Comparative study on the role of the source concentration on phosphate solubilisation by *Bacillus subtilis* and *Bacillus licheniformis*

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***Abstract*—** The global depletion of natural phosphate fertilizer resources, coupled with the rising demand for sustainable agriculture due to population growth, presents a major challenge in meeting the phosphate requirements for healthy plant development. This study explores a biological alternative by evaluating the phosphate-solubilising potential of two bacterial strains: *Bacillus subtilis* and *Bacillus licheniformis*. The experiment assessed their ability to solubilize phosphate from di-potassium hydrogen phosphate (K_2HPO4) in liquid broth media containing varying concentrations (2%, 3%, 5%, and 10%). Inoculated cultures were incubated at 37°C and 200 rpm for 24 hours in a B.O.D. shaker, followed by analysis of pH changes and phosphate solubilisation. Results indicated that both strains effectively solubilised phosphate at lower concentrations, primarily through acidification of the medium. However, concentrations beyond 3% K_2HPO4 inhibited bacterial growth, thereby limiting the phosphate solubilisation. These findings highlight the potential of these *Bacillus* species as biofertilizer agents and suggest an optimal phosphate concentration range for their effective application in agricultural systems.

***Keywords*—** *Phosphate solubilisation, Bacillus subtilis, Bacillus licheniformis, Phosphatase activity, (K_2HPO4) concentration, Plant growth-promoting bacteria (PGPB)*

I. Introduction

The global phosphorus crisis is intensifying with the rapid increase in population and food demand. This urgency necessitates the exploration of sustainable and environmentally friendly alternatives to chemical phosphate fertilizers. Among such alternatives, phosphate-solubilizing microorganisms (PSMs) have gained increasing attention due to their ability to convert insoluble forms of phosphorus into bioavailable forms through various biochemical processes, especially organic acid production [3].

Bacteria from the genus *Bacillus* are well-known for their robust nature, spore-forming ability, and high adaptability to diverse soil conditions, making them suitable candidates for biofertilizer development. Specifically, *Bacillus subtilis* and *Bacillus licheniformis* have demonstrated significant potential in phosphate solubilisation through acidification of their surrounding media and secretion of organic acids such as gluconic, lactic, and citric acid [4]. Moreover, their beneficial role extends to improving soil fertility and enhancing plant nutrient uptake in sustainable agricultural systems [5].

The phosphate solubilising ability of these bacteria is often associated with a decrease in pH of the surrounding environment, which enhances the dissolution of phosphate compounds. However, the efficiency of this solubilisation can vary depending on factors such as phosphate concentration, medium composition, and bacterial strain. High concentrations of phosphate salts, while increasing solubilisation substrates, may inhibit bacterial growth and activity due to osmotic or ionic stress [6]. Understanding the threshold at which phosphate concentration becomes inhibitory is thus crucial for optimizing the application of microbial inoculants in agriculture.

The present study aims to conduct a comparative evaluation of the phosphate solubilisation potential of *Bacillus subtilis* and *Bacillus licheniformis* using di-potassium hydrogen phosphate (K_2HPO4) as a model substrate at varying concentrations. Furthermore, the relationship between phosphate solubilisation and effect of phosphate source concentration on the solubilizing efficiency of these strains under controlled conditions. The outcomes of this research can contribute to the development of effective and eco-friendly phosphate biofertilizers for sustainable agriculture.

II. Materials and Methods

A. Bacterial Strain

Two phosphate-solubilizing bacterial strains—*Bacillus subtilis, Bacillus licheniformis,* were obtained from the Department of Microbiology culture collection. Pure cultures were maintained on nutrient agar slants at 4 °C and subcultured prior to use.

B. Media Preparation with varying K_2HPO4 Concentrations

A modified minimal salt medium [7] was used, composed of (per liter):

- Glucose – 10.0 g

- $MgSO_4 \cdot 7H_2O$ – 0.5 g
- NaCl – 0.5 g
- $(NH_4)_2SO_4$ – 1.0 g
- Yeast extract – 0.5 g
- Agar (for plates only) – 15.0 g
- K_2HPO4 – 2%, 3%, 5%, and 10% (w/v) as the variable phosphate source

The medium was adjusted to pH 7.0 and sterilized by autoclaving at 121 °C for 15 minutes.

C. *Inoculation and Incubation*

Each bacterial strain was inoculated into 100 mL of sterile broth containing the designated K_2HPO4 concentration (2, 3, 5 and 10%) in 250 mL Erlenmeyer flasks. The cultures were incubated at 30 °C for 28 hours on a rotary shaker at 150 rpm. All treatments were conducted in triplicates.

D. *Growth Measurement*

Bacterial growth was monitored by measuring the optical density at 600 nm (OD) using a UV–Visible spectrophotometer at an interval of 4 hrs for 28 hrs.

E. *Phosphatase Activity Assay*

At the end of incubation (24 h), the culture broth was centrifuged at 10,000 rpm for 10 minutes to obtain a clear supernatant. Acid and alkaline phosphatase activities were determined using p-nitrophenyl phosphate (pNPP) as substrate [8], the assay was performed at pH 9.0. The pH was maintained using glycine-NaOH buffer.

The reaction mixture (1 mL enzyme extract + 1 mL 1 mM pNPP + buffer) was incubated at 37 °C for 30 minutes and stopped by adding 1 mL of 1 N NaOH. The release of p-nitrophenol was measured at 405 nm. Enzyme activity was expressed in Units per mL (U/mL), where one unit is the amount of enzyme releasing 1 µmol of p-nitrophenol per minute.wo phosphate-solubilizing bacterial strains—*Bacillus subtilis*, *Bacillus licheniformis*, were obtained from the Department of Microbiology culture collection. Pure cultures were maintained on nutrient agar slants at 4 °C and subcultured prior to use.

F. *Soluble phosphate estimation*

Soluble phosphate content in the supernatant was estimated using Metavanadate method [9] of phosphorus analysis; the absorbance was measured by spectrophotometer (SHIMADZU, UV-VIS, 1900i) at 420nm.

G. ***Statistical Analysis***

All experimental data were recorded as mean ± standard deviation (SD) of triplicates. One-way ANOVA was performed to determine the significance of variation in growth and enzyme activities among different K_2HPO4 concentrations. Tukey's post hoc test was applied at $\mathbf{p < 0.05}$ for pairwise comparisons using SPSS version 25.

III. Result and discussion

A. *Growth Kinetics at Different K_2HPO4 Concentrations*

The growth pattern of *Bacillus subtilis* and *Bacillus licheniformis* was monitored over 28 hours under four concentrations of K_2HPO4 (2%, 3%, 5%, and 10%). The growth was measured as optical density at 600 nm at regular intervals.

For *Bacillus licheniformis* (Fig. 1) the maximum growth was observed at 3% K_2HPO4, where the OD reached approximately 1.56 by 20 hours. This was followed by 5% (OD ≈ 1.32), 10% (OD ≈ 1.10), and 2% (OD ≈ 1.02). The lag phase was shortest and exponential growth most prominent in 3% phosphate conditions, suggesting optimal utilization and tolerance at this concentration.

For *Bacillus subtilis* (Fig. 2), the highest OD (~1.24) was also recorded at 3% K_2HPO4, with moderate growth at 5% (~1.10) and lower OD values at 2% and 10% (~0.95 and ~0.85, respectively). The strain showed a sharp exponential phase between 4–16 hours, with the stationary phase initiating earlier in higher phosphate concentrations (5% and 10%), possibly due to phosphate-induced feedback inhibition or osmotic stress.

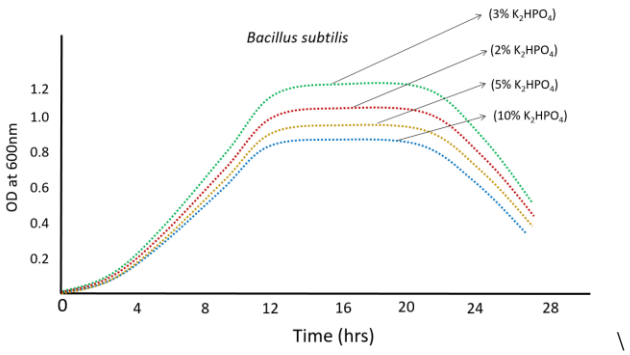

Fig. 1: Growth curve of *Bacillus subtilis* at different concentrations of K_2HPO_4 in the medium

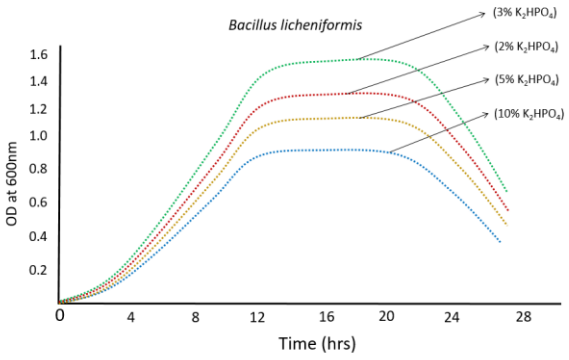

Fig. 2: Growth curve of *Bacillus licheniformis* at different concentrations of K_2HPO_4 in the medium

These results indicate that 3% K_2HPO4 provided optimal phosphate availability and minimal stress conditions for both strains, supporting maximal bacterial proliferation.

B. Alkaline Phosphatase Activity

Alkaline phosphatase activity, assayed at 24 hours post-inoculation, varied significantly across different phosphate concentrations. B. licheniformis consistently exhibited higher alkaline phosphatase activity than *B. subtilis* at all K_2HPO4 levels (Table 1). The highest enzyme activity for both strains was observed at 3% phosphate, indicating that moderate phosphate concentration supports optimal enzyme induction.
For *B. licheniformis*, the highest enzyme activity was observed at 3% K_2HPO4 (7.42 ± 0.28 U/mL), followed by 5% (6.51 ± 0.24 U/mL), 2% (4.85 ± 0.31 U/mL), and the lowest at 10% (3.92 ± 0.19 U/mL). A similar trend was seen in B. subtilis, with maximum activity at 3% (6.38 ± 0.21 U/mL), and reduced activity at both lower and higher phosphate concentrations.

TABLE I. ALKALINE PHOSPHATASE ACTIVITY (U/ML) AT 24 HOURS POST-INOCULATION.

K_2HPO4 Concentration	***B. licheniformis* (U/mL)**	***B. subtilis* (U/mL)**
2%	4.85 ± 0.31	3.74 ± 0.26
3%	**7.42 ± 0.28**	**6.38 ± 0.21**
5%	6.51 ± 0.24	5.42 ± 0.19
10%	3.92 ± 0.19	2.89 ± 0.23

The data suggests that excessive phosphate (10%) likely exerts negative feedback on phosphatase production, while moderate levels (3–5%) induce the highest enzyme synthesis. Lower phosphate (2%) likely limits metabolic activity due to insufficient phosphate availability [10].

C. luble Phosphate Quantification

Soluble phosphate released into the medium was estimated using the metavanadate method. For both strains, phosphate solubilization was highest at 3% K_2HPO4, correlating with growth and phosphatase activity. At 10%, solubilization was significantly lower despite the higher initial phosphate, indicating potential saturation effects or precipitation of excess phosphate as insoluble complexes [11].

IV. Conclusion

The present study highlights the influence of varying concentrations of K_2HPO4 on the growth, alkaline phosphatase activity, and phosphate solubilization efficiency of *Bacillus subtilis* and *Bacillus licheniformis*. Both strains exhibited optimal growth and enzymatic activity at 3% K_2HPO4, indicating that a moderate concentration of phosphate source promotes microbial proliferation and functional efficiency. Notably, *B. licheniformis* demonstrated consistently higher alkaline phosphatase activity and phosphate solubilization than B. subtilis, suggesting its superior adaptability and potential for use in phosphate-rich or slightly alkaline soils.

At higher phosphate concentrations (10%), a significant decline in growth and enzyme activity was observed, possibly due to feedback inhibition or osmotic stress. These findings emphasize the importance of optimizing phosphate levels in bioformulation strategies to maximize microbial performance and enhance sustainable phosphorus management in agriculture.

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