Exploring the phosphate solubilizing potential and acid-alkaline phosphatase activity of two multitrait heavy metal tolerant bacterial strains isolated in Northern Gujarat, India

Abstract

A study was conducted to isolate and characterize phosphate solubilizing bacteria (PSB) from soils of various locations in North Gujarat. A total of 40 PSB isolates were obtained, exhibiting a phosphate solubilizing index (PSI) ranging from 2.11 to 4.33. Interestingly, among 40 PSB isolates, a subset of 16 displayed an additional trait of potassium solubilization, as evidenced by their potassium solubilizing index (KSI) values falling within the range of 2.10 to 3.10. These 16 PSB isolates were screened for tolerance to heavy metal stressors, namely $CdCl_2$, $HgCl_2$, $Pb(NO_3)_2$, and $ZnSO_4$, spanning a concentration gradient from 0.1 mM to 10 mM. Remarkably, among the tested isolates, two, namely D4 and G2, exhibited exceptional resilience against the heavy metal stressors, as indicated by their minimum inhibitory concentration (MIC). Specifically, isolate D4 showed MIC values of 5 mM, 0.2 mM, 18 mM, and 12 mM, respectively, against $CdCl_2$, $HgCl_2$, $Pb(NO_3)_2$, and $ZnSO_4$. For isolate G2, the corresponding MIC values were 6 mM, 0.2 mM, 21 mM, and 11 mM. The isolates were identified as *Klebsiella pneumoniae* PHmD4 (D4) and *Enterobacter chuandaensis* PHmG2 (G2) through 16S rDNA sequencing.

In Pikovskaya's broth, *K. pneumoniae* PHmD4 and *E. chuandaensis* PHmG2 had phosphate solubilization values of 4.30 μ moles mL^{-1} and 3.51 μmoles mL^{-1}, respectively, with terminal pH values of 3.06 and 4.07. Enzymatic activity assays conducted after 96 hours of incubation in Pikovskaya's broth revealed that the highest acid phosphatase activity was 21.62 U mL^{-1} for *K. pneumoniae* PHmD4 and 21.09 U mL^{-1} for *E. chuandaensis* PHmG2. The highest alkaline phosphatase activity was 5.85 U mL^{-1} and 6.38 U mL^{-1}, respectively.

In tomato seed germinatin study, both non-bacterized and bacterized tomato seeds achieved a 100% germination rate. However, increasing metal concentrations diminished the germination percentage of heavy metal-treated seeds. Interestingly, bacterization with *K. pneumoniae* PHmD4 and *E. chuandaensis* PHmG2 ameliorated germination under cadmium stress but had no mitigative effect under mercury and zinc stress conditions.

Keywords: PSB, heavy metal tolerance, multitrait, phosphatase

1. Introduction

Soil contamination by heavy metals has emerged as a critical environmental concern, imposing significant challenges on ecological stability and sustainable agriculture [1]. While certain heavy metals serve as essential micronutrients at low concentrations, facilitating various metabolic activities in organisms [1], elevated levels disrupt the ecological equilibrium. High concentrations of heavy metals adversely affect the composition, function, and diversity of soil microbial communities, including those in the rhizosphere [2-4], ultimately compromising soil fertility and altering ecological dynamics [1].

In this context, the study explores the potential of phosphate solubilizing bacteria (PSB), particularly heavy metal-tolerant strains, which exhibit unique mechanisms to alleviate the toxic effects of heavy metals in the soil [5]. The PSBs can play a dual role in enhancing plant growth and mitigating heavy metal toxicity [5]. They employ metal expulsion, bioaccumulation, and biotransformation strategies to thrive in contaminated environments [5].

Furthermore, they enhance phytoremediation capabilities through growth-promoting traits such as organic acid production and siderophore secretion [6].

Phosphorus, an essential nutrient for plant growth, is often limited in soil due to low solubility, especially in alkaline and acidic soils [7]. To address this limitation, we investigated the phosphate solubilizing potential and acid-alkaline phosphatase activity of two multitrait heavy metal-tolerant PSB strains isolated from agricultural soils of Northern Gujarat, India [8]. These strains exhibit the ability to tolerate heavy metal contamination and the capacity to solubilize phosphates, making them potential candidates for biofertilizer production and environmental remediation [9,10].

The versatility of PSB, such as *Pantoea* strains, is well-documented, encompassing various plant growth promoting traits, including 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity and production of indole-3-acetic acid (IAA), hydrogen cyanide, ammonia, and siderophore [7]. These traits bolster plant growth and development, making PSBs ideal bioinoculants [7]. Additionally, the strains' solubilization of inorganic phosphates can lead to increased yields and reduced reliance on chemical fertilizers [10].

Phosphate solubilization by bacteria is influenced by several factors, including pH and carbon source availability [11]. Calcium phosphate solubilization is favored under alkaline conditions, while iron phosphate solubilization prevails under acidic conditions [11]. The choice of carbon source can also influence the solubilization of different phosphate compounds [11].

Beyond phosphate solubilization, the study delves into the intriguing aspect of acid-alkaline phosphatase activity in these bacteria. Acid phosphatase and alkaline phosphatase enzymes are pivotal in hydrolyzing organic phosphates, releasing inorganic phosphates vital for plant uptake [12]. While the positive association between alkaline phosphatase activity and certain disease conditions is documented, the relationship with acid phosphatase activity remains enigmatic [12].

2. Materials and methods

The research was executed in the Department of Microbiology, College of Basic Sciences and Humanities, Sardarkrushinagar Dantiwada Agricultural University, situated in Sardarkrushinagar, Gujarat, India. Soil samples from the North Gujarat region were gathered in polymeric containers. These containers were conveyed to the laboratory setting, where they underwent microbiological analysis within 24 hours of collection.

2.1 Screening of potent PSB isolates for heavy metal tolerance and additional traits

PSBs were selectively isolated from soil collected from agricultural lands of north Gujarat. The acquired soil samples were serially diluted and spread-plated on Petri plates, employing Pikovskaya's agar as the selective growth medium [13]. A mass of one gram of either rhizospheric or bulk soil was solubilized in 10 mL of aseptic distilled water contained within a sterilized test tube, followed by successive dilutions [14]. The resulting diluted samples were then inoculated onto agar plates containing Pikovskaya's medium and subjected to incubation. Colonies manifesting transparent clearance zones around were isolated and subjected to purification. Subsequently, the phosphate solubilization index (PSI) was calculated.

The PSB isolates were screened against $CdCl_2$, $HgCl_2$, $Pb(NO_3)_2$, and $ZnSO_4$ through agar well diffusion assay. The four wells were bored in bacterial pre-swabbed nutrient agar plates. The wells were filled with 100 µL heavy metal solution in 0.1, 1, 10, and 100 mM concentrations. The Petri plates were incubated at 30°C for 24h and observed for the presence or absence of bacterial growth around the wells.

In addition, the Aleksandrow medium was employed as the testing substrate to evaluate the potassium solubilizing capabilities of isolates, as per the methodology delineated in the previous study [15].

2.2 Characterization and identification of heavy metal tolerant PSB isolates

The bacteria were characterized through HiMedia® biochemical test kit, hydrolysis tests on sugar, and susceptibility profiling on antibiotic multi-discs. Particularly, the sugar test is based on the differences in the ability to utilize a particular substrate due to the metabolic variability among the bacterial world.

In addition, heavy metal tolerance profiling of PSB isolates was systematically conducted using a specialized HiAssorted™ Biochemical Test Kit (HiMedia Laboratories Pvt. Ltd., Mumbai, India). Heavy metal tolerant PSB cultures, aged 24 hours, were inoculated onto each segment of the assay kit using a sterilized inoculating loop. The cultures were incubated at 30±1°C for 24 hours. Post-incubation, the outcomes derived from the assays were cross-referenced with the manufacturer's catalog to validate their precision.

Concurrently, following the established procedure [16], culture tubes containing phenol red broth base were prepared. These tubes were subsequently inoculated with specific bacterial isolates, each accompanied by introducing a single carbohydrate disk into the medium. The incubation was performed at 30°C for 24 to 48 hours. Sugar metabolism by the bacterial isolates was monitored and denoted as positive upon a colorimetric transition from red to yellow, signifying efficacious fermentation of tested sugars (**Table 2**).

In addition, Mueller-Hinton agar-based plates were prepared to evaluate the antibiotic susceptibility of the bacterial isolates [17]. The test strains were uniformly swabbed across the agar surface using a sterile cotton applicator and then placed in a hexa disc impregnated with antibiotics. These plates were then incubated at a consistent temperature of 30°C for 24 hours. The observations were documented, thereby contributing to a holistic understanding of the biochemical and antibiotic profiles of the heavy metal-tolerant PSB isolates.

The sensitivity pattern of D4 and G2 isolates was determined against 20 antibiotics using the Kirby-Bauer disk diffusion method [18]. The antibiotic zones of inhibition (ZOI) were measured and compared with colony size, and sensitivity was interpreted using the sensitivity table furnished by the manufacturer (HiMedia Laboratories Pvt. Ltd., Mumbai, India), based on which they were graded as either sensitive or resistant. The 20 used antibiotics belonged to the ten different classes *viz.*, penicillin (ampicillin and carbenicillin), cephalosporins (cefotaxime, cephalothin, cefepime, and cefazolin), folate pathway antagonists (co-trimoxazole), aminoglycosides (gentamicin, amikacin, and streptomycin), macrolides (nitrofurantoin), quinolones (norfloxacin, ciprofloxacin, and moxifloxacin), fenicol (cefoperazone), β-lactam+β-lactamase inhibitor (piperacillin/tazobactam and ampicillin/sulbactam), tetracycline (doxycycline hydrochloride and tetracycline) and fluoroquinolones (gatifloxacin).

2.3 Measuring PSB mediated bioavailability of inorganic phosphate in growth medium

In the present investigation, inorganic phosphate (Pi) concentrations in the growth medium's supernatant were ascertained using the Fiske-Subbarow method [19]. To assure analytical accuracy, reagents were precisely prepared. All solutions were formulated using phosphorus-free double-distilled water.

Potassium dihydrogen phosphate weighing 340 mg was solubilized in 100 mL of water to generate a phosphorus calibration curve. The volume was subsequently augmented to 250 mL using distilled water, yielding a final 10 µ moles mL^{-1} phosphorus concentration.

Concurrently, a 10 N sulfuric acid solution was prepared by mixing 200 mL of 36 N sulfuric acid with 520 mL of distilled water.

An ammonium molybdate solution at a concentration of 2.5% was formulated by solubilizing 25 g of the compound in 200 mL of distilled water. The solution was mixed with 520 mL of 10 N H_2SO_4, and the final volume was equilibrated to 1 L using distilled water. The resultant reagent was stored in an amber-colored container to prevent photodegradation.

The 1-amino-2-naphthol-4-sulfonic acid (ANSA) reagent was prepared through a multi-step protocol involving the solubilization of bisulfite and sodium sulfite amalgamation with ANSA powder. The reagent was subsequently stored in a refrigerated setting in an amber-colored bottle.

A 10% trichloroacetic acid solution was formulated by solubilizing 10 g of the compound in 100 mL of distilled water. To quantify phosphate, aliquots of the standard phosphorus solution were dispensed into a series of tubes, and the volume was adjusted to 1 mL. To each tube, including the blank, sequential addition of 0.4 mL of 10% trichloroacetic acid, 0.4 mL of ammonium molybdate solution, 0.2 mL of ANSA reagent, and 4 mL of double-distilled water was done. After rigorous agitation, the tubes were allowed to stand for 5 minutes to facilitate the development of a blue-colored complex. The solutions were subsequently transferred to cuvettes, and their optical densities were measured at a wavelength of 640 nm.

2.4 Enzymatic assay of acid and alkaline phosphatase from heavy metal tolerant PSB isolates

We assayed acidic and alkaline phosphatase enzymes of bacterial isolates. An initial volume of 10 mL of aseptic Pikovskaya's broth was dispensed into a 20 mL sterile test tube. These tubes were then inoculated with the designated bacterial strains and incubated at 37°C for up to 96 hours. Periodic sampling was conducted at 24 hour intervals, followed by centrifugal separation at 5000 rpm for 10 minutes at 4°C. The ensuing cell-free supernatant was subsequently analyzed through an enzymatic assay to quantify the activities of unrefined acidic and basic phosphatases. The assay protocol was adapted from the methodology delineated by Bergmeyer, et al. [20].

The enzymatic evaluation was designed to measure the activities of acidic and alkaline phosphatases, considering their specific pH optima, thereby providing a nuanced understanding of their functional roles under diverse physiological states. A reaction mixture was formulated for the acidic phosphatase assay, comprising 0.5 mL of 100 mM acetate buffer at a pH of 4.8, fortified with 0.01M $MgCl_2$, along with 0.1 mL of the enzymatic source. In contrast, the alkaline phosphatase assay employed a reaction mixture of 0.5 mL of 100 mM glycine-NaOH buffer at a pH 8.0, augmented with 0.01M $MgCl_2$ and 0.1 mL of the enzymatic source. These mixtures were allocated into three distinct tubes labeled as the experimental, control, and reagent blank. The tubes were subsequently equilibrated in a water bath maintained at 37°C.

Post-equilibration, 0.5 mL of 15.2 mM p-nitrophenyl phosphate (PNP) substrate, pre-equilibrated at 37°C, was added to the experimental tube. The enzymatic reaction was permitted to ensue for 10 minutes, after which it was halted by incorporating 4 mL of 0.1N NaOH. Simultaneously, a time-zero control was established by adding the substrate after the quenching agent in the control tube. The reagent blank underwent a similar treatment as the experimental tube, albeit with the respective buffer replacing the PNP substrate. Spectrophotometric assessments were then carried out at a wavelength of 410 nm, and the readings were normalized to 100% transmission using the reagent blank. The absorbance values for control and experimental tubes were documented, and the concentration of p-

nitrophenol generated during the 10 minute incubation was ascertained using a millimolar extinction coefficient ($\varepsilon\lambda_{max}$) of 18.3 mM^{-1} cm^{-1}.

The enzymatic activities of acidic and basic phosphatases were subsequently computed as follows:

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

2.5 Effect of heavy metal tolerant PSB on tomato seed germination

In this study, tomato seeds (variety Azad T-5) were surface sterilized using a 6% hydrogen peroxide solution for five minutes, followed by thorough rinsing with distilled water five times. Subsequently, the surface sterilized seeds were primed for 30 minutes with heavy metal-tolerant PSB isolates maintained at one optical density (OD) growth. The inoculated seeds were then carefully positioned on water agar plates containing 0.1 – 10 mM heavy metals and incubated for three days. The seed germination rate was determined using the equation described below [21].

$$Germination\ rate\ (\%) = \frac{number\ of\ seeds\ germinated}{number\ of\ seeds} \times 100$$

3. Results and Discussion

3.1 Screening of potent PSB isolates for heavy metal tolerance and additional traits

The research led to the isolation of 40 distinct PSB isolates, possessing PSI between 2.11 and 4.33, highlighting the remarkable diversity within the cohort of PSB. Among these isolates, a select group of 16 showcased an additional trait of potassium solubilization with solubilizing index (KSI) values between 2.10 and 3.10, opening avenues for further exploration.

To fully appreciate the potential of these 16 PSB isolates, a rigorous screening process was initiated to test their survival ability against heavy metal stressors, specifically, $CdCl_2$, $HgCl_2$, $Pb(NO_3)_2$, and $ZnSO_4$. These heavy metals are notorious for deleterious effects on the environment. Remarkably, two standout isolates, D4 and G2, emerged against heavy metal toxicity. These resilient bacteria exhibited exceptional tolerance, as evidenced by their minimum inhibitory concentration (MIC) values. Isolate D4 displayed remarkable resistance with MIC values of 5 mM, 0.2 mM, 18 mM, and 12 mM, respectively, against $CdCl_2$, $HgCl_2$, $Pb(NO_3)_2$, and $ZnSO_4$. Similarly, isolate G2 demonstrated its mettle with impressive MIC values of 6 mM, 0.2 mM, 21 mM, and 11 mM for the same heavy metal stressors (**Table 1**). In particular, they displayed robust growth in low concentrations of $CdCl_2$ and $HgCl_2$, indicating their ability to thrive in environments with these heavy metals. However, their tolerance declined as the concentrations of these metals increased. Conversely, both isolates demonstrated remarkable tolerance for $Pb(NO_3)_2$, with their growth remaining unaffected up to 18 mM. Beyond this concentration, bacterial growth was inhibited. Interestingly, in the case of $ZnSO_4$, both D4 and G2 exhibited sensitivity, experiencing growth inhibition at concentrations exceeding 11 mM.

These findings provide valuable insights into the metal tolerance profiles of these PSB isolates, which are crucial for understanding their potential roles in agricultural soil remediation and nutrient solubilization. It is worth noting that several PSBs have been reported to grow at high concentrations of heavy metals, a phenomenon attributed to both induced and intrinsic mechanisms, as well as other environmental factors [22,23]. This knowledge enhances our understanding of the multifaceted abilities of PSBs in agricultural microbiology.

3.2 Characterization and identification of heavy metal tolerant PSB isolates

The isolates D4 and G2 were Gram-negative and non-motile short rods. D4 had capsulation, while G2 was non-capsulated. The D4 colonies were medium round, convex, waxy, translucent, and waxy. While G2 had medium, round, entire, raised, off-white, and opaque colonies.

The list of biochemical tests conducted using the HiMedia® biochemical test kit for isolates is shown in **Table 2** (Sr. 1-11)**.** The tests reveal the unique biochemical profiles of the isolates. Isolate D4 was positive for four tests, whereas G2 tested positive for three. Out of the 11 tests, both the isolates utilized citrate, reduced nitrate, and produced catalase. The isolates were negative for ornithine utilization, phenylalanine deamination, H_2S production, starch hydrolysis, gelatin hydrolysis, and indole production. The isolate D4 was also a urease producer.

Table 2 also shows the hydrolysis tests of the two isolates on 21 sugars (Sr. 12-32). The sugar hydrolysis test revealed unique biochemical profiles of the isolates. Isolate D4 used 19 sugars, whereas G2 hydrolyzed 16 sugars. None of the isolates hydrolyzed inulin in the growth medium. Similar to our study, biochemical characterization through sugar hydrolysis tests on phosphate solubilizing *Lysinibacillus varians* and *Pseudomonas putida* [24], *Bacillus* sp. [25], *Pseudomonas* sp. [26], *Proteus vulgaris, Pseudomonas aeruginosa, Acinetobacter radioresistens* and *Pseudomonas aeruginosa* [27] have been conducted.

The antibiotic susceptibility of the isolates was also assessed based on their zone of inhibition (**Table 3**). D4 and G2 were sensitive to aminoglycosides, quinolones, fenicol, tetracycline, and fluoroquinolones but exhibited macrolide resistance. This resistance is likely mediated by ribosomal target methylation [28,29]. Intriguingly, D4 was resistant to the folate pathway class of antibiotics, while G2 was sensitive. Bacterial resistance mechanisms may include mutations that induce genes coding for antifolate-targeted enzymes and other cellular modifications [30].

Both isolates were resistant to ampicillin but sensitive to carbenicillin. D4 was susceptible to all tested cephalosporins, while G2 was selectively resistant. Remarkably, both isolates were resistant to β-lactam antibiotics but sensitive to β-lactam + β-lactamase inhibitors, suggesting the production of β-lactamase enzymes, which are neutralized by the inhibitors in the latter class of antibiotics [31,32]. These findings are consistent with previous studies on antibiotic resistance in soil bacteria [33].

The 16S rDNA partial sequencing of the isolates was performed. Based on the National Center for Biotechnology Information (NCBI) BLAST search results of the 16S rDNA sequences, the isolates D4 and G2 were identified as *Klebsiella pneumoniae* and *Enterobacter chuandaensis,* respectively. The 16S rDNA sequence of the two isolates was submitted to the National Center for Biotechnology Information (NCBI) database, and the accession numbers OL839331 for *Klebsiella pneumoniae* PHmD4 (D4) and OL839334 for *Enterobacter chuandaensis* PHmG2 (G2) were obtained.

3.3 PSB mediated bioavailability of inorganic phosphate in growth medium

In our study, the bioavailability of Pi is demonstrated to be enhanced by heavy metal-tolerant PSB isolates (**Fig. 1**). A consistent increase in the rate of phosphorus solubilization is observed, which exhibits an inverse correlation with the pH alterations in the growth medium. Specifically, after 96 hours of incubation, *K. pneumoniae* PHmD4 solubilized 4.30 μ moles mL^{-1} of phosphorus in Pikovskaya's broth, with a pH decline to 3.06. Concurrently, *E. chuandaensis* PHmG2 solubilized 3.51 μ moles mL^{-1} of phosphorus, resulting in a final pH of 4.07. These observations are in alignment with Kumar, et al. [34], who reported that metal-tolerant plant growth-promoting *Enterobacter sp.* reduced the pH of the growth medium from

7 to 2, thereby achieving a peak phosphorus solubilization of 229 mg L^{-1}. The literature further substantiates these findings by indicating that the secretion of low molecular weight organic acids, such as lactic, oxalic, and citric acids, by PSBs contributes to the observed pH decline and enhances phosphorus availability from sources like monocalcium phosphate and rock phosphate [35-38].

3.4 Acid and alkaline phosphatase activity of the heavy metal tolerant PSB isolates

Our study observed a consistent upsurge in the acid phosphatase activity of bacterial isolates *K. pneumoniae* PHmD4 and *E. chuandaensis* PHmG2 over the designated 96 hour observation period (**Fig. 2**). Specifically, *E. chuandaensis* PHmG2 exhibited an acid phosphatase activity of 21.09 U mL^{-1}, while *K. pneumoniae* PHmD4 reached 21.62 U mL^{-1} at the 96 hour mark.

Acid phosphatases are a heterogeneous group of enzymes that are either secreted as soluble periplasmic proteins or retained as membrane-bound lipoproteins. These enzymes dephosphorylate a wide range of organic phosphodiesters, including nucleotides and sugar phosphates, to obtain Pi and organic byproducts [39,40]. Intriguingly, these enzymes are localized in the bacterial cell wall and are encapsulated by exopolysaccharides. Although they do not directly interact with inorganic phosphate mediums, they can modulate the medium's pH by releasing various organic acids [41]. The enzymatic reactions facilitate the hydrolysis of phosphodiester or phosphoanhydride bonds, releasing inorganic phosphate (Pi) [42].

Our study corroborates previous findings that suggest a significant correlation between phosphatase activity and the pH of the culture medium [43]. Moreover, acid phosphatase activity has been proposed as a reliable indicator for organic phosphorus mineralization [44,45]. However, we observed a lag in enzyme activity in the later phase, aligning with the observations made by Xiao, et al. [46]. This delay could be ascribed to the substrate specificity exhibited by the phosphatase enzyme, a characteristic that may differ across various bacterial strains [42]. In soil microbiology, several bacterial genera, including *Rhizobium*, *Enterobacter*, and *Klebsiella*, have been reported to express significant levels of acid phosphatase [47,48]. These findings are consistent with our study, which also focuses on isolates from the Enterobacteriaceae family.

Turning our attention to alkaline phosphatase activity, *K. pneumoniae* PHmD4 and *E. chuandaensis* PHmG2 exhibited activities of 5.85 U mL^{-1} and 6.38 U mL^{-1}, respectively, after 96 hours (**Fig. 2**). The literature suggests that lowering of pH levels influence alkaline phosphatase activity due to the accumulation of secondary metabolites [49,50].

Overall, our study adds to the growing body of evidence that underscores the pivotal role of acid and alkaline phosphatases in the solubilization of organic phosphates. Since agricultural soils predominantly exhibit a pH range from acidic to neutral, acid phosphatases are likely to play a more significant role in phosphorus solubilization than their alkaline counterparts [48].

3.5 Effect of heavy metal tolerant PSB on tomato seed germination

The present investigation provides an in-depth analysis of the impact of varying concentrations of heavy metals—cadmium, mercury, lead, and zinc—on tomato (variety Azad T-5) seed germination. The study employs PSB isolates *K. pneumoniae* PHmD4 and *E. chuandaensis* PHmG2 as treatment agents.

As documented in **Table 4**, initial observations confirm the high viability of tomato seeds, with the uninoculated control displaying a 100% germination rate. However, the germination rates markedly decreased when subjected to elevated concentrations of Hg and Pb. In contrast, the application of bacterial isolates appears to mitigate the decline. Specifically, *K.*

pneumoniae PHmD4 enhanced germination rates in seeds treated with Cd, while Zn treatment combined with *E. chuandaensis* PHmG2 either maintained or improved the germination rates.

The study also categorizes the toxicity levels of the heavy metals on germination. In the control group, the toxicity follows the order of Hg > Cd > Pb > Zn. The order changes to Hg > Pb > Cd > Zn when seeds were treated with *K. pneumoniae* PHmD4 and *E. chuandaensis* PHmG2. These findings align with previous researches. Baruah, et al. [51] reported a similar trend in reduced germination rates in pea and tomato seeds, while Abusriwil, et al. [52] observed this in *Madia sativa*. Additional studies, such as those by Muhammad, et al. [53] and Ahemad [54], have also documented the inhibitory effects of high mercury and cadmium concentrations on seed germination.

The scientific literature also describes mechanistic insights into the inhibition of seed germination by heavy metals. Altered cell membrane permeability accelerates the breakdown of stocked nutrients in the endosperm [55], further supported by Mittal, et al. [56], who reported decreased enzymatic hydrolysis of starch. Elevated levels of heavy metals disrupt cellular homeostasis, induce oxidative stress, and alter the expression of antioxidant enzymes [57]. Hormonal and nutritional imbalances also plays a role in germination rates, as evidenced by Atici, et al. [58], who reported that Pb and Zn alter concentrations of abscisic acid and gibberellic acid in germinating chickpea seeds. Several studies show that metal-tolerant PSB stimulates plant growth in the presence of toxic concentrations of Cd^{+2} [59-61], Cr^{+6} [26,61,62] and [3], Cu^{+2} [3], Ni^{+2} [35], Pb^{+2} [59] and Zn^{+2} [3].

4. Summary

The study obtained two PSB isolates, D4 and G2, with potassium solubilizing and heavy metal tolerating abilities. The isolates' characteristics, biochemical profiles, and sugar hydrolysis capabilities were examined, leading to their identification as *K. pneumoniae* PHmD4 and *E. chuandaensis* PHmG2 based on 16S rDNA sequencing.

Moreover, the study explored the crucial role of two PSBs in enhancing phosphorus bioavailability in the growth medium, demonstrating a direct correlation between phosphorus solubilization and pH alterations. Furthermore, the study delved into the isolates' acid and alkaline phosphatase activities, shedding light on their contributions to phosphorus solubilization and emphasizing the significance of pH levels in this process. The observed enzyme activity trends were discussed concerning substrate specificity and previous studies on acid phosphatase expression in specific bacterial genera.

Additionally, the research extended its focus to the effect of heavy metal-tolerant PSBs on tomato seed germination. The results revealed the inhibitory effects of heavy metals on germination rates and the mitigating impact of bacterial isolates *K. pneumoniae* PHmD4 and *E. chuandaensis* PHmG2. Mechanistic insights into the inhibition of seed germination by heavy metals were discussed, emphasizing the importance of cellular homeostasis and hormonal imbalances.

This comprehensive study offers valuable insights into the isolation, characterization, and functional aspects of heavy metal-tolerant PSBs, with potential applications in agriculture. It highlights PSBs role in improving phosphorus bioavailability and mitigating heavy metal toxicity in plants, making it a noteworthy contribution to agriculture.

5. Conclusion

This research unveils avenues for utilizing PSBs in agriculture. The isolation and characterization of PSBs, particularly the heavy metal-tolerant isolates D4 and G2, provide a

valuable resource for enhancing phosphorus availability in agricultural soils. PSBs ability to solubilize phosphorus, modulate pH levels, and resist various antibiotics underscores their potential as beneficial soil microorganism. Identifying these isolates as *K. pneumoniae* PHmD4 and *E. chuandaensis* PHmG2 through 16S rDNA sequencing further solidifies their taxonomic classification, facilitating future research and application in sustainable agriculture practices.

Moreover, the study of PSBs and their influence on tomato seed germination in environments contaminated with heavy metals underscores their capacity to alleviate the detrimental impact of pollutants on plant development. The findings offer mechanistic insights into the inhibitory effects of heavy metals on seed germination and the ameliorating influence of PSBs, which could have significant implications for soil remediation and crop production in contaminated environments. Overall, this research contributes valuable knowledge to agriculture and underscores the potential of PSBs as biofertilizers and bioremediation agents, opening doors to further research and practical applications in sustainable agriculture.

6. Future prospects

This study on PSBs in agriculture holds significant promise for future research and practical applications. The isolation and characterization of heavy metal-tolerant PSBs, identified as *Klebsiella pneumoniae* and *Enterobacter chuandaensis*, offer valuable resources for enhancing phosphorus availability in soils and mitigating heavy metal toxicity in plants. Future research can delve into microbial consortia interactions, genomic analysis for genetic optimization, and field trials to validate their practical benefits. Investigating ecological impacts, crop-specific applications, and biotechnological innovations can further optimize PSBs' contributions to sustainable agriculture. Promoting awareness among farmers and agricultural stakeholders is crucial for bridging the gap between scientific knowledge and on-the-ground implementation, ultimately revolutionizing farming practices for improved soil health and global food security.

Acknowledgments

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

Authors Contributions

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

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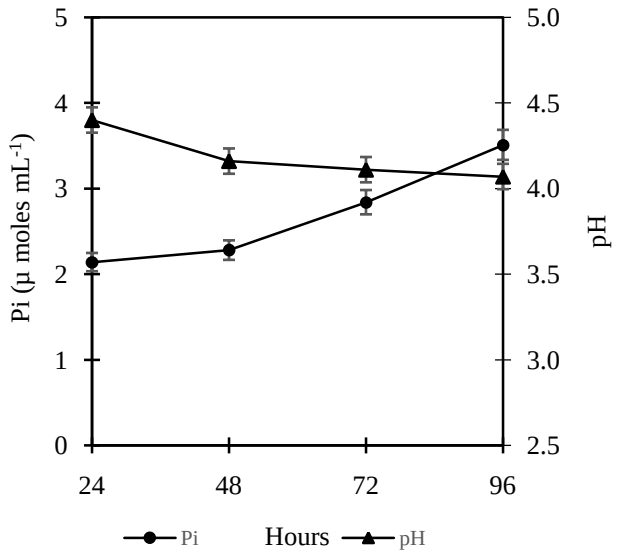
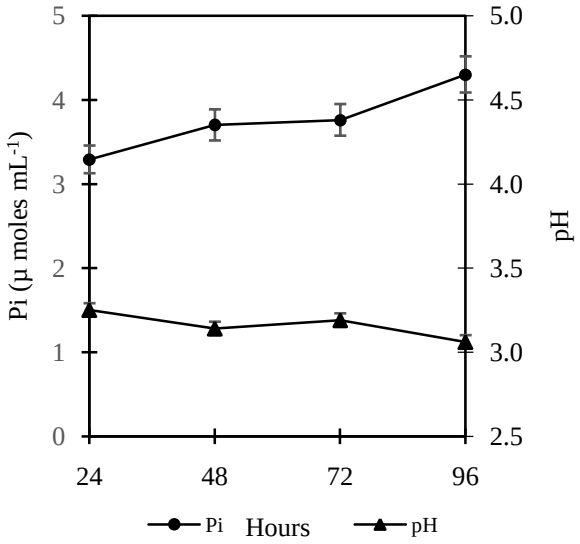


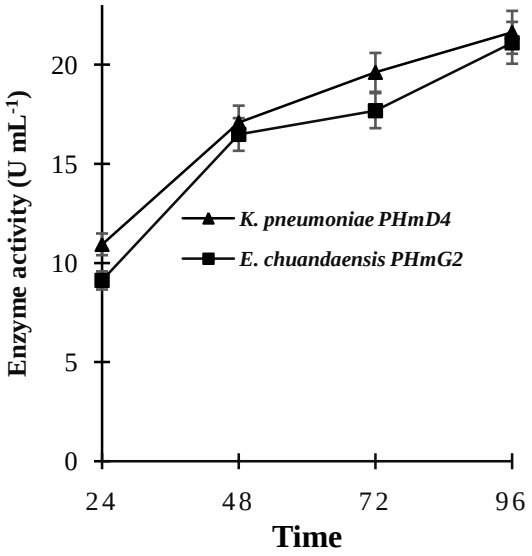
(i) *K. pneumoniae* PHmD4



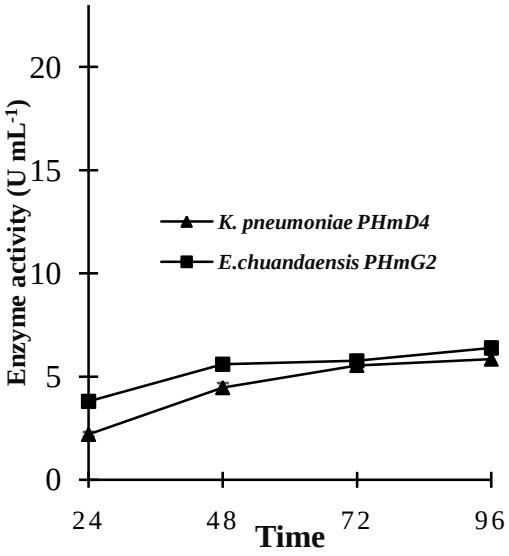
(ii) *E. chuandaensis* PHmG2

Fig: 1: Bioavailability of Pi favored by heavy metal tolerant PSB isolates in Pikovskaya's broth[*α]

***** Each value is a mean of three replicate
[α] Vertical bars represent SEm



Acid phosphatase activity



Alkaline phosphatase activity

Fig.: 2: Acid and alkaline phosphatase activity of heavy metal tolerant PSB isolates[*α]

***** Each value is a mean of three replicate
[α] Vertical bars represent SEm

Table 1: Growth of heavy metals tolerant PSB isolates under various concentrations of heavy metals

Heavy metal treatment (mM)		D4	G2	Heavy metal treatment (mM)		D4	G2
$CdCl_2$	1 mM	+	+	$HgCl_2$	0.1 mM	+	+
	2 mM	+	+		0.2 mM	+	+
	3 mM	+	+		0.3 mM	-	-
	4 mM	+	+		0.4 mM	-	-
	5 mM	+	+		0.5 mM	-	-
	6 mM	-	+		0.6 mM	-	-
	7 mM	-	-		0.7 mM	-	-
	8 mM	-	-		0.8 mM	-	-
	9 mM	-	-		0.9 mM	-	-
	10 mM	-	-		1.0 mM	-	-
$Pb(NO_3)_2$	11 mM	+	+	$ZnSO_4$	11 mM	+	+
	12 mM	+	+		12 mM	+	-
	13 mM	+	+		13 mM	-	-
	14 mM	+	+		14 mM	-	-
	15 mM	+	+		15 mM	-	-
	16 mM	+	+		16 mM	-	-
	17 mM	+	+		17 mM	-	-
	18 mM	+	+		18 mM	-	-
	19 mM	-	+		19 mM	-	-
	20 mM	-	+		20 mM	-	-
	21 mM	-	+		21 mM	-	-

+ = growth, - = non-growth

Table 2: The biochemical characterization of thermotolerant PSB isolates*

S. No.	Biochemical test	Isolate	
		D4	G2
1.	citrate utilization	+	+
2.	lysine utilization	+	-
3.	ornithine utilization	-	-
4.	urease production	+	-
5.	phenylalanine deamination	-	-
6.	nitrate reduction	+	+
7.	H_2S production	-	-
8.	starch hydrolysis	-	-
9.	gelatin hydrolysis	-	-
10.	indole production	-	-
11.	catalase	+	+
12.	adonitol	+	-
13.	arabinose	+	+
14.	cellulose	+	+
15.	dextrose	+	+
16.	dulcitol	-	+
17.	fructose	+	+
18.	galactose	+	+
19.	glucose	+	+
20.	inositol	+	+
21.	inulin	-	-
22.	lactose	+	-
23.	melibiose	+	+
24.	maltose	+	+
25.	mannitol	+	+
26.	mannose	+	+
27.	raffinose	+	+
28.	rhamnose	+	+
29.	sorbitol	+	-
30.	sucrose	+	+
31.	trehalose	+	-
32.	xylose	+	+

* Tests 1-11 were done through HiAssorted^{TM} biochemical test kit; 12-32 are sugar fermentation tests

Table 3: The antibiotic sensitivity tests of heavy metal tolerant PSB isolate

S. No.	Antibiotics	Antibiotic concentration ($\mu g\ mL^{-1}$)	Isolate zone of inhibition (mm)	
			D4	G2
1.	ampicillin	10	-	11^{r}
2.	cefotaxime	30	32^{s}	18^{r}
3.	cephalothin	30	22^{s}	18^{s}
4.	co-trimoxazole	25	11^{r}	18^{s}
5.	gentamicin	10	25^{s}	28^{s}
6.	nitrofurantoin	300	11^{r}	11^{r}
7.	norfloxacin	10	25^{s}	29^{s}
8.	cefoperazone	75	22^{s}	22^{s}
9.	amikacin	30	26^{s}	25^{s}
10.	piperacillin	10	11^{r}	19^{r}
11.	cefepime	30	30^{s}	33^{s}
12.	ciprofloxacin	5	27^{s}	27^{s}
13.	cefazolin	30	22^{s}	14^{r}
14.	streptomycin	10	23^{s}	23^{s}
15.	doxycycline	30	20^{s}	14^{s}
16.	moxifloxacin	5	24^{s}	26^{s}
17.	carbenicillin	100	22^{s}	24^{s}
18.	gatifloxacin	5	22^{s}	28^{s}
19.	tetracycline	30	24^{s}	24^{s}
20.	ampicillin/sulbactam	10	16^{s}	15^{s}

r-antibiotic resistance, s-antibiotic sensitivity

Table 4. Germination percentage of tomato seed treated with various concentrations of heavy metals and heavy metal tolerant PSB isolates*[α]

Heavy metal treatment	Concentrations (mM)	Germination percentage corresponding to bacterial treatments		
		Uninoculated control	*K. pneumoniae* PHmD4	*E. chuandaensis* PHmG2
$CdCl_2$	0.01	80.00±0.00	93.33±3.33	90.00±0.00
	0.10	80.00±0.00	86.67±6.67	90.00±0.00
	1.00	80.00±0.00	73.33±3.33	83.33±3.33
	10.00	60.00±0.00	63.33±6.67	70.00±0.00
$HgCl_2$	0.01	100.00±0.00	86.67±6.67	93.33±6.67
	0.10	100.00±0.00	36.67±8.82	40.00±0.00
	1.00	0	0	0
	10.00	0	0	0
$Pb(NO_3)_2$	0.01	100.00±0.00	90.00±0.00	90.00±0.00
	0.10	100.00±0.00	73.33±6.67	90.00±0.00
	1.00	100.00±0.00	66.67±6.67	83.33±3.33
	10.00	70.00±0.00	43.33±3.33	43.33±3.33
$ZnSO_4$	0.01	100.00±0.00	90.00±5.77	100.00±0.00
	0.10	100.00±0.00	83.33±3.33	100.00±0.00
	1.00	100.00±0.00	76.67±6.67	100.00±0.00
	10.00	90.00±0.00	73.33±3.33	96.67±3.33

* Non-bacterial and non-heavy metal treated seeds had 100% germination rates (not shown in table)

α results are presented as mean ± SEm of three replicates