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Fungal strain *Aspergillus flavus* F3 as a potential candidate for the removal of lead (II) and chromium (VI) from contaminated soil

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Abstract: Metal contamination of soil is a serious environmental problem due to mining and use of synthetic products (e.g. pesticides, paints, batteries, and industrial wastes), which are serious threat to human life. The current research is aimed at the remediation of soil contaminated with lead (II) and chromium (VI) using indigenous fungal strains through the comparative study of bioleaching and chemical leaching methods. The removal efficiencies of Pb (II) and Cr (VI) in bioleaching were higher than chemical leaching, where 99% Cr (VI) and 36% Pb (II) were removed by *Aspergillus flavus* (F3) in bioleaching through the production of approximately 332 mg L^{-1} malic acid, 213 mg L^{-1} succinic acid, and 35 mg L^{-1} citric acid. The removal efficiencies in chemical leaching were 21.30% for Pb (II) and 1.92% for Cr (VI) by malic acid, 29.30% for Pb (II) and 72% for Cr (VI) by succinic acid, 22.21% for Pb (II) and 60.70% for Cr (VI) by citric acid, and 2.20% for Pb (II) and 2.47% for Cr (VI) by oxalic acid. The sequential extraction procedure for Pb (II) and Cr (VI) before and after bioleaching showed that Pb (II) and Cr (VI) mostly bound to stable fractions after bioleaching. Scanning electron microscopy (SEM) with energy-dispersive X-ray analysis (EDX) helped to identify the characteristic changes in the morphology and elemental composition of *A. flavus* (F3) biomass before and after bioleaching, whereas Fourier transform infrared spectroscopy (FTIR) showed that fungal biomass contain hydroxyl, carboxyl, fatty acids, and amine groups on its surface. The results implied that the fungal strain *A. flavus* (F3) can be used to remediate soils contaminated with Pb (II) and Cr (VI).

Keywords: *Aspergillus flavus* (F3); bioleaching; contaminated soil; heavy metal.

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Introduction

The heavy metal contents of soil are increasing all over the world because of agricultural activities, domestic activities, and industrial developments (Vega et al., 2004; Sastre et al., 2006). In contrast to various other pollutants, it is relatively difficult to remove heavy metals from the environment. The surrounding environmental pollution in the past, such as soil and groundwater pollution, resulted in the improper management of heavy metals. Because of the significant effect of chromium and lead on the environment, they are known to be major toxic metals of common interest (Volesky, 1994). Chromium (VI) is toxic and is the chemical responsible for causing mutation to most organisms. It is responsible for causing lung carcinoma in humans, and it has corrosive effects on the skin and respiratory tract (Thacker and Madamwar, 2005). The U.S. Environmental Protection Agency (1998) has allowed the maximum permitted quantity of hexavalent Cr in natural water to only approximately 0.05 mg L^{-1}. Because of the toxic nature of this pollutant, it mainly forms negatively charged ion species and is found mostly in the oxyanion form. Therefore, it should be removed from soil before being distributed into the environment. However, mining, smelting, and improper disposal of Pb-containing wastes result in lead contamination. Uncontaminated soil contains lead concentrations <50 ppm, but soil lead levels in many urban areas exceed 200 ppm. (American Academy of

Pediatrics, 1993). The retention of lead in soil is due to many reasons, especially its mineralogical composition (Evans, 1989). There are many physiochemical methods available to remove heavy metals from soil, but these processes may not be so effective or very expensive, particularly when the metals have a range of 1–100 mg L^{-1} in solution (Ahluwalia and Goyal, 2007). Therefore, more appropriate and cost-effective technology should be developed to remove toxic heavy metals (Pb and Cr) in contaminated soil. Bioleaching technology has many advantages, such as mild reaction conditions, less environmental impact, and low energy use, and it is suitable for low-grade mine tailings and contaminated soils. In current decades, bioleaching has been quickly developed by applying microorganisms for metal extraction procedures (Olson et al., 2003; Rawlings et al., 2003; Ndlovu, 2008). Many microorganisms, including fungi (Bai and Abraham, 2002), yeasts (Padmavathy et al., 2003), bacteria (Thacker and Madamwar, 2005), and algae (Dönmez et al., 1999), were recognized as better candidates because of their capacity to sequester cationic and anionic metallic species. An indirect process is involved normally in the leaching of metals with heterotrophic microorganisms. In this process, amino acids and further metabolites, mainly organic acids such as gluconic acid, pyruvic acid, citric acid, oxalic acid, malic acid, and succinic acid, are produced by microorganisms (Ren et al., 2009; Amiri et al., 2011). Many species such as *Aspergillus niger*, *Penicillium simplicissimum*, *Penicillium purpurogenum*, *Rhodotorula rubra*, and *Acidithiobacillus ferrooxidans* have been found useful for the removal of heavy metals in soil (Valix et al., 2001; Mulligan and Cloutier, 2003). Fungal cell wall possesses a huge amount of polysaccharides and proteins. These biopolymers present several functional groups that may bind metal ions, e.g. carboxyl, sulfate, phosphate, and amino groups (Vegliò and Beolchini, 1997).

The objectives of this study were as follows: (i) the isolation and identification of indigenous resistant strains from heavy metal contaminated soil; (ii) the comparison of one- and two-step leaching processes by using different media; (iii) the comparison of the bioleaching method with chemical leaching by the induction of organic acid production by *A. flavus* (F3) and chemical acids; (iv) through scanning electron microscopy (SEM), studies on the morphology of *A. flavus* (F3) biomass; (v) the identification of the functional group on the biomass of *A. flavus* (F3) through Fourier transform infrared spectroscopy (FTIR) before and after bioleaching; and (v) studies on the chemical forms of Pb and Cr before and after bioleaching.

Results and discussion

Soil characterization

The initial soil pH in contaminated soil was found to be 6.92, and organic matter content was found to be 5.48% with an electrical conductivity of 3.08 $ms.cm^{-1}$. The total Pb (II) concentration in soil was 26 385 mg kg^{-1}, and Cr (VI) was 3014.3 mg kg^{-1} when measured by atomic absorption spectrophotometer (AAS). The textural analysis of the soils shows sand>silt >clay in contaminated soil, explaining sandy loam in contaminated soil.

Microscopic identification of different fungal strains

The morphological characteristics of six fungal isolates were studied. Colonies of the strain F1, F2, F3, F4, F5, and F6 when grown on Czapek yeast extract agar (CYA) plates showed the characteristics identical with those of *Rhizomucor pusillus* (Kiran et al., 2014), *Aspergillus flavus* (George and Mallery, 2010), *Aspergillus terreus* (Lass-Flörl et al., 2006), *Aspergillus tubingensis* (Houbraken et al., 2011), and *Neosartorya hiratsukae* (Samson et al., 2014).

Molecular identification

To construct the phylogenetic tree Internal transcribed spacer (ITS) sequence of all the fungal isolates (F1, F2, F3, F4, F5 and F6) were determined and aligned. Phylogenetic analysis of the 18S rDNA sequences for similarities between the ITS of fungal isolates and those in the NCBI database showed that most of them are phylogenetically related to different fungal genera (Figure 1). The topology of phylograms confirmed that fungal isolates used in this study were assigned to *R. pusillus* F1 WF |KM977883.1|, *R. pusillus* F2 YBF |KM977884.1|, *A. flavus* F3 LG |KM977885.1|, *A. terreus* F4 YF |KM977886.1|, *A. tubingensis* F5 BF |KM977888.1| and *N. hiratsukae* F6 DG |KM977889.1|.

Medium optimization and selection

Bioleaching of heavy metals in the one-step process

The one-step process experiment was conducted by growing fungal strains (F1–F6) along with 2.5% (w/v) soil. In the one-step process, a similar medium, i.e. Sabouraud

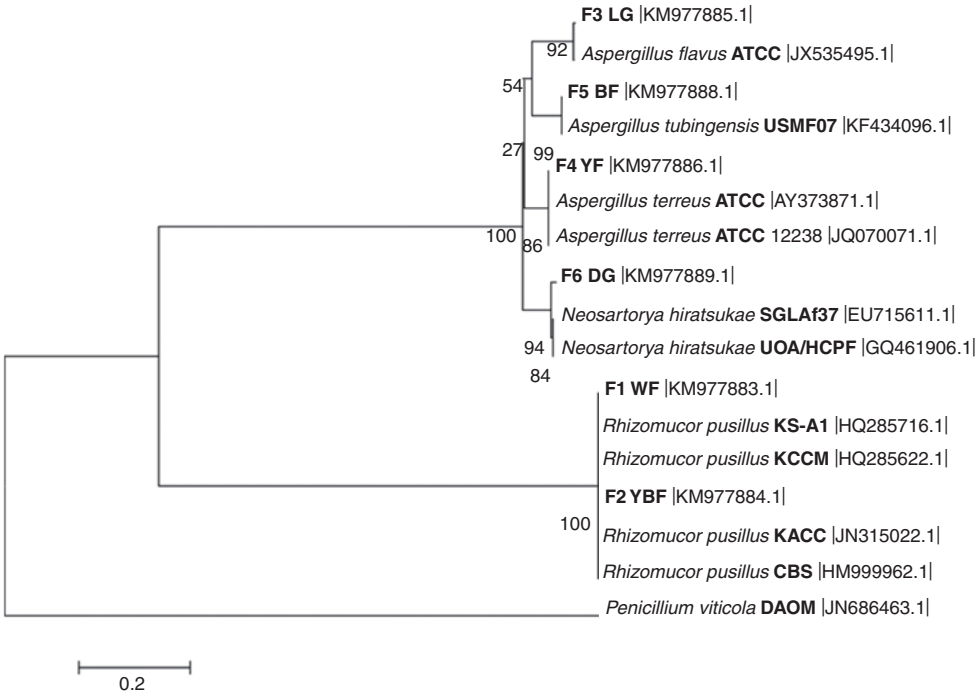

Figure 1: Phylogenetic tree of the fungal isolates with closest relatives based on a maximum parsimony analysis of ITS sequences. Length of ITS sequences for F1=578, F2=576, F3=548, F4=550, F5=543, and F6=548. *Penicillium viticola* DAOM was used as an out-group. Phylogenetic tree was constructed by using Mega 4.0 software.

dextrose broth (SDB), in F1 (*R. pusillus*) showed 6% removal for Pb (II) and 65% for Cr (VI) (Table 1), and F2 (*R. pusillus*) showed 4% for Pb (II) and 40% for Cr (VI). F3 (*A. flavus*) showed 36% removal for Pb (II) and 99% for Cr (VI), F4 (*A. terreus*) showed approximately 5% for Pb (II) and 82% for Cr (VI), F5 (*A. tubingensis*) showed 23.32% for Pb (II) and 78% removal for Cr (VI), and F6 (*N. hiratsukae*) showed 7% removal for Pb (II) and 31% removal for Cr (VI). The results (Table 1) clearly indicated that *A. flavus* examined in this study have the capacity to produce considerable concentrations of organic acids by removing approximately 99% in Cr (VI) and 36% in Pb (II). The removal percentage of Cr (VI) by using six different fungi are significantly different ($p<0.05$) by using different media in the one-step bioleaching method.

In comparison to the leaching step experiment, it was observed that the one-step leaching experiments have many advantages. The leaching efficiencies of 99% of Cr (VI) and 36% of Pb (II) were higher in the one-step process compared with the two-step process [39% Cr (VI) and 20.34% Pb (II)]. There may be two reasons for the result: first, in the fungal bioleaching, bioaccumulation occurs and improves metal leaching by changing the equilibrium metal concentration in the suspension (Bosshard et al., 1996). Both soluble and particulate types of heavy metals contain entire microbial cells alive or dead, and their

Table 1: Removal efficiency of heavy metals in the one-step process.

Fungi name	YPG		CM		SDB		SM	
	Pb (II) (%)	Cr (VI) (%)	Pb (II) (%)	Cr (VI) (%)	Pb (II) (%)	Cr (VI) (%)	Pb (II) (%)	Cr (VI) (%)
F1	4^{a}	17^{a}	0.70^{b}	21^{b}	6^{c}	65^{c}	2.52^{d}	1.90^{d}
F2	1^{a}	30^{a}	0.40^{a}	21^{b}	4^{b}	40^{c}	0.90^{a}	7^{d}
F3	3^{a}	40^{a}	2^{b}	20^{b}	36^{c}	99^{c}	3.10^{a}	11^{d}
F4	1^{a}	56^{a}	0.30^{a}	20^{b}	5^{b}	82^{c}	4.97^{c}	20^{d}
F5	2^{a}	51^{a}	1^{b}	16^{b}	23.32^{c}	78^{c}	11.00^{d}	16^{d}
F6	1^{a}	28^{a}	0.10^{b}	12^{b}	7^{c}	31^{c}	3.57^{d}	8^{d}

Given values are the average of triplicate samples. Significant differences are shown by different lowercase letters a–d in bioleaching with six different fungi in four different media, according to one-way ANOVA test at $p<0.05$.

products are highly efficient bioaccumulators (Silver and Phung, 1996). Capital and operating costs in the one-step process is low; in the two-step process, the production of metal leaching acids can be facilitated by producing organic acids in a separate step and by keeping away the difficulties related to (a) the treatment process in which the toxicity of soil is involved and (b) the field optimum fungal culture conditions that are difficult to maintain. However, capital costs would be increased because of the need for extra bioreactor tanks to produce the acids (Mulligan and Cloutier, 2003).

Bioleaching of heavy metals in the two-step process

After incubation for 15 days, among the four media used, SDB showed the removal of more heavy metals, i.e. approximately 7.6% for Pb (II) and 30% for Cr (VI) in F1 (*R. pusillus*), approximately 3.11% for Pb (II) and 23% for Cr (VI) in F2 (*R. pusillus*), 20.34% for Pb (II) and 39% for Cr (VI) in F3 (*A. flavus*), and 6.41% for Pb (II) and 22% for Cr (VI) in F4 (*A. terreus*). In the same way, F5 (*A. tubingensis*) and F6 (*N. hiratsukae*) showed 4.43% and 13.48% for Pb (II) and 36.98% and 18.58% for Cr (VI), respectively. Among all the six fungal strains, F3 (*A. flavus*) showed a maximum removal of 20.34% for Pb (II) and 39% for Cr (VI) (Table 2). The result could be because of the strong chemisorption of clays, soil humus, and oxides of lead and chromium (Ke et al., 2006). By contrast, soil mobility and metal retention are affected by the initial metal concentration, which is also an important factor (Reed et al., 1996). When the metal sorption capacity of most soils is ahead of the limit, contamination would be present as distinct metal-mineral phases as well (Davis and Singh, 1995). Such metal ions through the formation of insoluble precipitates can be immobilized in soil, incorporated into the crystalline structure of clays and metal oxides, and/or physically entrapped in the immobile water surrounding soil micro- and macropores (Pichtel and Pechtel, 1994). The heavy metal ions from contaminated soils adsorbed or entrapped are difficult to desorb even by using a strong chelating agent (ethylenediaminetetraacetic acid) for cases in which heavy metal ion complexes are somewhat constant (Davis and Singh, 1995).

Production of organic acids by *A. flavus* (F3) and gas chromatography (GC)

A. flavus (F3) showed the removal of more heavy metals due to the excreted metabolites (Figure 2). Different peaks were produced in different retention times on days 5, 10, and 15. Figure 2 shows the peaks for organic acids produced on day 15. At retention time, 'a' peaks for citric acids, 'b' peaks for malic acid, and 'c' peaks for succinic acid were produced in control and treatment. Peaks for oxalic acid were not so visible in both control and treatment. The results were consistent with those discussed by Molner-Perl and Morvai (1990). Many other microorganisms have been studied for the production of organic acids (Beauprez et al., 2010).

The amount of organic acid produced in milligrams per liter generated by *A. flavus* (F3) was analyzed in different periods of bioleaching (Table 3). Some other metabolites were also produced, but the major leaching agents were bioproduced organic acids. The amount of organic acids generated many factors such as medium buffering capacity, carbon source, and experimental states for fungi growth (Gadd and Sayer, 2000). The result explains that when the toxicity of heavy metals in soils is at the lower ratio of 2.5% (w/v), the basic metabolism of *A. flavus* (F3) is affected through bioleaching. The total content of organic acids formed in control was less than that formed in the bioleaching process (Table 3). During the bioleaching process, the contents of organic acids decreased in the order of citric acid≈succinic acid≈malic acid.

Furthermore, in the bioleaching process, the production of organic acids resulted in the pH decrease

Table 2: Removal efficiency of heavy metals in the two-step process.

Fungi name	YPG		CM		SDB		SM	
	Pb (II) (%)	Cr (VI) (%)	Pb (II) (%)	Cr (VI) (%)	Pb (II) (%)	Cr (VI) (%)	Pb (II) (%)	Cr (VI) (%)
F1	0.55	8	3.46	1.71	7.61	30.00	3.61	19.84
F2	2.43	17	2.02	0.49	3.11	23.00	1.01	10.15
F3	0.69	20	3.71	0.58	20.34	39.00	18.98	19.16
F4	1.09	18	1.83	1.46	6.41	22.00	2.59	13.04
F5	1.50	12	3.31	2.15	4.43	36.98	2.07	17.15
F6	1.17	8	0.25	4.51	13.48	18.58	1.62	8.58

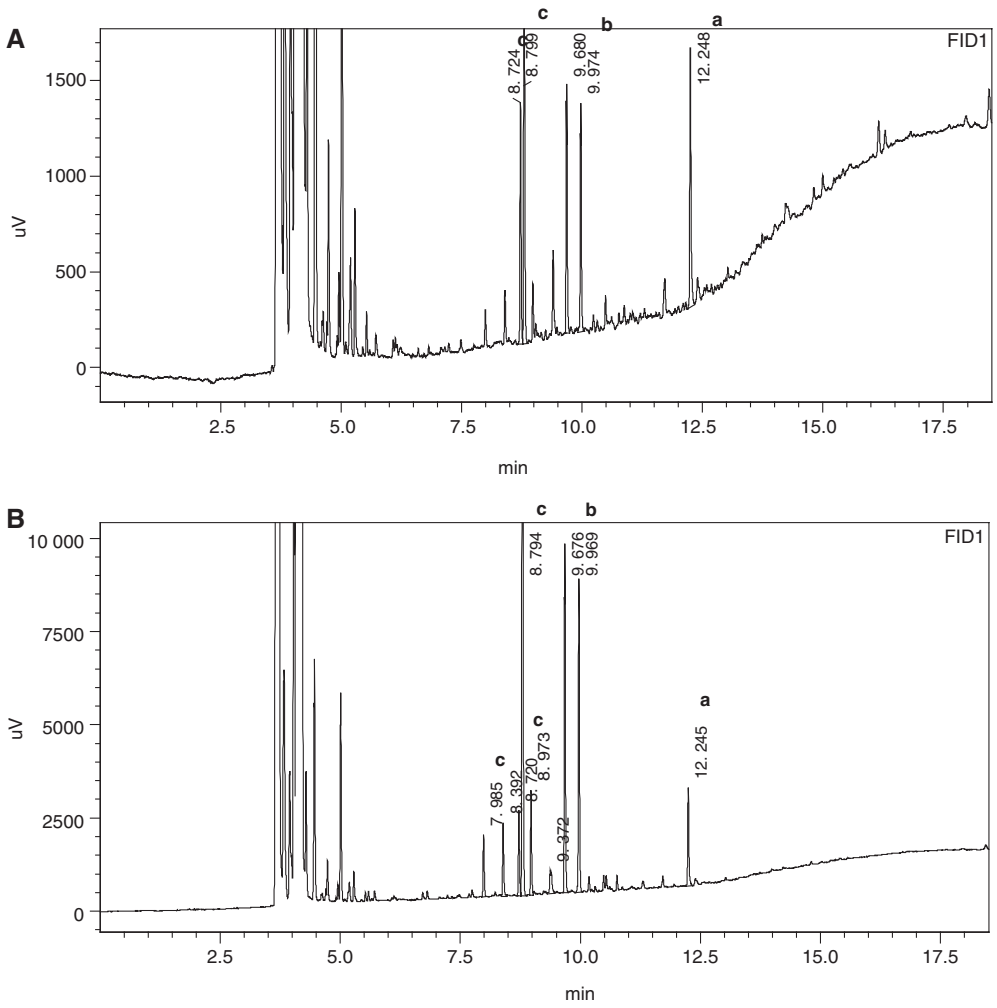

Figure 2: Chromatogram of organic acids produced by *A. flavus* (F3).
(A) Control (without heavy metals) and (B) test with heavy metals. Typical peaks for organic acids by GC/FID at different retention times: a – citric acid, b – malic acid, and c – succinic acid.

Table 3: Organic acids produced by *A. flavus* (F3) during different bioleaching processes.

Method name	Time	Malic acid (mg L^{-1})	Citric acid (mg L^{-1})	Succinic acid (mg L^{-1})
One-step bioleaching	5th day	174	35	144
	10th day	273	31	248
	15th day	332	35	213
Control	5th day	98	65	65
	10th day	192	85	114
	15th day	171	69	98

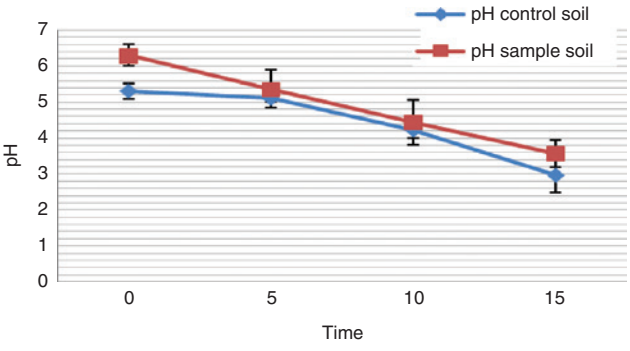

Figure 3: Changes of pH values in the one-step bioleaching process. pH levels are measured for 2 weeks for control and sample soils. Data are given as mean±SD, n=3.

(Ren et al., 2009). Figure 3 indicates that the filtrate pH values were reduced by 3–4 U throughout the growth of *A. flavus* (F3), which contain H^+ and organic acids (Burgstaller and Schinner, 1993; Castro et al., 2000; Brandl et al., 2001; Samson et al., 2014). The results clearly indicated that the *A. flavus* (F3) observed in this study have the capacity to produce major concentrations of organic acids. The final pH values were ±2.98 and 3.58 for

the control and treatment, respectively, which demonstrated that the growth of *A. flavus* (F3) is affected by the toxicity of contaminated soil (Paraszkiewicz et al., 2010).

Comparison of bioleaching with chemical leaching method

To compare the removal of heavy metals in bioleaching and chemical leaching, 0.6% citric acid, 0.25% oxalic acid, 0.05% malic acid, and 0.05% succinic acid were selected for chemical leaching because the pH levels of these acids ranged from 2 to 3.5 (Figure 3).

In bioleaching, the maximum removal of Cr and Pb were 99% and 36%, respectively, by *A. flavus* (F3) in SDB. However, during chemical leaching, malic acid had a removal efficiency of 21.30% for Pb (II) and 1.92% for Cr (VI); succinic and citric acids showed a removal efficiency of 29.30% and 22.21% for Pb (II) and 72% and 60.70% for Cr (VI), respectively; and oxalic acid showed 2.20% for Pb (II) and 2.47% for Cr (VI) (Figure 4). Results explained that malic acid, succinic acid, citric acid, and oxalic acid were mainly capable of removing heavy metals (Pb (II) and Cr (VI)) in chemical leaching, except that only 2.47% of Pb (II) and 2.20% of Cr (VI) were removed by oxalic acid and 1.92% of Pb (II) was removed by malic acid (Figure 4). The low removal efficiency of Pb (II) by oxalic acid could contribute to the precipitates of lead oxalate. The low removal efficiency of Cr can be by coordination reaction (Ding et al., 2010). Among the four organic acids, succinic acid filtered much Cr (VI) and Pb (II) (72% Cr (VI) and 29.30% Pb (II)) from metal-contaminated soil, followed by citric acid, malic acid, and oxalic acid during chemical leaching. These results were consistent with that reported by Bayat and Sari (2010). These results suggest that succinic acid should be the main and useful lixiviants of Cr (VI) and Pb (II) in the chemical leaching of the metal-contaminated soil. In comparison to chemical leaching, the metal removals during the one-step bioleaching method were 99% for Cr (VI) and 36% for Pb (II) using *A. flavus* (F3), whereas in the two-step process, metal removals reached up to 39% for Cr (VI) and 20.34% for Pb (II). In bioleaching, malic acid is produced more by *A. flavus* (F3), which helped to increase the efficiency of this fungus for removal. During bioleaching, in contrast to chemical leaching, the lower leaching removals of Pb (II) and Cr (VI) in the two-step process were probably because of the precipitation of lead and chromium in the presence of the sugars.

Generally, bioleaching by *A. flavus* (F3) using organic acid agents would be much efficient than chemical leaching because of elevated leaching removals and lesser cost.

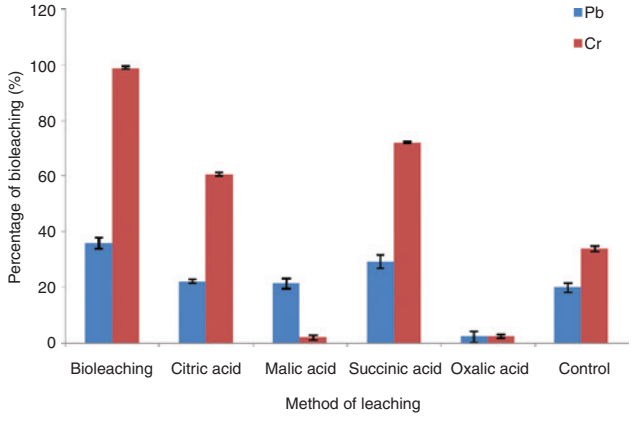

Figure 4: Comparing the removal percentage of heavy metals between *A. flavus* (F3) bioleaching and chemical leaching. Commercially obtained organic acids citric acid (6 g L^{-1}), malic acid (0.5 g L^{-1}), oxalic acid (2.5 g L^{-1}), and succinic acid (0.5 g L^{-1}) used for chemical leaching. Data are given as mean±SD, n=3.

Morphology of *A. flavus* (F3) before and after bioleaching

To observe bioleaching effects on surface of living cells, SEM with energy-dispersive X-ray analysis (EDX) were conducted. In SEM studies, before exposure to heavy metals, the hyphae of *A. flavus* (F3) were conidial, septate, and branched Figure 5A, whereas the hyphae of *A. flavus* (F3) showed typical change in morphology in Figure 5B in response to heavy metal stress by forming hyphal coils and its curling. Heavy metal toxicity to the fungi involves many factors, and other transport processes such as essential functional groups of enzymes are blocked. In the cells, the conformational changes of polymers occur, and essential metals are displaced (Ren et al., 2009). Same studies were conducted in response to other heavy metals exposure (Cánovas et al., 2004). These studies also examined that the presence of metal stresses had led to the formation of thiol compounds. The production of intracellular vacuoles is increased, which results in cell wall protrusions that perform the function of storage compartments for thiol-containing compounds, leading to the binding of metal ions into the intracellular regions and their storage in vacuoles. Cánovas et al. (2004) reported that because of the projections on the hyphae, the surface of *Aspergillus* sp. also had a rough surface on exposure to 50 mM of arsenate solution. Such alterations due to heavy metal stress on the surface of fungi show that the formation of intracellular compounds resulted in increased pressure inside mycelia, leading to the outward growth of the cell wall

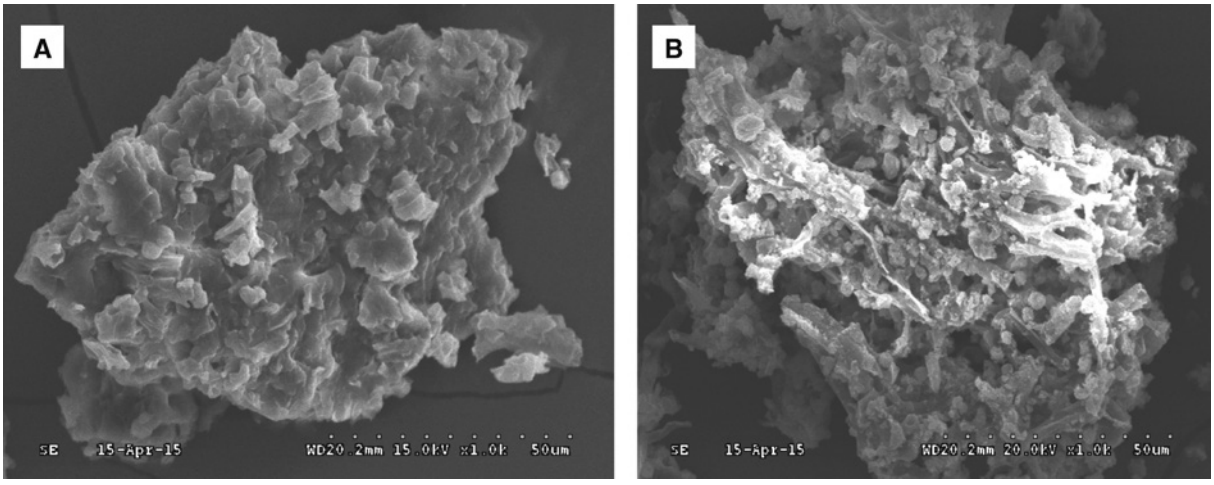

Figure 5: SEM of *A. flavus* (F3) biomass (A) before and (B) after bioleaching.
Two-week-old cultures were grown on SDB medium, and then the fungal biomass was dried in an oven at 60°C and treated with 10% glutaraldehyde and incubated for approximately 10–12 h at 4°C. The pretreated specimen was then sputtered with gold particles using a sputter coater under vacuum and observed under SEM at an accelerating voltage of 12 or 15 kV to capture images.

structures. To evaluate the metal ionic concentrations in the mycelia surface specifying mycofiltration, the energy-dispersive spectroscopy (EDS) analysis was performed. In Table 4, the results of EDS analysis for the control mycelia and mycelium treated with heavy metals are explained. No visible peaks for the metals Pb (II) and Cr (VI) are observed when EDS of the mycelia is exposed to these metals, indicating undetectable levels of metals on the surface of the mycelia by SEM-EDX. In the EDS spectra, the presence of traces or the absence of metal peaks shows that the metal removal by the mycelia of *A. flavus* (F3) instead of adopting adsorption on the surface may contain vigorous intracellular bioaccumulation mechanism.

Fourier transform infrared spectrum for functional group identification

To identify any functional groups involved, FTIR spectra of *A. flavus* (F3) before and after bioleaching experiment were examined. Figures 6A and B explain the comparison of metal-loaded biomass to control showing absorption bands of *A. flavus* (F3). During bioleaching, when metals binds, the protonated amido of protein and the *N*-acetyl glucosamine polymer of chitin are mostly involved. In Figure 6A, HCl (pH 1.0) was used to treat the control. In Table 5, the infrared absorption frequencies of peaks and the corresponding functional groups of the *A. flavus* (F3) are summarized. The -OH groups of glucose and the -NH groups of proteins were observed at approximately 3400 cm^{-1} possessing broad adsorption peak (Bai and Abraham, 2002; Zhou et al., 2005). The -CH stretching vibration of $C\text{-}CH_3$ was assigned in medium absorption band at 2923 cm^{-1}. These might be functional groups attributed to fatty acids found in membrane phospholipids (Yee et al., 2004). The amide I and amide II bands of amide bond, respectively, are because of the functional groups in *N*-acetyl glucosamine polymer or the protein peptide bond around the absorption peaks at 1645 and 1548 cm^{-1} (Bai and Abraham, 2002; Yee et al., 2004). The absorption peaks between 1460 and 1423 represent multiple weak aromatic bands, and 1033 cm^{-1} represents the glucose ring bands (Padmavathy et al., 2003). The moderately strong absorption bands at 1033 cm^{-1} might be assigned to the C-O stretching of sugar alcohol. The weak absorption band around 600–800 cm^{-1} is probably attributed to the C-Cl scissoring of alkyl halide. Thus, the *A. flavus* (F3) biomass contains hydroxyl, carboxyl, fatty acids, and amine

Table 4: EDX analysis of *A. flavus* (F3) biomass before and after bioleaching.

	Element	C	O	Mg	Al	Si	Ca
Before bioleaching	Weight (%)	57.43	42.54	–	–	–	–
After bioleaching	Weight (%)	35.67	50.76	1.56	1.87	3.27	3.78

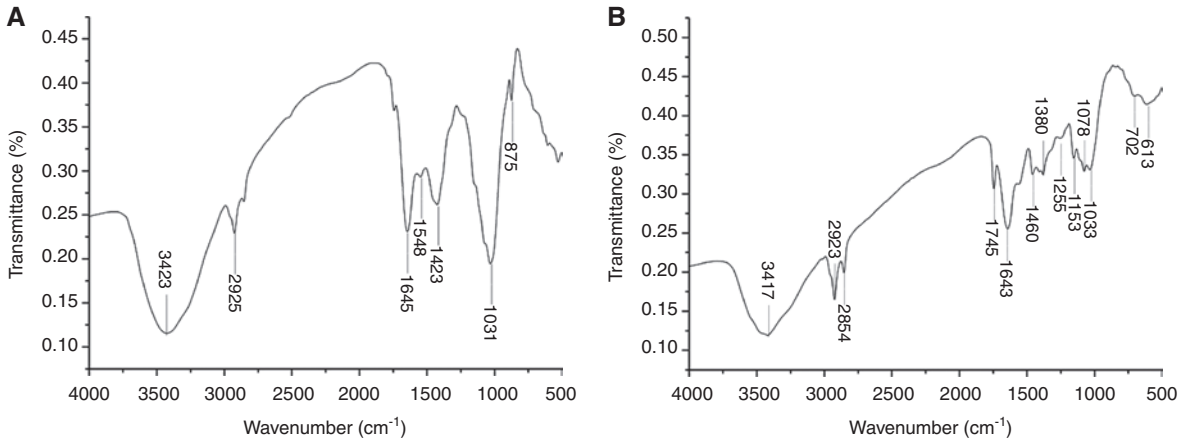

Figure 6: FTIR spectra of (A) control and (B) metal-loaded biomass.
Spectra of fungal biomass identifying different functional groups on the surface of fungus. The dried biomass was prepared with potassium bromide pellet technique and then analyzed with the range of scan wave from 400 to 4000 cm^{-1} with Thermo Nicolet 6700, FTIR spectrometer.

Table 5: Functional groups of the *A. flavus* (F3) biomass and corresponding FTIR adsorption frequencies.

FTIR peak	Frequency (cm^{-1})	Functional group	Assignment
1	3423–3417	Hydroxyl (–OH)	-NH stretching
2	2925–2923	-CH stretching	Vibration of C-CH3
3	1745–1645	C=O chelate stretching of O-C-NH_2	-NH wagging vibration of amide I
4	1460–1423	-CN stretching and -NH wagging vibration of O-C-NH_2	-NH wagging vibration of amide II
5	1078–1033	C-O stretching of alcohols	-CN stretching of -CH_2-NH_2
6	600–700	C-Cl	Scissoring vibration

groups on its surface. From the analysis of FTIR spectra presented in Figure 6, it can be seen that the mechanism for leaching for Cr (VI) and Pb (II) involves a chemical interaction between Cr and Pb ions and hydroxyl, amino acid, fatty acid, and carboxyl group from fungal biomass surface because the shifting of peak characteristics to these groups was registered. These results were very close to those reported for Pb(II), Cd(II), and Cu(II) onto the *Botrytis cinerea* fungal biomass (Anayurt et al., 2009).

Partitioning of heavy metals in different fractions before and after the one- and the two-step bioleaching processes

Figures 7 and 8 illustrate the speciation of heavy metals in soil before and after the one-step leaching processes. The Pb (II) in the soil sample was mainly in the forms of 27% exchangeable (step 1) and 39% residual fractions (step 5) before bioleaching. After the one-step leaching, the major content is intensively found in residual fractions (step 5), i.e. 53%, where the metal contents in other fractions are reduced. In particular, the exchangeable fraction (step 1)

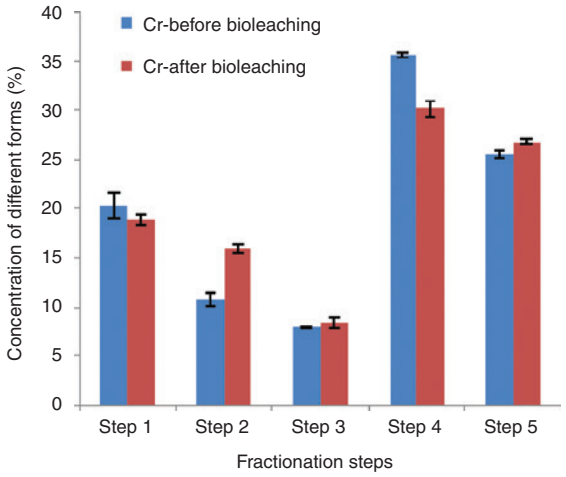

Figure 7: Variation in the partitioning of chemical forms for Pb (II) before and after the one-step bioleaching process.
Metals in five fractions (step 1=exchangeable, step 2=bound to carbonate fraction, step 3=bound to metal oxide, step 4=organically bound fraction, and step 5=residual fraction) were analyzed by AAS.

reduced remarkably at approximately 15.23%. However, Cr (VI) mainly exists in organic (step 4), residual (step 5), and exchangeable fractions (step 1) before leaching.

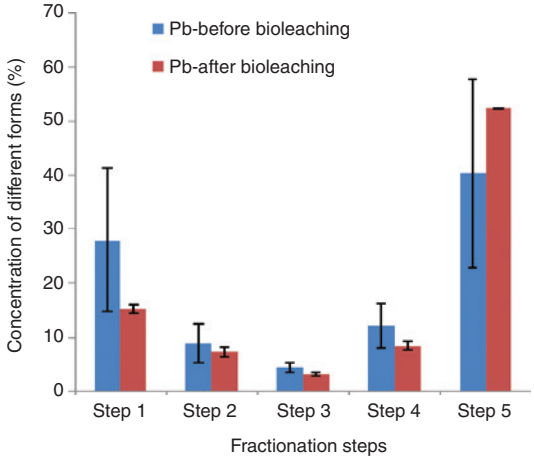

Figure 8: Variation in the partitioning of chemical forms for Cr (VI) before and after the one-step bioleaching process.
Metals in five fractions (step 1=exchangeable, step 2=bound to carbonate fraction, step 3=bound to metal oxide, step 4=organically bound fraction, and step 5=residual fraction) were analyzed by AAS.

Among them, organic fraction (step 4) takes the largest proportion at approximately 40.34%. After leaching, only exchangeable fractions (step 1) were reduced to 22.45%, whereas those bound to the Fe/Mn fraction (step 3) do not have an obvious change; the proportion of other fractions increased.

Bioleaching (one-step process) had a significant influence on the speciation of heavy metals. Pb (II) mostly bound to exchangeable and organic matter fractions after the one-step bioleaching, whereas Cr (VI) was found mostly bound to residual fractions. Therefore, Pb (II) and Cr (VI) left in soil were more constant and nonbioavailable. After the one-step bioleaching process, metals left in the soil were thus mostly bound in stable fractions.

Conclusions

Lixiviant inoculation with *A. flavus* (F3) producing organic acid has resulted in a high leaching efficiency. In the one-step bioleaching, the maximum removals were 99% in Cr (VI) and 36% in Pb (II). In contrast to chemical leaching, the bioleaching process illustrated enhanced removal rate. This confirmed that the possibility of using the bioleaching remediation method to remove heavy metals from soil increases. In SEM-EDX, trace amounts of metal ions are examined in the surface, indicating the primary adsorption step in accumulation. The FTIR analysis also clarified a possible bioaccumulation mechanism because specific chemical bond often has a unique energy absorption band. Sequential extraction results indicated the existence of Pb (II) in residual and exchangeable Cr (VI) after bioleaching by *A. flavus* F3. Bioremediation using indigenous fungal strains to remove heavy metals from soil can be regarded as an alternative to other known methods because of its short action period and better accumulation efficiency. This technology can be integrated with advanced agronomical and engineering skills to transform mycoremediation as a competitive remediation tool. Thus, the previously mentioned results specified that by using *A. flavus* (F3), the bioremediation method might be cost-effective for the leaching of heavy metals such as Pb (II) and Cr (VI) from industrial soil and toward the biological detoxification of soils, making it a promising technology.

Experimental

Soil sample collection

The contaminated soil used in this study was collected from the surface layer (0–20 cm) of Weifang ZTE Battery Co., Ltd., located in Weifang City, Shandong Province, China. It is a smelting industry that mainly produces and sells lead-acid batteries and accessories. In the process of lead-acid battery production and assembly, the transport of raw materials, the leaking of the production process, and stacking behavior may cause the site soil to be contaminated with lead, mainly from lead-contaminated wastewater, lead dust, and lead scrap ointment.

The contaminated soil sample was air-dried in laboratory conditions for 2 weeks, ground, sieved through a 2-mm polyethylene sieve, and dried to constant mass in an oven at 75°C and kept in desiccators for further analysis.

Preliminary examination of soil sample

Some properties such as pH, electrical conductivity, and total metal concentration were initially analyzed. Soil pH was measured potentiometrically in 1 M KCl with a soil/extractant ratio of 1:5 in three replicates per sample. For the determination of the total metal contents of the soil, the digestions were conducted with a mixture of 7 mol L^{-1} of nitric acid (HNO_3) concentrate, 2 cm^3 of hydrofluoric acid concentrate, and 1 cm^3 of 40% hydrogen peroxide (H_2O_2) solution on a sand bath at a temperature of 200–230°C, and then the AAS analyses for the determination of the total metal contents of the soil samples were conducted.

Fungal strains and growth medium

The media used for the growth and seed culture for all the fungal isolates used in the bioleaching process were as follows: SDB; yeast peptone glucose (YPG) containing 1% yeast extract, 2% peptone, and 2% glucose; chashi media (CM) containing 30 g sucrose, 1 g K_2HPO4, 3 g $NaNO_3$, 0.5 g $MgSO_4$, 0.5 g KCl, 0.01 g $FeSO_4$, and 1 L H_2O; and

sucrose media (SM) containing 100 g L^{-1} sucrose, 1.5 g L^{-1} $NaNO_3$, 0.5 g L^{-1} KH_2PO_4, 0.025 g L^{-1} $MgSO_4 \cdot 7H_2O$, 0.025 g L^{-1} KCl, and 1.6 g L^{-1} yeast extract. Cultivation time and temperature were 15 days and 30°C, respectively, at 130 rpm.

Isolation of resistant fungi: The isolation of indigenous fungal strains from soil samples by serial dilution method using Sabouraud dextrose agar (SDA) was conducted. Twenty-milliliter SDA plates were prepared; each sample up to 10^6 was used to make a serial dilution, and 1 mL of each 10^4 and 10^6 dilutions was plated with 0.5 g L^{-1} chloramphenicol in sterilized Petri plates in duplicate using spread plate technique and incubated at 28°C for 96 h. The colonies of the predominant genera of fungi were picked up and purified by the streak plate method. Individual colonies from the mixed or mother culture were subcultured on fresh SDA plates for obtaining pure culture of each isolates.

Morphological analysis: All the fungal strains were inoculated in CYA for morphological analysis and colony characterization. The plates were incubated in the dark at 30°C for 7 days.

Molecular identification: Isolation and purification of the total genomic DNA of the fungal isolates were conducted by using the method as described by (Rohlf, 1990). From fungal strains, the amplification and the sequencing of internal transcribed spacer of ITS were performed using the primers IT5: 5′-TCCGTAGGTGAACCTGCGG-3′ and 5′-TCCTCCGCTTATTGATATGC-3′ (Pedersen et al., 1997). The sequence obtained was aligned by using BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST) for comparison with currently available sequences. The sequences that showed 98% or more similarities with currently accessible sequences were regarded to be the same species. Furthermore, multiple alignments were conducted using Clustal X 1.83 and MEGA 4.0 for the construction of phylogenetic tree (Tamura et al., 2007).

Biological leaching experiments

One-step bioleaching: Each strain was separately cultivated in a seed culture medium in a 250-mL flask at 30°C and 130 rpm for 48 h. One milliliter of spore suspension (approximately 108 mL^{-1}) of each strain was inoculated in separate flasks containing presterilized 49 mL of YPG, CM, SDB, and SM medium along with 2.5 g of contaminated soil, and one control without soil was also run for each medium. The flasks were placed in a shaking incubator (IS-RDS3C, USA) at 30°C for 15 days at 130 rpm.

Two- step bioleaching: In the two-step process, spore suspension approximately 1 mL of each strain was first inoculated in 49 mL of four different media without soil (the first step). After 5 days of incubation, 2.5 g of soil was added to the conical flask (the second step). The bioleaching experiment was performed by plummeting the mixture in a shaking incubator at 130 rpm and 30°C for 15 days. All the experiments were run in triplicate.

GC-FID analysis of fungal metabolites

In different stages of bioleaching, the metabolites of *A. flavus* (F3), such as succinic acid, malic acid, oxalic acid, and citric acid, were determined by GC analysis (Katona et al., 1999). The 2-mL sample from the one-step bioleaching was placed on a 100-mL flask, and 40 mL of 5% sulfuric acid methanol mixture solution was added. The flask was connected to the condenser tube, refluxed for 2 h in a water bath at 60°C, and cooled to room temperature. Then 10 mL backflow liquid was taken into 100 mL separatory funnel, and 20 mL distilled water was added and mixed evenly with 10 mL dichloromethane extraction, and then 3.5 g anhydrous sodium sulfate was added and dried overnight. The supernatant extract was analyzed by GC.

The GC (2010PLUS, USA) conditions were as follows: specifications of chromatographic column used were RTX-5.60 m×0.25 µm×0.25 µm thickness. The flame ionization detector (FID) was set at 300°C, and the injector port was set at 280°C; 1 µL was injected using a split ratio of 10:1, temperature was 50°C for 2 min, and then a 20°C min^{-1} rate, up to 280°C, was kept for 10 min.

Comparison between bioleaching and chemical leaching experiments

After the selection of the best medium, bioleaching steps, and metabolites of *A. flavus* (F3) identification, the one-step bioleaching experiment was performed in 250 mL autoclaved conical flask by adding 1 mL of spore suspension in 49 mL of SDB with 2.5 g autoclaved soil and incubated for 15 days at 30°C and 130 rpm.

To assess the leaching effectiveness, chemical leaching was performed with different commercially available chemicals, i.e. citric acid (6 g L^{-1}), malic acid (0.5 g L^{-1}), oxalic acid (2.5 g L^{-1}), and succinic acid (0.5 g L^{-1}) of 2.5% (w/v) metal-contaminated soil and shaken at 130 rpm on 30°C for 24 h. Liquid samples were obtained at the end of the experiment and filtered, and heavy metal concentration was analyzed using the atomic absorption spectrophotometer M6 AAS, USA.

SEM with EDX

The morphology and elemental composition of the mycelium was observed by an SEM with EDX after bioleaching. The fungal biomass was dried in an oven at 60°C and then treated with 10% glutaraldehyde and incubated for approximately 10–12 h at 4°C. Further, to remove water content, the biomass was treated with alcohol gradations of 10%, 30%, 50%, 80%, and 100% for 2 min (Srivastava and Thakur, 2006). The pretreated specimens were then sputtered with gold particles using a sputter coater under vacuum and observed under SEM (Hitachi S-4800, USA) at an accelerating voltage of 12 or 15 kV to capture images. The EDX of these images was performed at 10 kV.

FTIR for functional group analysis

To recognize functional groups and bonds present in fungi, which were responsible for metal accumulation in cytosol, FTIR was performed. The best among six strains was *A. flavus* (F3), so the fungal mycelia of *A. flavus* (F3) were isolated, washed with distilled water, and oven-dried at 80°C for 4 h to achieve constant weight. The dried biomass was then prepared with potassium bromide pellet technique and then analyzed with the range of scan wave from 400 to 4000 cm^{-1} with Thermo Nicolet 6700, FTIR spectrometer, USA.

Sequential extraction of heavy metals in soil before and after bioleaching experiments

Dried soil samples acquired from the one-step bioleaching experiments along with control soil were fractioned by sequential extraction procedure adopted from Ren et al. (2009). The quantity of metals in all five fractions was analyzed by using AAS (M6 AAS), and the proportions of these fractions with the total of samples were calculated.

Statistical analysis

To compare the difference of methods, a one-way ANOVA at $p<0.05$ level of significance was applied by using a least significant difference test in SPSS 16 software (SPSS, Inc.). Each method was performed in triplicate.

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