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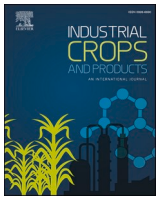

Functionalisation of organosolv lignin by enzymatic demethylation for bioadhesive formulation

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ABSTRACT

Bio-based adhesives offer a sustainable alternative to formaldehyde-based adhesives, which is the most common product applied in wood-based panel manufacturing. Despite the undeniable interest in lignin as a bulk chemical for the synthesis of bio-based adhesives, the main problem is its low reactivity. Therefore, it is crucial to study techniques to increase their reactivity, such as demethylation. One of the parameters to monitor the success of the demethylation is the methanol concentration measured in the reaction medium and complementing these results with the use of non-quantitative analyses such as FT-IR, MALDI-ToF and SEC. In order to maximize the action of the enzyme, a preliminary screening was performed with laccases with different redox potentials, which were added to organosolv lignin in the form of purified enzymes and cocktails. *Trametes versicolor* laccase was selected as the best candidate for the demethylation of organosolv lignin, with a methanol released concentration of 107 mg/L. Based on these results, parameters affecting both enzyme action and enzyme-substrate interaction were evaluated. Optimal working conditions were strongly defined by the use of surfactants that enhanced lignin solubility (Tween-80) and mediators that favored enzyme catalysis to participate directly in the enzyme catalytic cycle (HBT). However, it was also observed that factors such as pH, temperature and aeration have a direct impact on the enzymatic demethylation of lignin. Considering the operational variables that allowed better demethylation values, the amount of methanol released increased to 363 mg/L, which means an improvement of 239 %. These results were also supported by the increase in molecular weight observed in the SEC analysis and the reduction of the intensity of the FT-IR bands related to the methyl/methoxyl groups.

1. Introduction

The development of the adhesive market is increasingly widespread (Dinte and Sylvester, 2018). The most widely used alternatives are formaldehyde-based adhesives, such as urea-formaldehyde, phenol formaldehyde and melamine-formaldehyde (Liu et al., 2018; Zhu et al., 2018). However, the evolution of technological processes and legislation in favour of the use of non-volatile adhesives are some of the factors that have driven a trend towards more environmental-friendly processes (Dinte and Sylvester, 2018).

Within the wood and furniture sector, the growing concern for environmental aspects (such as the use of hazardous materials and chemicals, VOC emissions and waste management), as well as the production of formulations capable of competing with synthetic adhesives, has given a boost to adhesives of natural origin, also called bio-adhesives. Their application in the manufacture of panels in the wood industry would be a great ecological improvement as they would be products free of formaldehyde, a compound that is usually included in high percentages in wood adhesives. Changes in legislation on formaldehyde emissions, as well as consumer interest in buying green, sustainable and environmentally friendly products, are encouraging the development of studies and research to find alternatives to synthetic adhesives (Hemmilä et al., 2017).

Soy protein is one of the first bio-based adhesives with commercial projection for plywood (Li et al., 2004; Ye et al., 2019). There are also references with promising results for different types of bio-based materials: corn starch, chitosan; but undoubtedly, the most widely used raw material for the formulation of natural-based adhesives is lignin, the second most abundant natural polymer in the world after cellulose (Lange et al., 2013). This is the main reason for the widespread interest

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in finding potential applications for lignin, including the manufacture of bio-adhesives that can replace synthetic resins (Domínguez-Robles et al., 2018; El Mansouri et al., 2011; Ji and Guo, 2018). Different reports have identified the feasibility of partial substitution of phenol by lignin or the combination of tannin and lignin (Saražin et al., 2020), as well as different crosslinkers to replace formaldehyde, such as aromatic aldehydes, glyoxal, furfuryl alcohol, caprolactam, glycol compounds and hexamine (Pizzi, 2014). However, the incorporation of unmodified lignin in the formulation of phenolic resins is limited due to poor substitution performance and shows a reduction in bond strength with longer pressing time (Newman and Glasser, 1985) because lignin has lower reactivity compared to phenol.

Depending on the type of wood or the chemical pulping process (kraft, organosolv, sulphite), the structure and properties of lignin vary (Kalami et al., 2018). Its macromolecular structure is mainly composed of monomeric units such as 2-methoxy-4-propylphenol (guaiacol) in softwood and a mixture of guaiacol and 1,5-dimethoxy-4-propylphenol (syringol) in different proportions in hardwood (Chen et al., 2009). However, due to its low content of hydroxyl groups, the substitution of the aromatic ring and the steric barrier of its molecular structure, the reactivity of lignin is relatively poor (Kuo et al., 1991; Vázquez et al., 1999). Therefore, it is necessary to modify its main functional groups to increase its reactivity. Many studies have focused on different ways to improve the reactivity of lignin, such as demethylation (Venkatesagowda, 2019), oxidation (Yu et al., 2019) or phenolation (Li et al., 2018). However, demethylation seems to be one of the best methods, converting part of the methoxy groups into phenolic hydroxyl groups (Chen et al., 2021). The use of lignin in phenol based resin synthesis has been evaluated in different studies, using lignosulphonates, Kraft, organosolv and soda lignin (Alonso et al., 2004; Çetin and Özmen, 2002; Domínguez-Robles et al., 2018; Ghorbani et al., 2016). After chemical modifications of lignin, phenol can be replaced by organosolv lignin with acceptable physical and mechanical properties, such as internal bond strength, water absorption and material swelling.

Enzymatic demethylation opens up a biotechnological approach for lignin modification (Venkatesagowda, 2019), provided that the selected enzymes are able to cleave the O-methyl/methoxyl groups of phenolic and some non-phenolic compounds. Accordingly, methanol release is detected as an indicator of the progress of the reaction. Four main types of extracellular ligninolytic enzymes are known to be involved in lignin modifications, namely laccase, manganese peroxidase (MnP), lignin peroxidase (LiP) and versatile peroxidase (VP). The most studied enzyme is laccase (EC 1.10.3.2), since it is the only commercially available enzyme (Venkatesagowda, 2019). More specifically, laccase catalyses the oxidation of lignin-related phenol-vanillyl glycol, leading to the initial formation of dimers and subsequent polymerization reactions (Lundquist and Kristersson, 1985). Occasionally, it may be necessary to use redox mediators, which act as electron transporters with the purpose of increasing the redox potential of the enzyme (Bourbonnais and Paice, 1990). In fact, the first references of lignin demethylation by laccase considered the use of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as mediator for the treatment of Kraft pulp (Bourbonnais and Paice, 1996, 1992). However, in recent years, other synthetic and natural mediators have been studied to improve laccase performance in different biocatalytic reactions. Among them, the use of 1-hydroxybenzotriazole (HBT) in the modification of lignocellulosic compounds has been reported by different authors (Díaz-González et al., 2011; Navas et al., 2019). Unlike laccase, both MnP and VP require the presence of a specific mediator for each catalytic pathway, so it is necessary to oxidise the substrate (Chauhan, 2020). While MnP catalyses the oxidative depolymerization of lignin through the oxidation of Mn^{2+} to Mn^{3+} as a natural redox mediator, VP exhibits bifunctional catalytic properties of MnP and LiP (Pérez-Boada et al., 2005; Wong, 2009). Other enzymes such as o-demethylases present additional drawbacks such as the difficulty of their isolation, mainly due to their sensitivity to oxygen and also their tendency to bind to cell membranes (Venkatesagowda, 2019).

Based on these assumptions, the present study considers the enzymatic modification of organosolv lignin by various types of laccases, which is verified by monitoring the degree of demethylation and modification of phenolic groups enabling the cross-linking of lignin monomers for the production of bio-adhesives. The effects of different conditions and factors were evaluated with the enzyme which showed the highest lignin modification and the best demethylation results. In order to obtain more representative results, the defined as optimal conditions were evaluated in a 150 mL-bioreactor with the continuous supply of aeration. To the best of our knowledge, this is the first study that evaluate the joint action of aeration and other key factors for a fungal laccase performance in the demethylation of an organosolv lignin.

2. Materials and methods

2.1. Organosolv lignin and other reagents

Organosolv lignin, obtained after an organosolv treatment of beech wood, was kindly provided by the Fraunhofer Center for Chemical-Biotechnological Processes (CBP) (Leuna, Germany). In the fractionation of the process two phases are obtained: one solid fraction rich in cellulose and a black liquor mainly composed by lignin, C-5 sugars, and degradation products. Lignin was precipitated from this stream by the addition of water and filtered for separation. The enzymes used for the methanol measurement protocol were purchased from Sigma-Aldrich: horseradish peroxidase (HRP) (Sigma-Aldrich P6782, 950–2000 units/mg solid) and alcohol oxidase (AO) solution from *P. pastoris* (Sigma A2404, 10–40 units/mg protein). Pullulan standards (ReadyCal-Kit Pullulan, PSS-pulkitr1) used in the SEC analysis were purchased from GmbH. Vanillin was purchased from Merck, 1-hydroxybenzotriazole (HBT) was purchased from Fluka, Folin-Ciocalteu reagent was purchased from VWR Chemicals and other analytical chemicals from Sigma-Aldrich.

2.2. Laccase

Three different types of laccases were used for lignin demethylation: Two commercial laccase from *Trametes versicolor* (Ref. No. 53739; Sigma-Aldrich) and *Myceliophthora thermophila* (Novozymes 51003®) and a laboratory-produced laccase from *Ganoderma lucidum* (isolated from spent mushroom substrate, kindly provided by Hifas da Terra S.L., Pontevedra, Spain). The latter was obtained from submerged cultures of *G. lucidum* in a 5 L BIOSTAT® B fermenter (Sartorius Biotech). The fermentation medium (4 L) consisted of a diluted medium based on a wood industry residue (black liquor) diluted 1:37 with distilled water and supplemented with 4 g/L glucose, 2 g/L peptone, 0.5 mmol $MnSO_4$ and 0.15 mmol $CuSO_4$. The extracellular culture was harvested at the peak of laccase activity (about 3 days) and filtered through Whatman No. 1 filters (Maidstone, UK). After harvesting, the filtered culture broth was concentrated by microfiltration and ultrafiltration (Filtron Minisette System, Pall Corporation; 10 kDa cutoff) to the final activities around 150 U/mL, and finally stored at 5 °C until use.

2.3. Enzymatic modification of lignin in test tubes

The experiments of organosolv lignin transformation were conducted in 12 mL polypropylene tubes with 50 mg of lignin added to 5 mL of citrate-phosphate buffer (lignin concentration 1 % w/v). The tubes were sealed with plastic caps and incubated under agitation (1500 rpm, mixing orbit 2 mm) and constant temperature (30 °C) in a thermoshaker incubator (Land Technics MSC-100). The laccase dosage was 50 U/g lignin by adding 15–70 μL of the enzyme. All experiments, as well as the controls with thermally inactivated laccase, were performed in triplicate. Samples were taken at 0, 0.5, 1, 2, 4, 8 and 24 h, which were

centrifuged at 10,000 rpm for 10 min to measure methanol content and laccase activity in the supernatant. After that, the solid was washed twice with distilled water and freeze-dried for further analysis.

The variables tested on a small scale were a) laccase source (*T. versicolor*, *M. thermophila* and *G. lucidum*), b) pH (3, 4, 5, 6 and 7), c) temperature (20, 30 and 40 °C), d) addition of surfactants (150 mM PEG-600 %, and 1 % v/v Tween-80, which correspond with 398 and 50 μL, respectively) and e) addition of mediators (HBT and vanillin 30 mM, which correspond with 20.3 and 22.8 mg, respectively). The objective of this preliminary screening to increase the solubility of lignin and to evaluate laccases with different redox potentials. The parameters were evaluated following a one factor at a time (OFAT) approach in the given order.

2.4. Enzymatic modification of lignin in 150-mL reactor

A 150 mL-reactor (Biostat Q, B. Braun Biotech) was used to study the impact of aeration on laccase activity under more controlled conditions such as those afforded by an automated fermenter with numerous sensors and ports to monitor lignin modification. The reactor allowed continuous measurement of redox potential, pH, dissolved oxygen and temperature, and had the option of supplying air at a maximum flow rate of 110 mL/min.

In these experiments, the oxygen requirements for lignin oxidation by *T. versicolor* laccase, the addition of a mediator and the use of surfactants to improve lignin solubility were evaluated under optimised pH and temperature conditions at test-tube scale (pH 4 and 30 °C). Controls were performed with thermally inactivated enzyme. Triplicate test samples were withdrawn at 0, 0.5, 1, 2, 4, 8 and 24 h to measure the methanol released and check the enzyme activity described above.

2.5. Analytical protocols

2.5.1. Determination of enzyme activity

Laccase activity was determined by measuring the oxidation of 0.267 mM ABTS to its cation form ($ABTS^{+\cdot}$) at 420 nm ($\varepsilon_{420} = 36000\ M^{-1}\ cm^{-1}$) in McIlvaine buffer (pH 3) at room temperature (Muñiz-Mouro et al., 2017). During oxidation (Fig. S1) the reaction medium turns green due to the oxidised form of ABTS, so that the higher intensity of this colour (measured spectrophotometrically) is directly related to higher enzyme activity. One unit (U) of activity was defined as the amount of enzyme forming 1 μmol of $ABTS^{+\cdot}$ per min. All spectrophotometric measurements were carried out on a Shimadzu UV-1800.

2.5.2. Measurement of methanol content

The degree of lignin demethylation was measured indirectly by the release of methanol during modification by laccase. The methanol concentration was measured using the enzymatic method reported by Mangos and Haas (1996) with some modifications. This protocol uses two enzymes in cascade, an alcohol oxidase (AO) with high selectivity towards methanol, and a horseradish peroxidase (HRP), which uses the hydrogen peroxide generated by AO as a cofactor in the oxidation of ABTS, whose product is monitored spectrophotometrically. The protocol uses three solutions, which were prepared as follows. Solution A was prepared by dissolving ABTS diammonium salt, Sigma A1888) in distilled water (10 mg/mL). Solution B corresponds to 0.65 mg of horseradish peroxidase (HRP) (Sigma-Aldrich P6782) dissolved in distilled water and diluted to 1 mL, 20 μL of that solution diluted to 2 mL with distilled water. Solution C was an alcohol oxidase (AO) solution, which was prepared by adding 30 μL of alcohol oxidase solution from *Pichia pastoris* (Sigma A2404) to 2 mL of distilled water.

Solution A (30 μL, ABTS), solution B (10 μL, HRP) and solution C (10 μL, AO) were pipetted into one well of a microplate. Aliquots (150 μL) of methanol standards (0.1–2.0 mg of methanol/L) or sample diluted in 0.20 M potassium phosphate buffer (pH 7.5) were added. The microplates were placed in a Biotek Powerwave XS2 spectrophotometer, shaken for 2 s, and the kinetic software was started instantly. The absorbance at 420 nm was recorded for 60 s, which was then used to calculate the maximum slope (AU/min) using R statistical software (R-CRAN). A calibration curve was obtained by correlating methanol concentration and the slope of the absorbance at 420 nm vs time. To counteract the sample matrix effect, a volume of the initial sample was added to the methanol standards to achieve the same concentration in the samples. An example of a calibration curve for the experiment of lignin activation with air supply in the bioreactor is shown in Fig. S2. The concentration of methanol for preparing the calibration curve was in the range of 0–2 mg/L.

2.5.3. Indirect measurement of lignin solubility

Two indirect analytical methods were used to determine lignin solubility: ultraviolet absorption at 280 nm and determination of total phenolic content. The phenolic content of the modified lignin and its controls was measured using the Folin-Ciocalteu protocol (Singleton et al., 1965). First, 6 mg of freeze-dried treated lignin was dissolved in 1 mL of 0.2 M NaOH, and then different dilutions were prepared. 75 μL of diluted samples or gallic acid (used as standard) were added to 625 μL of Folin-Ciocalteu reagent (diluted at a ratio of 1:10 and 500 μL of Na_2CO_3 (7.5 % w/v)). The mixture was kept in the dark at room temperature for 1 h to complete the reaction, and then the absorbance was measured at 760 nm ($\varepsilon_{760} = 1.45 \times 10^3\ M^{-1}\ cm^{-1}$). The sample blank was prepared with 75 μL of methanol instead of the sample. Results were expressed as mg gallic acid equivalent (GAE) per g lignin as an average of two replicates. All spectrophotometric measurements were carried out on a Shimadzu UV-1800.

2.5.4. Fourier Transform Infrared Spectroscopy (FT-IR)

FT-IR spectra of the freeze-dried samples of unmodified and demethylated organosolv lignin were recorded on a VARIAN FT-IR 670 spectrometer provided by an attenuated total reflection (ATR) Miracle diamond crystal. The powder samples were placed in the diamond eye (1.8 mm) of the ATR equipment and the sample contact was secured by tightly screwing the clamping device. Each extract was scanned by recording the spectrum with 32 scans with a resolution of 4 cm^{-1} and the spectral width from 400 to 4000 cm^{-1}.

2.5.5. Size exclusion chromatography (SEC) analysis

Lignin was analysed by SEC in order to determine the weight-average molar mass (M_w) and number-average molar mass (M_n). Prior to analysis, 1 g of freeze-dried sample was dissolved in 1 mL of 0.1 M NaOH. An HP-1100 HPLC system was applied to measure the molecular weights of the different lignin samples, using 10 mM NaOH aqueous solution as mobile phase on MCX 100 A 5 μm 8 × 300 mm (PSS) and MCX 1000 A 5 μm 8 × 300 mm (PSS) columns coupled in series and with a MCX precolumn 5 μm 8 × 50 mm (PSS). A flow rate of 1 mL/min, a sample injection volume of 20 μL and an IR 1047 A-HP detector were used. Calculations were based on calibration curves obtained from monodisperse pullulan standards (with specific molecular weights of 180–708000 Da). Quantification of the peak signals was performed with Unichrom software. The determination of M_w and M_n was carried out using an R script programmed based on the algorithm reported by López-Abelairas et al. (2015). The molar mass dispersity ($Đ_M$) was calculated according to the equation:

$$Đ_M = M_w/M_n \quad (1)$$

2.5.6. Matrix-Assisted Laser Desorption/Ionization (MALDI) analysis

Samples for matrix-assisted laser desorption ionization time-of-flight (MALDI-ToF) analysis were analysed using 2,5-dihydroxy benzoic acid (DHB) as matrix and following the protocol described by Chen et al. (2021). A Shimadzu Biotech Axima-Performance mass spectrometer

(Kratos Analytical Shimadzu Europe Ltd.) was used for all measurements using a linear polarity positive fitting mode. A detailed protocol can be found in the Supplementary Material (Text S1).

2.6. Statistical analysis

Statistical analysis of the experimental results was performed using R software (version 4.0.5, R Core Team, 2021). To determine whether there were significant differences between the values released methanol from laccase and the temperature optimisation experiments, a one-way analysis of variance (ANOVA) was applied. If the ANOVA confirmed the difference, a post hoc analysis (Tukey's HSD) was applied to determine between which values the difference was significant. A significance level (α) of 0.05 was considered for all the statistical analysis. The main results of the statistical analysis were included in the Supplementary Material.

3. Results and discussion

The main objective of this study is the removal of methyl or methoxyl groups from lignin, in order to promote its reactivity for the synthesis of bio-adhesives (Hu et al., 2011). In addition, laccase treatment further leads to oxidation, polymerization and depolymeration of lignin (Venkatesagowda and Dekker, 2021). Therefore, further analysis of the transformed lignin, considering the total phenolic content and changes in structure by FT-IR, SEC and MALDI-ToF, should be addressed.

To improve the performance of the demethylation reaction, different parameters influencing both the enzymatic activity and the enzyme-substrate interaction were optimised. Lignin demethylation has been directly correlated with methanol release (Bourbonnais and Paice, 1992). For this reason, the methanol released was taken as a key parameter indicative of the conditions that allow progress in each of the optimisation steps.

3.1. Selection of laccase for lignin modification

The demethylation of organosolv lignin was studied using laccases of different redox potentials. For this purpose, two commercial laccases from *T. versicolor* and *M. thermophila*, with high (E_0 = 780 mV) and low (E_0 = 470 mV) redox potentials, respectively, were used (Singh et al., 2015). Furthermore, crude laccase from *G. lucidum* was evaluated, which has mid-redox potential (Kumar et al., 2021).

As shown in Fig. 1a, a higher amount of methanol was released after 24 h in the enzymatic modification of organosolv lignin with *T. versicolor* laccase (107 mg/L), which is related to a higher degree of lignin demethylation (Venkatesagowda, 2019). Previous studies have demonstrated that *T. versicolor* laccase is capable of attacking the methyl/-methoxyl groups of lignin, but in most of them this enzyme requires the use of a mediator compound that increases the catalytic potential of the enzyme (Venkatesagowda, 2019). The low-redox potential laccase reached a slightly lower value of methanol released after 24 h (90 mg/L), although it seemed that the demethylation with this enzyme was faster during the first hours of the reaction.

The lower amount of methanol released with *G. lucidum* laccase compared to that obtained with the other enzymes may be a consequence of the presence of inhibitors in the enzyme crude, since a black liquor-based medium from the organosolv treatment of beech wood was used for laccase production. This behaviour was also reported by Ibrahim et al. (2011), who showed that the use of a laccase crude was less efficient in the demethylation of lignin than the use of the purified enzyme.

In terms of phenolic content (Fig. 1b), all three enzymes led to a decrease in phenolic groups. This decrease is similar for the experiments with *T. versicolor* and *M. thermophila* laccases (22 % and 17 %, respectively). Whereas, in the case of the experiments with *G. lucidum* laccase, the reduction in phenolic content was about 3 times lower (6 %). These results show a direct relationship between the methanol released and the reduction of phenolic hydroxyl groups, with a higher reduction of phenolic hydroxyl groups with higher methanol release. In contrast, many studies have reported an increase in phenolic content after chemical oxidation of lignin (Song et al., 2016; Wang et al., 2018), which may be attributed to the transformation of oxidised methoxy groups into catechol intermediates. However, M. Wang et al. (2018); F. Wang et al. (2018) suggested that the absence of catechol intermediates after enzymatic treatment with laccase may be caused by the rapid oxidation of catechol to quinones. In view of the results obtained with *G. lucidum* laccase, we can conclude that the use of a crude laccase produced in a complex medium is not suitable for the enzymatic transformation of lignin.

A statistical analysis (Fig. S3) of the results of methanol released with *T. versicolor* and *M. thermophila* laccases was performed. It was found that the methanol released in the *T. versicolor* laccase treatment was significantly higher than that of *M. thermophila* (p-value 0.03885). For this reason, optimisation of the enzymatic demethylation of organosolv lignin was continued with *T. versicolor* laccase.

3.2. Influence of pH and temperature on lignin demethylation

The enzymatic modification of organosolv lignin was evaluated in the pH and temperature range most typical of laccase-catalysed

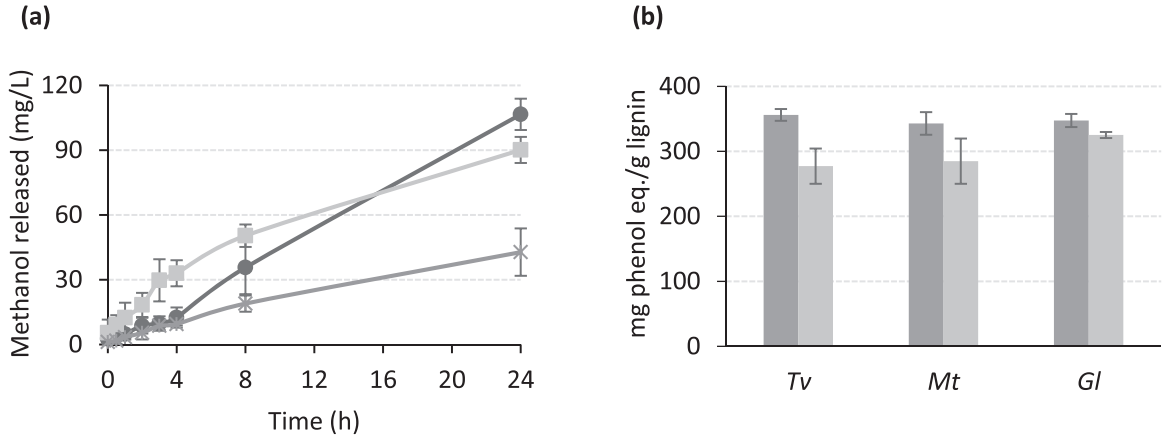

Fig. 1. Parameters measured during the enzymatic treatment of organosolv lignin at pH 6 and 30 °C in 5 mL experiments: (a) methanol released with *T. versicolor* (●), *M. thermophila* (■) and *G. lucidum* (×) laccase; (b) phenolic content after 24 h of enzymatic transformation with *T. versicolor* (Tv), *M. thermophila* (Mt) and *G. lucidum* (Gl) laccase (■) and their respective controls (■) with inactivated laccases.

reactions. Although lignin is more soluble at basic pH, enzymatic demethylation of lignin by laccase requires the use of neutral or acidic pH (Hämäläinen et al., 2018), as fungal laccase enzymes have an optimal pH range between 2.5 and 7, depending on the type of substrate (Gavrilaş et al., 2012; Kurniawati and Nicell, 2008; Morozova et al., 2007). Hence, pH values from 3 to 7 were evaluated in this study.

As shown in Fig. 2a, the highest lignin demethylation was obtained at acidic pH values. In this regard, values of 120–130 mg/L methanol were detected at pH 3–4 (Fig. S4). At neutral pH, no methanol release was observed during 24 h. At the same time, the enzyme showed the highest stability, with only 6 % loss of activity after 24 h, while at pH 3 laccase lost 75 % of the initial activity (Table S1). It is well known that laccase activity generally decreases with increasing pH due to inhibition by hydroxide anion, which binds to the trinuclear T2/T3 site to interfere with electron transfer from the T1 to T2/T3 sites (Xu, 1997).

In the case of temperature, the optimal activity of fungal laccases is between 30 °C and 55 °C (Kumar et al., 2012), although considering the duration of the reaction (24 h), it was decided to use temperatures between 20 °C and 40 °C. As shown in Fig. 2b, the highest methanol release was achieved at 40 °C (132 mg/L), while at 20 °C the methanol released was less than 75 % of the value achieved at other temperatures. Statistical analysis (Fig. S5) shows that there was no significant difference between methanol released at 30 °C and 40 °C (p-value 0.1391). Taking this into account and considering that the loss of enzymatic activity was lower at 30 °C than at 40 °C (Table S1), 30 °C was chosen as the optimal temperature for further optimisation of the enzymatic transformation of lignin.

Lignin treated with *T. versicolor* laccase at optimum pH (pH 4) and temperature (30 °C) was analysed by FT-IR and compared with the original lignin and with a control using inactivated laccase. The observed band assignments and their relative intensity are listed in Table 1 and were based on Hansen et al. (2016). The band at 1595 cm^{-1}, corresponding to aromatic ring vibrations, was used as the reference to calculate the relative intensities (Rashid et al., 2016). The absorption spectra of the different lignin samples show a similar profile (Fig. S6), although the relative intensity of certain bands showed differences. The control and untreated lignin (Fig. S6-a,b) showed similar relative intensities, suggesting that the addition of buffer and inactivated enzyme slightly affected the absorption spectrum. The band at 1216 cm^{-1}, that is related with phenolic groups, showed a decrease in the relative intensity, confirming that the phenolic content is affected after the enzymatic transformation at optimum pH and temperature. Methyl and methoxyl groups (with bands at 2935, 2841 and 1456 cm^{-1}) showed a decrease in relative intensities. On the other hand, the band associated with unconjugated C═O (1708 cm^{-1}) increased the relative intensity.

Table 1
Signal assignment and relative intensities in FT-IR spectra of untreated lignin, control (with inactivated laccase) and treated with laccase at optimum pH and temperature.

Wavenumber (cm^{-1})	Assignment	Relative intensity[a]			
		untreated	control	treated	Δ[b] (%)
3388	Phenolic OH + aliphatic OH	0.74	0.72	0.69	-3.5
2935	$CH_3 + CH_2$	0.71	0.71	0.66	-8.1
2841	$CH_3 + OCH_3$	0.56	0.56	0.52	-7.4
1708	C═O (Aromatic-Carbonyl)	0.43	0.38	0.44	+ 15.3
1456	$CH_3 + CH_2$	1.34	1.32	1.09	-17.4
1213	Phenolic OH	1.63	1.60	1.38	-13.7

[a] Relative intensity was calculated as the ratio of intensity of each band to the intensity of the reference band at 1595 cm^{-1}.
[b] Change in relative intensity; a negative value means a decrease, whereas a positive value means an increase.

3.3. Improvement of lignin solubility

Most of the tests performed on the chemical demethylation of lignin have been carried out in alkaline media to enhance lignin solubility (Evstigneev, 2011). However, when laccase is used to carry out the demethylation, an alkaline pH would negatively affect the activity of the enzyme as it would cause its inactivation within a short period of time. It has been shown that, at basic pH, protein denaturation occurs as a result of the disruption of enzymatic hydrogen bonds by excess OH^- ions (Saoudi et al., 2017). For this reason, enzymatic treatment must be performed at neutral or acidic pH, with the disadvantage of ensuring poor performance as the amount of dissolved lignin is lower.

To mitigate the poor enzyme-substrate contact resulting from this lower lignin solubility, it is important to evaluate the impact of the addition of different surfactants that can increase the amount of solubilised lignin. In this case, Tween-80 and PEG-600 were evaluated as surfactants that can increase the apparent solubility of lignin (Saoudi and Ghaouar, 2019; Xu et al., 2016). First, the impact of the addition of these compounds on the solubility of lignin was evaluated after 17 h. Through two analytical techniques: measurement of the soluble lignin fraction at 280 nm (Fig. 3a) and determination of the total phenolic content of the dissolved fraction (Fig. 3b) it was possible to identify the effect of the surfactants considered. The first technique is a direct measurement, while the second is an indirect measurement, in which a higher phenolic content in the soluble fraction can be related to a higher

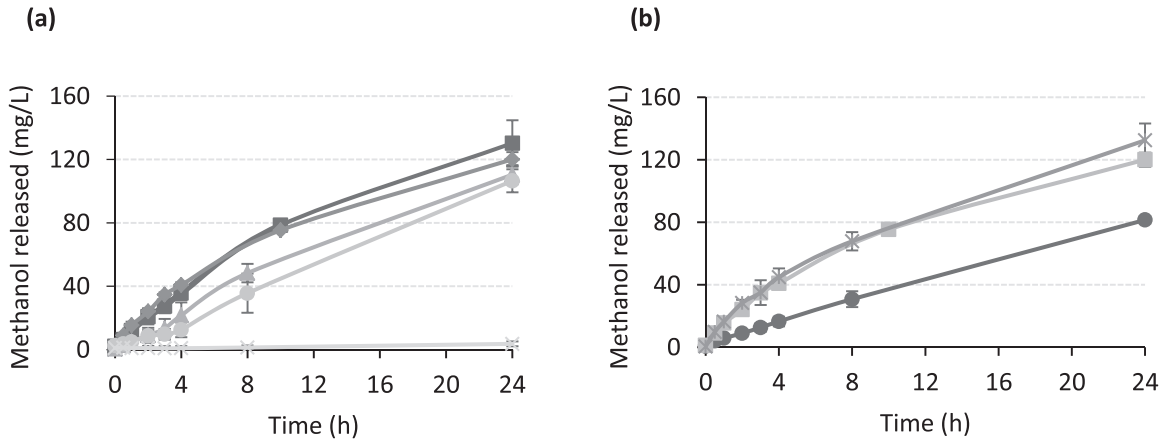

Fig. 2. Methanol released during the enzymatic treatment of organosolv lignin by *T. versicolor* laccase in 5-mL experiments: (a) at pH 3 (■), 4 (♦), 5(▲), 6 (●) and 7 (×); (b) at 20 (●), 30 (■) and 40 °C (×).

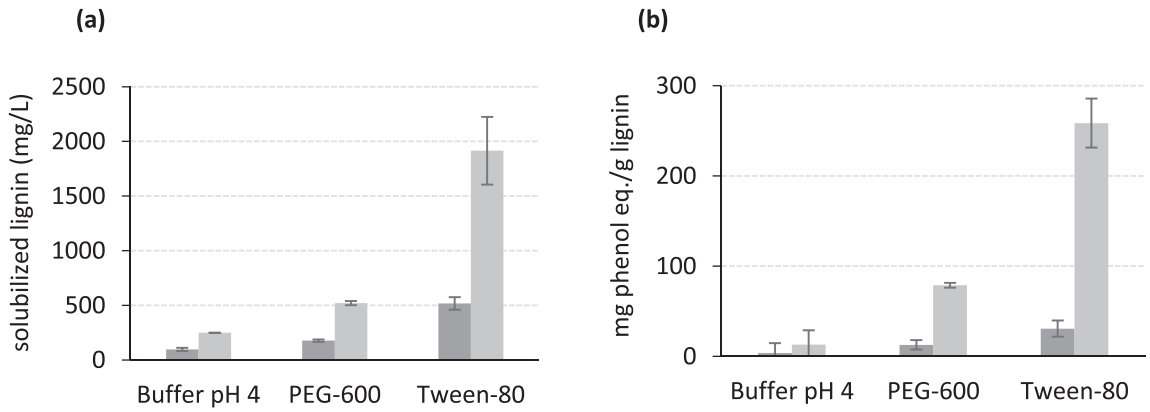

Fig. 3. Measurements of lignin solubility in buffer and different solubilising agents by (a) measurement of soluble lignin through absorbance at 280 nm and (b) determination of total phenolic content of dissolved fraction, at 0 h (■) and 17 h (■).

amount of dissolved lignin, since the only compound in the medium that has phenolic groups is lignin.

As shown in Fig. 3, Tween-80 and PEG-600 improve lignin solubility relative to the citrate-phosphate buffer at pH 4 used previously. Compared to samples at pH 4, the initial solubility increased 1.8 and 5.3-fold using PEG-600 and Tween-80, respectively, when measuring soluble lignin via absorbance at 280 nm. After 17 h, the improvement in solubility compared to the buffered sample was slightly higher, with 2.1 and 7.7-fold increases for PEG-600 and Tween-80, respectively. The same effect was observed when measuring total phenolic content (Fig. 3b), with a more pronounced effect for Tween-80 and in particular, after 17 h of incubation (19.8-fold higher, while the initial solubility was 8.0-fold higher than the buffered sample).

After evaluating the positive impact of Tween-80 and PEG-600 on lignin solubility, lignin demethylation was performed in the presence of both compounds. Fig. 4 shows that the methanol concentrations detected in the experiments with Tween-80 (202 mg/L), and PEG-600 (165 mg/L) were higher than that of the control experiment (120 mg/L). It is known that small length PEG can act as a stabiliser of the laccase structure, while increasing PEG chain length is associated with a decrease in laccase activity (Saoudi and Ghaouar, 2019). The loss of activity in the experiments with Tween-80, PEG-600 and the buffer control was 5.8 %, 28 % and 68.6 %, respectively (Table S1). Both polymers protected the enzyme from inactivation (Saoudi and Ghaouar, 2019; Wang et al., 2018), although Tween-80 exerted a more pronounced effect. Therefore, both the increase in apparent solubility and the stabilisation of laccase were more favourable when Tween-80 was used, so that a higher methanol release was observed.

FT-IR analysis of laccase treated samples containing Tween-80 (Table 2) showed a decrease in phenolic (1213 cm^{-1}) and hydroxyl groups (3338 cm^{-1}) and an increase of the carboxyl groups (1708 cm^{-1}) as observed previously. However, not all relative intensities corresponding to methyl/methoxyl groups decreased, since the intensities at 2935 and 2841 cm^{-1} corresponding to $CH_3 + CH_2$ and $CH_3 + OCH_3$, respectively, increased after treatment. However, it should be noted that the presence of the surfactant increased the intensity of these bands, as observed when comparing the control (presence of Tween-80) and the original lignin. Therefore, Tween-80 could be masking may reduce part of the expected reduction in the content of methyl/methoxyl groups, which may be obscured in the FT-IR spectra of Tween-80 treated lignin and its control (Fig. S6-c).

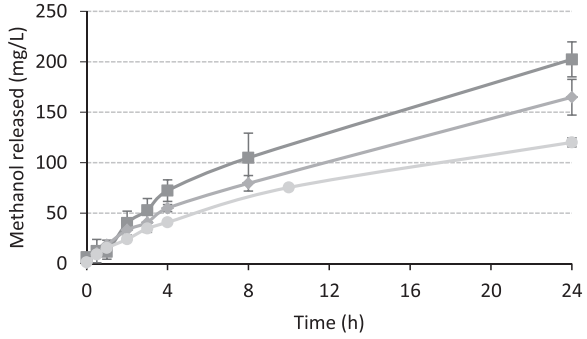

Fig. 4. Methanol released during the enzymatic treatment of organosolv lignin by *T. versicolor* laccase in 5-mL experiments using Tween-80 (■) and PEG-600 (♦) as solubilising agents, and in the experiment at pH 4 and 30 °C without addition of solubilizing agents (●).

Table 2
Signal assignment and relative intensities in FT-IR spectra of untreated lignin, control (with inactivated laccase) and treated with laccase at optimum pH and temperature and the presence of Tween-80.

Wavenumber (cm^{-1})	Assignment	Relative intensity[a]			
		untreated	control	treated	Δ[b] (%)
3388	Phenolic OH + aliphatic OH	0.74	0.64	0.57	-11.9
2935	$CH_3 + CH_2$	0.71	0.99	1.07	+ 7.4
2841	$CH_3 + OCH_3$	0.56	0.81	0.88	+ 8.7
1708	C═O (Aromatic-Carbonyl)	0.43	0.56	0.64	+ 13.0
1456	$CH_3 + CH_2$	1.34	1.47	1.31	-10.6
1213	Phenolic OH	1.63	1.65	1.34	-18.9

[a] Relative intensity was calculated as the ratio of intensity of each band to the intensity of the reference band at 1595 cm^{-1}.
[b] Change of the relative intensity with respect to the control with inactivated laccase; a negative value means a decrease, whereas a positive value means an increase.

3.4. Laccase mediator system (LMS)

The use of compounds acting as mediators is common in polymer oxidation by laccase, due to the ability of these compounds to increase the redox potential of the enzyme (Nguyen et al., 2016). To increase lignin demethylation, two types of mediators were evaluated: a natural compound (vanillin) and a synthetic compound (HBT). In the presence of these compounds, laccase generates radicals that are capable of oxidising phenolic and some non-phenolic lignin models (Crestini et al., 2003; Kupski et al., 2019).

Fig. 5 shows the methanol released both with LMS and with the joint use of HBT and Tween-80. Methanol released (86.2 mg/L) in the LMS with vanillin was lower than that achieved in the experiment at the same pH and temperature without mediator (120.1 mg/L). In the HBT

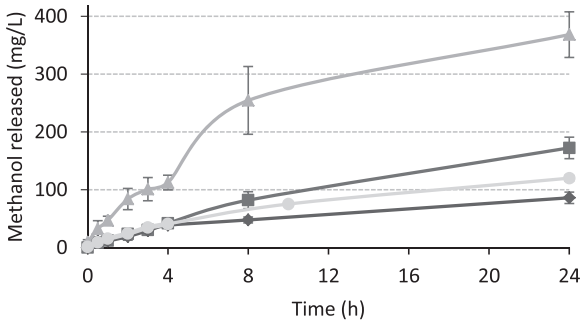

Fig. 5. Methanol released during the enzymatic treatment of organosolv lignin by *T. versicolor* laccase at pH 4 and 30 °C in 5-mL experiments using HBT (■) and vanillin (♦) as mediators, in the experiment with the addition of Tween-80 and HBT (▲) and in the experiment at pH 4 and 30 °C without the addition of surfactant or mediator (●).

experiment, the methanol released increased to 172 mg/L, which is 1.43 times the amount of methanol released without mediator. The combined use of HBT and Tween-80 resulted in 368 mg/L methanol released. This high increase is an indicator of the high yield of both compounds in the demethylation reaction. The role of HBT in the oxidation of laccase together with the increase of lignin solubility by Tween-80 had a high impact on the demethylation process.

It is also notable that the loss of laccase activity due to the addition of HBT was somewhat reduced due to the presence of Tween-80 (Table S1). In this regard, the presence of HBT resulted in the loss of 45.8 % of the initial activity; whereas when Tween-80 was also added, this value was reduced to 19.8 %. This loss of activity was lower than that reported in previous studies, even using longer reaction times (Bourbonnais et al., 1997). FT-IR analysis of the LMS-treated lignin showed a similar trend, with a slight increase in carbonyl groups, and a decrease in methyl/-methoxyl and hydroxyl groups (Table 3). The complete lignin spectra after enzymatic treatment with the addition of HBT and its control are included in the Supplementary Material (Fig. S6-d).

3.5. Aeration

It has been shown that oxygen supply has a remarkable effect on laccase-mediated oxidation of organosolv lignin (Ortner et al., 2015). For this reason, after optimisation of the main parameters related to laccase activity, the impact of adding an oxygen source to the reaction medium was evaluated. For this purpose, the demethylation process at pH 4 and 30 °C was carried out in a 150 mL-reactor to ensure better control of the continuous air supply and thus maintain the oxygen concentration above 80 % of its saturation value throughout the experiment.

The first set of experiments were carried out in the absence of mediator and surfactant in the reaction medium. The methanol released in the 150 mL non-aerated experiment (76.15 mg/L, Fig. 6) was substantially lower than the previous control (120.1 mg/L). This difference may be associated with an under-quantification of the methanol release, due to its partial vaporisation. It should be noted that, unlike the experiments performed in 5 mL tubes, the reactor system is open, which probably contributes to the loss of methanol in the vapour phase. In fact, when comparing the FT-IR results of both experiments under the same operating conditions but at different scales, the spectra in the fingerprint region are very similar, suggesting a similar effect (Fig. S6-b, e).

However, in comparative tests performed in the presence and absence of oxygen, methanol release increased from 76 mg/L to 107 mg/L when air was supplied (Fig. 6), even though no significant differences were observed at the initial times, which is indicative of the fraction of oxygen that existed in the reactor headspace. In contrast, at longer reaction times, the need for an external oxygen supply to ensure optimal enzyme activity is noticeable (Fig. S7).

Analysis of FT-IR spectra of lignin treated with and without air supply compared to untreated lignin shows an increase in the relative intensity of the 1708 cm^{-1} band, related to carbonyl groups (Table 4, Fig. S5-e). Furthermore, it is remarkable the decrease in the intensity of the 3650–3250 cm^{-1} bands for lignin treated with *T. versicolor* laccase under aeration, which is more relevant than that of lignin treated under the same conditions in the absence of aeration. The band at 750–850 cm^{-1} is also of interest, because the higher intensity of treated lignin in the presence of aeration may suggest an increase of aromaticity in the final sample, which may be related to a polymerisation process (Fig. S6-e).

3.6. Optimal conditions

After optimisation of all variables in the organosolv lignin enzymatic transformation experiments, the reaction was carried out in the 150 mL-reactor under the optimal conditions (aeration, pH 4, T 30 °C, addition of Tween-80 and use of HBT). As shown in Fig. 7 and according to the values of methanol released, it is observed that Tween-80 and HBT are the key parameters to increase methanol release, presenting a greater impact than other elemental variables, such as pH, temperature, or aeration. However, this increase in lignin demethylation is more remarkable due to the synergistic action between HBT and Tween-80. In this case, methanol release reached 363 mg/L, compared to values of 202 and 173 mg/L when both compounds are considered individually. If it is taken into account that methoxyl groups represents 23.94 % of the total weight of organosolv lignin (Lê et al., 2016) and the relation

Table 3
Signal assignment and relative intensities in FT-IR spectra of untreated lignin, control (with inactivated laccase) and treated with laccase at optimum pH and temperature and the presence of HBT.

Wavenumber (cm^{-1})	Assignment	Relative intensity[a]			
		untreated	control	treated	Δ[b] (%)
3388	Phenolic OH + aliphatic OH	0.74	0.71	0.67	-6.1
2935	$CH_3 + CH_2$	0.71	0.72	0.68	-6.1
2841	$CH_3 + OCH_3$	0.56	0.57	0.55	-3.3
1708	C═O (Aromatic-Carbonyl)	0.43	0.38	0.41	+ 7.7
1456	$CH_3 + CH_2$	1.34	1.36	1.09	-19.5
1213	Phenolic OH	1.63	1.64	1.24	-24.2

[a] Relative intensity was calculated as the ratio of intensity of each band to the intensity of the reference band at 1595 cm^{-1}

[b] Change of the relative intensity with respect to the control with inactivated laccase; a negative value means a decrease, whereas a positive value means an increase

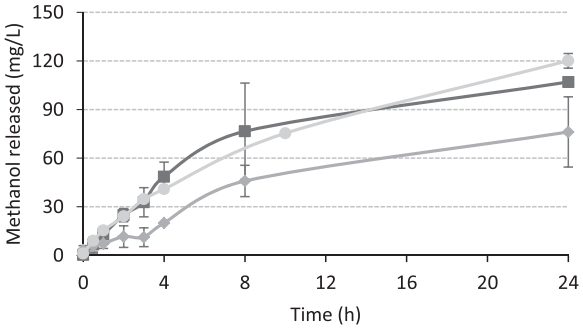

Fig. 6. Methanol released during the enzymatic treatment of organosolv lignin by *T. versicolor* laccase at pH 4 and 30 °C in 150 mL-reactor with aeration (■) and without aeration (♦), and in 5 mL scale at pH 4 and 30 °C without the addition of surfactant or mediator (●).

Table 4
Signal assignment and relative intensities in FT-IR spectra of untreated lignin, control (with inactivated laccase) and treated with laccase at the optimum pH and temperature with and without aeration.

Wavenumber (cm^{-1})	Assignment	Relative intensity[a]					
		untreated	control	no aeration	aeration	Δ[b] (%) no air	Δ[b] (%) air
3388	Phenolic OH + aliphatic OH	0.74	0.72	0.63	0.57	-12.2	-20.1
2935	$CH_3 + CH_2$	0.71	0.71	0.64	0.64	-10.2	-10.6
2841	$CH_3 + OCH_3$	0.56	0.56	0.50	0.48	-10.1	-12.9
1708	C═O (Aromatic-Carbonyl)	0.43	0.38	0.44	0.43	+ 14.9	+ 11.2
1456	$CH_3 + CH_2$	1.34	1.32	1.14	1.17	-14.2	-11.6
1213	Phenolic OH	1.63	1.60	1.42	1.48	-10.8	-7.5

[a] Relative intensity was calculated as the ratio of intensity of each band to the intensity of the reference band at 1595 cm^{-1}.
[b] Change of the relative intensity with respect to the control with inactivated laccase; a negative value means a decrease, whereas a positive value means an increase.

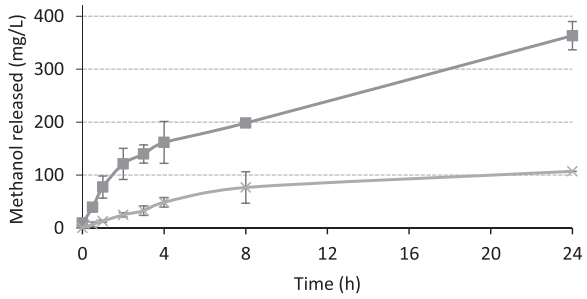

Fig. 7. Comparison of methanol released in 150 mL-reactor-scale experiments: addition of aeration at pH 4 and 30 °C (×) and use of all optimized conditions (pH 4, 30 °C, Tween-80, HBT and aeration) (■).

between methoxyl and methanol molecular weights, it can be stablished that the 363 mg/L released in this experiment represents a 14.7 % of demethylation of the original lignin. This value is in the same range that the reported by Wang et al., 2018 in the study of kraft lignin demethylation with *T. versicolor* laccase and HBT during 24 h and working with the same concentration of lignin and also with the addition of Tween-80 as solubilizing agent (19.2 % of demethylation). It is also remarkable that in the reactor the loss of activity was slightly higher (29.6 %, Table S1) than in the previous optimal conditions without aeration (19.8 %), but with a lower enzyme inactivation value than in the experiment with HBT and without addition of Tween-80, which confirms the protective effect of this compound on the enzyme even under aerated conditions.

The FT-IR analysis of the lignin treated in the reactor experiments shows the same trend as that of the lignin obtained in the small-scale optimisation (Fig. S6-f). The band related to the aromatic carbonyl (1708 cm^{-1}) is more intense for the treated lignin, and the decrease of the signals related to the phenolic hydroxyl group (1213 cm^{-1}) and methyl/methylene groups (1456 cm^{-1}) is also remarkable. All these results, in addition to the methanol released during the enzymatic treatment, reinforce the hypothesis of a two-step oxidation of the methoxy groups, with an initial transformation into hydroxyl groups, which are then oxidised to carbonyl groups. Table 5.

The SEC results (Fig. S8) show a slight increase in the molecular weight (791 Da) of the lignin treated under optimal conditions relative to the original lignin (Fig. S8-a). This polymerisation is probably obtained mainly through the formation of aromatic bonds of the hydroxyl group. These phenolic groups can be oxidised by laccase with a corresponding yield of lignin radicals that interact with other radicals to form phenyl-ether or carbonyl-ether bonds, with a corresponding increase in molecular weight (Antúnez-Argüelles et al., 2020). Whereas the lignin backbone, in which most of the aliphatic hydroxyl groups are located, was not degraded by laccase. This preference for aromatic hydroxyl groups over aliphatic groups in laccase has already been described by other studies (Gouveia et al., 2018), including studies using LMS with HBT as a mediator (Nugroho Prasetyo et al., 2010).

Table 5
Signal assignment and relative intensities in FT-IR spectra of untreated lignin, control (with inactivated laccase) and treated with laccase at optimum pH, temperature, the presence of Tween-80, HBT and aeration.

Wavenumber (cm^{-1})	Assignment	Relative intensity[a]			
		untreated	control	treated	Δ[b] (%)
3388	Phenolic OH + aliphatic OH	0.74	0.64	0.60	-5.9
2935	$CH_3 + CH_2$	0.71	0.99	0.92	-7.2
2841	$CH_3 + OCH_3$	0.56	0.80	0.74	-8.0
1708	C═O (Aromatic-Carbonyl)	0.43	0.53	0.61	+ 16.7
1456	$CH_3 + CH_2$	1.34	1.53	1.27	-17.0
1213	Phenolic OH	1.63	1.73	1.35	-22.0

[a] Relative intensity was calculated as the ratio of intensity of each band to the intensity of the reference band at 1595 cm^{-1}.
[b] Change of the relative intensity with respect to the control with inactivated laccase; a negative value means a decrease, whereas a positive value means an increase.

It is also noteworthy that in Fig. S8-b, comparing the control with the inactivated laccase and the lignin treated under optimal conditions, the molecular weight of the control is higher than that of the original lignin, which is attributed to the presence of Tween-80.

MALDI-ToF spectra of the original untreated lignin and the modified lignin under the optimal conditions in the reactor experiments are presented as Supplementary Material (Fig. S9 and S10). Although MALDI-ToF is not a quantitative technique, it can provide information of interest for predicting the architecture of the lignin molecule.

The spectra of modified lignin (Fig. S10) show the presence of higher dimers, trimers or oligomers with the addition of the lignin unit defined in Table S2 (176 Da). This pattern is indicative of the achievement of lignin polymerisation after enzymatic treatment. For example, in the scan range of *m/z* 300–600 and 600–900 Da a series was characterised starting from the precursor ion 360.96 (*m/z*), which gives rise to the ions of 536.6, 712.1 and 888.0 (*m/z*). Another series is that corresponding to 1035.7, 1210.9 and 1686.6 (*m/z*). The formation of these lignin oligomers could explain the decrease of 177.2 (*m/z*). The signal of the lignin monomer at 137.2 (*m/z*), related to guaiacyl units (Saito et al., 2005), also diminished in the treated lignin. Dimers and trimers of this compound, corresponding to signals at 223.2 and 408.8 (*m/z*), were detected in the enzymatically transformed lignin.

It is also important to note the systematic increase in 44 Da for the different *m/z* signals starting from 744.7 (Fig. S10 and S11) and covering the whole *m/z* range, which is related to the ethoxy repeat units of Tween-80 present in the control, the treated sample but not in the original untreated lignin (Fig. S9).

In order to compare the effect of the different parameters, Fig. 8 summarises the results of methanol release obtained throughout the optimization experiments. It is clearly observed that the addition of Tween-80 had a noticeable effect on methanol release, but the combined

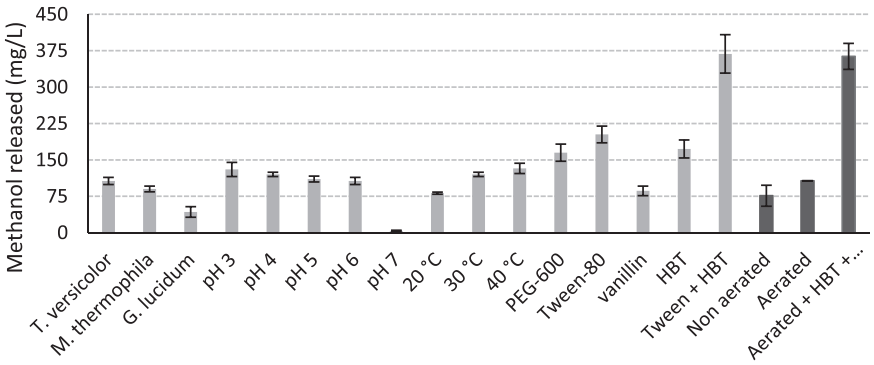

Fig. 8. Comparison of methanol released in all performed experiments: at 5 mL scale (light grey) and at 150 mL-reactor (dark grey).

action of the LMS and the addition of the surfactant seemed to have a synergistic effect, reaching almost double the methanol concentration in the medium. Tween-80 not only acts as a surfactant but is also a source of unsaturated lipids. Böhmer et al. (1998) demonstrated that the laccase/HBT system can catalyse the peroxidation of unsaturated fatty acids. In this mechanism, peroxyl radicals (ROO-) are strong oxidants that propagate lipid peroxidation, allowing the oxidation of recalcitrant compounds (Camarero et al., 2008). Elegir et al. (2005) demonstrated that LMS can oxidise fully methylated non-phenolic lignin substructures, but only in the presence of surface-active agents.

4. Conclusions

The demethylation of organosolv lignin has been achieved with different fungal laccases, concluding that commercial laccases produced higher lignin demethylation than the enzymatic crude obtained in complex fermentation media. A demethylated organosolv lignin suitable for use in the synthesis of bio-adhesives was obtained using *T. versicolor* laccase. It was shown that the most relevant parameters for the enzymatic demethylation reaction are those directly involved in the enzyme-substrate contact. In this case, these are the addition of solubilising agents to increase lignin solubility and the use of mediator compounds. The combined action of unsaturated fatty acids and LMS could favore lignin demethylation by lipid peroxidation mechanism. In addition, the optimal operating range of other key parameters in lignin demethylation (such as pH, temperature and aeration) was experimentally determined. The SEC and MALDI analyses revealed that the enzymatic treatment promoted the oligomerization of lignin.

The feasibility of using enzymatically demethylated lignin in the formulation of bio-adhesives is based on the transformation of part of the methoxy groups of the original lignin into hydroxyl groups and finally into carbonyl groups which are more reactive and may involve the formation of reinforced networks. Furthermore, the evaluation of the optimal conditions in the bioreactor scale paves the way to an industrial application of the process.

CRediT authorship contribution statement

S.G., TA.L. and G.E. designed the experiments; S.G. and TA.L. performed the experiments S.G., TA.L., X.C., A.P. and G.E. analysed the data, S.G., G.E. and MT.M. prepared the original draft; TA.L., G.E., G.F. and MT.M performed the revision and editing of the original draft. All authors have read and agreed to the published version of the manuscript.

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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.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.indcrop.2022.115253.

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