Holzforschung, Vol. 63, pp. 699–704, 2009 • Copyright © by Walter de Gruyter • Berlin • New York. DOI 10.1515/HF.2009.066

# Syringyl-type simple plant phenolics as mediating oxidants in laccase catalyzed degradation of lignocellulosic materials: Model compound studies

## 10th EWLP, Stockholm, Sweden, August 25–28, 2008

**Paula Nousiainen[1], Pekka Maijala[2], Annele Hatakka[2], Angel T. Martínez[3] and Jussi Sipilä[1,]***

[1] Department of Chemistry, University of Helsinki, Helsinki, Finland

[2] Department of Applied Chemistry and Microbiology, University of Helsinki, Helsinki, Finland

[3] CIB, CSIC, Ramiro de Maeztu 9, Madrid, Spain

*Corresponding author.
Department of Chemistry, P.O. Box 55, 00014 University of Helsinki, Helsinki, Finland
E-mail: jussi.sipila@helsinki.fi

## Abstract

The utility of simple syringyl-type phenolics of plant origin and some common synthetic compounds were compared with regard to their ability to act as laccase mediators in the oxidation of non-phenolic lignin model compounds. It was demonstrated that syringyl-type phenols, especially acetosyringone and methyl syringate, were able to mediate the oxidation of substrates of high oxidation potential by a laccase with a low redox potential. A mediator dose around 10% showed good performance compared to the equimolar quantities needed when the synthetic compounds 1-hydroxybenzotriazole or *N*-hydroxyacetanilide were used.

**Keywords:** acetosyringone; 1-hydroxybenzotriazole (HBT); laccase; natural mediators; N-hydroxyacetanilide (NHA); syringyl.

## Introduction

One of the most important challenges in modern industrial biotechnology is the eco-efficient and sustainable conversion of plant biomass into value added products and to substitute fossil fuels as a primary source for energy and chemicals. In such processes, the conversion of renewable organic materials often includes oxidation steps, either to degrade the material or to bring new reactive sites on it. These reactions are usually performed in the presence of inorganic chemical oxidants, but they can also be carried out enzymatically (Burton 2003). The enzymatic modification of lignocellulose could be based on the natural process of lignin degradation (a key step for carbon recycling in forest ecosystems) that has been defined as an 'enzymatic combustion' where fungal oxidoreductases play a central role (Kirk and Farrell 1987; Martínez et al. 2005). Until today, most oxidative enzymatic applications have focused on fungal laccases (EC 1.10.3.2), which can be produced at industrial scale.

Laccases are multicopper oxidases widely distributed in fungal and plant species, and they are involved in both lignin biosynthesis and biodegradation (Mayer and Staples 2002). Contrary to enzymes in general, laccases are quite unspecific with a wide substrate-specificity including substituted phenols, polyphenols, aromatic amines and thiols and even inorganic salts, and are able to convert substrates to their corresponding radicals with concomitant reduction of oxygen to water (Xu 1996). The active research on laccases spanning over decades has revealed that these enzymes require chemical substances with low molecular weight, the so-called mediators (Morozova et al. 2007). In combination with mediators, the substrate spectrum is large including even non-phenolic aromatic species and the recalcitrant lignin polymer with its predominantly non-phenolic substructures. In the absence of mediators, the access of laccase to lignin is hampered because it is embedded in the compact structure of the cell wall (d'Acunzo et al. 2006).

Most of the studied mediators are synthetic compounds based on nitrogen heterocyclics. Usually, these compounds are required in high amounts (Cantarella et al. 2003b) to enhance the catalytic effect, as shown for 1-hydroxybenzotriazole (HBT) due to the instability of the mediating radical (Bourbonnais et al. 1998). Accordingly, the process costs are high. In addition, many of the synthetic compounds are potentially problematic in environmental aspects (Xu 2005).

Recently, it has been found that some compounds derived from syringyl-type plant phenolics are good substrates for laccases, and they seem to be able to effectively mediate electron transfer between the oxidized laccase and target substrates (Cho et al. 2004; Camarero et al. 2005, 2007). As by-products of chemical pulping of hardwood, such phenolics are readily available, and their use already has resulted in promising effects in biobleaching of paper pulps (Camarero et al. 2007). These substances were also successful in decolorization of recalcitrant dyes (Camarero et al. 2005; Dupé 2008) and in removal of lipophilic extracts from kraft pulp (Gutiérrez et al. 2007). The properties of these new type of mediators are not fully known. Their application in other processes – such as in remediation of xenobiotics in waste water effluents and wood fiber functionalization (Widsten and Kandelbauer 2008) – will open interesting new fields for further investigations.

In the present paper, mediated laccase oxidation has been investigated on monomeric and dimeric lignin model compounds mimicking the most abundant substructures both in hardwoods and softwoods. The aim of

the present study was to test the real potential of promising "natural mediators", such as G-type and S-type lignin fragments, in oxidative degradation of lignocellulosic material. For this purpose, the oxidation of high redox potential non-phenolic 4-*O*-methylated lignin model compounds with a low redox potential laccase from *Myceliophthora thermophila* was examined using various syringyl-type lignols as mediators. According to the literature, the non-phenolic model compounds possess oxidation potentials higher than 1.4 V (Galli and Gentili 2004), whereas the oxidation potential for the *M. thermophila* laccase is approximately 0.5 V (Xu et al. 1996). For comparison purposes, similar oxidations were then also performed on common synthetic mediators, HBT and *N*-hydroxyacetanilide (NHA).

## Materials and methods

### Model compounds and mediators

Veratryl alcohol (*1*) (Acros Organics), acetosyringone (*10*) (Aldrich), syringaldehyde (*11*) (Aldrich) and HBT (*14*) (Aldrich) were of commercial grade. NHA (*15*) was synthesized following the procedure by Oxley et al. (1989). 5-Hydroxyvanillin (*12*) was prepared from vanillin by bromination and subsequent copper catalyzed basic hydrolysis (Ellis and Lenger 1998), 3,4,5-trimethoxybenzylalcohol (*2*) was obtained from corresponding aldehyde by reaction with $NaBH_4$ in ethanol. Arylglycerol β-ether compounds adlerol (pure *erythro* and mixture of *erythro and threo*) (*4*), erol (mixture of *e/t*) (*3*), (*5*) and (*6*), were synthesized according to the method by Nakatsubo et al. (1975). In some instances, 4-*O*-methylation was also performed by $CH_3I/K_2CO_3$ in acetone or dimethyl formamide. 4-*O*-methylated dehydrodiconiferyl alcohol derivative, non-phenolic phenylcoumaran-type model (Me-PC, *7*), was isolated from oxidative dehydrogenation-mixture (by the *zulauf*-method) of methyl ferulate followed by reduction with $LiAlH_4$ and methylation. Accordingly, dibenzodioxocin (Me-DBD, *8*) was prepared from coniferyl alcohol and dehydrodivanillyl alcohol, and syringaresinol (SR, *9a*) from sinapyl alcohol, its methylated derivative also being prepared (*9b*). Methyl 6-*O*-sinapoyl-α-D-glucopyranoside (*16*) was prepared according to Kylli et al. (2008). Methyl syringate was obtained by esterification of the corresponding acid. Sulfonylated model compounds (*18–21*) were synthesized by the microwave method modified according to Glennie (1966), in microwave reactor Biotage Initiator 8 (Figure 1). Veratraldehyde and 3,4,5-trimethoxybenzaldehyde, which were used for quantification of the oxidation mixtures were of commercial grade (both from Fluka) and adlerone [2-(2-methoxyphenoxy)-3-hydroxy-1-(3,4-dimethoxyphenyl)propan-1-one] was prepared in the laboratory by us. 1,3,5-trimethoxybenzene, which was used as internal reference in HPLC, was purchased from Fluka. All the synthesized compounds were purified with silica column chromatography before further use. For identification, NMR spectra were recorded with Varian Inova 500 and Varian Mercury Plus 300 spectrometers ($^1H$: 500 and 300 MHz, and $^{13}C$: 125 and 75 MHz, respectively) in $CDCl_3$, $D_2O$, DMSO-$d_6$ or acetone-$d_6$. The 2D NMR techniques (HSQC, HSQC-TOCSY and HMBC) were used for the identification of products using standard pulse sequences provided by the manufacturer in acquisition and data processing.

### Mediator screening

Screening of natural mediators was based on decolorization of Reactive Black 5 (DyStar, Germany) according to Camarero et al. (2005). The effect of acetosyringone, syringaldehyde, methyl syringate, sinapic acid, methyl 6-*O*-sinapoyl-α-D-glucopyranoside, vanillin, 5-hydroxyvanillin and 5-hydroxyacetovanillone as mediators on decolorization of Reactive Black 5 with laccase was monitored at 598 nm with a Varian Cary spectrophotometer. The reaction of 50 μM Reactive Black 5 with 50 μM of mediator and 5 nkat $ml^{-1}$ of laccase in 3 ml 50 mM sodium succinate buffer at pH 4.5 was followed for 30 min at 22°C.

### Enzyme activity assay and stability testing

A commercial laccase, obtained from the ascomycete *Myceliophthora thermophila* (NS 51003), kindly provided by Novozymes (Bagsvaerd, Denmark) was applied. Laccase activity was determined at 25°C by following the oxidation of 2,6-dimethoxyphenol (DMP) in 50 mM malonate buffer at pH 4.5, to the corresponding dimeric product ($\varepsilon_{420}$ 27 500 $M^{-1}$ $cm^{-1}$, referred to substrate concentration) (Jaouani et al. 2005). The laccase stability was then tested using the same procedure in the 50 mM malonate buffer with different concentration of methanol or freshly distilled 1,4-dioxane (% vol/vol) (Figure 2). Laccase was clearly less reactive in the presence of dioxane than with methanol. Interestingly, approximately 40% of activity was still present at the concentration of 20% dioxane. As the dimeric model compounds were scarcely soluble even in 30% of methanol, all model compound experiments were then performed in 20% dioxane. Also, in order to ascertain the activity of laccase in the reaction systems for longer reaction times, the amounts of oxidation products were in some cases followed by HPLC as a function of time for 5–7 days. In all of those experiments, the oxidation was found to have proceeded for longer than 2 days. In addition, the laccase activity (originally 5 nkat $ml^{-1}$) in 20% buffered dioxane/water solution was also determined as a function of time (samples of 0 h, 5 h, 24 h, 48 h, and 7 days) in separate assays by measuring the oxidation rate of ABTS [2,2′-azino-bis-(3-ethylthiazoline-6-sulfonate), Sigma] at 22°C followed at 420 nm (Eggert et al. 1996; see also Cantarella et al. 2003a). The results clearly showed that after 2 days in the aqueous dioxane, 90% of the activity of the original laccase solution still remained and in 7 days 20–30% of the activity was left.

### Model compound experiments

The oxidation of model compounds was performed in vials covered with parafilm under magnetic stirring at room temperature (22°C). Each reaction in total volume of 1 ml was a mixture of 50 mM succinate buffer (pH 6) and freshly distilled 1,4-dioxane as a co-solvent (20% vol/vol), 12 mM in substrate (lignin model compound), 1.2 mM or 12 mM in mediator, 12 mM in 1,3,5-trimethoxybenzene as internal standard, and 5 nkat (in reactions of low substrate to mediator ratio 12 mM:12 mM, referred to as 10:1) or 200 nkat (high substrate to mediator ratio 12 mM:12 mM, 1:1) laccase. In all solutions MilliQ-water was used. The reaction was followed for 0 min (control sample), 5 h, 24 h, and 48 h periods. Samples (0.1 ml) were diluted to 1.0 ml with 1:1 MeOH:$H_2O$ containing 1 mM of $NaN_3$ in order to inactivate the enzyme (Johannes and Majcherczyk 2000), after which the samples were filtered through 45 μm GHP Membrane HPLC filter (Acrodisc) and analyzed by HPLC.

### HPLC analysis

The conversion of the model compounds was followed by HPLC Agilent 1200 with detection wavelength of 270 nm. Conversion products were separated on Agilent reverse-phase Zorbax Eclipse XDB-C18 column (2.1 mm×100 mm, 3.1 μm) connected to a pre-column. Diode array UV-detector was used in the detection and peaks were identified and quantified by compar-

ison of the UV spectra and the retention times, and referenced when possible to commercially available or synthetically produced compounds. The quantification was made with external standard curves in the case of veratryl alcohol, veratraldehyde, adlerol, adlerone, 3,4,5 trimethoxybenzyl alcohol, and 3,4,5 trimethoxybenzaldehyde. In the case of methylated dibenzodioxocin, only the disappearance of the starting material was followed. In the HPLC studies, a sample of 5 μl was injected with autosampler and eluted with MeOH:$H_2O$ (MilliQ) gradient: 0–10 min 50:50 isocratic 0.15 ml $min^{-1}$; 10–20 min linear gradient 0.15 ml $min^{-1}$ from 50:50 to 100:0 and 21 min 0.2 ml $min^{-1}$. The column was equilibrated by applying 22–28 min linear gradient from 100:0 to 50:50 0.2 ml $min^{-1}$; 28–31 min 50:50 0.15 ml $min^{-1}$ and 31–40 min 50:50 0.15 ml $min^{-1}$.

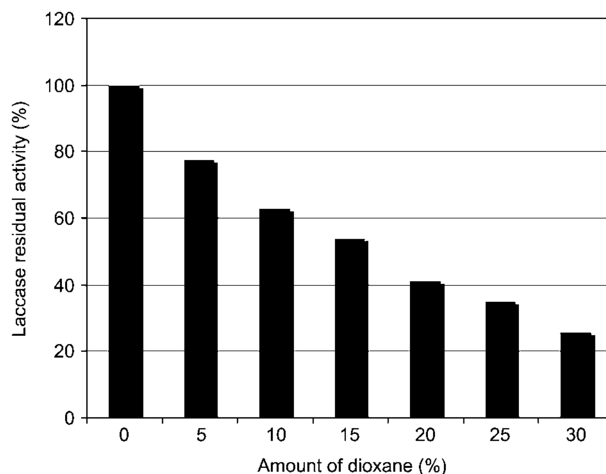


**Figure 2** *Myceliophthora thermophila* laccase activity in different water-to-dioxane ratio at pH 4.5 based on the oxidation of 2,6-dimethoxyphenol (DMP).

## Results and discussion

The ascomycete *Myceliophthora thermophila* laccase retains activity through a wide pH range (pH 4–9) and

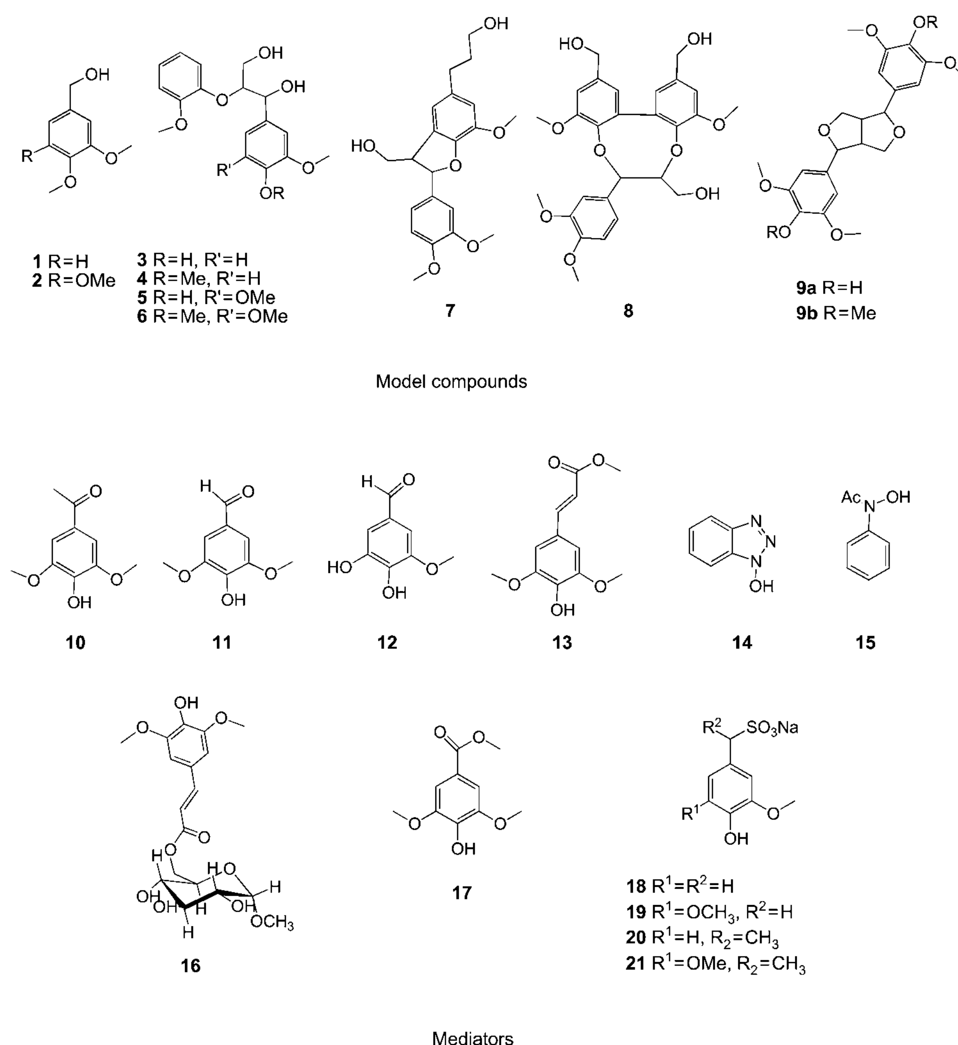


**Figure 1** The model compounds and the mediators used in oxidation systems: *1*, veratryl alcohol; *2*, 3,4,5-trimethoxybenzyl alcohol; *3–4*, phenolic (*3*) and methylated (*4*) guaiacylglycerol-β-guaiacyl ether dimers; *5–6*, phenolic (*5*) and methylated (*6*) syringylglycerol-β-guaiacyl ether dimers; *7*, methylated phenylcoumaran-type β-5 dimer (Me-PC); *8*, methylated dibenzodioxocin (Me-DBD); *9a*, syringaresinol (SR); and *9b*, methylated syringaresinol (Me-SR), *10*, acetosyringone (AS); *11*, syringaldehyde (SA); *12*, 5-hydroxyvanillin; *13*, methyl sinapate; *14*, 1-hydroxybenzotriazole (HBT); *15*, *N*-hydroxyacetanilide (NHA); *16*, methyl 6-*O*-sinapoyl-α-D-glucopyranoside (Sglp); *17*, methyl syringate (MeS); *18–21*, α-sulfonylated derivatives of vanillin (*18*), syringaldehyde (*19*), acetovanillone (*20*), and acetosyringone (*21*; AS-$SO_3$Na).

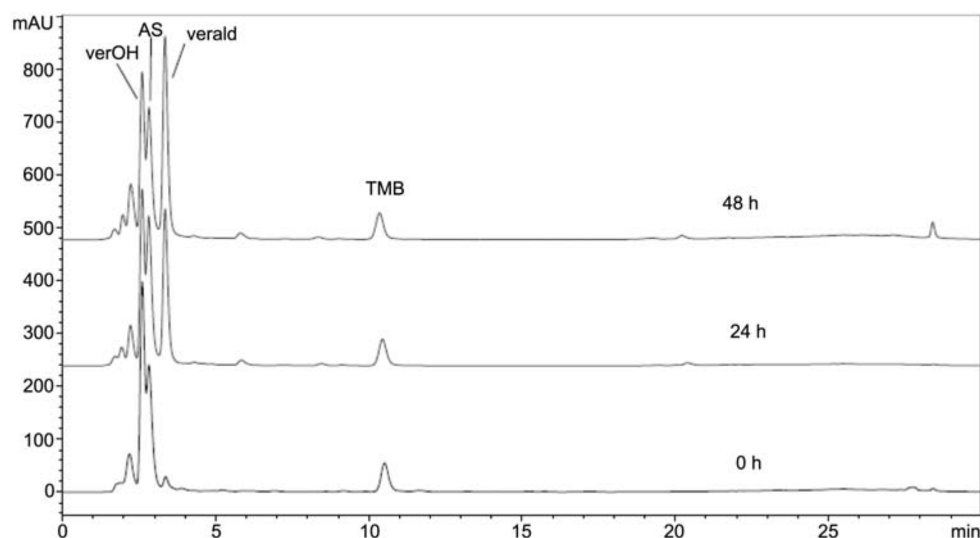


**Figure 3** HPLC analysis of products formed by *M. thermophila* laccase during the conversion of veratryl alcohol (verOH) with mediator acetosyringone (AS), within 0, 24, and 48 h. The quantification of the conversion products was made by external standards by following the conversion of veratryl alcohol (verOH) to veratraldehyde (verald). 1,3,5-Trimethoxybenzene (TMB) was used as an internal standard.

has notable thermostability (Xu et al. 1996). It is therefore an interesting enzyme for industrial applications, for example, in the pulp and paper technologies, when the target substrates have low redox potentials. The target substrates, lignin-type natural phenols in this study, are assayed as mediators.

To elucidate new mediating systems in oxidation of lignocellulosic materials with *M. thermophila* laccase, the oxidation system was studied first without a mediator. In the absence of the mediator, the laccase was not able to oxidize any of the non-phenolic model compounds. All experiments were performed at pH 6 which is close to the reported pH optimum for this laccase (Xu et al. 1996) and the reaction conditions were not optimized for each substrate in the reaction set-ups. To reveal the oxidation capacity, the same substrate concentration was used for each model compound and the substrate-to-mediator ratio was low (10:1) and/or high (1:1). In view of expected limitations for mediator loads in practical applications, higher substrate-to-mediator ratios were not studied.

In the first mediator screening, the assay of Reactive Black 5 decolorization showed the highest effect and decolorization rate for methyl syringate and acetosyringone, followed by syringaldehyde. Also, sinapic acid and methyl 6-*O*-sinapoyl-α-D-glucopyranoside showed some mediating capacity, but vanillin and 5-hydroxy analogs proved to be not suitable as effective mediators, as indicated by the low decolorization rate in comparison to the syringyl analogs.

The oxidation of veratryl alcohol (*1*) to veratraldehyde by the laccase from *M. thermophila* in aqueous dioxane (Table 1) was then followed by HPLC (Figure 3). We found that acetosyringone (*10*) and especially methyl syringate (*17*), which also acted as a good laccase substrate in the study by Kulys et al. (2002), showed marked ability to mediate the oxidation already at low mediator concentration levels. Interestingly, they seem to be even more effective as the commonly used synthetic mediators NHA and HBT at low mediator-to-substrate ratios.

The results clearly demonstrate that syringaldehyde and acetosyringone, but also NHA, disappeared during the oxidative reactions, as expected according to findings by Kawai et al. (1989). In the case of syringaldehyde (10:1) this took place already in 4 h, explaining the low mediating effect with this phenol. At high mediator level (1:1) it was able to mediate the reaction up to 5% conversion. It can be concluded that the observed stabilities for the natural mediators in our reaction system with *M. thermophila* laccase were methyl syringate ≫ acetosyringone > syringaldehyde.

The high redox potential synthetic mediator HBT was stable in our reaction conditions with the low redox laccase, but although possessing good mediating effect, the oxidation reaction was quite slow also at equimolar

**Table 1** Oxidation of veratryl alcohol (*1*) to veratraldehyde by *Myceliophthora thermophila* laccase in the presence of five phenolic mediators (*10*, *11*, *16*, *17*, and *21*) and two classical mediators (*14* and *15*) after 2 days in aqueous dioxane, using two different substrate-to-mediator ratios (10:1 and 1:1).

| Mediator codes (see Figure 1) | Ratio substrate/ mediator | Oxidation to veratraldehyde, % | Mediator oxidation[a] |
|---|---|---|---|
| AS (*10*) | 10:1 | 5% yield | + |
| AS (*10*) | 1:1 | 20% yield | + |
| SA (*11*) | 10:1 | (no reaction) | ++ |
| SA (*11*) | 1:1 | 5% yield | ++ |
| Sglp (*16*) | 1:1 | <5% (slow reaction) | + |
| Sglp (*16*) | 10:1 | (very slow reaction) | + |
| MeS (*17*) | 10:1 | 10% yield | - |
| MeS (*17*) | 1:1 | 25% yield | - |
| AS-$SO_3$Na (*21*) | 10:1 | (no reaction) | + |
| AS-$SO_3$Na (*21*) | 1:1 | 5% yield | + |
| HBT (*14*) | 10:1 | (very slow reaction) | - |
| HBT (*14*) | 1:1 | 20% yield | - |
| NHA (*15*) | 10:1 | (very slow reaction) | + |
| NHA (*15*) | 1:1 | 40% yield | + |

[a]Tentatively estimated from HPLC chromatograms.
For mediator abbreviations, see Figure 1.

1:1 conditions. On the other hand, NHA-laccase system oxidized model compounds faster and more effectively than other laccase-mediator systems in this study. Although NHA was gradually degraded in the reaction mixtures, it still worked for several days (up to 7 days in some instances).

To widen the substrate range, similar experiments were then performed with veratryl alcohol as oxidation target and syringaresinol (*9a*), syringylglycerol β-guaicyl ether (*5*) and monomeric 5-hydroxyvanillin (*12*) as mediators but now only at low mediator-to-substrate ratio. In the presence of syringylglycerol β-ether, the system produced slowly veratraldehyde and the β-ether concomitantly and slowly disappeared (data not shown). Syringaresinol, on the other hand, showed no mediating effect in the system, although it has earlier been demonstrated to mediate the *Pycnoporus cinnabarinus ss3*-laccase catalyzed oxidation of Reactive Black 5 (Camarero et al. 2008). In the case of 5-hydroxyvanillin (*12*) only a very rapid disappearance of the compound was observed and the veratryl alcohol was not oxidized. Also, lignosulfonate model compounds (*18–21*) were not particularly active as mediators. Only at high concentration, α-methyl syringyl sulfonate was able to induce the conversion veratryl alcohol to veratraldehyde (5%).

To investigate the reactivity of non-phenolic syringyl structures in the oxidation system, a similar set of experiments was performed with 3,4,5-trimethoxybenzyl alcohol (*2*), which is the syringyl analog to veratryl alcohol. This time, the experiments were performed only with acetosyringone (*10*), syringaresinol (*9a*), and syringylglycerol β-ether (*5*) as mediators. The dose of the mediator was uniformly low (1:10). Practically, similar results were obtained as in the case of veratryl alcohol. Again, acetosyringone showed marked activity, β-ether model was less reactive, and syringaresinol showed nearly no activity.

The oxidation of some dimeric non-phenolic model compounds representing different substructures of lignin were also tested at the same reaction conditions (Table 2).

Non-phenolic guaiacylglycerol β-guaiacyl ether adlerol (*4*) was oxidized in the 1:1 laccase-acetosyringone system and yielded the α-carbonyl derivative of guaiacylglycerol β-guaiacyl ether (adlerone) approximately to the same extent as in laccase-HBT system. Yet, NHA (*15*) proved to be the most effective mediator system with adlerol giving adlerone in 15% yield. This value is markedly lower than that (45%) obtained by Barreca et al. (2003) in high redox potential *Trametes villosa*-laccase/HBT system. The systems are, however, not comparable since the reaction conditions were completely different (for instance, the substrate-to-laccase ratio and the substrate concentration). It was also found that methylated phenylcoumaran (*7*) was not oxidized in any system studied; but interestingly, methylated dibenzodioxocin (*8*) showed astonishingly good reactivity in the acetosyringone-laccase system, although at the moment the reaction mechanism is not known. Various studies on isolated lignins have shown that dibenzodioxocin structure is most probably the main branching point in softwood lignin with comparatively high abundance (Heikkinen et al. 2003; Ralph et al. 2004). In this light, its high reactivity in our experimental system further increases the potential of the tested “laccase-natural mediator concept” in various applications, as already shown by Camarero et al. (2007). Methylated syringaresinol (*9b*), on the other hand, did not react at all with laccase-HBT 1:1 and laccase-AS 1:1 systems.

## Conclusions

The results demonstrate that syringyl-type compounds, especially acetosyringone and methyl syringate, are able

**Table 2** Oxidation of five dimeric/trimeric model compounds (*3*, *4*, *7*, *8*, and *9b*) by *Myceliophthora thermophila* laccase in the presence of two phenolic mediators (*10* and *17*) and two classical mediators (*14* and *15*) after 2 days in aqueous dioxane, using two different substrate-to-mediator ratios (10:1 and 1:1).

| Model compound (see Figure 1) | Mediator (see Figure 1) | Ratio model/ mediator | Characterization of the model oxidation, % | Mediator oxidation[a] |
|---|---|---|---|---|
| Adlerol (*4*) *e/t* mixture | AS (*10*) | 10:1 | (very slow reaction) | + |
| Adlerol (*4*) *e/t* mixture | MeS (*17*) | 10:1 | (very slow reaction) | - |
| Adlerol (*4*) *e/t* mixture | HBT (*14*) | 10:1 | (no reaction) | - |
| Adlerol (*4*) *e/t* mixture | AS (*10*) | 1:1 | <5% (slow reaction) | + |
| Adlerol (*4*) *e/t* mixture | MeS (*17*) | 1:1 | <5% (slow reaction) | ± |
| Adlerol (*4*) *e/t* mixture | HBT (*14*) | 1:1 | <5% (slow reaction) | - |
| Adlerol (*4*) *e/t* mixture | NHA (*15*) | 1:1 | 12% oxidized | + |
| Adlerol (*4*) *erythro* | AS (*10*) | 10:1 | (no reaction) | + |
| Adlerol (*4*) *erythro* | AS (*10*) | 1:1 | <5% (slow reaction) | + |
| Adlerol (*4*) *erythro* | MeS (*17*) | 1:1 | <5% (slow reaction) | ± |
| Adlerol (*4*) *erythro* | NHA (*15*) | 1:1 | 15% oxidized | + |
| Erol (*3*) | AS (*10*) | 10:1 | + | + |
| Erol (*3*) | AS (*10*) | 1:1 | + | + |
| Me-PC (*7*) | AS (*10*) | 1:1 | (no reaction) | + |
| Me-PC (*7*) | HBT (*14*) | 1:1 | (no reaction) | - |
| Me-SR (*9b*) | AS (*10*) | 10:1 | (no reaction) | + |
| Me-DBD (*8*) | AS (*10*) | 1:1 | 30% oxidized | + |

[a]Tentatively estimated from HPLC-chromatograms.
For model compound and mediator abbreviations, see Figure 1.

to mediate the oxidation of substrates of high oxidation potential by a laccase with low redox potential even in small quantities (1:10). Interestingly, the results suggest that such natural mediators are rather close in mediating property to those of commonly used synthetic mediators. This result opens an interesting field for further evaluation whether these syringyl-type phenolics could be of commercial value in degradation of lignocellulosic materials.

## Acknowledgements

EU contract NMP2-CT-2006-026456 (BIORENEW), and the Academy of Finland, contract 124160, are gratefully acknowledged for financial support of this work. The authors also wish to thank Mr. Jussi Kontro for technical assistance.

## References

Barreca, A.M, Fabbrini, M., Galli, C., Gentili, P., Ljunggren, S. (2003) Laccase-mediated oxidation of lignin model for improved delignification procedures. J. Mol. Cat. B: Enzym. 26:105–110.

Bourbonnais, R., Leech, D., Paice, M.G. (1998) Electrochemical analysis of the interactions of laccase mediators with lignin model compounds. Biochim. Biophys. Acta Gen. Subjects 1379:381–390.

Burton, S.G. (2003) Laccases and phenol oxidases in organic synthesis – a review. Curr. Org. Chem. 7:1317–1331.

Camarero, S., Ibarra, D., Martínez, M.J., Martínez, A.T. (2005) Lignin-derived compounds as efficient laccase mediators for decolorization of different types of recalcitrant dyes. Appl. Environ. Microbiol. 71:1775–1784.

Camarero, S., Ibarra, D., Martínez, A.T., Romero, J., Gutierrez, A., del Rio, J.C. (2007) Paper pulp delignification using laccase and natural mediators. Enzyme Microbial Technol. 49:1264–1271.

Camarero, S., CaÑas, A.I., Nousiainen, P., Record, E., Lomascolo, A., Martínez, M.J., et al. (2008) *p*-Hydroxycinnamic acid as natural mediators for laccase oxidation recalcitrant compounds. Environ. Sci. Technol. 42:6703–6709.

Cantarella, G., d'Acunzo, F., Galli, G. (2003a) Determination of laccase activity in mixed solvents: comparison between two chromogens in a spectrometric assay. Biotechnol. Bioeng. 82:395–398.

Cantarella, G., Galli, G., Gentili, P. (2003b) Free radical versus electron-transfer routes of oxidation of hydrocarbons by laccase/mediator systems: catalytic or stoichiometric procedures. J. Mol. Cat. B: Enzym. 22:135–144.

Cho, N.-S., Shjin, W., Jeong, S.-W., Leonowicz, A. (2004) Delignification of lignosulfonate by fungal laccase with low molecular mediators. Bull. Korean Chem. Soc. 25:1551–1554.

d'Acunzo, F, Galli, C., Gentili, P., Sergi, F. (2006) Mechanistic and steric issues in the oxidation of phenolic and non-phenolic compounds by laccase or laccase-mediator systems. The case of bifunctional substrates. New J. Chem. 30:583–591.

Dupé, E., Shareck, F., Hurtubise, Y., Beauregard, M., Daneault, C. (2008) Decolourization of recalcitrant dyes with a laccase from *Streptomyces coelicolor* under alkaline conditions. J. Ind. Microbiol. Biotechnol. 35:1123–1129.

Eggert, C., Temp, U., Eriksson, K.-E.L. (1996) The ligninolytic system of the white rot fungus *Pycnoporus cinnabarinus*: purification and characterization of the laccase. Appl. Environ. Microbiol. 62:1151–1158.

Ellis, J.E., Lenger, S.R. (1998) A convenient synthesis of 3,4-dimethoxy-5-hydroxybenzaldehyde. Synth. Comm. 28:1517–1524.

Galli, C., Gentili, P. (2004) Chemical messengers: mediated oxidations with the enzyme laccase. J. Phys. Org. Chem. 17: 973–977.

Glennie, D.W. (1966) Chemical structure of lignin sulfonates III. Some reactions of monomeric sulfonates. Tappi 49:237–243.

Gutiérrez, A., del Río, J.C., Ibarra, D., Rencornet, J., Romero, J., Speranza, M., et al. (2007) Enzymatic removal of free and conjugated sterols forming pitch deposits in environmentally sound bleaching of eucalypt paper pulp. Environ. Sci. Technol. 40:3416–3422.

Heikkinen, S., Toikka, M.M., Karhunen, P.T., Kilpeläinen, I.A. (2003). Quantitative 2D HSQC (Q-HSQC) via suppression of *J*-dependence of polarization transfer in NMR spectroscopy: application to wood lignin. J. Am. Chem. Soc. 125:4362–4367.

Jaouani, A., Guillen, F., Pennickx, M.J., Martinez, A.T., Martinez, M.J. (2005) Role of *Pycnoporus coccineus* laccase in the degradation of aromatic compounds on the olive oil mill wastewater. Enzyme Microb. Technol. 36:478–486.

Johannes, C., Majcherczyk, A. (2000) Laccase activity tests and laccase inhibitors. J. Biotechnol. 78:193–199.

Kawai, S., Umezawa, T., Higuchi, T. (1989) Oxidation of methoxylated benzyl alcohols by laccase of *Coriolus versicolor* in the presence of syringaldehyde. Wood Res. 76: 10–16.

Kirk, T.K., Farrell, R.L. (1987) Enzymatic "combustion": the microbial degradation of lignin. Annu. Rev. Microbiol. 41: 465–505.

Kulys, J., Krikstopaitis, K., Ziemys, A., Schneider, P. (2002) Laccase catalyzed oxidation of syringates in presence of albumins. J. Mol. Cat. B 18:99–108.

Kylli, P., Nousiainen, P., Biely, P., Sipilä, J., Tenkanen, M., Heinonen, M. (2008) Antioxidant potential of hydroxycinnamic acid glycoside esters. J. Agric. Food Chem. 56:4797–4805.

Martínez, A.T., Speranza, M., Ruiz-Dueñas, F.J., Ferreira, P., Camarero, S., Guillén, F., et al. (2005) Biodegradation of lignocellulosics: microbiological, chemical and enzymatic aspects of fungal attack to lignin. Int. Microbiol. 8:195–204.

Mayer, A.M., Staples, R.C. (2002) Laccase: new functions for an old enzyme. Phytochemistry 60:551–565.

Morozova, O.V., Shumakovich, G.P., Shleev, S.V., Yaropolov, Y.I. (2007) Laccase-mediator systems and their applications: a review. Appl. Biochem. Microbiol. 43:523–535.

Nakatsubo, F., Sato, K., Higuchi, T. (1975) Synthesis of guaiacylglycerol-β-guaiacyl ether. Holzforschung 29:165–168.

Oxley, P.W., Adger, B.M., Sasse, M.J., Forth, M.A. (1989) N-Acetyl-N-phenylhydroxylamine via catalytic transfer hydrogenation of nitrobenzene using hydrazine and rhodium on carbon. Org. Synth. 67:187–192.

Ralph, J., Lundquist, K., Brunow, G., Lu, F., Kim, H., Schatz, P.F., et al. (2004) Lignins: natural polymers from oxidative coupling of 4-hydroxyphenylpropanoids. Phytochem. Rev. 3:29–60.

Widsten, P., Kandelbauer, A. (2008) Laccase applications in the forest products industry: a review. Enzyme Microbial Technol. 42:293–307.

Xu, F. (1996) Oxidation of phenols, anilines, and benzenethiols by fungal laccases: correlation between activity and redox potentials as well as halide inhibition. Biochemistry 35:7608–7614.

Xu, F. (2005) Applications of oxidoreductases: recent progress. Ind. Biotechnol. 1:38–50.

Xu, F., Shin, W., Brown, S.H., Wahleithner, J.A., Sundaram, U.M., Solomon, E.I. (1996) A study of a series of recombinant fungal laccases and bilirubin oxidase that exhibit significant differences in redox potential, substrate, specificity, and stability. Biochim. Biophys. Acta 1292:303–311.

Received December 15, 2008. Accepted February 3, 2009.
Previously published online July 31, 2009.