1 *Original Research Article*

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3 Multifaceted proficiencies of thermotolerant 4 phosphate solubilizing bacteria from the Kutch 5 desert

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11 **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.

12
13 *Keywords: PSB, thermotolerance, multitrait, phosphatase*
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16 1. INTRODUCTION

17

18 The pursuit of sustainable agriculture has led to an increasing focus on the role of microorganisms in enhancing soil
19 fertility and crop yield. Among these, thermotolerant phosphate solubilizing bacteria (PSB) have emerged as a critical
20 factor for agricultural sustainability, particularly in challenging environments like arid and semi-arid regions. These bacteria
21 are not just academic curiosities but essential players in the ecosystem, especially in areas with unique climatic
22 conditions, such as the Kutch region in Gujarat [1].
23 Phosphorus, a vital macronutrient, is indispensable for plant growth and metabolic processes like energy transfer and
24 nutrient uptake. However, the majority of phosphorus in the soil exists in insoluble forms, such as calcium phosphate, iron
25 phosphate, and aluminum phosphate, rendering it inaccessible to plants [2,3]. Traditional methods to enhance
26 phosphorus availability have relied on chemical fertilizers, which have long-term environmental repercussions [4,5].
27 Thermotolerant PSBs offer a sustainable alternative to enhance phosphorus availability in the earth's tropical and warm
28 temperate zones. They are commonly found in the rhizosphere, the soil region surrounding plant roots and possess the
29 unique ability to convert insoluble forms of phosphates into soluble ones. The phosphate solubilization is facilitated by
30 producing organic acids like gluconic acid and phosphatase enzymes, which lower the soil pH and increase phosphate
31 solubility [6-8]. The significance of thermotolerant PSBs extends beyond phosphorus solubilization. These bacteria are
32 multifunctional; they can fix atmospheric nitrogen into a form that plants can use, thereby reducing the need for synthetic
33 fertilizers. They also synthesize phytohormones, plant growth regulators crucial in various physiological processes like

root development and flowering [1,9]. Furthermore, thermotolerant PSBs are non-pathogenic and can be biofertilizers in sustainable agriculture [10,11].
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 [1,12,13]. Thermotolerant PSBs as biofertilizers can enhance crop yield and soil fertility and have broader ecological implications [14]. 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 [4,9]. 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. MATERIAL AND METHODS

2.1 Screening of PSB isolates

The PSB were isolated from soil samples by serial dilution and plate count method using Pikovskaya's medium [15], a selective medium for P solubilizers. One gram of soil was dissolved in 10 mL sterilized distilled water in the test tube and serially diluted [16]. 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 index (PSI) 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. 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 seeded 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 [17]. We employed a standardized procedure to assess the bacterial strains' potential for solubilizing zinc [18]. 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, H_2S 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 [19]. Different sugar disks were pressed onto the surface of the plates and incubated at 30°C for 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**).

89 Furthermore, plates containing the Mueller-Hinton agar medium were prepared to assess the antibiotic effect on bacterial
90 growth [20]. The test organisms were streaked thrice onto the entire agar surface of the plate with a cotton swab. After
91 that, the hexa disc of antibiotics was pressed onto the plates, followed by incubation at 30°C for 24 hours. The results
92 were recorded, contributing to the comprehensive characterization of the isolates.
93

94 2.4 Measuring PSB mediated bioavailability of inorganic phosphate in growth medium

95 In our study, we quantified soluble phosphate levels in the supernatant of the growth medium using the Fiske-Subbarow
96 method [21]. The process required several solutions, each carefully prepared to ensure accuracy. We began using double
97 distilled water, devoid of phosphorus, for all our solution preparations.
98 To prepare a standard phosphorus solution, 340 mg of KH_2PO_4 was dissolved in 100 mL of water, followed by volume
99 adjustment to 250 mL with distilled water, resulting in a solution containing 10 µmoles mL^{-1} of phosphorus. In addition, 10
100 N sulfuric acid was prepared by combining 200 mL of 36 N concentrated H_2SO_4 with 520 mL of distilled water.
101 The 2.5% molybdate solution was prepared by dissolving 25 g of ammonium molybdate in 200 mL of distilled water.
102 Subsequently, the solution was transferred to a flask containing 520 mL of 10 N H_2SO_4, and the final volume was adjusted
103 to 1 L using distilled water. The resulting solution was carefully stored in a brown bottle.
104 The preparation of the 1-amino-2-napthol-4-sulfonic acid (ANSA) reagent involved a series of steps, including the
105 dissolution of bisulfite and sodium sulfite, their mixture with ANSA powder, and storage in a refrigerated environment in a
106 brown bottle.
107 Furthermore, a 10% trichloroacetic acid solution was prepared by dissolving 10 g of trichloroacetic acid in 100 mL of
108 distilled water. For phosphate estimation, aliquots of the standard phosphorus solution were pipetted into a series of tubes
109 and adjusted to a total volume of 1 mL. In each tube, including the blank, we sequentially added 0.4 mL of 10%
110 trichloroacetic acid, 0.4 mL of the ammonium molybdate solution, 0.2 mL of ANSA reagent, and 4 mL of double-distilled
111 water. After thorough mixing, we allowed the tubes to stand for 5 minutes to observe the development of a blue color in
112 the solution.
113 Subsequently, the solution was transferred into cuvettes, and optical density (OD) was measured at 640 nm. The data
114 allowed us to construct a standard graph, with the concentration of phosphorus on the x-axis and OD on the y-axis,
115 facilitating our quantitative analysis of soluble phosphate levels in the supernatant.
116

117 2.5 Quantification of acidic and alkaline phosphatase from thermotolerant PSB

118 In the study, we assayed acid and alkaline phosphatase enzymes of PSB isolates for analysis. We first utilized 10 ml of
119 sterilized Pikovskaya broth within a 20 ml sterilized test tube. Subsequently, these tubes were inoculated with the target
120 bacterial isolates and incubated at 37°C for up to 96 hours. At regular intervals of 24 hours, samples were withdrawn and
121 processed by centrifugation at 5000 rpm for 10 minutes at 4°C. The resulting cell-free supernatant was then subjected to
122 an enzymatic assay to determine the activity of crude acid and alkaline phosphatases. The methodology for the assay
123 was adapted from Bergmeyer, et al. [22].
124 The enzymatic assay assessed the activity of acid and alkaline phosphatases while considering their specific pH
125 preferences, offering insights into their functionality under various physiological conditions. For the acid phosphatase
126 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
127 $MgCl_2$ and 0.1 mL of the enzyme source. Conversely, the alkaline phosphatase assay utilized a reaction mixture
128 containing 0.5 mL of 100 mM glycine-NaOH buffer with a pH of 8.0, enriched with 0.01M $MgCl_2$, and 0.1 mL of the
129 enzyme source. These mixtures were then distributed into three tubes labeled the test, control, and reagent blank.
130 Subsequently, they were placed in a water bath at a constant temperature of 37°C for pre-equilibration.
131 Following pre-equilibration, we introduced 0.5 mL of 15.2 mM p-nitrophenyl phosphate (PNP) substrate, which had also
132 been pre-equilibrated at 37°C, into the test tube. The enzymatic reaction was allowed to proceed for 10 minutes before
133 being terminated by adding 4 mL of 0.1N NaOH. In parallel, a zero-minute control was established by introducing the
134 substrate after the quenching agent in the control tube. The reagent blank was treated similarly to the test but by
135 substituting the respective buffer with the PNP substrate. Subsequently, spectrophotometric measurements were
136 conducted at a wavelength of 410 nm and calibrated to 100% transmission using the reagent blank as a reference. The
137 resulting absorbance values for both the control and test tubes were recorded, and the concentration of p-nitrophenol
138 generated during the 10 minute incubation period was quantified using a millimolar extinction coefficient ($\varepsilon_{\lambda max}$) of 18.3
139 mM^{-1} cm^{-1}.
140 The acid/alkaline phosphatase activity was calculated as:

$$Enzyme\ Units\ L^{-1} = \frac{(A_{410\ nm}\ \text{Test} - A_{410\ nm}\ \text{Control})\ \text{x total volume of assay x dilution factor}}{milli\ molar\ extinction\ coefficient\ of\ \ PNP\ x\ volume\ of\ enzyme\ x\ incubation\ time\ (min)}$$

141 This equation calculates the enzyme activity (acid or alkaline phosphatase) in a sample based on the change in
142 absorbance at 410 nm due to the conversion of the substrate (PNP) into a product. The formula considers factors such as
143 the difference in absorbance between the test and control samples, the volume of the assay, dilution factor, extinction

144 coefficient of PNP, the volume of enzyme, and incubation time. It quantitatively measures enzyme activity, usually
145 expressed as units (U) L^{-1} enzyme, essential for various biochemical and microbiological studies.
146
147
148 ## 3. RESULTS AND DISCUSSION
149
150 ### 3.1 Screening of potent PSB isolates

151 Out of several PSB isolates screened, the top three phosphate solubilizers were PT26, PT31, and PT35, with a phosphate
152 solubilizing index of 3.12, 2.90, and 2.42, respectively.
153

154 ### 3.2 Screening of thermotolerance and additional traits PSB isolates

155 It is imperative to underscore the thermotolerant attributes of the bacterial isolates PT35, PT26, and PT31, as
156 corroborated by their ability to endure a temperature of 75°C for 30 minutes. Thermotolerance is a pivotal bacterial trait
157 that could significantly affect their applicability in biotechnological interventions within agriculture microbiology [23,24].
158 To ascertain whether the isolates exhibited thermophilic characteristics, they were cultured at 30°C, 45°C, and 60°C for
159 24 hours. Remarkably, all isolates—PT26, PT31, and PT35—exhibit peak optical densities at the lower end of the
160 temperature spectrum, precisely at 30°C. PT26 and PT31 register optical densities of 0.795 and 0.824, respectively. This
161 data is a pivotal reference point, establishing 30°C as the most conducive temperature for metabolic activity and growth,
162 thereby setting the standard for subsequent thermotolerance assessments. Specifically, PT35 displays a notable optical
163 density of 0.74 when subjected to a temperature of 45°C. While this might superficially imply enhanced thermotolerance, it
164 is imperative to situate this finding within the larger experimental framework. As the temperature escalates to 60°C, a
165 discernible decline in optical density is observed across the isolates, underscoring the inverse relationship between
166 elevated temperature and optical density. Specifically, PT26 and PT35 experience a drastic reduction in optical density,
167 plummeting to values of 0.21 and 0.182, respectively. This marked decrease indicates suppressed metabolic activity
168 under hyperthermal conditions, effectively dispelling any assumptions of thermophilic tendencies. In stark contrast, PT31
169 demonstrates exceptional thermal resilience, maintaining a nearly invariant optical density with readings of 0.295 at 45°C
170 and 0.293 at 60°C. This relative stability across a broad temperature range may suggest a more expansive
171 thermotolerance spectrum, thereby meriting further in-depth research.
172 While the isolates exhibit thermotolerance, it is salient to demarcate them from thermophiles, as their metabolic activity is
173 compromised at elevated temperatures. This nuanced understanding is vital for accurately characterizing these bacterial
174 isolates and elucidating their potential roles in phosphate solubilization under varying thermal conditions.
175 The isolated thermotolerant PSB exhibited distinct additional traits. Specifically, PT31 demonstrates the capacity to
176 solubilize potassium with a notable K solubilizing index of 1.09. In addition, PT26 showcases its ability to synthesize
177 indole-3-acetic acid (IAA) from tryptophan. This is substantiated by RF values of 0.53 for the standard and 0.46 for the
178 sample on thin-layer chromatography (TLC) plates. Notably, these outcomes align seamlessly with research conducted by
179 Thomas, et al. [25], who utilized analogous methodologies, including TLC and colorimetry, to discern and quantify IAA
180 production in bacteria isolated from soil samples. These findings underscore the multifaceted capabilities of these
181 thermotolerant PSBs, shedding light on their potential significance in various ecological and agricultural contexts.
182

183 ### 3.3 Characterization and identification of thermotolerant PSB isolates

184 The biochemical properties of bacterial isolates PT26, PT31, and PT35 can be analyzed in detail based on the data
185 provided in Table 1.
186 The isolate PT26 utilized citrate and reduced nitrate but was negative for all other biochemical tests, including lysine
187 utilization and urease detection. In an evaluation of carbohydrate metabolism, isolate PT26 metabolized a specific array of
188 sugars, including arabinose, dextrose, fructose, galactose, melibiose, maltose, mannitol, mannose, raffinose, rhamnose,
189 sucrose, and glucose. Conversely, it exhibited metabolic inactivity towards adonitol, cellobiose, dulcitol, inositol, inulin,
190 lactose, sorbitol, trehalose, and xylulose.
191 The isolate PT31 was metabolically inactive in citrate utilization, nitrate reduction, and all other assessed biochemical
192 assays. However, it displayed a broad metabolic versatility in sugar utilization, effectively metabolizing adonitol,
193 arabinose, dextrose, fructose, galactose, inositol, lactose, melibiose, maltose, mannitol, mannose, raffinose, rhamnose,
194 sorbitol, sucrose, trehalose, and glucose. It did not, however, metabolize cellobiose, dulcitol, inulin, and xylulose.
195 In contrast, isolate PT35 was metabolically active in citrate utilization and nitrate reduction, akin to PT26. It also remained
196 metabolically inactive across other biochemical assays. Concerning carbohydrate metabolism, PT35 metabolized
197 arabinose, dextrose, fructose, galactose, dulcitol, inulin, lactose, melibiose, maltose, mannitol, mannose, raffinose,

198 rhamnose, sucrose, glucose, while showing metabolic inactivity towards adonitol, cellobiose, inositol, sorbitol, trehalose,
199 and xylulose.
200 Upon comparative analysis, PT26 and PT35 were metabolically active in citrate utilization and nitrate reduction, whereas
201 PT31 was metabolically inactive. PT26 displayed a narrower metabolic spectrum in sugar utilization compared to PT31
202 and PT35. PT31 and PT35 exhibited similar metabolic versatility but diverged in their ability to metabolize specific sugars
203 such as adonitol and dulcitol. All three isolates had general metabolic inactivity towards amino acid utilization and other
204 biochemical assays, including urease and H_2S production.
205 Overall, PT26 specializes in metabolizing a specific subset of carbohydrates and is proficient in citrate utilization and
206 nitrate reduction. PT31, while lacking in citrate and nitrate metabolism, compensates with a broader spectrum of sugar
207 utilization. PT35 demonstrates a metabolic profile similar to PT31 regarding sugar utilization and additionally possesses
208 the capability for citrate utilization and nitrate reduction, rendering it the most metabolically versatile among the three
209 isolates.
210 Antibiotic susceptibility tests revealed that all three isolates were most susceptible to 30 µg levofloxacin-containing disks,
211 which produced the highest zones of inhibition. In comparison, 30 µg disks of amikacin and cefotaxime yielded slightly
212 lower inhibition zones. Moderate inhibitory effects were observed with 10 µg imipenem and 30 µg ceftazidime disks
213 across all isolates. Interestingly, 30 µg aztreonam disks manifested moderate inhibition zones with isolates P31 and P35
214 but showed the least efficacy against isolate P26. These findings collectively contribute to a comprehensive
215 understanding of these thermotolerant PSB isolates' biochemical and antibiotic susceptibility profiles, thereby providing
216 valuable insights for their potential applications in agriculture microbiology.
217

218 ### 3.4 PSB mediated bioavailability of inorganic phosphate in growth medium

219 In studying soluble phosphorus concentrations and pH levels in nutrient broth across three bacterial isolates, distinct
220 patterns emerge over 96 hours (**Fig. 2**). All isolates demonstrate increased soluble phosphorus concentrations, albeit at
221 varying rates. PT26 shows a moderate increase from 1.8 to 2.6 µ moles mL^{-1}, PT31 exhibits the slowest rate of increase,
222 moving from 1.5 to 2 µM/ml, and PT35 records the fastest increase, from 1.7 to 2.7 µ moles mL^{-1}.
223 Concomitantly, all bacterial isolates manifest a decrease in pH levels, indicating increased acidity of the nutrient broth.
224 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
225 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,
226 almost aligning with PT31 despite its faster soluble P increase.
227 It becomes evident that the isolates with moderate to high increases in soluble P (PT26 and PT35) also exhibit significant
228 drops in pH, which suggests that these isolates may produce more acidic byproducts as they metabolize phosphorus (**Fig.
229 2**). On the other hand, PT31, which has the slowest increase in soluble P, shows a moderate decrease in pH, indicating
230 less acidic byproduct formation compared to PT26 and PT35.
231 The observed trends in soluble P and pH levels could have implications for understanding these bacterial isolates'
232 metabolic pathways and ecological roles. The data suggest that PT26 and PT35 may be more efficient in phosphorus
233 uptake and produce more acidic metabolites, whereas PT31 appears less aggressive in phosphorus metabolism and acid
234 production.
235

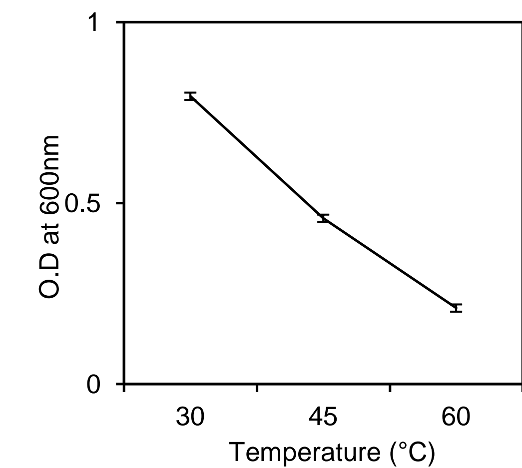
236 ### 3.5 Acid and alkaline phosphatase activity of the thermotolerant PSB isolates

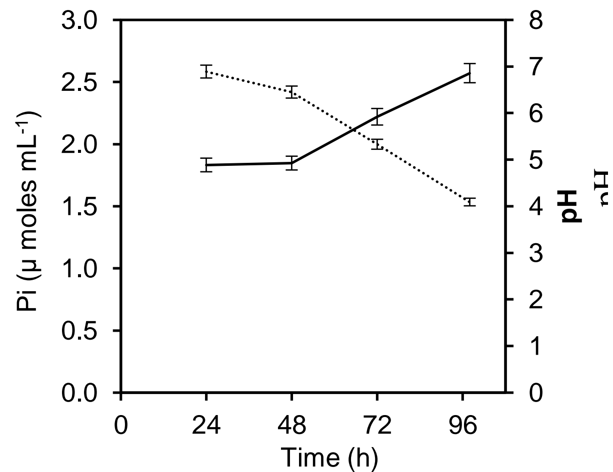
237 In the study of bacterial isolates PT26, PT31, and PT35, distinct patterns of enzymatic activity were observed for both acid
238 and alkaline phosphatases over 96 hours (**Fig. 3**). PT26 showed a significant increase in acid phosphatase activity,
239 starting at 5.06 Units L^{-1} and peaking at 26.9 U L^{-1}, suggesting that PT26 might prefer acidic environments or that the acid
240 phosphatase enzyme plays a crucial role in its metabolic processes. In contrast, its alkaline phosphatase activity started
241 at 2.22 U L^{-1} and peaked at a lower value of 5.15 U L^{-1}.
242 PT31 exhibited the highest peak in acid phosphatase activity, starting at a mere 1.1 U L^{-1} and reaching a remarkable
243 28.41 U L^{-1} in 96 hours, which could indicate that PT31 is the most metabolically active among the isolates, particularly in
244 acidic conditions. Its alkaline phosphatase activity also showed a significant increase, starting at 1.18 U L^{-1} and peaking at
245 7.56 U L^{-1}, the highest among the alkaline phosphatase readings.
246 PT35 started with relatively high enzyme activities for acid and alkaline phosphatases at 11.7 and 5.32 U L^{-1}, respectively.
247 However, both enzymes showed a plateauing trend after 72 hours, with acid phosphatase peaking at 26.3 U L^{-1} and
248 alkaline phosphatase at 6.06 U L^{-1}, which could suggest that PT35 is well-adapted to its environment from the outset and
249 may not require significant metabolic changes over time.
250 The intricate enzymatic behaviors exhibited by distinct bacterial isolates contribute to our understanding of soil health and
251 nutrient cycling and serve as a cornerstone for advancing research in agricultural microbiology. Specifically, the study
252 delves into the metabolic adaptability of these bacterial isolates under varying environmental conditions, thereby enriching
253 the existing body of literature [26].

254 One of the pivotal findings of the study pertains to the expression of acid phosphatases, a class of specialized enzymes
255 that facilitate the hydrolysis of phosphorus esters in acidic milieus. These enzymes are predominantly found in soil
256 bacteria belonging to genera such as *Rhizobium*, *Enterobacter*, and *Pseudomonas* [27]. While acid phosphatases do not
257 directly affect inorganic phosphorus, their enzymatic activity influences the culture medium's pH through
258 dephosphorylation, which generates organic acids [27]. These observations align with prior research that has documented
259 variable levels of acidic phosphatase activity across different bacterial isolates [28,29].
260 In addition to acid phosphatases, the study also sheds light on the heterogeneity observed in the production of alkaline
261 phosphatases. This variability can be ascribed to factors such as the ambient pH and the accumulation of specific
262 secondary metabolites. Notably, a direct correlation has been established between the activity of alkaline phosphatases
263 and incremental shifts in pH [30,31].
264 Contextualizing these findings within a broader research landscape, the study corroborates antecedent research that
265 reported a higher prevalence of alkaline phosphatase activity in Enterobacter sp. compared to acid phosphatase activity
266 [29]. In contrast, an inverse relationship was observed in *Bacillus* species, where acid phosphatase activity was more
267 prevalent [32].
268 The study comprehensively explores the multifactorial determinants influencing enzymatic activities in bacterial isolates. It
269 augments our current understanding and provides a robust framework for future research endeavors in microbial
270 enzymology. The implications of these findings transcend academic boundaries, offering invaluable insights into the
271 complex interplay between environmental variables and microbial enzymatic functions. Therefore, the study holds
272 significant potential for shaping future research trajectories in agricultural microbiology.
273

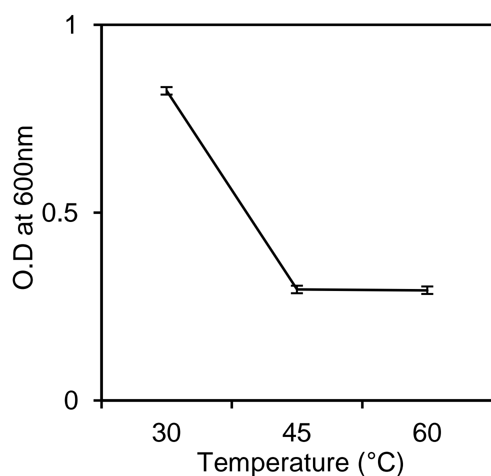
274 ### 3.6 Molecular identification of isolates

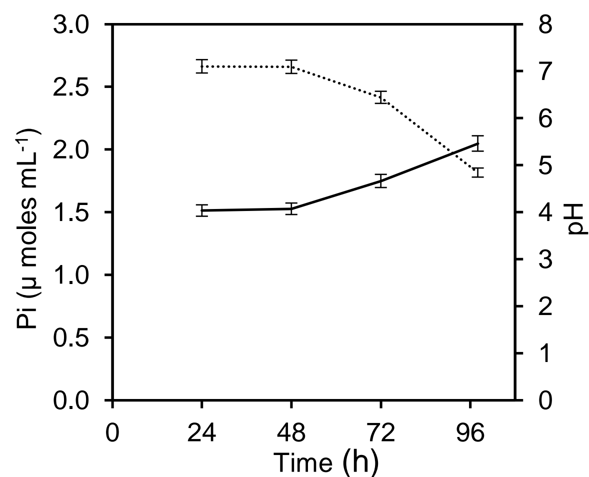
275 Utilizing 16S rRNA gene sequencing, the bacterial isolates designated as PT26, PT31, and PT35 were identified. The
276 obtained 16S rRNA gene sequences were submitted to the National Center for Biotechnology Information (NCBI)
277 database, and the accession numbers were obtained. The isolate PT26 was identified as *Enterobacter cloacae* with the
278 NCBI GenBank® accession number MW857282, PT31 was found to be *Klebsiella quasipneumoniae* under the accession
279 number MW857283, and isolate PT35 was determined to be *Enterobacter bugandensis* with the accession number
280 MW857284.
281
282





PT26





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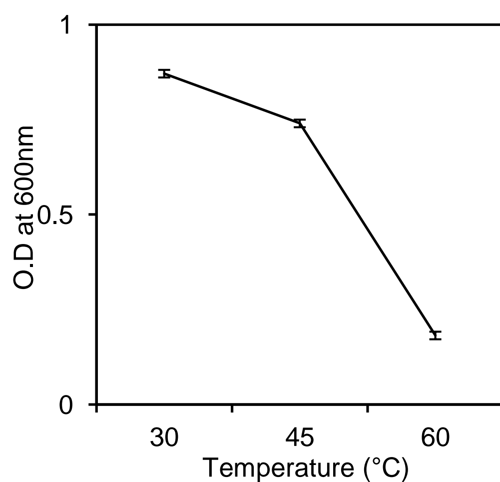
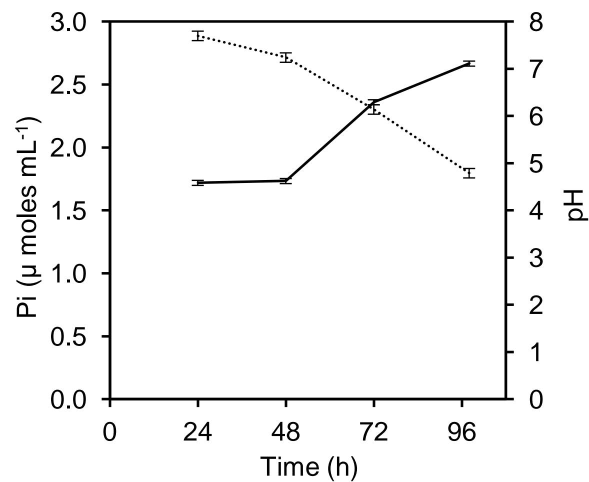




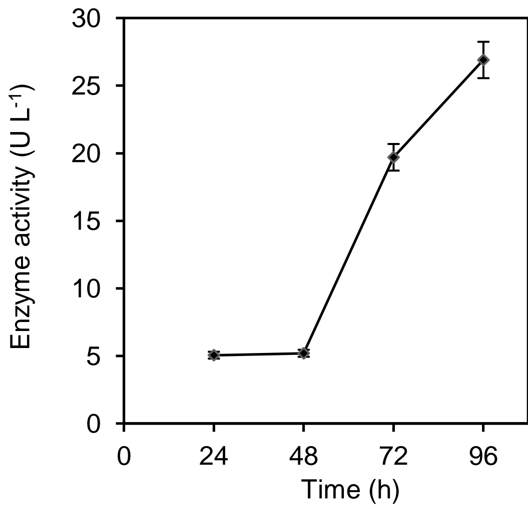
PT35

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

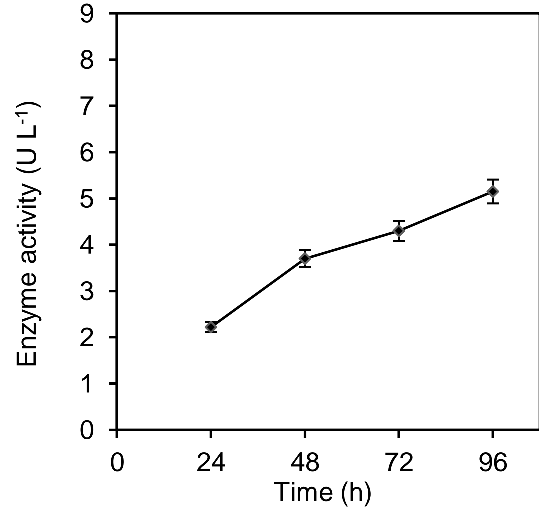
*-Each value is the mean of three replicates
ά -Vertical bars represent SEm

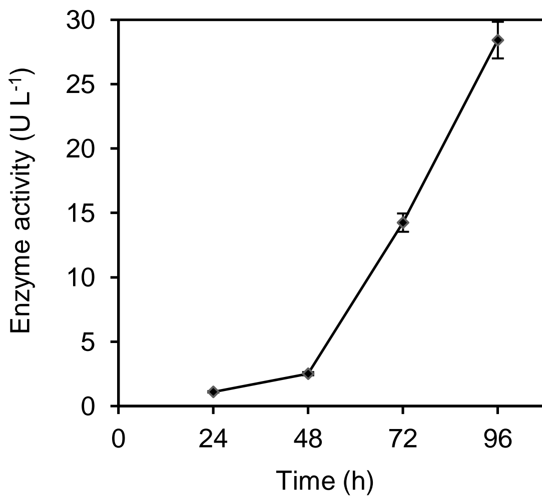
Fig. 2: Released Pi and pH change in Pikovskaya's broth of three thermotolerant PSB isolates (continuous lines – Pi values, dotted lines – pH.)*ά

* -Each value is the mean of three replicates
ά-Vertical bars represent SEm, Pi- inorganic phosphate



PT26





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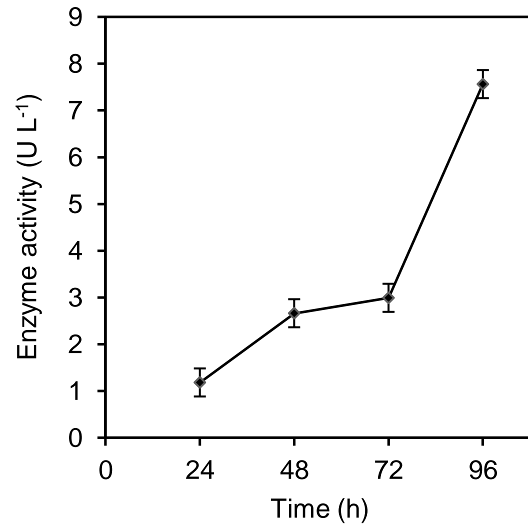
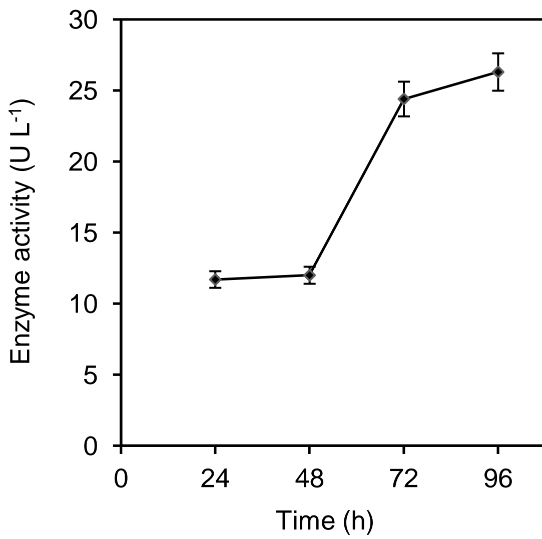
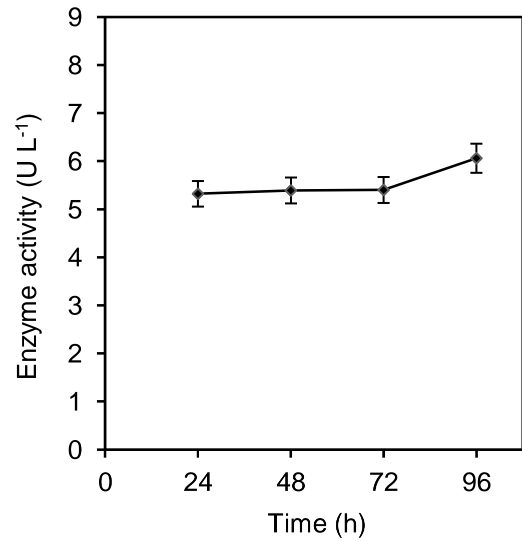




PT35



Acid phosphatase activity　　　　Alkaline phosphatase activity

Fig. 3: Acid and alkaline phosphate activity of three thermotolerant PSB isolates* [α]

*-Each value is the mean of three replicates, [α] -Vertical bars represent SEm

Table 1: The biochemical characterization and antibiotic sensitivity test of thermotolerant PSB isolates

Characteristics	S. No.	Biochemical test	Isolate		
			P26	P31	P35
Biochemical tests through HiAssorted™ biochemical test kit	1.	citrate utilization	+	-	+
	2.	lysine utilization	-	-	-
	3.	ornithine utilization	-	-	-
	4.	urease detection	-	-	-
	5.	phenylalanine deamination	-	-	-
	6.	nitrate reduction	+	-	+
	7.	H_2S 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

284

285 ## 4. CONCLUSION

286

287 The study successfully identifies and characterizes three thermotolerant phosphate-solubilizing bacterial isolates—PT26,
288 PT31, and PT35—regarding their phosphate solubilizing capabilities, thermotolerance, additional traits, and biochemical
289 profiles. These isolates demonstrate distinct phosphate solubilization and temperature tolerance abilities and exhibit
290 unique enzymatic activities and metabolic traits. PT35 stands out for its thermotolerance, PT31 for its broad temperature
291 range and potassium solubilizing ability, and PT26 for its synthesis of indole-3-acetic acid. Their distinct biochemical
292 profiles and enzymatic activities offer valuable insights into their potential roles in soil health and nutrient cycling.
293 Molecular identification through 16S rRNA gene sequencing further validates the uniqueness of each isolate, categorizing
294 PT26 as *Enterobacter cloacae*, PT31 as *Klebsiella quasipneumoniae*, and PT35 as *Enterobacter bugandensis*. The study
295 provides a comprehensive understanding of these isolates' metabolic adaptability and ecological roles, laying a robust

296 foundation for future research in agricultural microbiology. The findings have significant implications for enhancing soil
297 fertility and plant growth, making them highly relevant for sustainable agricultural practices.
298

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304 **COMPETING INTERESTS**

305 All authors declare no competing interest.
306

307 **AUTHORS' CONTRIBUTIONS**

308

309 Author 1 designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the
310 manuscript. Author 2 managed the analyses of the study, and author 3 managed the biochemical aspects of the study.
311 Author 4 managed the literature search of the entire study. All authors read and approved the final manuscript.
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