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# The Oxidative Stress of *Phanerochaete chrysosporium* Against Lead Toxicity

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**Abstract** Among the technologies for heavy metal remediation, bioremediation technology has gained extensive attention because of its low processing costs and high efficiency. The white-rot fungus *Phanerochaete chrysosporium* (*P. chrysosporium*) which has a good tolerance to heavy metals has been widely used in the heavy metal bioremediation. In order to figure out the molecular mechanisms involved in the oxidative stress of *P. chrysosporium* against metal toxicity, we examined the effect of $Pb^{2+}$ on the levels of reactive oxygen species and the production of malondialdehyde. Results showed that *P. chrysosporium* could adjust Pb-stressed condition by regulating the unique oxidation-antioxidation process in cells and kept a balance between oxidation and antioxidation when it was threatened by a different dose of $Pb^{2+}$. Investigations into the oxidative stress of *P. chrysosporium* to lead could not only provide a better understanding of the relationship between lead and oxidative stress in *P. chrysosporium*, but also offer important informations on the development of fungal-based remediation technologies to reduce the toxic effects of lead.

**Keywords** *Phanerochaete chrysosporium* · Malondialdehyde · Superoxide anion · Hydroxyl radical · Hydrogen peroxide

**Abbreviations**

| | |
|---|---|
| Pb | lead |
| ROS | reactive oxygen species |
| $O2^{-}$ | superoxide |
| $H_2O_2$ | hydrogen peroxide |
| •OH | hydroxyl radical |

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|---|---|
| *P. chrysosporium* | *Phanerochaete chrysosporium* |
| MDA | malondialdehyde |
| TBA | thiobarbituric acid |
| $R^2$ | linearly dependent coefficient |

## Introduction

Lead (Pb) is one of the most serious environmental pollutants as it is toxic in all its chemical forms [1–3]. The widespread use of Pb in many countries and districts has caused global contamination in air, water, and soil [4–9]. High concentrations of Pb are found in the plants, animals, microorganisms, and other species living on the earth [10]. The introduction of Pb, in various forms in the environment, can cause considerable detriment in the structure and function of cells [11]. Recent studies have reported lead's potential for inducing oxidative stress and evidence is accumulating in support of the role for oxidative stress in pathophysiology of Pb poisoning [12]. Consequently, it is suggested that oxidative stress in cells can be partially responsible for the toxic effects of Pb [13].

Reactive oxygen species (ROS) such as superoxide ($O_2^-$), hydrogen peroxide ($H_2O_2$), and the hydroxyl radical (•OH), produced intracellularly in all aerobic organisms, are well recognized for playing a dual role in biological systems since they can be either harmful or beneficial to living systems [14–16]. On one hand, ROS plays an important role in defense against infectious agents and in the function of a number of cellular signaling systems, for example, ROS at low concentrations is the induction of a mitogenic response [15]. On the other hand, at high concentrations, ROS can be important mediators of damage to cell structures, including lipids and membranes, proteins, and nucleic acids, resulting in a condition known as oxidative stress [13, 15]. Under normal conditions, the ROS are normally in balance with antioxidant molecules owing to the antioxidant action of non-enzymatic antioxidants in addition to antioxidant enzymes [14, 15, 17, 18]. Recent studies indicated that the heavy metals can cause an increase in the production of ROS [13, 19]. The cumulative production of ROS through either endogenous or exogenous insults is termed oxidative stress [15]. It has also been proven that heavy metals have inhibitory effects on the antioxidant enzymes which may destroy the antioxidant defense systems, causing an oxidative stress in cells [13].

White-rot fungus *Phanerochaete chrysosporium* (*P. chrysosporium*) is characterized by its unique ability to tolerate the low and middle concentration of Pb and remove Pb from wastewater with its mycelium [10, 20, 21]. So *P. chrysosporium* can be adapted to the complex polluted environment, and the application of *P. chrysosporium* in the treatment of Pb-polluted water has supposedly been a promising strategy [1, 10, 22, 23]. Although *P. chrysosporium* are increasingly appreciated as an agent in controlling Pb pollution and Pb is known to cause an oxidative stress in cells, there is a distinct lack of knowledge about the mechanisms for Pb-induced oxidative stress in *P. chrysosporium* [1, 13].

The aim of the present investigation was to reveal oxidative stress of *P. chrysosporium* against lead toxicity. Specifically, we investigated the effect of increased concentrations of Pb ions on the production of ROS and malondialdehyde (MDA) in cells of *P. chrysosporium* in a definite period of time. Our present evidence indicated that *P. chrysosporium* can adjust oxidation-antioxidation process in cells through self-regulation to make changes of ROS and MDA levels, and kept a balance between oxidation and antioxidation in cells when it is threatened by a different dose of $Pb^{2+}$, which suggested a relevant role of the $Pb^{2+}$ concentration and exposure time in Pb resistance of the *P. chrysosporium*.

## Materials and Methods

### Test Fungus and Culture Conditions

The test fungal strain BKMF-1767 of *P. chrysosporium* (obtained from the Type Culture Collection Center of China in Wuhan University) was used throughout and maintained at 4 °C on potato dextrose agar slants. These fungal strains were then incubated at 37 °C in the biological incubator for 24 h to resume its physiological activity.

After activated process, the fungal spores were gently transferred from the agar surface to a glass tube and suspended in an amount of sterile ultrapure water to form a spore suspension at the concentration of $2.5\times10^6$ spores per milliliter. All the containers and equipments were washed extensively with ultrapure water and sterilized by autoclaving at 115 °C for 20 min before they were used.

### Pb-Stress Experiment

For liquid-state cultivation, 500 μL aliquot from spore suspension ($2.5\times10^6$ spores per mL) was submerged into sterile potato dextrose broth of composition: 20 g of potato, 2.0 g of dextrose, an amount of ultrapure water diluted to a final volume of 100 mL in a 250-mL Erlenmeyer flask. Cultivation was performed on a shaker (120 rpm) at 30 °C for 48 h. Then, an exponentially growing culture of *P. chrysosporium* was supplemented with the sterile stock solution of Pb, prepared from its analytical grade salts $Pb(NO_3)_2$, to achieve selected concentrations of Pb (0, 50, 100, 200, and 400 mg/L) in the medium. After cultivation on the shaker (120 rpm) at 30 °C periodically (2, 8, and 24 h), 10 mL of the culture medium and 0.8 g of the fungal (wet weight) were taken from the medium; the former was used for pH determination and the latter was equally divided into sample A and sample B, which was used for measuring the intracellular MDA and ROS content, respectively. The concentration of Pb and the exposure time chosen in this study were on the basis of our previous study and pre-experiments, ensuring that the cell viability of the highest-concentration group after 24 h exposure was more than 70 %.

### Acquisition of Cell-Free Extract

After the addition of 2.5 mL trichloroacetic acid [10 % (*w*/*v*)] to sample A and 2.5 mL phosphate buffer solution (50 mM, pH=7.8) to sample B, both of the two samples were ultrasonicated for 2 min to make the fungal cells disrupted completely. The homogenate was centrifuged at 10,000 rpm for 5 min, then the resulting supernatant was collected into their respective centrifuge tube named A and B, and kept at 4 °C, preparing for the determination experiments. All steps during the acquisition of cell-free extract were performed at 4 °C.

### Determination of MDA

MDA, generally used as the indicator of lipid peroxidation, was determined by the thiobarbituric acid (TBA) reaction according the method of Dhindsa et al. with some modifications [24]. Two milliliters of 0.6 % TBA was added to 0.5 mL aliquot of the cell-free extract from tube A. The blank probe was the same mixture but supplemented with 0.5 mL ultrapure water instead of cell-free extract. The mixture was heated at 100 °C for 15 min then cooled in ice bath. After centrifugation at 10,000 rpm for 5 min, the absorbance of the supernatant was

measured at 450, 532, and 600 nm, using the absorbance coefficient of 155 $mM^{-1}$ $cm^{-1}$. The concentration of MDA was calculated by the following formula:

$$C_{MDA} = 6.45 \times (A_{532} - A_{600}) - 0.56 \times A_{450} \left(\mu\text{mol} \cdot \text{L}^{-1}\right)$$

Where the $A_{450}$, $A_{532}$, and $A_{600}$ represent the absorbance of the supernatant at 450, 532, and 600 nm, respectively.

Measurement of Superoxide Anion ($O_2^-$)

The intracellular content of $O_2^-$ was estimated by measuring the formation of $NO_2^-$ according to Oracz et al. [25]. The reaction of the initial mixture which contained 0.5 mL of 50 mM phosphate buffer solution (pH=7.8), 1.0 mL of 1 mM hydroxylamine hydrochloride, and 0.5 mL aliquot of the cell-free extract from tube B in a final volume of 2.0 mL was proceed at 25 °C for 60 min, then the resultant mixture reacted with 1.0 mL of 17 mM sulfanilic acid and 1.0 mL of 7 mM α-naphthylamine at 25 °C for 20 min before reading the absorbance at 530 nm. A control reaction was always performed wherein all the steps and components were exactly the same as described above, except that cell-free extract was replaced with an equal volume of ultrapure water. For calculations, a standard curve with $NO_2^-$ concentrations (from 0 to 50 μmol/L) was used.

Hydroxyl radical (•OH) Scavenging Activity

Hydroxyl radical scavenging activity of the cell-free extract was evaluated according to the procedure described by Smirnoff and Cumbes [26]. The assay mixture consisted of 1.0 mL $FeSO_4$ (1.5 mM), 0.7 mL hydrogen peroxide (6 mM), 1.2 mL sodium salicylate (20 mM), and 0.3 mL cell-free extract from tube B. After incubation for 30 min at 37 °C under dark conditions, the absorbance of the complex was measured at 562 nm. A blank, devoid of sodium salicylate, was prepared for background subtraction and a control without cell-free extract was for calculating the hydroxyl radical scavenging ability, using the following equation:

$$\bullet\text{OH scavenging ability (\% of control)} = \left[1 - \frac{A_2 - A_1}{A_0}\right] \times 100\%$$

Where $A_0$ is the absorbance of the control, $A_1$ is the absorbance of the blank, and $A_2$ is the absorbance in the presence of the extract.

Hydrogen Peroxide ($H_2O_2$) Production Assay

In order to determine the hydrogen peroxide production, a modified method previously described by Wang et al. [27] was used. A 1.0-mL aliquot of the cell-free extract from tube B was incubated with 0.2 mL 20 % $TiCl_4$ (*v*/*v*) and 0.2 mL stronger ammonia water at room temperature until precipitation appeared. After centrifugation at 3,000 rpm for 10 min to remove the supernatant, 3 mL of 2 M $H_2SO_4$ was added to the pellet to make them dissolved completely. Then the spectrum measurement was made at 410 nm against a blank which consisted of the same components but in the absence of cell-free extract. The production of hydrogen peroxide was calculated by using a standard curve measured before, preparing from known concentration of $H_2O_2$ (from 0 to 40 mM).

## Statistical Analyses

The experiment was conducted in triplicate at the same time and the results are expressed as mean±standard deviation.

# Results

## Effect of Pb on Lipid Peroxidation

The accumulation of lipid peroxides in *P. chrysosporium* indicated the enhanced production of oxygen species [28–30] and it was estimated by the content of MDA. Figure 1 depicts the variations of MDA content in *P. chrysosporium* exposing to various concentrations of $Pb^{2+}$.

As shown in the Fig. 1, after 2 h of the treatment with 50, 100, 200, and 400 mg/L Pb ions, the MDA content experienced a reduction of 21.9, 44.3, 58.6, and 29.6 % in comparison to the untreated control cells, respectively. It was also observed that increased MDA production upon exposure to 200 or 400 mg/L $Pb^{2+}$ was evident after 8 h for *P. chrysosporium* treated cells, while the MDA content of the untreated cells decreased with the extend of time. Furthermore, the treatment of fungal cells with 400 mg/L $Pb^{2+}$ for 8 h resulted in a maximum MDA content of this study, about 3-fold over the content of the control, which was an indicative of hyper-oxidative stress. On the other hand, a significant reduction of MDA content was seen as stress continued for 24 h and appeared to be the minimum content of MDA in each treated or untreated sample.

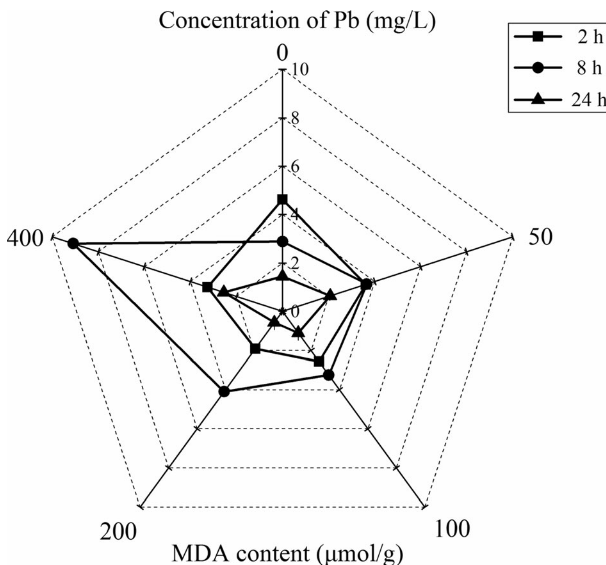


**Fig. 1** Accumulation of lipid peroxides (MDA content) in *P. chrysosporium* cells treated by enhanced Pb ions concentrations. The fungal strains were cultivated under submerged conditions with or without stress agents (50, 100, 200, and 400 or 0 mg/L $Pb^{2+}$, respectively) for 2, 8, and 24 h, respectively

Effect of Pb Stress on $O_2^-$ Generation

To investigate the potential involvement of oxidative stress on Pb ion stress, the generation of $O_2^-$ in *P. chrysosporium* was determined. Figure 2 shows the effect of $Pb^{2+}$ on superoxide anion production in *P chrysosporium* cells taken from 2, 8, and 24 h culture, respectively.

As seen in Fig. 2, the $O_2^-$ production decreased with the increase of $Pb^{2+}$ concentrations after 2 h of treatment. Thus, the $O_2^-$ content was up to 0.06 μmol/g in untreated cells, apparently much higher than those $Pb^{2+}$ treated cells, indicating a hyper-oxidative state. A sizable time-dependent decrease in superoxide anion was detected after exposure to 50 and 100 mg/L $Pb^{2+}$, whereas the $O_2^-$ content experienced a growth (from 0.01999 to 0.02569 μmol/g) then a decrease (from 0.02569 to 0.00946 μmol/g) at 400 mg/L. The control cells showed a sharp decrease in $O_2^-$ level after 8 and 24 h of incubation (56–64 % reduction), demonstrating a time-dependent stress condition. Moreover, the minimum superoxide anion content for each tested sample was found after 24 h of cultivation, through a remarkable

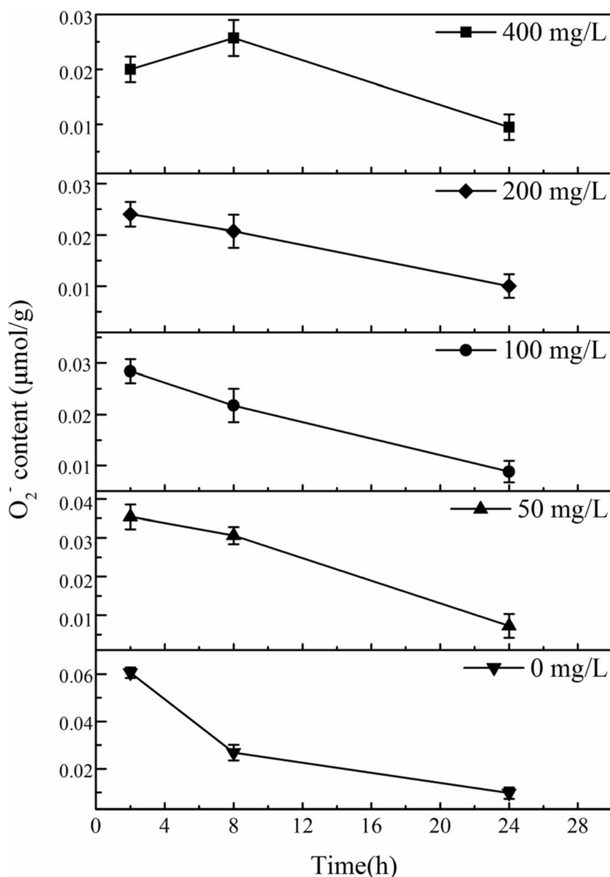


**Fig. 2** Effect of enhanced $Pb^{2+}$ concentrations on $O_2^-$ production by *P. chrysosporium*. The fungal strains were cultivated under submerged conditions with or without stress-agents (50,100, 200, and 400 or 0 mg/L $Pb^{2+}$, respectively) for 2, 8, and 24 h, respectively. The results are representative of five independent determinations in triplicate. The *bars* represent the standard deviation of the means

decline as compared with other culture periods (2 and 24 h). A similar trend has been demonstrated in the experiment of MDA described above.

## Effect of Pb on •OH Scavenging Activity

•OH was one of the most representative ROS as its damaging action was the strongest among the ROS produced in cells [31]. Measurement of the scavenging activity of •OH allowed estimation of the antioxidant defense capacity of *P. chrysosporium*. The •OH scavenging activity of *P. chrysosporium* was assessed by its ability to compete with salicylic acid for hydroxyl radical •OH in the •OH generating/detecting system. Intracellular •OH scavenging ability of *P. chrysosporium* in relation to $Pb^{2+}$ concentration and exposure time is shown in Fig. 3.

From Fig. 3, it could be seen that a 2-h exposure to $Pb^{2+}$ caused a concentration-dependent decline of •OH scavenging activity, which implies that a higher Pb concentration may result in a notable attenuation of •OH scavenging activity in *P. chrysosporium* at initial stage. The •OH scavenging activity of the untreated cells remained unchanged up to 24 h, while it was obviously increased with increase in the duration of exposure of cells to 100 and 200 mg/L $Pb^{2+}$. As a result of an 8-h exposure to 50 mg/L $Pb^{2+}$ treatment, the hydroxyl radical scavenging activity of *P. chrysosporium* was elevated by more than 49 % and then a sharp decline (41 % reduction) was experienced. When compared with all the test samples, the cells treated with 100 mg/L $Pb^{2+}$ for 24 h had the highest •OH scavenging activity (123 % of that of the control) followed by the cells exposed to 50 mg/L $Pb^{2+}$ for 8 h (114 % of control). The highest •OH scavenging activity was approximately 3-fold over the lowest level that occurred in the cells stressed by 400 mg/L $Pb^{2+}$ for 2 h (38 % of that of the control), respectively.

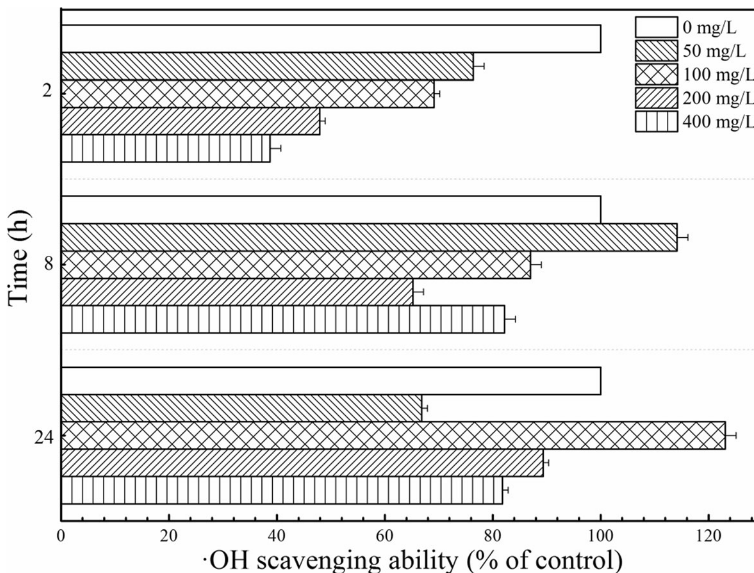


**Fig. 3** Percent of hydroxyl radical (•OH) scavenging ability in *P. chrysosporium* treated by enhanced Pb ions concentrations. The fungal strains were cultivated under submerged conditions with or without stress agents (50, 100, 200, and 400 or 0 mg/L $Pb^{2+}$, respectively) for 2, 8, and 24 h, respectively. The results are representative of five independent determinations in triplicate. The *bars* represent the standard deviation of the means

## Effect of Pb Stress on $H_2O_2$ Content

The formation of $H_2O_2$ is a marker of oxidative stress in $Pb^{2+}$ treated cells, determined by measuring the brown polymerization product produced by $Ti(SO_4)_2$ with $H_2O_2$. Figure 4 illustrates the effect of enhanced $Pb^{2+}$ concentrations on $H_2O_2$ production in cells of *P. chrysosporium*.

Results in Fig. 4 demonstrated a more active process of $H_2O_2$ generation in untreated and higher Pb concentrations (400 mg/L) in treated cells than other treated cells. Furthermore, a higher concentration (400 mg/L) of Pb ion resulted in a mutable change of $H_2O_2$ production apparently: while the Pb stress caused a significantly increase (11-fold of control) after 8 h, a markedly decrease was observed after exposure for 2 and 24 h, approximately 49 and 94 % reduction, respectively, as compared with the control. Pb ion concentrations of 50, 100, and 200 mg/L showed a slightly change of $H_2O_2$ content between 8 and 24 h incubation. The dependence of $H_2O_2$ generation on Pb concentration of up to 200 mg/L was obviously observed after exposed for 2 h. However, it was worth noting that the maximum value of $H_2O_2$ content was obtained from the untreated cells since heavy metals are known to be involved in many ways in the production of ROS including $H_2O_2$ [32, 33].

## Linear Analysis of $Pb^{2+}$ Concentrations and ROS Production

To study the effect of Pb stress on *P. chrysosporium* cells in more depth, a linear analysis was made to reveal the relationship of $Pb^{2+}$ concentration with ROS production and the pH in the culture medium. The data obtained was shown in Fig. 5.

The results from Fig. 5a, b demonstrate that the pH has an excellent negative correlation with $Pb^{2+}$ concentration after exposure for 2 and 8 h within a linearly dependent coefficient ($R^2$) of 0.954 and 0.975, respectively. The $O_2^-$ content and •OH scavenging ability of *P. chrysosporium* showed a better negative correlation with $Pb^{2+}$ concentration after 2 h treatment, with the same linearly dependent coefficient at 0.839 (Fig. 5a). In Fig. 5b, the positive correlation between both of MDA, $H_2O_2$ content, and Pb ion concentration was seen

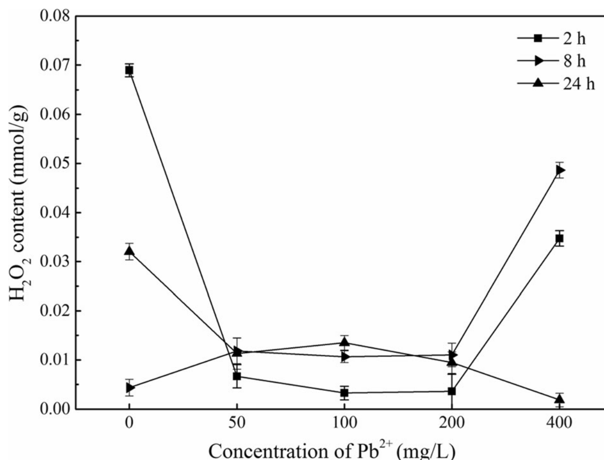


**Fig. 4** Changes of $H_2O_2$ content in *P. chrysosporium* cells treated with enhanced concentrations of $Pb^{2+}$. The fungal strains were cultivated under submerged conditions with or without stress agents (50,100, 200, and 400 or 0 mg/L $Pb^{2+}$, respectively) for 2, 8, and 24 h, respectively. The results are representative of five independent determinations in triplicate. The *bars* represent the standard deviation of the means

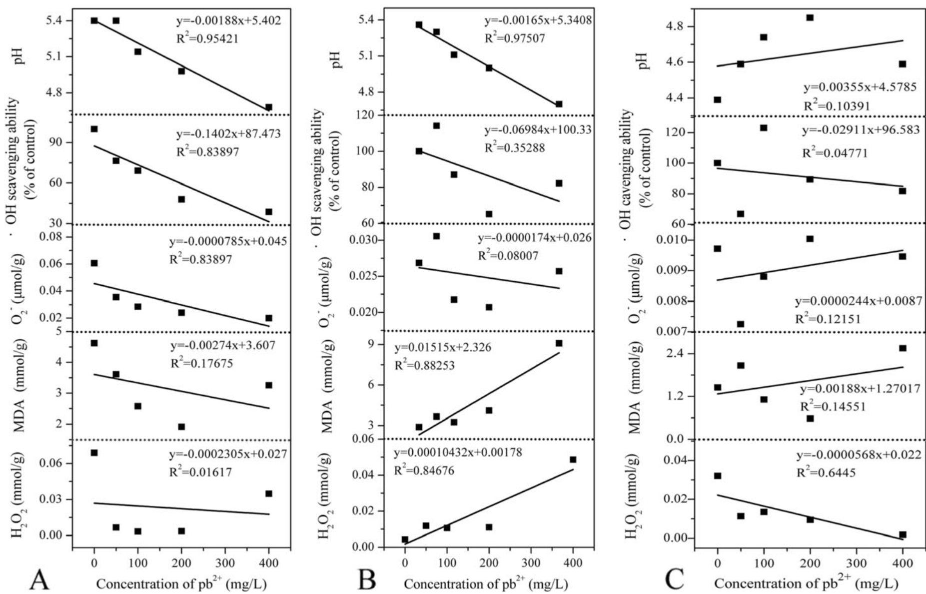


**Fig. 5** Linear relationship of enhanced $Pb^{2+}$ concentrations with ROS production and the pH of culture medium. **a**, **b**, **c** The exposure time of 2, 8, and 24 h, respectively. $R^2$ represent the linearly dependent coefficient

clearly; the linearly dependent coefficient of which was 0.883 and 0.847, respectively. However, the similar phenomenon was not observed in Fig. 5c since the linearly dependent coefficient of which was all below 0.800, probably due to the excessive accumulation of metabolic product secreted by cells as stress continued for 24 h. In general, the concentration of Pb did not have a good linear relation to ROS production.

## Discussion

Even though it is well known that Pb is a nonessential element for the normal growth and development of all organisms and it can be highly toxic by participating in many physiological processes, to date, relatively little is known as to the extent to the direct analyses of the relationship between external Pb concentration and oxidative stress in *P. chrysosporium*. The studies present here showed that increased concentrations of Pb affected the oxidant levels in *P. chrysosporium* by variating the formation of ROS and MDA in different manners. Simultaneously, these results are in agreement with the findings of Pinto et al. [34] who have concluded that the heavy metals appeared to be related to production of ROS. The critical toxicity level of $Pb^{2+}$ toward fungal *P. chrysosporium* spores is above 400 mg/L [35] and is thus much larger than that for other microorganisms. A similar trend of Pb tolerance has been established for the model strain under conditions of submerged cultivation.

The main finding of this study was that Pb stress can change redox levels in the cells of *P. chrysosporium*. First, our results indicated a correlation between external Pb concentrations and MDA and ROS levels in cells. Furthermore, MDA and ROS also can be observed in unstressed fungal cells, which presumably result from the normal aerobic metabolism in fungal cells [33, 36] but can be extremely harmful to organisms at high concentrations although it plays an important role in signaling and controlling the redox status of organisms [29]. On the

other hand, our result showed that the MDA and ROS content decreased with the increase of Pb concentrations in a linear relationship after treated for 2 h. In Fig. 5a, the negative correlation was enhanced in $O_2^-$ content ($R^2$=0.839) and the •OH scavenging activity ($R^2$= 0.839) than in the accumulation of MDA (lipid peroxide; $R^2$=0.177) and $H_2O_2$ ($R^2$=0.016). This phenomenon demonstrated that untreated fungal cells even faced a more critical oxidation state than the treated cells in the first 2 h, that is to say, $Pb^{2+}$ can diminish the MDA and ROS levels in cells at the beginning of exposure. Similar direct analyses of ROS content in fungal cells have not often been reported. According to Ercal [13], heavy metals could induce oxidative damage by directly increasing the production of ROS and reducing the cellular antioxidant capacity. Either way, the above may result in an unbalanced cellular redox status in fungal cells. Existing evidence [14, 28] suggested that aerobic organisms (eukaryotic and prokaryotic) have developed through evolutionary processes antioxidant defense mechanisms designed to prevent cellular damage from ROS insult. Accordingly, it has been proven that algae can produced several antioxidants (e.g., superoxide dismutase and glutathione peroxidase) to cope with unbalanced cellular redox status [34]. Presumably, possible explanations for the decrease of ROS and MDA levels in *P. chrysosporium* at the beginning of exposure could be that $Pb^{2+}$ enhanced the capacity of cellular antioxidant defense systems by stimulating the secretion of excess antioxidase which scavenges the exceed ROS. At the superficial level, this conjecture may disagree with Ercal who had demonstrated that Pb could destroy the antioxidant defense of cells and make them more sensitive to oxidative attacks as a result of its inhibitory effects on antioxidants enzymes [13]. In fact, although great efforts have been made to explore the oxidative stress of the cells against Pb toxicity, the exact molecular mechanisms that enable Pb to induce oxidative stress are not clearly understood. Two reaction mechanisms may be proposed for the response of cells to Pb stress: (1) Pb induces the cells' overproduction of ROS, and therefore, depletes the antioxidant capacity of the cells. (2) Pb accelerates the secretion of antioxidant enzymes to cope with the exceed ROS in cells, providing a protective effect against Pb toxicity. It can be assumed that these two mechanisms may occur at different moments or they occur at the same moment but react at different rate at the beginning of exposure that means the mechanism (2) react more quickly than mechanism (1) in the first 2 h; thus, a decrease in ROS and MDA levels in cells was observed. This phenomenon may relate to the enhanced expression of the genes that encode the enzymes and ROS in production since the oxidative stress induced by Pb can result in DNA damage in aerobic organisms [37, 38]. The genes expression and the production of antioxidase should be taken into account in further studies, in order to get a better understanding of the specific molecular mechanism involved in Pb-stressed cells.

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