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ORIGINAL ARTICLE

Treatment of wood fibres with laccases: improved hardboard properties through phenolic oligomerization

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Abstract
Laccase-treated wood fibres were tested for small-scale wet-process manufacture of hardboards. Two laccases of distinct redox potentials, one from *Pycnoporus cinnabarinus* and one from *Myceliophthora thermophila*, were compared in terms of their effect on the physical–chemical properties of the treated fibres and hardboards. Wood fibres were produced from Norway spruce (*Picea abies*) by thermomechanical pulping. The thermomechanical pulp was treated with each laccase in parallel, in the presence or absence of the synthetic laccase mediator 1-hydroxybenzotriazole (HBT). High-performance size-exclusion chromatography revealed that the ethanolic extractable phenolic compounds in the fibres underwent oligomerization upon enzymatic treatment, and that the extent of oligomerization was dependent on the enzyme source and concentration and on the presence or absence of mediator. Lower lignin oligomerization levels led to higher (up to two-fold) fibre internal bonding, whereas higher lignin oligomerization levels led to higher fibre hydrophobisation. X-ray photoelectron spectroscopy revealed a significant change in surface lignin content. These results demonstrate pre-treatment of spruce fibres with laccase–mediator systems prior to hot processing can improve the mechanical resistance of hardboards while using lower amounts of enzyme.

1 Introduction

Spruce softwood is a highly valuable lignocellulosic renewable raw-material resource for the future production of fuels, materials, and chemicals. This complex material is mainly composed of cellulose, hemicelluloses, and lignin, together with wood extractives that account for 1–5% of dry softwood weight (Baeza and Freer 2001; Spence et al. 2010; Stefanidis et al. 2014). Wood extractives, or 'resins', are the non-structural low-molecular-weight components of lignocellulose. They include terpenoids, waxes and fats, but also phenolics and inorganics. In wood, phenolic extractives are found in the bark and heartwood (Pecha and Garcia-Perez 2015). They are not essential for plant growth, but they protect the wood against pathogens and confer durability (Aloui et al. 2004). Phenolic extractives share the same chemical nature as lignin, and in softwood they are largely composed of guaiacol (Pecha and Garcia-Perez 2015). Softwood lignin is mainly synthesized by the polymerization of coniferyl alcohol units that are linked together through carbon–carbon and carbon–oxygen bonds (Adler 1977). The mechanical properties of wood fibres are mainly determined by the cellulose–hemicellulose matrix. This matrix is glued by polyphenolic lignin, which acts as an adhesive between the different components (Winandy and Rowell, 2005; Gea et al, 2011).

Laccases (benzenediol–oxygen oxidoreductase, EC 1.10.3.2) belong to a large and heterogeneous group of enzymes called polyphenol oxidases. In nature, they are found in plants (Yoshida 1883), fungi (Hatakka 1994; Thurston 1994; Schneider et al. 1999; Pandey et al. 2001; Baldrian 2006), bacteria (Claus 2003; Enguita et al. 2003; Bains et al. 2003; Sharma et al. 2007; Kumar and Chandra 2018), and insects (Kramer et al. 2001). Higher fungi are the main source of today's commercial laccases, and the majority of

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these fungi secrete them into the extracellular environment, where they act on their lignocellulosic substrate. Laccases contain a mononuclear T1 and trinuclear T2/T3 copper centres that catalyse the one-electron oxidation of substrates at the mononuclear Cu site and reduce oxygen to water at the trinuclear Cu site (Baldrian, 2006; Riva 2006). Laccase-mediated oxidation of substrates, such as reactive phenolic monomers, generates phenoxy radicals on the substrate that can then undergo a number of non-enzymatic reactions including covalent coupling to form phenolic dimers, oligomers and polymers through C–C, C–O and C–N bonds (Kudanga et al. 2011). Laccases catalyse not only the oxidation and polymerization of lignin phenolic monomers (monolignols) but also the depolymerization and ring cleavage of complex aromatic polyphenolic compounds if small aromatic molecules acting as mediators are present (Thurston 1994; Breen and Singleton 1999). These mediators can be either synthetic, like 2,2´-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 1-hydroxybenzotriazole (HBT) and violuric acid, or natural compounds, like syringaldehyde, acetosyringone, 2,6-dimethylphenol, 2,4,6-trimethylphenol, acetovanillone, vanillin, vanillyl alcohol, *p*-coumaric acid, and tannins (Cañas and Camarero, 2010; González-Arzola et al. 2009). As initiators of lignin depolymerization, laccase-mediator systems (LMS) can work as a tool for the delignification of recalcitrant biomass. For the wood industry, laccase-based treatments offer "green" alternatives to the toxic adhesives based on urea and phenol formaldehyde resins that are conventionally used to improve macroscopic properties of wood panels, such as swelling and internal bonding (Gonzalez-Garcia et al. 2011).

Rising population growth is driving greater global demand for wood-based products such as building materials, wood-based panels, and furniture. Hardboard (HB), medium-density fibreboard (MDF) and high-density fibreboards (HDF) are the most common fibreboards used today for furniture fabrication. Wet-process hardboards are currently one of the main types of fibreboard manufactured by hot-pressing wood fibres at high pressure with no additional (chemical) adhesives, but MDF or HDF are dry process wood-based board materials that require approximately 8–15% of urea–formaldehyde (UF) and other formaldehyde-based adhesives or isocyanate-based adhesives. Wet-process hardboard production thus has ecological advantages over dry-process fibreboard, particularly in terms of toxic gaseous emissions (Gonzalez-Garc ía et al. 2009; Widsten et al. 2009; Que et al. 2013).

However, the weaker fibre bonding achieved with wet-process hardboard manufacturing leads to worse mechanical properties than those obtained with the addition of an adhesive. Consequently, the hot-pressed hardboards fail to meet the requirements of standard EN 622–2 (i.e. bending strength, internal bonding, and moisture resistance). This problem is currently solved by post-heating the boards in an oven at 165 °C for 2–4 h (Myer and McNat 1985). This thermal treatment enhances fibre–fibre interactions and, thus, board performances, but also increases the required process energy (Luszczak 1979; Gonzalez-Garcia et al. 2011). Therefore, the development of alternative routes to improve hardboard properties is a particularly active and relevant field of research for the wood panel industry.

Laccase pre-treatment of lignocellulosic material prior to hot-pressing is one of the potential alternatives for more energy-friendly technological processes. The incubation of wood fibres with phenol-oxidizing enzymes results in the cross-linking of lignin moieties by means of covalent bonds (Felby et al. 2002, 2004). This mechanism improves adhesion of the wood fibres in the manufacture of future commercial acoustical ceiling tiles (Pelletier et al. 2017), MDF (Widsten et al. 2009; Euring et al. 2011) and fibreboard (Felby et al. 2004; Álvarez et al. 2011) and enables to engineer novel properties by functionalizing lignocellulosic materials (Filgueira et al. 2017).

Here, a hardboard process based on the enzymatic activation of wood fibres from Norway spruce (*Picea abies*) was investigated and optimized at laboratory scale. Two laccases, one sourced from the filamentous fungi *Pycnoporus cinnabarinus* (*Pc*Lac) and another from *Myceliophthora thermophila* (*Mt*Lac), were applied to a wood fibre slurry in the presence of a synthetic mediator (HBT). The low-redox-potential *Mt*Lac (E° of 0.47 V, Xu et al. 1998) is a commercially available enzyme that has been used in many studies focused on fibreboard manufacture (Widsten et al. 2009), organic synthesis (Hollman et al. 2008), conversion of plant biomass (Pardo et al. 2013) and eucalyptus pulp delignification (Babot et al. 2011). It retains activity through a wide pH range (from pH 4–7) and is thermostable up to 60 °C (Berka et al. 1995), which makes it one of the favoured candidates to date for biotechnological applications (Xu 1999). *Mt*Lac has already been applied in the textile industry (Pedersen and Kieruff 1998; Nyman and Hakala 2011), as a biocatalyst for iodine production (Xu 1998), in the beverage industry (Conrad et al. 2002), and in the tobacco industry (Kierulff et al. 2001). The high-redox-potential *Pc*Lac (E° of 0.75 V, Li et al. 1999) was previously shown to be active at acidic pH (3–5) and temperatures up to 65 °C (Record et al. 2002). The fact that *Pc*Lac can be produced at grams-per-litre (Lomascolo et al. 2003) has spurred extensive research into its potential application to the delignification of paper pulps (Poppius-Levlin et al. 1999; Camarero et al. 2004), food and pharmaceuticals, the textile industry (Georis et al. 2003; Labat et al. 2000), and to bioremediation (Prasad et al. 2012; Cañas et al. 2007). HBT has already been tested for pulp and paper applications (Poppius-Levlin et al. 1999; Widsten and Kandelbauer 2008; Gutiérrez et al. 2009; Ravalason et al. 2012; Singh et al. 2015) and was shown to be an

efficient mediator for MDF preparation (Felby et al. 2004). While a number of studies have focused on the reactivity of model phenolic substrates in the presence of laccase or LMS (Kawai et al. 1988; Kawai et al. 1999a, b; Rittstieg et al. 2003; Areskogh et al. 2010; Christopher et al. 2014; Hilgers et al. 2018), only a few studies have focused on the reactivity of phenolic extractives naturally present in lignocellulosic biomass. Therefore, the role of phenolic compounds in the modification of native lignin or raw lignocellulosic material is still not fully understood. There is some evidence that low-molar-mass phenolics enhance laccase-based processes (Felby et al. 2004; Camarero et al. 2005; Schubert et al. 2015), but their reactivity to lignocellulose is still not fully elucidated.

To guide further optimization of industry-scale hardboard preparation, this study focuses on the role and reactivity of the extractible phenolics from Norway spruce fibres during a short application of laccase in the presence of HBT. As no study using LMS for wet-process hardboard preparation is so far available, the present goal was to provide new insight into laccase and LMS-induced oligomerization of phenolics and their possible contribution to the properties of the resulting fibreboard. The wet-process hardboards obtained were characterized through a multi-scale approach in terms of chemical composition, ultrastructure and macroscopic properties via a combination of physical, chromatographic and spectrometric methods.

2 Materials and methods

2.1 Wood fibres

Thermomechanical pulp (TMP) fibres with a moisture content of 14% were produced using Norway spruce (*Picea abies*) woodchips. The spruce chips were refined using a thermomechanical refining process on a reefing pilot plant (Andritz, Austria). Process pressure and temperature were 2.8 bars and 140 °C, respectively. The refining energy applied was 433 kWh ton^{-1} of fibres produced.

2.2 Enzymes

*Pc*Lac was produced and purified as previously described (Berka et al. 1995). Novozym 51,003 *Mt*Lac was kindly provided by Novozymes (Denmark). Laccase standard activity was measured by monitoring oxidation of 500 µmol L^{-1} ABTS at 420 nm ($\varepsilon = 36{,}000$ L cm^{-1} mol^{-1}) in tartrate buffer (50 mmol L^{-1}, pH 4) at 25 °C for 1 min. Number of standard enzyme activity units (U) was expressed as µmol of ABTS oxidized per minute (Herpoël et al. 2000).

2.3 Enzymatic treatment of wood fibres

Each test used the equivalent of 80 g oven-dried fibres. Enzymatic treatment was performed in aqueous medium at room temperature (25 °C) for 2 h under stirring, using a fibre consistency of 3.0% (w/v) and different enzyme concentrations (8, 13 and 22 U laccase.g^{-1} fibres). Reaction pH was adjusted to 4.0 using 0.1 M H_2SO_4. HBT was used at a concentration of 2.0% (w/w fibres). The reaction medium was filtered to recover the aqueous liquid phase, and the solid fibres were washed with water and dried at 30 °C for 3 days. Controls were obtained by treating the fibres under the same reaction conditions without any added enzyme or mediator. Reactions were performed in duplicate, and treated fibres were used to make single-layer boards.

2.4 Preparation of hardboard panels

Hardboard panels were produced following the standard protocol routinely used at the FCBA technical centre. In the first step, the fibres (80 g, moisture content of 14%) were shaped into a mat with a handsheet former (Rapid Köthen, Frank-PTI, Germany). The fibre mat was then cold-pressed for 30 s at 1.0 bar to remove excess water, and hot-pressed using a small laboratory press at 200 °C, starting the press cycle with a peak pressure of 15 MPa. As hydraulic pressure drops rapidly due to compression of the web, 15 MPa pressure was reapplied for around 40 s and held for 7.20 min. The panels produced had a diameter of 20 cm and an average thickness of about 2.5 mm. The hardboard samples were prepared in triplicate for each condition. From each panel, five samples of 5 cm by 5 cm were cut out for mechanical tests.

2.5 Hardboard physical and mechanical properties

The physical and mechanical properties of the obtained hardboards were studied, i.e. fibre density, internal bond strength (IB) and thickness swelling (TS). Board density profiles were obtained using a GreCon DAX5000 analyser (Fagus-Grecon, Alfeld-Hannover, Germany). An Instron Type 3340 system (Instron, Norwood, MA) was used to measure IB strength with a maximum load capacity of 5 kN. The IB test was performed according to EN 319. The TS test was performed by immersing the board samples in water for 24 h according to EN 317. Sample dimensions were 5×5 cm. Significant differences were inferred by one-way ANOVA (Tukey's honest significant difference, $P < 0.05$).

2.6 Extraction of phenolic compounds

The aqueous liquid phase recovered by filtering the fibre reaction medium (60 mL) was extracted by 3×20 mL of a dichloromethane/ethyl acetate (50/50 v/v) mixture.

Enzyme-treated fibres and hot-pressed fibre panels were milled using a M20 Universal mill (IKA, Staufen, Germany) and the obtained powder was run through a two-step extraction (10 min by water, then 10 min by 95% (v/v) aqueous ethanol) at 40 °C in an accelerated solvent extractor (ASE) (Dionex ASE 350, Thermo Fisher Scientific, Waltham, MA). Ethanol extracts were then stored at – 20 °C for further analysis.

2.7 High-performance size-exclusion chromatography

Aliquots of the ASE ethanol and aqueous-phase extracts were evaporated to dryness, dissolved in tetrahydrofuran (THF), and filtered (GHP Acrodisc filters, 0.45 µm) before analysis by high-performance size-exclusion chromatography (HPSEC) using a polystyrene-divinylbenzene PL-gel column (5 µm, 100 Å, mixed-C pores, 600 mm × 7.5 mm I.D., Polymer Laboratories, Church Stretton, UK) with THF as eluent, and a 280-nm UV detector (Dionex Ultimate 3000 UV/vis detector, Thermo Fisher Scientific) as previously described (Baumberger et al. 2007). Degree of polymerization (DP) was assessed from the apparent molar masses determined by a calibration curve based on polyethylene oxide standards (Igepal, Sigma Aldrich, Saint-Quentin-Fallavier, France) and injection of pure coniferyl alcohol monomers, dimers, and tetramers.

2.8 LC–MS analysis

Aliquots of the ASE ethanol extracts were evaporated to dryness and dissolved in methanol and then ultra-filtered (0.45 µm, GHP Acrodisc, Pall Gelman, Merck, Molsheim, France) before injection onto an UHPLC apparatus (Thermo Fisher Scientific) combined with an electrospray ionization mass spectrometer (ESI–MS) and photodiode array (PDA) co-detection. UHPLC analysis was performed using a C18 column (2.7 µm, 50 mm × 2 mm I.D.mm; high purity, Thermo Fisher Scientific), a 12–95 vol.% aqueous acetonitrile, 1% HCOOH gradient (Millipore, Saint-Quentin-en-Yvelines, France) for 30 min and a 1 mL min^{-1} flow rate. Negative-ion ESI–MS spectra (120–2000 m/z) were acquired using a quadrupole–time-of-flight (Q-TOF) spectrometer (Impact II, Bruker, Leipzig, Germany) with a needle voltage at 4 kV and desolvation capillary temperature at 350 °C. DP and amount of β–O–4 bonds in the analysed oligolignols were determined according to the mass of deprotonated ions, the fragmentation patterns and theoretical masses expected for different types of coniferyl alcohol oligomers (Demont-Caulet et al. 2010).

2.9 Lignin determination and structural analysis

The Klason lignin content of fibres and panels was determined as previously described (Dence 1992) using two-step acidic hydrolysis and gravimetric determination after correction for ash content. Lignin structure was investigated by thioacidolysis, as previously described (Lapierre et al. 1995). Lignin-derived thioacidolysis monomers were determined by GC–MS as their trimethylsilylated derivatives. Thioacidolysis yields were expressed with respect to the obtained Klason lignin content. All analyses were performed in triplicate. One-way ANOVA was used to test for significant differences (Tukey's honest significant difference, $P < 0.05$).

2.10 X-ray photoelectron spectroscopy (XPS)

XPS surface analysis was performed on spruce fibres that had first been formed into small handsheets (diameter 50 mm, thickness 1 mm^2) using a glass filter (Millipore, 0.45 µm) and then dried at room temperature using a XR3E2 instrument (Thermo Fisher Scientific) equipped with a Mg K_α source (1253.6 eV). The X-ray source was operated at 15 kV and 20 mA. Prior to collecting data, the samples were equilibrated for 12 h in an ultra-high-vacuum chamber (10^{-10} mbar) to control desorption of the samples. Photoelectrons were collected by a hemispherical analyser at a constant take-off angle of 90°. Spectra were calibrated with respect to the C_{1s} peak at 284.6 eV as described previously (Dorris and Gray 1978). The chemical shifts relative to the C–C (C1, 284.6 eV) transition were 1.7 ± 0.1, 3.1 ± 0.1 and 4.4 ± 0.1 eV for C–O (C2), O–C–O or C=O (C3) and O=C–O (C4), respectively. Deconvolutions were performed using a 10% Lorentzian/Gaussian function after background subtraction by the Shirley method. Surface lignin was quantified here based on C1 percentages, using the equation (Swift 1982):

$$\Phi lignin = \frac{(C1 extracted pulp - X) * 100\%}{49\%}$$

where 49% is the amount of C1 in pure milled wood lignin, and X is level of contamination (2%) (Munk et al. 2017). For each sample, two handsheets were analysed at ten different positions. The values reported are the mean values from all measurements.

3 Results and discussion

3.1 Improvement of hardboard properties

To assess the benefit of enzymatic treatment for hardboard properties and define conditions relevant to industrial processes, two laccases, from either *P. cinnabarinus* (*Pc*Lac, high redox potential) or *M. thermophila* (*Mt*Lac, low redox potential), were applied to Norway spruce wood fibres at different concentrations (8, 13 and 22 U g^{-1} fibres) and in the presence or absence of the HBT mediator. A water reaction medium at pH 4 was chosen to preserve enzyme stability and performance and to decrease process costs. Enzymatically treated fibres and controls (including addition of HBT alone) were incubated in parallel. All fibre samples were then pressed into hardboards, and their physical and mechanical properties were characterized. The average density of control and laccase-treated hardboards was 960.60 kg m^{-3}, which was consistent with the 800 to 1120 kg m^{-3} density range characteristic of wet-process hardboards (fibreboards) (EN 316; Kamke 2001) but revealed no density differences ($P < 0.05$) between panels obtained from differently treated fibres. The benefit of

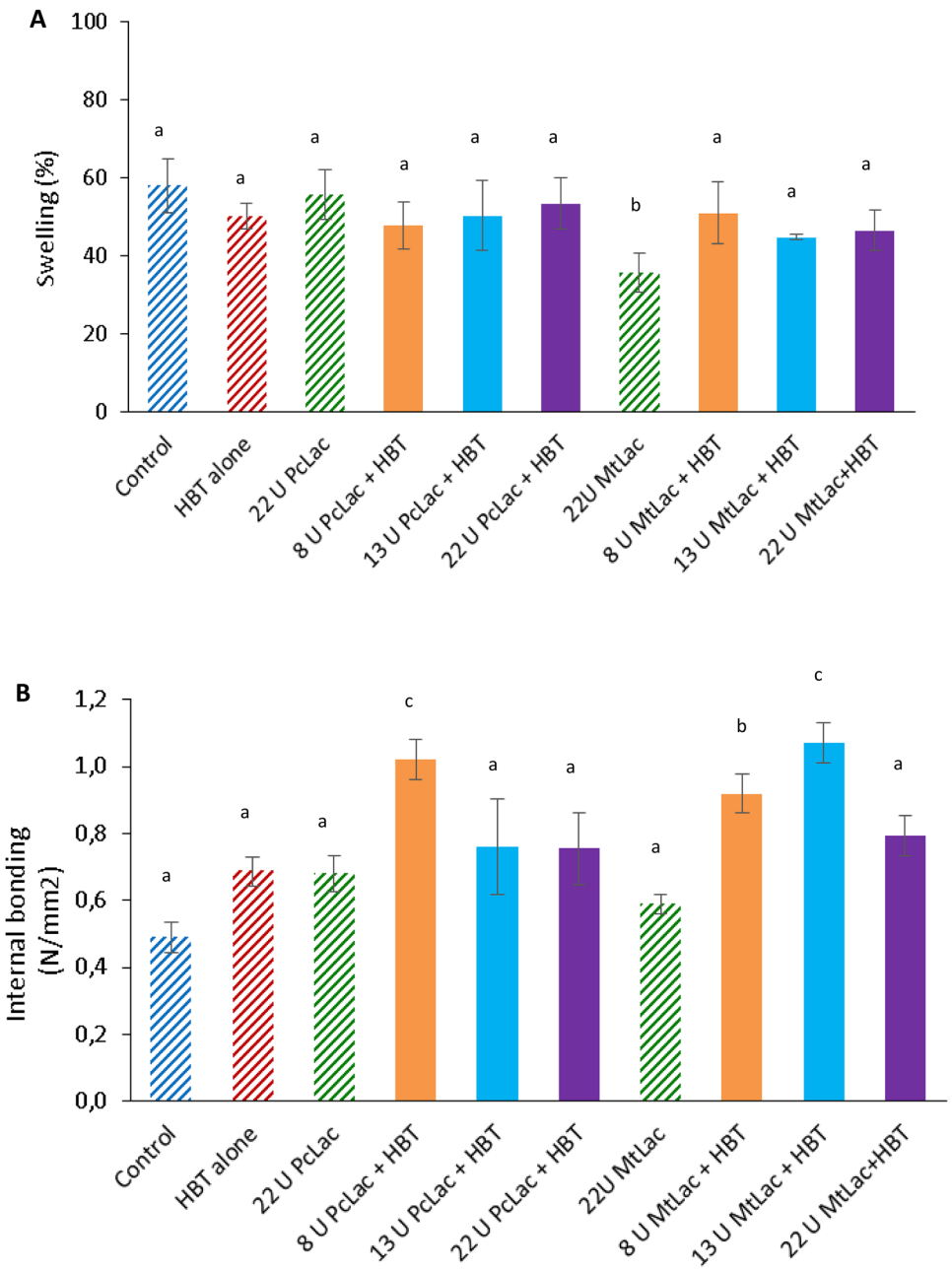

Fig. 1 Swelling (**A**) and internal bonding (**B**) of panels produced with fibres that underwent different laccase treatments. The buffer and HBT controls equate to wood fibre pre-treatment without laccase. Three replicates were performed for each treatment. Data are presented as means ± SD. Means flagged with different letters (a, b, c) are significantly different at $p < 0.05$

laccase-based treatments was assessed based on two key mechanical hardboard properties: TS and IB (Fig. 1). The highest effect on TS was a 40% reduction observed under treatment with 22 U g^{-1} *Mt*Lac and no HBT (Fig. 1A), whereas this treatment had no effect on IB (Fig. 1B). However, laccase–HBT systems implemented at low enzyme concentrations (8 U g^{-1} for *Pc*Lac and 13 U g^{-1} for *Mt*Lac) led to two-fold higher IB ($P < 0.05$; Fig. 1B). These results show that the mechanisms responsible for TS and IB improvement were distinct and demanded different treatment conditions, including enzyme concentration when the same enzyme or LMS is used. TS improved at higher enzyme concentration and in the absence of HBT, whereas IB increased at lower enzyme concentration and in the presence of HBT. In both cases, the reactivity of phenolic compounds as laccase substrates seems to be involved (Hollmann and Arends 2012; Nasir et al. 2013; Zhou et al. 2013; Yang et al. 2017). Due to their phenolic nature, both lignin and phenolic extractives are likely to react in the presence of oxidative laccase systems. Enzymatic action may lead to both structural modifications and the formation of novel or modified compounds. Changes in lignin structure and ethanol-extractible compounds were therefore analysed in parallel.

3.2 Fibre bulk composition

Lignin structure within the fibres reflects lignin composition in the raw material, and consequently varies depending on plant origin. Hardwood-fibre lignin contains guaiacyl (G) and syringyl (S) units in various proportions, whereas softwood-fibre lignin is mainly composed of G-units (Sjöström 1993; Brunow 2001; Guerra et al. 2006; Esteves et al. 2011; Du et al. 2013).

Klason lignin content accounted for 28% (±0.5) of the total dry weight of enzymatically treated and untreated control fibres and panels (data not shown). This value is consistent with the 27–32% previously obtained for TMP fibres and with softwood lignin content (Qiu et al 2006; Zoia et al. 2008; Esteves et al. 2011). The low standard deviation (±0.5) between sample averages and duplicates indicates that enzymatic lignin modification does not take place during acidic Klason hydrolysis. Investigation of lignin structure by thioacidolysis (Du et al. 2013) confirmed that spruce lignin is mainly made of G units [more than 98% of Klason lignin, compared to 1.5% for *p*-hydroxyphenyl units (H), and traces for syringyl units (S)] and confirmed the absence of detectable structural changes in the lignin mediated by the laccase treatment. This result is consistent with previous findings showing that laccase can activate fibre surface with minimal penetration into deeper layers of fibre (Álvarez et al. 2011; Moniruzzaman and Ono 2013). Unlike bulk lignin properties, changes in phenolic extractives from different samples clearly showed that these compounds demonstrated reactivity to enzymatic treatments.

3.3 Reactivity of phenolic extractives to enzymatic fibre treatment

The total amount of extractives in control fibres was 1.0–1.5%, estimated from dry weight loss after sequential water and ethanol extractions. This content is similar to the 1.9% extractive content reported for TMP fibres (Spence et al. (2010)) and for spruce wood (Overend et al. 1985; Caron et al. 2013). HPSEC analysis of the ethanolic extractives from control fibres revealed the presence of a mixture of phenolic oligomers assigned in the chromatogram (Fig. 2) according to their apparent DP: dodecamers

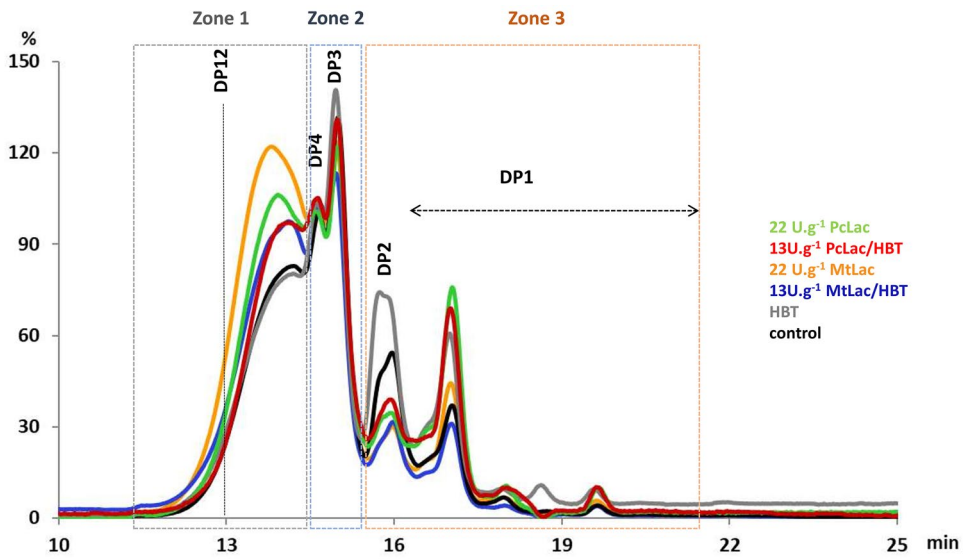

Fig. 2 Normalized HPLC-SEC profile of mono- and oligo-phenolic compounds in ethanol extracts from fibres pre-treated by 22 U g^{-1} of *Pc*Lac (green), 13 U g^{-1} of *Pc*Lac with 2%HBT (red), 22 U g^{-1} of *Mt*Lac (orange), or 13 U g^{-1} of *Mt*Lac with 2%HBT (blue). The buffer (black) and 2% HBT (grey) controls contain extractives from wood fibres pre-treated without laccase

(DP12) to tetramers (DP4) (zone 1, 13–14.6 min), two ill-resolved peaks of tetramers (DP4) and trimers (DP3) (zone 2, 14.6–15.5 min), as well as dimers (DP2) and monomers (DP1) (zone 3, starting at 15.5 min). The influence of enzymatic treatments was assessed for enzymatic formulations (22 U *Mt*Lac; 8 U *Pc*Lac + HBT; 13 U *Pc*Lac + HBT) that improved TS or IB and for 22 U *Pc*Lac (for comparison with 22 U *Mt*Lac). These changes were highlighted by normalization of the chromatograms with respect to the tetramer (DP4) peak eluted at 14.6 min (Fig. 2), which facilitated comparison. The proportion of different peaks changed with enzymatic treatment depending on enzyme source and concentration and on the presence of HBT. Comparison of the chromatograms revealed an increased proportion of high-molar-mass phenolics (zone 1, DP > 4) for all enzymatic treatments, with maximum polymerization observed with 22 U g^{-1} *Mt*Lac without HBT. This condition, like fibre treatment with 13 U g^{-1} *Mt*Lac HBT, not only caused an increase in peak surface for high-molecular-weight species (zone 1) but also peak broadening and shift towards lower retention times (higher molecular weights), which also points to polymerization of phenolics (Fig. 2), as previously observed with other laccase systems (Hollmann et al 2008; Hollmann and Arends 2012). Polymerization can stem from the coupling of radicals formed by laccase-catalysed oxidation of phenolics, according to previously described mechanisms (Brunow et al. 1998; Dermont-Caulet et al. 2010). For *Pc*Lac treatments, polymerization was less pronounced (smaller peaks in zone 1) and accompanied by a concomitant increase in the main monomer peak (17 min), suggesting that depolymerization also took place. These results show that both *Mt*Lac and *Pc*Lac enzymatic systems modify the chemical composition of fibre extractives, but via different molecular mechanisms.

HPSEC analysis of phenolic water extractives confirmed that all the low-molecular-weight species recovered (DP ≤ 4: Fig. 3, 15–22 min) disappeared from the reaction medium upon enzymatic treatments, thus confirming the reactivity of the phenolics to polymerization upon laccase activation. An ill-shaped tailing peak was detected after 22 min in chromatograms from all HBT-treated samples and was assigned to HBT itself. In one condition only (13 U g^{-1} *Pc*Lac + HBT), another peak appeared in the chromatogram at 18.3 min. The content of this peak had a UV spectrum similar to that of HBT (absorption maxima at 252 and 280 nm; Masoud et al. 2015) and was finally assigned to a HBT dimer using LC–MS analysis (m/z 251.0684, Supplementary Fig. 1). Only traces of HBT dimer were detected in the reaction media after *Mt*Lac + HBT treatments. This suggests that HBT reactivity differs when either *Pc*Lac or *Mt*Lac are employed. To summarize, both *Pc*Lac and *Mt*Lac treatments changed the composition of phenolic ethanolic and water extractives present in the reaction medium, but with distinct mechanisms.

To further elucidate the molecular mechanisms underpinning the differences between enzymatic treatments, a more precise investigation was carried out by LC–MS of fibre phenolic extractives after the following treatments: 22 U g^{-1} *Pc*Lac, catalysing the highest apparent depolymerization and monomer accumulation by HPSEC (Fig. 2); 13 U U g^{-1} *Mt*Lac + HBT leading to the lowest apparent depolymerization and monomer accumulation; and 13 U g^{-1} *Pc*Lac + HBT for comparison with 13 U g^{-1} *Mt*Lac + HBT. Twenty main compounds were detected in the ethanol extract of control fibres using negative ionization mode. These compounds exhibited maximal m/z ratios ranging from 265 to 571 amu (Fig. 4), which corresponds to monomeric to tetrameric coniferyl alcohol, the main precursor of softwood lignin. Some of these compounds disappeared from chromatograms

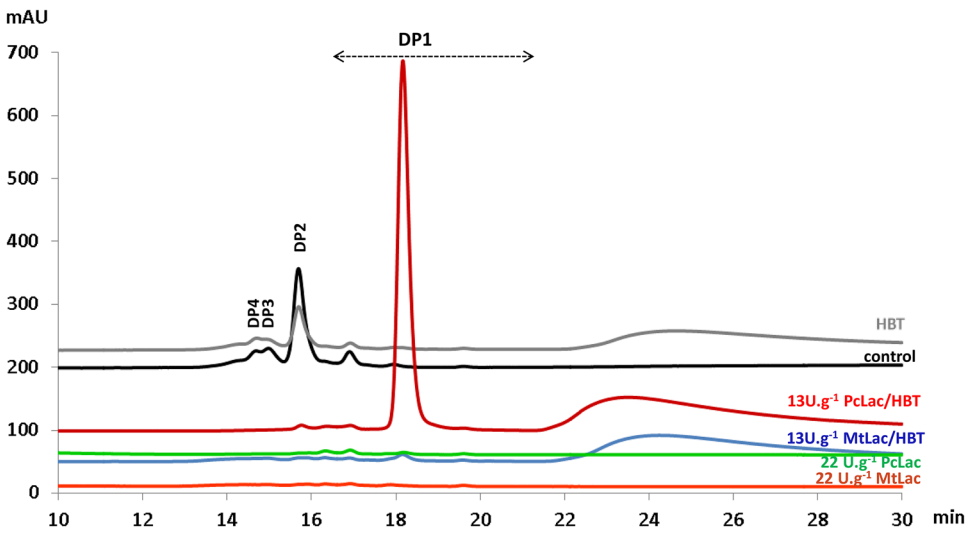

Fig. 3 HPLC-SEC profile of mono- and oligo-phenolic compounds obtained from water solutions of fibres treated by 22 U g^{-1} of *Pc*Lac (green), 13 U g^{-1} of *Pc*Lac with 2%HBT (red), 22 U g^{-1} of *Mt*Lac (orange), or 13 U g^{-1} of *Mt*Lac with 2%HBT (blue). The buffer (black) and 2% HBT (grey) controls contain extractives from wood fibres pre-treated without laccase

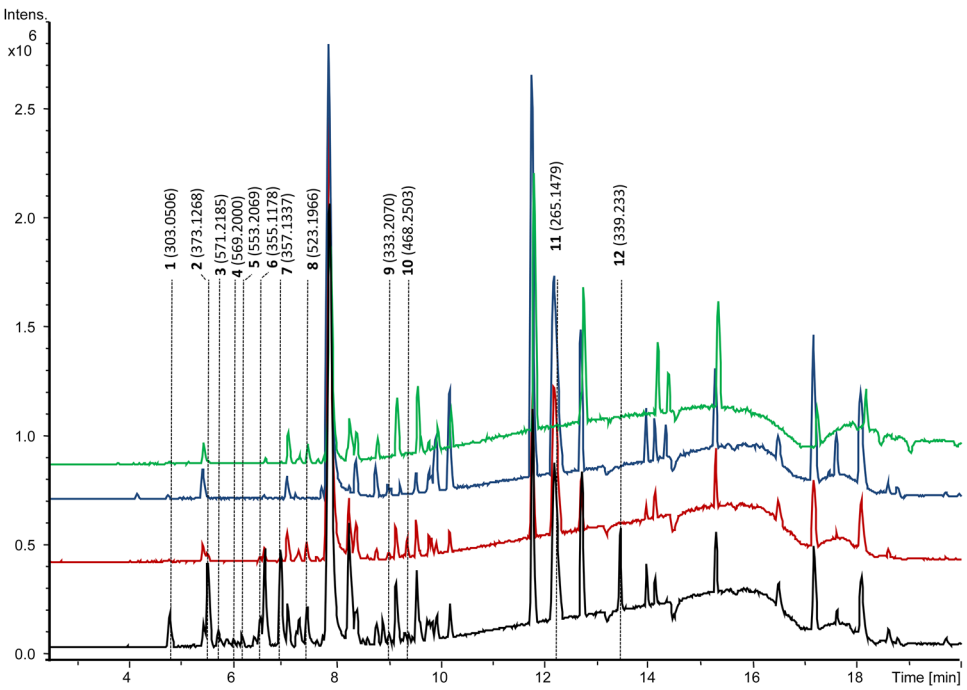

Fig. 4 Negative ionization-mode UHPLC-ESI-Q-TOF–MS ion chromatograms of ethanol-extractible phenolic monomers, dimers and oligomers released from fibres pre-treated by 22 U U g^{-1} *Pc*Lac (green), 13 U g^{-1} *Pc*Lac with 2%HBT (red), and 13 U g^{-1} *Mt*Lac with 2% HBT (blue). The buffer (black) control contains extractives from wood fibres pre-treated without laccase. Peaks of interest are numbered from 1 (lower retention time) to 12 (higher retention time), and the exact m/z value is given in brackets

Table 1 Analysis of phenolic compounds whose appearance change in the ethanol extract of control and enzymatically-treated fibres

Peak n°	Retention time (min)	m/z	Formula	DP	Number of β-O-4 bonds	Theoretical mass
1	4.8	303.0506	C15H11O7	2	0	303.0505
2	5.6	373.1268	C20H21O7	2	1	373.1287
3	5.7	571.2185	C30H35O11	3	2	571.2179
4	6.0	569.2000	C30H33O11	3	2	569.2023
5	6.2	553.2069	C30H33O10	3	1	553.2074
6	6.7	355.1178	C20H19O6	2	0	355.1182
7	7.0	357.1337	C20H21O6	2	0	357.1338
8	7.3	523.1966	C29H31O9	3	1	523.1968
9	8.9	333.2070	C20H29O4	3	0	333.2066
10	9.4	468.2503	C23H22N3O8	2	0	468.1407
11	12.2	265.1479	C17H13O3	2	0	265.0865
12	13.5	339.2331	C23H31O2	2	0	339.2324

Degree of polymerization and number of β–O–4 bonds were determined by comparing measured m/z values with theoretical m/z values of coniferyl dimer or trimer

in either all or some of the enzymatically treated samples (Fig. 4) (Table 1). Peaks with m/z 373.1286, 523.1966, 553.2069, 569.1579, and 571.2185 amu were tentatively assigned to guaiacyl dimers and trimers with at least one β-*O*-4 linkage (Fig. 4 and Table 1), while peaks with m/z 303.0506 and 339.2331 amu were assigned to guaiacyl and vanillyl C–C bonded dimers (Fig. 4 and Table 1). Other peaks with m/z 265.1479, 333.2070, 355.1178 and 357.1337 amu disappeared in an enzyme-dependent manner and were assigned to guaiacyl or vanillyl C–C bonded dimers (Fig. 4 and Table 1). In other words, the present LMSs were more efficient on β–*O*–4 bonds that are more readily oxidized than on β -5 or β–β (C–C) bonds. This result is also in agreement with a previous electrochemical study on coniferyl alcohol dimers (Cottyn et al. 2015) and structural characterization of LMS-delignified lignocellulosic biomass using the combination of py-GC–MS, 2D NMR, SEC and RP-UHPLC-MS (Hilgers et al. 2020).

A new peak with m/z 468.2503 amu appeared exclusively with the *Pc*Lac + HBT treatment (Fig. 4 and Table 1). It could be assigned as an adduct of HBT and a phenolic compound. Efficient grafting of N–OH mediators (such as HBT

and N-hydroxyphthalimide) onto lignin has already been observed when using other high-redox-potential laccases (Kleen et al. 2002; Tamminen et al. 2003; Munk et al. 2017; Hilgers et al. 2018, 2020) than *Pc*Lac. In addition to HBT grafting, Hilgers et al. (2020) also proved on native lignin that phenoxyl radicals may also undergo radical coupling to other lignin-derived radicals (repolymerization), and thereby suggested lignin (re)polymerization. This result supports the present hypothesis set out above that HBT could be involved in cross-linking reactions between fibres, thus leading to mechanical reinforcement.

3.4 Composition and reactivity of the fibre surface

XPS was employed to determine the content of oxygen and carbon (O/C) ratio in hemicellulose, cellulose, extractives, and lignin at the surface of 5-nm-thick fibre layers. The first component of the C1s spectrum of each sample was shifted to adventitious carbon binding energy (285.0 eV) (González-Arzola et al. 2006). Peaks were fitted using four components providing the following interpretation. The first peak (C1) corresponds to aliphatic and aromatic carbon bonds and is related to adventitious carbon from lignin. The second component (C2) corresponds to C–O bonding and originates from all wood compounds, especially cellulose and hemicellulose. The third component (C3) is associated with carbon atoms bound to either a carbonyl or two non-carbonyl oxygen atoms and originates mainly from cellulose and hemicelluloses. The fourth component (C4) corresponds to carboxylic acids, which are mainly found in spruce wood extractives. The distribution between the different carbon types reflects the overall chemical composition of the fibre surface. Since the C1 component is contributed by lignin only, the relative amount of surface lignin can be inferred by XPS and compared between controls and laccase-treated fibres. XPS data (Table 2) indicated that chemical modifications took place at the fibre surface during treatment and that these modifications differed between the enzymatic systems employed. The control fibres showed an O/C ratio of 0.33, consistent with the literature (Migneault et al. 2015). Treatment with HBT alone led to a decrease in O/C, i.e. an apparent increase in lignin content. This can be due to adsorption of HBT on the fibres, resulting in increased aromatic carbon bonds and consequently an increase in aromatic C1 signal. In contrast, all treatments with enzymes led to an apparent decrease in lignin content compared to the HBT control, suggesting that some lignin fragments were released in the reaction medium. The effect was more pronounced for 13 U g^{-1} *Pc*Lac + HBT than 13 U g^{-1} *Mt*Lac + HBT, which is consistent with the higher depolymerization capacity of *Pc*Lac. Note that the O/C ratio decreased with *Mt*Lac + HBT treatments but not with *Pc*Lac + HBT. Although these differences can hardly be assigned to precise molecular mechanisms, they confirmed that *Mt*Lac, *Pc*Lac and HBT have different actions at the fibre surface level.

3.5 Relationships between extractives reactivity and panel properties

HPSEC analysis of ethanol extractible phenolics obtained from hardboard panels showed an increase in the oligomeric fractions with DP > 4 for all enzymatically treated fibres compared to the HBT and buffer controls (Fig. 5). However, the differences between the chromatograms obtained from different panels were less pronounced than for the chromatograms obtained from treated fibres. This indicated that the panel production process levelled out the molar mass variation observed for enzymatically treated fibres and suggested that some enzymatically activated phenolic compounds were further polymerized by hot-pressing at 200 °C and 15 MPa.

It was possible to establish a clear relationship between TS and reactivity of extractives. Indeed, the only investigated laccase system leading to a decreased TS was 22 U g^{-1} *Mt*Lac (Fig. 1), which also gave the most pronounced polymerization, as revealed by HPSEC both after fibre treatment (Fig. 2) and after panel processing (Fig. 5). The TS parameter reflects the capacity of water to penetrate within the fibre network. Formation of phenolic polymers during fibre treatment and embedding of fibres within these polymers during hot pressing may account for increased panel hydrophobicity and reduced water absorption. In line with the present results, extractive grafting at the fibre surface and changes in fibre surface energy have already been reported for laccase-treated fibres (Hassingboe et al. 1998; Felby et al. 2004; Schroeder et al. 2007).

IB improvement was observed after fibre treatments with HBT and low enzyme doses (Fig. 1), i.e. the conditions resulting in the lowest polymerization levels before

Table 2 XPS data of laccase-treated and control spruce fibres

Sample	O/C	C1 (%)	C2 (%)	C3 (%)	C4 (%)	Φ_{lignin} (%)
Control	0.33 (0.020)	18.9 (2.0)	41.4 (4.1)	31.1 (3.5)	8.6 (1.1)	34.5
HBT	0.30 (0.015)	24.3 (2.2)	42.8 (3.2)	25.7 (2.5)	7.3 (0.8)	45.5
13U *Pc*Lac + HBT	0.35 (0.025)	12.5 (1.695)	42.0 (4.069)	35.3 (3.760)	10.1 (0.9)	21.4
13U *Mt*Lac + HBT	0.26 (0.012)	18.4 (1.751)	45.3 (4.256)	34.0 (3.805)	6.8 (0.7)	38.8
22U *Mt*Lac	0.26 (0.011)	10.1 (1.029)	53.0 (4.854)	30.4 (3.125)	6.5 (0.5)	16.5

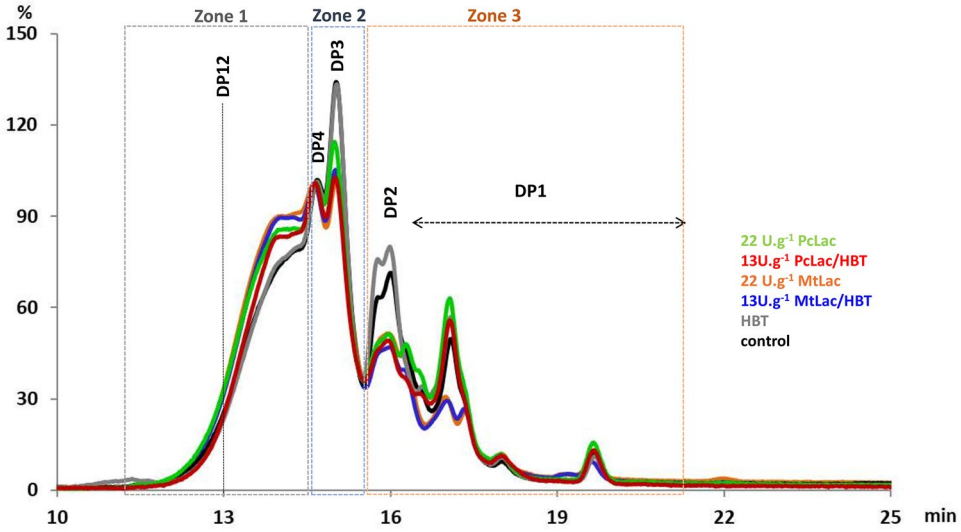

Fig. 5 Normalized HPSEC profile of ethanol-soluble mono- and oligo-phenolic compounds extracted from hardboard panels obtained from fibres pre-treated by 22 U g^{-1} of *Pc*Lac (green), 13 U g^{-1} of *Pc*Lac with 2%HBT (red), 22 U g^{-1} of *Mt*Lac (orange), or 13 U g^{-1} of *Mt*Lac with 2%HBT (blue). The buffer (black) and 2% HBT (grey) controls contain extractives from panels obtained from wood fibres pre-treated without laccase

hot-pressing (Fig. 2) and the same polymerization levels thereafter (Fig. 5). During hot-pressing, two phenomena are likely to determine the cohesion of laccase-treated panels: physical entanglement of the fibres, and creation of covalent and non-covalent interactions between the fibres (Unbehaun et al. 2000; Felby et al. 2004). The present results show that polymerization of activated low-molecular-mass phenolics during hot pressing improves mechanical properties (IB) of the panel. This improvement is possibly due to better proximity of fibres that possibly couples chemical grafting of extractives at the fibre surface with inter-fibre crosslinking, favouring crosslinking. Thus, whereas conditions favouring extractive polymerization during fibre treatments (high concentration of *Mt*Lac in the absence of HBT) improve water resistance, conditions leading to depolymerization or at least more controlled polymerization (low enzyme concentration and the presence of HBT) improve mechanical resistance, probably due to the favourable coupling of phenolic extractives, polymerization and cross-linking of fibre surfaces during hot-pressing. Moreover, these results demonstrate that the presence of HBT had a positive influence on fibre network reinforcement regardless of the enzyme used. It is likely that HBT grafted to the phenolic compounds by the N–OH function group may act as a 'polymerization chain-stopper' and thus help control the polymerization. Nevertheless, differences between the two enzymes exist, largely due to two main factors: different redox potentials (0.75 V for *Pc*Lac *vs* 0.47 V for *Mt*Lac) and possibly different substrate specificities including their capacity to oxidize HBT and recruit it as a mediator. Indeed, only *Pc*Lac induced HBT dimerization (Fig. 3), suggesting a likely different mechanism for *Mt*Lac-mediated HBT oxidation.

The propensity of *Pc*Lac to induce depolymerization, both in the presence and absence of HBT, is consistent with its higher redox potential. Indeed, depolymerization relies on oxidation of phenolic oligomers, which have a higher redox potential than phenolic monomers (Hilgers et al. 2018). Moreover, phenolic monomers, such as vanillin, vanillic acid and other water-soluble molecules apparently released from Norway spruce TMP fibres by depolymerization with *Pc*Lac, are likely to contribute to laccase action as natural mediators and thereby improve laccase-based processes (Hassingboe et al. 1998; Camarero et al. 2014).

In *Pycnoporus* LMS, HBT is not required for the oxidation of phenolic compounds but is oxidized and undergoes radical–radical coupling, resulting in new covalent bonds with phenolics. In fact, HBT oxidation by PcLac + HBT LMS is apparently beneficial for fibre crosslinking (IB), possibly by reaching and activating lignin domains in deeper layers of the fibres that are inaccessible to *Pc*Lac, whereas on the fibre surface, the additional creation of phenolic and HBT radicals evidenced polymerization/depolymerization of phenolic compounds and HBT (Figs. 2 and 4; Table 1), seen as a decrease in the relative amount of surface lignin and a slight increase in O/C ratio (Table 2).

Unlike *Pc*Lac, the absence of HBT dimers in the case of *Mt*Lac + HBT LMS (Fig. 3) suggests that HBT is not oxidized by the enzyme directly. It is likely activated through laccase-generated phenoxy radicals in the reaction medium, meaning that natural phenolic extractives would play the role of mediators. This may explain why the impact of 22 U g^{-1} *Mt*Lac on TS was influenced by the presence of HBT that might compete with the lignin surface as an alternative target of phenoxy radicals. Laccase-based oxidative treatments can generate phenoxy radicals not only on soluble phenolics but also on surface-exposed lignin. Laccase-mediated modification of surface lignin was previously shown for TMP and kraft pine lignin treated with the high-redox-potential laccases from *Fusarium proliferatum* (Hassingboe et al. 1998) and *Trametes villosa* (Euring et al. 2011). Euring

et al. (2011) showed that adding HBT or 4-hydroxybenzoic acid (HBA) to laccase accelerated the turnover of aromatic groups on surface lignin, with a positive effect on the mechanical properties (higher IB) of the resulting MDF. In addition, lignin oxidation at the fibre surface would generate radicals available for cross-coupling with radicals formed on soluble phenolic compounds. This hypothesis was supported by the reduced proportion of high-molar-mass extractives after hot-pressing (Figs. 2 and 5), and could explain the observed improved mechanical characteristics of the panels produced here.

4 Conclusion

The objective of this study was to compare two fungal enzymes in terms of their potential to improve the properties of wet-process wood fibreboard panels. The investigation was carried out at molecular, fibre and macroscopic level. All results pointed to distinct effects of the two fungal enzymes, reflecting different mechanisms of action on phenolics within the studied systems. Although previous studies showed that laccase-mediated treatment of fibres may enhance hardboard panel properties, likely by transforming wood extractives, the parameters controlling the reactivity of phenolic extractives and its impact on both water and mechanical resistance had never before been investigated. Here, it was demonstrated that two opposite reaction pathways need to be implemented to improve each of these two properties: fibreboard resistance to water swelling (TS) was improved by fibre enzymatic treatments that induce polymerization of phenolics before hot pressing (22 U g^{-1} *Mt*Lac, no HBT), whereas mechanical reinforcement (IB) was improved by fibre enzymatic treatments that limit lignin polymerization (low enzyme concentration plus HBT), thus favouring an accumulation of low-molecular-mass phenolic extractives and their polymerization for inter-fibre cross-linking during the subsequent hot-pressing step of panel manufacture. A major technical outcome of this study is that it raises the prospects for improving wood fibre IB by LMS-mediated activation using a 3 times smaller biocatalyst dose than usually practiced (22 U g^{-1} of laccase). This improvement in enzymatic doses will allow further process development for optimized industrial applications and improve their environmental and economic impact. It was also demonstrated that the laccase mediator HBT plays a role in the control of laccase-mediated lignin polymerization/depolymerization and that it is required for panel reinforcement whatever the laccase (either *Mt*Lac or *Pc*Lac) used. Despite their differences in redox potential, both enzymes were able to double the internal bond strength (IB) of the resulting wood panels. However, the same IB improvement was obtained at a lower *Pc*Lac concentration (8 U g^{-1}) than *Mt*Lac (13 U g^{-1}) concentration, which makes *Pc*Lac a better candidate for industrial applications. Moreover, its ability to catalyse mediator grafting may be useful for laccase-assisted modification of lignin and enable the development of novel lignin-based materials with environmental, economic and health benefits via binderless (synthetic resin-free) board production process routes.

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