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Characterization of Cd- and Pb-resistant fungal endophyte *Mucor* sp. CBRF59 isolated from rapes (*Brassica chinensis*) in a metal-contaminated soil

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ABSTRACT

To better understand the characteristics of fungal endophytes in the development of effective phytoremediation of heavy metals, the objectives of this study were to isolate a fungal endophyte tolerant Cd and Pb from rape roots grown in a heavy metal-contaminated soil, to characterize the metal-resistant fungal endophyte, and to assess its potential applications in removal of Cd and Pb from contaminated solutions and experimental soil. The isolate CBRF59 was identified as *Mucor* sp. based on morphological characteristics and phylogenetic analysis. From a Cd solution of 2.0 mM, the maximum biosorption capacity of Cd by dead biomass of *Mucor* sp. CBRF59 was 108 mg g^{-1}. Under the same conditions, the bioaccumulation capacity of Cd by active biomass of the strain was 173 mg g^{-1}. The bioaccumulation capacity of Pb by active biomass of the strain was significantly lower than that by dead biomass in the initial Pb concentrations from 1.0 to 2.0 mM. The ratio of Pb to Cd and initial pH values in the mixed Cd + Pb solutions affected the bioaccumulation and biosorption capacities of the metals by CBRF59. The addition of the active mycelia of CBRF59 significantly increased the availability of soil Pb and Cd by 77% and 11.5-fold, respectively. The results showed that the endophytic fungus was potentially applicable for the decontamination of metal-polluted media.

1. Introduction

Heavy metals, especially cadmium (Cd) and lead (Pb), generated from industrial wastewater and other human activities, exert a significant impact on human health and other living organisms in the environment [1,2]. Most physicochemical methods to remove heavy metals are expensive, inefficient, and labor-intensive [3]. Phytoremediation is considered as a highly promising technology for remediation of polluted sites. However, most hyperaccumulating plants are not suitable for field phytoremediation applications due to their small biomass and slow growth rates. Therefore, it is necessary to further develop phytoremediation strategies for heavy metal contaminated soils [4,5]. In this regard, interactions among metals, microbes, and plants have attracted much attention because of the biotechnological potential of microorganisms to remove metals directly from polluted media and the possible role of microorganisms in promoting plant growth in metal contaminated soils. Since the endophytes of metal accumulating plants can endure high concentrations of certain heavy metals [3], the plants in metal contaminated soils may harbor various endophytes with bioremediation potentials. However, such potentials have not been well explored for heavy metal bioremediation. While current studies mainly focus on bacterial endophytes [3,6,7], research of endophytic fungi is still in infancy. Fungi display a high ability to immobilize toxic metals by either insoluble metal oxalate formation, biosorption, or chelation onto melanin-like polymers, and fungal isolates are able to compete with the indigenous bacterial microflora in difficult situations. The use of filamentous fungi may provide some advantages over bacterial bioaugmentation [8]. Many fungi, such as *Trichoderma*, *Aspergillus*, and the arbuscular mycorrhizal fungi (AMF), have shown the potential to improve phytoremediation in metal-contaminated soils [9–12]. Among them, AMF associated with hyperaccumulating or non-hyperaccumulating plants have been mostly studied for their roles in phytoremediation [9,13–15]. Most of the known hyperaccumulators belong to non-mycorrhizal plants *Brassicaceae* family [9]. The isolation and characterization of fungal endophytes of *Brassica* plants is essential for phytoremediation of heavy metal contaminated soils. Nevertheless, there is little information about the potential of fungal endophytes isolated from plants grown in heavy metal-contaminated soils on the phytoremediation of these soils [3,6,11].

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Metal accumulation processes by microorganisms generally fall into two categories [16]: biosorption by non-living or non-growing biomass and bioaccumulation by living and growing cells. Although heavy metal biosorption by dead or living (in resting state) cells has been extensively studied [17,18], heavy metal bioaccumulation by growing microorganisms from single or particularly multi-metal systems has been rarely studied [1]. A better understanding of the characteristics of heavy metal-resistant fungal endophytes is a critical prerequisite for the development of effective phytoremediation of heavy metals. Therefore, the objectives of this study were to characterize a metal-resistant fungal endophyte isolated from rape roots grown in a heavy metal-contaminated soil and to assess the potential applications of the fungal endophyte in bioremoval of Cd and Pb from contaminated solutions and experimental soil.

2. Materials and methods

2.1. Isolation and identification of fungal endophytes resistant to Pb and Cd

Healthy roots of rape were collected from a heavy metal-contaminated site in the Dabaoshan mine, Guangdong Province, China (24°33′36.6″N, 113°43′14.0″E). Irrigated with mining wastewater, the study site had elevated levels of several heavy metals, including total Cd (9.17 mg kg^{-1}), total Pb (496 mg kg^{-1}), and total Cu (246 mg kg^{-1}). The available (or water-soluble) metal concentrations of Cd, Pb, and Cu in the soil were 0.17, 0.67, and 0.66 mg kg^{-1}, respectively. The Cd and Pb concentrations in the rape root were 224 and 666 mg kg^{-1}, respectively.

To isolate endophytic fungi, the root samples were washed with tap water to remove soil particles and sterilized by sequential immersion in 75% (v/v) ethanol for 2 min, and sodium hypochlorite solution (5% available chlorine) for 1 min, then washed in sterile water three times to remove the surface sterilization agents [11]. After the last step, 0.3 mL of the final washing water was plated on potato dextrose agar (PDA), incubated at 28 °C, then examined for microbial growth. The washed roots were cut into small pieces using a sterile blade and placed on plates containing the PDA medium for incubation for 1–2 weeks at 28 °C. The hyphal tips of endophytic fungi growing out from the samples were cut by a sterile Pasteur pipette and transferred to a new PDA plate with 10 mg L^{-1} of chloromycetin to prevent the bacterial growth. The colonies were purified and stored at 4 °C.

Isolates with Cd- and Pb-resistance were examined using the PDA medium added with gradually increasing concentrations of $CdCl_2$ and $Pb(NO_3)_2$. The products for standard Cd^{2+} and Pb^{2+} solutions were purchased from the National Analytical Center of Iron and Steel (NACIS), China. For each concentration, endophytic fungi were incubated at 28 °C for 7 days. With an increased metal concentration, if no apparent growth of fungi was observed on the plates, the metal concentration was considered as the highest metal concentration tolerated by the tested fungus.

The isolates were identified by morphological traits and fungal ITS1-5.8S-ITS2 region sequence analysis. The ITS1-5.8S-ITS2 region was amplified using fungal-specific ITS1F (5′-CTTGGTCATTTAGAG GAAGTAA-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) primers [12]. The DNA region was amplified and sequenced as described previously [19]. The sequences were matched with those already known using the BLAST search option at NCBI Genbank (http://www.ncbi.nih.gov/index.html). The sequences of the ITS region were aligned with the sequences of similar fungi retrieved from databases using CLUSTAL X and a phylogenetic tree was constructed using the neighbor-joining algorithm (PHYLIP, version 3.69) with the bootstrap analysis of 1000 replicates [20]. All phylogenetic trees were displayed using the program TreeView 32.

2.2. Metal bioaccumulation by active mycelia

Initial concentrations of Cd (as $CdCl_2$) or Pb ions (as $Pb(NO_3)_2$) in the potato dextrose broth (PDB) ranged from 0.1 to 2.0 mM. Initial mixed Cd + Pb concentrations used were as follows: 1.0 mM Cd + 2.0 mM Pb, 1.5 mM Cd + 1.5 mM Pb, and 2.0 mM Cd + 1.0 mM Pb. Initial pH values of 4.0, 5.0, or 6.0 were used. The medium without adding any heavy metals was used as a control. For each treatment, 100 mL PDB was put in a 250-mL Erlenmeyer flask and inoculated with a mycelial disc (7 mm in diameter) removed from the margins of 3-d-old colonies. The flasks with the different treatments were cultivated in a rotatory shaker (150 rpm) at 30 °C for 120 h. The mycelia were harvested with filtration through a 150 μM sieve and dried for 24 h at 65 °C to determine the dry weight. The filtrates were determined for the metal concentrations. Three replications of all assays were performed.

2.3. Metal biosorption by dried mycelia

The PDBs (pH 6.0) without any metals were inoculated with the identified fungus and incubated at 30 °C for 4 d in flasks on a rotatory shaker (150 rpm). After being harvested by filtration, the biomass was thoroughly washed with distilled water three times to remove the residual media. The biomass was dried for 24 h at 65 °C and ground in a mortar, then sieved through a sieve with 150 μM openings. The conditions (medium, dry weight of biomass, initial metal concentration, pH etc.,) for the metal biosorption assay by dried mycelia were the same as those for bioaccumulation by active mycelia above. The flasks were shaken on a rotatory shaker (150 rpm) for 2 h at 30 °C, then the biomass was separated with filtration and the filtrate was measured for metal ion concentrations. Note that the same experimental conditions (30 °C and incubation time 120 h) were designed for determining bioaccumulation and biosorption capacities. However, the biosorption process reached the equilibrium within 2 h.

2.4. Effects of Pb- and Cd-tolerant endophyte on the mobility of soil Cd and Pb

Soil samples were collected from the site where the plant samples were collected. The soil was air-dried and sieved (1.5 mm) to remove plant residuals, soil macrofauna, and stones. The identified endophyte was inoculated into the soil by the method of Du et al. [21]. The fungal biomass was harvested by filtration as described above. Then, 0.1 g (fresh weight) washed biomass in 1 mL sterilized water or 1 mL spore suspension (OD_{570} = 0.2) was added to 1 g autoclaved soil in 100-mL polypropylene centrifuge tubes. Sterile water was added to the autoclaved soil as an axenic control. All tubes were weighed, wrapped in brown paper, and placed on a rotatory shaker (150 rpm) at 30 °C. After 1 week, the tubes were again weighed and sterile water was added to compensate for evaporation. The tubes were incubated at 30 °C, 150 rpm for 4 h to extract the soil water-soluble Pb and Cd. Soil suspensions were centrifuged at 4000 rpm for 15 min and filtrated. Concentrations of Pb and Cd in the filtrate were measured using an inductively coupled plasma optical emission spectrometry (ICP-OES) (PerkinElmer Optima, 5300DV) [22]. The IDLs of the equipment are 0.024 and 0.19 mg kg^{-1} for Cd and Pb, respectively.

2.5. Metal determination

The residual Cd and Pb concentrations in the PDB were measured using the ICP-OES. The metal biosorption capacity is cal-

culated as follows:

$$q_j^s = \frac{V(C_{ji} - C_{jf}^s)}{W} \quad (1)$$

$$q_t^s = \frac{V\sum(C_{ji} - C_{jf}^s)}{W} \quad (2)$$

where q_j^s is the biosorption capacity of metal j (j = Cd, Pb) (mg g^{-1}), C_{ji} is the initial concentration of metal j (mg L^{-1}), C_{jf}^s is the final concentration of metal j in the PDB with the biosorption process (mg L^{-1}), V is the volume of the liquid medium (L), W is the biomass of mycelia (g), and q_t^s is the total metal biosorption capacity (mg g^{-1}). Similarly, the metal bioaccumulation capacity is calculated as follows:

$$q_j^a = \frac{V(C_{ji} - C_{jf}^a)}{W} \quad (3)$$

$$q_t^a = \frac{V\sum(C_{ji} - C_{jf}^a)}{W} \quad (4)$$

where q_j^a is the bioaccumulation capacity of metal j (j = Cd, Pb) (mg g^{-1}), C_{jf}^a is the final concentration of metal j in the PDB with the bioaccumulation process (mg L^{-1}), and q_t^a is the total metal bioaccumulation capacity (mg g^{-1}). The metal biosorption or bioaccumulation percentage is calculated by:

$$r = 100\%\frac{(C_i - C_f)}{C_i} \quad (5)$$

$$r_t = 100\%\frac{\sum(C_i - C_f)}{\sum C_i} \quad (6)$$

where r is the metal biosorption or bioaccumulation percentage, C_i is the initial concentration of heavy metal used (mg L^{-1}), C_f is the final concentration of heavy metal in the PDB with the biosorption or bioaccumulation process (mg L^{-1}), and r_t is the biosorption or bioaccumulation percentage of total metals.

2.6. Statistical analysis

Statistical analysis of data was carried out using the SPSS statistical package (version 16.0 for Windows, SPSS Inc.). Analyses of variances between bioaccumulation and biosorption capacities under the same treatment and variances between the mixed Cd + Pb and single metal treatments under the same conditions were conducted using the one-way analysis of variance (ANOVA). Significance analyses of different initial metal concentrations under the same pH and of different mixed Cd + Pb treatments under the same pH were performed with a 5% least significant difference (LSD test, $P < 0.05$).

3. Results

3.1. Identification of isolate CBRF59

From the surface-sterilized rape roots, fungi were isolated, belonging to *Fusarium*, *Trichoderma*, *Mucor*, and *Penicillium* genera. Among the isolates, the isolate CBRF59 could tolerate 5.0 mM Cd and 10.0 mM Pb, respectively, and was further identified. Colonies of CBRF59 grew rapidly at 28–30 °C and quickly covered the agar surface with fluffy appearance. The color of the front side was white initially and became tawny or black in time. The hyphae of CBRF59 had no septum, rhizoids, and prostrate branches. Sporangiophores were short, erect, and tapered towards their apices. Sporangia at the end of sporangiophores were round, gray to black in color, and filled with a large number of sporangiospores. Following the rupture of the sporangia, sporangiospores were freely spread. The sporangiospores varying in size and diameter were round or slightly elongated. The ITS1-5.8S-ITS2 region sequence analysis (Fig. 1) indicated that the strain CBRF59 [GenBank accession number: GU569095] clustered together with the 16 representative taxa of *Mucor* sp. with a 100% bootstrap support. Within this clade, the strain CBRF59 and other 5 representative taxa of *Mucor racemosus* formed a subclade with a relatively strong bootstrap support of 75%. According to the morphological characteristics and phylogenetic analysis, CBRF59 was categorized as the *Mucor* genus, especially close to *M. racemosus*.

3.2. Effects of Cd and Pb on biomass production of Mucor sp. CBRF59

As shown in Fig. 2, initial Cd or Pb concentrations affected the growth of CBRF59 differently. As the initial concentrations of Cd increased from 0.1 to 2.0 mM, the biomass (dry weight) of CBRF59 decreased significantly. With the initial Cd concentrations <0.5 mM, the pH effect on the biomass of CBRF59 was not significant (Fig. 2A). However, with the higher initial Cd concentrations, the toxicity of Cd to CBRF59 increased when the initial pH values of medium increased from 4.0 to 6.0. The initial Pb concentrations from 0.1 to 2.0 mM and the pH values (4.0–6.0) did not affected the growth of CBRF59 significantly (Fig. 2B). The biomass of CBRF59 cultivated in the medium with mixed Cd + Pb was higher than that in the medium with Cd, but lower than that in the medium with Pb (Table 1). For example, at pH value of 4.0, the biomass of CBRF59 cultivated in the medium containing 1.0 mM Cd + 2.0 mM Pb was 0.43 g, while the biomasses in the media containing 1.0 mM Cd and 2.0 mM Pb were 0.26 and 0.51 g, respectively.

3.3. Bioaccumulation capacity and percentage of Mucor sp. CBRF59

The bioaccumulation capacities and percentages of Cd and Pb by CBRF59 were studied in relation to the heavy metal initial concentrations. When the initial Cd concentrations increased from 0.1 to 2.0 mM, the Cd bioaccumulation percentage of CBRF59 at different pH values (4.0, 5.0, 6.0) showed the same change pattern (Fig. 3A). The Cd bioaccumulation percentage increased when the initial Cd concentrations increased from 0.1 to 0.5 mM, but the percentage declined to the minimum at the initial concentration of 1.0 mM. Then the percentage increased again with the initial concentrations. The maximum Cd bioaccumulation percentages at pH 4.0, 5.0, and 6.0 were all observed at the initial concentration of 0.5 mM, and were 67%, 72%, and 68%, respectively. The differences among Pb bioaccumulation percentages of CBRF59 at different pH values (4.0, 5.0, and 6.0) were not significant when the metal concentration increased from 0.1 to 2.0 mM (Fig. 3B). However, at the concentration of 0.1 mM, the Pb bioaccumulation percentages at pH 5.0 and 6.0 were significantly higher than that at pH 4.0.

As shown in Fig. 4A, the bioaccumulation capacities of Cd were not significantly different when the initial Cd concentration increased from 0.1 to 1.0 mM; however, when the initial Cd concentration increased from 1.0 to 2.0 mM, the bioaccumulation capacities increased significantly ($P < 0.05$). The maximum bioaccumulation capacities were obtained at the initial concentration 2.0 mM, and were 74.4, 122, and 173 mg g^{-1} at pH 4.0, 5.0, and 6.0, respectively. The bioaccumulation capacities of Pb at the different pH values (4.0, 5.0 and 6.0) increased with the initial concentrations from 0.1 mM to 2.0 mM (Fig. 4B). The maximum bioaccumulation capacities at pH 4.0, 5.0, and 6.0 were 41.0, 50.9, and 46.0 mg g^{-1}, respectively.

As shown in Table 1 and Fig. 4, the bioaccumulation capacity of Cd in the Cd + Pb solution was significantly lower than that in the

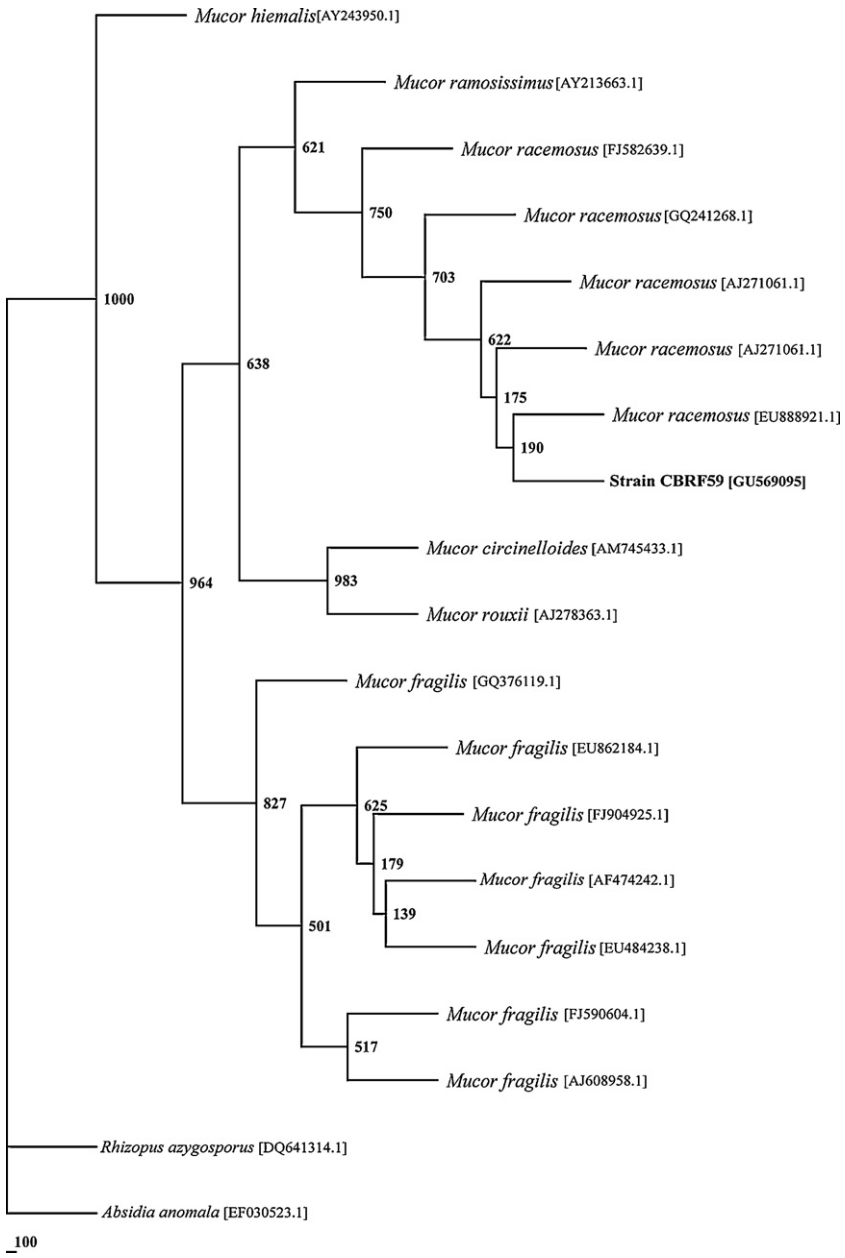

Fig. 1. Phylogenetic relationships of strain CBRF59 and 18 other related sequences including the outgroup *Absidia anomala* [EF030523.1]. The number in each branch indicates the number of trees from 1000 bootstrap replications in which the branch occurs.

Table 1
Biosorption and bioaccumulation capacities of *Mucor* sp. CBRF59 in the treatments of mixed Cd + Pb at initial pH values of 4, 5, and 6.

Treatment	Initial pH	Dry biomass (g)	q^a_{Cd}	q^s_{Cd}	q^a_{Pb}	q^s_{Pb}	q^a_t	q^s_t
1.0 mM Cd + 2.0 mM Pb	4.0	0.43 ± 0.02A	1.85 ± 0.05A/a	11.6 ± 0.48A/b	62.6 ± 7.48A/a	78.0 ± 3.81A/b	64.5 ± 7.48A/a	89.6 ± 4.23A/b
	5.0	0.43 ± 0.01A	5.11 ± 0.11B/a	15.5 ± 0.87B/b	70.5 ± 1.93AB/a	73.2 ± 4.18A/a	75.6 ± 1.94B/a	88.7 ± 5.05A/b
	6.0	0.39 ± 0.01B	7.45 ± 0.21C/a	19.4 ± 1.08C/b	79.4 ± 1.19B/a	88.2 ± 2.66B/b	86.8 ± 1.13C/a	108 ± 3.69B/b
1.5 mM Cd + 1.5 mM Pb	4.0	0.45 ± 0.03A	6.02 ± 2.40A/a	15.0 ± 0.64A/b	60.6 ± 4.33A/a	49.8 ± 8.34A/b	66.6 ± 6.24A/a	64.8 ± 8.60A/a
	5.0	0.37 ± 0.01B	7.41 ± 1.27A/a	24.9 ± 0.46B/b	65.8 ± 1.52AB/a	62.6 ± 0.37B/b	73.2 ± 2.73A/a	87.5 ± 0.24B/b
	6.0	0.36 ± 0.02B	8.18 ± 0.58A/a	30.1 ± 1.52C/b	56.9 ± 2.10AC/a	68.9 ± 3.39B/b	65.1 ± 1.94A/a	98.9 ± 4.90C/b
2.0 mM Cd + 1.0 mM Pb	4.0	0.39 ± 0.03A	7.04 ± 0.99A/a	20.3 ± 0.33A/b	44.2 ± 3.05A/a	47.6 ± 1.02A/a	51.2 ± 4.03A/a	67.9 ± 1.28A/b
	5.0	0.33 ± 0.00B	7.51 ± 0.34A/a	37.1 ± 0.73B/b	47.1 ± 1.06AB/a	54.2 ± 0.72B/b	54.6 ± 1.24AB/a	91.3 ± 1.33B/b
	6.0	0.24 ± 0.03C	11.9 ± 2.65B/a	55.0 ± 0.79C/b	53.0 ± 5.99B/a	72.8 ± 0.41C/b	65.0 ± 8.16B/a	128 ± 0.93C/b

q^s_{Cd} and q^s_{Pb} (mg g^{-1}) are the biosorption capacities of Cd and Pb, respectively; q^a_{Cd} and q^a_{Pb} (mg g^{-1}) are the bioaccumulation capacities of Cd and Pb, respectively; and q^a_t and q^s_t (mg g^{-1}) are the total metal bioaccumulation and biosorption capacities, respectively.
The values are mean ± standard deviation (n = 3).
Different capital letters indicate that multiple comparison differences are significant under the same treatment of metals at different initial pH (LSD test, $P < 0.05$). Different small letters indicate that comparison differences between q^s and q^a are significant under the same treatment of metals and initial pH (ANOVA, $P < 0.05$).

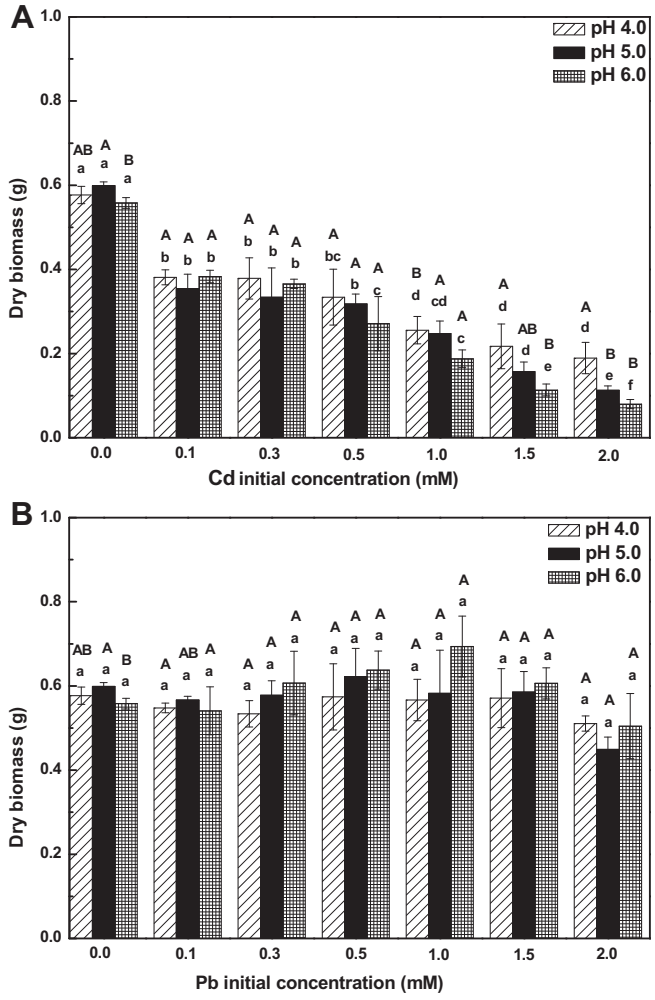

Fig. 2. Effects of Cd (A), Pb (B) on *Mucor* sp. CBRF59 biomass at initial pH values of 4, 5, and 6. Different capital letters on the bars indicate that the multiple comparison differences are significant with different initial pH values and the same initial metal concentrations ($P < 0.05$). Different small letters on the bars indicate that the comparison differences are significant under different initial metal concentrations and at the same initial pH ($P < 0.05$). The vertical line on each bar shows the standard deviation.

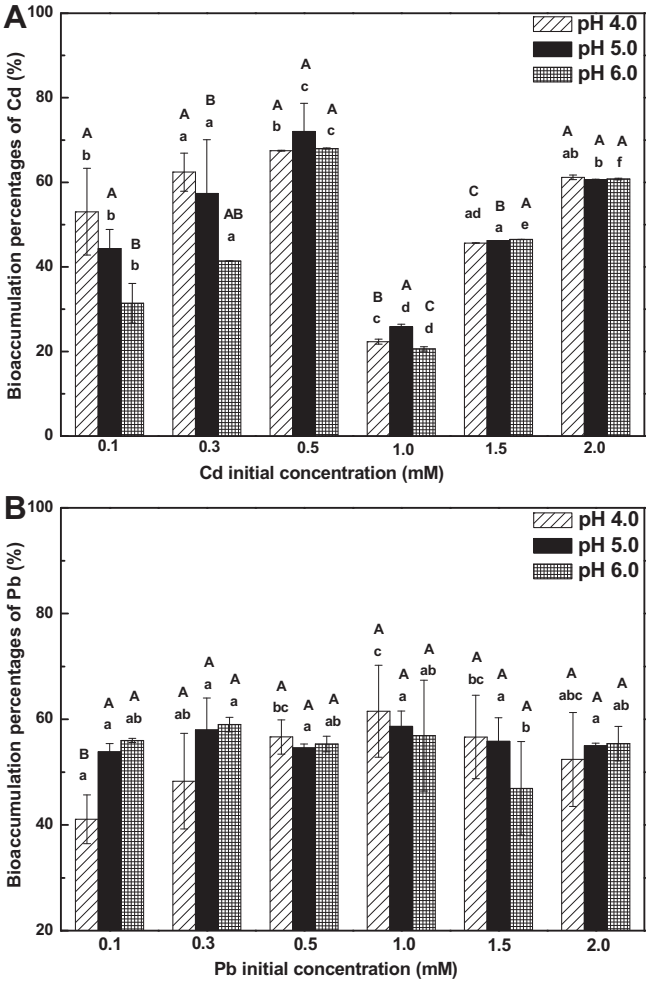

Fig. 3. Effects of initial concentrations of Cd (A) and Pb (B) on their bioaccumulation percentages by *Mucor* sp. CBRF59 at initial pH values of 4, 5, and 6. Different capital letters on the bars indicate that the multiple comparison differences are significant with different initial pH values and the same initial metal concentrations ($P < 0.05$). Different small letters on the bars indicate that the comparison differences are significant under different initial metal concentrations and at the same initial pH ($P < 0.05$). The vertical line on each bar shows the standard deviation.

single Cd solution. However, the bioaccumulation capacity of Pb in the Cd + Pb solution was significantly higher than that in the single Pb solution. For example, at pH 4.0, the bioaccumulation capacities of Cd and Pb in the solution of 2.0 mM Cd + 1.0 mM Pb were 7.04 and 44.2 mg g^{-1}, respectively, whereas the bioaccumulation capacities of Cd and Pb were 74.4 and 22.5 mg g^{-1} in the solutions of 2.0 mM Cd and 1.0 mM Pb, respectively. The sum of bioaccumulation capacities of both metals from the Cd + Pb combined treatment was also higher than that from the single ion solutions. In the Cd + Pb combined treatments, the highest bioaccumulation capacities of Cd and Pb were 11.9 and 79.4 mg g^{-1}, respectively.

3.4. Biosorption capacities of Mucor sp. CBRF59

The biosorption capacities of Cd and Pb increased significantly with the initial Cd and Pb concentrations from 1.0 to 2.0 mM (Fig. 5). The maximum biosorption capacities of Cd and Pb by the dead biomass of CBRF59 were 108 and 74.4 mg g^{-1}, respectively (Fig. 5). Compared with the single metal treatments, the biosorption capacity of Cd by CBRF59 decreased in the presence of Cd + Pb, while the biosorption capacity of Pb increased significantly (Table 1). The sum of biosorption capacities of both metals by CBRF59 in the Cd + Pb solutions was larger than that in the single metal solutions. The highest biosorption capacities of Cd and Pb absorbed in Cd + Pb solution were 55.0 and 88.2 mg g^{-1}, respectively.

3.5. Influence of CBRF59 on the mobility of soil Pb and Cd

To further facilitate the availability of Cd and Pb, the active mycelia and spores of the strain CBRF59 were added to the contaminated soil. The active mycelia of CBRF59 showed the ability to mobilize soil Cd and Pb significantly ($P < 0.05$) (Table 2). Compared to the non-inoculated control soil, the availability of Pb and Cd with the active mycelia of CBRF59 increased 77% and 11.5-fold, respectively. However, the addition of spore suspension did not increase the amount of water-soluble Pb and Cd significantly compared to the control.

4. Discussion

In Table 3, the maximum Cd and Pb uptake capacities by CBRF59 were compared with those by several fungi and bacteria reported in the literature, inluding *Rhizopus arrhizus*, *Mucor rouxii*, *Cupriavidus taiwanensis* TJ208, *Bacillus jeotgali*, *Aspergillus niger*, and

Table 2
Content of water-soluble Pb and Cd in soil treated with and without *Mucor* sp. CBRF59.

Treatment	Concentrations of water-soluble Pb (mg kg^{-1})	Ration in total Pb (%)	Concentrations of water-soluble Cd (mg kg^{-1})	Ration in total Cd (%)
Control (water + soil)	0.67 ± 0.05a	0.13	0.17 ± 0.05a	1.85
Spore suspension + soil	0.78 ± 0.09a	0.16	0.16 ± 0.03a	1.78
Live mycelium + soil	1.15 ± 0.28b	0.23	2.13 ± 0.38b	23.2

The values presented in columns 2 and 4 are mean ± standard deviation (n = 3).
Different small letters indicate that comparison differences are significant (ANOVA, P < 0.05).

Table 3
Comparison of uptake capacities of Cd and Pb between CBRF59 and microorganisms reported in the literature.

Microorganisms	Maximum Cd uptake capacities (mg g^{-1})	Maximum Pb uptake capacities (mg g^{-1})	Reference
Growing *Rhizopus arrhizus*	17.7	16.9	[23]
Live *Mucor rouxii* biomass	8.46	35.7	[24]
Cupriavidus taiwanensis TJ208	19.6	50.1	[25]
Bacillus jeotgali	57.9		[26]
NaOH pretreated *Aspergillus niger*		34.9	[27]
Pseudomonas veronii 2E	54		[28]
Growing endophytic fungus *Mucor* sp. CBRF59	173	50.9	This study
Dry biomass of endophytic fungus *Mucor* sp. CBRF59	108	74.4	This study

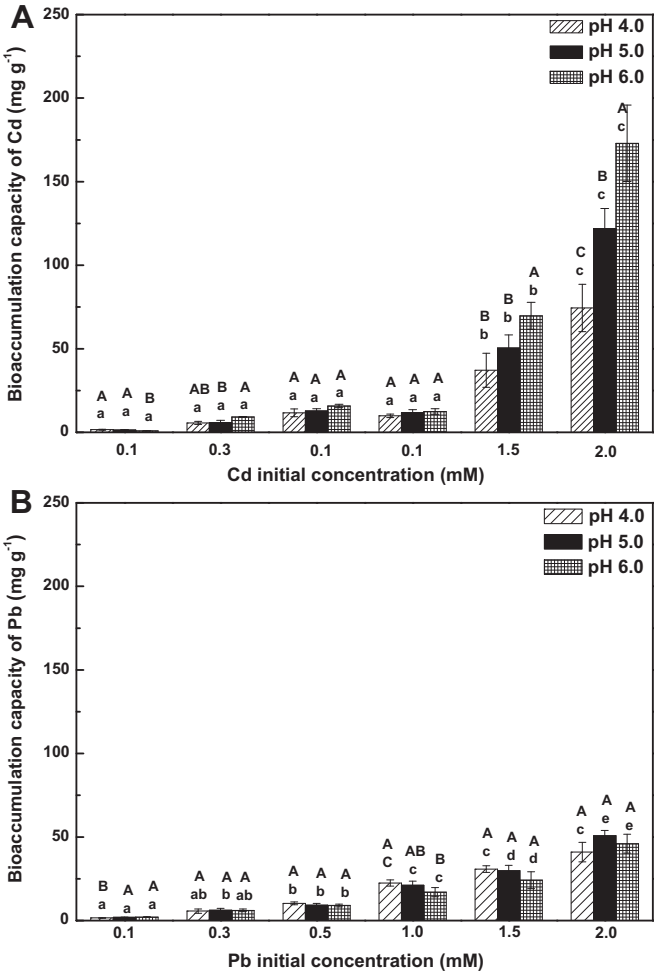

Fig. 4. Effect of initial concentrations of Cd (A), Pb (B) on bioaccumulation capacities of *Mucor* sp. CBRF59 at initial pH values of 4, 5, and 6. Different capital letters on the bars indicate that the multiple comparison differences are significant with different initial pH values and the same initial metal concentrations (P < 0.05). Different small letters on the bars indicate that the comparison differences are significant under different initial metal concentrations and at the same initial pH (P < 0.05). The vertical line on each bar shows the standard deviation.

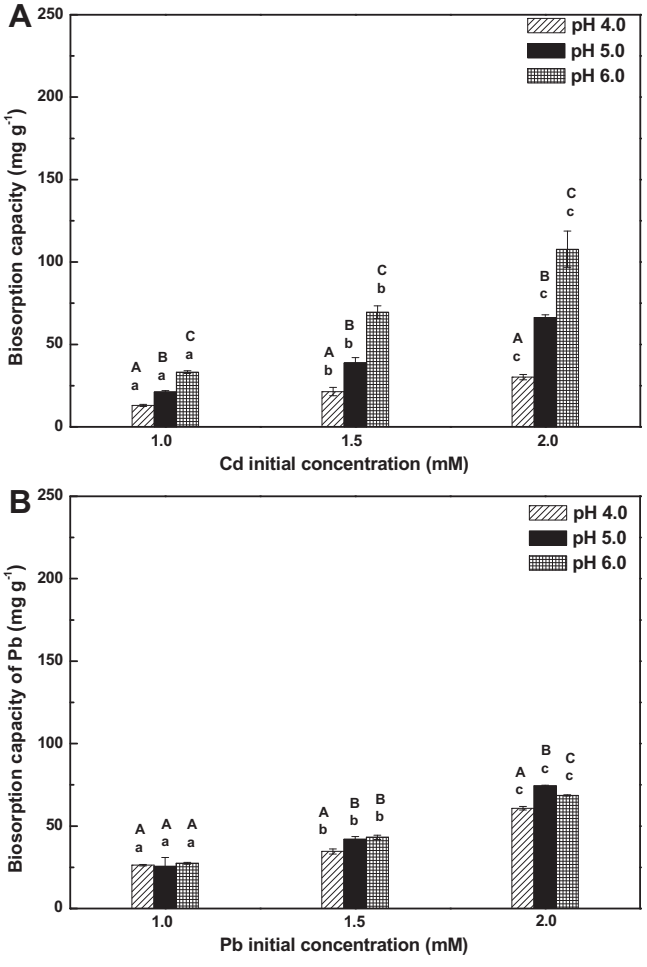

Fig. 5. Effect of initial concentrations in the treatments of mixed Cd + Pb of Cd (A), Pb (B) on biosorption capacities of *Mucor* sp. CBRF59 at initial pH values of 4, 5, and 6. Different capital letters on the bars indicate that the multiple comparison differences are significant with different initial pH values and the same initial metal concentrations (P < 0.05). Different small letters on the bars indicate that the comparison differences are significant under different initial metal concentrations and at the same initial pH (P < 0.05). The vertical line on each bar shows the standard deviation.

Pseudomonas veronii 2E. [23–28]. The maximum Cd uptake capacities by the fungi and bacteria were in the range from 8.46 to 57.9 mg g^{-1}. In our study, the maximum uptake capacities of Cd by the dead and active biomasses of CBRF59 were 108 and 173 mg g^{-1}, respectively. The biosorption value of Cd is higher than that by *Mucor* sp. from other sources. However, the value is lower than that by dead mycelia of endophytic fungus *Microsphaeropsis* sp. LSE10 of Cd hyperaccumulator *Solanum nigrum* L. [3], which might due to the different compositions of cell wall of the fungal species. Similarly, the maximum Pb uptake capacities by fungi and bacteria were between 16.9 and 50.1 mg g^{-1} [23–25,27], while the maximum uptake capacities of Pb by the dead and active biomasses of CBRF59 were 74.4 and 50.9 mg g^{-1}, respectively. The results indicated that the endophyte of hyperaccumulator might be a potential microorganism resource to remove heavy metals through biosorption or bioaccumulation [3].

So far most studies of microbial systems for removal of heavy metals have focused on biosorption by non-living or non-growing biomass [17,18]. These biosorbents need various biomass production processes, such as cultivation, harvesting, drying, processing and storage prior to use, which limit continuous operation of the systems [23,29]. Therefore, using living microorganisms for bioremoval should be a better option because of their abilities of self-replenishment, continuous metabolic uptake of metals after physical adsorption, and no need for separated biomass production processes [2]. Nevertheless, several factors (such as high metal concentrations and extreme pH) may limit the bioaccumulation in the living systems [30]. Therefore, it is crucial to examine these factors in the isolation and selection of metal-resistant strains as done in this study with various metal concentrations and pH values. If the problem of metal toxicity to the growing cell is resolved by the use of metal-resistant organisms, a self-replenishing system can be continuously operated [16]. The growing endophytic fungus CBRF59 could accumulate larger amount of Cd and Pb from solutions, which showed the potential to remediate contaminated solutions continuously.

The metal resistant microbes can tolerate the heavy metals by accumulation, efflux or other resistant mechanisms. In living cells, accumulation is the main intracellular accumulation realized by the transport process across the cell membrane. During the process of metal accumulation, an efflux mechanism can be functioning at a certain metal concentration preventing more metal accumulation [31,32]. Therefore, the removal efficiency of living cells was affected by the ambient metal concentration. When the initial Cd concentrations increased from 0.1 to 0.5 mM, the accumulation maybe predominated. The similar results were obtained from growing *Tetraselmis suecica*, when the Cd concentration increased from 0.6 to 45 mg L^{-1} in the medium, total Cd removed by growing *T. suecica* increased, although Cd was toxic and affected its growth [33]. With the initial Cd concentrations increased from 0.5 to 1.0 mM, the efflux probably was the predominated process, leading to the decline in bioaccumulation percentages. When the Cd concentration increased up to 1.5 mM, some enzymes or compounds (such as metal-binding peptides) was synthesized by the fungus to detoxify the metals in cells, the bioaccumulation percentages increased again [29].

The metal uptake by microorganisms from contaminated media can be divided into two categories [2]. One is biosorption by non-living or non-growing biomass, which is a metabolism-independent and passive uptake process. Another is bioaccumulation by living and growing cells, which is mainly an intracellular accumulation. In most cases of this study, the biosorption capacities by dead biomass of CBRF59 were higher than the bioaccumulation capacities by active biomass. The result could be attributed to the increase in both surface area and exposure of intracellular binding sites caused by heat-dried cells and to the absence of competition by H^+ ions as produced by living cells [31,34,35]. Our results also showed that the pH decreased when the fungus CBRF59 was cultivated in PDB with heavy metals (the data were not shown). It was also probable that, in the active biomass, the metal bioaccumulation could happen inside the cells but the efflux mechanisms might predominate, thus affecting the uptake efficiency [31,34]. Other reports suggested that the bioaccumulation capacities of heavy metals were higher than the biosorption capacities by dead biomass [2,36,37]. The different results may be attributable to the different resistant mechanisms of microorganisms to heavy metals. In the combined Cd + Pb solutions, the bioaccumulation and biosorption capacities of Cd or Pb increased with the increase of their ratios to the total concentration (3.0 mM). The highest bioaccumulation and biosorption capacities of Cd by biomass in the mixed solutions were obtained in the solution 2.0 mM Cd + 1.0 mM Pb. The highest bioaccumulation and biosorpton capacities of Pb were found in the 1.0 mM Cd + 2.0 mM Pb solution. The possible explanation of the results was that the higher initial concentrations of metals resulted in a strong driving force [1]. The biosorption or bioaccumulation capacities of both metals in the combined Cd + Pb solutions increased with the initial pH values. The dependence of the biosorption or bioaccumulation capacities on pH values should be attributed to hydrogen ions that compete with metal ions on the sorption sites [3,24].

It is shown that low bioavailability of soil metals may be a rate limiting factor for metal absorption by plants [21,22]. Microorganisms with high activities and large specific surface areas can potentially act as microbial chelates to affect heavy metal bioavailability in soils [6,21,22,38]. Many microbes, which have the ability to increase heavy metal bioavailability, have also showed profound effects on phytoremediation of heavy metals from soils. For example, rhizosphere and endophytic bacteria greatly increase the bioavailability of Cu and Pb in soils and enhance metal uptake by plants from soils [6,22]. The inoculation with the ectomycorrhizal fungus *Paxillus involutus* Pax2 can increase the bioavailability of Cd and Zn in soils and increased phytoextraction of Cd and Zn by willows [39]. More such studies have been reported in the literature [40,41]. Our results showed that the addition of the active mycelia of CBRF59 significantly increased the availability of soil Pb and Cd compared to the non-inoculated control soil. The Pb and Cd mobility in the soil was probably directly associated with the fungal activity [6,42]. During the processes, the active strain CBRF59 might synthesize some chemical compounds (such as siderophores, organic acids) to improve heavy metal availability [22,42,43]. Therefore, the strain *Mucor* sp. CBRF59 may have the potential for the phytoremediation of Pb and Cd from the soil.

5. Conclusions

A fungal endophyte CBRF59 tolerant Cd and Pb was isolated from rape roots and was identified as *Mucor* sp. The active mycelia of the endophytic fungi could accumulate Cd and Pb from solutions and enhance the amount of water-soluble metals in soil. Therefore, the results provide new insight into the plant-endophyte partnerships for improving bioremediation of the solutions and soils contaminated by Cd and Pb.

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