A novel, simple screening method for investigating the properties of lignin oxidative activity

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Enzymatic lignin degradation represents a key challenge for integrated biorefineries. Notwithstand-ing the rich content in aromatic compounds, lignin's complex structure has hampered identification of an effective and cost-efficient enzymatic procedure to transform it into less complex product families. Advancements in enzymatically modifying or degrading lignin require a simple and reliable analytical method to quickly screen diverse lignin samples by employing different enzymes and conditions. Here, we report on a novel, rapid, and economic colorimetric assay for lignin oxidation based on the reaction of 2,4-dinitrophenylhydrazine with the carbonyl groups generated by enzymatic oxidation. The assay was validated on monomeric and dimeric lignin model compounds by comparison with HPLC analysis. The colorimetric method was used to investigate the activity of ten laccases and eight peroxidases on three technical lignins under different experimental conditions (e.g., by altering pH and mediator used). The colorimetric method was also coupled to a size-exclusion chromatographic separation of the lignin sam-ple obtained after the enzymatic treatment in order to verify whether the enzymatic treatment resulted in lignin depolymerization, too. On the basis of this novel procedure, appropriate enzymatic treatments can now be identified to generate valuable lignin product streams.

Keywords:
Colorimetric assay
Lignin oxidation
Ligninolytic enzymes
Screening method
Lignin valorization

1. Introduction

Lignin is an aromatic heteropolymer showing a structural complexity highly dependent on the biomass source and lignin isolation process [1,2]. Owing to this heterogeneity, lignin contains a number of aromatic compounds of potential interest for the production of bio-based materials, fuels, and chemicals [3]. The monomer composition affects lignocellulosic biomass processing, and thus the design of lignin valorization strategies.

Detailed studies of lignin degradation are also hampered by a lack of suitable methods to detect lignin breakdown [4]. The use of ^{14}C-labeled lignin has been reported to be limited to activity assays while lignin-degrading enzymes are being purified: the method requires the preparation of radioactively labeled lignin and 7–14 days for each assay. A rapid colorimetric assay involving the reaction between diazotized sulfanilic acid and an alkaline solution of lignin has been developed and used in studies on lignin degradation by bacteria [5]. Here, Ahmad's group reported two spectrophotometric assays for lignin breakdown that can be carried out on 96-well microtiter plates and used for rapid screening of the lignin-degrading ability of a micro-organism [6]. The first assay involves attaching a fluorophore to the lignin polymer: breakdown of the lignin structure changes the environment of the fluorophore and hence fluorescence is also altered. The second assay employs chemically nitrated lignin: breakdown of the lignin structure releases nitrated phenols, leading to an increase in UV/Vis absorbance.

Here we report on the set up of a novel rapid screening procedure to test enzymatic lignin oxidation based on the reaction with 2,4-dinitrophenylhydrazine (2,4-DNP): this assay can be carried out on 96-well plates and does not require the use of modified lignins. In the assay, 2,4-DNP can be used to detect the carbonyl function of a ketone or aldehyde group: this is an addition-elimination reaction in which the nucleophilic addition of the $-NH_2$ group to the C=O carbonyl function of the enzyme product is followed by the removal of a water molecule (see Fig. 1A). A positive response is represented by the development of an orange or red color for aliphatic and aromatic carbonyl compounds, respectively. Here, 2,4-DNP does not react with other carbonyl-containing

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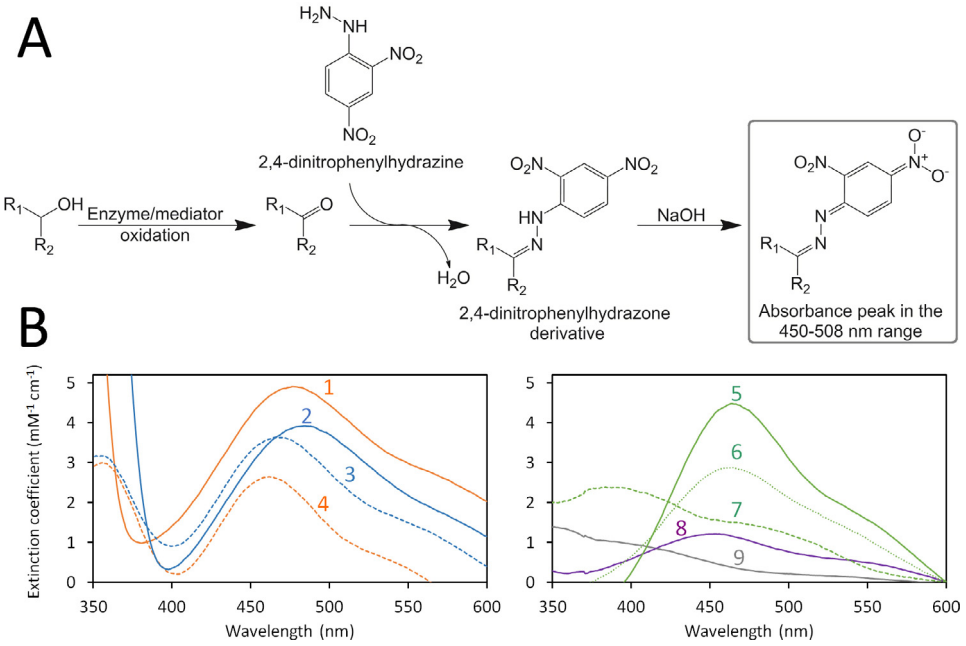

Fig. 1. (A) Reaction overview of 2,4-DNP with carbonyl groups. An alcoholic group in lignin can be oxidized to ketone or aldehyde by a ligninolytic enzyme/mediator system. Carbonyl groups spontaneously react in acidic conditions with 2,4-DNP, giving a dinitrophenylhydrazone derivative: this compound is subjected to a large bathochromic shift of the absorbance maximum in a basic solution and thus easily detected. (B) Absorbance spectra of dinitrophenylhydrazones obtained from different compounds at 0.25 mM final concentration. Left: 1) 4-hydroxybenzaldehyde; 2) vanillin; 3) isovanillin; 4) 3-hydroxybenzaldehyde; Right: 5) veratryl aldehyde (3,4-dimethoxybenzaldehyde); 6) 3-methoxybenzaldehyde; 7) 3,5-dimethoxybenzaldehyde; 8) 3,4-dimethoxyacetophenone; 9) butanal.

functional groups such as carboxylic acids, amides, and esters [7]. According to proposed biocatalytic models, an increase in carbonyl function in lignin samples was related to production of phenol monomers and a higher level of oxidation [8,9]. Indeed, this colorimetric assay can be coupled to a size-exclusion chromatography separation to evaluate whether lignin oxidation is coupled to its depolymerization.

In this work, we describe the application of the colorimetric assay to compare the activity of different lignin-oxidative enzymes and to study their substrate specificity. This rapid, simple, and reproducible assay provides a convenient alternative method for the study of enzyme-based lignin modification.

2. Materials and methods

2.1. Reagents and enzymes

The laccases from *Trametes versicolor* (Tv-L) and *Pleurotus ostreatus* (Po-L), the manganese peroxidase from *Phanerochaete chrysosporium* (Pc-MnP), the versatile peroxidase from *Bjerkandera adusta* (Ba-VP), and the lignin peroxidase from *Bjerkandera adusta* (LiP) were purchased from Sigma-Aldrich (Milano, Italy). LAC enzymes, horseradish peroxidase (HRP), and peroxidases from *Mycetinis scorodonius* (Ms-MnP) and *Pleurotus sapidus* (Ps-MnP) were supplied by ASA Spezialenzyme GmbH [10]. The manganese peroxidases from *Nematoloma frowardii*, reclassified as *Phlebia* sp. Nfb19 [11] (Nf-MnP), and *Bjerkandera adusta* (Ba-MnP) were purchased from Jena Bioscience GmbH (Jena, Germany). Recombinant laccase from *Bacillus licheniformis* (BALL), basidiomycete PM1 (OB-1), and polyphenol oxidase from *Marinomonas mediterranea* (MmPPOA-695-His) were expressed and purified as reported in [10,12]. See Table S1 for details.

Phenyl alcohols were purchased from Sigma-Aldrich and GGE (guaiacylglycerol-β-guaiacyl ether) from Zenteck s.r.l (Milano, Italy). The mediators 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), (2,2,6,6-tetramethylpiperidinyl-1-oxy (TEMPO), 1-hydroxybenzotriazole (HBT), N-hydroxyphthalimide (HPI), violuric acid (VA), and 3-hydroxyanthranilic acid (3-HAA) were purchased from Sigma-Aldrich. Kraft, Indulin AT, and Protobind lignins were purchased from Sigma-Aldrich, MeadWestvaco (Richmond, Virginia, USA), and GreenValue SA (Orbe, Switzerland), respectively.

Retentate and permeate fractions of Protobind lignin were obtained by dialyzing 0.5% (w/v) of lignin in 50 mM sodium acetate buffer, pH 5.0, containing 0.05% (v/v) Tween-80 using a 3-kDa dialysis tube, at 25 °C for 7 days.

2.2. Visible absorbance spectra analysis of phenylhydrazone derivatives

Visible absorbance spectra of hydrazone compounds were recorded on a Jasco V-580 spectrophotometer (Cremella, Italy) from 350 to 600 nm. A total of 25 μL of 10 mM carbonyl compound solutions were added to 225 μL of water; then, 250 μL of a 1 mM 2,4-DNP solution dissolved in 0.1 M HCl were added and the reaction mixtures were incubated for 5 min at room temperature. Spectra were recorded immediately after adding 500 μL of 1 M NaOH solution. The final concentration of each carbonyl compound was 0.25 mM.

2.3. Enzymatic assays

Enzymatic activities were assayed spectrophotometrically at 25 °C as follows. Laccase activity was measured by monitoring for 5 min at 25 °C the oxidation of 0.5 mM ABTS ($\varepsilon_{420\,nm}$ = 36 mM^{-1} cm^{-1}) at 420 nm in 50 mM sodium acetate, pH 5.0. The manganese peroxidase activity was assayed in the presence of 0.1 mM H_2O_2 and 2 mM $MnCl_2$ in 50 mM sodium malonate buffer, pH 4.5 [13]. The extinction coefficient at 270 nm was 11.59 mM^{-1} cm^{-1} for Mn^{3+}-malonate complex. The lignin peroxidase activity was assayed in the presence of 0.1 mM H_2O_2 and

2.5 mM veratryl alcohol in 50 mM sodium acetate buffer, at pH 3.0 or 5.0 [14].

One unit (U) of activity was defined as the amount of enzyme that oxidized 1 μmol of substrate per min at 25 °C.

2.4. Screening procedure

Phenyl alcohols were dissolved at 2 mM final concentration in 50 mM sodium acetate, pH 5.0, 0.05% (v/v) Tween-80 and 0.2% (v/v) dimethyl sulfoxide (DMSO). Incubations were carried out in a final volume of 1 mL: to each sample 0.1 U of Tv-L and 2 mM TEMPO were added. The samples were incubated on a rotatory wheel at 25 °C; after 24 h of incubation, 20 μL of sample were withdrawn, to which 30 μL 100 mM HCl were added and used for colorimetric screening in a 96-well microtiter plate. In each well, 50 μL of 1 mM 2,4-DNP dissolved in 100 mM HCl were added. The plate was incubated at 25 °C for 5 min, and then 100 μL of 1 N NaOH were added to allow color to develop. In parallel, control samples (not containing substrate or enzyme) were also analyzed. Each sample was analyzed in triplicate; values are reported as mean ± standard deviation.

Lignin samples were dissolved at a concentration of 1% (w/v) in 0.1 M NaOH and then diluted at final concentrations of 0.1, 0.05, or 0.01% (w/v) in 50 mM sodium acetate, pH 5.0, 0.05% (v/v) Tween-80 (the reaction pH was corrected with 0.1 M HCl, if not specified otherwise). Under these conditions, all technical lignin solutions appear homogeneous with no evidence of precipitate. Incubations were carried out in a final volume of 1 mL: to each sample, Tv-L was added at 0.2 U/mg lignin in 50 mM sodium acetate, pH 5.0, containing 0.05% (v/v) Tween-80 and ABTS (0 or 1 mM).

When the behavior of the different enzymes on lignin was studied, laccases were used in the presence of 2 mM of different mediators, while for Mn-peroxidase and LiP, 2 mM of $MnCl_2$ and 0.1 mM H_2O_2 were added. Due to the known pH preference [10,12,14], MmPPOA-695-His and BALL were also assayed at pH 7.0 and LiP at pH 3.0. The samples were incubated on a rotatory wheel at 25 °C; at fixed times, 20 μL of the sample were withdrawn, 30 μL 100 mM HCl was added, and the samples were then used for colorimetric screening in a 96-well plate.

Products formed by reaction of 2,4-DNP and the carbonyl groups of model compounds or lignin show an absorption peak (or a shoulder) at around 450–485 nm: the absorbance was measured using a microtiter plate reader (Tecan, Männedorf, Switzerland) at 450 nm. The color was stable for 15 min after the development.

2.5. HPLC analyses

The oxidation activity of Tv-L on the model compounds 3,5-dimethoxybenzyl alcohol and veratryl alcohol (at 2 mM final concentration) was determined by HPLC analysis. The 1-mL assay mixture containing 0.1 U of laccase in 50 mM sodium acetate, pH 5.0, 0.05% (v/v) Tween-80, 0.2% (v/v) DMSO, and 2 mM TEMPO was incubated on a rotatory wheel at 25 °C. After 24 h, 20 μL of reaction mixture were withdrawn, 40 μL of mobile phase was added, the mixture centrifuged, and 20 μL of supernatant analyzed by HPLC. Analyses were performed on a Merck Hitachi (Tokyo, Japan) apparatus with a Symmetry C8 column 100 Å (5 μm, 3.9 × 150 mm) (Waters, Milano, Italy), and an UV detector set at 254 nm; 34.5% methanol (v/v), 5.4% acetonitrile (v/v), and 0.1% formic acid (v/v) was used as mobile phase at a flow rate of 0.8 mL/min. The retention times of veratryl alcohol, veratryl aldehyde (3,4-dimethoxybenzaldehyde), 3,5-dimethoxybenzyl alcohol, and 3,5-dimethoxybenzaldehyde were 8.6, 15.1, 16.7, and 40.4 min, respectively.

The oxidation/degradation activities of different laccase/mediator combinations on the dimeric model compound 1-(4-hydroxy-3-methoxyphenyl)-2-(2-methoxyphenoxy) propane-1,3-diol (GGE) at 10 mM final concentration were also determined by HPLC analysis. The 0.5-mL assay mixtures containing 0.2 U of laccases in 50 mM sodium acetate, pH 5.0, and 2 mM mediator (TEMPO, ABTS, or HBT) were incubated on a rotatory wheel at 25 °C. At fixed times, 20 μL of the sample were withdrawn, 30 μL 100 mM HCl were added, and the sample was used for colorimetric screening in a 96-well plate. At the same time, 20 μL of reaction mixture were withdrawn, added of 40 μL of mobile phase, centrifuged, and 20 μL of supernatant were analyzed by HPLC. HPLC analyses were performed on a Phenomenex Luna C18(2) column (5 μm, 4.6 × 150 mm) (Castelmaggiore, Italy) and 0.1% (v/v) trifluoroacetic acid, 5.1% (v/v) CH_3CN, and 32.8% (v/v) CH_3OH in H_2O, at a flow rate of 0.8 mL/min; detection was at 254 nm.

2.6. Size-exclusion chromatography

A fast and simple size-exclusion chromatography procedure to separate lignin components originating from the enzymatic treatments according to their hydrodynamic radius was set up. The samples were fractionated on a Sephadex G25 column (1.3 × 26.5 cm) at a flow rate of 5 mL/min using an Akta purifier system (GE Healthcare, Milano, Italy): with this system the analysis could be automated and the eluted fractions recovered. The column was calibrated using PEG at different molecular masses (200–2000 Da). The column was loaded with 0.25 mg of a lignin sample dissolved in 0.1 M NaOH and diluted in 50 mM sodium acetate buffer, pH 5.0, 0.1 M LiCl, and 0.05% (v/v) Tween-80 (elution buffer).

3. Results

3.1. Set up of the screening procedure for lignin oxidation activity

A schematic representation of the 2,4-DNP assay is shown in Fig. 1A. The absorbance spectrum of the phenylhydrazone derivative depends on the compound used. Benzyl aldehydes show the highest extinction coefficient (centered at 460–480 nm) while the ketones show a 4-fold lower extinction coefficient, with a maximum at ~450 nm, see spectrum 8 in Fig. 1B for the hydrazone derived from 3,4-dimethoxyacetophenone. In contrast, aliphatic aldehydes react with 2,4-DNP yielding an adduct with a very low absorbance intensity, see spectrum 9 in Fig. 1B. All the benzaldehyde-derivate hydrazones show an absorbance peak at around 470 nm and differ in intensity depending on the substituents at positions 3, 4, and 5. These hydrazone-derivatives show an ε_{450nm} between 1.6 (for 3,5-dimethoxybenzaldehyde, spectrum 7) and 4.8 $mM^{-1}\,cm^{-1}$ (for 4-hydroxybenzaldehyde, spectrum 1). This demonstrates that the absorbance increase at 450 nm in the 2,4-DNP-based assay detects preferentially aromatic aldehydes; thus, it is useful to qualitatively follow the oxidation of benzylic alcohols, as the ones present in lignin. Indeed, the absorbance intensity at 450 nm (corresponding to the wavelength of the microtiter reader filter) shows a 2.7-fold change, depending on the benzyl aldehyde used; employing an average extinction coefficient of $2.6 \pm 1.4\ mM^{-1}\,cm^{-1}$, a semi-quantitative analysis can be performed, which is useful for comparing different enzymes belonging to the same class on the identical lignin sample. Notably, at 445 nm a lower variability (≤1.8-fold) of the extinction coefficient on the benzyl aldehyde used is apparent.

Different benzyl alcohols (see Fig. 2A) were chosen as model substrates to assay the oxidation by *Trametes versicolor* laccase (Tv-L), a commercial enzyme with well-known kinetic parameters and properties [10,12,15,16]. As summarized in Fig. 2A, an increase in absorbance at 450 nm was observed for all the substrates tested

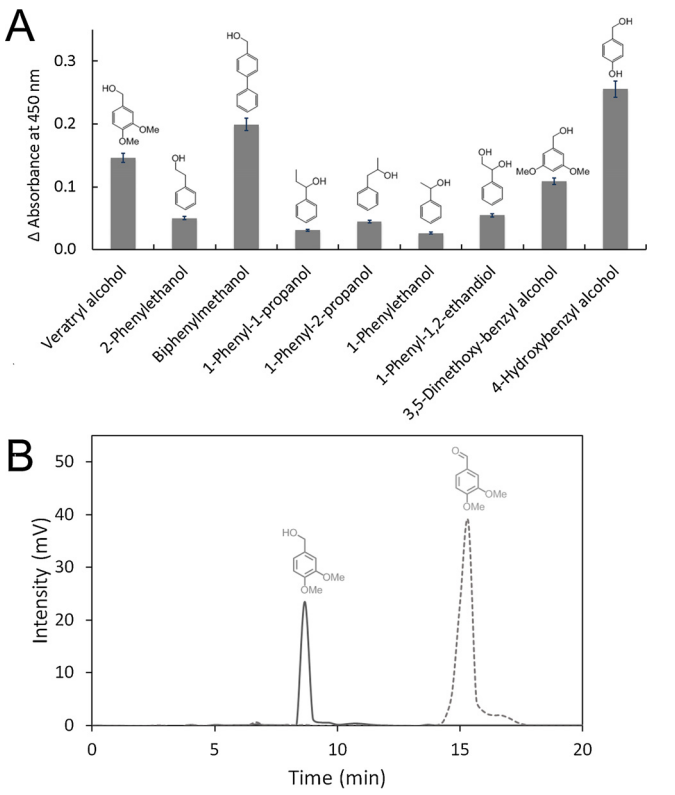

Fig. 2. Screening for phenyl alcohol oxidation performed on a 96-well microtiter plate and employing Tv-L. (A) The different alcohols were treated with Tv-L (0.5 U/μmol of substrate) in the presence of 2 mM TEMPO for 24 h, giving an increase in absorbance at 450 nm following reaction with 2,4-DNP. The absorbance values determined in the absence of Tv-L were subtracted from all samples. The values are reported as mean ± standard deviation (n = 3). (B) HPLC chromatograms of 2 mM veratryl alcohol oxidation in the presence of 2 mM TEMPO prior (continuous lines) and 24 h after adding 0.1 U Tv-L laccase (dotted lines), at 25 °C and pH 5.0. The peaks contain: 8.6 min, veratryl alcohol; 15.1 min, veratryl aldehyde (3,4-dimethoxybenzaldehyde).

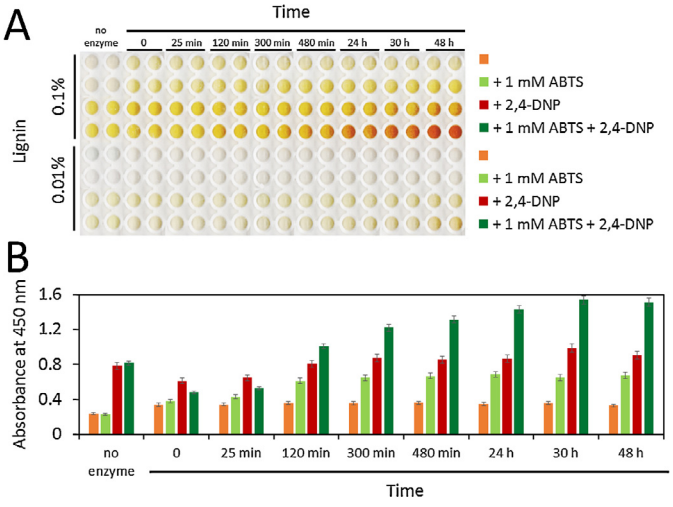

Fig. 3. Screening for lignin oxidation by Tv-L performed on a 96-well microtiter plate. (A) Protobind lignin at 0.01 or 0.1% (w/v) was treated with Tv-L (0.2 U/mg lignin) with or without 1 mM ABTS, resulting in a time-dependent increase in absorbance at 450 nm following reaction with 2,4-DNP at basic pH. (B) Absorbance values detected at increasing times during the screening assay on 0.1% (w/v) lignin reported in (A). The values are reported as mean ± standard deviation (n = 3). No absorbance change with time was apparent in the control wells with no enzyme added.

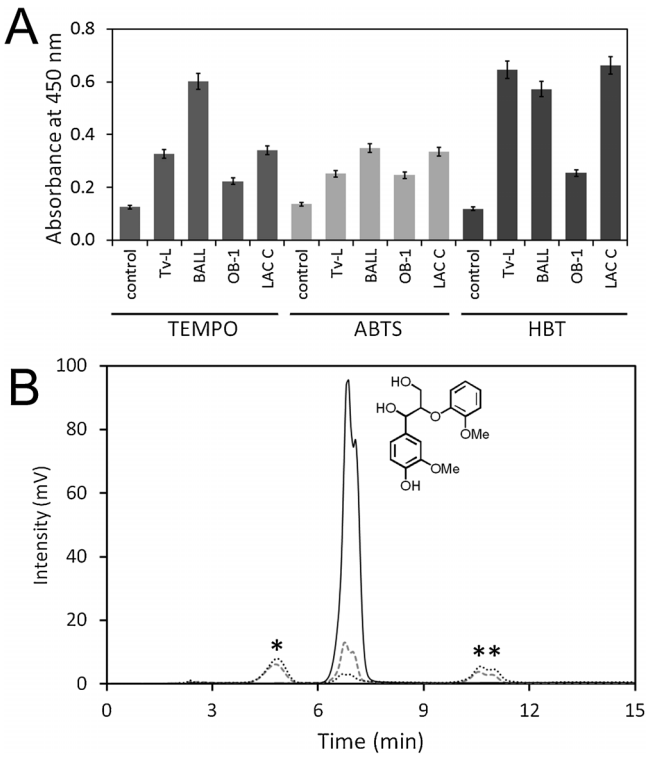

Fig. 4. (A) Colorimetric screening for oxidation/degradation of the dimeric lignin model compound GGE by employing different laccases and mediators (4 h of incubation). Control: absorbance assayed when no enzyme was added. The values are reported as mean ± standard deviation (n = 3). (B) HPLC analysis of the time course of reaction of 5 mL of 10 mM GGE with 1 U BALL laccase and 2 mM TEMPO, at 25 °C and pH 5.0. Continuous line: reaction mixture prior to adding BALL; dashed and dotted line: reaction mixture 2 and 4 h after adding BALL laccase.

following laccase oxidation and reaction with 2,4-DNP: in all cases standard deviation was <5%. The different absorbance values detected for each compound depend on: a) the extinction coefficient of the phenylhydrazone product; and b) the extent of the enzymatic oxidation reaction (from alcohol to carbonyl group), which is position dependent. As a general rule, substituted benzyl alcohols gave a higher response (i.e., veratryl and 4-hydroxybenzyl alcohols as compared to 1-phenylethanol or 1-phenyl-1-propanol). Interestingly, in the presence of two alcohol groups on the same molecule (i.e., 1-phenyl-1,2-ethandiol), a higher absorbance value was apparent with respect to the molecule carrying one —OH group only (i.e., 1-phenylethanol). Reaction controls were carried out in absence of substrate or enzyme and nonalcoholic molecules were used as well: no color development was observed in any of the cases.

In order to confirm that an oxidation reaction took place using Tv-L, the formation of the corresponding aldehyde was also determined by HPLC analysis starting from veratryl alcohol or 3,5-dimethoxybenzyl alcohol as substrate. As shown in Fig. 2B, after 24 h of incubation with Tv-L, a peak corresponding to 3,4-dimethoxybenzaldeyde (veratryl aldehyde) or 3,5-dimethoxybenzaldeyde, respectively, was apparent. A very close estimation of the aldehyde production was determined using the colorimetric assay (based on the extinction coefficients reported in Fig. 1B) and the HPLC method (i.e., ~7 and 8 nmol/min and ~10 and 8 nmol/min for veratryl alcohol or 3,5-dimethoxybenzyl alcohol, respectively), thus showing that the colorimetric method represents a quantitative assay for single compounds.

Treatment of the Protobind lignin with Tv-L at pH 5.0 and 25 °C resulted in a time-dependent increase in absorbance at 450 nm fol-

lowing reaction with 2,4-DNP (see Fig. 3), especially when 1 mM ABTS was used as mediator. The laccase reaction on lignin is facilitated by the use of a mediator: after the enzymatic oxidation and the conversion into stable radicals, this small molecule acts as electron shuttle that can access complex polymers such as lignin [27]. Notably, an initial decrease in absorbance at 450 nm was observed at the shorter times (Fig. 3B), probably arising from polymerization of low-molecular-mass compounds present in the lignin fraction; subsequently, a time-dependent increase in absorbance at 450 nm was apparent. Moreover, the absorbance change clearly depended on the enzymatic units added (Fig. S1). As a control, the same reaction was carried out at pH 9.0, a value at which Tv-L is inactive [12,15,16]: in this case no absorbance changes were observed (data not shown).

Taken together, these results demonstrate that the oxidation by Tv-L of alcoholic groups in lignin model compounds and in technical lignin can be easily detected by the 2,4-DNP-based colorimetric assay.

3.2. Screening of oxidation/degradation activity on a lignin model compound

The oxidation by different enzymes and in the presence of diverse mediators was evaluated on a well-known dimeric lignin model compound (GGE, Fig. 4A). During the first 4 h of incubation, a time-dependent increase in absorbance at 450 nm was observed while a decrease was apparent at longer times (6 and 24 h, not shown): this effect could arise from repolymerization of degradation compounds and/or over-oxidation to carboxylic acids.

Under the same experimental conditions, the degradation of GGE was also evaluated by HPLC analysis. As shown in Fig. 4B, the significant increase in the absorbance value that was observed when GGE was incubated for 4 h with recombinant BALL laccase and TEMPO was also evident in HPLC analysis: reduction of the quantity of GGE was paralleled by formation of additional peaks (retention time of 4.78 and 10.6–10.8 min, see stars in Fig. 4B).

3.3. Screening of ligninolytic degrading enzymes

On the basis of results obtained during the set up procedure with Tv-L, we evaluated the oxidation of lignin groups by different enzymes and in the presence of different mediators. In detail, the assay was carried out on two different lignin fractions treated with a number of laccases (at 0.2 U/mg lignin) in the absence or in the presence of 1 mM ABTS. In order to obtain homogeneous starting material, Protobind lignin was extensively dialyzed against 50 mM sodium acetate, pH 5.0, 0.05% (v/v) Tween-80 (using a membrane with a cut-off of 3-kDa): the permeate and retentate fractions were then used for the enzymatic assay employing ten different laccases [10,12].

A time-dependent increase in absorbance at 450 nm, corresponding to product accumulation, was observed for both lignin fractions and higher values were apparent in the presence of the redox mediator ABTS (Fig. 5). In particular, a significant absorbance increase at 450 nm was observed when the retentate fraction was incubated with the recombinant laccase BALL or the commercial laccases Po-L, LAC C, and LAC 3 (Fig. 5D). The highest lignin oxidation observed on the retentate fraction could result from the ABTS ability to act as an electron mediator on high-molecular-mass lignin fractions. Product formation frequently stops at times >48 h or when the absorbance change is >0.5: this can be related to different phenomena such as enzyme inactivation or product inhibition. The decrease in absorbance observed at 144 h strongly suggests repolymerization of the generated monomeric species.

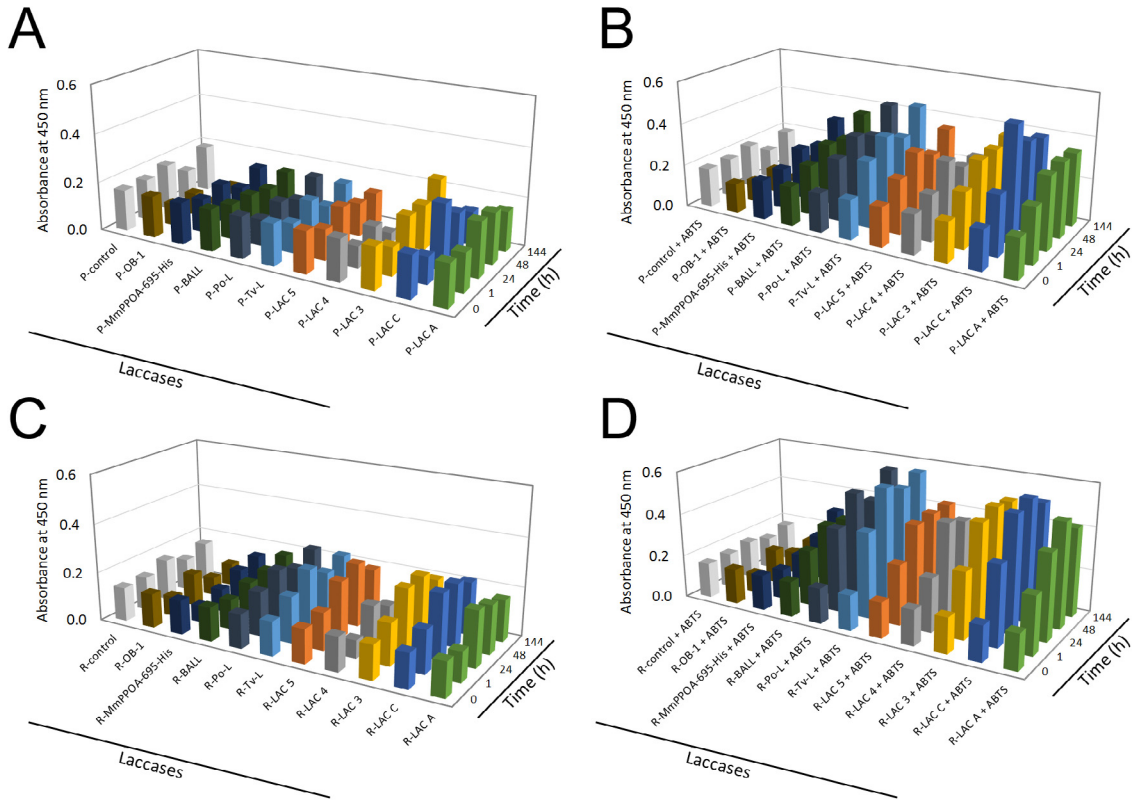

Fig. 5. Screening for lignin oxidative activities performed on 96-well microtiter plates. Permeate (A,B) and retentate (C,D) Protobind lignin fractions (P and R fractions, respectively) were treated with ten different laccases (0.2 U/mg lignin) in the absence (A,C) or in the presence of 1 mM ABTS (B,D): in all cases, a time-dependent increase in absorbance at 450 nm was observed following reaction with 2,4-DNP, whose intensity was higher when the mediator was also present. Control: no enzyme added. The values represent the mean of three measurements; in all cases, standard deviation was <5% (not shown).

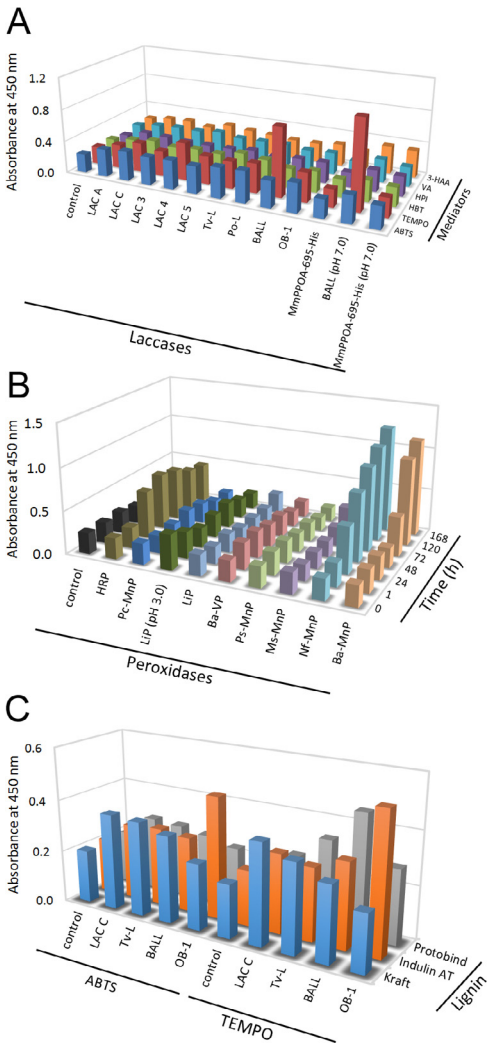

Fig. 6. Screening for lignin oxidation by different enzymatic activities performed on a 96-well microtiter plate. (A) The Protobind retentate fraction was treated with ten different laccases (0.2 U/mg lignin) in the presence of different redox mediators (1 mM) for 48 h, at 25 °C and pH 5.0. For BALL and MmPPOAA-695-His only, the activity was also assayed at pH 7.0. (B) The retentate fraction of Protobind lignin was treated with eight different peroxidases (0.2 U/mg lignin) giving a time-dependent increase in absorbance at 450 nm. Every 24 h, 0.1 mM H_2O_2 was added to the reaction mixture. For LiP only, the activity was also assayed at pH 3.0. (C) Kraft, Indulin AT, and Protobind (retentate fraction) were treated with four different laccases (0.2 U/mg lignin) in the presence of ABTS and TEMPO as redox mediators (2 mM) for 48 h. Control: no enzyme added. The values represent the mean of three measurements; in all cases, standard deviation was <5% (not shown).

Furthermore, the colorimetric assay was also suitable to evaluate the effect of different redox mediators. In this case, the assay was performed on the retentate lignin fraction (containing the high-molecular-mass compounds only) that was incubated with ten different laccases in the presence of various mediators: ABTS, TEMPO, HBT, HPI, VA, and 3-HAA. A significant change in the absorbance value was apparent when lignin was incubated for 48 h with the recombinant BALL laccase and TEMPO as redox mediator, at pH values 5.0 and 7.0 (see Fig. 6A).

In order to evaluate whether this assay could be applied to different classes of oxidative enzymes, lignin oxidation by different peroxidases was also investigated. Results shown in Fig. 6B on the retentate fraction of Protobind lignin to which 0.1 mM H_2O_2 was added (every 24 h) showed a significant increase in the absorbance value in the presence of Nf-MnP and Ba-MnP. However, no change

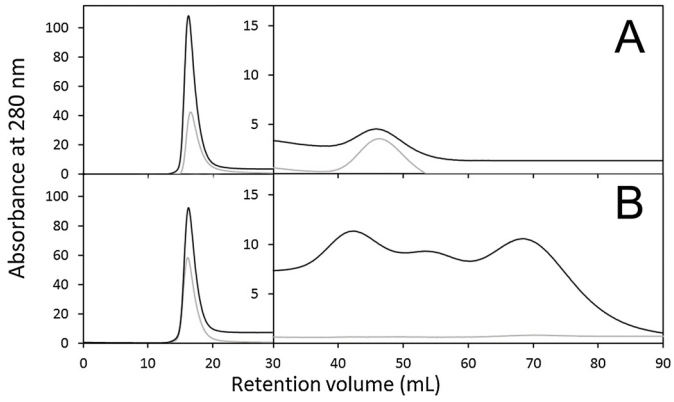

Fig. 7. Size-exclusion chromatography of lignins treated with laccases. (A) Kraft lignin treated for 24 h with 2 mM TEMPO (gray line) or TEMPO and 0.1 U of LAC C (black line), at 25 °C and pH 5.0. (B) Indulin AT lignin treated for 24 h with 2 mM ABTS (gray line) or ABTS and 0.1 U of OB-1 (black line), at 25 °C and pH 5.0.

in absorbance was apparent in control experiments performed in the absence of enzyme.

Finally, in order to evaluate the application of the screening procedure on different technical lignins, the colorimetric assay with laccases was performed under the same experimental conditions on three feedstocks. As shown in Fig. 6C, behavior of the four tested laccases differed depending on the lignin used: the highest absorbance value was detected on Indulin AT as substrate using OB-1 laccase while Tv-L and LAC C laccases showed a higher response when Kraft lignin was employed. Interestingly, Protobind lignin seems to be less susceptible to laccase/mediator oxidation, the only exception being the combined use of BALL enzyme and TEMPO mediator.

3.4. Analysis of lignin depolymerization by size-exclusion chromatography

The effect of the enzymatic treatment on the molecular mass distribution of the lignin sample was investigated using a fast and simple size-exclusion chromatography system. The lignin components were separated on a Sephadex G-25 column selected as stationary phase because of the tolerance to acids, bases, and solvents. Standards of polyethylenglycol were used to calibrate the column (inset of Fig. S2): lignins and monomers (i.e., vanillic acid and sinapyl alcohol) can be well separated by chromatography (Fig. S2). Treatment of Kraft lignin with LAC C and TEMPO resulted in an increase in the absorbance intensity at 280 nm of the peak corresponding to high-molecular-weight lignin components (Fig. 7A, peak at 15 mL), which agrees with the oxidation of lignin as observed by the 2,4-DNP-method, in particular to α-position phenyl-conjugated ketone group formation. With this treatment, monomer formation was marginal. On the other hand, when Indulin AT was treated with OB-1 and ABTS, monomers formed (see peaks at 40–70 mL elution volume) and significant oxidation of high-molecular-weight lignin was observed (Fig. 7B). Notably, the monomer formation identified by size-exclusion chromatography is in good agreement with the increase in absorbance intensity at 450 nm observed in the 2,4-DNP screening assays (lignin oxidation): actually, when no color formation was observed during the screening procedure (e.g., Kraft lignin incubated with OB-1 laccase in the presence of 2 mM TEMPO), little or no change in the size-exclusion chromatography profile in terms of intensity and elution peaks was apparent (data not shown).

4. Discussion

After more than 30 years of investigating lignin degradation, there is no known biocatalytic process for lignin depolymerization. A number of fungal and microbial strains have been reported to degrade monomeric [17–21] and in a few cases, and with a low efficiency, polymeric lignin [22–25]. Proficient lignin degradation requires the use of different enzymatic activities under specific reaction conditions [17,26,27] whereby identification is hampered by the fact that a simple and high-throughput assay is not available. The first step in microbial lignin degradation is the oxidation of hydroxyl groups in the aromatic components of lignin that facilitates its breakdown, the so-called "enzymatic combustion" [28].

Here, we developed a colorimetric assay based on reaction with 2,4-DNP, which can be easily managed on a 96-well microtiter plate, to screen the activity of lignin oxidative enzymes on different lignin samples and under different experimental conditions. The reliability of the 2,4-DNP assay was validated on monomeric (see Fig. 2B) and dimeric (see Fig. 4B) lignin model compounds by comparison with HPLC analysis. Notably, the assay is able to provide a quick and semi-quantitative figure of the relative activity of different laccases and peroxidases on true technical lignins, with no need to use labeled or modified lignins [5,6]. The colorimetric method clearly highlighted the discrepancies related to the use of different lignin samples (due to the distinctive origin and pre-treatments): the best laccase-mediator couple was thus identified for each technical lignin (see Fig. 6C). Indeed, the assay was also easily modified to investigate peroxidase activities, i.e., by adding 0.1 mM H_2O_2 to the assay mixture (see Fig. 6B). Notably, the colorimetric assay on lignin can be coupled to a size-exclusion chromatography separation performed on a Sephadex G25 column using an Akta purifier system. By this analysis, it is possible to verify whether the enzymatic treatment, in addition to lignin oxidation, releases low-molecular-weight compounds. The appearance of peaks at high elution volumes for Indulin AT treated with ABTS and OB-1 laccase was indicative of the presence of monomers (Fig. 7B).

At present, the use of this colorimetric assay for high-throughput screening of metagenomic complex libraries is hampered both by the background signal associated with the use of bacterial cultures and by the detection limit: we used a fairly large amount of enzyme per assay, corresponding to 0.1 U/mL = 100 U/L. Overall, this assay can be effectively used to investigate the specificity of different enzymes towards lignin linkage model compounds and different technical lignins, an issue of great relevance for future biotreatment applications. Indeed, a systematic investigation has not previously been reported in the literature because of the drawbacks related to the methodologies used (see Introduction section). The power of this method is its simplicity, reliability, and potential to comparatively screen different enzymatic activities under a variety of experimental conditions (in terms of mediators, pH, temperature, ionic strength, effectors, etc.) and on various lignin samples. This is expected to push the optimization of the conditions for enzymatic lignin treatment, thus encouraging the formation of low-molecular-weight products and the exploitation of lignin as a source of renewable aromatic chemicals.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.enzmictec.2016.10.013.

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