Influence of mediators on laccase catalyzed radical formation in lignin

Laccases (EC 1.10.3.2) catalyze oxidation of phenolic groups in lignin to phenoxyl radicals during reduction of O₂ to H₂O. Here, we examine the influence on this radical formation of mediators which are presumed to act by shuttling electrons between the laccase and the subunits in lignin that the enzyme cannot approach directly. Treatments of three different lignins with laccase-mediator-systems (LMS) including laccases derived from *Trametes versicolor* and *Myceliophthora thermophila*, respectively, and four individual mediators, 1-hydroxybenzotriazole (HBT), N-hydroxyphthalimide (HPI), 2,2,6,6-tetramethylpiperidin-1-yloxy (TEMPO), and 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) were assessed by real time electron paramagnetic resonance measurements. Radical steady state concentrations and radical formation rates were quantified. LMS treatments with 500 μM N-OH type mediators (HPI or HBT) did not affect the lignin radical formation, but increased doses of those mediators (5 mM) surprisingly led to significantly decreased radical formation rates and lowered steady state radical concentrations. Laccase-TEMPO treatment at a 5 mM mediator dose was the only system that significantly increased steady state radical concentration and rate of radical formation in beech organosolv lignin. The data suggest that electron shuttling by mediators is not a significant general mechanism for enhancing laccase catalyzed oxidation of biorefinery lignin substrates, and the results thus provide a new view on laccase catalyzed lignin modification.
Direct rate assessment of laccase catalysed radical formation in lignin by electron paramagnetic resonance spectroscopy

Laccases (EC 1.10.3.2) catalyse removal of an electron and a proton from phenolic hydroxyl groups, including phenolic hydroxyls in lignins, to form phenoxy radicals during reduction of O₂. We employed electron paramagnetic resonance spectroscopy (EPR) for real time measurement of such catalytic radical formation activity on three types of lignin (two types of organosolv lignin, and a lignin rich residue from wheat straw hydrolysis) brought about by two different fungal laccases, derived from Trametes versicolor (Tv) and Myceliophthora thermophila (Mt), respectively. Laccase addition to suspensions of the individual lignin samples produced immediate time and enzyme dose dependent increases in intensity in the EPR signal with g-values in the range 2.0047–2.0050 allowing a direct quantitative monitoring of the radical formation and thus allowed laccase enzyme kinetics assessment on lignin. The experimental data verified that the laccases acted upon the insoluble lignin substrates in the suspensions. When the action on the lignin substrates of the two laccases were compared on equal enzyme dosage levels (by activity units on syringaldazine) the Mt laccase exerted a
significantly faster radical formation than the Tv laccase on all three types of lignin substrates. When comparing the equal laccase dose rates on the three lignin substrates the enzymatic radical formation rate on the wheat straw lignin residue was consistently higher than those of the organosolv lignins. The pH-temperature optimum for the radical formation rate in organosolv lignin was determined by response surface methodology to pH 4.8, 33 °C and pH 5.8, 33 °C for the Tv laccase and the Mt laccase, respectively. The results verify direct radical formation action of fungal laccases on lignin without addition of mediators and the EPR methodology provides a new type of enzyme assay of laccases on lignin.

General information
State: Published
Organisations: Center for BioProcess Engineering, Department of Chemical and Biochemical Engineering, University of Copenhagen
Authors: Munk, L. (Intern), Andersen, M. L. (Ekstern), Meyer, A. S. (Intern)
Pages: 88-96
Publication date: 2017
Main Research Area: Technical/natural sciences

Publication information
Journal: Enzyme and Microbial Technology
Volume: 106
ISSN (Print): 0141-0229
Ratings:
BFI (2018): BFI-level 1
Web of Science (2018): Indexed yes
BFI (2017): BFI-level 1
Scopus rating (2017): SJR 0.754 SNIP 0.944
Web of Science (2017): Indexed yes
BFI (2016): BFI-level 1
Scopus rating (2016): CiteScore 2.83 SJR 0.774 SNIP 1.028
Web of Science (2016): Indexed yes
BFI (2015): BFI-level 1
Scopus rating (2015): SJR 0.846 SNIP 0.95 CiteScore 2.63
Web of Science (2015): Indexed yes
BFI (2014): BFI-level 1
Scopus rating (2014): SJR 1.063 SNIP 1.212 CiteScore 3.12
Web of Science (2014): Indexed yes
BFI (2013): BFI-level 2
Scopus rating (2013): SJR 1.17 SNIP 1.377 CiteScore 3.2
Web of Science (2013): Indexed yes
BFI (2012): BFI-level 2
Scopus rating (2012): SJR 1.166 SNIP 1.27 CiteScore 2.78
ISI indexed (2012): ISI indexed yes
Web of Science (2012): Indexed yes
BFI (2011): BFI-level 2
Scopus rating (2011): SJR 1.057 SNIP 1.262 CiteScore 2.74
ISI indexed (2011): ISI indexed yes
Web of Science (2011): Indexed yes
BFI (2010): BFI-level 2
Scopus rating (2010): SJR 1.207 SNIP 1.559
Web of Science (2010): Indexed yes
BFI (2009): BFI-level 2
Scopus rating (2009): SJR 1.304 SNIP 1.504
Web of Science (2009): Indexed yes
BFI (2008): BFI-level 1
Scopus rating (2008): SJR 1.214 SNIP 1.35
Web of Science (2008): Indexed yes
Scopus rating (2007): SJR 0.937 SNIP 1.259
Laccase catalyzed grafting of-N-OH type mediators to lignin via radical-radical coupling

Lignin is an underexploited resource in biomass refining. Laccases (EC 1.10.3.2) catalyze oxidation of phenolic hydroxyls using O2 as electron acceptor and may facilitate lignin modification in the presence of mediators. This study assessed the reactivity of four different synthetic mediators by laccases from Trametes versicolor and Pleurotus ostreatus by quantitative analysis of the reaction outcome by pyrolysis gas chromatography mass spectroscopy. The two laccases were equally efficient in catalyzing grafting, but only-N-OH type mediators grafted. HPI (N-hydroxyacetanilide) grafted 7-10 times better than HBT (1-hydroxybenzotriazole). Three different mechanisms are suggested to explain the grafting of HPI and HBT, all involving radical-radical coupling to produce covalent bonding to lignin. Lignin from exhaustive cellulase treatment of wheat straw was more susceptible to grafting than beech organosolv lignin with the relative abundance of grafting being 35% vs. 11% for HPI and 5% vs. 1% for HBT on these lignin substrates. The data imply that lignin can be functionalized via laccase catalysis with-N-OH type mediators.
A new laccase gene (mrlac) from *Meiothermus ruber* DSM 1279 was successfully overexpressed to produce a laccase (Mrlac) in soluble form in *Escherichia coli* during simultaneous overexpression of a chaperone protein (GroEL/ES). Without the GroEL/ES protein, the Mrlac overexpressed in *E. coli* constituted a huge amount of the total cellular protein, but the enzyme was localized in the insoluble fraction with no activity in the soluble fraction. Co-expression of the Mrlac with the *E. coli* GroEL/ES drastically improved proper folding and expression of active Mrlac in the soluble fraction. Spectroscopic analysis of the purified enzyme by UV/visible and electron paramagnetic resonance spectroscopy confirmed that the Mrlac was a multicopper oxidase. The Mrlac had a molecular weight of ~ 50 kDa and exhibited activity towards the canonical laccase substrates 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), syringaldazine (SGZ), and 2,6-dimethoxyphenol (2,6-DMP). Kinetic constants $K_m$ and $k_{cat}$ were 27.3 µM and 325 min$^{-1}$ on ABTS, 4.2 µM and 106 min$^{-1}$ on SGZ, and 3.01 µM and 115 min$^{-1}$ on 2,6-DMP, respectively. Maximal enzyme activity was achieved at 70°C with ABTS as substrate. In addition, Mrlac exhibited a half-life for deactivation at 70°C and 75°C of about 120 min and 67 min, respectively, indicating that the Mrlac is intrinsically thermostable. Finally, Mrlac was efficient in catalyzing the removal of 2,4-dichlorophene (DCP) in aqueous solution, a trait which makes the enzyme potentially useful for environmentally friendly applications.
Can laccases catalyze bond cleavage in lignin?
Modification of lignin is recognized as an important aspect of the successful refining of lignocellulosic biomass, and enzyme-assisted processing and upcycling of lignin is receiving significant attention in the literature. Laccases (EC 1.10.3.2) are taking the centerstage of this attention, since these enzymes may help degrading lignin, using oxygen as the oxidant. Laccases can catalyze polymerization of lignin, but the question is whether and how laccases can directly catalyze modification of lignin via catalytic bond cleavage. Via a thorough review of the available literature and detailed illustrations of the putative laccase catalyzed reactions, including the possible reactions of the reactive radical intermediates taking place after the initial oxidation of the phenol-hydroxyl groups, we show that i) Laccase activity is able to catalyze bond cleavage in low molecular weight phenolic lignin model compounds; ii) For laccases to catalyze inter-unit bond cleavage in lignin substrates, the presence of a mediator system is required. Clearly, the higher the redox potential of the laccase enzyme, the broader the range of substrates, including o- and p-diphenols, aminophenols, methoxy-substituted phenols, benzenethiols, polyphenols, and polyamines, which may be oxidized. In addition, the currently available analytical methods that can be used to detect enzyme catalyzed changes in lignin are summarized, and an improved nomenclature for unequivocal interpretation of the action of laccases on lignin is proposed. (C) 2015 Elsevier Inc. All rights reserved.

General information
State: Published
Organisations: Department of Chemical and Biochemical Engineering, Center for BioProcess Engineering
Authors: Munk, L. (Intern), Sitarz, A. K. (Intern), Kalyani, D. (Intern), Mikkelsen, J. D. (Intern), Meyer, A. S. (Intern)
Number of pages: 12
Pages: 13-24
Publication date: 2015
Main Research Area: Technical/natural sciences

Publication information
Journal: Biotechnology Advances
Volume: 33
Issue number: 1
ISSN (Print): 0734-9750
Ratings:
BFI (2018): BFI-level 2
Web of Science (2018): Indexed yes
BFI (2017): BFI-level 2
Scopus rating (2017): SJR 3.006 SNIP 3.531
Web of Science (2017): Indexed yes
BFI (2016): BFI-level 2
Scopus rating (2016): CiteScore 11.05 SJR 2.747 SNIP 3.141
Web of Science (2016): Indexed yes
BFI (2015): BFI-level 2
Scopus rating (2015): SJR 2.915 SNIP 3.396 CiteScore 10.56
Enzymatic polishing and modification of lignin

Department of Chemical and Biochemical Engineering

Period: 01/10/2012 → 18/04/2018

Number of participants: 6

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**Financing sources**
Source: Internal funding (public)
Name of research programme: Institut stipendie (DTU) Samf.
Project: PhD