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Journal of Molecular Catalysis B: Enzymatic 41 (2006) 61–69
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
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In search for practical advantages from the immobilisation of an enzyme: the case of laccase

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Received 20 December 2005; received in revised form 14 March 2006; accepted 20 April 2006
Available online 24 May 2006

Abstract

This study investigated the efficiency of four different immobilised laccase preparations in the mediator-assisted oxidation of a non-phenolic lignin model compound. To this aim, *Trametes villosa* laccase was either covalently bound onto Eupergit® C or activated carbon, or entrapped within copper or calcium alginate. The benchmark reaction, consisting in the side-chain oxidation of 4-methoxybenzyl alcohol (4-MBA) to 4-methoxybenzaldehyde, was taken as the criterion to compare the efficiency of the immobilised laccases, and to assess the possible advantages of their use in alternative to the native enzyme. For each laccase preparation, four mediators (including HBT, HPI, VLA and TEMPO) were comparatively investigated. Comparisons were made by using the same amount of activity (i.e. 10 U) for each laccase preparation, and showed that the native enzyme generally led to higher *p*-anisaldehyde yields than the immobilised laccases. The only exception was observed with Cu-alginate-laccase, which led to a 85% conversion of 4-MBA in the presence of HBT as the mediator. When testing the reusability of this immobilised system, a significant catalytic efficiency was maintained along three consecutive reaction cycles.

Keywords: Laccase; Immobilised enzymes; Mediators; Selective oxidation; Enzyme recycle

1. Introduction

The multi-copper oxidase laccase (EC 1.10.3.2, *para*-benzenediol:oxygen oxidoreductase) is an ubiquitous enzyme among basidiomycetes [1,2], and its direct involvement in the biological degradation of lignin has been reported [3–5]. The low substrate specificity of laccase, associated with its good intrinsic stability properties, has prompted interest for application in biobleaching [6,7], wastewater treatment [8], cathode fuel cells [9] and biosensors [10].

Phenols and anilines are typical substrates for the monoelectronic oxidation by laccase, in view of the appropriate values of redox potential. However, the use of low molecular weight compounds, often referred to as mediators, has been shown to enable laccase to oxidise even non-phenolic substrates indirectly [1,11,12], through the intervention of the oxidised form of the mediator as the reactive intermediate [13] (Scheme 1).

The oxidation of bulky and insoluble non-phenolic derivatives, or of the structurally complex lignin polymer, however, may require the addition of co-solvents where the activity of laccase is substantially depressed [14,15]. Moreover, any biotechnological use of laccase requires time-consuming procedures for the separation of the end-products from the enzyme.

In principle, immobilisation of laccase on appropriate polymeric supports may provide advantages with respect to these drawbacks [16–20]. In fact, immobilisation can protect laccase from denaturation by organic co-solvents, thereby extending its half-life, and allows the reuse of the enzyme in several reaction cycles [16,20]. An additional advantage due to immobilisation is the ease of separation of reaction products from the heterogeneous catalyst. Such advantages have been reported for several immobilised laccase preparations [21]. However, most of studies have been focused on the use of immobilised laccases in the oxidation of phenolic compounds, anilines and some dyes [16,18,20].

The present study is aimed at exploring the prospective advantages arising from laccase immobilisation in the mediator-

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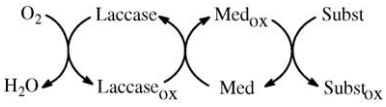
doi:10.1016/j.molcatb.2006.04.012



Scheme 1. The oxidation cycle of a laccase/mediator system.

assisted oxidation of non-phenolic compounds, and at assessing in this respect any possible superiority over the native enzyme. In fact, the biotechnological implications of the laccase/mediator concept are extremely relevant [11–13,15]. To this aim, four different immobilised laccases were prepared, their reactivity evaluated and compared towards a benchmark reaction, consisting in the mediator-assisted oxidation of the non-phenolic lignin model compound 4-methoxybenzyl alcohol (4-MBA). This benchmark reaction, which previously served to rank the efficiency of various laccase mediators [22,23], was used in the present study to assess the efficacy of differently immobilised laccases, and to compare them with the native enzyme. In the current investigation, laccase was either covalently bound onto Eupergit® C or activated carbon, or else entrapped within copper or calcium alginate (ionotropic trapping). These selected supports were previously shown to result in a significant stabilization of laccase from different sources, and allowed the use of the catalyst in both fixed and fluidised bed reactors [16,20,24]. Because the exposure of enzyme molecules to the reaction solution varies substantially in these immobilized preparations, results obtained in this study might have a quite general significance.

2. Materials and methods

2.1. Materials

All the substrates, mediators, salts and solvents were commercially available from either Carlo Erba or Aldrich.

2.2. Enzyme purification

Crude laccase from *Trametes villosa* (viz. *Polyporus pinsitus*) was a kind gift of Novo Nordisk Biotech. The enzyme was purified by anion-exchange chromatography on Q-Sepharose Fast Flow as previously reported [23] The preparation had an absorption ratio A_{280}/A_{610} of 20–30 and its activity, determined by the 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) spectrophotometric assay [25], was 6000 U/mL. One unit (U) is defined as the amount of enzyme producing 1 μmol of product per min under the assay conditions. The method of Lowry was employed for the determination of the concentration of laccase in the purified sample [26].

2.3. Immobilisation of laccase on activated carbon (AC)

Laccase was immobilised on AC by slightly modifying the procedure described by Davis and Burns [24]. In particular, 2.5 g of activated carbon (Darco® 20–40 mesh, Aldrich) were aminopropylated with 12.5 mL of a 2% (v/v) solution of aminopropyltriethoxysilane (APTES) in acetone under stirring for 12 h at 15 °C. Excess of APTES was removed by centrifugation and washing with 0.1 M phosphate buffer at pH 7.0 (buffer A). The pellet was then suspended in 12.5 mL of buffer A, containing 5% of glutaraldehyde, and stirred for 1 h. Excess of glutaraldehyde was removed by three cycles of centrifugation/washing with buffer A. The pellet was then suspended in 15 mL of buffer A, added with 6000 U of purified laccase, and stirred for 24 h at 5 °C. Non-bound enzyme was removed by centrifugation/washing cycles with buffer A, until no more activity could be detected in the washings. The activity of this preparation, determined by spectrophotometric assay with ABTS, was 115 U/g of dry support.

2.4. Immobilisation of laccase on Eupergit® C

Laccase was immobilised on the epoxy-activated polyacrylic support Eupergit® C (Rohm Pharma Polymers, Weiterstadt D) as already reported [16]. In particular, 2.5 g of support were suspended in 10 mL 1.0 M phosphate buffer pH 7.0 (buffer B) and added with 4000 U of laccase. The reaction mixture was then incubated at 5 °C for 48 h under orbital shaking (70 rpm). At the end of the incubation, the support was recovered by centrifugation (5000 × *g*, 10 min) and the non-covalently bound enzyme removed by centrifugation/washing cycles with buffer B, until no more activity was detected in the washings. The activity of this preparation was 623 U/g of dry support.

2.5. Immobilisation of laccase on alginate (Ca or Cu)

Ionotropic trapping of laccase in the 'egg-box' structure of either Ca- or Cu-alginate has been carried out as described elsewhere [19,20]. In particular, a sonicated 3% solution of sodium alginate (low viscosity alginic acid from *Macrocystis pyrifera*, Sigma) was added with 4000 U of laccase and loaded in a syringe fitted with a luer-lock needle (Gauge 18, Aldrich). The solution was extruded drop by drop from the syringe into a 0.15 M solution of $CaCl_2$ (or else of $CuSO_4$) in a 5 mM acetate buffer pH 5.0 (buffer C) under magnetic stirring. The formed spherules were filtered, taken up in fresh buffer C, and kept at 4 °C for 4 h (hardening phase). The spherules, ca. 3–4 mm in diameter, were finally washed with distilled water. To test the activity of this preparation, a known amount of alginate spherules was dissolved in phosphate buffer (pH 6.5) and subsequently assayed for activity with ABTS. The activity of laccase immobilised within copper- and calcium alginate was 2600 and 1700 U/g of dry support, respectively.

2.6. Determination of kinetic constants

For kinetic studies, enzymatic activity was determined by measuring the oxygen uptake rate with a SA 520 Clark oxygen electrode (Orion Instruments, Boston MA) connected with a LKB 481 single-channel potentiometric recorder. The reaction mixture (10 mL) containing variable concentrations (generally from 0.5 to 35 mM) of the tested mediator in 0.05 M citrate buffer pH 5.0 was equilibrated at 25 °C in the electrode chamber,

then the reaction was initiated by adding appropriate amounts of either native or immobilised laccase. Activity towards N–OH compounds was calculated from the oxygen uptake rate by assuming a stoichiometric ratio of 4 moles of oxidized mediator per mole of oxygen consumed [27]. Maximum reaction velocity (V_{max}), apparent K_m and specificity constant (V_{max}/K_m) were calculated (Table 3) by non-linear regression according to the Michaelis–Menten relationship. To this aim, the Enzfitter software (Elsevier Biosoft, Cambridge) was used.

2.7. Enzymatic oxidation

The oxidation reactions were performed under magnetic stirring in 3 mL 0.1 M citrate buffer pH 5; the citrate buffer was purged with O_2 for 30 min prior to the addition of the reagents. The concentrations of the reaction components were: [substrate] 20 mM; [mediator] 6 mM and 10 U of either native or immobilised *T. villosa* laccase. Incubations were carried out at room temperature for 24 h under oxygen (filled latex balloon). After a conventional work-up with ethyl acetate (containing the internal standard), the molar amount of oxidation product was determined by GC analysis with respect to an internal standard (acetophenone or 4-methoxy-acetophenone), suitable response factors being determined from authentic compounds; the yields were reckoned with respect to the molar amount of the substrate (Table 2). A VARIAN 3400 Star instrument, fitted with a 30 m × 0.25 mm methyl silicone gum capillary column, was employed in the GC analyses. The identity of the product was confirmed by GC–MS, run on a HP 5892 GC, equipped with a 30 m × 0.25 mm methyl silicone gum capillary column, and coupled to a HP 5972 MSD instrument, operating at 70 eV.

To determine residual activity at the end of the incubation, immobilised catalysts were recovered from the reaction mixture by either filtration (as for alginate-immobilised preparations) or centrifugation (5000 × *g*, 5 min), and subsequently washed with 0.05 M citrate buffer pH 5.0 (buffer C). On the other hand, the native enzyme was recovered by passing incubation mixtures through PD 10 columns packed with Sephadex G-25 (Amersham Pharmacia, Uppsala Sweden) and pre-equilibrated with buffer C. Residual laccase activity was determined with the Clark electrode using a saturating concentration of ABTS (5.0 and 50 mM for free and immobilised laccase, respectively) dissolved in buffer C.

3. Results and discussion

3.1. Benchmark oxidation of 4-methoxy-benzyl alcohol

The aerobic oxidation of the non-phenolic compound 4-MBA (Fig. 1) was performed at room temperature for 24 h with

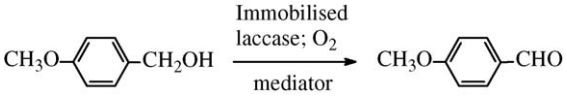


Fig. 1. Aerobic conversion of 4-methoxybenzyl alcohol (4-MBA) into 4-methoxybenzaldehyde by laccase in mediated oxidations.

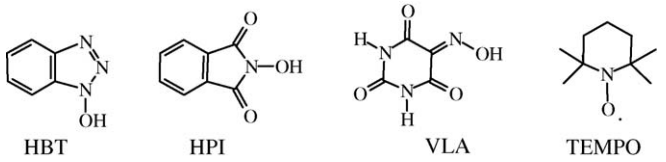


Fig. 2. Chemical structures of mediator compounds employed in the present study. HBT, 1-hydroxybenzotriazole; HPI, *N*-hydroxyphthalimide; VLA, violuric acid; TEMPO, 2,2,5,5-tetramethyl-4-piperidin-1-oxyl radical.

each one of the four immobilised laccases in combination with four different mediators, the structures of which are shown in Fig. 2.

Mediators 1-hydroxybenzotriazole (HBT), *N*-hydroxyphthalimide (HPI) and violuric acid (VLA), were representative cases of the ⟩N–OH mediators that, once oxidised by laccase, give rise to the aminoxyl radical form (⟩N–O•) as the reactive intermediate (Med_{ox}; cf. Scheme 1) [13,23]. The aminoxyl radical abstracts a benzylic hydrogen from the non-phenolic substrate, giving the aldehyde as the final end-product through a radical oxidation mechanism. Mediator TEMPO, instead, is known to be oxidatively converted by laccase into the oxoammonium form (⟩N=O+) which, in turn, oxidises alcohols very efficiently into carbonyl products by a polar mechanism [23]. The efficiency of these mediators in the oxidation of 4-MBA under catalysis by the immobilised laccases was investigated. Table 1 summarizes immobilisation yields [28] and catalytic capabilities, i.e. activity referred to support dry weight, of immobilised laccases employed in this study.

To facilitate comparison among the four immobilised systems, and to avoid ambiguity in the assessment of results, oxidation reactions were performed by using the same amount of activity (i.e. 10 U, determined by using ABTS as the substrate). Yields of *p*-anisaldehyde are reported in Table 2, and compared with those obtained by using the same amount of activity of native *T. villosa* laccase. The incubation time was limited to 6 h in the reactions mediated by TEMPO, which is the most efficient mediator in the oxidation of benzyl alcohols [22,23]. This was done in order to avoid the attainment of uniformly high yields that would impede a clear differentiation in terms of reactivity among the immobilised laccases.

No other oxidation products besides *p*-anisaldehyde were detected, the residual mass balance being made by the recovered *p*-anisyl alcohol. Table 2 shows that none of the immobilised sys-

Table 1
Immobilisation yield and catalytic capability of *Trametes villosa* laccase either covalently immobilised onto activated carbon (AC) and Eupergit® C or entrapped within Cu- and Ca-alginate

Immobilisation support	Immobilisation yield (%)[a]	Catalytic capability (U/mg of dry support)
AC	4.8	0.10
Eupergit® C	39	0.66
Cu-alginate	65	2.6
Ca-alginate	43	1.7

[a] Calculated according to [28].

Table 2
Yields (%) of *p*-anisaldehyde in the aerobic oxidation of *p*-anisyl alcohol with the supported laccases and the four mediators[a]

Immobilisation support	Yield (%)[b]			
	HBT	HPI	VLA	TEMPO[c]
None[d]	76 ± 3	70 ± 2	65 ± 2	90 ± 4
Cu-alginate	85 ± 3	11 ± 0.5	26 ± 1	71 ± 3
Ca-alginate	51 ± 2	24 ± 1	44 ± 2	62 ± 2
Eupergit® C	84 ± 3	55 ± 2	6.9 ± 0.3	71 ± 3
AC	74 ± 3	42 ± 2	48 ± 2	51 ± 2

[a] Conditions: [substrate] 20 mM, [mediator] 6 mM, immobilised laccase 3 U/mL, in 3 mL buffered water solution (0.1 M citrate buffer, pH 5) saturated with O_2. Reaction time: 24 h at room temperature under O_2 (filled latex balloon).
[b] GC yields were calculated vs. the molar amount of substrate.
[c] Reaction time, 6 h.
[d] Purified *Trametes villosa* laccase as the reference case [22].

tems led to significantly higher *p*-anisaldehyde yields than those obtained by using the same amount of activity of the native enzyme. The only exceptions were observed for Cu-alginate- and Eupergit® C-immobilised laccases, which provided slightly better results upon mediation by HBT. By contrast, in HPI- and VLA-mediated oxidations the performances of the immobilised systems were notably lower than those of the free enzyme, this effect being very relevant for the alginate-immobilised systems. Results shown in Table 2 point out that no major advantages arose from laccase immobilisation, regardless of the support and/or the technique employed. Once again, the benchmark reaction showed its merits by enabling to unambiguously single out advantages or disadvantages from the various combinations of mediator and catalyst.

Our results might reflect the ability with which each laccase preparation led to the formation of the oxidized mediator species responsible for 4-MBA oxidation (Med_{ox}, in Scheme 1). In this respect, these findings might be partially explained by either diffusional limitations of the mediator within the porous structure of the immobilisation supports (as for alginates), or conformational restrictions arising from multi-point covalent attachment of the enzyme onto the support. To assess this hypothesis, the affinity constants of the immobilised systems for each one of the mediators under study were determined and compared with those of the free enzyme.

Table 3 shows that apparent K_m values of the immobilised enzymes were invariably higher than those of the free enzyme, with the only exception of the affinity constant for HBT of laccases immobilised on AC and Ca-alginate. Apparent K_m values for HBT of those immobilised enzymes which provided highest *p*-anisaldehyde yields (in Table 2), i.e. laccase immobilised within Cu-alginate and onto Eupergit® C, were about 5.5- and 1.8-fold higher than those of the free counterpart. Similarly, Eupergit® C-laccase, the use of which resulted in remarkably higher *p*-anisaldehyde yields than Ca-alginate-laccase (55% versus 24%, respectively; Table 2) in HPI-mediated reactions, exhibited a significantly lower affinity for HPI than Ca-alginate-laccase (39 mM versus 7.9 mM, respectively; Table 3). These findings suggest that the affinity of the immobilised enzyme for the mediator is not a sufficient basis to account for the different performances observed among immobilised enzymes

Table 3
Kinetic constants of *Trametes villosa* laccase (TvL) in solution or immobilised onto several supports for mediators *N*-hydroxyphtalimide (HPI), violuric acid (VLA), 2,2,5,5-tetramethyl-4-piperidin-1-oxyl radical (TEMPO) and 1-hydroxybenzotriazole (HBT)

Mediator/support	K_m (mM)	V_{max} (U (g support)$^{-1}$)	V_{max}/K_m (min^{-1} (g support)$^{-1}$)
HPI/native TvL	5.4 ± 0.8	0.34 ± 0.02[a]	0.06[b]
HPI/Ca-alginate	7.9 ± 1.2	0.4 ± 0.02	0.051
HPI/Cu-alginate	15.4 ± 1.4	2.9 ± 0.3	0.188
HPI/Eupergit® C	39.5 ± 3.2	0.4 ± 0.03	0.010
HPI/AC	7.1 ± 0.4	0.2 ± 0.02	0.028
VLA/native TvL	5.2 ± 0.6	6.84 ± 0.4[a]	1.31[b]
VLA/Ca-alginate	6.4 ± 0.7	0.8 ± 0.06	0.125
VLA/Cu-alginate	16.0 ± 0.1	1.8 ± 0.2	0.112
VLA/Eupergit® C	21.5 ± 2.3	4.0 ± 0.2	0.186
VLA/AC	6.4 ± 0.9	1.2 ± 0.3	0.187
TEMPO/native TvL	3.0 ± 0.2	9.14 ± 1.3[a]	3.04[b]
TEMPO/Ca-alginate	4.6 ± 0.3	0.4 ± 0.03	0.086
TEMPO/Cu-alginate	7.2 ± 0.6	3.3 ± 0.1	0.458
TEMPO/Eupergit® C	3.9 ± 0.3	11.8 ± 1.6	3.025
TEMPO/AC	3.2 ± 0.5	4.3 ± 0.3	1.34
HBT/native TvL	15.0 ± 1.0	1.08 ± 0.06[a]	0.07[b]
HBT/Ca-alginate	11.2 ± 0.3	1.2 ± 0.07	0.107
HBT/Cu-alginate	82.2 ± 4.5	2.8 ± 0.2	0.034
HBT/Eupergit® C	26.4 ± 1.4	1.6 ± 0.3	0.060
HBT/AC	11.0 ± 0.7	0.6 ± 0.04	0.054

Experiments were performed in duplicate and data are the mean ± standard deviation.
[a] V_{max} of reactions catalyzed by native TvL is expressed in U (mg protein)$^{-1}$.
[b] V_{max}/K_m ratios for reactions conducted with native TvL are expressed in min^{-1} (mg protein)$^{-1}$.

in the benchmark reaction (Table 2). Regardless of the laccase preparation, *p*-anisaldehyde yields obtained in the benchmark reaction were not simply correlated with V_{max} values determined for each one of the mediators. This was expected, since apparent Michaelis–Menten constants for most of laccase/mediator combinations were generally higher than the mediator's concentration (i.e. 6.0 mM) used in the benchmark reaction. This implies that oxidative reactions were carried out at a concentration of the mediator which was far from being saturating.

The restrictions imposed by immobilisation might also be potentially beneficial for the retention of laccase activity in mediated reactions, due to the protective effect exerted by the support itself. In fact, it should be borne in mind that radical species from mediator compounds can undergo chemical reactions with aromatic side-chains of laccase, thereby inactivating the enzyme [29–31]. For example, it has been suggested that the aminoxyl radical from HPI, known as PINO, might lead to a degradation of the polypeptide backbone due to its ability to oxidise amides [32]. From previous studies [23], the stability of the aminoxyl radical from HPI and VLA is known to be higher than that of the aminoxyl radical from HBT, this feature possibly enabling the former two reactive species to diffuse and cause stronger damage to the enzyme during the incubation time. Alternatively, reaction of the oxidised mediator with the polymeric support employed could lead to undesired side processes; for example, oxidation of the alcoholic groups in alginates by TEMPO cannot be ruled out [33].

To deal with this aspect, residual activity was determined at the end of 24 h incubation in reaction mixtures containing 20 mM 4-MBA and 6 mM of each one of the tested mediators. Fig. 3 shows the extent of activity retention for both the free enzyme and immobilised systems.

The lowest residual activity was observed in VLA-mediated reactions, in agreement with the reported harmful interaction of this mediator with laccases from several sources [29]. In fact, a nearly total loss of activity was observed for the free enzyme, while immobilisation led to a lower extent of protection in VLA-mediated reactions than that observed in the presence of HBT (Fig. 3). In HBT-mediated reactions, the free enzyme only retained about 2% of the initial activity, whereas laccase immobilised within Cu-alginate and on Eupergit® C retained 39 and 58% of the initial activity, respectively. In HPI- and TEMPO-mediated reactions, the highest retention of activity was observed for immobilisation onto Eupergit® C. In particular, laccase immobilized onto this support exhibited the highest retention of activity in the presence of the majority of mediators, with the only exception of VLA. Fig. 3 also shows that the native enzyme retained a higher amount of activity in HPI- than in HBT-mediated reactions. Overall, the results do not provide a clear-cut correlation between half-life of the radical species of the mediator and extent of activity retention, and lend only moderate support to the idea of the immobilisation as a way of protection of the enzyme.

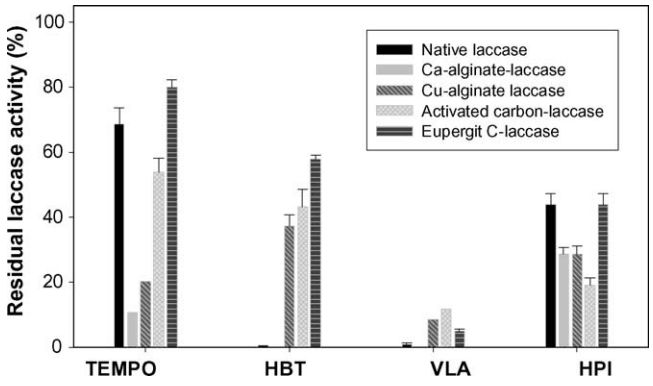


Fig. 3. Residual activity retained by laccase after 24 h incubation in 0.1 M citrate buffer pH 5.0 added with 4% CH_3CN (v/v), containing 20 mM 4-MBA, in the presence of 6 mM of each one of the following mediators: TEMPO, HBT, VLA and HPI.

Copper-alginate proved to be a better support than calcium-alginate for laccase immobilisation, a higher residual activity being generally maintained within the former support than with the latter (Fig. 3). This confirms the adequacy of Cu^{2+} ions as gelifying agents for the entrapment of fungal laccase [20]. Contrasting evidence is reported in the literature about the effect that some cations may have over the activity of laccase. Either stimulation or slight inhibition upon addition of Cu^{2+} ions are reported for laccases of different sources [34,35]. Because ionotropic trapping into the egg-box structure of alginate leads to the exposure of laccase molecules to the presence of either Cu^{2+} or Ca^{2+} ions, it appeared sensible to test for any effect of the two cations over the activity of the native enzyme. To this aim, $Cu(OAc)_2$ and $Ca(OAc)_2$ were selected, since acetate, unlike other counter-ions such as the halides, does not affect laccase activity [36]. Initial rate measurements in the oxidation of ABTS, performed by using [laccase]:[cation] molar ratios of 1:1, 1:10 and 1:1000, showed that laccase activity was not affected at all by both Ca^{2+} and Cu^{2+} (data not shown). To further support these data, the benchmark oxidation of 4-MBA by native laccase was investigated by using a [laccase]:[cation] molar ratio 1:10, that can be considered as a realistic ratio within the alginate-based immobilized systems. Results confirmed that the performances of the enzyme in mediated reactions was not generally affected by the presence of the two cations, with the exception of VLA and TEMPO-mediated reactions in the presence of Ca^{2+} (Table 4). Consequently, it might be concluded that the presence of the cations in the egg-box structure of alginate did not substantially influence the reactivity of the supported enzyme with respect to the free counterpart, apart from minor effects specifically exerted on single mediators. Therefore, the diversity of *p*-anisaldehyde yields observed by using laccase immobilized on either Ca- or Cu-alginate (Table 2) were not likely due to the presence of Cu^{2+} or Ca^{2+} cations.

Additional factors which might affect the oxidation yields in a laccase/mediator system are: (i) the initial substrate/mediator ratio [37], and (ii) the intrinsic stability of the oxidized mediator species throughout the reaction time. With regard to the latter factor, the highly unstable N–O$^{\bullet}$ intermediate [23], generated from HBT by laccase-catalyzed oxidation, might decay into secondary products, including benzotriazole, unable to feed

Table 4
Effect of the concentration of two cations upon the reactivity of native laccase with four mediators, evaluated from the yield of *p*-anisaldehyde in the benchmark oxidation of 4-MBA

Additive	Yield (%)[a]			
	HBT	HPI	VLA	TEMPO[b]
None[c]	76 ± 3	70 ± 2	65 ± 2	90 ± 4
$Cu(OAc)_2$[d]	77 ± 3	62 ± 2	66 ± 1	92 ± 3
$Ca(OAc)_2$[d]	77 ± 1	66 ± 2	53 ± 2	80 ± 3

[a] Conditions: [substrate] 20 mM, [mediator] 6 mM, 3 U/mL, in 3 mL buffered water solution (0.1 M citrate buffer, pH 5) saturated with O_2. The concentration of the enzyme was 1×10^{-9} M. Reaction time: 24 h at room temperature under O_2 (filled latex balloon). GC yields were calculated vs. the molar amount of substrate.
[b] Reaction time: 6 h.
[c] Data from Table 2.
[d] The concentration of the salt was 1×10^{-8} M.

the redox cycle [38]. However, these two factors were not dealt with in the present study since they are not tightly associated with the form in which the enzyme is employed, be it either native or immobilised.

3.2. Recycling the supported enzyme

The possibility of reuse of the catalyst is one of the most relevant advantages arising from immobilisation. To investigate this aspect, experiments were conducted by selecting the combination of mediator and immobilised catalyst which led to the highest *p*-anisaldeyde yield, i.e. HBT with laccase supported on Cu-alginate (in Table 2). An additional criterion for the selection of Cu-alginate-immobilised laccase was the ease of its recovery from the reaction mixture, due to the large dimensions of the spherules. At the end of each oxidation cycle (24 h), the spherules of Cu-alginate were recovered by filtration, washed with citrate buffer and exposed to a new oxidation cycle with fresh aliquots of substrate and mediator. The procedure was repeated five times, the production of *p*-anisaldehyde being determined for each oxidation step by GC (Table 5). The same experiments were also performed with laccase immobilised on Ca-alginate, for comparison.

Table 5
Yields (%) of *p*-anisaldehyde from repetitive oxidations with the same sample of immobilised enzyme; in each cycle, fresh aliquots of *p*-anisyl alcohol and mediator are added

Batch number	Yield (%)	
	Laccase on Cu-alginate	Laccase on Ca-alginate
I cycle	85 ± 3	51 ± 2
II cycle	66 ± 3	2 ± 0.1
III cycle	74 ± 3[a]	1 ± 0.05
IV cycle	27 ± 1	–
V cycle	10 ± 0.5	–

Experimental conditions as in Table 2.
[a] A modest amount of *p*-anisaldehyde was possibly a residual from the previous cycle.

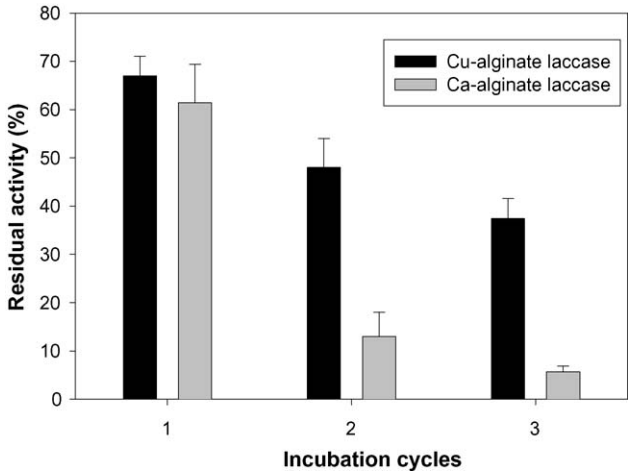


Fig. 4. Percent activity retained by *T. villosa* laccase immobilized within Ca-alginate and Cu-alginate along three consecutive incubation cycles of 24 h in 0.05 M citrate buffer pH 5.0 added with 4% (v/v) CH_3CN. Data are the mean of two replicates, and error bars indicate standard deviation of data.

Table 5 shows that the enzyme entrapped within Cu-alginate maintained a good catalytic efficiency over three oxidation cycles. By contrast, a dramatic loss of efficiency of laccase entrapped within Ca-alginate was observed after the first reaction cycle.

To assess whether these results might be ascribed to a different extent of activity retention throughout consecutive cycles, the two immobilized systems were incubated for 24 h in the reaction buffer (i.e. 0.1 M citrate buffer at pH 5.0) in the absence of both substrate and mediator, recovered and subsequently assayed for their residual activity versus ABTS at the end of each incubation cycle. Fig. 4 shows that laccase immobilized within Cu- and Ca-alginate retained a similar amount of activity at the end of the first incubation cycle. However, residual activity dropped dramatically in the latter immobilized system along successive incubation cycles.

Therefore, the lower reusability of Ca-alginate- than Cu-alginate-laccase might likely depend on the higher porosity and lower chemical stability of the former support, leading to a higher extent of enzyme leakage. As a matter of fact, alginate gels have been reported to be stabilized by replacing Ca^{2+} ions with other divalent cations, such as Cu^{2+}, which display a markedly higher affinity for this polysaccharide [39]. In addition, it has been suggested that several cations other than Ca^{2+} might reduce alginate porosity [20,39].

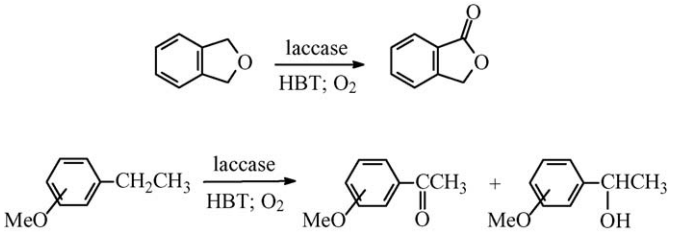


Fig. 5. Laccase-catalyzed oxidation products of phthalane and 4-methoxyethylbenzene obtained in HBT-mediated reactions.

Table 6
Aerobic oxidation of non-phenolic substrates (cf. Fig. 5) with immobilised laccases under mediation by HBT, at room temperature for 24 h[a]

Immobilisation support	Oxidation yield (%) from		
	4-Anisyl alcohol[b]	Phthalane[c]	4-MeO-ethylbenzene[d]
None[e]	76 ± 3	65 ± 2	20 ± 1
Cu-alginate	85 ± 3	70 ± 3	13 ± 0.5
Ca-alginate	51 ± 2	23 ± 1	11 ± 0.5
Eupergit® C	84 ± 3	59 ± 2	17 ± 0.5
AC	74 ± 3	1 ± 0.05	12 ± 0.5

[a] Reaction conditions: [substrate] 20 mM, [mediator] 6 mM, immobilised laccase 3 U/mL, in citrate buffer containing 4% CH_3CN for solubility reasons.
[b] Yield of *p*-anisaldehyde, from Table 2.
[c] Yield of lactone [40].
[d] Yield of *p*-MeO-acetophenone plus minor amounts of 1-(*p*-MeO-phenyl)-ethanol [37].
[e] Purified *T. villosa* laccase as the reference case [22].

3.3. Oxidation of other non-phenolic substrates

To assess whether the immobilised laccases had better proficiency towards the mediated oxidation of non-phenolic substrates *other than* benzyl alcohols, additional experiments were performed. To this aim, a cyclic benzyl ether, such as phthalane, and an alkylarene, such as 4-methoxy-ethylbenzene, were selected. These two substrates had given a moderate-to-good conversion into the corresponding oxidation products by the use of free laccase and HBT (Fig. 5) [37,40].

The yields in the aerobic oxidation of these two substrates, performed with the four supported laccases under mediation by HBT, are given in Table 6, and compared with the yields obtained with the native enzyme. For comparison, the results of the oxidation of 4-MBA from Table 2 are also reported. Once again, Cu-alginate and Eupergit® C were the supports that enabled laccase to perform better. In general, the mediated oxidation of the alkylarene substrate led to the lowest product yields, regardless of the use of the native or immobilised form of the enzyme.

3.4. Oxidations in mixed solvents

Several immobilised laccases have been shown to be less susceptible to denaturation by organic co-solvents than the native enzyme [21]. To provide support to this finding, the benchmark aerobic oxidation of 4-MBA was performed in the presence of four organic co-solvents, mixed in a 1:1 ratio (v/v) with the aqueous citrate buffer, with both HBT and HPI as radical mediators. The oxidation yields from laccase immobilised on either Cu-alginate or Eupergit® C were compared with those obtained from the native enzyme. The co-solvents under study were: isopropanol, dioxane, acetonitrile and ethylene glycol. The activity of both native and immobilised laccases was recalibrated in each water/organic co-solvent mixture by using the 3-hydroxyanthranilate (HAA) assay method. In fact, this method was shown to provide a more reliable activity determination in water–organic mixed solvents than the one based on ABTS [41]. The results of 4-MBA conversion are summarized in Table 7.

Yields of *p*-anisaldehyde were markedly reduced in buffer/organic co-solvent mixtures with respect to those obtained in buffer alone, as expected [41]. Among the immobilised systems, only Cu-alginate-laccase compared favourably with the performances of the native enzyme. In all cases, HBT resulted to be a better mediator than HPI.

4. Conclusions

This study provides evidence about a few advantages arising from immobilisation of an enzyme, but also disproves common beliefs in this field. Four different methods of immobilisation of laccase have been comparatively evaluated. Because of its novelty and potential applications, the oxidation of non-phenolic substrates was deliberately chosen. This kind of oxidation requires the use of mediators, which perform as a sort of shuttle between the enzyme and the substrate. As a consequence, our conclusions truly concern the efficiency with which the immobilised enzyme converts the mediator into the reactive intermediate (Med_{ox}). No attempts towards the oxidation of simple phenolic compounds, these being natural substrates of laccase, have been made. In fact, this point has already been investigated [16,18,20,28], and we also feared that the oxidation reactivity could be uniformly too high with the four immobilised enzymes, preventing any discrimination among the performances of each preparation. Moreover, the oxidation of non-phenolic substrates has more synthetic value, and positive results would make any particular immobilised enzyme attractive from a practical viewpoint.

Among the non-covalents supports tested, Cu-alginate led to an immobilized system that provided high *p*-anisaldehyde yields in mediated reactions, maintained high activity in the

Table 7
Yields (%) of *p*-anisaldehyde from the aerobic oxidation of *p*-anisyl alcohol in 0.1 M citrate buffer in the presence of 50% of organic co-solvents such as 2-propanol, dioxane, MeCN and ethylene glycol[a]

Organic co-solvent	NL/HBT	NL/HPI	CAL/HBT	CAL/HPI	EL/HBT	EL/HPI
None	76 ± 3	70 ± 3	85 ± 3	11 ± 0.3	84 ± 3	55 ± 2
Propanol	10 ± 0.4	8 ± 0.3	8 ± 0.3	5 ± 0.2	5 ± 0.2	6 ± 0.2
Dioxane	21 ± 0.4	11 ± 0.3	24 ± 0.4	4 ± 0.2	6 ± 0.2	10 ± 0.4
MeCN	10 ± 0.3	–	9 ± 0.3	8 ± 0.3	5 ± 0.2	11 ± 0.3
Ethylene glycol	40 ± 2	28 ± 0.5	32 ± 0.5	5 ± 0.2	18 ± 0.4	23 ± 0.4

Either native laccase (NL) or immobilised laccase on Cu-alginate (CAL) or Eupergit® C (EL), in the presence of either HBT or HPI as mediators, were used.
[a] Conditions as in Table 2.

presence of organic co-solvents and showed an appreciable operational stability. Among the covalent supports, Eupergit® C exhibited better performances than activated carbon, and gave results comparable with those of Cu-alginate. The similar performances observed with these supports in mediated reactions is somewhat peculiar and unexpected. In fact, the entrapment of the enzyme within the egg-box structure of Cu-alginate might suggest a restriction to enzyme/mediator interactions, and a limitation in the diffusion of the radical species of the mediator (Med_{ox}) from the support to the bulk solution. These mass-transfer effects were expected to be markedly attenuated in an immobilized system such as Eupergit® C-laccase, where enzyme molecules are predominantly immobilized onto the external surface of the support and, consequently, exposed to the bulk solution. Therefore, it ought to be hypothesized that other effects, such as non-productive orientation [42], decreased protein flexibility resulting from multi-point attachment and/or enzyme overcrowding on the surface of the support [43] might negatively affect the performances of this immobilized system. By and large, our expectations that the supported enzymes could outdo the performances of the native enzyme were not met. Because laccase is a rather inexpensive enzyme, it appears that the effort required to immobilise it does not pay back in terms of substantially enhanced performances, not even in view of any scaled-up oxidation procedure. This statement clearly concerns the immobilized *T. villosa* laccase systems investigated in the present study, and do not obviously extend to other immobilized preparations and/or distinct applications of these catalysts. In fact, several matrices, such as cross-linked cationic polymer [21], controlled porosity glass [17], nylon membranes [44] and Eupergit® C itself [21] were successfully used as immobilisation supports, and enabled laccase to perform an efficient removal of phenols from both synthetic and industrial wastewater. However, the present study describes an unambiguous way to compare the merits of an immobilised enzyme with respect to the native counterpart, and to rank the performances of four immobilized laccase preparations in the mediated oxidation of non-phenolic substrates under various conditions. Our approach enables to fight common beliefs about immobilised enzymes that have no clear-cut experimental support, and warns about a naive expectation of uniformly good results from use of the immobilisation technique.

Acknowledgment

The Authors thank the Italian MIUR for financial support (COFIN and FIRB projects).

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