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Plasma membrane behavior, oxidative damage, and defense mechanism in *Phanerochaete chrysosporium* under cadmium stress

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ABSTRACT

Microorganisms are essential for maintaining ecosystem balance, and understanding their response to toxic pollutants is important in assessing the potential environmental impacts of such releases. In this study, the response to the heavy metal cadmium and the potential defense or adaptive mechanisms of the widely used white-rot fungus, *Phanerochaete chrysosporium*, were investigated. The results indicated that cadmium causes plasma membrane damage, including rigidification of lipids, a decrease in H^+-ATPase activity, and lipid peroxidation. The cellular death may be mediated by oxidative stress with mitochondria membrane potential (MMP) breakdown and reactive oxygen species (ROS) formation. Parts of the cells were able to survive by activating antioxidant defense systems (antioxidant agents and enzymes). Extracellular synthesis of cadmium crystal particles was observed after exposure to dissolved cadmium ion, which is probably another detoxification mechanism in which the dissolved metal is precipitated, thus reducing its bioavailability and toxicity. These physiological responses of *P. chrysosporium* to cadmium together with the defense mechanisms can provide useful information for the development of fungal-based technologies to reduce the toxic effects of cadmium.

1. Introduction

The use of microorganisms in bioremediation has been extensively studied. *Phanerochaete chrysosporium*, a white-rot fungus, has been found to be efficient in treating wastewater containing heavy metals and toxic organic pollutants because of its unique ability to degrade xenobiotics and bioabsorb heavy metals [1–3]. However, the ability of *P. chrysosporium* to remove pollutants varies substantially with the types of pollutants and reaction conditions [3–5]. Moreover, its colonization and bioactivity depend largely on the types of pollutants and reaction conditions [1,6].

Generally, the objects disposed by *P. chrysosporium* are often toxic. For example, cadmium, a non-essential heavy metal widely present in the environment, is one of the most toxic environmental pollutants to all living cells [7]. Extensive studies have revealed that cadmium is toxic to aquatic plants at all levels—cellular, physiological, biochemical, and molecular [8]. The uptake and accumulation of cadmium in plant usually causes ROS burst by displacing Fe from proteins and inhibiting the electron transport chain in the mitochondria, as well as by inhibiting antioxidative systems in living cells. The excess ROS generated reacts with lipids, proteins, and pigments, and finally results in membrane damage and enzyme inactivation [9,10]. Other symptoms of cadmium toxicity include growth inhibition, proteins and DNA oxidation, and ultrastructural changes [8,11]. In the microorganism bioremediation research field, cadmium is also regarded as one of the most toxic heavy metals. Our previous study also demonstrated cadmium reduced protein production and enzymatic activities (lignin peroxidase and manganese peroxidase) of *P. chrysosporium* [3].

The toxic effects of pollutants affect microbial physiological changes, bioactivity, and colonization, resulting in decreased treatment ability [6] and limiting the development and popularization of biological treatment technologies. Therefore, understanding the response of *P. chrysosporium* to toxic pollutants stress in the medium is essential. Previous studies on cadmium removal using *P. chrysosporium* have provided some useful information on the relationship between fungal activities and cadmium removal [12,13]. Our previous study also reported that *P. chrysosporium* can bind Cd^{2+} ions by its mycelium and extracellular polymeric substances and remove them from wastewater [5]. However, information on

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the physiological responses of *P. chrysosporium* under cadmium stress is still limited. In particular, the effects of cadmium stress on the resistance, and adaptive responses of *P. chrysosporium* have not yet been reported in the literature.

In view of this, the main purpose of the present study was to explore cadmium toxicity in *P. chrysosporium* using physiological measurements. This study investigated the oxidative damage induced by cadmium and the defense mechanism of *P. chrysosporium* against these stresses. To assess physiological responses, we investigated plasma membrane fluidity, H^+-ATPase variation, and oxidative damage following exposure to cadmium because these variations can be used as indicators of microbial fitness and survival [14]. In addition, the antioxidative responses or defense mechanisms were evaluated.

2. Materials and methods

2.1. Strain and treatment

The *P. chrysosporium* strain BKMF-1767 (CCTCC AF96007) used in this study was obtained from the China Center for Type Culture Collection (Wuhan, China). Stock cultures were maintained on malt extract agar slants at 4 °C. Spores were gently scraped from the agar surface and blended in sterile distilled water to obtain a spore suspension. The spore concentration was adjusted to 2.0×10^6 spores/ml. Aqueous suspensions of fungal spores were inoculated into Kirk's liquid culture medium [15] in 500-ml Erlenmeyer flasks. Flasks containing 8×10^6 spores were incubated at 37 °C in an incubator. After 2 days of growth in liquid culture, the mycelia were treated with various concentrations of $Cd(NO_3)_2$ for 24 h. The mycelia were then harvested and washed twice with ultrapure water for further analysis. All chemicals used in this study were at least of analytical reagent grade.

2.2. Plasma membrane H^+-ATPase activity assay

The plasma membrane was obtained according to the methods described by Perlin et al. [16]. Plasma membrane H^+-ATPase activity was determined as the amount of P_i released by hydrolysis of ATP in the reaction mixture [17]. Released phosphate comes from the hydrolysis of ATP by ATPase in plasma membrane, as well as by apyrases and alkaline phosphatases, which are resistant to the inhibitors for mitochondrial ATPase (sodium azide), vacuolar ATPase (potassium nitrate), acid phosphatase (ammonium molybdate) and plasma membrane ATPase (Na_3VO_4). Accordingly, H^+-ATPase activity in plasma membrane was determined as the difference between the activities in the presence and absence of the inhibitor Na_3VO_4. Plasma membrane H^+-ATPase activity was measured using 5 ml disposable plastic tubes with 24 μl of purified plasma membrane and 500 μl of reaction mixture containing 50 mM 2-(N-morpholino)ethanesulfonic acid adjusted to pH 5.7 with Tris, 5 mM magnesium sulfate, 50 mM potassium nitrate, 5 mM sodium azide, 0.2 mM ammonium molybdate, and 1 μl of water or inhibitor (0.1 M Na_3VO_4). After 3 min of incubation at 30 °C, the assay was started by adding 10 μl of 0.1 M Na_2ATP. Color was developed for 10 min, and the absorbance at 690 nm was measured in a UV–vis spectrophotometer (Model UV-2550, Shimadzu Company, Tokyo, Japan). A calibration curve for phosphate was obtained for amounts of phosphate from 0.5 to 0.01 μmol supplied in a 0.1 M solution of Na_2HPO_4 as standard. One unit of activity corresponded to 1 μmol P_i released per min. The specific activity was expressed in units per g protein.

The protein concentration was determined by colorimetrically using a UV–vis spectrophotometer (Model UV-2550; Shimadzu Company, Tokyo, Japan) at 595 nm according to the method of Bradford [18] using bovine serum albumin as a standard.

2.3. Membrane fluidity determination

Fluidity of the plasma membrane was determined according to a previously described method [19]. Briefly, 282 mM mannitol (pH 7.2) and 50 μM 8-anilino-1-naphthalene-sulfonic acid (ANS) were added into the assay medium. An appropriate volume of plasma membrane preparation was then added to the medium to ensure that the amount of protein was identical in every determination. After 1 min of ANS addition and mixing, the fluorescence intensity was recorded at 22 °C using a fluorescence spectrometer (FluoroMax-4; Horiba Scientific, Tokyo, Japan). Fluorescence intensity was inversely proportional to membrane fluidity.

2.4. Cell viability assay

Cell viability was assessed using the MTT assay according to Luo et al. [20] with some modifications. MTT is a yellow water-soluble tetrazolium dye that is reduced by living cells to a water-insoluble purple formazan. The conversion of the MTT into purple formazan occurs only in living cells with active mitochondria and is directly proportional to the number of viable cells. Briefly, 0.2 g *P. chrysosporium* pellets were mixed with 1 ml MTT solution (5 g/l) and incubated at 50 °C. The reaction was stopped by adding 0.5 ml hydrochloric acid (1 M) to the mixture. The mixture was centrifuged ($10{,}000 \times g$, 5 min), the supernatant was discarded, and the pellets were agitated in 6 ml propan-2-ol for 2.5 h. The centrifugation process was repeated and the absorbance of the supernatant was recorded at 534 nm. In the test, an antioxidant agent (vitamin C, 0.1 mM) was used to explore the compensating effects against to cadmium toxicity.

2.5. ROS generation

The degree of ROS generation induced by cadmium was determined using a fluorometric indicator, 2,7-dichlorodihydrofluoresceindiacetate (H_2DCF-DA), as previously described [21]. H_2DCF-DA could be transformed into 2,7-dichlorodihydrofluorescein (H_2DCF) by intracellular esterase if they enter cells. When intracellular ROS generated, 2,7-dichlorofluorescein (DCF) would be converted from H_2DCF. Thus we measured the fluorescence intensity of DCF, which indicated the extent of ROS generation. Before cadmium exposure, the cells were incubated with H_2DCF-DA (5 μM) in incubation medium for 2 h. The H_2DCF-DA was then removed, and the mycelia were treated with the indicated cadmium concentrations. The medium was then removed, and the cells were washed with phosphate-buffered saline (PBS). Fluorescence was measured using a fluorescence spectrometer (FluoroMax-4; Horiba Scientific, Tokyo, Japan) with filters for excitation at 485 nm and emission at 525 nm.

2.6. Membrane lipid peroxidation and integrity assay

The cadmium-induced membrane liquid peroxidation was estimated by measuring the concentrations of malondialdehyde (MDA), which is a cytotoxic product of lipid peroxidation and an indicator of free radical production and consequent tissue damage. The harvested *P. chrysosporium* were homogenized in 10% trichloroacetic acid and centrifuged at $10{,}000 \times g$ for 15 min. The supernatant was boiled with thiobarbituric acid for 20 min. The heated supernatant was centrifuged at 5000 rpm for 5 min, and the absorbance was measured at 532 and 600 nm [22].