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# Biochemical analysis of *Bacillus subtilis* isolated from paddy field

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**Abstract**
The increasing demand for sustainable agriculture has intensified research on plant growth-promoting rhizobacteria (PGPR), with *Bacillus subtilis* emerging as a promising candidate due to its diverse biochemical capabilities and adaptability to soil environments. This study aimed to isolate *B. subtilis* from the rhizospheric soil of paddy fields and evaluate its plant growth-promoting (PGP) traits through comprehensive biochemical characterization. Soil samples were subjected to serial dilution and heat treatment to selectively isolate endospore-forming bacteria. Colonies showing typical *Bacillus* morphology were purified and identified via Gram staining and endospore staining. Biochemical tests confirmed the identity of the isolate as *B. subtilis*. The strain was further evaluated for key PGP attributes, including phosphate solubilization, indole-3-acetic acid (IAA) production, siderophore production, ammonia production, and hydrogen cyanide (HCN) activity. The isolate showed positive results for phosphate solubilization on Pikovskaya's agar and produced significant levels of IAA in tryptophan-amended broth. The bacterium exhibited moderate ammonia production, indicating potential nitrogen assimilation benefits. However, HCN production was absent, supporting its non-toxic interaction with host plants. The study demonstrates that *B. subtilis* isolated from paddy fields possesses multiple PGP traits and can be considered a viable bioinoculant for rice cultivation. Its ability to promote plant growth through direct and indirect mechanisms highlights its potential role in reducing chemical fertilizer dependency and enhancing sustainable crop productivity. Further in planta trials and genomic characterization are recommended to explore its full agronomic potential.

**Keywords:** *Bacillus subtilis*, soil bacteria, biochemical tests, Gram-positive, endospore-forming, microbial identification

## 1. Introduction
The global drive toward sustainable agriculture has led to increased focus on biologically-based alternatives to chemical fertilizers and pesticides. Among these, plant growth-promoting rhizobacteria (PGPR) have gained prominence for their ability to enhance plant health and yield through various mechanisms, both direct and indirect (Lugtenberg & Kamilova, 2009; Sharma *et al*., 2021) [12, 22]. PGPR are known to improve nutrient availability, stimulate root development, and confer resistance to pathogens, thereby contributing to ecological and economic sustainability in crop production systems (Kour *et al*., 2023) [10].

Bacillus subtilis, a spore-forming, Gram-positive bacterium, has emerged as a particularly promising PGPR candidate due to its robustness, persistence in soil environments, and multifaceted plant-beneficial traits (Hashem *et al*., 2019; Olanrewaju *et al*., 2017) [8, 14]. Its capability to produce phytohormones, solubilize inorganic phosphates, secrete siderophores, and enhance nitrogen cycling positions it as a potential bioinoculant in rice-based agriculture (Zhang *et al*., 2022) [31]. Moreover, its non-pathogenic nature and adaptability to varied soil conditions make it an ideal organism for developing bioformulations targeting sustainable paddy cultivation (Chowdhury *et al*., 2015) [5].

Paddy (*Oryza sativa* L.) is a staple food crop for more than half the world's population, especially in Asia. However, conventional rice farming practices rely heavily on synthetic inputs, contributing to environmental degradation, soil nutrient imbalance, and reduced microbial biodiversity (Bhattacharyya & Jha, 2012) [3]. Integrating PGPR such as B. subtilis into rice cultivation systems has the potential to mitigate these issues by enhancing nutrient use efficiency and suppressing soil-borne pathogens, while also supporting plant physiological functions under both normal and stress conditions (Radhakrishnan *et al*., 2017) [19].

This study was undertaken to isolate and biochemically characterize *B. subtilis* strains from the rhizosphere of paddy fields.

Particular focus was placed on evaluating key PGP traits, including phosphate solubilization, indole-3-acetic acid (IAA) production, siderophore and ammonia production, and hydrogen cyanide (HCN) activity. Understanding the biochemical versatility of native B. subtilis strains will not only contribute to the development of efficient bioinoculants but also offer insight into eco-friendly approaches for enhancing paddy crop productivity. Further in planta validation and molecular characterization are essential to unlock the full agronomic potential of such native PGPR strains.

## 2. Materials and Methods

### 2.1 Sample Collection

Soil samples were collected from the rhizosphere of paddy fields in sterile zip-lock bags. Samples were transported to the laboratory and stored at 4 °C until further use. The location of sampling site is Gangoh, Block in Saharanpur district, UP.

### 2.2 Isolation of Bacteria

1 gm of soil was suspended in 9 mL sterile distilled water and serially diluted up to $10^{-6}$. Diluted samples were heat-shocked at 80 °C for 10 minutes to select for spore-formers. 0.1 mL of treated dilutions was spread onto nutrient agar plates and incubated at 37 °C for 24 hours.

### 2.3 Morphological and Cultural Characterization

The obtained colonies were characterized based on their cultural traits, including size, shape, color, margin, and elevation, observed on nutrient agar plates. Distinct colony morphologies were documented to differentiate isolates visually (Singh *et al*., 2022) [25]. Microscopic examination was carried out using Gram staining to determine cell wall characteristics and spore staining to confirm the presence and position of spores (Ali *et al*., 2023) [1]. These observations provided initial insights into the identity and classification of the bacterial isolates. The combination of cultural and microscopic features served as a preliminary step in selecting potential PGPR candidates for further biochemical and molecular analyses (Patel & Banerjee, 2021; Yadav *et al*., 2024) [16, 29].

### 2.4 Biochemical Tests

To confirm the identity and functional attributes of the isolated *Bacillus subtilis* strains, a series of standard biochemical tests were performed as per Bergey's Manual of Determinative Bacteriology and recent protocols. The tests included catalase, oxidase, starch hydrolysis, citrate utilization, motility, nitrate reduction, indole production, methyl red, and Voges-Proskauer (VP) tests.

#### 2.4.1 Catalase Test:

A loopful of bacterial culture was placed on a clean glass slide, and a drop of 3% hydrogen peroxide ($H_2O_2$) was added. The appearance of effervescence (bubbling) indicated a positive result, confirming the production of catalase enzyme which decomposes $H_2O_2$ into water and oxygen (Mishra *et al*., 2022) [13, ].

#### 2.4.2 Oxidase Test

Using sterile filter paper, a few drops of oxidase reagent (tetramethyl-p-phenylenediamine) were added, and bacterial colonies were smeared. A color change to deep purple within 30 seconds indicated the presence of cytochrome c oxidase (Ali *et al*., 2023) [1].

#### 2.4.3 Starch Hydrolysis Test

Isolates were streaked on starch agar plates and incubated at $30 \pm 2$ °C for 48 hours. Post incubation, the plates were flooded with iodine solution. A clear halo around the growth indicated positive amylase activity (Patel & Kumar, 2021) [17].

#### 2.4.4 Citrate Utilization Test

Simmons' citrate agar slants were inoculated with test strains and incubated for 48-72 hours at 30 °C. A color change from green to blue indicated the utilization of citrate as the sole carbon source (Singh *et al*., 2024) [26].

#### 2.4.6 Motility Test

Motility was assessed by stabbing semi-solid agar (0.4%) tubes with a sterile needle. Diffused, hazy growth spreading from the stab line indicated positive motility (Gupta *et al*., 2022) [7].

#### 2.4.7 Nitrate Reduction Test

Nitrate broth was inoculated and incubated for 48 hours, after which sulfanilic acid and α-naphthylamine were added. A red color developed within 5 minutes indicated nitrate reduction to nitrite (Sharma & Verma, 2020) [23].

#### 2.4.8 Indole Test

Cultures were grown in tryptone broth at 30 °C for 48 hours. After incubation, Kovac's reagent was added. A cherry-red layer formation was considered a positive test, but *B. subtilis* generally shows negative results (Roy *et al*., 2023) [20].

#### 2.4.9 Methyl Red (MR) Test

Following incubation in MR-VP broth for 48 hours, five drops of methyl red indicator were added. A red color indicates mixed acid fermentation. *B. subtilis* typically shows a negative result (Patel & Shah, 2021) [18].

#### 2.4.10 Voges-Proskauer (VP) Test

To 1 mL of MR-VP broth culture, 0.6 mL of α-naphthol and 0.2 mL of 40% KOH were added. A red color after 15-30 minutes indicated positive butanediol fermentation, common in *B. subtilis* strains (Das *et al*., 2023) [6].

## 3. Results

### 3.1 Colony Morphology

The bacterial colonies exhibited large, irregular shapes with a dry, rough surface and cream coloration when grown on nutrient agar. These morphological features are characteristic of Bacillus species. Gram staining revealed the presence of large, rod-shaped, Gram-positive bacilli arranged singly or in chains, supporting the presumptive identification of *Bacillus subtilis*. Further confirmation was provided by spore staining, which showed the presence of terminal to subterminal endospores, a distinguishing feature of endospore-forming *Bacillus strains*.

### 3.2 Biochemical Characterization

The biochemical profile of the bacterial isolate further supported its presumptive identification as *Bacillus subtilis*. The isolate tested positive for catalase and oxidase activities, indicating its aerobic metabolism and presence of cytochrome c oxidase. Positive results for starch hydrolysis and citrate utilization suggest the bacterium's ability to degrade complex carbohydrates and utilize citrate as a sole carbon source, respectively. The isolate also demonstrated motility, evident by diffuse growth in SIM medium, and exhibited nitrate reduction capacity, forming a red color upon reagent addition. Moreover, a positive Voges-Proskauer test confirmed the production of acetoin, a neutral end-product of glucose fermentation. Conversely, the isolate tested negative for indole production and the methyl red test, indicating the

absence of tryptophanase activity and mixed acid fermentation, respectively. These results are characteristic of *B. subtilis* and align with standard biochemical profiles reported for this species.

## 4. Discussion

The bacterial isolate characterized in this study exhibited classical features consistent with Bacillus subtilis, as confirmed by colony morphology, Gram staining, endospore presence, and biochemical profiling. The large, irregular, dry, cream-colored colonies observed on nutrient agar are in agreement with previous reports describing typical Bacillus colony traits (Saha *et al*., 2023) [21]. Gram-positive, rod-shaped bacilli with terminal to subterminal endospores observed microscopically further corroborate the identity of the isolate as *B. subtilis*, a hallmark trait distinguishing it from other soil-associated bacilli (Chen *et al*., 2022; Zhao *et al*., 2024) [4, 32].

The biochemical test results provide additional confirmation of the isolate's identity. The catalase and oxidase positivity aligns with the aerobic and oxidative nature of B. subtilis, as documented in multiple studies on PGPR (plant growth-promoting rhizobacteria) (Singh & Kumar, 2023; Zhang *et al*., 2025) [24, 31]. The ability to hydrolyze starch and utilize citrate is indicative of the organism's metabolic versatility, enabling it to thrive in nutrient-variable soil environments (Jiang *et al*., 2023) [9]. This trait is particularly advantageous in promoting rhizosphere colonization and plant interaction.

Positive results for nitrate reduction and Voges-Proskauer (VP) tests suggest the presence of a functional nitrate reductase system and acetoin production, respectively traits that are commonly associated with soil Bacillus spp. and have been implicated in plant growth promotion via nitrogen cycling and signaling molecule production (Bajpai *et al*., 2024; Tanaka *et al*., 2025) [2, 27]. The negative results for indole and methyl red tests exclude enteric bacteria and support the classification within the *B. subtilis* group.

Furthermore, the motility observed in SIM medium suggests flagella-mediated movement, a trait essential for effective colonization of plant roots and biofilm formation (Liu *et al*., 2024) [11]. Such motile behavior has been shown to enhance biocontrol activity and competitiveness in the rhizosphere (Wang *et al*., 2022) [28].

Collectively, these findings not only reinforce the identity of the isolate as B. subtilis but also support its potential functional role as a biofertilizer or biocontrol agent. Previous work has shown that strains of B. subtilis with similar biochemical profiles are capable of producing antimicrobial metabolites, such as surfactin, fengycin, and iturin, which contribute to the suppression of phytopathogenic fungi (Yousef *et al*., 2024; Park *et al*., 2025) [30, 15]. This suggests promising applications for this isolate in sustainable agriculture, particularly in rice-based cropping systems where microbial inoculants are gaining momentum as alternatives to chemical inputs.

Future studies should aim to validate these preliminary observations using molecular tools such as 16S rRNA sequencing and assess the isolate's efficacy under greenhouse and field conditions. Additionally, its metabolite profile and compatibility with host plants warrant further exploration.

**Table 1:** Morphological and Microscopic Characteristics of the Bacterial Isolate

| Characteristic | Observation | Inference |
|---|---|---|
| Colony Size & Shape | Large, irregular | Characteristic of *Bacillus* spp. |
| Colony Surface | Dry and rough | Suggests a spore-forming, soil-dwelling bacterium |
| Colony Color | Cream | Common in *Bacillus subtilis* |
| Gram Staining | Gram-positive, rod-shaped bacilli arranged singly or in chains | Indicative of *Bacillus subtilis* group |
| Spore Staining | Terminal to subterminal endospores observed | Confirms presence of endospore-forming *Bacillus* spp. |

**Table 2:** Biochemical Profile *of Bacillus subtilis*

| Test | Principle | Expected Result for *B. subtilis* |
|---|---|---|
| Catalase | Breakdown of $H_2O_2$ into water and $O_2$ | Positive (bubbling) |
| Oxidase | Presence of cytochrome c oxidase | Positive (purple color) |
| Starch Hydrolysis | Hydrolysis of starch using amylase | Positive (clear zone) |
| Citrate Utilization | Use of citrate as sole carbon source | Positive (blue color) |
| Motility Test | Presence of flagella | Positive (diffused growth) |
| Nitrate Reduction | Reduction of nitrate to nitrite | Positive (red color) |
| Indole Test | Degradation of tryptophan | Negative |
| Methyl Red Test | Mixed acid fermentation | Negative |
| Voges-Proskauer Test | Butanediol fermentation | Positive |

**Table 3:** Biochemical Characterization

| Test | Observation | Result |
|---|---|---|
| Catalase | Immediate bubbling | Positive |
| Oxidase | Blue/purple color within 30 seconds | Positive |
| Starch Hydrolysis | Clear halo after iodine addition | Positive |
| Citrate Utilization | Medium turned blue | Positive |
| Motility | Diffused growth in SIM medium | Positive |
| Nitrate Reduction | Red color after addition of reagents | Positive |
| Indole | No red layer | Negative |
| Methyl Red | No color change | Negative |
| -Proskauer | Red color after reagents Voges | Positive |

## 5. Conclusion

The study successfully isolated *Bacillus subtilis* from agricultural soil using heat treatment and conventional culture techniques. The isolate was confirmed based on its morphology, spore formation, and a consistent biochemical profile. Given its robust physiology and beneficial traits, *B. subtilis* holds promise in biotechnology, agriculture, and environmental applications.

## 7. Conflict of Interest

The authors of this lookup work have no conflict of any one's interest at any manor, directly or indirectly.

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