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# Pb Tolerance and Bioaccumulation by the Mycelia of *Flammulina velutipes* in Artificial Enrichment Medium

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**Mushrooms have the ability to accumulate high concentrations of heavy metals, which gives them potential for use as bioremediators of environmental contamination. The $Pb^{2+}$ tolerance and accumulation ability of living mycelia of *Flammulina velutipes* were studied in this work. Mycelial growth was inhibited when exposed to 1 mM $Pb^{2+}$. The colony diameter on solid medium decreased almost 10% compared with the control. Growth decreased almost 50% when the $Pb^{2+}$ concentration increased to 4 mM in the medium, with the colony diameter decreasing from 80 mm to 43.4 mm, and dry biomass production in liquid cultures decreasing from 9.23±0.55 to 4.27±0.28 g/L. Lead ions were efficiently accumulated in the mycelia. The amount of $Pb^{2+}$ in the mycelia increased with increasing $Pb^{2+}$ concentration in the medium, with the maximum concentration up to 707±91.4 mg/kg dry weight. We also show evidence that a large amount of the $Pb^{2+}$ was adsorbed to the mycelial surface, which may indicate that an exclusion mechanism is involved in Pb tolerance. These results demonstrate that *F. velutipes* could be useful as a remediator of heavy metal contamination because of the characteristics of high tolerance to $Pb^{2+}$ and efficient accumulation of $Pb^{2+}$ ions by the mycelia.**

***Keywords***: bioaccumulation, heavy metal, Pb, mushroom, *Flammulina velutipes*

## Introduction

Heavy metal contamination has become a worldwide problem. Metal ions can be released into ecosystems through industrial effluents and other wastes, agricultural fungicides, mining, and other human activities. Heavy metal ions do not degrade and thus persist for a long time in nature. As a consequence, they can be accumulated through the food chain, creating a threat to public health (Ayres, 1992).

Several metals, such as Ca, Cu, Fe, Mg, Ni, and Zn, are essential to living organisms; however, they become toxic when present at concentrations higher than those required for fulfilling their physiological functions. In contrast, metals such as Cd and Pb, do not have any known cellular function even at low concentrations. Pb can provoke a variety of adverse responses in humans (Woolf, 2007), involving the central and peripheral nervous systems, haemopoietic system, cardiovascular system, kidneys, liver, and reproductive systems.

Compared with green plants, mushrooms are capable of accumulating higher concentrations of heavy metals (Kalac and Svoboda, 2000). Many studies have revealed this high bioaccumulation ability by measuring the concentrations of heavy metals in the fruiting bodies of mushrooms collected from various places (Demirbaş, 2002; Doğan *et al*., 2006; García *et al*., 2009; Wang and Hou, 2011; Nnorom *et al*., 2012). This capacity was also studied to evaluate their potential as bioindicators of environmental contamination, or with the aim of potential bioremediation (Sugiyama *et al*., 2008; Dai *et al*., 2012; Flouty and Estephane, 2012; Zhang *et al*., 2012). With the increasing amount of published research, there have been more and more studies on bioaccumulation mechanism, which is leading to a better understanding of metal-microbe interactions and will facilitate the application of this technology (Gadd, 2008; Olguín and Sánchez-Galván, 2012). What's more, heavy metal removal using living cells is recognized to have significant advantages because the cells are metabolically active, using nutrients to grow and increase (Malik, 2004; Chojnacka, 2010). The process occurs in two stages: initially heavy metals accumulate at the cell surface by physical or chemical interactions, and then a fraction of the metals is transported into the interior of the cell. Thus the metal removal mechanism has two components: bioadsorption of metals onto the cell surface and bioaccumulation of metals inside the cell (Olguím *et al*., 2005; Olguín and Sánchez-Galván, 2012).

Living organisms used for heavy metal removal should be selected from the species that not only are resistant to high doses of these pollutants, but also can bioaccumulate metals inside the cells using detoxification mechanisms that respond to toxic heavy metals. Here we report that the mushroom, *Flammulina velutipes*, one of the major cultivated mushrooms in Asia, is highly resistant to lead, being able to grow in artificial medium with a $Pb(NO_3)_2$ concentration of 4 mM. Bioadsorption and bioaccumulation data are reported in this paper. These results will provide information needed to discuss the Pb tolerance mechanisms and support the potential utility of *Flammulina velutipes* in remediation of environmental contamination.

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## Materials and Methods

### Mushroom strain and media

*Flammulina velutipes* was used in this study. Mushroom mycelia were cultivated on a dish containing 200 g/L potato extract, 20 g/L dextrose, 3 g/L $KH_2PO_4$, 1.5 g/L $MgSO_4$, and 20 g/L agar (PDA), at 25°C. After 7 days of growth, the dish was stored at 4°C, and the stock was transfered to a fresh PDA dish every three months.

### Lead tolerance tests

The strain was first cultivated in a PDA dish at 25°C for 5 days. Agar plugs (about 10 mm diameter) were removed from the edge of the colony and placed on fresh medium with the addition of $Pb(NO_3)_2$ solutions to different final concentrations, and incubated at 25°C. The radial growth of the strain was measured every day from day 2 to day 9 to evaluate the lead tolerance of the strain. The diameters were measured in three directions that passed through the center of the inoculation plug, and the results given are the average values. The fungal strain grown in parallel on a PDA dish without lead addition was set as the control.

### Mycelial growth in liquid medium

Liquid PDB medium (same as PDA but no agar) was used in shaken cultures. The pre-cultures were prepared by homogenization of a 10 mm-diameter, agar plug containing mycelia of *Flammulina velutipes* in 100 ml PDB medium in a 250 ml flask, agitated at 160 rpm, 25°C, for 7 days. The pre-cultures were homogenized again, then 5 ml of homogenized pre-cultures were transferred to the fresh liquid media containing different concentrations of $Pb(NO_3)_2$, and incubated at 25°C and 160 rpm.

Dried mycelial masses were determined daily, from the third day of growth to day 12 to examine mycelial growth. The mycelia were separated from liquid medium by centrifugation at 3,000 rpm for 5 min and washed three times with $ddH_2O$ after each centrifugation. Then the mycelia were dried at 70°C until constant weight (n=5). Samples were prepared in triplicate, and error is expressed as the standard deviation.

### Measurement of Pb content

Flame atomic absorption spectrometry (FAAS) was employed to determine the quantity of lead. *Flammulina velutipes* mycelia were cultured as described above. The amount of Pb adsorbed onto the cell surface and accumulated inside the cell was determined by the method described by Olguím *et al.* (2005). The mycelia were collected by centrifugation at 3,000 rpm for 5 min, washed with $ddH_2O$ to remove the residues of the medium, and then washed with 150 ml 0.1 M EDTA for 60 min shaking at 100 rpm. After that, the mycelia were again rinsed with $ddH_2O$. Pb adsorbed onto the cell surface was quantified in both solutions. Mycelia were lyophilized and resuspended in 2 ml nitric acid and digested at 85°C until the solution became clear, and then used to quantify the concentration of Pb that had been inside the cell. The quantity of lead was determined by a SOLAAR S4 AAS device (Thermo, Ltd, USA). The assay was performed in triplicate, and error is expressed as the standard deviation.

## Results

### Effect of Pb on mycelial growth and colony morphology

Pb is a toxic heavy metal that has no obvious biological functions. However, mushrooms have the ability to tolerate and accumulate heavy metals and a substantial amount of data on the metal content in mushroom fruiting bodies have been published. These published results show that developing mushroom fruiting bodies can accumulate various metal ions, with the ion contents varying in different mushroom species, and in heavily polluted areas, the metal concentrations are considerably elevated. Few data on the maximum metal ion concentrations that mushrooms can tolerate have been reported. Thus we first tested the Pb tolerance of *F. velutipes* living mycelia. The variation of the colony diameters are shown in Fig. 1. When the strain grew in PDA without lead, the colony diameter reached almost 80 mm on the 7th day, the average growth rate was 11.3 mm/day. The mycelial growth was slightly inhibited when 1 mM $Pb^{2+}$ was added to the medium. On day 7, the colony diameter was about 71.2 mm. The average growth rate was 10.1 mm/day. The mycelial growth decreased when the $Pb^{2+}$ concentration in the medium increased, and 4 mM $Pb^{2+}$ inhibited mycelia growth by half as compared with the control (Fig. 1A and 1B) The colony diameter was only 43.4 mm on the 7th day. The $ED_{50}$ value (the effective dose inhibiting growth by 50%) can be used to report the tolerence level. The higher the $ED_{50}$, the greater the Pb tolerance. The growth was strongly inhibited when the $Pb^{2+}$ concentration in the medium reached to 10 mM, yielding only 15 mm of colony growth by 7 days. When

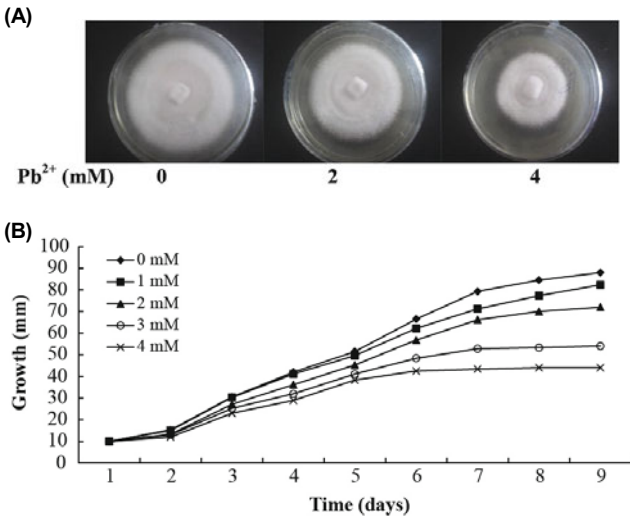


**Fig. 1. Colony morphology and mycelial growth of *F. velutipes* on PDA agar medium.** (A) Colony morphology of *F. velutipes* grown on PDA agar medium with different concentrations of lead ion. Fresh mycelia that grew on PDA or PDA supplemented with lead were photographed after 7 days of incubation. The plates containing 0, 2, 4 mM Pb are shown. (B) Mycelial growth of *F. velutipes* exposed to different concentration of Pb. Data are the average value of the radial growth on the agar plates.

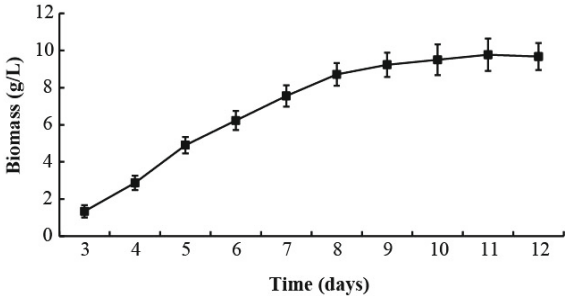


**Fig. 2. Mycelial growth of *F. velutipes* in liquid PDB medium.** Values represent mean±SD of dried mycelial biomass.

the $Pb^{2+}$ concentration was between 0 and 1 mM in the medium, mycelial growth did not differ from that of mycelia grown in normal PDA medium (data not shown).

### Biomass and Pb bioaccumulation by mycelia in liquid medium

The *F. velutipes* mycelial growth curve in control liquid medium is shown in Fig. 2. The growth of *F. velutipes* followed a typical growth curve, reaching a plateau at the eighth day of cultivation; the highest biomass was obtained by day 11; however, the biomass did not differ significantly from day 9 to 11, thus the ninth cultivation day was chosen to carry out the analysis of mycelial growth and Pb bioaccumulation in liquid, lead-containing media.

The dry weights of mycelia grown in the liquid media with different concentrations of Pb were measured after 9 days of culture (Fig. 3). Similar to the results of the previous tolerance test, with the increase of lead concentration in the medium, the biomass production decreased. The control dried mycelial weight was 9.23±0.55 g/L in the nine-day cultures; while the dried mycelial weights were 8.54±0.70, 6.01±0.93, and 5.04±0.29 g/L when grown in 1, 2, and 3 mM Pb-containing media respectively. When exposed to 4 mM Pb ($ED_{50}$), the dried mycelial weight was reduced to 4.27±0.28 g/L, less than half that of the control.

The quantities of lead absorbed into the interior of the

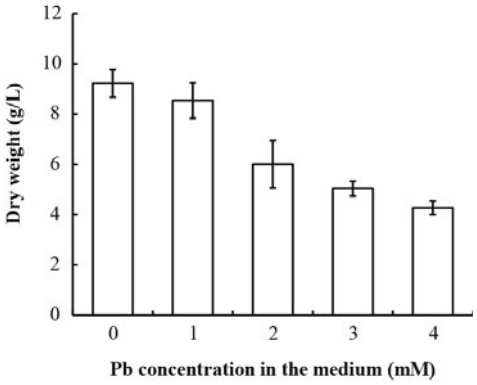


**Fig. 3. Effect of Pb on the growth of *F. velutipes*.** Mycelia cultivated in PDB media containing different concentrations of Pb. Values represent mean±SD of dried mycelial biomass.

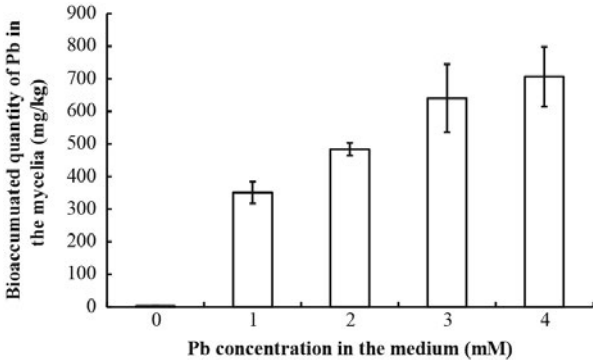


**Fig. 4. The amount of lead accumulated inside *F. velutipes* mycelia.** The mycelia were collected by centrifugation, washed with 0.1 M EDTA three times and with $ddH_2O$ three times. The quantity of lead was determined by flame atomic absorption spectrometry (FAAS). Data are the average values, and the error bar indicates standard deviation.

cells were determined and results are shown in Fig. 4. Lead ions were efficiently bioaccumulated by the *F. velutipes* living mycelia; the quantity of $Pb^{2+}$ in the mycelia increased with the increased concentration of $Pb^{2+}$ in the medium. In the presence of 1 mM and 4 mM lead ions, the $Pb^{2+}$ concentrations in the mycelia were 351±33.3 mg/kg and 707±91.4 mg/kg, respectively, increases of 78 times and 157 times respectively as compared with the control.

### Lead bioadsorption by the cellular surface of *Flammulina velutipes*

*Flammulina velutipes* showed high tolerance to lead; had an $ED_{50}$ for lead of 4 mM. But the FAAS results of the lead concentrations in mycelia were not much higher than for other strains we tested. For example, another popular mushroom, *Pleurotus eryngii* had an $ED_{50}$ for lead of 300 μM; the quantity of lead in the mycelia was 596±38.4 mg/kg when exposed to 300 μM $Pb^{2+}$ (unpublished data). One hypothesis for the high tolerance of *F. velutipes* is that it blocks lead ions from entering the cell. We then examined the quantity of lead adsorbed to the cellular surface. EDTA is the most common chelating agent used for desorbing metals, and the

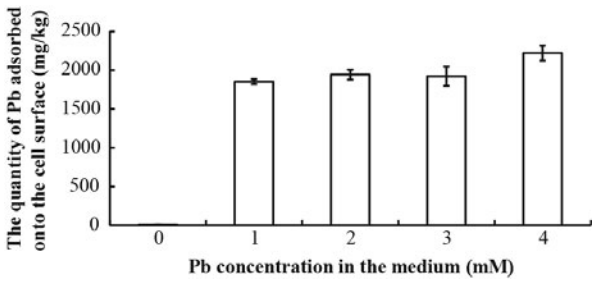


**Fig. 5. The amount of lead adsorbed by the mycelial surface of *F. velutipes*.** The mycelia were collected and washed with $ddH_2O$ to remove the residues of the medium, and then washed with 150 ml 0.1 M EDTA for 60 min, with shaking at 100 rpm. After that, the mycelia were rinsed with $ddH_2O$. Pb adsorbed onto the cell surface was measured in both solutions. Data are the average values, and the error bar indicates standard deviation.

amount of Pb adsorbed onto the cell surface can be quantified directly in the EDTA solution after washing (Olguín *et at*, 2005; Sánchez-Galván *et al*., 2008 and see 'Materials and Methods'). As we expected, a large amount of lead was adsorbed onto the cellular surface, the amount was much greater than that inside the mycelia (Fig. 5). The quantity of lead adsorbed by the mycelial surface of *F. velutipes* was between 1,850±33.3 mg/kg and 2,220±97.5 mg/kg when exposed to the medium with lead concentrations ranging from 1 mM to 4 mM, with the average bioadsorption amount being 1980 mg/kg. The amount of lead adsorbed by the cellular surface was 3–5 times that of lead taken into the cell.

## Discussion

It is well accepted that biosorption and bioaccumulation can have application for remediation of heavy metal contamination. Generally, biosorption is described as a passive process and dead biomass is often used for remediation; the metals only bind to the cellular surface. In contrast, bioaccumulation is an active process that needs living cells. Metals are not only bound to the cellular surface, but also are transported into the cell (Chojnacka, 2010). Nutrients are required to sustain cell growth. Organisms that are designed to be used for metal bioaccumulation should be highly resistant to these pollutants (Deng and Wilson, 2001). From this perspective, *F. velutipes* has potential to serve as a $Pb^{2+}$ bioaccumulator. Under the study conditions, *F. velutipes* exhibited high tolerance to $Pb^{2+}$ with an $ED_{50}$ up to 4 mM. Even exposed to higher $Pb^{2+}$ concentration, such as 6 mM or 8 mM, the mycelia still exhibited some slow growth (unpublished data). The mycelia grew normally in media containing $Pb^{2+}$ concentrations up to 1 mM.

Biosorption is a simple physicochemical process similar to conventional adsorption. The advantages of this process are simple operation and quick reaction. Limitations include the short life of the sorbents and the fact that it is a metabolically-passive process. Once the biosorption equilibrium is reached, the sorbents must be discarded or regenerated. Bioaccumulation goes further. Metals not only interact with the cell surface, but a fraction is also transported into the cell. With cell growth and multiplication, more metals are transported; and eventually, with sufficient biomass increase, the residual metal concentration becomes lowered. Our results suggest the potential utility of *F. velutipes* for bioremediation in view of its large accumulation capacity. Together with its high metal tolerance, we think *F. velutipes* has a huge potential for application in metal pollution remediation.

In the bioaccumulation process, the toxic effect caused by excess metals must be considered, and a good understanding of metal-microbe interactions, and the mechanisms that microorganisms use to cope with the toxic metals, will be very helpful for developing applications. Different mechanisms have been developed to reduce the toxic effect of metals in fungi (Bellion *et al*., 2006; Wysocki and Tamás, 2010). These mechanisms include reducing the uptake of metals into the cytosol by extracellular chelation through extruded ligands, and binding onto cell-wall components using intracellular ligands such as glutathione and metallothioneins for chelating metals in the cytosol; increased efflux from the cytosol out of the cell or into sequestering compartments and expression of antioxidant enzymes. Compared with the other strains we tested (see 'Materials and Methods' and data not shown), it is tempting to hypothesize that an exclusion mechanism may be involved in the case of *F. velutipes*. The cell wall and membrane play an important role in protecting cells against the metal toxicity. The amount of lead ions binding with the cellular surface is 3–5 times that found in the interior of the cell, which may be what gives *F. velutipes* the highest tolerance among the mushroom strains we tested. Some evidence shows that there is no direct relationship between tolerance and metal uptake (Pan *et al*., 2009; Chojnacka, 2010). That might be explained as different mechanisms between tolerance and bioaccumulation. Bioaccumulating organisms should be selected from species that are resistant to high doses of metals and do not have the mechanisms to protect themselves from excessive transport into the cell. Rather, they should have very effective detoxification mechanisms (Kocberber and Donmez, 2007; Chojnacka, 2010). It is reasonable to speculate that the intracellular ligands such as glutathione and antioxidant enzyme systems are expressed properly since the lead concentration in mycelia is up to 707±91.4 mg/kg. The toxicity of excess metals in the cell is usually due to the generation of oxygen radicals, while the antioxidant enzymes function to clear away ROS, provide protection or repair the oxidative damage. Tolerance and bioaccumulation ability of mushrooms is associated with the ability to clear away ROS. The actual mechanism in *F. velutipes* needs to be further investigated.

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