

Full Length Research Paper

Isolation, identification and cadmium adsorption of a high cadmium-resistant *Paecilomyces lilacinus*

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Microorganisms play important roles in bioremediation. The present study was carried out to investigate metal resistance and adsorption strains. A cadmium-resistant fungus M1 was isolated from Zhuzhou smelter in China. The 18S rRNA, internal transcribed spacers (ITS) region and β -tubulin gene of the strain were sequenced and aligned with the high similar sequences published in GenBank. The morphology was analyzed under scanning electron microscopy (SEM). The growth in the 100 mg/l cadmium medium was investigated and the biomass was analyzed by energy dispersive X-ray (EDX) spectroscopy. The results showed that strain M1 which tolerated 80 mM cadmium belong to *Paecilomyces lilacinus*. Except for cadmium, the fungus showed resistance to zinc, manganese, copper, lead, and cobalt. The cadmium biosorption capacity of the fungus reached 24.23 mg/g during growth. EDX confirmed that cadmium biosorption occurred on the fungus biomass. The work suggested that *P. lilacinus* M1 was a potential strain on cadmium bioremediation.

Key words: *Paecilomyces lilacinus*, cadmium-resistant, biosorption.

INTRODUCTION

Heavy metals pollution is increasing and poses a serious threat to environment and organisms. As a heavy metal, cadmium widely exists in industry and agriculture fields. Cadmium contamination mainly comes from mining and metallurgical processes, electroplating, stabilizing plastics, batteries, alloy manufacturing, pigment and high-phosphate fertilizers (Udo et al., 2001). Cadmium is a non-essential element and highly toxic to organisms even at very low dosages. Cadmium damages cells by strong affinity to glutathione and sulfhydryl groups in proteins and displacement of zinc and iron ions from proteins (Banjerdkij et al., 2005). Furthermore, researches indicated that cadmium

is related to reactive oxygen species (ROS) and cancer. Manca et al. (1994) reported that cadmium might interact with lipids and cause lipid peroxidation with consequential production of ROS. Hussain et al. (1987) found that cadmium reacts with intrinsic thiol-containing molecules and causes an increase in ROS by reducing the cellular antioxidative capacity. Cadmium may cause mutations even at low levels by inducing oxidative DNA damage and it decreases genetic stability by inhibiting the repair of endogenous and exogenous DNA lesions, resulting in increasing the probability of mutations and consequently cancer initiation (Filipic et al., 2006).

Cadmium pollution has attracted more attention and many scholars have done lots of work in this area. Among the measures of treatment of heavy metals pollution, bioremediation is a potential way as it has the advantages of low energy consumption, environmentally-friendly and low cost. The organisms used in environmental bioremediation mainly include microbe, algae and plant (Dostalek et al., 2004; Cruz et al., 2004). With the characteristics of quick reproducible velocity and easy control, microbe is preferable to use in environmental treatment. As a microorganism, fungus has been reported to be used in heavy metals

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Abbreviations: ITS, Internal transcribed spacers; SEM, scanning electron microscopy; EDX, energy dispersive X-ray; ROS, reactive oxygen species; PDA, potato dextrose agar; PCR, polymerase chain reaction; MIC, minimum inhibitory concentration; BLAST, basic local alignment search tool; PHB, poly-3-hydroxybutyrate; PCL, polycaprolactone.

bioremediation. *Phanerochaete chryso-sporium* mycelium was used as a biosorbent for heavy metals Cd(II), Pb(II) and Cu(II) from solution (Say et al., 2001; Li et al., 2004). Under appropriate processing, *Penicillium oxalicum* var. *Armeniaca* and *Tolypocladium* spp. exhibited better uptake of cadmium, lead and mercury (Svecova et al., 2006). *Aspergillus niger* has been shown to be capable of removing lead, cadmium, copper and nickel ions from wastewater (Kapoor et al., 1999). *Rhizopus* and *Aspergillus* sp. were thought as promising biosorbents of cadmium and chromium (Zafar et al., 2007).

In heavy metals bioremediation, the specialty of microbes closely related to treatment efficiency and seeking for suitable microbe is important. Zhuzhou smelter has produced heavy metals for more than 50 years. Especially, the cadmium pollution has become a potential threat to ecological environment and human health through food chain. The surrounding site and water have been polluted by heavy metals for a long time. The purpose of this investigation is to obtain high cadmium-resistance indigenous strains from polluted sites for their possible exploitation in bioremediation.

MATERIALS AND METHODS

Samples and culture media

Soil samples were collected from the cadmium plant in Zhuzhou smelter (Hunan province, south of China). 0.2 M stock solution of cadmium was prepared by dissolving Cd (NO₃)₂ in deionized water and sterilized by using 0.22- μ m pore-size sterile filters. Beef extract-peptone medium (g/l): (beef extract 3.0, peptone 10.0, NaCl 10.0), Czapek medium (g/l) (sucrose 30, NaNO₃ 2.0, K₂HPO₄ 1.0, KCl 0.5, MgSO₄ 0.5, FeSO₄ 0.01), potato dextrose agar (PDA) medium (g/l) (potato extract 200, glucose 20 and pH 6.5 \pm 0.3) were used.

Isolation and morphological characterization

Using the conventional plate method to isolate cadmium-resistant strains, soil suspensions were added to the beef extract-peptone liquid medium containing 2 mM cadmium ion. The cultures were incubated on a rotary shaker at 30°C, 180 rpm for 3 - 7 days and spread on plates. In order to isolate the strains with higher cadmium-resistant ability, the cadmium concentrations of medium were gradually increased. A series of solid media which contained different cadmium concentrations from 5 to 82 mM were prepared and strains were orderly inoculated and cultivated from low concentration to high level.

The fungus with the highest cadmium-resistant was selected as the study object in this work. The strain was inoculated on solid Czapek and PDA medium. The mycelia were observed at visual inspection. Mycelia, conidia and pycnidia of the fungus were observed with a scanning electron microscope (SEM).

18S rRNA, ITS and β -tubulin gene amplification

The fresh mycelia were harvested and rapidly ground in a mortar under liquid nitrogen (N₂) for 5 - 10 min, using a commercial kit; the genomic DNA was extracted from the mycelia. 18S rRNA gene was

amplified with universal primers f (5'-TGCCAGCMGCCGCGGTA-3') and r (5'-GACGGGCGGTGTGTRCAA-3') (Bundt et al., 2001). The polymerase chain reaction (PCR) conditions were as follows: 95°C for 5 min; 32 cycles: 94°C for 30 s, 55°C for 30 s and 72°C for 1 min; 72°C for 7 min and 4°C pause. The internal transcribed spacers (ITS) region sequence was amplified by the fungal universal primers ITS-1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS-4 (5'-CCTCCGCTTATTGATATGC-3') (Henry et al., 2000). The reaction conditions were as follows: 95°C for 5 min; 32 cycles: 94°C for 30 s, 56°C for 30 s and 72°C for 1 min; 72°C for 7 min and 4°C pause. Base on the β -tubulin sequences of GenBank, a pair primers were designed as: f (5'-CATGCGTGAGCTTGTATGC-3') and r (5'-TAG CCGAAAGCGAAGTTG-3'). The reaction conditions were: 95°C for 5 min; 32 cycles: 94°C for 30 s, 52°C for 30 s and 72°C for 1 min; 72°C for 7 min and 4°C pause.

The above PCR products were purified and then sequenced by Shanghai Sangon Biotechnologies Co. Ltd. to sequence. The 18S rRNA, ITS and β -tubulin sequences of the fungus were submitted to GenBank and analyzed with the basic local alignment search tool (BLAST) search tool. The phylogenetic tree was constructed on the basis of 18S rRNA sequence alignment.

Resistant to other heavy metals

The minimum inhibitory concentration (MIC) was measured to evaluate the tolerance of the fungus to other heavy metals. CuCl₂, CoCl₂, ZnSO₄, MnSO₄ and Pb(Ac)₂ were used to prepare 0.2 M single metal stock solutions, and sterilized by filters. PDA solid medium was amended with various amount of metal stock solutions to achieve the desired concentration. According to Zafar et al. (2007) method, each heavy metal plate was subdivided into three equal sectors, and the spore suspension was spotted on metal containing agar plates. The PDA medium without metal was also inoculated as control plate. The plates were incubated at 30°C for 3 - 7 days to observe the growth of fungus.

Biosorption experiment

250 ml Czapek liquid media contained 0 and 100 mg/l cadmium and were added in 500 ml shake flasks, and 1 \pm 0.1 g wet fungus biomass was inoculated in each flask. In order to eliminate the interference of medium on cadmium biosorption, the control experiment was performed in 100 mg/l cadmium Czapek liquid medium without cells inoculation. All the shake flasks were incubated on a rotary shaker at 30°C and 180 rpm. At intervals of 24 h (1 day), equal volumes of samples were picked up from the flasks and centrifuged at 8000 rpm for 20 min. The cadmium concentrations of the supernatant were analyzed using a flame atomic adsorption spectrophotometer. The biomass was dried at 60°C to constant weight, and the dry biomass weight was used to plot the growth curves. All the above experiments were performed in triplicates and average values were obtained. The biomass was analyzed by energy dispersive X-ray (EDX). The cadmium removal rate Re was obtained by the following equation:

$$Re = (C_{i-c} - C_{f-e}) / C_i \times 100\%$$

C_i (mg/l) is initial concentration, C_{f-c} (mg/l) is final concentration from control group, and C_{f-e} (mg/l) is final concentration from experimental group. The cadmium adsorption Q (mg/g) was obtained by the following equation:

$$Q = (C_{i-c} - C_{f-e}) \times V/m.$$

V is the volume (l) and m is the dried biomass (g).

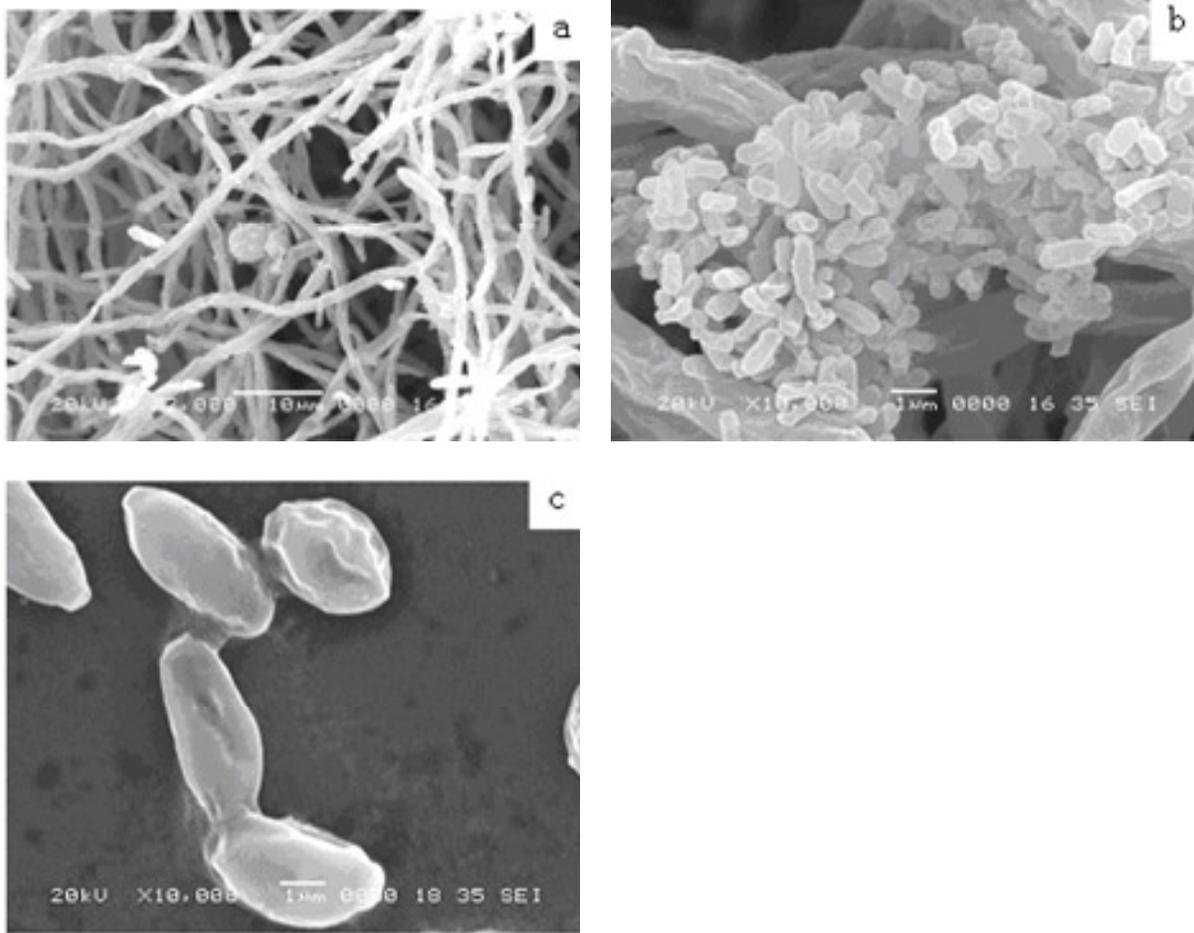


Figure 1. The morphological characteristics of fungus M_1 under scanning electron microscope. The fungus was cultured in PDA medium at 30°C, 180 rpm for 3 days. (a) Septal mycelia, (b) pycnidium, and (c) coidia.

RESULTS

The cadmium-resistant fungus

Some strains can grow on cadmium medium. Among them, the highest cadmium-resistant strain named M_1 can tolerate 80 mM cadmium on plate. When the metal concentration reached 82 mM, the fungus, M_1 , failed to grow after culturing for 7 days, and 82 mM was thought as the MIC of cadmium.

On Czapek and PDA solid medium, strain M_1 appeared white density mycelia and the bottom of plates showed yellow at the first 3 - 5 days. After then, the mycelia gradually became yellow or brown. SEM micrographs showed that the aerial mycelia had septate and branches; slender conidiophores grew out from aerial mycelia and conidia present oval shape (Figure 1).

18S rRNA, ITS and β -tubulin sequence analysis

18s rRNA, ITS region and β -tubulin gene sequence

amplified from the genomic DNA were submitted to GenBank and the accession number are FJ461772, FJ461773 and FJ502248, respectively. The sequences were aligned with sequences published in GenBank by BLAST tool and the result suggested that they have more than 99% similarity to *Paecilomyces lilacinus*. The phylogenetic tree of 18s rRNA confirmed the very close relationship between the fungus M_1 and *P. lilacinus*. (Figure 2)

MIC of heavy metals

MIC of heavy metals for *P. lilacinus* M_1 was investigated and the result is as shown in Table 1. The strain possessed marked resistance to cadmium, zinc, manganese, copper, lead, and cobalt. In comparison to the other metals, cobalt exhibited more toxicity to the fungus.

Cadmium adsorption in growing and EDX analyses

Though the fungus can grow in high cadmium (80 mM),

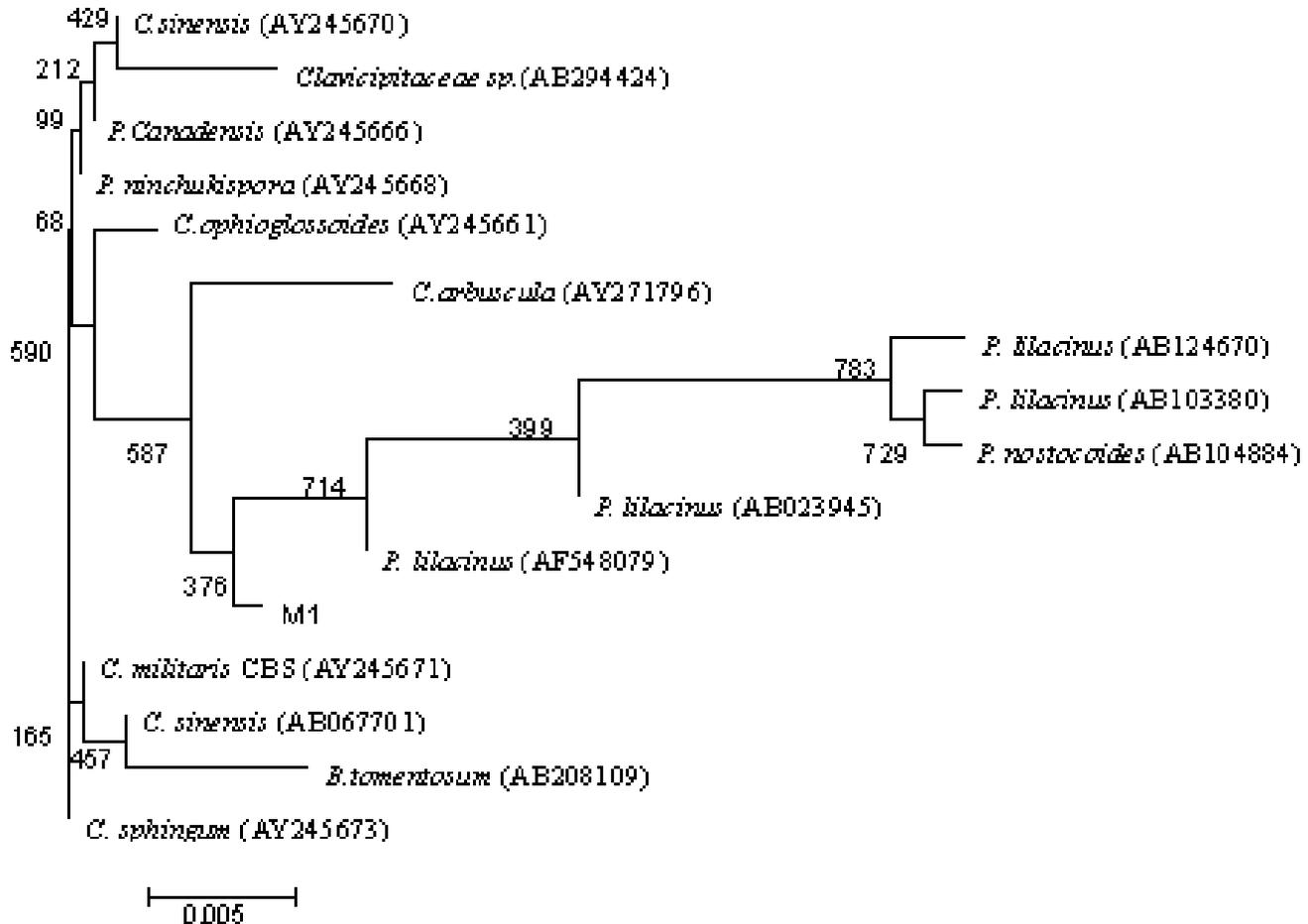


Figure 2. Phylogenetic tree based on 18S ribosome RNA sequence of the fungus M₁. This phylogenetic tree was produced using Clust X.

Table 1. The minimum inhibitory concentration (MIC) of heavy metals for *P. lilacinus* M₁.

Heavy metals	MIC (Mm)
Cd	82
Zn	95
Mn	60
Cu	35
Pb	35
Co	8

MIC was measured in amended PDA solid medium at 30°C for 3 - 7 days.

in high metal medium the biomass growth became slow. Considering the cadmium concentration of practical polluted soil and water, 100 mg/L was selected as the initial cadmium concentration in the fungus growth. Previous work suggested that wet biomass as inoculum was a better option than spore suspension in this experiment, and the former was used in this experiment. From the

growth curve (Figure 3), it was found that 100 mg/l cadmium exerted a little influence on fungus biomass; the strain entered log phase after culturing for 24 h and stationary phase on day 4. When compared to growth in the absence of cadmium, the biomass was gently decreased.

Growing in 100 mg/l cadmium medium, the fungus exhibited preferable cadmium adsorption capacity (Figure 4). The cadmium concentration of residual culture dropped from 100 to 28.97 mg/l in 4 days, after which there was a slight increase while the metal concentration was kept at 97.5 ± 1 mg/l in the control flasks. It also implied that the medium can adsorb trace cadmium and the interference to the result was slight.

To actually reflect the adsorption ability of the fungus, the cadmium adsorption by medium was excluded in calculating cadmium removal rate and adsorption amount (Figure 5). During the log phase, the cadmium removal by fungus displayed a rise from 8.09 to 68.8% and reached a plateau phase on day 4. The biosorption amount also presented an increasing trend, from 17.98 mg/g on day 1 to 24.23 mg/g on day 3, and subsequently

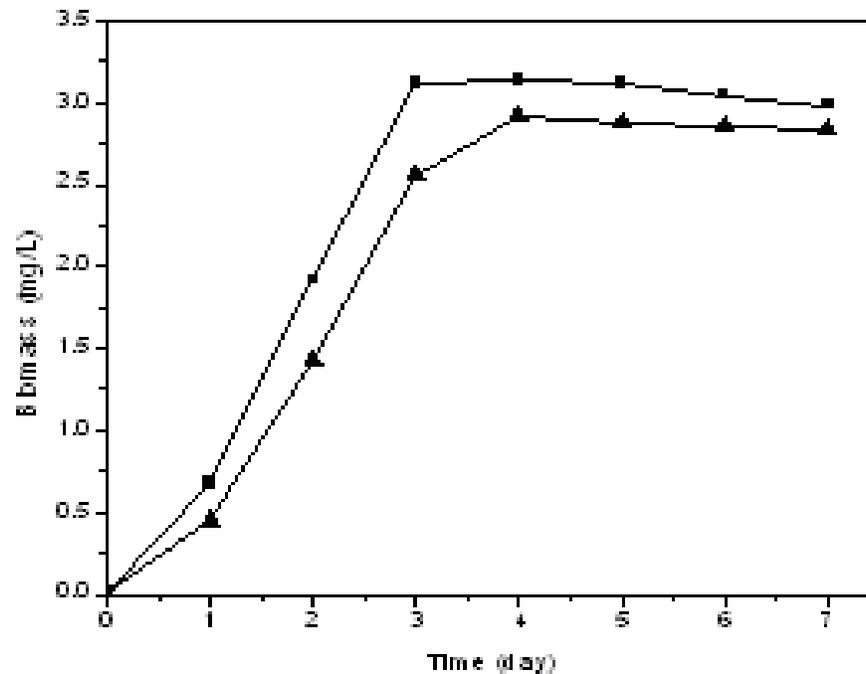


Figure 3. Growth curve of fungus M_1 . ■ = Inoculated without cadmium; ▲ = inoculated with 100 mg/L cadmium.

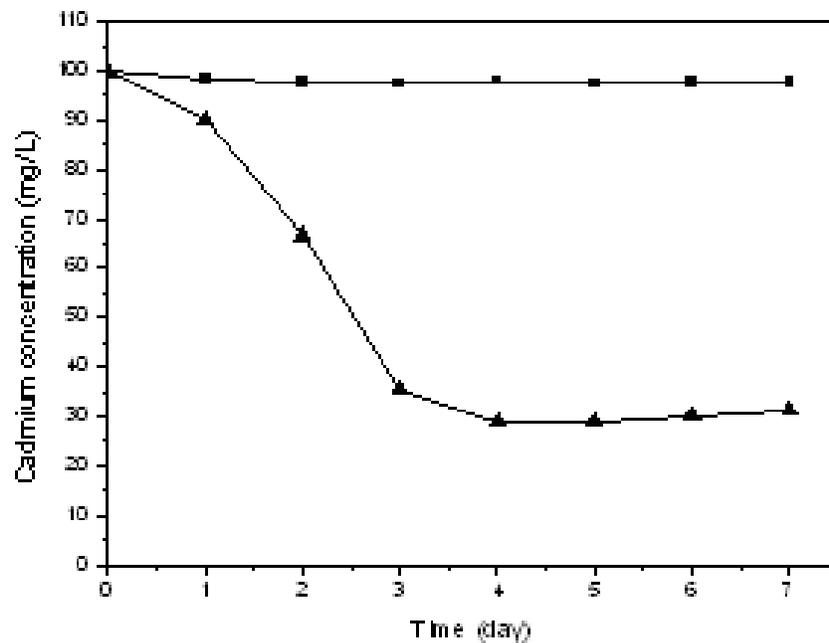


Figure 4. Cadmium concentration in medium. ▲ = M_1 was inoculated 100 mg/L cadmium medium; ■ = 100 mg/L cadmium medium without M_1 .

followed a minimal decrease. The result showed that the better cadmium adsorption by the fungus occurred after culturing for 3 - 4 days.

The fungus biomass grown both in the presence and

absence of cadmium was analyzed by EDX (Figure 6). The experimental result showed that cadmium was adsorbed by the fungus biomass, which directly suggested the presence of the metal on the cells. The EDS analysis

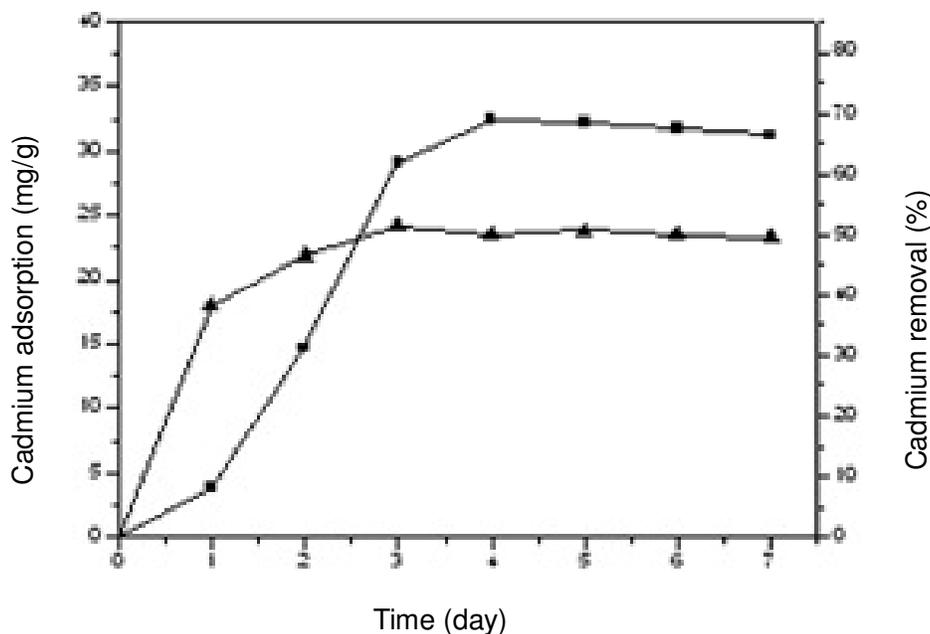


Figure 5. Cadmium removal and adsorption ability of fungus M₁. ■ = Cadmium removal; ▲ = cadmium adsorption.

confirmed the cadmium adsorption by fungus.

DISCUSSION

Organisms are often damaged or find it difficult to grow in an environment contaminated with cadmium due to the extreme toxicity of the metal. But some microbes that survived on cadmium pollution could have developed a cadmium-resistant mechanism to tolerate cadmium (Abou-Shanab et al., 2007). Strains isolated from heavy metal polluted sites generally possess resistant ability to metals (Patra et al., 2004). Thus the smelter was selected as sampling sites in this work.

The high cadmium-resistant ability of fungus M₁ possibly resulted from the long exposure to high cadmium concentration environment. It is an effective method for isolation of metal-resistant strain from the metal-polluted sites (Hu et al., 2007). In our previous work, *Paecilomyces aeruginosa* E₁ tolerated 18 mM cadmium ion were isolated from other sites in Zhuzhou smelter (Zeng et al., 2009). In this study a higher cadmium-resistant fungus was obtained.

In order to further study and apply the high cadmium-resistant fungus in biotechnology and environmental bioremediation, it is of great practical importance to correctly identify the strain. Morphological identification of fungus is the traditional method, but it is frequently too limited to allow application. The PCR technology has stimulated the use of molecular techniques. Molecular techniques have become an important reliable identification method

(Guarro et al., 1999). Some researches on identification and taxonomy have reported at the species level by 18s rRNA, ITS regions and beta-tubulin gene sequences (Green et al., 2004; White et al., 2006; Mello et al., 2006; Zuccaro et al., 2008).

On the basis of the 18s rRNA, ITS regions, beta-tubulin gene analysis and the morphological characteristics, the fungus M₁ can be identified as *P. lilacinus*. *P. lilacinus* has the greatest potential for use in combination with selected fungicides and nematicides for the control of potato cyst nematodes (Jacobs et al., 2003). *P. lilacinus* strain 251 showed significant control of the root-knot nematode *M. incognita* on tomato (Kiewnick and Sikora, 2006). In the field, *P. lilacinus* has been developed into commercial products and used to control nematodes for years (Zou et al., 2007). Moreover, it has been studied in environment treatment. Oda et al. (1995) found that *P. lilacinus* D218 excreted PHB (poly-3-hydroxybutyrate) and PCL (polycaprolactone) depolymerises to degraded PHB and PCL. *P. lilacinus* as biphenyl oxidizing organisms can oxidize chlorinated biphenyl derivatives (Sietmann et al., 2006). To our knowledge, there are seldom reports about *P. lilacinus* resistance to cadmium. This work has given information about the cadmium resistance and bio-absorption of *P. lilacinus*. It would contribute to the enrichment of fungus species in bioremediation and the expansion of the application of *P. lilacinus*.

Study of the cadmium-resistant strains has important role in treating cadmium contamination. It is helpful to understand the resistance mechanism, utilize absorption to remove metal, get the information of cadmium transfer

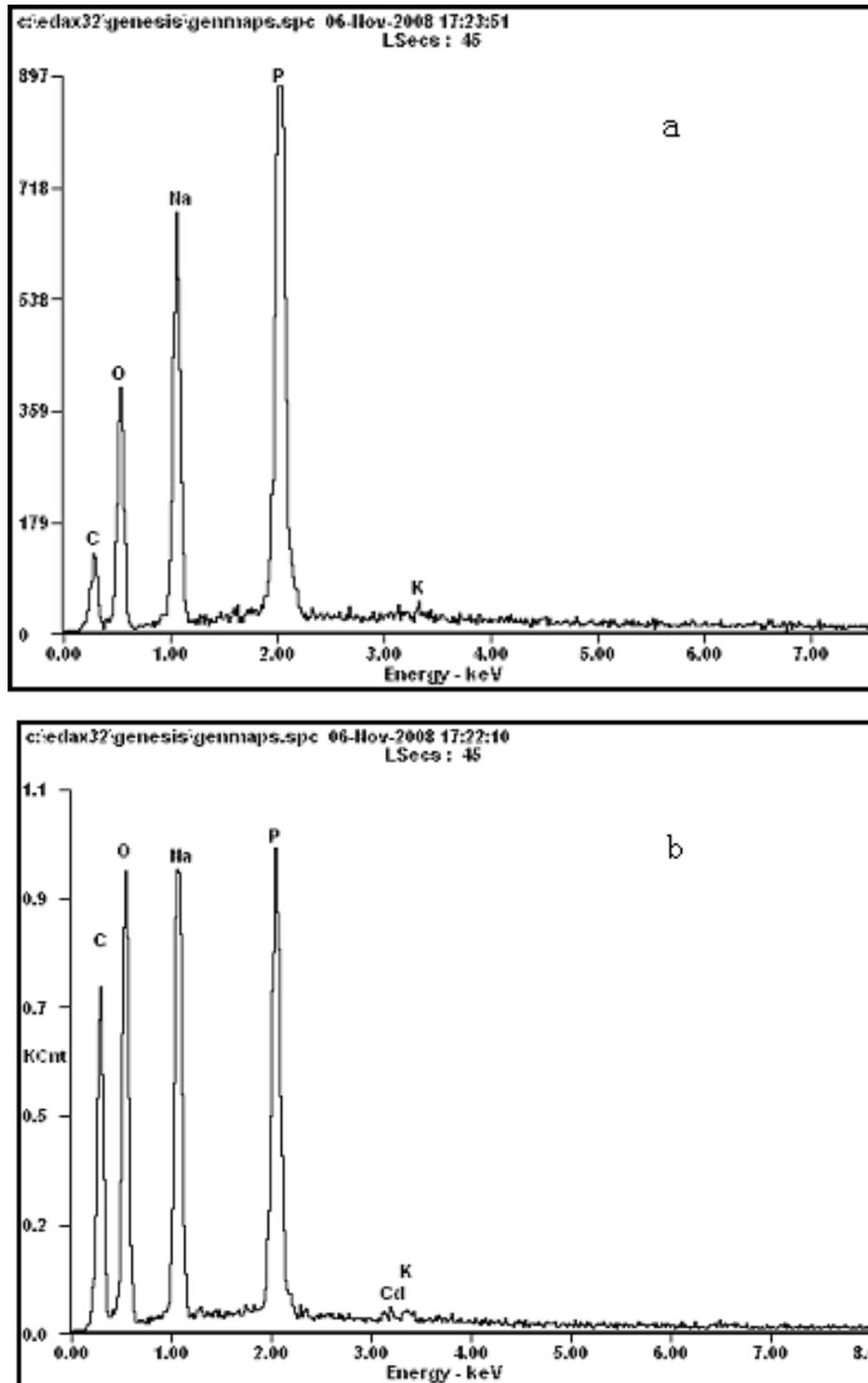


Figure 6. EDX images of biomass. (a) Fungus *M*₁ cultured in Czapek medium without cadmium, at 30°C, 180 rpm for 4 days. (b) Fungus *M*₁ cultured in Czapek medium with 100 mg/l cadmium, at 30°C, 180 rpm for 4 days.

in soil and water, and so on. Plenty of bacteria were studied on their resistance to heavy metal, such as *Staphylococcus aureus*, *Pseudomonas putia*, and *Bacillus*

circulans (Hetzer et al., 2006; Lee et al., 2001; Seip et al., 1990). Most mycorrhizal fungi were reported possessing cadmium-resistant ability (Blaudez et al., 2000). *Aspergillus*,

Penicillium, *Alternaria*, *Geotrichum*, *Fusarium*, *Rhizopus*, *Monilia* and *Trichoderma* were found resistance to cadmium from 0.2 to 5 mg/ml (Zafar et al., 2007). When compared to the previous cadmium-resistant strains, M₁ showed higher cadmium-resistance (Baldrian and Gabriel, 2002; Tanous et al., 2006). Moreover, the fungus M₁ was found well resistant to other metals such as zinc, manganese, copper, lead, and cobalt. The wide resistance to metals would contribute to the application of the fungus on varied metals bioremediation.

Fungus biomass has been reported in bioremediation of metals pollution. *P. chrysosporium* was used to biosorb cadmium (II), lead (II), copper (II) and the adsorption capacities reached 23.04, 69.77 and 20.33mg/g dry biomass, respectively (Say et al. 2001). The maximum experimental biosorption capacities for entrapped live and dead fungal mycelia of *L. sajur-caju* were found to be 104.8 ± 2.7 mg Cd (II) /g and 123.5 ± 4.3 mg Cd (II)/g, respectively (Bayramoglu et al., 2002). Akar and Tunali (2005) found that maximum biosorption capacities of Cd (II) and Cu(II) ions on *B. cinerea* were found to be 17.03 ± 0.76 mg/g and 9.23 ± 0.64 mg/g, respectively. The study of *P. chrysosporium* biosorption showed that the maximum biosorption capacity for immobilized and free biomass were 89 and 74 mg/g Cd (II), respectively (Iqbal and Edyvean, 2005). The cadmium biosorption ability of *P. lilacinus* M₁ can reach 24.23 mg/g during growth in this work. From the data, the biosorption capacity of *P. lilacinus* M₁ was moderate. However, the biosorption ability often varied with the test conditions, such as initial metal concentration, solution pH, contact time, biomass dosage, processing method, and so on (Selatnia et al., 2004; Svecova et al., 2006). Thus it is hardy to compare the adsorption capacity under varying conditions.

Moreover, with cadmium-resistant and biosorption capacity of the fungus, it is possible to seed the strain in polluted environment to directly adsorb metal. With respect to the polluted field water which made it difficult to centralized treatment, the seeding fungus may be a potential way to bioremediation.

In this work, the high cadmium-resistant fungus was isolated from Zhuzhou smelter and identified as *P. lilacinus*. Except for cadmium, the fungus showed well and wide metals resistance. During the growth in 100 mg/l cadmium medium, the fungus received less toxicity and can adsorb cadmium. The preliminary study suggested that the fungus is a potential species on cadmium bioremediation.

ACKNOWLEDGEMENTS

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