Singh et al | Article 9786
J Pure Appl Microbiol. 2024;18(4):2582-2591. doi: 10.22207/JPAM.18.4.31
Received: 03 August 2024 | Accepted: 16 September 2024
Published Online: 25 November 2024

P-ISSN: 0973-7510; E-ISSN: 2581-690X

RESEARCH ARTICLE OPEN ACCESS

Synergistic Enhancement of Cauliflower Yield: Harnessing Phosphate-Solubilizing Bacteria and Nitrogen-Fixing Microbes for Sustainable Agriculture

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Abstract

Cauliflower (*Brassica oleracea* var. *botrytis*) is a crucial cash crop predominantly consumed as a vegetable. High-yielding varieties are favored to maximize productivity and income, but they require substantial nutrients, leading to heavy reliance on chemical fertilizers and pesticides. This practice poses health risks and causes environmental pollution. Adequate nutrient availability, particularly for phosphorus (P) and nitrogen (N), is essential for optimal cauliflower growth. Phosphate-solubilizing bacteria (PSB) enhance P availability by solubilizing insoluble phosphates, whereas nitrogen-fixing microbes (NFM) convert atmospheric nitrogen into usable forms. These microbial inoculants are eco-friendly alternatives to chemical fertilizers, which promote nutrient availability and plant growth. The purpose of this study was to separate, identify, and describe PSB from the soil of the cauliflower rhizosphere in the Uttar Pradesh district of Lucknow, Unnao, and Kanpur. Selected PSB isolates were screened, characterized using 16S rRNA, and evaluated for their phosphate solubilization capacity at different phosphorus concentrations. The results showed increased phosphate solubilization up to 72 h, with tricalcium phosphate (TCP) solubilized most effectively at 500 ppm and rock phosphate (RP) or bone meal (BM) at 250 ppm. *Bacillus pumilus* exhibited the highest phosphate solubilization ability. This research highlights the potential of PSB and NFM as sustainable solutions for reducing chemical fertilizer dependency, enhancing soil fertility, and promoting cauliflower growth, thereby offering a promising approach to sustainable agriculture.

Keywords: *Bacillus pumilus*, Cauliflower (*Brassica oleracea* var. *botrytis*), Nitrogen-Fixing Microbes (NFM), Phosphate-Solubilizing Bacteria (PSB), Rhizosphere, 16S rRNA sequencing

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Citation: Singh P, Meshram A, Singh RK, ur Rahman L, Morya VK. Synergistic Enhancement of Cauliflower Yield: Harnessing Phosphate-Solubilizing Bacteria and Nitrogen-Fixing Microbes for Sustainable Agriculture. *J Pure Appl Microbiol.* 2024;18(4):2582-2591. doi: 10.22207/JPAM.18.4.31

INTRODUCTION

Cauliflower (*Brassica oleracea* var. *botrytis* L.), a significant member of the Brassicaceae family, is extensively cultivated as a high-value cash crop consumed primarily as a vegetable. Certain cultivars have also been used in seed production. Farmers typically use high-yielding cauliflower varieties to maximize productivity and income.[1] However, these varieties often require substantial amounts of nutrients and growth promoters, necessitating the use of various chemical fertilizers and pesticides. This practice poses significant health risks and contributes to environmental pollution.[2] Cauliflower is particularly valued for its nutrient-rich inflorescence, which necessitates adequate nutrient availability, especially phosphorus and nitrogen, for optimal growth and yield.[3] Phosphorus is essential for several physiological functions in plants but is often present in soil in insoluble forms, making it inaccessible to plants. Phosphate-solubilizing bacteria (PSB) can solubilize insoluble phosphates, thereby enhancing plant phosphorus uptake.[3] Similarly, nitrogen-fixing microorganisms (NFM) play a crucial role in nitrogen fixation by converting atmospheric nitrogen (N_2) into ammonia (NH_3) and other nitrogenous compounds that plants can assimilate.[4] Microbial inoculants containing PSB and NFM are gaining attention as eco-friendly alternatives to chemical fertilizers for improving nutrient availability and promoting plant growth.[5] The efficacy of these inoculants depends on the selection of efficient strains and their compatibility with the target crop.[5,6] Bioinoculant of rhizobia can effectively improve agricultural yield and productivity which indicates that Rhizobium is an effective Plant growth promoting microbe.[7,8] Additionally, investigating the synergistic relationship between PSB and NFM could enhance their ability to stimulate plant development and productivity. In this study, PSB from cauliflower rhizosphere soil was isolated, identified, and characterized. The synergistic effects of PSB and nitrogen-fixing microorganisms on cauliflower development and productivity were evaluated (Figure 1).

Purpose of the study

The primary goal of this study was to explore sustainable agricultural practices for cauliflower farming by reducing dependency on synthetic fertilizers through the application of plant growth-promoting rhizobacteria

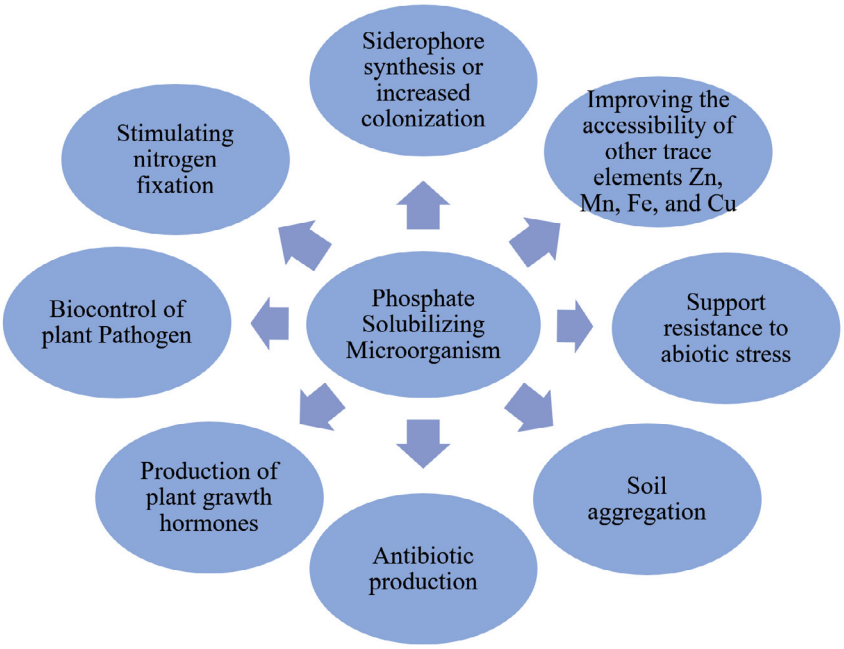

Figure 1. Functions of bacteria that solubilize phosphate during plant development and growth

(PGPR). These PGPR strains exhibit diverse functionalities, including nitrogen fixation, phosphate solubilization, and the production of vital compounds like indole acetic acid (IAA).[9] Specifically, the study aimed to identify and characterize effective PGPR strains suitable for commercial cauliflower cultivation in Uttar Pradesh. Soil samples were collected from different agricultural zones, followed by ecological modeling based on established protocols. The selected PGPR strains were used to develop customized biofertilizers with suitable carrier materials, tailored to specific crops and zones. The overarching goal is to integrate these biofertilizers into conventional agricultural practices, thus promoting sustainable farming by enhancing soil fertility, improving crop yields, and reducing environmental degradation. This research is expected to contribute to the global challenge of food security by optimizing agricultural systems through the use of eco-friendly inputs.

Assessment of soil PGPR

The first step in evaluating soil PGPR involves identifying key beneficial traits, as it is impractical to test numerous bacterial isolates directly in plants. Instead, PGPR strains are selected based on *in vitro* assays, where pure bacterial cultures are isolated and their activity is assessed in different media.[10,11] Beneficial traits such as nitrogen fixation, phosphate solubilization, potassium mobilization, siderophore production, phytohormone secretion, and pathogen suppression are evaluated by observing changes in the growth medium, such as color or transparency shifts around bacterial colonies.[12,13] The combined effects of bacterial consortia can significantly enhance plant health by promoting nutrient availability and protecting against pathogens. These cumulative benefits contribute to improved soil biological activity and, consequently, better plant growth and development (Figure 2).

METHODOLOGY

Study Design

Soil and root samples from the rhizosphere of the cauliflower planting area were collected from the Uttar Pradesh district of Unnao, Lucknow, and Kanpur. After excavating the rhizosphere while maintaining an intact root system, samples were sealed in plastic bags and kept cold (4°C) at the SIIC IIT Kanpur Soil Microbiology Laboratory until further examination. The analysis consisted of counting CFUs, group characteristic analysis, and microbe isolation from the root-soil sample. These samples, rich in diverse microbial communities, were subjected

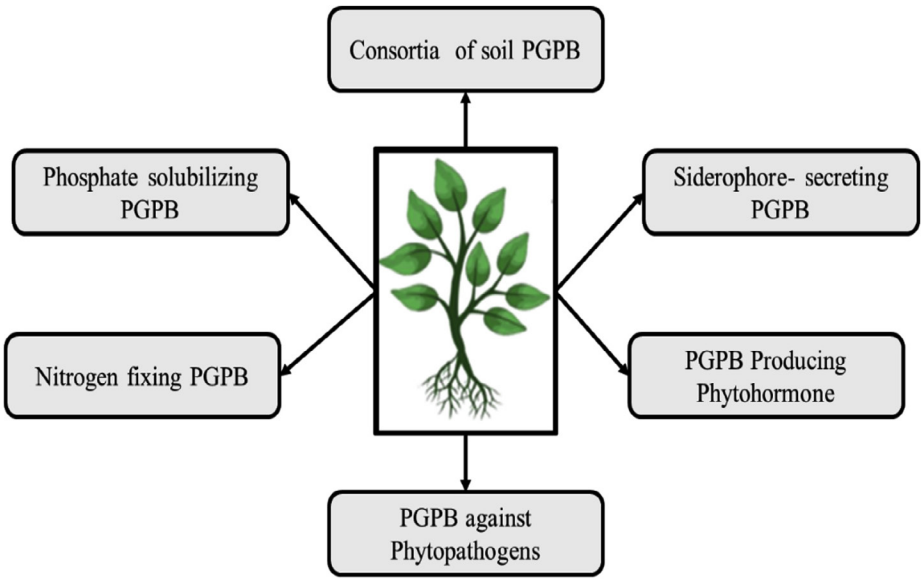

Figure 2. Different soil PGPBs promote plant development and growth in several ways

to selective enrichment on specialized media in the lab to isolate PSB. The isolated strains were further confirmed using molecular techniques, ensuring their relevance to local soil ecology and their potential for enhancing crop productivity. A 16S rRNA gene-based approach was utilized for the identification and characterization of bacterial isolates. This involved a series of morphological and biochemical analyses, followed by DNA extraction and PCR amplification using universal primers. Sequencing and phylogenetic analysis of *Bacillus pumilus* strains with significant phosphate-solubilizing capabilities were conducted, with results compared against the NCBI GenBank database to confirm strain identity. Additionally, PSB were screened by staining and characterized using 16S rRNA. The synergistic effect of PSB with commercially available biofertilizers was examined.

Collection of soil samples and isolation of PSB

The collection of soil samples from Unnao, Kanpur, and Lucknow initiated the isolation of PSB, which is vital for sustainable agriculture. These samples capture diverse soil microbial communities that are essential for understanding local ecology. In the laboratory, samples underwent selective enrichment on specialized media to isolate PSB, as confirmed through molecular techniques. This regional approach identified indigenous PSB strains adapted to local conditions, facilitating tailored biofertilizer development. This process enhances soil fertility and crop productivity, which are both crucial for sustainable agriculture. Isolation involves meticulous protocols for the selective cultivation of soil microbial populations. This leverages PSB's ability of PSB to solubilize phosphorus, which is vital for nutrient management. Geographically specific PSB isolation provides insights into local microbial ecology and adaptation, aiding tailored biofertilizer development. Overall, this process is pivotal for advancing agricultural sustainability by harnessing microbial nutrient cycling and enhancing soil health to improve crop yield.

Identification (16S rRNA Method) & characterization of the bacterial isolates

The process of identification and characterization of bacterial isolates using the 16S rRNA method involves a comprehensive analysis aimed at understanding the taxonomic composition and functional attributes of microbial populations. Following the isolation of bacterial colonies, a series of morphological and biochemical analyses were conducted to elucidate their characteristics. Initially, colony morphology served as a visual indicator that provided insights into the size, shape, texture, and pigmentation of bacterial colonies. Microscopic examination revealed the cell morphology, including shape, size, and arrangement. Gram staining distinguishes between Gram-positive and Gram-negative bacteria based on differences in the composition of their cell walls providing first taxonomic information.

To identify the isolated bacteria, morphological traits, such as size, shape, motility, spore presence or absence, and Gram staining were used. The bacteria were molecularly identified after DNA extraction by heat lysis. Sterile distilled water (200 µl) was used to suspend colonies from a 24 hour young culture on agar media in an Eppendorf tube. The suspension was subsequently centrifuged for 10 minutes at 14000 rpm after being incubated for 10 min at 100°C. Before PCR analysis, the DNA-containing supernatant was carefully collected and stored at -20°C.

Sequencing of *Bacillus pumilus* 16S Ribosomal RNA gene

To determine the sequence of the *Bacillus pumilus* 16S ribosomal RNA gene, bacterial isolates were first cultured from soil samples collected from the rhizosphere of cauliflower plants. Isolates with significant phosphate-solubilizing activity were selected for further analysis. DNA was extracted from the isolates using a standard bacterial genomic DNA extraction kit. The 16S rRNA gene was then amplified by polymerase chain reaction (PCR) using the universal bacterial primers 27F and 1492R. PCR products were purified using a commercial purification kit and sent for sequencing. The obtained sequences were compared against the NCBI GenBank database using BLAST for the identification and confirmation of *Bacillus pumilus*. Phylogenetic analysis was performed to ascertain the evolutionary relationships among the isolates

and known *Bacillus pumilus* strains. The sequence data were further analyzed for any unique genetic marker that could be associated with enhanced phosphate-solubilizing capabilities. This methodological approach ensured the accurate identification and characterization of *Bacillus pumilus* strains from the cauliflower rhizosphere. For this study, we selected 10 strains of *Bacillus pumilus* 16S ribosomal RNA gene based on the E-value obtained after BLAST analysis and analyzed these sequences for phylogenetic analysis of *Bacillus pumilus* using the pairwise alignment tool CLUSTAL W (Figure 3).

Isolation and screening of PSB

In agricultural microbiology, the process of isolating and screening PSB is essential to improve soil fertility and plant nutrition. Soil samples from the rhizosphere were suspended in distilled water to extract microbial communities associated with plant roots and identify phosphate-solubilizing bacteria. Decimal dilutions were prepared from this suspension and aliquots were inoculated onto Picovskaya's medium, a specialized growth medium designed to select bacteria capable of solubilizing phosphate compounds. Picovskaya's medium contains insoluble phosphate as the sole source of phosphorus, creating an environment in which only bacteria with the ability to solubilize phosphate can thrive.[14,15] After incubation, bacterial colonies exhibiting clear zones around them, indicating phosphate solubilization, were selected for further analysis. The isolated microbial colonies were subjected to staining to assess their characteristics. Staining techniques such as Gram staining provide insights into the cellular

Table. Phosphate solubilization analysis

Phosphorus Source	Concen. (ppm)	Absorbance (nm)	Solubilized Phosphate (mg/L)
Tricalcium Phosphate (TCP)	250	0.35	35
Tricalcium Phosphate (TCP)	500	0.42	42
Tricalcium Phosphate (TCP)	750	0.50	50
Tricalcium Phosphate (TCP)	1000	0.58	58
Rock Phosphate (RP)	250	0.30	30
Rock Phosphate (RP)	500	0.38	38
Rock Phosphate (RP)	750	0.45	45
Rock Phosphate (RP)	1000	0.52	52
Bone Meal (BM)	250	0.32	32
Bone Meal (BM)	500	0.40	40
Bone Meal (BM)	750	0.48	48
Bone Meal (BM)	1000	0.55	55

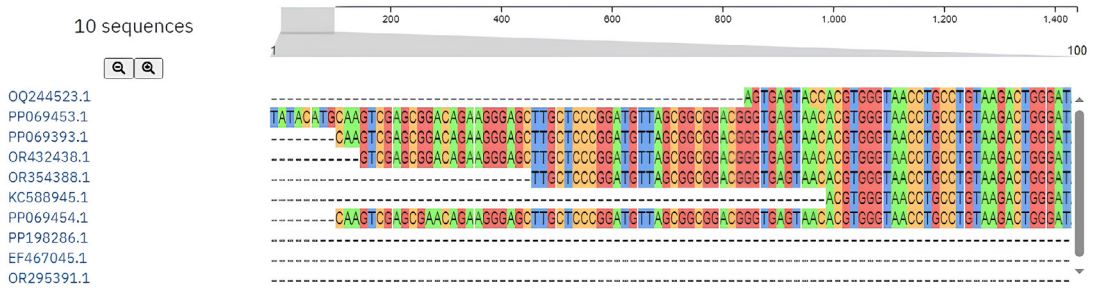

Figure 3. Pairwise alignment of 16S ribosomal RNA gene sequences from *Bacillus pumilus* isolates using CLUSTAL W tool

morphology and structure of bacteria. The positive staining results suggested the presence of PSB among the isolated colonies.

To characterize the isolated bacteria, 16S rRNA sequencing was performed. This molecular technique targets the highly conserved 16S rRNA gene, allowing the precise taxonomic identification of bacterial species.[16-18] By comparing the obtained sequences with reference databases, the identity of PSB isolates can be determined, providing valuable information about their phylogenetic relationships and evolutionary history. The isolation and screening of phosphorus-solubilizing bacteria involves a systematic approach to identify microbial populations capable of enhancing soil phosphorus availability. Through these methods, researchers can select and characterize bacteria with the potential to contribute to sustainable agriculture by improving plant nutrient uptake.

Estimation of phosphate solubilization

To determine the optimum concentration of phosphorus that can be effectively solubilized by the selected isolates, different concentrations

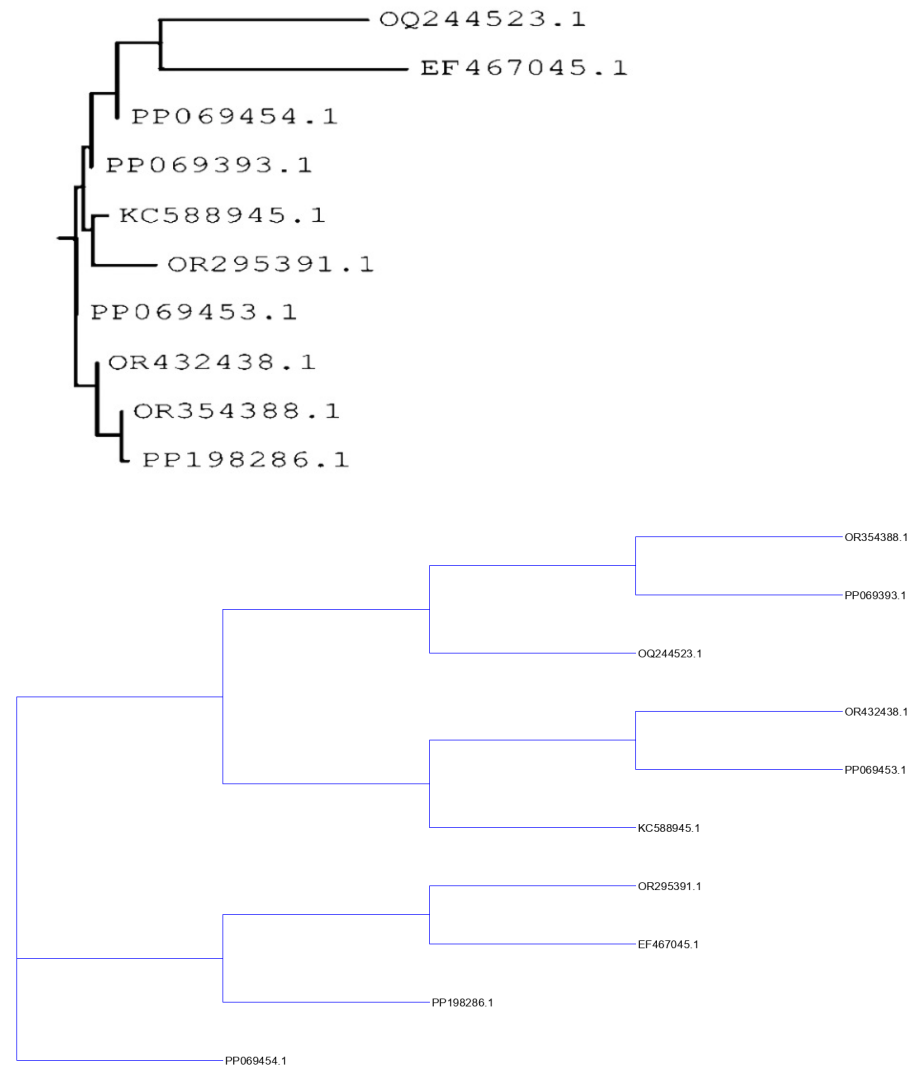

Figure 4. Phylogenetic trees illustrating the evolutionary relationships among the 16S rRNA sequences followed by the analysis of various strains of *Bacillus pumilus* sequences with the dendrogram (left) showing genetic distance-based clustering and the cladogram (right) detailing branching patterns and shared characteristics

of tricalcium phosphate (TCP) or bone meal (BM) were used as phosphorus sources. Concentrations of 250, 500, 750, and 1000 ppm were separately added to PVK (Pikovskaya's) broth. Next, a spectrophotometric technique was used to quantify the phosphate-solubilizing activity.

The procedure for estimating phosphate solubilization began with the preparation of PVK broth, in which varying concentrations (250, 500, 750, and 1000 ppm) of tricalcium phosphate (TCP), rock phosphate (RP), and bone meal (BM) were added as phosphorus sources. This was followed by inoculation of the broth with the selected phosphate-solubilizing bacterial isolates. The inoculated broth was incubated under suitable conditions for a specified period of time. After incubation, the samples were collected from each broth for analysis. The amount of solubilized phosphate was measured using a spectrophotometer, which involved reacting the sample with a molybdate reagent to form a blue complex that was measured at a specific wavelength.

RESULTS AND DISCUSSION

The 16S ribosomal RNA gene sequences derived from a range of *Bacillus pumilus* strains offer a fascinating glimpse into the intricate tapestry of the microbial diversity within this species. With each partial sequence representing distinct strains like JUBCH08, DKS1, KA-01, KKP 1402, KKP 3900, KKP 3902, KKP 3898, TNMMS-12, AAA, and ASOI-02, these genetic fragments paint a vivid picture of the evolutionary pathways and phylogenetic nuances present among these bacterial populations as shown in Figure 3. Through meticulous analysis of these sequences, researchers gain invaluable insights into the underlying genetic variations and adaptive strategies employed by *Bacillus pumilus* strains, which, in turn, illuminate their ecological niches, potential ecological roles, and even their applications across various environmental contexts. Analysis of these sequences provides valuable insights into the genetic variations and adaptations of *Bacillus pumilus*, as shown in Figure 4, shedding light on its ecological roles and potential applications in diverse environments.

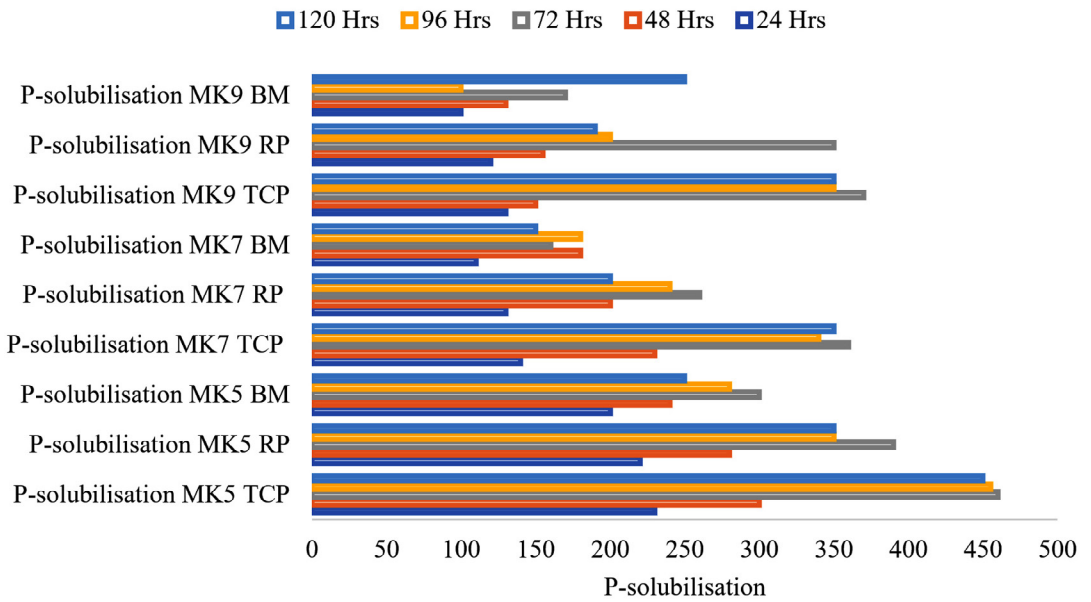

Figure 5. The graph illustrates that phosphate solubilization by various bacterial isolates with *Bacillus pumilus* showed the highest solubilization across different phosphorus sources and incubation durations. Isolate MK7[14] followed a consistent trend regardless of the amount of phosphorus source, and demonstrated a negative correlation between medium pH and solubilized phosphate content over time

In Figure 3, the alignment compares 10 sequences identified by their accession numbers (OQ244523.1, PP069453.1, PP069393.1, OR432438.1, OR354388.1, KC588945.1, PP069454.1, PP198286.1, EF467045.1, and OR295391.1). Nucleotide positions are indicated at the top, with conserved regions highlighted across sequences. Color coding represents nucleotide identities where adenine (A) is green, thymine (T) is red, cytosine (C) is blue, and guanine (G) is yellow, illustrating the genetic similarity and variation among the isolates. This alignment aids in understanding the phylogenetic relationships and confirming the identity of *Bacillus pumilus* strains.

The phylogenetic trees illustrated the evolutionary relationships among various strains of *Bacillus pumilus* sequences of the 16S rRNA gene. The dendrogram (left panel) shows hierarchical clustering based on genetic similarities, where branch lengths are proportional to genetic distances, indicating closer evolutionary relationships among clustered sequences. The cladogram (right panel) details the branching patterns of these relationships, emphasizing the shared-derived characteristics. Each branch is labeled with a unique specimen identifier showing notable clusters, such as OR354388.1 and PP069393.1, which are closely related and more distantly related sequences, such as PP069454.1 and PP198286.1. These trees offer valuable insights into the evolutionary history and genetic diversity of the specimens.

Phosphate solubilization analysis

The solubilization measurement, indicated by absorbance in Table, refers to the amount of phosphate released into the solution by the bacterial isolates, which was quantified using a spectrophotometer. The solubilized phosphate combines with a molybdate reagent to generate a blue complex, which is the basis for the spectrophotometric technique. Absorbance at a certain wavelength (usually approximately 880 nm) is a measure of the intensity of the blue color and indicates the quantity of solubilized phosphate.

All the selected isolates showed a consistent increase in phosphate solubilization with different phosphorus sources at varying concentrations (i.e., 250.00, 500.00, 750.00, and 1000.00 ppm) up to 72 h of incubation. Figure 5 illustrates the abrupt decrease in phosphate solubilization that followed this period. The solubility of the different phosphorus sources varied after incubation for 72 h; tricalcium phosphate (TCP) showed the highest solubilization at 500 ppm, whereas bone meal (BM) and rock phosphate (RP) were most effectively solubilized at 250 ppm. The isolate HWR48 was shown to be the most capable of solubilizing phosphate with levels as high as 88.42 mg/L from TCP, 33.54 mg/L from RP, and 4.84 mg/L from BM.

When the amounts of the three phosphorus sources and different incubation durations (24, 48, 72, 96, and 120 h) were examined, *Bacillus pumilus* showed the greatest phosphate solubilization. The second step was to Isolate MK7.[14] This suggests that regardless of the amount of phosphorus present, the trend in phosphate solubilization by various bacterial isolates was constant. The increased viable count of *Bacillus pumilus* under conditions containing TCP, RP, and BM may be the cause of its improved phosphate solubilization. A mean decrease in the final pH of the supernatant was observed in all instances between 24 and 72 h, which corresponded with the isolates' enhanced solubilization of phosphate. By lowering the pH of the medium and releasing low molecular weight organic acids that disassemble the phosphorus structure and release it into the medium, soil-isolated bacterial species may dissolve phosphate in both soil and culture media. With longer incubation times, a negative correlation was observed between the pH of the medium and solubilized phosphate content.

DISCUSSION

This study aimed to isolate, identify, and characterize phosphate-solubilizing bacteria (PSB) from the cauliflower rhizosphere. Morphological and biochemical analyses of the bacterial isolates were conducted, with molecular identification using 16S rRNA gene sequencing. The genetic diversity of the *Bacillus pumilus* strains isolated from the rhizosphere was significant. Phylogenetic analysis, supported by pairwise alignment using CLUSTAL W, revealed distinct evolutionary pathways among the isolates, providing insights into their ecological

roles and potential applications in improving soil fertility and nutrient availability.[19-21]

Our results showed that Bacillus pumilus exhibited the highest phosphate solubilization, particularly with tricalcium phosphate (TCP) as the phosphorus source. Solubilization was most effective at 500 ppm for TCP and 250 ppm for both rock phosphate (RP) and bone meal (BM), underscoring Bacillus pumilus's superior phosphate-solubilizing ability. The quantification of phosphate solubilization was performed using spectrophotometry, with the highest solubilization observed up to 72 hours of incubation, followed by a decline. This trend suggests that the bacterial activity is optimal during this period, indicating the potential need for periodic re-application of biofertilizers to ensure sustained nutrient availability.[8,10,14]

Although this study did not directly evaluate the growth or yield of cauliflower, it highlights the potential synergistic effects of PSB and nitrogen-fixing microorganisms (NFM) on nutrient uptake and soil fertility. Previous research has demonstrated that microbial inoculants such as PSB and NFM can enhance plant growth by improving nutrient availability, which may indirectly suggest similar benefits for cauliflower cultivation.[14,15,20] For example, co-inoculation with PSB and NFM has been shown to significantly increase nutrient absorption and biomass in other crops such as chickpeas, indicating the potential for similar results in cauliflower.[14] The integration of PSB and NFM into biofertilizer formulations can help reduce reliance on chemical fertilizers, mitigate environmental pollution, and support sustainable agricultural practices.[16,17,19] While this study focused on microbial isolation and characterization, the findings lay the groundwork for future research into tailored biofertilizer applications, potentially improving soil health and crop productivity in cauliflower farming. This work advances our understanding of microbial nutrient cycling and highlights the importance of microbial diversity in sustainable agriculture, with significant implications for eco-friendly farming systems and global food security efforts.

CONCLUSION

This study successfully isolated and characterized phosphate-solubilizing bacteria (PSB) from the cauliflower rhizosphere, with *Bacillus pumilus* identified as a particularly effective strain for phosphate solubilization. The use of 16S rRNA sequencing allowed for precise identification and taxonomic classification of the isolates. The results demonstrated that PSB has significant potential to enhance soil fertility by improving nutrient availability, which could reduce the need for chemical fertilizers. This research lays the foundation for developing tailored biofertilizers that promote sustainable agricultural practices. By leveraging indigenous microbial strains, future applications can focus on improving soil health and nutrient cycling, contributing to more eco-friendly farming systems.

ACKNOWLEDGMENTS

The authors would like to thank Adhita Biosciences Pvt. Ltd., Lucknow, for their support.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest

AUTHORS' CONTRIBUTION

All authors listed have made substantial direct and intellectual contribution to the work and approved it for publication.

FUNDING

None.

DATA AVAILABILITY

All the datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

Not applicable.

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