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# Role of oxidizing mediators and tryptophan 172 in the decoloration of industrial dyes by the versatile peroxidase from *Bjerkandera adusta*

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**Abstract**

A purified preparation of the versatile peroxidase from *Bjerkandera adusta* was tested for industrial dye decoloration. This ligninolytic enzyme efficiently oxidizes Mn(II) to Mn(III) and also exhibits Mn(II)-independent activity in the oxidation of aromatic substrates. It was able to decolorize 27 of the 41 industrial dyes tested. The presence of manganese in the reaction mixture did not enhance the decoloration rate, in fact for some dyes the transformation rate was inhibited. On the other hand, the presence of mediator molecules in the reaction mixture generally enhanced the decoloration of dyes that showed low activity without mediators, while the dyes that were decolorized rapidly without mediator were unaffected by the presence of veratryl alcohol, acetosyringone or TEMPO as oxidizing mediators. The role of tryptophan 172 on dye decoloration was investigated. Chemical modification of tryptophan residues using *N*-bromosuccinimide drastically reduced Mn(II)-independent activity and dye decoloration, while the Mn(II)-dependent activity was maintained. The loss of Mn(II)-independent and decoloration activities after tryptophan modification was not reversed by the addition of mediators. These results strongly suggest that the tryptophan 172 is involved in both mediator oxidation and dye decoloration activities, and in Mn(II)-independent activity.


*Keywords:* *Bjerkandera adusta*; Enzymatic decoloration; Dye; Oxidizing mediator; Tryptophan; Versatile peroxidase

## 1. Introduction

Since the end of the XIX century several thousands of synthetic dyes have been produced. The world annual textile dye production has been estimated at 800,000 tonnes [1]. In the textile industry, it is estimated that 15% of the dyestuff is released into wastewater effluent [2]. Synthesized dyes are designed to resist water, sunlight, sweat, some chemicals and microbial degradation. Thus, many industrial effluents are hardly decolorized by conventional wastewater treatment processes [3,4]. In addition, many of the industrial dyes are toxic, mutagenic or carcinogenic [5,6].

White rot fungi have been demonstrated to be good candidates for a biotechnological process for xenobiotic decontamination. These ligninolytic organisms are able to metabolize a wide range of chemical molecules, including chlorinated phenols, pesticides, PCBs, dioxins, organophosphorus compounds, nitrotoluenes, chloranilines, dyes, and other compounds of environmental concern [7–9]. Their extracellular enzymatic systems, which are involved in lignin degradation, consist mainly of oxidative enzymes: laccases, lignin peroxidases (LiP) and manganese peroxidases (MnP) [10]. Both peroxidases and laccases carry out substrate oxidation by a mechanism involving free radicals, and show low substrate specificity. These ligninolytic enzymes are directly involved in the degradation of various xenobiotic compounds, including industrial dyes. Recently, Wesenberg et al. [11] have published an extensive review on industrial dye decoloration by ligninolytic fungi. This review summarizes the research results and prospective use of fungi and their enzymes for the treatment of dye-containing industrial effluents.

A hybrid peroxidase, different from *Phanerochaete chrysosporium* lignin- and manganese-peroxidases, was first found in active lignin-degrading strains of *Pleurotus eryngii* [12]. This novel hybrid enzyme, called versatile peroxidase (VP), was also described in *Bjerkandera* sp. BOS55. It can both efficiently oxidize Mn(II) to Mn(III) and can also carry

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out Mn(II)-independent activity on aromatic substrates, and is also able to oxidize various phenolic and non-phenolic substrates such as 2,6-dimethoxyphenol, guaiacol, ABTS, and veratryl alcohol, in the absence of Mn(II) [13]. Similar versatile peroxidases have also been reported in *P. eryngii* [14–17], *Pleurotus pulmonarius* [18], *Pleurotus ostreatus* [19], as well as in *Bjerkandera adusta* [13,14,20,21]. Versatile peroxidase shows high identity with LiP (58–60%) and MnP (55%) both from *P. chrysosporium* [16]. The heterologous expression of VP in *Aspergillus nidulans* confirmed the ability of this hybrid enzyme to oxidize Mn(II) and different aromatic compounds in the absence of manganese [17]. This enzyme seems to have a long-range electron transfer pathway similar to those postulated for LiP [18]. The spectroscopic characterization of VP by EPR and electronic absorption techniques showed a protein-centered radical in the presence of hydrogen peroxide, which was identified as a tryptophan radical [22].

In this work, the capacity of this versatile peroxidase from *B. adusta* to decolorize industrial dyes is demonstrated and the role of Trp172 on these reactions is investigated.

## 2. Materials and methods

### 2.1. Chemicals

Industrial dyes were obtained from different manufacturers as indicated in Table 1, and under different commercial names. Acetosyringone, *N*-bromosuccinimide (NBS), TEMPO (2,2,6,6-tetramethyl-1-piperidinyloxy, free radical) and veratryl alcohol were purchased from Sigma–Aldrich Co. All buffer salts and hydrogen peroxide were obtained form J.T. Baker.

### 2.2. Protein preparation

Versatile peroxidase (VP) from *B. adusta* UAMH 8258 was obtained and purified to a single band on a Coomassie-stained electrophoresis gel as described previously [22,23]. The purified preparation showed Reinheitzahl ratio (Rz) ($A_{407}/A_{280}$) of 2.5, and specific activities for manganese peroxidase (MnP) of 105 U/mg protein measured by the $H_2O_2$-dependent formation of Mn(III)–malonate complex [24], and for lignin peroxidase (LiP) of 7.1 U/mg protein measured as veratryl alcohol oxidation [25]. Protein concentration was estimated by using both the BioRad protein reagent and also using an extinction coefficient at 407 nm of 77,000 $M^{-1}$ $cm^{-1}$ for the purified preparation.

### 2.3. Decoloration activity

Decoloration activity was determined by measuring the decrease of the dye absorbance at their maximum visible absorbance wavelength. Dye concentration in the reaction mixture was adjusted to 1.0 absorbance unit at the maximum wavelength in the visible spectrum (Table 1). Reaction mixtures were prepared with 0.5 mM $MnSO_4$ at pH 3.0 and without manganese at pH 4.0 according the optimal pH reported for pesticide transformation for both conditions [26]. The reactions were started by adding 0.1 mM hydrogen peroxide and the decoloration monitored in a Beckman spectrophotometer model DU 650. All the experiments were performed in triplicate.

The effect of three mediators, acetosyringone, veratryl alcohol and TEMPO, on dye decoloration was determined by adding different concentrations of the mediator to the reaction mixture and monitoring the dye decoloration at the maximal dye wavelength. The reactions were performed in 50 mM malonate buffer pH 4.0 and in the presence of 0.1 mM hydrogen peroxide. Kinetic constants were determined in a 50 mM malonate buffer pH 4.0 without manganese for Reactive black 5 ($\varepsilon_{595\,nm} = 37.0 \times 10^6\ M^{-1}\ cm^{-1}$), Reactive blue 38 ($\varepsilon_{620\,nm} = 16.4 \times 10^6\ M^{-1}\ cm^{-1}$), Reactive blue 72 ($\varepsilon_{665\,nm} = 25.1 \times 10^6\ M^{-1}\ cm^{-1}$), and Reactive violet 5 ($\varepsilon_{555\,nm} = 11.7 \times 10^6\ M^{-1}\ cm^{-1}$). The experimental data was plotted and the constants calculated by using the EnzFitter software (Biosoft, Cambridge, UK).

### 2.4. Tryptophan chemical modification

The chemical modification of tryptophan residues was performed using *N*-bromosuccinimide (NBS), as described by Spande and Witkop [27]. The effect of the extent of modification on VP activity was determined as follows, VP (25.9 μM) was mixed with different molar excesses of NBS in 0.1 M sodium acetate, pH 4.0. After 30 min of incubation at room temperature, reactions were centrifuged and extensively dialyzed against 10 mM sodium acetate, pH 4.0. Then, both specific Mn(II)-dependent and Mn(II)-independent activities were determined. The extent of NBS modification on tryptophans was spectrophotometrically determined at 280 nm after alkalinization of the modified VP sample with 10 N NaOH [28]. Because VP contains no tyrosine, absorbance values were considered to correspond to tryptophan. The measurements were carried out in triplicate and expressed as percentage of the unmodified protein.

## 3. Results and discussion

Forty-one industrial dyes were assayed for decoloration by purified VP from *B. adusta* UAMH 8258 (Table 1). Two different reaction conditions were assayed; pH 3.0 with 0.5 mM Mn(II) and pH 4.0 without manganese. These conditions were reported as the optimal for lignin peroxidase-like (Mn(II)-independent activity) and manganese peroxidase-like (Mn(II)-dependent activity) reactions on pesticide [26] and polycyclic aromatic hydrocarbon [23] transformations. Twenty-seven dyes were decolorized at pH 4.0 without Mn(II), and only 15 were decolorized at pH 3.0 in the presence of manganese. In general, no significant differences were found in most dyes in both conditions. However, Acid black 194, Direct green 6, Reactive blue 18 and Reactive green 19 showed significantly less decoloration rate, and no decoloration activity could be detected on Acid red 51, Reactive blue 198, and Reactive red 141 in the presence of 0.5 mM $MnSO_4$ at pH 3. Control experiments showed that VP is needed as a catalyst for the decoloration.

By far the most efficient single class of microorganisms to break down synthetic dyes are the white-rot fungi, which are mostly basidiomycetes [11]. The main challenge for enzymatic

Table 1
Decoloration rate of different industrial dyes by versatile peroxidase from *Bjerkandera adusta* UAMH 8258

| Dye | Source[a] | $\lambda_{max}$ | Decoloration rate ($\Delta A\ min^{-1}\ nmol^{-1}$) | |
|---|---|---|---|---|
| | | | pH 4 without Mn(II) | pH 3 with Mn(II) |
| Acid black 194 | A | 570 | 2.05 (±0.19) | 0.19 (±0.14) |
| Acid blue 185 | A | 620 | 12.98 (±0.55) | 12.50 (±0.74) |
| Acid red 51 | B | 525 | 1.17 (±0.09) | NR |
| Direct black 22 | A | 485 | 0.31 (±0.02) | 0.43 (±0.05) |
| Direct blue 199 | C | 620 | 25.64 (±0.81) | 16.43 (±0.31) |
| Direct blue 2 | C | 570 | 2.14 (±0.26) | 2.57 (±0.09) |
| Direct green 6 | C | 620 | 1.24 (±0.14) | 0.19 (±0.07) |
| Direct orange 26 | C | 495 | 1.76 (±0.21) | 1.93 (±0.05) |
| Direct red 23 | C | 510 | 3.79 (±0.14) | 1.55 (±0.09) |
| Direct yellow 58 | A | 415 | 0.08 (±0.01) | NR |
| Disperse black 1 | B | 560 | NR | NR |
| Disperse blue 56 | A | 545 | NR | NR |
| Disperse blue 73 | B | 580 | NR | NR |
| Disperse blue 79 | A | 560 | 0.21 (±0.01) | NR |
| Disperse orange 30 | A | 455 | NR | NR |
| Disperse red 1 | B | 440 | NR | NR |
| Disperse red 60 | A | 535 | 0.01 (±0.00) | NR |
| Disperse red 72 | B | 510 | NR | NR |
| Disperse red 161 | A | 470 | NR | NR |
| Disperse red 167 | B | 620 | NR | NR |
| Disperse yellow 3 | B | 440 | NR | NR |
| Disperse yellow 54 | A | 430 | NR | NR |
| Reactive black 5 | D | 595 | 5.36 (±0.09) | 5.95 (±0.50) |
| Reactive blue 18 | B | 605 | 2.19 (±0.26) | 1.17 (±0.10) |
| Reactive blue 19 | A | 590 | 1.81 (±1.09) | 3.05 (±0.17) |
| Reactive blue 38 | E | 620 | 10.78 (±0.07) | 20.57 (±0.86) |
| Reactive blue 72 | E | 665 | 0.47 (±0.07) | 1.43 (±0.14) |
| Reactive blue 198 | A | 625 | 2.67 (±0.05) | NR |
| Reactive green 19 | B | 610 | 1.05 (±0.12) | 0.69 (±0.05) |
| Reactive orange 16 | B | 485 | 0.27 (±0.01) | NR |
| Reactive red 4 | B | 540 | 1.35 (±0.08) | NR |
| Reactive red 141 | A | 540 | 1.02 (±0.12) | NR |
| Reactive red 180 | A | 540 | 0.52 (±0.04) | NR |
| Reactive yellow 2 | B | 400 | 0.14 (±0.02) | NR |
| Reactive yellow 84 | A | 410 | 0.07 (±0.01) | NR |
| Reactive violet 5 | F | 555 | 5.36 (±0.07) | 6.83 (±0.49) |
| Solvent yellow 16 | B | 490 | NR | NR |
| Sulfur black 1 | A | 635 | NR | NR |
| Vat blue 7 | A | 605 | 0.03 (±0.00) | NR |
| Vat red 10 | A | 520 | NR | NR |
| Vat yellow 46 | A | 440 | NR | NR |

The reaction mixture contained 1 absorbance unit of dye, at the maximal visible absorbance wavelength, in 50 mM malonate buffer and 0.42 nM enzyme, with or without 0.5 mM Mn(II). The reactions were started by adding 0.1 mM hydrogen peroxide.

[a] Dyes obtained from: (A) BASF (Ludwigshafen, Germany). (B) Colorantes Orion S.A. (Cuernavaca, México). (C) Colorfran S.A. (Monterrey, México). (D) Chemitalia (Milano, Italy). (E) Dystar (Milano, Italy). (F) Sigma–Aldrich-Fluka (St. Louis, Missouri, USA).

decoloration of industrial dyes is the large diversity of chemical structures among the synthetic dyes, which include polyenes, polymethines, diarylmethines, nitro and nitroso compounds, anthraquinones, and azo compounds. The last represent half of total dyes used in industry. Several works on dye decoloration have been published using whole cultures [29,30] or using the well-characterized lignin peroxidases, manganese peroxidases [31,32], laccases [33,34], and VP from *B. adusta* and *P. eryngii* [15,35]. As expected, enzymatic decoloration rate is dependent of the dye chemical structure [36,37].

Versatile peroxidase is different from *P. chrysosporium* lignin- and manganese-peroxidases, because it can both efficiently oxidize Mn(II) to Mn(III), and carry out Mn(II)-independent activity on aromatic substrates, and has been found in several lignin-degrading fungi [12–14,16,19,20,38]. As in the case of polycyclic aromatic hydrocarbons [23] and pesticides [26], the presence of manganese reduces the decoloration rate in some tested dyes (Table 1). Versatile peroxidase from *B. adusta* is able to bleach Reactive black 5, an azo dye which was not oxidized by manganese peroxidase from *P. chrysosporium*, in the presence or in the absence of $Mn^{2+}$ [14]. Paszczynski et al. [37] found that some different substituted sulfonated azo dyes were better oxidized in the presence of Mn(II) whereas others were better oxidized in the absence of Mn(II). In addition, it has been demonstrated [35] that $Mn^{2+}$ acts as a noncompetitive inhibitor for the oxidation of Reactive black 5 by VP from *B.*

Table 2
Kinetic constants for the oxidation of different dyes by versatile peroxidase from *B. adusta* UAMH 8258

| Dye | $k_{cat}$ ($min^{-1}$) | $K_M$ $H_2O_2$ (μM) | $K_M$ dye (μM) |
|---|---|---|---|
| Reactive black 5 | 286 | 7.9 | 22.0 |
| Reactive blue 38 | 1187 | 7.5 | 89.1 |
| Reactive blue 72 | 597 | 6.8 | 26.6 |
| Reactive violet 5 | 1012 | 10.9 | 47.3 |

Reactions were carried out in a 50 mM malonate buffer pH 4.0 without manganese. The decoloration was monitored at the maximal absorbance of each dye.

*adusta*, according to the presence of two substrate binding sites, one for Mn(II) and another on the protein surface connected to the heme by long-range electron transfers [39].

A manganese-binding site has been described for manganese peroxidases (MnP) from *Pleurotus*, *Bjerkandera* and *P. chrysosporium* [40], and a long-range electron transfer has been proposed for organic substrate oxidations [22,41]. These two different catalytic sites, one for Mn and another for veratryl alcohol, have been demonstrated in VP from *P. eryngii* [41]. High molecular weight substrates are unable to access the heme edge and they may be oxidized indirectly by superficial residues of the protein where the substrate electrons are conducted through an intramolecular pathway to the heme active site, as in the case of lignin peroxidase [42,43].

Two azo dyes (Reactive black 5 and Reactive violet 5) and two phthalocyanine dyes (Reactive blue 38 and Reactive blue 72) were selected for determination of kinetic constants under Mn(II)-independent reactions at pH 4. Catalytic constants ($k_{cat}$) varied from 286 to 1187 $min^{-1}$ (Table 2). On the other hand, affinity constants ($K_M$) varied only slightly for hydrogen peroxide (6.8–10.9 μM) and more significantly, from 22 to 89 μM, for the different dyes.

Versatile peroxidase from *B. adusta* has been named "dye-peroxidase" [44] because it is able to oxidize dyes directly in the absence of redox mediator. We have been exploring the use of oxidizing mediators with this enzyme, as used for laccases [45,46]. Veratryl alcohol (the model substrate for Mn(II)-independent or ligninase activity), acetosyringone and TEMPO were tested at different concentrations (Figs. 1–3). While the dyes showing high specific decoloration activity were, in general, unaffected by the presence of mediators, the dyes showing low activity were decolorized significantly faster when mediators were added to the reaction mixture. This decoloration rate enhancement is dependent of the mediator concentration (Figs. 1–3). The maximal increments on specific decoloration rates were up to 2500% for Direct yellow 58, 2000% for Dispersed red 60 and 1700% for Vat blue 7 with the presence of veratryl alcohol. On the other hand, the decoloration rate of Reactive yellow 2 increased 390% with acetosyringone, and 630% with TEMPO as mediator. However, none of the dyes showing low decoloration rate without mediator and showing significant increase in the decoloration in the presence of mediator, reach the decoloration activity values of those that are rapidly decolorized without oxidizing mediator. Nevertheless, these results show that the use of oxidizing mediators could enhance the peroxidase transformation of recalcitrant pollutants, as in the case of laccases [45,47]. It is important to point out that 13 of the 41 tested dyes, specially the disperse type, were not decolorized by VP with or without mediator (Table 1). These recalcitrant industrial dyes are: Disperse black 1, Disperse blue 56, Disperse blue 73, Disperse orange 30, Disperse red 1, Disperse red 72, Disperse red 161, Disperse red 167, Disperse yellow 3, Disperse yellow 54, Solvent yellow 16, Sulfur black 1, Vat red 10, and Vat yellow 46. The absence of reaction with certain dyes could be due to the higher redox potentials of dyes than those of VP and oxidized mediators.

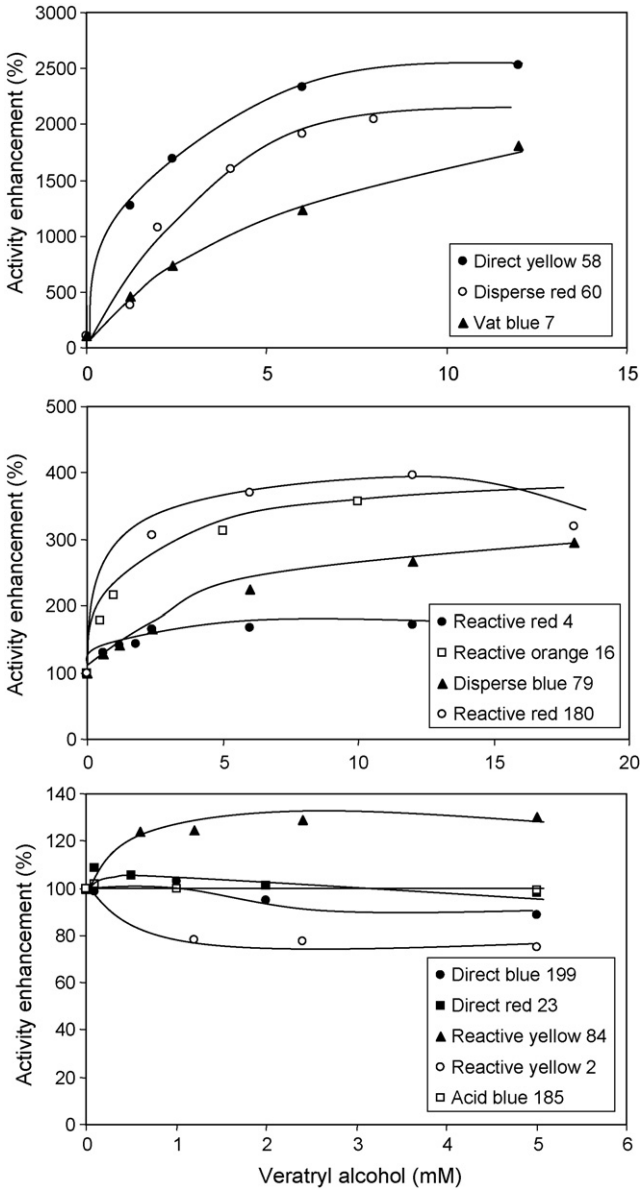


Fig. 1. Activity enhancement by veratryl alcohol as oxidizing mediator. The decoloration specific rates ($\Delta A$ $min^{-1}$ $nmol^{-1}$) without mediator are considered as 100%, and they are as follows: Acid blue 185, 12.98; Direct blue 199, 25.64; Direct red 23, 3.79; Direct yellow 58, 0.08; Disperse blue 79, 0.21; Disperse red 60, 0.01; Reactive orange 16, 0.27; Reactive red 4, 1.35; Reactive red 180, 0.52; Reactive yellow 2, 0.14; Reactive yellow 84, 0.07; Vat blue 7, 0.03.

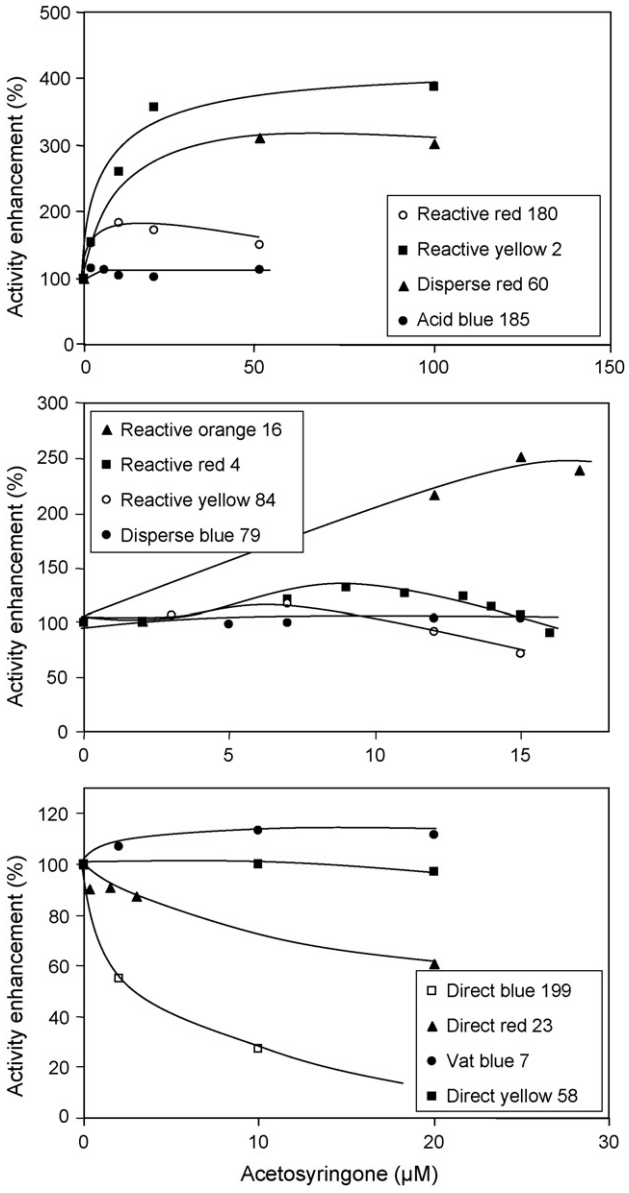


Fig. 2. Activity enhancement by acetosyringone as oxidizing mediator. The decoloration specific rates without mediator are considered as 100%, and they are shown in the legend of Fig. 1.

A redox mediator should have three properties: (i) it should be able to be oxidized by one-electron directly from the enzyme to produce a free radical, (ii) the produced radical should be stable enough to diffuse and react with the target compound, and (iii) it should have an appropriate redox potential. The three mediators used are chemically different. Acetosyringone is a phenolic compound, veratryl alcohol is a primary alcohol and TEMPO is compound able to form amoxyl radical. The different mechanisms of oxidation mediated by several mediators have been recently elucidated in the case of laccase [48,49]. It seems probable that for the VP-TEMPO system the dyes are oxidized via a persistent oxoammonium ion [49], the formation of a phenoxyl radical could be involved in the VP-acetosyringone system, and the VP oxidation of veratryl alchol produces veratryl aldehyde.

Recently, the nucleotide sequence of VP from *B. adusta* UAMH 8258 (GenBank DQ060037) has been listed together with those previously reported for VP's from *Bjerkandera* sp. B33/3 (GenBank AY217015) [38,50], *Pleurotus sapidus* (GenBank AM039632) and *Lepista irina* (GenBank AJ515245) [51]; as well as *P. eryngii* PS1 (GenBank AF175710) [52], VPL1 (GenBank AF007223) [16] and VPL2 (GenBank AF007224) [16]. The sequence of VP from *B. adusta* UAMH 8258 is similar to these from *Bjerkandera* sp. B33/3 (98% homology), showing only four different residues; Ile57, Ser93, Thr167 and Thr323. Considering the high sequence homology, the phylogenetic proximity and the availability of crystallographic structure, a molecular model has been constructed of VP from *B. adusta* UAMH 8258 [22]. The crystallographic structures of versa-

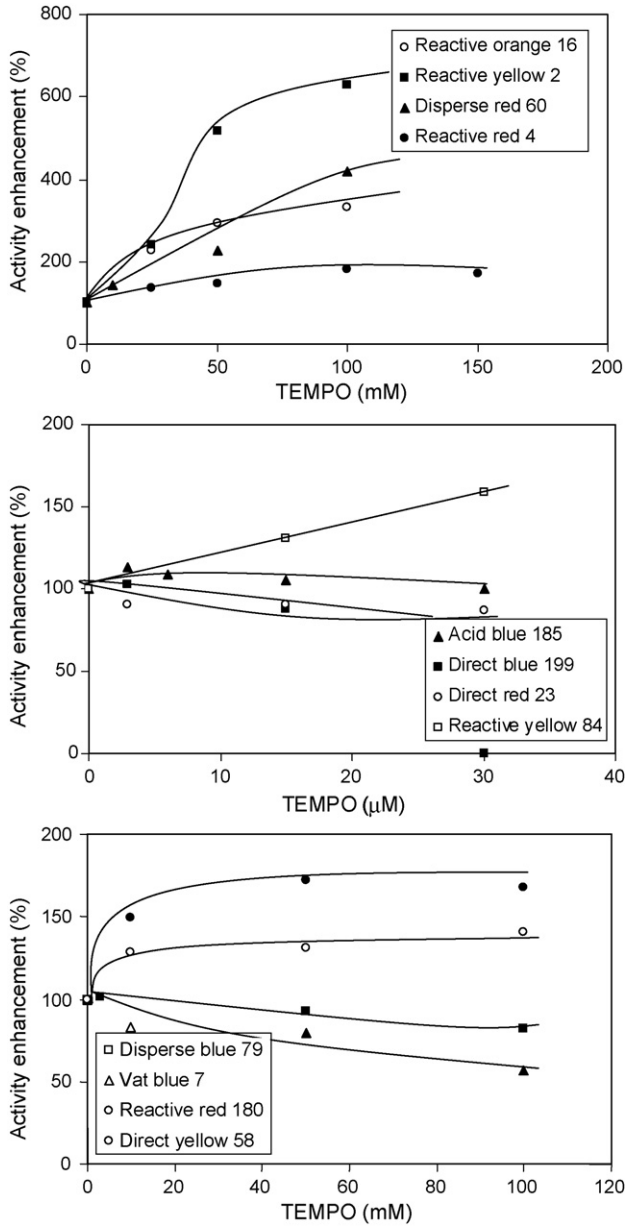


Fig. 3. Activity enhancement by TEMPO as oxidizing mediator. The decoloration specific rates ($\Delta A\ min^{-1}\ nmol^{-1}$) without mediator are considered as 100%, and they are shown in the legend of Fig. 1.

tile peroxidase from *P. eryngii* (PDB 2BOQ), and the lignin (PDB 1B80) and manganese (PDB 1MNP) peroxidases from *P. chrysosporium* were used as template. The VP shows a typical plant peroxidase folding and, as in the case of VP from *P. eryngii*, contains a binding site for Mn(II) constituted by the residues Glu37, Glu41 and Asp183. The existence of two different binding sites, one for the manganese ion and another for the dye, could contribute to the differences in the pH profiles in the presence and in the absence of manganese in the reaction media. Manganese acted as a noncompetitive inhibitor for pesticide transformation [26] and for the oxidation of azo dyes [15], showing similar values of inhibition constants for both substrates.

The nucleotide sequence of VP from *B. adusta* UAMH 8258 shows two Trp residues and no Tyr, and from the molecular model, VP contains a surface Trp172 that is structurally and functionally equivalent to Trp171 of lignin peroxidase from *P. chrysosporium* [53] and to Trp164 of versatile peroxidase VPL2 from *P. eryngii*. Compound I oxidizes this residue generating a radical which is stabilized by the coupled oxidation of a reducing substrate [21,22,54,55]. This is the opposite of Mn(II), which transfers the electrons directly to the heme through the propionates, and Trp172 is oxidized through a long-range electron transfer pathway as in the case of LiP [41,56]. A tryptophanyl radical also has been detected in this enzyme and seems to be functionally analogous to LiP W171 [22,57].

In order to determine the role of tryptophan 172 in dye decoloration, a specific chemical modification with NBS was performed. At a NBS/VP ratio of 9.6 only 6.8% (±5.3) of tryptophans remained unmodified, the Mn(II)-dependent activity was unaffected 113% (±11), and remarkably the Mn(II)-independent activity (ligninase activity) was drastically decreased to 20.6% (±1.0) (Fig. 4). These results confirm the catalytic role of Trp172 in the Mn(II)-independent activity as previously reported for VP [22,55,57]. Four different industrial dyes were selected to study the effect of chemical modification on tryptophans on their decoloration. Two showing high activity without mediator and in which the presence of mediator does not enhance the decoloration rate (Direct blue 199 and Acid blue 185), and two showing low decoloration rate and in which the presence of mediator significantly increase the decoloration rate (Direct yellow 58 and Disperse red 60). Table 3 shows the specific activities of unmodified and modified VP preparations on these dyes, with and without 10 mM veratryl alcohol. The chemical modification of tryptophans significantly decreased the enzyme capacity to decolorize the dyes showing high activity. The capacity of VP to decolorize Direct blue 199 and Acid blue 185 decreased 99% and 94%, respectively. The decoloration reduction was not reversed by the presence of veratryl alcohol as an oxidizing mediator. On the other hand, for the industrial dyes showing low decoloration rate, tryptophan modification also reduced the activity but to less extent, 75% and 70% for Direct yellow 58 and Disperse red 60, respectively, and no decoloration enhancement was detected in the presence of 10 mM veratryl alcohol. These results suggest an important role for tryptophan 172 in the direct decoloration of dyes and also in the oxidation of oxidizing mediators, and thus the effect of mediators for the enhancement of dye decoloration. Though the absolute rate of decoloration of some dyes, even in the presence of mediators, is so low as to be of little practical use.

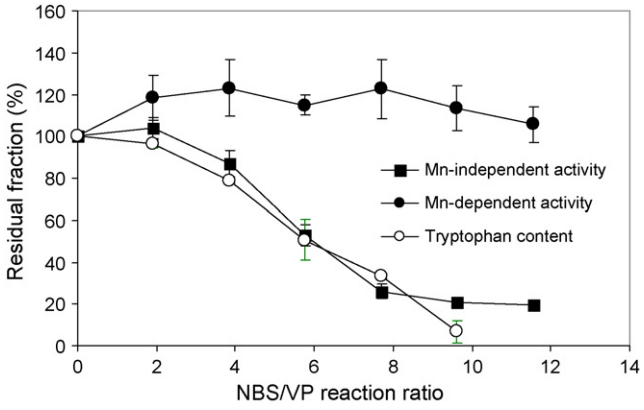


Fig. 4. Effect of the chemical modification of tryptophan residues of versatile peroxidase on the Mn(II)-dependent and Mn(II)-independent activities. The chemical modification was performed in 0.1 M sodium acetate pH 4.0 using *N*-bromosuccinimide (NBS) as modifying agent. VP solution was mixed with different molar excesses of NBS and, after 30 min incubation at room temperature, the reaction was centrifuged and dialyzed against 10 mM sodium acetate pH 4.0. The Trp content was calculated as percentage of the unmodified protein.

We can conclude so far that VP from *B. adusta* is able to oxidize a wide range of industrial dyes. Dye decoloration by VP does not require manganese(II) ion, and uses hydrogen peroxide as electron acceptor. The presence of oxidizing mediators enhances the decoloration activity of dyes showing low decoloration rates, while no effect was found for dyes that are highly decolorized in the absence of mediator. The chemical modification of the two tryptophans of the VP molecule disables it for both dye direct oxidation and mediator oxidation. Thus the surface Trp172 seems to be involved in VP-mediated industrial dye decoloration, in agreement with a long-range electron transfer

Table 3
Effect of the chemical modification of the tryptophan on the dye transformation with and without the presence of veratryl alcohol (VA) as mediator

| Dye | Decoloration activity ($\Delta A$ min$^{-1}$ nmol$^{-1}$) | | | |
|---|---|---|---|---|
| | Unmodified VP | Unmodified VP + 10 mM VA | NBS-modified VP | NBS-modified VP + 10 mM VA |
| Direct blue 199 | 25.6 (±0.8) | 22.7 (±0.4) | 0.31 (±0.01) | NRD |
| Acid blue 185 | 13.0 (±0.6) | 9.2 (±0.3) | 0.82 (±0.02) | 0.47 (±0.06) |
| Direct yellow 58 | 0.08 (±0.01) | 2.0 (±0.02) | 0.02 (±0.002) | 0.12 (±0.02) |
| Disperse red 60 | 0.01 (±0.00) | 0.22 (±0.01) | 0.003 (±0.0005) | 0.015 (±0.001) |

NRD: no reaction detected.

pathway [22,55]. As for laccases, the use of oxidizing mediators by versatile peroxidase may improve the transformation of some recalcitrant pollutants.

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