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Immobilized laccase for decolourization of Reactive Black 5 dyeing effluent

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Abstract

Reactive Black 5 industrial dyeing effluent was decolourized by free and immobilized laccase. The stability of the enzyme (194 h free and 79 h immobilized) depended on the dyeing liquor composition and the chemical structure of the dye. In the decolourization experiments with immobilized laccase, two phenomenons were observed – decolourization due to adsorption on the support (79%) and dye degradation due to the enzyme action (4%). Dyeing in the enzymatically recycled effluent provided consistency of the colour with both bright and dark dyes.

Introduction

Laccase (EC 1.10.3.2) is a multicopper oxidase which reduces O_2 to water and simultaneously performs one-electron oxidation of many aromatic substrates (Bourbonnais & Paice 1990). Laccases catalyze the removal of a hydrogen atom from the hydroxyl group of methoxysubstituted monophenols, *ortho*- and *para*-diphenols. They also oxidize other substrates such as aromatic amines, syringaldazine, and non-phenolic compounds to form free radicals (Robles *et al.* 2000). Laccases can degrade several dye structures (Abadulla *et al.* 2000) to transform toxic compounds into safer metabolites and may be useful to control environmental pollution (Gianfreda *et al.* 1999). About 90% of reactive textile dyes that enter sewage treatment plants pass through unchanged and are discharged to rivers (Pierce 1994). The traditional textile finishing industry consumes large amounts of water to process textile materials. The use of new closed-loop technologies such as the recycling of microbial or enzymatically treated dyeing effluents can decrease the water consumption (Spadaro *et al.* 1994). However, several limitations prevent the use of free enzymes for these applications. The stability and catalytic ability of free enzymes are dramatically decreased by highly polluted wastewaters. The use of immobilized enzymes can overcome some of these limitations and provide stable catalysts with long life times. In particular, immobilization by covalent coupling retains very high enzyme activity and is effective in removing phenolic compounds and colour over wide ranges of pH and temperature (Davis & Burns 1992, Rogalski *et al.* 1995).

The most realistic approach from which valuable information can be gained about the performance of the enzymes in industrial applications is to determine the enzyme half-life under process conditions. The objective of this work was to investigate the stability and decolourization efficiency of free and immobilized laccase in Reactive Black 5 industrial dye effluent and respective pure dye solution. The decolourization of the effluent would enable its reuse in dyeing processes providing water and energy savings in textile wet processing.

Materials and methods

Enzyme, dye and effluent

Trametes villosa laccase (EC 1.10.3.2), 6.8 g protein l^{-1}, supplied from Novo Nordisk, Denmark, was used for dye decolourization at pH 5 (0.1 M acetate buffer), 45 °C, as recommended by the provider. Reactive Black 5 (RB5) dye from Sigma (0.04 g l^{-1} in 0.1 M acetate buffer, pH 5) and the respective dyeing effluent (wavelength of maximum dye adsorption in both dyeing effluent and pure dye solution was 595 nm) were substrates for enzymatic decolourization. The composition of the RB5 dye-bath, from which the corresponding effluent was discharged, was 1 g RB5 l^{-1} and 30 g NaCl l^{-1}.

Laccase immobilization

Alumina (Al_2O_3) spherical pellets (3 mm diam.) from Sigma were silanized with 2.5% (v/v) γ-aminopropyltriethoxy silane (Sigma) in acetone at 45 °C for 24 h (Cho & Bailey 1979). The silanized carriers were washed with distilled water and treated with 2% (v/v) aqueous glutaraldehyde (Aldrich) for 2 h at room temperature, washed again and dried at 60 °C for 1 h. Ten g of support were immersed in 50 ml laccase preparation (0.8 g protein/l) in 0.1 M acetate buffer (pH 5), for 5 h at room temperature (Leonowicz *et al.* 1988, Costa *et al.* 2002). The amount of protein in the supernatant solution after immobilization was determined using the Bradford method. Bound protein was determined as a difference between initial and residual protein concentrations (immobilization yield ∼50%, 0.02 g of protein on the support).

Immobilized laccase stability in dyeing effluent

RB5 effluent sample and respective pure dye solution (100 ml) were adjusted to pH 5 and incubated with 10 g of alumina support with immobilized enzyme at 45 °C in a shaker bath (90 rpm). The support was previously saturated in a concentrated solution of RB5 (1 g l^{-1} in 0.1 M acetate buffer pH 5, for 1 h) in order to minimize the decolourization due to dye adsorption on the support.

The immobilized enzyme was removed at different times (1, 24, 48 and 72 h) and used to decolourize another solution of Reactive Blue 19 (RB19 from Sigma, 50 ml, 0.1 g l^{-1} in 0.1 M acetate buffer pH 5, 45 °C; wavelength of maximum dye adsorption is 595 nm) for 40 min. The percentage of RB19 decolourization as a function of the time was used to define the relative enzyme activity. The relative enzyme activity was plotted vs. time and from the derived exponential equation ($Y = Ai \times \exp(-k \times X)$; where Y = relative activity; X = time; Ai = initial activity; k = rate constant) the rate constant was provided. The half-life was calculated as ln2/k.

Free enzyme stability in dyeing effluent

RB5 dye solution and the effluent solution (100 ml) were adjusted to pH 5 and individually incubated with enzyme (0.2 g protein l^{-1}) in a shaker bath, at 45 °C. Sample (1 ml) were removed from the reaction mixture at 1, 24, 48 and 72 h, and used to decolourize RB19. The relative enzyme activity and the half-life were calculated as previously described.

Decolourization experiments

RB5 dye solution and effluent (100 ml, pH 5) were individually incubated with free enzyme (0.2 g protein l^{-1}) in a shaker bath (45 °C, for 24 h). The above experiment was repeated using 10 g alumina with immobilized enzyme (2 mg protein g^{-1} support). Samples of the reaction mixture were collected at different times to measure the dye absorbance and the percentage of effluent decolourization was calculated. In the case of free enzyme, samples were collected at 1, 2, 3, 4, 5, 24, 48, 72 and 140 h. Measurements of the decolourization with the immobilized enzyme were performed at 1, 2, 3, 4, 5 and 24 h, during 4 cycles.

Dye/protein/support interaction

BSA was immobilized on alumina support (∼ 2 mg protein g^{-1} support) saturated with RB5 (1 g l^{-1} for 1 h) to evaluate the effect of the support and protein adsorption in the decolourization process. Alumina support with and without immobilized BSA was incubated with RB5 pure dye solution and effluent (100 ml, pH 5, 45 °C) for 4 cycles of 24 h. Decolourization was measured at 1, 2, 3, 4, 5 and 24 h.

Re-dyeing experiments

Re-dyeing experiments using the enzymatically decolourized RB5 effluent were carried out in bright and dark colours, respectively – Reactive Orange 70 and Reactive Blue 214. The dyes were applied on

bleached cotton fabrics in two concentrations −0.25 g l^{-1} and 1.5 g l^{-1}, in the presence of 20 g Na_2CO_3 l^{-1} and 60 g Na_2SO_4 l^{-1}. The dyeing was performed in an Ahiba Spectradye dyeing apparatus (Datacolor) at 80 °C, for 1 h. Dyed fabrics were thoroughly washed afterwards by boiling to remove any unfixed dye. The colour differences (ΔE^*) on the fabrics dyed using enzymatically recycled effluent and fresh water were determined using a reflectance-measuring apparatus Spectraflash 600 (Datacolor), according to the CIELab colour difference concept at standard illuminant D65 (LAV/Spec. Excl., d/8, $D_{65}/10°$). We assumed a colour difference tolerance interval of one CIELab unit as acceptable.

Results and discussion

Laccase stability

The immobilized laccase had a higher stability than the free laccase in the buffer and salt solutions (Table 1). Comparatively, the stability of the free laccase in the effluent, containing both dye and salt, increased. The industrial dyeing effluent contains not only unfixed and hydrolyzed dyestuff (initially 1 g RB5 l^{-1}) but also NaCl (0.5 M). The ionic strength of the enzymatic solutions is one of the most important factors affecting the biocatalyst performance. The relatively high amounts of salt in the dyeing effluent enhance the electrostatic coupling of the anionic dyes and the positively charged proteins, thereby forming stable dye/enzyme aggregates. Various authors have reported enzyme stabilization above 0.5 M $(NH_4)_2SO_4$ and NaCl (Göller & Galinski 1999, Dötsch *et al.* 1995, Carpenter & Crowe 1988). Such stabilization occurred with both free and immobilized enzyme in the RB5 effluent compared to the RB5 pure solution and in the salt solution compared to the buffer (see Table 1).

In the presence of dye the stability of the immobilized enzyme unexpectedly decreased. The RB5 is a di-azo sulphonic dye (see Figure 1) that binds to enzyme molecules forming ion pairs between negatively charged sulphonic groups and positively charged protein groups. Anionic sulphonic dyes are known to protect the enzymes from inactivation (Matulis *et al.* 1999). Sulphonic dye stabilization was effective only on free laccase.

The sulphonate dye and the salt present in RB5 dyeing effluents probably exert a synergistic stabilization effect on free laccase.

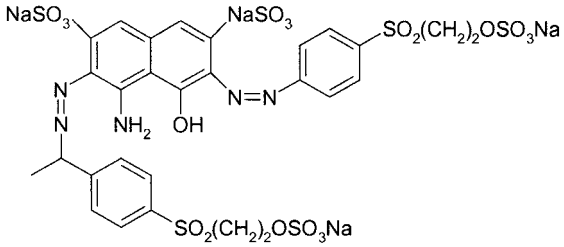

Fig. 1. Chemical structure of Reactive Black 5.

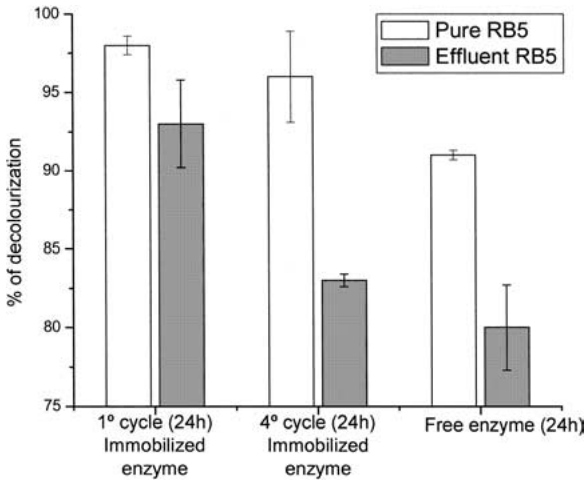

Fig. 2. Decolourization (%) of 100 ml Reactive Black 5 pure dye (0.04 g l^{-1}) and respective dyeing effluent with immobilized (10 g support, 0.002 g protein g^{-1} support) and free laccase (0.2 g protein l^{-1}) in 0.1 M acetate buffer pH 5, 45 °C, shaker bath (90 rpm), 4 decolourization cycles, 24 h each. Decolourization was followed spectrophotometrically at 595 nm.

Surprisingly, the immobilized enzyme showed lower stability than the free form in dyeing effluents. Normally the enzyme immobilization is expected to provide stabilization effect (Rogalski *et al.* 1995) restricting the protein unfolding process as a result of the introduction of random intra- and intermolecular crosslinks. The immobilization procedure has a variety of effects on protein conformation as well as on the state of ionization and dissociation of the enzyme and its environment (Emine & Leman 1995). The laccase structure became possibly less available after the immobilization for interaction with anionic dyes and thereby stabilization. The immobilization process, depending on the environment, might have a stabilization/destabilization effect on the enzyme.

Table 1. Half-life (h) of free (0.2 g protein l^{-1}) and immobilized laccase (10 g support, 0.002 g protein g^{-1} support) in 100 ml Reactive Black 5 pure solution (0.04 g l^{-1}) and respective dyeing effluent in 0.1 M acetate buffer pH 5, 45 °C, with shaking at 90 rpm.

	Half-life (h) ± S.D.	
	Immobilized laccase	Free laccase
Pure dye RB5 solution (0.04 g l^{-1})	57 ± 8	105 ± 16
Effluent RB5 solution (~0.04 g l^{-1})	79 ± 3	194 ± 38
Acetate buffer (0.1 M, pH5)	110 ± 26	85 ± 9
NaCl solution (30 g l^{-1})	148 ± 34	122 ± 19

Table 2. Colour differences (ΔE^*) on fabrics dyed (1 h, at 80 °C) in dye-baths (20 g Na_2CO_3 l^{-1}, 60 g Na_2SO_4 l^{-1} and 0.25 ÷ 1.5 g l^{-1} Reactive Orange 70 or Reactive Blue 214), prepared with laccase-recycled Reactive Black 5 dyeing effluent.

	Reactive Orange 70		Reactive Blue 214	
Dye concentration (g l^{-1})	0.25	1.5	0.25	1.5
ΔE^* ± S.D.	0.91 ± 0.02	0.34 ± 0.01	0.17 ± 0.03	0.13 ± 0.02

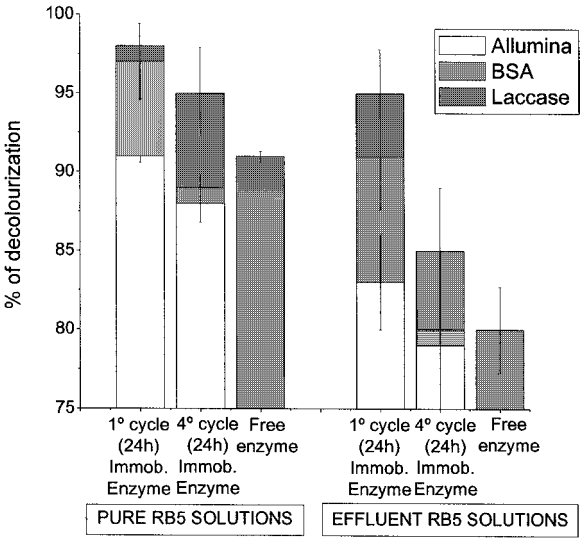

Fig. 3. Alumina (10 g), BSA (0.002 g protein g^{-1} support) and laccase (0.002 g protein g^{-1} support) contribution in the decolourization of 100 ml Reactive Black 5 pure solution (0.04 g l^{-1}) and dyeing effluent in 0.1 M acetate buffer pH 5, 45 °C, shaker bath (90 rpm), 4 decolourization cycles, 24 h each. Decolourization followed spectrophotometrically at 595 nm.

Decolourization of pure dyes and coloured effluents with free and immobilized laccase

The enzymatic decolourization of RB5 (~90%) took 24 h. This relatively slow decolourization can be explained by the hydrophilic nature of the RB5, which favors the equilibrium distribution in direction to the aqueous phase (Churchley *et al.* 2000). The decolourization of the pure dye solution was in all cases higher than for the effluent, with both free and immobilized laccase. The immobilized laccase, even after the 4th cycle of reuse, showed greater decolourization efficiency than the free enzyme (Figure 2). The greater decolourization performance of the immobilized enzyme in comparison to the free enzyme could be explained by a high dye adsorption on the alumina support. The difference in the decolourization capacity of immobilized laccase in pure and effluent solutions, from the first to the last cycle of utilization might be attributed to the presence of unfixed or hydrolyzed dyestuff and salt in the dyeing effluent.

Dye/protein/support interactions in decolourization

A series of experiments were carried out to evaluate the effect of the support/protein/dye interactions in the decolourization process. Alumina support, immobilized BSA and immobilized laccase were used for decolourization experiments in RB5 solutions. This will allow the effects of support adsorption, dye/protein interaction and enzymatic degradation of the dye to be quantified. In the first cycle of decolourization with immobilized laccase, the colour removal was mostly due to adsorption on the support and on the protein

(Figure 3). In the next cycles, partial saturation of the support occurred; the extra dye adsorption, due to the BSA protein decreased and the contribution of laccase increased. Even though the support was saturated with dye, further adsorption occurs and appears to be an important factor for decolourization. After 24 h it was still difficult to distinguish the laccase decolourization from the alumina adsorption of RB5. Decolourization due to adsorption on the support continues even after loss of the enzymatic activity. The decolourization with immobilized laccase is a complex process, which consists of concomitant dye-support adsorption, dye-protein adsorption and enzymatic dye degradation.

Dyeing using enzymatically recycled dyeing effluents

The dyeing in dye-baths prepared with laccase decolourized RB5 effluent, showed ΔE^* values for both dyes and dye concentrations, within the acceptable range of one unit. As might be expected dyeing in dark colour with decolourized dyeing liquor yielded slightly better results than dyeing in bright colour (Table 2).

Conclusions

Reactive Black 5 dye and salt present in the dyeing effluent stabilized free laccase. Immobilized laccase had a lower stability in dyeing liquors, this may be due to the enzyme structure being less accessible for interaction with salts and anionic dyes. Decolourization with immobilized laccase was due to adsorption on the support, and enzymatic degradation of the dye. Dyeing using an enzymatically decolourized RB5 effluent is comparable with conventional dyeing processes.

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