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ENVIRONMENTAL BIOTECHNOLOGY

Cadmium induced oxalic acid secretion and its role in metal uptake and detoxification mechanisms in *Phanerochaete chrysosporium*

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Abstract This study examines the role of oxalic acid in the uptake of Cd and participation in detoxification process in *Phanerochaete chrysosporium*. Cd-induced oxalic acid secretion was observed with growth inhibition and enzyme inactivation (LiP and MnP) of *P. chrysosporium*. The peak value of oxalic acid concentration was 16.6 mM at initial Cd concentration of 100 mg L^{-1}. During the short-term uptake experiments, the uptake of Cd was enhanced and accelerated in the presence of oxalic acid and resulted in alleviated growth and enzyme inhibition ratios. The formation of a metal-oxalate complex therefore may provide a detoxification mechanism via effect on metal bioavailability, whereby many fungi can survive and grow in environments containing high concentrations of toxic metals. The present findings will advance the understanding of fungal resistance to metal stress, which could show promise for a more useful application of microbial technology in the treatment of metal-polluted waste.

Keywords *Phanerochaete chrysosporium* · Cd uptake · Detoxification · Oxalic acid · Ligninolytic enzymes

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Introduction

White-rot fungi have been widely applied as biosorbents for heavy metals in wastewater treatment due to their capability of accumulating high concentrations of heavy metals. Particularly, *Phanerochaete chrysosporium*, as a representative species of white-rot fungi, has been most extensively studied based on its favorable heavy metal adsorption ability (Pakshirajan and Swaminathan 2009; Chen et al. 2011; Xu et al. 2012a, 2013). The use of *P. chrysosporium* in contaminated aquatic system bioremediation is of considerable interest as a branch of exciting low-cost and eco-friendly technology. Despite these advances, however, important questions involving heavy metal-induced toxicity to biosorbents and improvement of their metal-accumulating capacity and metal tolerance during environmental applications tend to be important (Arinia et al. 2014; Vadas and Ahner 2009). The exposure of microorganisms to heavy metals always inhibits microbial growth and metabolism because of the adverse effect on fungal reproduction and physiological metabolism (Baldrian et al. 2000; Say et al. 2001). The potential toxicity of metals therefore should be taken into account when considering any biological treatment of polluted soil or water.

To alleviate the damage caused by excess heavy metal, fungi evolved active defense mechanisms which are usually based on immobilization of heavy metals via extracellular and intracellular chelating compounds (Baldrian 2003). The mechanisms of immobilization by fungi included simple chelation or precipitation in their cell walls or extracellular matrices, e.g., by the formation of organic acid crystals (Gadd 1993; Jarosz-Wilkołazka and Grąz 2006). Among all chelating compounds, oxalic acid has been shown to be the predominant organic acid produced by many white-rot fungi as the typical extracellular metal chelator (Jin et al. 2014; Mäkelä

et al. 2010). The extracellular secretion of oxalic acid by *P. chrysosporium* under metal stress provides a means of metal detoxification by immobilizing soluble metal ions as metal-oxalate crystals (Shimadaa et al. 1997; Dutton, et al. 1993). Consequently, the production of oxalate-metal complexes may provide a mechanism whereby oxalic acid-secreting fungi can tolerate environments (wood or soil) containing high concentrations of toxic metals in either soluble or insoluble compounds.

Indeed, the efficient use of *P. chrysosporium* in heavy metal removal would be facilitated by investigating the effect of heavy metal on *P. chrysosporium* during heavy metal bioremediation. Previous researches mostly focused on the toxicity of Cd on the growth, extracellular protein, and extracellular ligninolytic enzymes of fungi (Baldrian and Gabriel 1997; Hatvani and Mécs 2003), but no details on the role of oxalic acid in the heavy metal uptake and fungi detoxification mechanisms have been discussed so far. Therefore, the main aim of this study was to gain insights into the toxicity and the detoxification of oxalic acid for *P. chrysosporium* at different initial concentrations of Cd during liquid fermentation. Real-time changes in mycelia dry biomass, Cd concentrations, lignin peroxidase (LiP), and manganese peroxidase (MnP) of *P. chrysosporium* under different Cd stress were studied in the present study. The mediated role of oxalic acid in Cd uptake in *P. chrysosporium* was also evaluated by determining the removal efficiency with the addition of oxalic acid.

Materials and methods

Materials and strains

The *P. chrysosporium* strain BKMF-1767 (ATCC 24725) was purchased from the China Center for Type Culture Collection (Wuhan, China). Stock cultures were maintained on potato dextrose agar (PDA) slants at 4 °C and then transferred to PDA plates at 37 °C for 6 days. To obtain inoculum, spore suspensions were prepared by gently scraping the spores on the agar surface and then blending in sterile distilled water. The spore suspension concentration was measured by a microscope with a blood cell counting chamber and adjusted to 2.0×10^6 mL^{-1}.

All the chemicals and reagents used in the experiment were of analytical reagent grade except for H_3PO_4 and CH_3OH which were of chromatographic reagent grade and were used without any further purification. Throughout this study, ultrapure water was used for the preparation of all the solutions.

Liquid-state cultivation conditions

Liquid-state cultivation was performed in 250 mL Erlenmeyer flasks containing 100 mL Kirk's liquid culture medium. Liquid culture medium was mixed thoroughly with $Cd(NO_3)_2$ solutions prepared from analytical grade and controlled the final Cd levels at 0, 20, 50, and 100 mg L^{-1}, respectively; 2.0 mL of aqueous spore suspension of *P. chrysosporium* was inoculated into 100 mL growth medium as described by Kirk et al. (1986). Finally, the mixture was incubated in a constant temperature incubator with a constant speed of 120 rpm at 30 °C for 18 days. All the experiments were carried out in triplicate and data presented were the mean values from these independent experiments.

Cd content determination

Cd concentration was not only a vital factor for the growth of fungi but also on the bioavailability of the metal (Huang et al. 2006). After fermentation for 2, 4, 6, 9, 12, and 18 days, liquid growth media were filtered through a 0.45 μm cellulose filter paper for Cd concentration assays. In consideration of the chelation of Cd by oxalic acid, deionized water and HNO_3 (2 %) were used as extraction solution for insoluble or soluble oxalic acid release. Cd concentrations were determined by an atomic absorption spectrometer (AAS, Agilent 3510, USA).

Biomass assay

The biomass level is a very important factor for toxicity analysis of cadmium to *P. chrysosporium*. After fermentation for 2, 4, 6, 9, 12, and 18 days, *P. chrysosporium* mycelium pellets were harvested from the flasks for biomass assay. The mycelium pellets were filtered through preweighted Whatman No. 1 filter papers and rinsed thoroughly thrice with deionized water and then dried to a constant weight as a measure of fungal dry weight (DW) using a vacuum freeze dryer (FD-1C-50, China) at −60 °C for 24 h. In order to better understand the Cd-induced growth inhibition of *P. chrysosporium*, growth inhibition ratios were defined and calculated as follows:

$$\text{Inhibition ratio}(\%) = \left(M_{\text{control}} - M_{\text{sample}}\right)/M_{\text{control}} \times 100 \quad (1)$$

where M_{control} and M_{sample} represent the dry biomass in the absence (M_{control}) and presence (M_{sample}) of Cd, respectively.

Ligninolytic enzyme analyses

Liquid growth medium samples were filtered through 0.45 μm syringe filters using cellulose filter paper for ligninolytic peroxidase activity analyses at 2, 4, 6, 9, 12, and 18 days. In this assay, two main ligninolytic peroxidases, LiP and MnP, were measured using a UV–vis spectrophotometer (UV-2250, SHIMADZU, Japan). All ligninolytic peroxidase activities were expressed in units per milliliter. LiP activity was determined by monitoring the formation rate of veratryl

aldehyde in the presence of H_2O_2 via the change in absorbance of reaction mixture at 310 nm (Huang et al. 2008). MnP activity was determined according to the method described by Huang et al. (2008), which was based on the oxidation of Mn^{2+} to Mn^{3+}.

Analysis of oxalic acid production

Oxalic acid in the extracellular secretions of *P. chrysosporium* was detected through high-performance liquid chromatography (HPLC) performed on an Agilent 1100 apparatus equipped with a UV–vis variable wavelength detector (VWD) (Li et al. 2011). A spare sample was centrifuged at 5,000 rpm for 15 min at 20 °C and then filtered through 0.45 μm syringe filters using cellulose filter paper for HPLC analysis. Phosphoric acid (0.2 %*v*/*v*) was used to be the mobile phase and was applied to a 4 min period at a flow rate of 0.5 mL min^{-1} with a constant detection wavelength at 210 nm. The analytical column was reversed-phase C_{18} column and maintained at 30 °C. Ten microliters of aqueous standard samples were injected into the liquid chromatograph via a 20 μL loop at ambient temperature.

Short-term Cd uptake experiments

In order to confirm the modulated role of oxalic in Cd uptake and *P. chrysosporium* detoxification process, short-term Cd uptake in the presence of oxalic acid has been investigated. *P. chrysosporium* were exposed to various solution treatments containing Cd and oxalic acid. The *P. chrysosporium* were grown on Kirk's liquid culture for 3 days and then washed with distilled ultrapure water three times and collected for Cd uptake experiments; 1.0 g (wet biomass) of *P. chrysosporium* pellets were added to a 50 mL Cd-containing solution. Time course of Cd uptake was also conducted in the presence of 1.0 mM Cd and 0.5 mM oxalic acid. The oxalic acid concentration was controlled at 0, 0.2, 0.5, 1.0, 2.0, and 4.0 mM together with 1.0 mM Cd to investigate the effect of oxalic acid on Cd uptake. LiP and MnP activities of *P. chrysosporium* exposed to 0.5 mM Cd in the presence of 0.5 mM oxalic acid were detected. Meanwhile, *P. chrysosporium* biomass was also exposed to various initial Cd concentrations at 0.2, 0.5, and 2.0 mM, with the addition of 1.0 mM oxalic acid.

Exposure of *P. chrysosporium* spores to Cd

Further study was conducted via investigation of the spore batch growth condition in the presence of Cd and/or oxalic acid on the PDA medium to determine the detoxification role of oxalic acid. *P. chrysosporium* spores were inoculated to PDA media containing 0.5 mM oxalic acid, 0.2 mM Cd, and both 0.2 mM Cd and 0.5 mM oxalic acid. PDA media without Cd and oxalic acid were used as control samples. After 3 days of incubation, images of those samples were collected to investigate the growth conditions of those tested samples.

Results

Removal of Cd at different initial concentrations

P. chrysosporium were grown for 18 days in the presence of Cd (0–100 mg L^{-1}). In all the tested Cd concentrations, Cd uptake occurred during the whole growth stage. As shown in Fig. 1, Cd concentrations in all groups went down sharply with increasing time at the early stage of fermentation (first 2 days). A slow decrease of Cd concentration was then observed from 4 to 9 days. After 9 days of cultivation, the removal efficiencies of Cd were 40.85, 41.76, and 47.04 % at 20, 50, and 100 mg L^{-1} Cd, respectively. Data on removal efficiency of Cd concentration indicated that *P. chrysosporium* could accumulate metals from the substrate. After this equilibrium period, the concentration of Cd appeared to have a slight increase from 12 to 18 days. It suggested that desorption took place, and the reason possibly was that the heavy metal-binding capacity is dependent on the mycelia age (Yetis et al. 2000). Actually, release of Cd has been observed after acid treatment, which further confirmed the formation of chelate Cd. The differences of Cd biosorption capacities after HNO_3 treatment were 3.75, 2.99, and 2.07 mg g^{-1} (dry biomass) at concentrations of 20, 50, and 100 mg L^{-1} Cd, respectively, after reaching the adsorption equilibrium on day 9. The difference was mainly because nitric acid extraction strongly released soluble cadmium oxalate crystals.

Cd-induced growth inhibition in *P. chrysosporium*

As one of the most popular complex phenomena, microbial growth was studied from the point of heavy metal toxicity (Baldrian 2003). The specific growth rate of *P. chrysosporium* exposed to various concentrations of Cd is shown in Fig. 2, in

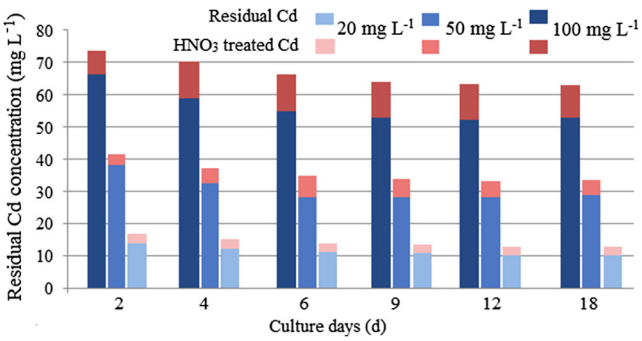

Fig. 1 Changes in Cd concentration during Kirk's liquid-state fermentation by *P. chrysosporium* in the treatments with different initial Cd concentrations of 0, 20, 50, and 100 mg L^{-1}

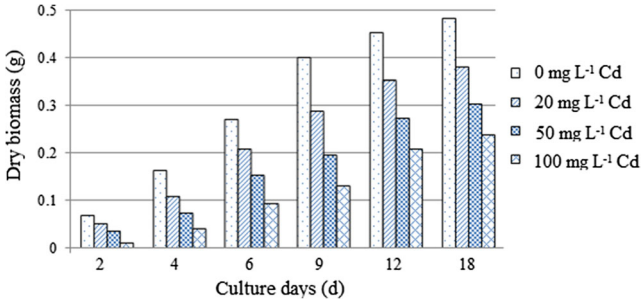

Fig. 2 Biomass of *P. chrysosporium* grown in Kirk's liquid culture media with different initial Cd concentrations after 2, 4, 6, 9, 12, and 18 days of growth

the case of dry biomass of *P. chrysosporium*. The addition of Cd immediately affected the growth of *P. chrysosporium*. Growth inhibition was greatest in *P. chrysosporium* grown with high Cd concentrations and was most marked at 100 mg L^{-1} Cd. Exposure to Cd at 20, 50, and 100 mg L^{-1} caused reductions in dry biomass at 24.7, 47.6, and 85.3 %, respectively, within 48 h. With the increase of fermentation time, the dry weight biomass increased under all Cd concentrations, with a decrease in growth inhibition. As example, the dry weight of fungi pellets was reduced by 21.4, 37.3, and 50.7 % at Cd concentrations of 20, 50, and 100 mg L^{-1} after 18 days of incubation, respectively, as compared to the control.

Effect of Cd on ligninolytic enzymes

Heavy metals in general are potent inhibitors of enzymatic reactions, by directly interacting with extracellular enzymes of fungi (Stohs and Bagchi 1995; Vallee and Ulmer 1972). In white-rot fungi, most attention has been paid to the metal toxicity toward extracellular enzymes LiP and MnP. The activities of LiP and MnP during liquid-state fermentation at different initial Cd concentrations are shown in Fig. 3. Low LiP activities were observed in the initial stage, and a weak decrease appeared from days 2 to 4 with a wave trough on day 4, then there was a dramatic increase from day 4 to day 9, and the maximum level of activity was observed on day 9, after which the level of activity decreased rapidly. Meanwhile, a local maximum level of MnP activity occurred on day 4. The maximum level of MnP activity, which was detected by monitoring the formation of Mn(III)-malonate complexes, was 0.61 U mL^{-1}, and the respective LiP activity level was 0.21 U mL^{-1}.

In each case, however, the LiP and MnP activities were considerably lower than those in the absence of Cd, which indicated the Cd-induced enzyme inhibition in *P. chrysosporium*. For example, the maximal MnP activities were 0.61, 0.54, and 0.50 U mL^{-1} on day 4 at Cd concentrations of 0, 20, and 50 mg L^{-1}, respectively, whereas a peak value at 100 mg L^{-1} of Cd was delayed to 6 days at the value

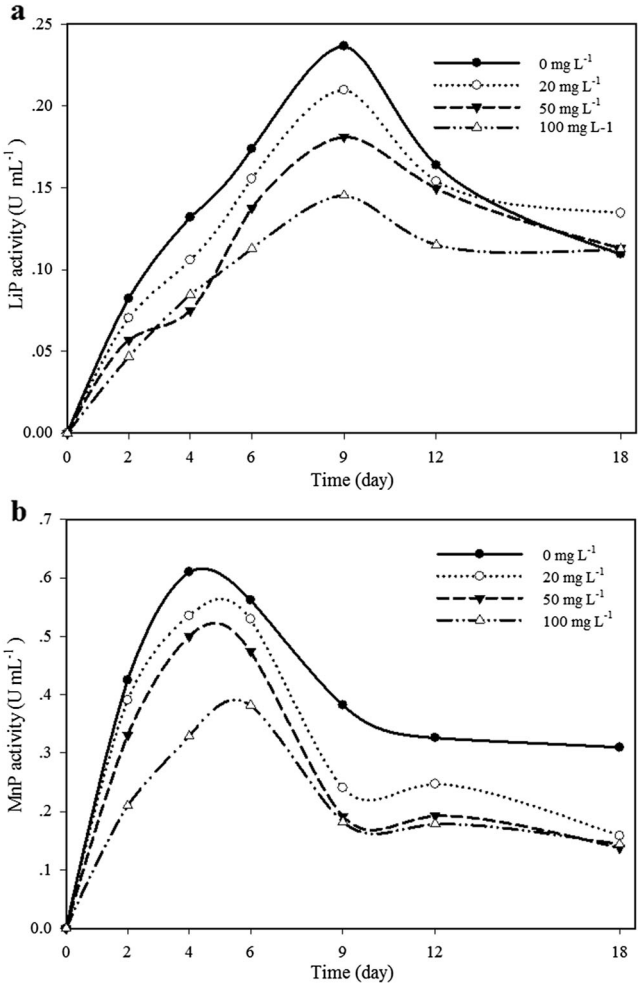

Fig. 3 Activities of LiP (**a**) and MnP (**b**) in *P. chrysosporium* under the exposure of various Cd concentrations (0, 20, 50, and 100 mg L^{-1})

of 0.38 U mL^{-1} (Fig. 3b). A distinct inhibition ratio was therefore calculated at the value of 0.377 compared with the control sample and sample in the presence of 100 mg L^{-1} Cd.

Oxalic acid production under Cd exposure

It has been pointed out that oxalic acid was the main and important metabolite with an elaborated response to metal exposure in white-rot fungi (Hastrup et al. 2006; Huang et al. 2008). Among fungi, oxalic acid is a very common metabolite as consequent byproducts in the tricarboxylic acid (TCA) cycle, which was biosynthesized by either oxaloacetate or glyoxylate (Shimadaa et al. 1997). In all experiments, oxalic acid concentrations varied with the grown mycelia during incubation (Fig. 4a). In the initial stage of liquid fermentation, there was a faint fluctuation before 4 days and then a sharp increase appeared. There was a small amount of accumulation for oxalic acid before 4 days possibly because of the production of absorbed oxalic acid and insoluble oxalate-metal complexes, which were formed by chelating metal

cations (Jarosz-Wilkołazka and Grąz 2006). Overall, in the case of fungal growth, large amounts of oxalic acid were detected in the growth medium. Exogenous Cd induced oxalic acid secretion at a concentration of 100 mg L^{-1}, with peak values at 16.63 mM on day 12, while others were 6.3 (0), 10.9 (20 mg L^{-1}) and 13.2 mM (50 mg L^{-1}), respectively. Moreover, negative correlations have been found between oxalic acid concentration and Cd-induced growth inhibition ratios. As examples, negative correlation coefficients at values of 0.8795, 0.6866, and 0.7809 have been observed at the initial Cd concentrations of 20, 50, and 100 mg L^{-1} Cd, respectively (Fig. 4b). Results confirmed that oxalic acid plays an important role in Cd detoxification, based on the fact that the higher oxalic acid secretion was correlated with the lower growth inhibition ratios.

Modulated role of oxalic acid in Cd uptake in *P. chrysosporium*

The time course of Cd removal in the absence and presence of oxalic acid is presented in Fig. 5. Biosorption capacity after Cd treatments was calculated based on wet biomass. The results showed that the biosorption of Cd in *P. chrysosporium* varied significantly and was apparently related to the exposure period with an almost linear increase with duration of exposure. The samples with oxalic acid addition were found to have considerably higher Cd biosorption capacity than those without oxalic acid at all times. In the absence of oxalic acid, the biosorption capacity remained 1.17 mg g^{-1}, while the biosorption capacity elevated to 1.79 mg g^{-1} after 6 h of exposure with the addition of 1 mM oxalic acid. Besides biosorption capacity, mean uptake rate also augmented in the presence of oxalic acid, and the mean uptake rate was increased from 0.1953 to 0.2987 mg g^{-1} h^{-1} after the addition of oxalic acid, indicating that oxalic acid addition might enhance and accelerate Cd uptake. In contrast to free Cd, Cd in partially chelated oxalic acid as Cd-oxalate complex, the initial biosorption capacity (0.16 mg g^{-1}) might be due to the chelate of Cd-oxalate.

In addition, we also examined the uptake of Cd by *P. chrysosporium* in the presence of increasing oxalic acid, with increasing metal/oxalic acid ratios from 1:0.2 up to 1:4. As shown in Fig. 6a, the Cd concentration decreased in all cases. The Cd concentrations decreased continuously with the elevation of exogenous oxalic acid addition. As the oxalic acid increased from 0.2 to 2.0 mM, the Cd uptake promoted from 1.41 to 2.63 mg g^{-1}. Importantly, there was a positive correlation between initial oxalic acid concentration and Cd uptake capacity (Fig. 6a, R^2=0.8892). Meanwhile, pH variations at the different oxalate concentrations after Cd biosorption were further examined. As shown in Fig. 6a, decrease of pH value after Cd uptake occurred. Commonly, the decrease of solution pH was mainly ascribed to the release of proton due to the ion exchange-induced biosorption mechanism in microorganisms ($2RH+M^{n+}=R_nM+nH^+$). In addition, a previous study also reported that secretion of organic acids also attributed to the acidification of the solution. The release of organic acids that both sequester cations and acidify the microenvironment is thought to be a major mechanism of extracellular chelation in fungi (Gadd 1993). However, remittent decline in pH value was found with exogenous oxalic acid. The results might be ascribed to the fact that the biosorption of Cd was accompanied with uptake of oxalic acid, resulting in the decrease of oxalic acid in the Cd-containing solution contributing to the faint decline in solution pH. In an acidic environment, the conjugated Cd-oxalate form of oxalic acid is transported

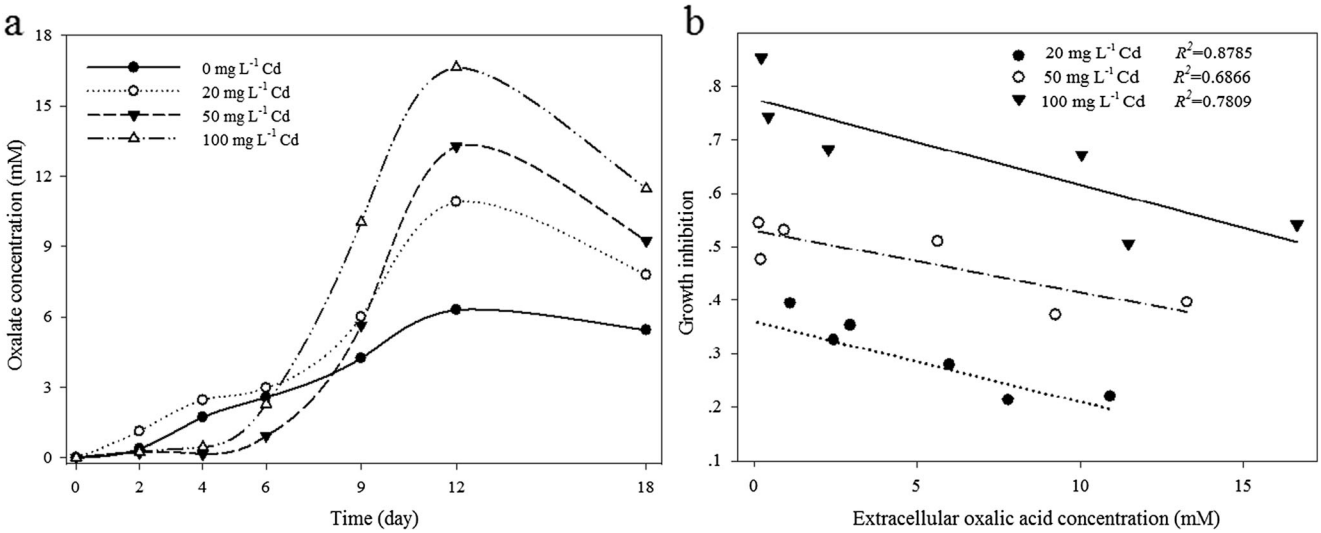

Fig. 4 **a** Oxalic acid secretion during Kirk's liquid-state fermentation by *P. chrysosporium* in the treatments with different initial Cd concentrations of 0, 20, 50, and 100 mg L^{-1}; **b** correlation between extracellular oxalic acid secretion and growth inhibition ratios

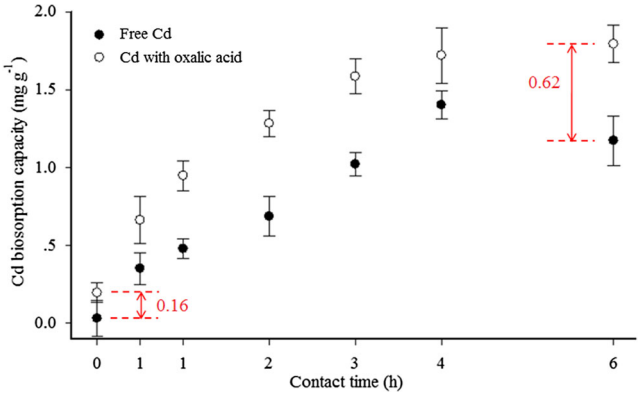

Fig. 5 Time course of Cd uptake in the absence and presence of oxalic acid. pH 5.0, temperature 35 °C, Cd concentration 1.0 mM, biosorbent dosage 2.0 g L^{-1}

through the cell membrane into the *P. chrysosporium* with the accumulation of Cd intracellularly. Moreover, enhancement of Cd uptake was also observed at various initial Cd concentrations. Especially, at the concentration of 2.0 mM Cd, the uptake capacity increased from 2.02 to 3.49 mg g^{-1} in the presence of 0.5 mM oxalic acid (Fig. 6b).

Detoxification role of oxalic acid in Cd-exposed *P. chrysosporium*

Ligninolytic enzymes (LiP and MnP) under Cd exposure (0.5 mM) were investigated in the absence and presence of oxalic acid (0.5 mM). Results shown in Fig. 7a revealed that Cd exposure caused the reduction of both LiP and MnP activities, and the ratios of change at the values of 68.62 and 51.23 % were calculated for LiP and MnP, respectively. However, alleviated LiP and MnP activities have been observed, which indicated that cells have undergone less severe toxicity in the presence of oxalic acid than those grown in the Cd-containing medium. The toxic effects of Cd at their respective concentrations were prevented by the addition of oxalic acid, indicating the detoxification role of oxalic acid. In order to further confirm the possible function of oxalic acid in Cd detoxification, *P. chrysosporium* spores were inoculated into the PDA medium with Cd (0.2 mM) and oxalic acid (0.5 mM). The growth of cells in the PDA medium was used as a sensitive measure of the toxic effects of heavy metals. As shown in Fig. 7b, after 3 days of incubation, a well-proportioned growth of *P. chrysosporium* covered the PDA culture dishes, and a similar growth condition has also been observed with the addition of oxalic acid (0.5 mM). However, distinct differences were observed with the presence of 0.2 mM Cd, confirming that the presence of Cd inhibited the growth of *P. chrysosporium*. An interesting result was found where the addition of oxalic acid to Cd-containing PDA medium promoted the growth of *P. chrysosporium* compared to those exposed to Cd ions, due to the remittent toxicity of Cd in the presence of oxalic acid.

Discussion

In recent years, applying biotechnology in controlling and removing metal pollution tends to be an attractive alternative to traditional technologies and gradually becomes a hot topic in the field of metal pollution control because of good affinity and low costs of biomaterials (Wang and Chen 2009; Xu et al. 2012b; Yetis et al. 1998). Nowadays, the potential application of white-rot fungi in the treatment of heavy metal-contaminated wastewater and in the recovery of metals in mining wastes or in metallurgical effluents is widely reported. In our study, uptake of Cd has been found during the growth stage of *P. chrysosporium*. It was widely reported that white-rot fungi are capable of accumulating metal ions in their cells

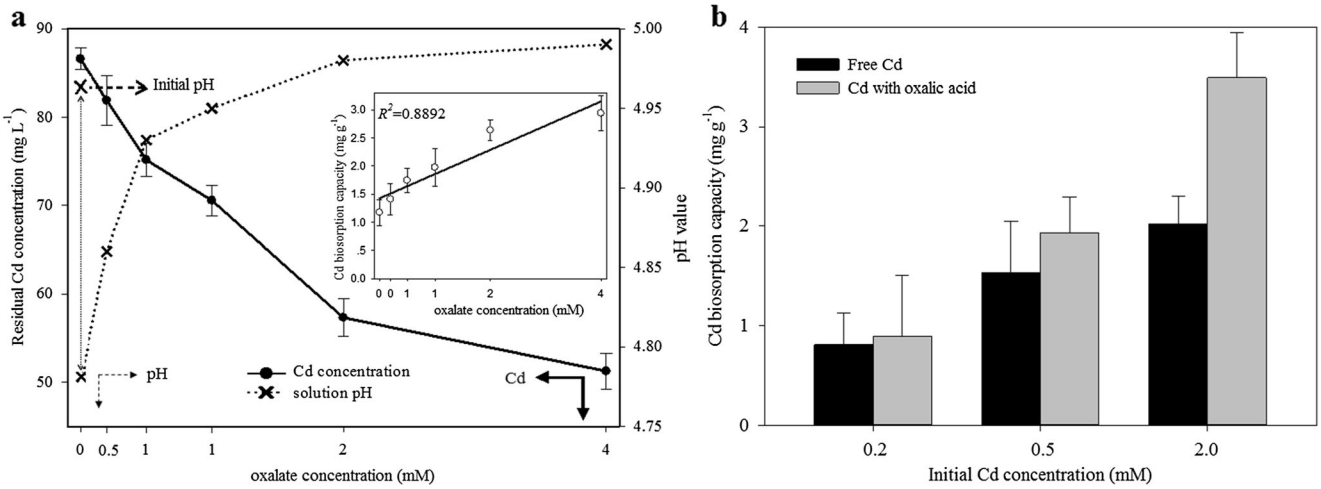

Fig. 6 **a** Cd uptake at various oxalic acid concentrations ranging from 0 to 4.0 mM; **b** Cd uptake at initial Cd concentrations of 0.2, 0.5, and 2.0 mM in the absence and presence of 1 mM oxalic acid

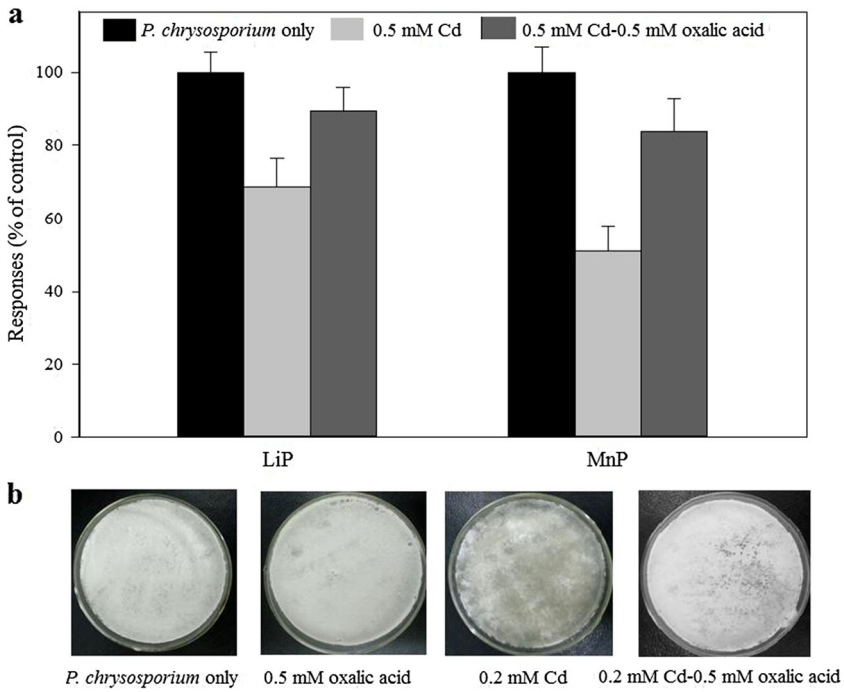

Fig. 7 **a** Responses of ligninolytic enzyme activity of *P. chrysosporium* exposed to 0.5 mM Cd in the absence and presence of oxalic acid (0.5 mM). Responses were calculated as $A_{sample}/A_{control} \times 100$ % and ligninolytic enzyme activity in the absence of Cd and/or oxalic was defined as 100 %; **b** growth images of *P. chrysosporium* at various conditions

by intracellular uptake, as many researchers validated, and can also be chelated with metal ions by the carboxyl, hydroxyl, or other active functional groups on cell wall surface and extracellular secreta (Baldrian 2003; Say et al. 2001).

Inhibition of fungal growth by heavy metals is a known phenomenon. Cd is an abundant, nonessential element that is generating concern due to its accumulation in the environment and nondegradability with a half-life of 10–30 years as a result of its increasing use in industrial applications. Cd and its associated toxicity are well known for its various adverse effects, e.g., growth inhibition, disturbances of enzyme functions, and even cell death. Indeed, Cd exposure inhibited growth and metabolism in *P. chrysosporium* and, as the result, affected extracellular enzyme levels. In our study, it was apparent that throughout the whole fermentation period, strong inhibition occurred at high levels of Cd. However, no significant effect on LiP and MnP activities was found at low Cd concentration (e.g. 20 mg L^{-1}), which was mainly ascribed to the tolerance of *P. chrysosporium* (Fig. 3). This observation was in good agreement with the previous reports that Cd in concentrations of 0.5–1.0 mM (56–112 mg L^{-1}) remarkably inhibited the activities of MnP and LiP of *P. chrysosporium*, whereas Cd at 20 mg L^{-1} did not affect the ligninolytic enzymes dramatically (Baldrian 2003). Accordingly, Cd is a competitive inhibitor of MnP, by inhibition of MnPI and MnPII reduction processes with generation of the MnP-Cd^{2+} complex. The flexibility of the Mn-binding site in MnP allows a wide variety of metal ions to bind and is the most likely site for Cd binding, accompanied with similar ionic radii (0.95 Å for hexacoordinated Cd and 0.83 Å for Mn^{2+}) (Shannon 1976; Youngs et al. 2000). Meanwhile, the presence of heavy metals can interfere with carbon- and energy-supplying systems of ligninase of *P. chrysosporium* (Baldrian 2003), leading to the inhibition of ligninolytic extracellular enzymes (both LiP and MnP).

Metals could be immobilized outside the hyphae by simple adsorption or precipitation in their cell walls or extracellular matrices, e.g., in some cases, with the subsequent formation of insoluble organic acid crystals. Organic acid chelation tends to be regarded as an important tolerance mechanism. In *P. chrysosporium*, a previous study has reported that *P. chrysosporium* could produce organic acids (e.g., citric, oxalic, gluconic, fumaric, lactic, and malic acids), which may chelate toxic metals, resulting in the formation of metallo-organic molecules (Huang et al. 2008; Gadd 1993). In our study, rapid and Cd-dependent induction of oxalic acid occurred. Peak values of oxalic acid occurred on day 12 at the concentration of 100 mg L^{-1} Cd (Fig. 4). The substantially higher oxalic acid secretion accompanying a higher initial Cd concentration in *P. chrysosporium* signifies cellular demand for oxalic acid, probably based on the higher consumption of oxalic acid for extracellular Cd chelation. In some cases, substantial accumulation of insoluble metal-oxalate crystals adhering to the hyphae has been found in many fungi, including both the brown and white-rot fungi (Tait et al. 1999). Actually, the release of oxalic acid participated in the sequestration of cations extracellularly via the formation of oxalate crystals. For example, calcium, zinc, and cobalt oxalate crystals formed in *Trametes versicolor* have been observed and characterized when grown on a medium amended with a wide range of insoluble metal compounds, such as ZnO, $Co_3(PO_4)_2$, and $CaCO_3$ plates (Jarosz-Wilkolazka and Gadd

2003). In view of the possible roles of extracellular chelation and surface adsorption in biosorption, the concentrations of Cd in solution after acid extraction were observed in relation to the initial Cd concentration. It was clearly observed that after acid extraction, the soluble Cd contents increased, mainly due to the chelation role of extracellular secreta (Fig. 1). In our previous study, the precipitation of strip-shaped crystals containing both lead and carbon around mycelia has been observed from Pb-polluted substrate, with a Pb-rich composition, facilitating the accumulation of heavy metals by chelating as insoluble oxalates (Huang et al. 2008).

To better understand the mediated role of oxalic acid in Cd uptake and possible function in Cd detoxification, it is necessary to fully understand the pathways of oxalic acid detoxification in *P. chrysosporium*. For that, it will be particularly relevant to increase our knowledge about the regulation of oxalic acid under Cd exposure. It was apparent that exogenous oxalic acid could promote the Cd uptake in *P. chrysosporium* (Fig. 5). However, Cd toxicity correlated to Cd accumulation was found palliated even though with the higher Cd uptake, based on the remittent inhibition ratio of LiP and MnP under Cd exposure.

Although the toxicity of Cd was correlated with the concentration, the addition of oxalic acid alleviated the Cd toxicity via the variation in Cd bioavailability. Generally, a major factor governing the toxicity of a metal is its bioavailability, which is considered as the fraction of the total contaminant in the interstitial water and soil particles that is available to the receptor organism. The toxicity and bioavailability of a heavy metal, in turn, depend in part on reactivity and solubility, i.e., the extent to which they are absorbed or stored in internal organs. For example, acetates of Cd, Pb, and Zn were less toxic than the respective sulfates to ammonification and nitrification (Vig et al. 2003). Additionally, the bioavailability of heavy metals is also governed by the nature of microorganisms and microbially mediated processes. Oxalic acid, a kind of organic acid with particular chemical and biological activity, often played a major role in alleviating metal toxicity via variation of the mobility and bioavailability of heavy metals (Brown et al. 1999; Chojnacka et al. 2005). In the present study, the secretion of oxalic acid in *P. chrysosporium* provides a means of decreasing bioavailability and conferring tolerance via immobilization of soluble Cd ions to an insoluble metal-oxalate complex. Meanwhile, palliative growth inhibition and ligninolytic enzyme inactivation have been found with the addition of oxalic acid. The concentration of Cd (0.5 mM) that inhibited MnP activity by 51.23 % either stimulated or caused only a modest inhibition of 83.87 % with the exogenous addition of 0.5 mM oxalic acid. Meanwhile, *P. chrysosporium* grown in binary combinations of oxalic acid and Cd in PDA medium was observed with the remittent growth inhibition of *P. chrysosporium* (Fig. 7). Results from this study were consistent with the reports that oxalic acid could facilitate the immobilization of soluble metal ions and form metal oxalates with Cd.

In summary, Cd was discovered to be a strong inhibitor of enzymes, and this inhibition increased with increasing Cd concentrations. High inhibition ratios of dry biomass and ligninolytic extracellular enzymes (LiP and MnP) were observed under Cd exposure. Supplementation of oxalic acid significantly relieved inhibition but also accompanied by the promoted Cd uptake in *P. chrysosporium*, due to the variation of Cd bioavailability via the formation of the Cd-oxalate complex. The findings reported herein would greatly help in better understanding the tolerance to heavy metals and designing optimal conditions for heavy metal uptake during wastewater treatment.

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