International Journal of Plant & Soil Science

Volume 35, Issue 20, Page 43-55, 2023; Article no.IJPSS.106489
ISSN: 2320-7035

Multifaceted Proficiencies of Thermotolerant Phosphate Solubilizing Bacteria from the Kutch Desert, Gujarat, India

Anurag Yadav [a*], Ravikumar Jadav [a], Kavin Soni [b] and Kusum Yadav [c]

[a] Department of Microbiology, College of Basic Science and Humanities, Sardarkrushinagar Dantiwada Agricultural University, Sardrarkrushinagar, Dist. Banaskantha, Gujarat-385506, India.
[b] Bioscience Research Laboratory, Sardarkrushinagar Dantiwada Agricultural University, Sardrarkrushinagar, Dist. Banaskantha, Gujarat-385506, India.
[c] Department of Biochemistry, University of Lucknow, Lucknow, Uttar Pradesh-226007, India.

Authors' contributions

This work was carried out in collaboration among all authors. Author AY designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Author RJ managed the analyses of the study and author KS managed the biochemical aspects of study. Author KY managed the literature search of the entire study. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/IJPSS/2023/v35i203784

Open Peer Review History:
This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc. are available here: https://www.sdiarticle5.com/review-history/106489

Original Research Article

Received: 08/07/2023
Accepted: 11/09/2023
Published: 16/09/2023

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

**Corresponding author: E-mail: anuragyadav123@gmail.com;*

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 also monitored over time.

Methodology: Screened multiple PSB isolates, selecting PT26, PT31, and PT35. Conducted growth measurements under elevated temperatures, assessed biochemical profiles, and tested the antibiotic susceptibility. Also monitored soluble phosphorus and pH of PSB isolates over 96 hours and measured acid and alkaline phosphatase activities. Molecular identification was done 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, and thus can benefit soil health and nutrient cycling. These findings contribute to agricultural microbiology research, potentially enhancing sustainable farming practices and soil fertility.

Keywords: PSB; thermotolerance; multitrait; acid phosphatase; alkaline phosphatase.

1. INTRODUCTION

The pursuit of sustainable agriculture has led to an increased 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 aluminum phosphate, rendering it inaccessible to plants [2,3]. Traditional methods to enhance phosphorus availability have relied on chemical fertilizers, which have long-term environmental repercussions [4,5].

Thermotolerant PSBs offer a sustainable alternative to enhance phosphorus availability in the earth's tropical and warm temperate zones. 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 [6-8]. 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, and 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, rendering 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, and synthesize phytohormones render them invaluable assets in the quest for agricultural sustainability.

2. MATERIALS 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 phosphate 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 Petri plate containing 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 also determined.

2.2 Screening for Thermotolerance and Additional Traits among PSB Isolates

A systematic temperature gradient experiment was conducted to assess the PSB 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 serially diluted, and respective dilutions were seeded on nutrient agar Petri plates through spread plating. The Petri plates were incubated at 30°C for 48 hours to evaluate their viability and thermal resilience.

A growth 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 and matched with control to assess the growth of the bacterial isolates.

The three PSB isolates underwent an 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, 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 was initiated using a HiMedia® test kit (HiMedia Laboratories Pvt. Ltd., Mumbai, India), specifically designed to identify Gram-negative bacteria. The kit was used for distinct tests, including citrate, lysine, and ornithine utilization, urease production, phenylalanine deamination, nitrate reduction, H_2S production, starch hydrolysis, gelatin hydrolysis, and 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 kit results were compared with the catalog to ensure accuracy.

In parallel, Petri plates containing phenol red agar medium were prepared and seeded 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 period, sugar utilization by the bacterial isolates was observed and marked as positive, indicated by a distinct color change from red to yellow around the disk, signifying the successful fermentation of the sugars as shown in Table 1.

Furthermore, plates containing the Mueller-Hinton agar medium were prepared to assess the antibiotic effect on the PSB growth [20]. The test organisms were streaked thrice onto the entire agar surface of the plate with a sterile 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 [21]. The

process required several solutions, each carefully prepared to ensure accuracy. We used double distilled water, devoid of phosphorus, for all our solution preparations.

To prepare a standard phosphorus solution, 340 mg of KH_2PO_4 was dissolved in 100 mL of water, followed by volume adjustment to 250 mL with distilled water, resulting in a solution containing 10 µ moles mL^{-1} of phosphorus. In addition, 10 N sulfuric acid was prepared by mixing 200 mL of 36 N concentrated H_2SO_4 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 H_2SO_4, and the final volume was adjusted to 1 L using distilled water. The resulting solution was carefully stored in a brown bottle to minimize light exposure.

The preparation of the 1-amino-2-napthol-4-sulfonic acid (ANSA) reagent involved a series of steps, including the dissolution of bisulfite and sodium sulfite, and mixing with ANSA powder, and storage in a refrigerated environment in a brown bottle to prevent photodegradation and ensure stability.

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 blue color in the solution.

Subsequently, the solution was transferred into cuvettes, and optical density (OD) was measured at 640 nm spectrophotometrically using a calibrated instrument.

2.5 Quantification of Acidic and Alkaline Phosphatase from Thermotolerant PSB

In the study, we assayed acid and alkaline phosphatase enzymes of PSB isolates. We first added 10 ml of sterilized Pikovskaya broth within the 20 ml sterilized test tubes. 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. [22].

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 $MgCl_2$ 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 $MgCl_2$, and 0.1 mL of the enzyme source. These mixtures were then distributed into three tubes labelled test, control, and reagent blank. Subsequently, the solutions were placed in a water bath maintained 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 tubes. 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 by substituting the respective buffer with the PNP substrate. Following this, spectrophotometric analyses were carried out at a wavelength of 410 nm. The instrument was calibrated to achieve 100% transmission, utilizing the reagent blank as the standard reference point for accurate measurements.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 ($\varepsilon\lambda_{max}$) of 18.3 mM^{-1} cm^{-1}.

The acid and alkaline phosphatase activity of the thermotolerant PSB isolates was calculated using the following formula:

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

This equation calculates the enzyme activity (acid or alkaline phosphatase) in a sample based on the change in absorbance at 410 nm due to the conversion of the substrate (PNP) into a product. The formula considers factors such as the difference in absorbance between the test and control samples, the volume of the assay, dilution factor, extinction coefficient of PNP, the volume of enzyme, and incubation time. It quantitatively measures enzyme activity, usually expressed as units (U) L^{-1} enzyme, essential for various biochemical and microbiological studies.

3. RESULTS AND DISCUSSION

3.1 Bacterial Screening for Phosphate Solubilization, Thermotolerance and Additional Traits

In total, 128 PSB isolates with various degrees of PSI were obtained in the study. It is imperative to underscore the thermotolerant attributes PSB isolates, as corroborated by their ability to endure a temperature of 75°C for 30 minutes. Thermotolerance is a pivotal bacterial trait that could significantly affect their applicability in biotechnological interventions within agriculture microbiology [23,24].

To ascertain whether the isolates exhibited thermophilic characteristics, they were cultured at 30°C, 45°C, and 60°C for 24 hours (Fig. 1). Remarkably, three isolates—PT26, PT31, and PT35—exhibited 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. These isolates had PSI of 3.12, 2.90, and 2.42, 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 experienced 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.

The isolated thermotolerant PSB exhibited distinct additional traits. Specifically, PT31 demonstrates the capacity to solubilize potassium with a notable potassium 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 research conducted by Thomas et al. [25], who utilized analogous methodologies, including TLC and colorimetry, to discern and quantify IAA production in bacteria isolated from soil samples. The findings underscore the multifaceted capabilities of thermotolerant PSB isolates, shedding light on their potential significance in various ecological and agricultural contexts.

3.2 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 production. 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 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 H_2S 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, producing the highest inhibition zone. 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 thermotolerant PSB isolates' biochemical and antibiotic susceptibility profiles, thereby providing valuable insights for their potential applications in agriculture microbiology.

3.3 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 emerged over 96 hours (Fig. 2). All isolates increased soluble phosphorus concentrations 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 µ moles mL^{-1}, and PT35 records the fastest increase, from 1.7 to 2.7 µ moles mL^{-1}.

Concomitantly, all bacterial isolates manifested 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.

It becomes evident that the isolates with moderate to high increases in soluble phosphate (PT26 and PT35) also exhibit significant drops in pH, which suggests that these isolates may produce more acidic byproducts as they metabolize phosphorus (Fig. 2). On the other hand, PT31, which was the slowest phosphate solubilizer, showed 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.

3.4 Acid and Alkaline Phosphatase Activity of the Thermotolerant PSB Isolates

In the study, three PSB isolates, namely 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 Units (U) L^{-1} and peaking at 26.9 U L^{-1}, 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 U L^{-1} and peaked at a lower value of 5.15 U L^{-1}.

PT31 exhibited the highest peak in acid phosphatase activity, starting at a mere 1.1 U L^{-1} and reaching a remarkable 28.41 U L^{-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 U L^{-1} and peaking at 7.56 U L^{-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 U L^{-1}, respectively. However, both enzymes showed a plateauing trend after 72 hours, with acid phosphatase peaking at 26.3 U L^{-1} and alkaline phosphatase at 6.06 U 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 [26].

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* [27]. While acid phosphatases do not directly affect inorganic phosphorus, their enzymatic activity influences the culture medium's pH through dephosphorylation, which generates organic acids [27]. These observations align with prior research that has documented variable levels of acidic phosphatase activity across different bacterial isolates [28,29].

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 reduction in pH [30,31].

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 [29]. In contrast, an inverse relationship was observed in *Bacillus* species, where acid phosphatase activity was more prevalent [32].

Our 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. The study holds significant potential for shaping future research trajectories in agricultural microbiology.

3.5 Molecular Identification of Isolates

Utilizing 16S rRNA gene sequencing, the bacterial isolates designated as PT26, PT31, and PT35 were identified. The obtained 16S rRNA gene sequences were submitted to the National Center for Biotechnology Information (NCBI) database, and the accession numbers were acquired. The isolate PT26 was identified as *Enterobacter cloacae* with the NCBI GenBank® 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.

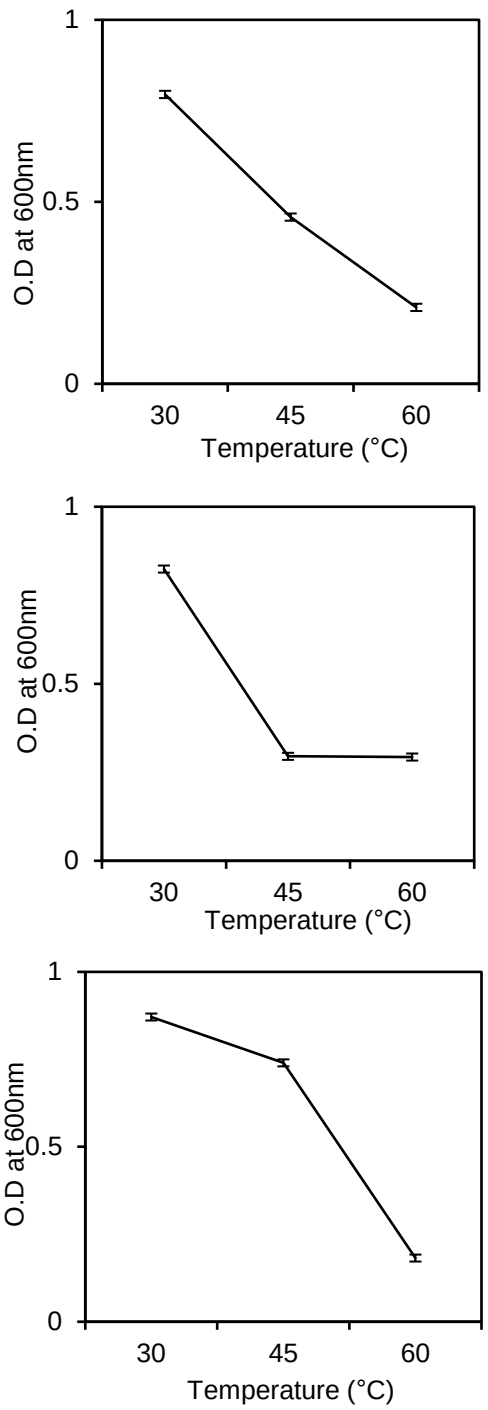

Fig. 1. Growth of thermotolerant PSB isolates on nutrient broth at elevated temperatures for 24 hours*α

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

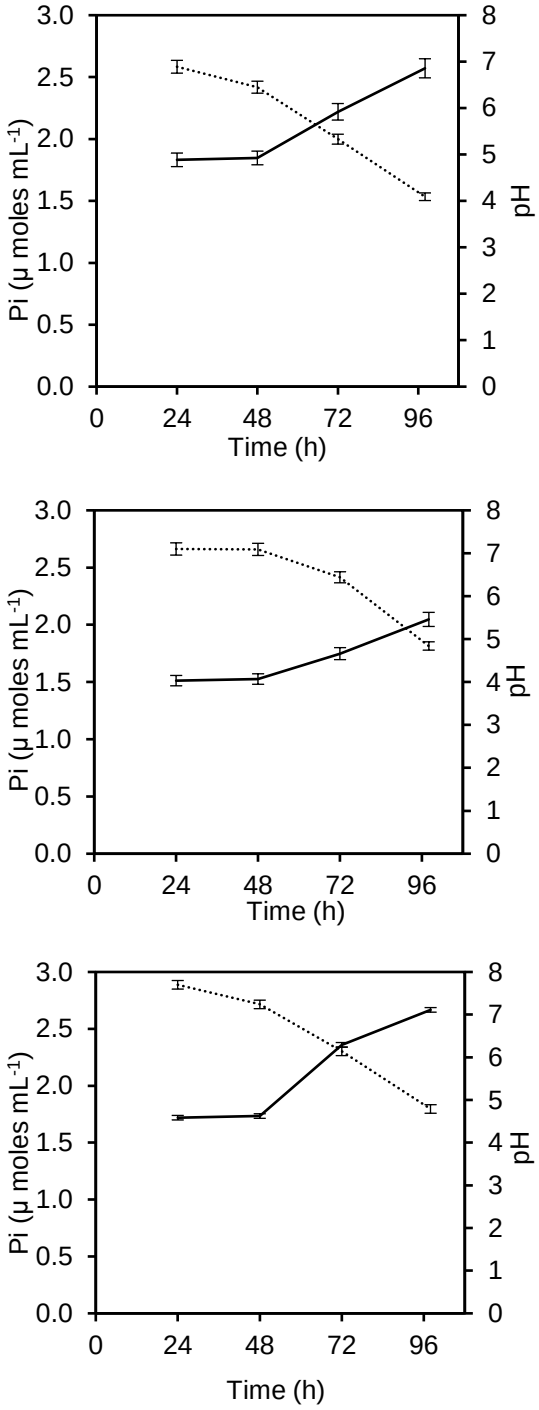

Fig. 2. Released Pi and pH change in Pikovskaya's broth of thermotolerant PSB isolates*α

(Continuous lines – Pi values, dotted lines – pH)
* -Each value is the mean of three replicates
α -Vertical bars represent SEm

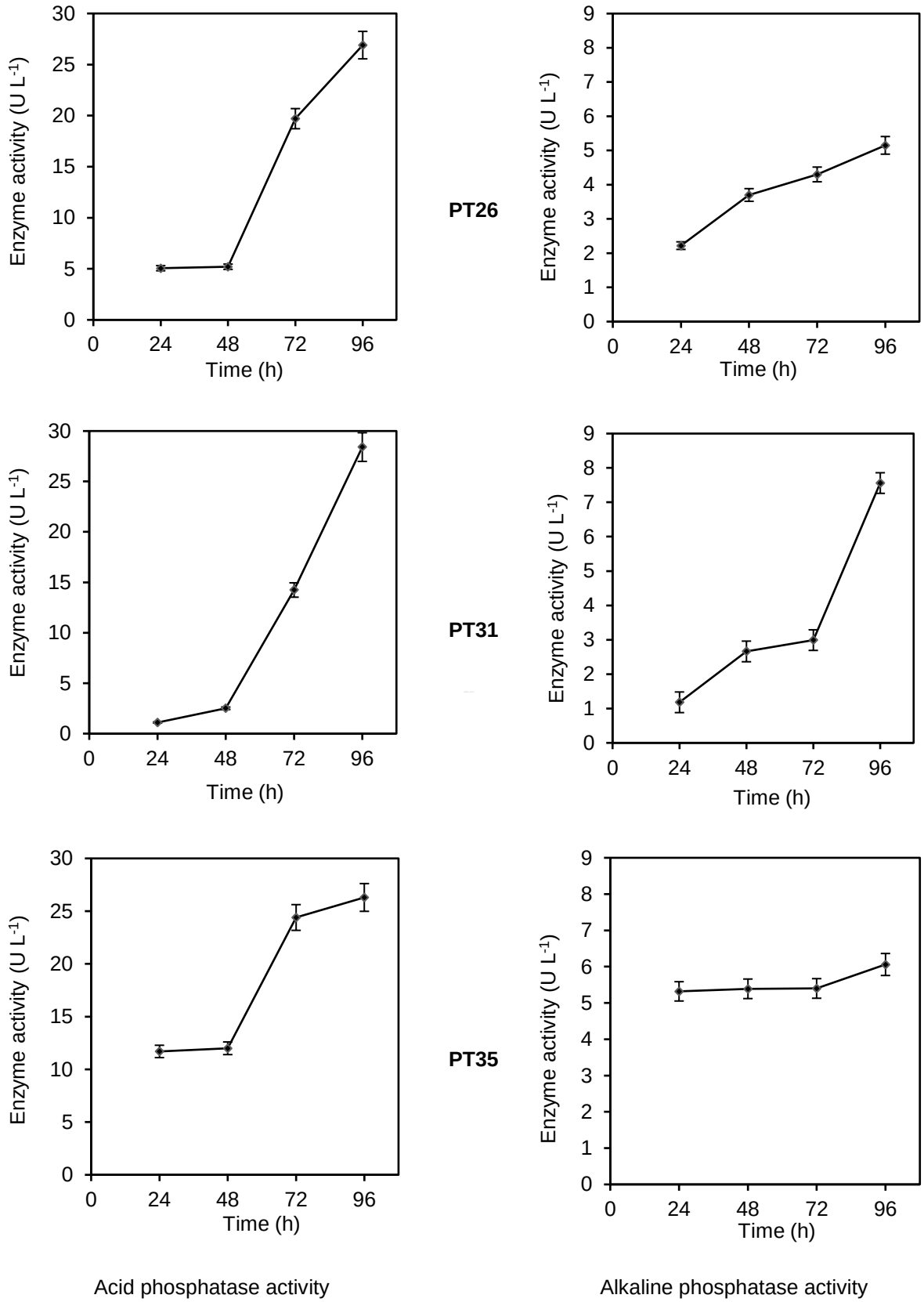

Fig. 3. Acid and alkaline phosphate activity of three thermotolerant PSB isolates* [α]

*-Each value is the mean of three replicates,

[α] -Vertical bars represent SEm

Table 1. Biochemical characterization and antibiotic sensitivity 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 tests	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

4. CONCLUSION

The study identified and characterized three thermotolerant phosphate solubilizing bacterial isolates—PT26, PT31, and PT35—with additional traits. The isolates exhibited distinct abilities for phosphate solubilization and temperature tolerance, along with 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. The distinct biochemical profiles and enzymatic activities of isolates 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 can significantly enhance soil fertility and plant growth, making them highly relevant for sustainable agricultural practices.

ACKNOWLEDGEMENTS

The authors acknowledge the Principal, College of Basic Science and Humanities, Sardrkrushinagar Dantiwada Agricultural University, Gujarat, India, for providing facilities for this research.

COMPETING INTERESTS

The authors have declared that no competing interests exist.

REFERENCES

1. Tarigan DM, Barus WA, Munar A, Lestami A. Exploration and Morphological Characterization of Phosphate-Solubilizing and Nitrogen-Fixing Bacteria in Saline Soil. SABRAO Journal of Breeding and Genetics. 2023;52(2): 550-563. DOI:10.54910/sabrao2023.55.2.27.
2. Wang H-y, Shen L, Zhai L-m, Zhang J-z, Ren T-z, Fan B-q, LIU H-b. Preparation and utilization of phosphate biofertilizers using agricultural waste. Journal of Integrative Agriculture. 2015;14(1):158-167.
3. Pradhan M, Das R, Sahoo R, Adak T, Pradhan C, Mohanty S. Comparative P solubilizing efficiencies of five acid soil bacteria incubated with calcium, aluminium and iron phosphates. Indian Journal of Biochemistry and Biophysics (IJBB). 2022;59(10):947-955.
4. Patel G, Singh S, Saxena DSK, Kaur DKJ. Isolation, biochemical characterization and production of biofertilizer from *Bacillus megaterium*. The International Journal of Life-Sciences Scientific Research. 2016; 2(6): 749-752. DOI:10.21276/ijlssr.2016.2.6.16.
5. Jha A, Pathania D, Damathia B, Raizada P, Rustagi S, Singh P, Rani GM, Chaudhary V. Panorama of biogenic nano-fertilizers: A road to sustainable agriculture. Environmental Research. 2023;116456.
6. Mei C, Chretien RL, Amaradasa BS, He Y, Turner A, Lowman S. Characterization of phosphate solubilizing bacterial endophytes and plant growth promotion in vitro and in greenhouse. Microorganisms. 2021;9(9): [Article 1935]. DOI:10.3390/microorganisms9091935.
7. Pramanik K, Ghosh PK, Ray S, Sarkar A, Mitra S, Maiti TK. An in silico structural, functional and phylogenetic analysis with three dimensional protein modeling of alkaline phosphatase enzyme of *Pseudomonas aeruginosa*. J Genet Eng Biotechnol. 2017;15(2):527-537. DOI:10.1016/j.jgeb.2017.05.003.
8. Rawat P, Das S, Shankhdhar D, Shankhdhar S. Phosphate-solubilizing microorganisms: mechanism and their role in phosphate solubilization and uptake. Journal of Soil Science and Plant Nutrition. 2021;2149-68.
9. Rodríguez H, Fraga R, Gonzalez T, Bashan Y. Genetics of phosphate solubilization and its potential applications for improving plant growth-promoting bacteria. Plant and Soil. 2006; 287(1-2):15-21. DOI:10.1007/s11104-006-9056-9.
10. Maitra S, Pramanick B, Dey P, Bhadra P, Shankar T, Anand K. Thermotolerant soil microbes and their role in mitigation of heat stress in plants. Soil Microbiomes for Sustainable Agriculture: Functional Annotation. 2021;203-242.
11. Sharma C, Khurana S, Megha K, Thakur A, Bhatia A, Gupta A. Assessment of pathogenic potential of Acanthamoeba isolates by in vitro and in vivo tests. Parasitology Research. 2023;1-10.
12. Qian Y, Shi J, Chen Y, Lou L, Cui X, Cao R, Li P, Tang J. Characterization of phosphate solubilizing bacteria in sediments from a shallow eutrophic lake and a wetland: isolation, molecular identification and phosphorus release ability determination. Molecules. 2010;15(11): 8518-8533. DOI:10.3390/molecules15118518.

13. Swain MR, Laxminarayana K, Ray RC. Phosphorus solubilization by thermotolerant *Bacillus subtilis* isolated from cow dung microflora. Agricultural Research. 2012;1(3):273-279. DOI:10.1007/s40003-012-0022-x.
14. Ait-Ouakrim EH, Chakhchar A, El Modafar C, Douira A, Amir S, Ibnsouda-Koraichi S, Belkadi B, Filali-Maltouf A. Assessment of Potent Phosphate-Solubilizing Bacteria Isolated from the Olive Tree Rhizosphere Grown on Phosphate Sludge and Their Effect on Common Bean Growth. Geomicrobiology Journal. 2023;1-13.
15. RI P. Mobilization of phosphorus in soil in connection with the vital activity of some microbial species. Microbiologiya. 1948; 17362-370.
16. Ranjan A, Mahalakshmi MR, Sridevi M. Isolation and characterization of phosphate-solubilizing bacterial species from different crop fields of Salem, Tamil Nadu, India. International Journal of Nutrition, Pharmacology, Neurological Diseases. 2013;3(1):29-33.
17. Raji M, Thangavelu M. Isolation and screening of potassium solubilizing bacteria from saxicolous habitat and their impact on tomato growth in different soil types. Archives of Microbiology. 2021; 203(6):3147-3161.
18. Fasim F, Ahmed N, Parsons R, Gadd GM. Solubilization of zinc salts by a bacterium isolated from the air environment of a tannery. FEMS Microbiol Lett. 2002;213(1): 1-6. DOI:10.1111/j.1574-6968.2002.tb11277.x.
19. Mazhar H, Abbas N, Hussain Z, Sohail A, Ali S. Extracellular lipase production from *Bacillus subtilis* using agro-industrial waste and fruit peels. Punjab Univ. J. Zool. 2016;31(2):261-267.
20. Saffari N, Salmanzadeh-Ahrabi S, Abdi-Ali A, Rezaei-Hemami M. A comparison of antibiotic disks from different sources on Quicolor and Mueller-Hinton agar media in evaluation of antibacterial susceptibility testing. Iran J Microbiol. 2016;8(5):307-311.
21. Fiske CH, Subbarow Y. The colorimetric determination of phosphorus. J. biol. Chem. 1925;66(2):375-400.
22. Bergmeyer HU, Bernt E, Lachenicht R. Alkaline phosphatase in serum determination with automatic analysers. In Methods of Enzymatic Analysis; Elsevier. 1974;864-868.
23. Désiré K, Léonce NG, Constant K, Ysidor K, Marius BGH. Microbiological safety and quality assessment of maize (*Zea mays* L) produced and stocked from rural conditions in Côte D'ivoire. Journal of Advances in Microbiology. 2021;21(6):60-74.
24. Farda B, Djebaili R, Vaccarelli I, Del Gallo M, Pellegrini M. Actinomycetes from caves: an overview of their diversity, biotechnological properties, and insights for their use in soil environments. Microorganisms. 2022;10(2):453.
25. Thomas S, Mathew L, Rishad K. Isolation and molecular identification of phosphate solubilizing bacteria, *Bacillus licheniformis* UBPSB-07 capable of enhancing seed germination in *Vigna radiata* L. Phytomorphology. 2018;68(1&2):13-18.
26. Chen X, Han X, Lu X, Yan J, Biswas A, Zou W. Long-term continuous cropping affects ecoenzymatic stoichiometry of microbial nutrient acquisition: a case study from a Chinese Mollisol. Journal of the Science of Food and Agriculture. 2021;101(15):6338-6346.
27. Araújo AP, Plassard C, Drevon JJ. Phosphatase and phytase activities in nodules of common bean genotypes at different levels of phosphorus supply. Plant and Soil. 2008;312(1-2):129-138, doi:10.1007/s11104-008-9595-3.
28. Chen Q, Liu S. Identification and characterization of the phosphate-solubilizing bacterium *Pantoea* sp. S32 in reclamation soil in Shanxi, China. Front Microbiol. 2019;10[Article 2171]. DOI:10.3389/fmicb.2019.02171.
29. Park JH, Bolan N, Megharaj M, Naidu R. Isolation of phosphate solubilizing bacteria and their potential for lead immobilization in soil. Journal of hazardous materials. 2011;185(2-3):829-836.
30. Fraser T, Lynch DH, Entz MH, Dunfield KE. Linking alkaline phosphatase activity with bacterial phoD gene abundance in soil from a long-term management trial. Geoderma. 2015;257:115-122.
31. Zheng Z, Tang A, Guan Y, Chen L, Wang F, Chen P, Wang W, Luo Y, Tian Y, Liang G. Nanocomputed tomography imaging of bacterial alkaline phosphatase activity with an iodinated hydrogelator. Analytical chemistry. 2016;88(24):11982-11985.

32. Ibarra-Galeana JA, Castro-Martínez C, Fierro-Coronado RA, Armenta-Bojórquez AD, Maldonado-Mendoza IE. Characterization of phosphate-solubilizing bacteria exhibiting the potential for growth promotion and phosphorus nutrition improvement in maize (*Zea mays* L.) in calcareous soils of Sinaloa, Mexico. Annals of Microbiology. 2017;67: 801-811.

Peer-review history:
The peer review history for this paper can be accessed here:
https://www.sdiarticle5.com/review-history/106489