Biocatalysis and Agricultural Biotechnology 5 (2016) 111–115

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

journal homepage: www.elsevier.com/locate/bab

Laccase-mediator catalyzed conversion of model lignin compounds ☆

Joseph O. Rich [a,*], Amber M. Anderson [a], Mark A. Berhow [b]

[a] Renewable Products Technology Research, US Department of Agriculture, Agricultural Research Service, National Center for Agricultural Utilization Research, 1815 N. University Street, Peoria, IL 61604, USA

[b] Functional Foods Research, US Department of Agriculture, Agricultural Research Service, National Center for Agricultural Utilization Research, 1815 N. University Street, Peoria, IL 61604, USA

ARTICLE INFO

Article history:
Received 15 October 2015
Received in revised form
2 December 2015
Accepted 3 January 2016
Available online 7 January 2016

Keywords:
2,6-Dimethoxybenzoquinone
p-Hydroxybenzaldehyde
Laccase
Mediator
Lignin bioconversion

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.

Published by Elsevier Ltd.

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*-hydroxybenzyaldehyde; 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.

* Corresponding author.
E-mail address: joseph.rich@ars.usda.gov (J.O. Rich).

http://dx.doi.org/10.1016/j.bcab.2016.01.001
1878-8181/Published by Elsevier Ltd.

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 *Pycnoporous 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 presiously 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 N_2. 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 ^{1}H 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 N_2 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 ^{1}H 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 Intertsil 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).