FINAL TECHNICAL REPORT

DOE BER Grant DE-SC0006929: Fungal Biodegradative Oxidants in Lignocellulose: Fluorescence Mapping and Correlation With Gene Expression

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Spatial mapping of extracellular oxidant production by a white rot basidiomycete during its deconstruction of lignocellulose

Oxidative cleavage of the recalcitrant plant polymer lignin is a crucial step in global carbon cycling, and is accomplished most efficiently by fungi that cause white rot of wood. These basidiomycetes secrete many enzymes and metabolites with proposed ligninolytic roles, and it is not clear whether all of these agents are physiologically important during attack on natural lignocellulosic substrates. One new approach to this problem is to infer properties of ligninolytic oxidants from their spatial distribution relative to the fungus on the lignocellulose. We grew *Phanerochaete chrysosporium* on wood sections in the presence of oxidant-sensing beads based on the ratiometric fluorescent dye BODIPY 581/591. The beads, having fixed locations relative to the fungal hyphae, enabled spatial mapping of cumulative extracellular oxidant distributions by confocal fluorescence microscopy. The results showed that oxidation gradients occurred around the hyphae, and data analysis using a mathematical reaction-diffusion model indicated that the dominant oxidant during incipient white rot had a half-life under 0.1 second. The best available hypothesis is that this oxidant is the cation radical of the secreted *P. chrysosporium* metabolite veratryl alcohol. This work was published in a peer reviewed journal:

Hunt CG, Houtman CJ, Jones DC, Kitin P, Korripally P, Hammel KE (2013) Spatial mapping of extracellular oxidant production by a white rot basidiomycete on wood reveals details of ligninolytic mechanism. Environ. Microbiol. 15:956-966. doi:10.1111/1462-2920.12039

In additional work, we have correlated gene expression in *P. chrysosporium* with oxidation of fluorescent BODIPY beads on wood. At intervals, specimens were harvested for bead imaging and for whole transcriptome analysis by Illumina RNAseq. Two major findings emerged from the study: (1) Bead oxidation was not continuous with time, but rather commenced abruptly several days after fungal inoculation onto the wood. This result indicates a transition from nonligninolytic to ligninolytic metabolism by the fungus. (2) Concurrent with this oxidative burst, several classes of fungal genes were highly up-regulated. Chief among these were genes encoding several peroxidases that have been shown to cleave lignin *in vitro*, as well as hydrogen peroxide-producing oxidases that support catalytic turnover of these peroxidases. By contrast, a

cellobiose dehydrogenase and two glycopeptides that have been proposed to produce ligninolytic hydroxyl radicals were not up-regulated. These results support a mechanism for incipient ligninolysis in which fungal peroxidases react with lignin to cleave it. This work was published in a peer reviewed journal:

Korripally P, Hunt CG, Houtman CJ, Jones DC, Kitin PJ, Cullen D, Hammel KE (2015) Regulation of gene expression during the onset of ligninolytic oxidation by *Phanerochaete chrysosporium* on spruce wood. Appl. Environ. Microbiol. 81:7802-7812. doi: 10.1128/AEM.02064-15.

Elucidation of biodegradative mechanism used by a recently sequenced brown rot fungus

Basidiomycetes that cause brown rot of wood are essential biomass recyclers in coniferous forest ecosystems and are potentially useful in new biomass processing technologies. Recent work indicates that distinct lineages of brown rot fungi have arisen independently from ligninolytic white rot ancestors via loss of lignocellulolytic enzymes. Brown rot thus proceeds without significant lignin removal, apparently beginning instead with oxidative attack on wood polymers by Fenton reagent produced when fungal hydroquinones or catechols reduce Fe^{3+} in colonized wood. Since there is little evidence that white rot fungi produce these metabolites, one might infer that independent lineages of brown rot fungi would not employ the same Fe^{3+} reductants. Recently, the genome of *Serpula lacrymans*, a brown rot member of the Boletales, was sequenced and the catechol variegatic acid was proposed to have a key role in its Fenton chemistry [Eastwood DC *et al.* (2011) Science 333:762-765]. We found that variegatic acid was undetectable in wood undergoing decay by *S. lacrymans*, that it was unable to reduce *in vitro* the Fe^{3+} oxalate chelates that predominate in brown-rotting wood, and that it did not drive Fenton chemistry *in vitro* under physiological conditions. Instead, the decaying wood contained physiologically significant levels of 2,5-dimethoxyhydroquinone, a reductant with a demonstrated biodegradative role when wood is attacked by certain brown rot fungi in two other divergent lineages, the Gloeophyllales and the Polyporales. Our results suggest that the pathway for 2,5-dimethoxyhydroquinone biosynthesis may have been present in ancestral white rot basidiomycetes, but do not rule out the possibility that it appeared multiple times via convergent evolution. This work was published in a peer reviewed journal:

Korripally P, Timokhin VI, Houtman CJ, Mozuch MD, Hammel KE (2013) Evidence from *Serpula lacrymans* that 2,5-dimethoxyhydroquinone is a lignocellulolytic agent of divergent brown rot basidiomycetes. Appl. Environ. Microbiol. 79:2377-2383. doi:10.1128/AEM.03880-12

Comparative analysis of oxidant production on lignocellulose by fungi that use different biodegradative strategies

Lignocellulose oxidation by diverse fungi involves oxidation, and a variety of oxidants is apparently employed. We have compared BODIPY bead oxidation on wood by a white rot basidiomycete (*Ceriporiopsis subvermispora*), a brown rot basidiomycete (*Gloeophyllum trabeum*), a soft rot ascomycete (*Daldinia concentrica*), and a non-decay ascomycete (*Ophiostoma piliferum*). Scanning electron microscopy showed that all three decay fungi

degraded the wood in our culture system, whereas *O. piliferum* did not. Confocal fluorescence microscopy of fluorescent beads placed on the wood showed that *C. subvermispora* and *G. trabeum* oxidized the beads rapidly, which is consistent with literature that indicates the production of ligninolytic peroxidases by the first fungus and of hydroxyl radicals by the second. However, *D. concentrica* did not oxidize the beads significantly, giving results no different than those obtained with the non-decay fungus *O. piliferum.* Since *D. concentrica* degraded the wood in these experiments, we conclude that it does not employ water-soluble, diffusible oxidants as decay agents, because these would have contacted the BODIPY beads if they were produced. Our results do not rule out the possibility that *D. concentrica* employs ligninolytic oxidants that are bound to the fungal hyphae, such as lipophilic reactive oxygen species associated with the fungal cell membrane. **A paper reporting the above results will be submitted to a peer-reviewed journal by December 2016.**

In additional experiments on *C. subvermispora*, we have investigated the nature of the oxidants that it produces on lignocellulose. The ability of this fungus to remove lignin before the substrate porosity has increased enough to admit enzymes suggests that small diffusible oxidants contribute to delignification. A key question is whether these unidentified oxidants attack lignin via single electron transfer (SET), in which case they are expected to cleave its propyl side chains between C1 and C2 and to oxidize the *threo*-diastereomer of its predominating structures more extensively than the corresponding *erythro*-diastereomer. We used two-dimensional solution-state nuclear magnetic resonance (NMR) techniques to look for changes in partially biodegraded lignin extracted from spruce wood after white rot caused by *C. subvermispora*. The results showed that residues indicative of C1-C2 cleavage were the major identifiable truncated structures in lignin after decay and that depletion of the predominating lignin subunits was markedly diastereoselective with a *threo* preference. Our results show that the ligninolytic oxidants of *C. subvermispora* are collectively more diastereoselective than currently known fungal ligninolytic oxidants and suggest that SET oxidation is one of the chemical mechanisms involved. This work was published in a peer reviewed journal:

Yelle DJ, Kapich AN, Houtman CJ, Lu FC, Timokhin VI, Fort RC, Ralph J, Hammel KE. 2014. A highly diastereoselective oxidant contributes to ligninolysis by the white rot basidiomycete *Ceriporiopsis subvermispora*. Appl. Environ. Microbiol. 80:7536-7544. doi: 10.1128/AEM.02111-14.

Influence of oxidant availability on lignin degradation in a natural environment

Seeking to extend our methods to real world situations, we have begun microcosm studies in collaboration with soil scientists at the University of California-Berkeley and Iowa State University. As biological ligninolysis is invariably oxidative, and our research has focused on the nature of these oxidants, we were interested in the fate of lignin in natural environments where oxygen is present but limiting. Over a period of 10 weeks, we tested the impact of redox fluctuations on lignin breakdown in humid tropical forest soils that undergo cyclic periods of water saturation and hypoxia. We used synthetic lignins labeled with ^{13}C in either of two positions (aromatic methoxyl or propyl side chain C2) to provide highly sensitive and specific measures of lignin mineralization seldom employed in soils. Four-day redox fluctuations increased the percent contribution of methoxyl C to soil respiration relative to static aerobic

conditions, and cumulative methoxyl-C mineralization was statistically equivalent under static aerobic and fluctuating redox conditions despite lower soil respiration in the latter treatment. Contributions of the less labile lignin C2 to soil respiration were equivalent in the static aerobic and fluctuating redox treatments during periods of O2 exposure, and tended to decline during periods of O_2 limitation, resulting in lower cumulative C2 mineralization in the fluctuating treatment relative to the static aerobic treatment. However, cumulative mineralization of both the C2- and methoxyl-labeled lignins nearly doubled in the fluctuating treatment relative to the static aerobic treatment when total lignin mineralization was normalized to total O_2 exposure. Oxygen fluctuations are thought to be suboptimal for canonical lignin-degrading microorganisms. However, O_2 fluctuations drove substantial Fe reduction and oxidation, and reactive oxygen species generated during abiotic Fe oxidation might explain the elevated contribution of lignin to C mineralization. To investigate this possibility, we supplemented some of the microcosms with Fe, but this stabilized lignin rather than enhancing its breakdown. Other factors, yet to be determined, apparently enhance lignin degradation beyond the extent expected from the amount of O_2 exposure our microcosms received. This work was published in two peer reviewed journals:

Hall SJ, Silver WL, Timokhin VI, Hammel KE. 2015. Lignin decomposition is sustained under fluctuating redox conditions in humid tropical forest soils. Glob. Chang. Biol. 21:2818-2828. doi:10.1111/gcb.12908

Hall SJ, Silver WL, Timokhin VI, Hammel KE. 2016. Iron addition to soil specifically stabilized lignin. Soil Biol. Biochem. 98:95-98. doi:10.1016/j.soilbio.2016.04.010

Development of a new fluorescence staining technique for early detection of biomass oxidation

We have found that the metachromatic dye Acridine Orange (AO) is extremely sensitive and useful in detecting fungal attack of wood. The literature, however, was mixed regarding the mechanism and what the stain response meant. Therefore we conducted model studies to elucidate the mechanism of AO staining on wood during early oxidation, finding that on mildly oxidized lignin, the association between AO and lignin was reduced such that stained wood sections emitted less green light during fluorescence microscopy. We describe how to use this property to provide a novel method for identifying wood oxidation. This change was detectable after less than a week, an interval that past work has shown to be too short for significant delignification of wood. Although fungal hyphae were observed in only a few wood lumina, oxidation was widespread, appearing relatively uniform over regions several hundred micrometers from the hyphae. This observation suggests that both classes of fungi release low molecular weight mild oxidants during the first few days of colonization. This work was published in a peer reviewed journal:

Houtman CJ, Kitin P, Houtman JCD, Hammel KE, Hunt CG (2016) Acridine orange indicates early oxidation of wood cell walls by fungi. PLoS ONE 11(7): e0159715. doi:10.1371/journal.pone.0159715

Development of new sensors to map fungal oxidation of lignocellulose

There is a need for improved sensors that can distinguish between different reactive oxygen species and oxidative enzymes. The problem is how to distinguish those oxidants that can depolymerize lignin from those that cannot. Our goal is to develop fluorescent probes that can be used to map gradients of only those oxidants capable of ligninolysis. When planning experiments using fluorescent dyes in oxidative environments, one must always be aware that environmental degradation of the dyes may influence results. The literature on the relative oxidative stability of dyes is haphazard and fragmented, with dye manufacturers providing the bulk of the available data. To assist us in choosing appropriate dyes and also to help the community at large, we have systematically recorded the degradation rate of 24 common dyes, covering all the major chemical backbones, under photo and chemical oxidation conditions. We intend to present side by side dye performance characteristics with all dyes exposed to the same conditions, to assist researchers in choosing appropriate dyes for their specific applications. **We are currently preparing a manuscript for submission to an open access, peer reviewed journal.**