

Multifaceted proficiencies of thermotolerant phosphate solubilizing bacteria from the Kutch desert

Abstract

Aims: This study aimed to isolate and characterize thermotolerant phosphate solubilizing bacteria (PSB) for potential agricultural applications. We aimed to identify promising PSB isolates, and evaluate their traits.

Study Design: Conducted a comprehensive screening of PSB isolates and selected three top candidates based on their phosphate-solubilizing index. These isolates underwent various tests, including thermotolerance assessment, biochemical profiling, and enzymatic assays. Soluble phosphorus and pH levels were monitored over time.

Methodology: Screened multiple PSB isolates, selecting PT26, PT31, and PT35. Conducted optical density measurements, assessed biochemical profiles, and tested antibiotic susceptibility. Also monitored soluble phosphorus and pH over 96 hours and measured acid and alkaline phosphatase activities. Molecular identification was achieved through 16S rRNA gene sequencing.

Results: Among the selected PSB isolates, PT35 showed superior thermotolerance and potassium solubilization capacity, while PT26 exhibited indole-3-acetic acid synthesis ability. Soluble phosphorus levels varied among isolates, affecting pH. Acid and alkaline phosphatase activities differed significantly. Molecular identification confirmed PT26, PT31, and PT35 as *Enterobacter cloacae*, *Klebsiella quasipneumoniae*, and *Enterobacter bugandensis*, respectively.

Conclusion: This study highlights the potential significance of PT35, PT26, and PT31 in agriculture. Their diverse traits and metabolic activities offer adaptability to varying conditions, benefiting soil health and nutrient cycling. These findings contribute to agricultural microbiology research, potentially enhancing sustainable farming practices and soil fertility.

Keywords: PSB, thermotolerance, multitrait, phosphatase

1. Introduction

The pursuit of sustainable agriculture has led to an increasing focus on the role of microorganisms in enhancing soil fertility and crop yield. Among these, thermotolerant phosphate solubilizing bacteria (PSB) have emerged as a critical factor for agricultural sustainability, particularly in challenging environments like arid and semi-arid regions. These bacteria are not just academic curiosities but essential players in the ecosystem, especially in areas with unique climatic conditions, such as the Kutch region in Gujarat [1].

Phosphorus, a vital macronutrient, is indispensable for plant growth and metabolic processes like energy transfer and nutrient uptake. However, the majority of phosphorus in the soil exists in insoluble forms, such as calcium phosphate, iron phosphate, and aluminium phosphate, rendering it inaccessible to plants [2]. Traditional methods to enhance phosphorus availability have relied on chemical fertilizers, which have long-term environmental repercussions [3].

Thermotolerant PSBs offer a sustainable alternative. They are commonly found in the rhizosphere, the soil region surrounding plant roots and possess the unique ability to convert insoluble forms of phosphates into soluble ones. The phosphate solubilization is facilitated by

producing organic acids like gluconic acid and phosphatase enzymes, which lower the soil pH and increase phosphate solubility [4,5]. The significance of thermotolerant PSBs extends beyond phosphorus solubilization. These bacteria are multifunctional; they can fix atmospheric nitrogen into a form that plants can use, thereby reducing the need for synthetic fertilizers. They also synthesize phytohormones, plant growth regulators crucial in various physiological processes like root development and flowering [1,6]. Furthermore, thermotolerant PSBs are non-pathogenic and can be biofertilizers in sustainable agriculture [7].

The resilience to temperature fluctuations sets thermotolerant PSBs apart, making them ideal candidates for biofertilizer formulations targeted for arid regions. Their cellular mechanisms are fine-tuned to withstand temperature stress, ensuring their phosphate solubilizing activity remains unhampered even in extreme conditions. This adaptability is particularly significant for regions where traditional mesophilic PSBs may lose efficacy due to high temperatures. Several genera of bacteria, including *Bacillus*, *Pseudomonas*, *Rhizobium*, *Enterobacter*, and *Burkholderia*, have been identified as potent phosphate solubilizers. These bacteria have been isolated from diverse environments like saline soil, cow dung, and lake sediments, highlighting their adaptability and wide distribution [1,8,9]. Thermotolerant PSBs as biofertilizers can enhance crop yield and soil fertility and have broader ecological implications. By reducing dependency on chemical fertilizers, these bacteria lower soil and water pollution, thereby promoting environmental sustainability. Therefore, understanding the genetics and mechanisms of phosphate solubilization in these bacteria is of great interest in agricultural research and can lead to improved agricultural inoculants and biofertilizers [3,6]. Thermotolerant PSBs offer a multifaceted approach to sustainable agriculture, especially in regions with challenging climatic conditions. Their ability to solubilize phosphorus, fix nitrogen, and synthesize phytohormones makes them invaluable assets in the quest for agricultural sustainability.

2. Materials and methods

The study was conducted in the Department of Microbiology, College of Basic Science and Humanities, Sardarkrushinagar Dantiwada Agricultural University, Sardarkrushinagar, Gujarat, India. The desert soil of the Kutch region of Gujarat, India, was collected in plastic vials. The vials were transported to the laboratory within 24 hours and microbiologically processed.

2.1 Screening of PSB isolates

The PSB were isolated from soil samples by serial dilution and plate count method using Pikovskaya's medium [10], a selective medium for P solubilizers. One gram of rhizosphere or bulk soil was dissolved in 10 mL sterilized distilled water in the test tube and serially diluted [11]. The diluted sample was spread onto Pikovskaya's agar medium and incubated. All the bacterial colonies displaying clear halo around were picked and pure cultured. The phosphate solubilization efficiency (PSE) of isolates was determined.

2.2 Screening of thermotolerance and additional traits of PSB isolates

A systematic temperature gradient experiment was conducted to assess the microbial isolates' thermotolerance rigorously. The isolates were exposed to a series of elevated temperatures—30°C, 45°C, 60°C, and 75°C—for 30 minutes in a calibrated water bath. Subsequently, the treated samples were cultured on nutrient agar plates and incubated at 30°C for 48 hours to evaluate their viability and thermal resilience.

A quantitative assay was also conducted to investigate the thermophilic nature of isolates. An aliquot of 0.1 mL from the overnight culture of each bacterial isolate was inoculated into separate tubes of nutrient broth. These tubes were subsequently incubated at 30°C, 45°C and

60°C for 24 h. Following the incubation period, each culture's optical density (OD) was measured to assess the growth of the bacterial isolates.

The three isolates underwent additional assessment to determine their ability to solubilize potassium using Aleksandrow Agar [12]. We employed a standardized procedure to assess the bacterial strains' potential for solubilizing zinc [13]. Specifically, tris-minimal agar medium was prepared and supplemented with D-glucose and various. Specifically, we prepared a tris-minimal agar medium and supplemented it with D-glucose and various insoluble zinc compounds. The selected zinc compounds included zinc oxide 1.244 g L⁻¹, equivalent to 15.23 mM; zinc phosphate 1.9882 g L⁻¹, equivalent to 5.0 mM; and zinc carbonate at 1.728 g L⁻¹, equivalent to 5.2 mM.

2.3 Characterization and identification of thermotolerant PSB isolates

The biochemical characterization provides a nuanced understanding of their metabolic capabilities, pivotal for tailoring their application in agriculture microbiology. The biochemical characterization was initiated using a HiMedia[®] test kit, specifically designed to identify Gram negative bacteria. The kit was used for distinct tests, including the following: citrate utilization test, lysine utilization, ornithine utilization, urease detection, phenylalanine deamination, nitrate reduction, H₂S production, starch hydrolysis, gelatin hydrolysis, indole production. The 24 hour old cultures of thermotolerant PSB were carefully streaked with an inoculating loop on every section of the HiAssorted™ test kit, followed by incubation at a controlled temperature of 30±1°C for 24 hours. Subsequently, the results of the kit strips were compared with the catalog to ensure accuracy.

In parallel, Petri plates containing phenol red agar medium were prepared and streaked with PSB isolates through spread plating [14]. Different sugar disks were pressed onto the surface of the plates and incubated at 30°C for a period ranging from 24 to 48 hours. Upon the incubation, sugar utilization by the isolates was observed and marked positive with a color change from red to yellow, indicating successful fermentation of the 21 sugars (**Table 1**).

Furthermore, plates containing the Mueller-Hinton agar medium were prepared to assess the antibiotic effect on bacterial growth [15]. The test organisms were streaked thrice onto the entire agar surface of the plate with a cotton swab. After that, the hexa disc of antibiotics was pressed onto the plates, followed by incubation at 30°C for 24 hours. The results were recorded, contributing to the comprehensive characterization of the isolates.

2.4 Measuring PSB mediated bioavailability of inorganic phosphate in growth medium

In our study, we quantified soluble phosphate levels in the supernatant of the growth medium using the Fiske-Subbarow method [16]. The process required several solutions, each carefully prepared to ensure accuracy. We began using double distilled water, devoid of phosphorus, for all our solution preparations.

To prepare a standard phosphorus solution, 340 mg of KH₂PO₄ was dissolved in 100 mL of water, followed by volume adjustment to 250 mL with distilled water, resulting in a solution containing 10 μmoles mL⁻¹ of phosphorus. In addition, 10 N sulfuric acid was prepared by combining 200 mL of 36 N concentrated H₂SO₄ with 520 mL of distilled water.

The 2.5% molybdate solution was prepared by dissolving 25 g of ammonium molybdate in 200 mL of distilled water. Subsequently, the solution was transferred to a flask containing 520 mL of 10 N H₂SO₄, and the final volume was adjusted to 1 L using distilled water. The resulting solution was carefully stored in a brown bottle.

The preparation of the 1-amino-2-naphthol-4-sulfonic acid (ANSA) reagent involved a series of steps, including the dissolution of bisulfite and sodium sulfite, their mixture with ANSA powder, and storage in a refrigerated environment in a brown bottle.

Furthermore, a 10% trichloroacetic acid solution was prepared by dissolving 10 g of trichloroacetic acid in 100 mL of distilled water. For phosphate estimation, aliquots of the standard phosphorus solution were pipetted into a series of tubes and adjusted to a total volume of 1 mL. In each tube, including the blank, we sequentially added 0.4 mL of 10% trichloroacetic acid, 0.4 mL of the ammonium molybdate solution, 0.2 mL of ANSA reagent, and 4 mL of double-distilled water. After thorough mixing, we allowed the tubes to stand for 5 minutes to observe the development of a blue color in the solution.

Subsequently, the solution was transferred into cuvettes, and optical density (OD) was measured at 640 nm. The data allowed us to construct a standard graph, with the concentration of phosphorus on the x-axis and OD on the y-axis, facilitating our quantitative analysis of soluble phosphate levels in the supernatant.

2.5 Quantitification of acidic and alkaline phosphatase from thermotolerant PSB

In the study, we assayed acid and alkaline phosphatase enzymes of PSB isolates for analysis. We first utilized 10 ml of sterilized Pikovskaya broth within a 20 ml sterilized test tube. Subsequently, these tubes were inoculated with the target bacterial isolates and incubated at 37°C for up to 96 hours. At regular intervals of 24 hours, samples were withdrawn and processed by centrifugation at 5000 rpm for 10 minutes at 4°C. The resulting cell-free supernatant was then subjected to an enzymatic assay to determine the activity of crude acid and alkaline phosphatases. The methodology for the assay was adapted from [Bergmeyer, et al. \[17\]](#).

The enzymatic assay assessed the activity of acid and alkaline phosphatases while considering their specific pH preferences, offering insights into their functionality under various physiological conditions. For the acid phosphatase assay, we prepared a reaction mixture of 0.5 mL of 100 mM acetate buffer with a pH of 4.8, supplemented with 0.01M MgCl₂ and 0.1 mL of the enzyme source. Conversely, the alkaline phosphatase assay utilized a reaction mixture containing 0.5 mL of 100 mM glycine-NaOH buffer with a pH of 8.0, enriched with 0.01M MgCl₂, and 0.1 mL of the enzyme source. These mixtures were then distributed into three tubes labeled the test, control, and reagent blank. Subsequently, they were placed in a water bath at a constant temperature of 37°C for pre-equilibration.

Following pre-equilibration, we introduced 0.5 mL of 15.2 mM p-nitrophenyl phosphate (PNP) substrate, which had also been pre-equilibrated at 37°C, into the test tube. The enzymatic reaction was allowed to proceed for 10 minutes before being terminated by adding 4 mL of 0.1N NaOH. In parallel, a zero-minute control was established by introducing the substrate after the quenching agent in the control tube. The reagent blank was treated similarly to the test but with substituting the respective buffer in place of the pNPP substrate. Subsequently, spectrophotometric measurements were conducted at a wavelength of 410 nm and calibrated to 100% transmission using the reagent blank as a reference. The resulting absorbance values for both the control and test tubes were recorded, and the concentration of p-nitrophenol generated during the 10-minute incubation period was quantified using a millimolar extinction coefficient ($\epsilon_{\lambda_{max}}$) of 18.3 mM⁻¹ cm⁻¹.

The acid/alkaline phosphatase activity was calculated as:

$$\text{Units per Lenzyme} = \frac{(A_{410 \text{ nm}} \text{ Test} - A_{410 \text{ nm}} \text{ Control}) \times \text{total volume of assay} \times \text{dilution factor}}{\text{millimolar extinction coefficient of PNP} \times \text{volume of enzyme} \times \text{incubation time (min)}}$$

3. Results and Discussion

3.1 Screening of potent PSB isolates

Out of several PSB isolates screened, the top three phosphate solubilizers were PT26, PT31, and PT35, with a phosphate solubilizing index of 3.12, 2.90, and 2.42, respectively.

3.2 Screening of thermotolerance and additional traits PSB isolates

It is imperative to underscore the thermotolerant attributes of the bacterial isolates PT35, PT26, and PT31, as corroborated by their ability to endure a temperature of 75°C for 30 minutes. Thermotolerance is a pivotal trait that could significantly affect their applicability in biotechnological interventions within agriculture microbiology.

To determine if the isolates were thermophilic, they were cultured for 24 hours at temperatures of 30, 45, and 60 °C. Remarkably, all isolates—PT26, PT31, and PT35—exhibit peak optical densities at the lower end of the temperature spectrum, precisely at 30°C. PT26 and PT31 register optical densities of 0.795 and 0.824, respectively. This data is a pivotal reference point, establishing 30°C as the most conducive temperature for metabolic activity and growth, thereby setting the standard for subsequent thermotolerance assessments. Specifically, PT35 displays a notable optical density of 0.74 when subjected to a temperature of 45°C. While this might superficially imply enhanced thermotolerance, it is imperative to situate this finding within the larger experimental framework. As the temperature escalates to 60°C, a discernible decline in optical density is observed across the isolates, underscoring the inverse relationship between elevated temperature and optical density. Specifically, PT26 and PT35 experience a drastic reduction in optical density, plummeting to values of 0.21 and 0.182, respectively. This marked decrease indicates suppressed metabolic activity under hyperthermal conditions, effectively dispelling any assumptions of thermophilic tendencies. In stark contrast, PT31 demonstrates exceptional thermal resilience, maintaining a nearly invariant optical density with readings of 0.295 at 45°C and 0.293 at 60°C. This relative stability across a broad temperature range may suggest a more expansive thermotolerance spectrum, thereby meriting further in-depth research.

While the isolates exhibit thermotolerance, it is salient to demarcate them from thermophiles, as their metabolic activity is compromised at elevated temperatures. This nuanced understanding is vital for accurately characterizing these bacterial isolates and elucidating their potential roles in phosphate solubilization under varying thermal conditions.

The isolated thermotolerant PSB exhibited distinct additional traits. Specifically, PT31 demonstrates the capacity to solubilize potassium with a notable K solubilizing index of 1.09. In addition, PT26 showcases its ability to synthesize indole-3-acetic acid (IAA) from tryptophan. This is substantiated by RF values of 0.53 for the standard and 0.46 for the sample on thin-layer chromatography (TLC) plates. Notably, these outcomes align seamlessly with prior research conducted by [Thomas, et al. \[18\]](#), who utilized analogous methodologies, including TLC and colorimetry, to discern and quantify IAA production in bacteria isolated from soil samples. These findings underscore the multifaceted capabilities of these thermotolerant phosphate solubilizing bacteria, shedding light on their potential significance in various ecological and agricultural contexts.

3.3 Characterization and identification of thermotolerant PSB isolates

The biochemical properties of bacterial isolates PT26, PT31, and PT35 can be analyzed in detail based on the data provided in Table 1.

The isolate PT26 utilized citrate and reduced nitrate but was negative for all other biochemical tests, including lysine utilization and urease detection. In an evaluation of carbohydrate metabolism, isolate PT26 metabolized a specific array of sugars, including arabinose, dextrose, fructose, galactose, melibiose, maltose, mannitol, mannose, raffinose, rhamnose, sucrose, and glucose. Conversely, it exhibited metabolic inactivity towards adonitol, cellobiose, dulcitol, inositol, inulin, lactose, sorbitol, trehalose, and xylulose.

The isolate PT31 was metabolically inactive in citrate utilization, nitrate reduction, and all other assessed biochemical assays. However, it displayed a broad metabolic versatility in

sugar utilization, effectively metabolizing adonitol, arabinose, dextrose, fructose, galactose, inositol, lactose, melibiose, maltose, mannitol, mannose, raffinose, rhamnose, sorbitol, sucrose, trehalose, and glucose. It did not, however, metabolize cellobiose, dulcitol, inulin, and xylulose.

In contrast, isolate PT35 was metabolically active in citrate utilization and nitrate reduction, akin to PT26. It also remained metabolically inactive across other biochemical assays. Concerning carbohydrate metabolism, PT35 metabolized arabinose, dextrose, fructose, galactose, dulcitol, inulin, lactose, melibiose, maltose, mannitol, mannose, raffinose, rhamnose, sucrose, glucose, while showing metabolic inactivity towards adonitol, cellobiose, inositol, sorbitol, trehalose, and xylulose.

Upon comparative analysis, PT26 and PT35 were metabolically active in citrate utilization and nitrate reduction, whereas PT31 was metabolically inactive. PT26 displayed a narrower metabolic spectrum in sugar utilization compared to PT31 and PT35. PT31 and PT35 exhibited similar metabolic versatility but diverged in their ability to metabolize specific sugars such as adonitol and dulcitol. All three isolates had general metabolic inactivity towards amino acid utilization and other biochemical assays, including urease and H₂S production.

Overall, PT26 specializes in metabolizing a specific subset of carbohydrates and is proficient in citrate utilization and nitrate reduction. PT31, while lacking in citrate and nitrate metabolism, compensates with a broader spectrum of sugar utilization. PT35 demonstrates a metabolic profile similar to PT31 regarding sugar utilization and additionally possesses the capability for citrate utilization and nitrate reduction, rendering it the most metabolically versatile among the three isolates.

Antibiotic susceptibility tests revealed that all three isolates were most susceptible to 30 µg levofloxacin-containing disks, which produced the highest zones of inhibition. In comparison, 30 µg disks of amikacin and cefotaxime yielded slightly lower inhibition zones. Moderate inhibitory effects were observed with 10 µg imipenem and 30 µg ceftazidime disks across all isolates. Interestingly, 30 µg aztreonam disks manifested moderate inhibition zones with isolates P31 and P35, but showed the least efficacy against isolate P26. These findings collectively contribute to a comprehensive understanding of these thermotolerant PSB isolates' biochemical and antibiotic susceptibility profiles, thereby providing valuable insights for their potential applications in agriculture microbiology.

3.4 PSB mediated bioavailability of inorganic phosphate in growth medium

In studying soluble phosphorus concentrations and pH levels in nutrient broth across three bacterial isolates, distinct patterns emerge over 96 hours (**Fig. 2**). All isolates demonstrate increased soluble phosphorus concentrations, albeit at varying rates. PT26 shows a moderate increase from 1.8 to 2.6 µ moles mL⁻¹, PT31 exhibits the slowest rate of increase, moving from 1.5 to 2 µM/ml, and PT35 records the fastest increase, from 1.7 to 2.7 µ moles mL⁻¹.

Concomitantly, all bacterial isolates manifest a decrease in pH levels, indicating increased acidity of the nutrient broth. PT26 starts at a pH of 6.89 and experiences a substantial decline to 4.09, particularly after 48 hours. PT31 begins at a pH of 7.1 and drops to 4.84, showing a moderate rate of decrease. PT35, starting at a higher initial pH of 7.7, ends at 4.79, almost aligning with PT31 despite its faster soluble P increase.

Upon juxtaposing the data from both graphs, it becomes evident that the isolates with moderate to high increases in soluble P (PT26 and PT35) also exhibit significant drops in pH, which suggests that these isolates may produce more acidic byproducts as they metabolize

phosphorus. On the other hand, PT31, which has the slowest increase in soluble P, shows a moderate decrease in pH, indicating less acidic byproduct formation compared to PT26 and PT35.

The observed trends in soluble P and pH levels could have implications for understanding these bacterial isolates' metabolic pathways and ecological roles. The data suggest that PT26 and PT35 may be more efficient in phosphorus uptake and produce more acidic metabolites, whereas PT31 appears less aggressive in phosphorus metabolism and acid production.

3.5 Acid and alkaline phosphatase activity of the thermotolerant PSB isolates

In the study of bacterial isolates PT26, PT31, and PT35, distinct patterns of enzymatic activity were observed for both acid and alkaline phosphatases over 96 hours (**Fig. 3**). PT26 showed a significant increase in acid phosphatase activity, starting at 5.06 UnitsL⁻¹ and peaking at 26.9 Units per liter, suggesting that PT26 might prefer acidic environments or that the acid phosphatase enzyme plays a crucial role in its metabolic processes. In contrast, its alkaline phosphatase activity started at 2.22 UnitsL⁻¹ and peaked at a lower value of 5.15 UnitsL⁻¹.

PT31 exhibited the highest peak in acid phosphatase activity, starting at a mere 1.1 UnitsL⁻¹ and reaching a remarkable 28.41 UnitsL⁻¹ in 96 hours, which could indicate that PT31 is the most metabolically active among the isolates, particularly in acidic conditions. Its alkaline phosphatase activity also showed a significant increase, starting at 1.18 UnitsL⁻¹ and peaking at 7.56 UnitsL⁻¹, the highest among the alkaline phosphatase readings.

PT35 started with relatively high enzyme activities for acid and alkaline phosphatases, at 11.7 and 5.32 UnitsL⁻¹, respectively. However, both enzymes showed a plateauing trend after 72 hours, with acid phosphatase peaking at 26.3 UnitsL⁻¹ and alkaline phosphatase at 6.06 Units L⁻¹, which could suggest that PT35 is well-adapted to its environment from the outset and may not require significant metabolic changes over time.

The intricate enzymatic behaviors exhibited by distinct bacterial isolates contribute to our understanding of soil health and nutrient cycling and serve as a cornerstone for advancing research in agricultural microbiology. Specifically, the study delves into the metabolic adaptability of these bacterial isolates under varying environmental conditions, thereby enriching the existing body of literature.

One of the pivotal findings of the study pertains to the expression of acid phosphatases, a class of specialized enzymes that facilitate the hydrolysis of phosphorus esters in acidic milieus. These enzymes are predominantly found in soil bacteria belonging to genera such as *Rhizobium*, *Enterobacter*, and *Pseudomonas* [19]. While acid phosphatases do not directly affect inorganic phosphorus, their enzymatic activity influences the culture medium's pH. It is achieved through a process known as dephosphorylation, which generates organic acids [19]. These observations align with prior research that has documented variable levels of acidic phosphatase activity across different bacterial isolates [20,21].

In addition to acid phosphatases, the study also sheds light on the heterogeneity observed in the production of alkaline phosphatases. This variability can be ascribed to factors such as the ambient pH and the accumulation of specific secondary metabolites. Notably, a direct correlation has been established between the activity of alkaline phosphatases and incremental shifts in pH [22,23].

Contextualizing these findings within a broader research landscape, the study corroborates antecedent research that reported a higher prevalence of alkaline phosphatase activity in *Enterobacter* sp. compared to acid phosphatase activity [21]. In contrast, an inverse relationship was observed in *Bacillus* species, where acid phosphatase activity was more prevalent [24].

The study comprehensively explores the multifactorial determinants influencing enzymatic activities in bacterial isolates. It augments our current understanding and provides a robust framework for future research endeavors in microbial enzymology. The implications of these findings transcend academic boundaries, offering invaluable insights into the complex interplay between environmental variables and microbial enzymatic functions. Therefore, the study holds significant potential for shaping future research trajectories in agricultural microbiology.

3.6 Molecular identification of isolates

Utilizing 16S rRNA gene sequencing, the bacterial isolates designated as PT26, PT31, and PT35 were taxonomically characterized. The partial sequences were submitted to NCBI database, and the accession numbers were obtained. The isolate PT26 was identified as *Enterobacter cloacae* with the accession number MW857282, PT31 was found to be *Klebsiella quasipneumoniae* under the accession number MW857283, and isolate PT35 was determined to be *Enterobacter bugandensis* with the accession number MW857284.

4. Summary

The study presents a comprehensive analysis of three thermotolerant phosphate-solubilizing bacterial (PSB) isolates—PT26, PT31, and PT35—focusing on their phosphate solubilizing indices, thermotolerance, additional traits, biochemical profiles, and enzymatic activities. PT35 exhibited superior thermotolerance, while PT31 showed a broader temperature tolerance range and the ability to solubilize potassium. PT26 was notable for its synthesis of indole-3-acetic acid (IAA). All isolates were Gram-negative, nonmotile, and non-endospore-forming, with distinct biochemical profiles. PT26 and P35 were proficient in nitrate reduction, an essential trait for soil nitrogen cycling.

The study also delved into the soluble phosphorus concentrations and pH variations in nutrient broth for these isolates. PT35 showed the fastest increase in soluble phosphorus concentrations, while PT26 and PT35 produced more acidic byproducts. Enzymatic activity analysis revealed distinct patterns for acid and alkaline phosphatases, contributing to our understanding of soil health and nutrient cycling. Molecular identification was achieved through 16S rRNA gene sequencing, identifying PT26 as *Enterobacter cloacae*, PT31 as *Klebsiella quasipneumoniae*, and PT35 as *Enterobacter bugandensis*.

The findings offer a robust framework for understanding the metabolic adaptability of these bacterial isolates under varying environmental conditions, thereby enriching the existing body of literature in agricultural microbiology.

5. Conclusion

The study successfully identifies and characterizes three thermotolerant phosphate-solubilizing bacterial isolates—PT26, PT31, and PT35—regarding their phosphate solubilizing capabilities, thermotolerance, additional traits, and biochemical profiles. These isolates demonstrate distinct phosphate solubilization and temperature tolerance abilities and exhibit unique enzymatic activities and metabolic traits. PT35 stands out for its thermotolerance, PT31 for its broad temperature range and potassium solubilizing ability, and PT26 for its synthesis of indole-3-acetic acid. Their distinct biochemical profiles and enzymatic activities offer valuable insights into their potential roles in soil health and nutrient cycling.

Molecular identification through 16S rRNA gene sequencing further validates the uniqueness of each isolate, categorizing PT26 as *Enterobacter cloacae*, PT31 as *Klebsiella quasipneumoniae*, and PT35 as *Enterobacter bugandensis*. The study provides a comprehensive understanding of these isolates' metabolic adaptability and ecological roles,

laying a robust foundation for future research in agricultural microbiology. The findings have significant implications for enhancing soil fertility and plant growth, making them highly relevant for sustainable agricultural practices.

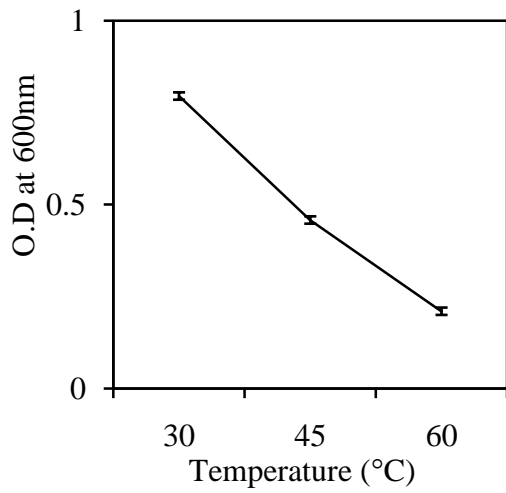
6. Future prospects

The study opens up several avenues for future research in agricultural microbiology, particularly in sustainable agriculture. Given the unique capabilities of the identified bacterial isolates in phosphate solubilization, thermotolerance, and additional traits like potassium solubilization and indole-3-acetic acid synthesis, these isolates could be further explored for their potential in biofertilizer formulations. Their distinct enzymatic activities also warrant deeper investigation into their roles in soil health and nutrient cycling. Moreover, the study's findings on the isolates' biochemical profiles and molecular identification offer a starting point for targeted genetic engineering to enhance specific traits, such as thermotolerance or phosphate solubilizing efficiency. The isolates' antibiotic susceptibility profiles could also be leveraged to develop microbial solutions compatible with existing agricultural practices. The study sets the stage for translational research to harness these bacterial isolates for real-world applications to enhance soil fertility, improve crop yield, and contribute to sustainable agricultural ecosystems.

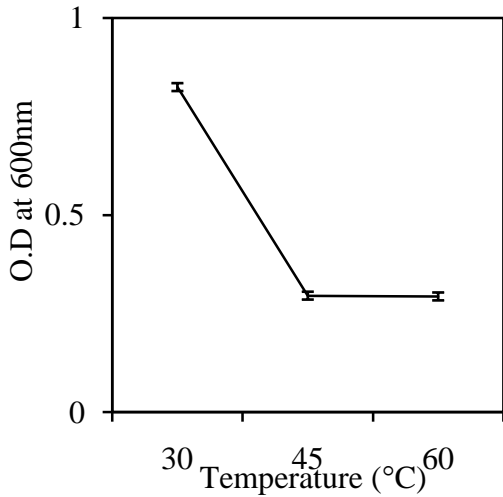
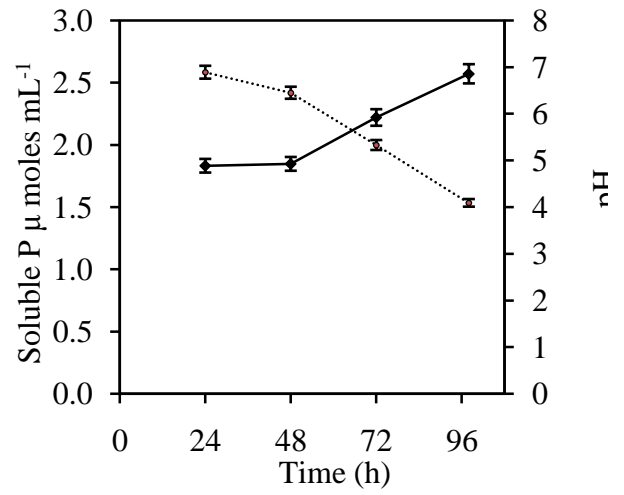
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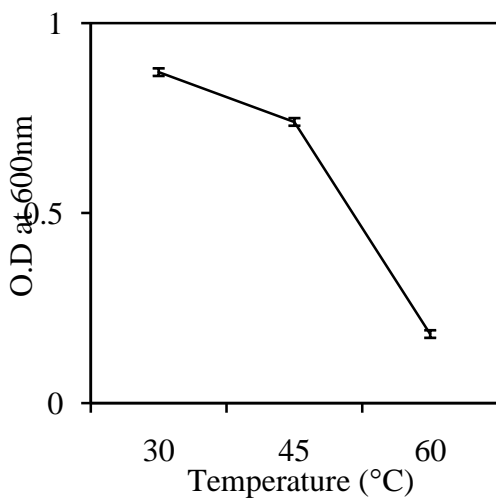
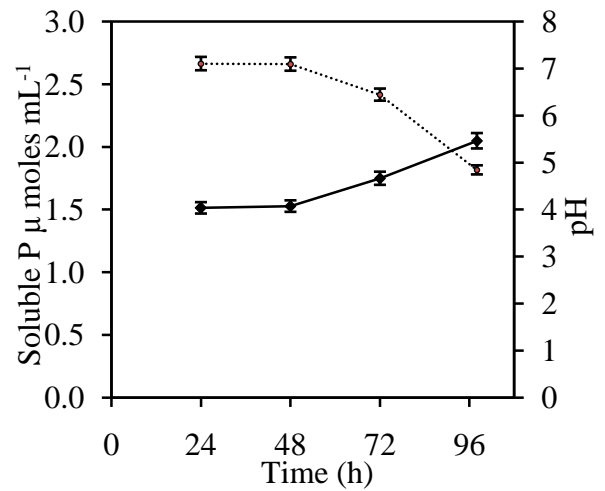
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PT26



PT31



PT35

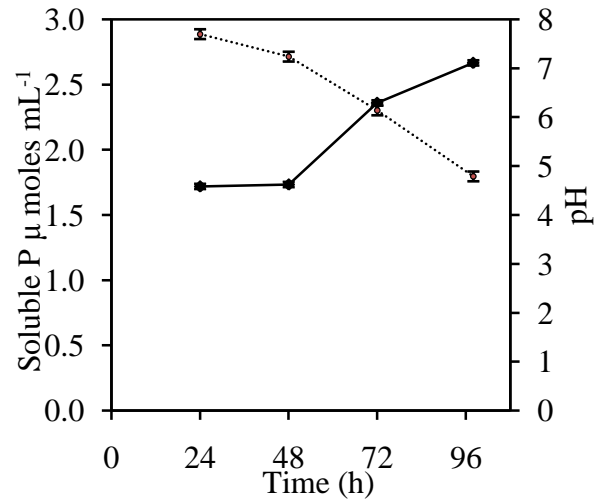


Fig. 1: Growth of three thermotolerant PSB on nutrient broth subjected to elevated temperatures

*-Each value is mean of three replicates

α -Vertical bars represent SEM

Fig. 2: Released Pi and pH change in Pikovskaya's broth containing three thermotolerant PSB isolate

*-Each value is mean of three replicates

α -Vertical bars represent SEM, Pi- inorganic phosphate

Pi pH

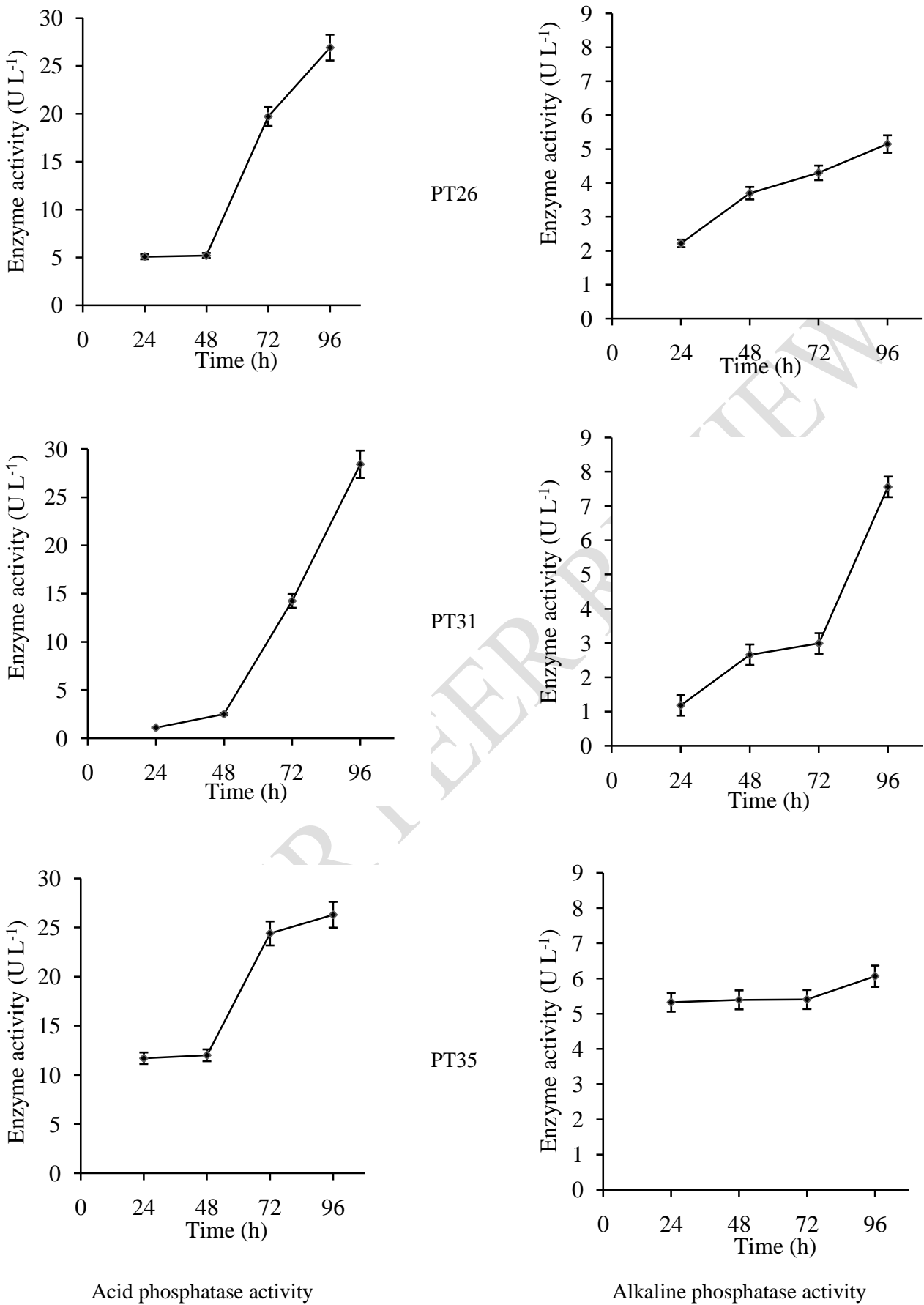


Fig. 3: Acid and alkaline phosphate activity of three thermotolerant PSB isolates*
 *Vertical bars represent SEM

Table 1: The biochemical characterization of thermotolerant PSB isolates

Characteristics	S. No.	Biochemical test	Isolate		
			P26	P31	P35
Biochemical tests through HiAssorted™ biochemical test kit	1.	citrate utilization test	+	-	+
	2.	lysine utilization	-	-	-
	3.	ornithine utilization	-	-	-
	4.	urease detection	-	-	-
	5.	phenylalanine deamination	-	-	-
	6.	nitrate reduction	+	-	+
	7.	H ₂ S production	-	-	-
	8.	starch hydrolysis	-	-	-
	9.	gelatin hydrolysis	-	-	-
	10.	indole production	-	-	-
Sugars utilization tests	11.	adonitol	-	+	-
	12.	arabinose	+	+	+
	13.	cellobiose	-	+	-
	14.	dextrose	+	+	+
	15.	dulcitol	-	-	+
	16.	fructose	+	+	+
	17.	galactose	+	+	+
	18.	inositol	-	+	+
	19.	inulin	-	-	+
	20.	lactose	-	+	+
	21.	melibiose	+	+	+
	22.	maltose	+	+	+
	23.	mannitol	+	+	+
	24.	mannose	+	+	+
	25.	raffinose	+	+	+
	26.	rhamnose	+	+	+
	27.	sorbitol	-	+	-
	28.	sucrose	+	+	+
	29.	trehalose	-	+	+
	30.	xylulose	+	+	+
	31.	glucose	+	+	+
Antibiotic sensitivity test	Antibiotics with concentrations		Inhibition zone in mm		
	32.	amikacin 30 µg ⁻¹	28	24	22
	33.	imipenem 10 µg ⁻¹	18	20	15
	34.	ceftazidime 30 µg ⁻¹	12	13	12
	35.	cefotaxime 30 µg ⁻¹	26	26	24
	36.	levofloxacin 5 µg ⁻¹	36	37	32
37.	aztreonam 30 µg ⁻¹	10	19	14	