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MOLECULAR CHARACTERIZATION OF BENEFICIAL MICROFLORA FROM COMMERCIAL VERMIWASH AND ITS EFFICACY ON SEED GERMINATION OF *Vigna radiata* L.

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ABSTRACT

Vermiwash, a liquid extract derived from vermicompost, plays a crucial role in organic farming, which is integral to sustainable agriculture. This study aimed to isolate and characterize plant-beneficial microbial strains from vermiwash and evaluate their potential as plant growth-promoting rhizobacteria (PGPR). Using standard microbiological techniques, bacterial colonies were isolated from vermiwash samples, resulting in the identification of twelve distinct strains (VW1 to VW12). These strains were subjected to biochemical characterization, including analysis of cell wall composition and various metabolic tests. The isolated strains were assessed for key plant growth-promoting traits, such as indole-3-acetic acid (IAA) production, ammonia synthesis, and phosphate solubilization. Additionally, their biocontrol capabilities were evaluated through amylase and cellulase activity assays and by testing their antagonistic effects against the fungal pathogen *Aspergillus niger*. Based on these qualitative assessments, strains VW-1, VW-4, and VW-9 were selected for further investigation in seed germination studies using *Vigna radiata* seeds. Furthermore, different concentrations of vermiwash extract (10%, 20%, and 30%) were tested to evaluate their impact on seed germination. Results indicated that both the vermiwash extract and the bacterial strains enhanced seedling growth, with notable improvements in hypocotyl and root length. Among the strains tested, VW-9 demonstrated superior performance in promoting shoot and root elongation compared to other microbial treatments and the control, highlighting its potential as an effective PGPR. Phylogenetic analysis identified strain VW-9 as *Bacillus zanthoxyli* (accession number PP694489). These findings suggest that the application of vermiwash, enriched with beneficial microorganisms, can serve as an effective bioinoculant in crop cultivation, supporting eco-friendly agricultural practices.

Keywords: Mungbean, Organic farming, PGPR, seed germination

The liquid extract obtained from vermicomposting organic waste, known as vermiwash, is characterized by its brownish-red coloration and consists of earthworm exudates and mucoid secretions. This extract is a rich source of micronutrients and organic compounds, making it an effective foliar spray with plant growth-promoting properties (Ansari *et al.*, 2010). Vermiwash combines beneficial microbes, enzymes, and water-soluble nutrients (Haynes and Swift, 1990), contributing significantly to agricultural productivity by enhancing plant growth and resilience to diseases. Its application increases the nutrient and organic matter content in the soil, which is readily available for plant uptake (Sivasubramanian and Ganeshkumar, 2004). Vermiwash is also abundant in beneficial microbial strains that can be harnessed as biofertilizers (Vessey, 2003). These microorganisms offer several advantages, including mineral solubilization, improved nutrient absorption, phytohormone secretion, and siderophore production. Regular application of these microbial strains can reduce the reliance on chemical fertilizers, lower costs, improve soil fertility, and foster environmentally friendly farming practices (Maheshwari *et al.*, 2012). Such methods are valuable for developing effective inoculants and biocontrol strategies, ultimately leading to improved crop systems and enhanced resistance (Flint *et al.*, 1998; Heimpel *et al.*, 2017).

The mungbean (*Vigna radiata*) is known for its ability to fix atmospheric nitrogen, a process essential for its growth but not accomplished independently. To enhance its productivity and robustness, mungbean relies on the presence of beneficial rhizosphere-dwelling bacteria (Friesen *et al.*, 2011). However, both biotic and abiotic factors have significantly impacted mungbean yields. In this context, the present study aimed to isolate advantageous microbial strains from commercial vermiwash and evaluate their plant growth-promoting traits on *Vigna radiata* seeds. The ultimate goal is to identify these traits and apply them to sustainable agricultural practices.

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MATERIALS AND METHODS

Sample collection

Three vermiwash samples were collected from the Bio-Resource Development Centre (R&D) in Shillong, Meghalaya. The samples were gathered in 500 ml bottles.

Isolation of bacterial strains from vermiwash

The samples were serially diluted, and 0.1 ml of each dilution was plated onto Nutrient Agar medium. The plates were incubated at 28±2°C for 24 to 48 hours. From the countable plates, twelve representative colonies exhibiting diverse morphological characteristics were selected. Pure cultures of these colonies were then prepared and preserved as glycerol stocks for subsequent analysis (Cappuccino & Sherman, 1992).

Identification of isolates

Based on molecular characterization and cultural traits, the isolates were shortlisted. The evaluation included a series of tests: starch hydrolysis, gelatin hydrolysis, carbohydrate fermentation, the IMViC series (Indole, Methyl Red, Voges-Proskauer, and Citrate tests), mannitol motility test, catalase test, oxidase test, and Gram staining (Cappuccino & Sherman, 1992).

In vitro screening of the isolated bacterial strains for multiple plant growth promoting and biocontrol activity

Indole 3- Acetic Acid Production

Five milliliters of Luria-Bertani broth medium (which contains tryptophan) were inoculated with the test bacterial strains. The cultures were incubated for 24 hours at 28°C on a rotary shaker set at 120 rpm. Following incubation, the cultures were centrifuged at 3000 rpm for 30 minutes. To 2 milliliters of the resulting supernatant, 4 milliliters of Salkowski's reagent and a few drops of orthophosphoric acid were added. The development of a pink colour indicated the production of indole-3-acetic acid (IAA) (Bric *et al.*, 1991).

Production of Ammonia

The capability of the isolates to produce ammonia in peptone water was assessed using the following procedure. Each test tube was inoculated with 10 milliliters of freshly prepared peptone water and incubated for 48 to 72 hours at 28 ± 2°C. After incubation, 0.5 milliliters of Nessler's reagent were added to each tube. A colour change from brown to yellow in the solution indicated a positive result for ammonia production (Cappuccino *et al.*, 1992).

Phosphate Solubilizing Activity

In the phosphate solubilization test, tri-calcium phosphate was used as the phosphate source to evaluate the conversion of insoluble phosphate to a soluble form that is available for plant uptake (Gouda *et al.*, 2018). Modified Pikovskaya's medium, supplemented with bromophenol blue, was prepared to facilitate the detection of phosphate solubilization by visualizing the clearance zone. After incubating the plates at 37°C for 48 hours, the test isolates were streaked onto the medium and observed for the presence of a distinct yellow hydrolysis zone, which indicates effective phosphate solubilization (Pikovskaya, 1948).

Protease activity

Protease activity was screened using the skimmed milk agar plate assay method. The plates were incubated at 28±2°C for 24 to 48 hours. Clear zones surrounding the microbial colonies indicated the presence of proteolytic activity (Kaur *et al.*, 1989).

Screening for cellulase

Each isolate was separately streaked onto carboxymethyl cellulose (CMC) agar plates, which were then incubated at 37°C for 72 hours. After incubation, the plates were stained with 0.1% (w/v) Congo red reagent. The presence of a clear zone of hydrolysis around the bacterial colonies was observed to confirm cellulose degradation (Apun *et al.*, 2000).

Screening for amylase

Bacterial isolates were inoculated onto starch agar plates, which were then incubated at 30°C for 48 hours. After incubation, the plates were treated with Lugol's iodine solution. The presence of a clear zone of hydrolysis around the bacterial colonies, indicating starch degradation, was observed (Cappuccino & Sherman, 1992).

Preparation of vermiwash and microbial isolates for seed germination

Vermiwash was prepared at three concentrations: 10%, 20%, and 30%. Seeds intended for germination were surface sterilized using 1% $HgCl_2$ and then thoroughly washed with sterile distilled water. The seeds were soaked in the various vermiwash concentrations and placed in Petri dishes lined with sterile filter paper moistened with distilled sterile water. Germination was allowed to proceed for five days, after which hypocotyl and root lengths were measured. Additionally, bacterial strains VW1, VW4, and VW9, selected based on plant growth-promoting (PGP) traits and biochemical studies, were utilized in seed germination experiments. These strains were grown in nutrient broth, incubated for

24 hours at 37°C, until reaching an optical density of 1×10^8 cfu/mL. The seeds were then coated with these bacterial cultures by incubating them for 1 hour in a rotary shaker at 120 rpm and room temperature. Seeds treated only with nutrient broth served as the control. On the fifth day, measurements of root and hypocotyl lengths were taken and compared with those of the control group to evaluate the effects of vermiwash and bacterial treatments.

Antagonistic studies against test pathogens

The antagonistic activity of various bacterial isolates against the fungus *Aspergillus niger* was assessed using the dual culture technique (Shehata *et al.*, 2016). In this method, bacterial isolates were streaked onto one side of a Petri dish containing potato dextrose agar (PDA), approximately 1 cm from the edge. *Aspergillus niger* mycelia were then placed perpendicular to the bacterial streak. The plates were incubated for five to seven days at 27±2°C. Control plates were prepared with only fungal mycelium and no bacterial isolates. The extent of mycelial growth and the width of the inhibition zone around the bacterial streak were measured to evaluate the antagonistic effect against the pathogen.

PCR and 16S rRNA Sequencing

Strain VW9, selected for its biochemical and plant growth-promoting rhizobacteria (PGPR) traits, was further analyzed through 16S rRNA sequencing (Woese, 1987). DNA was isolated using the conventional salting-out method. PCR amplification was performed with universal primers 27F-5′-AGAGTTTGATCMTGGCTCAG-3′ and 1492R-5′-GGTTACCTTGTTACGACTT-3′. The PCR mixture consisted of 1.0 µL of each primer (20 µM), 2.5 µL of 10× $MgCl_2$ buffer, 2.0 µL of each 2.5 mM dNTP, 0.5 µL of Taq DNA polymerase (3 U/µL), 1 µL of template DNA, and 18 µL of distilled water. The PCR conditions included: initial denaturation at 94°C for 5 minutes, followed by 30 seconds of annealing at 56°C, extension at 72°C for 1 minute, and a final extension at 72°C for 10 minutes. The molecular weight of the amplified product was assessed using a 100 bp ladder on a 1.5% agarose gel electrophoresis. Sequencing was conducted using a PCR heat cycler (GeneAmp PCR System 9700, Applied Biosystems) and the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA), following the manufacturer's instructions. The sequence quality was verified with Applied Biosystems' Sequence Scanner Software v1. The 16S rRNA gene sequence was compared against the NCBI BLAST database to identify homologous sequences. Species-level identification was based on a ≥98% similarity with sequences in GenBank. Sequence alignment and any necessary modifications were performed using MEGA 11 (Tamura *et al*., 2021). The final sequence was deposited in the GenBank database to obtain an accession number.

Statistical Analysis

The analysis of variance (ANOVA) was employed to statistically evaluate all data, and the least significant difference (LSD) at ($p = 0.05$) was used to compare the treatment means.

RESULTS AND DISCUSSION

Twelve bacterial strains, designated VW1 through VW12, were isolated from vermiwash and characterized based on their morphological and biochemical properties. The cultural characteristics of these isolates on nutrient agar are summarized in Table 1. The results of Gram staining and biochemical tests, including IMViC, catalase, oxidase, starch hydrolysis, gelatin hydrolysis, mannitol motility, citrate utilization,

Table 1. Cultural characteristics of the selected bacterial strains

Sr. No.	Strains	Shapes	Elevation	Margin	Transparency	Color	Consistency
1.	VW-1	Spherical	Flat	Smooth	Opaque	Off White	Mucoid
2.	VW-2	Spherical	Concave	Smooth	Opaque	Off White	Mucoid
3.	VW-3	Round	Concave	Smooth	Opaque	Light Yellow	Mucoid
4.	VW-4	Round	Flat	Smooth	Opaque	Pink	Mucoid
5.	VW-5	Round	Concave	Smooth	Transparent	Off White	Mucoid
6.	VW-6	Round	Flat	Smooth	Opaque	Off White	Mucoid
7.	VW-7	Round	Concave	Smooth	Opaque	Off White	Mucoid
8.	VW-8	Round	Concave	Smooth	Opaque	Light Yellow	Mucoid
9.	VW-9	Spherical	Flat	Smooth	Transparent	Creamy	Mucoid
10.	VW-10	Spherical	Flat	Smooth	Opaque	White	Mucoid
11.	VW-11	Spherical	Flat	Smooth	Opaque	White	Mucoid
12.	VW-12	Spherical	Flat	Smooth	Transparent	Creamy	Mucoid

Table 2. Biochemical tests to identify the bacterial strains

Sr. No.	TESTS	VW-1	VW-2	VW-3	VW-4	VW-5	VW-6	VW-7	VW-8	VW-9	VW-10	VW-11	VW-12
1	Gram Staining	+	+	-	+	-	+	+	-	+	+	+	+
2	Indole	-	-	-	-	-	-	-	-	-	-	-	-
3	Methyl Red	-	-	-	-	-	-	-	-	-	-	-	-
4	VP	-	-	-	-	-	-	-	-	-	-	-	-
5	Citrate	+	-	+	+	+	-	+	-	+	+	+	+
6	Mannitol	-	+	+	+	-	+	-	-	-	-	-	+
7	Carbohydrate	-	+	-	-	-	-	-	-	-	-	-	-
8	Gelatin	-	-	-	-	-	-	-	-	-	-	-	-
9	Starch	+	-	+	+	-	+	-	+	+	+	-	+
10	Oxidase	+	+	+	+	+	-	+	+	+	+	+	+
11	Catalase	+	+	+	+	+	+	+	+	+	+	+	+

and carbohydrate fermentation, are detailed in Table 2. The bacterial strains were further assessed for plant growth-promoting (PGP) and biocontrol traits to identify potential candidates for use as commercial biocontrol agents or plant growth promoters.

Ammonia Production: Ammonia production, a key intermediate in biological nitrogen fixation (Babalola, 2010; Mbai *et al.*, 2013), was observed in seven isolates (VW1, VW2, VW7, VW8, VW10, VW11, and VW12). These strains produced a brown to yellow colour upon the addition of Nessler's reagent, indicating positive ammonia production.

Phosphate Solubilization: Phosphorus is an essential nutrient often found in insoluble forms in the soil. Phosphate solubilization, which involves the production of metabolites such as phosphate-binding organic acids that convert insoluble phosphate into soluble forms, was demonstrated by eight isolates (VW4, VW5, VW6, VW7, VW8, VW9, VW10, VW11, and VW12). The formation of a clear zone around the colonies in the phosphate solubilization test indicated the successful degradation of calcium triphosphate, highlighting the strains' ability to enhance phosphorus availability to plants (Sherathia *et al.*, 2016).

Indole-3-Acetic Acid (IAA) Production: IAA, a critical phytohormone and auxin, plays a significant role in plant growth and development. Five strains (VW3, VW6, VW9, VW10, and VW12) were positive for IAA production, as evidenced by a reddish-brown colour after the addition of Salkowski's reagent. In Salkowski's reagent, a reddish-brown colour is produced due to the formation of bi-sulphonic acid of bi-cholestadiene. Several microorganisms, particularly within the *Bacillus* genus and other Gram-positive strains, are recognized for their ability to produce indole-3-acetic acid (IAA), a key phytohormone that regulates various aspects

Table 3. Plant growth promoting ability of the test bacterial strains

Sr. No.	TESTS	VW-1	VW-2	VW-3	VW-4	VW-5	VW-6	VW-7	VW-8	VW-9	VW-10	VW-11	VW-12
1	Ammonia	+	+	-	-	-	-	+	+	-	+	+	+
2	IAA	-	-	+	-	-	+	-	-	+	+	-	+
3	Phosphate	-	-	-	+	+	+	+	+	+	+	+	+

Table 4. Biocontrol potential of the test bacterial strains

Sr. No.	TESTS	VW-1	VW-2	VW-3	VW-4	VW-5	VW-6	VW-7	VW-8	VW-9	VW-10	VW-11	VW-12
1	Amylase	+	-	+	+	-	+	-	+	+	+	-	+
2	Protease	+	+	+	-	+	+	-	-	+	+	+	+
3	Cellulase	+	+	-	+	+	+	+	-	+	+	+	+

+ positive, - negative

Table 5. Effect of vermiwash on seedling characteristics of *Vigna radiata*

Sr. No.	Treatment	Hypocotyl length (cm)	Root Length (cm)
1	Vermiwash – 1 (10%)	5.0 ± 0.3a	3.0 ± 0.3a
2	Vermiwash – 2 (20%)	6.0 ± 0.5b	5.0 ± 0.3b
3	Vermiwash – 3 (30%)	8.0 ± 0.5c	5.5 ± 0.9b
4	Control	5.0 ± 0.3a	3.0 ± 0.2a

Within a column, means followed by different letters are significantly different at p≤0.05

of plant growth and development (Datta & Basu, 2000; Wahyudi *et al.*, 2011). IAA plays a crucial role in both short-term responses, such as enhanced cell elongation, and long-term developmental processes, including cell division and differentiation.

Proteolytic Enzyme Production: The proteolytic activity of the isolates was assessed using a skimmed milk agar plate assay. Nine strains (VW1, VW2, VW3, VW5, VW6, VW9, VW10, VW11, and VW12) demonstrated positive protease activity, indicated by the formation of clear zones around the colonies. This proteolytic activity is vital as it converts insoluble nitrogenous compounds into soluble forms, making them available for plant uptake.

Amylase Activity: The presence of amylase was determined using starch agar plates. Eight strains (VW1, VW3, VW4, VW6, VW8, VW9, VW10, and VW12) exhibited clear zones of hydrolysis following the addition of Lugol's iodine, confirming amylase activity. These results are consistent with findings by Ravichandran *et al.* (2021), who documented the role of enzymes such as phosphatases, amylases, and proteases in nutrient cycling processes.

Cellulase Activity: The ability of the isolates to degrade cellulose was tested using carboxymethyl cellulose (CMC) agar plates. CMC, a water-soluble cellulose derivative, is employed to detect cellulase-producing microorganisms. Clear zones around the colonies on CMC agar indicate cellulase activity. Ten strains (VW1, VW2, VW4, VW5, VW6, VW7, VW8, VW9, VW10, and VW11) demonstrated positive cellulase activity, as evidenced by the formation of clear zones around the colonies (Baharuddin *et al.*, 2010; Gomashe *et al.*, 2013; Das *et al.*, 2014). Cellulase production was confirmed by the presence of clear zones around the colonies on Carboxymethyl Cellulose (CMC) agar plates. Ten isolates (VW1, VW2, VW4, VW5, VW6, VW7, VW8, VW9, VW10, and VW11) demonstrated positive cellulase activity.

Antagonistic Activity: The antagonistic potential of the isolates against the plant pathogen *Aspergillus niger* was evaluated. Two strains, VW11 and VW12, exhibited significant inhibitory activity, as indicated by the zone of inhibition. This activity suggests that these strains can potentially inhibit fungal growth through antimicrobial mechanisms. Previous studies have noted similar antagonistic characteristics in microorganisms isolated from organic solutions (Ram *et al.*, 2022).

Volatile Organic Compounds (VOCs): The production of VOCs, such as ammonia and hydrogen cyanide, by the bacterial strains was considered a potential biocontrol mechanism. VOCs can damage fungal cell membranes, inhibit spore germination, or induce oxidative stress, thereby suppressing fungal growth (Yang *et al.*, 2023).

Seed Coating Experiments:

1. **Vermiwash Coating:** Green gram seeds (*Vigna radiata*) were coated with vermiwash at concentrations of 10%, 20%, and 30%. The 30% vermiwash concentration resulted in the highest growth, as detailed in Table 5.
2. **Microbial Coating:** Seeds were coated with bacterial isolates (VW1, VW4, and VW9) and compared to a control group coated with distilled water. Seeds treated with VW9 showed superior growth in both hypocotyl and root length, as compared to VW1 and VW4, indicating VW9's effective plant growth-promoting capabilities (Table 6).

Table 6. Effect of seed bacterization on seedling characteristics of *Vigna radiata*

Sr. No.	Treatment	Hypocotyl length (cm)	Root Length (cm)
1	W-1	5.0±0.3a	3.0 ± 0.3a
2	VW-4	6.0 ± 0.6ab	4.0 ± 0.6ab
3	VW-9	10.0 ± 0.7c	6.5 ± 0.8c
4	Control	5.0±0.3a	3.0±0.2a

Within a column, means followed by different letters are significantly different at p≤0.05

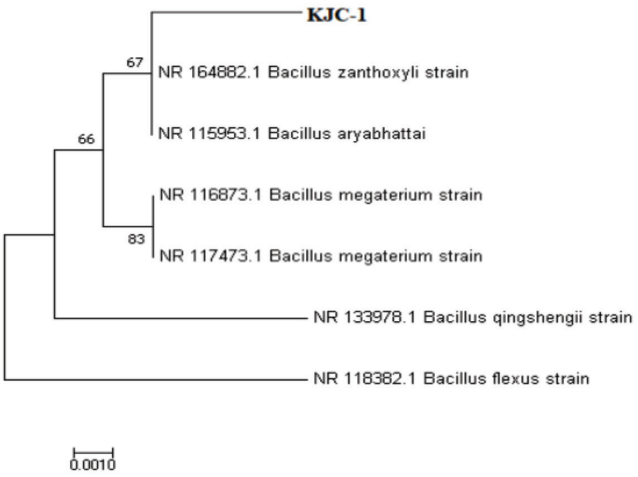

Fig. 1. Phylogenetic tree of strain KJC 1(VW-9) based on 16S rRNA gene sequences, constructed using the neighbour-joining method. The tree includes closely related Bacillus strains, with bootstrap values (based on 1000 replications) shown at the branching points, indicating confidence levels above 50%. GenBank accession numbers are provided in parentheses. *Bacillus flexus* (NR 118382.1) was used as the outgroup. The scale bar represents 0.001 substitutions per nucleotide position.

Molecular Identification: 16S rRNA sequencing identified the strain VW9 as *Bacillus zanthoxylli*. Phylogenetic analysis revealed a 99.4% sequence similarity with *Bacillus aryabhattai* and 99.2% with *Bacillus megaterium*. The strain exhibited over 98% homology with *Bacillus zanthoxyli*, a relatively under-studied plant growth-promoting (PGP) bacterium. This strain's physiological and biochemical traits suggest it may represent a novel species within the *Bacillus* genus. The GenBank accession number for VW9 is PP694489. This is the first reported study of *Bacillus zanthoxyli* isolated from vermiwash, showcasing its potential as an effective plant growth promoter and a sustainable option for organic farming (Dihazi *et al.*, 2012).

CONCLUSION

Vermiwash, a liquid fertilizer derived from vermicomposting with worms such as *Eisenia foetida* and *Lumbricus rubellus*, is rich in essential nutrients, plant hormones, and beneficial microorganisms. It is widely recognized for enhancing plant growth, improving crop yield, and protecting against pathogens, making it a valuable tool in organic farming. In our study, we investigated the effects of different concentrations of vermiwash on the seed germination of green gram (*Vigna radiata*). The results indicated that vermiwash significantly improved seed germination and provided pathogen protection, likely due to its nutrient content and beneficial microbial population.

Additionally, we explored the impact of various microbial strains on seed germination by coating seeds with these strains. Our findings showed that seeds coated with Bacillus strains, particularly VW9, demonstrated superior growth in terms of hypocotyl and root length compared to controls. This highlights the potential of these Bacillus strains as effective plant growth promoters and bio-fertilizers.

Given the adverse effects associated with chemical fertilizers and pesticides, there is a growing shift towards organic farming. The use of vermiwash and beneficial microorganisms from it provides a sustainable and environmentally friendly alternative. These findings highlight the potential of vermiwash and specific microbial strains to enhance agricultural productivity while supporting organic farming practices. The application of these organic solutions can meet the increasing demands for healthier and more sustainable agricultural methods, offering significant advantages in promoting plant growth and soil health.

Authors' contribution

Conceptualization and designing of research work (CMR, RP); Execution of field/lab experiments and data collection (DL, EL,PW); Analysis of data and interpretation (CMR, DL, EL,PW); Preparation of manuscript (CMR, RP, DL, EL,PW).

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