Journal of Hazardous Materials 185 (2011) 829–836

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

Isolation of phosphate solubilizing bacteria and their potential for lead immobilization in soil

Jin Hee Park[a,b], Nanthi Bolan[a,b,*], Mallavarapu Megharaj[a,b], Ravi Naidu[a,b]

[a] Centre for Environmental Risk Assessment and Remediation, University of South Australia, Mawson Lakes, SA, Australia
[b] Cooperative Research Centre for Contamination Assessment and Remediation of the Environment, Adelaide, SA, Australia

ARTICLE INFO

Article history:
Received 29 June 2010
Received in revised form 17 August 2010
Accepted 11 September 2010
Available online 1 October 2010

Keywords:
Pb contamination
Pb immobilization
Phosphate solubilizing bacteria (PSB)
Rock phosphate
P solubilization

ABSTRACT

Lead (Pb), a highly toxic heavy metal forms stable compounds with phosphate (P). The potential of phosphate solubilizing bacteria (PSB) to immobilize Pb by enhancing solubilization of insoluble P compounds was tested in this research. Eighteen different PSB strains isolated from P amended and Pb contaminated soils were screened for their efficiency in P solubilization. The PSB isolated from P amended soils solubilized 217–479 mg/L of P while the PSB from Pb contaminated soil solubilized 31–293 mg/L of P. Stepwise multiple regression analysis and P solubility kinetics indicated that the major mechanism of P solubilization by PSB is the pH reduction through the release of organic acids. From the isolated bacteria, two PSB were chosen for Pb immobilization and these bacteria were identified as *Pantoea* sp. and *Enterobacter* sp., respectively. The PSB significantly increased P solubilization by 25.0% and 49.9% in the case of *Pantoea* sp., and 63.3% and 88.6% in the case of *Enterobacter* sp. for 200 and 800 mg/kg of rock phosphate (RP) addition, respectively, thereby enhancing the immobilization of Pb by 8.25–13.7% in the case of *Pantoea* sp. and 14.7–26.4% in the case of *Enterobacter* sp. The ability of PSB to solubilize P, promote plant growth, and immobilize Pb can be used for phytostabilization of Pb contaminated soils.

1. Introduction

Phosphorus (P) is one of the major macronutrients required for plant growth, and both soluble and insoluble P compounds are used as a fertilizer source [1]. Phosphate compounds are also used to immobilize heavy metal contaminated environments, especially Pb contaminated soils, through phosphate-heavy metal precipitation [2–4]. Lead is a highly toxic element attracting particular attention because of its widespread occurrence in mine sites and its historical use as a fuel additive and pesticide. The presence of Pb in soils can pose risk to environment and human health. It is widely recognized that the mobility and bioavailability of Pb in soil is more important than the total Pb concentration [5]. Therefore, the reduction of Pb bioavailability is critical for the management and remediation of Pb contaminated soils.

Most P compounds are not readily soluble in soils and hence P is not easily accessible for both plant growth and metal immobilization. Phosphorus is sequestered by adsorption to the soil surface and precipitation by reaction with soil cations, particularly iron, aluminum and calcium [6]. Therefore, a large amount of P fertilizer is used to increase plant growth, which is likely to cause negative impact with respect to both environment and economy. Eutrophication is the main environmental problem that can be caused by excess P application and it is a common phenomenon in inland rivers of many countries including Australia [6].

In soils, insoluble P compounds can be solubilized by organic acids, phosphatase enzymes and complexing agents produced by plants and microorganisms [7]. For example, phosphate solubilizing bacteria (PSB) have been shown to enhance the solubilization of insoluble P compounds [8] and hence PSB have been widely used as inoculants to increase P uptake and crop yield [9]. Some of these PSB have been shown to exhibit plant growth promoting ability as measured by indole acetic acid (IAA) and siderophore production [10,11].

It has often been noticed that the distribution and activity of PSB and their subsequent effect on P solubilization are regulated by exogenous P level [12]. Mikanová and Nováková [13] showed the effect of exogenous level of P in the bacterial medium on P solubilization by PSB. In the presence of soluble P, the solubilization of insoluble P by some PSB was repressed and some PSB was not affected. However, there is a lack of studies on the number of total PSB and their P solubilization ability affected by available P concentration in soil.

Phosphate solubilizing bacteria also have the potential to remediate metal contaminated land by enhancing phosphate-induced immobilization of metals. For example, Wilson et al. [14] demon-

* Corresponding author at: CERAR, Building X, University of South Australia, University Boulevard, Mawson Lakes, SA 5095, Australia. Tel.: +61 8 8302 6218; fax: +61 8 8302 3124.
E-mail address: Nanthi.Bolan@unisa.edu.au (N. Bolan).

0304-3894/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.jhazmat.2010.09.095

strated that application of microbial and phosphate amendments reduced the Pb availability of contaminated soil. Although there have been many studies on the role of PSB to increase P fertilizer value [15,16], there has been no study reported on the environmental application of PSB in relation to the solubilization of P and subsequent Pb immobilization.

Even though metals are toxic to microorganisms, the ability to resist metals can enhance their potential application for the remediation of contaminated soils [17]. Since one of the objectives of this study is to test Pb immobilization by the isolated PSB strains in Pb contaminated soil, it is desirable for these strains to have both P solubilization activity and resistance to Pb so that they can survive and remain active in Pb contaminated soils. Bacteria-induced immobilization of metals is unlikely to occur if heavy metals are present at concentrations that are toxic to the inoculated bacteria [18]. Therefore, PSB were isolated from Pb contaminated soils as well as P amended soils and their P solubilization and growth promoting activities (IAA and siderophore) were compared in this study.

The objectives of our research are: (i) to isolate PSB from P amended soils and Pb contaminated soils with different characteristics, (ii) to evaluate their P solubilization capacity and solubilization mechanisms, and (iii) to evaluate the possible role of PSB and rock phosphate (RP) in Pb immobilization in soil.

2. Materials and methods

2.1. Soils used

Soils used to isolate PSB were locally collected from P amended sites and Pb contaminated sites. Two samples were collected from non-amended sites (virgin soil and agricultural soil), four soils from various P fertilizer amended sites, and three soils from Pb contaminated sites. Phosphate fertilizer amendments include triple superphosphate (TSP), single superphosphate (SSP), diammonium phosphate (DAP) and North Carolina rock phosphate (NCPR). The Pb contaminated sites include a shooting range and a Pb smelter. Soils were dried at room temperature and sieved through a 2 mm mesh.

The soil samples were analyzed for pH, total Pb, NH_4NO_3 extractable Pb and Olsen P. To measure pH, air dried soil sample was extracted with Milli-Q water (soil:water = 1:5) for 1 h. The pH was measured with a pH electrode after calibration. For total heavy metal content, 0.5 g of air dried soil was weighed into Teflon microwave soil digest vessels, 5 mL of aqua regia (HNO_3:HCl = 1:3) was added, and digested in a microwave digestion system (MARSX, CEM, USA). To measure bioavailable Pb concentration, soil sample was extracted with 1 M NH_4NO_3 solution (soil:solution = 1:2.5) for 2 h and Pb was analyzed with Inductively Coupled Plasma Mass Spectroscopy (ICP-MS, Agilent, Japan) [19]. Olsen P was measured by extracting soil with 0.5 M $NaHCO_3$ (soil:solution ratio = 1:20), at pH 8.5 [20]. Phosphorus concentration was measured with a spectrophotometer using the phosphomolybdate method [21].

2.2. Isolation of phosphate solubilizing bacteria

To extract bacteria from soil, field moist soil was mixed with sterile 0.2% NaCl solution (soil:solution = 1:100) and shaken for 16 h [22]. Suitable dilutions were inoculated using the standard National Botanical Research Institute's Phosphate (NBRIP) growth agar medium containing (per L) 10 g glucose, 5 g $Ca_3(PO_4)_2$, 5 g $MgCl_2{\cdot}6H_2O$, 0.25 g $MgSO_4{\cdot}7H_2O$, 0.2 g KCl, 0.1 g $(NH_4)_2SO_4$ and 1.5% agar [23]. The pH of the agar medium was adjusted to 7.0. Tricalcium phosphate was autoclaved separately and the other sterile ingredients were aseptically mixed after autoclaving. After 14 days of incubation of plates at 25 °C, the colonies with clear halos considered to be PSB [23] were counted and predominant colonies were further purified by re-streaking on the NBRIP agar plate. The halo and colony diameters were measured after 14 days of the incubation of plates at 25 °C.

2.3. Phosphorus solubilization

Solubilization of P by PSB was quantified using insoluble $Ca_3(PO_4)_2$ in NBRIP broth medium. One mL bacterial suspension (ca. 1×10^8 CFU/mL) was transferred to a 250 mL Erlenmeyer flask containing 100 mL of the medium and incubated for 14 days. A separate broth medium inoculated with sterile Milli-Q water served as the control treatment. The PSB culture medium was filtered with 0.45 μm syringe filter to analyze P concentration of the medium. Solubilized P concentration was measured by the phosphomolybdate method [21]. The pH of the each filtrate was measured and organic acid content of the filtrate was analyzed using ion chromatography (IC, ICS-2000, Dionex, USA). Lactate, acetate, propionate, pyruvate, malonate, maleate, tartarate, oxalate, succinate, fumarate, citrate and trans-aconite were analyzed [24].

2.4. Phosphatase activity

The acid phosphatase activity in the culture medium used for P solubilization experiment was measured by the method based on the hydrolysis of *p*-nitrophenyl phosphate as described by Tabatabai and Bremner [25]. Culture filtrate was incubated with *p*-nitrophenyl phosphate and modified universal buffer. After 1 h the hydrolysis reaction of *p*-nitrophenyl phosphate by phosphatase was terminated by adding 0.5 M $CaCl_2$ and 0.5 M NaOH solution. The mixture was centrifuged and the yellow color of supernatant was measured at 410 nm.

2.5. Indole acetic acid production

Selected PSB strains based on their ability to solubilize P were analyzed for IAA production [26]. The selective bacterial strains were grown in a minimal medium (50 mM KH_2PO_4, 50 mM K_2HPO_4, 5 mM $MgSO_4$, 25 mM $(NH_4)_2SO_4$, 1% glucose) amended with 0.05% of L-tryptophan at 25 °C for 14 days in a shaking incubator at 180 rpm. A separate broth medium inoculated with sterile Milli-Q water served as the control treatment. The culture filtrates were collected by a syringe filter. One mL of aliquot of the supernatant was mixed vigorously with 2 mL of Salkowski's reagent [27]. The mixture was incubated for 30 min at 25 °C in the dark. The absorbance of the resulting solution was measured at 535 nm. The concentration of IAA in the culture medium was determined using a standard curve prepared with various concentrations of analytical grade IAA.

2.6. Siderophore production

Siderophore production by selected PSB strains was determined in a Fe-deficient mineral salt medium. The mineral salt medium contained: 0.36 g KH_2PO_4, 1.4 g K_2HPO_4, 0.25 g $MgSO_4{\cdot}7H_2O$, 0.2 g NaCl, 0.02 g $CaCl_2{\cdot}2H_2O$, 15 mg EDTA, 0.16 mg $ZnSO_4$, 0.25 mg H_3BO_3, 0.2 mg Na_2MoO_4, 0.2 mg $MnSO_4{\cdot}4H_2O$, 0.02 mg $CuSO_4{\cdot}5H_2O$/L of water with 20 mM of mannitol and 10 mM of NH_4Cl_2 [26]. The selected strains were inoculated in the mineral salt media and incubated in a shaking incubator at 25 °C for 14 days at 180 rpm. A separate broth medium inoculated with sterile Milli-Q water served as the control treatment. The culture supernatants were filtered using 0.45 μm syringe filter. The filtrate was assayed for siderophore production using the Chrome Azurol S (CAS) assay [28]. Positive reactions were qualitatively estimated by changes in the color of the assay reagent from blue to orange. The

assay was considered negative when no change in the blue color was observed within 3 h. The absorbance was measured at 630 nm.

2.7. Identification of bacteria

Crude DNA extractions from selected bacterial strains were prepared [29]. Crude DNA extract was diluted in sterile Milli-Q water before carrying out PCR analysis under the following conditions [30]. A 25 μL PCR mixture contained Taq DNA polymerase buffer (Promega, Sydney), 2.5 mM $MgCl_2$, 200 μM betaine (Sigma, Sydney), 0.2 mM of each deoxynucleoside triphosphate, 25 pmol of each forward and reverse primers, 1 U of DNA polymerase (Promega, Sydney), and 1 μL of the diluted DNA extract as template. Almost complete 16S rRNA genes were amplified with the forward primer E8f (5′-AGAGTTTGATCCTGGCTCAG-3′) and the reverse primer 1541r (5′-AAGGAGGTGATCCANCCRCA-3′). The DNA was amplified with an iCycler thermocycler (BioRad, Sydney) with the following program: 5 min of pre-heating at 95 °C, 30 cycles of 30 s of denaturation at 95 °C, 30 s of primer annealing at 55 °C, 2 min of elongation at 72 °C, and 10 min of extension step at 72 °C. Successful amplification of a ~1525 bp DNA fragment was confirmed by running 5 μL of the PCR product on a 1% agarose gel. The PCR products were purified using MoBio UltraClean PCR Purification Kit (Geneworks, Adelaide), and sequencing of 16S ribosomal RNA (rRNA) was conducted at the Flinders DNA Sequencing Facility (Adelaide). Acquired 16S rRNA sequences were assessed through the Greengenes website. 16S rRNA sequences from the isolate were aligned and bootstrapped neighbour-joining relationships were estimated with MEGA version 4.1 [31].

2.8. Solubilization of rock phosphate and immobilization of Pb

To test the effect of PSB on solubilization of RP, 0.5 mL of bacterial suspension in sterile water (ca. 1.0×10^9 CFU/mL) was added to 5 g of sterile soil samples treated with various levels of RP (0, 200 and 800 mg P/kg soil) in a 50 mL tube, and incubated for 14 days at 25 °C. A control sample was prepared by adding the same amount of sterile Milli-Q water instead of bacteria inoculation. After incubation, samples were analyzed for Olsen P concentration as an index of P solubilization. Percentage increase in P solubilization resulting from PSB inoculation was estimated from the following equation (Eq. (1)):

$$\%\text{P solubilization} = \frac{\text{Olsen P[(Soil + RP + PSB)]}}{\text{Olsen P(Soil + RP)}} \times 100 \quad (1)$$

In a separate experiment, the soil samples used for solubilization of RP were spiked with $Pb(NO_3)_2$ at a concentration of 2000 mg Pb/kg soil and incubated with three times of wetting-drying cycle over a period of 20 days at 37 °C [32]. Various levels of RP (0, 200 and 800 mg P/kg soil) were amended to 5 g of sterile Pb-spiked soil and inoculated with bacterial strains CS2-B1 and SM1-B1. After 14 days of incubation, NH_4NO_3 extractable Pb concentration was analyzed using ICP-MS. Ammonium nitrate extractable Pb represents soil solution concentration (i.e. unimmobilized Pb) [19] and the percentage increase in Pb immobilization resulting from PSB inoculation was estimated from the following equation (Eq. (2)):

$$\%\text{Pb immobilization} = \frac{\text{NH}_4\text{NO}_3 \text{ extractable Pb [(Soil + RP) − (Soil + RP + PSB)]}}{\text{NH}_4\text{NO}_3 \text{ extractable Pb (Soil + RP)}} \times 100 \quad (2)$$

Ammonium nitrate extractable Pb has often been used to estimate Pb immobilization by various amendments including phosphate compounds [33].

2.9. Statistical analysis

All data were expressed as an average of three replicates. The data collected were analyzed statistically using SPSS 17 software. Relationships between different data were evaluated by means of simple linear regression analysis. Stepwise multiple regression analysis was computed using solubilized P concentration as a dependent variable, and pH, acid phosphatase and organic acid as independent variables to determine important factors for P solubilization by PSB. The acceptance limit was set to 95% significance level. Duncan's multiple range test was used to compare the means of the treatments, variability in the data was expressed as the standard deviation and a $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. Soil characteristics

The various physicochemical properties of soils are presented in Table 1. The two control soils (CS1 and CS2) were slightly acidic and NH_4NO_3 extractable Pb was not detected in these soils (detection limit: 0.003 mg/kg). The Olsen P content of CS2 was higher than CS1, but it was lower than that of the P amended soils. The Olsen P content of P amended soils (NCPR, SSP, DAP and TSP) was higher than 20 mg P/kg and pH ranged from acidic to neutral. The Olsen P content of Pb contaminated soils (SR, SM1 and SM2) was relatively lower than P amended soils, except SM1 for which Olsen P content was 53.1 mg P/kg. Ammonium nitrate extractable Pb concentration for the Pb contaminated soils ranged from 0.08 mg/kg to 29 mg/kg and 8.3% of total Pb in SR soil was NH_4NO_3 extractable Pb, indicating the high bioavailability of Pb in this soil. Ammonium nitrate extractable Pb concentration of SM1 was very low which may be attributed to the high Olsen P content of this soil. While the pH of SR was slightly acidic, the pH of SM1 and SM2 was alkaline.

The number of viable PSB colonies isolated from various soils is shown in Table 1. There was no significant correlation between Olsen P and number of PSB in the present study. However, it emerges that the number of PSB was relatively higher in P amended soils than in Pb contaminated and control soils, which may be attributed to either the supply of soluble P or Pb toxicity.

3.2. Isolation of phosphate solubilizing bacteria and their P solubilization characteristics

Eighteen morphologically different PSB strains based on halo formation in NBRIP medium were isolated from 9 different control, P fertilizer amended and Pb contaminated soils. The halo formation around the colonies provides the first qualitative indication of PSB [23]. The size of the halo ranged from 0.1 mm to 3 mm after 2 weeks of incubation.

The amount of P solubilized by various bacterial strains isolated from different soils is shown in Table 2. In the absence of PSB (i.e. control), 6.6 mg/L of P was solubilized in the culture medium. Among 14 different bacterial strains isolated from non-amended control soils and P fertilizer amended soils, CS2-B1, NCPR-B3, SSP-B1, and TSP-B4 solubilized more than 300 mg/L of P from insoluble $Ca_3(PO_4)_2$. SM1-B1 showed the highest P solubilization (293.1 mg/L) among the 4 strains isolated from Pb contaminated soils.

Bacterial growth reduced pH in the bacterial medium compared to the control medium except CS1-B1 strain. The pH of culture ranged from 4.31 to 7.60. Acid phosphatase activity produced by the isolated bacteria ranged from 0.0034 mM to 0.1420 mM as measured by *p*-nitrophenol production (Table 2). The PSB strains isolated from P amended soils showed relatively higher acid phos-

Table 1
Physicochemical properties of soils used for isolating PSB and number of viable colonies of PSB isolated from soils.

Sources	Soils	Description	pH	Total Pb (mg/kg)	NH_4NO_3 extractable Pb (mg/kg)	Olsen P (mg/kg)	Viable colonies of PSB
Non-amended sites	CS1	Virgin soil	5.78	–	–	4.3	1.60×10^5
	CS2	Agricultural control soil	5.32	–	–	18	3.54×10^6
P fertilizer amended sites	NCPR	North Carolina Phosphate rock amended soil	5.49	–	–	28	1.85×10^7
	SSP	Single Super Phosphate amended soil	5.25	–	–	28	1.36×10^7
	DAP	Diammonium phosphate amended topsoil (0–10 cm)	7.36	–	–	41	5.39×10^5
	TSP	Diammonium phosphate amended subsoil (10–20 cm)	7.55	–	–	120	8.69×10^6
Pb contaminated sites	SR	Pb contaminated soil from shooting range	5.88	346.1	29	3.8	1.40×10^4
	SM1	Pb contaminated soil from Zn, Pb smelter	8.20	619.0	0.077	53	2.20×10^4
	SM2	Pb contaminated soil from Zn, Pb smelter	9.26	2479	1.5	7.7	6.00×10^3

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

Bacterial strains	Source soil	Halo formation on solid medium (mm)	pH	Solubilized P (mg/L)	Acid phosphatase activity (*p*-nitrophenol (mM))	Organic acid (mg/L)[a]
Control	No bacteria	–	7.10	6.6	0.0144	49
CS1-B1	CS1	3	7.60	14.9	0.0147	248
CS1-B2	CS1	0.1	6.87	5.7	0.0102	181
CS1-B3	CS1	3	6.84	5.2	0.0134	125
CS2-B1	CS2	2	4.31	479.2	0.1420	601
CS2-B2	CS2	2	6.73	30.1	0.0105	333
NCPR-B1	NCPR	2	5.26	281.8	0.0112	204
NCPR-B2	NCPR	0.5	6.83	8.2	0.0131	124
NCPR-B3	NCPR	2	4.37	387.0	0.1104	1026
SSP-B1	SSP	2	4.53	380.5	0.1198	1112
DAP-B1	DAP	2	4.66	248.3	0.0837	347
TSP-B1	TSP	1	4.64	259.9	0.1286	263
TSP-B2	TSP	2	4.75	273.2	0.1286	598
TSP-B3	TSP	1	4.86	216.8	0.0824	401
TSP-B4	TSP	3	5.1	336.2	0.1045	778
SR-B1	SR	0.1	6.96	6.6	0.0173	36
SR-B2	SR	0.1	5.78	40.6	0.0082	44
SM1-B1	SM1	3	4.60	293.1	0.1401	314
SM2-B1	SM2	0.1	5.46	31.0	0.0073	20

[a] Organic acid was the 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.

phatase activity compared to the PSB from non-amended soils and Pb contaminated soils.

The amount of organic acids produced by the isolated bacteria ranged from 20 mg/L to 1112 mg/L. The PSB strains which solubilized more than 300 mg/L of P produced more than 600 mg/L of total organic acids (Table 2). The PSB strains isolated from P amended soils produced more organic acid than PSB from Pb contaminated soils. The most commonly produced organic acids by the 18 isolated bacteria were acetic, pyruvic, fumaric and citric acids.

3.3. Relationship between solubilized P concentration and pH, phosphatase activity and organic acid

The pH, phosphatase activity and organic acid concentration in bacterial cultures were analyzed and their correlation with solubilized P was evaluated to describe the mechanism of P solubilization through bacterial action. The simple regression analysis between solubilized P and acid phosphatase activity ($R^2 = 0.76$, Fig. 1(a)), organic acid ($R^2 = 0.61$, Fig. 1(b)) and pH ($R^2 = 0.79$, Fig. 1(c)) showed a significant linear relationship ($p < 0.001$). However, the stepwise multiple regression (Eq. (3)) indicated that pH was the most important contributor in P solubilization followed by organic acid concentration. The regression equation (Eq. (3)) showed that 88.4% of variability in P solubilization can be accounted for by these two variables.

$$P_{solubilized} = 655(\pm 106) - 97.8(\pm 16.3)\,pH + 0.191(\pm 0.0550)\,\text{organic acid} \quad (3)$$

3.4. Plant growth promoting effect of isolated bacteria

The plant growth promoting effect of selected PSB was evaluated by analyzing IAA and siderophore production (Table 3). All isolated bacteria revealed the ability to produce IAA, indicating that these strains could utilize L-tryptophan as a precursor for growth. Bacterial strain NCPR-B3 showed the highest IAA production (34.1 mg/L), and CS2-B1, SSP-B1 and SM1-B1 showed significantly higher siderophore production than the control treatment.

3.5. Identification of bacteria

The two bacterial strains that showed the highest P solubilization capacity and plant growth promoting effect from

Table 3
Indole acetic acid and siderophore production by 5 bacterial strains.

Samples	IAA production (mg/L)[a]	Siderophore production[a] (abs at 630 nm)
Control	0	0.0500 ± 0.0013 a
CS2-B1	12.83 ± 1.10 bc	0.0628 ± 0.0050 b
NCPR-B3	34.12 ± 1.49 a	0.0560 ± 0.0005 ab
SSP-B1	11.63 ± 0.18 b	0.0853 ± 0.0005 c
TSP-B4	13.37 ± 0.21 bc	0.0598 ± 0.0078 ab
SM1-B1	16.22 ± 0.37 d	0.0652 ± 0.0046 b

[a] Values are mean ± standard deviation of triplicate and the different letters within a column indicate a significant difference at $p < 0.05$ according to Duncan's multiple range tests using SPSS 17.

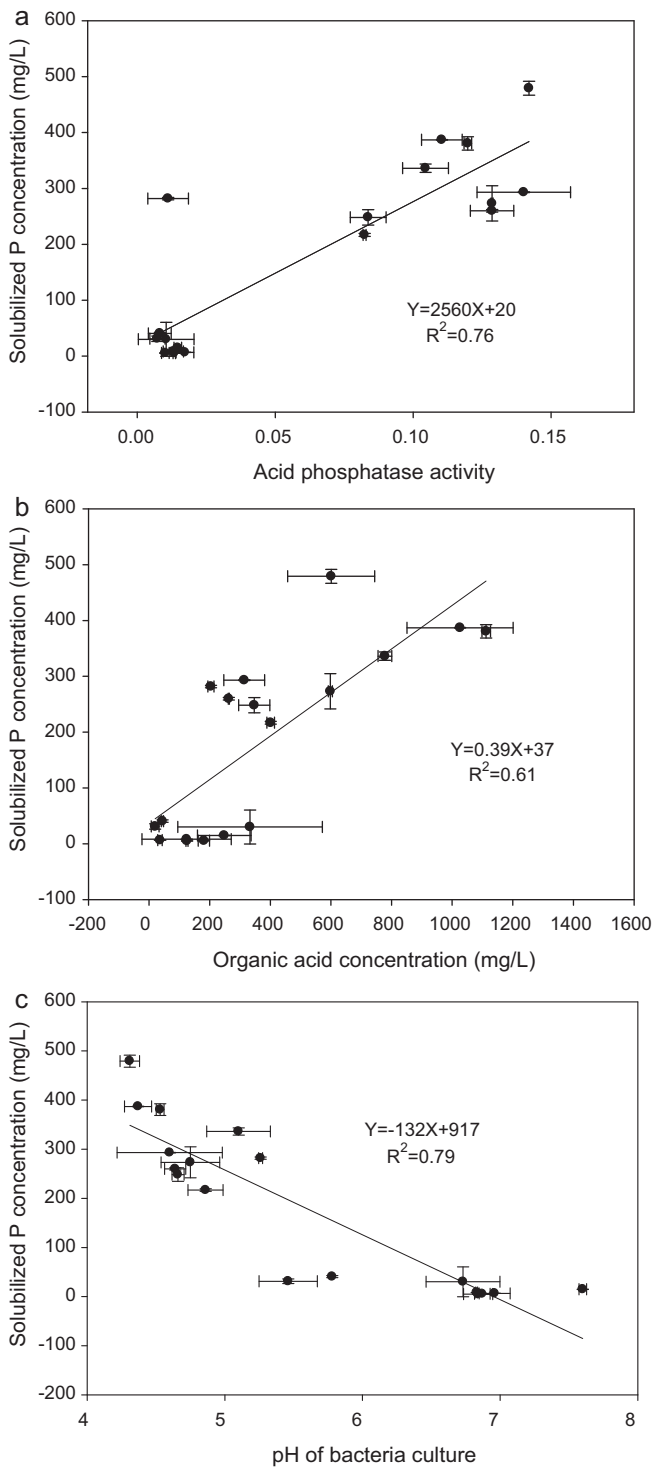

Fig. 1. Relationship between acid phosphatase activity (a), organic acid (b) and pH (c) of bacterial culture and solubilized P. Each value represents the mean of three replicates with standard deviation shown by error bars.

non-amended soil and Pb contaminated soil, CS2-B1 and SM1-B1 were putatively identified as *Pantoea* sp. and *Enterobacter cloacae*, respectively. Additionally the obtained sequences were deposited in the GenBank with accession number GQ414734 and GQ414735, respectively. Phylogenetic tree in Fig. 2 based on 16S rRNA sequence revealed a relationship between isolated bacteria in this research and other PSB strains reported in the literature. Two isolated bacteria were closely related to each other in phylogenetic tree.

3.6. PSB-enhanced solubilization of rock phosphate and immobilization of Pb

Phosphorus solubility as measured by Olsen P increased significantly with PSB inoculation. Phosphate solubilizing bacteria inoculation increased P solubilization by 25.0% and 49.9% for CS2-B1 and 63.3% and 88.6% for SM1-B1 at 200 and 800 mg/kg of RP addition, respectively (Fig. 3).

Phosphate solubilizing bacteria in the absence of RP amendment decreased NH_4NO_3-extractable Pb concentration and immobilized Pb by 7.6% in soil compared to the non-inoculated soil (Fig. 4). Rock phosphate addition decreased NH_4NO_3-extractable Pb concentration, indicating the immobilization of Pb. Phosphate solubilizing bacteria inoculation decreased NH_4NO_3-extractable Pb concentration in RP amendment and the decrease in Pb concentration increased with increasing levels of P addition. Phosphate solubilizing bacteria inoculation increased Pb immobilization by 8.25% and 13.7% for CS2-B1 and 14.7% and 26.4% for SM1-B1 at 200 and 800 mg/kg of RP addition, respectively (Fig. 4).

4. Discussion

Although the mechanism of P solubilization by organic acid is not well understood, it was reported that the pH decrease resulting from the release of organic acid could be related to P solubilization [34]. For example, RP is dissolved by the acid produced in the soils (Eq. (4)), which is a major reason of RP effectiveness as a P source mainly in acid soils (pH < 6.5) [35] and as a metal immobilizing agent in acid mine drainage and wastes [36,37].

$$Ca_{10}(PO_4)_6F_2 + 12H^+ \rightarrow 10Ca^{2+} + 6H_2PO_4^- + 2F^- \quad (4)$$

Bolan et al. [38] reported that low-molecular weight organic acids such as lactic, oxalic and citric acids increased availability of P from monocalcium phosphate and RP. The use of naturally occurring low-molecular weight organic acids produced in soil as microbial metabolites or plant exudates from dead or living cells is effective in releasing P from RP [39]. Kumar et al. [40] isolated a metal tolerant plant growth promoting bacterium (*Enterobacter* sp.) which decreased the pH of the growth medium from 7 to 2, thereby achieving the maximum solubilization of P (229 mg/L). Similarly, Schneider et al. [41] suggested that citric acid and oxalic acid produced by *Aspergillus niger* mobilized P from RP.

An attempt was made to predict the solubilization of $Ca_3(PO_4)_2$ from the changes in the pH in the broth culture medium. Bohner et al. [42] proposed diffusion controlled dissolution kinetics for $Ca_3(PO_4)_2$ in aqueous medium. They showed that the logarithm of the dissolution rate (k_s; mmol m^{-2} s^{-1}) is a linear function of the pH at zero saturation of Ca^{2+} and PO_4^{3-} ions in initial solution (Eq. (5)).

$$\mathrm{Log}(k_s) = 2.02 - 0.82\,\mathrm{pH}, \quad R^2 = 0.993 \quad (5)$$

The dissolution rates at different final pH in the culture media were calculated according to Eq. (5). Based on the particle size of $Ca_3(PO_4)_2$ used in our experiment we assumed a specific surface area of 0.15 m^2/g [43]. The predicted amount of dissolved P concentration was calculated by multiplying the calculated dissolution rate by specific surface area and reaction time. There was a significant correlation between the predicted P concentration based on the final pH value and measured P concentration (Fig. 5), indicating that the decrease in pH resulting from the release of organic acids by PSB is an important process regulating the solubilization of $Ca_3(PO_4)_2$.

It is known that phosphatase activity contributes to the solubilization of organic phosphates in soil [1]. Normally the pH of agricultural soils ranges from acidic to neutral, so acid phosphatases

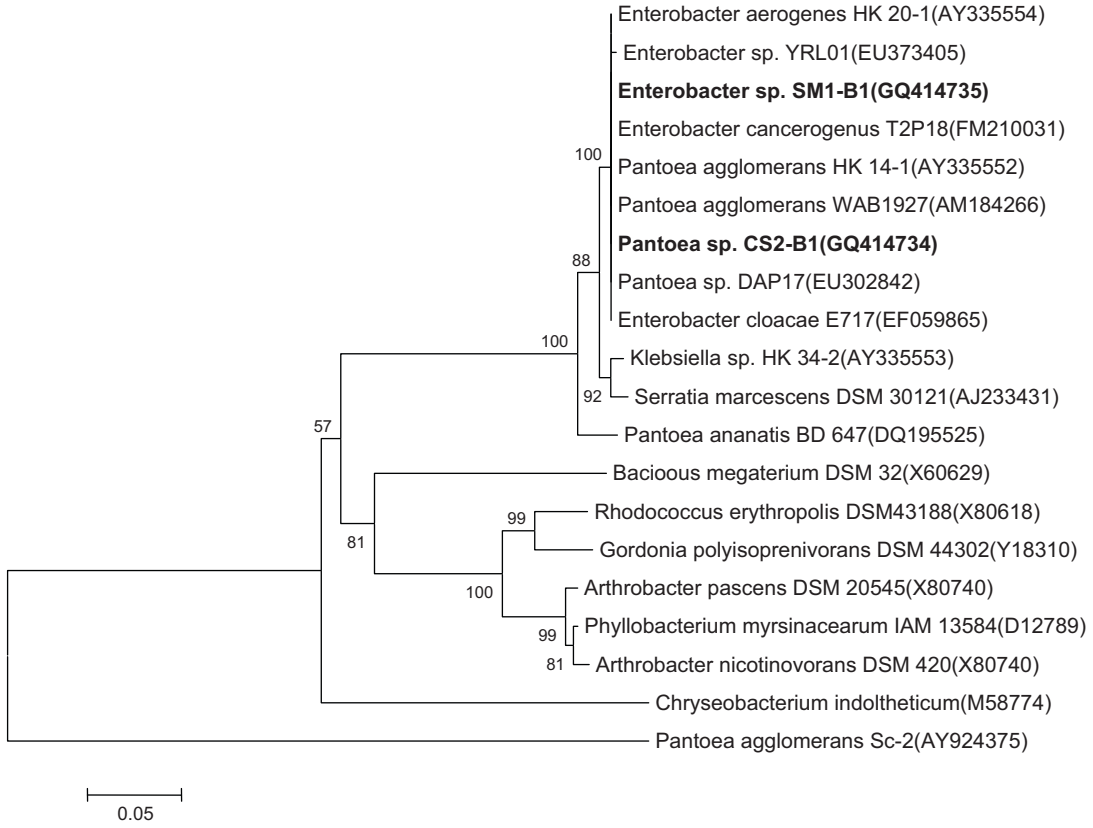

Fig. 2. Phylogenetic tree based on16S rRNA sequence showing the relationships between isolated PSB in this research and other published PSB isolates (accession numbers are given in parentheses). The tree was clustered with the neighbour-joining method using MEGA4.1 package. Bootstrap values based on 1000 replications are listed as percentages at the nodes. The scale bar indicates 0.05 substitutions per nucleotide position. Bacterial strains in bold indicate isolated PSB in this study.

are thought to play a major role in P solubilization [12]. Phosphatase does not act directly on inorganic P solubilization, but phosphatase activity may participate in lowering the pH of the culture medium by the dephosphorylating action and the production of acids [44]. This was supported by a significant correlation between phosphatase activity and pH of culture (R^2 = 0.69, Fig. not shown.).

Bacteria can enhance plant growth directly or indirectly by increasing available P, fixing nitrogen, sequestering iron by siderophores, producing antibiotics and plant hormones [45,46]. This group of bacteria is known as plant growth promoting bacteria (PGPB). Plant growth promoting bacteria can also be used to improve phytoremediation efficiency by increasing biomass production and uptake of contaminants [47]. Production of IAA by isolated PSB ranged from 12 mg/L to 34 mg/L indicating that the isolated PSB have plant growth promoting effect. It has been reported that P deficiency often induces N starvation, thus leading to higher IAA production [48]. Therefore, the ability to produce IAA of PSB is

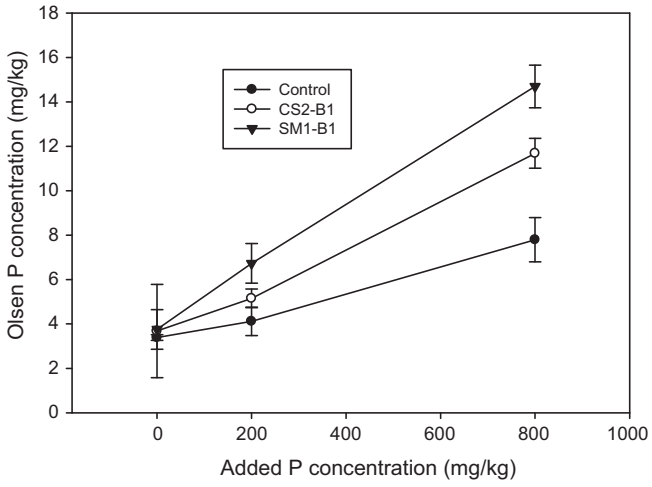

Fig. 3. Olsen P concentration of soils after incubation of CS2-B1 or SM1-B1 with various amounts of rock phosphate. Each value represents the mean of three replicates with standard deviation shown by error bars.

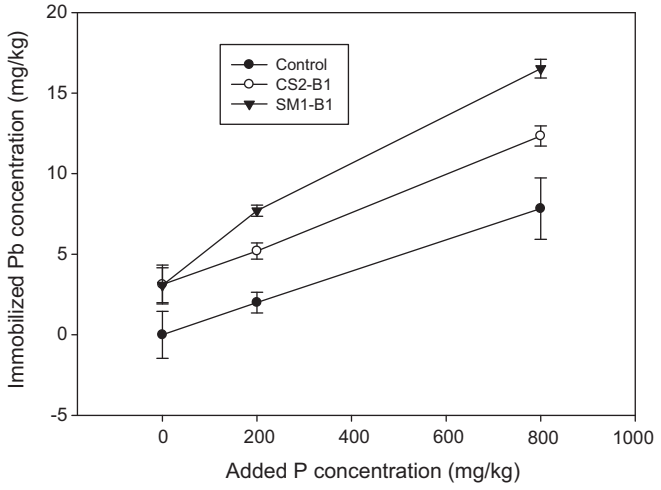

Fig. 4. Lead immobilization as measured by NH_4NO_3 extractable Pb with various amounts of rock phosphate addition and PSB (CS2-B1 or SM1-B1). Each value represents the mean of three replicates with standard deviation shown by error bars.

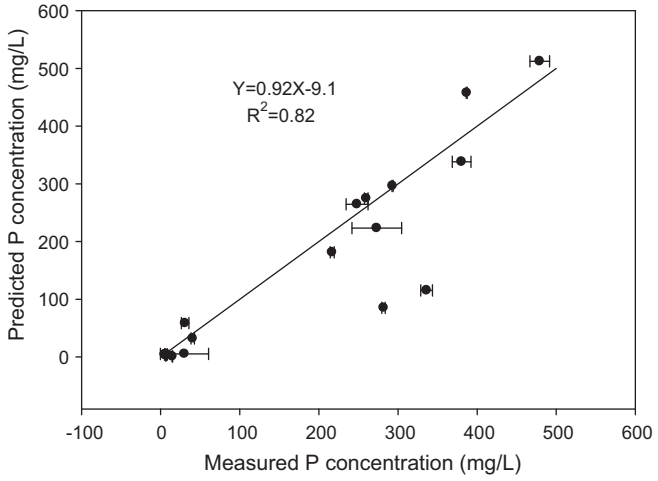

Fig. 5. Relationship between measured P concentration and predicted P concentration. Line indicates 1:1 correlation.

important in the application of the isolated PSB to phytostabilization. A copper resistant PGPB, *Achromobacter xylosoxidans* produced 6.4 mg/L of IAA [49]. Ma et al. [50] noticed that IAA production by nickel resistant bacteria ranged from 22.0 mg/L for *Bacillus* sp. to 87.7 mg/L for *Psychrobacter* sp. and high IAA production resulted in high plant biomass. Siderophores solubilize and sequester iron from the soil, but siderophores may also play a role in mobilizing heavy metals because they show a great affinity for divalent heavy metal ions [51,52]. Bacterial strain SSP-B1 showed the highest siderophore production. Indole acetic acid production contributes to phytostabilization by increasing root and shoot biomass [53], but siderophore production may increase phytoextraction capacity [54].

The isolated PSB in this research are closely related to other reported PSB isolates. *Pantoea* sp. and *Enterobacter* sp. were reported as PSB by other authors. Chung et al. [55] isolated bacteria from rhizosphere soils of various crops in South Korea and reported the genera *Enterobacter* and *Pantoea* as PSB. Pérez et al. [56] isolated PSB from iron-rich acidic soil in Venezuela and identified the genera belonging to *Burkholderia*, *Serratia*, *Ralstonia* and *Pantoea* by partial sequencing analysis of their respective 16S rRNA genes.

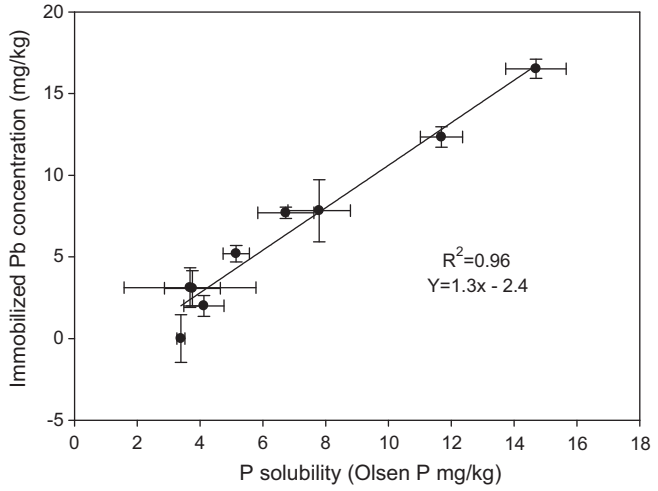

Fig. 6. Relationship between P solubility and Pb immobilization in soil. Each value represents the mean of three replicates with standard deviation shown by error bars.

Ghani et al. [57] investigated the enhancement of RP solubility through biological processes for 4 weeks and showed an increase in water and bicarbonate-extractable P with time. The solubility of P and Pb sources determines the immobilization of Pb by regulating the kinetics of pyromorphite precipitation process which is very rapid once both P and Pb ions become available in solution [58]. In our experiment, PSB have been shown to increase the solubilization of RP, thereby releasing P to solution. Bacteria have also been shown to increase the solubilization of Pb in soils [59,60]. Lead solubilization resulting from PSB is likely to facilitate the subsequent immobilization of Pb by P. Therefore, we included a soil sample without RP amendment (Fig. 4) and it was noticed that the isolated PSB immobilized Pb in soil even in the absence of RP addition. This may be attributed to the immobilization of Pb by native P in the soil (with an Olsen P level of 3.7 mg/kg).

The effectiveness of RP in immobilizing Pb from contaminated soils increased with increasing levels of RP because more dissolved P became available with increasing RP loading [61]. Therefore, dissolution of RP is an important process to enhance Pb immobilization as shown by the significant positive correlation between P solubility and Pb immobilization (Fig. 6). The dissolution of RP and precipitation of Pb as a carbonated fluoropyromorphite-like mineral is the primary mechanism of Pb immobilization by RP [4].

5. Conclusions

The results have demonstrated that: (i) PSB increase the solubilization of insoluble P compounds and the principal PSB-induced P solubilization mechanism is pH reduction by organic acid production; (ii) PSB provide plant growth promoting potential through the release of IAA and siderophores; and (iii) PSB enhance the P-induced immobilization of Pb through the release of P from insoluble P compounds.

The ability of selected PSB to solubilize insoluble P sources and promote plant growth can be used for phytostabilization of Pb contaminated soils by immobilizing Pb with insoluble P sources, thereby overcoming environmental degradation resulting from soluble phosphate sources used for Pb immobilization.

Acknowledgements

This study was sponsored by Cooperative Research Centre for Contamination Assessment and Remediation of the Environment (CRC CARE), Australia in collaboration with University of South Australia. The Fostering Project of Hub University of Industrial Collaboration (HUNIC), Jinju National University, Korea with CRC CARE partly supported this work. The authors thank Dr. Seidu Malik and Dr. Michael Beer for their assistance with identification of bacteria and Dr. Mohammad Rahman for technical assistance with the ICP analysis.

References

[1] J.T. Sims, A.N. Sharpley, Phosphorus: Agriculture and the Environment, American Society of Agronomy, 2005.
[2] N.S. Bolan, D.C. Adriano, R. Naidu, Role of phosphorus in (im) mobilization and bioavailability of heavy metals in the soil–plant system, Rev. Environ. Contam. Toxicol. (2003) 1–44.
[3] J. Cotter-Howells, S. Caporn, Remediation of contaminated land by formation of heavy metal phosphates, Appl. Geochem. 11 (1996) 335–342.
[4] Q.Y. Ma, T.J. Logan, S.J. Traina, Lead immobilization from aqueous solutions and contaminated soils using phosphate rocks, Environ. Sci. Technol. 29 (1995) 1118–1126.
[5] G.R. MacFarlane, M.D. Burchett, Toxicity, growth and accumulation relationships of copper, lead and zinc in the grey mangrove *Avicennia marina* (Forsk.) Vierh, Mar. Environ. Res. 54 (2002) 65–84.
[6] J.N. Harris, P.B. New, P.M. Martin, Laboratory tests can predict beneficial effects of phosphate-solubilising bacteria on plants, Soil Biol. Biochem. 38 (2006) 1521–1526.

[7] R. Duponnois, A. Colombet, V. Hien, J. Thioulouse, The mycorrhizal fungus *Glomus intraradices* and rock phosphate amendment influence plant growth and microbial activity in the rhizosphere of *Acacia holosericea*, Soil Biol. Biochem. 37 (2005) 1460–1468.
[8] H. Son, G. Park, M. Cha, M. Heo, Solubilization of insoluble inorganic phosphates by a novel salt- and pH-tolerant *Pantoea agglomerans* R-42 isolated from soybean rhizosphere, Bioresour. Technol. 97 (2006) 204–210.
[9] Z. Chen, S. Ma, L.L. Liu, Studies on phosphorus solubilizing activity of a strain of phosphobacteria isolated from chestnut type soil in China, Bioresour. Technol. 99 (2008) 6702–6707.
[10] E. Gamalero, L. Fracchia, M. Cavaletto, J. Garbaye, P. Frey-Klett, G.C. Varese, M.G. Martinotti, Characterization of functional traits of two fluorescent pseudomonads isolated from basidiomes of ectomycorrhizal fungi, Soil Biol. Biochem. 35 (2003) 55–65.
[11] A. Zaidi, M.S. Khan, M. Ahemad, M. Oves, Plant growth promotion by phosphate solubilizing bacteria, Acta Microbiol. Immunol. Hung. 56 (2009) 263–284.
[12] H. Rodriguez, R. Fraga, Phosphate solubilizing bacteria and their role in plant growth promotion, Biotechnol. Adv. 17 (1999) 319–339.
[13] O. Mikanová, J. Nováková, Evaluation of the P-solubilizing activity of soil microorganisms and its sensitivity to soluble phosphate, Rost. Vyroba 48 (2002) 397–400.
[14] C. Wilson, R.L. Brigmon, A. Knox, J. Seaman, G. Smith, Effects of microbial and phosphate amendments on the bioavailability of lead (Pb) in shooting range soil, Bull. Environ. Contam. Toxicol. 76 (2006) 392–399.
[15] Y.P. Chen, P.D. Rekha, A.B. Arun, F.T. Shen, W. Lai, C.C. Young, Phosphate solubilizing bacteria from subtropical soil and their tricalcium phosphate solubilizing abilities, Appl. Soil Ecol. 34 (2006) 33–41.
[16] S.N. Sahu, B.B. Jana, Enhancement of the fertilizer value of rock phosphate engineered through phosphate-solubilizing bacteria, Ecol. Eng. 15 (2000) 27–39.
[17] T.M. Roane, Lead resistance in two bacterial isolates from heavy metal-contaminated soils, Microb. Ecol. 37 (1999) 218–224.
[18] K. Sani, Assessment of lead toxicity to *Desulfovibrio desulfuricans* G20: influence of components of lactate C medium, Adv. Environ. Res. 5 (2001) 269–276.
[19] M. Pueyo, J.F. López-Sánchez, G. Rauret, Assessment of $CaCl_2$, $NaNO_3$ and NH_4NO_3 extraction procedures for the study of Cd, Cu, Pb and Zn extractability in contaminated soils, Anal. Chim. Acta 504 (2004) 217–226.
[20] S.R. Olsen, C.V. Cole, F.S. Watanabe, L.A. Dean, Estimation of available phosphorus in soils by extraction with sodium bicarbonate, in: L.C. Blakemore, P.L. Searle, B.K. Daly (Eds.), Soil bureau laboratory methods: A. methods for chemical analysis of soils, Soil Bureau Scientific Report 80, New Zealand, 1954, pp. 35–36.
[21] J. Murphy, J.P. Riley, A modified single solution method for the determination of phosphate in natural waters, Anal. Chim. Acta 27 (1962) 31–36.
[22] P. Illmer, F. Schinner, Solubilization of inorganic phosphates by microorganisms isolated from forest soils, Soil Biol. Biochem. 24 (1992) 389–395.
[23] C.S. Nautiyala, An efficient microbiological growth medium for screening phosphate solubilizing microorganisms, FEMS Microbiol. Lett. 170 (1999) 265–270.
[24] R. Baziramakenga, R.R. Simard, G.D. Leroux, Determination of organic acids in soil extracts by ion chromatography, Soil Biol. Biochem. 27 (1995) 349–356.
[25] M.A. Tabatabai, J.M. Bremner, Use of p-nitrophenyl phosphate for assay of soil phosphatase activity, Soil Biol. Biochem. 1 (1969) 301–307.
[26] M. Rajkumar, R. Nagendran, K.J. Lee, W.H. Lee, S.Z. Kim, Influence of plant growth promoting bacteria and Cr^{6+} on the growth of Indian mustard, Chemosphere 62 (2006) 741–748.
[27] S.A. Gordon, R.P. Weber, Colorimetric estimation of indoleacetic acid, Plant Physiol. 26 (1951) 192–195.
[28] B. Schwyn, J.B. Neilands, Universal chemical assay for the detection and determination of siderophores, Anal. Biochem. 160 (1987) 47–56.
[29] J.M. Bell, J.C. Paton, J. Turnidge, Emergence of vancomycin-resistant *Enterococci* in Australia: phenotypic and genotypic characteristics of isolates, J. Clin. Microbiol. 36 (1998) 2187–2190.
[30] T. Cáceres, M. Megharaj, S. Malik, M. Beer, R. Naidu, Hydrolysis of fenamiphos and its toxic oxidation products by *Microbacterium* sp. in pure culture and groundwater, Bioresour. Technol. 100 (2009) 2732–2736.
[31] S. Kumar, M. Nei, J. Dudley, K. Tamura, MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences, Brief Bioinform. 9 (2008) 299–306.
[32] J. Si, B. Tian, H. Wang, Effect of incubation temperature and wet-dry cycle on the availabilities of Cd, Pb and Zn in soil, J. Environ. Sci. 18 (2006) 1119–1123.
[33] S. Brown, B. Christensen, E. Lombi, M. McLaughlin, S. McGrath, J. Colpaert, J. Vangronsveld, An inter-laboratory study to test the ability of amendments to reduce the availability of Cd, Pb, and Zn in situ, Environ. Pollut. 138 (2005) 34–45.
[34] B. Fuentes, M. Jorquera, M.d.L.L. Mora, Dynamics of phosphorus and phytate-utilizing bacteria during aerobic degradation of dairy cattle dung, Chemosphere 74 (2009) 325–331.
[35] N.S. Bolan, M.J. Hedley, Dissolution of phosphate rocks in soils. 2. Effect of pH on the dissolution and plant availability of phosphate rock in soil with pH dependent charge, Nutr. Cycl. Agroecosys. 24 (1990) 125–134.
[36] V.P. Evangelou, Y.L. Zhang, A review: pyrite oxidation mechanisms and acid mine drainage prevention, Crit. Rev. Env. Sci. Technol. 25 (1995) 141–199.
[37] D.L. Harris, B.G. Lottermoser, Evaluation of phosphate fertilizers for ameliorating acid mine waste, Appl. Geochem. 21 (2006) 1216–1225.
[38] N.S. Bolan, R. Naidu, S. Mahimairaja, S. Baskaran, Influence of low-molecular-weight organic acids on the solubilization of phosphates, Biol. Fertil. Soils. 18 (1994) 311–319.
[39] K. Kpomblekou-A, M.A. Tabatabai, Effect of low-molecular weight organic acids on phosphorus release and phytoavailabilty of phosphorus in phosphate rocks added to soils, Agric. Ecosyst. Environ. 100 (2003) 275–284.
[40] K.V. Kumar, N. Singh, H.M. Behl, S. Srivastava, Influence of plant growth promoting bacteria and its mutant on heavy metal toxicity in *Brassica juncea* grown in fly ash amended soil, Chemosphere 72 (2008) 678–683.
[41] K.D. Schneider, P. van Straaten, R.M. de Orduña, S. Glasauer, J. Trevors, D. Fallow, P.S. Smith, Comparing phosphorus mobilization strategies using *Aspergillus niger* for the mineral dissolution of three phosphate rocks, J. Appl. Microbiol. 108 (2009) 366–374.
[42] M. Bohner, J. Lemaître, T.A. Ring, Kinetics of dissolution of β-tricalcium phosphate, J. Colloid Interface Sci. 190 (1997) 37–48.
[43] M.P. Ginebra, F.C.M. Driessens, J.A. Planell, Effect of the particle size on the micro and nanostructural features of a calcium phosphate cement: a kinetic analysis, Biomaterials 25 (2004) 3453–3462.
[44] V. Achal, V.V. Savant, M.S. Reddy, Phosphate solubilization by a wild type strain and UV-induced mutants of *Aspergillus tubingensis*, Soil Biol. Biochem. 39 (2007) 695–699.
[45] B.R. Glick, D.M. Penrose, J. Li, A model for the lowering of plant ethylene concentrations by plant growth-promoting bacteria, J. Theor. Biol. 190 (1998) 63–68.
[46] S. Mantelin, B. Touraine, Plant growth-promoting bacteria and nitrate availability: impacts on root development and nitrate uptake, J. Exp. Bot. 55 (2004) 27–34.
[47] A.G. Khan, Role of soil microbes in the rhizospheres of plants growing on trace metal contaminated soils in phytoremediation, J. Trace Elem. Med. Biol. 18 (2005) 355–364.
[48] M. Malhotra, S. Srivastava, Stress-responsive indole-3-acetic acid biosynthesis by *Azospirillum brasilense* SM and its ability to modulate plant growth, Eur. J. Soil Biol. 45 (2009) 73–80.
[49] Y. Ma, M. Rajkumar, H. Freitas, Inoculation of plant growth promoting bacterium *Achromobacter xylosoxidans* strain Ax10 for the improvement of copper phytoextraction by *Brassica juncea*, J. Environ. Manage. 90 (2009) 831–837.
[50] Y. Ma, M. Rajkumar, H. Freitas, Improvement of plant growth and nickel uptake by nickel resistant-plant-growth promoting bacteria, J. Hazard. Mater. 66 (2009) 1154–1161.
[51] A. Evers, R.D. Hancock, A.E. Martell, R.J. Motekaitis, Metal ion recognition in ligands with negatively charged oxygen donor groups. Complexation of iron(III), gallium(III), indium(III), aluminum(III), and other highly charged metal ions, Inorg. Chem. 28 (1989) 2189–2195.
[52] G.M. Gadd, Microbial influence on metal mobility and application for bioremediation, Geoderma 122 (2004) 109–119.
[53] M.S. Liphadzi, M.B. Kirkham, G.M. Paulsen, Auxin-enhanced root growth for phytoremediation of sewage-sludge amended soil, Environ. Technol. 27 (2006) 695–704.
[54] M. Rajkumar, N. Ae, M.N.V. Prasad, H. Freitas, Potential of siderophore-producing bacteria for improving heavy metal phytoextraction, Structure 13 (2005) 29–41.
[55] H. Chung, M. Park, M. Madhaiyan, S. Seshadri, J. Song, H. Cho, T. Sa, Isolation and characterization of phosphate solubilizing bacteria from the rhizosphere of crop plants of Korea, Soil Biol. Biochem. 37 (2005) 1970–1974.
[56] E. Pérez, M. Sulbarán, M.M. Ball, L.A. Yarzábal, Isolation and characterization of mineral phosphate-solubilizing bacteria naturally colonizing a limonitic crust in the south-eastern Venezuelan region, Soil Biol. Biochem. 39 (2007) 2905–2914.
[57] A. Ghani, S.S.S. Rajan, A. Lee, Enhancement of phosphate rock solubility through biological processes, Soil Biol. Biochem. 26 (1994) 127–136.
[58] M. Chrysochoou, D. Dermatas, D. Grubb, Phosphate application to firing range soils for Pb immobilization: the unclear role of phosphate, J. Hazard. Mater. 144 (2007) 1–14.
[59] L.Y. He, Z.J. Chen, G.D. Ren, Y.F. Zhang, M. Qian, X.F. Sheng, Increased cadmium and lead uptake of a cadmium hyperaccumulator tomato by cadmium-resistant bacteria, Ecotoxicol. Environ. Saf. 72 (2009) 1343–1348.
[60] C.Y. Jiang, X.F. Sheng, M. Qian, Q.Y. Wang, Isolation and characterization of a heavy metal-resistant Burkholderia sp. from heavy metal-contaminated paddy field soil and its potential in promoting plant growth and heavy metal accumulation in metal-polluted soil, Chemosphere 72 (2008) 157–164.
[61] L.Q. Ma, G.N. Rao, Aqueous Pb reduction in Pb-contaminated soils by Florida phosphate rocks, Water Air Soil Pollut. 110 (1999) 1–16.