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Influence of mediators on laccase catalyzed radical formation in lignin

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ABSTRACT

Laccases (EC 1.10.3.2) catalyze oxidation of phenolic groups in lignin to phenoxyl radicals during reduction of O_2 to H_2O. Here, we examine the influence on this radical formation of mediators which are presumed to act by shuttling electrons between the laccase and the subunits in lignin that the enzyme cannot approach directly. Treatments of three different lignins with laccase-mediator-systems (LMS) including laccases derived from *Trametes versicolor* and *Myceliophthora thermophila*, respectively, and four individual mediators, 1-hydroxybenzotriazole (HBT), N-hydroxyphthalimide (HPI), 2,2,6,6-tetramethylpiperidin-1-yloxy (TEMPO), and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) were assessed by real time electron paramagnetic resonance measurements. Radical steady state concentrations and radical formation rates were quantified. LMS treatments with 500 µM N-OH type mediators (HPI or HBT) did not affect the lignin radical formation, but increased doses of those mediators (5 mM) surprisingly led to significantly *decreased* radical formation rates and lowered steady state radical concentrations. Laccase-TEMPO treatment at a 5 mM mediator dose was the only system that significantly increased steady state radical concentration and rate of radical formation in beech organosolv lignin. The data suggest that electron shuttling by mediators is not a significant general mechanism for enhancing laccase catalyzed oxidation of biorefinery lignin substrates, and the results thus provide a new view on laccase catalyzed lignin modification.

1. Introduction

Lignin, a complex, heterogeneous, and water-insoluble aromatic polymer, is one of three major components in lignocellulosic material. The structure of lignin arises from radical coupling reactions of primarily three hydroxycinnamyl alcohols: p-coumaryl, coniferyl, and sinapyl alcohols resulting in an entangled network of phenolic and non-phenolic subunits (mono-aromatic phenylpropanoid units) [1]. The water-insoluble, recalcitrant nature of lignin makes modification, separation, and general upgrading of lignin streams from biorefineries challenging [2,3]. Currently, this abundant and renewable carbon source is thus the most poorly exploited lignocellulosic biopolymer, except for being used for energy production by combustion, regardless of its potential as a raw material for sustainable manufacturing of various new products and materials [2–4].

White-rot fungi are efficient lignin degrading organisms, and enzymes expressed by them serve as biocatalysts enabling lignin modification under mild conditions [5]. Laccases (benzenediol:oxygen oxidoreductases, EC 1.10.3.2) are capable of modifying lignin in a catalytic cycle which involves oxidation of four moles of mono-phenolic substrate (e.g. phenolic lignin subunits) by simultaneous reduction of one mole of O_2 to two moles of H_2O. Laccases are of particular interest as biocatalysts for lignin modification since they only require O_2 as opposed to other lignin modifying enzymes, which require H_2O_2. The oxidizing capacity of laccase is defined by the redox potential of the T1 copper site in the enzyme. Fungal laccases have particularly high redox potentials ranging from 0.5 to 0.8 V vs. the Normal Hydrogen Electrode (NHE) [6,7]. The laccase catalyzed oxidation of phenolic subunits, which represent 10–30% of the units in native lignin, leads to formation of phenoxyl radicals and thus "activation" of the lignin polymer [8–10]. Stabilized by the lignin matrix, these phenoxyl radicals linger in the polymer long enough to be detected and quantified by EPR spectroscopy [11,12]. The phenoxyl radicals increase the reactivity of lignin to facilitate polymerization, depolymerization, and grafting processes which are not governed by laccase catalysis [11,13,14].

The action of laccase on lignin may be expanded by use of mediators. Mediators are low weight molecular compounds which are relatively stable, although reactive, in their oxidized as well as in their reduced form (depending on the mechanism of oxidation, the oxidation of mediators may produce radical intermediates). Mediators of natural origin are already present in biomass, whilst synthetic mediators include various types of chemical compounds [15,16]. It is claimed that mediators enhance the

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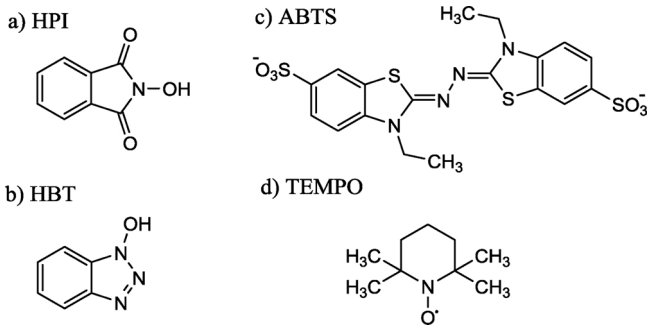

Fig. 1. Structures of synthetic mediators included in LMS treatments.

laccase catalyzed lignin modification by two mechanisms: i) The mediators act as electron transfer agents between laccase and substrate, where the oxidized form of the mediator, i.e. after being catalytically oxidized by laccase, diffuses away from the catalytic pocket and, due to its limited size, is capable of oxidizing substrates being inaccessible or too bulky for the laccase to oxidize directly [17–22]. ii) The mediator expands the oxidizing capability towards oxidation of higher-redox potential non-phenolic subunits in lignin (> 1.3 V vs. NHE) by introducing alternative reaction pathways for oxidation [18,22–25]. The different reaction pathways for oxidation of non-phenolic model substrates (e.g. benzyl alcohols) catalyzed by laccase-mediator systems via use of synthetic mediators, have been thoroughly studied [15,21,26,27]. TEMPO is a resonance-stabilized stable radical compound (Fig. 1d) which has been widely explored as a mediator: Once oxidized by laccase, the TEMPO radical forms an oxoammonium ion which reacts to oxidize non-phenolic compounds by polar addition-elimination reactions. The generated hydroxyl amine returns to the radical state either by acid induced disproportionation or by laccase oxidation. Laccase catalyzed oxidation of ABTS and N-OH type mediators, i.e. HBT and HPI (Fig. 1a–c), results in formation of reactive mediator species. The resulting $ABTS^+$ radical may react with non-phenolic substrates to create benzylic cation radical intermediates by one-electron transfer (ET) pathways, while N-oxyl radicals from oxidation of HBT and HPI create benzylic radical intermediates by hydrogen abstraction (HAT) [28,29]. These radical intermediates are less stable than phenoxyl radicals and cannot be detected by EPR without use of spin trapping techniques [11,30]. Despite the vast amount of well performed studies of laccase mediator systems (LMS) treatments on lignin and lignin model compounds, the currently available results concerning LMS treatments on lignins are often inconsistent, and a comprehensive understanding of how laccase-mediator-systems impact lignin modification is lacking [14,23]. Whether the enhancing effect by mediators on laccase catalyzed lignin modification is primarily caused by the claimed ability of mediators to function as electron shuttles or is a result of their ability to facilitate oxidation of non-phenolic units in lignin is for example rarely addressed. If mediators act as electron shuttles to oxidize bulky and inaccessible parts of lignin this would mean that a LMS treatment would enhance the rate of the radical response compared to the corresponding neat laccase induced radical formation in lignin. In this framework the objective of the present study was to investigate the influence on laccase catalyzed radical formation in lignin by synthetic mediators representing different oxidation routes. This assessment was done by measuring if any quantitative differences in stable lignin derived radicals detectable by EPR could be observed.

2. Materials and methods

2.1. Lignins

Organosolv lignin, SOL, was obtained from Sigma-Aldrich (Milwaukee, WI, USA). The beech organosolv lignin, BOL, was produced at Thünen Institute of Wood Research (Hamburg, Germany) [31]. Both SOL and BOL lignins had a high lignin content comprising 94 wt% and 87.4 wt% of Klason lignin, respectively.

The lignin from wheat straw, WSL, was obtained from hydrothermally pretreated wheat straw that had been exhaustively enzymatically treated with a Cellic® CTec2 enzyme cocktail from Novozymes (Bagsværd, Denmark): The wheat straw was first hydrothermally pretreated in a pilot plant facility for 10 min at 190 °C according to [32]. The resulting insoluble fiber fraction was then repeatedly treated (3 rounds of treatment) with Cellic® CTec2 for 48 h at 4.5% of wheat straw dry matter, pH 5.1, 50 °C with 0.02 wt% sodium azide added as preservative. The Cellic® CTec2 was added in a ratio of 0.5% by weight (liquid) to the pretreated wheat straw fiber (dry matter). In between each enzyme treatment the suspension was centrifuged for 20 min at 5350*g* and the resulting pellet was washed twice with deionized water. The final wheat straw lignin pellet was washed, dried at 70 °C, ground and sieved. This treatment increased the content of Klason lignin in the wheat straw lignin (WSL) sample from 25.9% to 43.7 wt%. The remaining content consisted of 37.8 wt% cellulose, 9.2 wt% hemicellulose, and 6.1 wt% ash.

2.2. Laccases

The fungal laccases were derived from the basidiomycete *Trametes versicolor* (Tv) and the ascomycete *Myceliophthora thermophila* (Mt). The Tv laccase was purchased from Fluka (St. Gallen, Switzerland), and the Mt laccase kindly donated from Novozymes (Bagsværd, Denmark).

2.3. Laccase activity assay

Determination of laccase doses were based on spectrophotometric monitoring of the oxidation of syringaldazine at 530 nm ($\varepsilon = 6.5 \cdot 10^4\ M^{-1}\ cm^{-1}$) at 25 °C. The assay reaction was carried out in 0.25 mM syringaldazine, 10 vol% ethanol, 25 mM sodium acetate (pH 5.0) using a suitable amount of enzyme. Enzyme activity was expressed in units: One Unit (U) was defined as the amount of enzyme required to catalyze conversion of 1 µmol of substrate (syringaldazine) per minute at the assay reaction conditions.

2.4. Mediators

Four mediators, 1-hydroxybenzotriazole (HBT), N-hydroxyphthalimide (HPI), 2,2,6,6-tetramethylpiperidin-1-yloxy (TEMPO) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) (Fig. 1), all obtained from Sigma-Aldrich (Steinheim, Germany), were tested in combination with each of the laccases on each of the 3 lignin samples. Each mediator was solubilized in Milli-Q water to 10 mM and adjusted to pH 5 with 0.5 mM NaOH. The mediator solutions were then added individually in equimolar amounts to a resulting concentration of 500 µM in the relevant reaction mixtures. The experiments employing 5 mM of final mediator concentration were prepared similarly, but on the BOL lignin substrate only.

2.5. Laccase-mediator study of radical formation in lignin suspensions

Lignin suspensions were prepared by adding Milli-Q water resulting in a 10 wt% lignin suspension, including pH adjustment to pH 5 with 0.5 M NaOH. The slurry was heated to 60 °C, left overnight at 5 °C and re-adjusted to pH 5. The laccase treatments were conducted in aliquots of 800 µl slurries carried out in darkness at 25 °C. To ensure oxygen saturation, the samples were vigorously shaken at 900 rpm and sealed with gas permeable, water proof membranes (ABgene, Surrey, UK). Laccases were dosed in 2.0 U/g of solid (DM) (Units according to the syringaldazine assay) and solutions of mediator was added to a concentration of 500 µM (or additionally for assessment on the BOL lignin substrate to 5 mM). For each sample, an amount of 50 µl of suspension was repeatedly drawn from the slurry for immediate EPR measurements

in a time span of 0–120 min. Reference treatments were carried out at similar conditions, where addition of mediator was replaced with addition of Milli-Q water. Additionally, enzyme-free controls in all combinations with mediators and lignins were included.

2.6. Electron paramagnetic resonance spectroscopy (EPR)

The radical formation in the lignin suspensions were determined directly by Electron Paramagnetic Resonance (EPR) spectroscopy. During the laccase catalyzed reactions, samples were drawn into 50 µl capillary tubes under vigorous shaking at 900 rpm, while care was taken that the sensitive part of the capillary tube was free of air bubbles. To avoid any possible measurement variation caused by sedimentation in the capillary tube, the EPR measurement was always done at the same time immediately after the sample had been drawn into the capillary tube. EPR detection was carried out immediately after sampling at 20 °C with a MiniScope MS200 (Magnettech, Berlin, Germany) operating at a frequency of 9.4 GHz using the following settings: Modulation amplitude of 0.2 mT; sweep width 10 mT, and a sweep time of 30 s. The number of spins was calculated by double integration of the resonance signal lines after subtraction of the background signal (i.e. the resonance signal line of the reference treatment without any laccase and mediator addition). The area under the absorption signal was converted to a radical concentration through a linear standard curve ($R^2 = 0.99$) based on solutions with 5 µM–5 mM of the stable nitroxyl radical TEMPO (Sigma-Aldrich, Milwaukee, WI, USA).

2.7. Determination of g-values by EPR

The g-values of the resonance signals measured by EPR were calculated by resolving the field strength where the spectrum, presented as the first derivative of the absorption signal, passed through zero. The values were calculated relative to the g value for TEMPO. Only EPR signals with high intensities were used for establishing the g-values of the EPR signals.

3. Results and discussion

3.1. EPR signal response in lignin by LMS treatment

The EPR analysis of the lignin suspensions treated with laccase alone or in combination with a mediator provided EPR spectra with signals changing in response to the progress of the reactions (Fig. 2). The three different lignin samples, two organosolv lignins (BOL, SOL) and a wheat straw lignin residue (WSL) treated with either *Trametes versicolor* (Tv) laccase or *Myceliophthora thermophila* (Mt) laccase provided similar EPR spectra with a single broad signal typical of lignin derived stable radicals (data not shown) i.e. signals indicating phenoxyl and semiquinone radicals as previously reported for lignin rich substrates [30,33,34]. Lignin samples treated with an LMS (laccase-mediator-system) of laccase (Tv laccase) plus 500 µM HPI or HBT showed spectra resembling those generated from samples treated with laccase only (Fig. 2a–c). EPR spectra from corresponding LMS treatments with Tv laccase and 500 µM ABTS gave similar signals, but the signal intensity increased much faster with the ABTS added as compared to the plain laccase treatments (Fig. 2). This faster intensity increase was due to the overlapping signal from the additional formation of $ABTS^{\cdot+}$ radicals also generated by laccase oxidation, which contributed to a higher intensity in signal response (Fig. 2d). The laccase catalyzed generation of $ABTS^{\cdot+}$ radicals was confirmed by control experiments with laccase and 500 µM ABTS in absence of lignin, which resulted in a rapidly increasing signal within the same magnetic field range of 332–337 mT (Fig. 2e). In contrast to previously published low field EPR spectra of $ABTS^{\cdot+}$ radicals, no hyperfine interactions were detectable, since the EPR parameters were optimized for detection of lignin radicals. LMS treatments of lignin samples with 500 µM TEMPO also resulted in an EPR spectral response with additional signal pattern from the TEMPO radicals overlapping the signal from the lignin derived radicals (Fig. 2f). Control experiments with laccase and TEMPO in absence of lignin confirmed that the overlapping signal were caused by the TEMPO radical (Fig. 2g). The lignin derived radical signal became visible as the signal from the TEMPO radical declined over time and the signal from lignin derived radicals increased (Fig. 2f).

The g-values derived from the EPR spectra of the lignin suspensions after sole laccase treatment ranged from 2.004-2.005 (Table 1). These data are in accordance with g-values reported previously for various lignin samples [30]. As previously observed, the slightly higher g-values recorded for the organosolv lignins (BOL > SOL > WSL) may be caused by increased amounts of semiquinones known to be present in processed lignin such as organosolv lignin [30,34,35]. Additionally, the linewidth of the EPR signals were broader for the organosolv lignins (max 0.82 −0.91 mT) compared to the linewidth of WSL lignin (max 0.75 mT) and the linewidth expanded during the reaction, which indicated a change in the radical composition (data not shown). The g-values derived from the EPR spectra of lignin samples treated with LMS of HPI or HBT were similar to the values derived from the spectra of samples treated with laccase alone (Table 1). The g-values of lignin derived radicals from spectra of LMS treated samples with ABTS and TEMPO were not obtainable, as the g-values of LMS treated samples were influenced by the presence of either the TEMPO radical (g = 2.0060) [36] or the $ABTS^{\cdot+}$ radical (g = 2.0043) [28]. Consequently, g-values determined for LMS samples with TEMPO had increased g-values closer to that of the TEMPO radical, whereas LMS samples with ABTS had slightly decreased g-values closer to that of the $ABTS^{\cdot+}$ radical (BOL and SOL only) (Table 1).

Regardless of the inclusion of mediators, no evident differences between treatment with Tv laccase and Mt laccase were observed in any of the lignin samples, neither in the signal responses nor the g-values. The presence of different radicals affects the overall g-value of an EPR spectra, hence the consistency between the spectra generated by Tv and Mt laccase suggests that the two laccases catalyze the formation of the same type of radicals in the lignins detectable by EPR measurement. Additionally, the presence of mediators known to facilitate alternative oxidation pathways did not affect the distribution of detectable radicals in the lignin samples.

3.2. Impact of LMS treatments on formation of radicals in lignin

The evolution of lignin radical concentrations for each of the LMS treatments was compared to those obtained in the reference treatments with laccase only on each of the three lignin substrates (Fig. 3). The time frames for each of the combinations of laccase and lignin were set to include measurements where a steady state in radical formation was reached and a slight decay was observed. Initial rates were determined for each of the progress curves in the linear time interval (Table 2) and the maximal steady state concentrations were determined by calculating the averages of the highest concentrations of lignin derived radicals attained for each treatment (Table 3).

With regard to oxygen consumption during laccase catalysis the treatments were carried out using gas permeable membranes with vigorous shaking and extra head space to optimize oxygen saturation. At 25 °C the oxygen solubility in water is approximately 250 µM, which in theory allows laccase catalyzed formation of radicals up to 1 mM before oxygen is depleted from the system [37]. Initial radical formation rates with and without mediators were determined well before potential depletion of oxygen would influence the rate. In our previous study, a linear dose-response relation between laccase and radical formation rate was confirmed, which verifies that oxygen depletion does not influence the radical formation rate within the time span of the assay[12]. However, when an increased dose of the mediator ABTS was applied it cannot be excluded that the total amount of obtained radicals may be limited by oxygen solubility.

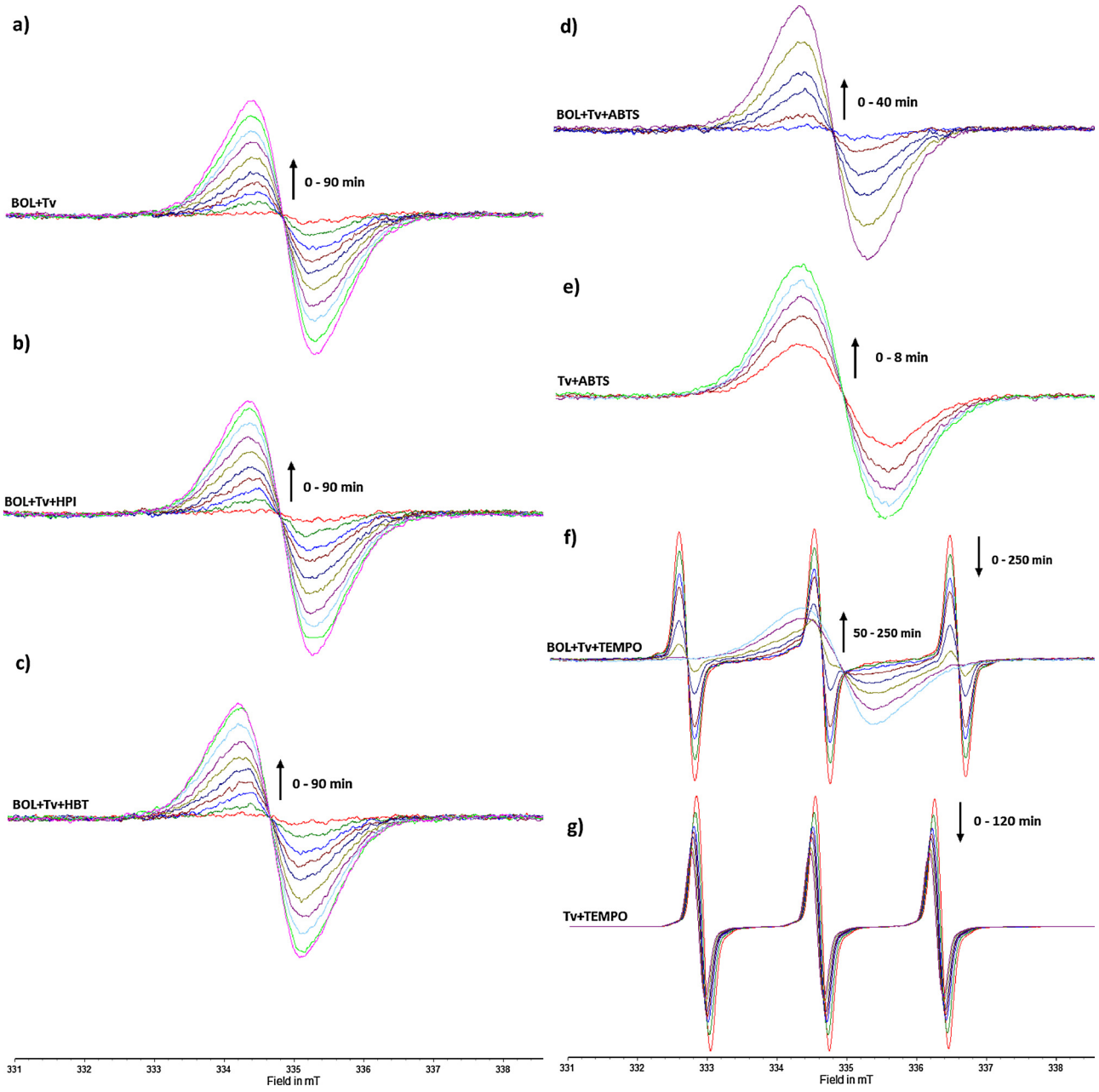

Fig. 2. Representative EPR spectra overlay showing signal response of radicals in BOL suspensions (10 wt% DM solid) measured at varying time points during laccase and LMS treatments with Tv laccase (2U/g DM solid) and 500 μM mediator. a) Treatment with Tv laccase; b) LMS treatment with HPI; c) LMS treatment with HBT; d) LMS treatment with ABTS, where signals developed faster due to the overlapping signal from additional formation of $ABTS^{\cdot +}$ radicals generated by laccase oxidation (see 2e); e) Laccase and $ABTS^{\cdot +}$ incubated without lignin. Since radicals were generated by laccase oxidation the signal increased even faster than when lignin was present (see 2d); f) Signals from LMS treatment with TEMPO. The response is a combination of the signal of the TEMPO radical and the lignin radicals. Over time, the TEMPO radicals response declined gradually as the signal from lignin derived radicals increased (see 2 g); g) Laccase and TEMPO incubated without lignin. The decline of the TEMPO radical occurred slower than when lignin was present (see 2f). Similar spectra were generated with SOL and WSL as lignin substrates regardless of the type of laccase used.

The order of lignin samples in which the highest rate of laccase catalyzed radical formation was achieved was WSL > SOL > BOL (Table 2). This order was inversely correlated with the highest steady state *amount* of radicals formed; BOL > SOL > WSL (Table 3). The difference between the apparent reaction rates and the maximal level of radicals formed may be ascribed to the differences in lignin content and phenolic subunits among the three lignin samples.

The radical formation rate facilitated by the MT laccase catalysis was consistently significantly faster than that of the Tv laccase catalysis on all three lignins, and inclusion of mediators in the LMS treatments did not affect this trend (Table 2). On the contrary, Tv laccase catalysis alone facilitated a significantly higher maximal steady state concentration of lignin derived radicals in BOL and SOL than Mt laccase treatment (Table 3). The latter may be due to the higher redox potential of the Tv laccase compared to that of the Mt laccase (790 mV versus 465 mV vs NHE). However, taking into account that two extremities of redox potential of laccases are represented by the Tv and the Mt laccase, the relative difference in obtained maximal steady state concentration was limited. The higher maximal steady state concentration achieved by the Tv laccase catalysis compared to the Mt laccase catalysis was much less pronounced for LMS treatments than for mere laccase treatments (Table 3). Observations related to laccase catalyzed

Table 1
Recorded g-values of EPR signals of the lignin suspensions upon laccase and LMS treatments. Standard deviations ≤0.00008.

	BOL		SOL		WSL	
	Tv lac	Mt lac	Tv lac	Mt lac	Tv lac	Mt lac
–	2.0048	2.0049	2.0048	2.0047	2.0043	2.0044
HPI	2.0049	2.0049	2.0047	2.0047	2.0044	2.0045
HBT	2.0048	2.0049	2.0047	2.0047	2.0044	2.0045
ABTS[a]	2.0046	2.0046	2.0046	2.0046	2.0044	2.0045
TEMPO[b]	2.0059	2.0060	2.0061	2.0061	2.0059	2.0059

[a]The g-value reported for the $ABTS^{\cdot+}$ radical in the literature is g = 2.0043 [28].
[b] The g-value reported for the TEMPO radical in the literature is g = 2.0060 [36].

radical formation without mediators were in accordance with results obtained previously with the same lignins [12].

3.3. Impact of LMS treatments with N-OH type mediators on radical formation

In LMS treatments with laccase and 500 µM of HPI or HBT on the lignins, the progress kinetics of the measurable lignin derived radicals were similar to those of the respective reference treatments with either Tv laccase or Mt laccase (Fig. 3). This similarity was also reflected in the initial rates, where no significant differences between reference treatments with Tv or MT laccase and the corresponding LMS treatments with HPI or HBT were observed (Table 2). Likewise, no significant differences were observed in maximal steady state concentrations of lignin derived radicals between reference treatments with Tv and MT laccase and the corresponding LMS treatments with HPI or HBT (except for the LMS treatment of BOL with Mt laccase and HBT) (Table 3), but a tendency of reaching slightly lower maximal steady state values in presence of HPI and HBT was observed.

Table 2
Initial rates (v_i) of radical formation (µM/min) in BOL, SOL and WSL lignin substrates upon laccase and LMS treatments. All slopes were calculated by linear regression and had a correlation coefficient corresponding to a value of $R^2 \geq 0.91$ and standard deviations were in the range of 0.04–0.90. The significant differences ($p \leq 0.05$) between the slopes of radical formation within each column are shown as superscript letters (a–d) and significant difference ($p \leq 0.05$) between the slopes within rows for each substrate are shown as superscript letters (x–y).

	BOL		SOL		WSL	
	Tv lac (µM/min)	Mt lac (µM/min)	Tv lac (µM/min)	Mt lac (µM/min)	Tv lac (µM/min)	Mt lac (µM/min)
–	$1.30^{b,x}$	$1.98^{bc,y}$	$1.98^{b,x}$	$2.94^{b,y}$	$2.51^{b,x}$	$4.15^{b,y}$
HPI	$1.30^{b,x}$	$1.88^{c,y}$	$2.09^{b,x}$	$2.57^{b,y}$	$2.31^{b,x}$	$3.97^{b,y}$
HBT	$1.32^{b,x}$	$2.19^{b,y}$	$2.26^{b,x}$	$2.75^{b,y}$	$2.49^{b,x}$	$4.18^{b,y}$
ABTS	$4.29^{a,x}$	$7.47^{a,y}$	$9.06^{a,x}$	$13.06^{a,y}$	$5.55^{a,x}$	$9.69^{a,y}$
TEMPO	$1.13^{c,x}$	$1.59^{d,y}$	$1.40^{c,x}$	$1.97^{c,y}$	$1.51^{c,x}$	$3.33^{b,y}$

Both HPI and HBT have been used as -N-OH type mediators in laccase catalyzed reactions where they are presumed to be enzymatically oxidized to N-oxyl radicals to act on high potential substrates (such as non-phenolic alcohols in e.g. lignin) via a HAT oxidation route [15,27]. The absence of an EPR signal from oxidized HBT has been reported before by Astolfi et al. [21] and was ascribed to the instability of the radical, which causes it to rapidly decompose into inactive, non-radical compounds at such speed that the current method used in the EPR analysis will not be able to detect it [21,38]. However, the inability to detect the radical formation of both HBT and HPI might also be due to the elevated reactivity of the N-oxyl radical [27], which could lead to self-condensation reactions and hence extinguishing of the

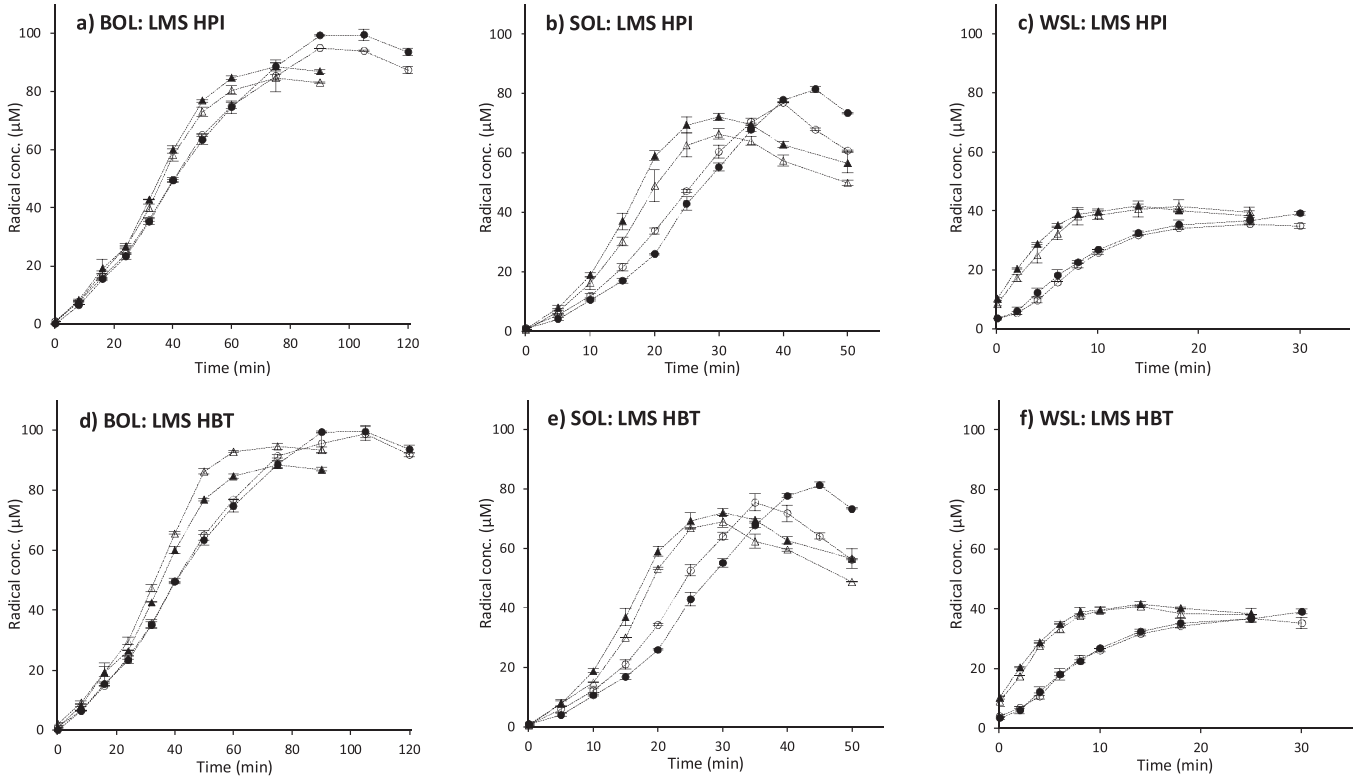

Fig. 3. Each graph show progression curves of lignin derived radical formation during LMS treatments with Tv lac (○) or Mt lac (△) compared to sole treatments of Tv lac (●) or Mt lac (▲). The treatment with each N-OH mediator, 500 µM HPI a–c) and 500 µM HBT d–f), are presented row-wise and treatments of each lignin (BOL, SOL WSL) are presented column-wise.

Table 3
The maximal steady state radical concentrations obtained by laccase and LMS treatments with mediator concentrations of 500 μM. The values were determined as average at the time point of the highest radical concentrations were achieved. Standard deviations were in the range of 0.1–7.7 μM. The significant differences ($p \leq 0.05$) between the values within each column are shown as superscript letters (a–d) and significant difference ($p \leq 0.05$) between the values within rows for each substrate are shown as superscript letters (x–y).

	BOL		SOL		WSL	
	Tv lac (μM)	Mt lac (μM)	Tv lac (μM)	Mt lac (μM)	Tv lac (μM)	Mt lac (μM)
–	$100^{c,x}$	$89^{c,y}$	$81^{b,x}$	$72^{b,y}$	$39^{b,x}$	$42^{b,x}$
HPI	$95^{c,x}$	$85^{c,y}$	$77^{b,x}$	$66^{bc,y}$	$35^{c,x}$	$42^{b,y}$
HBT	$99^{c,x}$	$95^{b,x}$	$76^{b,x}$	$69^{b,x}$	$37^{bc,x}$	$41^{b,x}$
ABTS	$221^{a,x}$	$234^{a,x}$	$247^{a,x}$	$244^{a,x}$	$125^{b,x}$	$189^{a,y}$
TEMPO	$115^{b,x}$	$89^{c,y}$	$59^{c,x}$	$52^{c,y}$	$37^{bc,x}$	$46^{b,y}$

EPR signal. Regardless, the inability to detect the *N-oxyl* radicals of either HPI and HBT as well as the observed lack of impact by LMS treatments with the N-OH type mediators (HBT and HPI) on the radical response of stable lignin derived radicals altogether suggests that the these type of mediators are unlikely to diffuse into the lignin polymer to oxidize inaccessible sites. We therefore doubt that radical shuttling by these mediators promotes the reaction of laccase catalyzed lignin oxidation. Consequently it is unlikely that either HPI or HBT enhance laccase modification by oxidizing phenolic subunits in lignin otherwise not available to the catalytic pocket of laccase.

3.4. Impact of LMS treatments with ABTS and its confounding signals

The signal from lignin derived radical in the LMS treatments with 500 μM ABTS, had overlapping signal from the formation of $ABTS^{\cdot+}$ radicals as illustrated in Fig. 2d and e. At the EPR parameters employed, which were optimized for detection of lignin derived radicals, any distinction between the $ABTS^{\cdot+}$ radicals and the lignin derived radicals was infeasible. Consequently, the quantification of radical formation by LMS treatments with ABTS, providing the basis for the progress curves (Fig. 3), initial rates (Table 2), and maximal steady state values (Table 3), was based on the sum of lignin derived radicals and $ABTS^{\cdot+}$ radicals. This implies substantially increased values and the values are therefore not directly comparable to the values of the other LMS treatments. Hence, an assessment of ABTS as an electron carrier to enhance laccase oxidation of lignin to form radicals was not possible, but the formation of a more stable $ABTS^{\cdot+}$ radical compared to the *N-oxyl* radicals might indicate a potential of ABTS to enhance laccase catalyzed lignin modification through an electron carrier mechanism.

In order to compare progress curves from LMS treatments with ABTS and the reference treatments with only laccase, the control experiments with laccase and ABTS without lignin were included (Fig. 4). Evident differences in the progress curves of radical formation were observed. Laccase catalyzed oxidation of ABTS in absence of lignin (control) thus showed a typical Michaelis-Menten curve with a linear part with constant rate followed by a decrease in rate. Due to the confounding signal from the $ABTS^{\cdot+}$ radicals, the progress curves of radical formation by the LMS treatments with ABTS were based on the sum of $ABTS^{\cdot+}$ radicals and lignin derived radicals and these curves had substantially increased numbers compared to lignin radical formation by laccase alone. The progress curves from the LMS treatments of lignin had an S-shaped form with an initial lag-phase. Compared to the control experiments with laccase and ABTS only, the apparent initial formation of $ABTS^{\cdot+}$ radicals in the LMS treatments with lignin appeared to be hampered. This apparent difference in progress of radical formation suggests that the presence of lignin affects the observed net radical formation of ABTS. This impact may be caused by a reaction between lignin and the rapidly formed $ABTS^{\cdot+}$ radicals, which in turn will decrease the net number of $ABTS^{\cdot+}$ radicals ending up with a lower net formation of radicals. Alternatively, the presence of lignin may imply an actual inhibition of the radical formation due to adsorption of laccases onto the surface of lignin, which has been demonstrated previously [39,40].

Although the initial rates of ABTS oxidation catalyzed by Tv laccase and Mt laccase were similar, 6.8 μM/min vs. 6.5 μM/min, respectively, the initial rates from the LMS treatments of the all three lignins implied a significant difference between Tv laccase and Mt laccase activity on lignin (Table 2). It might be speculated that the lower radical formation rates exhibited by Tv laccase compared to Mt laccase on all three lignins may be caused by a higher adsorption of the Tv laccase to lignin.

3.5. Impact of LMS treatments with TEMPO

LMS treatments with TEMPO had overlapping signal of the TEMPO radical (Fig. 2f). Due to the distinct shape of the TEMPO radical signal, the contribution to the overall radical concentration could be excluded. The exclusion was done by subtracting the area of the double integrated TEMPO radical signal from the total area of the entire double integrated signal, by which only increase in the lignin derived radicals was reflected. The progress of lignin derived radical formation facilitated by LMS treatments with 500 μM TEMPO was decreased compared to the reference treatments with Tv laccase or Mt laccase alone (Fig. 5); correspondingly, the initial rates of LMS treatments with TEMPO were significantly decreased and were significantly lower than the reference treatments as well as the other LMS treatments in all three lignins (except for the Mt-WSL treatments) (Table 2). However, in progress curves of BOL and WSL treated with LMS with TEMPO, it was observed that lignin derived radical formation did not stagnate within the measured timeframe (Fig. 5a, c). If the continued progression of radical formation was estimated, it would result in a steady state concentration of stable lignin derived radicals, which exceeded the steady state concentrations achieved by the reference treatments with Tv laccase or Mt laccase alone, as already observed for the Tv-TEMPO treatment of BOL (Table 3). On the other hand, the maximal steady state concentrations of lignin derived radicals achieved on SOL was significantly reduced (27% with Tv and 28% with Mt), signifying that the lignins responded differently to LMS treatments with TEMPO.

A considerable decrease in the TEMPO radical concentration was observed in the control experiments where TEMPO was incubated with the different lignins in absence of laccase (data not shown). This indicates that TEMPO reacts with lignin independently of laccase without the necessity of being oxidized to the oxoammonium ion by laccase catalysis. TEMPO is a well-known oxidizing agent and has been shown to react with lignin and cause a drop in lignin content of pulp [41]. However, in contrast to the LMS treatments with TEMPO of the lignins, the control experiments with TEMPO and lignin only did not cause any a detectable formation of lignin derived radicals indicating that the reaction induced by TEMPO alone is different from that of laccase catalysis, and it was assumed that it did not interfere with the radical formation of the LMS treatments, once the signal from TEMPO had been subtracted. Despite, the overlapping signal from the TEMPO radical and the observed apparent non-enzymatic reaction between TEMPO and the lignin substrates, assessment of the LMS treatments with 500 μM TEMPO on initial rates and steady state concentration were feasible. As the only LMS treatment in this study, Laccase-TEMPO treatments indicated an enhancing effect in the lignin derived radical steady state concentration of WSL and BOL, when the progression curves were extrapolated beyond the experimental timeframe. Even though a wider experimental timeframe would provide more distinct results, these observations indicate that TEMPO may pave a way for increasing the radical formation of stable lignin derived radicals, not accessible to the laccase.

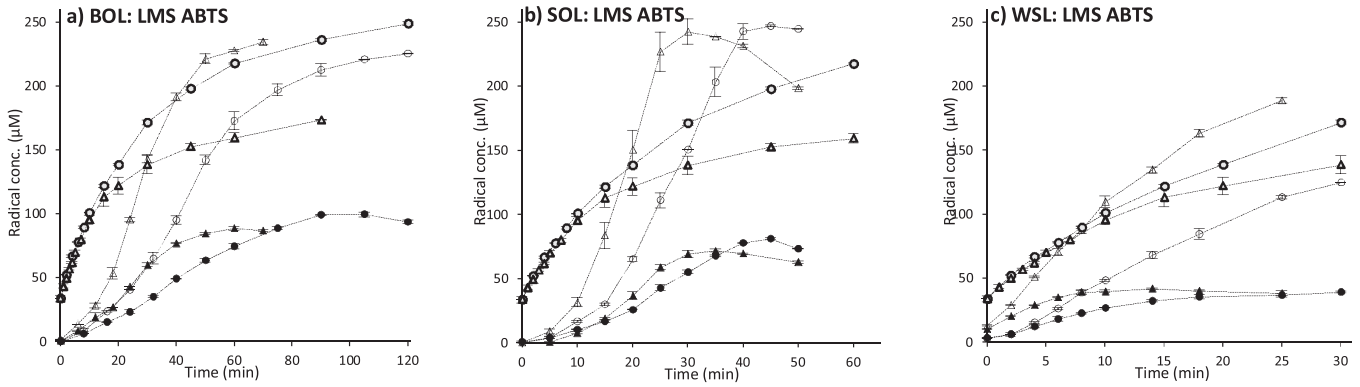

Fig. 4. Progression curves of radical formation in each lignin: a) BOL; b) SOL; c) WSL – all during LMS treatments with 500 µM ABTS and Tv lac (○) or Mt lac (△) compared to sole treatments with Tv lac (●) or Mt lac (▲) and compared to control treatments with 500 µM ABTS and Tv lac (○) or Mt lac (▲) in absence of lignin. The radical responses in samples with ABTS were higher due to the contribution from the $ABTS^{\cdot+}$ radicals (see Fig. 2).

3.6. Impact of LMS treatments on radical formation at increased mediator dose

Studies of various LMS treatments on lignin containing bio-streams (e.g. lignocellulosic biomass conversion and pulp from paper production) employ mediator doses in the range of 1–10 mM [42–44]. Consequently we expanded the LMS treatments of BOL to include increased mediator doses of 5 mM but with the laccase addition level remaining the same.

EPR measurements of LMS treatments with 5 mM mediator doses provided spectra similar to those obtained at 500 µM doses. As for the treatments with 500 µM mediator doses, treatments with ABTS and TEMPO resulted in overlapping signals from $ABTS^{\cdot+}$ radicals and TEMPO radicals. As previously discussed, the impact of ABTS on lignin derived radicals was not possible to assess, since only the sum of lignin derived radicals and $ABTS^{\cdot+}$ radical was obtainable (Fig. 6c). The increased mediator doses of the LMS treatments with 5 mM TEMPO provided progress curves (Fig. 6d) suggesting a significant increase in radical formation rate and maximal steady state concentration of lignin derived radicals as compared to the reference treatments with laccase alone (Table 4). The enhancing effect of LMS treatments with 5 mM TEMPO on BOL were coherent with the findings with LMS treatment at 500 µM and supported the suggestion that TEMPO may contribute to increase the radical formation of stable lignin derived radicals in lignin. Even though addition of TEMPO provides a significant increase in lignin derived radicals, the extent becomes somewhat less significant when compared to the amount of TEMPO radicals required to obtain a substantial increase. For example, when the maximal steady state concentration of lignin derived radicals in BOL generated by the reference treatments with Tv laccase and MT laccase (Table 4 – 87 and 76 µM, respectively) are compared to the total amount of detectable radicals (sum of lignin derived and TEMPO radicals) at corresponding times in the LMS treatments with 5 mM TEMPO (2863 and 2629 µM), the amount of TEMPO radicals are 30 times higher compared to the lignin derived radicals. Percentwise, the amount of lignin derived radicals only constitute 3% of the total amount of detectable radicals, and the LMS treatments with 5 mM TEMPO (Table 4 – 103 and 105 µM) only increase the share to 4%. Given that a mediator is added to function as an assisting component to enhance the laccase catalysis of lignin modification, mediator doses seems remarkably high when compared to the concentration of lignin derived radicals facilitated by laccase catalysis.

Unexpectedly, LMS treatments with HPI or HBT caused a profound and significant decrease in lignin radical formation rate and maximal steady state concentration, thus emphasizing that these type of mediators do not function well as electron carriers under the tested laccase catalysis conditions. The observed decrease in lignin derived radicals was more pronounced for LMS treatments with HPI than for LMS treatments with HBT (Fig. 6a and b). Assuming that the N-OH mediators have been oxidized by laccase followed by deprotonation to form the N-oxyl radicals, it is a possibility that these radicals inactivate the laccases. However, in LMS treatments with TEMPO where the total radical concentration was substantially higher, the inactivation of laccase did not occur to an extent that can justify this explanation. In a former study where samples with organosolv lignin were treated with 16 mM of HPI and HBT (at 50 °C for 24 h) the mediators were found to graft onto the lignin, and the extent of grafting was demonstrated to be substantially higher for HPI compared to HBT [45]. It was proposed that once the N-oxyl radicals were formed, they could undergo radical coupling with phenoxyl radicals generated by laccase catalysis and

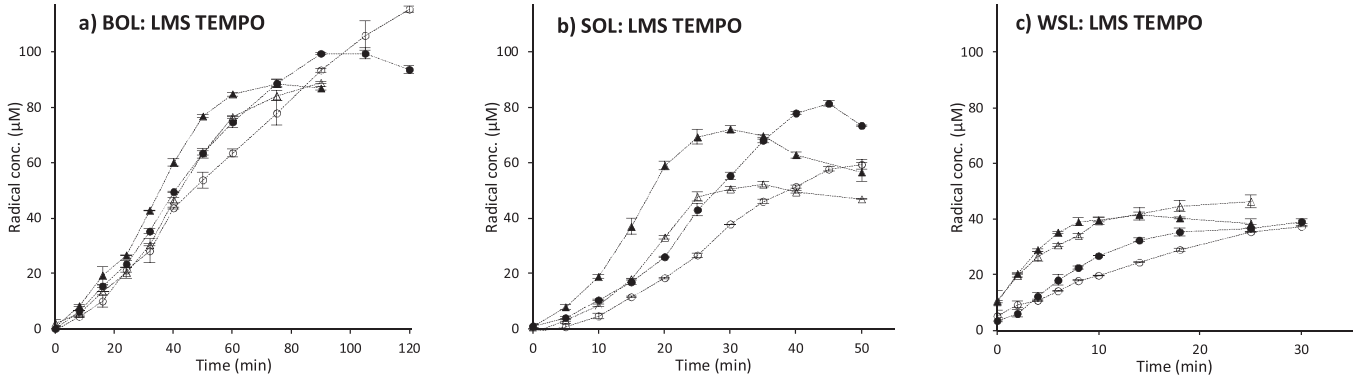

Fig. 5. Progression curves of lignin derived radical formation in each lignin a) BOL; b) SOL; c) WSL – all during LMS treatments with 500 µM TEMPO and Tv lac (○) and Mt lac (△) compared to sole laccase treatments with Tv lac (●) and Mt lac (▲).

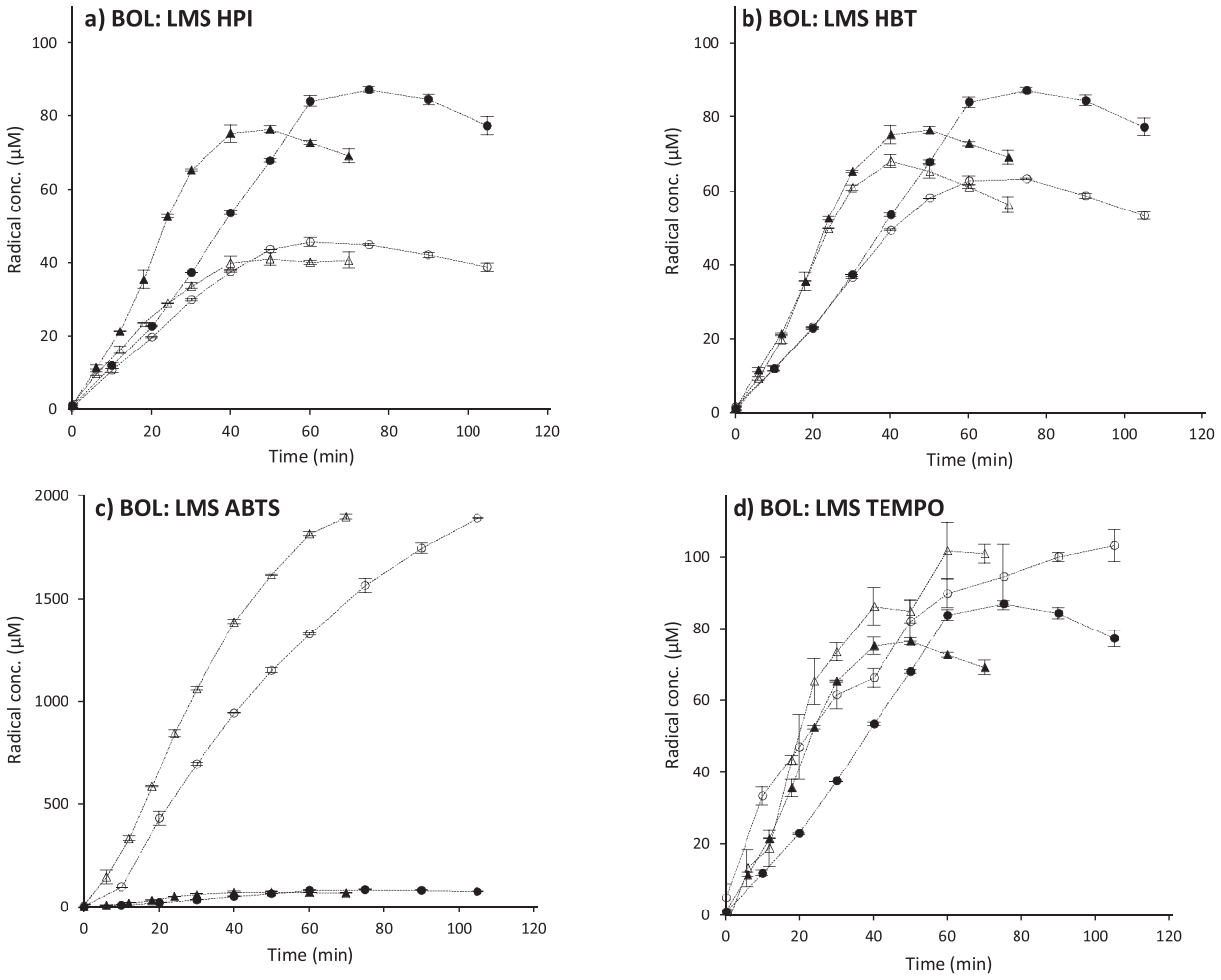

Fig. 6. Progression curves of radical formation in BOL during LMS treatments with Tv lac (○) or Mt lac (△) compared to sole treatments of Tv lac (●) or Mt lac (▲). The mediator doses were 5 mM HPI a), HBT b), ABTS c) and TEMPO d). The radical responses in samples with ABTS were higher due to the contribution from the $ABTS^{\cdot+}$ radicals.

Table 4
Initial rates (v_i) of radical formation (µM/min) and maximal radical concentrations (µM) at steady state in BOL facilitated by laccase and LMS treatments with a mediator concentration of 5 mM. All slopes had a correlation coefficient corresponding to a value of $R^2 \geq 0.93$ and standard deviations were in the range of 0.02–1.71. Standard deviations for maximal values were in the range of 0.2–11.9. The significant differences ($p \leq 0.05$) among the slopes and the max values within each column are shown as superscripted letters (a–d) and significant difference ($p \leq 0.05$) among the slopes and max values within each row are shown as superscripted letters (x-y).

	Initial rate (v_i)		Maximal radical concentration	
	Tv lac (µM/min)	Mt lac (µM/min)	Tv lac (µM)	Mt lac (µM)
–	$1.36^{c,x}$	$2.11^{c,y}$	$87^{c,x}$	$76^{c,y}$
HPI	$0.95^{e,x}$	$1.25^{e,y}$	$46^{e,x}$	$41^{e,x}$
HBT	$1.20^{d,x}$	$2.05^{d,y}$	$64^{d,x}$	$68^{d,x}$
ABTS	$24.76^{a,x}$	$35.05^{a,y}$	$1890^{a,x}$	$1897^{a,x}$
TEMPO	$1.43^{b,x}$	$2.79^{b,x}$	$103^{b,x}$	$105^{b,x}$

thereby graft onto lignin. Radical coupling between an N-oxyl and a phenoxyl radical would indeed decrease the amount of EPR detectable phenoxyl radicals in lignin, and may explain why LMS treatments with HPI decreased the steady state concentration of lignin derived radicals more than the LMS treatments with HBT.

4. Conclusion

Real time EPR measurements of lignin suspensions treated with different laccase-mediator-systems were employed to investigate the ability of different mediator additions to enhance steady state concentrations and rates of laccase catalyzed formation of stable lignin derived radicals. When added at reasonable dosage levels none of the mediators had any impact on the performance of Tv laccase and Mt laccase with regard to the radical formation in the three lignin substrates. Assessment by EPR measurements of LMS treatments of lignin suspensions cannot unequivocally clarify whether LMS treatments introduce alternative reaction routes that enables catalytic oxidation of non-phenolic radicals in lignin. However, EPR measurements can clarify if mediators function as electron carriers to oxidize non-phenolic subunits in lignin otherwise inaccessible to the catalytic pocket of laccase. The data obtained render it particularly unlikely that N-OH type mediators (HPI and HBT) impact laccase catalyzed lignin modification by acting as electron shuttles. There was thus no increase in the lignin radical steady state concentrations or formation rates and no oxidized forms of the mediators (N-oxyl radicals) were detected. Rather, at a 10 time increased mediator dose the N-OH type mediators led to significantly decreased lignin radical formation rates and steady state concentrations in the reactions compared to the respective reference treatments without mediators, which is suggested to be due to grafting

of the mediators into lignin. LMS treatments with TEMPO as mediator were the only treatments that increased the lignin radical steady state concentrations and formation rates of radicals in one of the lignin samples (beech organosolv lignin), suggesting that this mediator may oxidize stable radicals in lignin not available to laccase. This result was however only obtained at a high mediator dose, where the concentration of the TEMPO radical was more than 30 times higher than the level of radicals formed in the lignin. The results obtained in this study thus suggest that LMS treatments with feasible mediator doses do not enhance laccase catalyzed activation of lignin. It is therefore unlikely that general observations of enhanced lignin modification after LMS treatments are primarily caused by electron shuttling with mediators acting as carriers. The results highlight that data obtained with laccase-mediator systems on simple phenol substrates may not always be valid models for laccase catalysis on genuine lignin processing streams. These findings have implications for development of laccase assisted valorization of lignin in biorefining of lignocellulose.

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