Ecological Engineering 75 (2015) 110–115

Contents lists available at ScienceDirect

Ecological Engineering

journal homepage: www.elsevier.com/locate/ecoleng

Cadmium induced hydrogen peroxide accumulation and responses of enzymatic antioxidants in *Phanerochaete chrysosporium*

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ARTICLE INFO

Article history:
Received 11 August 2014
Received in revised form 24 November 2014
Accepted 29 November 2014
Available online xxx

Keywords:
Phanerochaete chrysosporium
Cadmium
Toxicity
Enzymatic antioxidants
Detoxification

ABSTRACT

The white-rot fungi *Phanerochaete chrysosporium* have been widely applied in heavy metal bioremediation, which commonly results in a stress exposure. Exposure studies showed that Cd bioaccumulation triggered H_2O_2 generation. Thereafter, the H_2O_2, as signal molecules, activated the enzymatic antioxidant response in the case of the conjunction of SOD and CAT. High levels of R (SOD/CAT) were found initially under Cd exposure, and then a significant decline occurred in time-course Cd exposure. Antioxidant activities also provided an alternative as an indication of the existence of antioxidant response upon Cd exposure, via coupling H_2O_2 level with the variation in antioxidant activities. This finding potentially has implications for the understanding of tolerance behavior and detoxification mechanisms, which is beneficial to expand the application of *P. chrysosporium* in bioremediation biotechnology.

1. Introduction

Heavy metals, such as cadmium, lead, copper, chromium, and arsenic, are widely occurring environmental pollutants. Among these, cadmium (Cd) is listed as a priority pollutant by the US environmental protection agency, due to its high water solubility, without known biological function, high toxicity and easy enrichment in food chain and pose a threat to human beings (Pérez-Chaca et al., 2014; Balestri et al., 2014; Xu et al., 2012a). Biosorption is an emerging cost-effective and ecofriendly technology that utilizes microorganisms to remove, transform, or stabilize a variety of heavy metals. Many workers have reported the application of *Phanerochaete chrysosporium* (*P. Chrysosporium)* in biosorption of heavy metals, in the case of high efficiency and low cost (Xu et al., 2012b, 2013; Iqbal and Edyvean, 2004; Çeribasi and Yetis, 2001). The biosorption capacity of *P. chrysosporium* partly depends on their ability to survive potentially toxic treatments. Studies carried out in different microorganisms have revealed that Cd is strongly toxic and causes growth inhibition and even cell death (Chen et al., 2008). Recent studies further reported that metals such as iron, copper, cadmium, chromium, lead and mercury exhibited the ability to produce reactive oxygen species (ROS), resulting in oxidative stress alteration of calcium homeostasis and DNA damage (Klaunig et al., 1998).

The question as how microorganisms may defend themselves against heavy metals is therefore receiving increasing attention. It has been widely reported that exposure to heavy metals led microorganisms to develop a series of defense mechanisms, including antioxidant defenses (Cadenas, 1997; Mishra et al., 2006). Fungi, like many other microorganisms, rely on antioxidant defense mechanisms to protect against oxidative damage (Chagas et al., 2008; Pacini et al., 2013). However, scarce study is underway to determine the antioxidant response under heavy metal exposure in *P. chrysosporium*. In order to prove the importance of using antioxidants in heavy metal detoxification, pertinent biochemical detoxification mechanisms contributing to stress tolerance is necessary to understand.

To understand tolerance and maximize the potential application of *P. chrysosporium* in bioremediation, it is essential to understand the toxicity of the heavy metals and the detoxification mechanisms of *P. chrysosporium*. The present research work was

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http://dx.doi.org/10.1016/j.ecoleng.2014.11.060
0925-8574/

therefore focused on Cd induced H_2O_2 generation and the antioxidant response of *P. chrysosporium* on the metabolic and physiological level compared for cells cultured at Cd concentrations of 0, 20, 50 and 100 ppm. We further investigated the feasibility of the antioxidant mechanism of *P. chrysosporium* to Cd exposure, with particular attention given to the constitution and response of superoxide dismutase (SOD) and catalase (CAT). Meanwhile, effect of Cd on the antioxidant activity of *P. chrysosporium* was determined by detection the variations in total antioxidant activity (TAA) and radical scavenging activity (RSA).

2. Materials and methods

2.1. Strain and chemicals

The *P. chrysosporium* strain BKMF–1767 (ATCC 24,725) was purchased from the China Center for Type Culture Collection (Wuhan, China) and maintained by subculturing on potato dextrose agar (PDA) slants at 4 °C. All reagents used in the experiment were of or above analytical reagent grade.

2.2. Exposure to Cd

Spore suspensions of *P. chrysosporium* were prepared in the sterile distilled water at a concentration of 2.0×10^6 cell mL^{-1}. Liquid cultures, consisted of 100 mL growth medium as described by Kirk et al. (1986) were inoculated with 2 mL the defined spore suspensions with desirable concentrations of Cd. For the toxicity experiments, Cd in the mixture was controlled at the concentration of 0, 20, 50 and 100 ppm via the addition of $Cd(NO_3)_2{\cdot}4H_2O$. All experiments were conducted under constant stirring at 30 °C and 120 rpm and performed in three culture replicates.

At the selected time intervals, *P. chrysosporium* biomass was collected and weighted, washed three times in 20 mL phosphate buffer solution (PB, 0.05 M, pH 7.0) solution, and then homogenized in 10 mL of phosphate buffer solution (PB, 0.05 M, pH 7.4). The homogenate was centrifuged at 10,000 rpm at 4 °C for 10 min, and the supernatant was filtered by 0.45 μm filter membranes and collected for further use. Removal of Cd was determined by detect the residual Cd in the growth medium by an atomic absorption spectrometer (AAS, Agilent 3510, USA). The concentrations of the Cd in the prepared *P. chrysosporium* extracts were tested to evaluate the bioaccumulation level of Cd.

2.3. H_2O_2 generation detection

During the chronic exposure time, the ROS and H_2O_2 generation, were frequently measured. H_2O_2 was detected according to H_2O_2 assay kit purchase from Beyotime institute of biotechnology.

2.4. Enzymatic antioxidant responses detection

Briefly, CAT activity was assayed by measuring the rate of decrease in H_2O_2 (100 mM) at 240 nm by UV–vis spectrophotometer (UV-2250, SHIMADZU, Japan). 0.1 mL extract was added to 2.5 mL PB solution (50 mM, pH 7.4), the reaction was started by addition of 0.5 mL H_2O_2. CAT activity was monitored through H_2O_2 consumption. Data were expressed in U mg $prot^{-1}$ (Nemmiche et al., 2007). The biological activities of SOD were tested in supernatants according to Marklund and Marklund (1974) by quantifying the inhibition of superoxide-dependent pyrogallol (PAPG) self-oxidation with the addition of *P. chrysosporium* extracts. One SOD unit is defined as the enzyme quantity that inhibits the autoxidation of PAPG by 50%. The reaction mixture contained 2.5 mL Tris–HCl buffer solution (100 mM, pH 8.2), 0.15 mL PAPG (10 mM) and 0.1 mL *P. chrysosporium* extracts. After intensive mixing, the variation in absorbance of the mixture was recorded via ultraviolet spectrophotometer at 320 nm for 3 min. Meanwhile, R (SOD/CAT) was defined as the ratios of enzyme activity between SOD and CAT. Protein concentrations were measured by the Bradford method (Bradford, 1976) using bovine albumin as the standard.

2.5. Antioxidant activity analysis

Antioxidant activity estimation was characterized as TAA, $O_2^{\bullet-}$ and $^{\bullet}OH$ RSA (Liu et al., 2014). Total antioxidant activity of *P. chrysosporium* extracts were conducted by measuring the variation in absorbance of $ABTS^{\bullet+}$ solution at 734 nm vs time for 3 min after the addition of 100 μl *P. chrysosporium* extracts. The $ABTS^{\bullet+}$ solution was prepared via the dilution of 2,2′-azino–bis(3-ethylbenzthiazoline-6- sulfonic acid) (ABTS) in acetate buffer to the absorbance of 0.70 (±0.02) at 734 nm using a UV–vis spectrophotometer. Meanwhile, the inhibition ratio between autoxidation and oxidation rate of pyrogallic acid, at the absence and presence of *P. chrysosporium* extracts, was detected to determine the $O_2^{\bullet-}$ scavenger activity. $^{\bullet}OH$ scavenger activity was performed by orderly mixing 1.5 mM $FeSO_4$, 6 mM H_2O_2, 20 mM sodium salicylate and *P. chrysosporium* extracts, and then incubated for 30 min in dark. The $^{\bullet}OH$ scavenger activity was conducted by calculation the variation in absorbance at the absence and presence of *P. chrysosporium* extracts at 562 nm.

2.6. Statistical analysis

All of the above analytical experiments were done in triplicate, and the results were analyzed as the mean value with the standard deviation. Correlation and regression analyses were carried out using the regression program in SPSS software (SPSS 18.0, Germany). All reported error bars represent one standard error of the arithmetic mean.

3. Results

3.1. Cd uptake and bioaccumulation in P. chrysosporium

P. chrysosporium has been identified as an effective bioremediation agent for its biosorption and degradation ability. Cd concentration curves shown in Fig. 1a demonstrated that Cd contents in all tested concentrations declined in the early calculation stage (first 4 days). As the concentration of Cd increased, there was a concomitant increase in Cd removal efficiency. It was mainly because that the initial concentration supplied a kind of important driving force, to overcome the existing mass transfer resistance of Cd, simultaneously reinforce the active uptake of *P. chrysosporium* (Xu et al., 2012b). Concomitantly, the disappearance of Cd was accompanied with a gradual bioaccumulation of Cd in *P. chrysosporium*. In all tested Cd concentrations, Cd bioaccumulation increased in relation to the Cd content of the growth medium as shown in Fig. 1b. A large increase in Cd bioaccumulation occurred in *P. chrysosporium* grown at high Cd concentration (100 ppm), while Cd contents increased slightly at concentrations of 50 ppm Cd. Interestingly, there was a distinct decrease in Cd contents after 6 d of growth at 20 ppm Cd, which might be ascribed to the active efflux mechanism.

3.2. Cd-induced H_2O_2 accumulation in P. chrysosporium

It is known that Cd exposure is inevitably connected with an internal oxidative stress, even though Cd is a non redox-active element (May et al., 1998). The excessive production of ROS, via consumption of oxygen and antioxidants result in a so-called