Enhancing the solubility of insoluble phosphorus compounds by phosphate solubilizing bacteria

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Abstract
Phosphorus (P) is one of the major nutrients for plant growth, and also can be used for metal immobilizing amendment in soil. However, the uptake of P by plants is limited due to its strong adsorption onto soil particles and low solubility of phosphate compounds in soil solution. The objective of this research was to isolate phosphate solubilizing bacteria (PSB) from various soils and evaluate their potential for P solubilization from insoluble P compounds. Nineteen PSB were isolated from various soil samples and six strains solubilized more than 250 mg/L of P from tricalcium phosphate amended National Botanical Research Institute's Phosphate (NBRIP) medium. Direct inoculation of PSB to rock phosphate increased the citric acid solubility of rock phosphate indicating that the isolated PSB strain was effective for P solubilization in soil.

Key Words
Phosphate solubilizing bacteria, Phosphorus, Rock phosphate

Introduction
Phosphorus (P) is one of the major nutrients required for plant growth but most of the P compounds are not readily soluble in soil, hence P is not easily accessible for plant growth. Insoluble phosphate compounds such as rock phosphate are cheaper than soluble P fertilizer, but the solubility of these compounds is a limiting factor for their use as a source of P. Insoluble phosphate compounds can be solubilized by organic acids, phosphatase enzymes and complexing agents produced by plants and microorganisms (Duponnois *et al*. 2005). For example, phosphate solubilizing bacteria (PSB) have been shown to enhance the solubilization of insoluble P compounds through the release of organic acids and phosphatase enzymes (Sahu and Jana 2000). Therefore, PSB have been widely used as inoculants to increase crop yield by solubilizing insoluble P in soils (Sundara *et al*. 2002; Dey *et al*. 2004). The objective of this research was to isolate PSB from various soils and evaluate their potential for P solubilization from insoluble P compounds.

Methods
Isolation of PSB from various soils
Soils used for PSB isolation were locally collected from P amended and Pb contaminated sites. To extract bacteria from soil, 0.5 g of soil was mixed with 50 mL of autoclaved 0.2 % NaCl solution (soil: solution = 1: 100) and shaken for 16 hours (Illmer and Schinner 1992). Suitable dilutions were inoculated using the NBRIP growth agar medium containing (per L) glucose, 10 g; $Ca_3(PO_4)_2$, 5 g; $MgCl_2 \cdot 6H_2O$, 5 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; KCl, 0.2 g and $(NH_4)_2SO_4$, 0.1 g, agar 1.5 % (Nautiyala 1999). The pH of the agar medium was adjusted to 7.0 before autoclaving. Tricalcium phosphate was autoclaved separately. Then, the other sterile ingredients were aseptically mixed after autoclaving. The soil solution (0.1 mL NaCl extract) was spread on the NBRIP agar medium and incubated for 14 days at 25 □. The colonies with clear halo were considered as PSB (Nautiyala 1999) and these colonies were picked up and further purified by re-streaking on an agar plate. The halo and colony diameters were measured after 14 days of the incubation of plates at 25 °C.

Identification of PSB
Crude DNA was extracted from bacterial strains, CS2-B1 and SM1-B1 and almost complete 16S rRNA genes were amplified. Successful amplification of DNA fragment was confirmed by running 5 µL of the PCR reaction on a 1 % agarose gel. The sequencing of 16S rRNA was conducted at the Flinders DNA Sequencing Facility (Adelaide). Acquired 16S rRNA sequence were assessed through the Greengenes website. The 16S rRNA sequences from the isolate were aligned and bootstrapped neighbor-joining relationships were estimated with MEGA version 4.1 (Kumar *et al*. 2008).

Analysis of P solubilization, phosphatase activity and organic acid
The isolated bacteria were cultured in 250 mL Erlenmeyer flask containing 100 mL of tricalcium phosphate-based NBRIP medium and incubated for 14 days. A separate broth medium inoculated with sterile water was served as a control. The PSB culture was harvested by centrifugation at 4000 rpm for 10 minutes followed by filtration with 0.45 μm syringe filter. Phosphorus concentration of the filtrate was measured by spectrophotometer using phosphomolybdate method (Murphy and Riley 1962).
The acid phosphatase activity in the culture media used for P solubilizaiton experiment was measured by following the method based on hydrolysis of ρ-nitrophenyl phosphate (Tabatabai and Bremner 1969). pH and organic acid content of the filtrate were measured using pH meter and ion chromatography (IC, ICS-2000, Dionex), respectively.

Phosphorus solubility after incubation of rock phosphate with PSB
To test the effect of PSB on P solubilization of rock phosphate, 0.2 mL of bacterial suspension (SM1-B1, ca. $1.0X10^9$ CFU/mL) was added to 2 g of rock phosphate in sterile 50 mL tube, 0.8 mL of water was added into the tube and incubated for 7, 14 and 28 days at 25 ℃. Control sample was prepared by adding same amount of sterile water instead of bacterial inoculation. After incubation, samples were analyzed for bacterial population by plate counting method, water- and citric acid-extractable P concentration by phosphomolybdate method using ascorbic acid as the reducing agent (Murphy and Riley 1962).

Results
Isolation of PSB from various soils
Most of the bacteria isolated from P amended soil showed halo formation on NBRIP medium indicating that they are PSB, but only one bacterium showed halo formation from Pb contaminated soils. Among 19 different bacterial strains, CS2-B1, NCPR-B3, JRP-B1, SSP-B1, and TSP-B4 solubilized more than 300 mg/L of P from insoluble tricalcium phosphate and SM1-B1 showed the highest P solubilization among those isolated from Pb contaminated soils (Table 1). In control, 6.6 mg/L of P was solubilized without PSB. Acid phosphatase acivity produced by the isolated bacteria ranged from 0.0034 mM to 0.1420 mM as measured by p-nitrophenol production (Table 1). The PSB strains isolated from P amended soils showed relatively higher acid phosphatase activity compared to the PSB from non-amended soils and Pb contaminated soils. Phosphatase enzymes are produced mostly by microorganisms and play a key role in the solubilization of organic P compounds (Rodríguez and Fraga 1999; Jakobsen *et al*. 2005). The total amount of organic acid produced by the isolated bacteria ranged from 20 mg/L to 112 mg/L (Table 1). The PSB strains isolated from P amended soils produced more organic acid than PSB from Pb contaminated soils and there was a strong relationship between amount of organic acid produced and the extent of P solubilizaiton. The most commonly produced organic acids by the nineteen isolated bacteria were acetic, pyruvic, fumaric and citric acids.

Identification of PSB
The two bacterial strains which showed the highest P solubilization capacity, CS2-B1 and SM1-B1 were putatively identified as *Pantoea sp*. and *Enterobacter cloacae,* respectively and the obtained sequence deposited in the Genbank with accession number GQ414734 and GQ414735, respectively. Phylogenetic tree in Figure 1 based on16S rRNA sequence showed the relationship between isolated bacteria in this research and published PSB.

Table 2. Isolated PSB and their characterization in NBRIP broth medium.

Bacterial strains	Source soil	Halo formation on solid medium	pH	Solubilized P (mg/L)	Acid phosphatase activity (ρ-nitrophenol (mM))	Organic acid (mg/L)*
Control	No bacteria	-	7.10	6.6	0.0144	49
CS1-B1	Control soil1	Yes (3 mm)	7.60	14.9	0.0147	248
CS1-B2	Control soil1	No	6.87	5.7	0.0102	181
CS1-B3	Control soil1	Yes (3 mm)	6.84	5.2	0.0134	125
CS2-B1	Control soil2	Yes (2 mm)	5.68	479.2	0.1420	601
CS2-B2	Control soil2	Yes (2 mm)	6.73	30.1	0.0105	333
NCPR-B1	NCPR	Yes (2 mm)	5.26	281.8	0.0112	204
NCPR-B2	NCPR	Yes (0.5 mm)	6.83	8.2	0.0131	124
NCPR-B3	NCPR	Yes (2 mm)	4.37	387.0	0.1104	1026
SSP-B1	SSP	Yes (2 mm)	4.53	380.5	0.1198	1112
JRP-B1	JRP	Yes (3 mm)	5.20	493.0	0.0034	791
DAP-B1	DAP	Yes (2 mm)	4.66	248.3	0.0837	347
TSP-B1	TSP	Yes (1 mm)	4.64	259.9	0.1286	263
TSP-B2	TSP	Yes (2 mm)	4.75	273.2	0.1286	598
TSP-B3	TSP	Yes (1 mm)	4.86	216.8	0.0824	401
TSP-B4	TSP	Yes (3 mm)	5.1	336.2	0.1045	778
SR-B1	Shooting range	No	6.96	6.6	0.0173	36
SR-B2	Shooting range	No	5.78	40.6	0.0082	44
SM1-B1	Smelter1	Yes (3 mm)	4.60	293.1	0.1401	314
SM2-B1	Smelter2	No	5.46	31.0	0.0073	20

*Organic acid was sum of lactic acid, acetic acid, propionic acid, pyruvic acid, malonic acid, maleic acid, tartaric acid, oxalic acid, succinic acid, fumaric acid, citric acid and trans-aconitic acid.

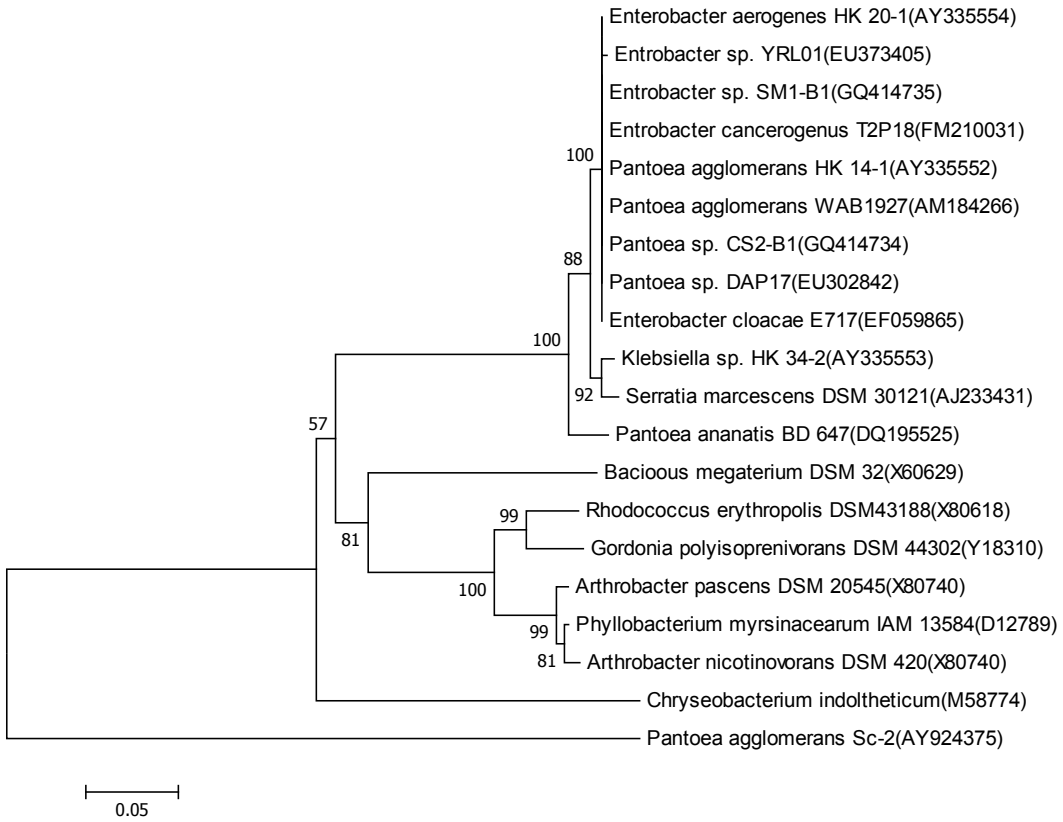

Figure 1. Phylogenetic tree based on16S rRNA sequence that shows the relationships between isolated PSB in this research and other published PSB isolates (accession numbers are given in parentheses). The tree was clustered with the neighbour-joining method using MEGA 4.1 package. Bootstrap values based on 1000 replications were listed as percentages at the nodes. The scale bar indicates 0.05 substitutions per nucleotide position.

Phosphorus solubility after incubation of rock phosphate with PSB

Phosphate solubilizing bacterial inoculation increased the concentration of citric acid-soluble P by 70 - 100 times compared to the control treatment (Figure 2). Citric acid-soluble P increased with incubation period and PSB inoculation achieved 0.93 % (w/w) citric acid-soluble P (9.3 % of total P) compared to 0.013 % (w/w) for control (0.13 % total P) at days 28.

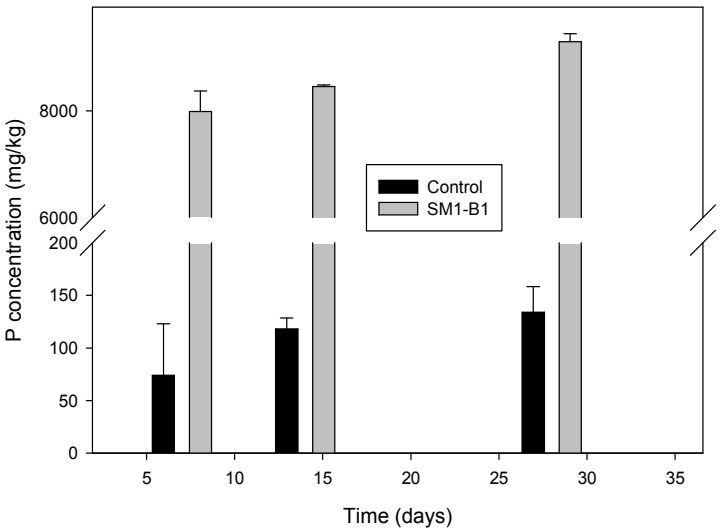

Figure 2. Citric acid extractable P concentration after incubation of rock phosphate with PSB.

Conclusion

Six PSB strains isolated from Pb contaminated and P amended soils solubilized more than 250 mg/L of P from tricalcium phosphate amended NBRIP medium. Direct inoculation of PSB to rock phosphate increased the solubilization of rock phosphate as shown by an increase in citric acid solubility.

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