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Improvement of tolerance to lead by filamentous fungus *Pleurotus ostreatus* HAU-2 and its oxidative responses

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HIGHLIGHTS

- The tolerance of fungus to Pb was enhanced significantly after acclimatization.
- The acclimated strain could accumulate Pb as high as 165.0 mg g^{-1}.
- Pb could induce the mycelia to produce malonaldehyde and H_2O_2.
- Catalase and glutathione might play an important role in elimination of the toxicity.

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ABSTRACT

Wastewater contaminated with heavy metals is a world-wide concern. One biological treatment strategy includes filamentous fungi capable of extracellular adsorption and intracellular bioaccumulation. Here we report that an acclimated strain of filamentous fungus *Pleurotus ostreatus* HAU-2 can withstand Pb up to 1500 mg L^{-1} Pb, conditions in which the wildtype strain cannot grow. The acclimated strain grew in liquid culture under 500 mg L^{-1} Pb without significant abnormity in biomass and morphology, and was able to remove significant amounts of heavy metals with rate of 99.1% at 200 mg L^{-1} and 63.3% at 1500 mg L^{-1}. Intracellular bioaccumulation as well as extracellular adsorption both contributed the Pb reduction. Pb induced levels of H_2O_2, and its concentration reached 72.9–100.9 μmol g^{-1} under 200–1000 mg L^{-1} Pb. A relatively higher malonaldehyde (MDA) concentration (8.06–7.59 nmol g^{-1}) was also observed at 500–1500 mg L^{-1} Pb, indicating that Pb exposure resulted in oxidative damage. The fungal cells also defended against the attack of reactive oxygen species by producing antioxidants. Of the three antioxidant enzymes superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT), CAT was the most responsive and the maximal enzyme activity was 15.8 U mg^{-1} protein. Additionally, glutathione (GSH) might also play a role (3.16–3.21 mg g^{-1} protein) in detoxification under relatively low Pb concentration (100–200 mg L^{-1}). Our findings suggested that filamentous fungus could be selected for increased tolerance to heavy metals and that CAT and GSH might be important components of this tolerance.

1. Introduction

Wastewater contaminated with heavy metals is a world-wide concern and efficient and inexpensive remediation methods are needed. Conventional methods for the removal of dissolved heavy metals from polluted environments include chemical precipitation

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and sludge separation, chemical oxidation or reduction, ion exchange, reverse osmosis, filtration, adsorption using activated charcoal, electrochemical treatment and evaporative recovery (Corey et al., 1987; Volesky, 1994). However, physico-chemical techniques can be very costly, consume a lot of energy, and may cause secondary pollution (Price et al., 2001). Microbiological strategies to remove heavy metals in wastewater using bioaccumulation present an alternative method of heavy metal removal, and potentially can performed with minimal costs and are more environmentally friendly (Zafar et al., 2007). Generally, microbes can remove heavy metals by two mechanisms: extracellular biosorption, a metabolism-independent binding of negatively charged free groups to the fungal cell wall, and intracellular bioaccumulation, an energy-dependent metal influx (Malik, 2004; Raspanti et al., 2009; Xu et al., 2014). Various partially understood mechanisms allow heavy metal bioaccumulation in microorganisms, including cell wall cation exchange, extracellular chelation with organic acids, and intracellular bioaccumulation (Huang et al., 2012). Heavy metals that are accumulated intracellularly can cause serious cellular damage by thiol-binding and protein denaturation, primary displacement of essential metals required for biological reactions, or due to secondary effects resulting from oxidative stress (Flora et al., 2008; Stijve and Roschine, 1974).

Filamentous fungi typically have large biomass and some strains are able to uptake single or multiple heavy metals simultaneously (Mishra and Malik, 2012). Therefore, these organisms could theoretically be utilized in water treatment. Effective use of these fungi would require that they have high tolerance to the metals and that they are efficiently able to remove heavy metals. However, in spite of several reports on the ability of filamentous fungi to remove heavy metals, the response of fungi to heavy metal stress was not well understood.

We examined the ability of the filamentous fungus, *Pleurotus ostreatus* HAU-2, to remove and tolerate Pb, a heavy metal with wide distribution in the environment and high toxicity. Firstly, we improved the tolerance of the strain to Pb by a acclimazation process. Secondly, we assessed the growth and removal efficiency of Pb by the strain in liquid culture. Finally, the oxidative damage and antioxidant levels of the strain during Pb stress were determined.

2. Materials and methods

2.1. Microorganism, chemicals and media

Fungus *P. ostreatus* HAU-2 was supplied by the Henan Academy of Agricultural sciences, China. The organism was stored on solid potato dextrose agar (PDA) plates at 4 °C.

Solid PDA medium (pH4.5) included the following: glucose 20.0 g L^{-1}, potato extraction 200 mL L^{-1}, agar 18.0 g L^{-1}.

The liquid medium used (pH4.5) included the following: glucose 5.0 g L^{-1}, KH_2PO_4 0.2 g L^{-1}, $MgSO_4$ 0.05 g L^{-1}, $(NH_4)_2SO_4$ 0.32 g L^{-1}, $CaCl_2$ 0.01 g L^{-1}, inorganic solution (1 mL L^{-1}), and vitamin solution (0.5 mL L^{-1}).

2.2. Acclimazation experiment

The strain was inoculated onto solid PDA media. After 7 d incubation (28 °C, 150 rpm), an agar plug (5 mm diameter) cut out from the margin was inoculated onto solid PDA media that contained 100 mg L^{-1} Pb. After 7 d incubation (28 °C, 150 rpm), another agar plug (5 mm diameter) cut out from the margin was inoculated onto solid PDA media contained 150 mg L^{-1} Pb. The above process was repeated several times and the Pb concentration was elevated 50 mg L^{-1} for each time till the strain was unable to grow.

To evaluate the effects of the acclimazation process on the Pb tolerance of *P. ostreatus* HAU-2, a comparative experiment was performed. Briefly, both the wildtype and the selected strain were inoculated onto solid PDA media containing different Pb concentrations (100, 200, 500, 1000, or 1500 mg L^{-1}). The diameter of the colony was compared after 7 d incubation (28 °C).

2.3. Growth in liquid culture

After 7 d growth on the solid PDA media (28 °C), an agar plug (5 mm diameter) of the selected strain was cut out from the margin and was used to inoculate flasks containing 100 mL liquid media with Pb concentrations of 0, 100, 200, 500, 1000 or 1500 mg L^{-1}. Each treatment was carried out in at least triplicate. After 10 d incubation (28 °C, 150 rpm), the liquid cultures were filtered. To determine the Pb removal efficiency, the Pb concentration in the supernatant was analyzed by atomic absorption spectrometer (AAS, Agilent 3510, USA).

The mycelia in some flasks was collected and dried in the oven (80 °C, 24 h) to measure their biomass. In order to investigate the morphological characteristic of the strain under Pb stress, image of mycelia under different Pb concentration was done using SEM (S-3400N-II) under the following analytical condition: EHT = 25.00 kV, WD = 7.5 mm, SignalA = SE. Samples for SEM were prepared using standard protocol (Tyagi and Malik, 2010).

To determine the adsorptive Pb content, the mycelia in some flasks was treated with 50 mL 0.2 mol L^{-1} HNO_3 (30 min) solution for three times to desorb the adsorbed metals. The washed acid solutions were combined and digested with nitric acid and sulphuric acid (3:1 ratio) before analysis by AAS. To determine the accumulative Pb content, the washed mycelia were rinsed with ultrapure water three times, homogenized in a glass homogenizer, digested and analyzed by AAS.

2.4. H_2O_2 and MDA content analysis

To determine the H_2O_2 concentration, the homogenized mycelia was suspended in 100 mM PBS buffer (pH7.8) that contained 1% (w/v) polyvinylpyrrolidone (PVP). After centrifugation (20 min, 12,000 g), the supernatant was used to measure the H_2O_2 levels as described by Verma and Mishra (Verma and Mishra, 2005).

To determine the MDA concentration, the homogenized mycelia were resolved in 0.1% (w/v) trichloroaceticacid (TCA) solution. After centrifugation (15 min, 12,000 g), an aliquot of the supernatant was added to 0.5% thiobarbituricacid (TBA) in 20% TCA and heated at 90 °C for 30 min. After cooling on ice, the mixture was centrifuged at 8000 g for 5 min. The absorbance was determined at 532 and 600 nm and the MDA concentration was calculated from the difference between these absorbance values (Nader et al., 2005).

2.5. SOD, CAT and POD activity analysis

To measure antioxidative enzyme activity, 0.5 g fresh mycelia were ground in liquid nitrogen to extract the total protein. The resulting powder was suspended in 3 mL of extraction buffer that contained 50 mM sodium phosphate buffer (pH7.5), 1% (w/v) PVP, and 0.1 mM EDTA. The enzyme activities of SOD and CAT were measured as previously described (Xu et al., 2010). The POD activity was determined by measuring the increase in the absorbance at 470 nm as a result of the oxidation of guaiacol. The reactive ion mixture contained 25 mM sodium phosphate buffer (pH7.0), 0.1 mM EDTA, 0.05% guaiacol, 1 mM H_2O_2, and 50 μl enzyme in a