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Bioremediation of Pb-contaminated soil by incubating with *Phanerochaete chrysosporium* and straw

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Abstract

The bioremediation of the simulated lead (Pb)-contaminated soils by incubating with *Phanerochaete chrysosporium* and straw was studied at laboratory-scale. The soil pH, Pb concentration, soil microbial biomass, microbial metabolic quotient, microbial quotient and microbial biomass C-to-N ratios were monitored. The above indicators were to study the stress of Pb on soil and the microbial effects during the bioremediation process. It was found that the soils treated with *P. chrysosporium* and straw showed a much lower concentration of soluble-exchangeable Pb, lower metabolic quotient and biomass C-to-N ratios (0 mg kg^{-1} dry weight soil, 1.9 mg CO_2-C mg^{-1} biomass carbon and 4.9 on day 60, respectively) and higher microbial biomass and microbial quotient (2258 mg kg^{-1} dry weight soil and 7.86% on day 60, respectively) compared with the controls. In addition, the kinetic parameters in the model based on logistic equation were calculated by the BIOLOG data. By analyzing those kinetic parameters some information on the metabolic capacity of the microbial community could be obtained. All the results indicated that the bioavailability of Pb in contaminated soil was reduced so that the potential stress of Pb was alleviated, and also showed that the soil microbial effects and the metabolic capacity of microbial community were improved.

Keywords: *P. chrysosporium*; Inoculation; Bioremediation; Pb-contaminated soil

1. Introduction

Heavy metal contamination in soil becomes a widespread problem. Lead (Pb) has been recognized as one of the most hazardous heavy metal among environmental pollutants. The primary sources of Pb-contamination come from the mining and smelting activities, combustion of leaded gasoline, land application of sewage sludge, battery disposal and Pb-bearing products [1]. Such irregular inputs of Pb result in the high concentrations of Pb in soils. Lin et al. [2] reported that soils with Pb concentration higher than 1000 mg kg^{-1} occurred in the south-western part of Falun, Sweden, where large amount of the industrial wastes was deposited. Buatier et al. [3] found that the Pb concentrations were 460–2670 mg kg^{-1} in the surface horizons of a polluted site in France. The toxicity and bioavailability of Pb are affected by soil pH, redox potential and Pb species. It is commonly accepted that Pb compounds in soil mainly exist in exchangeable, carbonate-bound, Fe/Mn oxide-bound, organic and residual phases [4,5]. The serious negative impacts of Pb in the soluble and exchangeable phases easily leached out of soils on groundwater and surface water and even crops have been observed, while Pb in the organic and residual phases is inactive due to the strong binding capacity of organic matter and sulfides, especially in the heavily contaminated soils [5,6]. Consequently, active Pb in soluble and exchangeable phases poses more threat to environment, ecosystem and human, compared with immobilized Pb in other phases, and how to reduce active Pb effectively receives much more concerns in the remediation of Pb-contaminated soils [6].

Comparing with conventional physico-chemical approach, bioremediation is a technology not to aggravate other environmental problems but to remediate the polluted soil partially or fully to the original state, which relies on the natural soil community or addition of exogenous organism (plant or microorganism)

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with exceptional metal-binding capacity to remove the heavy metals from soil or alleviate the toxicity of metals by reducing the bioavailability and mobility of metals largely. Previous studies focused on the bioremediation of soils contaminated with heavy metals (such as Pb, Cd, Cr and so on) by phytoremediation [7,8], however, little information is available on the application of inoculum of microorganism to stabilize metal-contaminated soils. But in fact, heavy metals are non-biodegradable pollutants and not easily removed from soils by regular treatments under some conditions, so the total metal content is difficult to decrease greatly. To prevent metal ion in soil from entering food chain or groundwater, microorganisms adsorbing and accumulating metal ion are expected to be applied to immobilize metals in soils. In previous studies, it was proved that *P. chrysosporium* was good at absorbing metal from dilute solutions by its mycelium and less Pb ion transferred [9]. *P. chrysosporium* are capable of accumulating metal ions in their cells by intracellular uptake, as many researchers validated, and can also be chelated with metal ions by the carboxyl, hydroxyl or other active functional groups on cell (including the dead cell) wall surface [10]. Meanwhile *P. chrysosporium* is able to grow in both solid and liquid environment and degrade a wide range of xenobiotic effectively even in the nutrient-limited condition [11]. So it could be adapted to complex polluted environment and grow better than other microorganisms after inoculation into soils. As a result, it is of its own advantage to be applied to the bioremediation of metal-contaminated soil. But there are few reports about its application to inactivate metals in soils.

The aim of this study was to inoculate *P. chrysosporium* as the exogenous microorganism, together with some straws, into Pb-contaminated soil for reducing the solubility and bioavailability of Pb and improving the soil microbial activity. The changes of Pb content and microbial indices that took place in bioremediation process were analyzed systematically, all of which were used to evaluate the remediation effects on Pb-contaminated soils by incubating with and without inoculum of *P. chrysosporium*. These results are expected to provide useful references on alleviating the environmental impact of metal-contaminated soils by inoculation with *P. chrysosporium* and straw.

2. Materials and methods

2.1. Microorganisms preparation

The white-rot basidiomycete, *P. chrysosporium* strain BKM-F-1767 was used. Stock cultures were maintained on malt extract agar slants at 4 °C. Spore suspensions were prepared in sterile distilled water. The fungal concentration was measured and adjusted to 2.0×10^6 CFU ml^{-1}.

2.2. Soil properties and preincubation

Uncontaminated soils were collected from about 100 cm underground on the unfrequented hillside of Yuelu Mountain (Changsha, China), from which gravels and large organic scraps were removed. The soil was air-dried and ground to pass through a 2 mm nylon screen, and its main physico-chemical characteristics were measured and listed as follows (dry weight): 39% of clay, an organic C content of 0.83%, total N of 0.059%, a pH value of 4.9, and the total contents of Cu, Cd, Pb were 11.5, 0 and 17.9 mg kg^{-1}. Then this soil was mixed and homogenized with $Pb(NO_3)_2$ solution for adding Pb^{2+} 400 mg kg^{-1} dry weight soil, which was preincubated for 5 weeks so that the stimulated Pb-contaminated soil became relatively stabile.

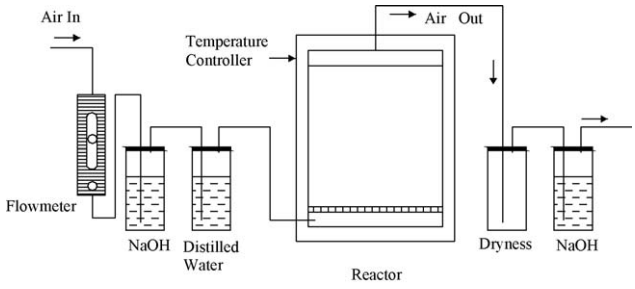

Fig. 1. Schematic diagram of experimental apparatus.

2.3. Experimental design

The experimental apparatus used for this research consisted of a lab-scale column reactor, a CO_2 removal trap, a humidifier, and a trap for collecting CO_2 generated in biodegradation as shown in Fig. 1. Blower was used for aeration, and the air flow was controlled at 0.1 m^3 h^{-1} by flow meter. CO_2 was removed from the incoming air by reacting with an alkali solution of 2 M sodium hydroxide (NaOH) so that the collected CO_2 was entirely from decomposition [12]. The humidifier containing distilled water was used to prevent any aspirated alkali solution from entering the compost reactor, and to raise the moisture content of the incoming air. Air-tight glass vessel of 5 l was used as reactor. The air with CO_2 removed and being humidified entered the reactor from bottom through perforated plastic plate. Emitted CO_2 was continuously trapped in a solution of 2 M NaOH which was renewed every 3 days. Two identical sets of experimental apparatuses were prepared and labeled as Reactors A and B.

Each reactor was set up by adding 1.5 kg of the contaminated soil as prepared above. Equal straws were put into each reactor to mix thoroughly with soil in the ratios of 1:6, and this mixture was adjusted to 60% water content. The spore suspension prepared as above was inoculated in the weight ratio of 2% into the mixture in Reactor B, and Reactor A without inoculum was performed as control. Contaminated soil without *P. chrysosporium* was co-incubated with the added straws in Reactor A, while the contaminated soil with inoculum of *P. chrysosporium* was co-incubated with the added straws in Reactor B. The addition of straws could enhance soil porosity for better aeration and offer metabolizable substrate as necessary nutriments for microorganisms. The additional control reactor with soil and *P. chrysosporium* only was prepared and labeled as Reactor C. The Reactor D was also prepared with the contaminated soil, but without inoculum and straw, and is thus, another control. Such two controls were used for better indication of the intrinsic immobilization of Pb in soil. Both soils were incubated 60 days in this study.

Five individual 10 g fresh samples were taken from different sites in the reactor periodically and mixed together homogeneously for routine analysis. Some of the samples were air-dried, loosed, and passed through a 2 mm nylon sieve. After removal of the straw the samples were used for Pb content measurement. On days 0 and 60, additional samples (three 10 g samples) were taken from reactor and subjected to homogenization for BIOLOG analysis. All the analyses were performed in duplicate.

2.4. Soil pH and Pb content determinations

Soil pH value was measured in 1:10 sample–H_2O extract after shaking for 30 min [13]. Because the toxicity of metal was associated with metal bioavailability, the soils were analyzed for Pb-fractions according to the continual extract procedure of Tessier et al. [14]. Five fractions of Pb were extracted in turn as follows:

(i) *Soluble-exchangeable*: The 1 g dry soil was extracted for 1 h with 8 ml of 1 M $MgCl_2$ (pH 7.0) with continuous agitation.
(ii) *Bound to carbonates*: The residue from (i) was extracted for 5 h with 8 of 1 M NaOAc adjusted to pH 5.0 with acetic acid (HOAc).
(iii) *Bound to Fe–Mn oxides*: The residue from (ii) was extracted for 6 h with 20 of 0.04 M $NH_2OH{\cdot}HCl$ in 25% (v/v) HOAc at 96 °C with occasional agitation.
(iv) *Bound to organic matter*: The residue from (iii) was added 3 of 0.02 M HNO_3 and 5 of 30% H_2O_2 adjusted pH 2 with HNO_3, and the mixture was heated to 85 °C for 2 h with occasional agitation. A second 3 ml aliquot of H_2O_2 (pH 2 with HNO_3) was then added and the sample was heated again to 85 °C for 3 h with intermittent agitation. After cooling, 5 of 3.2 M NH_4OAc in 20% (v/v) HNO_3 was added, and the sample was diluted to 20 and agitated continuously for 30 min.
(v) *Residual*: The residual Pb was calculated by subtracting the other four fractions from total Pb. Additional 1 g dry soil was digested with a 5:1 mixture of hydrofluoric and perchloric acids for total Pb measurement.

Between each successive extraction, separation was effected by centrifuging and Pb concentrations in the supernatant were determined by an atomic absorption spectrometer (Agilent 3510, USA). The distribution coefficient γ_i of each Pb-fraction showed the ratios of Pb content in the different fractions and was calculated as follows:

$$\gamma_i = \frac{f_i}{f} \quad (1)$$

In Eq. (1), f_i and f represent content of each Pb-fraction and total amount of Pb in soils, respectively.

2.5. Microbial physiological indices analysis

Studies on soil microbial biomass carbon (C_{mic}), microbial metabolic quotient (qCO_2), microbial quotient (C_{mic}/C_{org}) and microbial C-to-N ratio (C_{mic}/N_{mic}) can provide information on the biochemical processes occurring in the soil, and there is growing evidence that soil biological parameters may be of great potential as the early and sensitive indicators of soil ecological stress and restoration [15–17]. So we analyzed these microbial physiological indices listed above.

The C_{mic} and biomass N (N_{mic}) were measured by fumigation of the sample with ethanol-free chloroform and extraction with 0.5 M K_2SO_4, according to Brooks et al. [18] and Vance et al. [19]. The soil qCO_2 was the ratio of CO_2 produced by soil basal respiration and C_{mic} [20]. The CO_2 production was measured by titration with 1 M HCl after adding an excess of barium chloride and phenolphthalein indicator to the alkali solution trapping CO_2 as previously described. The samples were dried to constant weight in the oven at 80 °C, and then ignited in muffle at 550 °C for 5 h. Afterwards, the organic carbon (C_{org}) could be estimated from the organic matter content calculated by the weight loss on ignition, as described by Huang et al. [21]. The microbial quotient was calculated by the ratio of C_{mic} to C_{org} (C_{mic}/C_{org}).

2.6. BIOLOG community-level physiological profiling

The potential metabolic diversity of soil microbial communities was assessed using BIOLOG plates as described by Kell and Tate [22], which showed the sole carbon source utilization of soil biota. Fresh soil equivalent to 5 g dry weight was added to 100 sterile water in a flask and shaken with a rotary shaker for 10 min, and 10-fold serial dilution was made for this soil suspension. The 10^{-3} dilution (150 μl) was then used to inoculate into each well of BIOLOG GN plate. These plates were incubated at 25 °C for 156 h. Color development was measured as optical density (OD) at 590 nm, and the OD_{590} was read for each well at 12 h intervals. The OD_{590} of control well containing no carbon source was subtracted from the OD_{590} of each of the other wells. The average absorbance (average well color development, AWCD) was then calculated for each plate at each reading time [23] and plotted against time to give AWCD curves. To evaluate AWCD of each soil sample, kinetic model parameters and curves fitting to the color development time-course data were also considered.

2.7. Statistical analysis

For each treatment, three parallel sets of experimental reactors were run. The results to be presented were mean value for the three reactors, and the standard deviations were used to summarize experimental data. Statistical analyses were performed on the kinetic parameters obtained from BIOLOG analysis, which used the software package SPSS 12.0 for Windows (SPSS, Germany). These tests included: (1) non-linear regression analysis performed to describe the AWCD of each soil sample for showing the kinetic parameter value and suggesting a density-dependent logistic growth curve and (2) a one-way analysis of variance (ANOVA) for the single time-point OD value and kinetic model parameters.

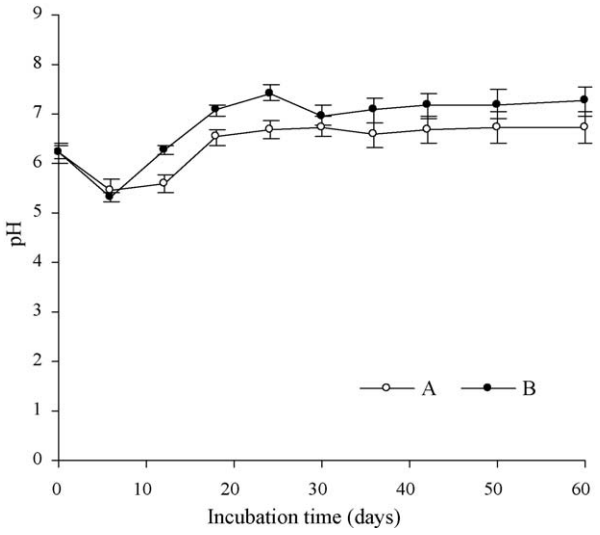

Fig. 2. pH changes in A and B soils during incubation: (A) control without inoculum and (B) treatment with inoculum and straw. The bars represent the standard deviation of the means ($n = 3$).

3. Results

3.1. Soil pH changing with incubation

In the early stages of soil incubation, pH of samples from Reactors A and B decreased slightly. After 9 days, the pH values of both samples increased significantly and later tended to stabilize (Fig. 2). On day 60, pH turned to be 6.7, 7.3 for the Reactors A and B, respectively. The pH value in B soil was neutral and higher than that in A soil (Fig. 2).

3.2. Soil Pb concentrations and distribution coefficient (γ_i)

Pb concentrations in all soils varied during incubation (Table 1). The soluble-exchangeable Pb content in A–C soils displayed the highest value on day 6, and then remarkably decreased with incubation. After 60 days of incubation, the concentration of soluble-exchangeable Pb in B soil even dropped to 0 mg kg^{-1}, while that in A soil and C soil remained 100.5 and 77.0 mg kg^{-1}, respectively. It was shown that in D soil a slight decrease on the content of soluble-exchangeable Pb and a few changes in the other four Pb-fractions. The carbonate-bound Pb, organic-bound Pb and residual Pb in A–C soils increased obviously after 6-day incubation, whereas Fe–Mn oxides-bound Pb were found to increase after 18 days. Compared with the control soils, the highest concentration of carbonate-bound Pb, organic-bound Pb and residual Pb, and the lowest concentration of soluble-exchangeable Pb were found in B soil after incubation (Table 1), which offered the evidence for the lowest mobility and bioavailability of Pb in B soil.

The higher the distribution coefficient (γ_i) of each Pb-fraction, the more the corresponding Pb-fraction will be. The γ_i of soluble-exchangeable Pb on day 0 was the highest among all γ_i of Pb-fraction on day 0 in both A and B soils, which showed Pb existed mainly in the soluble-exchangeable form in both soils before remediation (Fig. 3). After 60-day incubation, in both A and B soils, the γ_i of soluble-exchangeable Pb reduced, whereas that of the other four forms increased, which showed one form of Pb had been transformed into another (Fig. 3). It can be also observed that the order of abundance of the five fractions of Pb in B soil on day 60 was residual Pb > organic-bound Pb > Fe–Mn oxides-bound Pb > carbonate-bound Pb > soluble-exchangeable Pb. These results revealed that the active Pb in B soil had been transformed into inactive Pb indicating the alleviation of Pb-contamination after 60-day remediation with *P. chrysosporium* and straw.

3.3. Microbial physiological indices

Microbial biomass, being the living part of soil organic matter, can be a good index for comparing the toxicity of Pb in soil. The C_{mic} changed significantly during the incubation period (Table 2). It was also observed that the C_{mic} was much higher in B soil relative to that in A soil throughout after 6 days of incubation (Table 2). The qCO_2, as calculated by the CO_2-C-to-C_{mic} ratio, was higher in A soil compared to that in B soil throughout after 12-day incubation (Table 2). After 6-day incubation, the C_{mic}/C_{org}, being the ratio of the C_{mic} to soil organic carbon, was much lower in A soil than that in B soil. And the C_{mic}/C_{org} in B soil displayed the highest value on day 24 (Table 2). The change of C_{mic}/N_{mic} in A and B soils was similar, which showed a downtrend on the whole during incubation. Much lower C_{mic}/N_{mic} ratio throughout incubation process was found in A soil (Table 2), compared with that in B soil.

3.4. Kinetic model and parameters

The samples' AWCD determined by BIOLOG were nonlinear with the incubation time for microplates inoculated with the soil samples, and the shape of the color development curve was generally sigmoidal (Fig. 4), which can be described by the kinetic model based on the density-dependent logistic growth equation. We used the equation modified by Lindstrom et al. [24]

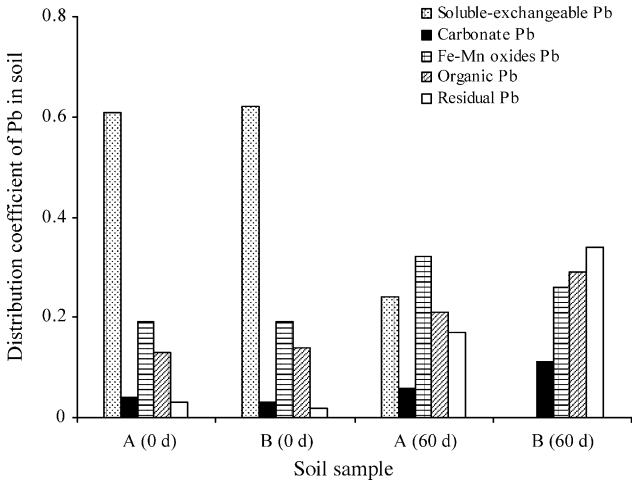

Fig. 3. Distribution coefficient (γ_i) of Pb in A soil on day 0, B soil on day 0, A soil on day 60 and B soil on day 60, respectively: (A) control without inoculum and (B) treatment with inoculum and straw.

Table 1
Pb content of five fractions in A–D soils during incubation

Pb-fractions	Time (days)									
	0	6	12	18	24	30	36	42	50	60
Soluble-exchangeable Pb (mg kg^{-1})[a]										
A	254.7 (1.5)	259.0 (1.9)	238.4 (2.1)	213.1 (1.2)	183.6 (1.7)	162.9 (4.2)	137.9 (3.8)	121.1 (0.5)	104.5 (0.8)	100.5 (1.3)
B	258.9 (1.8)	266.7 (1.1)	200.5 (2.0)	154.3 (1.1)	96.2 (1.7)	54.4 (1.3)	29.3 (1.0)	12.5 (0.7)	0.0 (0.2)	0.0 (0.1)
C	260.1 (2.6)	261.3 (2.4)	236.6 (2.5)	192.1 (2.6)	155.3 (1.9)	131.3 (2.0)	112.4 (2.7)	93.5 (1.2)	81.2 (0.9)	77.0 (1.7)
D	259.4 (2.1)	260.2 (1.3)	252.4 (3.1)	240.9 (2.3)	231.3 (2.2)	220.2 (3.4)	211.5 (3.6)	203.5 (2.8)	201.7 (2.3)	200.1 (2.2)
Carbonate-bound Pb (mg kg^{-1})[a]										
A	16.7 (0.4)	8.4 (0.2)	12.6 (0.3)	29.3 (0.1)	33.4 (0.3)	45.9 (0.2)	50.2 (0.2)	41.8 (0.1)	29.3 (0.1)	25.1 (0.2)
B	12.5 (0.3)	4.2 (0.1)	37.6 (0.4)	62.6 (0.6)	87.8 (0.9)	66.9 (0.3)	71.1 (1.2)	66.9 (1.1)	54.2 (0.5)	45.9 (0.8)
C	18.0 (0.6)	12.1 (0.3)	17.4 (0.6)	31.4 (0.8)	34.0 (0.7)	32.2 (0.4)	33.2 (0.3)	34.9 (0.5)	28.7 (0.6)	24.1 (0.5)
D	17.2 (0.4)	15.7 (0.5)	17.0 (0.4)	18.2 (0.5)	19.9 (0.6)	18.4 (0.2)	19.1 (0.1)	20.7 (0.2)	19.6 (0.4)	19.1 (0.4)
Fe–Mn oxides-bound Pb (mg kg^{-1})[a]										
A	79.3 (1.4)	87.7 (1.6)	75.3 (0.9)	66.9 (1.2)	70.9 (1.3)	83.5 (1.9)	100.3 (1.6)	116.9 (2.1)	129.5 (2.3)	134.0 (1.0)
B	79.3 (0.7)	100.0 (1.8)	71.0 (1.2)	45.9 (0.4)	62.7 (0.8)	87.8 (1.4)	87.9 (0.6)	96.1 (1.3)	104.2 (2.5)	108.5 (1.2)
C	80.3 (2.2)	88.0 (2.1)	71.8 (1.5)	64.1 (1.3)	76.0 (1.6)	86.4 (1.5)	87.4 (2.0)	92.4 (1.9)	96.4 (1.4)	100.3 (1.5)
D	80.9 (2.0)	82.2 (1.7)	80.6 (1.4)	81.0 (1.8)	79.9 (1.3)	83.2 (1.9)	83.5 (1.2)	85.0 (1.5)	86.2 (1.0)	86.9 (1.1)
Organic-bound Pb (mg kg^{-1})[a]										
A	54.3 (0.2)	46.0 (0.6)	46.0 (0.4)	50.1 (0.9)	58.4 (1.0)	62.6 (1.4)	71.0 (1.9)	75.2 (1.5)	83.6 (1.6)	87.9 (0.8)
B	58.5 (0.5)	37.5 (0.1)	45.9 (0.3)	54.2 (0.7)	66.9 (1.1)	83.7 (1.8)	104.6 (2.0)	112.8 (1.9)	120.9 (2.3)	121.0 (1.3)
C	53.7 (0.4)	51.2 (0.2)	57.1 (0.6)	65.9 (1.1)	78.7 (1.3)	84.8 (2.0)	100.5 (2.2)	106.4 (2.1)	113.7 (2.5)	116.3 (1.2)
D	52.0 (0.3)	50.9 (0.4)	50.7 (0.3)	52.4 (0.8)	55.7 (1.1)	57.8 (1.2)	58.2 (0.9)	60.8 (1.4)	61.9 (1.2)	62.3 (0.8)
Residual Pb (mg kg^{-1})[a]										
A	12.5 (0.1)	16.7 (0.2)	46.0 (0.5)	58.5 (1.6)	70.9 (1.3)	62.6 (1.7)	58.5 (1.2)	62.6 (1.4)	71.0 (1.1)	71.2 (0.9)
B	8.4 (0.1)	8.3 (0.2)	62.6 (0.9)	100.1 (1.3)	104.5 (1.1)	125.5 (1.8)	125.5 (2.1)	129.6 (2.2)	137.5 (2.4)	141.9 (1.7)
C	9.1 (0.3)	8.9 (0.1)	39.2 (0.6)	68.1 (1.2)	77.2 (1.7)	86.8 (1.4)	87.5 (1.6)	93.3 (1.1)	100.8 (1.2)	103.2 (1.0)
D	10.1 (0.2)	10.8 (0.4)	19.0 (0.5)	27.2 (0.9)	32.9 (0.6)	40.0 (0.5)	47.4 (0.7)	49.6 (0.9)	50.4 (0.8)	51.2 (0.6)

(A) Control without inoculum; (B) treatment with inoculum and straw; (C) control without straw; (D) control without inoculum and straw.
[a] Values are means ($n = 3$) with standard deviation in parentheses.

as below, which is a form providing parameters being interpreted more readily with respect to the shape of the OD kinetic curve and to:

$$y = \mathrm{OD}_{590\mathrm{nm}} = \frac{K}{1 + e^{-r(t-s)}} \quad (2)$$

underlying microbiological behavior driving its shape. In Eq. (2), K represents the asymptote ($y = K$) approached by the mean test well OD curve that means the highest OD in the culture course, the unitless coefficient r determines the exponential rate of OD change, and t is the time following inoculation of the microplate, the exponential parameter s in the denom-

Table 2
The microbial parameters of A and B soils during incubation

Time (days)	Biomass carbon (mg kg^{-1} soil)[a]		Metabolic quotient (mg CO_2-C mg^{-1} C_{mic})[a]		Biomass C/organic C (%)[a]		Biomass C/N[a]	
	A	B	A	B	A	B	A	B
0	979 (10)	1034 (34)	–	–	2.12 (0.18)	2.27 (0.24)	7.5 (1.1)	8.9 (1.2)
3	840 (8)	763 (17)	4.2 (0.5)	4.5 (0.4)	1.89 (0.22)	1.71 (0.13)	7.9 (0.9)	9.3 (1.5)
6	892 (12)	991 (26)	4.4 (0.4)	5.1 (0.9)	2.23 (0.29)	2.44 (0.18)	8.3 (1.2)	10.2 (0.5)
9	965 (15)	1535 (79)	4.5 (0.8)	5.5 (0.7)	2.55 (0.35)	3.99 (0.49)	8.9 (0.7)	10.3 (0.4)
12	1158 (31)	1714 (51)	5.4 (1.1)	3.9 (0.2)	2.98 (0.38)	4.57 (0.33)	8.1 (0.6)	9.5 (0.3)
15	1397 (54)	1935 (76)	4.6 (1.0)	3.3 (0.2)	3.65 (0.42)	4.96 (0.48)	6.8 (0.5)	8.7 (0.7)
18	1547 (55)	2473 (93)	3.7 (0.7)	2.7 (0.4)	3.91 (0.31)	6.20 (0.54)	5.9 (0.8)	8.2 (0.6)
24	1364 (67)	2789 (68)	3.7 (0.3)	2.1 (0.1)	3.43 (0.42)	8.46 (0.73)	6.4 (0.7)	7.4 (0.8)
27	1276 (62)	2271 (72)	4.0 (0.6)	2.5 (0.3)	3.34 (0.26)	7.25 (0.70)	6.6 (0.9)	7.7 (0.2)
30	1331 (59)	2171 (80)	3.7 (0.4)	2.6 (0.5)	3.78 (0.23)	6.60 (0.55)	5.7 (0.3)	6.6 (0.4)
36	1301 (48)	2090 (55)	3.5 (0.5)	2.2 (0.1)	3.69 (0.36)	6.10 (0.44)	6.1 (0.4)	6.8 (0.5)
42	1379 (75)	2297 (83)	3.2 (0.3)	2.0 (0.2)	4.04 (0.40)	6.82 (0.67)	4.9 (0.2)	5.6 (0.3)
50	1351 (64)	2287 (69)	3.1 (0.4)	1.9 (0.3)	4.31 (0.19)	7.47 (0.36)	4.5 (0.1)	5.1 (0.3)
60	1311 (73)	2258 (81)	3.1 (0.2)	1.9 (0.1)	4.50 (0.25)	7.86 (0.43)	4.2 (0.2)	4.9 (0.1)

(A) Control without inoculum and (B) treatment with inoculum and straw.
[a] Values are means ($n = 3$) with standard deviation in parentheses.

inator is the time to the midpoint of the highest mean OD (when $y = K/2$).

Each plot in Fig. 4 presented the mean OD change over time in the blank-corrected test wells of the three replicate plates. The curve fitting technique fitted the time-course OD data for microplate test wells closely and was useful for estimating kinetic parameter data that reflect the response of culturable organisms in the microplate inoculum. All kinetic parameter values from fit curves fitting to the OD time-course provided a good ($R^2 > 0.99$) fit to the sigmoidal kinetics of color development data (Fig. 4). The parameters K and r values estimated for A soil and B soil on day 60 were much higher than those on day 0, and the parameter K for B soil increased more than that for A soil. The parameter s for A and B soils was reduced from days 60 to 29.4 and 30, respectively, compared with that on day 0 (Fig. 4), which meant much less time needed to reach the midpoint of the highest mean OD in B soil after bioremediation. These results showed the improvement of the metabolic activity of microbial community in A and B soils by remediation, and the microbial community in B soil was of better metabolic capacity after bioremediation with *P. chrysosporium* and straw, compared with that in A soil as control (Fig. 4). One-way ANOVA was performed to the OD time-course data and the kinetic parameters generated by the model. It is found that there was significant difference ($P < 0.05$) between the kinetic model parameters K and s in A soil on day 60 and those in B soil, whereas the OD time-course data and the r in A soil on day 60 was not significantly different from ($P = 0.128$ and 0.293, respectively) those in B soil.

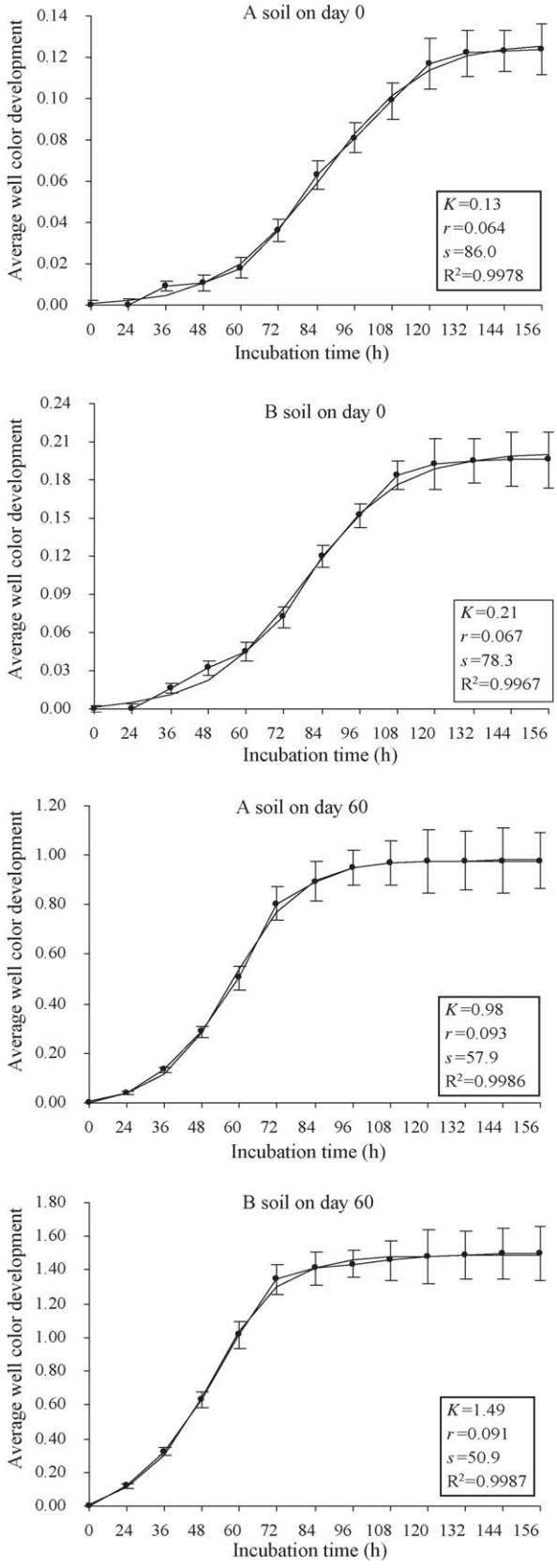

Fig. 4. Kinetics of average well color development for A and B soils: (A) control without inoculum and (B) treatment with inoculum and straw on days 0 and 60. Absorbance values (—●—) are the measured values. Bars are standard deviation of the means ($n = 3$). The solid line (—) is a plot of the equation fit to the mean absorbance data. Kinetic model K, r and s parameter data for the fit equation are presented along with the R^2 value of the fit to the absorbance data for each curve.

4. Discussion

Previous studies have shown that the total amount of heavy metals cannot reflect the mobility and bioavailability of metals well, whereas the effective concentration of metal has significant relationships with the toxicity and plant availability of metal [25,26]. To the same kind of heavy metal, soluble salt in exchangeable phase is the easiest to be assimilated by plants and as a result the toxicity of Pb in soil to environment can be evaluated by observing in which phase the metal existed [27]. The bioavailability and transfer ability of heavy metal is reduced with the turn of extraction [14]. After 60-day bioremediation the Pb in B soil was mainly bound to residual fraction and organic fraction with less mobility and activity, while the Pb in A soil was mainly bound to Fe–Mn oxides fraction and soluble-exchange fraction. Our results showed the least toxicity of Pb to living organisms, the least stress from Pb on environment for the significant reduction of active Pb in B soil by co-incubating the soil with *P. chrysosporium* and the added straws, compared with those in the control soils (Fig. 3 and Table 1). Reasons for these results could be as follows: (i) white-rot fungi inoculated into B soil can be chelated with Pb by the carboxyl, hydroxyl or other active functional groups on cell wall surface to reduce Pb activity [9] and (ii) white-rot fungi could improve the organic

matter decomposition and nutrient cycling, as reported previously [21], which promoted the formation of humus, while that Pb was chelated with humus is the mechanism responsible for Pb immobilization.

Another mechanism that could account for the lower Pb activity in B soil after bioremediation was the higher pH in B soil (Fig. 2). The pH of the original soil (no addition) was only 4.9. The pH is known to affect the ionic form and chemical mobility. A high pH may facilitate the decrease of the solubility of metals in the medium [28], which was also validated by the findings in our research that the less soluble-exchangeable Pb concentration was found as the pH in both soils increased (Table 1 and Fig. 2). The lower active Pb concentrations in soil with higher pH might be due to the increasing pH, which facilitates the cationic heavy metal retention to soil surfaces via adsorption, inner sphere surface complexation, and/or precipitation and multinuclear type reactions [29]. Guttormsen et al. [30] found that soil pH affected metal hydrolysis, ion-pair formation, organic matter solubility, as well as surface charge of iron and aluminum oxides, organic matter, and clay edges. Appel and Ma [31] reported that soil pH plays a major role in the sorption of heavy metals as it directly controls the solubilities of metal hydroxides, as well as metal carbonates and phosphates, and as a result the higher pH facilitates the precipitation and immobilization of metals. The solubilization of the ammonia formed by organic nitrogen ammonification led to the formation of ammonium and an increase in the pH values in soil. This might explain the increasing pH observed in both A and B soils during incubation time (Fig. 2). The pH in B soil increased more than that in A soil (Fig. 2), the reason for which might be that *P. chrysosporium* in B soil facilitated the degradation of organic matter [21] and the solubilization of ammonia or the volatilization of organic acid. The effective concentration of Pb in B soil was lower than the controls (Table 1), which benefited the reduction of toxicity.

Brookes [32] suggested that the relative effects of heavy-metal contamination of soils on ecosystem function can be evaluated by comparing microbial parameters, and an increasing body of evidence suggests that soil microflora plays an important role in ecosystemlevel nutrient cycling processes and microorganisms are far more sensitive to heavy metal stress than soil animals or plants growing on the same soils [33–35]. So we analyzed some microbial parameters to evaluate the growth of microorganisms and the microbial activity in our study. Microbial biomass is a much more sensitive indicator of changing soil conditions than is the total organic matter content, and microbial biomass synthesis is inhibited in heavy metal-contaminated soils generally [36]. Long-term exposure of soil microflora to high heavy metal concentrations would decrease the C_{mic}/C_{org}, because the toxicity of metal reduced microbial biomass and metabolic efficiency [37,38]. So the reduction of Pb stress on soil microflora could facilitate the growth of microorganisms and might be responsible for the increase of C_{mic} and C_{mic}/C_{org} in A soil and B soil (Tables 1 and 2). The increasing C_{mic} and C_{mic}/C_{org} in both soils during incubation (Table 2) indicated that the metabolic efficiency in the conversion of carbon substrates into biomass increased. Our data also showed the C_{mic} and the C_{mic}/C_{org} were higher obviously in B soil than those in A soil (Table 2), which suggested the higher metabolic efficiency in carbon mineralization in B soil. With the addition of *P. chrysosporium*, the total amount of soil microorganisms in B soil increased (Table 2). The inoculated *P. chrysosporium* could accumulate metal on cell wall and deposited metal intracellularly [10], and as a result the toxicity of metal was reduced so as to provide a condition favorable to the growth and the carbon utilization of the soil microorganisms. The above two reasons might be responsible for the higher C_{mic} and C_{mic}/C_{org} in B soil.

There is accumulating evidence that the higher qCO_2 is observed in most of metal-contaminated soil, and the qCO_2 was about 2.0 times greater in contaminated soil than that in uncontaminated soil [32,39]. Dai et al. [40] also found that the qCO_2 decreased with the alleviation of metal toxicity. The qCO_2 in both soils decreased markedly after 12 days of bioremediation (Table 2), which might indicate the Pb toxicity in both soils was alleviated. The qCO_2 in A soil was about 1.6 times greater than that in B soil after remediation. Microbial metabolic efficiency was inhibited in the presence of metals, and more energy was required for microbial synthesis. Microorganisms mainly utilized organic carbon in soil as maintenance energy for growth under metal contamination, so the CO_2 release increased and the conversion of organic carbon into biomass decreased. On the contrary, microorganisms could convert substrates into biomass effectively with less or no metal contamination. These findings might explain the change of the qCO_2 observed in our study, the similar results were also shown by Dai et al. [40].

The microorganisms (such as fungus and bacteria) with different C/N ratio contributed to the biomass C/N ratio for soil. Generally, there is the dominance of fungi in polluted soils and bacteria in the uncontaminated soils, due to the tolerance of fungi to metal [41]. Because of the difference among the C/N ratios of microorganisms (e.g. fungi, bacteria, etc.) the biomass C/N ratio in soil varied with the unequal growth of different microorganisms. The C_{mic}/N_{mic} in the samples displayed a decrease when the C_{mic} increased (Table 2), which might be because the microorganisms with low C/N ratio increased faster than those with high C/N ratio. Joergensen et al. [42] also confirmed that large microbial biomass C/N ratios were caused by increased fungal to microbial biomass ratios under metal contamination, and they reported that C/N ratio for bacteria was as low as 3.5:1 and fungi, from 10 to 15:1. Although the C_{mic}/N_{mic} in B soil decreased more than that in A soil during incubation, our results showed the lower C_{mic}/N_{mic} in A soil with higher contamination level and the higher C_{mic}/N_{mic} in B soil with lower contamination level on day 60 (Tables 1 and 2), which was opposite to the previous studies and the conclusions we obtained above. The C_{mic}/N_{mic} was not suitable to be used as an index for estimating metal toxicity and contamination level in contaminated soil with inoculation of microorganism, which might be because the C_{mic}/N_{mic} from the inoculated microorganism would affect this estimation.

The microbial indices mentioned above just reflect the difference of growth of soil microorganisms and microbial activity under Pb-contamination, but not show the changes in microbial

community status. The BIOLOG procedure is a useful method for the characterization of the metabolic capacity of soil microbial communities. The differences between the metabolic activities of microbial communities can be assessed by analyzing the utilization of carbon sources in BIOLOG plates [43,44]. The higher K and the lower S were observed in B soil after remediation (Fig. 4), which showed the co-incubation method adopted in our study improved the utilization of carbon sources by soil microflora. It was also observed that there was a significant difference between kinetic parameters K and S fitted to logistic growth curve in A soil and those in B soil, while no significant difference between the AWCD in A soil and that in B soil was found (Fig. 4). The above results indicated the kinetic parameters were more sensitive to the changes of metabolic capacity of microbial community than the AWCD, which was used to evaluate microbial community activity and soil ecological status, in accordance with the previous reports [24].

5. Conclusions

In conclusion our results showed that incubating contaminated soil with the inoculated *P. chrysosporium*, together with the added straws as nutrient, could reduce the active Pb, alleviate the Pb stress, and stabilize the Pb-contaminated soil. In addition, the treatment improved the remediation of the soil in comparison to the controls. All these results might be because the Pb ion was absorbed by the mycelia of *P. chrysosporium* and chelated by the humus formed in the incubation process. However, further studies are needed to investigate and confirm the immobilization mechanisms of Pb, because the mechanisms were not very certain in this study. The traditional bioremediation technology relying on plants might be difficult to remediate some lean soil contaminated with heavy metal because the soil conditions are unfavorable to the growth of the plants. In addition, when soil microflora had deteriorated, conventional physico-chemical approach could not improve soil microbial activities effectively but only removed metal from contaminated soils. The approach used in our study could not only immobilize Pb ions in soils, but also effectively improve soil microbial activities and the metabolic capacity of microbial community. However, metal ions were just immobilized for complexation and not removed from soil in our study, so the approach needs improvements and deserves further researches.

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