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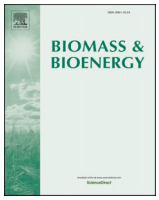

# Laccase-mediator system for the ionic liquid-assisted treatment of a technical lignin with partial dissolution

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ABSTRACT

The valorisation of lignin is a key aspect in the optimal exploitation of the paradigm of a biorefinery based on lignocellulosic biomass. In this work, the treatment of the technical lignin Indulin AT with a recombinant laccase, or with a combination of this enzyme with the mediator 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), has been carried out at 25 °C in a mildly acidic buffered aqueous medium. The presence of the ionic liquid 1-ethyl-3-methylimidazolium acetate ([$C_2$mim][OAc]) has also been investigated. Due to the limited solubility of Indulin AT in water, only partial dissolution occurred. In the lignin recovered after the treatments, a preferential diminution of the guaiacyl units was observed. In the analysis of the aqueous phases, a variety of phenolic compounds were identified, deriving from either guaiacyl or *p*-hydroxyphenyl units, although in moderately low concentrations. The highest assigned concentrations, always achieved when using the laccase-ABTS system (versus the treatment with no mediator), corresponded to different phenolic compounds, depending on whether the ionic liquid was involved or not in the treatment. In general, the overall concentration of phenolic compounds (measured as equivalents of gallic acid) was much greater after treatments involving the ionic liquid.

## 1. Introduction

Lignin is one of the major components in lignocellulosic biomass, constituting an abundant, biorenewable, and geographically distributed resource. Despite its potential as a raw material for producing valuable and more sustainable compounds, it has often been regarded by the chemical industry as a low-value by-product or even as a residue due to the difficulty in transforming it into valuable substances in a viable way. For this reason, on an industrial scale, most processes dealing with lignin are focused on its usage as a simple fuel [1] or in its degradation to reduce the oxygen demand in wastewater emissions [2,3].

Most alternative methods investigated to obtain value-added products from lignin are based on its depolymerisation using strong solvents and/or high temperatures and pressures [4,5], which hamper the environmental benefit of valorising a biorenewable resource. Enzyme-based methods, such as laccases, involve milder conditions. Laccase is a type of enzyme commonly used to transform lignin via oxidation, and its effect can be improved with the assistance of mediators to configure the so-called laccase-mediator systems, LMS [6]. In the scientific literature to date, these systems have been essentially applied under homogeneous conditions, with the tested lignin fully solubilised in aqueous media, due to the use of highly sulfonated lignin or as a result of a sufficiently acid or alkaline pH [7]. Investigation of LMS for processing lignins with a low sulfonation degree (and hence with limited water solubility) under mild pH conditions remains unexplored, despite their potential to lead to lignin valorisation processes with improved green credentials.

Ionic liquids (ILs) [8] are salts with a melting or glass transition temperature below or near ambient temperature (a mark of 100 °C is typically considered). As a result of the interesting set of properties that many of them typically exhibit (including great solvation ability, negligible vapour pressure, and thermally stable liquid character at usual process temperature ranges), ILs have received increasing attention over the last couple of decades as alternative solvents. Among the many and varied applications for which they have been proposed [9], the treatment of lignocellulosic biomass and its major biopolymeric

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constituents holds great promise, thanks to the ability of some ILs to solvate (or deeply interact with) such materials. Besides a strong focus on cellulose dissolution and transformation, as well as on pretreatment and fractionation of lignocellulosic matrices, ILs have also emerged as alternative solvents and/or catalysts that provide new opportunities to convert lignin efficiently into high added-value compounds via its depolymerisation [10–14].

Thus, in this work, the action of an LMS, in a mildly acid aqueous medium, on technical lignin with a low degree of sulfonation and poor solubility in water has been investigated, with the optional presence of an IL. In particular, a recombinant laccase derived from the fungus *Myceliophthora thermophila* was selected, along with 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as a mediator, which is a popular choice for this type of LMS. In addition, the presence of an IL, namely 1-ethyl-3-methylimidazolium acetate ([$C_2$mim][OAc]), in the aqueous medium was also explored. This IL is an archetypal choice for lignocellulose dissolution and fractionation, which has gained relevance over the last few years. However, the potential build-up of lignin in the IL due to incomplete precipitation upon the addition of an antisolvent (typically water or an aqueous mixture of solvents) represents a significant problem in the scalability and continuous operation of such fractionation strategies in the context of the real application [15]. Therefore, the study of the direct modification of lignin in a medium composed of water and IL may be an alternative to overcome this issue [16]. Moreover, it is worth noting that [$C_2$mim][OAc] is fully miscible with water and exhibits an outstanding lignin dissolution capacity [16]; and, although it induces a reduction in laccase activity [17,18], this reduction can be offset by its action as an adjuvant in the improvement of lignin treatment with an LMS [19,20].

## 2. Materials and methods

### 2.1. Materials

Indulin AT, a pine kraft lignin commercialised by Meadwestvaco, was used as received. Its sulphur content was determined by X-ray spectrometry with an Oxford Instruments Lab-X3500S spectrometer, and it was found to be 2.12%. Acetic acid, sodium acetate, and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were supplied by Sigma-Aldrich with nominal purities greater than 99.5%, 99%, and 98%, respectively. Molecular oxygen, $O_2$, was supplied as compressed gas by Praxair with a purity of 99.5%. The ionic liquid [$C_2$mim][OAc] was purchased from Iolitec with a nominal purity greater than 95%. Due to its high hygroscopicity, its water content was determined by Karl Fischer titration in a Metrohm 899 coulometer, and it was found to be 0.05%, which was interpreted as low enough to neglect it in the solution calculations. A commercial (recombinant) fungal laccase from the ascomycete *Myceliophthora thermophila* was supplied by Novozymes (code NS51003). The laccase activity of the enzyme extract, as determined via the procedure described in section 2.2, was 945 U/mL. Gram-positive bacterium *Staphylococcus aureus* EG17 and Gram-negative bacterium *Escherichia coli* CECT 736 were obtained from the Centre of Biological Engineering collection of the University of Minho.

### 2.2. Enzyme activity assay

The laccase activity was determined according to a slightly modified version of the procedure reported by Buswell et al. [21], based on monitoring the oxidation reaction of the ABTS at 420 nm. First, the enzyme extract was diluted in 50 mM acetate buffer, pH 5.0 (made with acetic acid and sodium acetate), and then the reaction was carried out at room temperature by adding 200 μL of the diluted enzyme to 2.0 mL of a buffered ABTS solution (same buffer) to a final concentration of 1 mM in the assay. Mixing was done directly in a quartz cell placed in the measuring chamber of a Jasco V-560 UV–vis spectrophotometer, and absorbance measurements were recorded every 10 s for 1.5 min. The enzyme activity was calculated with the following expression:

$$Activity\ (\mathrm{U/L}) = \frac{\Delta E \times V_t}{\varepsilon \times l \times V_s} \times 10^6$$

where $\Delta E$ is the change in the extinction of light [$min^{-1}$] at 420 nm (obtained as the slope of the linear fit of data points in the absorbance versus time plot); $\varepsilon$ is the molar absorption coefficient of ABTS, with a value of $3.6 \times 10^4\ M^{-1}\ cm^{-1}$ [21]; $l$ is the layer thickness (1.00 cm) that the light must pass in the cell; and $V_t$ and $V_s$ refer to the total volume of the assay and the volume of the enzyme extract solution, respectively. One enzymatic activity unit (U) was defined as the amount of enzyme required to oxidase 1 μmol of the substrate (ABTS) per minute at the assay reaction conditions.

### 2.3. Enzyme stability

The stability of the laccases over time was performed under the treatment conditions for lignin modification, described in section 2.4. Briefly, the properly diluted enzyme extract was pre-incubated, at room temperature, with 0.1 M acetate buffer, pH 5.0, or a mixture of 90:10 (w/w) of 0.1 M acetate buffer and the IL [$C_2$mim][OAc]. Then, a volume of this mixture was withdrawn at predetermined times throughout approximately 12 h, and the residual laccase activity was measured as already described in section 2.2.

### 2.4. Modification of lignin with LMS

For the treatment of lignin with the laccase-ABTS system, a solid load of 5 g of Indulin AT per 100 g of the buffered aqueous medium was systematically used. Different buffered aqueous media were tested: a 0.1 M acetate buffer, pH 5.0 (treatment code 1); the same buffer with a concentration of 25 mM ABTS mediator (treatment code 2); these two aforementioned treatments with oxygen pre-saturation (treatment codes 1O and 2O); and each of these four media mixed in a 90:10 (w/w) ratio with the IL [$C_2$mim][OAc] (treatment codes 1+IL, 2+IL, 1O+IL, and 2O+IL). Both the lignin and the media were placed in a glass Erlenmeyer flask and, in those cases including pre-saturation with oxygen, a stream of oxygen gas was bubbled for 30 min before the addition of the enzyme. An enzyme load of 25 $U/g_{lignin}$ was then added. Incubation was carried out at 25 °C (a controlled near-ambient temperature compatible with keeping the energy consumption of the procedure to a minimum) in a thermostated orbital shaker at ca. 150 rpm for 12 h, with pH monitoring to ensure a maximum variation of ±0.1 units. After this time, the treatment was terminated by immersing the Erlenmeyer flasks in boiling water for 20 min to denaturalise the enzyme. This time was precisely controlled to prevent different solubilisations or degradations of lignin induced by the high temperature at this stage. Control runs were performed in the absence of laccase, ABTS, and oxygen pre-saturation, by subjecting the combination of lignin and acetate buffer (code Ctrl), or lignin and the 90:10 mixture of buffer and [$C_2$mim][OAc] (code Ctrl+IL), to the same conditions as described above, including the final step by immersion in boiling water. All treatments were carried out in triplicate.

In those experiments without IL, the separation of non-solubilised lignin from the reaction medium after treatment was carried out by simple centrifugation at 9000 rpm for 20 min in a Centurion Pro-Xtra3r refrigerated centrifuge. In treatments involving IL, filtration through an S3 sinter funnel in a Kitasato flask under a soft vacuum was used. In both cases, the aqueous phase (filtrates) was kept for subsequent analyses (see section 2.6), and the solids were washed with water twice, using 15 mL each time, to guarantee full removal of residual laccase and IL (if potentially present). Finally, the washed solids were dried by lyophilisation in a LaboGene CoolSafe 100-9 lyophiliser and subjected to subsequent characterisation, as described in section 2.5 below.

### 2.5. Characterisation of the modified lignin samples

The solid samples of lignin obtained after the different treatments, as well as the raw Indulin AT for comparison purposes, were characterised by several techniques as described in the following subsections.

#### 2.5.1. NMR spectroscopy

Lignin samples were dissolved in deuterated dimethyl sulfoxide (DMSO-$d_6$, provided by Sigma-Aldrich with a purity of 99% and an atomic deuteration level of 99.5 D %), and transferred to 5-mm NMR tubes for their NMR analysis. A procedure similar to that reported by Rencoret et al. [22] and Feng et al. [23] was used. An 11.7 T Bruker DRX-500 spectrometer operating at a frequency of 500 MHz for $^{1}$H, and equipped with a BBI probe with PFG gradient on the z-axis, was used to record the spectra at 300 K. The 2D $^{1}$H–$^{13}$C HSQC spectrum was measured for each sample with the centre at 4.7 ppm and 100 ppm, and spectral widths of 11 ppm and 240 ppm, for the $^{1}$H and $^{13}$C dimensions, respectively. The number of collected complex points was 2048 for the $^{1}$H dimension, and 256 for the $^{13}$C dimension. The spectra were acquired with a recycle delay ($d_1$) of 5 s and 64 scans per $t_1$ increment. The nominal value of $^{1}J_{CH}$ used for the INEPT periods was 140 Hz. The processing of the spectra was carried out with the software Mestrenova v.14.0 (Mestrelab Research Inc.). Before the 2D Fourier transformation, the FIDs were apodised with a 90°-shifted sine-bell function in both dimensions and with line broadening of 8 Hz and 14 Hz for the $^{1}$H and $^{13}$C dimensions, respectively. The FIDs were then zero-filled to 2048 and 512 points in the $^{1}$H and $^{13}$C dimensions, and subsequently the Fourier transformation was applied in both dimensions. Next, the spectra were phased and baseline-corrected in both dimensions and finally subjected to a processing operation of $T_1$ noise reduction.

#### 2.5.2. Thermogravimetric analysis (TGA)

TGA of the samples was performed in a TA Instruments TGA Q500 thermogravimetric analyser, using flow rates of 40 mL/min and 60 mL/min of nitrogen gas (Nippon Gases, 99.999%) as balance purge gas and sample purge gas, respectively. For each case, an open platinum pan with ca. 1.5–2.5 mg of sample was automatically introduced by the apparatus into the furnace chamber. The thermal program consisted of quick heating from room temperature to 105 °C, followed by a 15-min isotherm at this temperature to eliminate traces of humidity or other volatiles, and completed with a heating ramp at a rate of 10 °C/min up to 800 °C. The software Universal Analysis 2000 by TA Instruments was used to process the spectra.

#### 2.5.3. Differential scanning calorimetry (DSC)

DSC analyses were carried out in a TA Instruments DSC Q2000 differential scanning calorimeter, equipped with an RCS 90 refrigerated cooling system, and using a flow rate of 50 mL/min of nitrogen gas as the purge gas. For each run, ca. 5–15 mg of sample was placed in an aluminium pan, which was subsequently closed with a pierced lid of the same material. An analogous empty pan with a lid was used as a reference. The thermal program included three cycles, each of which comprised the following steps: a heating ramp at a rate of 10 °C/min up to 200 °C, a 5-min isotherm, a cooling ramp at a rate of −10 °C/min down to 0 °C, and another 5-min isotherm. The repetitiveness of at least the second and third cycles was ensured. Glass transition (midpoint) temperatures were determined from the signal of the heating ramp of the third cycle with the corresponding function of the software Universal Analysis 2000 by TA Instruments.

#### 2.5.4. Attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy

ATR-FTIR spectra were collected in a Bruker Alpha II spectrometer with a diamond crystal attenuated total internal reflectance (ATR) accessory, from 4000 to 400 cm$^{-1}$, at a resolution of 2 cm$^{-1}$ and 4 scans per sample through Opus software.

#### 2.5.5. Antioxidant activity

The radical scavenging activity (RSA) of the lignin samples was determined by adapting a previously reported method [24], with a 50% (v/v) mixture of water and acetone (Fisher Scientific, ≥ 99.8%) as the solvent instead of 80% (v/v) aqueous ethanol. The method is based on the reduction of the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH, provided by Sigma-Aldrich with Quality Level 100) by an antioxidant, decreasing its absorbance at a wavelength of 515 nm. A commercial standard antioxidant known as 2, 6-di-tert-butyl-4-methylphenol, commonly referred to as butylated hydroxytoluene (BHT, supplied by Sigma-Aldrich with a nominal purity of 99%), was used as a reference. The absorbances were measured in a Biotek Cytation3 UV–vis spectrophotometer after incubating the samples in microplates for 30 min at room temperature in a light-protected environment.

#### 2.5.6. Minimal inhibitory concentration (MIC)

The evaluation of MICs was carried out through the micro-dilution methodology described by the Clinical and Laboratory Standards Institute [25], with some modifications. Initially, *S. aureus* and *E. coli* strains were grown into nutrient agar (NA, Oxoid) at 37 ± 2 °C for 24 h. Subsequently, a single colony of each strain was placed into 20 mL of Nutrient Broth (NB, Oxoid) and incubated at 37 °C, 150 rpm, for 18 h, and then both bacterial cell suspensions were adjusted to an optical density (80–82% in the McFarland standards) between 0.09 and 0.11, measured at 620 nm, indicating a concentration of 1 × 10$^{8}$ CFU/mL. After that, the inocula were properly diluted in NB to 1 × 10$^{6}$ CFU/mL. On the other hand, solutions of the lignin samples diluted in DMSO (anhydrous, Invitrogen, >99.5%) were prepared from concentrations of 300 mg/mL and serially diluted to obtain the following concentrations: 234.50, 117.25, 58.63, and 29.50 μg/mL. Concentrations above 234.50 μg/mL were not considered due to their too-high absorbance.

Experiments were carried out in a sterile 96-well microplate, in which 100 μL of inoculum suspension was added to a 100 μL sample. The microplate was incubated at 37 ± 2 °C for 24 h, and the absorbance was measured at 620 nm using a spectrophotometric microplate reader (Sunrise Tecan, Grödig, Austria). The behaviour of the samples was evaluated against growth and sterility controls, which consisted in using 100 μL of NB plus 100 μL of inoculum suspension as a microbial growth control (negative control) and 200 μL of NB as a sterility control (positive control). Finally, the concentrations of each treatment capable of inhibiting growth were determined when compared with the negative control.

### 2.6. Characterisation of the aqueous phase

#### 2.6.1. HPLC chromatography

Aqueous phases obtained as filtrates after the lignin treatments were analysed by high-performance liquid chromatography (HPLC) in an HP Series 1100 HPLC chromatograph equipped with an HP G1315A diode array detector. A reversed-phase KromaPhase 100 C18 column by Scharlau (4.6 mm × 200 mm, 5 μm particle size) and a pre-column of the same material were used, at 40 °C. The injection volume was 4.0 μL, and the flow rate of the mobile phase was 1.0 mL/min. The HPLC grade solvents used were aqueous 0.1% formic acid (purchased from Scharlau as an aqueous solution with nominal purity >98%) as solvent A and acetonitrile (Supelco, ≥ 99.9%) as solvent B. The elution gradient for solvent B was as follows: from 0.0 to 15 min, eluent B at 5%; from 15 to 50 min, a linear increase to 60%; from 50 to 55 min, a linear increase to 100%; and column equilibration from 55 to 60 min at 5%. Phenolic compounds were identified by comparing their retention times with those of different standards, namely: benzyl phenyl ether (Aldrich, 98%), caffeic acid (Sigma, >98%), catechin hydrate (Sigma-Aldrich, 98%), chlorogenic acid (Aldrich, 95%), *trans*-cinnamic acid (Sigma-Aldrich, 99%), *p*-coumaric acid (Sigma, >98%), 3,4-dihydroxybenzoic acid (Aldrich, ≥99%), ellagic acid (Alfa Aesar, 97%), epicatechin

(Sigma-Aldrich, 97%), *trans*-ferulic acid (Aldrich, 99%), gallic acid (Merck, ≥98.5), guaiacol (Sigma-Aldrich, 99%), 4-hydroxybenzoic acid (Sigma-Aldrich, 99%), isoeugenol (Aldrich, 98%), naringin (Sigma, ≥90%), quercetin dihydrate (Sigma-Aldrich, >98%), syringic acid (Sigma-Aldrich, ≥95%), vanillic acid (Alfa Aesar, 98%), and vanillin (Sigma-Aldrich, 99%). Several wavelengths were evaluated, selecting for each compound the wavelength that offered the best resolution of its peak. Samples were run in triplicate, and the concentration results were expressed as average values after discarding possible outliers based on a statistical treatment at a 95% confidence level.

#### 2.6.2. Total phenolic content

The concentration of total phenolic compounds in the aqueous phases was determined through a procedure adapted from that described by Makkar et al. [26]. According to this procedure, 0.5 mL of the conveniently diluted sample was mixed in test tubes with 0.25 mL of a 1 N aqueous solution of the Folin-Ciocâlteu reagent, which was supplied by Sigma (Quality Level 100) with an original concentration of 2 N, and then 1.25 mL of a 20% (w/w) solution of $Na_2CO_3{\cdot}10H_2O$ (Sigma-Aldrich, 99%) was added. After 40 min of incubation at room temperature, the absorbance was recorded at 725 nm in a Biotek Cytation3 UV–vis spectrophotometer. The total content of phenolic compounds was then obtained as equivalents of gallic acid using the appropriate calibration curve (using anhydrous gallic acid purchased from Sigma with nominal purity >98%).

## 3. Results and discussion

### 3.1. Laccase stability

The commercial laccase from *M. thermophila* used in this work has an acidic optimum pH of 5.0. Maximal laccase activity was found in 0.1 M sodium acetate buffer, pH 5.0, while no significant difference in the enzyme activity was verified from 25 °C to 35 °C (data not shown). Upon addition of a 10% (w/w) of the IL [$C_2$mim][OAc], the pH was found to rise to 6.1 (the IL is contributing acetate anions to the medium), while the residual activity of the enzyme in the presence of IL corresponded to 52% of the initial activity in the buffered medium with no IL, after 12 h incubation. At higher IL concentrations (particularly 20% and 30%, in which the medium pH changes to 6.6 and 7.1, respectively), the enzyme was not stable at the given concentration of sodium acetate buffer. Dominguez et al. [27] reported that the stability of laccase from *T. versicolor* decreases at high concentrations of imidazolium ILs. Stevens et al. [28] reported that a laccase from the hyperthermophilic bacterium *Thermus thermophilus* lost more than 50% activity in only 2% (w/v) [$C_2$mim][OAc], despite its thermophilicity, with the cation favouring the binding of the IL to a region close to the active site. On the other hand, Harwardt et al. [17] reported that laccase stability from *T. versicolor* at 15% (v/v) [$C_2$mim][OAc] and [$C_2$mim][$EtSO_4$] in buffer solution was higher compared to laccase in buffer, and was also depended on the anionic part of the ILs toward the enzyme. Thus, besides IL, the source of laccase (microorganism) also seems to be an important point to be considered, once microorganisms present the capability to bio-synthesise enzymes with different specificities.

### 3.2. Modification of lignin with LMS

#### 3.2.1. Solid recovery yield

The percentages of lignin recovered after the different treatments of Indulin AT are shown in Fig. 1. In general, all percentages are quite high, roughly lying in the range of 80–90%. The fraction of sample lost is a result of the inherent solubility of part of Indulin AT in the tested media (ca. 8–9%) and the action of laccase in generating additional soluble materials. No big differences are observed among treatments, although a slightly higher solid recovery percentage can be noticed for the treatments with IL compared to their corresponding pairs. This may be partly due to the increase in pH caused by the presence of IL in the treatment medium, which results in a decrease in enzyme activity.

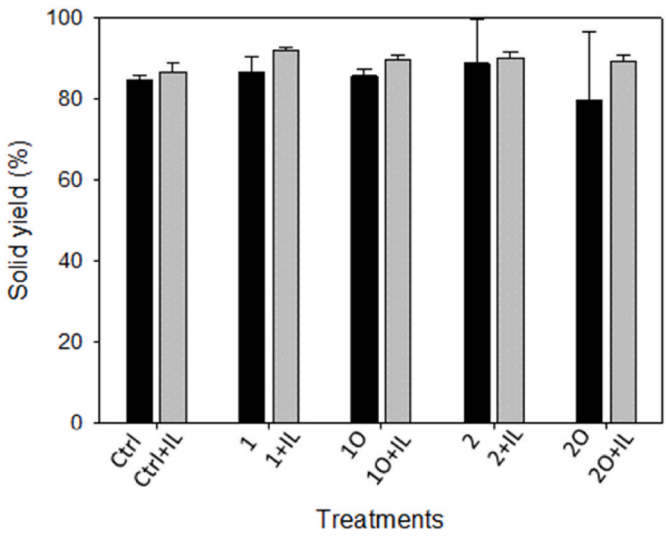


**Fig. 1.** Percentage of lignin sample recovered after the treatments. Black and grey bars correspond to the treatments without and with ionic liquid (IL), respectively. 'Ctrl' and 'Ctrl+IL' correspond to treatments with only lignin and the reaction medium (acetate buffer or 90:10 mixture of buffer and ionic liquid, respectively) (controls); '1' correspond to lignin treatments with laccase in the reaction medium; '2' correspond to lignin treatments with laccase and mediator (ABTS) in the reaction medium; and 'O' correspond to treatments with pre-oxygenation of the reaction medium.

#### 3.2.2. Structural NMR characterisation

HSQC NMR spectroscopy is one of the most commonly used methods to determine molecular structures in lignin. Fig. 2 shows an example of a spectrum, namely the Ctrl+IL sample, with the identification of signal regions associated with specific structural features [22,29,30]. The analogous spectra for the other lignin samples recovered from the different treatments, as well as for raw Indulin AT, can be inspected in Figs. S1-S3 in Supplementary Data. The areas under the peaks in the labelled regions were integrated in all the spectra. However, the absolute values of these integrated areas were ruled out for quantitative comparison among treatments, since such values were affected by variations in the concentration of the NMR sample prepared for analysis and by processing (particularly the step of phase correction of the Fourier-transformed signal) of each spectrum in the specialised software to achieve an optimised peak visualisation. Instead, the area ratios related to the largest area (systematically the $C_\beta$ region) were calculated for subsequent comparison. Table S1 in Supplementary Data shows that most ratios are relatively small (lower than 0.15). The ratio between the $G_2$+$G_5$+$G_6$ region (or simply the $G$ region) and the $C_\beta$ region is the exception. Such ratio, denoted as the $G/C_\beta$ ratio, is shown in Fig. 3 for all treatments carried out. The $G$ region is constituted by signals from the atoms in the aromatic ring of guaiacyl units ($G$-units). Thus, the lower values of the $G/C_\beta$ ratios of the treated lignin, compared to the raw Indulin AT, indicate that the treatments tested cause a proportionally greater disappearance, in the lignin, of these $G$-units with regard to the non-aromatically bonded $C_\beta$ atoms. This is consistent with the reported consideration of the β-O-4 bond as being the most easily cleavable bond in lignins through oxidation, which in turn may facilitate the release of $G$-units from the solid phase [31]. Similar conclusions to those from the $G/C_\beta$ ratios in Fig. 3 would be achieved if calculating an "expanded ratio" using all the identified aromatic/unsaturated regions ($G_2$+$G_5$+$G_6$ plus $FA_2$ plus $H_{2,6}$+$J_\beta$) in the numerator and all the identified aliphatic oxygenated side-chain regions ($A_\alpha$+$A'_\alpha$ plus $A_\gamma$ plus $B_\gamma$ plus $C_\beta$ plus $C_\gamma$) in the denominator [32] – see numerical values in the bottom row of

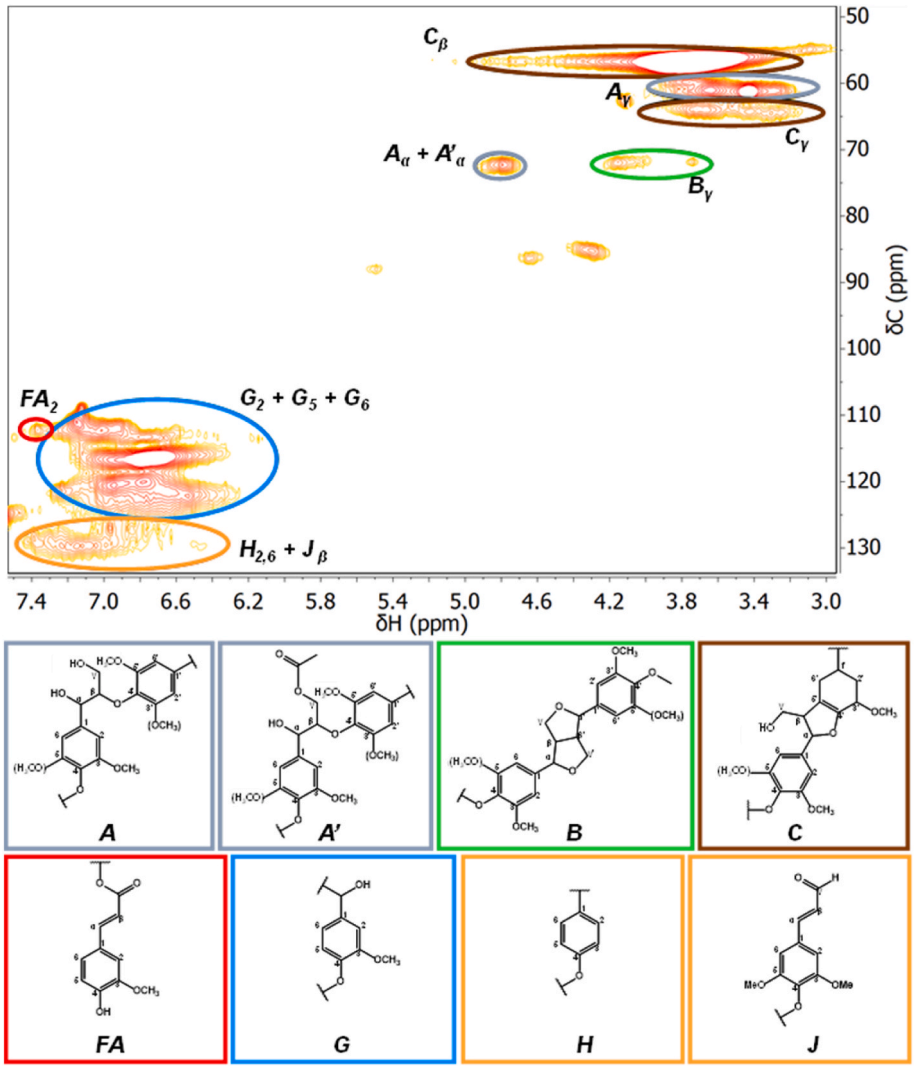


**Fig. 2.** 2D $^{1}H–^{13}C$ HSQC NMR spectrum of the Ctrl+IL recovered lignin sample, showing the structures identified and the integration areas used to compare the effect of lignin treatments.

Table S1 in Supplementary Data. Longe et al. [32] reported some changes in lignin structure after treatment with laccase and ABTS or violuric acid as mediators, such as a general decrease in the relative amount of *G*-units, and these changes appeared to be mediator-dependent. Rico et al. [33] also reported a decrease in *G*-units after treatment of lignin with *M. thermophila* laccase and methyl syringate mediator.

If comparing the set of treatments without IL and the set of treatments with IL, clearly different trends can be observed in Fig. 3. In the treatments carried out in absence of IL, an evident decreasing trend of the $G/C_\beta$ ratio occurs in the order: Ctrl >1 > 2 > 1O > 2O; which corresponds in general with the intensity of the treatment: laccases only (1), laccases with ABTS (2), laccases with an $O_2$ pre-saturation of the medium (1O), and laccases with ABTS and the $O_2$ pre-saturation (2O). A specific comparison of the $G/C_\beta$ ratio for treatments 1 and 2 reveals that the redox potential of the ABTS mediator is more suitable than that of the laccases to react on the *G*-units. Similarly, direct comparisons of treatments 1 and 1O and treatments 2 and 2O manifest the relevance of supplementation with $O_2$ over naturally dissolved $O_2$ in the transformation of these aromatic units with the investigated laccase (-mediator) system. In any case, it is important to note that the laccase from *M. thermophila* is a low redox potential laccase (465 mV; [34]), showing the importance of the ABTS to increase the oxidative capacity of the enzyme. Regarding the set of treatments involving the IL, a similar value of $G/C_\beta$ ratio was obtained, independently of the treatment conditions, even for the control run (in the absence of enzyme). This points to the presence of [$C_2$mim][OAc] as the main responsible for the elimination of the *G*-units from the treated lignin, with the potential action of the enzyme being masked by such IL effect (for example, via the decrease of the laccase activity as a result of increased pH or ionic strength due to the presence of [$C_2$mim][OAc] in the medium, among other possible effects).

### 3.2.3. Thermal characterisation and FTIR analysis

Thermal stability and decomposition rate are greatly influenced by the internal structure of the lignin [35]. Fig. 4 shows the TG curves and the corresponding derivative (DTG) curves for raw Indulin AT and the lignin samples recovered from treatments 2 and 2+IL, as representatives of treatments without and with IL, respectively (the entire set of TGA thermograms can be inspected in Fig. S4 and S5 in Supplementary Data). Different steps in the decomposition process can be identified [29,35]. An initial decomposition, caused by dehydration of the lignin and removal of low molar mass volatiles, is observed in all thermograms up to ca. 200 °C. In the approximate range of 200–500 °C, mass loss is related to the decomposition of the side chains and the decomposition of the C–C linkages. In the case of raw Indulin AT, two peaks in the DTG curve can be identified in this range, the first one (associated with the side chains) being much larger than the second one (associated with the

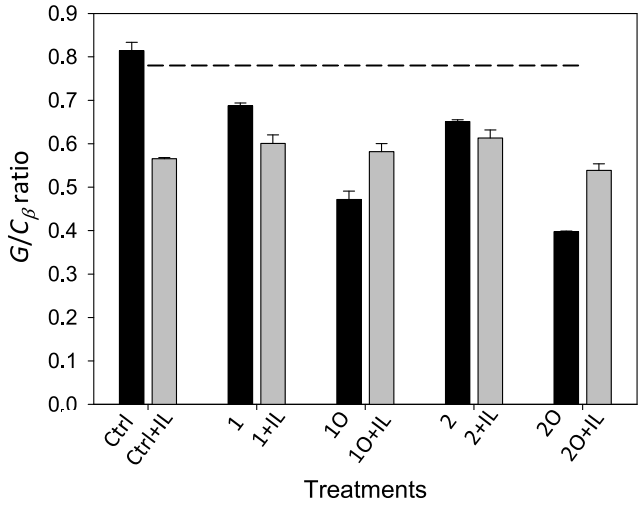


**Fig. 3.** $G/C_\beta$ ratio calculated from the integration of selected areas in the HSQC NMR spectra. The horizontal dashed line represents the value for raw Indulin AT, the black columns represent the lignin samples from the treatments without ionic liquid, and the grey columns are those lignin samples from the treatments with ionic liquid.

C–C linkages). A shift of the first peak, overlapping the second small peak, is observed in the case of treatment 2; so, probably in this treatment, the side chains associated with lower thermal stability in raw Indulin AT are transferred to the liquid phase. Interestingly, the decomposition pattern in this range is notably different for the sample recovered from treatment 2+IL, where the two independent peaks are observed again in the derivative curve, but with the second one being larger than the first one, and the first one not being shifted for higher temperatures compared to raw Indulin AT. This invites to think of a more extensive transfer of side-chain moieties to the aqueous phase and even a possible rearrangement of the bonds in the lignin structure as a result of the presence of [$C_2$mim][OAc] in the treatment medium. Finally, above ca. 500 °C, which is the step where the decomposition of the aromatic rings occurs, all samples behave quite similarly. Thus, it can be assumed that aromatic rings are not particularly affected by any of the treatments investigated herein.

The large amount of residue (char) observed at the end of all TGA thermograms, at 800 °C, is likely a result of the high percentage of *G*-units in Indulin AT, since these units can easily undergo ionic condensation and radical coupling reactions at the ortho and meta positions for phenolic OH groups in aromatic rings [29,36]. At a 95% confidence level, there is no statistical difference between the residues at 800 °C for the samples from the treatments without the IL (1, 2, 1O, and 2O), nor between those for the samples from the treatments with the IL (1+IL, 2+IL, 1O+IL, and 2O+IL). The mean residuals for these two sets were 43.0 ± 0.7% and 44.6 ± 0.8%, respectively. In both cases, these char values are comparatively lower than the 48.1 ± 0.8% obtained for raw Indulin AT. This is compatible with the transformation of some *G*-units from the raw material into other value-added compounds in processing with the treatments tested herein.

A glass transition ($T_g$) value of 156 °C was found for raw Indulin AT by DSC, which is a value similar to that reported by other researchers for Indulin AT (although the water content of the sample highly influences its $T_g$, and therefore values in a range as wide as 90–180 °C can be found in the literature) [37,38]. For the lignin samples recovered from all treatments without IL, the $T_g$ values obtained from the corresponding DSC thermograms are somewhat higher and lie in the narrow range 167–173 °C, including the control sample. This slight increase in $T_g$ may be due to the dissolution of low molecular weight moieties in the aqueous phase [39], while the presence of the enzyme and mediator in

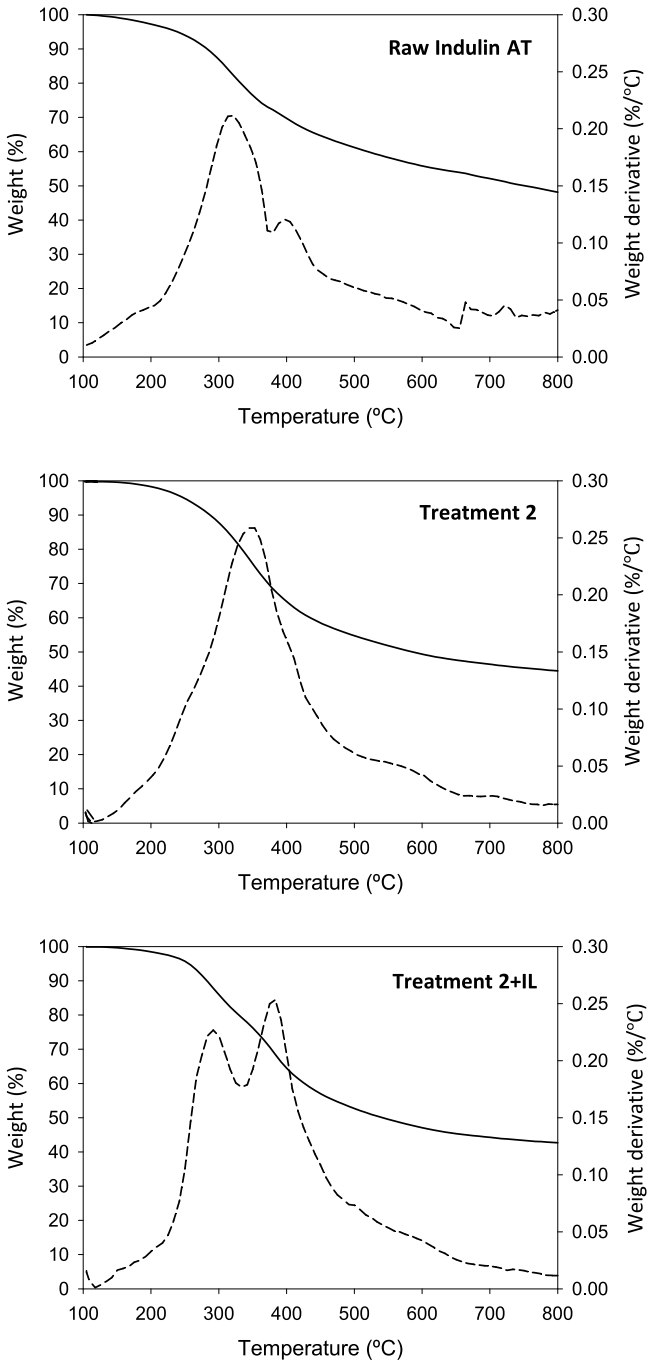


**Fig. 4.** TG (solid lines) and DTG (dashed lines) curves for raw Indulin AT (top) and the lignin samples recovered after treatments 2 (centre) and 2+IL (bottom).

the treatments would have little influence. However, for the lignin samples recovered from treatments involving the IL, the $T_g$ obtained was essentially the same as that of raw Indulin AT: in the range 155–157 °C, including the corresponding control sample (Ctrl+IL). This might be a result of the balance between a greater degree of deconstruction of the original lignin structure due to the applied treatment and the loss of those low molecular weight moieties getting into the aqueous phase. What can be ruled out is the existence of a potential reaction of the lignins with the IL, as evidenced by the FT-IR spectra in Fig. 5: all of them (for treatments involving the IL or not) are analogous to the spectrum of raw Indulin AT, and show characteristic signals associated with different structural features of lignin. Among these, the following can be particularly cited (and marked with vertical dashed lines in

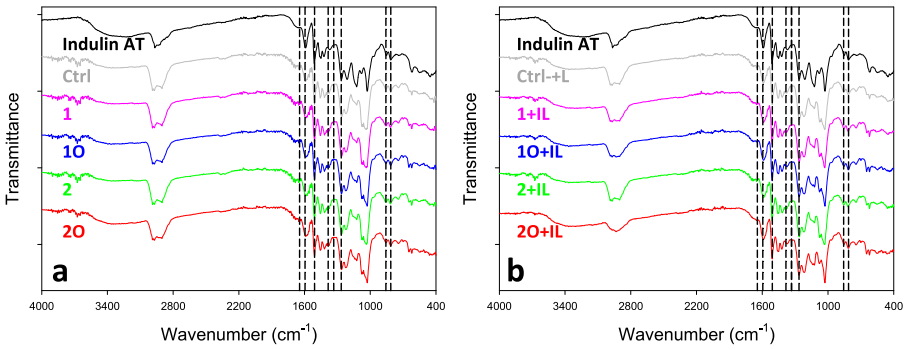


**Fig. 5.** FT-IR spectra of raw Indulin AT and of the solid samples recovered after each treatment: a) treatments in the absence of ionic liquid; b) treatments with the presence of ionic liquid [$C_2$mim][OAc]. Treatment codes are displayed on the corresponding spectral lines in the same colour. Dashed vertical lines in plots c and d correspond to the characteristic signals of lignin (see the main text for details). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Fig. 5): quinonoid structures at 1645 $cm^{-1}$, aromatic skeletal vibrations and C=O stretch at 1595 $cm^{-1}$, aromatic skeletal vibration at 1509 $cm^{-1}$, phenolic hydroxyl at 1384 $cm^{-1}$, syringyl ring vibration at 1333 $cm^{-1}$, guaiacyl ring breathing at 1265 $cm^{-1}$, and C–H out-of-plane vibrations of carbons 2, 5 and 6 of the guaiacyl ring at 857 $cm^{-1}$ and 813 $cm^{-1}$ [23]. Stevens et al. [18] also reported that alkaline lignin treated with laccase, ABTS, and aqueous ILs (including [$C_2$mim][OAc]) showed few structural changes, although the lignin was partially solubilised and converted to degradation products. All individualised values of $T_g$ obtained for the different lignin samples are listed in Table S2 in Supplementary Data, and the entire set of DSC thermograms is displayed in Figs. S6 and S7 in Supplementary Data.

### 3.2.4. Antioxidant and antimicrobial capacities

The radical scavenging activity (RSA) was measured to evaluate the antioxidant capacity for all lignin samples recovered after the different treatments. The corresponding values, expressed as the percentage of total inhibition of DPPH oxidation, are listed in Table 1. A small decrease in the RSA percentage concerning raw Indulin AT (about 2–3%) is already observed for control runs. For the sets of treatments with laccases, a smaller reduction than for the corresponding control run, or even an increase in the RSA percentage, is observed. Nevertheless, all values lie in the range 73–79%, thus representing small variations, even statistically non-significant for several entries, with regard to the intrinsic antioxidant activity of the lignin model tested (RSA of 76.5%). Although somewhat lower, the RSA percentages of all lignin samples are still reasonably comparable to the value of 87.1% obtained for a widely recognised antioxidant such as BHT; so, it can be stated that the quite good antioxidant activity exhibited by Indulin AT, is barely affected by the treatments proposed in this work. In parallel, the concentrations of the raw or treated lignin to achieve a 50% inhibition of radical species ($IC_{50}$) were approximately double of those in BHT: 0.188–0.213 mg/mL versus 0.093 mg/mL. For this $IC_{50}$ parameter, again, the conditions of the different lignin treatments have little effect with respect to raw Indulin AT – see Table 1. On the other hand, Li et al. [40] have reported an improvement in the antioxidant capacity of alkali and enzymatic hydrolysis lignins treated with laccase from *Aspergillus oryzae* compared to untreated lignin, with the enzymatic hydrolysis lignin having an $IC_{50}$ lower than the untreated lignin and BHT (0.029 vs. 0.060 vs. 0.038 mg/mL, respectively).

**Table 1**
RSA and $IC_{50}$ of raw Indulin AT and lignin samples recovered after the corresponding treatments. Values for the standard antioxidant BHT are added for comparison. Values in parenthesis represent the radius of the confidence interval at a 95% confidence level.

| Sample | RSA (%) | $IC_{50}$ (mg/mL) |
|---|---|---|
| Raw Indulin AT | 76.5 (1.6) | 0.188 (0.001) |
| Ctrl | 74.1 (0.2) | 0.211 (0.001) |
| 1 | 75.0 (0.4) | 0.202 (0.012) |
| 1O | 76.2 (0.7) | 0.209 (0.008) |
| 2 | 76.2 (0.2) | 0.200 (0.009) |
| 2O | 79.0 (1.7) | 0.191 (0.008) |
| Ctrl+IL | 73.2 (0.3) | 0.198 (0.001) |
| 1+IL | 74.2 (0.1) | 0.213 (0.014) |
| 1O+IL | 75.9 (0.3) | 0.191 (0.008) |
| 2+IL | 74.8 (0.2) | 0.191 (0.006) |
| 2O+IL | 76.3 (0.4) | 0.194 (0.000) |
| BHT | 87.1 (4.1) | 0.093 (0.004) |

The antimicrobial capacity was evaluated for those treated lignin samples that exhibited a substantial modification of their structure according to the NMR results – namely the 2O and 2O+IL samples. In addition, raw Indulin AT was tested for comparison purposes. The obtained results are shown in Fig. 6. As can be seen, a decrease ($p < 0.05$) in the *E. coli* and *S. aureus* growth (~30% and ~10%, respectively) was observed when raw Indulin AT was used, independently of the concentration tested. The lignin sample recovered from the 2O treatment caused a significant inhibition ($p < 0.05$) against *S. aureus* growth from the concentration of 117.25 μg/mL, achieving ~80% inhibition at 234.5 μg/mL. This lignin also led to a reduction ($p < 0.05$) of *E. coli* growth at 234.5 μg/mL (24% inhibition) when compared with the growth control, while, for other concentrations studied, the inhibition detected was lower (~13%). The lignin sample recovered after the treatment 2O+IL showed an inhibition of 78% over the Gram-positive strain at the concentration of 117.25 μg/mL ($p < 0.05$), while with the Gram-negative strain, a 24% inhibition was found at 234.5 μg/mL ($p < 0.05$). It is remarkable that LMS treatments, with or without IL, improved the antimicrobial capacity of lignin against the Gram-positive bacterium *S. aureus*. This can be related to the number of functional groups and the molecular weight of the treated lignin, as reported by Yun et al. [41], who found that the studied original lignin and fractionated lignins can inhibit the growth of Gram-negative bacteria (*E. coli* and *Salmonella*) and Gram-positive bacteria (*Streptococcus* and *S. aureus*), in which the lignin fraction with the lowest molecular weight and highest phenolic hydroxyl content showed the best antimicrobial capacity.

### 3.3. Lignin-derived compounds in the liquid medium after treatment

The total concentration of phenolic compounds in the aqueous phase after each treatment, as determined by the method of Folin-Ciocâlteu, is shown in Fig. 7. For all treatments involving the IL, these concentrations are notably higher than for any of the treatments without IL. As stated before, the presence of [$C_2$mim][OAc] in the treatment fluid leads to a moderate increase in pH, which can facilitate the transfer of lignin material from the solid to the liquid phase in the form of phenolic compounds. Interestingly, among treatments with the presence of IL, samples from treatments without a mediator (1+IL and 1O+IL) led to a phenolic concentration similar to that of the control run (Ctrl+IL). In contrast, those treatments with the combination enzyme + mediator resulted in a higher concentration of phenolic compounds in the aqueous phase, highlighting the key role of ABTS in generating water-soluble phenolic compounds during the lignin treatment. On the other hand, pre-saturation with oxygen did not cause any relevant effect on

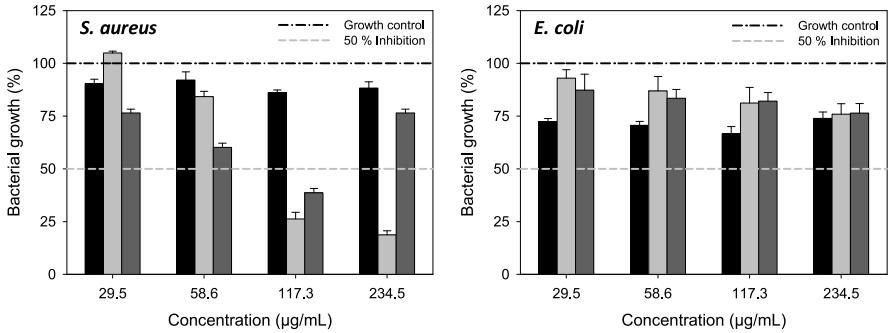


**Fig. 6.** Bacterial growth as a result of the effect of different concentrations of raw Indulin AT (black bars), lignin sample from treatment 2O (light grey bars) and lignin sample from treatment 2O+IL (dark grey bars) on *Staphylococcus aureus* and *Escherichia coli.*

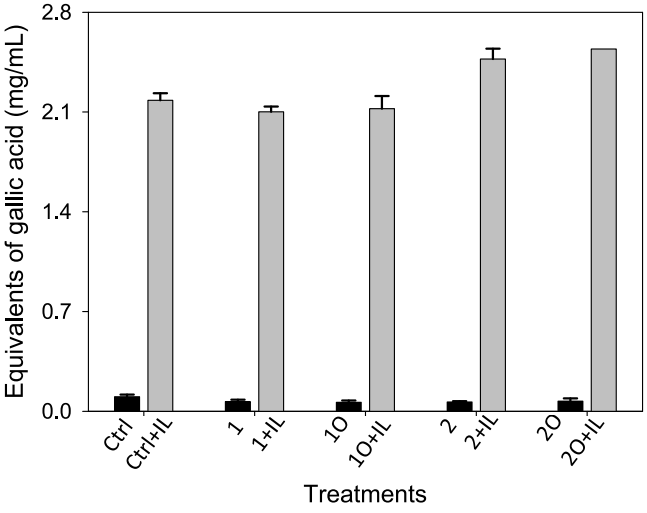


**Fig. 7.** Total phenolics concentration, expressed as equivalents of gallic acid, in the aqueous phase after each lignin treatment. Black and grey columns correspond to treatments without and with ionic liquid, respectively.

this total concentration of phenolic compounds in the aqueous phase, as evidenced by a comparison of the bars of treatments 1+IL and 1O+IL, and treatments 2+IL and 2O+IL. One further aspect to be mentioned is that, in some treatments, the control run (Ctrl) seems to provide a slightly higher concentration of phenolic compounds in the aqueous phase than all enzymatic treatments. This may result from the recondensation action of laccases on the lignin substrate since the depolymerisation process is an equilibrium reaction that also involves the repolymerisation of low molecular weight oligomers [42].

A considerable number of compounds contributing to the total phenolic concentration were identified by HPLC and individually quantified. Table 2 explicitly lists those for which concentrations higher than 10 mg/L were obtained in at least one of the treatments. Other phenolic compounds, namely caffeic acid, chlorogenic acid, 3,4-dihydroxybenzoic acid, isoeugenol, quercetin dihydrate, and syringic acid, were identified in some of the treatments, but always in concentrations lower than 10 mg/L. In agreement with the very small content of syringyl units (*S*-units) reported for lignin from conifers [43,44], the relevant phenolic compounds in Table 2 come from either *p*-hydroxyphenyl units (*H*-units) or *G*-units. This can be easily linked with the relative reduction of *G*-units in the recovered solid, as evidenced through the discussion of the evolution of the $G/C_\beta$ ratio – see section 3.2.2; but also, with a reduction that can be observed in Table S1 in Supplementary Data for the ratio $(J_\beta + H_{2,6})/C_\beta$. Although the latter is a much smaller ratio in magnitude (and hence it was not the object of the central discussion of section 3.2.2), it is significantly reduced in the treatments without IL, and less reduced in the treatments involving IL (in this case with affection in the Ctrl+IL treatment). This indicates a relative reduction of the *H*-units in the recovered solid (since the area of the $H_{2,6}$ structures corresponds to *H*-units, and the contribution of the area of the $J_\beta$ structures, connected with *S*-units, is systematically very small). Therefore, it is also compatible with the identification of phenolic compounds derived from *H*-units in the post-treatment liquid phases in appreciable concentrations. Given the fact that only about 20–30% of the peaks identified by HPLC could be typically assigned to available standards, any attempt to move from this qualitative analysis

**Table 2**
Concentration of phenolic compounds (expressed in mg/L) identified in the aqueous phase of the different treatments (at concentrations equal or higher than 10 mg/L in at least one of the treatments).

| Phenolic compound[a] | Treatment | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Ctrl | 1 | 1O | 2 | 2O | Ctrl+IL | 1+IL | 1O+IL | 2+IL | 2O+IL |
| Benzyl phenyl ether (H) | 49 | 46 | 44 | 43 | 33 | 33 | 34 | 33 | 33 | 34 |
| Catechin hydrate (H) | 116 | 73 | 63 | 494 | 596 | 92 | 87 | 80 | 78 | 63 |
| *trans*-Cinnamic acid (H) | 87 | 103 | 79 | 94 | 103 | 149 | 139 | 140 | 145 | 130 |
| *p*-Coumaric acid (H) | <10 | <10 | <10 | <10 | <10 | 10 | 10 | 10 | 380 | 300 |
| Ellagic acid (H) | <10 | <10 | <10 | <10 | <10 | 39 | 49 | 46 | 41 | 33 |
| Epicatechin (H) | 10 | <10 | 19 | 25 | 10 | 28 | 26 | 24 | 25 | 23 |
| *trans*-Ferulic acid (G) | <10 | <10 | <10 | <10 | <10 | 10 | 10 | <10 | 14 | 14 |
| Gallic acid (H) | <10 | 43 | <10 | 303 | 273 | 10 | 11 | 11 | 13 | 10 |
| Guaiacol (G) | 53 | <10 | <10 | <10 | <10 | 68 | 64 | 58 | 65 | 54 |
| 4-Hydroxybenzoic acid (H) | <10 | <10 | <10 | 212 | 244 | <10 | <10 | <10 | <10 | <10 |
| Naringin (H) | <10 | <10 | <10 | <10 | <10 | 93 | 88 | 76 | 82 | 67 |
| Vanillic acid (G) | <10 | <10 | <10 | <10 | <10 | 73 | 69 | 62 | 71 | 73 |
| Vanillin (G) | 86 | 97 | 74 | 26 | 25 | 76 | 95 | 94 | 173 | 95 |

[a] Letters in parentheses after the names of the compounds refer to the unit from which they originate: H for *p*-hydroxyphenyl units, and G for guaiacyl units.

to a more quantitative correlation of the disappearance of *G*-units or *H*-units in the recovered solid with the appearance of phenolic compounds derived from these *G*-units or *H*-units would be inappropriate. In any case, from a simultaneous consideration of the NMR data from section 3.2.3 and the HPLC data in the present section (Table 2), it can be presumed that a majority of the unidentified compounds in the liquid phase should likely derive from *G*-units.

In the general analysis of the data in Table 2 for the entire set of treatments, the treatments using enzyme without mediator (treatments 1/1O and 1+IL/1O+IL) do barely influence the concentration of the identified phenolic compounds with respect to the corresponding controls (treatments Ctrl and Ctrl+IL, respectively). However, adding the mediator ABTS leads to notably higher concentrations of specific phenolic compounds. In the case of treatments without IL (treatments 2 and 2O), the concentrations of catechin hydrate, gallic acid, and 4-hydroxybenzoic acid are boosted. For treatments 2+IL and 2O+IL, the relevant increase in the concentration of *p*-coumaric acid and vanillin is observed with respect to Ctrl+IL. It can be said, consequently, that the presence of the IL in the treatments with LMS is capable of conditioning the nature of the depolymerisation-derived phenolic compounds for which a boosted concentration in the aqueous phase is obtained, with respect to the corresponding control treatments with no LMS.

## 4. Conclusions

A representative technical lignin with limited water solubility, namely Indulin AT, was treated at 25 °C with a recombinant laccase from *Myceliophthora thermophila* and with a laccase-mediator system (consisting of this laccase and ABTS) in a mildly acid aqueous medium, in the presence and the absence of 10% IL [$C_2$mim][OAc]. In the recovered solids, a preferential decrease of guaiacyl units was observed with respect to raw Indulin AT, with an enhancement in this effect when a pre-saturation of the treatment media with $O_2$ was carried out. The solid from the studied treatments (2O and 2O+IL) presented enhanced antimicrobial activity against *S. aureus,* compared to raw Indulin AT. A good number of low-molecular-weight compounds were identified in the aqueous filtrates resulting from the treatments, originating from both guaiacyl and *p*-hydroxyphenyl units. Although relatively low concentrations have been typically found, the levels of some phenolic compounds (catechin hydrate, gallic acid, and 4-hydroxybenzoic acid for treatments in the absence of [$C_2$mim][OAc], and *p*-coumaric acid and vanillin for treatments in the presence of this IL) were particularly boosted when performing the treatment with the laccase-mediator system compared to the treatment with laccase alone. Pre-saturation with $O_2$ had little effect on the concentrations of phenolic compounds obtained in the aqueous phases.

## Authors' contributions

CAP: Methodology, Formal analysis, Investigation, Writing – Original draft, Visualisation. LFB: Methodology, Validation, Investigation, Writing – Review and editing. HR: Conceptualisation, Writing – Review and editing, Supervision, Project administration, Funding acquisition. ER: Writing – Review and editing, Funding acquisition. JAT: Funding acquisition, Writing – Review and editing. MM: Conceptualisation, Methodology, Funding acquisition, Writing – Review and editing, Supervision.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biombioe.2023.106928.

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