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The effect of violuric acid on the decolourization of recalcitrant dyes by laccase from *Trametes hirsuta*

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Abstract

In this paper, the effect of the redox mediator violuric acid (VA) on the decolourization of the two recalcitrant acid dyes C.I. Acid Red 97 and C.I. Acid Green 26 by crude laccase obtained from *Trametes hirsuta* cultures was investigated. The laccase–mediator system (LMS) led to a higher extent of decolourization in shorter times than that obtained without mediator addition, especially for C.I. Acid Red 97 infact the dye was almost totally decolourized (about 90%) in only 3 min. In addition, the stability of laccase against VA was also assessed.

Keywords: Decolourization; Laccase; Recalcitrant dyes; Redox mediator; *Trametes hirsuta*; Violuric acid

1. Introduction

Laccase (EC 1.10.3.2) is the generic name given to a family of multicopper oxidases that are capable of oxidizing several different substrates with the concomitant reduction of dioxygen to water. Although these enzymes exhibit specific affinity for oxygen as their electron acceptor, their specificity towards their reducing substrates is rather low [1]. Laccases catalyze the removal of a hydrogen atom from the hydroxyl group of methoxy-substituted monophenols, *ortho*- and *para*-diphenols; they also oxidize other substrates such as aromatic amines, syringaldazine and non-phenolic compounds to form free radicals [2].

The capability of laccase to degrade chromophores such as triarylmethane, indigoid, azo and anthraquinoid suggests that it offers potential application in textile dye bleaching processes [3,4]. However, these processes have been hindered due to unfavourable kinetics between the enzyme and the dye; the use of small molecules that are capable of acting as electron transfer mediators between the enzyme and the dye has been regarded as a feasible solution to this particular drawback [5].

Several organic and inorganic compounds, thiol and phenol aromatic derivatives, *N*-hydroxy compounds and ferrocyanide have been reported as effective mediators. When mediators are incorporated in laccase-assisted processes, electron transfer from the mediator to the enzyme is followed by electron donations from the target molecule to the oxidized mediator, which causes the regeneration of the mediator [5–7]. In this context, compounds other than laccase substrates capable of undergoing indirect catalytic oxidation reactions can be treated with a laccase–mediator system (LMS).

Redox-mediated laccase catalysis has been used in many applications, as exemplified by pulp delignification, polycyclic aromatic hydrogen (PAH) degradation, pesticide or insecticide degradation and organic synthesis [8]. Claus et al. [9] found that LMS enhanced dye decolourization and some dyes resistant to laccase degradation were also decolourized; mediators having the N–OH functionality were regarded to display

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optimum performance. Thus, Soares et al. [10,11] found that violuric acid (VA) was the most effective redox mediator for laccase oxidation reactions among several mediators tested. Also, Barreca et al. [12] determined that VA was the most valuable redox mediator for the α-oxidation of the model lignin Adlerol. The aim of the present paper was to test the efficiency of the compound VA as a redox mediator for the decolourization of the two recalcitrant acid dyes by laccase from *T. hirsuta*.

2. Materials and methods

2.1. Microorganism

Trametes hirsuta (BT 2566), obtained from Dr. G.M. Gübitz, Institute of Environmental Biotechnology, Graz University of Technology, Austria, was grown on PDA (potato dextrose agar) plates at 3 °C for about 10 days. Thereafter, the plates were maintained at 4 °C until used; the fungus was sub-cultured every three months.

2.2. Laccase production and crude enzyme preparation

T. hirsuta grown was immobilized on stainless steel sponges in a bioreactor [13]. Culture broth was collected at the maximum laccase activity (9 days), filtered, clarified by centrifugation at 8000 g for 15 min, frozen, defrosted and then filtered to remove the precipitated polysaccharides. The resulting clear filtrate was concentrated on an Amicon membrane with a molecular weight cut-off of 10 kDa. The experiments were performed with this concentrated clear filtrate.

2.3. Determination of laccase activity

ABTS (2,2′-azino-di-[3-ethyl-benzo-thiazolin-sulphonate]) was used as a substrate for spectrophotometric determination of laccase activity as described by Niku-Paavola et al. [14]. One activity unit was defined as the amount of enzyme that oxidized 1 μmol of ABTS per minute; activities are expressed in $U\,L^{-1}$.

2.4. Dye decolourization experiments

The dyes employed were *Sella Solid Red* (C.I. Acid Red 97; AR 97), a disazo dye, manufactured by TFL (Germany) and *Luganil Green* (C.I. Acid Green 26, AG 26), a copper phthalocyanine dye, manufactured by BASF (Germany); their structures are not disclosed in the Colour Index. Stock solutions (0.2% w/v in water) were stored in the dark at room temperature.

The reaction mixture for dye decolourization consisted of an aqueous solution of dye, crude laccase (final concentration, $500\,U\,L^{-1}$) and VA at different concentrations (1, 2 and 5 mM) in citrate phosphate buffer (pH 5.0 for AR 97 and pH 4.0 for AG 26, according to previous results by our research group) in a final volume of 1.5 mL.

Dye concentrations were selected in order to obtain around 1.5 absorbance units at the maximum wavelength in the visible spectrum ($40\,mg\,L^{-1}$ for AR 97 and $130\,mg\,L^{-1}$ for AG 26). All the reaction mixtures were incubated at room temperature, without shaking and in complete darkness.

Decolourization was measured spectrophotometrically from 350 to 750 nm, calculated by measuring the area under the plot and expressed in terms of percentage. A control test containing the same amount of a heat-denatured laccase was performed in parallel. The assays were done twice, the experimental error being below 3%.

2.5. Stability of laccase from T. hirsuta against VA

Laccase was incubated with VA at concentrations ranging from 0 to 10 mM at room temperature and in complete darkness. Samples were collected at convenient times and residual activities were determined at 30 °C using the ABTS assay .

3. Results and discussion

Most research related to LMS has aimed at delignification purposes; recently interest has been focused on its application in dye bleaching. In this paper, the effect of the addition of the redox mediator VA on two recalcitrant acid dye decolourization was assessed. Fig. 1 shows the chemical structure of VA.

3.1. Effect of VA on dye decolourization

As seen in Fig. 2, VA led to both higher decolourization percentage and higher decolourization rate of AR 97 than those obtained without redox mediator. From the different concentrations tested the best results were obtained at VA concentrations of both 2 mM and 5 mM. Thus, a decolourization percentage of about 90% in 3 min was obtained. This value is 3-fold higher and obtained in much shorter time than that found without mediator.

AG 26 showed much higher resistance to decolourization than AR 97 and could only be decolourized to a certain extent, even with the addition of VA (Fig. 3). This slow decolourization rate may be attributed to the complexity of the phthalocyanine chromophore [15]. Thus, at a VA concentration of 1 mM, decolourization supposed only a slight improvement (3.7% for 20 h and 6.2% for 24 h) in relation to that obtained without redox mediator. Interestingly, at higher VA concentrations (2 and 5 mM), dye decolourization was lower than those achieved without mediator. This could be due to

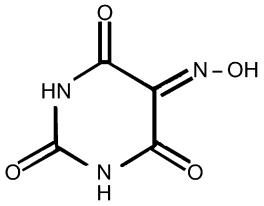

Fig. 1. Chemical structure of violuric acid.

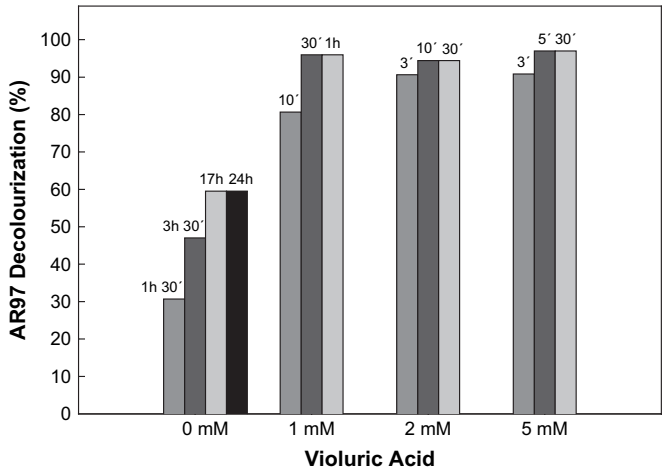

Fig. 2. Decolourization of the dye C.I. Acid Red 97 in the absence and presence of different violuric acid concentrations.

laccase inactivation by VA or due to laccase inhibition by radicals generated in the process.

According to the above results it can be asserted that the extend to which VA enhances the laccase catalyzed reactions depends on the nature of the dyes. This is in agreement with the investigations performed by Soares et al. [11]. Also, recently Camarero et al. [16] found that the efficiency of different natural mediators depended on the type of dye to be treated.

It is beyond the scope of the present work to provide mechanistic interpretations of the observed results as this would require analysis and identification of the reaction products. Nevertheless, it is worth mentioning that several researchers [8,17] compared fungal laccases with a variety of redox mediators and found that the redox potential of laccases varied depending on the laccase source. This could dictate the need and/or nature of redox mediator for the degradation of a particular dye to occur.

Despite VA is regarded to be toxic, taking into account the low concentration employed (2 mM) and the high improvement achieved its utilisation is more than justified.

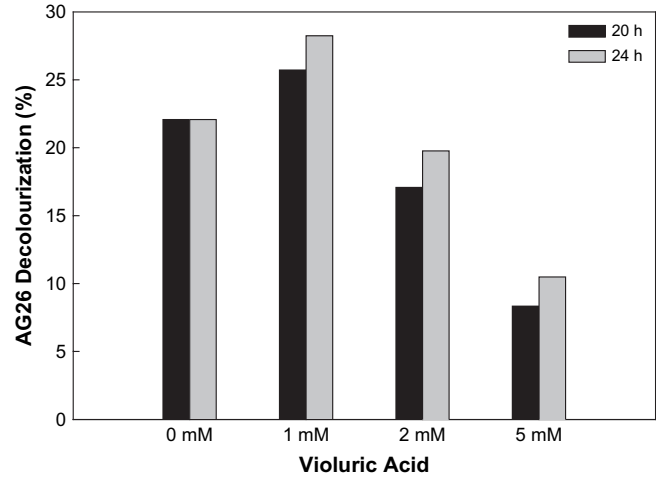

Fig. 3. Decolourization of the dye C.I. Acid Green 26 in the absence and presence of different violuric acid concentrations.

Table 1
Effect of violuric acid on *T. hirsuta* laccase stability

Violuric acid (mM)	Residual laccase (%)		
	2 h	7 h	4 days
0	89	64	48
1	56	45	11
2	50	40	2
5	47	24	1

3.2. Stability of laccase against violuric acid

It is known that VA is one of the most inactivating mediators for laccases [8,18], maybe due to the degradation of laccase's aromatic amino acids by the free radical of VA [8]. Hence, in the present paper the stability of laccase against VA at the concentrations employed for dye decolourization was assessed. It was found that laccase stability decreased as VA concentration increased, being this effect more acute along time. For the selected concentration of 2 mM, laccase stability diminished about 45% in 2 h in relation to the laccase without VA (Table 1). The decolourization of AR 97 was not influenced by this effect, since almost total decolourization occurs in 3 min; however, the decolourization rate of AG 26 was much slower. The inactivation of laccase would slow down the production of VA free radical affecting the oxidation of the dye and this could likely be the reason for the best results that were obtained at a lower VA concentration.

4. Conclusions

In view of the results obtained, it can be concluded that the redox mediator VA was very efficient in the decolourization of the disazo AR 97 dye; thus, by addition of this compound, AR 97 decolourization was augmented by 3-fold and its decolourization rate increased enormously, showing the efficiency of the LMS in dye decolourization.

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