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Original Research

Enhanced Bio-Immobilization of Pb Contaminated Soil by Immobilized Bacteria with Biochar as Carrier

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Abstract

This paper examines the potential value of phosphate-solubilizing bacteria(PSB) in the dissolution of soil phosphorus and in the subsequent immobilization of lead (Pb), both in bacterial growth medium and in soil. In growth medium, Pseudomonas chlororaphis showed both phosphate-solubilizing and Pb-immobilizing capability, the immobilization of Pb was attributed to pyromorphite formation, as indicated by X-ray diffraction analysis. P. chlororaphis cannot multiply in soil in the presence of indigenous soil bacteria; however, when the added content of PSB-immobilized biochar (PIB) was equal to or greater than 800 mg/kg, the PSB could proliferate effective and the NH_4NO_3-extractable Pb concentration was decreased to below 1 mg/kg. Therefore, the inoculation of PIB in soil can be used as an alternative technique to Pb immobilization, thereby avoiding secondary pollution arising from the addition of large amounts of phosphorus as a heavy-metal passivator.

Keywords: phosphate-solubilizing bacteria, biochar, Pb immobilization, Pb contaminated soil

Introduction

Lead (Pb) is a highly toxic heavy metal that attracts particular attention and has always been present in soils and in surface and underground waters. There are several sources of Pb contamination in the environment, such as flaking paint, the use of leaded gasoline, waste incineration, the application of pesticides, and mining operations [1]. It is widely recognized that the mobility and bioavailability of Pb in soil is more important than total Pb concentration [2]. Therefore, the reduction of Pb bioavailability is critical for the management and remediation of Pb-contaminated soils.

The bioavailability of Pb can be decreased by forming compounds with various materials so as to decrease its toxicity [3-4]. Studies on Pb immobilization have been undertaken using several types of phosphorus compounds, such as hydroxyapatites, rock phosphate (RP), diammonium phosphate, phosphoric acid, and combinations of these materials [5-6]. Phosphorus compounds can reduce the availability of Pb through the formation of stable pyromorphite, $Pb_5(PO_4)_3Cl$, which can be achieved by treatment with various phosphates [7-8].

Most phosphorus compounds are not readily soluble in soils and hence phosphorus is not easily accessible, either for plant growth or for the immobilization of

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Pb. Phosphorus is sequestered by adsorption to the soil surface and by precipitation by reaction with soil cations, particularly iron, aluminum, and calcium [9]. Therefore, large amounts of phosphorus-containing fertilizers are used to increase plant growth, which is likely to lead to the accumulation of a "phosphorus store" in soils [10]. The excessive input of phosphorus is likely to have a negative impact with respect both to the environment and to cost.

Phosphate-solubilizing bacteria (PSB) have been used as inoculants to increase crop yield by solubilizing insoluble phosphorus compounds in soils [11]. PSB facilitate the dissolution of phosphorus from soils by means of phosphatase enzyme activity and organic acid production [12]. Recent studies have shown that phosphorus dissolution by PSB can increase the efficiency of Pb immobilization in water and soils [13]. There have been few studies, however, that have reported the application of the immobilized microorganism technique to contaminated soil remediation. As PSB cannot multiply rapidly when they are in direct competition with indigenous soil microorganisms, the immobilization of PSB on carrier materials of some kind may be helpful for enhancing the efficiency of phosphorus dissolution and Pb immobilization.

The large-scale production of biochar as a means of carbon sequestration provides an opportunity for using such materials as inoculum carriers. Biochar is the product of the thermal degradation of organic materials in the absence of air (pyrolysis), and it has been recommended for carbon sequestration and to improve soil fertility [14-15]. Biochar can provide a protective niche for PSB and hence reduce competition from indigenous microorganisms [16]. Most biochars are suitable for use as inoculum carriers and possess high internal porosity, large specific surface area, and the ability to adsorb organic compounds and bacteria [17].

In this paper, we report a study on the immobilization of Pb by PSB immobilized on cow dung biochar, which was found to promote the rapid multiplication of PSB in soil. The objectives of this work were to demonstrate the possible role of PSB and their effects on Pb immobilization in soil.

Materials and Methods

Isolation of Phosphate-Solubilizing Bacteria

Bacterial strains were isolated from Pb-contaminated soils and from phosphorus-treated soils. To isolate bacteria from soil, screening and purification were carried out in a medium containing (per L): 10 g glucose, 5 g $Ca_3(PO_4)_2$, 5g $MgCl_2{\cdot}6H_2O$, 0.25 g $MgSO_4{\cdot}7H_2O$, 0.2 g KCl, 0.1 g $(NH_4)_2SO_4$, and 1.5% agar. The pH of the agar medium was adjusted to 7.0 [18]. $Ca_3(PO_4)_2$ was autoclaved separately and added to the other sterile ingredients aseptically after autoclaving. After 14 days of incubation of the plates at 28°C, the colonies with clear halos, which were considered to be PSB, were counted and the most prominent of the colonies were further purified by re-streaking on agar plates. The halo and colony diameters were then measured after an additional 14 days at 28ºC. Bacterial colonies were identified and selected based on their ability to liberate phosphorus from $Ca_3(PO_4)_2$. Species identification was conducted using 16S rDNA sequence analysis and a biologbacterial identification system (Biolog, USA); by these means, PSB colonies were identified as *Pseudomonas chlororaphis*.

Biochar Preparation and PSB Immobilization

Cow dung is a model organic fertilizer and represents a typical biomass that can be used for biochar preparation. After being washed with tap water and air-dried, the cow dung was pyrolyzed in a custom-made pyrolysis device at 200, 300, 400, or 500ºC under oxygen-limited conditions; heating was for 4 h and the biochar was then cooled for12 h. The resulting biochars were denoted as T200, T300, T400, and T500, respectively, according to the pyrolytic temperature. The basic chemical properties of the biochars, and of the cow dung starting material, are presented in Table 1.

To prepare biochar-carrying immobilized bacteria, biochar and liquid growth medium were first mixed at a ratio of 1:10 (w/v) and inoculated with PSB. The biochar-containing culture was then grown to log phase and the inoculated biochar was harvested using gauze and rinsed with sterile medium. It was then air-dried in a sterile cabinet and stored at 4ºC prior to use. This biochar preparation containing immobilized PSBwas denoted as PIB.

The specific surface area was measured using the Brunauere EmmetteTeller (BET) method (Micromeritics Gemini 2380, US), using N_2 as the adsorbate gas. The surface morphology of the biochar was analyzed using a Hitachi S-4100 scanning electron microscope (SEM) at 25 kV; samples were sputtered with gold prior to analysis [19].

Pb Immobilization Ability of PIB in Bacterial Growth Medium

Three groups of sterile 250 mL Erlenmeyer flasks were set up in triplicate. Control flasks (CK) contained100 mL of medium supplemented with 100 mg of Pb^{2+}; experimental flasks designated TRB contained, in addition, 1g of phosphate rock; and experimental flasks designated TRP were set up to simulate the pH of soil, and in these flasks the pH was adjusted to pH 6.2-7.2 every 6 hours using NaOH. All flasks were incubated in the dark at 28ºC on a shaker at 100 rpm. After 40 days of incubation we determined the amount of dissolved phosphorus, the residual Pb and the solution pH.

To examine the immobilization of Pb in the form of Pb-phosphate compounds, the Pb-contaminated soils before and after bio-immobilization were analyzed by X-ray diffraction (XRD). For XRD analysis, the samples were pulverized to less than 75 mm using a benchtop

Table 1. Table 1 pH, elemental composition (W%), CEC, and surface area of different biochars.

Sample name	pH	Organic C ($g\ kg^{-1}$)	Total N ($g\ kg^{-1}$)	Total P ($g\ kg^{-1}$)	CEC ($cmol\ kg^{-1}$)	BET ($m^2\ g^{-1}$)
Cow Dung	6.5	921.7	5.8	9.3	53.7	0.7
T200	6.8	810.1	6.7	12.3	89.4	2.48
T300	7.7	729.3	7.5	15.8	122.4	3.13
T400	8.5	672.7	8.3	19.6	193.9	5.48
T500	8.6	410.2	8.7	20.3	211.2	3.97

mill in order to reduce the preferential orientation of particles horizontally loaded to the sample holder. The XRD was operated at 1 kW (35 kV and 28.2 mA) and Cu Ka radiation (1.540 56 Å) was selected as the primary beam. The goniometer was operated under the following conditions: start angle 10, final angle 70, step size 0.02, scan rate 0.5/min.

Pb Immobilized by PIB in Pb-Contaminated Soil

To examine the effect of PIB on Pb-contaminated soil, 50 g of Pb-spiked (600 mg/kg, $Pb(NO_3)_2$) and phosphorus-spiked (1 g/kg, RP) soil samples were treated with bacterial suspension(5ml/kg of soil) in the log phase and various levels of PIB (400, 600,800, and 1,000 mg/kg of soil). The treated soils were incubated at 25ºC for 60 days.

The amount of NH_4NO_3-extractable Pb has often been used to estimate the degree of Pb immobilization achieved by various treatments [20, 21], including treatment with phosphate compounds. To measure the bioavailable Pb concentration, soil samples were extracted with 1M NH_4NO_3 solution (soil:solution = 1:2.5) for 2 hours and Pb was analyzed by ICP-MS (Agilent, Japan). Olsen-P was measured by extracting soil with 0.5M $NaHCO_3$ (soil:solution ratio = 1:20) [22], at pH 8.5. The pH was measured using a pH electrode and the bacterial population was measured by plate-counting using agar medium.

Results and Discussion

Bacterial Immobilization onto Biochar

Fig. 1 shows SEM images of the cow dung, biochar, and PIB. It can readily be seen that the surface of biochar (Fig. 1b) had an irregular texture, whereas the surface of the cow dung was smoother. There were numerous large channel-like structures on the surface of the biochar (Table 1); the porous structure of biochar is created during pyrolysis by the production of volatile material. As this material escapes, pores and cracks begin to appear on the surface of the biochar [23]. For PIB, a micrograph taken at 1,300× magnification (Fig. 1c) reveals a quite uniform and compact bacterial layer. At higher magnification (5,000×),

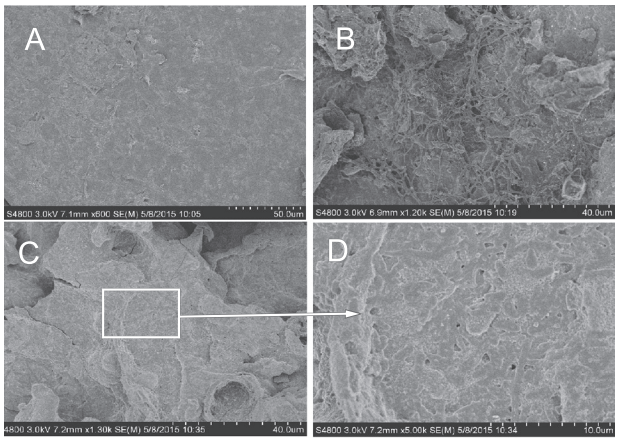

Fig. 1. SEM micrographs of different materials (a) original cow dung, (b) biochar P400, (c) PIB, (d) the target area of sample C at high magnification.

however, it can be seen that in some areas the bacterial layer does not completely cover the biochar surface; these discontinuities, which are not extensive, are caused by very small holes (Fig. 1d), and some studies have shown that these micropores are caused by the respiration of PSB [24].

Pb Immobilization in Bacterial Growth Medium

Fig. 2 shows the changes in pH, phosphorus concentration, and Pb concentration over time in bacterial growth media containing PIB. In both the TRB and the TRP flasks, the level of Pb dissolved in the medium decreased over time as the phosphorus concentration increased, whereas there was no significant change in the control flasks (CK). On the other hand, although throughout the time course the phosphorus concentration in the medium in the TRB flasks was appreciably higher than in the TRP flasks (Fig. 2b), the Pb concentration in the medium was also substantially higher in the TRB flasks than in the TRP flasks (Fig. 2a); this result is therefore contrary to previous studies indicating that Pb could be passified by dissolved phosphorus [25]. The Pb concentration in the medium in the TRP flasks decreased to lower than the detection limit, with pH maintained within a constant range, whereas in the TRB flasks the Pb concentration in the medium remained

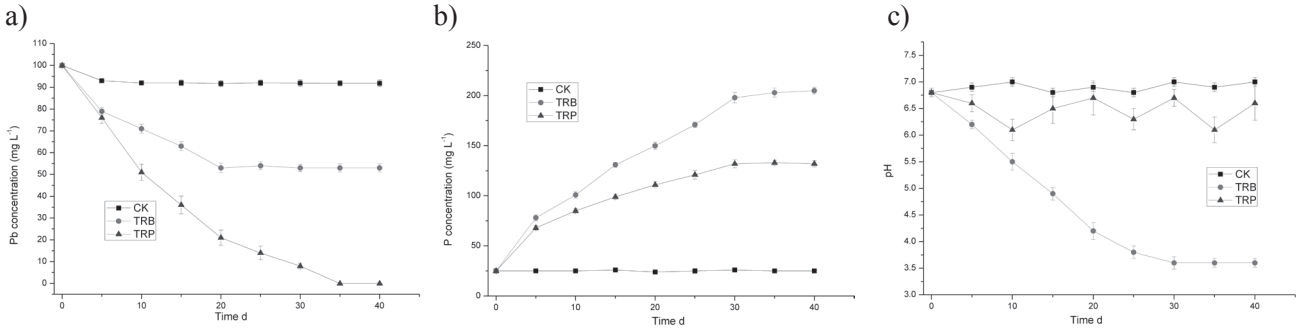

Fig. 2. Changes measured over time in the concentrations of a) Pb, b) phosphorus (P), and c) pH in bacterial growth media containing PIB.

at a relatively high level (around 55 mg/L, Fig. 2c). This indicates that PSB can effectively dissolve phosphate rock in acid and neutral environments, whereas the immobilization of Pb was inhibited under high acidic conditions.

This contrasted with the behavior in bacterial growth medium, in which there was no evidence for PSB-enhanced Pb immobilization because the growth of PSB reduced the pH of the solution, thereby inhibiting Pb-phosphate formation. It was demonstrated that in solution the amount of Pb immobilized increased if the pH was kept stable within a defined range (6.2-7.2). Accordingly, the pH of the solution is an important factor affecting the immobilization of Pb by phosphorus compounds.

Bio-Immobilization of Pb in Pb-Contaminated Soil

PIB significantly decreased the NH_4NO_3-extractable Pb concentration when the level of PIB treatment was equal to or greater than 800 mg/kg; the decrease in the Pb concentration increased with increasing levels of the PSB population and at higher values of Olsen-P. Although PSB decreased the pH of bacterial growth medium significantly, the soil used in this study has adequate pH buffering capacity and the pH changed only slightly (Table 2).

As shown in Table 2, the PSB population increased significantly in the B400-B1000 series of samples, in line with the amounts of PIB that had been added to the soil, whereas no PSB were detected in either the BM or the CK samples. This result indicates that biochar as a carrier for bacteria is favorable to the growth and reproduction of PSB in soil. The pH of the soil fell only slightly, on account of the buffering capacity of the soil. The increased population of PSB in the soil boosted the Olsen-P level, leading to a decrease in the NH_4NO_3-extractable Pb concentration to below 1mg/kg. The CEC, which is an indicator of soil fertility [26], was also increased in line with the amount of PIB added; this indicates that PIB is helpful in improving soil quality.

XRD Analysis of Pb-Contaminated Soils

Fig. 3 shows the XRD pattern of Pb-contaminated soils before and after bio-immobilization, indicating the formation of pyromorphite ($Pb_5(PO_4)_3Cl$), and a small amount of $Pb_5(PO_4)_3F$, which are the most stable lead compounds [5, 27]. The relative amount of $Pb_3(CO_3)_2$ and $Ca_3(PO_4)_2$ were decreased, while the relative amount of pyromorphite was significantly increased before and after bio-immobilization, which provided evidence of Pb immobilization by the P compound activated by PSB.

Table 2. Final physicochemical properties of experimental soil samples used to investigate Pb immobilization.

Sample	pH	Olsen-P (mg/kg)	NH_4NO_3 extractable Pb (mg/kg)	CEC (cmol/kg)	PSB population (CFU/g dry soil)
CK	6.78±0.008	8.72±0.02	21.23±0.07	6.73±0.08	ND
BM	6.77±0.013	9.13±0.07	19.48±0.06	6.81±0.09	ND
B400	6.72±0.011	11.88±0.11	16.28±0.10	9.89±0.11	2.5×10^3
B600	6.69±0.026	18.37±0.18	4.54±0.11	14.21±0.10	7.8×10^5
B800	6.67±0.018	28.74±0.53	0.98±0.23	17.75±0.15	1.8×10^6
B1000	6.66±0.02	29.98±0.57	0.87±0.21	18.84±0.14	1.9×10^6

CK denotes the Pb-spiked and P-spiked soil; BM denotes PSB suspension (5ml/kg) added to soil. B400, B600, B800, and B1000 denote PIB at 400, 600, 800, and 1000 mg/kg, respectively, added to soil.
"±" experimental error representing standard deviation of triplicates
ND: Not detected;detection limit was 250 CFU/g of dry soil.

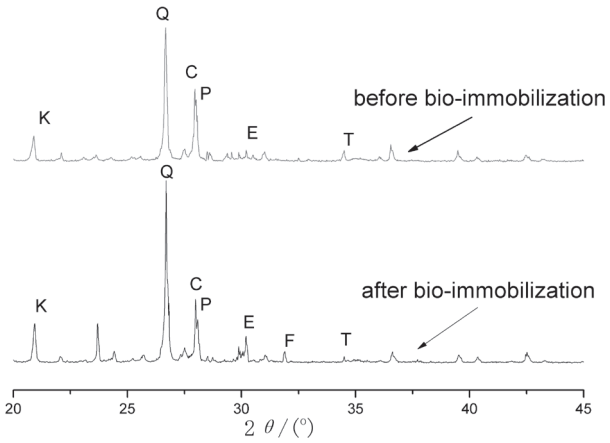

Fig. 3. XRD pattern of Pb-contaminated soils.
Q: SiO_2, K: kaolinite, C: $CaCO_3$, P: $Pb_3(CO_3)_2$, E: $Pb_5(PO_4)_3Cl$, F: $Pb_5(PO_4)_3F$, T:$Ca_3(PO_4)_2$

The dissolution of soil phosphorus and the subsequent formation of pyromorphite by PSB can be expressed as follows:

$$Ca_5(PO_4)_3X + 6H^+ \xrightarrow{PSB} 5Ca^{2+} + 3H_2PO_4^- + X^- \quad (1)$$

$$5Pb^{2+} + 3H_2PO_4^- + X^- \Longrightarrow Pb_5(PO_4)_3X + 6H^+ \quad (2)$$

X: F, Cl

Conclusions

In soil, the use of PIB promoted the growth and reproduction of PSB, enhanced the dissolution of phosphorus, and led to the pacification of Pb. The formation of pyromorphite was shown by XRD analysis in Pb-contaminated soil after bio-immobilization. The pH was not affected by PSB and the NH_4NO_3-extractable Pb concentration decreased with the time of incubation with PIB in Pb-contaminated soil. Using biochar as a carrier, PSB were effective in the immobilization of Pb. The PIB-mediated Pb immobilization technique can therefore be applied to Pb-contaminated soils to mitigate Pb bioavailability. However, the long-term stability of immobilized Pb needs to be investigated in the field.

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