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Biological leaching of heavy metals from a contaminated soil by *Aspergillus niger*

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ABSTRACT

Bioleaching of heavy metals from a contaminated soil in an industrial area using metabolites, mainly weak organic acids, produced by a fungus *Aspergillus niger* was investigated. Batch experiments were performed to compare the leaching efficiencies of one-step and two-step processes and to determine the transformation of heavy metal chemical forms during the bioleaching process. After the one or two-step processes, the metal removals were compared using analysis of variance (ANOVA) and least-significance difference (LSD). *A. niger* exhibits a good potential in generating a variety of organic acids effective for metal solubilisation. Results showed that after the one-step process, maximum removals of 56%, 100%, 30% and 19% were achieved for copper, cadmium, lead and zinc, respectively. After the two-step process, highest removals of 97.5% Cu, 88.2% Cd, 26% Pb, and 14.5% Zn were obtained. Results of sequential extraction showed that organic acids produced by *A. niger* were effective in removing the exchangeable, carbonate, and Fe/Mn oxide fractions of Cu, Cd, Pb and Zn; and after both processes the metals remaining in the soil were mainly bound in stable fractions. Such a treatment procedure indicated that leaching of heavy metals from contaminated soil using *A. niger* has the potential for use in remediation of contaminated soils.

1. Introduction

Throughout the world there is growing concern that the heavy metal content of soils are increasing as the result of industrial, mining, agricultural and domestic activities [1,2]. Unlike many other pollutants, heavy metals are difficult to remove from the environment. These metals can not be chemically or biologically degraded, and are ultimately indestructible. The toxic effects of heavy metals result mainly from the interaction of metals with proteins (enzymes) and inhibition of metabolic processes. When accumulated in soils, heavy metals such as copper, cadmium, lead, zinc, nickel, mercury and chromium can be present in concentrations toxic to plants, animals, humans and aquatic life [3], therefore reliable remediation techniques are required for site clean up [4]. In contrast to organic pollutants, metals are not mineralised by micro-organisms but can be oxidised or reduced (i.e., transformed to different redox stages), or complexed by organic metabolites. Currently, a bioleaching approach offers attractive features for the extraction of metals from solid materials, such as lower cost and energy requirements, environmental safety and operational flexibility.

Some species of heterotrophic micro-organisms, such as *Aspergillus* and *Penicillium*, have shown potential for metal bioleaching. Metal leaching by heterotrophic micro-organisms generally involves an indirect process with microbial production of amino acids, other organic acids, and other metabolites. These metabolites dissolve metals from minerals by displacement of metal ion from the ore or soil matrix by hydrogen ions, or by the formation of soluble metal complexes and chelates [5]. In this way, the most important species of fungi are *Aspergillus niger* (*A. niger*) and *Penicillium simplicissimum* because of their ability to excrete abundant amounts of organic acids [5].

A. niger is one of the most widely used fungi in bioleaching. The fungus has been used commercially in the production of organic acids, such as citric acid [6,7], oxalic acid [8] and gluconic acid [9]. These acids are well-known lixiviants for the leaching of heavy metals from ore materials and solid wastes [10], and may reduce the cost of commercial heavy metal decontamination and decrease any environmental impacts resulting from metal contamination [11,12]. *A. niger* has been found to overproduce organic acids that can serve as leaching agents for the solubilisation of Al, Fe, Mn, Ni, Pb, Cd, Cu, and Zn from fly ash [13].

Although the process of metal leaching using *A. niger* looks promising, only relatively few studies have been performed with actual metal-contaminated materials. These include fly ash [13], spent fluid catalytic cracking (FCC) catalyst [14,15] and nickeliferous laterites [16]. However, there is a lack of studies about leaching heavy metals from contaminated soil by using heterotrophic

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6micro-organisms such as *A. niger*. On the other hand, the toxic effects of heavy metals depend not only on concentrations but also on bioavailability [17]. It is essential to find out the dynamic transformation of chemical forms of heavy metals during bioleaching, which is necessary to clarify the reaction process and to forecast the environmental chemical behaviour of heavy metals (i.e., mobility, bioavailability and toxicity) after bioleaching using *A. niger*. However, there is limited information on the changes of heavy metals speciation during bioleaching by *A. niger*. The objectives of the present study are: (1) to investigate the effectiveness of fungal generated organic acids for leaching heavy metals from a contaminated soil; (2) to compare one and two-step leaching processes; and (3) to determine the transformation of chemical speciation of heavy metals before and after the one and two-step processes.

2. Materials and methods

2.1. Soil

The sandy soil used in this study was collected from the Shenyang Smelting Industry, 1ocated in the Tiexi district of Shenyang (123°49′411″E, 42°07′785″N), China, historically contaminated with heavy metals. The smelting industry was built originally in August 1936, and thus has been located in the city for over 60 years. However, it had stopped its smelting activities about 10 years ago. This area is well known in China for its notorious heavy metals contamination. The soil sample was air dried and sieved through a 2-mm sieve to break soil clumps and to remove rocks, and was then mechanically mixed to ensure homogeneity and stored in a plastic container for subsequent experiments. The total heavy metal contents in the soil were determined by an acid digestion method ($HNO_3 + HClO_4 + HF$) [18]. The digested liquid was filtered through Whatman No. 42 paper, and the filtrate was analysed for the heavy metals content using an atomic absorption spectrophotometer (Varian, Spectr AA 240, USA).

2.2. Spore and inoculum preparation

A. niger, a laboratory stock culture, originally isolated from a heavy metal-contaminated soil, was cultured according to the procedure of Bosshard et al. [13]. Adaptation of the fungus was carried out through a series of sub-cultures after exposure to the soil used in the study. For inoculum preparation, the adapted *A. niger* was incubated three times on potato dextrose agar (PDA) slants using a sterile platinum loop at 30 °C for 5 days. Five-day-old conidia were harvested from potato dextrose agar surface using sterile distilled water. The number of spores was counted using a haemocytometer and standardised to approximately 3.5×10^6 spores mL^{-1} of spore suspension. For the bioleaching experiments, 1 mL of spore suspension was added to 100 mL of sucrose medium with the composition: Sucrose 100 g L^{-1}; $NaNO_3$ 1.5 g L^{-1}; KH_2PO_4 0.5 g L^{-1}; $MgSO_4 \cdot 7H_2O$ 0.025 g L^{-1}; KCl 0.025 g L^{-1}; yeast extract 1.6 g L^{-1}.

2.3. Two-step bioleaching experiments

The production of organic acids can be performed within the soil piles (one-step process) or produced in separate reactors (two-step process). In the two-step leaching process, One ml of *A. niger* spore suspension was first cultivated in 250 mL autoclaved conical flasks containing 100 mL of sterile sucrose medium without adding soil (first step). The cell-free spent medium after centrifugation which was obtained after 15 days of fungal incubation was then used for the leaching process with 5% (w/v) autoclaved soil (second step). Leaching was carried out by tumbling the mixture in a rotary shaking incubator at 30 °C. At regular time intervals over 4 days, samples obtained from each flask were filtered through 0.45-μm Millipore membrane and the filtrate was analysed for organic acid concentrations by using HPLC (Agilent 1100 Series, USA) and heavy metal concentrations using an atomic absorption spectrometer (AAS) (Spectr AA 240, Varian, USA). All the experiments were carried out in duplicate.

2.4. One-step bioleaching experiments

The one-step bioleaching process was carried out using 250 mL autoclaved conical flasks with 5% (w/v) autoclaved soil in 100 mL of sucrose medium. One ml of *A. niger* spore suspension (3.5×10^6 spores mL^{-1}) was added aseptically to these conical flasks. The experimental control was sucrose medium without added soil. All flasks were incubated in a rotary shaking incubator at 120 r/min and 30 °C. At regular time intervals over 15 days, samples from each conical flask were filtered and analysed for organic acid and heavy metal concentrations. All experiments were run in duplicate.

Sterile experimental conditions were achieved by autoclaving each flask containing soil suspension separately at 121 °C for 20 min prior to inoculation.

2.5. Chemical leaching experiments

Chemical leaching of the metal-contaminated soil was carried out using gluconic acid (15 g L^{-1}), citric acid (6 g L^{-1}), oxalic acid (2.5 g L^{-1}) and malic acid (0.5 g L^{-1}) as well as a mixture of the these four acids (15 g L^{-1} gluconic acid, 6 g L^{-1} citric acid, 2.5 g L^{-1} oxalic acid and 0.5 g L^{-1} malic acid). These experiments were conducted under the same conditions as bioleaching described in Section 2.4 (but without inoculum and only one day incubation).

2.6. Speciation of heavy metals in soil before and after bioleaching experiments

The dried soil residues obtained from one (15 days) and two-step (15 days + 2 days) bioleaching experiments, together with untreated soil samples, were fractioned by a sequential extraction procedure [19]. Three replicates of 1 g of soil sample, sieved to 0.2 mm, were used. The sequential extraction procedure was as follows:

(i) Exchangeable fraction: 15 mL of 1 mol/L $MgCl_2$ was added, shaken continuously at room temperature for 2 h. The mixture was then centrifuged at 4000 r/min for 10 min and the supernatant was filtered through a 0.45 μm membrane;
(ii) Carbonate fraction: 15 mL of l mol/L CH_3COONa (adjusted to pH 5.0 with CH_3COOH) was added, shaken continuously at room temperature for 2 h. The mixture was centrifuged at 4000 r/min for l0 min. The supernatant was filtered through a 0.45 μm membrane;
(iii) Fe-Mn oxide fraction: 20 mL of 0.04 mol/L hydroxylamine hydrochloride (NH_2OH–HC1) in 25% (v/v) acetic acid was added, shaken occasionally at 96 ± 3 °C for 5 h, then another 10 mL of 0.04 mol/L NH_2OH–HCl in 25% (v/v) acetic acid was added. The mixture was centrifuged at 4000 r/min for 10 min after cooling, and the supernatant was filtered through a 0.45 μm membrane and then diluted to 10 mL;
(iv) Organic fraction: This extraction is divided into three stages—stage (I): 3 mL of 0.02 mol/L HNO_3 and 5 mL H_2O_2 (30%) (Adjusted to pH 2.0 with 0.1 mol/L HNO_3) was added, standing at room temperature for 1 h; stage (II): 3 mL H_2O_2 (at pH 2.0), intermittently agitated for 2 h at (85 ± 3)°C, another 3 mL H_2O_2 was added, and continue agitated for 3 h at (85 ± 3)°C; stage (III): 5 mL of 3.2 mol/L NH_4Ac was added after cooling,

diluted to 20 mL with deionised water, shaken continuously for l h. The mixture was centrifuged at 4000 r/min for l0 min. The supernatant was filtered through a 0.45 μm membrane;

(v) Residue fraction: the residue from (iv) was digested with10 mL HF and 2 mL $HClO_4$ in an oven according to the method of Lu [18].

The amounts of metals in various fractions were determined using an AAS (Varian, Spectr AA 240, USA) and the proportions of these to the total for that sample were calculated.

2.7. HPLC analyses

HPLC analyses organic acids were done as described in Van Hees et al. [20]. All data were corrected by blank sample values, i.e., deionised water, which were processed in the same way (centrifugation, filtration, and concentration). HPLC (Agilent 1100 Series, USA) conditions were: Zorbax C18 column, 250 mm × 4.6 mm; mobile phase 5 g L^{-1} $(NH_4)_2HPO_4$–H_3P04 (pH 2.5); flow rate 0.5 mL min^{-1}; temperature 35 °C; injection loop 10 μL; diode array detector (DAD) at 215 nm.

2.8. Statistical analyses

Means of individual parameters were compared using one-way analyses of variance (ANOVA) and least-significance difference (LSD) using statistical software, SPSS 11.5 (SPSS Inc., Chicago, USA), at 95% confidence. The homogeneity of the variances was tested with Games–Howell's test.

3. Results and discussion

3.1. Soil characterization

The initial soil pH was found to be near to neutral (7.8) (soil: water = 1:2.5), organic matter content was 3.42%. The soil was sandy and comprised 51.6% sand, 15.2% clay and 34.4% silt. The soil was highly contaminated with heavy metals and the concentrations of heavy metals in the soil were 1100 mg/kg Cu, 105 mg/kg Cd, 21138 mg/kg Pb and 7760 mg/kg Zn, respectively. Metal concentrations in these samples are extremely high, posing a significant hazard for human health and the environment.

3.2. Production of organic acids

To confirm that the *A. niger* was indeed producing organic acids in the sucrose medium, analyses of the culture medium was performed 15 days after inoculation. Gluconic acid (13,667 mg/L) was produced at the highest concentration, followed by citric (6089 mg/L), oxalic (2393 mg/L) and malic (456 mg/L) acids.

3.3. Removals of heavy metals in the two-step process

Contaminated soil (5%, w/v) was added to the organic acid solution obtained from the culture medium described in Section 3.2. The experiment was performed to evaluate the ability of the organic aids to remove the heavy metals in separated acid production and leaching steps. The results are shown in Table 1, which exhibits statistical values (means, standard deviations, and significant values) of the removals of heavy metals on Days 1, 2, 3 and 4, respectively. After incubation for 1 day, 73% of Cu, 70.8% of Cd, 15.6% of Pb, 14.2% of Zn was removed. It can be clearly seen from Table 1 that time after 2 days does not have a significant influence on the leaching efficiency in the two-step process; a prolongation of the second step did not increase the amount of metal removal. An incubation time of 2 days was sufficient for an effective leaching of Cu, Cd, Pb and Zn, and after incubation for 2 days, 84.3%, 84.4%, 25% and 14.4% for Cu, Cd, Pb and Zn, respectively, was removed. Table 1 also shows that copper and cadmium were most easily extracted from the soil followed by lead and zinc. This result may be due to strong chemisorption by clays, oxides and soil humus of lead and zinc [21]. Therefore the mobility of Pb and Zn was obviously lower than that of Cu and Cd. On the other hand, the initial metal concentration is also an important parameter affecting the metal retention and mobility in soils [22]. When the metal sorption capacity of most soils is exceeded, the contamination would additionally be present as discrete metal–mineral phases [23]. Such metal ions can be immobilised in soil by the formation of insoluble precipitates, incorporation into the crystalline structure of clays and metal oxides, and/or by physical entrapment in the immobile water surrounding soil micro- and macro-pores [24]. The heavy metal ions adsorbed or entrapped are difficult to desorb from contaminated soils even using the strong chelating agent EDTA for cases in which the heavy metal ion complexes are rather stable [23].

3.4. Removal of heavy metals in the one-step process

One-step process experiments were performed by growing the *A. niger* in the presence of 5% (w/v) soil. The filtrate pH and concentrations of copper, cadmium, lead and zinc were determined for samples taken from the soil-containing culture at regular time intervals. The concentrations of organic acids were determined at 3, 5, 9 and 13 days of the leaching experiment. Fig. 1 indicates that the filtrate pH values decreased by 2–4 U during the growth of *A. niger* due to the excreted metabolites, which included H^+ and organic acids [5,13,25,26]. The results clearly indicated that the *A. niger* examined in this study was capable of generating significant concentrations of organic acids. The final pH values were 3.07 and 2.23, respectively, for the treatment and control, which showed that the addition of the contaminated soil resulted in some toxicity to the growth of *A. niger*. In fact, in our study, pH was taken as an indication of growth since there was good correlation with cellular dry weight (r = 0.95).

Table 1
Comparison of means of heavy metal removals using one-way ANOVA and LSD[a] during the two-step bioleaching process.

Time(days)	Cu (%)		Cd (%)		Pb (%)		Zn (%)	
	Mean ± S.D.[b]	P[c]	Mean ± S.D.	P	Mean ± S.D.	P	Mean ± S.D.	P
1	73 ± 7.3A	0.013	70.8 ± 1.7A	0.015	15.6 ± 0.4A	0.003	14.2 ± 0.02A	<0.001
2	84.3 ± 8AB		84.4 ± 8B		25.0 ± 3B		14.4 ± 0.07A	
3	95.7 ± 5.8B		88.2 ± 2.2B		23.1 ± 0.8B		14.5 ± 0.07A	
4	97.5 ± 8.5B		86.1 ± 6.7B		26.1 ± 3.7B		13.0 ± 0.09A	

[a] n = 5, 95% confidence interval.
[b] Indicated by LSD that removal values in each day followed with different letters are statistically different.
[c] Significance values indicated by one-way ANOVA between different leaching times.

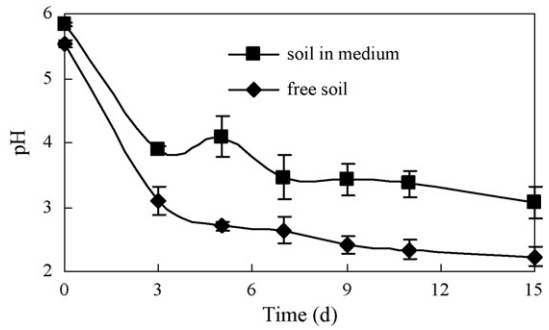

Fig. 1. Changes of pH values in the one-step bioleaching process.

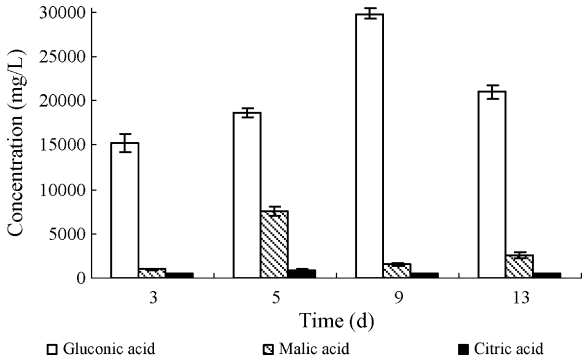

Fig. 2. Concentration of organic acids as a function of time in the one-step bioleaching process.

The concentrations of organic acids excreted by the fungus during the one-step process are shown in Fig. 2. In the presence of the soil, the fungus excreted a lower concentration of citric acid (450–950 mg/L) than malic (950–7500 mg/L) and gluconic (15,000–30,000 mg/L) acids. The fungus excreted higher concentrations of malic and citric acids on Day 5; gluconic acid concentration peaked on Day 9. However, oxalic acid concentration was very low and insignificant. Bioleaching with *A. niger* is mainly based on the acidolysis mechanism, that is, solubilisation of the material on account of the acidification [5,27]. It is known that the type and concentration of excreted organic acids by *A. niger* is dependent on the presence of certain heavy metals and trace elements [3,13].

Over the same time period, the extraction of metals from the soil by the fungus is shown in Table 2. It can be seen that the removal efficiencies varied substantially on any given day and also between days. Cu, Cd, Pb and Zn showed similar leaching behaviour. They leaching of Cu, Cd and Zn were almost maximum at 56%, 100% and 19%, respectively, on Day 13, whereas removal of Pb was found to peak on Day 7 (30%). The markedly reduced apparent leaching observed for Zn on Day 15 requires further investigation.

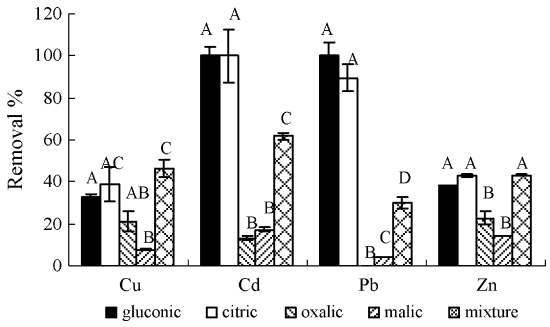

Fig. 3. Removal of various heavy metals over 24 h during chemical leaching using different organic acids.

In comparison to the separate acid production and leaching step experiment, the single step leaching experiments showed several advantages. Higher leaching efficiencies (100% of Cd, 30% of Pb and 19% of Zn) occurred in the one-step process than in the two-step process (84.4%, 25% and 14.4% for Cd, Pb and Zn). The result may be due to two reasons. Firstly, bioaccumulation occurs in the fungal bioleaching and enhances metal leaching via altering the equilibrium metal concentration in the suspension [13]. Secondly, a different metabolite was involved in two-step leaching (gluconic > citric > oxalic > malic) compared with one-step leaching (gluconic > malic > citric). However, the leaching efficiency of Cu in the one-step process was lower than in the two-step process which may be due to the weaker selective biosorption of Cu by mycelia of the *A. niger* than for the other metals. Intact microbial cells, live or dead, and their products can be highly efficient bioaccumulators of both soluble and particulate forms of heavy metals [28]. One-step processes can reduce capital and operating costs; the two-step process producing organic acids in a separate step can facilitate the production of metal leaching acids, and avoid the difficulties related to: (a) maintaining optimum fungal culture conditions in the field; and (b) the toxicity of soil involved in the treatment process. However, capital costs would increase due to the requirement for extra bioreactor tanks to produce the acids [29].

3.5. Removal of heavy metals in chemical leaching process

To determine the chemical leaching of contaminated soil using gluconic (15 g L^{-1}), citric (6 g L^{-1}), oxalic (2.5 g L^{-1}), malic (0.5 g L^{-1}) and a mixture of the four acids (15 g L^{-1} gluconic, 6 g L^{-1} citric, 2.5 g L^{-1} oxalic, 0.5 g L^{-1} malic) after 24 h incubation, the metal removals shown in Fig. 3 were compared. The concentration of the individual organic acids used were similar to that of the organic acids present in 15-day-old spent medium of *A. niger* grown in the sucrose medium (Section 3.2). Among the four organic acids, gluconic acid leached more Cd and Pb (100% Cd and 100% Pb) from the metal-contaminated soil. Citric acid showed more potential

Table 2
Comparison of means of heavy metal removals using one-way ANOVA and LSD[a] during one-step bioleaching process.

Time (days)	Cu (%)		Cd (%)		Pb (%)		Zn (%)	
	Mean ± S.D.[b]	*P*[c]	Mean ± S.D.	*P*	Mean ± S.D.	*P*	Mean ± S.D.	*P*
3	9.1 ± 2.2A	<0.01	60 ± 6.2A	<0.01	10 ± 6.8A	<0.01	6.7 ± 3A	<0.01
5	32.7 ± 8BC		86.3 ± 7.2B		19.4 ± 4.1B		14 ± 4.4AB	
7	25.2 ± 1.5B		98.3 ± 3C		30.2 ± 6.7C		15.7 ± 3.8B	
9	40.3 ± 3.8C		99.1 ± 7.3C		25.7 ± 3.1BC		15 ± 2.8B	
13	42 ± 11.4ABCD		100.0 ± 3.2C		27.4 ± 4.8C		19.0 ± 6B	
15	50.6 ± 4.5D		95.3 ± 5.8C		19.3 ± 2.1B		5.5 ± 0.8A	

[a] $n = 5$, 95% confidence interval.
[b] Indicated by LSD that removal values in each day followed with different letters are statistically different.
[c] Significance values indicated by one-way ANOVA between different leaching time.

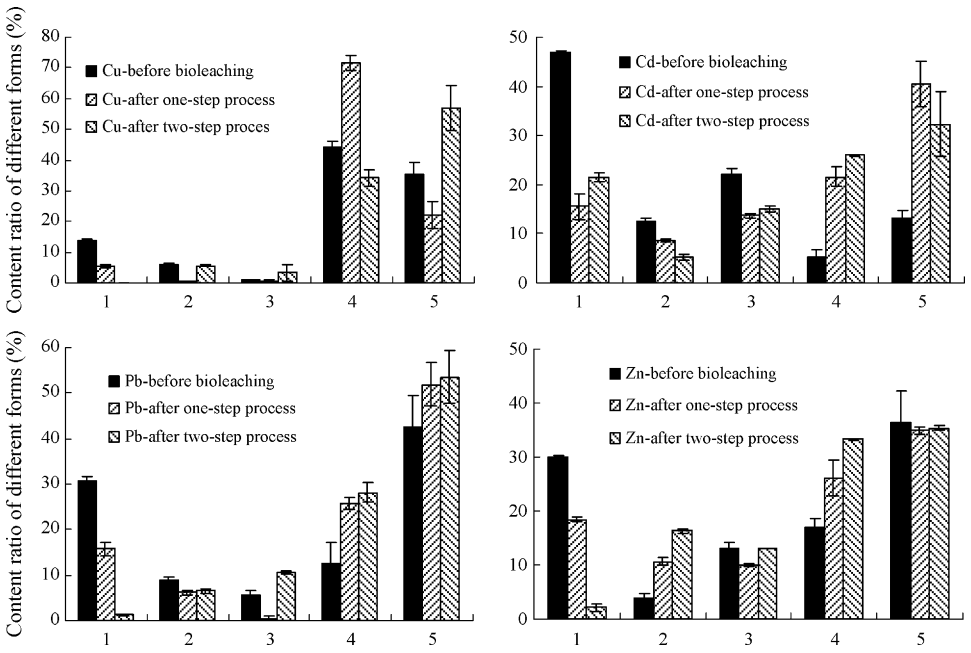

Fig. 4. Variation in partitioning of chemical forms for metal before and after one- (15 days) and two-step (15 days + 2 days) bioleaching processes.

than other acids to solubilise copper, cadmium, lead and zinc in the experiment providing higher removals (40% Cu, 100% Cd, 90% Pb, and 43% Zn). It is interesting to find that the removals of Cu and Zn obtained using gluconic and citric acids respectively were similar to the removals by the mixture of acids. These results suggest that gluconic and citric acids were the major and effective lixiviants of Cu and Zn in the chemical leaching of the metal-contaminated soil. The lower removals for Cd and Pb (62% and 30%, respectively) by the mixture of the four acids compared to gluconic and citric acids might be due to the precipitation of Cd and Pb as their oxalates.

During chemical leaching process, the heavy metal removals for the mixture of the four acids were up to 46.4% of Cu, 61.8% of Cd, 30.2% of Pb, and 43.3% of Zn. In comparison to the chemical leaching, the metal removals during two-step process using *A. niger* reached 84.3%, 84.4%, 25% and 14.4% for Cu, Cd, Pb and Zn, respectively. The lower leaching removals of Pb and Zn in the two-step process compared with chemical leaching were possibly due to the precipitation of lead and zinc in the presence of the sugars during bioleaching. Generally speaking, bioleaching using organic acids produced by *A. niger* would be more effective than chemical leaching using organic acid agents because of the higher leaching removals and lower cost in leaching agents.

3.6. Variation in chemical form of heavy metals before and after one and two-step process

The different forms of heavy metals represent different energy states, and this affects not only the efficiency of bioleaching but also the bioavailability of heavy metals after bioleaching. Metals in exchangeable, carbonate and Fe/Mn oxide-bound fractions are considered to be more mobile, dangerous and bioavailable. The organic matter and residual fractions are considered to be more stable and nonbioavailable compared with metals in exchangeable, carbonate and Fe/Mn oxide-bound fractions [30]. Therefore it is necessary to describe the partitioning of heavy metals into different fractions before and after the bioleaching. The partitioning of heavy metals in the soil before and after the one and two-step leaching processes is shown in Fig. 4. The bioleaching (both one and two-step processes) had a significant impact on partitioning of heavy metals. After one-step bioleaching, Cu, mainly bound to organic matter, in residual and in exchangeable fractions in raw soil, was found mainly bound to organic matter and in residual fractions. Cd, mainly in exchangeable and Fe/Mn oxide-bound fractions before bioleaching, was mainly left in the residual fraction. The greatest changes for Pb and Zn were in exchangeable and organic matter bound fractions, and for Zn, also the carbonate bound form. Hence Pb and Zn remaining in the soil were more stable and nonbioavailable. After two-step bioleaching, the exchangeable, carbonate and Fe/Mn oxide fractions of Cu, Cd, Pb and Zn were mainly left in organic matter and residual bound fractions. After both the one- and two-step bioleaching processes, metals remaining in the soil were thus mainly bound in stable fractions.

4. Conclusions

Inoculation of a lixiviant with organic-acid-producing *A. niger* can result in a high leaching efficiency. In the two-step process, the highest metal removal efficiencies were 97.5%, 88.2%, 26% and 14.5% for Cu, Cd, Pb and Zn, respectively. During one-step bioleaching, the highest removals were 56% of Cu, 100% of Cd, 30% of Pb and 19% of Zn. Compared to chemical leaching, the bioleaching process showed better removals and reduced cost. This proved that it was feasible to remove heavy metals from soil by using the bioleaching remediation method. The results also showed that there were significant differences in heavy-metal binding before and after bioleaching. After the bioleaching, metals remaining in the soil were mainly found in the stable fractions and were unavailable to the surrounding environment. The findings above indicate that the bioremediation process using *A. niger* could be effective for the leaching of heavy metals from the industrial soil contaminated by heavy metals and show a promising technology towards a biological detoxification of soils.

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References

[1] F.A. Vega, E.F. Covelo, M.L. Andrade, P. Marcet, Relationships between heavy metals content and soil properties in mine soils, Anal. Chim. Acta 524 (2004) 141–150.
[2] J. Sastre, G. Rauret, M. Vidal, Effect of the cationic composition of sorption solution on the quantification of sorption–desorption parameters of heavy metals in soils, Environ. Pollut. 140 (2) (2006) 322–339.
[3] R.H. Dowdy, V.V. Volk, Movement of heavy metals in soils, in: D.W. Nelsenetal (Ed.), Chemical Mobility and Reactivity in Soil Systems, SSSA Spec. Publ. 11, SSSA, Madison, WI, 1983, pp. 229–240.
[4] R. Abumaizar, L.I. Khan, Laboratory investigation of heavy metal removal by soil washing, J. Air Waste Manage. Assoc. 46 (1996) 765–768.
[5] W. Burgstaller, F.J. Schinner, Mini review: leaching of metals with fungi, J. Biotechnol. 27 (2) (1993) 91–116.
[6] M. Legisa, M. Gradisnik-Grapulin, Sudden substrate dilution induces a higher rate of citric-acid production by *Aspergillus niger*, Appl. Environ. Microbiol. 61 (7) (1995) 2732–2737.
[7] H.S. Grewal, K.L. Kalra, Fungal production of citric acid, Biotechnol. Adv. 13 (1995) 209–234.
[8] H. Strasser, W. Burgstaller, F. Schinner, High yield production of oxalic acid for metal leaching processes by *Aspergillus niger*, FEMS Microbiol. Lett. 119 (1994) 365–370.
[9] S.N. Dronawat, C.K. Svihla, T.R. Hanley, The effects of agitation and aeration on the production of gluconic acid by *Aspergillus niger*, Appl. Biochem. Biotechnol. 51–52 (1995) 347–354.
[10] A.B. King, A.W.L. Dudeney, Bioleaching of nephelin, Hydrometallurgy 19 (1987) 69–81.
[11] S.A. Wasay, S.F. Barrington, S. Tokunaga, Organic acids for the in situ remediation of soils polluted by heavy metals: soil flushing in columns, Water Air Soil Pollut. 127 (2001) 301–314.
[12] C.N. Mulligan, M. KamLi, B.F. Gibbs, Bioleaching of heavy metals from a low-grade mining ore using *Aspergillus niger*, J. Hazard. Mater. 110 (2004) 77–84.
[13] P.B. Bosshard, R. Bachofen, H. Brandl, Metal leaching of fly ash from municipal waste incineration by *Aspergillus niger*, Environ. Sci. Technol. 30 (1996) 3066–3070.
[14] K.M.M. Aung, Y.P. Ting, Bioleaching of spent fluid catalytic cracking catalyst using *Aspergillus niger*, J. Biotechnol. 116 (2005) 159–170.
[15] D. Santhiya, Y.P. Ting, Bioleaching of spent refinery processing catalyst using *Aspergillus niger* with high-yield oxalic acid, J. Biotechnol. 116 (2005) 171–184.
[16] P.G. Tzeferis, S. Agatzini, E.T. Nerantzis, Mineral leaching of non-sulphide nickel ores using heterotrophic micro-organisms, Lett. Appl. Microbiol. 18 (1994) 209–213.
[17] A.Y. Renoux, R.D. Tyagi, R. Samson, Assessment of toxicity reducing after metal removal in bioleaching sewage sludge, Water Res. 35 (2001) 1415–1424.
[18] R.K. Lu, Soil Agriculture Chemistry Analysis Method, Agriculture Science Press of China, Beijing, 1999.
[19] A. Tessier, P.G.C. Campbell, M. Bisson, Sequential extraction procedure for the speciation of particulate trace metals, Anal. Chem. 5l (1979) 844–851.
[20] P.A.W. Van Hees, A.M.T. Andersson, U.S. Lundström, Separation of organic low molecular weight aluminum complexes in soil solution by liquid chromatography, Chemosphere 33 (1996) 1951–1966.
[21] X. Ke, P.J. Li, Q.X. Zhou, Y. Zhang, T.H. Sun, Removal of heavy metals from a contaminated soil using tartaric acid, J. Environ. Sci. 18 (4) (2006) 727–733.
[22] B.E. Reed, P.C. Carriere, R.J. Moore, Flushing of Pb (II) contaminated soil using HC1, EDTA and $CaC1_2$, Environ. Eng. 122 (1996) 48–50.
[23] A.P. Davis, I. Singh, Washing of zinc (II) from contaminated soil column. I, Environ. Eng. (ASCE) 121 (2) (1995) 174–185.
[24] J. Pichtel, T.M. Pechtel, Comparison of solvents for ex-situ removal of chromium and lead from contaminated soil, Environ. Eng. Sci. 14 (2) (1997) 97–104.
[25] I.M. Castro, J.L.R. Fietto, R.X. Vieira, M.J.M. Tropia, L.M.M. Campos, E.B. Paniago, R.L. Brandao, Bioleaching of zinc and nickel from silicates using *Aspergillus niger* cultures, Hydrometallurgy 57 (2000) 39–49.
[26] H. Brandl, R. Bosshard, M. Wegmann, Computer-munching microbes: metal leaching from electronic scrap by bacteria and fungi, Hydrometallurgy 59 (2001) 319–326.
[27] F. Glombitza, U. Iske, M. Bullmann, Biotechnology based opportunities for environmental-protection in the Uranium mining-industry, Acta. Biotechnol. 12 (2) (1992) 79–85.
[28] S. Silver, L.T. Phung, Bacterial heavy metal resistance: new surprises, Ann. Rev. Microbiol. 50 (1996) 753–789.
[29] C.N. Mulligan, G.R. Cloutier, Bioremediation of metal contamination, Environ. Monit. Assess. 84 (2003) 45–60.
[30] C.N. Mulligan, R.N. Yong, B.F. Gibbs, Remediation technologies for metal-contaminated soils and groundwater: an evaluation, Eng. Geol. 60 (2001) 193–207.