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Biodeinking of flexographic inks by fungal laccases using synthetic and natural mediators

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ABSTRACT

The use of new printing technologies based on flexographic inks hampers ink elimination during paper recycling, making necessary the development of alternative methods. Decolorization of four flexographic inks has been evaluated by using fungal laccases, three of them from basidiomycetes (*Trametes villosa*, *Coriolopsis rigida*, and *Pycnoporus coccineus*), and one from the ascomycete *Myceliophthora thermophila* in the presence of synthetic and natural mediators.

The results obtained showed a higher capacity of the three basidiomycete laccases to decolorize flexographic inks as compared with *M. thermophila* laccase, a low redox potential laccase. Basidiomycete laccases decolorized inks without mediators at long reaction times, although the presence of natural or synthetic mediators (above all HBT) accelerated the process. On the other hand, *M. thermophila* laccase was unable to decolorize the inks assayed. The addition of syringyl-type mediators led to medium levels of decolorization except for R48 ink, which was almost completely decolorized.

Most decolorization was obtained during the first hours of treatment, when all the basidiomycete laccases were fully active in the presence of mediators. As opposed to other basidiomycete laccases, which become inactive in the presence of HBT after 24 h, the enzyme of *P. coccineus* was not deactivated by this mediator even after 48 h. A complete loss of *M. thermophila* laccase activity was observed at short times with acetosyringone and methyl syringate, the only two mediators able to promote ink decolorization with this enzyme.

1. Introduction

Recycled paper industry has experienced a great development in the last years due to its economical and environmental advantages. Ink elimination, the major difficulty in paper production, is usually performed by flotation because of its low water consumption. Its efficiency depends on factors like ink and fiber properties, printing process, and coating compounds. Although flotation is efficient in offset printed paper deinking, the use of new printing technologies, as flexographic inks, present recycling problems. Flexographic inks are made up of pigments, resin, solvent, and additives. Ink/fiber detachment in these inks is easy, because ink can be dispersed at alkaline pH due to carboxylate group's formation, and then pigment particles are liberated. Nevertheless, the flotation process is difficult in these conditions because acrylates act as dispersants and pigment particles are small and exhibit hydrophilic character. This behavior causes problems in black waters clarification and also produces ink particles redeposition in fibers.

Laccases are multicopper oxidases that catalyze the oxidation of phenolic compounds, aromatic amines, and other compounds by abstraction of one electron, using oxygen as electron acceptor. The oxidative process can subsequently progress through non-enzymatic reactions. The driving force of the enzymatic mechanism lies on the difference between the redox potential of the reducing substrate and that of the copper T1 site (where the reducing substrate is oxidized) that varies between +0.5 and +0.8 V. However, the study of these enzymes and their applications has increased during the last years, because they are able to oxidize aromatic compounds with high-redox potential (above +1.4 V) in the presence of redox mediator compounds [1]. Such recalcitrant substrates include a great variety of dyes, with a wide range of chemical structures, lignin, polycyclic aromatic hydrocarbons (PAHs), and other non-phenolic aromatic compounds that produce environmental problems [2–4]. Many efforts are being carried out to find the ideal mediators, from synthetic or natural sources. These compounds must be oxidized by laccase producing cyclic reactions and developing stable intermediates and reduced forms, which must not affect the enzyme activity, and in addition they should have a

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reasonable price to be used in industrial applications [5,6]. A large number of studies have been reported with synthetic mediators, as 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) [1], and N-hydroxy compounds as 1-hydroxybenzotriazole (HBT), violuric acid, and N-hydroxyacetanilide [7], as well as with natural compounds derived from lignin degradation, as acetosyringone or syringaldehyde, that can be obtained as by-products of the pulp industry or from industrial effluents [8,9]. Some fungal metabolites, as 3-hydroxyanthranilate produced by *Pycnoporus cinnabarinus* [10], were also tested as mediators.

Among laccases, high-redox potential laccases (HRPLs) produced by basidiomycete fungi ($E^\circ \geq 0.70$ V) are of great biotechnological interest due to their higher oxidative capabilities, including the oxidation of mediator compounds with high-redox potentials [11,12]. On the other hand, laccases for industrial applications need of robust expression systems to provide big amounts of enzyme, as well as to allow modulating their redox potential and catalytic properties through protein engineering. This would serve to produce tailor-made enzymes designed to degrade/transform recalcitrant compounds causing environmental problems. The commercially available laccase from the ascomycete *Myceliophthora thermophila* has lower redox potential ($E^\circ = +0.48$ V) [13] than those from basidiomycete laccases, as that from *Trametes villosa* ($E^\circ = +0.78$ V) [11]. However, *M. thermophila* can be heterologously expressed in industrial hosts with high yields [14], while the difficult heterologous expression of HRPLs [15] limits their large-scale commercialization.

In an attempt to contribute for the searching of alternative methods for deinking flexographic paper, we assayed the decolorization of flexographic inks (Blue 1, Red 48:4, Violet 3, and Flexiprint Magenta HX-E) by fungal laccases. *M. thermophila* laccase was compared with the HRPLs from *T. villosa*, *Coriolopsis rigida* and *Pycnoporus coccineus* alone or in the presence of synthetic (HBT and ABTS) or natural mediators (syringaldehyde, acetosyringone, methyl syringate, and *p*-coumaric acid).

2. Materials and methods

2.1. Inks, laccases, and mediators

The flexographic inks, Blue 1 (B1), Red 48:4 (R48), Violet 3 (V3), and Flexiprint Magenta HX-E (MG) (Table 1), were supplied by Quimovil SA and Flint Group Iberia SA (Spain).

Laccases from *T. villosa* (Novozym 51002) and *M. thermophila* (Novozym 51003) were supplied by Novozymes® (Denmark), and crude extracts containing laccases from *C. rigida* and *P. coccineus* were produced in a basal medium with glucose supplemented with copper and copper plus ethanol, respectively, as previously reported [16,17]. Peroxidase activity was not detected in these extracts.

Laccase activity was determined at 25 °C by measuring the oxidation of 5 mM 2,6-dimethoxyphenol (DMP)(Merck) to coerulignone ($\varepsilon_{469} = 27{,}500\ M^{-1}\ cm^{-1}$, referred to substrate concentration) in 100 mM sodium tartrate pH 4, for all basidiomycete laccases, and 100 mM sodium phosphate buffer pH 6 for *M. thermophila* laccase. These pH values were chosen on the basis of the stability of each enzyme. One unit of laccase was defined as the amount of enzyme oxidizing 1 μmol of substrate/min.

Synthetic mediators used in enzymatic treatments were 1-hydroxybenzotriazole (HBT, Fluka), and 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS, Roche), and natural mediators were syringaldehyde (Aldrich), acetosyringone (Aldrich), *p*-coumaric acid (Aldrich), and methyl syringate (Novozymes®).

2.2. Biodeinking treatments

Biodeinking treatments were performed in a total volume of 5 mL. Ink concentration was 0.1% (v/v) in B1 and MG treatments and 0.03% in V3 and R48. The treatments were carried out by using 5 U/mL of laccase (determined using DMP as substrate) and 500 μM of the different mediators in 50 mM sodium tartrate buffer, pH 4, except for the *M. thermophila* laccase that was performed in 50 mM sodium phosphate buffer, pH 6. Tween 80 at 0.01% was added in all cases and MG treatment contained in addition 4% ethanol to obtain a homogeneous and stable suspension. Enzymatic treatments were maintained in an orbital shaker at room temperature and 280 rpm. Each treatment was carried out twice and controls, without enzyme and mediator, were treated in the same conditions.

2.3. Ink and effluent properties

Ink samples were dissolved in 75% dimethyl sulfoxide (DMSO; Merck) (v/v), stirred 1 h at 40 °C and 800 rpm, and the degradation/transformation of ink components analyzed by changes in the absorption spectra between 400 and 750 nm at different incubation times in a Shimazdu UV-1800 spectrophotometer. Decolorization in samples was followed by absorbance changes at those wavelengths where maximum absorbance was observed, which depend on ink and pH (Table 1).

3. Results and discussion

3.1. Ink decolorization

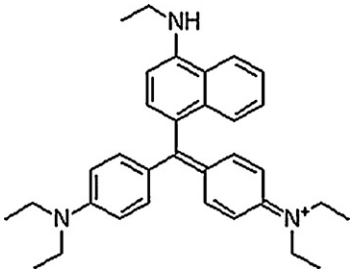
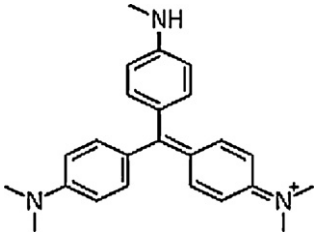
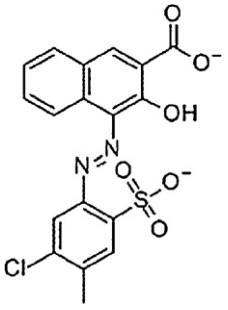
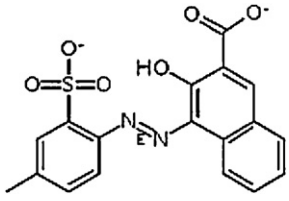
Ink decolorization process by using basidiomycete laccases was carried out at pH 4 since the enzymes were sufficiently stable at this pH, at least for 24 h at room temperature, and their maximum activity levels were found between pH 3.5 and 4.5 (using DMP as substrate). When using *M. thermophila* laccase the treatments were carried out at pH 6, since the enzyme is more stable and works efficiently at this pH, which agrees with data previously reported [18].

Due to the inks complexity, composed of different compounds as mentioned above, comparison of enzymatic efficiency in these treatments is a difficult task. Flexographic inks are not soluble in aqueous systems, forming a stable suspension whose absorbance cannot be measured. Several solvents, as methanol, ethanol, and acetonitrile were assayed to solubilize ink suspensions, being DMSO the most adequate since it gave a transparent solution with the four inks. Different ink concentrations in each treatment were chosen to avoid the saturation of the absorbance range of the spectrophotometer.

Ink decolorization led in general to an overall depletion of absorbance in the visible spectra (Fig. 1a). However, in some cases, a color change was observed and this is reflected in the corresponding spectrum by a shift in the maximum absorbance (Fig. 1b). This color change would be most probably due to the oxidation of different groups in ink pigment. Some mediators (as syringaldehyde, acetosyringone, and above all ABTS), once oxidized by laccase, give colored products that could interfere in the decolorization measurements. Fortunately, this color disappears after DMSO addition, thus allowing the pigment quantification without interferences. During the decolorization process an increase in turbidity was observed. It could be probably due to the effect of other ink compounds, like resins and additives, or to the formation of polymeric bioconversion products in the reaction.

In general, higher ink decolorization yields were found by using basidiomycete laccases than that obtained with *M. thermophila* laccase (Figs. 2 and 3), most probably due to their higher redox

Table 1
Characteristics of the flexographic inks used in this work.

Commercial ink and pigment characteristics	Pigment chemical classification	Pigment formulation	Maximum absorbance (nm) in DMSO	
			pH 4	pH 6
Blue 1 (B1) CAS 1325-87-7 CI[a] 42595:2	Triarylmethane pigment Phosphotungstomolybdic acid salt		616	620
Violet 3:1 (V3) CAS 68647-35-8 CI[a] 42535:4	Triarylmethane pigment Silicomolybdic acid salt		592	590
Red 48:4 (R48) CAS 5280-66-0 CI[a] 15865:4	Monoazo (chlorine substituted) on manganese base		492	480
Flexiprint Magenta HX-E (MG) CAS 5281-04-9 CI[a] 15850:1	Monoazo on calcium base		512	490

[a] Color index.

potentials [13]. *C. rigida* laccase almost completely decolorized inks without mediators at long treatment times, although the presence of natural or synthetic mediators accelerated the process. The effect was especially noticeable during the first four hours of treatment, reaching in most cases the maximum decolorization values. Inks based on triarylmethane pigments (B1 and V3) showed a similar deinking pattern. Laccase was able to decolorize up to 40–50% after 24 h of treatment (Fig. 4), although the maximum decolorization was reached using HBT after 2 h of treatment. None of the mediators assayed (synthetic or naturals) reached similar values to those found with HBT after 48 h. On the other hand, the other inks based on monoazo pigments (MG and R48), showed different decolorization patterns. MG ink was much more easily oxidized with all the mediators used (except using *p*-coumaric acid). By contrast only about 60% decolorization was reached in R48, using HBT and ABTS. This ink was the most recalcitrant, probably due to the presence of a –Cl group, which is an electron withdrawing radical that can negatively affect substrate oxidation. Ink decolorization patterns with laccases from *T. villosa* and *P. coccineus*, were very similar to that produced by *C. rigida* enzyme. The decolorization yields found in the present work comparing the use of HBT and natural mediators in treatments with high-redox potential laccases, agree with previous reports on the efficiency of these mediators for pulp biobleaching applications [5,8,19], but contrast with those obtained for enzymatic decolorization of synthetic organic dyes [20].

In the case of treatments with *M. thermophila* laccase (Fig. 3), the enzyme was unable to decolorize any of the inks assayed without mediators, suggesting a high-redox potential for these inks.

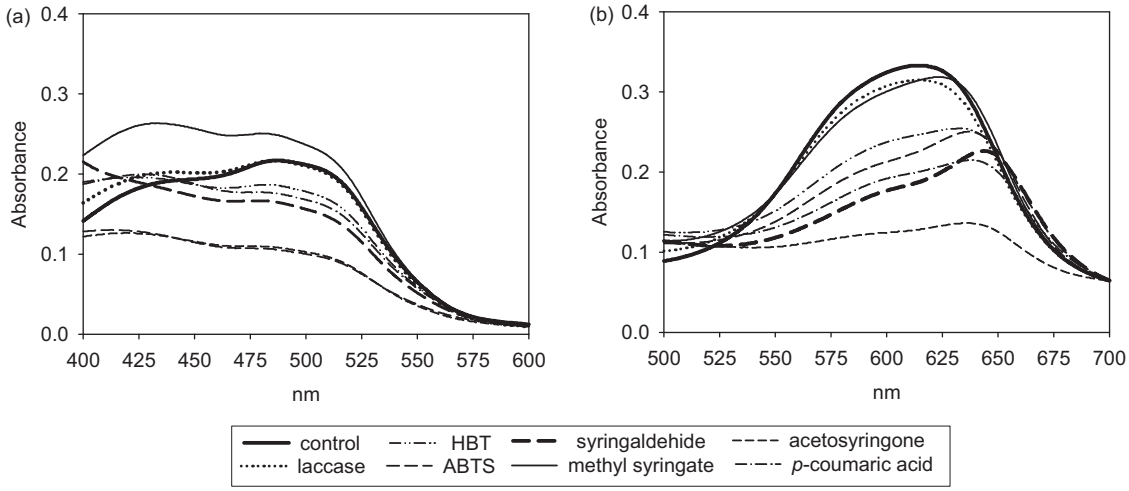


Fig. 1. Changes in the absorbance spectrums of R48 (a) and B1 (b) inks after treatments with *C. rigida* laccase, in the presence or absence of different mediators, for 20 min at pH 4.

However, in the presence of phenolic mediators such as acetosyringone and methyl syringate, R48 ink was almost completely decolorized and in the other inks only around 40–60% decolorization was attained. The other natural mediators, *p*-coumaric acid and syringaldehyde, as well as the synthetic mediators, did not show any effect on deinking, except syringaldehyde, in of R48 and MG inks (40% decolorization), both with azo pigments.

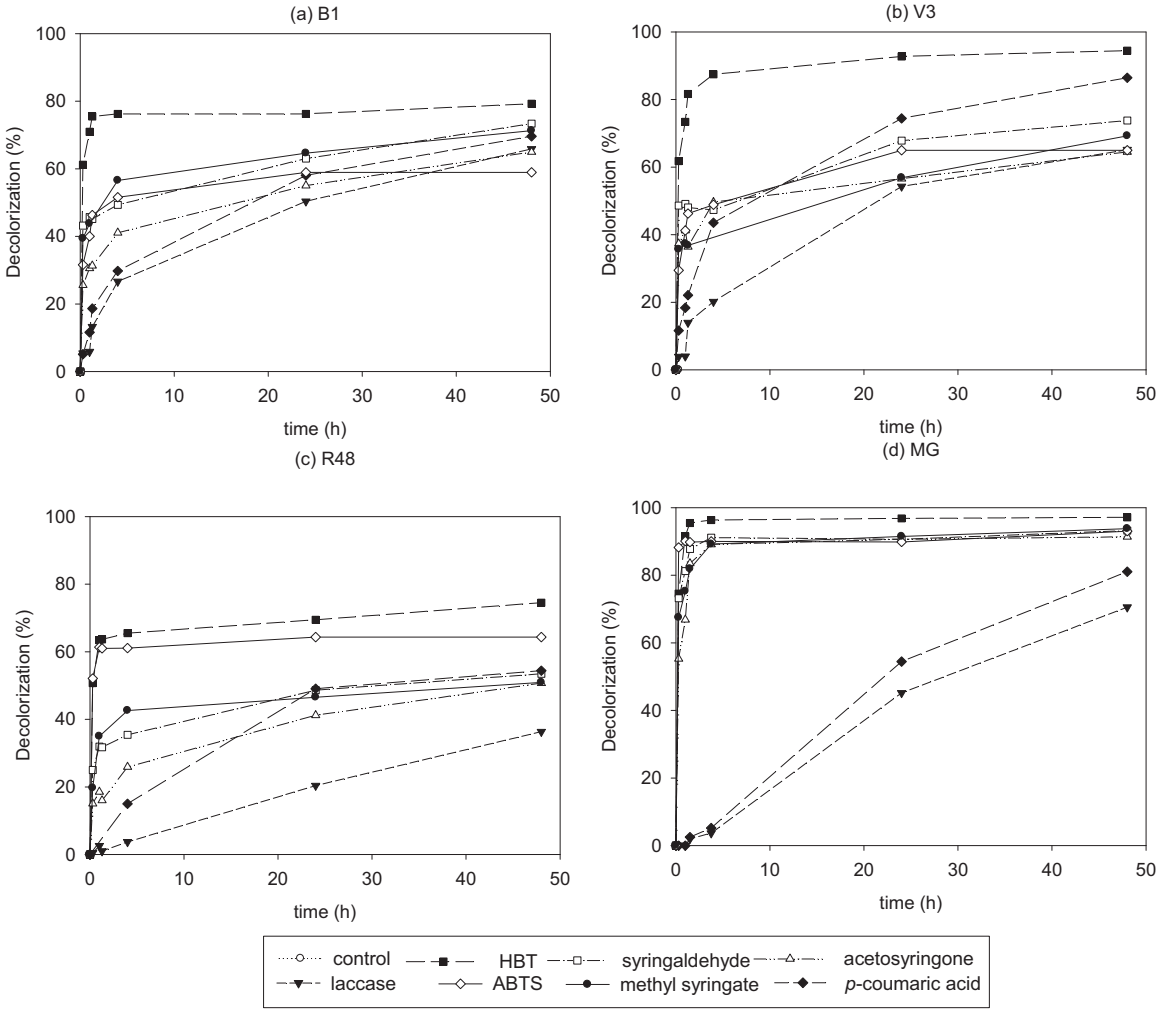


Fig. 2. Decolorization (%) of four flexographic inks after treatments with *C. rigida* laccase, in the presence or absence of different mediators, for 48 h at pH 4. The values represented are the mean of duplicates (±10%). Similar patterns were obtained with laccases from *T. villosa* and *P. coccineus*.

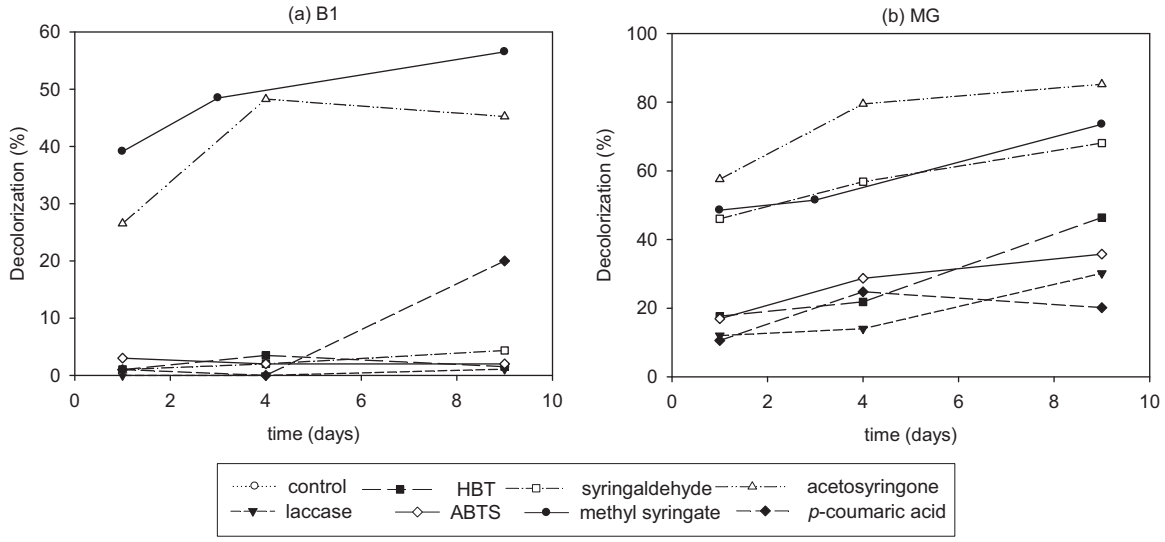


Fig. 3. Decolorization (%) of two flexographic inks after treatments with *M. thermophila* laccase, in the presence or absence of different mediators, for nine days at pH 6. The values represented are the mean of duplicates (±10%).

The degradation efficiency of the laccase–mediator system is expected to depend on several factors. The redox potential of the enzyme has to be enough to initiate the mediator oxidation, generating new oxidative species which should show affinity towards the substrate. Radicals and/or oxidation products formed from HBT were the most reactive towards the chromophoric groups of the pigments. The lack of ink decolorization with *M. thermophila* laccase and HBT is explained because the enzyme is unable to oxidize this mediator [18]. However, this laccase oxidizes ABTS to ABTS$^{\bullet+}$, as observed in this study (data not shown) and in accordance with previous reports [21]. Thus, how can be explained the absence of decolorization with the *M. thermophila* laccase–ABTS system while HRPLs–ABTS decolorize all the inks treated? The explanation would be related to the different oxidized species of ABTS formed by the different laccases.

As previously reported [22], the oxidation of ABTS by laccase occurs in successive steps. A quite stable cation radical (ABTS$^{\bullet+}$), is initially produced by one-electron oxidation of ABTS, being subsequently oxidized to other more oxidative by-products. Nevertheless, this radical is a weak oxidant (redox potential of 0.69 V vs. NHE) unable to react with recalcitrant substrates of high-redox potential [22]. So, the lack of decolorization with *M. thermophila* laccase suggests that this enzyme cannot perform the oxidation of ABTS beyond the cation radical, thus hindering the generation of more reactive species that would be involved in the ink decolorization by basidiomycete laccases–ABTS.

On the other hand, the natural mediators assayed, except *p*-coumaric acid, improve ink decoloration by using the four laccases, suggesting that these compounds can be oxidized by both high- and low-redox potential enzymes and act as mediators once oxidized by the enzyme. Basidiomycete laccases are able to oxidize *p*-coumaric acid, but this mediator decolorates the inks at rates slower than those showed by other natural mediators. The phenolic natural mediators follow a hydrogen transfer mechanism, like HBT, for oxidizing the target substrates [22]. The syringyl-type compounds (acetosyringone, methyl syringate, and syringaldehyde), with two methoxyl substituents in the ring, are oxidized by laccase faster than *p*-coumaric acid, indicating that methoxyl groups play a more important role as electron donors than the double bond of the lateral chain of *p*-coumaric acid [23].

The successful use of syringyl-type phenolics as mediators of *M. thermophila* laccase for delignification [19] and pitch removal of paper pulps [14] has been recently reported. An effective decolorization of inkjet inks (decolorized by *Trametes versicolor* laccase without mediator, but not by *M. thermophila* laccase) has been attained by using the ascomycete laccase in the presence of acetosyringone [24]. However, it is difficult to reach the efficiency of high-redox potential basidiomycete laccases such as those used here. Their oxidative capabilities allow not only the direct oxidation of recalcitrant aromatic compounds, but also the complete oxidation of high-redox potential mediators (HBT, ABTS, or *p*-coumaric acid) which significantly increase the oxidation of target substrates like the flexographic inks tested here.

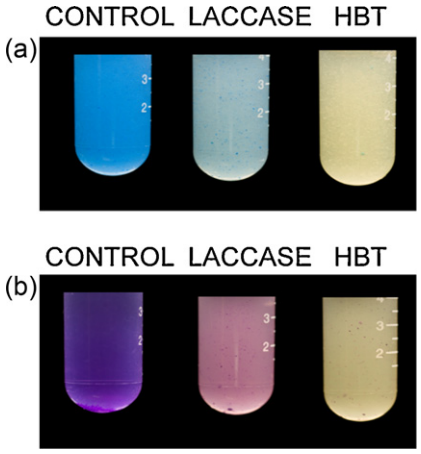


Fig. 4. Color changes observed after treatments of B1 (a) and V3 (b) inks with *C. rigida* laccase, in the presence or absence of HBT, for 24 h at pH 4 in aqueous media. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.2. Effect of mediators on laccase stability

The effect of mediators on laccase stability is shown in Fig. 5. Although basidiomycete laccases were stable at room temperature at short times (100% activity after 4 h), a slow activity decrease was observed with time, remaining 70% at 48 h even in the presence of most mediators. It has been reported that the presence of natural mediators does not inactivate *P. cinnabarinus* laccase [5].

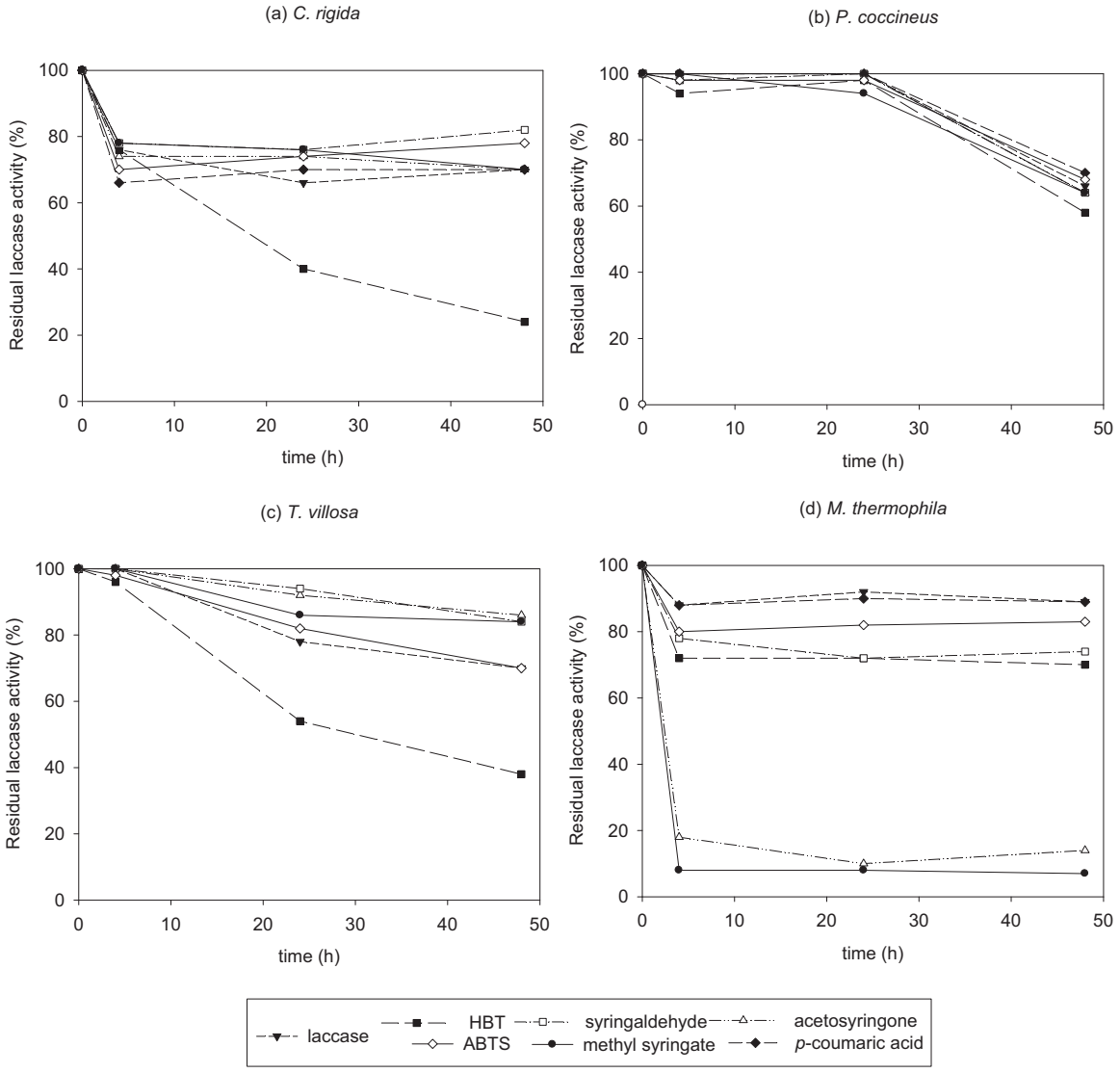


Fig. 5. Residual laccase activity (%) in the absence of ink, with or without mediators, during 48 h of reaction. Values are the mean of duplicates (±10%).

As an exception, addition of HBT caused around 50% of activity loss in laccases from *T. villosa* and *C. rigida* at long times (more than 24 h). Anyway, ink addition into the reaction did not affect enzymatic activity and our data indicate that most decolorization was obtained during the first 4 h of treatment, when all the assayed basidiomycete laccases were fully active. Regarding *P. coccineus* enzyme, similar activities were found between 4 and 48 h with and without mediators, suggesting that this laccase was the most stable in the presence of radicals from oxidized HBT. This could be of interest in several industrial applications where laccase recovery is an important factor.

Different results were reported by using *P. cinnabarinus* laccase at 50 °C with HBT and *p*-coumaric acid, since these mediators led to enzyme inactivation at very short times (1–2 h), although the presence of pulp during biobleaching protected enzymatic activity from HBT radicals [5,18]. Our findings suggest that basidiomycete laccases are more stable at room temperature in the presence of HBT, since enzymes inactivation occurs at longer reaction times.

On the other hand, *M. thermophila* laccase was very stable (Fig. 5d), but a complete activity loss was observed after 4 h in the presence of acetosyringone and methyl syringate. Only these two laccase–mediator systems were able to decolorize all the assayed inks to different extents. To the best of our knowledge, this is the first report on *M. thermophila* laccase inactivation by natural mediators. It was also observed that in the presence of ink, *M. thermophila* laccase activity with these two mediators was around 50–60% during the first 4 h of treatment, being completely lost after 24 h (Table 2). These results agree with previous reports suggesting that the presence of a substrate protects laccase activity, which otherwise would be quickly lost due to the radicals generated by the laccase–mediator system [5,18].

Table 2
Residual *M. thermophila* laccase activity (%) after 4, 24, and 48 h with or without mediators, in treatments of R48 ink. Laccase (LAC) and laccase in the presence of syringaldehyde (SA), acetosyringone (AS), *p*-coumaric acid (PCA), and methyl syringate (MS). Values are the mean of duplicates (±10%).

Time (h)	LAC	+HBT	+AS	+SA	+PCA	+ABTS	+MS
4	100	96	48	94	100	92	60
24	92	76	14	90	98	64	0
48	64	64	6	80	86	54	0

4. Conclusion

Flexographic paper cannot be deinked with the flotation methods currently used in recycled paper production. In this study we

demonstrate for the first time the feasible decolorization of flexographic inks by using fungal laccases. Basidiomycete HRPLs were able to decolorize these inks to some extent, although the addition of mediators improved the action of the enzyme at very short times. In general, the use of HBT produced the highest decolorization values. The *thermophila* laccase was not able to oxidize the assayed inks alone, but the presence of syringyl-type natural mediators overcame the limitations of this low-redox potential laccase to achieve the decolorization of flexographic inks.

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References

[1] R. Bourbonnais, M.G. Paice, Oxidation of non-phenolic substrates: an expanded role for laccase in lignin biodegradation, FEBS Lett. 267 (1990) 99–102.
[2] A.I. Cañas, M. Alcalde, F. Plou, M.J. Martínez, A.T. Martínez, S. Camarero, Transformation of polycyclic aromatic hydrocarbons by laccase is strongly enhanced by phenolic compounds present in soil, Environ. Sci. Technol. 41 (2007) 2964–2971.
[3] M. Alcalde, M. Ferrer, F.J. Plou, A. Ballesteros, Environmental biocatalysis: from remediation with enzymes to novel green processes, Trends Biotechnol. 24 (2006) 281–287.
[4] D. Wesenberg, I. Kyriakides, S.N. Agathos, White-rot fungi and their enzymes for the treatment of industrial dye effluents, Biotechnol. Adv. 22 (2003) 161–187.
[5] A. Fillat, J.F. Colom, T. Vidal, A new approach to the biobleaching of flax pulp with laccase using natural mediators, Bioresour. Technol. 101 (2010) 4104–4110.
[6] A. Cañas, S. Camarero, Laccases and their natural mediators: biotechnological tools for sustainable eco-friendly processes, Biotechnol. Adv. 28 (2010) 694–705.
[7] F. Xu, J.J. Kulys, K. Duke, K. Li, K. Krikstopaitis, H.-J.W. Deussen, E. Abbate, V. Galinyte, P. Scheneider, Redox chemistry in laccase-catalyzed oxidation of N-hydroxy compounds, Appl. Environ. Microbiol. 66 (2000) 2052–2056.
[8] S. Camarero, D. Ibarra, Á.T. Martínez, J. Romero, A. Gutiérrez, J.C. del Río, Paper pulp delignification using laccase and natural mediators, Enzyme Microb. Technol. 40 (2007) 1264–1271.
[9] F. Ismail, D.A. Mulholland, J.J. Marsh, An analysis of the water soluble components of Sappi Saiccor's effluent streams, Water SA 31 (2005) 569–574.
[10] C. Eggert, U. Temp, J.F.D. Dean, K.E.L. Eriksson, A fungal metabolite mediates degradation of non-phenolic lignin structures and synthetic lignin by laccase, FEBS Lett. 391 (1996) 144–148.
[11] O. Morozova, G. Shumakovich, M. Gorbacheva, S. Shleev, A. Yaropolov, Blue laccases, Biochemistry (Moscow) 72 (2007) 1136–1150.
[12] C. Galli, P. Gentili, Chemical messengers: mediated oxidations with the enzyme laccase, J. Phys. Org. Chem. 17 (2004) 973–977.
[13] A. Svendsen, F. Xu, *Myceliophthora thermophila* laccase variants, 09/732350 US 7622287 B2, USA, 2009.
[14] E.D. Babot, A. Rico, J. Rencoret, L. Kalum, H. Lund, J. Romero, J.C. del Río, A.T. Martínez, A. Gutiérrez, Towards industrially-feasible delignification and pitch removal by treating paper pulp with *Myceliophthora thermophila* laccase and a phenolic mediator, Bioresour. Technol. 102 (2011) 6717–6722.
[15] A. Kunamneni, I. Ghazi, S. Camarero, A. Ballesteros, F.J. Plou, M. Alcalde, Decolorization of synthetic dyes by laccase immobilized on epoxy-activated carriers, Process Biochem. 43 (2008) 169–178.
[16] A. Jaouani, F. Guillén, M.J. Penninckx, A.T. Martínez, M.J. Martínez, Role of *Pycnoporus coccineus* laccase in the degradation of aromatic compounds in olive oil mill wastewater, Enzyme Microb. Technol. 36 (2005) 478–486.
[17] M.C.N. Saparrat, F. Guillén, A.M. Arambarri, A.T. Martínez, M.J. Martínez, Induction, isolation, and characterization of two laccases from the white-rot basidiomycete *Coriolopsis rigida*, Appl. Environ. Microbiol. 68 (2002) 1534–1540.
[18] D. Ibarra, J. Romero, M.J. Martínez, J. Martínez, S. Camarero, Exploring the enzymatic parameters for optimal delignification of eucalypt pulp by laccase–mediator, Enzyme Microb. Technol. 39 (2006) 1319–1327.
[19] A. Fillat, M.B. Roncero, T. Vidal, Assessing the use of xylanase and laccases in biobleaching stages of a TCF sequence for flax pulp, J. Chem. Technol. Biotechnol. 86 (12) (2011) 1501–1507, http://dx.doi.org/10.1002/jctb.2662.
[20] S. Camarero, D. Ibarra, M.J. Martínez, A.T. Martínez, Lignin-derived compounds as efficient laccase mediators for decolorization of different types of recalcitrant dyes, Appl. Environ. Microbiol. 71 (2005) 1775–1784.
[21] M. Alcalde, T. Bulter, Colorimetric assays for screening laccases, in: F.H. Arnold, G. Georgiou (Eds.), Directed Enzyme Evolution: Screening and Selection Methods, vol. 230, Humana Press, Totowa, NJ, 2003, pp. 193–201.
[22] F. d'Acunzo, C. Galli, First evidence of catalytic mediation by phenolic compounds in the laccase-induced oxidation of lignin models, Eur. J. Biochem. 270 (2003) 3634–3640.
[23] A. Cañas, Diseño de nuevos sistemas lacasa-mediador: empleo de mediadores de origen natural y mejora de lacasas por evolución dirigida, Ph.D. Dissertation, Universidad de Alcalá, Departamento de Microbiología y Parasitología.
[24] K. Nyman, T. Hakala, Decolorization of inkjet ink and deinking of inkjet-printed paper with laccase–mediator system, Bioresources 6 (2011) 1336–1350.