterial isolates was measured qualitatively (16). Zinc oxide (ZnO) and zinc phosphate Zn₃(PO₄)₂ were added individually to isolates to solubilise zinc on Tris-minimal medium at a concentration equal to 0.1 % Zn. Pure endophytic bacteria were spotted on plates, cultured in the dark at 28 °C and after 7 days, the creation of a distinct halo zone around bacterial growth was monitored.

Zinc solubilisation efficiency (SE) was calculated as per standard methodology (17).

$$SE = \frac{\text{Diameter of solubilisation halo zone}}{\text{Diameter of colony}} \times 100$$

Phosphate solubilisation

Phosphate solubilisation ability of endophytic bacteria was determined qualitatively by streaking strains on National Botanical Research Institutes Phosphate growth (NBRIP) medium (18). Presence of yellow clear halo zone around bacterial growth after 5–7 days incubation period at 28 ± 2 °C was used as indicator for positive P solubilisation (19).

In vitro evaluation of promising pearl millet endophytic bacteria against *Magnaporthe grisea*

Isolation of pathogen from blast infected leaf sample of pearl millet

The pathogen *Magnaporthe grisea* was isolated from pearl millet leaves exhibiting typical blast symptoms collected from the field area of the Department of Plant Pathology. Isolation was performed under aseptic conditions using the spore drop method with slight modifications. Small, infected leaf bits (3–5 mm) were excised from typical blast lesions and surface sterilised in 1 % sodium hypochlorite (NaOCl) for 30 sec, followed by 3 rinses with sterile distilled water. The disinfected leaf pieces were placed on sterile cotton moistened with distilled water in sterile petri plates and incubated at 25 ± 2 °C for 48 hr to promote sporulation. After incubation, the leaf fragments were transferred to a Lesion Print (LP) setup. The bits were placed on sterile moist cotton fixed to the inner lid of a small petri plate (5.5 cm diameter), while the base contained rice straw extract agar (RSEA) medium. Plates were sealed and incubated at 25 ± 2 °C for 3 days until fungal colonies appeared on the medium as lesion prints (20, 21).

For purification, a single mycelial disc was macerated in rice straw extract broth and mixed with lukewarm RSEA

medium. After 3–4 days of incubation at 25 ± 2 °C, individual colonies developed. A single spore colony was selected and transferred to OMA slants for storage.

In vitro evaluation by using dual culture technique

The *in vitro* antagonistic effect of the endophytic bacterial isolates against the fungal pathogen was assessed following dual culture technique. Both the organisms were allowed to grow on dual culture media, King's B + PDA (1:1) in the petri dishes. Pure and fresh cultures of endophytic bacteria and fungi was obtained by culturing in King's B for 72 hr and PDA for 4–5 days respectively. For the antagonistic study, a mycelial disc of 4 mm diameter agar plug containing hyphae were placed in the centre of a petri dish containing dual media under aseptic conditions. Each isolate of bacteria was streaked in circular form maintaining 3 cm distance from the test fungal plug (22). The petri dishes containing only the test fungus was considered as the control. The petri dishes were incubated at 28 ± 2 °C for 6 days and the observations was recorded.

Treatments:

T₁ - PMSEB 3

T₂ - PMSEB 7

T₃ - PMSEB 10

Observation to be recorded:

Mycelial growth was recorded when there is 90 mm growth in check plates at 28 ± 2 °C. Percent growth inhibition was calculated, following the method described (23) as below.

$$SE = \frac{C - T}{C} \times 100$$

Where, C = colony diameter of fungal pathogen in control petri dishes (cm) and

T = colony diameter of fungal pathogen in the inhibition petri dishes (cm).

In vivo evaluation of promising pearl millet endophytic bacteria against blast disease of pearl millet

Field trial were carried out by seed treatment and foliar application with best endophytic bacteria alone and in combination at Research Farm, Department of Plant Pathology, Chaudhary Charan Singh Haryana Agricultural University, Hisar, India. Blast severities were recorded using 0–9 scale (24) as detailed in Table 1.

The experiment was conducted with 9 treatments during 2021 and 2022 in randomised block design each having 4 replications. The plot size was 4.5 m x 3 m the distance between rows and seedlings was 45 and 10 cm respectively. Six lines were maintained for each treatment (Plot) and 50 seeds were sown in each plot. Seeds of pearl millet cultivars viz HHB-67 Imp was surface sterilised with 0.1 % HgCl₂ for 2–3 min, washed with distilled water many times and dried with the use of sterile blotter paper. Seeds was inoculated with 1 mL inoculum of selected pearl millet root bacterial endophytic isolates independently and in combination. The foliar application of selected endophytes was applied in respective plots after 15 days of sowing. The observations on total number of plants, plants exhibiting symptoms of blast disease were recorded at 30, 45 and 60 days after sowing. Germination percentage disease incidence was calculated and yield data as well as test weight was recorded.

Table 1. List of blast severities recorded during field trial

Rating scale	Symptoms and lesions	Disease reaction
0	No lesion	Highly resistant
1	No lesion to small brown specks of pin head size without sporulating center	
2	Large brown specks	
3	Small, roundish to slightly elongated, necrotic gray spot, about 1–2 mm in diameter with a brown margin	Resistant
4	Elliptical lesions, 1–2 cm long, frequently confined to the area between main veins, covering <2 % of leaf area	Moderately resistant
5	Typical blast lesions covering <10 % of the leaf area	
6	Typical blast lesions covering 10–25 % of the leaf area	
7	Typical blast lesions covering 26–50 % of the leaf area	Susceptible
8	Typical blast lesions covering 51–75 % of the leaf area and many leaves dead	Highly susceptible
9	>75 % leaf area covered with lesions and most leaves dead	

Treatments:

T₁- Seed treatment with PMSEB 3 at 5 mL /kg seed

T₂- Seed treatment with PMSEB 7 at 5 mL /kg seed

T₃- Seed treatment with PMSEB 10 at 5 mL /kg seed

T₄- Seed treatment + Foliar spray with PMSEB 3 at 1×10⁸ mL⁻¹ till run off

T₅- Seed treatment + Foliar spray with PMSEB 7 at 1×10⁸ mL⁻¹ till run off

T₆- Seed treatment + Foliar spray with PMSEB 10 at 1×10⁸ mL⁻¹ till run off

T₇- Seed treatment with PMSEB 3 + PMSEB 7+ PMSEB 10

T₈- Seed treatment + Foliar spray with PMSEB 3 + PMSEB 7+ PMSEB 10

T₉- Control (Untreated)

Characterisation of promising endophytes on the basis of biochemical characters

Pearl millet root endophytic bacterial isolates found promising in screen house and field experiments were examined for various morphological, biochemical characteristics as per procedure described in Bergey's manual of Determinative Bacteriology (25).

Sequencing of 16S rRNA of pearl millet root bacterial endophytes

Promising bacterial endophytes retrieved from the pearl millet roots were identified based on partial 16S rRNA sequencing. The CTAB technique was used to extract the endophytic bacteria's genomic DNA, which was then amplified using the forward primer 5' AGA GTT TGA TCC CTC AG 3' and the reverse primer 5' AGA GAG GTG ATC CAG CCG CA 3'. 1 µL of template DNA, 400 ng of 16S Forward primer, 400 ng of 16S Reverse primer, 4 µL of dNTP (2.5 mM, each), 10 µL of 10X Taq DNA polymerase assay buffer and 1 µL of Taq DNA Polymerase Enzyme (3U/µl) were used in the PCR process. The reaction mixture's final volume was set at 100 µL. PCR was performed in a thermocycler with a 5 min denaturation at 95 °C, followed by 35 cycles of 30 sec at 94 °C, 15 sec at 50 °C, 1.30 min at 72 °C and a final extension of 7 min at 72 °C. The amplified PCR products were examined using a gel documentation system and electrophoresed on 1 % agarose gels using TAE buffer. Following sequencing, the 16S rRNA gene partial sequences of potential bacterial endophytes were acquired. The identified gene sequence was submitted to NCBI Gene Bank and accession number was obtained.

Statistical analysis

All experiments were conducted using a completely randomised

design (CRD) with a minimum of 5 replicates per treatment and repeated 3 times for consistency. Data were analysed as mean ± standard error. Analysis of variance (ANOVA) was used to determine significant differences in shoot length and shoot number using OPSTAT software (<http://14.139.232.166/opstat/>).

Results and Discussion**Isolation of pearl millet stem bacterial endophytes and their evaluation for biocontrol activities**

Plant samples from stem of pearl millet were collected from plant pathology experimental area of CCS Haryana Agricultural University, Hisar with latitude 29°14' N and longitude of 75°70' E, having sandy loam soil texture of the kharif 2021. A total of 13 pearl millet stem bacterial endophytes (PMSEB) were obtained (PMSEB 1, PMSEB 2, PMSEB 3, PMSEB 4, PMSEB 5, PMSEB 6, PMSEB 7, PMSEB 8, PMSEB 9, PMSEB 10, PMSEB 11, PMSEB 12 and PMSEB 13) (Fig. 1).

Out of 13 bacterial isolates, 4 isolates show positive for siderophore production, HCN production, ACC utilisation, 3 showed positive for IAA production, 5 showed positive for ammonia production, 6 showed positive for cellulolytic activity, 3 showed positive for Zn solubilisation and 6 showed positive for phosphate solubilisation (Table 2 and 3).

In vivo evaluation of promising pearl millet endophytic bacteria against blast disease of pearl millet

During kharif 2021, out of all treatments, T₄ (Seed treatment + Foliar spray with PMSEB 3 at 1×10⁸ mL⁻¹ till run off) showed minimum % disease incidence at 60 DAS (56.94 %) and maximum germination percentage of 71.4, which is at par with the T₈ (Seed treatment + Foliar spray with PMSEB 3 + PMSEB 7+ PMSEB 10) showing the minimum % disease incidence at 60 DAS (57.62 %) and germination 70.4 %, whereas, T₂ (Seed treatment with PMSEB 7 at 5 mL/kg seed) showed the maximum disease incidence at 60 DAS that is 61.21 % and minimum germination 66.5 % (Table 4 and 5).

During kharif 2022, Out of all treatments, T₄ (Seed treatment + Foliar spray with PMSEB 3 at 1×10⁸ mL⁻¹ till run off) showed minimum % disease incidence at 60 DAS (56.93 %) and germination % of 69.3, which is at par with the T₈ (Seed treatment + Foliar spray with PMSEB 3 + PMSEB 7+ PMSEB 10) showing the minimum disease incidence at 60 DAS (59.97 %) and germination percentage of 72.1 %, whereas, T₅ (Seed treatment + Foliar spray with PMSEB 7 at 1×10⁸ mL⁻¹ till run off) showed the maximum % disease incidence at 60 DAS that is 61.21 % (Table 6 and 7).

Table 2. Biocontrol activity shown by pearl millet bacterial endophytes

Bacteria	Siderophore production	HCN production	ACC utilisation	IAA production
PMSEB 1	-	-	-	-
PMSEB 2	-	-	+	-
PMSEB 3	+	+	-	+
PMSEB 4	-	-	-	-
PMSEB 5	+	-	+	-
PMSEB 6	-	-	-	-
PMSEB 7	+	+	+	-
PMSEB 8	-	-	-	-
PMSEB 9	-	-	-	-
PMSEB 10	+	+	-	+
PMSEB 11	-	-	-	-
PMSEB 12	-	+	-	+
PMSEB 13	-	-	+	-

“+” indicates positive reaction and “-” indicates negative reaction.

Table 3. Biocontrol activity shown by pearl millet bacterial endophytes

Bacteria	Ammonia production	Cellulolytic activity	Zn solubilisation	Phosphate solubilisation
PMSEB 1	-	+	-	-
PMSEB 2	-	-	-	+
PMSEB 3	+	+	+	+
PMSEB 4	-	-	-	-
PMSEB 5	-	-	-	-
PMSEB 6	+	+	-	+
PMSEB 7	+	+	-	+
PMSEB 8	-	-	-	-
PMSEB 9	-	-	-	-
PMSEB 10	+	+	+	+
PMSEB 11	-	-	-	-
PMSEB 12	+	+	+	+
PMSEB 13	-	-	-	-

Table 4. Effect of seed treatment and foliar spray on germination %, disease incidence, yield and test weight during kharif 2021

Treatments	Germination (%)	Disease incidence (%)			Yield at maturity (Kg/ ha)	Test weight (g)
		30 DAS	45 DAS	60 DAS		
T ₁ - Seed treatment with PMSEB 3 at 5 mL /kg seed	69.5	39.49	44.05	59.05	890.37	8.5
T ₂ - Seed treatment with PMSEB 7 at 5 mL /kg seed	66.5	39.67	46.21	61.21	857.79	8.4
T ₃ - Seed treatment with PMSEB 10 at 5 mL /kg seed	67.4	40.48	47.71	62.71	869.53	8.3
T ₄ - Seed treatment + Foliar spray with PMSEB 3 at 1×10 ⁸ mL ⁻¹ till run off	71.4	29.59	41.94	56.94	954.41	9.1
T ₅ - Seed treatment + Foliar spray with PMSEB 7 at 1×10 ⁸ mL ⁻¹ till run off	67.8	40.54	47.103	62.10	871.32	8.4
T ₆ - Seed treatment + Foliar spray with PMSEB 10 at 1×10 ⁸ mL ⁻¹ till run off	68.86	39.62	46.79	61.79	893.23	8.4
T ₇ - Seed treatment with PMSEB 3 + PMSEB 7+ PMSEB 10	69.5	39.81	44.91	59.91	893.33	8.6
T ₈ - Seed treatment + Foliar spray with PMSEB 3 + PMSEB 7+ PMSEB 10	70.4	37.04	42.62	57.62	906.42	8.7
T ₉ - Control (Untreated)	59.7	47.61	54.62	69.62	765.57	8.2
CD (<i>p</i> = 0.05)	0.37	0.632	0.522	0.492	8.652	0.08
SE (m)	0.13	0.221	0.183	0.172	3.031	0.03

Table 5. Effect of seed treatment and foliar spray on % disease index during kharif 2021

Treatments	% disease index		
	30 DAS	45 DAS	60 DAS
T ₁ - Seed treatment with PMSEB 3 at 5 mL /kg seed	32.43	39.36	49.36
T ₂ - Seed treatment with PMSEB 7 at 5 mL /kg seed	33.65	38.88	48.87
T ₃ - Seed treatment with PMSEB 10 at 5 mL /kg seed	31.33	39.54	49.54
T ₄ - Seed treatment + Foliar spray with PMSEB 3 at 1×10 ⁸ mL ⁻¹ till run off	24.47	36.57	46.57
T ₅ - Seed treatment + Foliar spray with PMSEB 7 at 1×10 ⁸ mL ⁻¹ till run off	33.33	43.68	53.68
T ₆ - Seed treatment + Foliar spray with PMSEB 10 at 1×10 ⁸ mL ⁻¹ till run off	32.16	41.10	51.10
T ₇ - Seed treatment with PMSEB 3 + PMSEB 7+ PMSEB 10	32.83	39.67	49.77
T ₈ - Seed treatment + Foliar spray with PMSEB 3 + PMSEB 7+ PMSEB 10	31.23	37.11	47.11
T ₉ - Control (Untreated)	42.67	51.36	61.36
CD (<i>p</i> = 0.05)	0.575	0.439	0.442
SE (m)	0.202	0.154	0.154

Table 6. Effect of seed treatment and foliar spray on germination %, disease incidence, yield and test weight during kharif 2022

Treatments	Germination (%)	Disease incidence (%)			Yield at maturity (Kg/ ha)	Test weight (g)
		30 DAS	45 DAS	60 DAS		
T ₁ - Seed treatment with PMSEB 3 at 5 mL /kg seed	68.4	38.76	47.67	57.67	874.53	8.4
T ₂ - Seed treatment with PMSEB 7 at 5 mL /kg seed	65.91	40.75	51.43	61.43	842.36	8.3
T ₃ - Seed treatment with PMSEB 10 at 5 mL /kg seed	66.77	41.54	52.61	62.61	861.48	8.2
T ₄ - Seed treatment + Foliar spray with PMSEB 3 at 1×10 ⁸ mL ⁻¹ till run off	69.33	35.26	46.93	56.93	901.63	8.8
T ₅ - Seed treatment + Foliar spray with PMSEB 7 at 1×10 ⁸ mL ⁻¹ till run off	66.86	41.38	53.79	63.79	862.52	8.3
T ₆ - Seed treatment + Foliar spray with PMSEB 10 at 1×10 ⁸ mL ⁻¹ till run off	70.4	40.84	50.87	60.68	906.65	8.7
T ₇ - Seed treatment with PMSEB 3 + PMSEB 7+ PMSEB 10	70.7	38.27	47.83	57.83	912.43	8.8
T ₈ - Seed treatment + Foliar spray with PMSEB 3 + PMSEB 7+ PMSEB 10	72.1	36.52	49.97	59.97	923.53	9.1
T ₉ - Control (Untreated)	62.4	49.23	62.43	72.43	779.94	7.7
CD ($p = 0.05$)	0.369	0.546	0.579	0.463	9.991	0.071
SE (m)	0.129	0.192	0.203	0.162	3.503	0.027

Table 7. Effect of seed treatment and foliar spray on % disease index during kharif 2022

Treatments	% disease index		
	30 DAS	45 DAS	60 DAS
T ₁ - Seed treatment with PMSEB 3 at 5 mL /kg seed	34.77	46.15	56.15
T ₂ - Seed treatment with PMSEB 7 at 5 mL /kg seed	35.66	47.27	57.27
T ₃ - Seed treatment with PMSEB 10 at 5 mL /kg seed	33.63	44.73	54.7
T ₄ - Seed treatment + Foliar spray with PMSEB 3 at 1×10 ⁸ mL ⁻¹ till run off	26.32	34.45	44.45
T ₅ - Seed treatment + Foliar spray with PMSEB 7 at 1×10 ⁸ mL ⁻¹ till run off	30.74	41.98	51.98
T ₆ - Seed treatment + Foliar spray with PMSEB 10 at 1×10 ⁸ mL ⁻¹ till run off	30.96	40.68	50.87
T ₇ - Seed treatment with PMSEB 3 + PMSEB 7+ PMSEB 10	33.21	41.52	51.52
T ₈ - Seed treatment + Foliar spray with PMSEB 3 + PMSEB 7+ PMSEB 10	30.16	40.49	50.49
T ₉ - Control (Untreated)	39.07	52.24	62.24
CD ($p = 0.05$)	0.541	0.524	0.524
SE (m)	0.19	0.184	0.184

Table 8. Pooled analysis of treatments on disease incidence during kharif 2021 and kharif 2022

Treatments	Germination (%)	Disease Incidence (%)			Yield at maturity (Kg/ ha)	Test weight (g)
		30 DAS	45 DAS	60 DAS		
T ₁ - Seed treatment with PMSEB 3 at 5 mL /kg seed	68.95	39.12	45.86	58.33	882.4	8.45
T ₂ - Seed treatment with PMSEB 7 at 5 mL /kg seed	66.233	40.21	48.82	61.32	850	8.35
T ₃ - Seed treatment with PMSEB 10 at 5 mL /kg seed	67.083	41.01	50.16	62.66	865.45	8.25
T ₄ - Seed treatment + Foliar spray with PMSEB 3 at 1×10 ⁸ mL ⁻¹ till run off	70.35	32.42	44.43	56.93	928	8.983
T ₅ - Seed treatment + Foliar spray with PMSEB 7 at 1×10 ⁸ mL ⁻¹ till run off	67.33	40.96	50.44	62.94	866.9	8.35
T ₆ - Seed treatment + Foliar spray with PMSEB 10 at 1×10 ⁸ mL ⁻¹ till run off	69.63	40.23	48.73	61.23	899.95	8.583
T ₇ - Seed treatment with PMSEB 3 + PMSEB 7+ PMSEB 10	70.10	39.04	46.37	58.87	902.967	8.733
T ₈ - Seed treatment + Foliar spray with PMSEB 3 + PMSEB 7+ PMSEB 10	71.25	36.78	46.95	58.75	914.867	8.933
T ₉ - Control (Untreated)	61.05	48.42	58.25	71.02	772.8	7.95
CD for Seasons	7.054	3.324	4.339	5.56	9.4097	0.883
CD for Treatments	7.054	0.544	0.44	0.441	8.61	0.073
CD for Season x Treatments	7.054	0.769	0.622	0.624	12.177	0.103

Table 9. Pooled analysis of treatments on % disease index during kharif 2021 and kharif 2022

Treatments	Per cent disease index		
	30 DAS	45 DAS	60 DAS
T ₁ - Seed treatment with PMSEB 3 at 5 mL /kg seed	33.61	42.75	52.77
T ₂ - Seed treatment with PMSEB 7 at 5 mL /kg seed	34.66	43.07	53.08
T ₃ - Seed treatment with PMSEB 10 at 5 mL /kg seed	32.48	42.12	52.12
T ₄ - Seed treatment + Foliar spray with PMSEB 3 at 1×10 ⁸ mL ⁻¹ till run off	25.39	35.51	45.51
T ₅ - Seed treatment + Foliar spray with PMSEB 7 at 1×10 ⁸ mL ⁻¹ till run off	32.03	42.83	52.83
T ₆ - Seed treatment + Foliar spray with PMSEB 10 at 1×10 ⁸ mL ⁻¹ till run off	31.56	40.89	50.95
T ₇ - Seed treatment with PMSEB 3 + PMSEB 7+ PMSEB 10	33.02	40.59	50.59
T ₈ - Seed treatment + Foliar spray with PMSEB 3 + PMSEB 7+ PMSEB 10	30.69	38.80	48.80
T ₉ - Control (Untreated)	40.87	51.80	61.80
CD for Seasons	2.635	3.67	3.674
CD for Treatments	0.515	0.445	0.474
CD for Season x Treatments	0.728	0.63	0.674

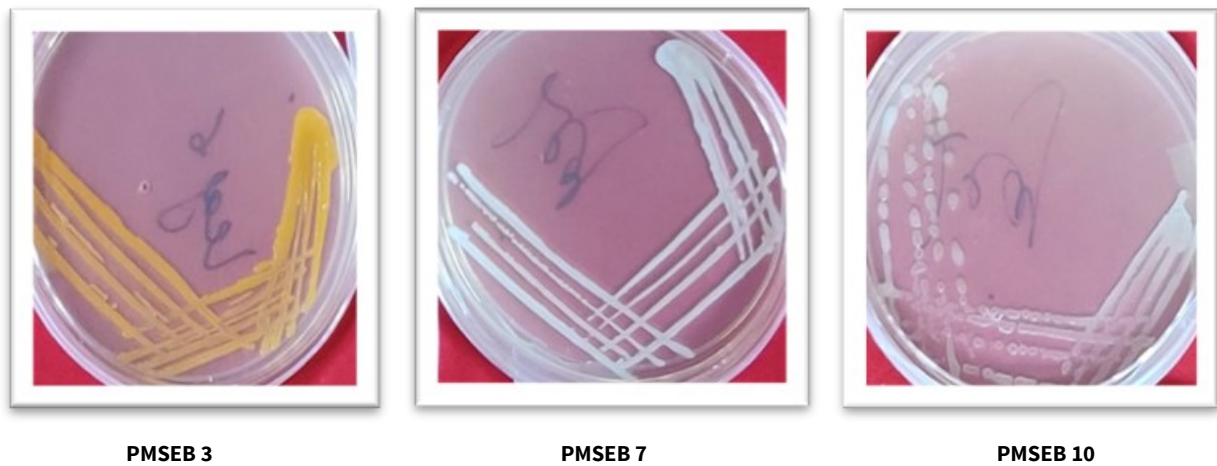


Fig. 1. Isolated bacterial colonies were evaluated for biocontrol activity under *in vitro* conditions.

Pooled analysis of Treatments

The data presented in Table 8 and 9 provides a comprehensive analysis of the impact of various treatments on disease incidence and % disease index during the kharif seasons of 2021 and 2022. The data shows that these treatments have a significant influence on germination rates, disease incidence at different growth stages (30 DAS, 45 DAS and 60 DAS), yield at maturity (Kg/ha) and test weight (g). For instance, the treatment T4, which involves seed treatment and foliar spray with PMSEB 3, demonstrated a notable increase in germination and a substantial reduction in disease incidence at 45 DAS. This data allows for the identification of effective treatment strategies for disease management and yield improvement in kharif crops. The results suggest that T4 significantly reduces disease index, particularly at 30 DAS, which indicates the importance of integrated treatment approaches. The critical difference (CD) values in both tables help researchers determine the significance of treatment effects, with variations among stages and parameters.

Morphological and biochemical characterisation of promising pearl millet endophytic bacteria of pearl millet cultivar

Morphological characteristics of a promising pearl millet bacterial endophyte identified as PMSEB 3. These morphological features offer insights into the nature and potential of this bacterium (Table 10).

Colony morphology: PMSEB 3 exhibits yellowish colonies that are circular and convex in shape. This suggests that it has distinctive visual characteristics, making it easy to distinguish from other bacterial strains.

Gram reaction: The endophyte is Gram-positive (+ve). Gram-positive bacteria have a thick peptidoglycan layer in their cell walls, which can impact their ability to interact with the host plant and may be significant for its role as an endophyte.

Colony diameter (mm): The colonies have a relatively small

diameter of 1.4 mm, indicating that it may not require an extensive amount of space to grow, which could be advantageous in a crowded plant environment (Fig. 2).

Growth: PMSEB 3 displays very fast growth, which could be a valuable characteristic for a bacterial endophyte, especially if it needs to quickly establish itself within the plant.

Shape: The bacterium is rod-shaped, indicating a specific cellular structure. This shape might have implications for its motility and interaction with host plant cells.

Length (μm) and width (μm): The bacterium has a length of 2.417 μm and a width of 0.770 μm , which is relevant for understanding its size and may influence its ability to penetrate plant tissues and interact with other microorganisms.

Spore production: PMSEB 3 does not produce spores. This information is essential, as spore formation can be a significant factor in bacterial survival and dispersal.

PMSEB 3, a potential pearl millet bacterial endophyte, displays a range of biochemical characteristics with implications for its role in plant health and growth. Notably, it is Methyl Red and

Table 11. Biochemical characters of promising pearl millet bacterial endophytes

Sl. No.	Test	PMSEB 3
1.	Indole production	-
2.	Methyl Red	+
3.	Vokges-Proskauer reaction	+
4.	Citrate utilisation	+
5.	Oxidase	+
6.	Catalase	+
7.	Starch hydrolysis	+
8.	Gelatin utilisation	-
9.	Motility	+
10.	Glucose utilisation	+
11.	Lactose utilisation	+
12.	Sorbitol utilisation	-
13.	Arabinose utilisation	-
14.	Rhamnose utilisation	+
15.	Sucrose utilisation	+
16.	Urease production	-

Table 10. Morphological characters of promising pearl millet bacterial endophytes

Sl. No.	Morphological characters	PMSEB 3
1.	Colony morphology	Yellowish, Circular, Convex
2.	Gram reaction	+ ve
3.	Colony diameter (mm)	1.4
4.	Growth	Very Fast
5.	Shape	Rod
6.	Length (μm)	2.417
7.	Width (μm)	0.770
8.	Spore production	NO

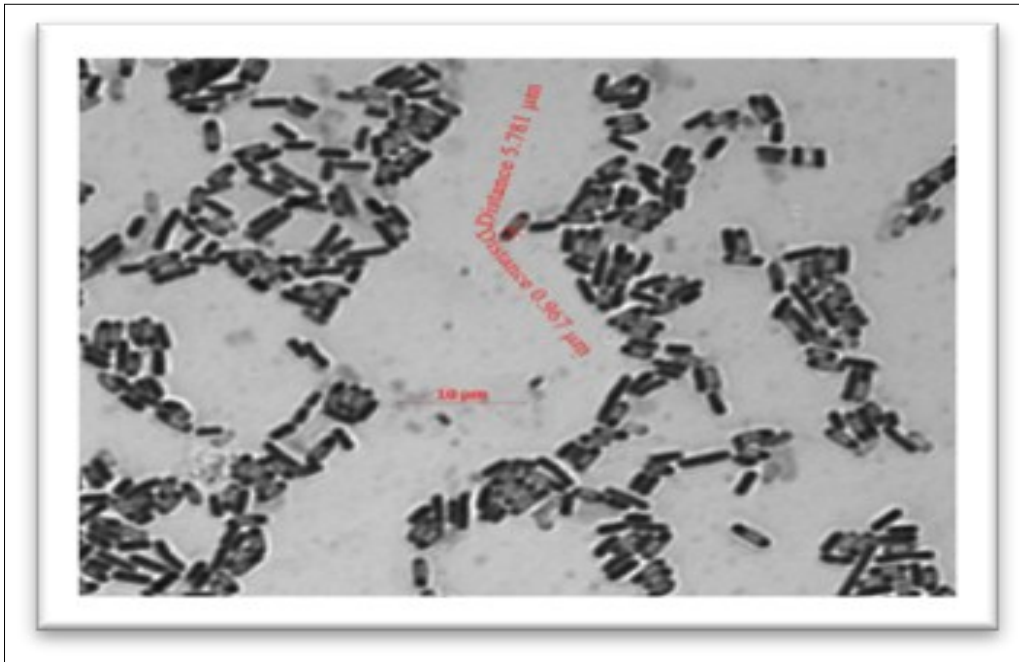


Fig. 2. Size of promising pearl millet endophytic bacteria.

Voges-Proskauer positive, indicating mixed acid fermentation and acetoin production. This metabolic flexibility is advantageous in diverse environments. PMSEB 3 can utilise citrate as a carbon source, potentially facilitating its survival within the host plant. Additionally, it exhibits positive traits such as motility, the utilisation of glucose, lactose and sucrose, which suggest adaptability and energy generation capabilities (Table 11). Its capacity to hydrolyse starch highlights its ability to break down complex carbohydrates. However, the bacterium does not produce indole or urease and does not utilise gelatin, sorbitol or arabinose. These characteristics may impact its interactions with the host plant and its ability to modify nitrogen environments.

Identification of the promising endophytic bacteria by partial sequencing of 16S rRNA genes

Molecular analysis of Promising isolate was performed using universal primer 16S rRNA gene. DNA was extracted from the bacterial culture and its amount and quality were assessed using a NanoDrop-2000 (Thermo Fisher Scientific, USA) and resolved in a 1% (w/v) agarose gel. A single band of high-molecular-weight DNA

was then confirmed by visualisation in gel documentation. A single distinct PCR amplicon of around 1500 bp was seen on a 1% (w/v) agarose gel after the fragment 16S rDNA was amplified using the 27F and 1492R primers. Purified PCR amplicons (16S rDNA) were subjected to forward and reverse DNA sequencing reactions using the BDT v3.1 Cycle sequencing kit on a genetic analyser reproduce gene sequences. The purified product was sequenced using Sanger's technique of DNA sequencing. Using aligner software, the gene's consensus sequences were produced from the forward and reverse sequencing data. The blast 2.2.9 system was used to compare the acquired sequences of both genes with the nucleotide sequences that were accessible in the NCBI. Twenty microliters of the reaction mixtures were analysed on 1.5% agarose in the presence of 0.5 µg of ethidium bromide per mL and photographed under UV illumination. PCR amplification of 16S region from bacterial sample showing amplification of ~1500 bp region in 1.5% agarose gel with 1KB ladder. Results showed that the sequenced PCR amplicons of PMSEB 3 showed up to 99.93%

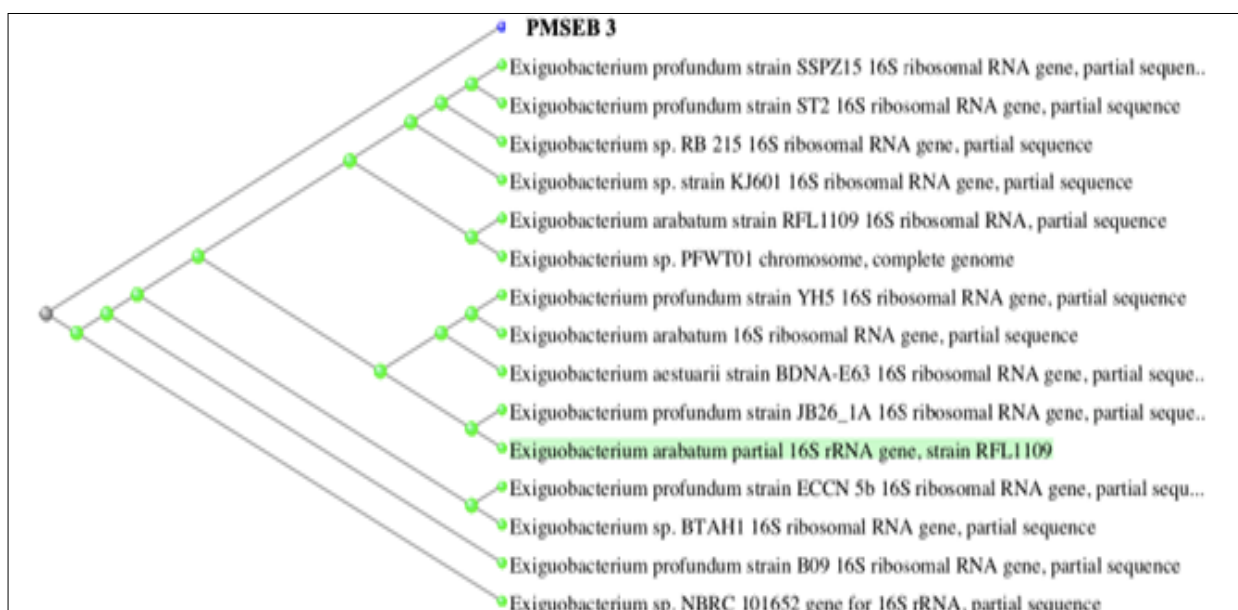


Fig. 3. Molecular phylogenetic analysis of *Exiguobacterium profundum*.

similarity with *Exiguobacterium profundum* 16s RNA nucleotide sequences (Fig. 3) (Accession no. OR09444).

Using 1000 repetitions for bootstrap testing in MEGA 7.0 software, the maximum likelihood technique based on the Tamura-Nei model was used to perform the molecular phylogenetic analysis. 16 nucleotide sequences were analysed and evolutionary distances were calculated. In a study, it was recovered and identified 12 bacterial isolates from the tomato rhizosphere using morphological and cultural parameters (26). Twelve antagonistic bacterial isolates were evaluated against the soil transmitted pathogens *P. debaryanum*, *R. solani* and *S. rolfsii*. In the present study, all pearl millet bacterial endophytes were tested for ACC utilisation by spotting them on ACC amended minimal medium plates, out of which 4 showed the positive results. These findings are consistent with the earlier findings (27), which screened 563 bacteria isolated from pea, lentil and chickpea roots and found that 5 % of the isolates had ACC deaminase activity. Similarly, reports are on 200 bacterial isolates from berseem clover plant rhizospheric soil and roots, nodules and discovered that only 72 isolates were positive for ACC deaminase synthesis (28). The results obtained are in line with (29), who said that ammonia was created by endophytic bacteria derived from medicinal plants that were taken from Saint Katherine Protectorate in South Sinai, Egypt. Nessler's reagent was added to growth media to demonstrate this production. It was also found that only 8 endophytic bacterial strains that were isolated from the roots of *Amaranthus hybridus*, *Solanum lycopersicum* and *Cucurbita maxima* plants had PGP characteristics (30). Biochemical and morphological analyses showed that most of these strains were rod-shaped, fast growers and Gram negative. Our findings are comparable to those of the 31 recovered brown sarson (*Brassica rapa* L.) isolates from 22 villages spread throughout 3 districts of the Kashmir valley, each of which had a unique morphology. Only 12 isolates viz. SB13, SB14, SB26, SB28, SB43, SB46, SB51, SB55, SB58, SB64, SB67 and SB73 having most outstanding attributes were identified up to genus level by their morphological, physiological and biochemical characters. All the selected endophytes were rod shaped except isolate SB73 was cocci in shape. Only 5 isolates were Gram positive while rest were Gram negative. Two isolates (SB51, SB26) were methyl red positive while none of the isolate was found to be positive with respect to Voges-Proskauer reaction however, citrate utilisation ability was found in SB13, SB58, SB26, SB46 and SB55. Isolate SB46 was negative to oxidase activity while rest were positive, however all the isolates were positive to catalase. Cellulose hydrolysing ability was observed in none of the isolate while isolates SB51, SB64 and SB28 showed starch hydrolysing ability. Acid production was observed in 3 isolates viz. SB14, SB58 and SB43 isolates.

The effects of plant growth-promoting (PGP) and biocontrol seen in this study are probably mediated by a number of complementary mechanisms. According to molecular identification, isolate PMSEB 3 exhibited 99.93 % similarity to *Exiguobacterium profundum*, suggesting that it may have a useful endophyte function. These isolates may suppress infections through competition, antibiosis and the generation of inhibitory metabolites, according to their antagonistic activity against soil-borne pathogens. Several endophytes demonstrated ACC deaminase activity, which plays a key role in lowering plant ethylene levels under stress conditions, thereby enhancing root

growth and stress tolerance (31). Ammonia production further indicates their ability to improve nitrogen availability and promote plant nutrition. Morphological and biochemical traits such as catalase and oxidase activity, citrate utilisation and starch hydrolysis suggest metabolic versatility, enabling better colonisation and survival within host tissues. Collectively, these mechanisms-including pathogen inhibition, stress modulation via ACC deaminase, nutrient mobilisation and effective root colonisation-contribute to enhanced plant growth, reduced blast incidence and improved yield in pearl millet.

Conclusion

The study revealed that PMSEB 3 has demonstrated potential as a stem endophyte to control pearl millet blast disease caused by *Magnaporthe grisea*. Molecular analysis confirmed its identity as beneficial endophyte with 99.93 % similarity with *Exiguobacterium profundum*. Our screening of promising isolates of endophytes with growth-inhibiting properties for pearl millet not only has tremendous potential regarding women empowerment but it also stands evidence to nature's creation to aid in the fight against diseases. Both *in vitro* and *in vivo* assessments have demonstrated their efficacy, offering tangible solutions to a pressing agricultural challenge. Seed treatment alone, and subsequent foliar spray of PMSEB 3 (T4) recorded lowest disease incidence at 60 DAS which was significantly less compared to other treatments during field evaluation in kharif 2021 and 2022 (56.94 % and 56.93 %) respectively. In the respective years, this helped to improve germination 71.4 % and 69.33 %. Additionally, characterisation of these endophytes from their morphological and biochemical perspective further allows us to understand their uniqueness which serves as a basis for getting their complete elucidation for enhanced biocontrol abilities. Overall, PMSEB 3 offers a sustainable and eco-friendly strategy for reducing blast severity and improving pearl millet productivity.

Authors' contributions

PV and VKM participated in analysing results of experiments carried out the field experiment and original draft writing. The conceptualisation and supervision of the research was carried out by VKM, TV and MS. VK and PY contributed to finalisation data and taught me how to use software. PY and MK helped in isolation of bacteria culture and molecular identification. MK and MJ helped in writing and review editing. All the authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest to declare.

Ethical issues: None

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