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Purification, characterization and decolorization of bilirubin oxidase from *Myrothecium verrucaria* 3.2190

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ABSTRACT

Myrothecium verrucaria 3.2190 is a nonligninolytic fungus that produces bilirubin oxidase. Both *M. verrucaria* and the extracellular bilirubin oxidase were tested for their ability to decolorize indigo carmine. The biosorption and biodegradation of the dye were detected during the process of decolorization; more than 98% decolorization efficiency was achieved after 7 days at 26 °C. Additionally, the crude bilirubin oxidase can efficiently decolorize indigo carmine at 30 °C ~ 50 °C, pH 5.5 ~ 9.5 with dye concentrations of 50 mg l^{-1} ~ 200 mg l^{-1}. Bilirubin oxidase was purified and visualized as a single band on native polyacrylamide gel electrophoresis (PAGE). Several enzymatic properties of the purified enzyme were investigated. Moreover, the identity of the purified bilirubin oxidase (BOD) was confirmed by matrix assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF-MS). These results demonstrate that the purified bilirubin oxidase in *M. verrucaria* strain has potential application in dye effluent decolorization.

Introduction

Synthetic dyes are mainly classified as azo, anthraquinone and indigo dyes by their typical chromophores (Zollinger 1987); numerous studies have been devoted to decolorization of azo and anthroquinone dyes, as in the case of Congo Red and Remazol Brilliant Blue R (RBBR), but rather less attention has been paid to biological decolorization of indigo dyes (Tatarko & Bumpus 1998). Indigo carmine is an indigoid dye considered to be resistant to degradation (Mittal *et al.* 2006). Indigo carmine is a popular dye that can be used in textile coloring, pharmaceutical tablets and capsules, as well as medical diagnostics. However, indigo carmine is very toxic and difficult to metabolize if it enters the human body. Consumption of this dye is fatal since it is a carcinogen that can lead to reproductive, developmental, and neurological toxicity. It can also cause skin and eye irritations through contact (Jenkins 1978; Yoshida *et al.* 1971).

Decolorization of synthetic dyes in industry is a significant practical problem. Many dyes are difficult to degrade due to their complex structure and synthetic origin and because they are stable against light, temperature, and chemical compounds (Chung & Stevens 1993). Therefore, extensive applications of these dyes lead to environmental pollution and health risks. Conventional physical and chemical techniques used for wastewater treatment produce highly toxic organic chemicals from dyes and require high levels of energy to degrade them, which limit dye application (Moreira-Gasparin *et al.*

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2009). Among the available physical and chemical methods, adsorption appears to be the most efficient technique for dye removal because of its easy operation, ability to concentrate dyes, and cost effectiveness. A number of waste materials such as bottom ash and deoiled soya have been used to remove dyes from wastewater (Gupta *et al.* 2009; Mittal *et al.* 2008, 2009, 2010a and 2010b). Alternatively, dye decolorization using microorganisms has received great attention in recent years due to its eco-friendly nature, cost effectiveness, and easy application (Fu & Viraraghavan 2001). Many researchers have reported decolorization of dye solutions by bacteria such as *Bacillus cereus* (Mechsner & Wuhrmann 1982) and *Pseudomonas* sp. (Zimmermann *et al.* 1982), and by the white-rot fungi *Phanerochaete chrysosporium* (Cripps *et al.* 1990) and *Trametes versicolor* (Mehna *et al.* 1995).

Bilirubin oxidase (BOD) (EC.1.3.3.5, bilirubin:oxygen oxidoreductase) is a multicopper oxidase that catalyzes the oxidation of bilirubin to biliverdin *in vitro* concomitantly with the four-electron reduction of molecular oxygen to water (Solomon *et al.* 1996). The conidial fungi *Myrothecium verrucaria* (Sulistyaningdyah *et al.* 2004) and *Penicillium janthinellum* (Seki *et al.* 1996) have been known as BOD-producing fungi. Recently BOD activity was even found in *Bacillus subtilis* CotA (Sakasegawa *et al.* 2006). This enzyme has been used to determine the concentration of bilirubin in serum to diagnose the degree of jaundice for clinical investigations of the liver (Doumas *et al.* 1999). Besides its wide clinical application, BOD plays an important role in biobattery and biosensor manufacturing, cloth bleaching, and degradation of effluents. A majority of studies have focused on white-rot fungi, such as *P. chrysosporium*, which are ligninolytic organisms capable of degrading various types of dyes. The degradation process by lignin peroxidase was found to require the presence of redox mediators, which is a major drawback in its application in wastewater treatment. However, there are limited reports about the application of nonligninolytic fungi and their enzymes in dye decolorization.

Myrothecium verrucaria 3.2190 is a nonligninolytic fungus producing BOD (Zhang *et al.* 2007). Although *Myrothecium* sp. cells were able to absorb Orange II, 10B (blue), RS (red) dyes (Brahimi-Horn *et al.* 1992) and RBBR (Zhang *et al.* 2007), very few studies have focused on *M. verrucaria* and its secreted BOD to treat dye effluents without redox mediators. In this paper, we reported that *M. verrucaria* 3.2190 and its major extracellular enzyme BOD can significantly decolor indigo carmine. The method for purification and characterization of the BOD from culture supernatants of *M. verrucaria* are described.

Materials and methods

Microorganism and culture conditions

Myrothecium verrucaria 3.2190 was provided by the Institute of Microbiology Chinese Academy of Science (IMCAS) in Beijing. The strain was maintained on potato dextrose agar (PDA) slants. The mycelium from a slant tube was transferred to PDA plates and allowed to grow for 7 days with alternating light and dark (12 h: 12 h). The strain on the plate was prepared for spore inoculum. One milliliter of spore suspension (10^7 spore ml^{-1}) was added to Erlenmeyer flasks (250 ml) containing 100 ml of liquid culture medium. The culture was incubated on a rotary shaker at 28 °C, 150 rpm for 7 days. The liquid culture medium in this experiment contained (per liter): 200 g peeled potatoes; 20 g glucose; 7.5 g soya peptone (Sinopharm Chemical Reagent Co. Ltd., China).

Enzyme assay and kinetic properties

The BOD activity assay was conducted by measuring the absorbance decrease of bilirubin (Weibian, Shanghai, China) at 440 nm ($\varepsilon_{440} = 1.8 \times 10^4\ M^{-1}\ cm^{-1}$) after 3 min with a spectrophotometer. The enzyme activity was determined by the following techniques: 10 μl of enzyme sample and 30 μl of 34.2 mM bilirubin dissolved in 3 ml of Tris-HCl buffer (0.1M, pH 8.1) was incubated at 25 °C (Murao & Tanaka 1981). The oxidation of 2, 2′-azino-*bis* (3-ethylbenzthiazoline-6-sulfonate) (ABTS; Sigma, St. Louis, MO, USA) was measured and determined as an absorbance increase at 420 nm ($\varepsilon_{420} = 3.6 \times 10^4\ M^{-1}\ cm^{-1}$) after 3 min. The reaction system (3 ml) incubated at 25 °C contained 20 μl of enzyme solution, 1.98 ml of citrate-phosphate buffer (0.1 M, pH 3.4), and 1 ml of 1 mM ABTS (He *et al.* 2003). One unit was defined as the amount of BOD that oxidized 1 μmol of substrate per minute. All assays reported were the average value from triplicate measurements. The standard protein concentrations were determined with bovine serum albumin by the method of Bradford (1976). Substrate specificity of the purified BOD was measured using bilirubin and ABTS. The rate of substrate oxidation was calculated by measuring the absorbance change at each wavelength and optimum pH. The Michaelis-Menten constant was determined from double-reciprocal plots of the initial oxidase rates and concentrations of substrates as described (Durand *et al.* 2012).

Decolorization ability of Myrothecium verrucaria

Dye decolorization ability of *Myrothecium verrucaria* was assayed in the liquid culture medium with indigo carmine at a final concentration of 100 mg l^{-1}. The absorption spectrum of indigo carmine was scanned from 200 nm to 800 nm using U-2800 spectrophotometer (Hitachi, Japan). Decolorization of indigo carmine was monitored at the wavelength of 600 nm. To evaluate biomass production, the mycelia were harvested by centrifugation at 8000×g for 20 min (Himac CF16RX, Hitachi, Japan), and weighed after dehydrating at 80 ºC for 24 h. To examine the absorbance of the dye, the mycelia collected by centrifugation were suspended in 40 ml methanol shaking at 150 rpm for 12 h. The mycelial suspension was centrifuged at 8,000×g for 20 min. The mycelial precipitate was resuspended in 20 ml methanol and then recentrifuged. The supernatants were pooled together, and the absorbance of the supernatants at the wavelength of 600 nm was measured. Decolorization percentage (P) was calculated as described previously (Liu *et al.* 2009).

Dye decolorization ability of the enzyme from Myrothecium verrucaria

The effects of temperature, pH, dye concentration, and enzyme activity on the decolorization of indigo carmine by