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ORIGINAL PAPER

Evaluation and improvement of phosphate solubilization by an isolated bacterium *Pantoea agglomerans* ZB

Lingling Li[1,2] · Rongbin Chen[1,2] · Zhenyu Zuo[1,2] · Zaosheng Lv[1,2] · Zhonghua Yang[1,2] · Wei Mao[1] · Yaoning Liu[1] · Yuxi Zhou[1] · Jiaqing Huang[1] · Zibing Song[1]

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Abstract

A phosphate solubilizing bacterium ZB was isolated from the rhizosphere soil of *Araucaria*, which falls into the species *Pantoea agglomerans*. Optimization for phosphate solubilization by strain ZB was performed. At optimum culture conditions, the isolate showed great ability of solubilizing different insoluble inorganic phosphate sources viz. $Ca_3(PO_4)_2$ (TCP), Hydroxyapatite (HP), $CaHPO_4$, $AlPO_4$, $FePO_4$ along with rock phosphates (RPs). Inoculation with planktonic cells was found to enhance dissolved phosphorous as compared to that achieved by symplasma inoculation. Besides inoculation with different status of cells, pre-incubation could also exert a great effect on phosphate solubilization ability of *P. agglomerans*. When isolate ZB was cultured with glucose as carbon sources, phosphorous was more efficiently dissolved from HP and RP without pre-incubation in comparison to that obtained with pre-cultivation. Pre-cultivation, however, was more suitable for P solubilization than no pre-cultivation when bacteria were grown with xylose. A positive correlation was detected between the production of organic acids and phosphate solubilization. *P. agglomerans* ZB possessed many plant growth promotion traits such as N_2 fixation and production of indole 3-acetic acid, phytase, alkaline phosphatase. Pot experiment showed inoculation with single isolate ZB or biofertilizer prepared from semi-solid fermentation of isolate ZB with spent mushroom substrate (SMS) compost could enhance plant growth with respect to number of leaves, plant leave area, stem diameter, root length, root dry mass, shoot dry mass and biomass when compared to the abiotic control, revealing strain ZB could be a promising environmental-friendly biofertilizer to apply for agricultural field.

Keywords Biofertilizer · Inoculation · *Pantoea agglomerans* · Phosphate solubilization · Pre-incubation · Symplasma · Spent mushroom substrate

Introduction

Phosphorous is a major constituent of some macromolecules such as nucleic acid and energy carrier NADPH. It is one of the most essential nutrients for plant metabolism, growth and development. Despite high total concentration of P, soluble P form was deficient in soil. To achieve the maximum plant productivity, phosphate fertilizers in great amounts were commonly applied to the soil, which were easily precipitated in insoluble complexes and unavailable to plants. The excessive applied phosphate fertilizers could lead to environmental problems such as groundwater contamination and waterway eutrophication (Kaur and Reddy 2013).

Several soil microorganisms, including bacteria and fungi, are known to have the ability of converting insoluble phosphates into soluble forms available to plants (Xiao et al. 2008; Coutinho et al. 2012; Alori et al. 2017). It is of significant interest in agriculture field to apply these phosphate solubilizing microorganisms (PSMs) for improving plant production without causing any environmental hazards. As we know, many bacteria of *Pantoea agglomerans* show prominent environmental versatility and adaptability, and possess a variety of bio-solubilization capabilities (Kim et al. 1997; Jung et al.

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✉ Lingling Li
moonletsmile@163.com

[1] School of Chemistry and Chemical Engineering, Wuhan University of Science and Technology, Wuhan 430081, Hubei, People's Republic of China

[2] Key Laboratory of Hubei Province for Coal Conversion and New Carbon Materials, Wuhan University of Science and Technology, Wuhan 430081, Hubei, People's Republic of China

2002; Son et al. 2006; Bhatia and Sharma 2010; Walterson and Stavrinides 2015). However, it is still a challenge to enhance phosphorus solubilizing ability of this bacterium. The species *P. agglomerans* is characterized by the formation of biofilm-like muliticellular symplasmata (Dutkiewicz et al. 2015). The structure symplasmata are formed due to the aggregation of individual single cells (Duan et al. 2007) and maintained by uniquely tight bindings between cells. The survival ability of symplasmata structure strengthens much more than those of planktonic cells (Yu et al. 2016). As we know, inoculation with cells at different stages could affect bacterial growth and metabolism. Since there was the physiological change between planktonic cells and aggregated cells in symplasmata, inoculation with different status of cells (planktonic cells or aggregative symplasma-structure cells) may provide them different phosphorous-solubilizing abilities. Thus, it is crucial for improving bio-solubilization capability of *P. agglomerans* to find out which inoculation was more effective, inoculated with aggregative symplasma-structure cells or with separated planktonic cells. Furthermore, some studies have revealed that pre-cultivation could exert great effects on the bio-solubilization of rock phosphates (Priha et al. 2014; Li et al. 2016, 2018). Until now, there is little understanding about whether pre-incubation could be an effective means to enhance the dissolution of insoluble phosphates by bacteria of *Pantoea*.

In present study, a phosphorous-solubilizing bacterium *P. agglomerans* was isolated from the rhizospheric soil. Further attempt had been made to enhance its solubilization ability by optimizing culture conditions and investigating the effect of inoculation with different status of cells and pre-cultivation on dissolution of insoluble phosphates. In addition, the influence on plant growth as a function of phosphate solubilization by *P. agglomerans* was also evaluated in pot experiments.

Materials and methods

Mineral samples

Rock phosphates obtained from Yichang phosphorite (Hubei, PR China) were ground and sieved to 200-mesh size for experiments. The chemical composition of samples is shown as following: 21.98% P_2O_5, 33.55% CaO, 24.94% SiO_2, 1.27% MgO, 5.08% Al_2O_3, 2.52% Fe_2O_3, 1.45% S, 0.86% Na_2O, 2.42% K_2O, 0.11% BaO, 0.025% MnO, 0.34% TiO_2, 2.09% F, 0.003% As, 0.0001% Mo, 0.00014% Hg.

Isolation of phosphorous-solubilizing bacteria

The rhizospheric soil (5–15 cm depth) of *Araucaria* growing in Qingshan River Beach (Wuhan, Hubei, PR China) was enriched in National Botanical Research Institute's phosphate (NBRIP) growth medium (glucose 10.0 g/L, $(NH_4)_2SO_4$ 0.15 g/L, KCl 0.2 g/L, $MgCl_2{\cdot}6H_2O$ 0.5 g/L, $MgSO_4{\cdot}7H_2O$ 0.5 g/L, pH 7.0) (Xiao et al. 2008) with tricalcium phosphate (TCP, 5.0 g/L) for a period of 3 days at 30 °C on a rotary shaker at 200 r/min. Serial dilutions of the suspension were then plated onto NBRIP solid medium with 5.0 g/L TCP. After incubation at 30 °C for up to 3 days, colonies with visible phosphorous-dissolving halo/zone were picked and streak culture was repeated on NBRIP agar plate containing 5.0 g/L TCP until pure culture was examined with an optical microscope. Solubilization Index (SI) of the pure isolate, the ratio of solubilization halo diameter and colony diameter, was measured after 3 days of incubation at 30 °C. (Ghosh et al. 2016).

Identification of phosphorous-solubilizing bacteria

The shape and size of cells were observed by Scanning electron microscopy (SEM, FEI Nova NanoSEM 400). Colony appearance and motility test of the strain were determined according to standard methods. Physiological characteristics were determined according to Bergey's Manual of Systematic Bacteriology (Brenner et al. 2001). Bacterial strain was plated onto Ashby's mannitol agar to check its nitrogen-fixing ability. IAA production, alkaline phosphatase activity and phytase activity of strain ZB were characterized as described (Iyer et al. 2017).

Phylogenetic analysis was carried out through 16S rDNA sequencing using primers 7F (5′-GAGAGTTTGATCCTG GCTCAG-3′) and 1541R (5′-AAGGAGGTGATCCAGCC-3′), which was sequenced by TSingKe Biological Technology Co. Ltd. (Wuhan, China) and then analyzed using nucleotide BLAST search program at NCBI databases. The alignment of sequences was performed with Clustal Omega software. Phylogenetic tree was constructed using Neighbor-Joining method and evolutionary distances were calculated according to Maximum Composite Likelihood model. Bootstrap analysis was conducted based on 500 replicates. Mega 5.05 package was used for phylogenetic analysis (Li et al. 2013).

The influence of PSM treatments on available phosphorous in soils

The influence of PSM treatments on soil properties (available P) was evaluated in comparison to the abiotic control treatments without the addition of TCP. Soil samples (0–20 cm depth, unfertilized) were collected from the neighborhood of Wuhan university of Science and Technology, sieved (< 2 mm) and then sterilized for use. Available phosphorous in soils was analyzed by sodium bicarbonate method (Sahay and Patra 2014) after mixing the culture of

active planktonic ZB cells at late exponential phase cultivated in NBRIP liquid medium with soil samples (10^7–10^8 cells/g) for 48 h. The same sterilized culture was applied to the soil for the abiotic control.

Optimization of culture conditions for bio-solubilization

The effects of initial pH, temperature and rotation speed on bacterial growth and bio-solubilization were tested in 250-mL shake flasks. Each flask contained 1 mL inocula of active ZB cells in the late exponential phase and 100 mL of NBRIP liquid medium with 5.0 g/L TCP. The incubation was performed at initial pH 7.0 and different temperatures of 10, 15, 20, 25, 28, 29, 30, 31, 32, 34, 36, 38, 40, 45 °C on a rotary shaker at 200 r/min; or at 30 °C and different initial pH of 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0 on a rotary shaker at 200 r/min; or at 30 °C and initial pH 7.0 on a rotary shaker at different rotation speeds of 100, 125, 150, 175, 200 and 225 r/min.

In order to investigate the influence of carbon sources and nitrogen sources on bacterial growth and bio-solubilization, strain ZB was tested by grown in NBRIP liquid medium with 5.0 g/L TCP containing alternative kinds of carbon sources and nitrogen sources at 30 °C and initial pH 7.0 on a rotary shaker at 200 r/min. Various carbon sources (10.0 g/L): glucose, xylose, sucrose, maltose, galactose, fructose and mannitol. Various nitrogen sources (0.15 g/L): $(NH_4)_2SO_4$, KNO_3, yeast extract, peptone and urea. Different concentrations (5.0, 7.5, 10, 12.5, 15, 20, 25, 30, 40 g/L) of glucose were tested to find out optimum concentration of carbon sources for bacterial growth and P solubilization. Different concentrations of ammonium sulfate at 0.05, 0.10, 0.15, 0.20, 0.25, 0.30, 0.40 g/L were added for studying the effect of nitrogen source concentration on bacterial growth and P solubilization. Moreover, different concentrations (1.0, 2.5, 5.0, 10.0, 15.0, 20.0. 25.0, 30.0, 40.0, 50.0 g/L) of TCP were added to investigate the concentration of P sources suitable for bacterial growth and bio-solubilization. The optimal NBRIP medium for bio-solublization of insoluble phosphates was then obtained on the basis of these experiments. The bacteria cultured in optimal NBRIP medium at 30 °C on a rotary shaker at 175 r/min were also tested for the ability to solubilize various types of insoluble inorganic phosphates ($Ca_3(PO_4)_2$, $CaHPO_4$, hydroxyapatite, rock phosphates, $FePO_4$ and $AlPO_4$) with the concentration of 5.0 g/L.

For all the optimizing test of culture conditions, optical densities of cultures (OD_{600}) were determined after incubation for 48 h. The supernatant content of P_2O_5 was detected by phosphomolybdate method with a UV-2000 spectrophotometer at 420 nm after 48 h incubation (Chi et al. 2006). Sterile abiotic control was used in each set of experiments to determine the contribution of the medium. The concentration of soluble P in bio-solubilization experiment was subtracted by that in the abiotic control. Each measurement was repeated in triplicate and the average values were reported.

The effect of inoculation with planktonic cell and symplasmata on bio-solubilization

The culture of isolate ZB cultivated in optimized NBRIP liquid medium with 5.0 g/L TCP for 3 days at 30 °C on a rotary shaker at 175 r/min was used for the test. Symplasmata and planktonic cells in the culture were successively separated using a G_2 sand-core funnel (intercept size 30 ~ 50 μm) and G_4 sand-core funnel (intercept size 4 ~ 7 μm) (Changchun Glass Instrument Company, China). For inoculation, symplasmata peeled off from G_2 filter cake were diluted to the approximately equivalent concentration of plantonic cells obtained from G4 filtrate according to the biomass as described previously (Yu et al. 2016). All the experiments were performed in triplicate in 250-mL shake flasks containing 5 mL of the adjusted inocula and 100 mL of optimized NBRIP liquid medium with 5.0 g/L TCP. OD_{600}, pH value and dissolved P were determined at intervals. The mean values were recorded. Specific growth rate (μ, h^{-1}) was calculated from the slopes of the linear portion of plots (Li et al. 2013).

The influence of pre-cultivation on bio-solubilization

Inocula of planktonic cells used in the test were prepared by filtrating 2-day culture of isolate ZB using G_4 sand-core funnel, which was cultivated in optimized NBRIP liquid medium with 5.0 g/L TCP at 30 °C on a rotary shaker at 175 r/min. All bio-solubilization experiments were performed in a 250-mL flask containing 100 mL phosphorous-limited optimized NBRIP liquid medium with KH_2PO_4 (0.02 g/L) as P source on a rotary shaker at 175 r/min incubated at 30 °C, in which glucose or xylose at a concentration of 12.5 g/L was added as carbon sources. The experiments consisted of two leaching methods: bio-solubilization with and without pre-cultivation. For bio-solubilization without pre-incubation, 5.0 g/L of TCP, 5.0 g/L of hydroxyapatite (HP) or 0.5 g/L of rock phosphate (RP) were initially added to these media with the inocula. For bio-solubilization with pre-incubation, however, these insoluble phosphates were added into the pre-grown cultures after 24 h of incubation. After 2 days of bio-solubilization, suitable amount of leaching solution was sampled from each flask to determine pH value, cell concentration (OD_{600}) and the supernatant content of P_2O_5. Values were given as means ± S.D. for triplicate samples.

HPLC analyses of organic acids produced

In order to analyze the component of organic acids, cell free supernatant was obtained for injecting to high-performance liquid chromatography (HPLC, Agilent 1100) after centrifugation at 10,000×r/min for 20 min and filtration through 0.45 µm membranes. Organic acids were separated on SB-C_{18} column (4.6 × 150 mm, 5 µm; Agilent) with the mixture of 3% methanol and 0.01 moL/L K_2HPO_4 as mobile phase in which pH was adjusted to 2.86 by phosphoric acid at a constant flow rate of 0.5 mL/min and at operating temperature of 30 °C. Retention time of each signal was recorded at 215 nm.

Pot experiment

Pot experiments were performed by inoculating isolate ZB to the soil or preparing biofertilizer from semi-solid fermentation of strain ZB with spent mushroom substrate (SMS) compost. The approximately same height (3–4 cm) Chili pepper seedlings were grouped for soil inoculation and SMS inoculation. All the experiments were conducted in triplicates. Before plantation, the seedlings were surface sterilized with 0.1% $HgCl_2$ and subsequently repeatedly washed with sterilized water. Sterilized soil samples (unfertilized) was used in each pot for plantation. For pot experiment with soil inoculation, active ZB cells at late exponential phase were applied in the soil (10^6–10^7 cells/g of soil) (Ghosh et al. 2016). For pot experiment with SMS biofertilizer, semi-solid fermentation was performed for 6 days with 6-h turning over by inoculating active ZB cells at late exponential phase (5%) into 30 g of autoclaved medium (5 g corn flour and 25 g 20-mesh sun-dried SMS) in 250-mL flask at an initial moisture content of 65–70% (Zhu et al. 2012). The rapid bacterial growth could lead to the maximum biomass concentration of 5×10^8 cells/g biofertilizer. The soil was then added with 1% (w/w) SMS biofertilizer for six times. The control set was not inoculated with bacteria. After planted in the soils, the seedlings was watered daily. All plants were uprooted after 28 days of plantation to measure number of leaves in the plant (NOL), plant leaf area (PLA), stem diameter (SD), shoot length (SL), shoot dry weight (SDW), root length (RL), root dry weight (RDW) and biomass (Bakhshandeh et al. 2014).

Results

Isolation of phosphate solubilizing bacteria

A strain named ZB was isolated from the rhizosphere soil of *Araucaria* growing in Qingshan River Beach, Wuhan, China. The clear phosphorous-dissolving halo appeared in the colonies of isolate ZB grown on NBRIP solid medium after 3 d of incubation with Solubilization Index (SI) ~ 4.1 (Fig. S1).

Characterization of morphology

For morphological observation, strain ZB is a rod-shaped, Gram-negative, motile bacterium without capsule and endospore observed under an optical microscope. As SEM image of cell morphology shown in Fig. 1, the size of cells is about 1.4–1.8 µm in length and 0.5–0.6 µm in width. The colonies grown on LB agar after 18 h of incubation were yellowish, round, drop-shaped, smooth, moist and convex which have regular margins with approximately ~ 2 mm in diameter (Fig. S2a). The culture grown in NBRIP liquid medium became pale yellow-pigmented turbid after 18 h of incubation, in which some ball aggregations began to be formed at the second day and grew gradually larger from bead-size to ball-size (Fig. S2b). The puncture test on LB semisolid medium at 30 °C showed the bacterium is facultatively anaerobic and motile.

Physiological characteristics of isolate ZB

Strain ZB exhibited similar biochemical properties to the reference strains (supplementary Table 1). It showed activities of catalase and gelatin liquefaction. The cells were oxidase, amylase, urease and lipase negative. It also possessed the ability of reducing nitrate to nitrous acid. It couldn't degrade tryptophan to produce indole but could produce hydrogen sulfide from the hydrolysis of cysteine. Strain ZB

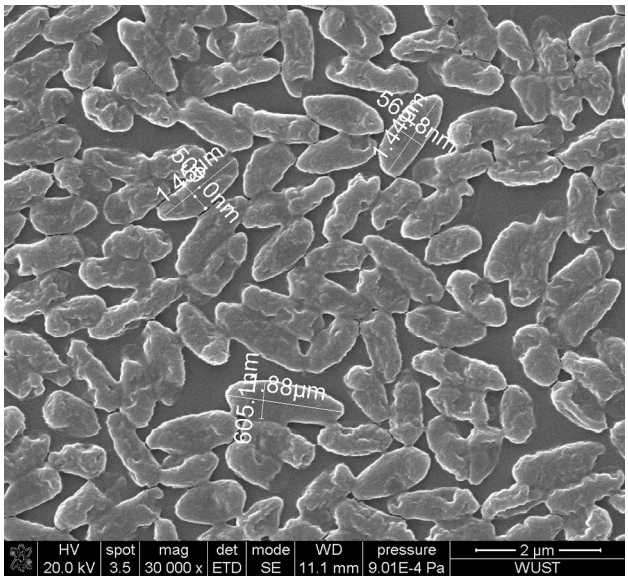

Fig. 1 Cell morphology of strain ZB under Scanning electron microscopy (×30,000)

could utilize sucrose, mannitol, maltose, galactose, glucose and xylose as sole carbon sources. Citrate could be utilized by the bacteria. Acid but little gas was produced from D-glucose. According to V.P. test, glucose could be hydrolyzed via glycolysis of strain ZB to produce pyruvic acid, which was further converted to acetylmethylmethanol. Methyl red test was negative for strain ZB. Optimum growth occurred at 30 °C in the range from 10 to 45 °C and at pH 6.0 over a range from 4.0–9.0. Bacterial growth could not be observed at 41 °C but at 37 °C.

Furthermore, strain ZB possessed many plant growth promoting characteristics (supplementary Table 2). It could grow on Ashby's mannitol agar, showing bacterial activities of N_2 fixation. Isolate ZB was positive for production of indole-3-acetic acid (IAA, 21 ± 0.35 ug/mL), phytase activity (1.31 ± 0.2 uM/min/mg protein) and alkaline phosphatase activity (4.2 ± 0.3 uM/min/mg protein). Strain ZB could also significantly increase available P content of soils from 8.25 ppm to 12.33 ppm (8.92 ppm in the control), revealing its potential as a promising bio-fertilizer (Sahay and Patra 2014).

16S rDNA phylogeny

The 16S rDNA sequence with 1441 bp size was deposited in GenBank database with accession number MG685887.1. Phylogenetic tree (shown in Fig. 2) summarizing the phylogenetic relationship among *Pantoea* species reveals that strain ZB is closely related to *P. agglomerans* with 98% similarity.

Considering morphological, physiological and biochemical characteristics, and phylogenetic analysis based on 16S rDNA gene sequence, strain ZB should be classified into *P. agglomerans*.

Optimization of culture conditions for bio-solubilization

Culture conditions for phosphate solubilization by strain ZB were optimized as shown in Fig. 3. The temperature, initial pH and rotation speed have certain effects on solubilization ability of tricalcium phosphate as bacterial growth. The highest soluble P of 629.74 mg/L P_2O_5 was dissolved at 30 °C in the range from 10 to 45 °C (Fig. 3a). The production

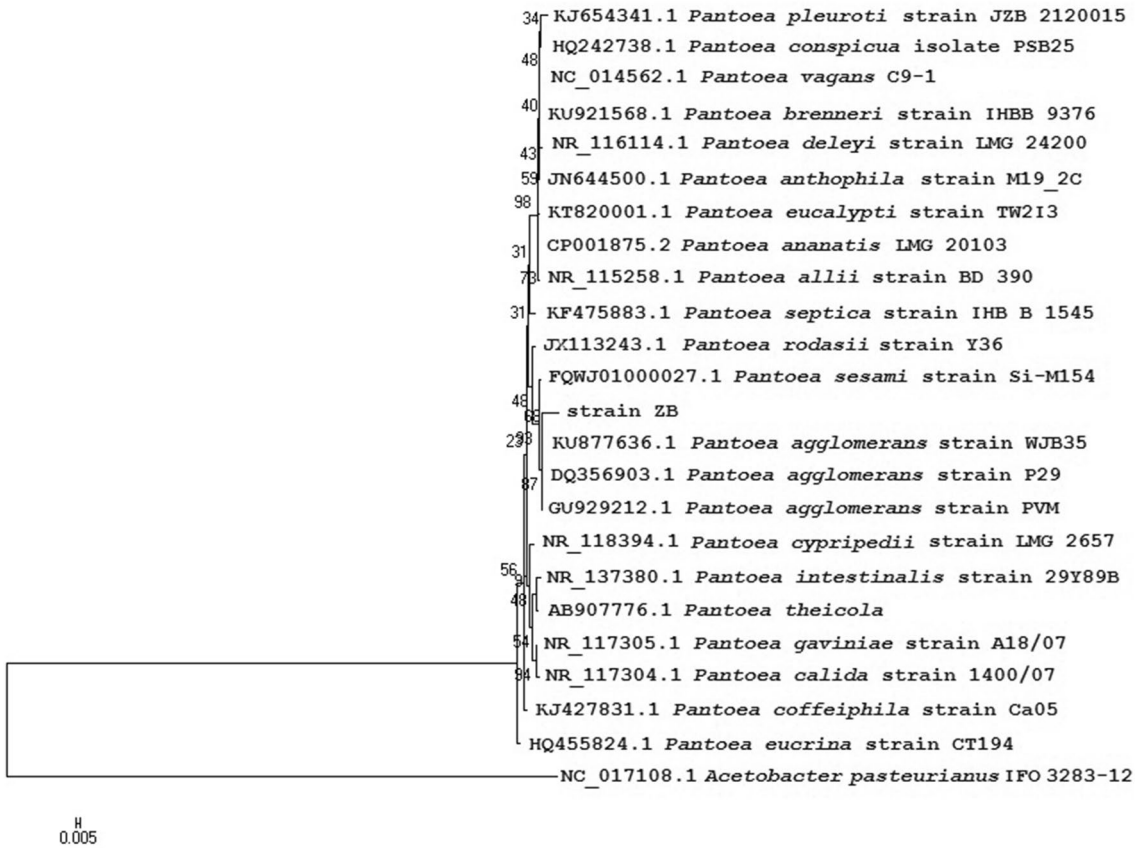

Fig. 2 Phylogenetic analysis derived from 16S rDNA gene sequence of strain ZB. Phylogenetic tree was constructed with Neighbor-Joining method and Maximum Composite Likelihood model taking bootstrap value as 500. Bar, 0.005 substitutions per nucleotide position

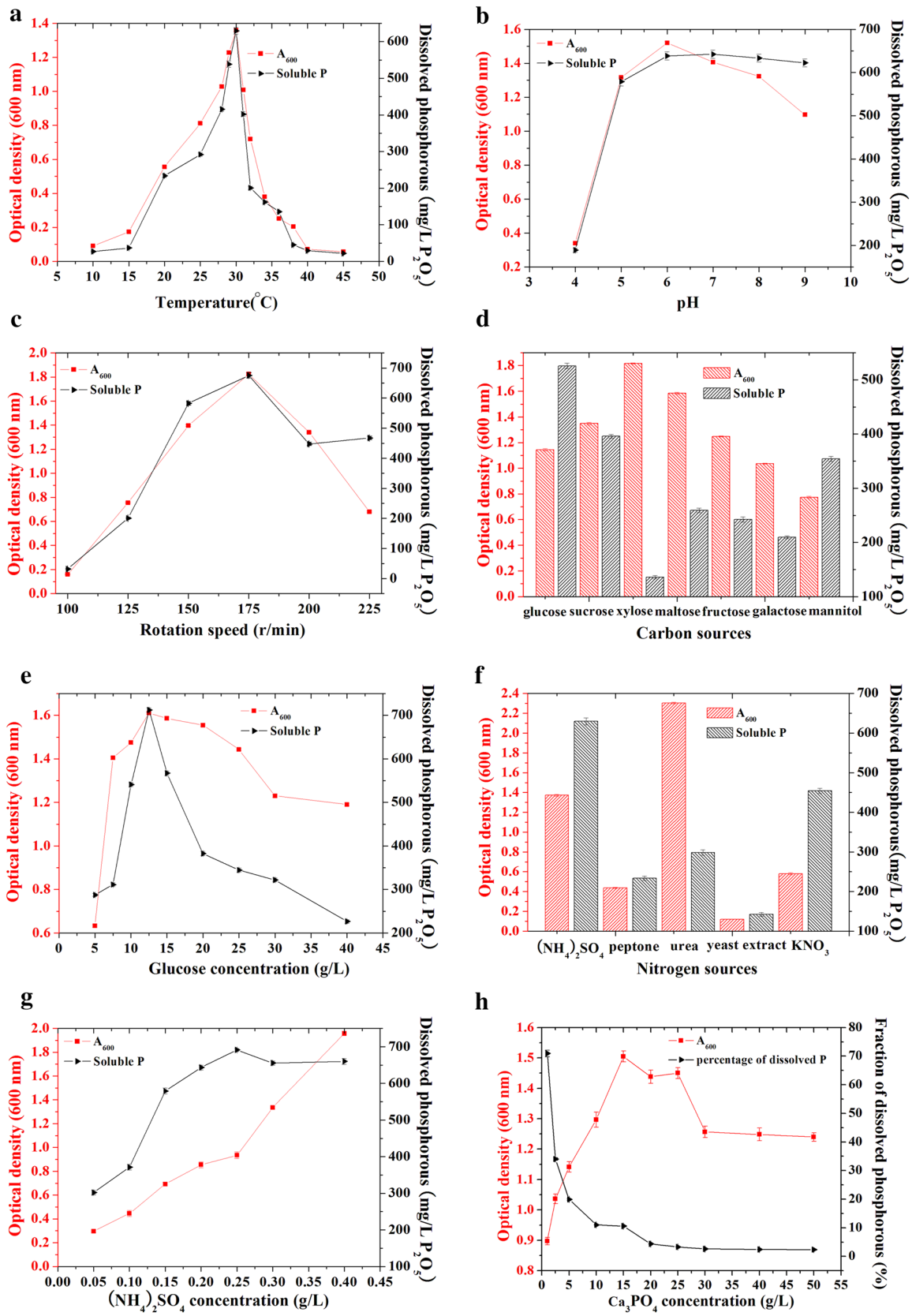

Fig. 3 Effect of temperature (**a**), initial pH (**b**), rotation speed (**c**), carbon sources (**d**), glucose concentration (**e**), nitrogen sources (**f**), $(NH_4)_2SO_4$ concentration (**g**) and TCP concentration (**h**) on bacterial growth and TCP solubilization by *P. agglomerans* ZB. Results are means of data from three independent trials. *Error bars* represent standard deviation

of soluble P could reach the highest (642.34 mg/L P_2O_5) at pH 7.0 ranging from 4.0 to 9.0 (Fig. 3b). The concentrations of dissolved phosphate from TCP (622.27 to 642.34 mg/L P_2O_5) were more or less unchanged over the range from pH 6.0 to pH 9.0. The maximum P solubilization was found to be 675.23 mg/L P_2O_5 at rotation speed of 175 r/min (Fig. 3c). From these figures, a positive correlation was observed between the concentration of soluble P and the population of the isolate.

Furthermore, various types of carbon source, nitrogen source was selected to evaluate their effects on phosphate solubilizing activity. The maximum phosphate solubilization (525.76 mg/L P_2O_5) was achieved when bacteria were cultivated with glucose as carbon source (Fig. 3d). Xylose was the best carbon source for bacterial growth, however, which bio-solubilization activity was the lowest among all carbon sources tested. There is no correlation between cell growth and soluble P production for the optimization test of carbon sources, which is similar to Son's report (Son et al. 2006). Glucose at the concentration ranging from 12.5 to 15 g/L was optimal for bacterial growth and bio-solubilization (Fig. 3e). The highest cell concentration (A_{600} 1.61) and soluble P (712.06 mg/L P_2O_5) was detected at 12.5 g/L of glucose concentration. P solubilization was found to increase as the concentration of glucose was increased from 5 to 12.5 g/L and then decrease at higher concentration. As shown in Fig. 3f, the optimum nitrogen source for bacterial growth was urea. The maximum phosphorous solubilization (629.74 mg/L P_2O_5), however, was observed with ammonium sulfate as nitrogen source which was significantly higher than other N sources. Moreover, the maximum P solubilization (691.21 mg/L P_2O_5) was observed at $(NH_4)_2SO_4$ concentration of 0.25 g/L (Fig. 3g). The change of insoluble phosphate solubilization from 643.29 to 691.21 mg/L P_2O_5 was not obvious with ammonium sulfate concentration ranging from 0.2 to 0.4 g/L. Bacterial growth was greatly enhanced when increasing ammonium sulfate concentration.

Various concentrations of TCP were also added to the culture to find out the effect of TCP concentration on phosphate solubilization, which was denoted in this experiment as faction of dissolved phosphorous (%) rather than the concentration of soluble P (mg/L P_2O_5). As shown in Fig. 3h, percentage of soluble phosphorous released by strain ZB was largely decreased with increasing the amount of TCP. The faction of dissolved P was approximately held constant at above 10 g/L of P source concentration. Although the highest faction of dissolved P was observed at 1.0 g/L of TCP, 5 g/L of TCP was added to NBRIP medium for optimization of bio-solubilization taking into account phosphorous-solubilizing capacity. Moreover, 15 g/L of TCP was also found to give the maximal bacterial growth. The highest soluble P production of 974.61 mg/L P_2O_5 was also achieved in the medium containing 15 g/L of TCP (data not shown in Fig. 3h).

Taken together, an optimized NBRIP medium was determined for bio-solubilization of TCP by strain ZB, which contained 12.5 g/L of glucose, 0.25 g/L of $(NH_4)_2SO_4$, 0.2 g/L of KCl, 0.5 g/L of $MgCl_2{\cdot}6H_2O$, 0.5 g/L of $MgSO_4{\cdot}7H_2O$, 5.0 g/L $Ca_3(PO_4)_2$ (pH 7.0). For optimization of bio-solubilization, the bacterium was cultured in a 250-mL flask at 30 °C on a rotary shaker at 175 r/min. Figure 4 shows the change of bacteria growth, pH and soluble P production over time under optimal culture conditions in optimized NBRIP liquid medium with TCP (5.0 g/L). As shown in Fig. 4a, the

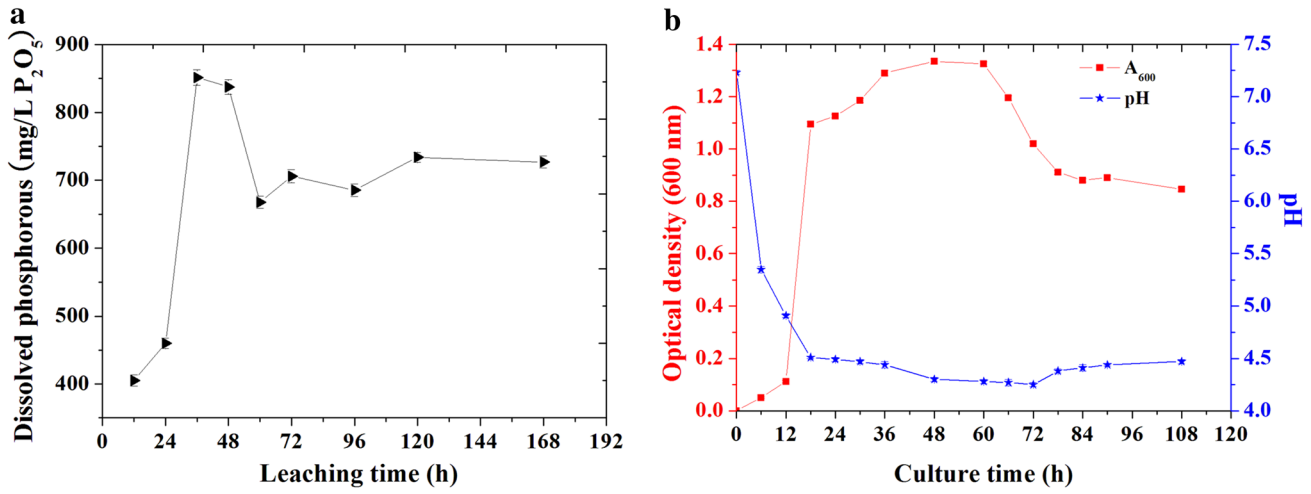

Fig. 4 The changes in dissolved phosphorous (**a**), bacterial growth and pH (**b**) over time during TCP solubilization by *P. agglomerans* ZB in optimized NBRIP medium (pH 7.0) with 5.0 g/L TCP at 30 °C on a rotary shaker at 175 r/min. Results are means of data from three independent trials. *Error bars* represent standard deviation

maximum soluble P production of 851.61 mg P_2O_5 L^{-1}, corresponding to 37.18% of the total phosphorous, was bio-solubilized from TCP after 36 h, which was approximately 1.6-fold by that in the unmodified NBRIP medium (532.26 mg P_2O_5 L^{-1}) inoculated with the same batch of culture at 30 °C on a rotary shaker at 200 r/min. *P. agglomerans* ZB entered the log phase at the 12th hour, sustained the exponential growth for 6 h and reached the stationary phase after 18 h with the highest cell concentration at the 48th hour (Fig. 4b). A sharp decrease in pH of the culture from 7.2 to ~ 4.5 was observed in the first 18 h of incubation and reached the lowest pH of ~ 4.25 after 72 h (Fig. 4b).

Bio-solubilization of different insoluble inorganic phosphates

There is no metal-P compound serving as the universal P source for phosphorous solubilizing microorganisms because soils vary in pH and several chemical properties (Bashan et al. 2013). In order to investigate the potential of *P. agglomerans* for applying in agronomic practices, bacterial ability of solubilizing various types of insoluble inorganic phosphate sources was determined as shown in Table 1. The production of soluble P (mg/L P_2O_5) released from $Ca_3(PO_4)_2$, Hydroxyapatite (HP), $CaHPO_4$, $AlPO_4$, $FePO_4$ and rock phosphates (RPs) was 851.61, 1355.23, 1236.13, 17.33, 25.08 and 23.24, respectively. The dissolution of $Ca_3(PO_4)_2$, HP, $CaHPO_4$ and RPs was significantly higher than $AlPO_4$ and $FePO_4$.

The effect of inoculation with symplasmata or planktonic cells on TCP solubilization

The species *P. agglomerans* is characterized by the formation of multicellular symplasmata, which are peculiar, elongated aggregates surrounded by a translucent exopolysaccharide sheath (Dutkiewicz et al. 2015) and formed due to the aggregation of individual single cells (Duan et al. 2007). There are still a large amount of planktonic cells coexisting with symplasma-structure bacteria in the culture. In this experiment symplasma-structure cells and planktonic cells separated from 3-day culture using sand-core funnel were respectively inoculated to optimized NBRIP medium for investigating the effect of inoculation with different status of cells on solubilization activity of strain ZB.

Table 1 Solubilization of various insoluble phosphates by *P. agglomerans* cultured in optimal NBRIP medium at 30 °C on a rotary shaker at 175 r/min

Phosphate source (5.0 g/L)	Soluble P (mg/L P_2O_5)	Fraction of solubilized phosphorous (%)
$Ca_3(PO_4)_2$	851.61 ± 9.75	37.18
Hydroxyapatite	1355.23 ± 13.0	63.88
$CaHPO_4$	1236.13 ± 9.75	47.36
$AlPO_4$	17.33 ± 3.25	0.6
$FePO_4$	25.08 ± 3.25	1.07
Rock phosphate	23.24 ± 6.50	2.11

As shown in Fig. 5a, bacterial growth (denoted by optical density at 600 nm) was negatively influenced by inoculation with symplasma. The extended period of lag phase, the longer log growth phase and lower concentration of planktonic cells were observed in the culture with symplasma inoculation in comparison to planktonic cell inoculation. Furthermore, specific growth rate constant (μ) of strain ZB under symplasma inoculation (0.0306 h^{-1}) was greatly lower than that under inoculation with planktonic cells (0.0701 h^{-1}) as presented in Table 2, revealing that inoculation with planktonic cells could enhance bacterial growth. When symplasmata were inoculated to fresh medium, environmental changes stimulated the detachment of aggregated cells inside the symplasmata and their dispersion to the medium (Ma et al. 2011), resulting in the extended lag phase and the decreased bacterial growth.

Changes of pH value and dissolved P in the culture inoculated with planktonic cells and symplasmata are presented in Fig. 5b, c, respectively. Symplasma inoculation could significantly exert a negative effect on phosphate solubilization along with culture acidification. As shown in Fig. 5b, the pH gradually decreased from initial value of 7.2 and reached the lowest value of 3.76 at 48 h and then slightly increased in the cultures with planktonic cell inoculation. By comparison, a initial gradual decrease of pH was also observed in the culture with symplasma inoculation and pH value dropped to the lowest value of 3.82 at 54 h. Afterwards a slight increase in pH value is also found in the culture with symplasma inoculation. Figure 5c showed the concentration of soluble P in the culture inoculated with planktonic cells increased significantly from 12 to 48 h and then began to drop gradually while a gradual increase of dissolved phosphorous concentration was observed from 12 to 84 h in the culture inoculated with symplasmata after which solubilization of TCP was decreased. Furthermore, the maximum concentration of dissolved phosphorous with inoculation of planktonic cells (943.84 mg P_2O_5 L^{-1} at 48 h) constitutes an increase of 21.89% over that achieved by symplasma inoculation (774.34 mg P_2O_5 L^{-1} at 84 h), revealing inoculation with planktonic cells were more suitable for phosphate solubilization.

The effect of pre-cultivation on bio-solubilization of insoluble inorganic phosphates

As described before there was a significant difference in phosphate solublization ability of bacteria cultured with

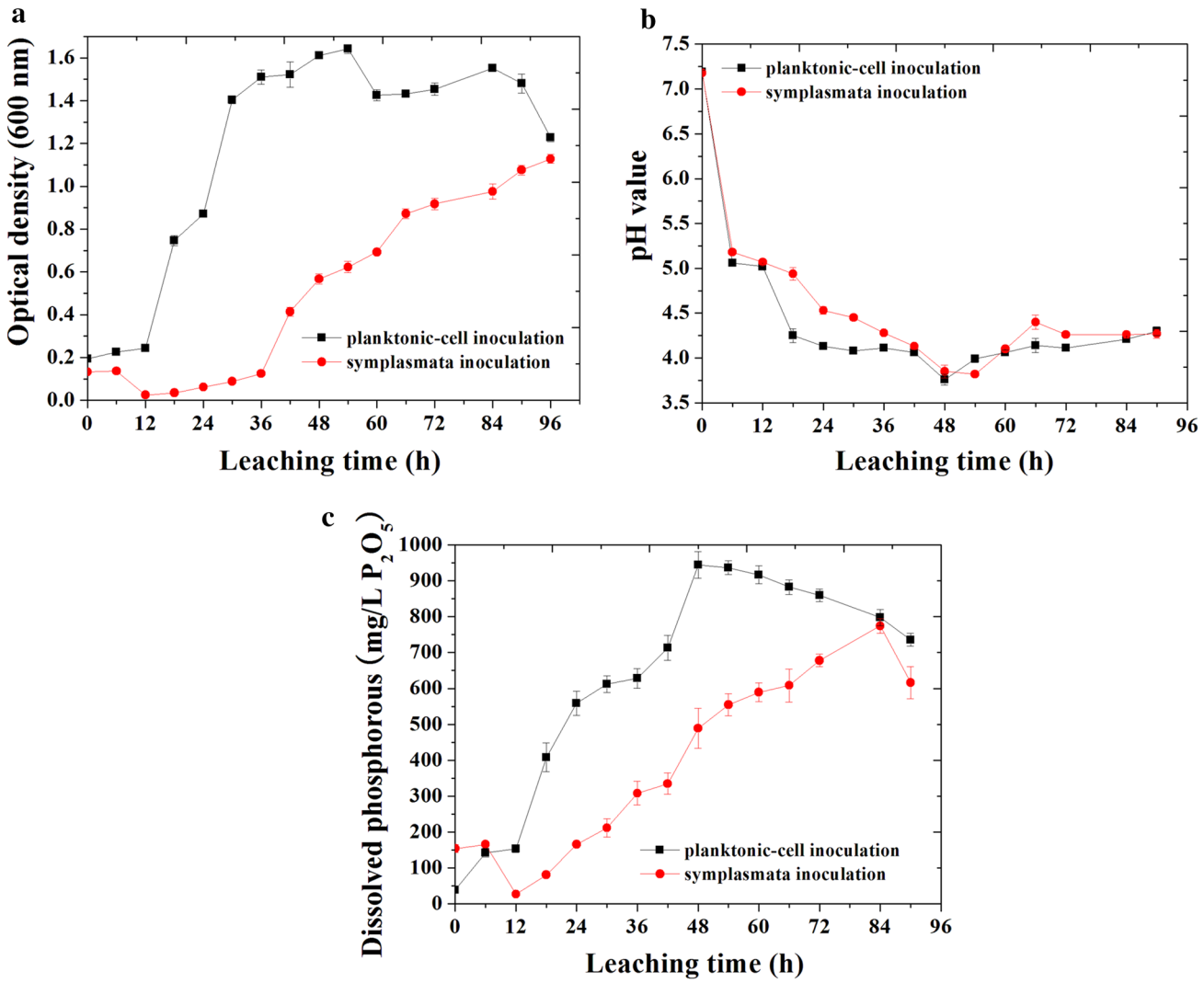

Fig. 5 The change mode of bacterial growth (**a**), pH value (**b**) and phosphate solubilization (**c**) when cultivated with planktonic cells inoculation (black rectangle) and symplasma inoculation (red circle), respectively. Results are means of data from three independent trials. *Error bars* represent standard deviation

Table 2 Specific growth rate constant of *P. agglomerans* ZB in the culture inoculated with planktonic cells and symplasma-structure cells

Samples	Specific growth rate constant (μ, h^{-1})
Culture inoculated with planktonic cells	0.0701
Culture inoculated with symplasmata	0.0306

glucose and xylose as carbon source. The influence of pre-incubation on P solubilization was explored in a series of bio-solubilization experiments without and with pre-cultivation of bacteria grown with glucose or xylose as carbon sources under inoculation of planktonic cells.

To investigate the effect of pre-cultivation on bio-solubilization of insoluble phosphates by strain ZB, a phosphorous-limited optimized NBRIP liquid medium was developed. Different concentrations of KH_2PO_4 were added into optimized NBRIP medium to determine optimal KH_2PO_4 concentration, which could supply P source for bacterial growth before the addition of insoluble P source but not have a negative influence on the subsequent bio-solubilization under the condition with pre-cultivation. As shown in Table 3, pH was gradually decreased from an initial 7.2 to a final ~ 3.7 in all the liquid media. The optimal KH_2PO_4 concentration was 0.02 g/L, which could reach the lower pH 3.56 after 24 h and meanwhile soluble P was decreased to an undetected level.

The comparison of phosphorous solubilization with or without pre-cultivation of the microorganism is shown in

Table 3 Variation of pH variation and remaining soluble P in optimized NBRIP liquid medium supplied with different concentration of KH_2PO_4

The concentration of KH_2PO_4 (g/L)	The change of pH in the culture						Remaining soluble P (mg/L) (after 24 h)
	0 h	6 h	12 h	18 h	24 h	30 h	
0.01	7.2	4.29	3.64	3.7	3.72	3.76	–
0.02	7.2	4.28	3.52	3.59	3.56	3.62	–
0.025	7.2	4.33	3.50	3.57	3.56	3.64	–
0.05	7.2	4.12	3.52	3.58	3.56	3.7	4.86

Table 4 Effect of pre-cultivation on tricalcium phosphate, hydroxyapatite and rock phosphate solubilization by *P. agglomerans* ZB

Leaching methods	Carbon sources	Insoluble phosphorous	Bacterial growth ($\times 10^7$cells/mL)	Soluble P (mg/L P_2O_5)	Final pH
No pre-cultivation	Glucose	$Ca_3(PO_4)_2$	10.6 ± 1.4	935.10 ± 9.75	4.24 ± 0.04
	Xylose	$Ca_3(PO_4)_2$	17.4 ± 1.4	217.71 ± 9.75	4.55 ± 0.03
	Glucose	Hydroxyapatite	16.4 ± 0.8	1442.75 ± 13.00	3.36 ± 0.02
	Xylose	Hydroxyapatite	21.0 ± 0.2	406.18 ± 3.25	4.81 ± 0.02
	Glucose	Rock phosphate	21.6 ± 0.4	64.99 ± 6.50	4.44 ± 0.02
	Xylose	Rock phosphate	18.4 ± 0.4	29.24 ± 3.25	4.83 ± 0.02
Pre-cultivation	Glucose	$Ca_3(PO_4)_2$	67.8 ± 1.0	1247.78 ± 13.00	4.18 ± 0.04
	Xylose	$Ca_3(PO_4)_2$	114.4 ± 1.6	701.88 ± 6.50	4.32 ± 0.03
	Glucose	Hydroxyapatite	87.4 ± 1.4	484.16 ± 9.75	4.56 ± 0.03
	Xylose	Hydroxyapatite	36.6 ± 0.6	1257.53 ± 9.75	3.51 ± 0.03
	Glucose	Rock phosphate	12.6 ± 0.6	9.75 ± 3.25	4.92 ± 0.04
	Xylose	Rock phosphate	20.0 ± 0.8	97.48 ± 6.50	4.04 ± 0.02

Table 4. Insoluble inorganic phosphorous was initially added to the media with the inocula for bio-solubilization without pre-incubation of bacteria. After 2-day of bio-solubilization, *P. agglomerans* ZB cultured with glucose as carbon sources could dissolve 935.10 mg P_2O_5 L^{-1} from tricalcium phosphate (TCP) at pH 4.24, 1442.75 mg P_2O_5 L^{-1} from hydroxyapatite (HP) at pH 3.36 and 64.99 mg P_2O_5 L^{-1} from rock phosphates (RPs) at pH 4.44, which corresponded to 40.83%, 68.01%, 59.14% of the total phosphorous in P sources, respectively. However, the bacteria cultivated with xylose as carbon sources could solubilize 217.71 mg P_2O_5 L^{-1} from TCP at pH 4.55, 406.18 mg P_2O_5 L^{-1} from HP at pH 4.81, and 29.24 mg P_2O_5 L^{-1} from RPs at pH 4.83, corresponding to 9.51%, 19.15%, 26.61% of the total P, respectively.

For bio-solubilization with pre-cultivation, insoluble inorganic phosphates were added to the 1-day pre-grown culture. The bacteria cultured with glucose as carbon sources could dissolve 1247.78 mg P_2O_5 L^{-1} from TCP at pH 4.18, 484.16 mg P_2O_5 L^{-1} from HP at pH 4.56 and 9.75 mg P_2O_5 L^{-1} from RPs at pH 4.92, which corresponded to 54.48%, 22.82%, 8.87% of the total phosphorous in P sources, respectively. When cultured with xylose as carbon sources, the bacteria could solubilize 701.88 mg P_2O_5 L^{-1} from TCP at pH 4.32, 1257.53 mg P_2O_5 L^{-1} from HP at pH 3.51 and 97.48 mg P_2O_5 L^{-1} from RPs at pH 4.04, corresponding to 30.65%, 59.28%, 88.70% of the total phosphorus, respectively.

The results showed that pre-incubation could exert a great effect on phosphate solubilization ability of *P. agglomerans*. No pre-cultivation was optimal for phosphate solubilization (except TCP) by bacteria grown with glucose. However, all the optimum P dissolution processes were treated with pre-incubation of bacteria cultured with xylose as carbon source. Glucose was more suitable for P dissolution under no pre-cultivation while xylose was preferable for bio-solubilization (except TCP) under pre-cultivation. For TCP, P solubilization was higher when bacteria were cultured with glucose than with xylose no matter under pre-incubation or not, which may be due to the enrichment and domestication of strain ZB in NBRIP medium with glucose as carbon source and TCP as phosphorous source.

HPLC analyses of organic acids produced

As we know, phosphate solubilizing ability of PSMs was associated with the release of low molecular weight organic acids (Gupta and Kumar 2017; Iyer et al. 2017; Khan et al.

Table 5 The kinds and concentrations of organic acids detected by HPLC analyses in the supernatants for inoculation and pre-cultivation experiments (preinc.=preincubation)

samples	C source	P source	Inoculation	Leaching methods	Organic acids (mg/L)							Total organic acids (mg/L)
					gluconic acid	oxalic acid	tartaric acid	malic acid	acetic acid	citric acid	succinic acid	
1	glucose	TCP	Planktonic cells	No preinc.	79.17	-	145.61	544.76	-	-	266.51	1036.05
2	glucose	TCP	symplasmata	No preinc.	14.62	-	-	390.199	-	-	346.67	751.489
3	glucose	TCP	Planktonic cells	No preinc.	11.99	13.05	-	581.53	-	-	617.48	1224.05
4	glucose	TCP	Planktonic cells	preinc.	6.6	-	-	747.53	-	-	803.1	1557.23
5	xylose	TCP	Planktonic cells	No preinc.	7.81	64.45	-	-	-	-	77.46	149.72
6	xylose	TCP	Planktonic cells	preinc.	7.17	-	-	214.04	-	-	205.87	427.08
7	glucose	HP	Planktonic cells	No preinc.	-	31.64	-	165.8	170.3	-	352.3	720.04
8	glucose	HP	Planktonic cells	preinc.	-	11.72	50.85	-	35.1	-	108.9	206.57
9	xylose	HP	Planktonic cells	No preinc.	-	8	34.81	-	-	29.99	-	72.8
10	xylose	HP	Planktonic cells	preinc.	-	38.05	231.77	-	-	-	-	269.82
11	glucose	RPs	Planktonic cells	No preinc.	-	-	17.98	32.67	24.9	151.1	-	226.65
12	glucose	RPs	Planktonic cells	preinc.	-	-	8.41	13.33	-	-	13.95	35.69
13	xylose	RPs	Planktonic cells	No preinc.	-	9.1	-	14.85	14.2	55.9	-	94.05
14	xylose	RPs	Planktonic cells	preinc.	-	12.81	-	214.87	-	79.06	-	306.74

2007), which could diffuse freely outside from the cells, decrease pH value of the culture and facilitate phosphate solubilization by supplying protons and chelating the cations (mainly calcium ions) bound to phosphate (Gadd, 1999; Wei et al. 2018). HPLC analyses detect different kinds and concentrations of organic acids in the cultures for inoculation and pre-cultivation experiments (Fig. S3 and Fig. S4). As shown in Table 5, a positive correlation was detected between organic acid production and the concentration of soluble P released from insoluble inorganic phosphates, which is in accordance with those of research papers (Son et al. 2006; Wei et al. 2018).

Pot experiment

Pot experiments were conducted under spent mushroom substrate (SMS) inoculation and soil inoculation with *P. agglomerans* ZB. Potted plants inoculated with strain ZB under SMS inoculation and soil inoculation were grown for 28 days after planting. Figure 6 represents plant growth promoting (PGP) traits of isolate ZB tested in the experiments. PGP parameters (number of leaves (NOL), leave length, maximum leave width, plant leave area (PLA), stem

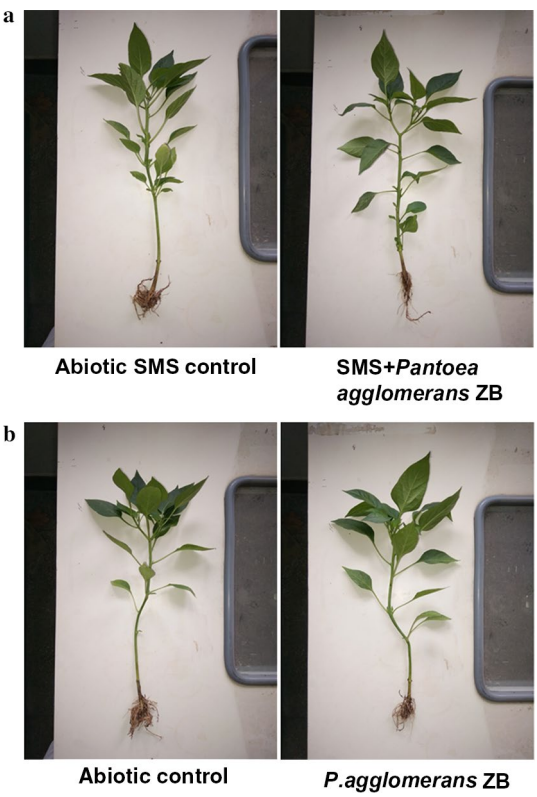

Fig. 6 Application of two methods (**a** Spent mushroom substrate inoculation; **b** soil inoculation) at an early growth stage of Chili pepper (The plants were harvested 28 d after planting). Left figure is abiotic control, and right one is inoculation with *Pantoea agglomerans* ZB

Table 6 Effect of spent mushroom substrate inoculation and soil inoculation with *P. agglomerans* ZB on Chili pepper growth measured 28 d after planting

Traits	Treatment (mean ± standard deviation)			
	Abiotic control	ZB	Abiotic SMS control	SMS + ZB
Numbers of leaves in plant	14.67 ± 0.56	16 ± 0.67	25.67 ± 0.44	28.33 ± 0.44
Leave length (cm)	6.692 ± 0.356	8.546 ± 0.41	6.634 ± 0.253	6.546 ± 0.28
Maximum leave width (cm)	3.198 ± 0.112	4.1 ± 0.157	2.816 ± 0.075	3.828 ± 0.09
Plant leaf area (cm^2)	240.76 ± 8.42	420.46 ± 11.94	364.29 ± 28.12	532.42 ± 20.69
Stem diameter (cm)	0.33 ± 0.021	0.384 ± 0.045	0.346 ± 0.023	0.422 ± 0.064
Shoot length (cm)	22.7 ± 1.05	20.5 ± 1.18	20.2 ± 1.315	18.9 ± 1.705
Root length (cm)	6.2 ± 0.51	7.1 ± 1.24	4.6 ± 0.35	7.5 ± 1.18
Biomass (mg/plant)	1297.4 ± 37.7	1437.3 ± 41.3	1478.3 ± 36.6	1567.8 ± 38.0
Root dry mass (mg/plant)	429.1 ± 25.1	619.3 ± 34.4	603.3 ± 33.8	621.2 ± 37.1
Shoot dry mass (mg/plant)	344.8 ± 31.3	365.5 ± 27.6	273.6 ± 18.9	448.0 ± 29.3

diameter (SD), shoot length (SL) and shoot dry mass(SDW), root length (RL) and root dry mass (RDW), biomass) were scored for the uprooted plants as shown in Table 6. The amount of all measured traits, except for SL, was significantly enhanced in bacterial inoculation set as compared to the control. Single *P. agglomerans* inoculation increased NOL, PLA, SD, RL, RDW, SDW and biomass by 9.07%, 74.64%, 16.36%, 14.52%, 44.33%, 6.0% and 10.78%, respectively, in comparison with the control. By comparison, the significant increase of NOL, PLA, SD, RL, RDW, SDW and biomass was 10.36%, 46.15%, 21.97%, 63.04%, 2.97%, 63.74% and 6.05%, respectively, when applied with SMS + ZB inoculation compared to abiotic SMS control. The values were greater under SMS inoculation than soil inoculation in all measured parameters with an increase of 77.06% (NOL), 26.63% (PLA), 9.90% (SD), 5.63% (RL), 0.31% (RDW), 22.57% (SDW) and 9.08% (biomass).

Discussion

Phosphorous is by far the least mobile and available to plants in most soil conditions compared with the other major nutrients (Khan et al. 2007). A great amount of P from chemical fertilizers become insoluble due to high reactivity of soluble phosphate with other elements (Al^{3+}, Fe^{3+} and Ca^{3+}) when applied into the soil, which are unavailable to plants. The microorganisms possessing a phosphate-solubilizing ability can convert soil phosphorous to soluble forms available to plants by solubilizing precipitated insoluble phosphatic compounds and mineralizing organic phosphorous (Xiang et al. 2011).

In this study, a phosphate-solubilizing bacterium *P. agglomerans* ZB was isolated from the rhizosphere soil of *Araucaria*. This bacterium has better phosphorous solubilizing ability of various insoluble phosphate compounds. Bashan et al. (2013) suggested that when selecting potential phosphorous-solubilizing bacteria applied for alkaline soils and acidic soils, Ca-P compounds (including rock phosphates), Fe–P and Al-P compounds were added for the metal-P candidates, respectively. Higher amount of dissolved P was obtained by *P. agglomerans* ZB for Ca-P compounds than Fe–P and Al-P compounds, revealing bacteria were more suitable for applying in alkaline soil.

Under optimized culture conditions, inoculation with planktonic cells, pre-incubation with bacteria for TCP and RPs or no pre-cultivation for HP, the maximum dissolved P obtained in present study was 1247.78 mg/L P_2O_5 from TCP, 1442.75 mg/L P_2O_5 from HP and 97.48 mg/L P_2O_5 from RPs, respectively, which was comparable to the previously reported dissolved P data of *P. agglomerans* (Kim et al. 1997; Jung et al. 2002; Son et al. 2006; Bakhshandeh et al. 2014).

Pantoea agglomerans ZB demonstrated the potential as a promising bio-fertilizer. It possessed many plant growth-promoting activities like N_2 fixation and production of IAA, phytase, alkaline phosphatase. Strain ZB could also significantly increase available P content of soil. Pot experiments with chili peppers showed significant improvement of plant growth by applied with *P. agglomerans* biofertilizer, especially under SMS inoculation. SMS, a lignocellulosic byproduct of mushroom industry, could provide nutrition for biofertilizer fermentation (Zhu et al. 2013). The results of our experiment showed *P. agglomerans* biofertilizer converted from SMS compost could not only develop an environmental-friendly biofertilizer with great phosphorous solubilizing ability but also recycle the agricultural waste material.

Conclusion

Pantoea agglomerans ZB revealed comparable phosphate solubilizing ability by optimizing culture conditions, inoculation with planktonic cells, treating with pre-cultivation for tricalcium phosphate and rock phosphates solubilization

and with no pre-incubation for hydroxyapatite dissolution. A promising bio-fertilizer prepared from semi-solid fermentation of isolate ZB with SMS compost might promote the sustainable development of agricultural industry.

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