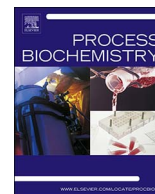




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Purification and characterization of a novel manganese peroxidase from white-rot fungus *Cerrena unicolor* BBP6 and its application in dye decolorization and denim bleaching

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ABSTRACT

Manganese peroxidase, with molecular mass of 45 kDa, was purified from the white-rot fungus *Cerrena unicolor* BBP6 and named MnP-BBP6. The optimum temperature and pH of MnP-BBP6 activity were 60 °C and 4.5, respectively. MnP-BBP6 showed high stability toward many metal ions. It could effectively decolorize many types of dyes including Congo red (53.9% in 12 h), methyl orange (77.6% in 12 h), Remazol brilliant blue R (81.0% in 5 h), bromophenol blue (62.2% in 12 h) and crystal violet (80.9% in 12 h). With gallic acid as the redox mediator, azure blue was decolorized by 63.1% in 24 h. In addition to dye decolorization, purified MnP-BBP6 also presented significant effect on denim bleaching, with up to 3-fold increase in reflectance without mediators. With such strong decolorizing activity, MnP-BBP6 was demonstrated to be a potential peroxidase in the textile industry, and its decolorization potential was greatly enhanced with the addition of redox mediators.

1. Introduction

Synthetic dyes, whose global production was estimated at about thousands of kilo tons, are intensively used in several industry areas such as textile, medicament, food, and leather industries [1,2]. The chemical structures of synthetic dyes used in the industries are designed to resist light, sweat, water, oxidizing agents, and microbial attack [3]. Many dyes are potential health hazards because of their possible conversion to toxic or cancer-causing products under certain specific conditions [4]. It is estimated that 10–15% of the dyes are lost in the effluent during dyeing processes. Because of the chemical structure and properties of dyes, it is difficult to degrade and decolorize dye-containing effluents through conventional biological wastewater treatments [4]. For these reasons, the environmental problems posed by dye-containing waste discharge have attracted increased public attention.

White-rot fungi (WRFs) have been shown to possess remarkable potential for degrading a wide range of pollutants including polycyclic aromatic hydrocarbons, synthetic dyes, and other priority pollutants [5]. This potential is mainly associated with the extracellular ligninolytic enzymes of WRFs including laccase, manganese-dependent peroxidase, lignin peroxidase, and manganese-independent peroxidase [6]. Manganese-dependent peroxidase or manganese peroxidase (MnP, EC 1.11.1.13) is an extracellular glycosylated heme protein that

catalyzes the H₂O₂-dependent oxidation of Mn²⁺ into Mn³⁺. Mn³⁺ is then stabilized by organic acid to form Mn³⁺ organic acid complex, which acts as a low-molecular-weight diffusible redox-mediator that breaks the aromatic rings of lignin polymers [7].

The unique degradative ability of MnP makes this enzyme attractive for various biotechnological applications, such as removal of hazardous wastes [8], bioremediation of organopollutants in water and soil [9], and bleaching and pulping of cellulose [10]. MnP is effective in dye decolorization, and this valuable application has attracted significant attention in recent years [5]. Free MnP could be easily denatured under industrially unfavorable conditions such as pH variation, unsuitable temperature, and presence of toxic byproducts; however, immobilization of MnP provides stability to the enzyme against these variable conditions and generates efficient biocatalysts in dye decolorization reactions [11,12]. This makes it possible to expand the use of MnP in industries with adverse conditions. It has been found that some syringyl-type phenols, which can form stable phenoxyradicals, could efficiently enhance the effect of laccase in its catalytic oxidation systems [13]. Recently, it was demonstrated that MnP catalytic oxidation reactions could be enhanced by the addition of mediators such as phenolic compounds [14]. The use of extracellular ligninolytic enzymes from WRFs in the textile industry is constantly growing, especially in the denim bleaching processes [15,16]. Currently sodium hypochlorite

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treatment is the main bleaching method for light washes on blue denim jeans because of its low cost and room-temperature reactions [15]. However, it also has disadvantages such as risk of chemical injuries, denim yellowness, and reduced denim strength [16]. Currently, enzymatic denim bleaching is mostly conducted using laccases [15,16]. Continuous efforts are made to search for new enzymes with good denim bleaching performance for industrial application. So far, there have been no reports on the use of MnP for such applications. With its high redox activity on dyes, MnP has the potential to be applied in denim bleaching.

In recent years, many MnPs were purified and characterized from different WRF strains such as *Trametes* sp. [5], *Irpex lacteus* [9], *Rhizoctonia* sp. [17], *Stereum ostrea* [18], *Schizophyllum* sp. [19], and *Phanerochaete chrysosporium* [20]. *Cerrena* sp. was reported to produce high-level activities of laccase and MnP, which are very efficient in dye decolorization [21]. However, studies on dye decolorization using enzymes from *Cerrena* sp. were limited to laccases, and no study on denim bleaching by purified MnP from *Cerrena* sp. was reported [21,22]. In this paper, we characterized MnP from *Cerrena unicolor* BBP6 (MnP-BBP6) and investigated its capability in dye decolorization and denim bleaching. We demonstrated that MnP-BBP6 purified from *C. unicolor* BBP6 was a valuable enzyme with superior performance in dye decolorization and denim bleaching. It has great potential in practical use in textile industry.

2. Materials and methods

2.1. Strain and culture conditions

The chemicals and reagents used in this study were of analytical grade and were obtained from Sigma-Aldrich (St. Louis, Missouri, USA) unless otherwise stated.

About 30 strains of WRFs were isolated from Singapore rain forest, and they were screened on agar plates containing three dyes, phenol red, Remazol Brilliant Blue R (RBBR), and azure blue (Azure B). Strain BBP6 was selected because of its superior capability for dye decolorization, in particular, Azure B decolorization. The 18S rDNA sequencing approach was used to identify strain BBP6. A 1776-bp fragment of 18S rDNA of strain BBP6 was amplified by PCR and sequenced. The sequence was deposited in NCBI GenBank under accession number KY400275. Our sequence showed 99% sequence identity with the 18S rDNA sequences of *C. unicolor* (AY850007) and *Cerrena* sp. WR1 (GQ899199). Our strain was therefore denoted as *C. unicolor* BBP6.

Stock culture of strain BBP6 was maintained at 4 °C on potato dextrose agar (PDA) (Difco, Franklin Lakes, New Jersey, USA). *C. unicolor* BBP6 was first inoculated in 100 ml potato dextrose broth (PDB) (Difco, Franklin Lakes, New Jersey, USA) in 250 ml Erlenmeyer flasks and cultured at 150 rpm and 28 °C for 5 days. For the production of MnP, the culture was transferred into the fermentation medium (24 g/l PDB, 10 g/l wheat bran and 20 g/l tryptone) with 10% (v/v) inoculum and incubated in a shaking incubator at 150 rpm and 28 °C. After 3 days of incubation, catechol with a final concentration of 100 mg/l was added to the culture. When MnP activity reached its maximum, the culture was harvested for enzyme purification and dye decolorization studies.

2.2. Measurement of MnP activity and protein content

The activity of MnP was measured by monitoring the formation of Mn^{3+} -malonate complexes at 270 nm ($\epsilon_{270} = 11,590/\text{M}/\text{cm}$) as described previously [23]. The reaction mixture contained 1 mM MnSO_4 , 0.1 mM H_2O_2 , and MnP solution in 50 mM sodium malonate buffer (pH 4.8) (Alfa Aesar, England). One unit of MnP activity was defined as the amount of enzyme that oxidized 1 μmol of substrate per minute. Protein content was determined by the Bradford method using bovine serum albumin (BSA) as the standard.

2.3. Purification of manganese peroxidase

Broth culture of *C. unicolor* BBP6 was harvested at the highest MnP activity and centrifuged to remove the mycelia. The crude enzyme was diluted and applied to a DEAE Sepharose Fast Flow anion-exchange column (GE Healthcare, Buckinghamshire, UK). The column was equilibrated with 20 mM sodium acetate buffer (pH 5.0) until the pH value was stabilized, and then eluted using a linear gradient of 0–1 M NaCl at a flow rate of 1 ml/min. Active fractions were collected, desalted, concentrated, and stored at 4 °C.

SDS-PAGE, containing a 5% polyacrylamide stacking gel and a 12% polyacrylamide resolving gel, was performed to verify the purity of the extracted MnP. The molecular mass of the purified MnP was estimated using unstained protein ladders (Bio-Rad Laboratories, Singapore).

2.4. Characterization of purified MnP-BBP6

The kinetic parameters of MnP-BBP6 in the presence of Mn^{2+} and H_2O_2 were calculated using the Lineweaver–Burk plot method. The experiments were performed in 50 mM malonate buffer (pH 4.8) at 30 °C using fixed H_2O_2 concentrations (0.1 mM) and varying Mn^{2+} concentrations (1–10 mM) or fixed Mn^{2+} concentrations (1 mM) and varying H_2O_2 concentrations (0.01–0.1 M).

The substrate specificities of MnP-BBP6 were examined using 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonate) (ABTS, $\epsilon = 36,000/\text{M}/\text{cm}$), 2,6-dimethoxyphenol (2,6-DMP, $\epsilon = 49,600/\text{M}/\text{cm}$), and guaiacol ($\epsilon = 6400/\text{M}/\text{cm}$) at wavelengths 420, 470, and 436 nm, respectively. The reaction mixture contained 4 mM of a substrate, 1 mM MnSO_4 , 100 U/L MnP-BBP6, and 0.1 mM H_2O_2 in 50 mM malonate buffer (pH 4.8). Reactions with all substrates were quantitated in 100 mM sodium tartrate buffer at pH 4.5.

MnP activity was measured at temperatures ranging from 20 °C to 80 °C and pH 4.8 to determine the optimum temperature of the purified MnP. The purified MnP was incubated at different temperatures (20–80 °C) for 3 h, and the residual activity was then measured at pH 4.8 and 30 °C to evaluate its thermal stability. The relative enzyme activity at different temperatures was calculated by setting the highest activity as 100%.

The effects of pH on MnP activity were determined in the pH range 3.0–6.5 at 30 °C. For pH stability studies, MnP-BBP6 was incubated in buffers with various pH values for 3 h, and the residual MnP activity was then measured at pH 4.8 and 30 °C. The relative enzyme activity at different pH values was calculated by taking the highest activity as 100%.

The effect of various metal ions (Merck, Germany) on MnP activity was studied using 10 mM of each metal ion at pH 4.8 and 30 °C. The enzyme was incubated with different metal ions for 3 h, and the residual activity was then measured to determine the stability of MnP-BBP6 in the presence of metal ions. MnP with no metal ions was used as the control. The relative enzyme activity was calculated by taking the activity of the control group as 100%.

All the characterization experiments were performed in triplicate.

2.5. Decolorization of different types of dyes by the purified MnP-BBP6

To evaluate the dye decolorization ability of the purified enzyme, MnP-BBP6 was used to decolorize different types of synthetic dyes including RBBR, Congo red (CR), brilliant blue R (BBR), methyl orange (MO), bromophenol blue (BPB), and crystal violet (CV). The maximal absorbance and the chemical structures of these dyes are shown in Table 1.

Two milliliters of the reaction mixture contained (final concentration) Mn^{2+} (1 mM), H_2O_2 (0.1 mM), malonate buffer (50 mM, pH 4.8), the purified MnP-BBP6 (100 U/l), and each individual dye (100 mg/l). The control samples were the decolorization mixture without the enzyme. All the decolorizing reactions were carried out at 30 °C, and the

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