

Original Research Article

Study of endophytic bacteria in pearl millet (*Pennisetum glaucum* (L.) R. Br.) roots

ABSTRACT

Aims: The current investigation aims to isolate and identify the root endophytic bacteria from Pearl millet (*Pennisetum glaucum*) variety MSH-1970, as well as evaluate the qualities that promote plant growth.

Study design: The study involves isolating endophytic bacteria, performing biochemical and molecular characterization, and evaluating their ability to stimulate plant development through biochemical tests.

Place and Duration of Study: Department of Bioscience and Biotechnology, Banasthali Vidyapith, Tonk, Niwai, Rajasthan, 2022–2023.

Methodology: Pearl millet MSH-1970 was planted in pots in the greenhouse of the Department of Bioscience and Biotechnology and endophytic bacterial strains were isolated. The isolates' morphological features were recorded using *Bergey's Manual of Systemic Bacteriology*, and biochemical testing was done. 16S rRNA gene PCR was used to do molecular characterization of the potential endophyte, and the PCR product was sequenced. Ammonia generation, phosphate solubilization, and HCN production were used to identify the properties that promote plant development.

Results: In the initial culture of endophytic bacteria isolated from the pearl millet variety MSH-1970 yielded 14 colonies on Nutrient agar medium. One of the colonies, BVCA-16 could solubilize phosphate, and generate hydrogen cyanide, and ammonia. Using 16f-16r and 63f-1244r primers, a molecular analysis of BVCA16 revealed 99.92% similarity with *Pseudomonas* sp. AUS22. The sequence was submitted to Genbank NCBI as *Pseudomonas* sp. strain BVCA-16, accession number OR350932.

Conclusion: In the Pearl millet variety MSH-1970, the endophytic bacteria *Pseudomonas* sp. strain BVCA-16 was identified and it shows potential to promote plant growth.

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Keywords: Endophytic bacteria, pearl millet, *Pseudomonas*, phosphate solubilisation.

1. INTRODUCTION

A crucial staple crop in arid and semi-arid areas, pearl millet (*Pennisetum glaucum* (L.) R. Br.) makes a substantial contribution to the world's food security. Interest in understanding the complexities of pearl millet's plant-microbe interactions has grown due to its unusual capacity to adapt to harsh climatic circumstances, such as drought and high temperatures (Numan et al., 2021). One important but less-studied aspect of the plant microbiome is the endophytic bacteria found in the root tissues. Endophytic bacteria are important regulators of plant development and health (del Carmen et al., 2021). They frequently get a variety of advantages from their symbiotic interactions with host plants, such as the ability to absorb nutrients and withstand stress (Chaudhary et al., 2022). *Aerobacter*, *Aeromonas*, *Agrobacterium*, *Bacillus*, *Curtobacterium*, *Enterobacter*, *Erwinia*, *Mycobacterium*, *Flavimonas*, *Pseudomonas*, *Sphingomonas*, and other endophytic bacteria have been

Comment [SA3]: Suggested revision: "Pearl millet (*Pennisetum glaucum* (L.) R. Br.), a crucial staple crop in arid and semi-arid regions, plays a significant role in global food security."

identified from bean (Struz et al., 1997). Even while endophytic bacteria have been investigated in great detail in a variety of plant species, little is known about their functions and diversity in the context of pearl millet (Watts et al., 2023).

It is well known that a dynamic microbial population inhabits the rhizosphere, the area where plant roots and soil meet, with endophytic bacteria playing a major role in maintaining the plant's general health. A thorough investigation of the endophytic bacteria linked to pearl millet roots is necessary to determine their functional capacities, taxonomic diversity, and their agronomic uses.

2. MATERIAL AND METHODS

2.1. Isolation of endophytic bacterial from pearl millet roots

2.1.1 Pearl millet root collection

The roots of pearl millet MSH-1970 were collected from the plants grown in greenhouse at the Department of Bioscience and Biotechnology, BanasthaliVidyapith, Rajasthan.

2.1.2 Isolation of endophytic bacteria

The surface of the roots was sterilized in a series of three solutions: 2.6% (w/v) sodium hypochlorite solution for 2 min, 75% (v/v) ethanol, and then 75% (v/v) ethanol sequentially for about half a min and then thoroughly cleaned with sterile distilled water. The roots were applied to nutritional agar (NA) plates and incubated at $28\pm 2^{\circ}\text{C}$ for 3 days to check for ~~and~~ surface contamination.

~~To isolate bacterial endophytes, the above procedure was followed~~ and then 1g root sample was ground ~~up~~ into a homogeneous paste in a pestle and mortar with 10 ml of sterile, distilled water and left to stand for 20 min. 10 μl of the diluted supernatant was placed over NA plates and incubated at $28\pm 2^{\circ}\text{C}$ and examined at periodic intervals. Bacterial colonies that were classified based on ~~several~~ visual observations and colony characteristics were noted (Don et al., 2005). Pure culture ~~was were~~ established. For subsequent studies, the bacterial plates were kept at $4\pm 1^{\circ}\text{C}$ and glycerol stock ~~was~~ prepared for long term storage.

2.3. Characterization of plant growth-promoting activities

2.3.1. Phosphate solubilization

To perform the phosphate solubilization test, Pikovskaya's agar was prepared and inoculated with isolated bacteria for 5-7 days at $28\pm 2^{\circ}\text{C}$. The plates were periodically checked for clear zones ~~have formed~~ around the bacterial growth (Karpagam et al., 2014).

2.3.2. Test for drought tolerance capacity of the bacterial strain:

To check the drought tolerant traits of bacterial isolates which were able to solubilize phosphate (as above 2.3.1), Nutrient agar media (NAM) was prepared with different PEG₆₀₀₀ concentrations for the drought tolerance assay. The corresponding concentrations (10%, 15%, 18%, 21%, and 24%) of polyethylene glycol (PEG₆₀₀₀) were then added (Muscolo et al., 2014). 100 μl of a 24h pre-culture (OD_{540nm}=0.2, 108cfu/ml) was added to PEG-supplemented broth, which was then continuously shaken at 150 rpm on a rotary flask shaker at 30°C . The absorbance was noted ~~at~~ till 78h (6-12h intervals) at 540 nm using a spectrophotometer. The bacterial isolate(s) that perform better in the drought tolerance test were taken forward for further experiments.

2.3.3. Ammonia production test

To conduct the ammonia production test, the suspension of bacterial isolate was added to 10 ml of peptone water, which was then incubated for 3-5 days at $28\pm 2^{\circ}\text{C}$ (Cappuccino et al., 2008). ~~After incubation, 0.5ml of~~ Nessler's reagent, ~~0.5ml,~~ was added drop by drop from the side of the wall ~~to the bacterial suspension~~ after the medium became turbid (Chaudhary et al., 2021).

2.3.4. HCN test

To perform the HCN test, Glycine (4.4 g/l) was added to the nutrient agar medium (Lorck, 2004; Manasa et al., 2017). The spreading plate technique was used to inoculate the bacterial culture onto the plate and after growth at $28\pm 3^{\circ}\text{C}$, for 4-6 days, a sterile

Comment [SA4]: Identified in

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Comment [SA12]: Suggested revision: Drought tolerance was assessed in bacterial isolates that solubilized phosphate (as described in section 2.3.1). Nutrient agar media (NAM) was prepared with varying concentrations of PEG6000 for the drought tolerance assay.

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Comment [SA15]: Suggested revision: Ammonia production was tested by adding a bacterial suspension to 10 mL of peptone water, which was then incubated for 3-5 days at $28\pm 2^{\circ}\text{C}$.

Whatman filter paper soaked in sodium bicarbonate (2%) and picric acid solution (0.5%) was placed over the culture and observed for change in color.

2.4. Biochemical characterization of isolated endophytic bacteria

The bacterial isolate(s) selected to have growth promoting activities from the abovementioned tests (2.3.1, 2.3.2, 2.3.3, 2.3.4) were taken and the following biochemical tests performed: indole test, MR-VP test, motility test, citrate test, catalase test, urea test, nitrate test, H₂S test (Aneja et al., 2007).

2.5 Molecular characterization

The Sigma-Aldrich Cat. No. NA2110 GenElute Bacterial Genomic DNA Kit was used to extract bacterial DNA. The following universal 16S rRNA primer set (Weisburg et al., 1991) was used in PCR to amplify DNA: fd1 (5'-AGAGTTTATCCTGGCTCAG-3') and rd1 (5'-AAGGAGGTGATCCAGCC-3').

In another set, the forward primer 63f (5'-AGGCCTAACACATGCAAGTC-3') and the reverse primer (5'-CTCGCTGCCACTGTAC-3') was also used to amplify the 16S rRNA gene (Srivastava et al., 2008).

A PCR tube containing 2.5mM dNTPs, 1XTaq buffer, 0.2µM primers, 0.5U/µL Taq polymerase, and 125ng/µg of DNA was used to conduct the reactions. Applied Biosystems' Verity Thermal Cycler was used for PCR amplification (Weisburg et al., 1991). 1.5% agarose gel containing ethidium bromide was used for gel electrophoresis, and the results were shown using a gel documentation system. The PCR product was sent for sequencing to NCMR-NCCS, Pune and Xcelris Genomics, ~~Ahmadabad~~Ahmedabad, India. EZTaxon (<https://www.ezbiocloud.net/>) and nucleotide BLAST (<https://blast.ncbi.nlm.nih.gov/>) was used to analyze the partial sequences.

3. RESULTS AND DISCUSSION

A total of 14 bacterial endophytes could be grown in pure culture from the pearl millet roots.

~~Among Out of~~ 14 bacterial isolates only 6 (BVCA 14, BVCA15, BVCA16, BVCA 17, BVCA18, BVCA19) showed phosphate solubilization capacity ~~as revealed~~ by formation of distinct clear zone ~~surrounding around the~~ bacterial ~~dis~~colony (Fig. 1a), demonstrating the capacity to dissolve insoluble phosphate compounds. Phosphates are a critical component of soil fertility and plant nutrient and by increasing plants' accessibility to insoluble phosphates, this characteristic raises agricultural output (Kumar et al., 2021).

Using a NAM medium supplemented with different PEG concentrations, the growth of (BVCA 14, BVCA15, BVCA16, BVCA 17, BVCA18, BVCA19) bacterial isolates was monitored at 540nm for 72h at regular intervals.

The absorbance value steadily decreased as the PEG concentration increased, indicating that the bacterial strains were susceptible to PEG at high concentrations. The growth suppression observed is consistent with research showing that chemicals such as PEG 6000 can cause osmotic stress, which can hinder the development of bacteria (Johnson et al., 2024).

It was observed that among the six isolates, BVCA-16 showed visible growth in even higher ~~PGG-PEG~~ concentrations, whereas the other strains were not able to survive. As such a detailed analysis of the bacterial isolate BVCA-16 was done and the data is represented in Fig.2.

All further experiments were performed with the bacterial isolate BVCA-16.

The ammonia production test was performed to evaluate the capacity of the bacterial isolate to metabolize amino acids and create ammonia as a metabolic byproduct (Devi et al., 2018).

In the present study, positive results were obtained when yellow medium turned brown, indicating ammonia production, which is typical of bacterial metabolism of amino acids. ~~when the yellow medium was converted to brown showing that ammonia synthesis was occurring~~ (Fig. 3a).

Hydrogen cyanide (HCN) production is an important metabolic characteristic of certain bacteria. ~~The ability of some bacteria to produce hydrogen cyanide (HCN), which is essential~~

Comment [SA16]: Mention the colours for positive reaction

Comment [SA17]: Suggested revision: Bacterial isolates selected for their growth-promoting activities in the tests described above (sections 2.3.1 to 2.3.4) were subjected to the following biochemical tests: indole, MR-VP, motility, citrate, catalase, urea, nitrate, and H₂S tests (Aneja et al., 2007).

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~~to their metabolic capacities, is a notable characteristic.~~ With implications for both microbial identification and interactions with the environment, the bacteria isolate BVCA-16 was ~~test tested~~ for its capacity to produce hydrogen cyanide. BVCA-16 showed a positive result in the HCN test, demonstrating the existence of hydrogen cyanide ~~generation~~ production (Fig.3b). According to Bumunang et al., 2014, hydrogen cyanide can operate as an effective antibacterial agent and participate in allelopathy, which affects how bacteria and other organisms interact.

The ~~biochemical characterization tests are shown~~ tests for biochemical characterisation are ~~represented~~ in Fig. 3c-j. The presence of indole in the broth's reddish-pink color indicates that the organism can produce the enzyme tryptophanase (Kabiraz et al., 2023). The MR-VP test yielded a negative result, showing ~~the lack of no~~ mixed acid fermentation and acetoin synthesis (Das et al., 2022). The diffusion of bacterial growth from the place of inoculation in a semisolid agar medium during the motility test indicated that the motility was positive. The capacity of the bacteria to use citrate as its only carbon source is shown by a positive citrate test, which helps identify Enterobacteriaceae (MacFaddin et al., 2000). The use of citrate is confirmed by the observed alkaline pH change. The existence of catalase in the bacteria was confirmed by a positive catalase test, which allows hydrogen peroxide to be broken down into water and oxygen (Wheelis et al., 2008). The urea test ~~yielded~~ ~~was~~ negative result, indicating the absence of urease activity in the isolated bacterial strain. This finding aids in the differentiation of urease-negative organisms (Clark et al., 2022). The positive result of the nitrate test indicated that the bacteria were reducing nitrate to nitrite or nitrogen gas (MacFaddin et al., 2000). The H₂S test produced a negative result, indicating that the bacterial isolate ~~does not create~~ ~~produce~~ hydrogen sulfide gas during the metabolism of sulfur-containing substances. This finding is pertinent to the classification and differentiation of bacteria, especially in the context of microbial identification and environmental investigations (Johnson et al., 2021). The liquefaction of gelatin showed a favorable result for the gelatinase test. This implies that the bacterial isolate can produce the enzyme gelatinase, which aids in the breakdown of gelatin into smaller peptides and amino acids.

~~Genomic DNA of the bacterial isolate BVCA-16 was extracted and amplified using universal primers targeting the 16S rRNA gene, a commonly used marker for bacterial identification. Genomic DNA of the bacterial isolate BVCA-16 strain was obtained and PCR was conducted with the use of universal primers, the 16S rRNA and the PCR amplicon sent for sequencing.~~

EZTaxon and NCBI BLAST tools were used to examine the partial sequence BVCA-16. The sequence exhibited >99% similarity to other strains ~~on in the~~ BLAST analysis at NCBI portal (Table 1) and was submitted to the NCBI database as *Pseudomonas sp.* (BVCA-16) with the accession code OR350932.

~~Endophytes, in addition to along with phyllosphere microorganisms, play a crucial role in contribute to~~ maintaining plant health. ~~These microbes, which are predominantly bacteria and fungi, can be found~~ It is common to find endophytes, which are mostly made up of bacteria and fungus, in all parts of the plant, including the roots, stems, leaves, fruits, and seeds. ~~Among the seed endophytic bacteria identified in pearl millet species such as Kosakoniacowanii, Bacillus subtilis, B. tequilensis, B. velezensis, Pantoeastewartii, Paenibacillus dendritiformis and Pseudomonas aeruginosa were among the seed endophytic bacteria that are have been reported from pearl millet seeds~~ (Kumar et al., 2021). Additionally, *P. aeruginosa* and other diazotrophic endophytic bacteria are associated with pearl millet (Gupta et al., 2022). The soil microbiome, ~~and as well as~~ the phyllospheric and endophytic elements of the millet-associated microbiome, ~~contribute significantly to~~ ~~are often engaged in~~ nutrient cycling, absorption, pathogen defence, and plant growth promotion. ~~Therefore, these microbes hold considerable~~ ~~as a result, they provide a great deal of~~ promise for ~~use in creating~~ ~~developing~~ microbial formulations ~~that will increase~~ ~~aimed at enhancing~~ agricultural productivity, yield and growth.

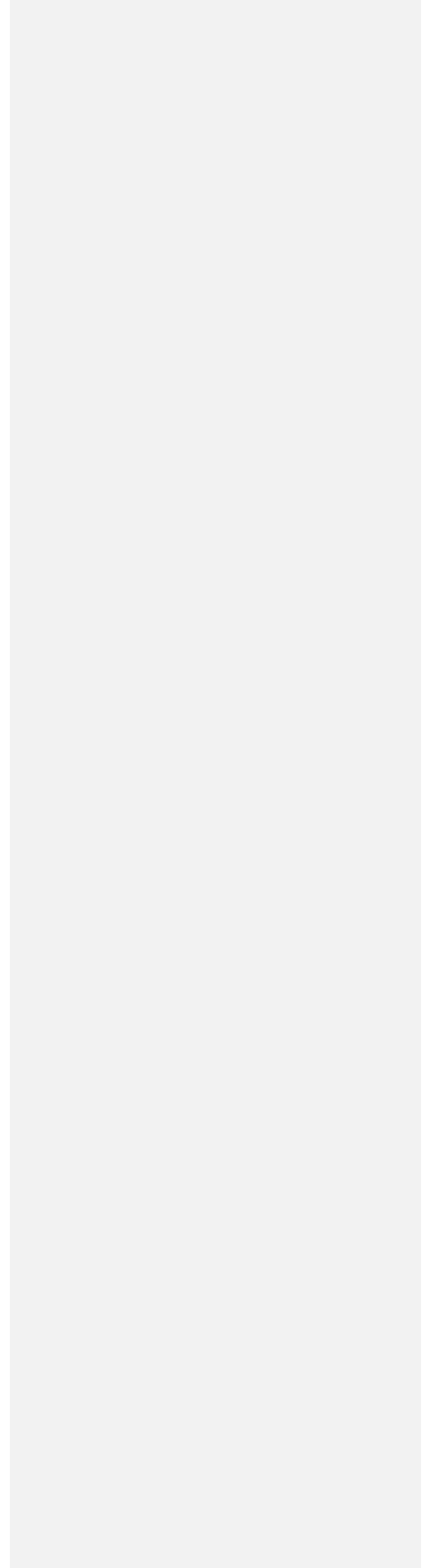
Comment [SA19]: The reddish-pink color in the broth indicates the presence of indole

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During the motility test, the diffusion of bacterial growth away from the inoculation point in semisolid agar indicated positive motility.

Comment [SA22]: Ensure consistent usage of terms like "bacterial isolate" versus "bacterial strain".

UNDER PEER REVIEW



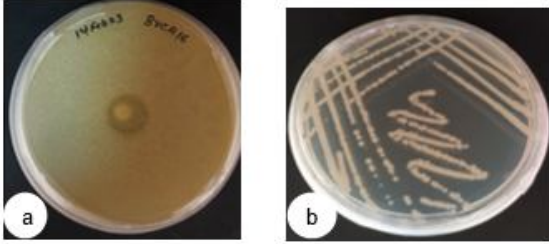


Figure 1. a) Clear zone around bacterial culture (BVCA-16) in phosphate solubilizing test, b) bacterial isolate BVCA-16 grown in NA medium.

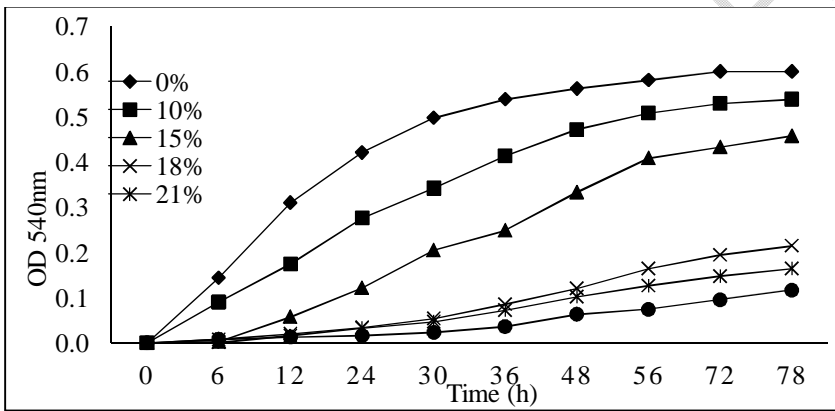


Figure 2. The strain BVCA-16 growth curve at 0% - 24% polyethylene glycol (PEG; PEG₆₀₀₀).

Non-inoculated Inoculated Non-inoculated Inoculated

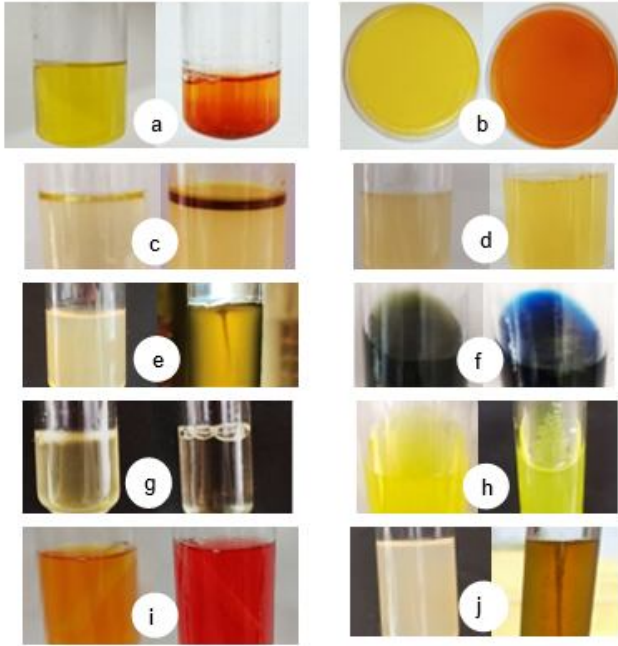


Figure 3. Biochemical analysis of a selected bacterial strain (BVCA-16): a) ammonia production, b) HCN production, c) Indole test, d) MR-VP test, e) motility test, f) citrate test, g) catalase test, h) urea test, i) Nitrate test j) H₂S test by selected bacterial strains.

4. CONCLUSION

In the Pearl millet variety MSH-1970, the endophytic bacteria *Pseudomonas* sp. strain BVCA-16 was identified and it shows potential to promote plant growth.

Comment [SA23]: Your conclusion is concise, but it can be enhanced by adding more detail about the significance of your findings.

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