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Laccase-mediator catalyzed conversion of model lignin compounds[☆]

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ABSTRACT

Laccases play an important role in the biological break down of lignin and have great potential in the deconstruction of lignocellulosic feedstocks. We examined a variety of laccases, both commercially prepared and crude extracts, for their ability to oxidize three model lignol compounds (*p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol). We identified both mediated and non-mediated laccase-catalyzed reactions that converted *p*-coumaryl alcohol and sinapyl alcohol to *p*-hydroxybenzaldehyde and 2,6-dimethoxybenzoquinone, respectively. Interestingly, the products produced by the concerted action of the laccase mediator system on lignol substrates are the same as those produced by chemical catalytic approaches. The enzymatic approach affords the opportunity for a biological approach to the conversion of lignin to valuable specialty chemicals that have use in a variety of industrial, consumer and pharmaceutical applications.

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1. Introduction

A biorefinery integrates unit operations to convert biomass into a variety of biobased products, including fuels, chemicals, energy, and feed. Government policy initiatives over the last decade have emphasized the production of biobased fuels, and consequently the number of dry-grind ethanol biorefineries has increased significantly. Historically, fuel ethanol biorefineries have produced only two primary products – ethanol and distillers grains. More recently, corn oil and carbon dioxide provided additional revenue streams. However, the growth and sustainability of the biorefining industry requires the development of more value-added products from abundant agricultural feedstocks, including lignocellulosics.

The modern ethanol dry-grind biorefinery generally produces two primary products – ethanol and distillers grains. Well-established conventional corn-to-ethanol plants have struggled with fluctuations in the price of corn and stagnant demand for transportation fuels, while the cellulosic biofuel industry remains in its infancy. Current gasoline blending rates restrict the growth of the

ethanol sector, and although distillers grains are a significant revenue stream to commercial ethanol plants, their value is generally limited to animal feed. To improve the economic viability of the biorefining industry, there is a need for novel products from renewable biomass-based feedstocks.

Combinatorial biocatalysis uses multiple catalysts and iterations to create focused libraries of complex natural products or synthetic compounds (Michels et al., 1998; Altreuter and Clark, 1999; Rich et al., 2002, 2009). This approach uses biocatalysts that have evolved for the synthesis of complex natural products and exploits the broad array of chemistries required for the production and degradation of organic biomolecules that occur under relatively mild conditions. Some of these reactions (e.g., oxidations, esterifications) are difficult to selectively reproduce using purely chemical means.

Fungal laccases used with oxidizable low-molecular-weight compounds (mediators) are able to oxidize some nonsubstrate compounds (Bourbonnais and Paice, 1990; Johannes and Majcherczyk, 2000; Riva, 2006; Morozova et al., 2007). While this laccase-mediator system (LMS) has received widespread attention for bleaching kraft pulp (Bourbonnais et al., 1997), these laccase-mediator systems can be applied to the oxidation of various compounds. Redox mediators accelerate the reaction rate by shuttling electrons from the biological oxidation of primary electron donors to the electron-accepting organic compound (Husain and Husain, 2008), and their mechanism of action has been the subject of significant research (Crestini et al., 2003; Wong, 2009).

Biocatalytic oxidative chemistries provide opportunities to

Abbreviations: LMS, laccase mediator system; pCA, *p*-coniferyl alcohol; 4HBA, *p*-hydroxybenzaldehyde; SA, sinapyl alcohol; DMQ, 2,6-dimethoxybenzoquinone

[☆]Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable. USDA is an equal opportunity provider and employer.

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selectively introduce new functionality into a structurally complex natural product (Rich et al., 2009). Using combinations of dozens of mediators with 10–20 commercially available laccase enzymes will provide hundreds of combinations that will be efficiently handled using automated synthetic and analytical methods (Larson et al., 2013). This approach will identify reaction systems, including biocatalyst and mediating substrate, that are efficient for introducing new functional moieties into structurally complex natural products, such as lignin.

2. Materials and methods

2.1. Chemicals

TEMPO free radical (TEMPO) was obtained from Aldrich (Steinheim, Germany), 5-aminosalicylic acid (ASA) from TCI America (Portland, OR), and 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) from Fluka (Switzerland). Acetonitrile and ethyl acetate were from EMD Chemicals (Darmstadt, Germany). *p*-Coumaryl alcohol, MS-2010 synthesized by Bionet Screening Compounds/Key Organics, was obtained through Ryan Scientific, Inc. (Mount Pleasant, SC). Coniferyl alcohol was from Alfa Aesar (Ward Hill, MA), sinapyl alcohol and 4-hydroxybenzaldehyde were from Sigma-Aldrich (St. Louis, MO), and 2,6-dimethoxybenzoquinone was from TCI America (Portland, OR).

2.2. Enzymes

Enzymes used in screening the lignols were previously described (Larson et al., 2013), including laccase C from ASA Spezialenzyme GmbH (Wolfenbüttel, Germany), laccase from *Trametes versicolor* from Fluka 38429 (Sigma-Aldrich) and laccase from *Pycnoporus cinnabarinus* (ATCC 200478). Laccase from *Aureobasidium pullulans* (NRRL 50381) was also prepared as previously described (Rich et al., 2013).

2.3. Screening of model compounds

Screening of laccase enzymes with each substrate was performed in a manner similar to our previous report (Larson et al., 2013), with reaction progress monitored by RP-HPLC equipped with PDA. As described below, pCA and sinapyl alcohol were screened with a limited number of enzyme–mediator pairs and enzymes without mediators, respectively. The initial testing of enzyme–mediator pairs and the analytical conditions immediately revealed successful reaction conditions for converting pCA and SA into new compounds. This eliminated the need for a larger screening effort with these substrates. Coniferyl alcohol was screened against the full enzyme and mediator libraries as previously described (Larson et al., 2013).

2.4. Synthesis of *p*-hydroxybenzaldehyde

A 12.5 ml reaction was performed using 9.4 mg *p*-coumaryl alcohol (5 mM), 4.55 mM ABTS, and 33.25 µg (4.62 U) laccase from *P. cinnabarinus* ATCC 200478 in McIlvaine buffer at pH 4.4 and 20% tert-butanol. The reaction was incubated 28 °C, 200 rpm with 1 in diameter rotation overnight and extracted three times with ethyl acetate. The organic phase was pooled and dried under a stream of N₂. The dried extract was re-suspended in 50% acetonitrile, loaded onto a silica 4 g column (RediSep Rf Gold), and dried under vacuum manifold for 1 h. The silica 4 g preparative column containing the dried sample was placed on top of a second silica 4 g column with the Teledyne 1800 Combi Flash Rf System and eluted

with a gradient of ethyl acetate in hexane. The fractions were run on HPLC as described previously and fractions containing the product were pooled and dried under vacuum to give 3.2 mg (34% isolated yield) of 4HBA as a pale peach powder. The isolated product was identical by GC–MS and ¹H NMR to the standard 4HBA (obtained from Sigma-Aldrich).

2.5. Synthesis of 2,6-dimethoxybenzoquinone

A 10 ml volume reaction was performed in a 25 ml Erlenmeyer flask with stopper using 10.5 mg sinapyl alcohol, 10% acetone, 10% acetonitrile, and 26.6 µg (0.139 U) laccase from *P. cinnabarinus* ATCC 200478 in McIlvaine buffer pH 4.4. The flask was incubated 28 °C, 200 rpm with 1 in diameter rotation overnight. After overnight incubation, the reaction was extracted 3 times with 15 ml ethyl acetate and transferred the organic phase to a vial and subsequently dried under a N₂ stream. The dried extract was re-suspended in 50% acetonitrile, loaded onto a silica 4 g column (RediSep Rf Gold), and dried under vacuum manifold for 1 h. The silica 4 g preparative column containing the dried sample was placed on top of a second silica 4 g column with the Teledyne 1800 Combi Flash Rf System and eluted with a gradient of ethyl acetate in hexane. The fractions were run on HPLC as described previously and fractions containing the product were pooled and dried under vacuum to give 1.5 mg (14% isolated yield) of DMQ as a yellow powder. The isolated product was identical by GC–MS and ¹H NMR to the standard DMQ (obtained from TCI America).

2.6. HPLC analysis

Reaction products were analyzed by HPLC (LC Prominence with a photodiode array detector, Shimadzu, Kyoto, Japan) using an Intersil ODS-3 5 µm column (4.6 × 250 mm). Separation was obtained in gradient mode from 10% to 80% aqueous acetonitrile with 0.025% trifluoroacetic acid at a flow rate of 1 ml/min. The quantitative analysis was performed by monitoring the UV absorption at 280 nm; and absorption spectra within the range of 200–800 nm were collected. Standard curves were prepared for *p*-coumaryl alcohol and 4-hydroxybenzaldehyde within the appropriate concentration range (five-point standard curve) and quantitative analysis performed using LC Solution (Shimadzu, Kyoto, Japan) and Microsoft Excel.

2.7. GC–MS analysis

GC–MS analysis was performed on an Agilent Technologies GC–MS (7890A GC System with 5975C inert MSD with Triple Axis Detector) with a HP 5MS (Agilent Technology) column (cross-linked 5% diphenyl and 95% dimethyl polysiloxane; 30 m × 0.25 mm i.d., film thickness 0.25 µm). Helium was used as carrier gas (60.7 kPa) at a flow rate of 20 ml/min. The samples were injected at 200 °C. The initial oven temperature was 70 °C and was held for 1 min, then raised at a rate of 20 °C/min to 270 °C and held for 1 min. The mass range *m/z* 50–550 was utilized and the data analyzed with MSD ChemStation (ver. E.02.00.493).

3. Results/discussion

Lignin is a major structural component of woody plants, and one of the most abundant organic compounds on earth (Lora and Glasser, 2002). At present, however, it has limited commercial value. In contrast to the readily hydrolysable bonds between subunits of other wood polymers (e.g., cellulose and hemicellulose), lignin degradation requires oxidative attack on the carbon–carbon and ether inter-unit bonds (Martínez et al., 2005).

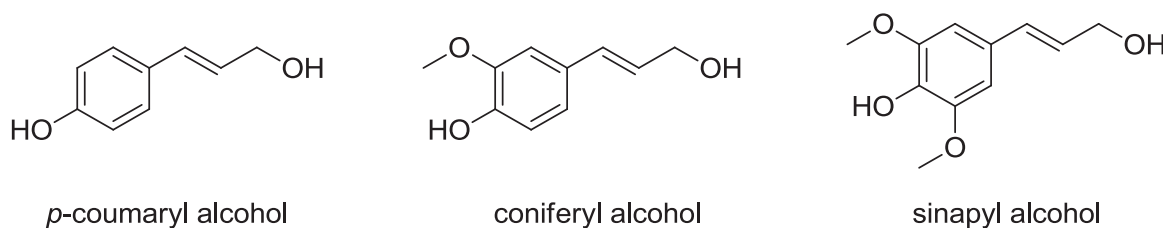


Fig. 1. Lignin model substrates for evaluation of the utility of laccase-mediator combinations for synthetically useful oxidations.

Extracellular peroxidases and oxidases are thought to play an important role in the initial depolymerization of lignin (Martínez et al., 2005).

The formation of monomeric phenolic compounds during various processes will be a direct result of lignin degradation (Martínez et al., 2005). Generally, three main aromatic constituents form the major part of the lignin with differing substitutions of the 3- and 5-positions of the aromatic nucleus. In order to improve the economics of ethanol production from lignocellulosic feedstocks, it will be necessary to identify valuable coproducts from lignin – the primary residual by-product. Currently, the commercial use for lignin is to burn for heating value.

We have previously shown that it is important to screen a wide-range of enzyme-mediator pairs (Larson et al., 2013), as predicting activity of the laccase mediator system with a given substrate has been elusive. We screened three model lignin substrates, the common lignols *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol (Fig. 1), to evaluate the utility of laccase-mediator combinations for synthetically useful oxidations. Enzymes were paired with mediators in a combinatorial fashion and reactions initiated by the addition of model lignin substrates. Samples were periodically withdrawn and products were determined by rapid LC/MS methods to determine the conversion and identify the type of transformation (Cotterill and Rich, 2005; Cotterill et al., 2008).

Initial evaluation of selected enzyme and mediator combinations with *p*-coumaryl alcohol as substrate quickly revealed two potential products by HPLC (Table 1). One of these products resulted from the direct action of the laccase on the substrate (non-mediated) and the other was produced by selected enzyme-mediator pairs. The product distribution for the selected enzyme-mediator pairs is described in Table 1. Employing ABTS as the mediator minimized the production of the non-mediated product (Fig. 2), while TEMPO and ASA tended to show greater levels of the non-mediated product

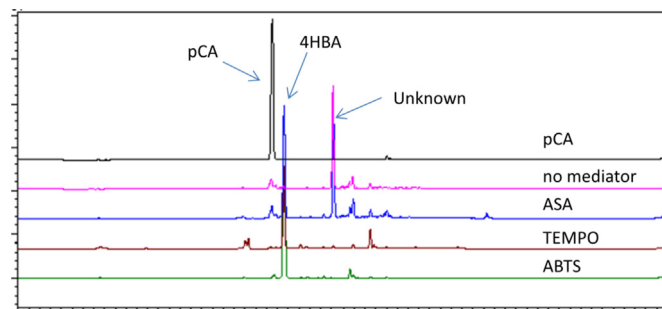


Fig. 2. HPLC chromatograms showing the product distribution of the laccase-mediated conversion of *p*-coumaryl alcohol. Abbreviations: *p*-coumaryl alcohol (pCA); 5-aminosalicylic acid (ASA); 2,2,6,6-tetramethylpiperidinyloxy free radical (TEMPO); 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS); *p*-hydroxybenzaldehyde (4HBA).

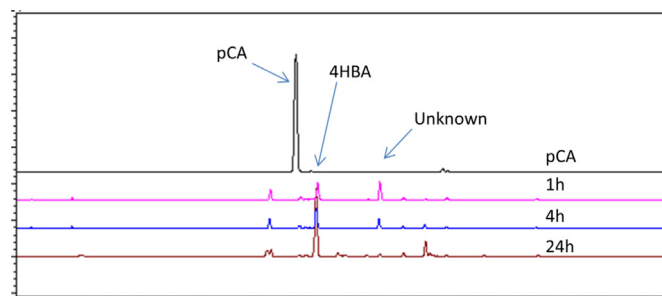


Fig. 3. Time course of *p*-coumaryl alcohol conversion with *P. cinnabarinus* laccase with ABTS as mediator. There is the initial appearance of an unknown product with the subsequent production of *p*-hydroxybenzaldehyde and disappearance of the unknown product. Abbreviations: *p*-coumaryl alcohol (pCA); *p*-hydroxybenzaldehyde (4HBA).

Table 1
Product distribution in the laccase-mediated conversion of *p*-coumaryl alcohol.

Enzyme	Source organism	Mediator	Compound remaining, %		
			pCA	4HBA	Unknown
Laccase C	<i>Trametes</i> sp.	ASA	8.0	51.9	40.1
		ABTS	2.2	94.2	3.6
		TEMPO	3.0	92.5	4.5
		None	13.8	0.0	86.2
38429	<i>T. versicolor</i>	ASA	1.8	60.9	37.3
		ABTS	28.1	70.9	1.0
		TEMPO	ND	ND	ND
		None	21.6	0.0	78.4
200478	<i>Pycnoporus cinnabarinus</i>	ASA	9.7	51.4	38.9
		ABTS	25.7	74.3	0.0
		TEMPO	6.2	76.4	17.4
		None	17.3	0.0	82.7
50381	<i>Aureobasidium pullulans</i>	ASA	62.9	34.6	2.5
		ABTS	36.2	61.7	2.1
		TEMPO	35.0	45.2	19.8
		None	51.6	0.0	48.4

ND – not determined.

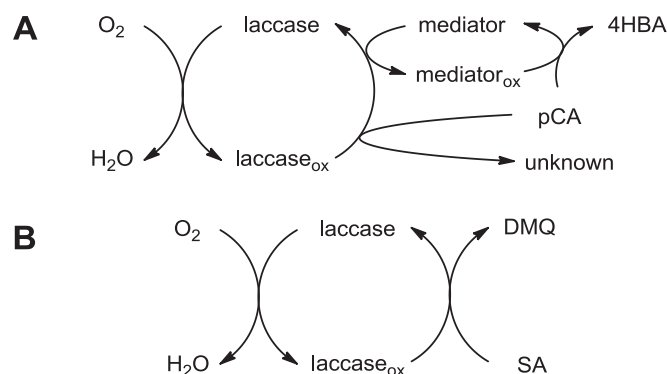


Fig. 4. Reaction mechanism for the laccase-catalyzed conversion of *p*-coumaryl alcohol (A) and sinapyl alcohol (B) to *p*-hydroxybenzaldehyde and 2,6-dimethoxy-1,4-benzoquinone, respectively.

(Table 1). Indeed, the ABTS mediated reaction catalyzed by laccase from *P. cinnabarinus* did not produce the non-mediated product up to 24 h. The non-mediated product appeared to be transient in the TEMPO-mediated reactions, as the time course shows the initial

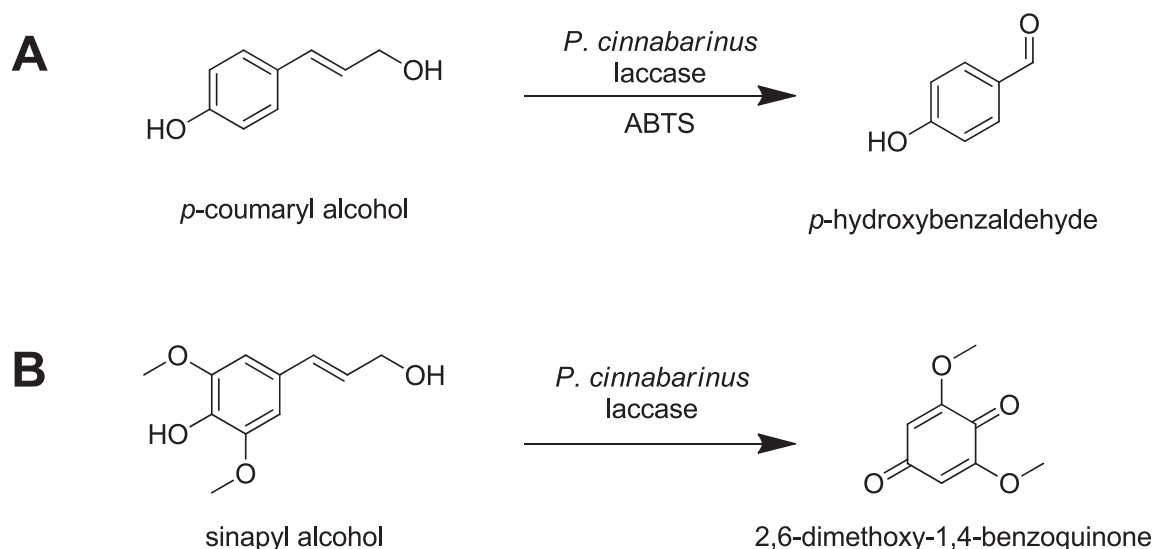


Fig. 5. Reaction scheme for the conversion of model lignols *p*-coumaryl alcohol (A) and sinapyl alcohol (B) to *p*-hydroxybenzaldehyde and 2,6-dimethoxy-1,4-benzoquinone, respectively.

appearance followed by the subsequent disappearance of this product (Fig. 3). While this could suggest that the unknown product is an intermediate in the route to 4HBA, the concomitant appearance of the 4HBA with the unknown product (data not shown) and the observation that *P. cinnabarinus* produced 4HBA in the absence of the unknown product indicate that these two products are likely formed by two parallel reaction pathways (Fig. 4A). A second unknown enzyme-catalyzed product does appear in longer incubations (Fig. 3, 4 h and 24 h timepoints), including in the no-mediator control; however, the synthesis of this unknown compound appears to be minimized in the reaction with ABTS as the mediator (Fig. 2). It is possible that these unknown products result from dimerization/oligomerization of the starting material in a fashion well known in laccase catalysis (Eriksson et al., 1990; Eggert et al., 1996; Sun et al., 2013). While it is difficult to ascertain the exact reaction mechanism, it is clear that the LMS is a useful catalyst for the synthesis of products from these lignin model compounds.

Ultimately, the mediated product was identified as *p*-hydroxybenzaldehyde (Fig. 5A) by GC–MS (comparison with authentic standard). We then synthesized *p*-hydroxybenzaldehyde using the *P. cinnabarinus* laccase and ABTS as the mediator and the product structure was confirmed by NMR. To date, the structures of the non-mediated products have not been established.

Initial screening of laccase enzymes with sinapyl alcohol as a substrate revealed a new product was formed by all the enzymes in the absence of any mediator (Fig. 4B). Furthermore, employing mediators did not enhance the production of this product. The product was synthesized using the *P. cinnabarinus* enzyme without any mediator. The isolated product was tentatively identified as 2,6-dimethoxybenzoquinone (Fig. 5B) using an Agilent GC–MS equipped with MSD ChemStation and confirmed with NMR and comparison with a commercially obtained standard.

Chemical conversion of lignin-related model compounds has also generated DMQ (Biannic and Bozell, 2013). Several novel Co-Schiff base complexes catalyzed the oxidative cleavage of monomeric and dimeric lignin model compounds. A syringyl subunit model was converted to DMQ in up to 82% isolated yield and *N*-benzyl catalysts were developed that were able to convert both syringyl and guaiacyl models to benzoquinones in moderate yields. Initial tests with one of these Co-catalysts was able to catalyze the oxidative conversion of organosolv lignin from poplar in MeOH/DMSO and resulted in a mixture of products that included benzoquinones and benzaldehydes.

Cu-doped Co-based catalysts have been demonstrated to convert steam exploded lignin from cornstalk to aromatic aldehydes (Deng et al., 2010). *p*-Hydroxybenzaldehyde, vanillin and syringaldehyde were produced from the H, G, and S subunits of lignin. However, the conversion to these products was < 3%, 5% and up to 13%, respectively using a variety of related catalysts.

A more comprehensive screen of enzymes and mediators (Larson et al., 2013) against coniferyl alcohol did not reveal any useful products. While coniferyl alcohol was consumed in many reactions, there were no products observed by HPLC or GC–MS. Many of these reactions did turn cloudy with a peach-colored insoluble material that was removed during centrifugation. This could suggest that coniferyl alcohol may have been polymerized, similar to reports with other enzymes, including horseradish peroxidase (Fournand et al., 2003) and other laccases (Eriksson et al., 1990; Eggert et al., 1996); possibly through the polymerization of a methoxy radical produced similar to compounds observed in the biodegradation of lignocellulosics (Martínez et al., 2005).

In summary, the laccase mediator system catalyzed the conversion of *p*-coumaryl alcohol to *p*-hydroxybenzaldehyde. The laccase also converted the starting material to an unidentified product that disappeared over time. Sinapyl alcohol was efficiently converted to 2,6-dimethoxybenzoquinone by *P. cinnabarinus* laccase. Interestingly, the products produced by the concerted action of the LMS on lignol substrates are the same as those produced by chemical catalytic approaches. The enzymatic approach affords the opportunity for a biological approach to the conversion of lignin to valuable specialty chemicals that have use in a variety of industrial, consumer and pharmaceutical applications.

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