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Assessing The Quality of Phosphate Solubilizing Bacteria from Rhizospheric Soil Of Kathmandu Valley

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Abstract

In the rhizosphere, phosphate-solubilizing bacteria (PSB) play a critical role in helping to promote the solubilization of inorganic phosphorus compounds into soluble forms that are suitable for plant nourishment. The aim of this study was to investigate the qualitative and quantitative activity of Phosphate Solubilizing Bacteria (PSB) isolated from the soil of Kathmandu, Nepal. This study was done from March to September 2024 in the microbiology laboratory of National Soil Science Research Center (NSSRC), Nepal Agricultural Research Council (NARC), Khumaltar, Lalitpur. A total of 40 soil samples were collected from the rhizosphere of 8 different types of plants (Aloe vera, potato, wheat, banana, maize, tomato, paddy and soybean). A total of 20 PSB isolates were obtained and identified Via Colony features, Gram's staining, and biochemical tests. The phosphate solubilization ability of the bacterial isolates was assessed using both solid and liquid Pikovskaya's (PVK) media containing insoluble tricalcium phosphate. The solubilization zone surrounding colonies were visually evaluated, and the solubilized phosphates in the liquid medium were determined using the molybdenum blue technique. In agar media, the solubilization index (SI) ranged from 1.33 to 3. Based on the results of qualitative evaluation, best 6 isolates (A_5 2, B_4, M_5, R_1, R_3, R_5) that showed the higher phosphate solubilization index was selected for the quantitative evaluation. In the liquid media, the PSB isolates showed phosphate solubilization ranging from 1.17 to 6.33 mg L^{-1} in the PVK medium, with highest value produced by *Pseudomonas aeruginosa.* Best 6 isolates were compared with commercial P fertilizer qualitatively as well as quantitatively where the commercial P fertilizer were less effective than the isolated PSB. This study suggests that these PSB isolates can solubilize phosphate and that can potentially be explored as production of phosphate biofertilizers.

Keywords Phosphate solubilizing bacteria, Phosphate solubilization, *Pseudomonas*, Biofertilizer

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Introduction

Phosphorus (P) is one of the macronutrients crucial for plant growth. Phosphorus can be found in soil in both organic and inorganic forms. Plant residuals, microbial tissues, and manures contain organic P; inorganic P, which is found in many soils, is inaccessible to plants because it complexes with metals like iron (Fe), aluminium (Al), and calcium (Ca) [1]. However, soil typically contains just 0.4–1.2 g of soluble P [2]. The majority of soil P (about 95–99%) can be found in insoluble forms, making it difficult for plants to use. In the past, agricultural fields have been fertilized with both organic and inorganic fertilizers in order to sustain nutritional balances while dealing with nutrient shortages [3,4].

Rock phosphate (RP) serves as the primary source of P_2O_5 in the production of the majority of phosphate fertilizers. There is a limited global supply of rock phosphate, despite the fact that demand is still rising [5]. The only thing preventing RP from being used directly as a soil amendment is its extremely low solubility [6]. As a substitute to phosphate fertilizers, several studies have confirmed the use of microorganisms to solubilize insoluble phosphate compounds [7].

In the rhizosphere, phosphate-solubilizing bacteria (PSB) serve an important part in assisting with the solubilization of inorganic phosphorus compounds into soluble forms that are suitable for plant nourishment [8]. Numerous PSB species, from genera: *Pseudomonas*, *Klebsiella*, *Enterobacter*, *Arthrobacter*, *Alcaligenes*, *Bacillus*, *Rhizobium*, *Azospirillum*, and *Serratia*, have been shown to promote growth and maturity in a variety of economically significant crops [9]. PSBs can raise plant P availability through altering rhizosphere soil activities, giving plants vital nutrients, and producing additional growth regulators in different stages of production [7,10]. Similarly, PSBs have the ability to formulate hormones that promote the development of plants, such as gibberellins and Indole Acetic Acid (IAA) [11–14].

Tricalcium phosphate (TCP) has long been the go-to insoluble phosphate source for PSB activity screening because of its uniformity and easy handling. However, in an effort to replicate more accurate soil conditions and increase our knowledge of PSB effectiveness, researchers have been using alternate substrates more and more. For instance, PSB behaviour in acidic and iron-rich soil conditions has been studied using aluminium phosphate ($AlPO_4$) and iron phosphate ($FePO_4$) [15]. Similar to this, it has been determined that rock phosphate, a naturally occurring mineral form of phosphate, can more closely resemble field conditions [16]. Furthermore, the ability of bacteria to mineralize organic phosphorus through phytase activity has been evaluated using organic phosphate sources such as phytate [17]. These investigations show that PSB's solubilizing capabilities can differ greatly based on the substrate, which has major implications for its usage in agriculture.

Despite being widely distributed in soils, a significant amount of phosphorus remains insoluble, and plants cannot use it. This has resulted in the overuse and unsustainable usage of chemical phosphate fertilizers in agricultural systems, which contribute to the degradation of soil and pollution. Therefore, it is becoming more and more important to investigate eco-friendly alternatives. The negative environmental impacts of chemical fertilizers and the growing understanding of the benefits of a healthy plant-soil relationship through the use of microbial inoculants have influenced farmers' perspectives in the modern period. As farmers are increasing the usage of chemical fertilizer significantly on the daily basis. The fertility of soil is decreased so in order to control it phosphate biofertilizer can be used as alternative which increases the accessibility of the phosphorus. Considering the environmental and public health, researching the microbial ability to provide P nutrients to the crop holds paramount significance as an alternative to use the chemically synthesised phosphate sources. Therefore, in this study we isolated and identified phosphate solubilizing bacteria and tested their ability to solubilize the insoluble phosphate.

Methods

Sample size

Soil samples were collected from the rhizosphere of Eight different types of plants as Aloe vera, potato (*Solanum tuberosum*), wheat (*Triticum aestivum*), banana (*Musa paradisiaca*), maize (*Zea mays*), tomato (*Solanum lycopersicum*), paddy (*Oryza sativa*) and soybean (*Glycine max*) with total of 40 samples for the study.

Sample collection

At a depth of 15 cm, soil samples weighing between 5 and 10 grams (estimated) were taken from the rhizosphere in sterile plastic envelop from different areas of Kathmandu and Lalitpur districts and samples were processed for isolation of phosphate solubilizing bacteria.

Isolation of phosphate solubilizing bacteria

Phosphate solubilizing bacteria were isolated from collected soil samples by serial dilution plate count method using Pikovskaya's (PVK) medium. The presence of phosphate solubilizing bacteria was indicated by the formation of transparent (halo) zone around the colonies in PVK agar plates. Colonies showing phosphate solubilizing zones were transferred to fresh Pikovskaya's agar (PVK) medium to obtain pure culture. Phosphate solubilizing bacteria exhibiting halo zone were selected from the total isolates and were further examined.

Identification of phosphate solubilizing bacteria

On the basis of colony morphology, microscopic and biochemical traits, the PSB were identified. Gram staining and various biochemical tests such as Catalase test, Oxidase test, Indole test, Methyl red test, Voges-Proskauer test, Citrate utilization test, Triple sugar iron test, and Urease test, and Nitrate reduction test were performed for identification of PSB.

Qualitative estimation of phosphate solubilization

The solubility of Tri-Calcium Phosphate (TCP) by PSB isolates in agar plates were tested further. Pikovskaya's agar medium were spot-inoculated with pure cultures of phosphate-solubilizing bacteria [18,19]. After six days of incubation at 30°C, the colonies with clear zones of phosphate solubilization were noted. By calculating the Solubilization Index (SI), which measures the solubilization zone surrounding the colony, the phosphate solubilizing capacity was analysed [20].

The phosphate solubilization index (PSI) were determined by obtaining the diameterof bacterial colony and halo zone. The following equation was used to calculate the solubilization index using the values:

$$\text{Phosphate Solubilization Index (PSI)} = \frac{CD + HD}{CD}$$

Where,

CD – Colony Diameter

HD – Halo zone Diameter

Quantitative estimation of phosphate solubilization

PSB isolates were further examined for their potential to solubilize phosphate in broth medium. A conical flask containing 100 ml of Pikovskaya's broth was inoculated with 200 µL of inoculum isolates, and the mixture was cultured for 12 days at 30°C in a shaking incubator at 120 rpm. As a control, sterile uninoculated Pikovskaya's broth was used. The cultures were extracted using a centrifuge and centrifuged at 10,000 rpm for ten minutes. The molybdenum blue technique was used to measure the total soluble phosphate in the culture supernatant using a UV-visible spectrophotometer set to 880 nm.

Solubilizing activity comparison of the isolates and commercially available phosphate biofertilizer

Commercially available phosphate biofertilizer was screened side by side along with the PSB isolates. Commercial P biofertilizer was spot inoculated at the center of Pikovskaya's agar medium. The plates were incubated at 30°C for 6 days and halo zone of P solubilization around the colonies were noted for the solubilization index (SI).

Commercial P biofertilizer were further examined for their ability to solubilize phosphate in broth medium. 200 µL of commercial P biofertilizer was inoculated in Pikovskaya's broth (100 ml) in conical flask and was incubated on a shaking incubator at 120 rpm for 12 days at 30°C. Then, P fertilizer was centrifuged at 10,000 rpm for 10 min using centrifuge. By measuring the supernatant at 880 nm in a UV-visible spectrophotometer, the total soluble phosphate was determined.

Data analysis

Microsoft Excel was used when entering all of the data, the interpretation of the data was shown visually for the purpose of appreciating various analytical features through the use of diagrams, graphs, and tables.

Results

Isolation of Phosphate Solubilizing Bacteria (PSB)

From 40 soil samples processed, PSB were isolated in 18 (45%) soil samples with total of 20 isolates.

Sample Wise Growth Distribution of PSB

Out of these PSB isolates highest percentage i.e. 20% (4) were isolated from maize, paddy and potato. The least percentage of PSB isolates were 5% (1) from banana and tomato (**Figure. 1**).

Identification of Phosphate solubilizing bacteria (PSB)

In this study, a total of 20 PSB isolates showed halo zone on Pikovskaya's agar media and they were further processed. These PSB isolates were identified by the colony characteristics, Gram's staining and biochemical tests. Out of 20 PSB isolates, 16 (80%) isolates were identified as *Pseudomonas aeruginosa* and remaining 4 (20%) were identified as *Pseudomonas fluorescens* (**Table 1**).

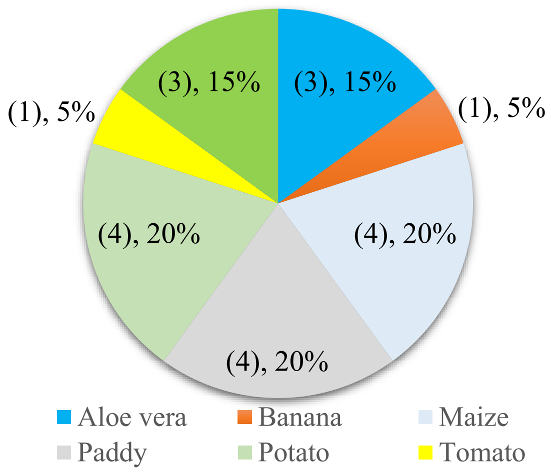


Figure 1: Growth status of different PSB isolates from soil samples

Table 1: Distribution of Identified Phosphate Solubilizing Bacteria

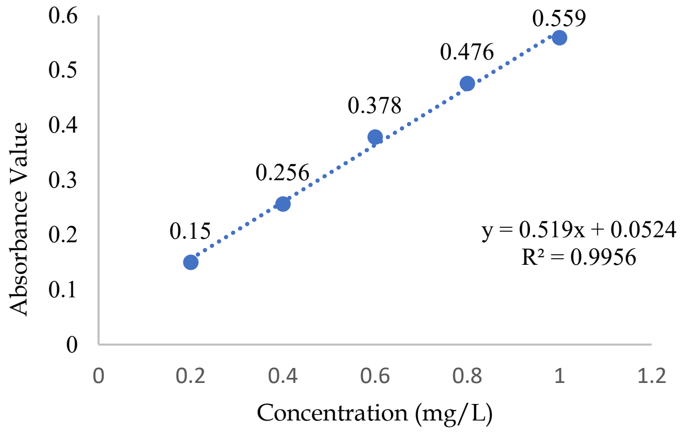
Bacteria among positive isolates	Number	Percentage (%)	Total
Pseudomonas aeruginosa	16	80%	20
Pseudomonas fluorescens	4	20%	

Table 2: Solubilization index (SI) of PSB isolates isolated from rhizosphere of different plant in Pikovskaya's agar plate.

Bacterial Isolates	Solubilization index		
	Day 2	**Day 4**	**Day 6**
A_4	1.71	2.14	2.25
A_5 1	1.75	2.25	2.5
A_5 2	2	2.66	3
B_4	1.42	1.75	2.6
M_1	1.62	2.33	2.44
M_2	1.33	1.62	2.4
M_4	1.66	1.75	2.3
M_5	1.83	1.88	2.6
P_3	1.6	2	2.14
P_4	1.66	1.71	2.12
P_5 1	1.71	2.28	2.5
P_5 2	1.71	2.37	2.44
R_1	1.55	2	2.63
R_2	1.5	2	2.33
R_3	2	2.33	2.75
R_5	1.5	1.88	2.54
T_4	1.5	1.6	2.08
W_3	1.62	2.22	2.33
W_4	1.71	2.25	2.44
W_5	1.75	1.88	2.11

Qualitative estimation of phosphate solubilization

In Pikovskaya's agar plates, all of the examined phosphate-solubilizing bacterial isolates produced distinct halo zones of varying sizes, indicating positive responses for phosphate solubilization (**Table 2**). All isolates showed signs of the halo zone after the 2^{nd} day of incubation. The solubilization index (SI) ranged from 1.33 to 3. Isolate A_5 2 showed maximum SI in day 6 with PSI of 3 and least PSI 1.33 was showed by isolate M_2 in day 2.



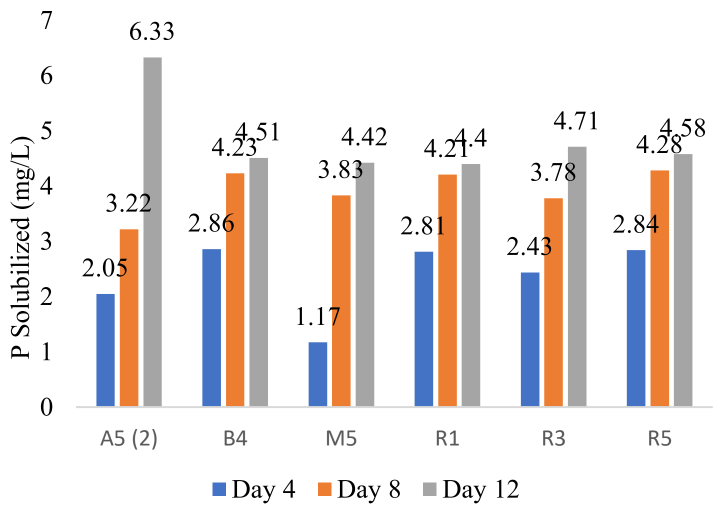


Figure 2: Calibration of phosphate standard curve

Figure 3: Quantitative estimation of P solubilized in PVK broth

Quantitative estimation of phosphate solubilization

Among the 20 isolates, best 6 isolates (A_5 2, B_4, M_5, R_1, R_3, R_5) were selected for the quantitative estimation. They were selected on the basis of SI value (>2.5). The amount of soluble P released after 4^{th}, 8^{th} and 12^{th} days of incubation are presented in **Figure 3**. The amount of phosphate solubilized is calculated with the help of phosphate standard curve (**Figure 2**).

Table 3: Comparative evaluation of the isolates and commercial P fertilizer in Pikovskaya's agar

Bacterial Isolates	Solubilization Index		
	Day 2	Day 4	Day 6
A_5 2	2	2.66	3
B_4	1.42	1.75	2.6
M_5	1.83	1.88	2.6
R_1	1.55	2	2.63
R_3	2	2.33	2.75
R_5	1.5	1.88	2.54
Commercial P fertilizer	1.6	1.83	2

Table 4: Comparative evaluation of the isolates and commercial P fertilizer in Pikovskaya's broth.

Bacterial Isolates	Phosphate solubilization (mg/L)		
	Day 4	Day 8	Day 12
A_5 2	2.05	3.22	6.33
B_4	2.86	4.23	4.51
M_5	1.17	3.83	4.42
R_1	2.81	4.21	4.4
R_3	2.436	3.78	4.71
R_5	2.84	4.28	4.58
Commercial P fertilizer	2.76	3.95	5.71

Qualitative comparison of the isolates and commercial P fertilizer

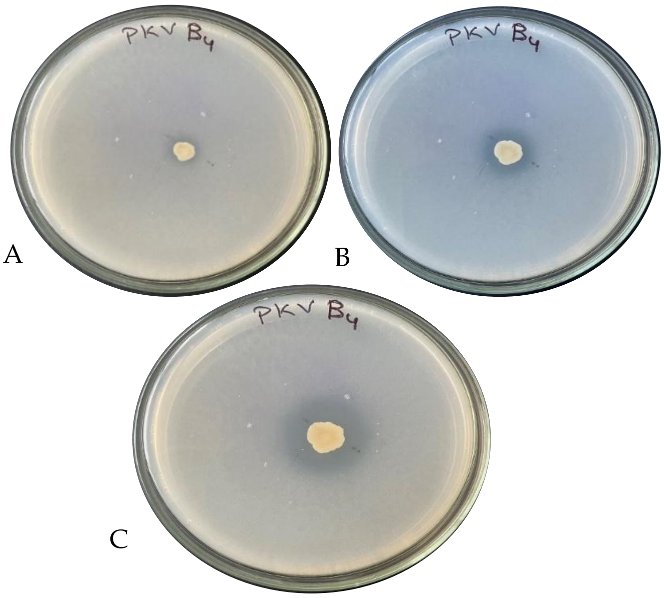
The solubilization index (SI) of six phosphate-solubilizing bacteria (PSB) isolates was tracked over six days and compared to that of a commercial phosphorus (P) fertilizer (**Table 3**). Isolate A_5 2 displayed the highest SI value of 3 on Day 6. The commercial P fertilizer showed a gradual increase in solubilization over time, but its maximum SI reached only 2 on Day 6.

Quantitative comparison of the isolates and commercial P fertilizers

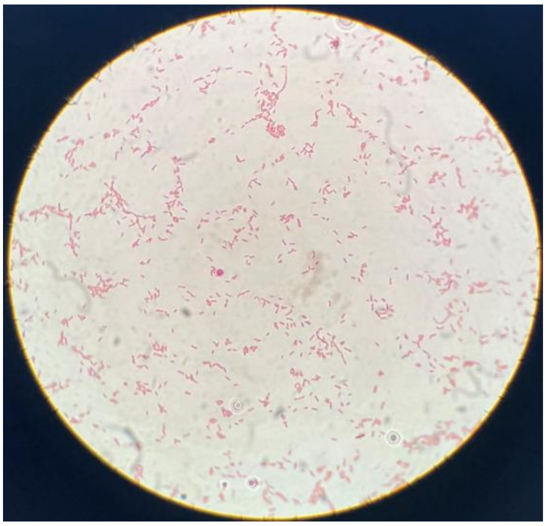
Phosphate solubilization levels (mg/l) of six bacterial isolates and commercial phosphorus (P) fertilizer were monitored on Days 4, 8, and 12 (Table 4). Among the isolates, A5 (2) demonstrated the highest solubilization activity, reaching 6.33 mg/l by Day 12, outperforming both the other bacterial isolates and the commercial P fertilizer. The commercial fertilizer achieved a maximum solubilization of 5.71 mg/l on Day 12, which was still lower than A5 (2) but higher than most other isolates.

Discussion

Phosphate solubilizing bacteria were isolated in 18 (45%) soil samples with total of 20 isolates. Out of these PSB isolates highest percentage i.e. 20% (4) were isolated from maize, paddy and potato. The least percentage of PSB isolates were 5% (1) from banana and tomato. Among the isolates obtained, all belonged to the genus *Pseudomonas*, and showed strong phosphate solubilization capabilities. Isolate A_5 2 exhibited superior performance both on solid (Pikovskaya's agar) and liquid (Pikovskaya's broth) media, surpassing even commercial phosphate biofertilizers in terms of solubilization index (SI) and soluble phosphate concentration.



Photograph 1: Halo zone produced by PSB isolate (B4) in Pikovskaya's (PVK) agar. **(A)** shows halo zone produced in 2nd day. **(B)** represents halo zone produced in 4th day. **(C)** shows halo zone produced in 6th day.



Photograph 2: Gram staining of PSB isolate (*Pseudomonas aeruginosa*)

In this study, phosphate solubilizing bacteria was high in maize and paddy. Both maize and paddy are known to secrete abundant root exudates, such as sugars, amino acids, organic acids, and phenolic compounds. These exudates act as carbon sources that promote PSB and other microbial development. Dakora & Phillips [21] explained that root exudation influences microbial community structure and diversity, often enhancing the abundance of functional groups like PSB. Similar finding was observed from the study by Gupta [22] and Pantigoso [23] where they revealed that phosphate solubilizing bacteria can be isolated from the rhizosphere of paddy and potato. According to Gupta [22] areas where rice is grown, phosphate solubilizing bacteria (PSB) are of significant class that are found in small amounts. PSB were isolated from the rhizosphere of a wild potato grown in a greenhouse and were studied by Pantigoso [23]. The isolated PSB were found to have P-solubilizing capabilities, that could dissolve both organic and inorganic phosphorus.

Photograph 3: Biochemical tests of *P. aeruginosa*. (**Urease**: -ve, **TSIA**: alk/alk, **Citrate**: +ve, **MR**: -ve, **VP**: -ve **Indole**: -ve)

All the isolates were rod-shaped gram-negative bacteria and they were motile. According to Meireles [24], this kind of bacteria has the highest prevalence in soils. Patten and Glick [25], reported that these rod-shaped, motile, non-spores forming, Gram-negative strains of bacteria are usually found in the rhizosphere and belongs under the genus *Pseudomonas*. Amri [8] mentioned that one of the best genera for solubilizing phosphates is *Pseudomonas* spp. Comparable outcomes were achieved by Meireles [24], declaring that *Pseudomonas* is considered to be the predominant PSB in soils. *Pseudomonas* spp. is well-known for producing a wide range of low molecular weight organic acids, such as: Gluconic acid, 2-ketogluconic acid, Citric acid, Oxalic acid. These acids aid in acidifying the environment, dissolving insoluble phosphate (such as rock phosphate or tricalcium phosphate), and chelating metal ions (Ca^{2+}, Fe^{3+}, and Al^{3+}) that bind phosphate, making it available for plants [17].

The SI value obtained by the test isolates in this study was similar to other researchers, such as Amri [8] screened the PSB belonging to genus *Pseudomonas, Pantoea, Stenotrophomonas* which showed maximum SI value in PVK medium. A distinct halo zone surrounded the colonies of the isolates, which may have been caused by phosphatase enzyme activity or the synthesis of organic acids or polysaccharides [26]. According to Amri [8] solubilization index can serve as the foundation for an initial comparison of the isolates' TCP solubilization capacities. It appears that *Pseudomonas* isolates are the most effective at solubilizing solid phosphate. One of *Pseudomonas*' most prevalent traits is the solubilization of solid phosphates.

Similar outcomes have been reported in earlier research. Mehta, Nautiyal and Zaidi [16,27] showed that *Pseudomonas* and *Bacillus* species isolated from rhizospheric soils exhibited halo zone development and increased SI values over time. Their results showed maximum SI values ranging from 2.5 to 3.2, which is quite similar to the greatest SI found in our investigation. The ability to solubilize TCP as the sole source of phosphate in PVK broth was shared by all isolates, despite variations in concentration of solubilization in broth. The P solubilization ability was enhanced by all the isolates over the period of time but maximum P solubilization was observed by A_5 2 at 12th day with concentration of 6.33 mg L^{-1}. All isolates showed a rising trend in soluble phosphate content from day 4 to day 12, which indicates that solubilizing agents-likely organic acids are continuously produced. This trend, where bacterial activity gradually lowers the medium's pH and increases the release of bound phosphates, has been commonly documented in previous investigations of Chen [28] and Kang [29]. The isolates in this investigation that were identified as *Pseudomonas* had the highest concentration of soluble phosphate in the PVK broth during a 12-day incubation period. Similar findings were observed from the study of Ahmad [30] who revealed that *Pseudomonas* spp. is an efficient phosphate solubilizer.

When six chosen phosphate-solubilizing bacterial (PSB) isolates and a commercial phosphorus (P) fertilizer were compared on Pikovskaya's agar, the solubilization index (SI) showed that the microbial isolates were more effective than the commercial fertilizer in terms of phosphate solubilization. With a SI value of 3 on Day 6, A_5 2 demonstrated the best solubilization ability among the studied isolates, whereas the commercial P fertilizer only reached a maximum SI of 2 within the same time frame. This indicates that, although commercial fertilizers are commonly used in agriculture, they may not solubilize phosphate as efficiently as naturally occurring phosphate-solubilizing bacteria (PSB). Additionally, the variation observed among the bacterial isolates suggests differences in their enzymatic functions or metabolic processes that contribute to phosphate solubilization. Prasad [31] revealed that phosphate solubilizing bacteria had the capacity to breakdown tricalcium phosphate in both NBRIP and Pikovskaya's media. The growth medium's rising pH and dissolved level of phosphate served as indicators of phosphate solubilization.

The results clearly show that bacterial isolates, particularly A_5 2, have the potential to surpass the phosphate solubilization efficiency of commercial P fertilizer. Isolate A_5 2 showed the P- concentration of 6.33 mg/L at day 12 which surpassed all the isolates along with the commercially available phosphate biofertilizer.

Similar results from several investigations have shown that bacterial isolates' capacity to solubilize phosphate tends to grow over time. For instance, during seven days of incubation, Khan [32] obtained phosphate solubilization values by *Pseudomonas* spp. ranging from 6.5 to 9.1 µg/ml. The well-known PSB strain *Bacillus megaterium* also showed solubilization efficiencies of up to 8.7 µg/ml, according to Chen [28]. By the seventh day of the current investigation, the isolates A5 2 and commercial phosphate biofertilizer had phosphate solubilization levels of 6.33 µg/ml and 5.71 µg/ml, respectively. This superior performance of A_5 2, in particular, could be attributed to the enhanced secretion of organic acids such as gluconic and citric acid, which are known to chelate cations bound to phosphate and release soluble P more effectively than synthetic fertilizers. According to studies by Rodríguez & Fraga [17] and Nautiyal [18], the generation of organic acid is a crucial component of PSBs' high efficiency. The consistent and comparatively higher solubilization by A5 2 position it as strong candidate for development into commercial biofertilizer, especially for phosphorus-deficient soils in Nepal and similar agroecological zones.

Conclusion

This study, PSB isolates from soil samples of various plants may have a role in the high availability of soluble phosphates that plants can absorb. It was discovered that every PSB that was isolated from eight distinct plants exhibited phosphate solubilizing activity in both the broth and agar media. In this study, the solubility of phosphate was screened for 20 bacterial isolates. *P. aeruginosa* and *P. fluorescens*, two species in the genus, demonstrated the greatest capacity for solubilizing phosphates. The result obtained with the PSB isolates from rhizosphere of different plants can be exploited for improved plant growth. Commercially used synthetic fertilizers, which are very expensive can be replaced by the PSB biofertilizer.

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Author contributions

All the authors have equal contribution in this manuscript. All the authors read and approved the final manuscript.

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Data availability

All the required data and materials of research is given in the manuscript.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Trial registration

Trial registration was not applicable.

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