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

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\textbf{A B S T R A C T}

Myrothecium verrucaria 3.2190 is a nonligninolytic fungus that produces bilirubin oxidase. Both \textit{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\^\circ C. Additionally, the crude bilirubin oxidase can efficiently decolorize indigo carmine at 30–50\^\circ C, pH 5.5–9.5 with dye concentrations of 50 mg l\textsuperscript{-1}–200 mg l\textsuperscript{-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 \textit{M. verrucaria} strain has potential application in dye effluent decolorization.

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\section*{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 anthraquinone 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 \textit{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 \textit{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 \textit{et al.}...
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 (10⁵ 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 (ε₄₄₀ = 1.8 × 10⁴ 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 (0.1 M, 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 (ε₄₂₀ = 3.6 × 10⁶ 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 °C 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
the crude BOD were examined. The 4-ml reaction system contained 0.1 M Tris-HCl buffer (pH 5.5–9.5), indigo carmine (final concentration) 50–200 mg l⁻¹, and enzyme solution (final enzyme activity 1–4 U ml⁻¹). The reaction temperature was from 30–60°C. Control samples were prepared with inactivated enzyme under the same conditions, and all experiments were performed in triplicate.

**BOD purification**

*Myrothecium verrucaria* cultures were grown in liquid medium for 7 days prior to BOD purification. To remove mycelia, 350 ml of the culture broth were centrifuged at 8000×g for 20 min. The supernatant was treated with ammonium sulfate (80% saturation) for 12 h, and the precipitate was dissolved in Tris-HCl (pH 6.0) buffer. The homogenate was dialyzed against distilled water for 24 h with periodic change of distilled water. The dialysis bag with the solution was embedded in polyethylene glycol 20,000 for concentration until only 10 ml liquid remained. This liquid was defined as crude BOD. The crude BOD was applied to a DEAE-Sepharose Fast Flow column (1.6×50 cm, Dingguo, Beijing, China), and equilibrated with 20 mM phosphate buffer (pH 6.0). After washing the column with the same buffer, the proteins were eluted by a linear gradient of NaCl (0.1 M, 150 ml) at a flow rate of 1 ml min⁻¹. Fractions containing the BOD activity were collected together and concentrated by polyethylene glycol 20,000 in a dialysis tube. The concentrated enzyme solution was added to a Sephacryl G-75 column (1.6×50 cm, Amersham Pharmacia, Uppsala, Sweden) and equilibrated with 20 mM phosphate buffer (pH 6.0). The bound proteins were eluted with the same buffer at a flow rate of 0.8 ml min⁻¹. The BOD positive fractions were pooled together and stored at −20°C until further use.

**Polyacrylamide gel electrophoresis and spectrum**

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed based on the protocol of Laemmli (1970) with 5% stacking gel and 15% resolving gel using a Hoefer mini VE vertical electrophoresis system (Amerham Biosciences, San Francisco, CA, USA). The SDS-PAGE gel was stained with Coomassie brilliant blue R-250 (Fluka, Buchs, Switzerland). The molecular weight of the purified BOD was estimated by the standard protein marker. In order to prove that the BOD enzyme was completely purified, and that it could catalyze the oxidation of bilirubin and ABTS, native PAGE was performed according to the procedure of Guo (2005). The gel was stained with Coomassie brilliant blue R-250 and citrate-phosphate buffer (pH 3.4) with 1 mM ABTS after separation of proteins on native PAGE. The native gel was also bisected; one-half was incubated in 0.2 M NaCO₃ solution with 50 mM bilirubin, the other half was soaked in the same citrate-phosphate buffer (pH 3.4) with 1 mM ABTS. The native gel was incubated in 50 mg l⁻¹ indigo carmine without redox mediators for decolorization to detect the enzyme activity.

**Analysis of N-terminal amino acid sequence**

The N-terminal amino acid sequence of the purified BOD was obtained by Shanghai GeneCore Biotechnologies (Shanghai, China) using a Procise® cLC Sequencing System (Model 492cLC, Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s instructions.

**Measure of MALDI-TOF-MS**

The purified BOD was analyzed by the Northeast Forestry University Biotechnology Research Institute of Daqing (Daqing, China) with a 4800 MALDI TOF/TOF mass spectrometer (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s instructions. Prior to sample spectra acquisition, optimal laser fluence tuning and other instrumental parameters (number of shots per subspectrum, number of subspectra) were determined. Protein identification was performed by searching extracted peak lists generated with one defined set of filter parameters against the NCBI database using the Mascot (Matrix Science, London, UK) search engine (MS/MS ion search, 1 mis cleavage, MS tolerance: 0.3 Da, MS/MS tolerance: 0.3 Da).

**Effects of pH and temperature on BOD activity and stability**

The optimum pH of the purified BOD was measured from pH 6–10 using bilirubin as substrate in 0.1 M Tris-HCl buffer, and pH 2.8 to pH 6.0 using ABTS as substrate in 0.1 M citrate-phosphate buffer, respectively. The optimum temperature of the purified BOD activity was monitored at temperatures from 20–70°C using bilirubin as the substrate. The effect of pH on the purified enzyme stability was determined by calculation of the activity remaining after incubation for 1 h at 25°C in 0.1 M phosphate buffer (pH 6.0–10.0). The thermal stability of bilirubin was measured in 0.1 M phosphate buffer (optimum pH) for 1 h at 20–70°C. Both experiments used bilirubin as substrate. All experiments were carried out in triplicate.

**Effect of inhibitors, metal ions, and other compounds on BOD enzyme activity**

The effects of potential inhibitors, several metal ions, and other compounds including urea and ethylenediaminetetra-acetic acid (EDTA) on purified BOD activity were determined using bilirubin as substrate. The possible inhibitors included l-cysteine, sodium azide, and dithiothretol (DTT). The metal ions and compounds primarily contained Na⁺, K⁺, Li⁺, Ag⁺, Cu²⁺, Ca²⁺, Mg²⁺, Mn²⁺, Zn²⁺, Ba²⁺, Fe³⁺, Fe²⁺, Fe³⁺, Al³⁺. All experiments were performed in triplicate.

**Results and discussion**

**Decolorization in batch liquid cultivation**

The decolorization of indigo carmine by *Myrothecium verrucaria* was evaluated in batch incubation for 7 days. The maximum absorption spectrum of indigo carmine was 600 nm. There was no change in absorbance in the abiotic controls. At the beginning of the liquid cultivation (1–3 days), both the decolorization rate (26–52%) and BOD activity (0.78–1.12 U ml⁻¹) were very low, respectively (Fig. 1). Whereas the mycelium grew vigorously (0.26 g–0.70 g) with
the dye, an absorption rate increase from 23 % to 45 % was generated (Fig 2). Therefore, biosorption played a major role in dye removal in the first 3 days. On the fourth day of cultivation, enzyme activity dramatically increased (2.7 U ml$^{-1}$) with an upward trend in the dye decolorization rate (91 %) (Fig. 1). In addition, the blue pigmentation of mycelium became faint. Biomass production increased slightly from day 4 to 7, while the dye absorption rate rapidly declined (Fig. 2). Thus, dye decolorization was mainly due to biodegradation by BOD at the later stage. The enzyme and biomass reached the maximal decolorization rate of 98 % after 7 days.

Dye decolorization ability of BOD enzyme from Myrothecium verrucaria

The BOD enzyme could efficiently decolorize indigo carmine under different conditions. The decolorization rate increased as BOD enzyme activity increased; the BOD enzyme activity (1~4 U ml$^{-1}$) could achieve 92 % decolorization within 50 min when the reactions were carried out at 40 °C with 0.1 M Tris-HCl buffer (pH 7.5) (Fig. 3A). In the following enzymatic dye decolorization experiments the BOD concentration was 1 U ml$^{-1}$. The effects of pH on dye decolorization are shown in Fig. 3B. These results revealed that the enzyme could decolorize indigo carmine over a pH range from 5.5 to 9.5. The decolorization rate exceeded 90 % after incubation at 40 °C for 2 h. At pH 7.5 the decolorization process was faster, while at pH 5.5 the decolorization process was slower. The effect of temperature on dye decolorization was not so obvious at pH 7.5 (Fig. 3C) with a 92 % decolorization rate obtained at 50 °C within 40 min. The dye decolorization process became slower with the increase of dye concentration (Fig. 3D). All the culture conditions had higher decolorization rates with a longer cultivation time.

BOD purification

As described in Table 1, 2.43 mg of BOD was purified from 350 ml culture supernatant. The final purification was 3.12-fold, and recovery of total enzyme activity was 27.92 %. The purified enzyme had two protein bands corresponding to molecular mass of 68.1 kDa and 48.6 kDa on SDS-PAGE gel, respectively (Fig. 4A); however, only a single protein band appeared on the native PAGE gel (Fig. 4B) where the band at lane 3 (stained with ABTS) and lane 4 (stained with Coomassie brilliant blue R-250) were at the same level, which can indicate that the enzyme was purified to electrophoretic homogeneity. The native PAGE stained with ABTS and bilirubin showed a clear green band against a transparent background and a white band against yellow background to identify whether the purified BOD was able to catalyze the oxidation of ABTS and bilirubin (Fig. 4C). As shown in Fig. 4D, the native PAGE stained with indigo carmine showed a clear white band of decolorized zone against blue background after incubation for 20 min without any redox mediators, thus further demonstrating that purified BOD indeed had the ability to degrade indigo carmine. The purified BOD enzyme showed maximum absorption at around 600 nm, and a small shoulder at 330 nm, indicating that the enzyme was a multicopper oxidase.

N-Terminal amino acid sequence of BOD

The N-terminal acid sequence of the purified BOD was determined to be VAQISPQYPMFTVPL. It showed 100 % identity with BOD from Myrothecium verrucaria (Koikeda et al. 1993). Homologies of up 86.7 % were exhibited with BOD from M. verrucaria 24G-4 (Sulistyaningdyah et al. 2004). However, the homology of BOD between M. verrucaria and Pleurotus ostreatus was only 30 % (Masuda-Nishimura et al. 1999).

Measurement of MALDI-TOF-MS

The purified bilirubin oxidase was subject to MALDI-TOF-MS analysis. The mass spectra and tandem mass spectra analysis of the purified BOD are shown in Fig. 5. Fig. 5A shows the first mass spectrum determination. Eight high peaks in Fig. 5A were chosen for secondary mass spectrum determination. The secondary mass spectra with corresponding eight peptides are shown in Fig. 5B-5I. The eight peptides detected by tandem mass spectra were compared with a database, and homologies of up 100 % were found with BOD from Myrothecium verrucaria. These results clearly demonstrate that the purified protein was indeed bilirubin oxidase.
Enzymatic properties

Effect of pH and temperature on enzyme activity and stability

The optimum reaction pH of the purified enzyme for bilirubin was 7.5; the enzyme activity showed slight decrease after incubation at 25°C for 1 h at pH 6.5–9.0. The optimum pH of the enzyme reaction for ABTS was pH 3.4. The BOD showed maximum enzyme activity at 40°C, when the reaction was observed at its optimum pH 7.5. The enzyme activity was stable after incubation for 1 h at a temperature up to 40°C, and thermal stability was decreased beyond 50°C.

Kinetic properties

Table 2 summarizes the kinetic constants of the purified BOD enzyme catalyzing substrates as bilirubin and ABTS. The apparent $K_m$ for bilirubin was 0.021 mM at the optimal pH 7.5 whereas $K_m$ for ABTS was 0.126 mM at the optimal pH 3.4. The $K_{cat}$ values for bilirubin (131.19 min⁻¹) and ABTS (118.96 min⁻¹) did not differ too much. Therefore, the catalytic efficiency of the enzyme ($K_{cat}/K_m$) for bilirubin ($K_{cat}/K_m = 6247.14$) was much higher than for ABTS ($K_{cat}/K_m = 944.12$).

Effect of inhibitors, metal ions, and other compounds on enzyme activity

The effects of several inhibitors on enzyme activity are shown in Table 3. The purified BOD activity was not affected significantly by 0.1 mM L-cysteine but was inhibited 10-fold with 1 mM L-cysteine. Similarly, the purified BOD activity was inhibited approximately 4-fold by 10 mM sodium azide. Dithiothreitol (DTT) also inhibited the BOD activity (36.77 %) as concentration increased to 1 mM. The effects of different metal ions, urea, and EDTA on BOD enzyme activity were also investigated (Table 4). BOD activity was inhibited to some extent by the addition of most metal ions except Na⁺, K⁺ and Li⁺. However, copper ions increased the enzyme activity remarkably, and BOD activity was not influenced by urea and EDTA.
Although previous studies have focused on ligninolytic white-rot fungi that are involved in degradation of industrial dye effluents (Wesenberg et al. 2003), our results demonstrated that a nonligninolytic strain Myrothecium verrucaria 3.2190 played an important role in dye removal. However, it is generally agreed that biological decolorization has mainly involved biodegradation and fungal adsorption (Tatarko & Bumpus 1998; Zimmermann et al. 1982). Various species of white-rot fungus such as Phanerochaete chrysosporium, Trametes versicolor, Irpex lacteus, and Phlebia tremellosa (Tatarko & Bumpus 1998; Wang & Yu 1998), can remove dyes by fungal adsorption and enzyme degradation. However, there are species variations on different processes. In the liquid culture of Aspergillus niger, dye decolorization was mostly attributed to fungal adsorption (Miranda et al. 1996), while biodegradation played a leading role in the dye decolorization process of Cyathus bulleri (Vasdev et al. 1995). Though dye decolorization of M. verrucaria 3.2190 consists of two major mechanisms, biodegradation by the extracellular BOD played a more important role than fungal bioadsorption. During the initial decolorization process of indigo carmine, M. verrucaria showed a great adsorption, so that in the beginning, adsorption of the fungal mycelia was the key mechanism. When a high level of BOD was excreted into the liquid culture, blue mycelia turned colorless, and the decolorization rate dramatically increased. Therefore, biodegradation was an important mechanism for dye removal. It is worth pointing out that the 98% decolorization rate obtained in M. verrucaria 3.2190 cultures after 7 days of liquid culture was higher than other M. verrucaria strain decolorization processes (Brahimi-Horn et al. 1992). As a result, this non-ligninolytic strain would be suitable for dye effluent decolorization. Several studies reported that decolorization by microorganisms was partly dependent on nutrient concentrations, especially nitrogen concentration, thus further research is needed to investigate the influence of the nutrient concentrations on the decolorization (Ambrósio & Campos-Takaki 2004; Vasdev et al. 1995).

SDS-PAGE analysis showed that the purified BOD contained two protein bands (68.1 and 48.6 kDa). The molecular weight of the large subunit was consistent with the molecular weight of BOD from M. verrucaria (Pan et al. 1991); however, two groups of investigators showed that the molecular weight of BOD from the same species was 66 kDa or 64 kDa (Hiromi et al. 1992; Shimizu et al. 1999). The small discrepancy might be due to a difference in the sugar content since Murao & Tanaka (1981) estimated the molecular weight at 52 kDa. BOD has two N-linked carbohydrate chains in addition to O-linked carbohydrate chains (Shimizu et al. 1999), and when BOD was produced in Aspergillus oryzae, both authentic and recombinant BOD appeared as two bands in SDS-PAGE (Kataoka et al. 2005). Our results showed that the native PAGE stained with Coomassie brilliant blue R-250 and ABTS revealed a single band at the same level, which shows that the enzyme had been purified to electrophoretic homogeneity. The native PAGE stained with indigo carmine showed a clear white band of decolorized zone against blue background after incubation for 20 min without any redox mediators, and this result further indicated that the purified enzyme played a major role in decolorization.
The native PAGE stained with ABTS and bilirubin were employed to characterize the ability of the purified BOD to catalyze the oxidation of ABTS and bilirubin. The apparent $K_m$ for bilirubin was lower than ABTS, which implied that the enzyme had a high affinity for bilirubin. It has been reported that enzymes could be characterized by native PAGE with staining based on specific substrates (Murugesan et al. 2007). The BODs from *M. verrucaria*, *Trachderma tsunodae*, and *Penicillium janthinellum* have been purified and biochemically characterized. The results revealed that BOD was a member of the multicopper oxidase family that contains four copper ions per functional unit and was classified as types I, II, III copper ions according to their spectroscopic and magnetic properties (Shimizu et al. 1999). Moreover, BOD was able to catalyze the oxidation of several typical laccase substrates such as ABTS and various phenolic compounds. The substrate specificities of BOD and other multicopper oxidases are slightly different, because BOD catalyzes reactions

![Figure 5](image)

**Table 2 – Kinetic constants.**

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<thead>
<tr>
<th>Substrate</th>
<th>Optimal pH</th>
<th>$\varepsilon_{\text{max}}$ (M cm$^{-1}$)</th>
<th>Wavelength (nm)</th>
<th>$K_m$ (mM)</th>
<th>$k_{\text{cat}}$ (min$^{-1}$)</th>
<th>$k_{\text{cat}}/K_m$ (mM$^{-1}$ min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bilirubin</td>
<td>7.5</td>
<td>18 000</td>
<td>440</td>
<td>0.021</td>
<td>131.19</td>
<td>6247.14</td>
</tr>
<tr>
<td>ABTS</td>
<td>3.4</td>
<td>36 000</td>
<td>420</td>
<td>0.126</td>
<td>118.96</td>
<td>944.12</td>
</tr>
</tbody>
</table>
Effects of metal ions and compounds on bilirubin oxidase activity. Our results are different from their study, since we found that copper ions could remarkably increased BOD activity as compared to other metal ions. This may be due to the fact that BOD from M. verrucaria contains copper in its active site that may help maintain the correct conformation and enzyme activity.

### Conclusion

In this study, the bilirubin oxidase from Myrothecium verrucaria 3.2190 was purified to homogeneity. Several enzymatic properties of the purified enzyme were investigated. Furthermore, the identity of the purified bilirubin oxidase (BOD) was confirmed by Matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF-MS). The bilirubin oxidase was tested for its ability to decolorize indigo carmine. Our results indicated that the purified BOD in M. verrucaria strain has potential application in dye decolorization in industry. Further research is needed to focus on the optimization of BOD production from M. verrucaria and heterologous expression of this protein to achieve higher production.

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### References


