Kinetic Properties of Manganese Peroxidase from the Mushroom *Stereum ostrea* and its Ability to Decolorize Dyes

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Manganese peroxidase (MnP) was isolated from the culture filtrate of the wood log mushroom *Stereum ostrea* (*S. ostrea*), grown on Koroljova medium, and then purified by ammonium sulfate [70% (w/v)] fractionation, DEAE-cellulose anion exchange chromatography, and Sephadex G-100 column chromatography, with an attainment of 88.6-fold purification and the recovery of 22.8% of initial activity. According to SDS-PAGE the molecular mass of the MnP was 40 kDa. The optimal pH and temperature were found to be 4.5 and 35°C, respectively. The enzyme was stable even after exposure to a pH range of 4.5 to 6.0, and at temperatures of up to 35°C at a pH of 4.5 for 1h. The $K_m$ and $V_{max}$ values for the substrate phenol red were found to be 8 µm and 111.14 U/mg of protein, respectively.

The MnP also oxidized other substrates such as guaiacol, DMP, and veratryl alcohol. Sodium azide, EDTA, SDS, Cu²⁺, and Fe²⁺, at 1–5 mM, strongly inhibited enzyme activity, whereas Ca²⁺ and Zn²⁺ increased enzyme activity. The participation of the purified enzyme in the decolorization of dyes suggests that *S. ostrea* manganese peroxidase could be effectively employed in textile industries.

Keywords: *Stereum ostrea*, manganese peroxidase, purification, characterization, decolorization

White-rot fungi are capable of degrading all basic wood polymers, and in particular lignin, owing to an ability to synthesize relevant hydrolytic and oxidative extracellular enzymes. These enzymes are responsible for the degradation of lignin into low-molecular-weight compounds that can be assimilated for fungal growth [12]. The most outstanding enzymes, which participate in lignin biodegradation, are laccase (Lac), manganese peroxidase (MnP), and lignin peroxidases (LiP). Owing to the low substrate specificity of lignolytic enzymes, they can oxidize a wide range of compounds with structural similarities to lignin, so they play an important role in the bioremediation of various toxic compounds in soils and waste waters [25]. White-rot fungi form a diverse group that contains a large number of genera, some of which have not been explored for use in lignolytic systems. Certain white-rot fungi such as *Phanerochaete chrysosporium*, *Pleurotus eryngii*, *Pleurotus ostreatus*, and *Trametes versicolor* have drawn more attention than other ligninolytic enzymes [4, 24, 30, 33]. However, in a recent study, the relatively less characterized white-rot fungus *S. ostrea* (false turkey-tail fungus), belonging to the family Stereaceae, has been shown to produce laccase in higher yields than *Phanerochaeta chrysosporium* [38]. The presence of manganese peroxidase has also been demonstrated in the lignolytic system of *S. ostrea* [28]. MnP (E.C. 1.11.1.13) catalyzes the $H_2O_2$-dependent oxidation of Mn(II) to Mn(III), which, in turn, acts as a nonspecific oxidant that attacks phenolic lignin structures by one electron oxidation [26]. MnP activity has been observed in *Earliella scabrosa* [13], *Lentimula edodes* [32], *Schizopyllum* sp. [6], *Lenzites betulina* [16], *Aspergillus terreus* [18], and *Trametes versicolor* [9]. A pattern of lignolytic enzymes is not common in all fungal cultures. For instance, lignin peroxidase is the dominant enzyme in the lignolytic system of *P. chrysosporium*, whereas laccase is the prominent enzyme in the lignolytic system of *S. ostrea* [28]. It should also be noted that some fungal cultures with positive MnP activity failed to decolorize dyes in growth media, whereas some other fungal cultures without MnP activity exhibited the effective decolorization of dyes [29]. There appears to be wide diversity in the kinetic properties of the lignolytic enzymes of fungal cultures, and this provides for a wider base for the application of lignolytic enzymes in the decolorization of dyes, pulp treatment, and the degradation of organic.
pollutants [7]. Direct evidence for the involvement of any enzyme in the decolorization of dyes comes from studies that utilize only a purified enzyme. Therefore, the main aim of the present study was to purify and characterize the kinetic properties of manganese peroxidase produced by S. ostrea, a white-rot fungus most commonly found in hardwood forests, and to subsequently test the participation of the MnP of S. ostrea in the decolorization of dyes.

**MATERIALS AND METHODS**

**Microorganism and Culture Conditions**

The S. ostrea was kindly supplied by Prof. M. A. Singaracharya, Department of Microbiology, Kakatiya University, Andhra Pradesh, India, and was isolated from wood logs. The fungal culture of S. ostrea was maintained on Koroljova medium [20] containing (g/l) 3.0 peptone, 10.0 glucose, 0.6 KH₂PO₄, 0.001 ZnSO₄, 0.4 KH₂PO₄, 0.0005 FeSO₄, 0.05 MnSO₄, 0.5 MgSO₄, and 20.0 agar (pH 5.0). Fungal mycelial suspension of the above culture, for purpose of inoculum, was prepared by adding 2 ml of sterile distilled water to the freshly (7 days old) grown slants of S. ostrea. For the production of MnP, the fungal culture was cultivated in liquid broth of Koroljova medium for 10 days at 30°C in an orbital shaker after inoculation in the same manner as has been previously detailed [28].

**Assay of Manganese Peroxidase Activity**

MnP activity was determined by the oxidation of phenol red at 610 nm [21]. The assay mixture included 0.5 ml of culture filtrate, 0.25 M sodium lactate (pH 4.5), 0.5% bovine albumin, 200 mM MnSO₄, 2.0 mM H₂O₂ (prepared in 0.2 mM sodium succinate buffer, pH 4.5), and 0.1% phenol red. The changes in absorbance of the reaction mixture was monitored at 610 nm (ε = 22,000 M⁻¹ cm⁻¹) for 5 min. MnP activity was expressed in IU, where one unit of MnP was defined as the amount of enzyme that oxidized 1 µmol of phenol red per minute.

**Purification of Manganese Peroxidase**

The S. ostrea exhibited peak production of MnP on the 6th day of incubation, and at that time the culture broth derived from the growth of S. ostrea was filtered through Whatman No. 1 filter paper, and the filtrate was centrifuged to remove any cells or clumps. The supernatant was fractionated by solid ammonium sulfate precipitation at the desired % saturation. The precipitate was collected by centrifugation at 20,000 × g for 30 min at 4°C (REMI-C24 Centrifuge, Mumbai, India). The supernatant was collected for further use and the pellet dissolved in 0.2 mM sodium succinate buffer, pH 4.5. The extract was dialyzed against 5 L of the same buffer for 24 h, with three changes, and was kept overnight at 4°C using a dialysis membrane with a 10 kDa cut-off (GeNei, Bangalore, India). The ammonium sulfate % saturation in the supernatant was increased in a stepwise fashion, with the addition of solid ammonium sulfate, and the collection of precipitate, and the supernatant steps already given were further repeated. The 70% saturated ammonium sulfate fraction (dialyzed enzyme solution) with the highest enzyme activity was applied to a column of DEAE-cellulose (2 × 20 cm), which was previously equilibrated with sodium succinate buffer (pH 4.5) and washed with a negative linear gradient of 0.2 to 1 M NaCl at a flow rate of 1 ml/min at 4°C. The active fractions were collected and loaded onto a Sephadex G-100 column (1.6 × 60 cm), and equilibrated with sodium succinate buffer (pH 4.5). The column was then eluted with the same buffer at a flow rate of 0.5 ml/min. The active fractions of MnP were pooled, dialyzed, concentrated by ultrafiltration, and stored at −20°C for enzyme characterization and decolorization experiments.

Purity and apparent molecular weights of the peaks were examined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using methods as described by Laemmli [22]. Molecular weight markers (GeNei, Bangalore, India) were run along with purified S. ostrea MnP on 10% gels in electrophoresis, followed by Coomassie Blue R-250 staining.

**Kinetic Properties of MnP**

MnP activity was measured at different temperatures ranging from 20–70°C. The influence of pH upon MnP activity was determined at room temperature using Na-tartrate (pH 2.5–5.5) and phosphate buffer (pH 6.0–8.5). Thermal stability was investigated by pre-exposing the purified enzyme to temperatures within a range of 20–70°C for 1 h of incubation, and the residual activity was tested at 30°C. The pH stability was determined by pre-incubating the enzyme at a different pH (pH 2.5–5.5, Na-tartrate, and pH 6.0–8.5 phosphate, buffer) for 1 h at 30°C. The remaining activities were measured under standard assay conditions.

Three substrates (Mn²⁺, H₂O₂, and phenol red) were included in the assay media. Concentrations of any two substrates were kept constant at 1 mM in the assay media and the concentration of the third substrate was varied in a range from 1 to 10 mM in 0.2 mM sodium succinate buffer, pH 4.5. An assay medium without enzyme served as the control. The initial velocity of the MnP reaction was measured in terms of phenol red transformation. The Michaelis constants Kₘ and Vₘₘₐₓ, were calculated from Lineweaver–Burk plots of the initial velocity of MnP vs. Mn²⁺, H₂O₂, or phenol red as substrate. All measurements were performed in triplicate and the mean value was subjected to statistical analysis. Enzyme activities were calculated using an extinction coefficient of 22,000 M⁻¹ cm⁻¹ and expressed in IU.

The substrate specificities of MnP were examined with guaiacol, 2,6-dimethoxyphenol (DMP), phenol red, and veratryl alcohol (2 mM each) under standard assay conditions at 470 nm (ε = 6,740 M⁻¹ cm⁻¹), 470 nm (ε = 49,600 M⁻¹ cm⁻¹), 610 nm (ε = 22,000 M⁻¹ cm⁻¹), and 310 nm (ε = 9,300 M⁻¹ cm⁻¹), respectively. MnP activity was expressed in IU, where one unit of MnP was defined as the amount of enzyme that oxidized 1 µmol of substrate per minute.

The influences of various inhibitors (sodium azide, SDS, and EDTA) at 0.1, 1, and 5 mM concentrations on MnP activity were tested by pre-incubating the enzyme with different inhibitors in 0.2 mM sodium succinate buffer, pH 4.5, for 30 min at room temperature before the addition of the substrate, and the enzyme activity was assayed in triplicate utilizing the standard MnP assay method.

The effects of various metal ions (Cu²⁺, Fe²⁺, Ca²⁺, Zn²⁺, Co²⁺, Mg²⁺) at 1, 5, and 10 mM concentrations were studied under standard assay conditions, and the obtained values were compared with those observed in the presence of Mn²⁺.

MnP activity in the presence of citrate, malate, oxalate, fumarate, and acetate (1 mM) was measured using standard activity assay conditions. The observed values were then compared with the activity in the presence of the control (devoid of carboxylic compounds).
Decolorization of Dyes by Purified MnP

The reactive dyes Rose Bengal (HiMedia, Mumbai, India), Remazol Brilliant Violet (Sigma-Aldrich, Mumbai, India), Remazol black-5, Remazol blue-19, and Remazol orange-16 were provided by the local textile industry “Dharmavaram,” Anantapur district of Andhra Pradesh, India. The chemical structures of the dyes used are specified in Fig. 1. Spectrophotometric scanning was performed and the absorption maxima were identified as 545, 577, 590, 590, and 530 nm, respectively.

Table 1. Manganese peroxidase production by S. ostrea.

<table>
<thead>
<tr>
<th>Incubation time (days)</th>
<th>MnP activity (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0.35 ± 0.05</td>
</tr>
<tr>
<td>4</td>
<td>0.98 ± 0.07</td>
</tr>
<tr>
<td>6</td>
<td>2.00 ± 0.10</td>
</tr>
<tr>
<td>8</td>
<td>1.53 ± 0.07</td>
</tr>
<tr>
<td>10</td>
<td>0.87 ± 0.07</td>
</tr>
</tbody>
</table>

Data in the figure are the mean of triplicate tests with standard error bars.

Stock solutions of dyes were diluted in sodium phosphate buffer (100 mM; pH 5.9), usually to a final concentration of 200 mg/l. Purified MnP was added to the dye solutions at a loading of 100 U/l. The experiments were conducted in a total volume of 10 ml (in 15 ml plastic screw cap tubes), in triplicate. Dye solutions devoid of enzymes, and enzyme solutions devoid of dye, served as controls. All the screw cap tubes were incubated at 30°C in a rotary shaker (100 rpm) and the color was measured spectroscopically in a Chemito spectrascan UV-2600 spectrophotometer (Mumbai, India) at the absorbance maximum of the dye at different time intervals.

RESULTS AND DISCUSSION

MnP Production

Growth of S. ostrea, a white-rot fungus of the most common occurrence in wood logs, on Koroljova media produced an extracellular lignolytic enzyme, manganese peroxidase (MnP), in the broth (Table 1). Peak production occurred under optimal growth conditions, with the highest titer of MnP at 2.0 U/ml yielded from the culture broth of S. ostrea on the 6th day of incubation.

Secretion of laccase by S. ostrea has only recently been confirmed [28, 38]. However, an element of doubt has remained about the presence of peroxidases such as MnP in the lignolytic system of S. ostrea because of conflicting results [27]. The demonstration of MnP activity in the culture media of S. ostrea in the present study should serve to remove this doubt. The titer of MnP yielded by S. ostrea in the present study was comparable, or better than (0.2–1.4 U/ml) the yield obtained with other cultures such as Phanerochaete chrysosporium [4, 36], P. ostreatus [17], and Phlebia radiata [37] under different growth conditions in liquid media. It has been previously shown that solid cultures of the basidiomycetous fungus Nematoloma frowardii on wheat straw [15], and Phlebia radiata on chopped wheat straw [37], produced MnP as the predominant lignolytic enzyme. MnP expression in fungal cultures appears to be controlled by Mn²⁺ concentrations in the media, and the inclusion of Mn²⁺ levels in a range of 16–40 ppm ensures the adequate expression for detection of MnP by the different cultures [2, 23]. Care was taken to grow S. ostrea on a medium with 50 ppm of Mn²⁺ in the present study for the production of MnP.
MnP Purification

The culture filtrate, derived after growth of *S. ostrea* for 6 days, was used as a source of crude enzyme for purification. The culture filtrate was sequentially subjected to ammonium sulfate precipitation in a stepwise fashion at the desired saturations of 40%, 50%, 60%, 70%, and 80% (w/v) (Fig. 2). A 70% ammonium sulfate saturation fraction made for good recovery of the enzyme with a high specific activity (Fig. 2). Anion-exchange chromatography of the above fraction on a column of DEAE-cellulose (2 × 20 cm) resulted in the enrichment of the MnP enzyme, with a specific activity of 11 U/mg protein (Table 2). Gel filtration of the anion-exchange chromatography fraction on a Sephadex G-100 column (1.6 cm × 60 cm) led to more than a 2-fold increase in the specific activity during the purification process of the MnP. The three-step protocol employed in this study for MnP purification enabled us to obtain the MnP enzyme at a purification of about 88.6-fold and a final yield of 22.8%. The results of the purification processes are summarized in Table 2. The extracellular MnP enzyme was purified to homogeneity, as was evident from the appearance of a single band on SDS-PAGE (Fig. 3). The molecular mass of the enzyme was estimated by SDS-PAGE to be 40 kDa. In a similar way, purification of MnP from the activities of other lignolytic fungi within the range of 5–80 folds has been previously achieved [5, 6]. The purified MnP of *S. ostrea* has a molecular mass of 40 kDa, thus falling within the usual range of 37–62.5 kDa exhibited by MnPs of other white-rot fungi such as *Schizophyllum* F17 (48 kDa) [6], *Irpex lacteus* (37 kDa) [2], *Trichophyton rubrum* (42 kDa) [3], and *Nematoloma frowardii* (44 kDa) [31]. Clear evidence for the presence of MnP in the lignolytic system of *S. ostrea* comes from the demonstration of activity with the purified MnP enzyme from the culture of *S. ostrea*.

Kinetic Properties of MnP

The influence of pH within a range of 2 to 8.5 on the MnP activity of *S. ostrea* is shown in Fig. 4. The display of maximal activity at a pH of 4.5 by the MnP indicated that a pH of 4.5 is the optimal pH for the enzyme. On either side of this pH, there was a decrease in enzyme activity. Similarly, MnP enzymes from other organisms, such as *Rhizoctonia* sp. [5] and *Schizophyllum* F17 [6], exhibited activity over a pH range of 2.0–6.0, with maximal activity seen at pHs of between 4.0–4.5. By contrast, the MnP of *Irpex lacteus* [2] and *Phanerochaete chrysosporium* exhibited an optimal pH 2–4 units higher than the usual range of most MnPs of fungi [36].

The effect of varying temperatures on the activity of the purified MnP of *S. ostrea* was examined (Fig. 5). The MnP had an optimum temperature of 35°C, as shown by maximal activity at this temperature. The incubation of the enzyme at temperatures other than 35°C, on either side, resulted in a drop in enzyme activity. In other studies, it was observed that the temperature optima of purified MnPs from the organisms *Aspergillus terrus* [18], *Phlebia floridensis* [1], *Schizophyllum* F17 [6], and *P. chrysosporium* fell

<table>
<thead>
<tr>
<th>Sl.No.</th>
<th>Purification step</th>
<th>Protein (mg)</th>
<th>Total activity (U)</th>
<th>Specific activity (U/mg)</th>
<th>Purification fold</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Crude filtrate</td>
<td>115</td>
<td>35</td>
<td>0.30</td>
<td>1.0</td>
<td>100.00</td>
</tr>
<tr>
<td>2</td>
<td>(NH₄)₂SO₄</td>
<td>40</td>
<td>26</td>
<td>0.65</td>
<td>2.1</td>
<td>74.20</td>
</tr>
<tr>
<td>3</td>
<td>DEAE-Cellulose</td>
<td>1.0</td>
<td>11</td>
<td>11.00</td>
<td>36.60</td>
<td>31.40</td>
</tr>
<tr>
<td>4</td>
<td>Sephadex G-100</td>
<td>0.3</td>
<td>8</td>
<td>26.60</td>
<td>88.66</td>
<td>22.80</td>
</tr>
</tbody>
</table>
**Stereum ostrea** and its ability to decolorize dyes

within a range of 25–40°C [33, 34]. A high optimal temperature of 60°C was found in respect of *Irpex lacteus* [2]. It is known that the rapid loss of activity in the MnPs of a variety of other organisms at high temperatures occurs as a result of the inactivation of the enzyme by a denaturation process [35].

Pre-exposure of the enzyme in 0.2 mM sodium succinate buffer (pH 4.5) to temperatures within a range of 20–70°C for 1 h, followed by an assay of activity values under standard assay conditions, indicated that the enzyme was stable at up to 35°C, but was inactivated rapidly at temperatures higher than this. The enzyme was inactivated totally at 65°C (Fig. 6). The MnP of *S. ostrea* exhibited relatively moderate temperature tolerance, retaining 40% of optimal activity after exposure of the MnP for 1 h to a 50°C environment. In other studies, the temperature stability of purified MnPs also differed widely; the enzyme from *P. chrysosporium* lost activity completely after 15 min at 60°C [8], whereas the MnP from *Lentinula edodes* still retained 62% of activity after 1 h of similar treatment [32].

The stability of the enzyme at different pHs was measured under standard activity assay conditions after 1 h of pre-incubation at different pH values in 50 mM Na-tartrate buffer for pH 2.5–5.5 and phosphate buffer for pH 6.0–8.5 at 30°C (Fig. 7). The purified MnP remained comparatively stable in the region of 4.5–6.0, but was inactivated rapidly outside of this range. The purified MnP was stored in 0.2 mM sodium succinate buffer, pH 4.5, at 4°C. Under the latter conditions, the activity was seen to be stable for a period of more than 1 month. Moreover, only a 15% activity decrease was observed for enzyme when stored at −20°C for 100 days.

Pre-steady state kinetic analysis was carried out as described in the Materials and Methods section using...
Mn\textsuperscript{2+}, H\textsubscript{2}O\textsubscript{2}, and phenol red as substrates. As the substrate concentration increased, a linear relationship between MnP activity and substrate concentration was observed. The \(K_m\) and \(V_{\text{max}}\) values of the MnP of \textit{S. ostrea} in the present study for phenol red were found to be 8 \(\mu\)M and 111.14 U/mg of protein, respectively. The \(K_m\) and \(V_{\text{max}}\) values towards Mn\textsuperscript{2+} were 50 \(\mu\)M and 81 U/mg of protein, respectively. The \(K_m\) and \(V_{\text{max}}\) values in respect to H\textsubscript{2}O\textsubscript{2} were 51 \(\mu\)M and 104 U/mg of protein, respectively. The MnP of \textit{S. ostrea} in the presence of Mn\textsuperscript{2+} and H\textsubscript{2}O\textsubscript{2} displayed a high affinity towards phenol red in a similar manner (\(K_m<10 \mu\)m) as exhibited by MnPs of other organisms, such as \textit{P. ostreatus} \[10\] and \textit{P. eryngii} \[24\] towards 2,6-dimethoxyphenol. It has been observed that all isoforms of MnPs of fungal cultures oxidized 2,6-dimethoxyphenol with a maximal rate in the range of 7–412 \(\mu\)M \[35\]. The higher side of this range of maximal velocity was registered in respect to isoforms of \textit{Bjerkandera} \textit{sp.} acting on 2,6-dimethoxyphenol in the presence of Mn\textsuperscript{2+} and H\textsubscript{2}O\textsubscript{2} \[35\]. It is also known that the MnPs of lignolytic cultures exhibit requirements for Mn\textsuperscript{2+} with \(K_m\) values in a range of 15–55 \(\mu\)M, and H\textsubscript{2}O\textsubscript{2} with \(K_m\) value in a range of 7–83 \(\mu\)M \[23, 35\]. A high affinity of MnP enzymes with low \(K_m\) towards Mn\textsuperscript{2+} and H\textsubscript{2}O\textsubscript{2} has been registered in respect to \textit{P ostreatus} and \textit{P. eryngii} \[24\]. On the other hand, the MnPs of \textit{Rhizoctonia} \textit{sp.} \[5\] and of \textit{S. ostrea} in the present study have a low affinity towards the same substrate. The MnPs of other lignolytic cultures promote oxidation reactions on substrates Mn\textsuperscript{2+} and H\textsubscript{2}O\textsubscript{2} at a maximal velocity in a broader range of 10–1,000 \(\mu\)M. The oxidation of Mn\textsuperscript{2+} and H\textsubscript{2}O\textsubscript{2} catalyzed by the MnP of \textit{S. ostrea} occurred at a rate falling in the above range. The wide range in kinetic properties of MnPs in organisms could be due to differences in assay conditions employed and the inherent capacities of the MnP enzymes of the organisms.

The \textit{S. ostrea} MnP in the present study showed the best oxidation efficiency on guaiacol (7.7 U/ml), whereas the oxidation of veratryl alcohol was the lowest (3.12 U/ml). The activities of the enzyme on phenol red and 2,6-DMP were 4.67 and 6.24 U/ml, respectively. Similar observations for the oxidation of dimethoxyphenol, ABTS, guaiacol, and Syringaldigaline by the MnPs of \textit{P. ostreatus} and \textit{P. eryngii} \[24\] in the presence of Mn\textsuperscript{2+} and H\textsubscript{2}O\textsubscript{2} have been made. The extent of oxidation is dependent on the nature of substrate and the source of the enzyme. The oxidation of DMP, guaiacol, and ABTS by the MnP of \textit{P. ostreatus} equally proceeded at a maximal rate of about 150 units of transformation \[13, 14\].

The effects of three inhibitory compounds, SDS, sodium azide, and EDTA, on the activity of the MnP of \textit{S. ostrea} are shown in Fig. 8. The inhibition of enzyme activity by all these compounds tested in this study was dose-dependent and exhibited maximal (80–98%) inhibition at the highest concentration (5 mM) used in this study. Amongst the various metal ions tested for effects on the activity of the MnP of \textit{S. ostrea} under standard assay conditions (Table 3), Zn\textsuperscript{2+} and Ca\textsuperscript{2+} significantly stimulated MnP activity. In contrast, Fe\textsuperscript{3+} and Cu\textsuperscript{2+} reduced the activity, and there was a slight decrease in MnP activity between 1 and 10 mM of Mg\textsuperscript{2+} and Co\textsuperscript{2+}. These observations are in agreement with the results of the influence of compounds and metal ions on the activity of the MnP of the \textit{Schizopyllum} F17 strain \[6\]. The inhibition in the activity of MnP by SDS could

![Table 3. Effects of metal ions on MnP activity.](image)

<table>
<thead>
<tr>
<th>Metal ions</th>
<th>Concentration (mM)</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu\textsuperscript{2+}</td>
<td>1</td>
<td>8.75</td>
</tr>
<tr>
<td></td>
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<td>17.90</td>
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<td>2.70</td>
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<tr>
<td>Fe\textsuperscript{3+}</td>
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<td>45.00</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>10.51</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Ca\textsuperscript{2+}</td>
<td>1</td>
<td>132.5</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>123.0</td>
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<td></td>
<td>10</td>
<td>110.8</td>
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<td>Zn\textsuperscript{2+}</td>
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<td>Mg\textsuperscript{2+}</td>
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<td>92.5</td>
</tr>
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<td></td>
<td>5</td>
<td>84.6</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>78.3</td>
</tr>
</tbody>
</table>

Control, assay medium devoid of inhibitors. % inhibition, difference of the activity of MnP of the control and the activity of MnP in the presence of inhibitor, expressed as % in relation to activity of MnP of the control. Data in the figure are the mean of triplicate tests with standard error bars. Absolute values of activity of MnP of the control (3.00 U/ml).
probably be due to the denaturation of the MnP enzyme protein by SDS. The inactivation of MnP by sodium azide is paralleled by the formation of azidyl radicals and the high-yield conversion of heme into the meso-azido adduct [11, 14]. EDTA may inhibit MnP activity by making Mn$^{2+}$ ions unavailable to enzymes because of its chelating with ions. The toxicity of metal ions (Fe$^{2+}$, Cu$^{2+}$) to the activity of MnP may be due to the non-replacement of Mn$^{2+}$ ions [14].

A comparison in the activity of the enzyme in the presence of citrate, malate, oxalate, fumarate, and acetate (1 mM) with activity in the presence of tartrate (Fig. 9) indicated that oxalate and malate are stimulatory to the activity of the MnP. These carboxylic compounds may bind manganese ions and stabilize them, resulting in an increase in MnP activity. A low MnP activity was obtained in the presence of citrate, fumarate, and acetate. The role of manganese is supported by the observation that $\alpha$-hydroxy acids, which stabilize Mn$^{2+}$, stimulate catalytic activity, whereas ligands that form strong multidentate chelates with manganese inhibit activity [14]. Production of organic acids has been demonstrated in the growth of N. frowardii, in either solid-state fermentation and submerged fermentation [15], and is thought to be responsible for more $^{14}$CO$_2$ evolution from the labeled polymer of coniferyl alcohol by N. frowardii [15].

Decolorization of Dyes with Purified MnP of S. ostrea
The incubation of the purified MnP of S. ostrea with five dyes, Rose Bengal, Remazol Brilliant Violet, Remazol black-5, Remazol blue-19, and Remazol orange-16, resulted in the decolorization of all the dyes tested in this study (Fig. 10). The decolorization percentages of the above dyes by the enzyme occurred within a range of 71–84% after 16 h incubation with the MnP at 100 U/l for all the dyes. The decolorization rate increased with an increase in incubation time (Fig. 10).

In previous literature, it has been observed that the growth of some cultures without MnP activity in the presence of dyes resulted in the decolorization of dyes, whereas in other instances, the growth of some other cultures with MnP activity failed to decolorize dyes [24]. However, it is worth bearing in mind that the majority of decolorization studies were carried out with the use of crude culture filtrates containing more than one lignolytic enzyme, and may not provide clear-cut evidence regarding the participation of individual enzymes in the decolorization of dyes [28, 38]. In the present study, only a single, purified MnP was used in the reaction to test for the decolorization of dyes. The MnP of S. ostrea was able to decolorize all five dyes tested in the present study within a range of 70–80% during 16 h of incubation. The decolorization of the dyes was not due to use of H$_2$O$_2$ in the assay reaction system. In a similar fashion, the decolorization of azo dyes, such as Congo Red, Orange G, and Orange IV, with the MnP of Schizophyllum F17 [6, 19], and of sulfonaphthalein dyes with the MnP of P. chrysosporium [7], has been reported. The difference in the decolorization of dyes is obviously related to differences in the chemical structure and kinetic properties of the individual MnP enzymes of cultures. Thus, this study clearly demonstrates the ability of the purified MnP of S. ostrea to effectively decolorize dyes, thus showing its potential to be developed in an enzyme-based process for the decolorization of dyes in industrial effluents, either in free form or in immobilized form.

The results of the present study clearly confirm the presence of MnP in the lignolytic system of S. ostrea, proved beyond any doubt by the demonstration of MnP activity with enzyme protein purified from its culture filtrate. The MnP of S. ostrea exhibited a requirement for Mn$^{2+}$ and H$_2$O$_2$ with moderate affinity, and was able to effectively decolorize dyes.
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REFERENCES


