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Isolation and characterisation of phosphate-solubilising bacteria from serpentine soils in Yen Bai Province

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Abstract. Phosphate-solubilising bacteria (PSB) contribute to plant phosphorus nutrition by transforming insoluble phosphates into bioavailable forms. In our work, PSB were isolated from serpentine soils in Yen Bai Province, Vietnam—an environment typified by low nutrient availability and elevated metal content. Forty-two bacterial isolates with phosphate-solubilising traits were screened in the NBRIP medium. Among them, strain VW132 demonstrated the highest solubilisation capacity and was taxonomically identified as *Burkholderia* sp. via 16S rRNA gene analysis. Strain VW132 reached maximum phosphate solubilisation of 359 mg/L when cultivated under optimised conditions, i.e, ammonium sulfate and glucose as nitrogen and carbon sources, 1% NaCl, and pH 7. However, phosphate solubilisation markedly declined at NaCl concentrations exceeding 1%, suggesting reduced salt tolerance. The relationship between soluble phosphate concentration and biofilm development was also investigated. Results show enhanced biofilm formation with increasing levels of K_2HPO_4, indicating a phosphate-dependent biofilm response.

Keywords: phosphate-solubilising bacteria, serpentine soil, *Burkholderia* sp., biofilm

1 Introduction

Phosphorus (P) is an indispensable macronutrient for plant productivity and is considered next in significance to nitrogen [1]. It is involved in vital plant metabolic functions, notably photosynthesis, signal transduction, and respiratory processes [2]. Despite its importance, the level of available phosphorus in soil is typically low, ranging from 400 to 1260 mg/kg [3]. This limited availability requires the application of fertilisers to sustain crop productivity and meet the growing food demand driven by global population growth. As a result, the global demand for phosphorus-based fertilisers has been rising 2.5–3.0% annually [4]. Chemical fertilisers typically provide phosphorus in several formulations, including monoammonium phosphate, triple superphosphate, ammonium polyphosphate, and diammonium phosphate. One major limitation of such fertilisers is that a large portion of the added soluble inorganic phosphate rapidly binds in the soil, making it inaccessible to plants [5]. In addition, the depletion of global phosphorus reserves underscores the urgency of working out alternative strategies to address low phosphorus availability caused by this immobilisation. Phosphate-solubilising bacteria (PSB) are capable of converting insoluble forms of phosphate, such as tricalcium phosphate and different phosphate minerals, into forms accessible to plants, thereby reducing reliance on synthetic fertilisers [6].

Therefore, employing PSB as microbial inoculants is regarded as a sustainable and eco-conscious strategy [7].

Previous studies have reported that various PSB-based bioinoculants are commercially available and distributed globally [8, 9]. However, the effectiveness of microbial inoculation remains a subject of debate [10], as its success is strongly affected by environmental conditions, particularly soil characteristics and the composition of native microbial communities. This challenge can be solved by expanding the diversity of culture-dependent PSB isolates that could offer a solution adapted to the wide range of soil types where inoculants are applied. This premise underscores the need for novel isolation strategies, such as targeting new environments and habitats. Each contains a unique microbial community, which may include previously unidentified or uncharacterised bacterial strains.

PSB have been recovered from different soil environments in previous work [11, 12]; however, investigations focusing on serpentine soils are still scarce. Serpentine soils are notable for their scarcity of essential nutrients and elevated levels of heavy metals, presenting a uniquely harsh habitat that supports specially adapted microbial communities. Studies on PSB in serpentine soils can provide new insights into the diversity of PSB under adverse conditions, the discovery of novel isolates, and potentially valuable genetic information related to heavy metal resistance that may be exploited. In our research, PSB were recovered from serpentine soils collected in Yen Bai Province. The role of environmental factors in regulating phosphate-solubilising activity and biofilm formation of potential PSB in relation to phosphate availability were studied. This study primarily aimed to discover phosphate-solubilising bacteria from serpentine soils that could serve as promising candidates for future use in agriculture.

2 Materials and methods

2.1 Soil sampling

The soil in this study was obtained from six separate points across serpentine outcrops located in Yen Bai Province, Vietnam. Approximately 100 g of topsoil from the A-horizon layer at a depth of 10 cm was collected from undisturbed plots. Each sample was transferred into sterile plastic bags and kept at 4 °C prior to the isolation of phosphate-solubilising bacteria.

2.2 Isolation of phosphate-solubilising bacteria with enrichment culture

One gram of soil was suspended in test tubes containing an NBRIP medium to recover phosphate-solubilising bacteria. The medium formulation (per liter) consisted of 10 g glucose, 5 g $Ca_3(PO_4)_2$, 5 g $MgCl_2{\cdot}6H_2O$, 0.25 g $MgSO_4{\cdot}7H_2O$, 0.2 g KCl, and 0.1 g $(NH_4)_2SO_4$ [13]. The medium was adjusted to pH 7 prior to autoclaving. The insoluble phosphorus components were sterilised independently by autoclaving, and the remaining sterile components were aseptically combined after autoclaving. Test tubes containing 5 mL of the medium inoculated with soil samples were incubated for 7 days at 30 °C on a shaker incubator at 200 rpm. In the following week, 0.5 mL of the incubated culture was transferred into fresh test tubes containing a new liquid NBRIP medium and incubated under the same conditions for another 7 days. This enrichment step was repeated three times. After every weekly incubation period in the NBRIP liquid medium, the culture was serially diluted tenfold by dispensing 1 mL portions into 9 mL of autoclaved 0.85% NaCl. From the 10^{-3} and 10^{-4} dilutions, 0.1 mL of suspension was plated onto Petri dishes

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containing the NBRIP medium. The suspension was uniformly distributed across the plates with a sterile glass rod, followed by incubation at 30 °C for 5 days [14]. Phosphate solubilisation was indicated by the formation of clear zones around bacterial colonies, and the diameters of these zones were measured [15]. Colonies showing distinct halos were picked and purified through multiple rounds of sub-culturing, and the resulting isolates were maintained at –80 °C in glycerol stocks until subsequent analysis.

The identification of phosphate-solubilising isolates was initially based on visual assessments of colony morphology, such as size, shape, and pigmentation [16]. Gram staining was performed on the purified PSB strains following conventional microbiological techniques [16].

2.3 16S rRNA gene and phylogenetic analysis

The PCR amplification of the 16S rRNA gene was performed with total genomic DNA as a template, employing the universal bacterial primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). Sequencing the PCR product was carried out by Phu Sa Genomics Joint Stock Company (Can Tho province, Vietnam). The sequence was analysed by using BLAST on NCBI, and a 16S rRNA phylogenetic tree was generated by MEGA version 11 through the neighbor-joining method with 1,000 bootstrap replicates [17].

2.4 Effect of culture conditions on the phosphate solubilisation by the bacterial strain

Inoculum preparation: Individual colonies were cultured in LB broth (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl) at 30 °C with shaking at 200 rpm for 24 h. The collected cells were rinsed and resuspended in sterile distilled water to achieve an OD_{600} of 1.0.

To investigate the effect of different factors on phosphate-solubilising activity, we evaluated four variables: NaCl concentration, initial pH, carbon source, and nitrogen source. Cultivation was performed in 100-mL Erlenmeyer flasks containing 30 mL of NBRIP broth. The levels of all factors, except for the one being examined, were kept constant (incubation temperature: 30 °C; inoculum volume: 2% v/v at OD_{600} = 1.0; agitation speed: 200 rpm; culture duration: 7 days). All experiments were conducted in triplicate. Seven days after incubation, phosphate-solubilising activity was analysed by determining the concentration of soluble phosphate in the culture supernatant with the vanado-molybdate yellow colorimetric technique at 410 nm, according to Mehta and Nautiyal [18]. Phosphate levels were quantified by means of a standard curve prepared from a KH_2PO_4 stock solution (r^2 = 0.997). Control flasks without bacterial inoculation served as blanks, and their readings were subtracted from the corresponding experimental values.

Salt concentration: To evaluate the influence of salinity on phosphate solubilisation, we grew the strain in the NBRIP medium supplemented with an NaCl solution of different concentrations (0, 1, 2, 3, and 5%).

Initial pH: The influence of pH on phosphate solubilisation was assessed by adjusting the initial pH of the NBRIP broth to 3.0, 5.0, 7.0, and 9.0.

Carbon source: To determine the effect of carbon sources, we cultured the strain in modified NBRIP broth (glucose omitted) supplemented with different sugars (glucose, sucrose, manitol, and starch), each supplied at 10 g per liter.

Nitrogen source: The strain was also cultured in NBRIP broth without a nitrogen source supplemented with various nitrogen compounds, including $(NH_4)_2SO_4$, NH_4NO_3, KNO_3, and pepton.

2.5 Biofilm formation by bacterial strain in response to phosphate availability

To examine the relationship between biofilm formation by bacterial strain and environmental phosphate availability, we modified the NBRIP medium by substituting the insoluble phosphorus source ($Ca_3(PO_4)_2$) with a soluble phosphorus source (K_2HPO_4) at varying concentrations (0, 25, 50, 100, 200, 300, 400, and 500 mg/L). Biofilm formation was then quantified under these different soluble phosphate conditions.

Quantitative analysis of biofilm formation was conducted by safranin staining, with the method described by O'Toole et al. [19] with slight modifications. A 2% (v/v) bacterial inoculum (OD_{600} = 1.0) was suspended in fresh culture broth, and 175 μL aliquots were inoculated into 96-well microtiter plates. The plates were maintained at 30 °C under static incubation for 48 h to enable biofilm growth. After incubation, the supernatants were carefully removed, and the adherent cells were stained by adding 175 μL of 50 mg/L safranin to each well and incubating at ambient temperature for 30 minutes. After the excess stain was discarded, each well was repeatedly rinsed with distilled water three times and air dried, and then the retained stain was dissolved in a 20% (v/v) acetone-ethanol solution. The content from four wells was combined to yield an adequate volume for absorbance measurement. Biofilm production was assessed by measuring dye absorbance at 490 nm with a Thermo Scientific Multiskan EX ELISA plate reader (UK). All tests were repeated three times, and the absorbance values were averaged.

2.6 Statistical analysis

The data were represented as mean ± the SE of three independent replicates. Statistical evaluation was carried out by means of one-way ANOVA with Tukey's HSD test when $p < 0.05$ to compare the treatments.

3 Results and discussion

3.1 Isolation and identification of phosphate-solubilising bacterial strain

All individual colonies obtained were evaluated for their phosphate-solubilising potential on the NBRIP solid medium. The formation of a clear halo around the colony in a medium where tricalcium phosphate served as the sole phosphorus source indicated phosphate solubilisation activity. The abundance of phosphate-solubilising bacteria depends on the nature of the isolated samples. Yahya and Azawi [20] reported that PSB are generally more abundant in agricultural and rangeland soils. Additionally, Kim et al. [21] demonstrated that various factors, such as land use practices and soil properties, including physical and chemical characteristics, organic matter content, and phosphorus availability, contribute to soil density of phosphate-solubilising bacteria. Overall, 42 strains of phosphate-solubilising bacteria were recovered from the serpentine soils collected in Yen Bai Province. The morphological profiles of the isolates revealed a uniform appearance, with round edges, white or off-white coloration, and a mucoid or viscous texture. Cell morphology analysis shows that 90.5% of the strains were rod-shaped, while 9.5% were spherical. Gram staining reveals that the majority of the strains were Gram-positive (80.9%), whereas only 19.1% were Gram-negative (Fig. 1A). The diameter of clear halos formed around the isolates varied from 2 to 13 mm after five days of incubation (Fig. 1B). The largest clear zone diameter (13 mm) was observed in isolate VW132 with promising phosphate-solubilising activity, and it was selected for detailed study.

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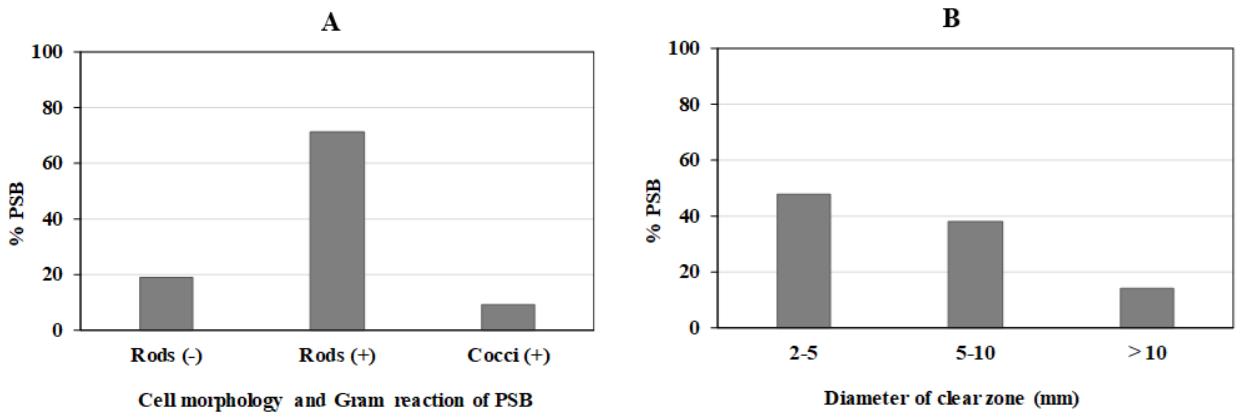

Fig. 1. (A) The proportion of PSB strains classified by cell morphology and Gram reaction ((+): positive-Gram, (–): negative-Gram); (B) The proportion of PSB strains with different clear zone diameters

The 16S rRNA gene sequence examination demonstrates that VW132 exhibited 99.78% similarity to *Burkholderia territorii* LMG 28158. A phylogenetic tree was generated from the 16S rRNA sequences of VW132 and closely related reference strains to determine its taxonomic position (Fig. 2). As shown in Fig. 2, strain VW132 is tightly clustered with the other strains of *Burkholderia*, indicating that this strain belongs to *Burkholderia*. The 16S rRNA sequence of this strain also reveals considerable similarities to those of *B. lata*, *B. contaminans*, *B. cenocepacia*, and *B. cepacia* (Fig. 2), indicating more robust analyses were needed. However, the close taxonomic relationship between strain VW132 and the species within the *Burkholderia cepacia* complex raises significant biosafety considerations. While certain *Burkholderia cepacia* complex strains have been utilised in biocontrol and environmental remediation, others are known to be plant or opportunistic human pathogens [22]. Therefore, further analyses, such as whole genome sequencing and virulence factor profiling, are essential to clarify the pathogenic potential and biosafety level of strain VW132 prior to its agricultural applications.

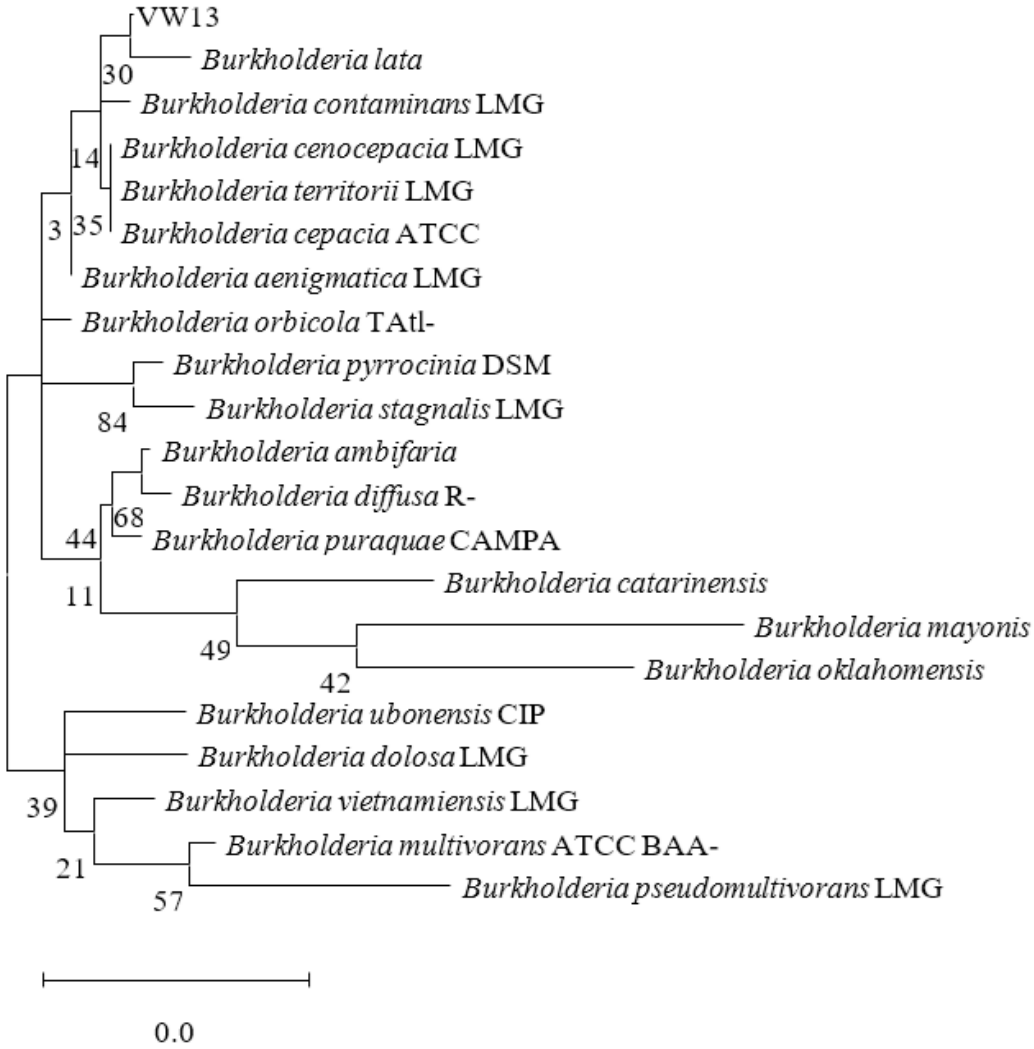

Fig. 2. Phylogenetic relationships inferred by means of neighbor-joining approach from 16S rRNA gene sequences and related reference strains (The scale bar indicates 0.01 substitutions per nucleotide site.)

3.2 Phosphate-solubilising capacity of VW132 strain under different culture conditions

Phosphate solubilisation is known as an intricate process influenced by multiple factors, particularly the nutritional, physiological, and cultivation conditions of the microbial cultures involved. The ability to tolerate various stress conditions is a crucial determinant of microbial survival and proliferation in soil environments. Although the VW132 strain exhibited the most significant phosphate dissolution in the NBRIP medium at an initial pH of 7 (297 mg/L), it also demonstrated the capability of solubilising phosphate across a wide pH range (3 to 9) (Fig. 3A). Statistically, phosphate solubilisation at pH 5 and pH 7 was significantly higher than that at pH 3 and pH 9, indicating that VW132 performs optimally under slightly acidic to neutral conditions.

The VW132 strain exhibited the highest phosphate solubilisation at an NaCl concentration of 1% (350 mg/L, labeled 'a'), followed by 0% (256 mg/L, labeled 'b'). This strain was completely incapable of solubilising phosphate at higher NaCl concentrations (2, 3, and 5%), with values close to zero (all labeled 'c') (Fig. 3B). Statistically,

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phosphate solubilisation in 1% NaCl was significantly higher than that in all other solutions ($p < 0.05$), indicating that mild salinity may enhance solubilisation with this strain. In contrast, concentrations above 1% resulted in a drastic and statistically significant decline in solubilisation activity, suggesting that VW132 is highly sensitive to salt stress beyond 1% NaCl. The ability of strain VW132 to solubilise phosphate most efficiently under mild salinity (1% NaCl) suggests its potential application in agricultural areas affected by low to moderate salt stress. Such conditions are commonly found in coastal regions influenced by seawater intrusion, as well as in post-salinised soils during the dry season. Furthermore, the strain's ability to maintain activity under slightly saline conditions could be beneficial for crop production in upland or hilly regions with poor nutrient availability and low phosphorus content. These findings underscore VW132 as a promising candidate for biofertiliser development, particularly for use in marginal lands where conventional chemical fertilisers may be inefficient or environmentally harmful.

Phosphate-solubilising microorganisms are capable of utilising various carbon sources to fulfill their energy requirements; however, their growth and phosphate-solubilising efficiency can differ depending on the carbon source provided. Carbon sources are essential for enhancing microbial proliferation and organic acid biosynthesis. Consequently, the type of available carbon source significantly affects both the composition and concentration of organic acids produced, which, in turn, determines the extent of phosphate solubilisation [23]. Fig. 3C shows that glucose supports the highest phosphate solubilisation, followed by mannitol and sucrose, whereas starch results in the lowest activity. Statistical analysis (indicated by different letters in Fig. 3C) shows that glucose significantly promotes phosphate solubilisation compared with sucrose and starch ($p < 0.05$).

In contrast, mannitol showed no significant difference compared with glucose or sucrose but was significantly more potent than starch. This confirms that the choice of carbon source significantly affects phosphate solubilisation efficiency. Regarding carbon source utilisation, our results show that monosaccharides such as glucose and mannitol are more effective in enhancing phosphate solubilisation than disaccharides or complex sugars. These findings align with earlier work by Dave and Patel, who reported superior phosphate-solubilising activity with monosaccharides to other carbohydrate types [24]. However, Patel and colleagues used *Citrobacter* sp. DHRSS and observed different trends, with maltose outperforming glucose and sucrose [23]. This suggests that the phosphate solubilisation efficiency depends on the specific carbon source and may vary among bacterial species.

Fig. 3D illustrates that phosphate solubilisation differs depending on the nitrogen source used. Among the various nitrogen sources tested, NH_4NO_3 was the most effective for phosphate solubilisation with the VW132 strain (359 mg/L), whereas peptone exhibited the lowest solubilisation (114 mg/L). Statistical analysis (as indicated by the different letters in Fig. 3D) revealed that phosphate solubilisation with NH_4NO_3 was significantly higher than with KNO_3 and peptone ($p < 0.05$), while $(NH_4)_2SO_4$ showed no significant difference compared with NH_4NO_3 or KNO_3. Peptone led to the lowest solubilisation and was statistically different from all other nitrogen sources. These observations are consistent with previous research showing that inorganic nitrogen is generally more effective than organic nitrogen in stimulating phosphate solubilisation [25, 26].

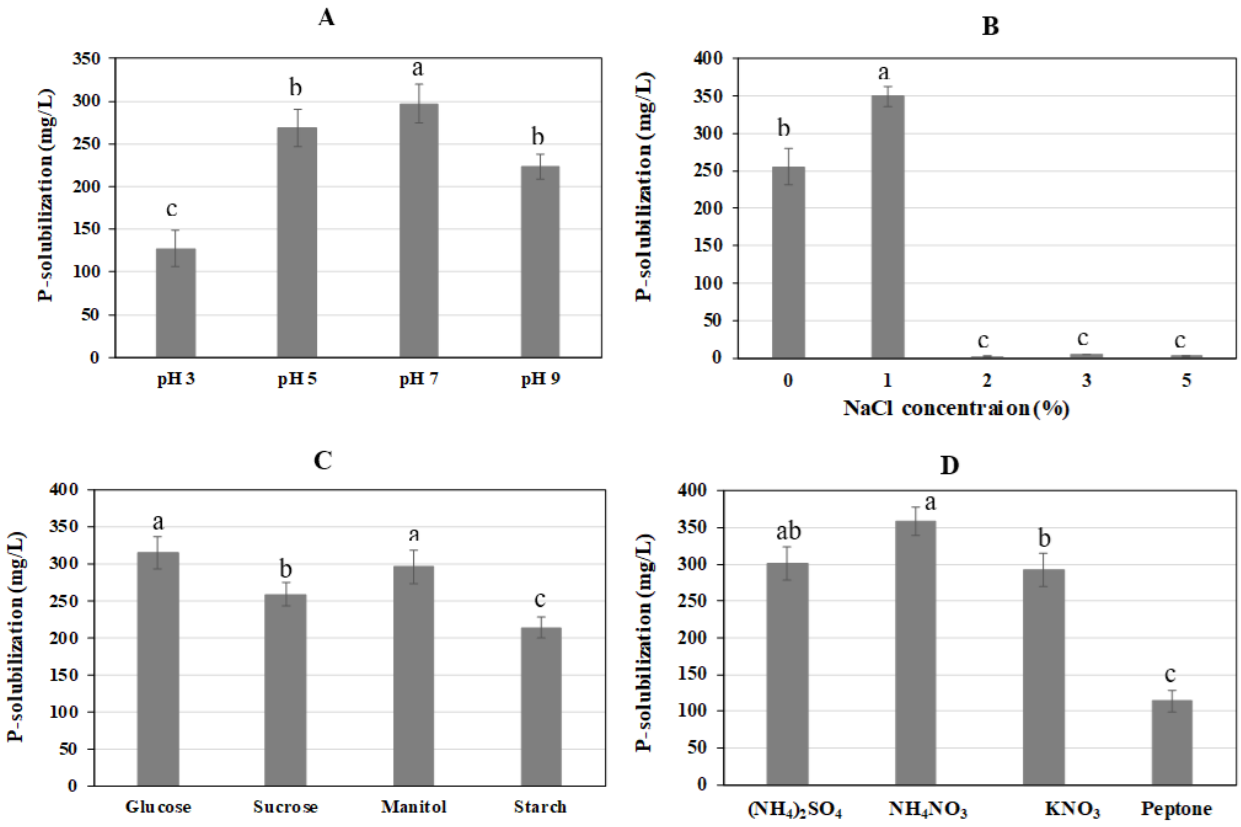

Fig. 3. Effect of different NaCl concentrations (A), initial pH (B), carbon sources (C), and nitrogen sources (D) on phosphate solubilisation with the VW132 strain (The bars indicate mean values ± SE of three replicates. Different letters on the bars represent statistically significant differences ($p < 0.05$, Tukey's HSD test).)

3.3 Changes in biofilm formation in response to different levels of available phosphate

Biofilm-forming ability in PSB is closely associated with their capacity to colonise the rhizosphere and persist in the soil environment. To assess how phosphate availability influences the development of biofilms in strain VW132, we carried out quantitative assays with varying concentrations of soluble phosphate (K_2HPO_4). The results shown in Fig. 4 indicate that the amount of biofilm formed by the VW132 strain increased in correlation with rising phosphate concentrations. The differences between groups with 200 ppm or higher concentrations and those with lower concentrations (0–100 ppm) were statistically significant. Similar to previous results, the findings indicate the biofilm formation decline under phosphorus-deficient conditions [27–29]. However, Ghosh et al. [30] reported that two strains, *Burkholderia unamae* and *Burkholderia tropica* P4, exhibited maximal biofilm formation under phosphate-limited conditions (25 mg/L). Upregulation of alkaline phosphatase activity in phosphorus-limited biofilms was reported by Huang et al. [31]. Limited phosphorus availability has also been shown to stimulate biofilm development with the phytopathogenic bacterium *Agrobacterium tumefaciens* [32]. These contrasting observations suggest that the effect of phosphate availability on biofilm formation may be strain-dependent and influenced by ecological adaptation or regulatory mechanisms. While some strains may respond to phosphate limitation by enhancing biofilm formation as a survival or nutrient acquisition strategy, others, such as the VW132 strain, may require sufficient phosphate

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availability to support the metabolic activity needed for robust biofilm development. In addition, the ability of VW132 to form biofilms under higher phosphate conditions may have significant biological implications in the rhizosphere. In soil environments, biofilm formation facilitates microbial adhesion to root surfaces and soil particles, thereby enhancing colonisation and persistence under abiotic stresses, such as salinity, drought, or nutrient fluctuations. Moreover, biofilms provide a protective matrix that improves the strain's ability to compete with indigenous soil microbes and tolerate hostile conditions [33]. For phosphate-solubilising bacteria, biofilm development in the rhizosphere supports sustained phosphate solubilisation and exhibits close interaction with root exudates, potentially improving plant nutrient uptake [33]. Therefore, the observed phosphate-responsive biofilm formation by VW132 may contribute to its ecological success and functional efficiency in plant-associated environments.

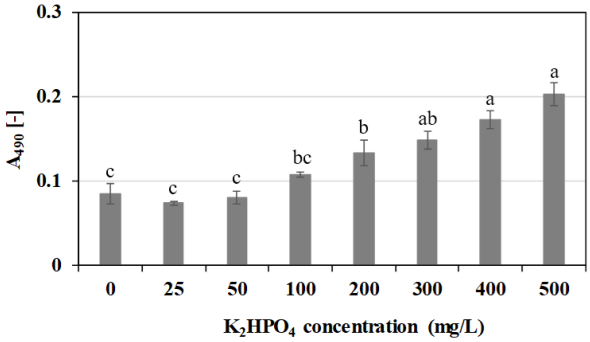

Fig. 4. Biofilm formation index of VW132 strain on plastic surface at different concentrations of K_2HPO_4 (0 to 500 mg/L) (The bars indicate mean values ± SE of three replicates. Different lettering on the bars represents statistically significant differences ($p < 0.05$, Tukey's HSD test).)

4 Conclusion

The challenge of phosphorus deficiency in croplands has led to growing interest in microorganisms that can transform insoluble phosphorus into forms accessible to plants. Forty-two phosphate-solubilising bacterial strains were obtained, among which *Burkholderia* sp., VW132, exhibited the most significant solubilisation ability. Phosphate solubilisation by the VW132 strain reached a maximum of 359 mg/L when cultured within a medium amended with glucose and $(NH_4)_2SO_4$ as the carbon and nitrogen sources, respectively, with 1% NaCl at pH 7. Biofilm formation by this strain at varying concentrations of available phosphate demonstrated a direct correlation between increased phosphate levels in the culture medium and biofilm biomass. Given its performance under mild salinity and origin from nutrient-poor, and metal-rich serpentine soils, strain VW132 holds promise as a biofertiliser candidate for agriculture in nutrient-poor, mildly saline, or metal-stressed soils. Our results lay the groundwork for subsequent greenhouse and field evaluations to verify its efficacy under real-world conditions and further explore its role in promoting sustainable agricultural production in degraded environments.

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