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ARTICLE TYPE

Role of Laccase as an Enzymatic Pretreatment Method to Improve Lignocellulosic Saccharification

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The recalcitrant nature of lignocellulose, in particular due to the presence of lignin, is found to decrease the efficiency of cellulases during the saccharification of biomass. The efficient and cost effective removal of lignin is currently a critical biotechnological challenge in order to improve the enzymatic digestibility of cellulose for bioethanol production. In this study the role and reactivity of laccase from *Trametes versicolor* (TvL) was assessed with and without mediators for the improved saccharification of acid-pretreated wheat straw. Lignin model compound studies using veratryl alcohol and β-O-4 dimers revealed that 1-hydroxybenzotrizole (1-HBT) was the most effective mediator. Combination of TvL and TvL+1-HBT treatments with an alkaline-peroxide extraction step increased the released glucose concentration following hydrolysis by up to 2.3g/L compared to an untreated control. Pyrolysis-gas chromatography-mass spectrometry (py-GC-MS) with tetramethylammonium hydroxide (TMAH) thermochemolysis analysis of the extracted lignin revealed structural changes that are consistent with lignin degradation mechanisms typical of fungi.

Introduction

Lignocellulosic substrates are attractive feedstocks for second generation biofuel production due to their high abundance and low cost. They are widely considered as waste or by-products from forestry and agricultural industries and unlike first generation biofuel feedstocks such as corn, they do not compete with food [1]. The removal of lignin from lignocellulosic substrates remains a critical issue in biomass processing for the production of bioethanol. The presence of residual lignin following conventional pretreatment methodologies is reported to negatively affect ethanol production via several mechanisms:

1) Lignin can bind non-specifically to the cellulases employed for saccharification, resulting in the reduced catalytic activity of these enzymes due to the potential blocking of active sites and the prevention of cellulose binding [2-4].
2) Lignin that remains bound to cellulose following pretreatment causes a reduction in the surface area of the cellulose that is available for enzymatic hydrolysis [4].
3) Lignin derived products such as aromatic phenols, acids and aldehydes are often toxic to the yeasts that perform the ethanol fermentation. Small concentrations of these inhibitors have been found to destroy the integrity of the yeast membrane systems preventing growth and sugar assimilation [5-8].

The first two issues directly affect the efficiency of cellulose hydrolysis during biomass processing and contribute to the recalcitrance-related obstacles that currently make second generation bioethanol production a costly and energy intensive process. There is a need for effective pretreatment technologies that can remove or reduce this recalcitrance issue whilst still maintaining cost competitive fuel production.

Biocatalytic approaches to lignin removal are currently being explored as greener alternatives to pre-existing chemical, thermal and physical pretreatment strategies. White-rot fungi from the basidiomycete phyla degrade all components in wood including lignin. Lignin degradation is carried out via the oxidative activities of a panel of ligninolytic enzymes from the fungal secretome. The majority of these enzymes are peroxidases such as lignin peroxidase (LiP EC 1.11.1.14) and manganese peroxidase (MnP EC 1.11.1.13), however laccases (EC 1.10.3.2; benzenediol: oxygen oxidoreductase) are also suggested to play a role in lignin degradation and are attractive enzymes industrially due to their catalytic dependence on molecular oxygen as opposed to hydrogen peroxide. This dependence results in the higher stability of most laccases compared to peroxidases which suffer from deactivation by hydrogen peroxide [9]. Laccases are phenol-oxidases that catalyse the one electron oxidation of phenolics, aromatic amines, di-amines and other electron rich substrates via the four-electron reduction of oxygen to water [9-11]. Interestingly the substrate range of laccase can be expanded through the oxidation of lower molecular weight molecules known as mediators or enhancers. Oxidation of these mediators by laccase generates charged intermediates which are able to act as chemical oxidants, overcoming the steric and redox limitations that prohibit laccases from oxidising bulky and/or nonphenolic substrates [12-14].

Interest into the role of laccases on lignin degradation has developed due to reports of some white rot basidiomycetes that are found to degrade lignin or lignin model compounds in the absence or deficiency of LiP and/or MnP [15-18]. This observation suggests that degradation mechanisms by other enzymes such as laccase or aryl-alcohol oxidase (AAO) can predominate in lignin depolymerisation. However, a typical lignin polymer is 10-15% phenolic in composition, rendering up to 90% of the polymer unreactive to laccase. Fungi are assumed to use laccase for non-phenolic lignin degradation through self-generation of reactive phenoxy radicals which act as natural mediators. This is said to occur from the laccase catalysed oxidation of phenolic lignin components and or/existing lignin degradation products [18-20] or through the oxidation of their secreted phenolic metabolites [16].

In this study we initially investigated the development of an effective laccase-mediator system (LMS) using laccase from *Trametes versicolor* (TvL) in the presence of both synthetic and

natural mediators. Selected systems were thereafter evaluated for their ability to improve the saccharification reaction of acid-pretreated wheat straw by analysing the concentration of glucose released. The mechanism of laccase and laccase-mediated lignin modification and degradation was explored using a series of model lignin β-O-4 linked dimers and py-GC-MS with TMAH.

Results and Discussion

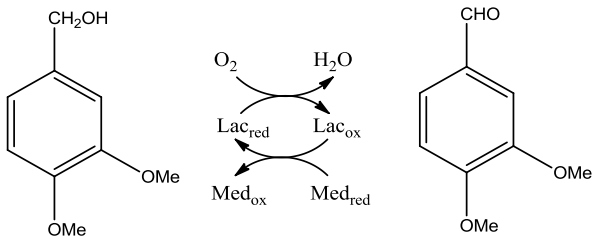
Redox mediator screening for lignin studies

A panel of 30 phenolic compounds were selected for assessment of their suitability as natural redox mediators with *Trametes versicolor* laccase (TvL redox potential ~800mV [21]). A preliminary high throughput screening assay was developed based on the previous work of Camarero *et al.*, [22] using the recalcitrant dye Reactive Black-5 (RB-5). The data revealed that under optimised mediator and dye concentrations most of the 30 phenolic compounds screened were successful at decolourising RB-5 to varying degrees within 3h. Syringaldehyde, acetosyringone and 2,4,6-trimethylphenol were found to be the best mediators for decolourising RB-5 (see SI Table 1). No decolourisation was observed in the absence of mediators.

The best 13 phenolic compounds from the decolourisation of RB-5 were further screened against the lignin model compound veratryl alcohol **1** along with synthetic compounds 1-hydroxybenzotriazole (1-HBT), 2,2'-azinobis(3-ethylbenzthiazoline-6-sulphonic acid) ABTS, and violuric acid which are reported to be effective mediators for laccase catalysed oxidations [14, 23, 24]. In addition, several dyes/indicators were screened following reports of these compounds acting as laccase substrates and mediators [20, 22, 25]. **1** is a widely utilised monomeric lignin model compound for laccase mediator studies [12, 13, 24, 26-28]. Oxidation leads to the formation of a single product veratryl aldehyde **2,** which is simple to detect by HPLC. Laccase is unable to oxidise **1** in the absence of a mediator making this a suitable candidate for assessing the non-phenolic oxidation ability of laccase mediators. Analysis of the oxidation of **1** over time showed that 1-HBT, violuric acid and ABTS were efficient redox mediators for this reaction, with conversions to **2** reaching up to 99% after 24h under optimal conditions (Table 1). Of the dyes investigated, only phenol red (PR) and remazol brilliant blue (RBB) acted as potential mediator substrates for TvL, resulting in 50 & 52% conversion respectively. No oxidation of **1** was observed in the absence of mediators.

In agreement with previous studies [19, 29], the natural phenolic mediators were not found to oxidise **1** to **2** in significant quantities which is in contrast to the results observed with RB-5. Presumably substrate **1** is less effective at reducing the intermediate phenoxy radicals produced by laccase compared with RB-5. This will explain the slower reaction rate observed with **1** which would promote undesired non-catalytic mechanisms leading to radical coupling and polymerisation, thus preventing the conversion of **1** to **2** [20, 30]. Under the assumption that lignin model compounds such as **1** can provide a reasonable indication of laccase reactivity towards natural lignin, then naturally occurring phenolic structures such as those investigated here would be expected to act poorly as mediators due to their instability in the catalytic cycle. The rapid decolourisation of RB-5 suggests a role for these compounds in technologies linked with dye detoxification/decolourisation in the textile industry.

Table 1: Oxidation of veratryl alcohol (**1**) by TvL in the presence of a redox mediator



Mediator	% Conversion to **2**[a]			
	A	B	C	D
1-HBT	**99**	94	98	92
ABTS	74	61	87	**88**
Violuric acid	31	71	24	**74**
Phenol red	**50**	19	17	33
Remazol Brilliant Blue	21	9	22	**52**

Oxidation of 3mM **1** under four different enzyme-mediator conditions. A: 1.6U/ml TvL, 3:1 lac-med ratio, B: 1.6U/ml TvL, 1:1, C: 0.4U/ml TvL, 3:1 D: 0.4U/ml 1:1[a]% conversion was determined by LC-MS after 24h, optimal conversions are shown in bold.

The effect of laccase and LMS on lignocellulosic saccharification

In order to determine the effectiveness of the successful laccase mediator systems (LMS) investigated with **1** towards a lignocellulosic bioprocess, laccase and LMS treatments were carried out on wheat straw that had previously been treated with dilute sulphuric acid. Dilute acid hydrolysis is a commonly employed pretreatment method that effectively solubilises the hemicellulose component within lignocellulose. Removal of hemicellulose improves the digestibility of cellulose by increasing the porosity of the substrate [31]. Wheat straw slurries treated with different concentrations of TvL were initially analysed for free phenol content using the Folin-ciocalteu total phenol assay [32]. In accordance with a previous study [33], a decrease in soluble phenol content was observed in wheat straw slurries treated with TvL (see SI, Figure 2) supporting the role of laccase in the oxidation of phenolic structures within biomass and the removal of the reactive intermediate phenoxy radicals by polymerisation.. A cellulase preparation (Genencor GC-220) was added to the slurries following treatment with the different TvL concentrations to allow hydrolysis of the cellulose fraction. Following quantification of the released glucose it was revealed that no increase in glucose concentration was observed in the TvL treated slurries compared to the laccase free and denatured laccase negative controls. The reaction was further investigated with TvL and the addition of synthetic mediator 1-HBT. An increase in glucose release was not observed following hydrolysis despite the incorporation of wash steps to remove potential inhibitory compounds and both the enzyme and mediator. In fact, it appeared that laccase treated slurries were inhibiting the saccharification process (Figure 1 top graph).

A recent study by Gutierrez *et al.*, [34] reported an increase in polysaccharide hydrolysis for eucalypt wood and elephant grass treated with *T. villosa* laccase and 1-HBT after the incorporation of an alkaline-peroxide extraction (APE) step. This step was therefore applied to all wheat straw slurries following laccase treatments. Quantification of released glucose during hydrolysis following this additional step revealed that slurries pretreated with TvL and TvL+1-HBT now released higher concentrations of glucose following hydrolysis compared to the no laccase no

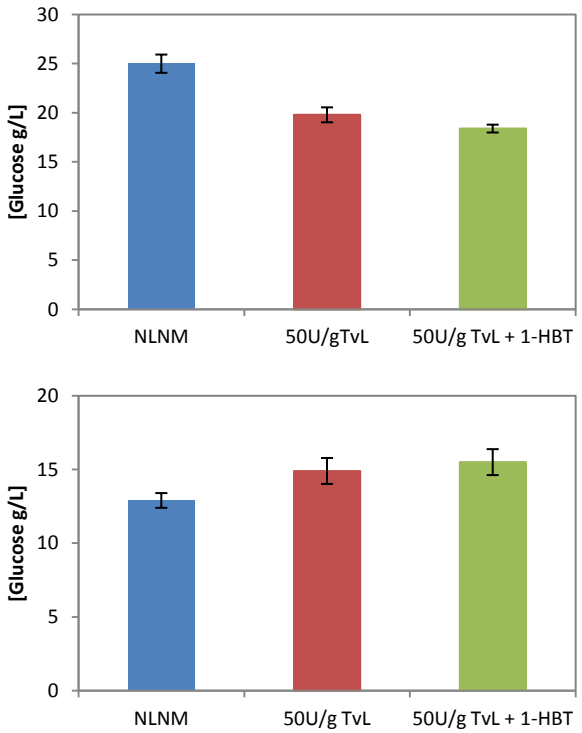


Figure 1. Concentration of glucose released from 0.6g (d.w) wheat straw hydrolysed by 2.9FPU GC220 for 48h following treatment with/without TvL and TvL+1-HBT. Top: No APE step after laccase treatment. Bottom: APE treatment performed after laccase treatment. Error bars represent standard error of three biological replicates.

mediator negative control (NLNM) (Figure 1 bottom graph). This LMS can induce a positive effect on the saccharification process of wheat straw. The results in Figure 1 also demonstrate that use of an APE step leads to a decrease in released glucose concentration both in the presence and absence of laccase (Figure 1). This can be explained by the additional biomass handling steps during three consecutive extractions and subsequent wash steps which results in biomass loss. In addition, the alkali oxidative environment can induce undesired cellulose cleavage reactions. The addition of magnesium sulphate has been reported to minimise peroxide induced cellulose degradation suggesting the opportunity to minimise these undesired reactions during process optimisation and development [35].

Optimisation of laccase loading and mediator concentration revealed that 500U/g d.w WS (units TvL per gram dry weight of wheat straw) was optimal for increased saccharification (see SI, Figure 4) however due to commercial enzyme costs, subsequent experiments were carried out using 150U/g (d.w WS). Use of the redox mediator 1-HBT at 5% (w/w) with 150U/g TvL proved optimal and lead to an increase of 1.4g/L (13%) glucose after 64h hydrolysis compared to the mediator free control (Table 2).

Based on the mediator screening data, some synthetic and natural mediators were applied to the optimised bioprocess to assess their suitability and reactivity on wheat straw lignin. 1-HBT was found to be the most effective mediator, increasing the concentration of released glucose following hydrolysis by 2.3g/L (35%) compared to the NLNM negative control (Table 3). This is in support of previous studies whereby 1-HBT was reported to be the most efficient mediator in the delignification and biobleaching of lignocellulose [14, 36-38]. Violuric acid also proved to be an effective mediator, increasing glucose concentration by 1.7g/L (26%). 1-HBT and violuric acid belong

Table 2: Investigating the optimum concentration of 1-hydroxybenzotriazole (1-HBT) with TvL to improve wheat straw saccharification

Biomass treatment[a]	Glucose concentration g/L[d]
150U/g[b] TvL	10.8 (0.13)
150U/g[b] TvL + 2.5%[b] 1-HBT[c]	11.0 (0.23)
150U/g[b] TvL + 5%[b] 1-HBT[c]	**12.2** (0.37)
150U/g[b] TvL + 7.5%[b] 1-HBT[c]	11.2 (0.80)

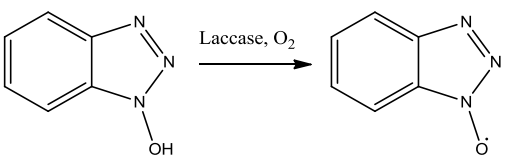
[a] 0.5g d.w wheat straw incubation for 40h, 28°C, 200rpm [b] enzyme units per g dry weight wheat straw biomass [c] % (g/g) mediator per dry weight wheat straw biomass [d] glucose concentration determined by HPLC-RID following 64h hydrolysis with 2.9 FPU GC220. Parentheses represent standard error of three biological replicates. Bold indicates most successful treatment.

Table 3: Screening of synthetic and natural mediators with TvL for the improvement of wheat straw lignocellulosic saccharification

Biomass treatment[a]	Glucose concentration g/L[d]
NLNM	6.6 (0.03)
150U/g TvL	7.8 (0.19)
150U/g TvL 1-HBT[b]	**8.9** (0.20)
150U/g TvL ABTS[b]	7.7 (0.22)
150U/g TvL violuric acid[b]	8.3 (0.10)
150U/g TvL syringaldehyde[c]	7.7 (0.08)
150U/g TvL acetosyringone[c]	7.5 (0.25)

[a] 0.5g d.w wheat straw incubation for 40h, 28°C, 200rpm [b] mediator concentration of 1-HBT was 5% (g/g) d.w biomass, molar concentration of ABTS and violuric matched to molar concentration of 1-HBT [c] mediator concentration 7mM [d] glucose concentration determined by HPLC-RID following 42h hydrolysis with 2.9 FPU GC220. Parentheses represent standard error of three replicates. Bold indicates most successful treatment.

the hydroxylamine laccase mediator class and share the N-OH structural feature. Previous studies have revealed that these mediators undergo laccase catalysed oxidation to produce aminoxyl radicals following deprotonation as outlined with 1-HBT in Scheme 1. Oxidation reactions promototed by these aminoxyl radicals are reported to follow a hydrogen-atom transfer (HAT) mechanism by abstraction of the benzylic hydrogen of the reduced substrate [28, 39].



Scheme 1. The oxidation of 1-hydroxybenzotriazole (1-HBT) by laccase to produce the corresponding nitroxy radical

The use of potential TvL mediators acetosyringone and syringaldehyde did not improve saccharification when compared to the mediator free control. This result was expected following the previous unsuccessful oxidation of model compound **1** by both LMS. Surprisingly, the reaction of TvL with the synthetic mediator ABTS did not improve saccharification despite the excellent conversion of **1** to **2** using this LMS (Table 1). A similar result was also observed by Chen [37], who reported little differences between the measured water soluble carbohydrate concentration released from hydrolysed ensiled corn stover after TvL and ABTS treatment. Similarly, the application of PR and RBB as mediators failed to result in an increased glucose concentration compared with the mediator free control (see SI, Figure 6). These observations suggest that the ability of a LMS to successfully oxidise the monomeric guaiacyl (G-type) lignin model compound **1**, does not necessarily correlate with an ability

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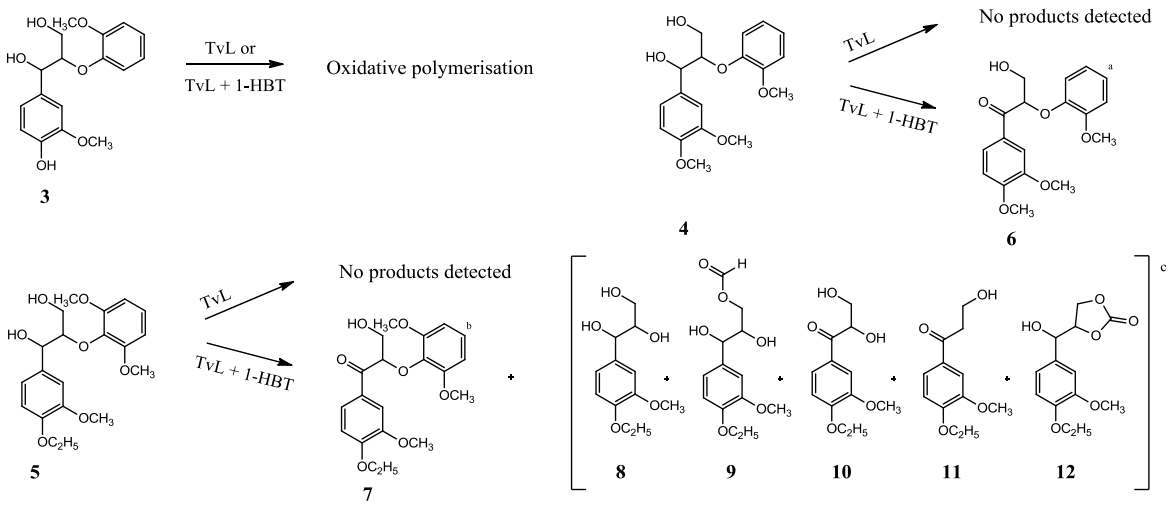


Scheme 2: Reactions of lignin model β-O-4 dimers **3**, **4**,and **5** [a] Product **6** characterised by MS and NMR following purification [b] Product **7** characterised by MS and by comparison with an authentic standard [c] Degradation products were identified by LC-MS and comparison with degradation products reported in the literature [40].

of the LMS to react with lignin to improve cellulose hydrolysis. Monomeric substrates such as **1** are likely to represent poor lignin model compounds due to the absence of β-linked structures that are predominant in natural lignin. Furthermore, the structural complexity of lignin regarding the heterogeneously linked G-type, S-type (syringly) and H-type (*p*-hydroxylphenyl) sub-structures highlights the challenges associated with drawing meaningful conclusions from model studies.

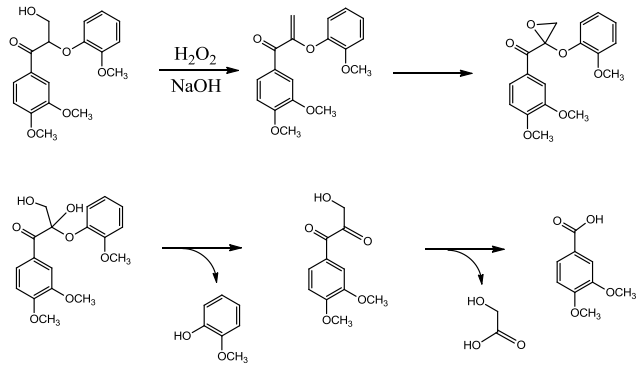
Interestingly, the incubation of wheat straw with TvL in the absence of mediator consistently improved saccharification. This increase in glucose release following laccase treatment alone has been previously reported [4, 34, 36]. It has been speculated that the binding of laccase to lignin sites within biomass competes with, and reduces the non-specific binding of cellulases therefore improving saccharification. Furthermore, electron spectroscopy for chemical analysis (ECSA) has been used to probe surface modifications of spruce lignin following treatment with *Trametes hirsuta* laccase [36]. The study revealed an increase in carboxylic acid residues following laccase treatment and suggests that this modification to lignin may decrease the non-specific adsorption of negatively charged cellulases due to electrostatic repulsion. Conversely, the treatment of lignin with laccase has been reported to increase undesired cellulase binding thus inhibiting saccharification [4]. Although the mechanism of this has not been described, this effect may explain the decrease in saccharification following TvL and TvL + 1-HBT treatment without APE reported in this study. Jurado *et al*., and Tabka *et al*., [33, 41] reported the same inhibitory effect with steam exploded wheat straw and two different fungal laccases, however they did not investigate the use of APE.

The results obtained in this study consistently revealed an improvement of saccharification following the incubation of wheat straw with TvL and a successful LMS followed by APE, demonstrating the reproducibility of this effect even when different batches of washed substrate were used. To further investigate reproducibility, the optimised bioprocess with TvL, 1-HBT and APE was repeated with two other agricultural residues, corn and sorghum stover. The same trends regarding the increase in glucose release was observed with both substrates (see SI Figure 7) demonstrating the success of the laccase and 1-HBT treatments with alternative substrates.

Investigating the reaction of TvL and TvL + 1HBT with lignin model β-O-4 linked dimers

β-O-4 linked dimers (**3**-**5**) were used in an attempt to gain an understanding of potential structural changes occurring within lignin as a result of laccase/LMS treatment. Dimers **3** and **4** were commercially available whilst dimer **5** was synthesised by a modified method of Kawai *et al*., [40, 42] (see SI section 1.9). Such structures have been used extensively to study lignin degradation mechanisms due to the predominance of this β-O-4 linkage within lignin [42-46].

All 3 dimers were incubated with TvL both with and without 1-HBT. Immediate sampling of the reaction of the phenolic dimer **3** with TvL in the absence of 1-HBT and subsequent analysis by LC-MS revealed a product peak *m*/*z* 661, consistent with oxidative dimerisation of **3**. After 24h, complete consumption of starting material **3** and initially formed dimerisation product was observed (see SI, Figure 8), presumably as a result of further polymerisation as anticipated with oxidised phenolic substrates. As expected, the reactions of non-phenolic dimers **4** and **5** with TvL in the absence of 1-HBT failed to result in the formation of oxidation products, with only starting material observed by LC-MS. Consistent with previous studies [13, 47], oxidation of dimer **4** with TvL in the presence of 1-HBT led to the formation of the uncleaved ketone product **6** as the sole oxidation product, providing evidence for a Cα oxidation mechanism (Scheme 1). The analogous Cα oxidation product was also observed when



Scheme 3. The proposed pathway for the degradation of a phenacylaryl ether (involving cleavage of the Cα-Cβ) by alkaline-peroxide treatment as investigated by Gierer *et al.*, [50]

dimer **5** (*threo*: *erythro* 5:2) was reacted with TvL + 1-HBT, however, in this instance a complex mixture of degradation products were also observed by LC-MS that were not present in the TvL and substrate only negative controls. Presumably these degradation products are derived from oxidation of the more electron rich aromatic ring in dimer **5** which bears an additional methoxy substituent. Comparison of the *m*/*z* values with the suite of oxidation products characterised by Kawai *et al.*, [44] from the same dimer allowed identification of degradation products **8-12** (*m*/*z* 265, 293, 263, 247 and 291) (Scheme 2, refer to SI figures 8-11 for all LC-MS traces and product characterisation). The production of Cα oxidation products **6** and **7** from the non-phenolic dimers **4** and **5** may provide a plausible explanation for the role of APE in the improvement of saccharification following LMS treatment. During APE, the hydroperoxide anions produced are reported to react with the carbonyl structures within lignin resulting in C-C bond cleavage [48, 49]. The mechanism of degradation of phenyacylaryl ethers (e.g. structure **6**) hydrogen peroxide in alkaline media has been studied previously and is outlined in Scheme **3** [50]. An increased presence of carbonyl structures in lignin following treatment with TvL + 1-HBT may correlate with increased lignin removal by C-C bond cleavage following APE. These studies conducted with non-phenolic dimers **4** and **5** may provide important mechanistic insights into the role of LMS towards lignin degradation.

Pyrolysis GC-MS with TMAH

Thermally-assisted hydrolysis and methylation using tetramethylammonium hydroxide (TMAH) thermochemolysis followed by gas chromatography-mass spectrometry (GC-MS) has rapidly developed as a tool for characterising the relative proportions of lignin monomers in plant material [51]. Pyrolysis of lignin with TMAH is reported to induce cleavage of propyl-aryl-ether bonds (β-O-4) and the methylation of both aromatic and alkyl side chain hydroxyl groups [52]. This technique has been used by researchers studying the degradation of lignin in the natural environment or by pure cultures of fungi [53-55] however in this study it is explored for the determination of the action of laccase and LMS on the lignin structure. The technique was used to investigate and compare the composition of organosolv extracted wheat straw lignin following pretreatment with TvL, TvL + 1-HBT and no enzyme and no mediator (NLNM).

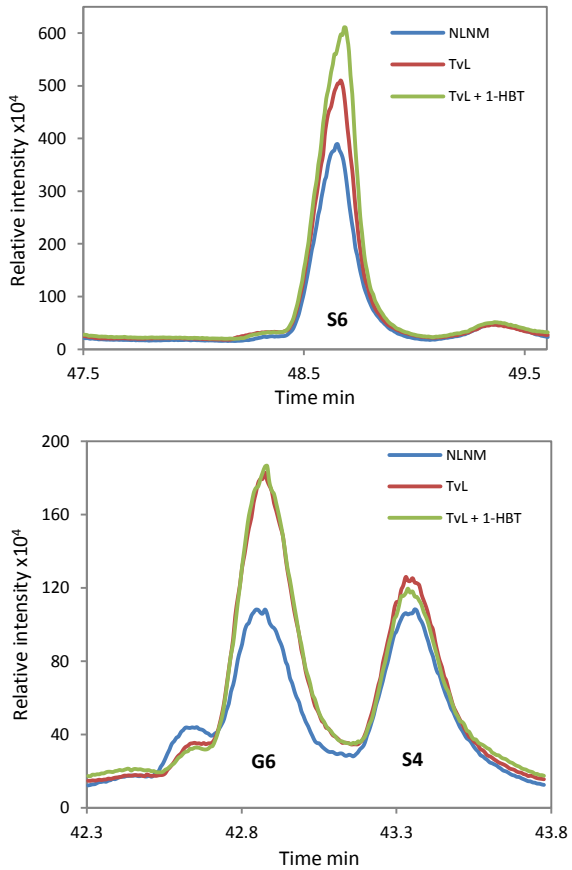


Figure 2: Partial chromatograms of the total ion current (TIC) for the TMAH thermochemolysis products methyl, 3, 4, 5-trimethoxybenzoate (S6) (top), and methyl, 3,4-dimethoxybenzoate (G6) and 3,4,5-trimethoxybenzaldehyde (S4) (bottom) for both laccase and laccase mediator treated wheat straw lignins and the untreated control.

A collection of guaiacyl (G) and syringyl (S) thermochemolysis products were liberated from the lignin samples. Peaks were identified by library searches and by the comparison of peak positions and characteristic mass ions from published data [53] (See SI Table 2). A peak of high abundance derived from the *p*-hydroxylphenyl (H) monolignol was also observed. Wheat straw lignin is reported to contain all 3 mono-lignols (G, S and H) with a predominance of G and S-type lignins (H-type <10%) [56, 57] and S/G ratios around 1.2-1.4 [53, 58]. A partial chromatogram displaying the identified TMAH thermchemolysis products of organosolv extracted wheat straw lignin without enzymatic pretreatment is provided in the SI (Figure 12).

The most significant observation from the total ion currents (TIC) was the differences observed in the peak intensities of methyl 3,4,5-trimethoxybenzoate (S6) and methyl 3,4-dimethoxybenzoate (G6) between the laccase treated samples and the untreated control (Figure 2). Oxidative lignin degradation mediated by white rot fungi such as *T. versicolor* is reported to occur via Cα-Cβ side chain cleavage at the Cα position. This oxidative cleavage leads to the production of aromatic aldehydes such as 3,4,-dimethoxybenzaldehyde (G4) and 3,4,5-trimethoxybenzaldehyde (S4) from alcohol groups which are reported to undergo further oxidation to their carboxylic acids (such as G6 and S6) [53, 59, 60]. Researchers examining the ratio between the G and S unit acids (G6 and S6) and the aldehydes (G4 and S4) by calculating [Ac/Ald]G and [Ac/Ald]S have found

Table 4. [Ac/Al] ratios of both G and S units from organosolv extracted wheat straw treated with/without TvL/TvL+1-HBT

	Treatment[a]	[Ac/Al]G	[Ac/Al]S
Exp 1	NLNM	0.79	3.44
	150U/g TvL	1.22	4.91
	150U/g TvL+1-HBT[b]	**1.55**	**5.69**
Exp 2	NLNM	0.76	3.96
	150U/g TvL	1.67	5.21
	150U/g TvL+1-HBT	**1.82**	**6.97**
Exp 3	NLNM	1.13	4.80
	150U/g TvL	1.67	5.36
	150U/g TvL+1-HBT	**1.82**	**7.62**
		Average increase in [Ac/Al]G	Average increase in [Ac/Al]S
	NLNM to TvL	0.63 (0.15)	1.09 (0.27)
	NLNM to TvL + 1-HBT	0.84 (0.11)	2.69 (0.23)
	TvL to TvL + 1-HBT	0.21 (0.06)	1.6 (0.43)

[a] Incubation of wheat straw for 40h, at 28°C and 200rpm [b] 5% (g/g) d.w biomass. Parenthesis represent standard error of three biological replicates. Bold represents highest ratio.

increased ratios in fungal-degraded lignin compared to native lignin [53, 55]. The same ratio increase was found when fungal-degraded lignin was analysed via alternative methods such as solid state ^{13}C-NMR, alkaline CuO and nitrobenzene oxidation [60-62]. Calculation of these ratios revealed an increase in the [Ac/Ald]G ratio from 0.79 (NLNM) to 1.22 (TvL) and a furtherincrease to 1.55 (TvL+1-HBT). A similar trend was observed for [Ac/Ald]S whereby the ratio increased from 3.44 (NLNM) to 4.91 (TvL) and 5.69 (TvL+1-HBT) (Table 4). The trend was reproducible across three separate experiments set up using wheat straw from different washed batches. The increase in both ratios following preincubation with TvL and TvL + 1-HBT is consistent with the reported Cα-Cβ oxidative cleavage mechanism of lignin by fungal enzymes [53, 59].

Conclusions

The saccharification of acid-pretreated wheat straw was successfully improved by the development of a bioprocess incorporating an alkaline-peroxide extraction step following the incubation with laccase and a laccase-mediator system. Mediator screening studies using the lignin model compound veratryl alcohol revealed that a correlation between the activity towards the substrate and real lignin could not be established for all mediators. 1-HBT was proven to be the most successful mediator for the bioprocess. Studies using non-phenolic β-O-4 dimers with the LMS provided evidence for a Cα oxidation mechanism and possible β-O-4 cleavage. We suggest that the increased formation of the Cα oxidised groups within lignin following LMS treatment positively assists with lignin removal by alkaline peroxide extractions. Extracted lignin from laccase and LMS treated wheat straw was found to contain a higher proportional of syringyl and guaiacyl acid monomers by py-GC-MS with TMAH thermochemolysis when compared to the aldehyde counterpart. This observation is consistent with reported fungal-mediated lignin degradation mechanisms providing further evidence for the role of laccase and a laccase mediator system in lignin degradation.

Notes and References

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