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Biochemical characterisation and plant growth-promoting potential of native *Bacillus* spp. isolated from Bhendi (*Abelmoschus esculentus* L. Moench) Rhizosphere

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Abstract
Bhendi (*Abelmoschus esculentus* L. Moench), an economically important vegetable crop, is widely cultivated across tropical and subtropical regions but often suffers from reduced productivity due to soil nutrient depletion and overuse of chemical fertilisers. In this study, fifteen native *Bacillus* spp. isolates were obtained from the rhizosphere soils of bhendi cultivated in major growing tracts of Tamil Nadu and evaluated for their biochemical and plant growth-promoting characteristics. Morphological and microscopic examinations confirmed that all isolates were endospore-forming, Gram-positive rods. Biochemical analyses revealed that all isolates produced indole acetic acid (IAA) and hydrogen cyanide (HCN) and tested positive for casein, starch, and gelatin hydrolysis, while negative for hydrogen sulphide (H_2S) production. Among the isolates, AUBs-1 exhibited superior performance in the roll towel assay, recording the highest germination percentage (95%), shoot length (12.5 cm), root length (9.6 cm), and vigour index (2097.5), followed by AUBs-2 and AUBs-14. The untreated control recorded significantly lower values for all parameters. The study highlights the potential of native *Bacillus* spp., particularly isolate AUBs-1, as effective bioinoculants for enhancing seed germination, early vigour, and sustainable growth of bhendi. Further molecular characterisation and field validation of these isolates could support their development into commercial biofertilizer formulations for eco-friendly crop management.

Keywords: *Bacillus subtilis*, bhendi, biochemical characterisation, PGPR, vigour index, bioinoculant, *Abelmoschus esculentus*

Introduction
Bhendi (*Abelmoschus esculentus* L. Moench), commonly known as okra or lady's finger, is one of the most important vegetable crops cultivated extensively in tropical and subtropical regions. It is highly valued for its tender pods, which are a rich source of essential micronutrients, including vitamins A, B, and C, calcium, and iron, thus contributing significantly to human nutrition and global food security [10]. As a member of the Malvaceae family, bhendi is a multipurpose crop, with its leaves, buds, flowers, pods, stems, and seeds all being utilized for various culinary, industrial, and traditional medicinal applications [8]. Bhendi is highly regarded by health organizations, including the World Health Organization (WHO), for its numerous disease-fighting abilities linked to its high content of fiber and antioxidants [14]. India is the world's largest producer of bhendi, occupying the fifth position among vegetable crops in terms of total area and production.

However, bhendi productivity is frequently hampered by factors like nutrient depletion, soil-borne pathogens, and the detrimental effects of excessive chemical inputs. While inorganic fertilisers temporarily boost yields, their continuous use leads to soil degradation, environmental pollution, and nutrient deterioration [20, 18]. To overcome these constraints and achieve sustainable production, there is a growing global emphasis on adopting eco-friendly, microbial-based approaches that effectively enhance both plant growth and soil fertility [6, 29].

The utilization of native Plant Growth-Promoting Rhizobacteria (PGPR), such as *Bacillus* species, isolated directly from the bhendi rhizosphere, presents a promising solution. These microbes naturally enhance nutrient availability and suppress pathogens through mechanisms like phytohormone (e.g., Indole Acetic Acid or IAA) and volatile compound (e.g., Hydrogen Cyanide or HCN) production. Although the general benefits of *Bacillus* are well-documented, specific strains native to a crop's rhizosphere are often more effective. This study, therefore, aimed to isolate, biochemically characterise, and evaluate the plant growth-promoting potential of indigenous *Bacillus* strains from the bhendi rhizosphere to identify superior isolates for developing sustainable bioinoculants. The overall goal was to select the most potent isolate capable of significantly enhancing the germination and early vigour of bhendi seedlings.

Materials and Methods

Seed Source

Bhendi (*Abelmoschus esculentus* L.) variety 'Sakthi' was used for the study. The seeds were procured from the Tamil Nadu Agricultural University (TNAU) Seed Sale Counter, Coimbatore, Tamil Nadu.

Isolation of PGPR from bhendi rhizosphere

Soil samples were collected from the rhizosphere region of bhendi plants from fifteen different bhendi growing tracts of Tamil Nadu. After removing the loosely adhering soil from the freshly excised roots, root segments (1g) were taken and suspended in 10 ml of sterile distilled water to get 10^{-1} dilution. Serial dilutions were made to get dilutions up to 10^{-7}. From 10^{-6} and 10^{-7} one ml of each aliquot was pipetted out and poured into a sterilized Petri plate containing nutrient agar medium for *Bacillus* and they were gently rotated clockwise and anti-clockwise for uniform distribution and incubated at room temperature (28±2 ºC) for 48 and 72 hours for bacteria. Colonies with characteristics of *Bacillus* spp were isolated and purified by the streak plate method [22] on a Nutrient agar medium. The pure cultures were maintained on the respective agar slants at 4 ºC.

Morphological Characterization of *Bacillus* spp.,

The morphological and microscopic characteristics of all Fifteen *Bacillus* sp*p*. isolates were examined. After incubating on Nutrient agar plates for 4 to 6 days at 28±2 °C, individual colonies were characterized based on various traits, including colour, shape, colony margins, irregular spreading edges, opacity and texture. Microscopic observations focused on identifying *Bacillus* sp*p*. by their endospore-forming, rod-shaped structures. The identified isolates were designated as AUBS 1 to AUBS 10.

Biochemical characterization of the *Bacillus* isolates

Well-grown prolonged cultures of *B. subtilis* were subjected to biochemical tests according to Bergey's Manual for Determinative Bacteriology [4].

Gram's staining technique

A loopful bacterial culture was transferred on a clean slide and a smear was made which was air dried and heat-fixed. The smear was flooded for one minute, with crystal violet. Excess stain was poured off and the slide was washed in a gentle stream of water. Lugol's iodine solution was applied and allowed to remain for one min, decolorized with 95 percent ethyl alcohol. The smear was washed in a gentle stream of water and counter-stained with safranin for 30 seconds. The Gram-negative cells appeared red and Gram-positive cells appeared violet in colour [13].

IAA Production test

All the test bacterial cultures were inoculated in nutrient broth with tryptophan (0.2%) or without tryptophan incubated at 28±2 °C for seven days. Cultures were centrifuged at 3000 rpm for 30 min, 2 ml of the supernatant was mixed with 2 drops of orthophosphoric acid and 4ml of Solawaski's reagent (50 ml, 35% perchloric acid; 1ml 0.5 FeCl3). The development of a pink colour indicates IAA production [2].

Casein hydrolysis

The bacterial cultures were streaked on skim milk agar and incubated at 25 °C or 37 °C. The milk agar culture shows the presence or absence of a clear area, or zone of proteolysis surrounding the growth of each of the bacterial test organisms [24].

HCN Production test

Production of HCN was determined by Wei *et al*. [31]. Bacteria were grown on TSA supplemented with 4.4 g/l of glycine, white filter paper strips soaked in picric acid solution (2.5 g of Na2CO3 and 1 lit of water) were placed in the lid of each Petri dish, sealed with parafilm and incubated for two to three days at 28±2 °C. After incubation HCN production was indicated by the presence of a coloured zone around the bacteria.

KOH Test

A loopful bacteria culture was placed on a glass slide. One drop of three percent KOH solution was added over it and thoroughly mixed with the help of an inoculation needle. Bacterial chromosomes separate as thin threads, indicating gram-negative bacteria [25].

Starch hydrolysis

Add starch to the Nutrient Agar medium. Streak the test bacteria and incubate for 45 hrs. Then the Petri dishes were flooded with 1% iodine solution. The Halo zone around the bacterial colonies indicates a positive result [28].

Gelatin liquefaction

The nutrient Agar medium was amended with Gelatin and the test bacteria was inoculated. Plates were incubated for 48 hrs. Melting of Nutrient agar media in the tubes indicated a positive result for Gelatin liquefaction [9].

Fluorescent pigmentation

The test tubes containing sterile King's B medium, inoculated with the isolates of P fluorescens incubated for five days after observed. Yellowish green fluorescent pigment observed under UV light (365 nm) indicated positive results [17].

Urease activity

Nutrient agar medium with urea was inoculated with test bacteria. A clear zone around the colonies indicated a positive result [5].

HS Production test

Test bacteria were inoculated in the media containing SIM (Sulfide Indole Motility) medium, Inoculated tubes were incubated at 37 °C for 24-48 hrs. The formation of black precipitation was observed for positive results [5].

Methyl red test

In clean test tubes, five milliliters of glucose phosphate broth (1g glucose, 0.5 percent KH_2PO_4, 0.5 percent Peptone and 100 ml distilled water) were dispensed and sterilized. After that, the tubes were infected with the test organism and incubated for 48 hours at 37°C. A red colour showed a favourable reaction at the end of the incubation period [19].

Preparation of the culture filtrate of *Bacillus subtilis* isolates:

B. subtilis isolates were inoculated into Erlenmeyer flasks containing 100 ml of sterile Nutrient broth. The flasks were kept on a rotary shaker at 100 rpm for 48 hrs. Bacterial culture broth was centrifuged at 10,000 rpm for 10 min. Then the supernatant was filtered through a 0.22 µm pore size filter and the filtrates obtained were used for further studies [15].

Plant Growth Promotion activity of *Bacillus* strains

Bacillus strains were cultured in nutrient broth at 28 ± 2 °C for 48 h on a rotary shaker (150 rpm), centrifuged at 6,000 rpm for 15 min, and resuspended in phosphate buffer (0.01 M, pH 7.0) to a concentration of 10^8 cfu/ml (OD_{595} = 0.3) (30). Bhendi seeds were soaked in the bacterial suspension for 2 h, blot-dried, and incubated in moist paper towels under controlled growth chamber conditions (25 ± 1 °C, 80% RH, 14 h light/10 h dark) (16). Seeds treated with sterile water served as controls. After 21 days, root and shoot lengths were measured, and germination percentage was calculated as:

$$\text{Germination\%} = \frac{\text{Number of seeds germinated}}{\text{Total Number of seeds sown}} \text{X100}$$

The vigour index was calculated using the following formula (1)

Vigour index = Percent germination × Seedling length (Shoot length + Root length)

Statistical analysis

The collected data were statistically analysed using WASP (Web Agri Stat Package) version 2.0, developed by ICAR-Central Coastal Agricultural Research Institute, Goa. Statistical evaluation was performed using analysis of variance (ANOVA) at a significance level of $P<0.05$, followed by Duncan's Multiple Range Test (DMRT) [11].

Results and Discussion

Biochemical Characterisation of Bacillus Isolates

The biochemical characterisation of *Bacillus* isolates revealed consistent results across all fifteen isolates (AUBs-1 to AUBs-15). All isolates produced indole acetic acid (IAA) and hydrogen cyanide (HCN) and showed positive reactions for casein hydrolysis, starch hydrolysis, gelatin liquefaction, and catalase activity. None of the isolates produced hydrogen sulphide (H_2S) or pigments. Furthermore, all isolates were uniformly Gram-positive, confirming their identity as *Bacillus* spp. The uniformity observed in the biochemical reactions indicates a high degree of metabolic stability among the isolates, reflecting their potential as efficient plant growth-promoting rhizobacteria (PGPR) (Table1).

Twenty-four PGPR strains isolated from rhizosphere soils were identified as Bacillus spp. based on biochemical and functional characteristics, including phosphate solubilisation, siderophore production, IAA synthesis, and HCN production [21]. Most Bacillus isolates also exhibited positive starch hydrolysis, while Bacillus subtilis CBS31 was characterised for its biochemical stability and thermodynamic properties, supporting its suitability for bio-industrial and agricultural applications [23]. These results are further supported by reports confirming the biochemical versatility of Bacillus spp. in various environmental and industrial contexts [7].

Plant Growth-Promoting Activity of Bacillus Isolates

The plant growth-promoting efficiency of the *Bacillus* isolates was evaluated using the roll towel method. Significant variations were observed among the isolates in terms of germination percentage, shoot length, root length, and vigour index (Table 2). Among the isolates, AUBs-1 recorded the highest germination percentage (95%), longest mean shoot length (12.5 cm), root length (9.6 cm), and maximum vigour index (2097.5), indicating its superior performance in promoting early seedling growth. Isolates AUBs-2 and AUBs-14 also exhibited high germination percentages (94% and 93.5%) and vigour indices (1926.0 and 1831.0, respectively), confirming their strong growth-promoting potential (Table 2).

In contrast, the untreated control exhibited the lowest performance, recording 83% germination, 10.2 cm shoot length, 8.2 cm root length, and a vigour index of 1529.6, clearly indicating the positive impact of *Bacillus* inoculation on seed germination and early seedling growth. These findings are consistent with previous reports, where treatments with bioagents such as *Bacillus* and *Pseudomonas* significantly enhanced seed germination, shoot and root length, and seedling vigour in okra [12]. Similarly, single inoculation with PGPR strains such as Okhm5-4 and Okhm10 markedly improved okra growth, particularly in shoot and root development, compared with co-inoculation and untreated controls [26]. PGPR have also been reported to promote plant growth through mechanisms such as nitrogen fixation, phosphate solubilisation, and ammonia production [3]. In the present study, Bacillus isolates from the bhendi rhizosphere exhibited comparable plant growth-promoting traits, including IAA and HCN production, enzymatic activity, and nutrient mobilisation, collectively enhancing seed germination and seedling vigour, thus demonstrating their potential as effective bioinoculants for sustainable crop production.

Table 1: Biochemical characteristics of different *Bacillus* isolates

SI. NO	Isolate NO	H_2S test	IAA production	HCN production	Casein hydrolysis	Pigment	Gram staining	Methyl Red test	Gelatin liquefaction	Starch hydrolysis	KOH TEST
1	AUBs-1	-ve	+ve	+ve	+ve	-ve	+ve	-ve	+ve	+ve	-ve
2	AUBs-2	-ve	+ve	+ve	+ve	-ve	+ve	-ve	+ve	+ve	-ve
3	AUBs-3	-ve	+ve	+ve	+ve	-ve	+ve	-ve	+ve	+ve	-ve
4	AUBs-4	-ve	+ve	+ve	+ve	-ve	+ve	-ve	+ve	+ve	-ve
5	AUBs-5	-ve	+ve	+ve	+ve	-ve	+ve	-ve	+ve	+ve	-ve
6	AUBs-6	-ve	+ve	+ve	+ve	-ve	+ve	-ve	+ve	+ve	-ve
7	AUBs-7	-ve	+ve	+ve	+ve	-ve	+ve	-ve	+ve	+ve	-ve
8	AUBs-8	-ve	+ve	+ve	+ve	-ve	+ve	-ve	+ve	+ve	-ve
9	AUBs-9	-ve	+ve	+ve	+ve	-ve	+ve	-ve	+ve	+ve	-ve
10	AUBs-10	-ve	+ve	+ve	+ve	-ve	+ve	-ve	+ve	+ve	-ve

Table 2: Plant growth-promoting activity of *Bacillus* sp isolates (Roll towel method)

Isolates	Germination Percentage (%)	Mean shoot length (cm)	Mean root length (cm)	Vigour index
AUBs-1	95.00a	12.5	9.6	2097.5
AUBs-2	94.00ab	11.8	9.2	1926.0
AUBs-3	90.00d	10.0	7.9	1611.0
AUBs-4	88.50de	9.8	7.8	1555.8
AUBs-5	91.5°Cd	10.5	8.3	1684.2
AUBs-6	88.00e	9.7	7.7	1531.2
AUBs-7	87.50e	9.5	7.6	1503.5
AUBs-8	89.00de	10.7	8.5	1703.8
AUBs-9	90.50d	10.3	8.4	1654.5
AUBs-10	87.50e	9.6	7.7	1504.5
AUBs-11	93.00bc	11.2	8.9	1836.0
AUBs-12	92.0°C	11.0	8.7	1781.4
AUBs-13	88.00e	9.6	7.8	1526.4
AUBs-14	93.50b	11.0	8.8	1831.0
AUBs-15	87.00e	9.4	7.5	1471.8
Control	83.00f	10.2	8.2	1529.6

*Values are means of three replications

Means followed by a common letter are not significantly different at the 5% level by DMRT

Fig 1: Axenic culture of AUBs-1

Conclusion

The present study successfully isolated and characterised fifteen *Bacillus* spp. from the bhendi rhizosphere, confirming their identity through morphological and biochemical analyses. All isolates exhibited key plant growth-promoting traits such as IAA and HCN production, casein, starch and gelatin hydrolysis, indicating their strong metabolic and functional potential. Among them, isolate AUBs-1 demonstrated superior growth-promoting ability, recording the highest germination percentage and vigour index. The overall performance of the isolates was significantly higher than the untreated control, highlighting their effectiveness as bioinoculants. These findings suggest that *Bacillus* isolates, particularly AUBs-1, hold promise for development into sustainable bioformulations for enhancing bhendi growth and productivity.

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