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Growth, metabolism of *Phanerochaete chrysosporium* and route of lignin degradation in response to cadmium stress in solid-state fermentation

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HIGHLIGHTS

- Cd stress affected the growth and metabolism activity of *P. chrysosporium*.
- The production of reactive oxygen species (ROS) increased under Cd exposure.
- Cd was a inhibitor of lignocelluloses degradation, especially lignin and cellulose.
- Partial Cd could be removed by *P. chrysosporium*.

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ABSTRACT

This study examines the growth, metabolism of *Phanerochaete chrysosporium* (*P. chrysosporium*) and route of lignin degradation in response to cadmium (Cd) stress in solid-state fermentation of rice straw. Less living fungi biomass was found under Cd exposure, suggesting that Cd had strong toxicity to *P. chrysosporium*. The maximum values of lignin peroxidase and manganese peroxidase were 0.34 and 5.21 U g^{-1} at the Cd concentration of 32 mg kg^{-1}, respectively, lower than that in control, which indicated Cd stress would inhibit ligninolytic enzymes. The production of reactive oxygen species (ROS) including hydroxyl radicals (·OH), superoxide anion radical ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) increased after Cd exposure. Higher concentration of oxalate was detected at high Cd concentrations. Cd stress also had influence on the rates of lignocelluloses degradation and the route of lignin degradation. Partial Cd could be removed by *P. chrysosporium*.

1. Introduction

Lignocellulose is mainly composed of cellulose, hemicelluloses and lignin. Lignin acts as a barrier to any solutions or enzymes by linking to both hemicellulose and cellulose, which prevents penetration of lignocellulolytic enzymes to the interior of lignocellulosic structure. While lignin is hard to degrade due to that it is highly irregular and insoluble. Therefore, the degradation of lignin is the key step to the lignocellulose transformation (Huang et al., 2010; Zhao et al., 2012). *Phanerochaete chrysosporium* (*P. chrysosporium*), a well-known white-rot fungus, has been shown to effectively degrade lignin, which has been correlated with the simultaneous secretion of extracellular oxidative enzymes including lignin peroxidase (LiP) and manganese peroxidase (MnP) (Kersten and Cullen, 2007; Zeng et al., 2013). Therefore, much attention has currently been drawn to the development of solid-state fermentation (SSF) with *P. chrysosporium* for the efficient treatment of lignocellulosic waste (Huang et al., 2008; Ganesh Kumar et al., 2006; Zeng et al., 2007).

The contamination of surface and groundwater by cadmium (Cd) as a result of industrial and domestic waste disposal has become one of the most important ecological problems in many countries and districts. Agricultural wastes which contain much lignocellulose in the Cd-polluted area are polluted to some extent according to various ways. Nowadays, the potential application of white-rot fungi in the treatment of heavy metal- contaminated wastewater and in the recovery of metals in mining wastes or in metallurgical effluents is widely reported. Previous studies confirmed that *P. chrysosporium* could remove Cd from wastewater through active absorption and surface adsorption (Li et al., 2004; Say et al., 2001; Zuo et al., 2014). The biosorption capacity of *P. chrysosporium* partly depends on their ability to survive potentially toxic exposure. However, there is limited and scattered information about treatment of Cd contaminated lignocellulosic waste

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with *P. chrysosporium*. Toxic heavy metal and the white-rot fungi would influence each other (Baldrian, 2003). The existence of toxic heavy metal can inhibit the growth of microorganism, lead to the morphology and physiology changes of microorganism, affect the metabolism of microorganism, and regulate the transcription and activity of ligninolytic enzymes (Chen et al., 2014; Gallego et al., 2012). This would disturb lignocellulose biodegradation process and slow degradation speed. Therefore, the understanding on the SSF process of lignocellulosic waste at the presence of Cd is necessary, which would provide useful information for the development of fungi-based technologies to improve the degradation of heavy metal-polluted lignocellulosic wastes.

The main purpose of our study was to understand the growth, metabolism of *P. chrysosporium* and route of lignin degradation in response to Cd stress in SSF. Herein, ROS levels, ligninolytic enzymes, and oxalate were detected for the first time in SSF process with *P. chrysosporium* under Cd stress, which could provide theory for the research on interaction between Cd and microbes. Meanwhile, the removal ability of Cd by *P. chrysosporium* in biodegradation process was also studied.

2. Materials and methods

2.1. Inoculum preparation and chemicals

P. chrysosporium strain BKMF-1767 (ATCC 24725) was obtained from China Center for type Culture Collection (Wuhan, China). Fungal cultures were maintained on potato dextrose agar (PDA) slants at 4 °C, and then transferred to PDA plates at 37 °C for several days. The spores on the agar surface were gently scraped and blended in the sterile distilled water as spore suspension. The spore concentration was measured and adjusted to 2.0×10^6 CFU mL^{-1}. All the chemicals used in this work were of analytical reagent grade.

2.2. Solid-state fermentation conditions and sampling

The straw obtained from suburban areas of Yuelu District (Changsha, China) was air-dried and cut into small pieces about 2 mm. The concentration of total Cd in the straw was 0 mg kg^{-1}. SSF was carried out in 500 mL flasks containing 30 g of straw and 167 mL basic nutrient solution. Flasks, labeled as A (control), B (2), C (4), D (8), E (16) and F (32), were supplemented and mixed thoroughly with $CdCl_2$ solutions by adding total Cd^{2+} 0, 2, 4, 8, 16, and 32 mg kg^{-1} (dry straw) respectively. They were used to simulate different degrees of Cd pollution. Each flask was stoppered and autoclaved twice for 30 min at 121 °C. Then 3 mL spore suspension was inoculated at room temperature. The fermentation experiments were performed at 37 °C for 40 days. The humidity was maintained at the initial level (85%) in the entire fermentation period. After inoculation, fermented straw was harvested from different sites in the flask periodically (0, 5, 10, 15, 20, 25, 30, 35 and 40 day) and mixed together homogeneously for routine analysis. All experiments were performed in three replicates.

2.3. Living fungi biomass determination

The total amount of ergosterol was measured after saponification. Freeze dried samples were homogenized and extracted with 1 mL cyclohexane and 4 mL 10% (w/v) KOH dissolved in methanol followed by sonication for 15 min. The samples were heated for 90 min at 70 °C, 1 mL deionised water and 2 mL cyclohexane were added. Samples were centrifuged (3000 rpm, 5 min) and the cyclohexane phase (upper phase) was transferred to new test tubes. Samples were washed with 2 mL cyclohexane, the two cyclohexane phases were combined, and evaporated under a stream of nitrogen at 40 °C. The samples were dissolved in 1 mL methanol at 40 °C for 15 min followed by sonication for 1 min and filtering through a 0.45 μm syringe filter and analyzed on a Pharmacia HPLC, using UV detection of the conjugated double bond (Nylund and Wallander, 1992). Standards and blanks were treated in the same manner. The two agar media used were also analyzed, and were found not to contain ergosterol (data not shown).

2.4. Ligninolytic enzymes activity assays

1 g sample was suspended at a 1:20 (w:v) ratio of sample-to-distilled water on a rotary shaker at 200 r min^{-1} for 30 min and then centrifuged at 3500 rpm for 15 min. The supernatant fluid was filtered through 0.45 μm filter papers. Substrate filtrate was used for ligninolytic peroxidase activity analyses with a Shimadzu 2550 UV–visible spectrophotometer. LiP activity was measured as described by Tanaka et al. (2009), one unit (U) of LiP activity was defined as the amount of the enzyme required to produce 1 M veratryl aldehyde from the oxidation of veratryl alcohol per minute. MnP activity was measured as described by Lopez et al. (2007), and one unit (U) of MnP was defined as the amount of enzyme required for producing 1 M Mn^{3+} from the oxidation of Mn^{2+} per minute.

2.5. Reactive oxygen species determinations

1 g sample was suspended at a 1:20 (w:v) ratio of sample-to-distilled water on a rotary shaker at 180 rpm for 30 min and then centrifuged at 5000 rpm for 10 min. 1 mL supernatant fluid was mixed with phosphate buffer solution (pH 7.8) and hydroxylamine hydrochloride (0.1 mL 10 mM L^{-1}), then warm bathed at 25 °C for 30 min, reacted with 0.5 mL α-naphthylamine for 20 min at 25 °C. At last, 4 mL ether was added to the mixture, centrifuged at 1500 rpm for 5 min. The supernatant fluid was measured with a Shimadzu 2550 UV–visible spectrophotometer at 530 nm (Cheng et al., 2014).

The improved thiobarbituric acid (TBA) method was used to assay ·OH (Anderson and Greenwald, 1985). TBA-reactive substances (TBARS) were determined as follows. The rest of the sample leach liquor from analysis of O_2^- was collected and filtered through 0.45 μm filter paper, 0.0022 g 2-deoxy-D-ribose was added into 4 mL filtrate and incubated at 37 °C for 1 h, then TBA (Sigma, 0.25%) and trichloroacetic acid (0.7%) were added, and the mixture was heated at 100 °C for 15 min. The absorbance at 532 nm was measured. The control was the same reaction mixture with no sample leach liquor added. The reductive activity of ·OH was indicated by the decrease of absorbance at 532 nm compared to that of the control.

In order to determine H_2O_2 production, a modified method previously described by Bian et al. (2007) was used. 1 g sample was suspended at 50 mL distilled water on a rotary shaker at 180 rpm for 30 min and then centrifuged at 5000 rpm for 10 min. 1.0 mL supernatant fluid 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 3000 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 sample extract. The production of H_2O_2 was calculated by using a standard curve measured before, preparing from known concentration of H_2O_2 (from 0 to 40 mM).

2.6. Extraction and analysis of oxalate

The extraction process was carried out in an ultrasound bath sonicator. Fermented straw was firstly incubated with deionized