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COSMO-RS assisted selection of eutectic solvents for lignin dissolution and enhanced laccase activity

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ABSTRACT

Enzymatic lignin depolymerization provides a sustainable route for its valorization. Typically conducted in aqueous media, it faces technical limitations due to lignin's low solubility in water. Alternative media, such as aqueous solutions of eutectic solvents (ES), show excellent potential. However, selecting an optimal formulation that achieves both high lignin solubility and enhanced enzymatic activity remains a major challenge. This study aimed to establish an innovative method for screening and assessing the potential of ES in facilitating efficient lignin solubilization and enzymatic depolymerization, assisted by COSMO-RS and followed by experimental validation. Lignin solubility was predicted by calculating activity coefficient at infinite dilution of a lignin model compound in ES, while laccase activity was correlated with the water activity of the ES. A total of 10,638 combinations were screened, from which 22 showed promising properties and were selected for the experimental trials. Among those, an unexpected combination Acetamide:1,6-Hexanediol exhibited the highest lignin solubility of 46.5%*w*/w, achieving 111 % relative laccase activity and enhancing laccase-mediated depolymerization in the presence of mediators. For the best of our knowledge, this ES formulation was never addressed in literature for this purpose. Therefore, these findings offer promising insights and advance sustainable lignin valorization *via* enzymatic depolymerization.

1. Introduction

In the pulp and paper industries and second-generation biofuel production, lignin (15–30%*w*/w) is treated as a by-product, with 98 % of the 70–80 million tons produced annually being burned for energy generation [1–3]. An emerging valorisation route involves its depolymerization into low-molecular-weight aromatic monomers (*e.g.* phenol, vanillin, acetovanillone, guaiacol, among others), which are valuable for chemical industries or new product development [4–6]. Among the available methods, enzymatic depolymerization stands out for its high selectivity, mild conditions, and reduced energy demand [3,7]. Laccases are particularly promising enzymes due to their use of O_2 as the final electron acceptor [8], although their activity is mostly limited to phenolic units and may cause undesired polymerization [9]. To overcome this, laccase mediator systems (LMS) have been developed to expand laccase reactivity towards non-phenolic units, enabling efficient depolymerization of Kraft and alkaline lignin [10,11].

However, the complexity and structural heterogeneity of lignin present challenges in lignin depolymerization [12], while additional obstacles arise from the low solubility of lignin in conventional solvents, including water, that compromise the mass transfer for an efficient biocatalysis [3,13], as well as the low selectivity and inefficient product stabilization provided by the solvent.

Therefore, finding new solvent systems capable of dissolving lignin, providing biocompatibility with enzymes, and enabling stabilization of depolymerized products to avoid repolymerization is required. In this regard, green solvents, particularly eutectic solvents (ES), may stand as promising alternative to achieve these goals. ES are a class of alternative solvents that have been demonstrating high potential to dissolve and valorise lignin [14–16]. These solvents can be prepared by combining a hydrogen bond acceptor (HBA) and a hydrogen bond donor (HBD) in specific molar ratios, resulting in a melting temperature lower than that of the individual compounds [17]. ES can be tailored to modify various physicochemical properties, offering environmentally friendly and

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sustainable alternatives to traditional solvents, depending on precursor characteristics [18].

Regarding enzymatic processes, ES have demonstrated biocompatibility with certain enzymes [19], preventing their denaturation and deactivation, which are commonly observed in the presence of conventional organic solvents [20]. In laccase-mediated processes, aqueous ES solutions have been shown to enhance enzymatic activity, as observed by Chan et al. [21] and Toledo et al. [20]. Specifically, systems containing cholinium dihydrogen citrate ([Ch]DHC) as HBA led to a 200 % increase in laccase activity at 25 °C compared to phosphate buffer (pH 7.0). These improvements are attributed to synergistic interactions among ES components and are influenced by the ES type, molar ratio, and concentration [20].

The exploration of laccase activity in ES and their aqueous solutions is at an early stage of development. Selecting appropriate ES remains challenging due to the extensive possibilities of combining various HBAs and HBDs, while enhancing enzymatic activity is dependent on the solubility of the substrate and on the enzyme stability in the applied solvent system. These challenges might be circumvented by conducting an initial screening with computational tools linked with thermodynamic models to optimize the process of solvent design [22]. Among these models, the COnductor-like Screening MOdel for Real Solvents (COSMO-RS), stands out as a powerful predictive tool. It is a thermodynamic model grounded in quantum chemistry and statistical thermodynamics for solvent selection [23] that is independent of experimental data [24]. Additionally, COSMO-RS allows for the prediction of the system's activity coefficient, enabling the correlation of a solute's solubility within the system [25,26].

Besides using the COSMO-RS model to correlate the solubility of solutes in solvents, it was also employed to correlate enzyme activity in ionic liquids (ILs) using COSMO-RS descriptors of the examined solvents. Pedersen et al. [27] assessed parameters including hydrogen bond moments, ion sizes, sigma moments, and water activity coefficients to attempt to correlate these with cellulase activity in ILs. Their findings revealed that cellulase activity correlated with cation size, with smaller cations having less influence on enzyme activity, as well as with both the hydrogen bond basicity and second sigma moment (polarity) of anions. High polarity and hydrogen bond basicity of anions led to higher enzyme activities [27]. However, establishing a quantitative correlation between solvents with potential for lignin solubility and laccase activity is a challenging achievement, since multiple factors, including reaction conditions, solvent properties, biocatalyst characteristics, enzyme-solute *vs* enzyme-solvent interactions, among others [28] are also involved beyond the solubility parameter.

Considering the interaction complexity between laccase, ES and lignin, this study aimed to apply the COSMO-RS model as a multiple screening tool to identify HBA:HBD combinations that allow for high lignin solubility and simultaneously keeping or improving laccase activity using different descriptors inherent to the solvent. For this purpose, the activity coefficients of a lignin model compound (guaiacylglycerol-β-guaiacyl ether - GG) at infinite dilution in ES (ln ($\gamma^{\infty}_{GG,ES}$)) were calculated, which can be directly correlated with lignin solubility. On the other hand, the correlation between seven COSMO-RS descriptors of ES (hydrogen bond basicity and acidity of the HBDs and HBAs, molecular sizes (area and volume), second and third sigma moments, and the logarithm of the water activity coefficient in ES ln ($\gamma^{\infty}_{water,ES}$)) with laccase activity was performed. After selecting the solvents using this *in silico* approach, sequential experimental trials were conducted to assess laccase activity and lignin solubility, and the most promising ES was tested for laccase depolymerization of GG as a proof of concept.

2. Material and methods

2.1. Materials

Sodium phosphate dibasic, laccase from *Trametes versicolor* (> 0.5 U·mg^{-1}), acetonitrile (HPLC grade), guaiacylglycerol-β-guaiacyl ether (GG), 1-hydroxybenzotriazole hydrate (HBT), 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), and formic acid were purchased from Honeywell Fluka, Sigma, PanReac AppliChem, TCI, Aldrich, Alfa Aesar, and Carlo Erba Reagents respectively. Citric acid monohydrate and dimethyl sulfoxide (DMSO) were obtained from Fisher Scientific. The chemical structure, molar mass, CAS number, purity and supplier of the compounds used as HBA or HBD in this work are detailed in Table S1 from supplementary material. Water Milli-Q quality (< 18.2 μS·cm^{-1}, Millipore Corp., United States) was used in HPLC analysis (mobile phases and samples). Kraft lignin [16] (Suzano, Brazil) used in solubility assays is from *Eucalyptus urograndis.*

2.2. COSMO-RS model

COSMO-RS, a quantum chemistry-based thermodynamic model, was applied for the identification of ES exhibiting high lignin solubility and laccase activity. The Turbomole software (TmoleX19 Version 4.5) was used for geometry optimization and charge density calculations of molecules (COSMO-BP-TZVP) for subsequent COSMO-RS analyses. In this respect, GG (Fig. S2A) was chosen as lignin model compound, representative of the β-O-4 aryl ether bond lignin subunit [29–31]. COSMO-RS computations were conducted using the COSMOtherm package (Version 21.0) with the BP_TZVP_21 parameterization. A comprehensive set of 10,658 possible ES was generated by combining 73 hydrogen bond acceptors (HBAs) and 146 hydrogen bond donors (HBDs), while the infinite dilution activity coefficient of GG in those combinations at infinite dilution ($\gamma^{\infty}_{GG,ES}$) was calculated at 25 °C. The most promising combinations for lignin dissolution were selected based on their natural logarithm ($\ln(\gamma^{\infty}_{GG,ES})$), from which low values suggest potential high lignin solvating capacity of the solvent [32].

On the other hand, given the absence of a parameter directly correlating with laccase activity, seven COSMO-RS descriptors of the examined ES, namely hydrogen bond moments (H-bond basicity (Hb_acc3) and H-bond acidity (Hb_don3)) of the HBDs and HBAs, molecular sizes (area and volume), second (Sig_2) and third (Sig_3) sigma moment, and the natural logarithm of water activity coefficient in screened ES ($\ln(\gamma^{\infty}_{water,ES})$) [20] at 25 °C, were investigated to address correlations with laccase activity. These parameters were calculated using the COSMO-RS method within the Chemical Potential section, specifically employing the Mixture tool. All calculations were performed using the COSMOtherm package (Version 21.0) with the BP_TZVP_21 parameterization.

2.3. ES preparation

A diverse selection of alcohols, amides, amines, amino acids, carboxylic acids, inorganic salts, halogenated salts, sugars, phenols, and terpenes was used to prepare ES (chemical structures are shown in Table S1). The HBAs and HBDs were weighed in a fixed molar fraction of 1:1 to glass flasks using a Mettler Toledo XS 205 model with a precision of $\pm 2 \times 10^{-4}$ g. The glass flasks were placed on an aluminium block holder coupled with a heated stirring plate (Heidolph MR Hei-TEC model) and were heated at 80 °C with continuous stirring at 500 rpm until a homogeneous liquid was formed. ES aqueous solutions were also prepared at distinct ES concentrations using ultrapure water.

2.4. Determination of laccase activity via ABTS oxidation

The determination of laccase activity (P.1) was based on the methodology described by Toledo et al. [20] and Ander & Messner [33] with slight modifications.

In the first place, laccases were incubated in aqueous solution of sodium phosphate dibasic buffer (pH 7.0) containing selected ES (25%*v*/v), to a final enzyme concentration of 0.25 $g \cdot L^{-1}$ at room temperature during 10 min. Afterwards, 50 μL of prepared enzymatic medium was mixed with 250 μL of ABTS solution in water (0.4 mM) and 700 μL of 46 mM citrate/ 100 mM phosphate buffer (pH 4.5). The laccase activity can be determined through the oxidation of ABTS into a stable radical at 25 °C [34]. The formation of the radical was monitored by UV–Vis spectrophotometer (UV-1700 model, Shimadzu) at 420 nm and laccase activity determined with a 5 min kinetic model with 12 measurements/ min. The Eq. (1) expresses the amount of enzyme able to oxidize ABTS at 1 $\mu mol \cdot min^{-1}$ (one unit of laccase activity, U) per liter (U/L).

$$U/L = \left(A_{min} \text{ x } f_{dil} \text{ x } V_r \text{ x } 10^6\right)/\varepsilon_{420 \text{ nm}} \quad (1)$$

where A_{min} is the angular coefficient of the slope of the kinetic curve and determined by linear regression; f_{dil} is the dilution factor, V_r is the volume of reaction (in mL), 10^6 is the conversion of M into μM; and $\varepsilon_{420\ nm}$ is the molar absorption coefficient of ABTS (36.000 $M^{-1} \cdot cm^{-1}$). Along the study, the results of laccase activity are expressed as relative activity (%) to the control sample containing phosphate buffer (36 mM, pH 7.0) free of ES.

2.5. Lignin solubility in ES

The solubility of lignin (P.2) was determined using the saturation method proposed by Sosa et al. [16] In this method, an amount (2.0 ± 0.1 g) of neat ES and aqueous solution with different ES concentrations (0, 10, 25, 40, 50, 60, 85%*w*/w) were weighed in a glass vial assisted by a Mettler Toledo XS205 balance (precision of $\pm 2 \times 10^{-4}$ g). The vial was then placed in an aluminium block holder that was coupled to a heated stirring plate (Heidolph MR Hei-TEC model) to maintain the temperature at 40 °C (PT100). Subsequently, excess Kraft lignin was added to the mixture and stirred magnetically at 150–200 rpm for 24 h to ensure saturation. The samples were then filtered using a nylon filter (0.45 μm pore size, 25 mm diameter) to separate insoluble lignin, while the filtrate samples were subsequently diluted in dimethyl sulfoxide (DMSO) for UV analysis (Biotek Synergy HT Microplate Reader) at a wavelength of 280 nm. The determination of lignin solubility in samples ($g_{lignin} \cdot 100 g_{ES}^{-1}$) was carried out by preparing a calibration curve with known concentrations of Kraft lignin in DMSO.

2.6. Enzymatic depolymerization of guaiacylglycerol-β-guaiacyl ether (GG) in laccase mediator systems (LMS)

This step was inspired by in study of Hilgers et al. [9] with modifications. Laccases were incubated in aqueous solution of sodium phosphate dibasic buffer (pH 7.0) containing selected ES (25%*v*/v), to a final enzyme concentration of 2.5 $g \cdot L^{-1}$, at room temperature and during 10 min. After incubation, the sample (50 μL) was mixed with solutions of GG, citrate/phosphate buffer solution (pH 4.5), and mediator (ABTS or HBT). The volume of GG (0.4 mM), buffer solutions (46/100 mM), and ABTS (2 mM) or HBT (2.2 mM) were 125 μL, 700 μL, and 125 μL, respectively. The solution was stirred (400 rpm) and heated (40 °C) in a thermomixer (ThermoMixer C, Eppendorf) for 24 h. The resulting mixture was cooled in an ice bath for 15 min and centrifuged (MICROSTAR 17, VWR) at 15,000 rpm for 10 min. The decanted supernatant was then filtered with a syringe filter (nylon, 0.45 μm pore size, 25 mm diameter) and analyzed by HPLC. Control solutions with only lignin model compound and mediator (ABTS or HBT) were prepared.

Moreover, a kinetic profile of the enzymatic reaction was carried out at 0, 0.25, 2, 4, 8, and 24 h. At each time point, 400 μL of the sample was extracted and processed using the same procedure as the 24-h method. The samples were subsequently analyzed by HPLC methodology.

2.7. High-performance liquid chromatography

Samples were analyzed using an HPLC apparatus equipped with analytical C18 reversed-phase column (250 × 4.6 mm), RP18 CoreShell 5 μm, from SunShell (ChromaNik Technologies, Inc.), and a diode-array detection system (VWR Hitachi Chromaster) set at a 280 nm wavelength detection. The HPLC analytical method was adapted from Hilgers et al. [31]. The separation was performed in gradient mode at a flow rate of 1.2 $mL \cdot min^{-1}$ with sample injection volume of 10 μL. The mobile phases, Milli-Q water (phase A) and acetonitrile (phase B), were both acidified with 0.1%*v/v* formic acid. The gradient elution was as follows: 0.0–1.5 min at 5 % B isocratic, 1.5–9.0 min at linear gradient to 19 % B, 9.0–10.0 min at 19 % B isocratic, 10.0–11.5 min at linear gradient to 22 % B, 11.5–12.0 min at 22 % B isocratic, 12.0–13.5 min at linear gradient to 26 % B, 13.5–15.0 min at linear gradient to 30 % B, 15.0–21.0 min at linear gradient to 60 % B, and finally, the column was cleaned and reconditioned to the initial conditions of 5 % B. The column oven was maintained at 45 °C, while the automatic injector operated at 20 °C. The compounds were identified based on their retention times and literature data [30,31,35,36].

2.8. Experimental error

All experiments were performed in triplicate and the obtained results were expressed as means with associated standard deviation errors.

3. Results and discussion

3.1. Theoretical screening

The solubility of lignin in ES was first assessed in COSMO-RS by calculating the logarithmic activity coefficient at infinite dilution of lignin model compound GG in ES ($\ln(\gamma^{\infty}_{GG,ES})$) at 25 °C. A comprehensive study was conducted on 73 HBAs (Table S2,) and 146 HBDs (Table S3) in an equimolar mixture (1:1), resulting in a total of 10,658 potential ES. This parameter expresses the relative strength of intermolecular interaction between solute (GG) and solvent (ES) at infinite dilution, where low $\ln(\gamma^{\infty}_{GG,ES})$ values indicate stronger interactions between solute and solvent [37], or in other words, higher solute solubility in the solvent [26]. The GG solubility trend in ES is depicted in Fig. 1A, labelling the examined HBAs (vertical axe) and HBDs (horizontal axe), while the blue and yellow colours means low and high $\ln(\gamma^{\infty}_{GG,ES})$ values, respectively. At first sight, GG solubility is clearly affected by chosen ES precursors (HBA:HBD), where a higher impact of HBA than HBD on defining the best solvent for GG solubility is notorious. The halogenated salts (34–63 - Table S2) are shown as promising HBAs to increase lignin solubility in ES (low logarithmic values), while among HBDs, amides (1–7 - Table S3), alcohols (8–35 - Table S3), phenols (36–56 - Table S3), carboxylic acids (73–123 - Table S3), amino acids (124–133 - Table S3) and terpenes (139–141 - Table S3) demonstrated the lowest $\ln(\gamma^{\infty}_{GG,ES})$ values. On the opposite, sugars showed a negative impact on lignin solubility as HBA (64–69 - Table S2) as depicted in yellow to orange colours, while as HBD (57–72 - Table S3) with halogenated salts they seem to increase lignin solubility (Fig. 1A).

As observed in the literature, the presence of alcohols, carboxylic acids, and amino acids in the composition of ES has shown a positive impact on lignin solubility [16]. This alignment with previously reported data suggests that the trends observed in COSMO-RS offer a reliable approximation to reality [16,38]. The main factor contributing to the high lignin solubility in ES composed of these families of compounds is mostly attributed to the capacity to establish strong hydrogen bonds with lignin polar groups [39]. Particularly, HBDs with functional groups capable of establishing hydrogen bonds, including hydroxyl and

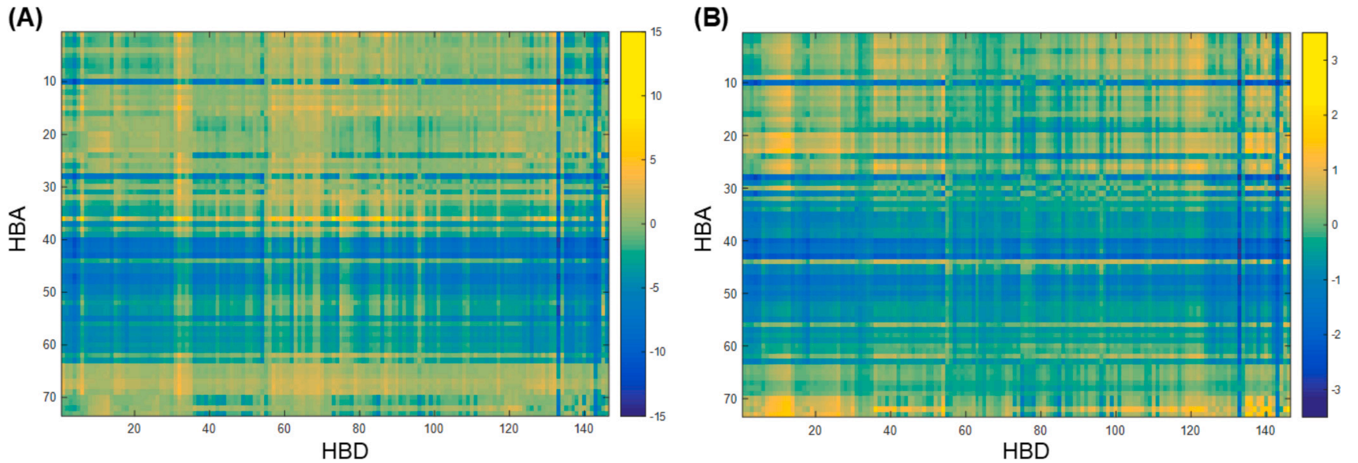

Fig. 1. Logarithmic activity coefficients at infinite dilution of (A) GG in ES ($\ln(\gamma_{GG,ES}^{\infty})$) and (B) water in ES ($\ln(\gamma_{water,ES}^{\infty})$), both predicted by COSMO-RS in 10,658 possible HBA:HBD combinations (1:1) at 25 °C. The structural families for HBA are: Amides (1–3), amino acids (4–16), carboxylic acids (17–22), inorganic salts (28–33), halogenated salts (34–63), sugars (64–69), phenols (70–71), terpenes (72–73); and for HBD are: Amides (1–7), alcohols, (8–35), phenols (36–56), sugars (57–72), carboxylic acids (73–123), amino acids (124–133), nitrogenated compounds (134–138), terpenes (139–141), and others (142–146).

carboxylic groups, favours lignin dissolution in ES [38]. Yet, the position and number of those groups in the HBD's structure may also exhibit negative effects. For instance, Sosa et al. [16] revealed that the position of hydroxyl group in malonic acid's structure increases steric hindrance with lignin interaction, decreasing lignin solubility in malonic-based ES [16]. Furthermore, a high number of hydroxyl groups (*e.g.* sugars) form a strong hydrogen bonding network increasing the competition of solvent-solvent interactions to the detriment of solute-solvent interactions [38]. This competition does not favour lignin dissolution in sugar-based ES, corroborating data shown in Fig. 1A.

The halogenated salts not only showed beneficial for lignin solubility when composing ES [40], but also when used alone as ionic liquids [41]. Particularly, cholinium chloride ([Ch]Cl - 34) has been used as HBA in ES formulation not only towards successful lignin solubility [15,16,40,42], but also for lignin extraction from biomass [43]. The high hydrogen basicity of chloride anion enables strong hydrogen bonding with lignin hydroxyl groups from lignin, being one of the reasons for its high capability for lignin solubility [44]. Moreover, the halide anion in ES was also observed to enhance the cleavage of aryl ether bond in lignin, suggesting a strong interaction with lignin macromolecular structure [45].

Additionally, due to the amphiphilic nature of lignin macromolecules, increasing the carbon chain length or giving aromaticity to the HBD might increase the dispersive and π-π interactions of ES with lignin [16,46]. In this sense, as suggested by data depicted in Fig. 1A, terpenes and phenols would provide dispersive and π-π interactions with lignin, respectively, leading to improved lignin solubility.

Although ES composed of HBA and HBD combinations that exhibit potential for effective lignin solubilization, as indicated by their low $\ln(\gamma_{GG,ES}^{\infty})$ values, do not necessarily correlate with enhanced laccase activity. In fact, while an ES may efficiently solvate lignin, it may not provide the required chemical environment to stabilize the enzyme. Furthermore, it is often observed in the literature that families of DES commonly reported to enhance laccase activity tend to exhibit lower lignin solubilization potential [20]. To address these challenges, an alternative COSMO-RS-based approach was employed to screen ES that are capable of maintaining or even enhancing laccase activity, while also optimizing lignin solubilization compared to conventional aqueous buffers.

As there is no single thermodynamic parameter directly correlating with enzyme activity, we examined seven descriptors, namely hydrogen bond moments (Hb), basicity (Hb_acc3) and H-bond acidity (Hb_don3), molecular sizes (area and volume), second (Sig_2) and third (Sig_3) sigma moments, and the logarithmic water activity of the ES ($\ln(\gamma_{water,ES}^{\infty})$). These descriptors were selected, while the relative laccase activity, measured *via* ABTS oxidation in the presence of an aqueous solution of ES at various water contents, was obtained from literature for correlation [20].

Among the examined COSMO-RS descriptors, $\ln(\gamma_{water,ES}^{\infty})$ exhibited the strongest correlation with experimental data (Fig. 2.), while no relevant parity was identified with other descriptors (Figs. S3 and S4). The most reasonable explanation relies on the fact that only $\ln(\gamma_{water,ES}^{\infty})$ effectively describes the behaviour of the ES mixture, whereas the other descriptors are associated with the individual molecules (HBAs and HBDs). Additionally, it is well-established in the literature that the water activity of the solution has a significant impact on enzyme activity [3,47].

Laccase activity tends to be higher for $\ln(\gamma_{water,ES}^{\infty})$ values close to zero (Fig. 2.). In other words, as $\ln(\gamma_{water,ES}^{\infty})$ approaches zero, the activity coefficient at infinite dilution of water in ES approaches unity, similar to pure water ($\gamma_{water,ES}^{\infty} \approx 1$). This observation aligns with the fundamental

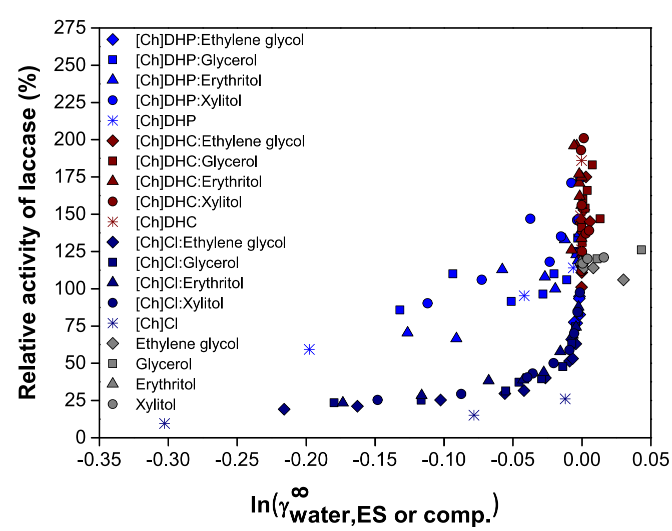

Fig. 2. Experimental data [20] of relative laccase activity (%) at 25 °C (*via* ABTS oxidation) correlated with the logarithmic water activity of the ES ($\ln(\gamma_{water,ES}^{\infty})$) and mixture of pure compounds in water ($\ln(\gamma_{water,comp}^{\infty})$) calculated by COSMO-RS.

understanding of enzymes behaviour in non-aqueous systems, where water content significantly influences enzyme-catalyzed reactions by impacting folding, structure, and function [3].

In a system composed of enzyme, water, and excess organic solvent (*e.g.* ES), water molecules will be distributed and constantly exchanging between two different regions, namely the protein vicinity layer and the bulk region. When the interaction of water and ES is weak, water molecules migrate to enzyme vicinity layer enabling a surface hydration shell capable of maintaining the enzyme stability (*e.g.* laccase) [47]. This apparently happens with ES composed of cholinium dihydrogen phosphate ([Ch]DHP) and citrate ([Ch]DHC) as HBA. Indeed, these ES not only provide stability to the laccase, but also increase its relative activity ($>$ 100 %). On the other hand, an opposite effect is expected in cases of strong interactions of water with ES ($\ln(\gamma^{\infty}_{water,ES}) < 0$), which decreases the surface hydration shell of the enzyme and consequently compromises its enzymatic stability. This is observed for ES composed of [Ch]Cl that show reduced laccase activity ($<$ 100 %, Fig. 2.). Although the chloride anion has shown to be a crucial element in lignin dissolution and extraction [15,16,40,42,43], its presence seems sometimes to be problematic for laccase activity, especially when combined with HBDs such as sugars and short carbon chain length alcohols [20]. This is due to its hydration capacity that reduces the number of water molecules surrounding the enzyme surface.

This reinforces that, while $\ln(\gamma^{\infty}_{water,ES})$ may not capture all aspects of enzyme–solvent interactions, it effectively reflects the interactions between water and the solvent, which are known to influence enzymatic activity. Therefore, water activity in ES can be a reasonable parameter for qualitatively screening ES capable of maintaining or enhancing laccase activity, in line with the ideas supported by Halling and Valivety [48] regarding enzymatic catalytic activity in non-aqueous media.

In this sense, $\ln(\gamma^{\infty}_{water,ES})$ were calculated for all HBA:HBD combinations proposed in this work and the obtained results are shown in Fig. 1B. The best HBA candidates with respect to laccase activity (values near zero, green region in the graph) were amides (1–3, Table S2), amino acids (4–16, Table S2), sugars (64–69, Table S2), and mainly halogenated salts (34–63, Table S2). The best HBD candidates were amides (1–7, Table S3), alcohols (8–35, Table S3), phenols (36–56, Table S3), sugars (57–72, Table S3), and carboxylic acids (73–123, Table S3).

To identify ES candidates that optimize both lignin solubility and laccase activity, the two matrices (Fig. 1A and B) were combined by multiplying the lignin solubility matrix ($\ln(\gamma^{\infty}_{GG,ES})$) by the absolute value of the laccase activity matrix ($\ln(\gamma^{\infty}_{water,ES})$). For lignin solubility, values below zero had a positive influence, highlighting solubility's importance. For the laccase activity matrix, only the range of $\ln(\gamma^{\infty}_{water,ES})$ values closest to zero were considered, as these values represent the highest enzymatic activity. Among these, a total of 22 combinations were selected, aiming to represent the most promising families of HBAs and HBDs, such as halogenated salts, inorganic salts, amides, amines, alcohols, amino acids, carboxylic acids, phenols, sugars, and terpenes. Organic salts ([Ch]DHC) were also chosen, as they have been reported in the literature as a high-potential HBA and will be used as a benchmark. Only 13 combinations were obtained in liquid form for the experimental screening of laccase activity, at a molar ratio of 1:1 (as described in Table S4).

3.2. Experimental screening: laccase activity

Fig. 3 illustrates laccase activity *via* ABTS oxidation at 25 °C in the presence of 13 selected ES aqueous solutions (25%*w*/w ES), each with a 1:1 M ratio of HBA to HBD, chosen from previous theoretical screening and benchmarked with [Ch]DHC:Erythritol (reference ES from Toledo et al. [20]). The results are expressed as relative activity (%) compared to the ES-free phosphate buffer (36 mM, pH 7.0). The detailed values and standard deviations are provided in the SI† (Table S5). At first sight,

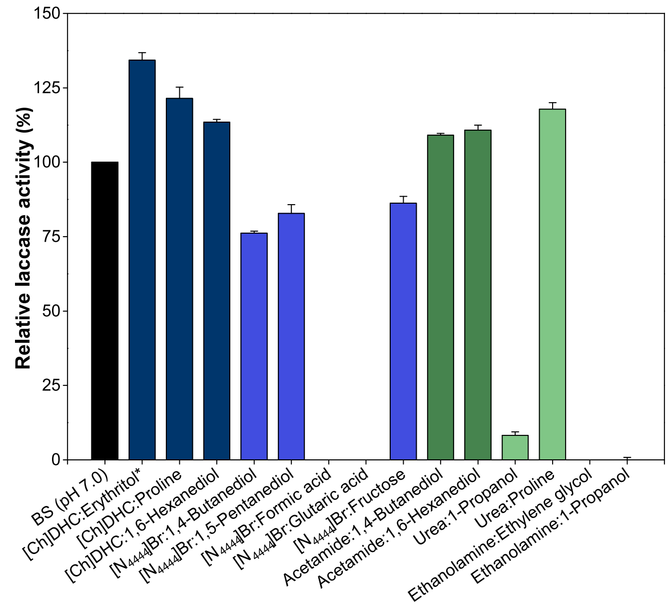

Fig. 3. Relative laccase activity (%) at 25 °C in the presence of selected HBA: HBD (1:1) combinations for the formation of ES aqueous solutions with a 25% w/w ES content. Different colours represent distinct HBAs composing the ES. * [Ch]DHC:Erythritol was included to compare with previously reported data [20].

the relative laccase activity ranged from 131 % to 0 % among the examined ES. [Ch]DHC:Proline and Urea:Proline exhibited the best chemical environment for the highest laccase activity, 121.46 ± 3.76 % and 117.86 ± 2.14 %, respectively, close to the highest value achieved with reference [Ch]DHC:Erythritol. On the opposite side, $[N_{4444}]$Br: Formic acid, $[N_{4444}]$Br:Glutaric acid, Ethalonamine:Ethylene glycol and Ethalonamine:1-Propanol provided null laccase activity.

As shown in Fig. 3, only the reference ES and five out of 13 ES combinations —[Ch]DHC:Proline, [Ch]DHC:1,6-Hexanediol, Acetamide:1,4-Butanediol, Acetamide:1,6-Hexanediol, and Urea:Proline, enabled laccase activity superior to the standard phosphate buffer (values >100 %). Among HBAs, [Ch]DHC exhibited the highest laccase activation. The presence of three carboxylate groups and one hydroxyl group in dihydrogen citrate structure is expected to provide a strong hydrogen bonding network with enzyme surface, allowing for enzyme stability [49]. On the other hand, despite urea being known as a protein denaturant through direct denaturation mechanisms, Urea:Proline exhibited a good medium for laccase, exceeding 100 % relative activity. In the investigation conducted by Monhemi et al. [50], concerning the stabilization of lipase in ES with high urea content, the formation of hydrogen bonds between urea (HBA) and the HBD was determinant on hindering the diffusion of urea molecules in the enzymatic structure. This allows maintaining urea's hydrogen bonding with amino acids at the enzyme surface and consequently amplifying its activity [50]. Furthermore, the presence of an amino acid, such as proline, could also be advantageous in the interaction with enzyme surface, since a beneficial impact is observed when proline is combined either with urea and [Ch]DHC. However, when using a different urea-based mixture (Urea:1-Propanol), a negative impact on enzyme activity was observed. This negative impact can be circumvented, when urea is substituted by acetamide. The mixture of acetamide with diols (1,4-butanediol or 1,6-hexanediol) showed also relative laccase activity superior to 100 %. The presence of hydroxyl groups in diols chemical structure will favour hydrogen bonding at the enzyme surface, similarly to carboxylate groups in [Ch]DHC. This is in line with conclusions reported by [20,51] that emphasized enhanced laccase activation with increasing number of hydroxyl groups in HBD composition. Molecular docking analysis [20] further support this statement, demonstrating that hydroxyl groups enhance hydrogen bonding with enzyme's amino acids, thereby increasing enzymatic activity. This effect allows stabilizing the enzyme by preventing destabilizing ions from penetrating its individual domains [21].

On the other hand, applying ES composed of halogenated salts ($[N_{4444}]$Br) decreased substantially the laccase activity independent of HBD, as demonstrated by experiments (Fig. 2.) with other halide salts ([Ch]Cl). Among these HBD families, the former exhibited the most negative influence on laccase activity (null activity), which can be caused by a drastic pH decrease of the catalytic medium. A similar pH shift can be also attributed to ethanolamine-based ES, but in this case caused by a substantial pH increase of the catalytic medium.

The discrepancy between the outcomes predicted by COSMO-RS data and the observed experimental results highlights the complexity of enzymatic reactions in aqueous environments. While COSMO-RS provides valuable insights into solvent behaviour, its lack of direct consideration for acid-base dissociation results in its inability to accurately predict the effects of pH variations on enzymatic activity, posing a significant challenge [23]. Furthermore, the drastic pH shifts experienced in certain ES compositions, like those containing halogenated salts or ethanolamine, emphasizes the importance of understanding pH-dependent enzymatic behaviour.

The pH is a crucial parameter in biocatalysis, since all enzymes have an optimal pH at which catalytic activity achieves maximal values [52]. The ABTS biocatalytic oxidation mediated by laccases is pH-dependent, therefore any external agent that might influence the pH of the enzymatic medium must be investigated [20]. In this regard, the effect of selected ES on the pH of enzymatic media, at both stages of incubation and reaction of laccases, was evaluated. For comparative purposes, Fig. 4. and Table S6 shows the pH of the neat ES aqueous solutions (50% *w*/w) in light blue bars as well as the pH of enzymatic media containing ES, either at incubation stage (dark blue bars) or at enzymatic reaction (orange bars). Furthermore, additional experiments with other ES besides the selected 13 ES were performed to complement this analysis.

At first sight, the natural pH of the studied ES aqueous solutions (25%w/w) varies from acidic to basic solutions, depending on the acidity or basicity of the resulting mixture of HBA and HBD. When these solvents are mixed with the phosphate solution containing laccase for the incubation stage, the pH must be set to 7.0. In most cases the pH is the same as the buffer, however few cases demonstrated substantial deviation thereof, which means that those ES present high acidity or high basicity. After incubation, the resulting solution is transferred to a citrate buffer with pH set to 4.5. In this case, the pH is set to or close to 4.5 when acidic ES are used, such as ES containing [Ch]DHC as HBA or those possessing organic acids as HBD. On the other hand, the alkaline ethanolamine-based ES presented a substantial pH shift from 4.5.

By correlating the pH data at different stages with laccase relative activity (red dots represent data depicted Fig. 4.), it can be inferred that high acidic or high alkaline ES exhibit a substantial negative impact on laccase activity. For instance, ES containing organic acids (formic and glutaric acids) causes drastic pH reduction at the incubation, probably inducing laccase inactivation at this stage, leading to a null enzyme activity. Similar results are observed for the alkaline ES based on ethanolamine, which influences both incubation and reaction stages. Indeed, under extreme pH conditions, catalytic reactions are known to be partially or completely affected due to rapid enzyme denaturation [53]. Interestingly, in case of [Ch]DHC-based ES, the observed pH shift from 7.0 to approximately 4.0 at the incubation stage did not influence laccase activity. These pH shift observations and similar laccase activity were also reported by Delorme et al. [51], when using [Ch]DHC-based ES. Overall, all ES that successfully exhibited laccase relative activity higher than 100 % share similar pH at catalytic stage, *i.e.*, equal or close to 4.5 (optimal pH for laccase *T. versicolor* activity).

Therefore, based on the obtained results, the top four ES exhibiting the highest laccase activity, namely Acetamide:1,6-Hexanediol, [Ch]DHC:1,6-Hexanediol, Urea:Proline, and [Ch]DHC:Proline, were selected for the subsequent experimental screening assessing lignin solubility.

3.3. Experimental screening: lignin solubility

The solubility of Kraft lignin in the selected four ES and reference ES ([Ch]DHC:Erythritol) [20] in the presence of different water contents was evaluated at 40 °C, producing distinct solubility curves as depicted in Fig. 5. Detailed solubility values and standard deviations are provided in the supplementary material (Table S7).

The obtained lignin solubility curves showed that the ability of the examined ES aqueous solutions in dissolving Kraft lignin can be ordered as follows: Acetamide:1,6-Hexanediol > [Ch]DHC:1,6-Hexanediol > Urea:Proline > [Ch]DHC:Proline > [Ch]DHC:Erythritol. Among [Ch]

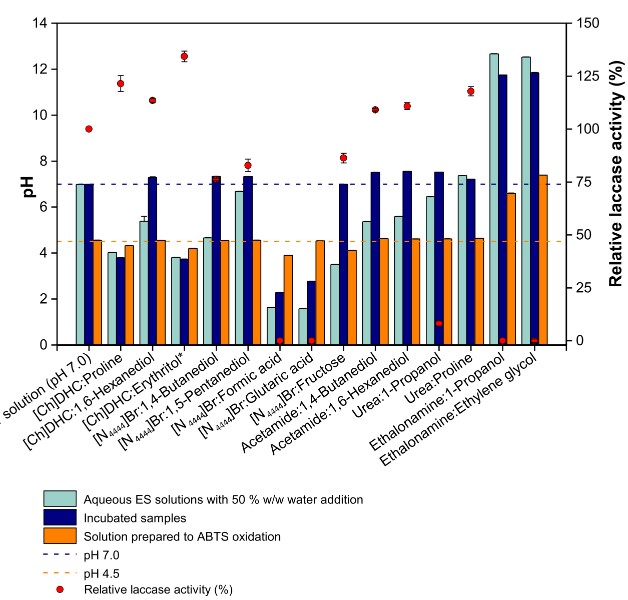

Fig. 4. Measured pH of ES aqueous solutions at 50%w/w content, incubated samples (ES aqueous solutions + phosphate buffer (pH 7.0) + laccase), and solution prepared for the enzymatic ABTS oxidation (incubated samples + citrate/phosphate buffer (pH 4.5) + ABTS solution). *[Ch]DHC:Erythritol was included to compare with previously reported data [20].

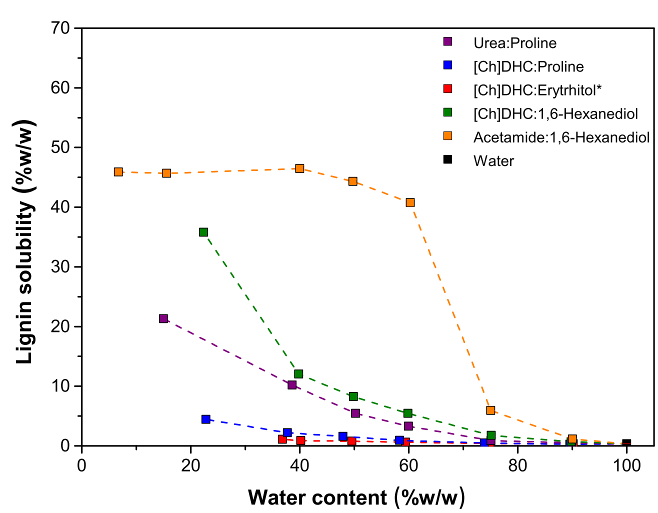

Fig. 5. Experimental screening of Kraft lignin solubility in selected ES aqueous solutions at 40 °C. [Ch]DHC:Erythritol* was included to compare with previously reported data [20]. Different colours represent different HBA:HBD (1:1) combinations.

DHC-based ES, 1,6-Hexanediol demonstrated the highest ability to dissolve lignin reaching up to 35.8 % solubility as maximal value. This particular HBD was also highlighted by Sosa et al. [16] as one of the most efficient diol-based HBDs towards lignin solubility when combined with [Ch]Cl. In addition, this result aligns with conclusions reported by other authors [16,39,54] stating that lignin solubility is favoured when HBD's alkyl chain length increases. However, when changing the HBD by proline or erythritol, lignin solubility drastically decreased. Impressively, <1 % was achieved as maximal lignin solubility using [Ch]DHC:Erythritol, the same ES that enabled the highest laccase activity in the study disclosed by Toledo et al. [20]. The high number of hydroxyl groups present in erythritol structure form a strong hydrogen bonding with water that almost nullify solute-solvent interactions [38]. This result means that an ES can show favourable conditions for enzyme activity, but it may not exhibit the required properties to dissolve lignin.

Finally, the replacement of [Ch]DHC by acetamide and urea exhibited a positive effect on lignin solubility in Acetamide:1,6-Hexanediol and Urea:Proline, respectively. Among examined ES, Acetamide:1,6-Hexanediol showed the highest lignin solubility up to 46.5%*w*/w as maximal value (126 times higher than water).

Regarding to the water content, in all cases the ability of ES aqueous solutions to dissolve lignin was considerably reduced with increasing water contents. This observation is in line with other published studies [16,39]. In the case of Urea:Proline and [Ch]DHC:1,6-Hexanediol systems, increasing water content had a notably detrimental effect on lignin solubility. For example, by adding water up to a water content of 40%w/w, a decrease in solubility of approximately 48 % and 34 % is observed for the Urea:Proline and [Ch]DHC:1,6-Hexanediol systems, respectively. On the other hand, Acetamide:1,6-Hexanediol demonstrated a different behaviour on lignin solubility at increasing water contents since lignin solubility is maintained at maximal values up to 60%w/w water content. This phenomenon has been characterized as an hydrotropic solubility mechanism [40], where the particular combination of Acetamide and 1,6-Hexanediol perform a strong interaction with the hydrophobic regions of the amphiphlic lignin macromolecules, forming ES-lignin aggregates that are soluble in water. Therefore, ES acts as a hydrotrope enabling the dissolution of water-insoluble lignin at high water contents.

The disclosed data underscores the need of carefully balancing factors that are important for enzymatic activity and simultaneously beneficial for lignin solubility in ES aqueous solutions. These factors are closely related to the chemical structure of ES precursors and their chemical interactions with both laccases and lignin macromolecules that can predicted through COSMO-RS quantum chemistry. Among >10,000 combinations, the proposed approach allowed the selection of a unique combination of acetamide and 1,6-hexanediol to produce an ES capable of increasing laccase relative activity (111 %) and lignin solubility (46.5%*w*/w). To the best of our knowledge, this ES was never reported as capable of dissolving lignin. Therefore, this result is a novelty in contrast to state-of-the-art. Moreover, these precursors are appropriate to make a benign solvent. FDA (Food and Drug Administration) has approved acetamide as food additive, acting as a flavouring agent or adjuvant, while 1,6-hexanediol is recognized by the same agency as an indirect food additive applied as a component of adhesives for food packaging [55].

3.4. Enzymatic depolymerization of guaiacylglycerol-β-guaiacyl ether (GG)

An exploratory enzymatic depolymerization of a lignin model compound (GG) using laccase mediator systems (LMS) in the presence of Acetamide:1,6-Hexanediol was investigated as a proof of concept. The experiments were performed under fixed reaction conditions. Two different laccase oxidation mediators, namely ABTS (2 mM) or HBT (2.2 mM), were used to evaluate their role in the enzymatic cleavage of GG. In addition, two other experimental setups were performed to better understand the observed trends: (i) a solution without GG and (ii) a solution without laccase. The enzymatic depolymerization was monitored by HPLC-DAD at 280 nm; acquired chromatograms are shown in Fig. 6A (ABTS) and Fig. 6B (HBT).

As shown in Fig. 6, the two mediators ABTS and HBT were consumed during the catalytic oxidation of the lignin model compound. In addition, GG was completely consumed in both LMS systems after 24 h. In the reactions with ABTS (Fig. 6A), oxidized ABTS ($ABTS_{ox}$) is formed after the reaction with the mediator and laccase, which is consistent with the results in the literature [31]. Furthermore, polymerization reactions seem to occur, resulting in reaction products (RP) such as GG-based dimers (RP1 – reaction products 1) and trimers (RP2 – reaction products 2), also reported in the literature [31]. This indicates that GG undergoes radical polymerization in the presence of the laccase/ABTS system, *via* C − C bond formation, enabling the formation of oligomeric species [36,56].

Although primarily indicative of polymerization, it is noteworthy that both polymerization and depolymerization can occur in similar

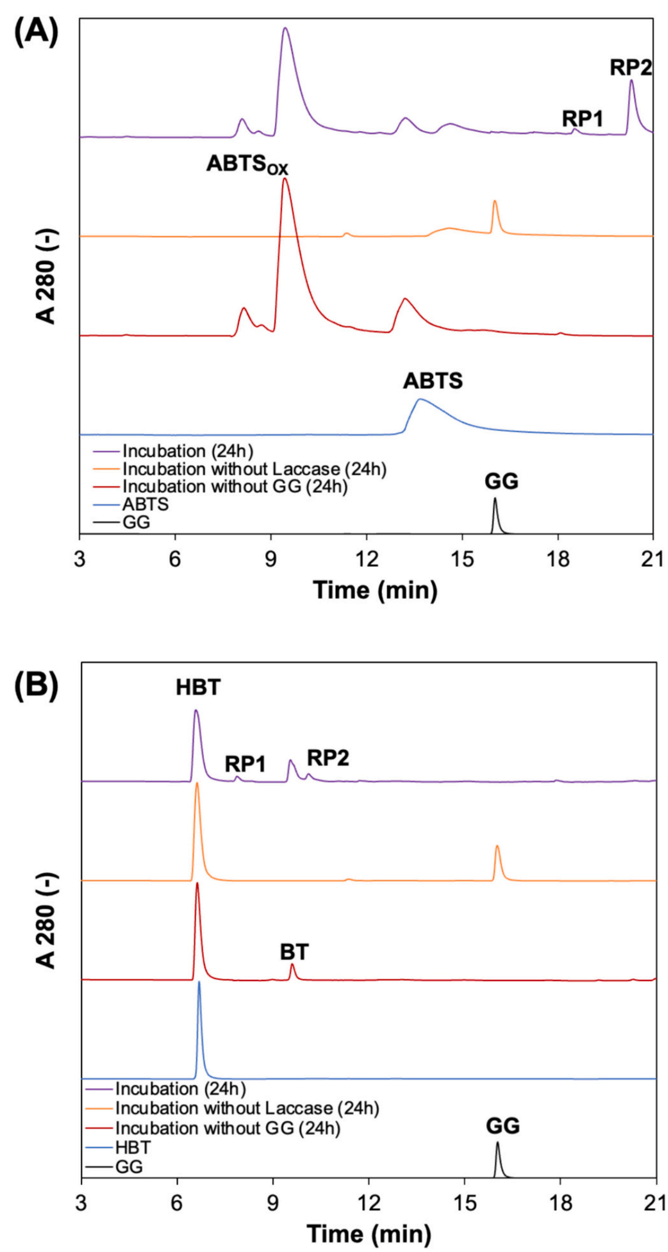

Fig. 6. HPLC-DAD$_{280}$ chromatograms of lignin model compound (GG) with LMS: (A) ABTS and (B) HBT incubated for 24 h.

systems. This dual behaviour is attributed to the rapid formation of $ABTS_{ox}$, enabling the mediator to either oxidize lignin, leading to bond cleavage (depolymerization), or facilitate radical coupling, resulting in bond formation (polymerization). When using HBT as the mediator Fig. 6B), HBT was partially converted to benzotriazole (BT) after 24 h, as noted in previous studies [9,30]. Furthermore, depolymerization reactions were found, since cleavage products RP3 ($C_{11}H_{14}O_5$) and RP4 ($C_{11}H_{14}O_4$) from GG were observed. This observation is in agreement with the findings of Hilgers et al. [9,30], who reported that HBT mediates the depolymerization of lignin through radical hydrogen atom transfer (HAT) mechanisms, leading to similar cleavage products.

In order to better understand the mechanisms and kinetics of the enzymatic reactions with the two mediators, the reactions were observed over 24 h at different time intervals (0, 0.25, 2, 4, 8 and 24 h). The kinetic chromatograms are shown in Fig. 7A (ABTS) and Fig. 7B (HBT).

When using ABTS as the mediator, GG is consumed in <0.25 h. As the

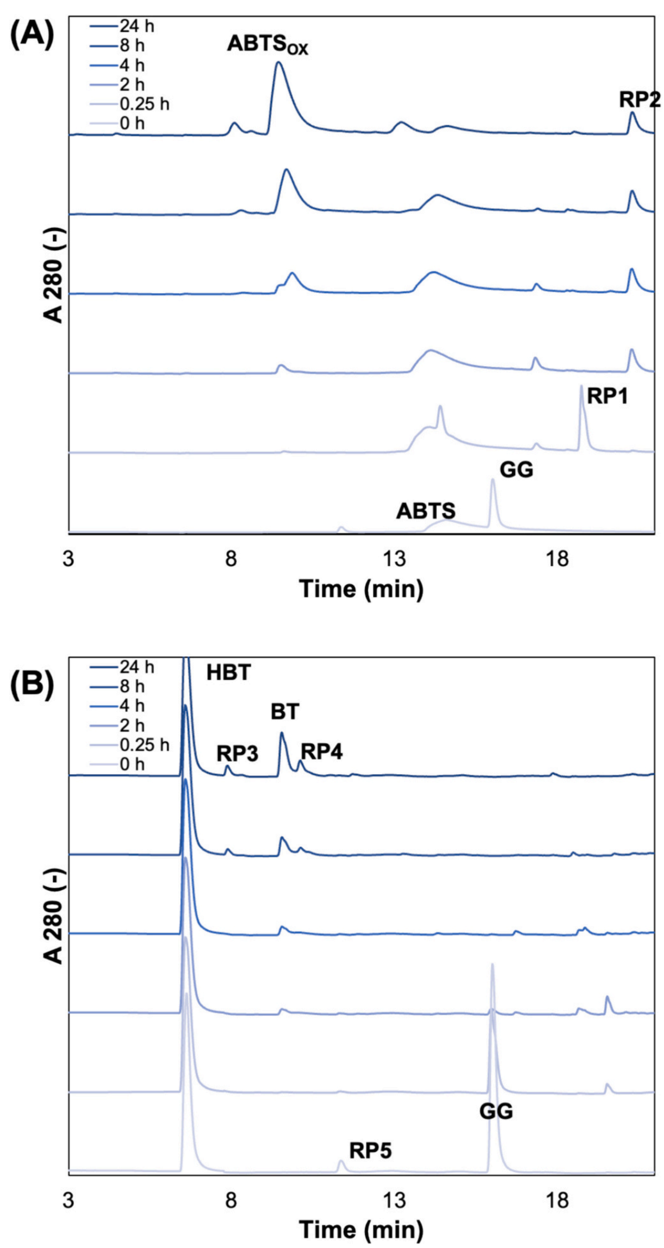

Fig. 7. Kinetic HPLC-DAD chromatograms acquired at 280 nm of lignin model compound (GG) with LMS: (A) ABTS and (B) HBT.

reaction time increases, the amount of oxidized mediator increases, as do the reaction products (RP1, RP2, RP3 and RP4). This rapid consumption and subsequent formation of reaction products is consistent with the kinetics described in the literature, where ABTS-mediated reactions exhibit rapid substrate turnover and subsequent polymerization [31].

In the reaction with HBT, the consumption of GG was slightly slower, but still rapid. After 0.25 h of reaction around 60 % of the substrate was consumed, reaching almost 90 % after 1 h. Only traces of the compound (<0.3 %) remained after 2 h and continued decreasing until complete consumption. With increasing reaction time, the concentration of BT and cleavage products (RP3 and RP4) increased. Previous findings [9,30,36] also demonstrated that HBT-mediated reactions lead to significant substrate breakdown within a short time frame.

Zhou et al. [3] emphasized that the crucial aspect of lignin depolymerization lies in directing the reaction towards the breakdown pathway rather than polymerization. The results obtained (Figs. 6 and 7) show that the presence of the laccase-mediator system (LMS) was essential to steer the reaction towards substrate degradation, as evidenced by the rapid consumption of GG and the increase in reaction products (RP1 to RP4). This aligns with the well-established role of mediators in facilitating the oxidation of non-phenolic lignin subunits, which laccase alone cannot efficiently oxidize [9,36]. Moreover, studies such as Chen et al. [35] support that natural syringyl-type mediators significantly accelerate β-O-4 bond cleavage and Cα oxidation, corroborating our finding that the addition of mediators enhances laccase activity in lignin depolymerization. The formation of mediator-substrate complexes through π-π stacking interactions suggests that these weak interactions play a key role in boosting laccase-catalyzed lignin biodegradation.

4. Conclusions

In this study, a robust methodology using COSMO-RS as a predictive tool to determine the optimal combinations of HBA and HBD for solubilization and enzymatic depolymerization of lignin was developed. The activity coefficient at infinite dilution of guaiacylglycerol-β-guaiacyl (GG) in ES ($\ln(\gamma^{\infty}_{GG,ES})$) allowed the screening of lignin solubility in these solvents. The ($\ln(\gamma^{\infty}_{water,ES})$) was identified as a key parameter capable of describing with laccase activity in aqueous ES solutions. By combining these two parameters ($\ln(\gamma^{\infty}_{GG,ES})$) and $\ln(\gamma^{\infty}_{water,ES})$) calculated by COSMO-RS, the selection process was simplified, narrowing down the possible combinations of HBA and HBD from 10,658 to 22 combinations with promising properties. Among those, the unexpected combination of Acetamide:1,6-Hexanediol showed the highest lignin solubility (46.5% *w*/w) and achieved 111 % relative laccase activity, also enhancing laccase depolymerization in the presence of mediators (ABTS or HBT). The enzymatic depolymerization of GG ether demonstrated that ABTS and HBT are effective mediators in the oxidation of lignin model compounds, each influencing the process differently: ABTS promotes polymerization, while HBT promotes depolymerization. Additionally, it was shown how pH can negatively affect laccase activity in ES formulations, revealing a complex relationship between ES precursors, pH, and laccase activity. This study highlights ES as a promising solvent with significant potential for efficient lignin depolymerization, marking a valuable advancement in lignin valorisation.

Abbreviations

[Bmim]Cl 1-butyl-3-methylimidazolium chloride
[Ch]Ac cholinium acetate
[Ch]Cl cholinium chloride
[Ch]DHC cholinium dihydrogencitrate
[Ch]DHP cholinium dihydrogen phosphate
$[N_{4444}]$Br tetrabutylammonium bromide

[PMpyrr][OAc]	(1-methyl-1-propylpyrrolidinium acetate)
ABTS	2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid
BT	benzotriazole
DMSO	dimethylsulfoxide
ES	eutectic solvent
GG	guaiacylglycerol-β-guaiacyl ether
HBA	hydrogen bond acceptor
HBD	hydrogen bond donor
HBT	1-hydroxybenzotriazole

CRediT authorship contribution statement

Paula V.A. Pontes: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Ana M. Ferreira:** Writing – review & editing, Methodology, Conceptualization. **João A.P. Coutinho:** Writing – review & editing, Project administration, Funding acquisition. **Jürgen Andreaus:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization. **André M. Costa Lopes:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Filipe H.B. Sosa:** Writing – original draft, Software, Investigation, Funding acquisition, Conceptualization.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2025.146048.

Data availability

No data was used for the research described in the article.

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