Identification of Phosphate-solubilizing Microorganisms and Determination of Their Phosphate-solubilizing Activity and Growth-promoting Capability

Ying-Ying Wang,[a] Pei-Shan Li,[a] Bi-Xian Zhang,[c] Yan-Ping Wang,[a] Jing Meng,[a] Yun-Fei Gao,[c] Xin-Miao He,[b,c,*] and Xiao-Mei Hu [a,*]

Phosphate-solubilizing microorganisms have been considered as a novel alternative approach to provide phosphate fertilizers that promote plant growth. In this study, three strains were isolated and identified as *Penicillium oxalicum* FJG21, *Penicillium oxalicum* FJQ5, and *Bacillus subtilis* BPM12, with a relatively high phosphate-solubilizing activity. Various phosphate sources were investigated, and $Ca_3(PO_4)_2$ was identified as the effective phosphate source. Factors governing the phosphate-solubilizing activity of the strains included carbon and nitrogen sources, initial pH, and fermentation time. A high soluble phosphorus content was achieved with 529.0 μg·mL^{-1}, 514.0 μg·mL^{-1}, and 330.7 μg·mL^{-1} for *Penicillium oxalicum* FJG21, *Penicillium oxalicum* FJQ5, and *Bacillus subtilis* BPM12, respectively. An inverse correlation of the quantity of soluble phosphorus content and the pH value of the medium was observed. In addition, *Bacillus subtilis* BPM12 displayed a prominent capability of producing indole acetic acid. *Penicillium oxalicum* FJG21 and *Penicillium oxalicum* FJQ5 exhibited high cellulase activities. These phosphate-solubilizing microorganisms with good phosphate-solubilizing capability and growth-promoting ability are the promising strains for agricultural utilization.

Keywords: Phosphate-solubilizing microorganisms; $Ca_3(PO_4)_2$; Indole acetic acid; Cellulase

*Contact information: a: College of Life Science, Northeast Agricultural University, Harbin, 150030, China; b: Key Laboratory of Combining Farming and Animal Husbandry, Ministry of Agricultural and Rural Affairs, 150086, P. R. China; c: Heilongjiang Academy of Agricultural Sciences, Harbin, 150086, China; *Corresponding authors: huxiaomei1982@163.com;13895777337@163.com*

INTRODUCTION

Phosphorus is one of the most essential nutrients for plant growth and development. It exists in soil as mineral salts or is incorporated into organic compounds. Although these phosphorus compounds are abundant in agricultural soils, most of them occur in an insoluble form, which is less available to plants (Miller *et al*. 2010). Therefore, large amounts of soluble phosphate fertilizers are widely applied to increase the agricultural production. However, over 15 million tons of phosphate fertilizer is applied worldwide every year, of which up to 80% is lost as insoluble forms (Gyaneshwar *et al.* 2002). This is because the soluble phosphorus that is applied to soil is quickly transformed into insoluble forms by combining with metal ions such as calcium (Ca^{2+}), aluminum (Al^{3+}), and iron(Fe^{3+}) (Sati and Pant 2018). The excess application of phosphate fertilizer also causes environmental problems, leading to the phosphorus pollution resulting from soil erosion and water runoff (Zeng *et al.* 2016).

In current years, phosphate-solubilizing microorganisms (PSMs) have been considered as a novel alternative approach to provide phosphate fertilizers that promote plant growth. A variety of PSMs, such as *Aspergillus* (Li *et al.* 2016), *Penicillium* (Efthymiou *et al.* 2018), *Pseudomonas* (Linu *et al.* 2019), *Burlkholderia* (Hsu *et al.* 2015), *Acinetbacter*, *Pantoea*, and *Bacillius* (Almoneafy *et al.* 2014), have been found capable of transforming the insoluble phosphates into their soluble forms in the soil through the process of acidification, chelation, and exchange reactions. A correlation between pH and soluble phosphorus has been found (Nahas 1996). It is generally accepted that a decrease in pH could cause the solid acidity and increase the phosphate solubilization. The PSMs can produce organic acids, such as gluconic acid, citric acid, oxalic acid, succinic acid, lactic acid, formic acid, and acetic acid, that convert $Ca_3(PO_4)_2$ into a bioavailable phosphate through protonation (Morales *et al.* 2011; Wei *et al.* 2016).

In addition to phosphate solubilization, PSMs may have other capabilities to promote plant growth, such as producing indole acetic acid, fixing nitrogen, and producing siderophore (Zaidi *et al.* 2009; Srinivasan *et al.* 2012). The PSMs are widely applied in increasing the yield of various crops such as rice (Bakhshandeh *et al.* 2017), wheat (Singh and Reddy 2011), maize (Vyas and Gulati 2009), and soybeans (Wang *et al.* 2007).

In this study, a series of PSMs were screened and identified. Their phosphate-solubilizing activities and other growth-promoting capabilities were investigated. Environmental factors, including carbon and nitrogen sources, phosphate sources, initial pH, and fermentation time were determined. A correlation between pH and soluble phosphorus was measured. This study will provide useful information on the application of PSM strains in practice.

EXPERIMENTAL

Methods

Sample collection

Soil samples were collected from the corn farm in the Jiagedaqi region of the Heilongjiang province, P.R. China (50°09’N,123°45’E) and stored in sealed, sterile bags at 4 °C (Singh *et al.* 2015). All chemicals used in the experiment were purchased from Aladdin Reagent Co., Ltd. (Shanghai, China).

Isolation of phosphate-solubilizing microorganisms

Distilled, sterile water (90 mL) at room temperature was mixed with 10 g of the soil sample for 30 min, which was then diluted. The resultant soil solution was plated on Pikovskaya's agar (PVK) medium that included glucose (10g), $(NH_4)_2SO_4$ (0.5 g), $MgSO_4 \cdot 7H_2O$ (0. 1 g), KCl (0.2 g), yeast extract (0.5 g), NaCl (0.2 g), $MnSO_4$ (0.002 g), $FeSO_4$ (0.002 g), Ca_3PO_4 (5 g), and distilled water (1000 mL) at pH 7.0, which was incubated at 30 °C for 7 days (Nautiyal 1999).The strains with clear halo zones were further studied.

Determination of phosphate-solubilizing activity

Next, 1 mL of the suspension was inoculated in 100 mL of the PVK medium using insoluble $Ca_3(PO_4)_2$ as a sole P source at pH 7.0, which was incubated at 30 °C on a rotary shaker at 150 rpm. Uninoculated PVK broth medium was used as a control. After 7 days, 5.0 mL of the solution was centrifuged at 10000 rpm for 10 min and passed through a 0.45-μm nylon filter. The quantitative measurement of phosphate solubilization was performed

on a UV-6100 spectrophotometer (Shanghai Metash Instruments Co., Ltd., Shanghai, China) at 700 nm based on a Mo-Sb colorimetry method (Guo *et al.* 2019) and was calculated according to the standard curve of KH_2PO_4. The pH value was analyzed by a pH meter (EL20; Mettler Toledo, Zurich, Switzerland). Each experiment was conducted in three triplicates. Organic acids in the culture medium were analyzed by a Waters 2489 high performance liquid chromatograph (HPLC; Waters Technology Co., Ltd., Milford, MA, USA) using a ZORBAX SB-Aq 250 mm × 4.6 mm column (Agilent Technologies Inc., CA, USA). The mobile phase consisted of 0.01 mol·L^{-1} KH_2PO_4 and 1% phosphoric acid with a flow rate of 1 mL·min^{-1}. Organic acids were detected by monitoring absorbance at 210 nm using an ultraviolet (UV) detector (Waters 2498; Waters Technology Co., Ltd., Milford, MA, USA).

Molecular identification of microorganisms

A DNA extraction of the isolates was conducted following the procedure specified by the manufactures of a bacterial DNA extraction kit (Omega Bio-tek, Inc., Morgan Hill, CA,USA) and a fungal DNA extraction kit (Omega Bio-tek, Inc., Morgan Hill, CA, USA). A 16S rDNA fragment was amplified by polymerase chain reaction (PCR) with 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'-GGT TAC CTT GTT ACG ACT T-3'). An internal transcribed spacer (ITS) rDNA fragment was amplified by ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCG CCT TAT TGA TAT GC-3'). The PCR was a 50 μL system, including template DNA 2 μL, forward primer 2 μL,reverse primer 2 μL, 2 × mastermix 25 μL, and DdH_2O 19 μL (Tiangen, Beijing, China). The conditions for PCR were as follows: 95 °C for 5 min in initial denaturation, 35 cycles of 95 °C for 30 s, 55 °C for 35 s, 72 °C for a 2 min denaturation annealing and extension, and 72 °C for a 10 min final extension of the amplified DNA. The PCR products were checked for the expected size on 1% agarose gel and were sequenced at Huada Gene Company (Beijing, China). The sequences were compared against the GenBank database using the NCBI BLAST program. Phylogenetic trees were constructed using MEGA 5.0 software (National Institutes of Health, Bethesda, MD, USA). The sequences were deposited into GenBank and the accession numbers were obtained.

Analysis of indole acetic acid production

Indole acetic acid (IAA) production of PSMs was determined according to the method of Gordon and Weber (1951) with some modifications. The strain was incubated in a potato dextrose agar (PDA) medium for fungi and a Luria-Bertan (LB) medium for bacteria supplemented with 2mg·mL^{-1} of tryptophan at 30 °C for 6 days. Uninoculated PDA or LB liquid medium was used as a control. Each experiment was conducted in three triplicates. After that, the fermentation broth was centrifuged at 10000 rpm for 10 min. Then, 2 mL of the supernatant was mixed with 4 mL of Salkowski solution including 35% of $HClO_4$ and 0.5 mol·L^{-1} $FeCl_3$. The mixture was incubated in the dark at 40 °C for 30 min. Finally, IAA was measured by a spectrophotometric method (UV-6100; Shanghai Metash Instruments Co., Ltd., Shanghai, China) at 530 nm and was calculated from the standard curve of pure IAA (Asghar *et al.* 2002).

Analysis of siderophore production ability

Quantitative estimation of siderophores was performed based on the Chrome Azurol S (CAS) method (Schwyn and Neilands 1987). The strain was inoculated in an iron-deficient CAS liquid medium and incubated on a rotary shaker (ZQLY-108S; Shanghai

Zhichu Instrument Co., Ltd, Shanghai, China) at 150 rpm at 30 °C for 5 days. Next, the suspension was centrifuged at 10000 rpm for 5 min. Then, 1 mL of supernatant was then mixed with 1 mL of CAS detection solution (10 mM HDTMA, 1 mM $FeCl_3$ solution, 2 mM CAS solution). The absorption value was measured at the wavelength of 630 nm after 1 h of standing. Uninoculatediron-deficient CAS liquid medium was used as a control.

Analysis of cellulase activity

Fungi were cultured in a PDA liquid medium for 3 days. Bacteria were cultured in LB liquid medium overnight. Then, 1 mL of fermentation broth was inoculated into 100 mL of Hutchison medium (KH_2PO_4 1.0 g, $MgSO_4$ 0.3 g, peptone 2 g, NaCl 0.1 g, $CaCl_2$ 0.1 g, $FeCl_3$ 0.01 g, and corn straw 10 g) at 30 °C and 150 rpm for 5 days. The resulting solution was then centrifuged at 8000 rpm for 5 min at 4 °C to give the crude enzyme solution. Filter paper cellulase (FPase), endoglucanase (CMCase), and β-glucosidase (Kazeem *et al.* 2017) were determined according to the International Union of Pure and Applied Chemistry (IUPAC) standard (Ghose 1987). The FPase was assayed by incubating the 0.5 mL of suitably diluted enzyme with Whatman No. 1 filter paper (1.0 × 6.0 cm) containing 1.5 mL of sodium citrate buffer (pH 4.8) for 60 min at 50°C. The CMCase activity was determined using sodium carboxymethyl cellulose (CMC-Na, 1%, w/v) for 30 min at 50 °C. β-glucosidase activity was measured using salicin solution (0.5%, w/v) for 30 min at 50 °C. The reducing sugars were measured at 540 nm. One unit (U) of enzyme activity was defined as the amount of enzyme that released 1 μ mol of glucose per minute under the assay conditions.

RESULTS AND DISCUSSION

Isolation and Identification of PSMs

Initially, 18 strains with halo zones in PVK agar medium were isolated as the positive microbes, indicating their ability to solubilize phosphate. Two fungal isolates named FJG21 and FJQ5, and one bacterial isolate named BPM12 with clear halo zones were selected and determined for their phosphate solubilizing activity. The amount of soluble phosphate by these strains was evaluated based on the Mo-Sb colorimetry method (Guo *et al.* 2019). The results showed that all the strains could solubilize $Ca_3(PO_4)_2$ in quantities. The soluble phosphorus content of the strains FJG21, FJQ5, and BPM12 was originally obtained at 343.2 $\mu g \cdot mL^{-1}$, 339.2 $\mu g \cdot mL^{-1}$, 189.1 $\mu g \cdot mL^{-1}$, respectively.

Molecular Identification of PSMs

Molecular identification was conducted with MEGA 5.0 software using a neighbor-joining method. The phylogenetic trees are shown in Fig. 1. The fungi were identified based on ITS rDNA sequence. Sequence FJG21 showed 100% similarity with *Penicillium oxalicum* NRRL787 (NR121232), which was identified as *Penicillium oxalicum* FJG21. Sequence FJQ5 showed 100% similarity with *Penicillium oxalicum* NRRL 787 (NR121232), which was identified as *Penicillium oxalicum* FJQ5. The bacteria were identified based on 16S rDNA sequence. Sequence BPM12 showed 97.8% similarity with *Bacillus subtilis* DSMO (AJ276351), which was identified as *Bacillus subtilis* BPM12. The obtained nucleotide sequences were submitted to NCBI GenBank under accession No. MN055969, No. MN058027, and No. MN086884, respectively.

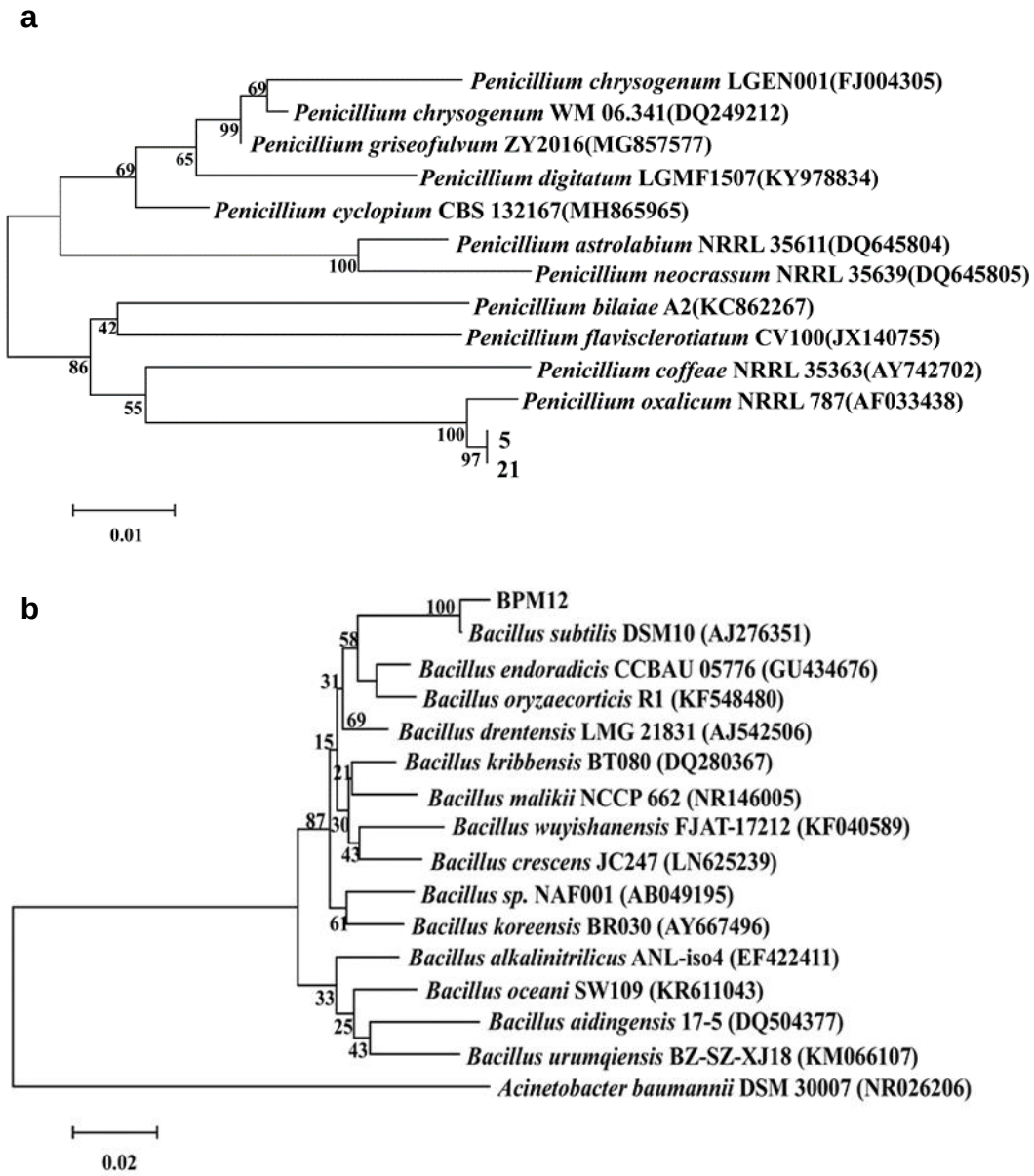


Fig. 1. The phylogenetic analysis: a: *Penicillium oxalate* FJG21 and *Penicillium oxalate* FJQ5 based on ITS rDNA sequence; b: *Bacillus subtilis* BPM12 based on 16S rDNA sequence

Carbon and Nitrogen Sources for the Phosphate-solubilizing Activity of the Strains

Various carbon sources were investigated for their effects on the insoluble phosphate solubilization at the concentration of 1% (w/v). As shown in Fig. 2, glucose and mannitol were the most effective carbon sources for the phosphate solubilization by all the strains. Specifically, more effective phosphate solubilizing activity was observed for *P. oxalicum* FJG21 with glucose (343.2 $\mu g \cdot mL^{-1}$) and mannitol (336.4 $\mu g \cdot mL^{-1}$) and *P. oxalicum* FJQ5 with glucose (339.2 $\mu g \cdot mL^{-1}$) and mannitol (332.9 $\mu g \cdot mL^{-1}$). *B. subtilis* BPM12 exhibited good phosphate-solubilizing ability with glucose (189.1 $\mu g \cdot mL^{-1}$) and mannitol (161.6 $\mu g \cdot mL^{-1}$). A similar result was obtained for *Penicillium sp.* PSM11-5, which was applied in a categorical experimental design to select glucose as the best carbon

source (Chai *et al.* 2011). In all cases, insoluble phosphate solubilization was accompanied by a noticeable pH decrease. The pH decrease of *P. oxalicum* FJG21 was from an initial 7.0 to 2.95 to 5.93, and the pH decrease of *P. oxalicum* FJQ5 was from an initial 7.0 to 3.33 to 5.78. The maximum phosphate-solubilizing activity was obtained with glucose as the carbon source for both *P. oxalicum* FJG21and *P. oxalicum* FJQ5 at pH 2.95 and pH 3.33, respectively. Similarly, the pH decrease of *B. subtilis* BPM12 was from an initial 7.0 to 4.25 to 6.02. The maximum phosphate-solubilizing activity was observed at pH 4.25 with glucose as the carbon source.

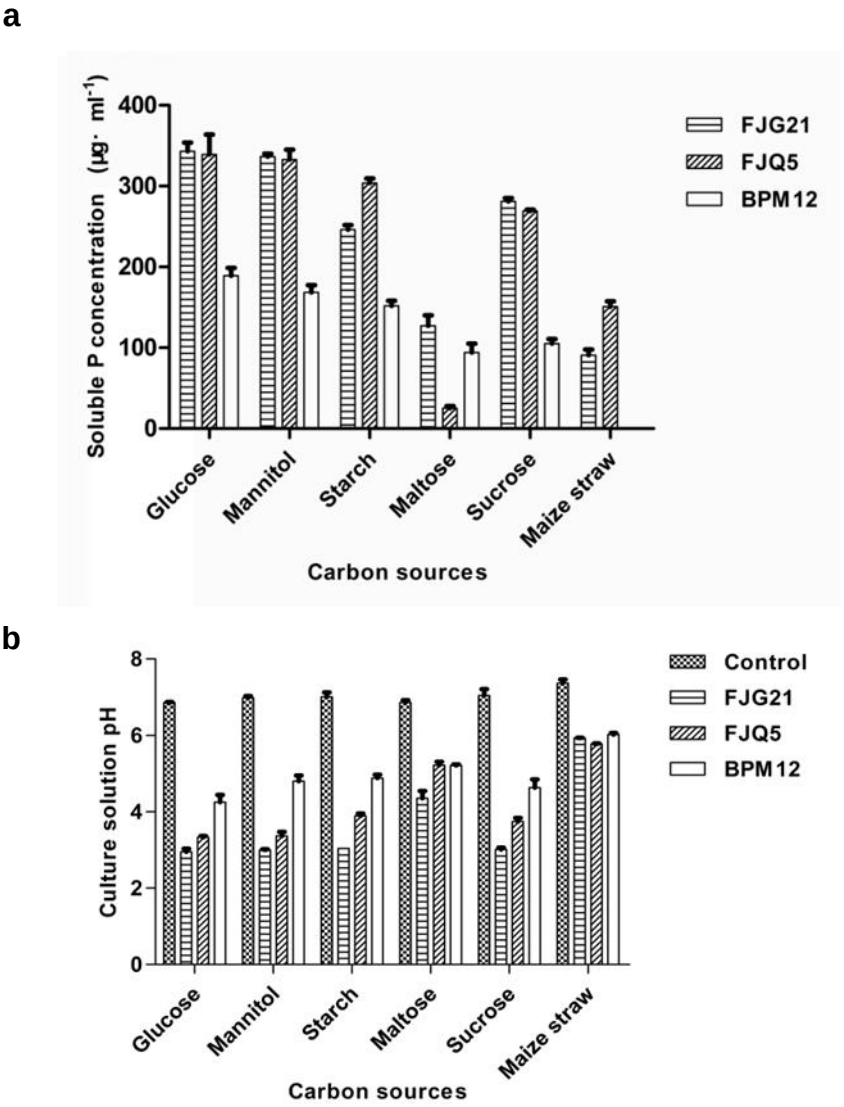


Fig. 2. Effect of carbon sources on the phosphate solubilizing activity: a: the phosphate-solubilizing activity of the strains; b: the correlation of pH value of the medium

Among the different nitrogen sources tested in the previous work, KNO_3 was the best nitrogen source for insoluble phosphate solubilization by *Aspergillus tubingensis* and their phenotypic mutants (Relwani *et al.* 2008). The best nitrogen source for *Penicillium* PSM11-5 and *Aspergillus aculeatus* was $(NH_4)_2SO_4$ (Narsian and Patel 2000; Chai *et al.* 2011). Various nitrogen sources were added separately to the medium at the concentration of 0.1% (w/v) to assess their effects on insoluble phosphate solubilization. As shown in

Fig. 3, yeast extract was the optimal nitrogen source for *P. oxalicum* FJG21 and *P. oxalicum* FJQ5 with a soluble phosphate content of 420.2 μg·mL^{-1} and 409.2 μg·mL^{-1}, respectively. Furthermore, $(NH_4)_2SO_4$ was the best nitrogen source for *B. subtilis* BPM12 with a soluble phosphorus content of 272.0 μg·mL^{-1}. No solubilization activity was detected with urea as the nitrogen source for *B. subtilis* BPM12. Meanwhile, the pH of the culture medium decreased notably as the insoluble phosphate solubilization was increased. The pH of *P. oxalicum* FJG21 and *P. oxalicum* FJQ5 were reduced from an initial 7.0 to 3.29 and 2.55 with yeast extract as the nitrogen source. The pH of *B. subtilis* BPM12 was decreased from an initial 7.0 to 4.2 when $(NH_4)_2SO_4$ was used.

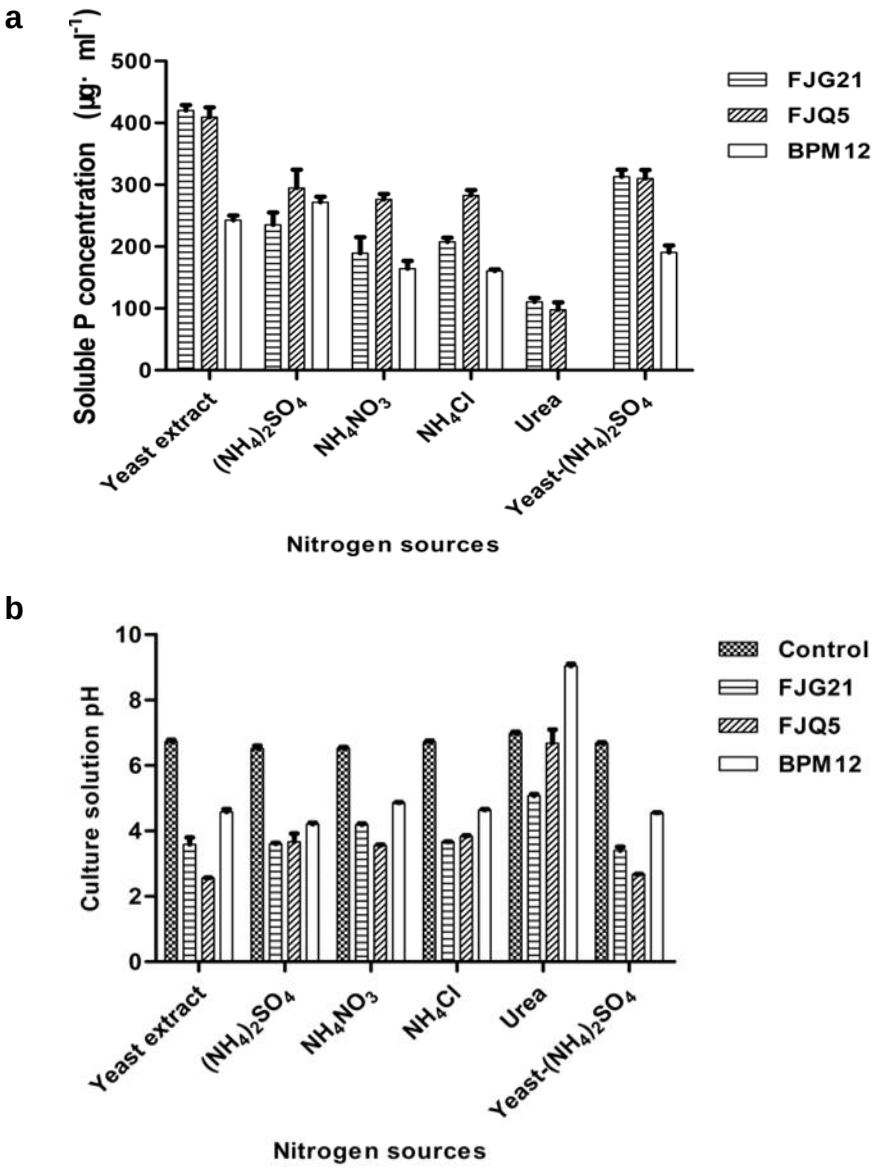


Fig. 3. Effect of nitrogen sources on the phosphate solubilizing activity: a: the phosphate-solubilizing activity of the strains; b: the correlation of pH value of the medium

Determination of the Capability of the Strains for Various Phosphate Sources

The use of PSMs could utilize insoluble phosphate sources and convert them into soluble phosphate forms. In this study, several phosphate sources were investigated at the concentration of 0.5% (w/v). As shown in Fig. 4, the solubilization of $Ca_3(PO_4)_2$, $CaHPO_4$, and hexacalcium by microbes was remarkably higher than $AlPO_4$ and $FePO_4$. All three strains had the strong capability to solubilize $Ca_3(PO_4)_2$. The soluble phosphorus content of the *P. oxalicum* FJG21, *P. oxalicum* FJQ5, and *B. subtilis* BPM12 was detected at 441.4 $\mu g \cdot mL^{-1}$, 439.9 $\mu g \cdot mL^{-1}$, and 276.3 $\mu g \cdot mL^{-1}$, respectively. However, none of the strains could solubilize $FePO_4$. Similarly, a distinct decrease of pH was obtained with the increased insoluble phosphate solubilization from initial 7.0 to 2.50 and 4.42 for various phosphate sources. When Ca_3PO_4 was used as the sole source of phosphorus, the lowest pH of the *P. oxalicum* FJG21, *P. oxalicum* FJQ5, and *B. subtilis* BPM12 was observed at 2.78, 2.50, and 4.00, respectively. Higher solubilization of $Ca_3(PO_4)_2$ and $CaHPO_4$ than iron phosphate and aluminium phosphate was also observed by Thakur *et al.* (2014).

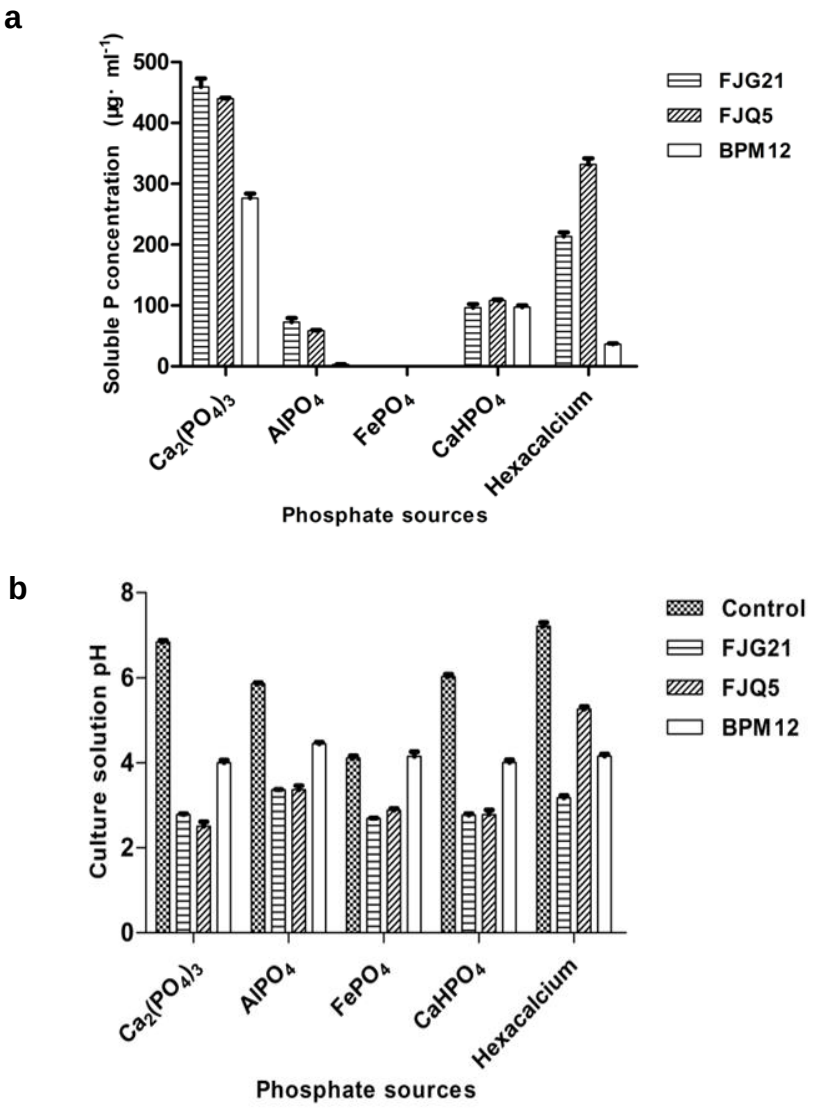


Fig. 4. The capability of the strains for various phosphate sources: a: the phosphate solubilizing activity of the strains; b: the correlation of pH value of the medium

Evaluation of Initial pH for Insoluble Phosphate Solubilization

The effect of initial pH on the phosphate solubility of the strains is illustrated in Fig. 5. When the initial pH was 5.0, the soluble phosphorus content of *P. oxalicum* FJG21 and *P. oxalicum* FJQ5 was achieved at 488.0 μg·mL^{-1} and 500.8 μg·mL^{-1}, respectively. However,the soluble phosphorus content of *B. subtilis* BPM12 was obtained at 299.5 μg·mL^{-1} at the initial pH of 6.0. A final pH range of 2.75 to 2.95 and 2.52 to 2.88 was observed for *P. oxalicum* FJG21 and *P. oxalicum* FJQ5, respectively. The final pH range of *B. subtilis* BPM12 was obtained with 3.94 to 4.53. A similar result was reported by Zhang *et al.* (2018). The pH of the fermentation broth of *Talaromyces aurantiacus* JX04 and *Aspergillus neoniger* JX16 changed from an initial pH of 1.5 to 6.5 to a final pH of 2.5 to 5.6 and 2.34 to 4.68. All the strains in this work possessed better phosphate solubility under acidic conditions.

Determination of Incubation Time for Insoluble Phosphate Solubilization

Initially, the longer incubation time was associated with an increase in soluble phosphorus content and with a decrease in pH in the medium. The maximum soluble phosphorus content was obtained at 529.0 μg·mL^{-1} for *P. oxalicum* FJG21 at pH 5.0 after 8 days and 514.0 μg·mL^{-1} for *P. oxalicum* FJQ5 at pH 5.0 after 6 days.

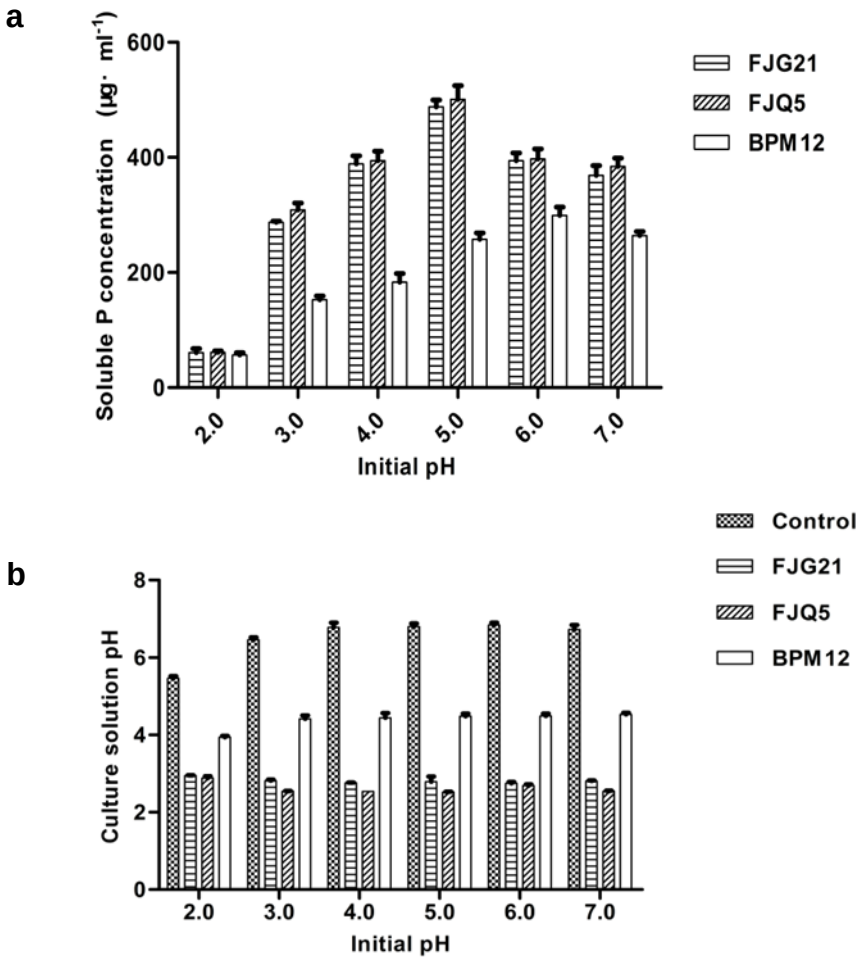
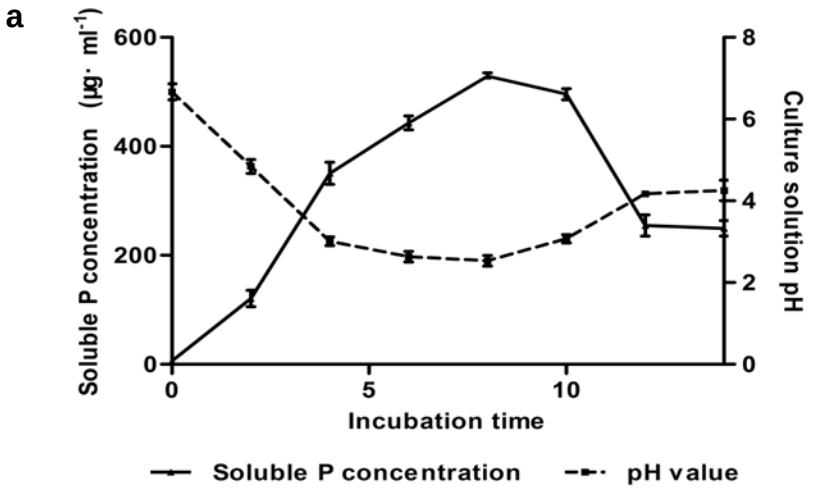


Fig. 5. Initial pH values for insoluble phosphate solubilization: a: the phosphate-solubilizing activity of the strains; b: the correlation of pH value of the medium

After incubation for 5 days, the soluble phosphorus content reached up to 330.7 μg·mL^{-1} for *B. subtilis* BPM12 at pH 6.0. The pH values showed an inverse correlation with the quantity of soluble phosphate. The largest drop in pH was accompanied with the highest phosphorus solubilization activity. However, with the further increase of culture time, the available phosphorus content decreased, and the pH value increased. As the fermentation time increased, the soluble phosphorus content improved. While furthering the extent of the incubation time, the soluble P content decreased because of the depletion of the nutrients in the culture solution. As reported, when the medium was inoculated for 5 days, *Burkholderia* SCAUKO309 achieved the maximum soluble phosphorus content (452 μg·mL^{-1}) at a minimum pH value of 3.12. After incubation for 7 days, the amount of dissolved phosphorus was 154 μg·mL^{-1} and the pH value of the medium was 4.95 (Zhao *et al.* 2014).

Analysis of Indole Acetic Acid Production of PSMs

Additionally, PSMs were examined for the production of plant growth-promoting substances, including indole acetic acid (IAA) and siderophore. As a result, *B. subtilis* BPM12 was found capable of producing IAA. No production of siderophore was found for PSMs in this work. In this study, *B. subtilis* BPM12 had the capacity to produce IAA with or without tryptophan as a precursor. As shown in Fig. 7, the production of IAA increased with the increasing tryptophan concentration in the medium. A high concentration of IAA was observed at 28.02 μg·mL^{-1}, when tryptophan was added at 10 g·L^{-1}. Several microorganisms, such as *Agrobacterium*, *Pseudomonas*, *Bacillus*, *Rhizobium*, and *Azospirillum*, are known to produce IAA (Mohite 2013; Mukhtar *et al.* 2017). The IAA was detected in quantities ranging from 2.7 to 31.8 μg·mL^{-1} from phosphate-solubilizing rhizobacteria (Jiang *et al.* 2018). Moreover, microbes, such as *Bacillus* Tp. 1B-7B and *Penicillium* Tp. 1F-5F, produced IAA, especially when growth media were supplemented with tryptophan, a precursor of IAA (Hassan 2017).



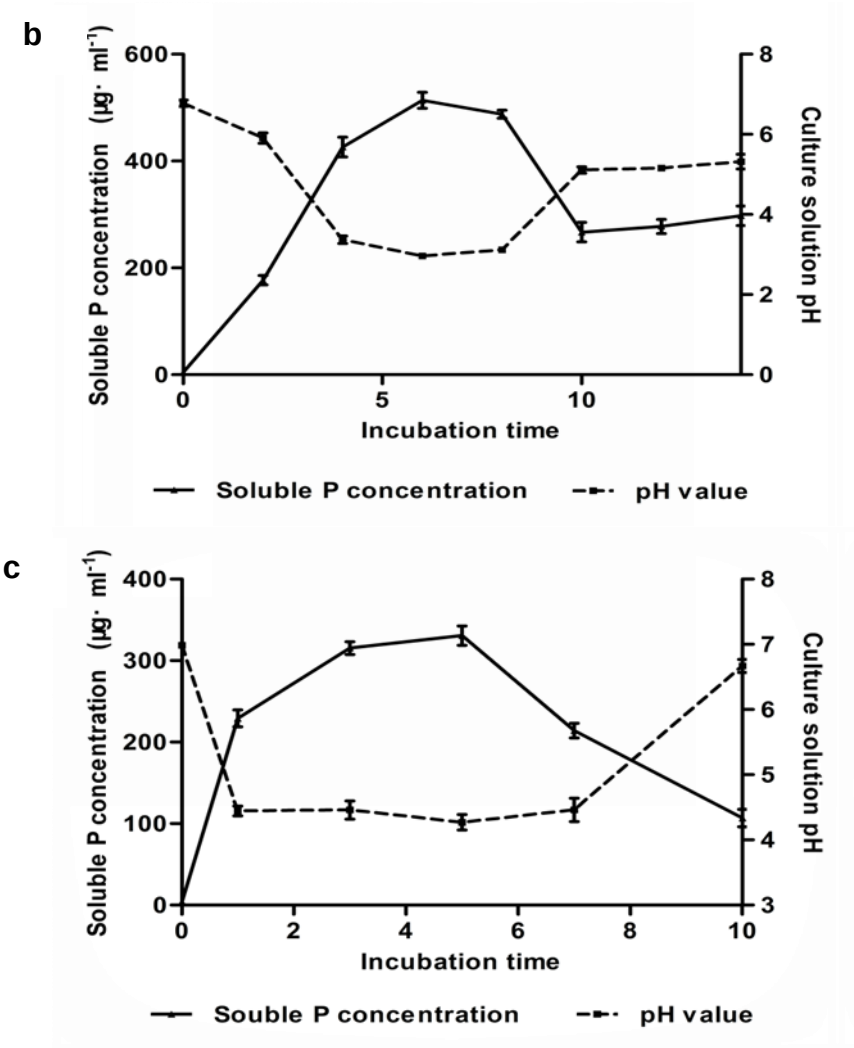


Fig. 6. Incubation time for insoluble phosphate solubilization and the correlation of pH value: a: *P. oxalate* FJG21, b: *P. oxalate* FJQ5, and c: *B. subtilis* BPM12

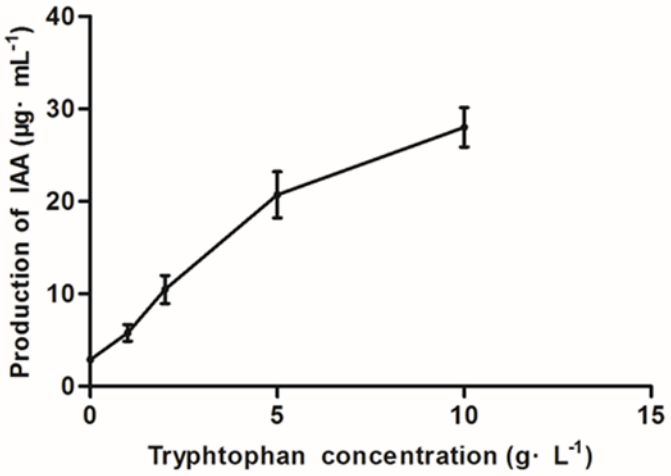


Fig. 7. Quantitative production of IAA with different tryptophan concentration

Analysis of Cellulase Activity of PSMs

A complete cellulase system is important to convert cellulose into monomeric sugars for the effective degradation of lignocellulosic biomass. In this study, enzymatic activities were observed and the results are illustrated in Fig 8. The cellulase activities of *P. oxalicum* FJG21 were achieved at 0.44 U·mL^{-1} (β-Gase), 0.08 U·mL^{-1} (CMCase), and 0.05 U·mL^{-1} (FPase). The cellulase activities of *P. oxalicum* FJQ5 were obtained at 0.25U·mL^{-1} (β-glucosidase), 0.09 U·mL^{-1} (CMCase), and 0.15 U·mL^{-1} (FPase). No cellulase activity was observed for *B. subtilis* BPM12. It has been stated that cellobiose accumulation would inhibit the cellulase activity; thus a high ratio of β-Gase to FPase could improve enzymatic hydrolysis of cellulose (Shah *et al.* 2015; Li *et al.* 2017). As reported, *Penicillium funiculosum* displayed remarkable enzymatic activity with FPase (0.354 U·mL^{-1}) and β-glucosidase (1.835 U·mL^{-1}) (Castro *et al.* 2010). *P. oxalicum* HC6 generated notable the following cellulase activity values: FPase (0.11 U·mL^{-1}), CMCase (0.21 U·mL^{-1}), and β-glucosidase (0.43 U·mL^{-1}) (Sun *et al.* 2018). In this study, *P. oxalicum* FJG21 and *P. oxalicum* FJQ5 exhibited a relatively high cellulase activity and a high ratio of β-Gase to FPase, which contributed to the enzyme hydrolysis of biomass. *P. oxalicum* FJG21 and *P. oxalicum* FJQ5 are potential strains for the effective degradation of biomass and the production of biofuel.

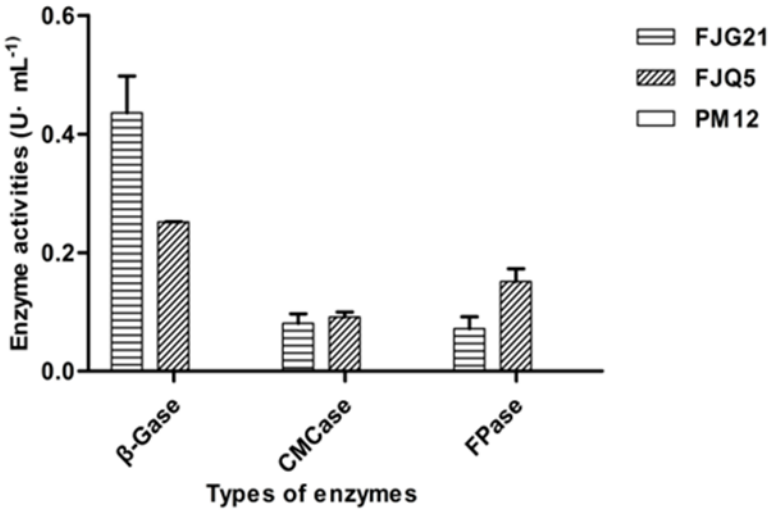


Fig. 8. Enzyme activities of PSMs

Discussion

A number of fungi and bacteria have been found to solubilize elemental phosphate from insoluble phosphate for plant growth such as *Penicillium, Aspergillus* (Li *et al.* 2016), *Pseudomonas*, *Bacillus*, *Burkholderia*, *Rhizobium*, *Agrobacterium*, *Micrococcus*, *Enterobacter*, and *Erwinia* (Anandham *et al.* 2007; Jha *et al.* 2008; Öğüt *et al.* 2011). Filamentous fungi, mainly *Penicilliums* including *Penicillium oxalicum* (Gong *et al.* 2014) and *Penicillium bilaii* (Gómez-Muñoz *et al.* 2018), are widely used to solubilize insoluble phosphates. In previous studies, six phosphate-solubilizing fungi were screened, including *Aspergillus awamori* and *Penicillum citrinum,* and their phosphate-solubilizing activity ranged from 38 to 760 μg·mL^{-1} (Mittal *et al.* 2007). Three phosphate-solubilizing bacteria

were isolated from the gut of earthworms with a stable phosphate-solubilizing activity of 222 μg·mL^{-1} (*Bacillus megaterium* PSB1), 213 μg·mL^{-1} (*Staphylococcus haemolyticus* PSB2), and 193 μg·mL^{-1} (*Bacillus licheniformis* PSB3) (Biswas *et al.* 2018). In this study, the soluble phosphorus content of *P. oxalicum* FJG21, *P. oxalicum* FJQ5, and *B. subtilis* BPM12 using $Ca_3(PO_4)_2$ was determined to be 529.0 μg·mL^{-1}, 514.0 μg·mL^{-1}, and 330.7 μg·mL^{-1} respectively. Compared with previous studies, all the new isolates in this work have a strong capability to solubilize the insoluble phosphate.

Several PSMs are reported to be able to utilize insoluble phosphate sources, such as $Ca_3(PO_4)_2$, $CaHPO_4$, fluorapatite, rock phosphates, iron, aluminium, and magnesium phosphate, and convert them into soluble phosphate forms (Thakur *et al.* 2014). In this study, all the strains could utilize $Ca_3(PO_4)_2$, $CaHPO_4$, $AlPO_4$, and hexacalcium. High P solubilization was obtained for $Ca_3(PO_4)_2$. No P solubilization was observed for $FePO_4$. This result agreed with data obtained by Banik and Dey (1983), which reported that rock phosphates, aluminium phosphates, and iron phosphates are less solubilized compared to $Ca_3(PO_4)_2$. Thus, the capacity of PSMs to solubilize P depended on the chemical properties of the P source. Zhang *et al.* (2018) and Son *et al.* (2005) reported that fungi exhibited low P solubilizing ability in media containing $AlPO_4$ and $FePO_4$. Islam *et al*.(2019) described $AlPO_4$ and $FePO_4$ have complex structure than $Ca_3(PO_4)_2$.

Moreover, all the strains showed the maximum soluble P concentration accompanied with a minimum pH value (Son *et al.* 2005). The minimal pH value of the *P. oxalicum* FJG21, *P. oxalicum* FJQ5, and *B. subtilis* BPM12 were 2.33, 2.96, and 4.27, respectively. An increase in the amount of solubilized phosphorus was followed by a pH drop. The mechanisms of phosphate solubilization by microorganisms are very complex and are not completely known yet. It is commonly accepted that microbial mechanisms used to solubilize phosphate include acidification, chelation, and exchange reactions. Organic acids play an important role in phosphate solubilization processes, which can help the release of P by providing protons and complexing anions, or ligand exchange reactions or complexion of metal ions release to solution (Nahas 1996). Tricarboxylic acids, such as citric and oxalic, and other lower molecular weight organic acids are considered to be the main contributors to phosphate solubilization and a decrease in pH. Some researches indicate that the type and amount of organic acids produced can be influenced by insoluble phosphate used in the cultures. In this study, malicacid and oxalic acid were detected for *P. oxalicum* FJG21 and *P. oxalicum* FJQ5 based on HPLC analysis. Phosphate solubilization could be the result of the combined effect of pH decrease and organic acids production (Yu *et al.* 2011). More research is needed to gain a better insight into the mechanism of phosphate-solubilization (Chai *et al*. 2011).

Furthermore, fungi were observed to be superior to bacteria in solubilizing calcium phosphate and rock phosphate (Sperber 1958). It was found that fungi presented good P-solubilizing capability and generated more stable genetic traits than those of bacteria (Whitelaw 1999). In this study, fungous *P. oxalicum* FJG21 (529.0 μg·mL^{-1}) and *P. oxalicum* FJQ5 (514.0 μg·mL^{-1}) displayed better phosphorus-solubilizing activity than bacterial *B. subtilis* BPM12 (330.7 μg.mL^{-1}). These fungi were able to retain P-solubilizing ability over many subculturing transfers. Fungi are generally good acid producers and consequently show greater phosphate solubilization activity than bacteria (Scervinoe *et al*. 2010). Among these organisms are species of *Aspergillus*, *Penicillium*, *Talaromyces*, and *Eupenicillium*, which are considered "key organisms" in the P cycle (Whitelaw 1999). Most of them solubilize inorganic calcium phosphates and have a limited capacity of solubilizing aluminum or iron phosphates (Illmer and Schinner 1995). However, after

several subcultures, the decrease of phosphorus-solubilizing activity of *B. subtilis* BPM12 was observed. A high percentage of the bacterial isolates lost their solubilizing ability when subcultured (Kucey 1983).

Naturally occurring phosphate solubilizing microorganisms have been recognized as a source of P fertilizer (Bhardwaj *et al*. 2014). Several authors have reported a notable increase in yield of wheat and soybean through inocubation of P-solubilizing fungi (Kucey 1987, 1988). Several phosphate solubilizing species of *Penicillium* have been evaluated for their plant growth promotion efficiency (Kucey 1988; Wakelin *et al*. 2004), For instance, *Penicillium bilaji* inoculation increased P availability and uptaken by canola. *Penicillium bilaiae* RS7B-SD1, *Penicillium sp.*1 KC6-W2, and *Penicillium Radicum* FRR4718 exhibited P-solubilizing activity and promoted the growth of wheat root. P solubilizing bacteria play a significant role in increasing the P efficiency of both native and applied P and improving the growth and yield of various crops (Thakur *et al*. 2014).

CONCLUSIONS

1. Two fungal isolates were identified as *P. oxalicum* FJG21 and *P. oxalicum* FJQ5. One bacterial isolate was identified as *B. subtilis* BPM12. All the strains exhibited a high phosphate-solubilizing activity.
2. A high soluble phosphorus content was observed, with values up to 529.0 $\mu g \cdot mL^{-1}$ for *P. oxalicum* FJG21 after 8 days, 514.0 $\mu g \cdot mL^{-1}$ for *P. oxalicum* FJQ5 after 6 days, and 330.7 $\mu g \cdot mL^{-1}$ for *B. subtilis* BPM12 after 5 days.
3. All the strains effectively utilized $Ca_3(PO_4)_2$. Glucose and NH_4Cl promoted the phosphate-solubilizing activity of both *P. oxalicum* FJG21 and *P. oxalicum* FJQ5. Glucose and $(NH_4)_2SO_4$ assisted the phosphate-solubilizing activity of *B. subtilis* BPM12. They possessed better phosphate solubility under acidic conditions.
4. 10.47 $\mu g \cdot mL^{-1}$ of IAA was achieved by *B. subtilis* BPM12. A production of 0.44 $U \cdot mL^{-1}$ (β-glucosidase), 0.08 $U \cdot mL^{-1}$ (CMCase), and 0.05 $U \cdot mL^{-1}$ (FPase) was obtained by *P. oxalicum* FJG21. 0.25 $U \cdot mL^{-1}$ (β-glucosidase), 0.09 $U \cdot mL^{-1}$ (CMCase), and 0.15 $U \cdot mL^{-1}$ (FPase) were observed by *P. oxalicum* FJQ5.

ACKNOWLEDGMENTS

This work was supported financially by the Harbin Science and Technology Innovative Talents Project (2017RAQXJ148), Key Laboratory of Combining Farming and Animal Husbandry, Ministry of Agriculture and Rural Affairs (KLTMCUAR2017-1).

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Article submitted: September 13, 2019; Peer review completed: January 14, 2020; Revised version received and accepted: January 22, 2020; Published: February 20, 2020.
DOI: 10.15376/biores.15.2.2560-2578