**Research Article**

# Insoluble phosphate solubilisation and acid phosphatase activity of bacteria isolated from organic paddy soils

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## Abstract

An increase in phosphorus (P) availability through the addition of microbial inoculant, such as phosphate-solubilising bacteria (PSB), in organic agricultural soils, could be a good option for increasing soil fertility. We evaluated phosphate-solubilising (PS) ability and phosphate-mineralising (PM) ability of PSB isolated from organic paddy soils. Total soil bacteria were isolated on LB agar and tested on NBRIP agar for tricalcium phosphate (TCP) solubilisation. Three PSB strains were selected, based on their colonial morphologies on LB agar, for the measurement of PS ability in NBRIP broth, and PM ability in the presence of acid phosphatase substrate, *p*-nitrophenyl phosphate in culture broth. PSB were identified by morphology and a molecular technique using 16S rRNA gene. The results indicated that the selected PSB were able to solubilise TCP and presented acid phosphatase activity. The TCP solubilisation was performed via acid production in NBRIP broth. The strain Ca-106, which was closely related to *Bacillus* sp. based on the identity of 16S rRNA gene sequences, presented the TCP solubilisation of 181.57 mg P $l^{-1}$ which was higher than that in the previous report. The other strains (Ca-203 and Ca-270) solubilised TCP with the values of 195.48 mg P $l^{-1}$ and 180.46 mg P $l^{-1}$ respectively. They were closely related to *Enterobacter* sp., based on the identity of 16S rRNA gene sequences. The activity of extracellular acid phosphatase presented the values of 8.12 µM *p*NP $ml^{-1}$ $h^{-1}$, 7.41 µM *p*NP $ml^{-1}$ $h^{-1}$ and 4.40 µM *p*NP $ml^{-1}$ $h^{-1}$ for the strains Ca-106, Ca-203 and Ca-270, respectively. The high activity of acid phosphatase was observed for the selected PSB when compared to the previous reports that used the same technique. Taken together, the results indicated that all selected PSB had both PS ability via the acidification process and PM ability via the production of extracellular acid phosphatase. They can be potentially applied as microbial inoculants for increasing, especially, organic P mineralisation in organic agricultural soils. Further, the PSB strains merit a further study with a target plant, in pot experiments, in order to verify their ability to promote plant growth.

**Keywords:** Organic paddy soils, Phosphate-solubilising bacteria, Tricalcium phosphate solubilisation, Organic phosphorus mineralisation, Acid phosphatase activity

## Introduction

Phosphorus (P) is an indispensable macronutrient for plant growth. This nutrient is the component of many biomolecules in plant cells, especially, ATP which is used to store and transfer the energy in the cells. P also presents in biomolecules such as DNA, RNA, phospholipids and sugar phosphate. Additionally, numerous functions of the plant cells are regulated by phosphorylation/dephosphorylation processes, therefore the presence of P in the cells is strictly necessary. Despite its importance, P is the one of limiting nutrients for plant growth in agricultural soils due to the high fixation of this element in soil components [1, 2].

Naturally, plant can uptake P only in the form of orthophosphate ions ($HPO_4^{2-}$, $H_2PO^{4-}$) in soil liquid phase, which presents in the pores of soil particles, called soil solution [3]. As available forms of P contain negative charges, they are immobile nutrients that are strongly fixed with all soil components that have positive charges leading to low P concentrations which range from 0.1 to 10 µM in the soil solution [3]. In addition, P is adsorbed on clay mineral, aluminium oxides, iron oxides and humic acids. It can also form complexes with metal ions such as $Ca^{2+}$ and $Mg^{2+}$ in the soil solution of alkaline soils and $Al^{2+}$, $Fe^{2+}$ and $Fe^{3+}$ in the soil solution of acid soils. Depending on the pH of the soil solution, P can precipitate with the metals ions to form solid rock phosphates such as tricalcium phosphate (TCP), aluminium phosphate and iron phosphate becoming unavailable for plant uptake [3].

The unavailable forms of P in rock phosphates and biomolecules in living soil organisms, could be available for plant uptake via solubilisation process and mineralisation process. These processes require phosphate-solubilising microorganisms that can be categorized into phosphate solubilising-bacteria (PSB) and phosphate-solubilising fungi (PSF) [4-6]. PSB and PSF have the ability to secrete low molecular weight organic acids (LMWOAs) and the proton ($H^+$) leading to rock phosphate solubilisation [2, 4, 6]. In addition, some PSB and PSF can also secrete extracellular phosphatase which provide a mineralisation of organic P such as ATP, DNA, RNA that are accumulated in the soils after the death of soil organisms and during litter decomposition [7, 8]. Some PSB in the genera *Bacillus* and *Enterobacter* possess both phosphate-solubilising (PS) ability and phosphate-mineralising (PM) ability [7].

PSB can be used microbial inoculants to improve P uptake by plants [9-11], especially in organic agriculture (OA) which depends on natural nutrient cycling [12]. Phosphate bio-fertilizer has shown improving P uptake and has stimulated plant growth in many studies [9-11]. In the context of sustainable agriculture, OA has increased in recent years [13]. A decrease in crop production in OA farms in many regions of the world was reported to be due to the reduction of nutrient availability [14-16]. It was reported that a decrease in available P in the soils from OA farms [17-19] was due to the removing of P during the harvest [17]. Study on phosphate-solubilising microorganisms could be a good option to mobilise unavailable nutrients, especially immobile P in OA farms, by the ability of microbial inoculants such as phosphate bio-fertilizer in order to increase crop production. The aims of this study was to isolate and to screen PSB that have both PS ability and PM ability from organic paddy soils.

## Materials and methods

### 1. Bacterial isolates

Three bacterial isolates were from organic paddy soils located at Ban Chanote, Klong Yong-Lantakfa Community Enterprise in Lantakfa, Nakhon Chaisri district, Nakhon Pathom province, Thailand. They were tested for TCP solubilisation on National Botanical Research Institute's phosphate growth medium (NBRIP) agar. Bacteria were isolated on Luria–Bertani (LB) agar (10 g $l^{-1}$ peptone, 5 g $l^{-1}$ yeast extract, 5 g $l^{-1}$ NaCl, 15 $l^{-1}$ agar) following Jorquera and co-workers [20]. Then, PSB were screened on NBRIP agar (10 g $l^{-1}$ D-glucose, 5 g $l^{-1}$ $Ca_3$ $(PO_4)_2$, 5 g $l^{-1}$ $MgCl_2$ $6H_2O$, 0.25 g $l^{-1}$ $MgSO_4$ $7H_2O$, 0.2 g $l^{-1}$ KCl, 0.1 g $l^{-1}$ $(NH_4)_2SO_4$, 15 g $l^{-1}$ agar) [21]. The isolates that formed clear zones on NBRIP agar were considered as PSB. Among colonies, we selected three PSB isolates which were commonly found in the soils based on morphology identification, in order to characterise their ability for TCP solubilisation in NBRIP broth and their production of extracellular acid phosphatase.

### 2. Identification of phosphate-solubilising bacteria

In PSB identification procedures, the selected PSB isolates were grown on LB agar and incubated at 30°C for 24h. Bacterial colonies were observed. Shape, margin, elevation and color of colonies were noted. PSB cells were stained with Gram's method and observed under the microscope in order to study cell morphologies. In addition, a molecular identification was performed by amplifying and sequencing 16S rRNA genes with the universal bacterial primers 27F (5'-GAGTTTGATCCTGGCTCAG-3') and 1492R (5'GGTTACCTTGTTACGACTT-3'). The primers amplify V1-V9 regions located on 16S rRNA gene and generate an approximately 1500-bp product [22]. Total DNA of the selected PSB isolates was extracted with GeneJET Genomic DNA Purification Kit (Thermo Scientific) and DNA concentrations were measured by a Nanodrop (Thermo Scientific). Polymerase Chain Reaction (PCR) was performed with the following condition: initial denaturation at 95°C for 3 minutes, denaturation at 95°C for 30 seconds, annealing at 56°C for 30 seconds, extension at 72°C for 3 minutes (35 cycles) and final extension at 72°C for 10 minutes. PCR products were checked on 1% agarose gel electrophoresis at 100 voltages for 40 minutes. PCR products were purified using PCR purification Kit (Invitrogen). The concentrations of PCR products were verified by a Nanodrop (Thermo Scientific) and the PCR products were then sequenced. The nucleotide sequences of 16S rRNA genes were corrected using the HVDR online program [23]. The corrected sequences were compared with reference sequences in GenBank database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify PSB genera.

### 3. Measurement of phosphate-solubilising activity

Three strains of PSB were selected for the evaluation of PS ability. PS ability was performed in NBRIP liquid culture. The PS measurement was tested by following the protocol of Johri and co-workers [24] with modification. The selected strains were grown in LB broth until the OD600 reached 0.3-0.4. Bacterial cells were collected by centrifugation at 5000 rpm for 10 min and the pellets were washed twice with 0.85% NaCl. Bacterial cells were resuspended in 0.85% NaCl. The turbidity of bacterial cell suspension was compared by McFarland standards procedure [25]. Then, the OD625 was adjusted to 0.08-0.1 to

obtain the cell density of $10^8$ CFU/ml. Five milliliters of the bacterial suspension of each PSB strain were inoculated in 45 ml of NBRIP broth in four replicate flasks. The final bacterial cell density was approximately $10^7$ CFU/ml. The samples were incubated at 30°C and shaken at 180 rpm on an orbital shaker. Three milliliters of bacterial suspension were withdrawn from three flasks at 0h, 24h and 32h. After each withdrawal, one milliliter of bacterial suspension was centrifuged at 10000 rpm for 5 min. This fraction was used to quantify water soluble P (Pi) concentration by the method of malachite green assay [26]. The last flask that contained bacterial suspension was used for pH measurement with a pH meter for each time of sampling. PS ability was calculated from the difference between Pi concentrations at the end of the experiment and at the inoculation time. The uninoculated control was also performed using four replicate flasks by the same protocol.

**4. Measurement of phosphate-mineralising activity**

PM ability was the evaluation of the activity of acid phosphatase secreted by PSB under *in vitro* condition. The modified protocol of De Freitas and co-workers was used for this measurement [27]. Three strains of PSB were grown in NBRIP broth supplemented with 1.5 g $l^{-1}$ of TCP until exponential phase. The quantity of TCP was reduced to induce the production of extracellular phosphatase under low P condition. Bacterial cells were collected at the exponential growth phase. Then, they were inoculated in 50 ml of NBRIP broth supplemented with 1.5 g $l^{-1}$ of TCP in three replicate flasks. The final bacterial cell density was approximately $10^7$ CFU/ml. The samples were incubated at 30°C with shaking (180 rpm) on an orbital shaker. Four milliliters of bacterial suspension were withdrawn at 0h, 24h, 48h, 72h and 96h from each flask. Among them, three milliliters of bacterial suspension were centrifuged (5000 rpm, 15 min) and filtered through 0.2 µm pore size membrane filters. The fractions were stored at -20°C. These fractions contained the crude extract of the enzyme and it is called “the phosphatase fraction”. One milliliter of bacterial suspension was serially diluted and spreaded on LB agar. Then, bacterial colonies were counted incubating at 30°C for 24h. Bacterial growth curve was calculated to monitor the end-point of exponential growth of bacteria. The phosphatase fraction, at end-point of exponential growth (24h) was selected to perform the measurement of phosphatase activity.

The phosphatase fraction (0.075 ml) of each strain was added into a 0.2 ml sterile micro centrifuge tube and then, 0.24 ml of 0.1M Acetate buffer, pH6, was added. 0.06 ml (0.05M) of *p*-nitrophenyl phosphate (*p*NPP) was added into the reaction mixture. Three replicates were conducted for each PSB strain. An auto hydrolysis of phosphatase fraction was controlled by replacing 0.06 ml of *p*NPP with ultrapure water. Additionally, the auto hydrolysis of substrate was also tested by replacing 0.075 ml of phosphatase fraction with ultrapure water. All samples were incubated at 30°C for 0-30 min. The enzyme reaction was stopped by adding 0.375 ml of 0.5N NaOH. The chromogenic product (yellow color) was measured at an absorbance of 405 nm using Tabatabai and Bremner method [28], by a spectrophotometer (CE1011, Cecil Instruments Ltd). The standard curve was prepared with para-nitrophenol (*p*NP) solution at the concentrations of 0, 10, 20, 30, 40, 50µM. Phosphatase activity was calculated from the difference between the concentrations of *p*NP

produced during 0-30 min and expressed as μM *p*NP $min^{-1}$.

**5. Statistical analysis**

The statistical analysis was conducted with R software (version 2.11.1, The R Foundation for Statistical Computing). In the measurement of PS ability, three PSB strains were compared in order to verify their effects on TCP solubilisation. The concentrations of released total Pi during 0-32h were compared. For this, the normal distribution of data was verified using Shapiro-Wilk test ($\alpha$ = 0.05). The data that presented non-normal distribution was transformed using log transformation. Then, one-way ANOVA was used and followed by a post hoc Duncan's test ($\alpha$ = 0.05). Additionally, the same statistical tests were used to compare the significant difference of phosphatase activity and the cell density of three PSB strains in the measurement of PM ability.

## Results

**1. Insoluble phosphate solubilisation of PSB**

The identification of cultivable PSB (Ca-106, Ca-203 and Ca-270) is presented in Table 1. In morphological identification, the isolate Ca-106 formed colonies with round, undulate margin and pulvinate elevation on LB agar. The color of colonies was opaque white and the colonies had smooth surface. The cell morphology indicated that this isolate was a gram-positive rod. The molecular identification based on sequence of 16S rRNA gene indicated that the isolate Ca-106 was closely related to *Bacillus* sp. (GenBank Accession no. MW785756) with 99.68% identity. The isolates Ca-203 and Ca-270 exhibited similar colonial morphology on LB agar. They formed round colonies with entire margin and convex elevation. The color of colonies was creamy white and the colonies had smooth surface. The cell morphology indicated that they were gram-negative rods. The molecular identification showed that they were closely related to the genus *Enterobacter*. The isolate Ca-203 was closely related to *Enterobacter* sp. (GenBank Accession no. MT893375) with 98.10% identity. The isolate Ca-270 was closely related to *Enterobacter* sp. (GenBank Accession no. OL348061) with 97.04% identity (Table 1).

**Table 1** Colony morphologies, Gram stain and 16S rRNA gene sequence identities of PSB isolates.

| **Isolates** | **[a]Colony M** | **[b]Gram C** | **Mostly closely related organisms** | | |
|---|---|---|---|---|---|
| | | | **Species** | **GenBank Accession no.** | **Sequence identity (%)** |
| Ca-106 | C,U,P,OW,S | P, R | *Bacillus* sp. | MW785756 | 99.68% |
| Ca-203 | C,E,Co,CW,S | N,R | *Enterobacter* sp. | MT893375 | 98.10% |
| Ca-270 | C,E,Co,CW,S | N,R | *Enterobacter* sp. | OL348061 | 97.04% |

[a] Colony M: colony morphology on LB agar, C=circular form, U=Undulate margin, E=entire margin, P=pulvinate, Co=convex, OW=opaque white, CW=creamy white, S=smooth surface.

[b] Gram C: Gram stain and cell shape, P=Gram positive, N= Gram negative, R=rod shape.

The inoculation of PSB strains in NBRIP broth, supplemented with 5 g $l^{-1}$ TCP, at the final cell density of $10^7$ CFU/ml, indicated that they presented the ability to solubilise TCP. The mean values of Pi increased with the inoculation time for inoculated NBRIP broth when compared to uninoculated NBRIP broth (Table 2). The mean values of Pi, calculated from the difference between Pi concentrations at the end of the experiment and at the inoculation time, were 181.57 mg P $l^{-1}$, 195.48 mg P $l^{-1}$ and 180.46 mg P $l^{-1}$ for the strains Ca-106, Ca-203 and Ca-270, respectively. The concentrations of Pi were not significantly different ($p$=0.94) according to one-way ANOVA analysis (Table 2). The results indicated that the selected PSB strains had the equal ability for TCP solubilisation. The result of pH measurement exhibited the decreasing pH in inoculated NBRIP broth when compared to uninoculated NBRIP broth. In uninoculated NBRIP broth, the pH values were the same for the whole experiment, ranging from 6.31 to 6.46. The pH values of NBRIP broth inoculated with the strain Ca-106 were 6.23, 4.83 and 4.69 at 0h, 24h and 32h, respectively. The pH values of NBRIP broth inoculated with the strain Ca-203 were 6.35, 4.77 and 4.65 at 0h, 24h and 32h, respectively. The pH value of NBRIP broth inoculated with the strain Ca-270 were 6.38, 4.72 and 4.68 at 0h, 24h and 32h, respectively.

**Table 2** Quantity of P (mg P $l^{-1}$) in NBRIP broth supplemented with 5 g $l^{-1}$ of TCP.

| **Strains** | **Time (h)** | | |
|---|---|---|---|
| | **0** | **24** | **32** |
| Control | 21.12 (1.29)e | 19.79 (0.62)e | 21.76 (0.30)e |
| Ca-106 | 22.75 (2.74)e | 177.12 (12.53)cd | 204.31 (15.50)ab |
| Ca-203 | 26.57 (1.64)e | 190.63 (11.02)bc | 222.00 (6.11)a |
| Ca-270 | 17.70 (2.01)e | 171.56 (13.10)d | 198.17 (6.73)b |

The data are the averages of three replicates. The values in brackets are the standard deviations. The control was uninoculated NBRIP broth. Different letters mean statistically significant differences at $p < 0.05$.

**2. Phosphatase activity of PSB**

In the measurement of PM ability, under *in vitro* condition, all selected PSB strains: Ca-106, Ca-203 and Ca-270 grew well in NBRIP broth supplemented with 1.5 g l-1 of TCP at 30 ° C. The initial cell densities were not significantly different among the selected strains ($p$-value=0.09). The inoculation of the PSB strains at $10^7$ CFU/ml indicated that all strains reached the stationary phase in one day. At the inoculation time (0h), the statistical analysis indicated that the cell densities were not different with the $p$-value of 0.09 (Figure 1A). The acid phosphatase fraction was collected at the end of exponential phase (24h) and it revealed that all strains presented acid phosphatase activity when adding *p*NPP as a substrate (Figure 1B). The auto hydrolysis of the phosphatase fraction of each PSB strain was performed by replacing *p*NPP with ultrapure water. The result indicated that this auto hydrolysis was varied and depended on PSB strains. The auto hydrolysis of the stains Ca-106, Ca-203 and Ca-

270 presented the values of 9.77, 12.14, 7.66 µM *p*NP $ml^{-1}$ $h^{-1}$, respectively. These values were used to normalize the acid phosphatase activity by subtracting from the values obtained from each sample when *p*NPP was added. The autohydrolysis of the substrate showed that *p*NPP did not release *p*NP during 30 min of incubation at 30 ° C. The statistical analysis showed that the hydrolysis of *p*NPP was significantly different depending on PSB strain in 30 min of incubation at 30 ° C (*p*-value=0.009). The high activity of acid phosphatase was observed for the stains Ca-106 and Ca-203 that were 1.85 times and 1.68 times higher than that of the strain Ca-270. The strains Ca-106 and Ca-203 exhibited the phosphatase activity of 8.12 µM *p*NP $ml^{-1}$ $h^{-1}$and 7.41 µM *p*NP $ml^{-1}$ $h^{-1}$, respectively. In addition, the strain Ca-270 displayed the phosphatase activity of 4.40 µM *p*NP $ml^{-1}$ $h^{-1}$ (Figure 1B).

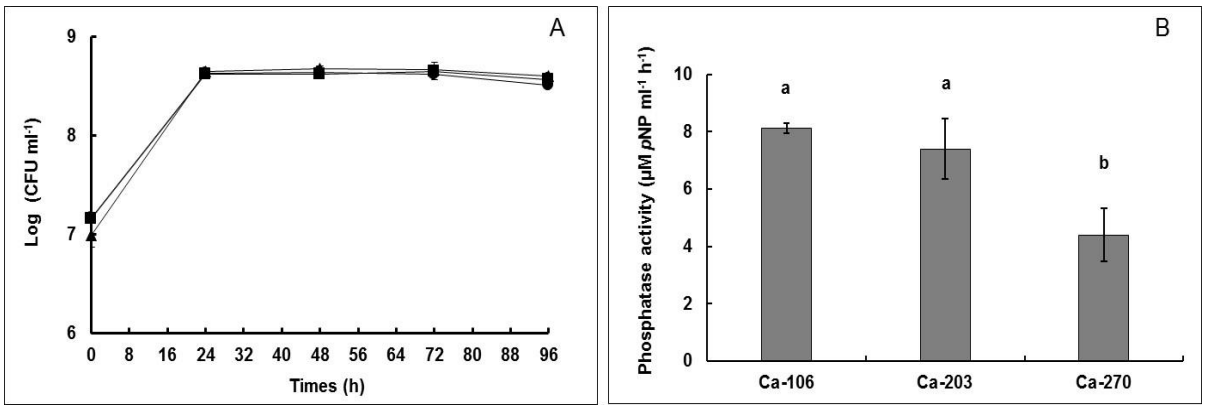


**Figure 1** Bacterial growth curve in NBRIP broth supplemented with 1g $l^{-1}$ TCP at different time intervals. Mean values (n=3) were plotted against times. Black triangle represented the strain Ca-106, black circle represented the strain Ca-203 and black square represented the strain Ca-270 (A). Graph bars show mean values of *in vitro* phosphatase activity (n=3) of the phosphatase fraction collected at 24h. Different letters indicated the differences of mean values (ANOVA, $p<0.05$) (B).

## Discussion

### 1. Phosphate-solubilising ability of PSB

It was reported that the decreasing of pH was the general trend in TCP solubilisation. The kinetic of TCP solubilisation and the release of Pi was concomitant with a decrease in pH in the liquid medium. Acidification in NBRIP broth was due to the secretion of LMWOAs and the proton ($H^+$) into the medium to solubilise insoluble TCP and release Pi [2, 6, 8]. The result of PSB identification indicates that the strain Ca-106 was closely related to *Bacillus* sp.. Li and co-workers reported that *B. flexus* could solubilise TCP at the value of 15 mg P $l^{-1}$ [29] which was twelve times lower than that of our strain. In addition, *B. flexus* secreted LMWOAs such as gluconic acid, citric acid, oxalic acid, malic acid, tartaric acid and hydroxypropionic acid [30]. The strains Ca-203 and Ca-270 belonged to the genus *Enterobacter.* According to the previous reports, the bacteria in the genus *Enterobacter* were PSB [31]. It was reported that *E. ludwigii* could solubilise TCP at 866 mg P $l^{-1}$ and produced citric acid, lactic acid and acetic acid [32]. The amount of Pi was four times higher than that of our strain. *E. tabaci* presented the amount of TCP solubilisation at 225 mg P $l^{-1}$ and produced lactic acid tartaric acid and quinic acid [33]. The amount

of Pi was also higher than our strain. Hinsinger and co-workers reported that the PS ability of PSB depended on the type of LMWOAs and their concentrations which varied among bacterial species [2]. In our research, the PS ability of selected PSB strains was not significantly different. The result suggests that even if PSB strains produced different LMWOAs at different concentrations, they might present the equal PS ability. In addition, to confirm this hypothesis, the research should be conducted to examine the type and the concentration of LMWOAs produced by the selected PSB. Besides the proposed hypothesis, the measurement of PS ability was not included the assimilation of Pi in NBRIP broth by PSB strains. The limitation of the method might has an effect on Pi concentration remained in the medium, leading to the equal PS ability in this study.

**2. Phosphate-mineralising ability of PSB**

The selected PSB strains presented phosphatase activity. This result is in agreement with the review of Hayat and co-workers which highlight that bacteria in the genus *Bacillus* and *Enterobacter* presented both PS ability and PM ability [7]. The PM ability of these bacteria in our study is associated with acid phosphatase production because the pH of the enzyme reaction was settled at 6. De Freitas and co-workers who used the same protocol as our research, to measure acid phosphatase activity, reported that the acid phosphatase activity of some species of *Bacillus* and *Xanthomonas* ranged from 0.15-0.84 μM *p*NP $ml^{-1}$ $h^{-1}$ [27] which were four to eight times lower than those of our PSB strains. However, the selected PSB in our study were not the same species as in their research. There is still no report on the quantity of *p*NP released from the molecule of *p*NPP using the crude extract of the extracellular acid phosphatase from the genera *Bacillus* and *Enterobacter*. The high acid phosphatase activity, observed in the PSB strains, could be a valuable data to select a microbial inoculant used in OA soils, especially, in an acidic soil, where the activity of the enzyme might be optimized.

## Conclusions

This research was able to verify that the selected PSB presented both PS ability and PM ability. They solubilise TCP via the acidification process and mineralise *p*NPP via extracellular acid phosphatase production. The strain Ca-106, which was closely related to *Bacillus* sp., presented higher PS ability than this species in the previous report. However, PS ability of the strains Ca-203 and Ca-270, which were closely related to the genus *Enterobacter*, was relatively low in our study. The most effective strains for acid phosphatase activity are the strains Ca-106 and Ca-203. In our study, the acid phosphatase activity was higher than the previous report. These selected PSB may have a potential to use as microbial inoculants for increasing organic P availability in OA farms, especially, in an acidic soil.

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