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Heavy metal-induced glutathione accumulation and its role in heavy metal detoxification in *Phanerochaete chrysosporium*

Piao Xu · Liang Liu · Guangming Zeng · Danlian Huang · Cui Lai · Meihua Zhao · Chao Huang · Ningjie Li · Zhen Wei · Haipeng Wu · Chen Zhang · Mingyong Lai · Yibin He

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Abstract *Phanerochaete chrysosporium* are known to be vital hyperaccumulation species for heavy metal removal with admirable intracellular bioaccumulation capacity. This study analyzes the heavy metal-induced glutathione (GSH) accumulation and the regulation at the intracellular heavy metal level in *P. chrysosporium*. *P. chrysosporium* accumulated high levels of GSH, accompanied with high intracellular concentrations of Pb and Cd. Pb bioaccumulation lead to a narrow range of fluctuation in GSH accumulation (0.72–0.84 μmol), while GSH plummeted under Cd exposure at the maximum value of 0.37 μmol. Good correlations between time-course GSH depletion and Cd bioaccumulation were determined (R^2>0.87), while no significant correlations have been found between GSH variation and Pb bioaccumulation (R^2<0.38). Significantly, concentration-dependent molar ratios of Pb/GSH ranging from 0.10 to 0.18 were observed, while molar ratios of Cd/GSH were at the scope of 1.53–3.32, confirming the dominant role of GSH in Cd chelation. The study also demonstrated that *P. chrysosporium* showed considerable hypertolerance to Pb ions, accompanied with demand-driven stimulation in GSH synthesis and unconspicuous generation of reactive oxygen stress. GSH plummeted dramatically response to Cd exposure, due to the strong affinity of GSH to Cd and the involvement of GSH in Cd detoxification mechanism mainly as Cd chelators. Investigations into GSH metabolism and its role in ameliorating metal toxicity can offer important information on the application of the microorganism for wastewater treatment.

Keywords Glutathione metabolism · Heavy metal · Intracellular chelator · Detoxification · *Phanerochaete chrysosporium*

P. Xu · L. Liu · G. Zeng (✉) · D. Huang (✉) · C. Lai · M. Zhao · C. Huang · N. Li · Z. Wei · H. Wu · C. Zhang · M. Lai · Y. He
College of Environmental Science and Engineering, Hunan University, Changsha 410082, People's Republic of China
e-mail: zgming@hnu.edu.cn
e-mail: huangdanlian@hnu.edu.cn

P. Xu · L. Liu · G. Zeng · D. Huang · C. Lai · M. Zhao · C. Huang · N. Li · Z. Wei · H. Wu · C. Zhang · M. Lai · Y. He
Key Laboratory of Environmental Biology and Pollution Control (Hunan University), Ministry of Education, Changsha 410082, People's Republic of China

Introduction

Heavy metal contamination is of great concern on account of the tendency for bioaccumulation and the toxic effect in plants, microorganisms, and human beings. Over the few decades, biosorption technology has been devised for the treatment and removal of heavy metals (Pointing 2001; Xu et al. 2012a; Zeng et al. 2007, 2013). Various partially understood mechanisms appear to be responsible for heavy metal bioaccumulation in microorganisms, which include cell wall cation exchange, extracellular chelation with organic acids, and intracellular bioaccumulation (Fourest and Roux 1992; Huang et al. 2012). Microorganisms exposure to potentially toxic heavy metals arises the entry of the metals intracellularly and results in significant concentration of certain metals intracellularly. Heavy metals accumulated intracellularly can cause serious damages by thiol-binding and protein denaturation replacing, primary displacement of essential metals involved in biological reactions, or a secondary effect of oxidative stress (Bridges and Zalups 2005; Flora et al. 2008). As example, cadmium can react with polythiol groups on cellular macromolecules and substitute for zinc in Zn-containing enzymes, e.g., carboxypeptidases and metallothioneins, causing oxidative stress indirectly (Howlett and Avery 1997; Price and Morel 1990).

Accordingly, metabolism-dependent intracellular bioaccumulation or transport commonly occurs in living cells via

some physicochemical interactions, for instance via the synthesis of particular cell constituents or metabolites (Casalino et al. 2006; Gadd 1990). Such metabolites may act as efficient metal chelators or the creation of a particular microenvironment in the vicinity of the cell that facilitates deposition or precipitation (Mendoza-Cózatl et al. 2005; Vatamaniuk et al. 2000). It is well known that one of the best described hyperaccumulation pathway involves their intracellular chelation by either glutathione (GSH) or low molecular weight sulfur-containing peptides derived from GSH (Clemens 2001; Mutoh and Hayashi 1988). Actually, an active detoxification mechanism developed by plants, algae, and fungi to avoid heavy metal poisoning, involving intracellular sequestration of heavy metal ions by means of GSH and peptides synthesized at the expense of GSH, the phytochelatins (PCs), has been widely reported (De Vos et al. 1992; Srivastava et al. 2006). GSH is the most abundant cellular SH in most living organisms and involved in alleviating metal stress in a number of ways, such as metal sequestration and antioxidant response (Sun et al. 2007). Vögeli-Lange and Wagner (1996) have speculated GSH as a first, rapidly responsive, thiol-based line of defense against Cd exposure. Some works have also shown that GSH molecules involved in Cd sequestration and metal toxicity amelioration in yeast and plant cells (Drążkiewicz et al. 2003; Herbette et al. 2006; Xiang and Oliver 1998). However, connections to the pathways for uptake, partitioning, and bioaccumulation of toxic metals result in multilevel regulation of cell homeostasis related to the toxicity and tolerance mechanism. The physiological range for GSH between binding-induced toxicity and detoxification is therefore extremely narrow and a tightly controlled metal homeostasis network to adjust to fluctuations in metal availability is a necessity for all organisms.

Phanerochaete chrysosporium are known to be vital hyperaccumulation species for heavy metal removal with admirable intracellular bioaccumulation capacity (Huang et al. 2008; Xu et al. 2012b, 2013). It has been reported that the presence of heavy metals may bring about, through selective pressure, microorganisms that might develop different kinds of defensive or reparative mechanisms to overcome metal toxicity. Coping with adversity environment is one of the prerequisites for the *P. chrysosporium*. To date, numerous data are available concerning the detoxification mechanisms in plant, bacterial, and fungi, such as chelation by metal-binding compounds, metal deposition, alterations of membrane structures, and synthesis of stress metabolites (Karlovsky 2011; Mishra et al. 2006; Wu et al. 2013). Nevertheless, our knowledge of the heavy metal hyperaccumulation and tolerance mechanisms of *P. chrysosporium* is quite limited at the molecular and cellular levels. Especially, little is known about GSH accumulation and possible involvement of GSH in heavy metal sequestration and their roles in heavy metal detoxification in *P. chrysosporium*. Investigation on heavy metal bioaccumulation and its tolerance/detoxification mechanism in a severe environment can offer important information for enhancing their ability to tolerate high metal concentrations and apply in heavy metal remediation.

In this study, experiments were pursued to determine how heavy metals accumulate at the cellular level and to analyze the role of GSH in the heavy metal bioaccumulation. GSH accumulation in *P. chrysosporium*, a typical white-rot fungus, was quantified under Pb and Cd exposure at various concentrations (0–400 mg L^{-1}). Our present study also extends observations about the role of GSH in heavy metal chelation and detoxification. An attempt is made to establish whether a relationship exists between the GSH accumulation and their capacity to accumulate heavy metals in *P. chrysosporium*. Simultaneously, GSH as indicators for metal chelator intracellularly were further determined by analyzing the time-and-concentration-dependent molar ratios of metals/GSH. Biotechnological relevance of this study resides in the possibility of developing organisms with high capacity of GSH accumulation for heavy metal removal.

Materials and methods

Strains and materials

All reagents used in the experiment were of analytical reagent grade. All solutions were prepared with ultrapure water (18.2 MΩ cm^{-1}). The *P. chrysosporium* BKMF-1767 (ATCC 24725) was obtained from China Center for Type Culture Collection (Wuhan, China). The *P. chrysosporium* spore suspensions were prepared by scraping and blending in the sterile ultrapure water and then adjusted to a concentration of 2.0×10^6 cells mL^{-1}. Two milliliters of spore suspensions was inoculated to 250 mL Erlenmeyer flasks containing 100 mL growth medium, the rotation speed was fixed at 120 rpm and then incubated at 30 °C. After 5 days of incubation, the *P. chrysosporium* biomass was collected for the exposure experiments.

Exposure studies

One gram of *P. chrysosporium* wet biomass was mixed with 50 mL aqueous solution at various initial Pb or Cd concentrations (0–400 mg L^{-1}) and agitated on a rotary shaker at a constant speed of 150 rpm at pH 5.0 at 30 °C. The quantification of heavy metals in the solution after biosorption was analyzed in an atomic absorption spectrometer (AAS, Agilent 3510, USA) at 0, 6, 12, 24, 36, 48, and 72 h.

P. chrysosporium extract preparation

The heavy metal-exposed *P. chrysosporium* biomass was collected at the selected time intervals and centrifuged at 12,000 rpm for 10 min. Afterwards, the biomass was treated with 50 mL 0.2 mol L^{-1} HNO_3 solution for 30 min to desorb the adsorbed metals at the *P. chrysosporium* surface and then rinsed with ultrapure water for three times. Thereafter, 5 mL of ultrapure water was added and homogenated in glass homogenizer to extract the soluble compounds. The soluble fraction was centrifuged at 12,000 rpm, 4 °C, for 10 min, and then filtered for the preparation of *P. chrysosporium* extract, applied for heavy metal analysis and GSH detection.

Intracellular heavy metal bioaccumulation analysis

Intracellular heavy metal bioaccumulation was followed in heavy metal biosorption for 0, 6, 12, 24, 36, 48, and 72 h. Samples (1.25 mL) from the above *P. chrysosporium* extract were filtered through Whatman paper and diluted with ultrapure water to 50 mL. Such samples were analyzed for intracellular heavy metal analysis ($M_{HM\ W}$).

Intracellular GSH detection

The GSH content was determined by the method of Ellman (1959) with minor modifications. *P. chrysosporium* extract (0.25 mL) was mixed with 0.5 mL Tris–HCl buffer solution (0.25 M, pH 8.0), and then added 0.25 mL methanal (3 %) for 20 min. Then the mixture was allowed to react with 3 mL 5,5-dithiobis-2-nitrobenzoic acid (DTNB) (1 mM). The solution was then kept at room temperature for 5 min and the extinction was read at 412 nm (Shimadzu 2550 UV–visible spectrophotometer). The GSH (Sigma, USA) were used as identification and curve calibration for intracellular GSH detection ($M_{GSH\ W}$).

Molar ratios of metal/GSH analysis

One milliliter of the *P. chrysosporium* extract was treated with 4 mL 0.2 mol L^{-1} HNO_3 at 30 °C for 4 h, and then collected for heavy metal analysis ($M_{HM\ H}$) and GSH detection ($M_{GSH\ H}$). The molar ratios of metal/GSH were defined as the ratio of the D value between water extract and HNO_3-treated extract of metals and GSH (Eq. 1).

$$\text{Molar ratio of metal/GSH} = (M_{HM\,H} - M_{HM\,W})/(M_{GSH\,H} - M_{GSH\,W}) \quad (1)$$

Where $M_{HM\ W}$ and $M_{HM\ H}$ is the amount of the bioaccumulated heavy metals (μmol g^{-1}) in water extract and HNO_3-treated extract, respectively; $M_{GSH\ W}$ and $M_{GSH\ H}$ are the amount of the intracellular GSH (μmol g^{-1}) in water extract and HNO_3-treated extract, respectively.

Intracellular ROS detection

ROS formation in *P. chrysosporium* was measured after treated with Pb and Cd for 36 h. Intracellular ROS detection was conducted by reactive oxygen species assay kit purchase from Beyotime Institute of Biotechnology. Briefly, combined with 15 μL of DCFH-DA solution (0.1 mM, diluted in DMSO solution) and 275 μL PB solution (0.1 M, pH 6.0), 10 μL of *P. chrysosporium* extract was then added into the mixture, and reacted in the dark at 37 °C for 30 min. Fluorescence intensity was immediately read with PerkinElmer LS-55 spectrofluorimeter (UK). The excitation wavelength was controlled at 480 nm, and the emission wavelength was at 525 nm.

Statistical analysis

The analysis of the same compound was made three replications and results were expressed as weighted mean and for each value were calculated the standard deviation. Statistical analyses were performed using the SPSS statistical package (version 18.0 for Windows). The level of significance was set at $P<0.05$ in all cases.

Results

Reduction of metal concentrations

As shown in Fig. 1, uptake of Pb was rapid in the initial stage of biosorption and a plateau was reached after 48 h. A significant increase in Cd biosorption capacity occurred in *P. chrysosporium* with concentration-dependent. As observed, the fungi removed about 90 % of the Pb at 50 mg L^{-1}, and almost uptook during the first 24 h. Higher concentrations supplied a kind of important driving force, to overcome the existing mass transfer resistance between heavy metals and biomass, therefore reinforce the active uptake of *P. chrysosporium*. Mean uptake rate at initial 6 h augmented with the increase of the test Pb concentration was 0.0547 mg g^{-1} h^{-1} (20 mg L^{-1}), 0.1524 mg g^{-1} h^{-1} (50 mg L^{-1}), 0.2943 mg g^{-1} h^{-1} (100 mg L^{-1}), 0.4803 mg g^{-1} h^{-1} (200 mg L^{-1}), and 0.7477 mg g^{-1} h^{-1} (400 mg L^{-1}). Similarly as in the case of higher concentrations, the uptake rate was rising during a shorter exposition time. The removal of Cd also increased considerably with increasing of the initial metal concentrations. The admirable ability to uptake Pb and Cd could be exploited in treatment of heavy metals from wastewater.

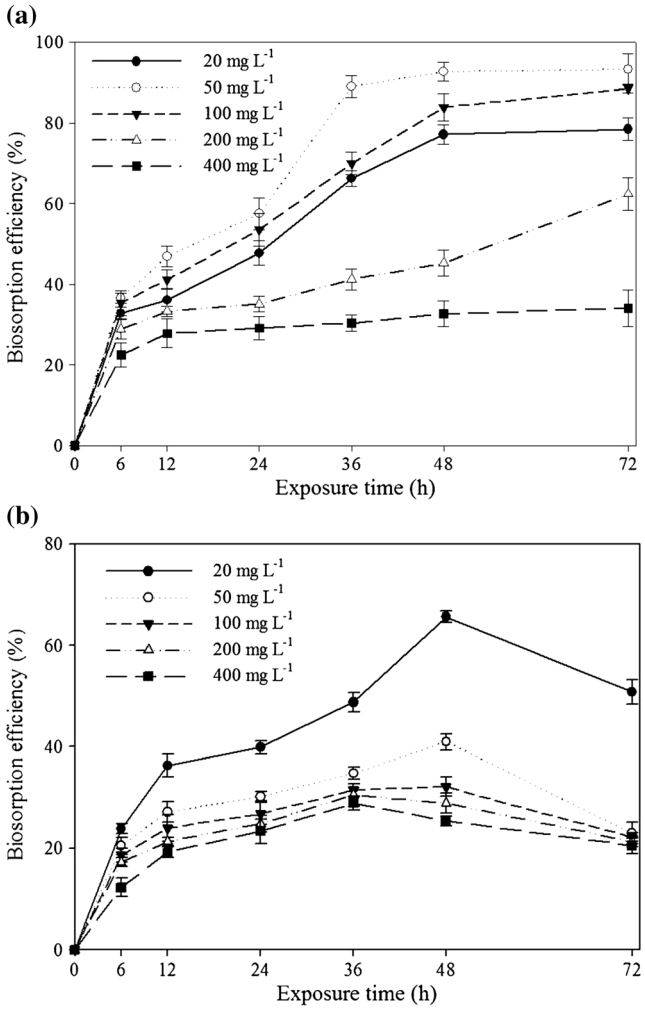

Fig. 1 Time-course of heavy metal uptake in *P. chrysosporium* at various concentrations of Pb (**a**) and Cd (**b**). The *bars* represent the standard deviations of the means (n=3)

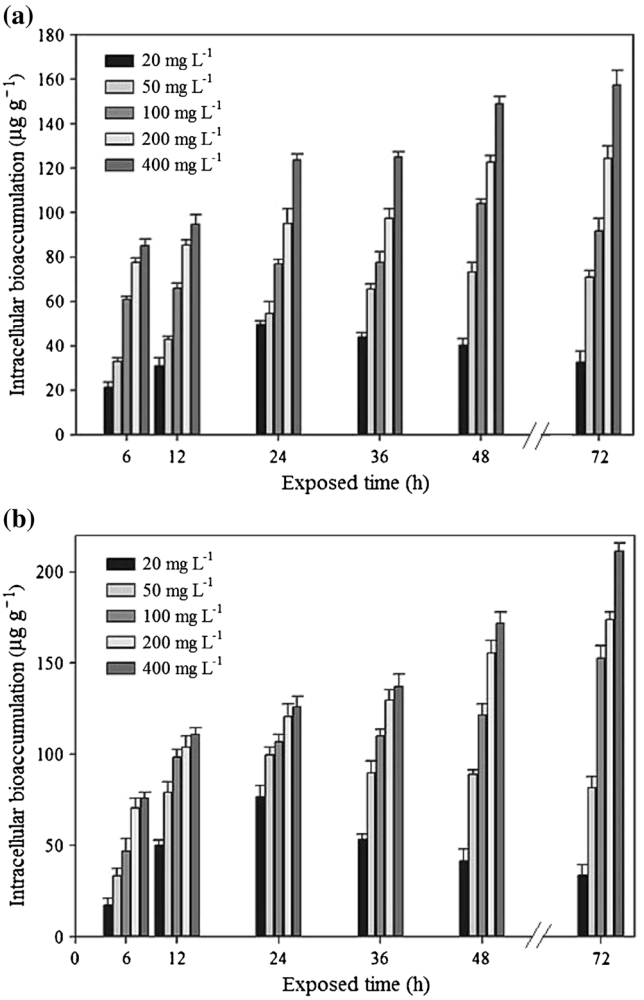

Fig. 2 Time-course of heavy metal bioaccumulation in *P. chrysosporium* at initial concentrations of 20, 50, 100, 200, and 400 mg L^{-1} Pb (**a**) and Cd (**b**). Mean values and standard deviation from three replicate samples. The *bars* represent the standard deviations of the means (n=3)

Intracellular bioaccumulation of Pb and Cd in *P. chrysosporium*

The intracellular bioaccumulation of metals in *P. chrysosporium* exposed to various concentrations of heavy metals is shown in Fig. 2. Initial strong bioaccumulation of both Pb and Cd has been found, with more pronounced Cd levels. The content of intracellular accumulated Pb increased from 106.8 to 126.0 μg g^{-1} at 24 h, while the initial Pb concentration varied from 100 to 400 mg L^{-1} (Fig. 2a). Simultaneously, the intracellular accumulated Cd above the concentration of 100 mg L^{-1} exposure after 24 h was more than 100 mg kg^{-1}, which is a widely accepted threshold for Cd hyperaccumulation (Milner and Kochian 2008). Interestingly, a consistent increase in Pb and Cd concentration of cells occurred at 200 and 400 mg L^{-1}, while a slight decrease was evident at their lower external concentrations of 20–100 mg L^{-1} (Fig. 2b). Especially, 17 μg g^{-1} of Pb decrease and 33 μg g^{-1} of Cd decrease has been found at 20 mg L^{-1} of Pb and Cd, respectively, demonstrating a distinct efflux of Pb and Cd in *P. chrysosporium*.

Concentration-dependent accumulation of GSH in *P. chrysosporium*

The quantification of intracellular GSH in *P. chrysosporium* exposed to various concentrations of Pb and Cd is shown in Fig. 3. Considerable amounts of GSH were accumulated in both Pb- and Cd-exposed *P. chrysosporium*, while with opposite tendency in consideration of the metal concentrations, which might be ascribed to the distinct toxicity and affinity of Pb and Cd. Exposing *P. chrysosporium* to moderate concentrations of Pb (less than 200 mg L^{-1}) resulted in a concentration-dependent increase in GSH content (Fig. 3(a)). GSH accumulation was highest in cells treated with 200 mg L^{-1} Pb. Oppositely, GSH concentration measured in *P. chrysosporium* individuals decreased with

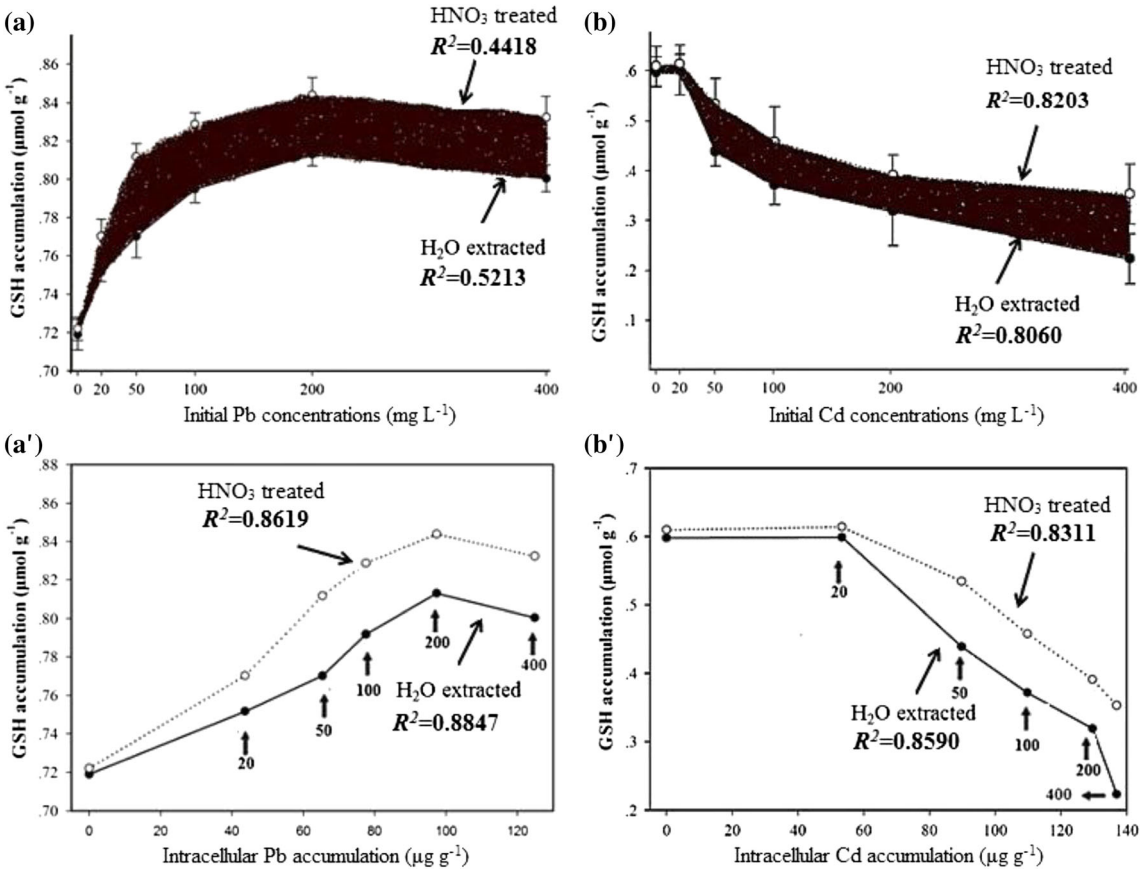

Fig. 3 Concentrations of intracellular GSH and GSH variations in *P. chrysosporium* exposed to a range of 0–400 mg L^{-1} Pb (**a**) and Cd (**b**) for 36 h and relationship between GSH accumulation and Pb exposure (*a′*) and Cd exposure (*b′*). *Brown areas* represent the *D* value of GSH between ultrapure water extract and HNO_3-treated extract

increasing Cd concentrations, except a trivial stimulation of GSH accumulation at 20 mg L^{-1} of Cd (Fig. 3(b)). This decrease in GSH level was dependent on the Cd concentration (R^2>0.80), with higher Cd concentrations causing incremental depletion in GSH levels. A maximum depletion of 0.37 μmol was observed at 400 mg L^{-1} of Cd, while a fluctuation within a narrow range of 0.72–0.84 μmol has been determined under Pb exposure at the concentration of 0–400 mg L^{-1}.

Time-course accumulation of GSH in *P. chrysosporium*

The time-course study showed that exposure to heavy metals resulted in changes in GSH concentration, with marked differences in two populations of heavy metals (Pb and Cd). It was observed in this study that the GSH depletion was induced significantly upon Cd exposure, while Pb exposure only resulted in a slight depletion of GSH at later intervals. Initial Pb exposure cause increase in GSH accumulation, then followed a slight depletion of intracellular GSH. GSH accumulation fluctuated within a narrow range of 0.75–0.55 μmol under Pb exposure, presenting a value of 0.202 and 0.197 μmol of GSH depletion in water extract and HNO_3-treated extract, respectively (Fig. 4(a)).

Comparatively, a distinct decline in GSH content under Cd exposure occurred in a time-dependent manner (Fig. 4(a′)). Remarkably, GSH decreased regularly over the Cd exposure period, and a decrease of 0.557 and 0.530 μmol in GSH was observed from 0 to 72 h in water extract and HNO_3-treated extract, respectively (Fig. 4(b′)). Additionally, HNO_3-treated samples exhibited higher levels of GSH, which was likely ascribed to the partial digestion of GSH in metal–GSH complexation in *P. chrysosporium*.

Relationship between GSH variation and metals bioaccumulation

To obtain further evidence for the functionality of GSH during the heavy metal exposure period, relationship between time-course GSH variation and heavy metal bioaccumulation was conducted by linear regression analysis. Apparently, there was a statistical difference between the values observed in the presence of Pb and in the experiment in all the Cd treatments (Fig. 5). Positive linear relationships were observed between

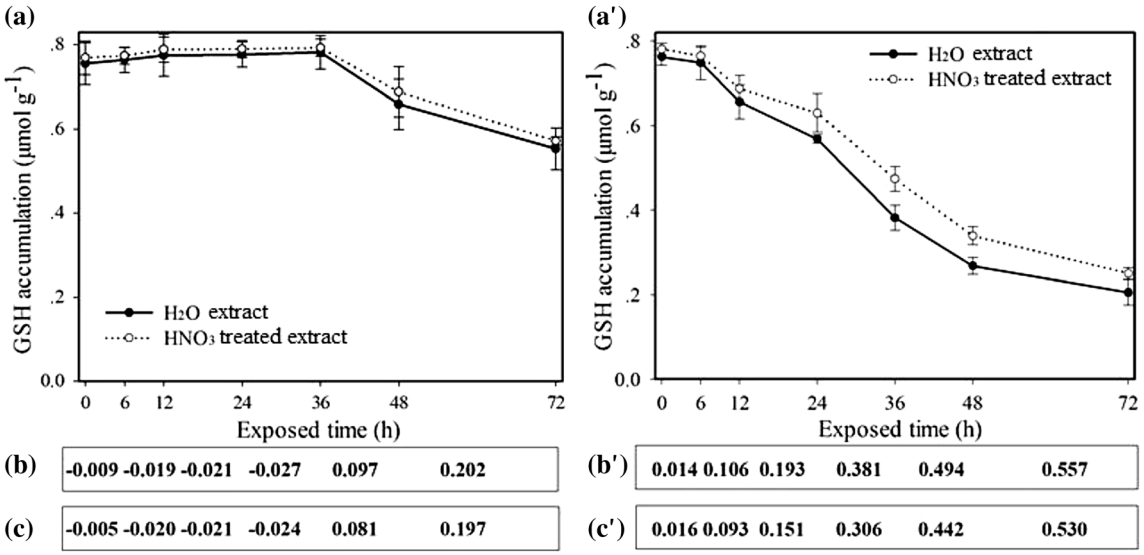

Fig. 4 Time-course of GSH accumulation in *P. chrysosporium* under Pb (*a*) and Cd (*a′*) exposure at the 100 mg L^{-1} of initial metals and time-course of GSH variation (μmol g^{-1}) in ultrapure water extract and HNO_3-treated extract during the biosorption process of Pb (*b*, *c*) and Cd (*b′*, *c′*)

GSH depletion and Cd bioaccumulation in *P. chrysosporium* (R^2=0.8741 and 0.8772), but no significant correlations have been found between Pb bioaccumulation and GSH depletion (R^2=0.3458 and 0.3879). Additionally, as shown in Fig. 3, GSH accumulation was significantly related to the intracellular metal bioaccumulation, both Pb and Cd (R^2=0.83).

Molar ratios of metal/GSH analysis

GSH as indicators for metal chelator in *P. chrysosporium* intracellularly were determined by analyzing the molar ratios of metal/GSH, defining as the amount of metal ions that are complexed to GSH molecules (Fig. 6). From Fig. 6a, it was apparent that Cd showed relatively higher ratios (0.62–2.13) than Pb (0.12–0.24) from 6 to 72 h. Results confirmed the GSH-mediated Cd chelation in *P. chrysosporium*. In addition, the molar ratios of metal/GSH were elevated with the increase of exposed heavy metals (Fig. 6b), which might be ascribed to the stronger driving force at the high concentrations. As shown in Fig. 6b, at the range of 20–400 mg L^{-1} heavy metals, molar ratios of Cd/GSH ranging from nearly 1.53 to 3.32 have been observed, while molar ratios of Pb/GSH were at the scope of 0.10 to 0.18 under Pb exposure. It is speculated from the calculated ratios that 1 mol GSH molecules can chelate 1.53–3.32 mol Cd and 0.10–0.18 mol Pb, respectively. The relatively high molar ratios of Cd/GSH were quite agreed with the above deduction that the Cd-induced GSH depletion was ascribed to the chelating role of GSH to Cd, based on the good correlations between time-and-concentration-dependent GSH depletion and Cd bioaccumulation (Fig. 3(b′) and Fig. 4(a′), R^2>0.80).

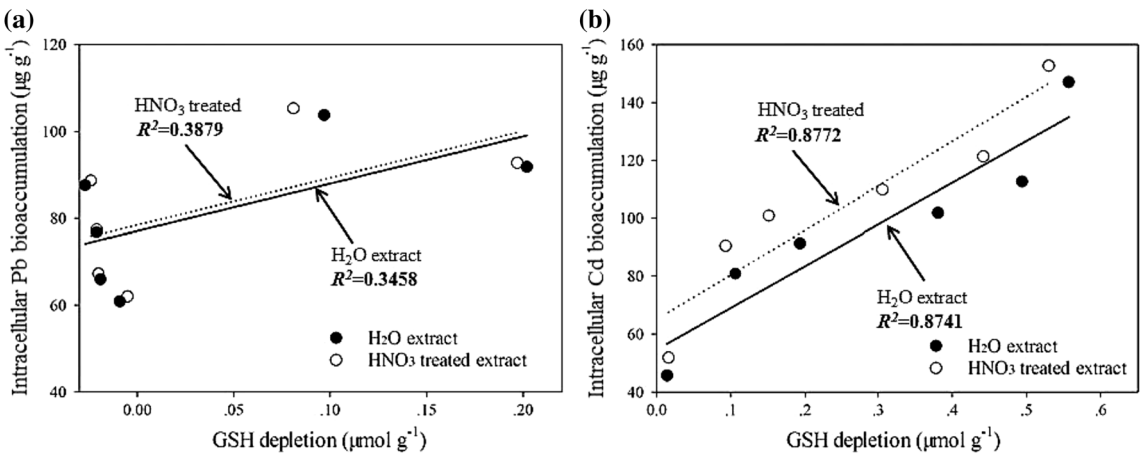

Fig. 5 Relationships between time-course GSH variation and Pb bioaccumulation (**a**) and Cd bioaccumulation (**b**) under the exposure of 100 mg L^{-1} metals

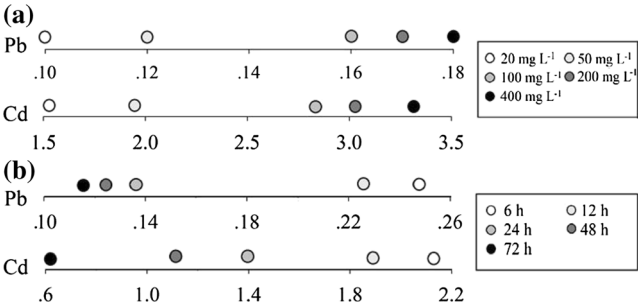

Fig. 6 **a** Molar ratios of metals/GSH in *P. chrysosporium* versus time of exposure during the 72 h time-course exposed to 100 mg L^{-1} of Pb and Cd. **b** Molar ratios of metal/GSH in *P. chrysosporium* while exposed to various concentrations of Pb and Cd for 36 h

Discussion

Pb and Cd are of particular concern and locally present in enormous quantities. Research interest into the potential of white-rot fungi for biosorption of heavy metals has been widely reported aimed at characterizing and quantifying the metal binding properties of white-rot fungi. It was observed in this study that rapid and efficient uptake and bioaccumulation of Pb and Cd occurred in *P. chrysosporium*. Basically, mechanisms responsible for biosorption may be one or combination of metabolism-dependent and metabolism-independent process due to the complex structure of microorganisms (Kamei et al. 2006; Tsezos and Volesky 1982). Surface adsorption via ion-exchange, hydrolytic adsorption, and surface precipitation is most approval biosorption mechanisms, further contributing to transport of ions through cell wall and membrane. Nevertheless, it tends to be only the first steps in metal uptake and bioaccumulation. Membrane possessing negative potential and the presence of intracellular metal binding or sequestration sites provided driving forces for the uptake of positive metal ions intracellularly. Metals trapping into the inside cells through selective binding sites with higher affinity than those at the cell surface and/or transfer into an intracellular compartment resulted in intracellular bioaccumulation.

Heavy metal ions that do enter intracellularly pose a potential threat to cells. Heavy metals with electron-sharing affinities, such as Pb and Cd, can result in the formation of covalent attachments mainly between sulfhydryl groups and metals intracellularly (Flora et al. 2008). Pb and Cd are clarified as the sulfhydryl-reactive metals with high affinity to thiols. The solubility product of CdS and PbS was at the value of 1.40×10^{-29} and 8.4×10^{-28}, respectively (Pauling 1988). Hence, intracellular accumulated Pb and Cd will preferentially bind to S donors. Possible S donors for heavy metal ions are GSH, a class of small thiol (SH)-rich peptides that are constitutively present or synthesized in response to heavy metal exposure (Williams et al. 2000). GSH is synthesized from cysteine in two consecutive ATP-dependent reactions. Initially, γ-glutamylcysteine (γ-EC) is formed from L-glutamate and L-Cys by the catalysis of γ-glutamylcysteine synthetase (γ-ECS), and then further adding glycine to the C-terminal of γ-EC by glutathione synthetase (GS) to form GSH (Meister 1995). Accordingly, the synthesis of GSH starting from inorganic sulfate is demand driven by sulfur assimilation and cysteine biosynthetic pathways, affected by different stress situations such as heavy metal exposure, oxidative stress, and sulfur or nitrogen deficiency (Mendoza-Cózatl et al. 2005; Xiang and Oliver 1998). Generally, there are vast differences for the various metals with respect to bioavailability, uptake activity, and efficiency of translocation, determining metal exposure levels and metal bioaccumulation. Initial Pb exposure enhanced GSH synthesis from 0 to 36 h, which might contribute to the consumption of the sulfhydryls in the *P. chrysosporium*, leading to the demand driven of GSH synthesis and incremental GSH accumulation. Upstream of GSH synthesis is in the case of the assimilation of sulfate as well as the demand-driven synthesis of GSH under Pb exposure has been also found at the concentrations of 0–400 mg L^{-1}. Metal-induced enhancement of intracellular GSH was also found in some metal-tolerant plants, such as *Arabidopsis trichome* (Gutiérrez-Alcalá et al. 2000) and *Sedum alfredii* (Sun et al. 2007). In overall, heavy metal ions are required for formation of thiolates of GSH, which result in the stimulation of GSH synthesis driven by increasing demand for GSH in response to appropriate metal exposure.

However, as soon as environmental stress was imposed in the form of ions, the amount of GSH plummeted, demonstrating the extremely restricted synthesis and incremental depletion of GSH, just as continuous exposure to Cd. As shown in Fig. 3 and Fig. 4, excess and continuous heavy metal exposure triggers the decrease in GSH accumulation, probably due to the potential threat and toxicity of heavy metals to microorganisms. It was widely accepted that the main heavy metal toxicity by intracellular ions could result from the disruption of metabolism homeostasis by bonding with atoms of sulfur, oxygen, and hydrogen present in the sulfhydryl groups, carboxyl, disulfide, or multiple amino compounds (Bertin and Averbeck 2006; Das et al. 2013). Nowadays, there is increasing evidence from experimental studies that a common consequence of heavy metal exposure is that they result, at some stage of stress exposure, in an increased production of ROS (Bussche and Soares 2011; Schützendübe and Polle 2002). The toxicity of Pb and Cd was therefore characterized as the induced ROS production in Pb and Cd-exposed *P. chrysosporium*. As shown in Fig. 7, Pb exposure caused unconspicuous ROS generation in *P. chrysosporium*, ratios at the range of 1.01 to 1.13 have been found at 20–400 mg L^{-1} Pb doses (Fig. 7(a′)). However, under Cd exposure, there was a significant increase in fluorescence intensity compared with control, intense stimulation ratios ranging from 1.13 to 1.66 were calculated at 20–400 mg L^{-1} Cd doses (Fig. 7(b′)). Apparently, severe toxicity related to reactive oxygen damage

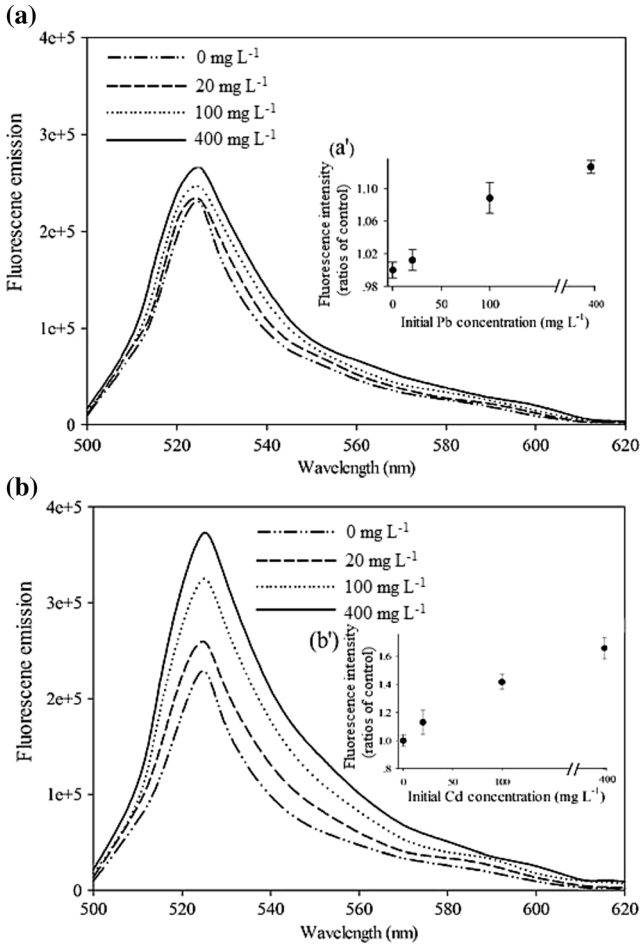

Fig. 7 Fluorescence intensity variation under various concentrations of Pb (**a**) and Cd (**b**) exposure and ratios of fluorescence intensity stimulation under Pb (*a′*) and Cd (*b′*) exposure

was found under Cd exposure with higher ROS levels while compared with Pb exposure. We guessed that the unconspicuous ROS generation may be attributable to the mild toxicity of Pb and the relative strong tolerance of *P. chrysosporium* to Pb. Numerous studies have also confirmed that *P. chrysosporium* could survive in Pb-rich environment and adapt to excess Pb ions (Baldrian and Gabriel 1997; Falih 1997; Huang et al. 2008). This could be called as naturally selected metal hypertolerance, which is again largely metal-specific. While compared with Pb, Cd is an extremely reactive heavy metal with affinity towards the functional groups of biomolecules, especially thiol groups, which made them intracellularly chelated by GSH forming as Cd-bis(glutathionate) (Cd–GS_2) (Vatamaniuk et al. 2000). Cd was found to be one of the most potent growth inhibitor and toxicant, representing a stringent response phase which is characterized by weight loss, synthesis of brown pigment, production of secondary metabolites, and production of idiopathic proteins (Broda et al. 1989; Dhawale et al. 1996). Additionally, Cd exposure commonly caused increased lipid oxidation products, which might disturb metabolisms contributing to GSH metabolism process. Moreover, GSH is also essential for the synthesis of metal-binding peptides such as PCs, which inactivate and sequester heavy metals such as Cd, Pb, and Hg by forming stable metal complexes intracellularly (Cobbett 2000; Estrella-Gómez et al. 2012), further exacerbating the GSH depletion. As a result, radual bioaccumulation of Cd cause severer toxicity to *P. chrysosporium* cells and GSH was consumed in combating the imposed stress; thereafter, GSH depletion occurred response to Cd exposure.

Although heavy metal-induced thiol binding is partially the cause for its high toxicity, this feature is also used by several organisms to rend the metal harmless to the cell, through sequestration with metal-detoxifying ligands, which converts it into a more innocuous form (Lima et al. 2006). A major strategy to detoxify nonessential heavy metals is the synthesis of specific low-molecular weight chelators, such as GSH, to avoid binding to physiologically important proteins. Initially, the most immediate answer for the role of GSH would be the alleviation of oxidative stress arising from heavy metal exposure (Corticeiro et al. 2006; Estrella-Gómez et al. 2012). But in other reports, it has been documented that GSH mainly served as metal chelator agent, rather than the reduction of oxidative stress (Corticeiro et al. 2006; Lima et al. 2006). Chelation and sequestration processes result in removal of the toxic ions from sensitive sites. Furthermore, previous research has reported that GSH was a potential cytosolic chelator for Cd ions, with a relatively high affinity for binding Cd ($K_{d\ Cd}>10^{10}$) (Perrin and Watt 1971; Vögeli-Lange and Wagner 1996). In our study, time–concentration-dependent increase in GSH accumulation with Pb ions appeared to be a compensatory response to ameliorate heavy metal toxicity. GSH showed weaker affinity to Pb ions, with lower molar ratios of Pb/GSH (0.10–0.24). The results was quite agreed with the previous result that Pb influence the GSH metabolism possibly in the case of demand driven of GSH synthesis via sulfate depletion, rather than chelation with GSH, attributed to the considerable hypertolerance to Pb ions. No significant correlations between Pb bioaccumulation and GSH depletion ($R^2<0.39$) also confirmed the previous conclusion. Detoxification of Pb occurs in a GSH-dependent manner therefore respond to metals by the upregulation of sulfur amino acid and GSH synthesis. At the same time, molar ratios of Cd/GSH (1.53–3.32) shown in Fig. 6 further demonstrated the vital role of GSH in Cd chelation. The results was also presented by the remarkable *D* value of GSH between ultrapure water extract and HNO_3-treated extract (Fig. 3) at various Cd concentrations, accompanied with positive linear relationships between GSH depletion and Cd bioaccumulation ($R^2>$ 0.87). Results presented the dominant role of GSH as an

effective donor of Cd. Recently, Mendoza-Cózatl et al. (2008) reported that Cd might exist in intracellular compartments in the forms of Cd-GSH and Cd-PCs complexes in *Brassica napus* further chelated by GSH and PCs. The result was agreed with the previous study conducted by Dameron et al. (1989), who reported that metal complexes with GSH formed in *Candida glabatra* presented a molar ratio at the range of 0.5–2.0. Results related to the accumulation of GSH suggested that GSH acted as intracellular metal buffers as well as the substrates as S donors for metal chelation, which tended to be principal mechanisms in heavy metal detoxification in *P. chrysosporium*. Besides intracellular sequestration via GSH chelation, active efflux mechanism to explain decreased accumulation commonly occurred via cation-specific export proteins by an energy-dependent process linked to the proton motive force (PMF) or adenosine triphosphate (ATP), which were characterized by a number of laboratories (Brey et al. 1980; Cohen et al. 1988). In our study, time-dependent reduced uptake of both Pb and Cd at lower concentrations attributed to active efflux mechanism as indicated in Fig. 2 tends to be the concurrent heavy metal tolerance mechanism in *P. chrysosporium*. Active efflux mechanism was initially proposed by Cohen et al. (1988), who suggested that a reduced accumulation of norfloxacin in *Escherichia coli* involved a carrier-mediated active efflux generated by proton motive force, with an apparent *K*m of 0.2 mM and a *V*max of 3 nmol min^{-1} mg of $protein^{-1}$.

In summary, the findings presented here demonstrated GSH-mediated heavy metal chelation as a novel mechanism of metal uptake in *P. chrysosporium*. GSH depletion was determined at 0.197–0.202 and 0.557–0.530 μmol g^{-1} at 100 mg L^{-1} Pb and Cd, respectively. Positive relationships between time-course GSH depletion and Cd bioaccumulation were observed ($R^2 > 0.87$). Higher time-and-concentration-dependent molar ratios of Cd/GSH were observed than Pb/GSH, while with relatively weak affinity to Pb. It can thus be concluded that GSH was an important metabolism response to heavy metals; the detail molecular mechanism and transformation of GSH warrants further study. These results are useful in developing biotechnological strategies for Cd bioremediation procedures and open novel prospective for the improvement of metal tolerance in white-rot fungi.

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