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Assessment of the joint effect of laccase and cellobiose dehydrogenase on the decolouration of different synthetic dyes

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ABSTRACT

In this paper the efficiency of the combined action of laccase and cellobiose dehydrogenase (CDH) to decolourise different synthetic dyes such as Remazol Brilliant Blue R (RBBR), Methyl Green (MG), Direct Violet (DV), Ponceau Xylidine (PX), Bismark Brown (BB) and Poly R-478 (PR) was assessed. It was found that the use of CDH could be a promising alternative to the utilisation of the expensive and poisonous chemical mediators such as HOBT although much research on this topic remains still to be done.

1. Introduction

Synthetic dyes are extensively used in several industries such as the textile, paper, pharmaceutical, cosmetics and food industries. Over 7×10^5 tonnes of approximately 10,000 different dyes and pigments are produced annually world-wide, of which about 50,000 tonnes are discharged into the environment [1]. The discharge of very small amounts of dyes (less than 1 mg/l for certain dyes) is aesthetically displeasing, impedes light penetration into water, retards photosynthesis, inhibits the growth of aquatic biota and interferes with gas solubility in water bodies [2]. For these reasons several countries are adopting stringent regulations for the discharge of coloured industrial effluents.

Synthetic dyes are usually treated by physical or chemical methods [3]. However, these processes are financially and often also methodologically demanding, time-consuming and mostly not very effective. Currently one of the possible alternatives for the treatment of dye-containing effluents is the use of ligninolytic fungi, which can oxidise a wide variety of organic pollutants including synthetic dyes [4–6]. This ability is due to an extracellular non-specific and non-stereoselective enzyme system consisting of lignin peroxidases (LiP, EC 1.11.1.14), manganese peroxidases (MnP, EC 1.11.1.13) and laccases (EC 1.10.3.2). The latter have been subject of recent research due to (i) laccases present a better thermostability than LiPs and MnPs and (ii) laccases only require the presence of oxygen from air but neither manganese nor hydrogen peroxide.

Laccases (*p*-diphenol:dioxygen oxidoreductases; EC 1.10.3.2) are multicopper-containing enzymes that catalyse the one-electron oxidation of phenolic substrates and aromatic amines with the simultaneous four electron reduction of molecular oxygen to water [7]. The broad substrate specificity of laccases together with the fact that they use molecular oxygen as the final electron acceptor, make these enzymes highly interesting for biotechnological applications. The range of chemical structures oxidised by laccases can be even increased by using different natural and synthetic redox mediators [8]. The basis of the laccase-mediator concept is the use of low-molecular weight compounds, which once oxidised by laccase to radicals act as redox mediators oxidising other compounds that are not substrates of laccases. Mediators having the N–OH functionality are regarded to give the best performances. Thus, Xu et al. [9] found that old newsprint could be deinked by laccase plus violuric acid (VA). Also, Rodríguez Couto and Sanromán [10] found that lac-

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case plus VA effectively decolourised the recalcitrant dyes Acid Red 97 and Acid Green 26. However, these compounds are expensive and toxic which limits their application to an industrial scale. The search for natural mediators is in progress [11]. Also, recent studies have shown that cellobiose dehydrogenase (CDH; EC 1.1.99.18), an extracellular haemo-flavo-enzyme, produced by a number of wood-degrading and phytopathogenic fungi, plays a role in the early events of lignocelluloses degradation and wood colonisation [12] by the generation of hydroxyl radicals in a Fenton type reaction in the presence of an electron donor [13]. Furthermore, hydroxy radicals should be involved in the demethylation of lignin, as they can transform non-phenolic in phenolic units and facilitate their degradation by other oxidative enzymes such as MnP or laccase [14]. In this regard, CDH has been reported to display *in vitro* a synergism with laccases in the decolouration of different classes of textile dyes [15]. In the present study, we investigated the joint effect of laccase and CDH on the decolouration of different synthetic dyes.

2. Materials and methods

2.1. Microorganism

Trametes pubescens MB89 (CBS 696.94; Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands) was obtained from the Institute of Applied Microbiology, University of Natural Resources and Applied Life Sciences (Vienna, Austria) and was maintained on malt extract agar (MEA) plates at 4 °C and subcultured every 3 months.

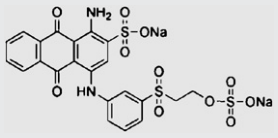
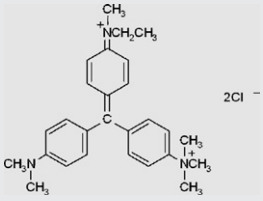
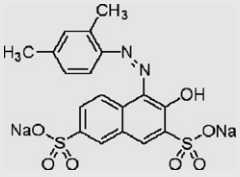
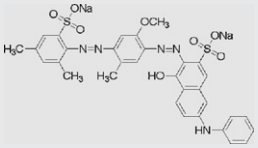
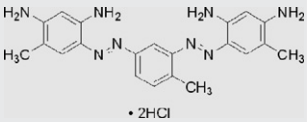
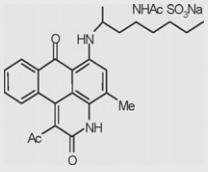
2.2. Culture conditions for laccase production

Laccase was produced as previously described [16]. Culture broth was collected at the maximum laccase activity (day 10) and clarified by centrifugation at 8000 × *g* for 15 min. After that, the supernatant was ultra-filtrated in an Amicon stirred cell apparatus (YM10 membrane). The resulting concentrated extract was used to perform the decolourising experiments.

2.3. Culture conditions for the simultaneous production of CDH and laccase

T. pubescens was cultivated as above except 10 g/l of microcrystalline cellulose powder was added as a carbon source instead of glucose to obtain the simultaneous production of CDH and laccase. The extracellular fluid was collected on day 10 when CDH activity was 49 U/l and laccase activity was 4808 U/l, centrifuged and concentrated as indicated above.

Table 1
Characteristics of the synthetic dyes used.

Dye	Class	λ_{max} (nm)	CI number	CI name	Structure
Remazol Brilliant Blue R (RBBR)	Anthraquinonic	595	61200	Reactive Blue 19	
Methyl Green (MG)	Tryarylmethane	630	42585	Basic Blue 20	
Ponceau Xylidine (PX)	Monoazo	503	16150	Acid Red 26	
Direct Violet 51 (DV)	Diazo	549	27905	Direct Violet 51	
Bismark Brown R (BB)	Diazo	468	21010	Basic Brown 4	
Poly R-478 (PR)	Polymeric	520	–	–	

CI = colour index.

2.4. Enzyme assays

Laccase activity was spectrophotometrically determined as described by Niku-Paavola et al. [17] with 2,2′-azino-di-[3-ethyl-benzo-thiazolin-sulphonate] (ABTS) (ε_{420} = 36,000/M cm), as a substrate. One activity unit (U) was defined as the amount of enzyme that oxidised 1 μmol of ABTS per min. The activities were expressed in U/l.

CDH activity was spectrophotometrically measured according to Baminger et al. [18] by following the decrease in absorbance of the electron acceptor, i.e. 2,6-dichlorophenol-indophenol (DCIP; Chem Service Inc., West Chester, PA, USA), at 520 nm ($\varepsilon_{520} = 6.8 \times 10^3$/M cm), pH 4.0 and 37 °C for 5 min. The reaction mixture, in a total volume of 1 ml, contained 100 μl DCIP (3 mM in water containing 10% v/v ethanol), 100 μl lactose (300 mM in 100 mM sodium acetate buffer, pH 4.0), and an appropriately diluted CDH sample (in 100 mM sodium acetate buffer, pH 4.0). To inhibit laccase activity sodium azide (30 mM) was added. One unit of CDH activity (U) was defined as the amount of enzyme that reduced 1 μmol of DCIP per min. The activities were expressed in U/l.

Manganese-dependent peroxidase activity was spectrophotometrically assayed at 468 nm by the method of Kuwahara et al. [19]. The reaction was started by adding 0.4 mM H_2O_2. One activity unit (U) was defined as 1 μmol of 2,6-dimethoxyphenol (DMP) oxidised per minute and the activities were expressed in U/l.

Lignin peroxidase activity was spectrophotometrically determined at 310 nm according to Tien and Kirk [20]. The reaction was starting by adding 0.4 mM H_2O_2. One activity unit (U) was defined as 1 μmol of veratryl alcohol oxidised in 1 min and the activities were reported as U/l.

All spectrophotometric measurements were carried out using a double-beam UV–Visible spectrophotometer Helios alfa (Thermo Spectronic, Cambridge, UK). All the results are the mean of three determinations.

2.5. Dye decolouration experiments

The dyes used were Remazol Brilliant Blue R (RBBR), Direct Violet 51 (DV), Ponceau Xylidine (PX), Bismark Brown R (BB) purchased from Sigma–Aldrich (St. Louis, MO, USA) and Methyl Green (MG), purchased from Merck (Germany). The characteristics of the dyes are summarised in Table 1. Stock solutions (0.1% w/v in water) were stored in the dark at room temperature.

The reactions (final volume 1.5 ml) were carried out directly in the spectrophotometer cuvette. The experimental setup was as follows: an aqueous solution of dye and extracellular liquid containing laccase (500 U/l, final concentration), laccase (500 U/l, final concentration) plus CDH (40 U/l, final concentration), laccase (500 U/l, final concentration) plus CDH (40 U/l, final concentration) plus lactose (30 mM, final concentration) or laccase (500 U/l, final concentration) plus HOBT (0.06%, final concentration) depending on the experiment in succinic buffer (pH 4.5). Dye concentrations were selected in order to obtain around 0.8–1.0 absorbance units at the maximum wavelength in the visible spectrum (0.133 g/l for RBBR, 0.015 g/l for DV, 0.022 g/l for PX and BB and 0.033 g/l for MG, final concentrations). All the reactions were incubated at room temperature (25 °C), in static conditions and in darkness.

The residual dye concentrations were spectrophotometrically measured and associated with the decrease in the absorbance at the peak of maximum visible wavelength for each dye (Table 1). Dye decolouration was expressed in terms of percentage. A control test containing the same amount of heat-denatured enzymes was performed in parallel.

2.6. Statistical data analysis

All the experiments were duplicated and samples were analysed three times. Statistical analysis was carried out using SPSS 16 for windows (SPSS, Chicago, IL, USA) and SAS 9.1 for windows (SAS Institute Inc., Cary, NC, USA), standard deviation being ≤10%. Data among treatments were analysed by one-way analysis of variance (ANOVA) using Microsoft Excel (Microsoft). Readings were considered significant when p was ≤0.05.

3. Results and discussion

3.1. Enzyme production

As shown in Fig. 1A in the glucose cultures laccase activity first appeared on day 4 (372 U/l) and then it sharply increased to 4304 U/l on day 7 reaching its peak on the last cultivation day (7974 U/l). CDH was not detected, since glucose inhibits CDH expression [21]. As for cellulose cultures (Fig. 1B), the laccase profile obtained was almost identical ($p > 0.05$) to that attained in the glucose cultures. Thus, laccase activity started on day 4 (418 U/l) and then it sharply increased reaching its peak on the last cultivation day (7771 U/l). CDH production started on day 5 (5 U/l) and then it increased reaching its peaks on days 9 (52 U/l) and 10 (49 U/l). At the same time laccase activity was 2335 U/l (day 9) and 4808 U/l (day 10). Neither MnP nor LiP activities were detected in any of the two cultures.

3.2. Decolouration of different synthetic dyes

As an alternative to the utilisation of the expensive and poisonous chemical mediators the action of laccase was combined to that of CDH, an enzyme able to indirectly generate hydroxyl radicals in a Fenton-type reaction. CDH was expressed by *T. pubescens*

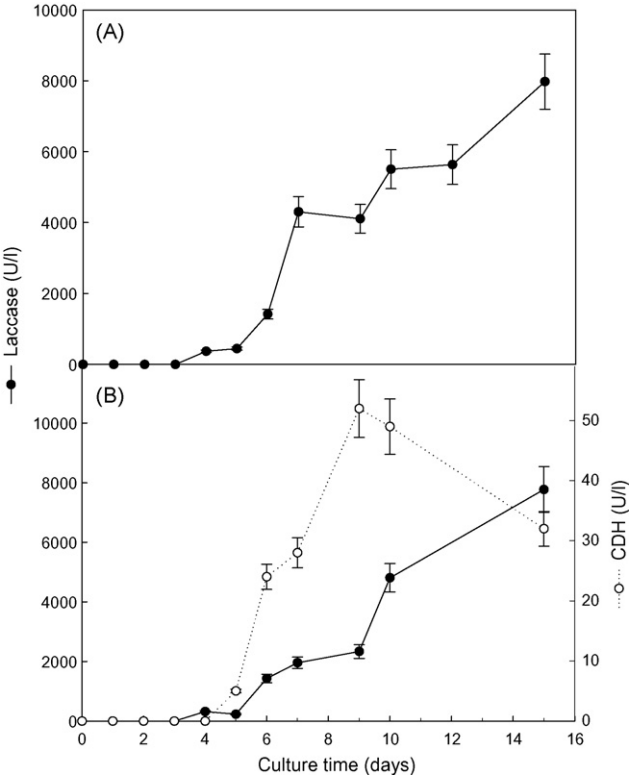


Fig. 1. Laccase and CDH production by semi-solid-state cultures of *Trametes pubescens* grown on dyed sunflower seed shells: (A) glucose cultures; (B) cellulose cultures.

only when grown on media containing cellulose without glucose, as reported above. In this medium the fungus also secreted laccase. The decolourising action of extracellular crude extracts containing CDH (40 U/l) and laccase (500 U/l) was compared to that of extracts containing only laccase (500 U/l). Furthermore, since the action of CDH is associated to the oxidation of cellobiose, lactose, or similar carbohydrates, decolouration experiments with extracts containing CDH were performed in the absence or in the presence of 30 mM lactose. Ciullini et al. [15] found that this carbohydrate was essential to activate the CDH to the production of hydrogen peroxide which further generates, in a Fenton type reaction, hydroxyl radicals. Decolouration in the presence of the well-known redox mediator 1-hydroxybenzotriazole (HOBT) was also performed for comparison.

As shown in Fig. 2, laccase plus CDH plus lactose resulted in the total decolouration of the dye RBBR in 22 h. The differences between the different treatments are no significant ($p = 0.93$) for the whole period but from 18 h onwards they are slightly significant ($p = 0.03$). However, no significant differences between using laccase plus CDH and laccase plus CDH plus lactose ($p > 0.05$) were found. This might be due to the RBBR dye can act as an electron donor for CDH when there is no lactose in the medium. Our results differ from that obtained by Soares et al. [22], who found that a commercial laccase (Novo Nordisk, Denmark) was only able to decolourise the dye RBBR in the presence of redox mediators.

As for the dye MG, laccase plus CDH plus lactose showed the slowest decolouration rate but from 16 h onwards almost the same decolouration percentages than those obtained by the other treatments was reached (Fig. 3). Anyway, the differences among treatments were no significant ($p = 0.09$). Therefore, CDH is not exerting any effect on MG decolouration which might be due to CDH inactivation by some sub-products of MG oxidation by laccase.

Fig. 4 shows the decolouration obtained for the dye DV. The most efficient combination in decolourising the dye DV was laccase plus HOBT, which led to a decolouration of about 84% in only 1.5 h. The values found for the different treatments showed no significant differences during the first 15 min ($p = 0.82$) but from here onwards they were significant different ($p = 0.009$).

The decolouration obtained for the dye PX is shown in Fig. 5. The system laccase plus HOBT was the most effective one. Thus, a decolouration percentage of around 80% in only 0.5 h was attained. However, from here onwards the absorbance increased likely due to the formation of coloured degradation products. The values found for laccase, laccase plus CDH and laccase plus CDH plus lactose were not significantly different during the first 5 h ($p = 0.07$) but afterwards they were significant ($p = 0.01$), the latter showing the best results (80% in 18 h).

As shown in Fig. 6 the dye BB was scarcely decolourised. The system laccase plus HOBT gave the highest decolouration percentage

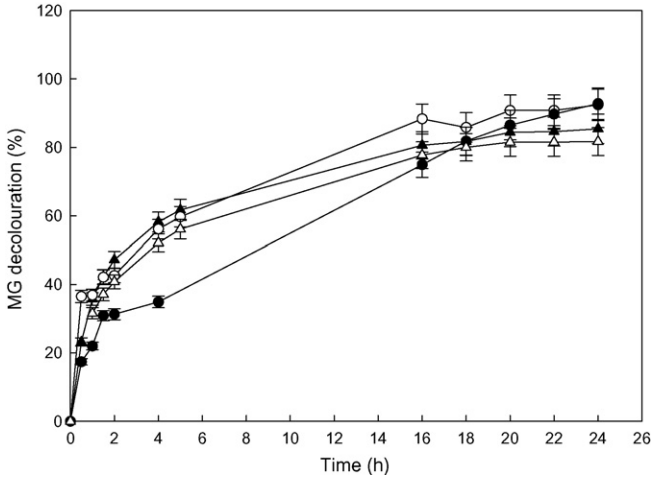


Fig. 3. Methyl Green decolouration by different laccase combinations: ▲ laccase; ○ laccase + CDH; ● laccase + CDH + lactose; △ laccase + HOBT.

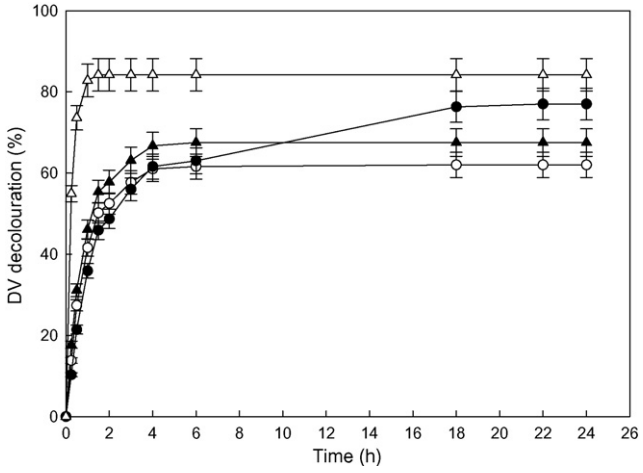


Fig. 4. Direct Violet 51 decolouration by different laccase combinations: ▲ laccase; ○ laccase + CDH; ● laccase + CDH + lactose; △ laccase + HOBT.

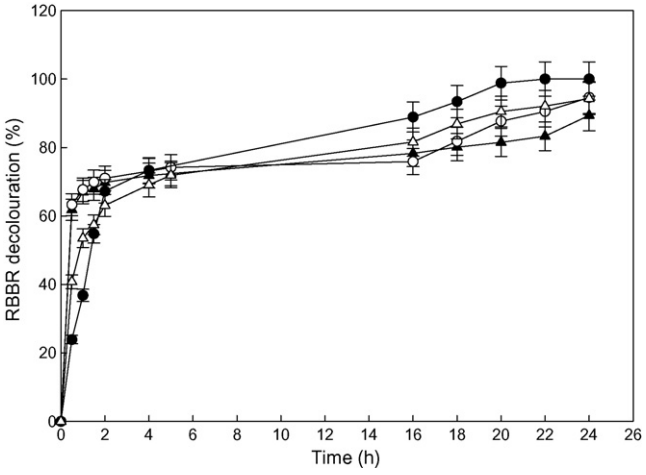


Fig. 2. Remazol Brilliant Blue R decolouration by different laccase combinations: ▲ laccase; ○ laccase + CDH; ● laccase + CDH + lactose; △ laccase + HOBT.

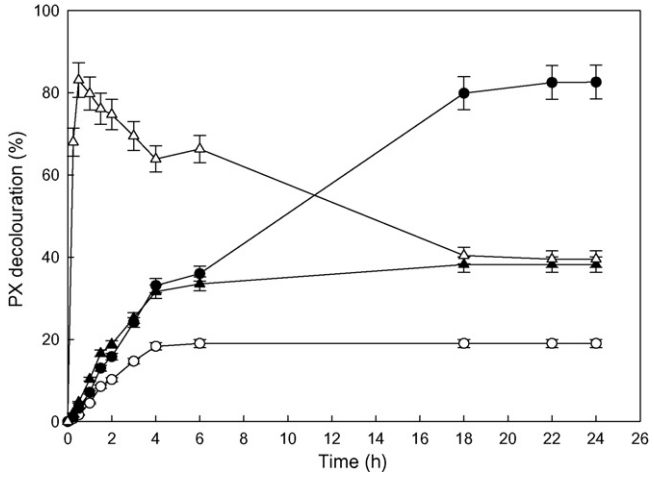


Fig. 5. Ponceau Xylidine decolouration by different laccase combinations: ▲ laccase; ○ laccase + CDH; ● laccase + CDH + lactose; △ laccase + HOBT.

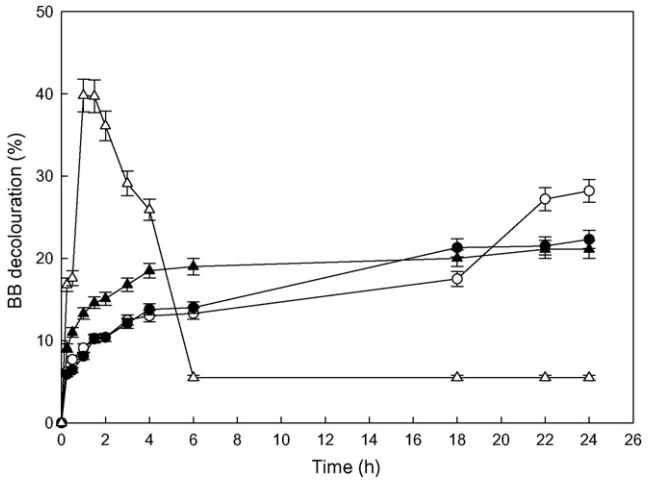


Fig. 6. Bismark Brown R decolouration by different laccase combinations: ▲ laccase; ○ laccase + CDH; ● laccase + CDH + lactose; △ laccase + HOBT.

(about 40% in 1 h) but from here onwards the absorbance increased as it occurred for the dye PX. This is likely due to the formation of coloured sub-products. Laccase, laccase plus CDH and laccase plus CDH plus lactose showed differences slightly significant ($p = 0.02$), being more marked at the end of the treatment ($p = 0.005$). Surprisingly, higher decolouration was obtained by laccase plus CDH than laccase plus CDH plus lactose. This might be due to the dye acts as an electron donor for CDH and when there is lactose in the medium both substrates are in competition for CDH lowering the reaction.

The polymeric dye PR was only decolourised in the presence of HOBT although to a very low extent, about 24% in 1 h (Fig. 7). In addition, thereafter decolouration hardly improved (Fig. 7), which could likely be due to the presence of some inhibiting sub-products generated in the dye degradation process.

The above results show that the individual dye structures influence the decolouration extent obtained by laccase, indicating the specificity of laccase towards different dye structures. The anthraquinonic and tryarylmethane dyes were easily decolourised by all the approaches used. Azo dyes, however, presented a different decolouration profile depending on both the dye structure and the approach used. In the case of azo dyes, it is known that the nature and position of the dye substituents strongly affect the decolouration extent [23]. Electron-withdrawing substituents were generally found to diminish reaction rates whereas electron-donating groups

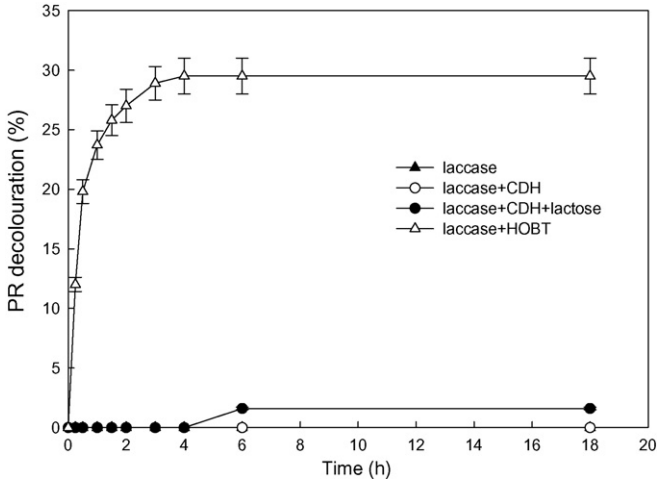


Fig. 7. Poly R-478 decolouration by different laccase combinations: ▲ laccase; ○ laccase + CDH; ● laccase + CDH + lactose; △ laccase + HOBT.

enhance the susceptibility of the dye towards oxidative attack by laccases [24]. In the present study, from the different azo dyes tested DV and PX were easily decolourised, especially by laccase plus HOBT and laccase plus CDH plus lactose. This might be due to the presence of a hydroxyl group in ortho position and the presence of soluble sulfonic groups. The resistance of BB to decolouration by laccase might be related to limited accessibility of the $-NH_2$ groups due to steric hindrance. The polymeric dye was the most recalcitrant to laccase decolouration due to its complex aromatic structure.

Although the dyes PX and BB were decolourised very quickly in the presence of HOBT the subsequent increase in absorbance (Figs. 5 and 6), likely due to the generation of coloured degradation products, make this system unsuitable. Thus, the use of CDH together with laccase could be a promising alternative to the use of the more expensive and less environmentally friendly chemical mediators such as HOBT. Anyway, much research remains to be done in order to find the optimal conditions for dye decolouration.

4. Conclusions

This study showed that CDH can be used instead of the redox mediator HOBT for the decolouration of some synthetic dyes by laccase enzymes. Therefore, the combination of laccase plus CDH could be an alternative in the treatment of dye-containing effluents to the expensive and poisonous chemical mediators. Anyway, more studies are required in order to optimise the decolouration process. Also, the high cost of the CDH enzyme discourages the application of this approach to an industrial scale. However, treatment with the whole organism could be a promising strategy. In addition, the heterologous expression of several CDHs in *Pichia pastoris* might open promising perspectives.

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