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Isolation and polyphasic characterization of *Bacillus altitudinis* Strain PUPB PSBP 2 from the legume rhizosphere with phosphate-solubilizing and other PGPR activities

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ABSTRACT

Phosphorus unavailability is a major constraint limiting crop productivity, necessitating sustainable alternatives to chemical fertilizers. In the present study, phosphate-solubilizing bacteria (PSB) were isolated from the rhizosphere soils of legume crops collected across Nalgonda, Mahabubnagar, and Rangareddy districts, Telangana, India. Among the ten efficient PSB isolates selected based on halo zone formation on Pikovskaya's agar (7.1–11.4 mm), isolate PSBP-2 exhibited the highest phosphate solubilization efficiency with a halo zone diameter of 11.4 mm. Quantitative estimation further confirmed its superior performance, releasing 58.7 ± 0.20 µg/mL of soluble phosphate in Pikovskaya's broth. PSBP-2 also demonstrated strong plant growth–promoting traits, including the highest indole-3-acetic acid (IAA) production (48.0 ± 2.0 µg/mL), notable phytase activity with a hydrolysis zone of 12 mm, maximum biofilm formation (OD_{600} = 0.78 ± 0.05), and the highest ammonia production (0.48 ± 0.03 OD_{450}). Morphological and biochemical characterization revealed that PSBP-2 is a Gram-positive, rod-shaped, capsulated, spore-forming bacterium with positive methyl red, citrate utilization, starch hydrolysis, and carbohydrate fermentation reactions. Molecular identification based on 16S rRNA gene sequencing confirmed the isolate as Bacillus altitudinis strain PUPB PSBP-2 (GenBank accession number: PX239710), which clustered within the Bacillus altitudinis clade in phylogenetic analysis. The integration of high phosphate solubilization, auxin production, organic phosphorus mineralization, and rhizosphere colonization traits establishes Bacillus altitudinis PUPB PSBP-2 as a promising multifunctional plant growth–promoting rhizobacterium with potential application as a biofertilizer for sustainable legume cultivation.

Keywords: *Phosphate-solubilizing bacteria; Legume rhizosphere; Bacillus altitudinis; Indole-3-acetic acid; Phytase activity; Biofilm formation; Plant growth–promoting rhizobacteria.*

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Introduction

Phosphorus (P) is a vital macronutrient required for multiple physiological functions in plants, including ATP-mediated energy transfer, nucleic acid formation, membrane integrity, and cellular signaling pathways. Despite its abundance in total soil reserves, only a minimal proportion is readily accessible to plants because phosphorus rapidly becomes immobilized through interactions with calcium, iron, or aluminum minerals, depending on soil type and pH conditions. As a result, poor phosphorus availability continues to limit crop yields across many agricultural systems, encouraging farmers to depend heavily on chemical phosphate fertilizers. However, these fertilizers often exhibit low use efficiency and contribute to several environmental concerns such as nutrient runoff, eutrophication, and the exhaustion of finite phosphate rock resources [1; 2]. In response to these limitations, phosphate-solubilizing bacteria (PSB) have emerged as promising biological tools for sustainable phosphorus management. PSB can convert insoluble inorganic and organic phosphorus sources into plant-available forms by secreting organic acids that acidify the rhizosphere and chelate metal ions. They also release enzymes such as phosphatases and phytases, which accelerate the mineralization of organically bound phosphorus [3; 1]. Current research highlights that beyond improving phosphorus availability, PSB significantly modulate soil microbial ecology and nutrient transformations, underscoring their important contribution to environmentally sustainable nutrient cycling [2].

Plant growth-promoting rhizobacteria (PGPR) comprise a diverse and functionally rich group of soil microorganisms known for enhancing plant growth through several complementary biochemical and physiological pathways. Beyond their ability to mobilize insoluble phosphorus, PGPR contribute to plant vigor by generating essential phytohormones such as indole-3-acetic acid (IAA), facilitating iron uptake through siderophore synthesis, fixing atmospheric nitrogen, producing ammonia, lowering stress-induced ethylene levels via ACC deaminase, and inhibiting plant pathogens through an array of antimicrobial compounds (4; 5). These multitiered mechanisms are particularly valuable in environments where nutrient scarcity or abiotic stress limits crop productivity, since relying on a single functional activity

often proves insufficient under challenging field conditions. Within this broader microbial group, Bacillus species have gained considerable prominence due to their resilience and formulation advantages. Their ability to form endospores ensures long-term survival, enhanced stress tolerance, and improved consistency in biofertilizer applications [6; 7]. Members of this genus are known to secrete a wide spectrum of organic acids, enzymes, siderophores, and bioactive metabolites that assist in nutrient mobilization, root stimulation, and the suppression of soil-borne diseases. Owing to these attributes, Bacillus-based inoculants are increasingly adopted in sustainable and climate-adaptive agricultural systems [8; 9]. Among them, *Bacillus altitudinis* has recently attracted research attention as an emerging rhizosphere inhabitant with notable plant growth-promoting capabilities. Studies have shown that this species can solubilize phosphate, synthesize plant-beneficial phytohormones, and enhance the resilience of plants to stresses such as drought and salinity. In addition, B. altitudinis has been associated with shifts in rhizosphere microbial composition, indicating its potential influence on broader soil ecological processes (10; 11). Nevertheless, as functional attributes can vary substantially among strains, detailed strain-specific assessment remains essential before large-scale agricultural deployment [4].

Methodology

Collection of rhizosphere soil samples

Rhizosphere soils were collected in a systematic manner from the root zones of actively growing legume crops cultivated in selected agricultural fields located across the Nalgonda, Mahabubnagar, and Rangareddy districts of Telangana, India. Sampling involved gently removing whole plants and collecting the soil firmly adhering to the root system within a depth of 0–15 cm, which corresponds to the metabolically active rhizosphere region. In total, 13 distinct rhizosphere samples were obtained, representing a wide range of cultivated legumes including chickpea, pigeon pea, black gram, green gram, lentil, soybean, pea, cowpea, horse gram, groundnut, red gram, Bengal gram, and green pea from various villages across the three districts. 500 g of rhizosphere soil from each site was collected using sterile spatulas to avoid external contamination. All samples were placed in sterile, properly labeled polyethylene bags indicating the crop type and collection location, and were transported to the microbiology laboratory under ambient conditions for subsequent analysis.

Isolation of phosphate-solubilizing bacteria

Phosphate-solubilizing bacteria (PSB) were isolated using Pikovskaya's (PVK) agar medium, which incorporates insoluble tricalcium phosphate as the exclusive phosphorus source to enable selective screening. For each soil sample, 100 µL of the appropriate serial dilution was aseptically dispensed onto PVK agar plates and evenly distributed using a sterile L-shaped spreader. The plates were incubated at 30 ± 2 °C for 5–7 days under aerobic conditions to allow microbial growth. Following incubation, the plates were carefully observed for colonies surrounded by distinct clear zones, which indicate the solubilization of tricalcium phosphate through microbial secretion of organic acids and subsequent dissolution of mineral phosphates. Bacterial colonies producing well-defined halo zones were considered putative phosphate-solubilizing isolates and were selected for further characterization.

Quantification of phosphate solubilization

Quantitative assessment of phosphate solubilization by the selected bacterial isolates was performed using the vanadomolybdate colorimetric method after culture growth in Pikovskaya's (PVK) broth. Actively growing bacterial cultures were inoculated into 100 mL of PVK broth containing 0.5% tricalcium phosphate (TCP) as the insoluble phosphorus source and incubated at 30 ± 2 °C for 5–7 days on a rotary shaker maintained at 120 rpm. Following incubation, the cultures were centrifuged at 10,000 rpm for 10 minutes to obtain a clear, cell-free supernatant. For phosphate quantification, 1 mL of the supernatant was mixed with vanadomolybdate reagent and subsequently treated with ammonium molybdate solution. The mixture was allowed to stand at room temperature for approximately 10 minutes to facilitate the formation of a yellow phosphovanadomolybdate complex. The intensity of the developed color was measured at 420 nm using a UV–Visible spectrophotometer, and the corresponding absorbance values were used to determine the concentration of solubilized phosphate.

Indole-3-Acetic Acid (IAA) Production

Indole-3-acetic acid (IAA) production by the bacterial isolates was assessed using a nitrocellulose membrane overlay technique. For this assay, each isolate was spot-inoculated onto Luria–Bertani (LB) agar plates supplemented with 5 mM L-tryptophan, followed by incubation at 28 ± 2 °C for 24–48 hours to allow growth and metabolite secretion. After incubation, sterile nitrocellulose membrane discs were carefully placed over the bacterial colonies so that the secreted indolic compounds could be absorbed onto the membrane surface. The membranes were then gently lifted and transferred onto filter papers pre-soaked with Salkowski reagent. The appearance of a pink to reddish coloration on the membranes signified the presence of IAA, and the depth of the color provided a qualitative measure of the auxin-producing capability of each isolate.

Quantification of Indole-3-Acetic Acid (IAA)

Quantitative determination of indole-3-acetic acid (IAA) produced by the bacterial isolates was carried out using the Salkowski colorimetric assay. Each isolate was inoculated into Luria–Bertani (LB) broth supplemented with 5 mM L-tryptophan and incubated at 28 ± 2 °C for 72 hours on a rotary shaker operating at 150 rpm to promote metabolite synthesis. After incubation, the cultures were centrifuged at 10,000 rpm for 10 minutes, and the resulting supernatant was carefully collected to obtain a cell-free extract. A measured volume of this supernatant was mixed with freshly prepared Salkowski reagent, and the mixture was kept in the dark at room temperature for approximately 30 minutes to allow the pink chromophore to develop. The absorbance of the reaction mixture was then recorded at 530 nm using a UV–Visible spectrophotometer. IAA concentrations were calculated by comparing absorbance values with a standard calibration curve generated using known concentrations of analytically pure IAA.

Phytase activity assay

Qualitative screening of phytase activity in the bacterial isolates was carried out using phytic acid agar containing sodium phytate as the sole phosphorus source. The medium was prepared, its pH was adjusted to 6.5–7.0, sterilized, and subsequently dispensed into sterile Petri dishes.

Actively growing cultures (18–24 hours old) were spot-inoculated onto the solidified medium and incubated at 37 ± 1 °C for 2–4 days under aerobic conditions. Following incubation, the plates were observed for clear halo zones surrounding the colonies, which signified the hydrolysis of sodium phytate by phytase-producing strains. The diameter of the hydrolytic zones was measured and used to qualitatively compare phytase-producing ability among the different isolates.

Quantitative biofilm formation assay

Biofilm-forming ability of the bacterial isolates was assessed through a crystal violet staining assay. Luria–Bertani (LB) broth was inoculated with 1% (v/v) of 24-hour-old cultures and incubated without agitation at 28 °C for 15 days to facilitate biofilm establishment. At the end of the incubation period, the planktonic phase was carefully decanted, and the tubes were rinsed gently with 0.85% sterile saline to remove loosely attached cells. The tubes were then air-dried to stabilize the adhered biofilm layer. Subsequently, a 1% crystal violet solution was added and allowed to stain the biofilm for 45 minutes at room temperature. After staining, excess dye was discarded, and the retained crystal violet was solubilized using 95% ethanol. The intensity of the extracted dye, corresponding to biofilm biomass, was measured at 600 nm using a UV–Visible spectrophotometer.

Quantification of ammonia production

Ammonia production by the bacterial isolates was evaluated using a colorimetric assay based on Nessler's reagent. Freshly grown bacterial cultures were inoculated into sterile peptone water and incubated at 28 °C for 48 hours under static conditions to allow ammonification. After incubation, the cultures were centrifuged at 10,000 rpm for 10 minutes, and the clarified supernatant was carefully collected. An equal volume of Nessler's reagent was then added to the supernatant, and the mixture was left to react at room temperature for approximately 10 minutes. The appearance of a yellow to orange color indicated ammonia production. The absorbance of the developed color was recorded at 450 nm using a UV–Visible spectrophotometer, and the obtained values were used to compare relative ammonia production among the isolates.

Morphological and biochemical characterization of isolate PSPB 2

Bacterial isolate PSBP-2 was subjected to a series of standard biochemical assays to assess its metabolic and enzymatic characteristics. Initial identification involved Gram staining to determine the organism's cellular morphology and Gram reaction. Following this, the isolate was evaluated using conventional biochemical tests, including starch hydrolysis, indole production, methyl red, Voges–Proskauer, citrate utilization, catalase activity, and glucose fermentation. Each assay was performed using appropriate microbiological media and reagents under strictly aseptic conditions. Incubation was carried out at 37 °C for the recommended time intervals specific to each test, and results were interpreted based on characteristic color changes or observable reactions. All experiments were conducted in duplicate to ensure reliability and reproducibility of the findings.

Molecular identification and phylogenetic analysis of PSBP 2

Molecular identification of the PSBP-2 isolate was carried out through 16S rRNA gene sequencing. Genomic DNA was extracted from freshly grown cultures using a standard extraction protocol. The 16S rRNA gene was amplified using universal primers 27F and 1492R, and successful amplification was verified by agarose gel electrophoresis. The purified PCR products were then subjected to Sanger sequencing. The resulting sequences were manually edited, aligned using commonly used bioinformatics software, and compared against reference sequences available in the NCBI BLASTn database to determine the closest taxonomic match. Phylogenetic analysis was performed using the Neighbor-Joining approach based on the Kimura 2-parameter model, and the robustness of the phylogenetic tree was assessed through bootstrap analysis with 1000 replicates.

Results

Screening and selection of efficient phosphate-solubilizing bacterial (PSB) isolates

A total of ten phosphate-solubilizing bacterial isolates were obtained from the rhizosphere soils of various legume crops grown in Nalgonda, Mahabubnagar, and Rangareddy districts, based on their ability to produce halo zones on Pikovskaya's (PVK) agar. The size of the clear zone surrounding each colony served as a qualitative measure of phosphate solubilization efficiency. Among all isolates, PSBP-2 collected from the pigeon pea rhizosphere in Miryalaguda (Nalgonda district) recorded the largest halo zone (11.4 mm), identifying it as the most efficient solubilizer. Strong activity was also exhibited by PSBP-8 (10.8 mm) isolated from red gram and PSBP-7 (10.5 mm) from groundnut, both of which were categorized as highly efficient PSB. Several other isolates, including PSBP-3 (9.5 mm), PSBP-4 (10.1 mm), PSBP-6 (9.2 mm), and PSBP-9 (9.9 mm), consistently produced well-defined halo zones, indicating robust phosphate-mobilizing ability across legume rhizospheres. In contrast, isolates PSBP-1 (8.2 mm), PSBP-5 (7.8 mm), and PSBP-10 (7.1 mm) generated comparatively smaller but clear halo zones and were therefore classified as moderately effective solubilizers. The range of halo zone diameters observed among the isolates demonstrates considerable functional diversity, with multiple strains showing strong potential for further biochemical and plant growth-promoting evaluation (Table 1).

Table 1. Screening and selection of efficient phosphate-solubilizing bacterial (PSB) isolates on Pikovskaya's agar medium

Isolate No.	Legume Crop	Location	Village Name	Halo Zone Diameter (mm)	Phosphate Solubilisation Efficiency (PSE)
PSBP-1	Chickpea	Nalgonda	Chityal	8.2	Moderate
PSBP-2	Pigeon pea	Nalgonda	Miryalaguda	11.4	Very High
PSBP-3	Green gram	Nalgonda	Narketpally	9.5	High
PSBP-4	Soybean	Mahabubnagar	Bhootpur	10.1	High
PSBP-5	Pea	Mahabubnagar	Narayanpet	7.8	Moderate
PSBP-6	Cowpea	Mahabubnagar	Devarkadra	9.2	High
PSBP-7	Groundnut	Rangareddy	Chevella	10.5	High
PSBP-8	Red gram	Rangareddy	Shankarpally	10.8	High
PSBP-9	Bengal gram	Rangareddy	Ibrahimpatnam	9.9	High
PSBP-10	Black gram	Nalgonda	Nakrekal	7.1	Moderate

Quantification of phosphate solubilization

Quantitative assessment of phosphate solubilization demonstrated clear differences in the ability of the ten PSB isolates to release soluble phosphorus into the medium. Among all isolates, PSBP-2 exhibited the highest measurable concentration of soluble phosphate (58.7 ± 0.20 µg/mL), reaffirming its prominence as the most efficient solubilizer. This was followed closely by PSBP-8 (54.1 ± 0.15 µg/mL) and PSBP-7 (51.3 ± 0.21 µg/mL), both of which also showed strong solubilization capacity. Substantial phosphate release was further observed in PSBP-4 (49.9 ± 0.15 µg/mL) and PSBP-9 (47.5 ± 0.20 µg/mL), indicating that these isolates also possess robust solubilizing activity. Moderate levels of solubilized phosphate were recorded for PSBP-3 (45.2 ± 0.23 µg/mL) and PSBP-6 (42.1 ± 0.18 µg/mL). In comparison, PSBP-1 (32.1 ± 0.20 µg/mL), PSBP-5 (29.3 ± 0.15 µg/mL), and PSBP-10 (28.4 ± 0.15 µg/mL) showed relatively lower, but still detectable, phosphate-releasing ability. Collectively, these findings align well with the qualitative plate assay results and clearly establish PSBP-2 as the most potent phosphate-solubilizing isolate among the strains evaluated (Fig. 1).

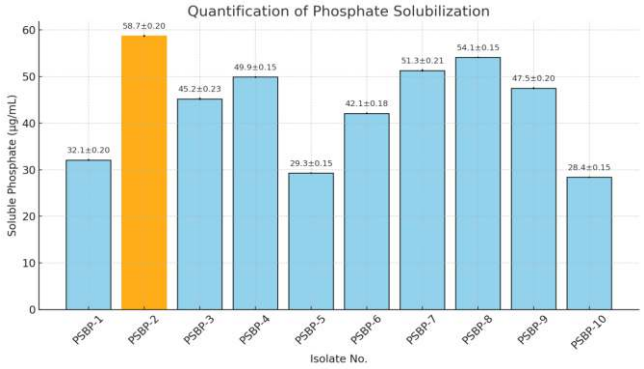


Figure 1. Quantitative estimation of phosphate solubilization by selected PSB isolates

Indole-3-Acetic Acid (IAA) production by bacterial isolates

Qualitative assessment of indole-3-acetic acid (IAA) production revealed clear differences among the PSBP isolates, as indicated by the intensity of coloration formed upon exposure to Salkowski's reagent. Of all the isolates examined, PSBP-2 generated the deepest pinkish-red color, signifying the highest level of auxin synthesis. Intermediate pigmentation, corresponding to moderate IAA production, was observed in PSBP-7, PSBP-8, and PSBP-3. In contrast, isolates such as PSBP-4, PSBP-5, PSBP-9, and PSBP-10 produced only faint coloration, reflecting relatively lower auxin output. The weakest response was recorded for PSBP-1 and PSBP-6, which showed minimal color development and therefore limited IAA production. Collectively, these observations clearly pinpoint PSBP-2 as the strongest IAA producer among the tested isolates, reinforcing its potential as an effective plant growth-promoting bacterium (Fig. 2).

Figure 2. Qualitative assessment of Indole-3-Acetic Acid (IAA) production by PSBP isolates

Quantification of indole-3-acetic Acid (IAA) production

Quantitative evaluation of indole-3-acetic acid (IAA) production showed considerable variability among the PSBP isolates. PSBP-2 exhibited the highest IAA yield, recording 48.0 ± 2.0 µg/mL, which clearly establishes it as the most efficient auxin producer. Relatively high levels of IAA were also detected in PSBP-8 (36.2 ± 1.8 µg/mL), PSBP-4 (33.1 ± 1.6 µg/mL), and PSBP-7 (31.0 ± 1.5 µg/mL). Moderate IAA synthesis was observed in PSBP-6 (29.4 ± 1.4 µg/mL) and PSBP-3 (27.2 ± 1.3 µg/mL). Lower concentrations were noted for PSBP-9 (25.0 ± 1.2 µg/mL), PSBP-5 (21.4 ± 1.1 µg/mL), PSBP-10 (20.6 ± 1.1 µg/mL), and PSBP-1 (18.5 ± 1.0 µg/mL). Taken together, these findings confirm PSBP-2 as the most proficient IAA-producing isolate, further highlighting its potential importance in plant growth promotion (Fig. 3).

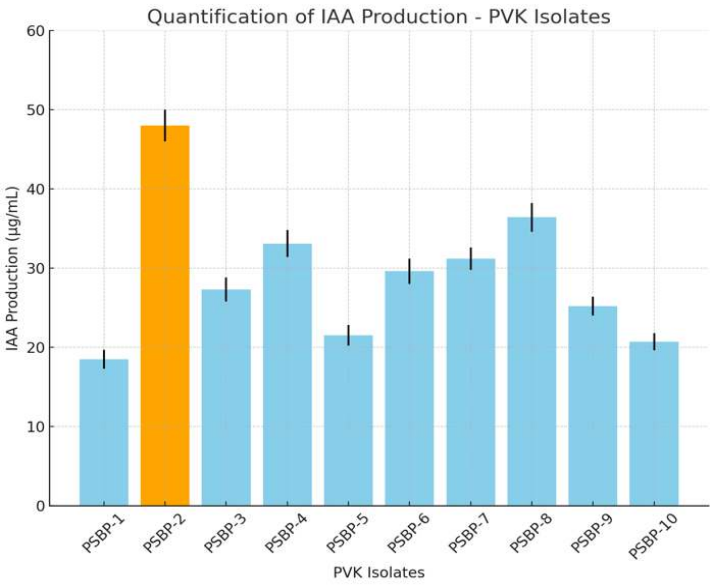


Figure 3. Quantitative estimation of Indole-3-Acetic Acid (IAA) production by PSBP isolates

Phytase Activity Assay

Qualitative assessment of phytase activity displayed clear variability among the PSBP isolates, as demonstrated by the differences in hydrolytic zone diameters on phytic acid–based agar medium. PSBP-2 produced the widest zone of clearance (12 mm), indicating strong phytase activity and efficient phytate hydrolysis. Moderate levels of activity were recorded for PSBP-4, PSBP-6, PSBP-7, and PSBP-9, each exhibiting hydrolysis zones between 6 and 7 mm. In comparison, isolates PSBP-1, PSBP-3, PSBP-5, PSBP-8, and PSBP-10 developed smaller zones of 4–5 mm, signifying relatively low phytase-producing capability. Collectively, these observations identify PSBP-2 as the most active phytase producer among the evaluated isolates, supporting its potential contribution to organic phosphorus mineralization (Fig. 4).

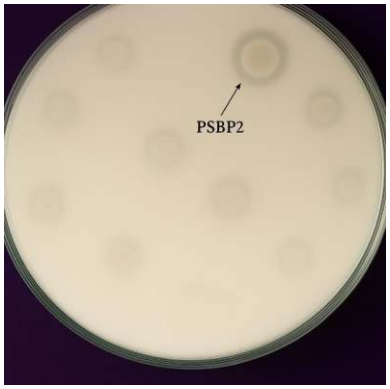


Figure 4. Phosphate solubilization by PSBP-2 on Pikovskaya's agar medium

Quantitative Assessment of Biofilm Formation

Quantitative assessment of biofilm development showed marked differences among the PSBP isolates. PSBP-2 demonstrated the greatest biofilm biomass, with an OD_{600} value of 0.78 ± 0.05, indicating a strong capability for surface attachment and matrix formation. Moderate levels of biofilm were produced by PSBP-6 (0.42 ± 0.04), PSBP-9 (0.36 ± 0.03), and PSBP-4 (0.35 ± 0.03). In comparison, PSBP-7 (0.28 ± 0.02), PSBP-10 (0.22 ± 0.02), PSBP-8 (0.21 ± 0.02), PSBP-3 (0.20 ± 0.02), PSBP-5 (0.18 ± 0.01), and PSBP-1 (0.15 ± 0.02) exhibited considerably lower levels of biofilm formation. These findings clearly establish PSBP-2 as the strongest biofilm-forming isolate among the group, a characteristic that contributes to improved persistence and colonization efficiency within the rhizosphere environment (Fig. 5).

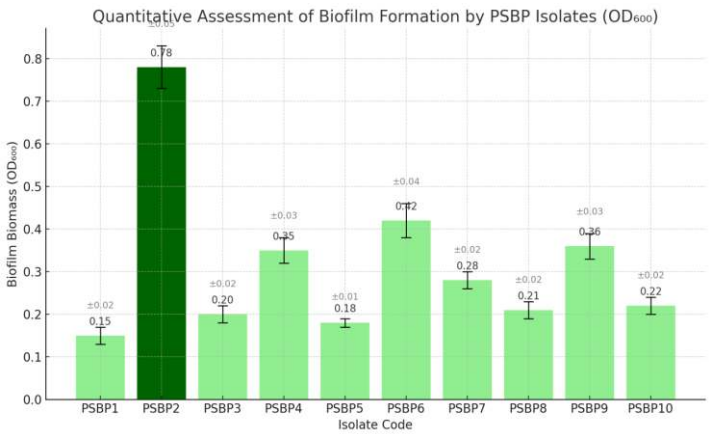


Figure 5. Quantitative assessment of biofilm formation by PSBP isolates

Quantification of Ammonia Production

Quantitative measurement of ammonia production revealed considerable variation in ammonification capacity among the PSBP isolates. PSBP-2 showed the highest absorbance value (0.48 ± 0.03), demonstrating its strong ability to produce ammonia. Moderate ammonia levels were recorded for PSBP-6 (0.25 ± 0.02), PSBP-9 (0.22 ± 0.01), and PSBP-4 (0.20 ± 0.01). In contrast, comparatively lower ammonia production was noted in PSBP-7 (0.18 ± 0.01), PSBP-8 (0.17 ± 0.01), PSBP-10 (0.16 ± 0.01), PSBP-3 (0.15 ± 0.01), PSBP-5 (0.14 ± 0.01), and PSBP-1 (0.12 ± 0.01). Taken together, these findings clearly identify PSBP-2 as the most proficient ammonia-producing isolate, reinforcing its potential contribution to nitrogen-mediated plant growth enhancement (Fig. 6).

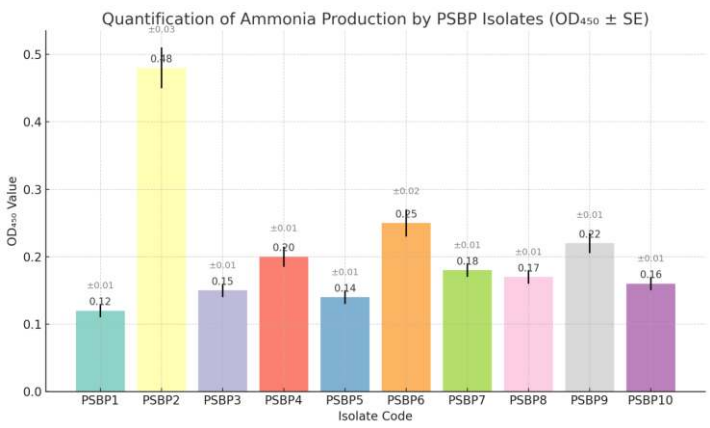


Figure 6. Quantitative Estimation of Ammonia Production by PSBP Isolates

Morphological & Biochemical characterization of isolate PSBP 2

Microscopic observation of isolate PSBP-2 using simple staining revealed that the cells were rod-shaped, confirming its bacillary form. Gram staining produced a clear Gram-positive reaction, indicative of a thick peptidoglycan-rich cell wall. Capsule staining showed the presence of a well-defined capsule surrounding the cells, suggesting its importance in enhancing environmental resilience and facilitating adherence to root surfaces. Spore staining confirmed that the isolate produces endospores, a feature commonly associated with increased stress tolerance and prolonged survival in soil habitats. Collectively, these staining outcomes characterize PSBP-2 as a hardy, spore-forming Gram-positive bacterium with traits well suited for rhizosphere colonization and persistence (Table 2).

Table 2. Microscopic and staining characteristics of isolate PSBP-2

Staining	PSBP 2
Simple staining	Bacilli
Gram staining	Positive
Capsule staining	Capsulated
Spore staining	Sporulated

Biochemical characterization of isolate PSBP-2 revealed a distinct enzymatic and metabolic profile. The isolate showed a negative response in the indole test, indicating that it does not convert tryptophan into indole. A positive methyl red reaction confirmed its ability to perform mixed acid fermentation, producing stable acidic metabolites. The Voges–Proskauer test was negative, suggesting that acetoin is not generated during glucose metabolism. PSBP-2 exhibited a positive citrate utilization reaction, demonstrating that it can grow using citrate as its sole carbon source. The isolate tested negative for catalase, urease, and hydrogen sulfide (H_2S) production, indicating the absence of these specific enzymatic functions. However, it showed a positive reaction for carbohydrate fermentation, confirming its ability to metabolize sugars, and a positive starch hydrolysis test, reflecting the secretion of extracellular amylase. Taken together, these biochemical features highlight the metabolic adaptability of PSBP-2 and align well with traits commonly reported for plant growth–promoting Bacillus species (Fig. 7 & Table 3).

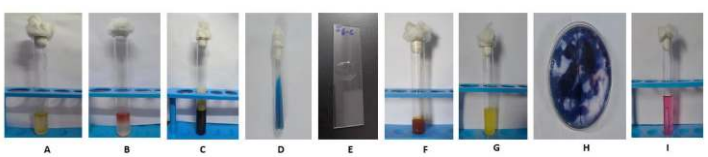


Figure 7: Biochemical studies of PSBP 2

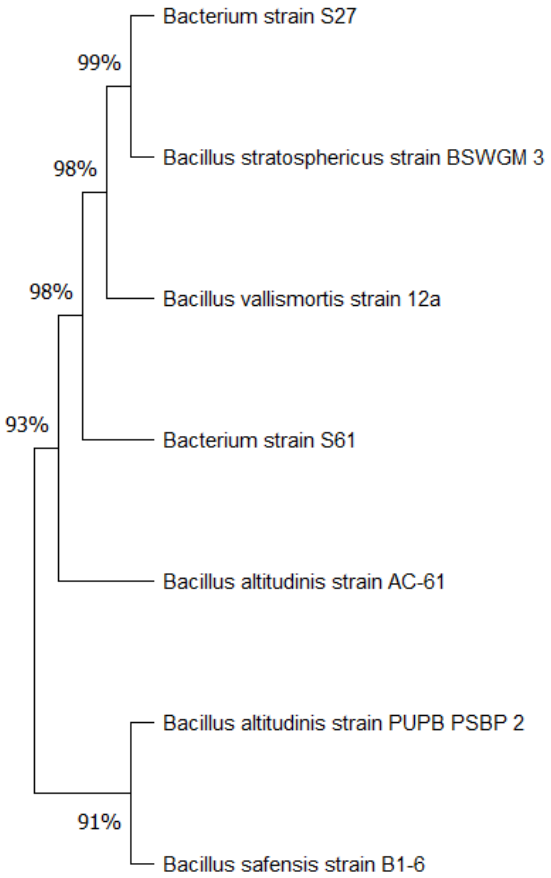
(Here A: Indole test, B: MR test, C: VP test. D: Cirate test, E: Catalase test, F: H2S test, G: Urease test, H: Starch hydrolysis and I: Carbohydrate fermentation test)

Table 3. Biohemical test of isolate PSBP 2

Test	Result
Indole	Negative
Methyl red	Positive
Voges Proskauer	Negative
Citrate	Positive
Catalase	Negative
Urease	Negative
H_2S	Negative
Carbohydrate fermentation	Positive
Starch hydrolysis	Positive

Molecular Identification & Phylogenetic analysis of PSBP 2

The potent isolate PSBP-2 was conclusively identified through amplification and sequencing of its 16S rRNA gene followed by sequence submission to the GenBank database. The gene was successfully amplified and sequenced using Sanger dideoxy sequencing, yielding a partial sequence of approximately 1400 base pairs. BLASTn comparison revealed a strong sequence match with members of the genus Bacillus, showing closest alignment to *Bacillus altitudinis*. Based on sequence similarity, bioinformatics analysis, and phylogenetic comparison with existing reference strains, the isolate was designated as *Bacillus altitudinis* strain PUPB PSBP-2. The validated sequence was uploaded to GenBank and assigned the accession number PX239710, with the corresponding taxonomic reference listed under taxon ID: 2933287. This classification reflected robust similarity values and affirmed the placement of PSBP-2 within the *B. altitudinis* lineage. Phylogenetic analysis further supported the molecular identity of PSBP-2. A phylogenetic tree constructed using the Neighbor-Joining algorithm and evaluated with 1000-replicate bootstrap analysis positioned the isolate within the *Bacillus altitudinis* clade. PSBP-2 demonstrated close grouping with *Bacillus altitudinis* strain AC-61, confirming its strong evolutionary association with this species. The isolate shared 91% sequence similarity with *Bacillus safensis* strain B1-6 and 93% similarity with Bacterium strain S61. Additionally, it showed remarkably high sequence similarity (98–99%) with other related species, including *Bacillus vallismortis* strain 12a, *Bacillus stratosphericus* strain BSWGM-3, and Bacterium strain S27. These findings collectively reinforce the taxonomic assignment of PSBP-2 to the *B. altitudinis* cluster and demonstrate its close genetic relationship with several agriculturally and environmentally significant Bacillus species.



Phylogenetic Tree Depicting the Evolutionary Relationship of Bacillus altitudinis Strain PUPB PSBP 2

Discussion

The findings of the present study confirm that legume rhizosphere soils support a diverse assemblage of phosphate-solubilizing bacteria exhibiting marked variation in functional efficiency and plant growth–promoting traits. Qualitative screening on Pikovskaya's agar revealed halo zone diameters ranging from 7.1 to 11.4 mm, reflecting substantial heterogeneity in phosphate solubilization capacity among the isolates. Among them, PSBP-2 produced the widest solubilization zone (11.4 mm), followed by PSBP-8 (10.8 mm) and PSBP-7 (10.5 mm), indicating strong mineral phosphate dissolution potential. Similar magnitudes of halo zone formation have been reported for efficient Bacillus isolates from crop rhizospheres, where enhanced phosphate solubilization was attributed to organic acid secretion and localized pH reduction [12; 13].

Quantitative phosphate solubilization assays further validated the superiority of PSBP-2, which released 58.7 ± 0.20 µg/mL of soluble phosphate in PVK broth. This value was notably higher than those recorded for PSBP-8 (54.1 ± 0.15 µg/mL) and PSBP-7 (51.3 ± 0.21 µg/mL), while isolates such as PSBP-10 (28.4 ± 0.15 µg/mL) and PSBP-5 (29.3 ± 0.15 µg/mL) showed limited activity. Comparable phosphate solubilization levels (50–65 µg/mL) have been reported for elite Bacillus strains isolated from legume-based cropping systems, reinforcing the classification of PSBP-2 as a highly efficient phosphorus mobilizer [14; 5]. The strong correlation between plate-based halo formation and broth-based quantification observed in this study supports the continued use of PVK agar as a reliable preliminary screening tool. Beyond phosphorus solubilization, PSBP-2 exhibited pronounced indole-3-acetic acid (IAA) production. The isolate produced the highest auxin concentration (48.0 ± 2.0 µg/mL), while moderate levels were recorded for PSBP-8 (36.2 ± 1.8 µg/mL) and PSBP-4 (33.1 ± 1.6 µg/mL). In contrast, PSBP-1 produced only 18.5 ± 1.0 µg/mL of IAA. IAA concentrations exceeding 30 µg/mL are widely recognized as sufficient to induce root elongation, lateral root initiation, and increased root hair density, thereby improving nutrient uptake efficiency [15; 16]. The elevated auxin synthesis by PSBP-2 suggests its strong capacity to modulate root system architecture in legume crops.

Phytase activity further distinguished PSBP-2 from the remaining isolates. The largest hydrolysis zone (12 mm) observed for PSBP-2 indicates efficient degradation of phytate-bound organic phosphorus, whereas moderate activity (6–7 mm) in PSBP-4, PSBP-6, PSBP-7, and PSBP-9 and low activity (4–5 mm) in other isolates suggest limited organic phosphorus mineralization. Phytase-producing Bacillus spp. have been shown to play a critical role in releasing bioavailable phosphorus from organic pools, particularly in intensively cultivated soils where phytate accumulation is common [17; 18]. The strong phytase activity of PSBP-2 therefore enhances its ecological relevance and functional versatility. Biofilm formation is a key determinant of rhizosphere competence and persistence of PGPR. In the present study, PSBP-2 demonstrated the highest biofilm biomass (OD_{600} = 0.78 ± 0.05), which was markedly greater than that of PSBP-6 (0.42 ± 0.04) and PSBP-9 (0.36 ± 0.03). Robust biofilm formation facilitates root surface colonization, protects bacterial cells from environmental stress, and supports sustained expression of beneficial traits in soil ecosystems [19].

This characteristic likely contributes to the consistent multifunctional performance of PSBP-2. Ammonia production also varied significantly among the isolates, with PSBP-2 exhibiting the highest absorbance value (0.48 ± 0.03). Moderate ammonification was observed in PSBP-6 (0.25 ± 0.02) and PSBP-9 (0.22 ± 0.01), while PSBP-1 showed minimal activity (0.12 ± 0.01). Although ammonia production does not equate to nitrogen fixation, it contributes to localized nitrogen availability and can enhance early plant growth, particularly in nitrogen-limited soils [20]. Morphological, biochemical, and molecular analyses confirmed PSBP-2 as Bacillus altitudinis strain PUPB PSBP-2, a Gram-positive, spore-forming bacterium with metabolic traits typical of plant-associated Bacillus species. Recent genomic and functional studies emphasize that strain-level variability within B. altitudinis significantly influences nutrient solubilization efficiency, stress tolerance, and rhizosphere colonization ability, highlighting the importance of polyphasic characterization for selecting elite PGPR candidates [19; 21].

Conclusion

The present study successfully isolated and characterized efficient phosphate-solubilizing bacteria from legume rhizosphere soils, highlighting the functional diversity of indigenous microbial communities. Among the ten screened isolates, PSBP-2 consistently demonstrated superior performance across multiple plant growth–promoting attributes. This isolate exhibited the highest phosphate solubilization capacity, producing a halo zone of 11.4 mm on Pikovskaya's agar and releasing 58.7 ± 0.20 µg/mL of soluble phosphate in broth culture. In addition, PSBP-2 showed strong auxin production (48.0 ± 2.0 µg/mL IAA), high phytase activity (12 mm hydrolysis zone), robust biofilm formation (OD_{600} = 0.78 ± 0.05), and the greatest ammonia production (0.48 ± 0.03 OD_{450}), collectively indicating its multifunctional plant growth–promoting potential. Morphological, biochemical, and molecular analyses confirmed PSBP-2 as *Bacillus altitudinis* strain PUPB PSBP-2, a spore-forming, Gram-positive bacterium well adapted to rhizosphere environments. The integration of inorganic and organic phosphorus mobilization, phytohormone production, and strong rhizosphere colonization traits underscores the suitability of this strain as a bioinoculant candidate. Overall, the findings suggest that *Bacillus altitudinis* PUPB PSBP-2 holds significant promise for improving phosphorus availability and enhancing plant growth in legume-based cropping systems. Further greenhouse and field evaluations are warranted to validate its agronomic effectiveness and support its development as a sustainable biofertilizer.

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