

Oxidative Degradation of Wood by Brown-Rot Fungi

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INTRODUCTION

Brown-rot fungi are Basidiomycetes that remove cellulose and other polysaccharides from wood, leaving an amorphous, brown, crumbly residue that is composed largely of lignin, hence the name brown-rot. Decay by brown-rot fungi is by far the most serious type of damage to wood in-service. These fungi cause structural failure before losses in total wood substance are detected. The effect of brown-rot fungi on wood strength properties is due to cellulose depolymerization. Worldwide losses of millions of dollars annually result from fungal deterioration of untreated *or* inadequately-treated wood (11). Several species of brown-rot fungi decay heartwood in living firs. These tree pathogens add to the losses in wood product utilization and threaten forest and plantation stands (9).

Brown-rot fungi represent an untapped resource for industrial bioconversion of lignocelluloses. With the unique ability to circumvent the lignin barrier in cellulose utilization, brown-rot fungi offer a potential cost-effective pretreatment process for saccharification of cellulose.

Relatively little research attention has been given to identifying the mechanism of wood cellulose depolymerization by brown-rot fungi. The actual biochemical system is not known, but evidence has accumulated in support of an oxidative mechanism of depolymerization. A model of cellulose depolymerization is based on fungal production of extracellular reduced oxygen species.

ALTERED PHYSICAL AND CHEMICAL PROPERTIES OF WOOD

Anatomically, wood is the cylinder of secondary xylem inside the bark of trees. Long, tapering tracheids constitute the dominant cell type in the conifer axial system. Tracheids have a secondary cell wall composed of three layers (S1, S2, S3) that consecutively encircle a central lumen. Attack of the cell wall by brown-rot fungi is initiated predominantly by hyphae growing in the lumen in contact with the S3 layer (Fig. 1). Hyphae appear in electron micrographs to be attached to the

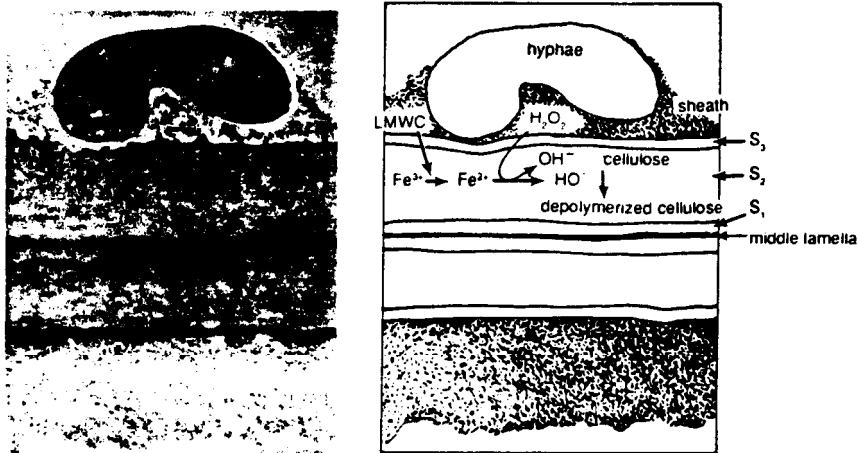


Figure 1. Early stage of wood decay by brown-rot fungi. Left, transmission electron micrograph of two adjacent cell walls of hemlock tracheids during early stage of *Postia placenta* degradation; 12,000x (Lydia Murmanis Collection, Forest Products Laboratory, Madison, Wisconsin). Right, diagrammatic representation of same with hypothetical scheme for the oxidative fragmentation of cellulose by brown-rot fungi. LMWC, low molecular weight chelator. Adapted from (6, 9, 10, 19).

xylem cell wall by a gelatinous sheath. The hyphae of brown-rot fungi are widespread in wood before any significant weight losses are detected. The thickest layer (S2) is intensely degraded whereas the S3 layer remains relatively undegraded until late in decay. Degradation is not localized near the hyphae. Apparently, there is widespread and deep diffusion of the initial degrading agents (9).

Chemically, wood consists of 40 to 60% cellulose, 10 to 30% hemicellulose, and 15 to 30% lignin. Water- and alkali-insoluble cellulose comprise the S2 layer in the amorphous as well as crystalline form and the S3 layer in the crystalline form. Hemicelluloses and lignin are located between cellulose microfibrils (9).

Brown-rot fungi cause a rapid and extensive reduction of the average number of glucosyl residues per wood cellulose molecule (degree of polymerization, DP) from about 10^4 to about 200 units. The sizes of the resulting fragments apparently correspond to cellulose "crystallite." Depolymerization of wood cellulose is measured by a substantial increase in alkali volatility products and a rapid drop in wood strength properties. The mechanism of depolymerization is a perplexing biochemical question. Brown-rot fungi produce cellulases and hemicellulases, but the enzyme complex does not mimic the action of brown-rot fungi in depolymerizing cellulose (9).

Accessibility of the cellulose in wood to brown-rot degradation remains a question. Only a very small percentage of the cell wall volume is accessible to even the smallest known cellulases. In a study of pore sizes in sound and brown-rotted

wood, Flournoy *et al.* (5) report that molecules greater than 6,000 D would be excluded from the pores of wood. This result is in agreement with previous studies (11). Much smaller cellulose components or other as-yet unidentified agents must also be involved in cellulose depolymerization.

Frustrating the solution to the depolymerization problem is the lack of information about the exact molecular structure and chemistry of the tracheid cell wall. The complexity of the fungus-wood system and culture requirements of the fungus have hindered the progress in designing an assay for a depolymerization agent. The fungi are readily grown in simple chemically-defined liquid media, but manifestation of the cellulose-depolymerizing system in such cultures has not been achieved. Consequently, no assay has been successfully developed for a depolymerizing agent. Cellulose has been the substrate of choice in the search for such agent(s).

Lignin is a potential substrate for a fungal agent that leads to cellulose depolymerization. This polymer surrounds wood cellulose microfibrils and is likely to come into contact with the fungal agent before cellulose. Lignin is chemically altered during brown-rot decay (11) and an oxidized polymeric lignin residue accumulates (1). The capacity of brown-rot fungi to degrade lignin model compounds has been correlated with their capacity to degrade cotton cellulose and wood (4). The hemicellulose components of wood are also potential substrates in a reaction with a fungal agent that results in cellulose degradation. Hemicellulose sugars surround cellulose microfibrils and are removed from wood by brown-rot fungi (11).

OXIDATIVE DEPOLYMERIZATION OF CELLULOSE

Evidence for an oxidative system of cellulose degradation for brown-rot fungi was shown when Highley (8) demonstrated that brown-rotted cellulose contained carbonyl and carboxyl groups. Recently, Highley *et al.* (10) chemically and physically characterized brown-rotted cellulose. Molecular weight determinations of cellulose degraded by the brown-rot fungus *Postia placenta* gave a symmetrical DP distribution with a fairly tight distribution around DP 200 from the original 2000. This is consistent with random cleavage of all the cellulose and must have occurred in the amorphous regions, not just at the fiber surfaces. The X-ray diffraction analysis indicated a preferential attack by the fungus on amorphous regions of cellulose, confirming earlier work (2). Infrared spectroscopy and carboxyl determinations with methylene blue showed that carboxyl groups were present in the degraded cellulose. Uronic acids were not detected in acid hydrolysates of the brown-rotted cellulose, indicating that oxidation did not occur at C-6.

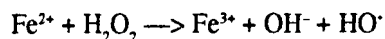
Cellulose decayed by *P. placenta* has been further chemically characterized and compared to cellulose depolymerized by 1) acid-hydrolysis; 2) Fenton's reagent (ferrous salts $[\text{Fe}^{2+}]$ and hydrogen peroxide $[\text{H}_2\text{O}_2]$); and 3) periodate/bromine oxidation (17). Fenton's reagent but not the other systems mimicked the

brown-rot system in all measured characteristics. Gas chromatographic and mass spectroscopic analysis of the acid hydrolysates of brown-rotted cellulose identified four major acids glyceric, erythronic, gluconic, and arabonic acids. No uronic acid was produced. The presence of glyceric and erythronic acids indicates oxidative cleavage of the vicinal diol carbon-carbon bond within glucosyl residues (17). Properties of the cellulose depolymerized by the brown-rot fungus differed markedly from that caused by isolated fungal cellulases and by cellulolytic white-rot fungi (9).

Oxidation of cellulose by the superoxide radical (O_2^-) is similar to that of brown-rot fungi (20). Both types of degradation rapidly decrease the DP of cellulose to about 200 and show symmetrical molecular weight distributions, which indicate random cleavage of all the cellulose, not just the fiber surfaces. For both types, the ratio of number average DP to weight average DP is about 1:2, which is also consistent with random cleavage. Since both types of degradation reach a "limit DP" similar to that obtained after acid hydrolysis, degradation must proceed through the amorphous regions. The rapidity of the degradation by brown-rot fungi and by potassium O_2^- compared with acid hydrolysis suggests that cellulose-metal complexes and oxidation reactions by oxygen radicals are involved in cellulose depolymerization by brown-rot fungi.

DEPOLYMERIZATION MODEL

The current model for cellulose depolymerization is based on evidence that rapid oxidation occurs during early stages of brown-rot decay, that pore size in wood is too small for known enzymes to access wood cellulose, and that Fenton's reagent mimics brown-rot degradation of cellulose (9, 11). The model predicts production of the hydroxyl radical (HO^\cdot) from H_2O_2 and ferrous salts by the Fenton-Haber-Weiss reaction.



This oxygen free radical would cleave long chain cellulose into smaller fragments.

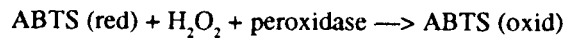
The model began with the suggestion by Cowling and Brown (2) that the depolymerization of cellulose with H_2O_2 and Fe^{2+} as described in 1965 by Halliwell (7) might also apply to the mechanism of brown-rot cellulose degradation. Koenigs (18) reported that some brown-rot fungi produce extracellular oxidizing agents, presumably H_2O_2 , and that wood contains enough iron for a possible involvement of the iron-peroxide Fenton system in cellulose degradation. However, no extracellular fungal agent has been isolated and demonstrated to depolymerize wood cellulose to "the limit" DP (9).

H_2O_2 Production

The first reports of H_2O_2 production by brown-rot fungi were not easily reproduced (9). Researchers have subsequently reported variable results in detecting H_2O_2 in cultures of brown-rot fungi; in contrast, H_2O_2 production by

white-rot wood decay fungi is easily detected (9). In addition to questioning whether brown-rot fungi produce extracellular H_2O_2 , the variable results have brought into question: 1) the specificity and sensitivity of detection methods; 2) the variability of fungal species and strains of a given species; 3) culture conditions for assays; 4) lack of H_2O_2 accumulation; and 5) growth-specific production of H_2O_2 by fungi.

A relatively sensitive enzymatic calorimetric assay has been used recently to detect extracellular H_2O_2 in culture filtrates (9, 11, 15). The assay is based on the oxidation of 2,2'-azino-di-3-ethylbenzothiazole-6-sulphonic acid (ABTS) by horseradish peroxidase in the presence of H_2O_2 . The H_2O_2 assay is based on the following reaction.



The peroxidase-catalyzed reaction has detected H_2O_2 in chemically-defined solid, liquid, and wood-based culture media of many brown-rot fungi under specific culture conditions (15). Production in stationary culture medium was induced by nitrogen and for carbohydrate starvation. The difficulty in consistently detecting H_2O_2 maybe due to the low concentration produced in the early stages of fungal development. The H_2O_2 maybe produced transiently, *i. e.* in small concentrations for a short period of time at a specific hyphal age during primary mycelial growth. If rapidly utilized or quenched by chemicals in the culture media, H_2O_2 may not be consistently detected. Inoculum source may also influence detection of H_2O_2 , since adjacent mycelial plugs taken from agar stock plates may exhibit variable hyphal growth rate (Illman, unpublished data).

Attempts to isolate oxidases and peroxidases from brown-rot culture filtrates have not been successful (9, 11). With employment of current culture conditions for H_2O_2 and $HO\cdot$ production, this may change. Alternatively, H_2O_2 may be generated by a nonenzymatic reaction in the brown-rot system.

Although it has been predicted that H_2O_2 is a diffusible DP agent (9), its role as an agent moving from tracheid lumen to an S2 site of depolymerization has not been demonstrated. An alternate explanation is that H_2O_2 is generated in close proximity to its site of action, in or near the hyphal sheath. A proposed model of the sheath as facilitator of degradation (6) could account for site-specific generation and oxidation of H_2O_2 . Green *et al.* (6) proposed a comprehensive transport model of the sheath for brown- and white-rot wood decay fungi. The model offers an alternative or supplemental hypothesis to simple diffusion of decay agents into the woody substrate during brown-rot decay. The utilization of H_2O_2 in site-specific oxidation reactions would prevent its accumulation in culture media,

One-electron oxidation of 2-keto-4-thiomethyl butyric acid (KTBA) as measured by ethylene production has been used as an assay for oxidizing agents in brown-rot culture filtrates (4). Oxidation of KTBA is nonspecific. It is oxidized by $HO\cdot$, ceric ammonium sulfate, or peroxidases in the presence of H_2O_2 . Enoki *et al.* (4) report that acetone precipitates from 30-d-old culture filtrates of four brown-rot species produced ethylene after NADH was added under 100% O_2 ,

atmosphere. Ethylene decreased when catalase was added. Ethylene was produced when H_2O_2 was added under 100% N_2 atmosphere.

Oxygen Free Radicals

The presumptive role of H_2O_2 is the generation of HO^\cdot in a reaction with a metal or a metal chelate. The superoxide radical is a precursor candidate for generation of H_2O_2 . Thus, assays for a depolymerization agent have been based on detection systems for oxygen free radicals.

The chemical assays that have been used to survey fungal culture media and fungi-degraded wood for the production of extracellular oxygen free radicals include the bleaching of the p-nitrosodium methylaniline spectrum and silver oxidation as measured by desilvering of silver-lined culture flasks (11).

A more direct detection of HO^\cdot in brown-rot systems was made by using electron spin resonance spectroscopy (ESR) and a spin trapping technique (13, 15). The oxygen radical spin trap 5,5-dimethylpyrroline-N-oxide (DMPO) was used to derivative HO^\cdot to a more stable adduct radical that gives a specific, four-line ESR signal. The ESR signal of the HO^\cdot -DMPO adduct was observed in wood slivers of Douglas fir and white fir when inoculated with *P. placenta* (15). The HO^\cdot -DMFO signal was also detected in 10-d-old nitrogen-limited liquid cultures of *P. placenta* supported by 0.1% cellobiose or 0.170 glucose (13, 15). The chemical reaction(s) generating HO^\cdot has not been determined.

The radical trapping molecule used in this study reacts with both HO^\cdot and $O_2^{\cdot-}$. The resulting radical adducts give different, specific ESR spectra for each radical. We did not detect the ESR spectra for the $O_2^{\cdot-}$ -DMPO adduct (13, 15). If formed, it could decay to the HO^\cdot -DMPO adduct (7). The chemical source of the radical is being investigated. Direct involvement of HO^\cdot in the decay process has not been shown.

Culture filtrates from several brown-rot fungi are reported to catalyze a one-electron oxidation of KTBA under an atmosphere of 100% O_2 but not 100% N_2 . Addition of an electron donor (NADH) under 100% O_2 or H_2O_2 under 100% N_2 increased KTBA oxidation. Superoxide dismutase decreased the O_2 -NADH oxidation by almost 50%, and catalase decreased oxidation almost completely. The radical scavengers, guaiacol and N,N'-dimethyl-4-nitrosoaniline (DMNA), decreased oxidation by about 80%.

The presumptive role of HO^\cdot as an agent to oxidize cellulose requires formation at its site of action (7). The hydroxyl radical is the most reactive oxygen species, is nonspecific, and, unlike H_2O_2 and $O_2^{\cdot-}$, would not diffuse from its site of generation (7). Reactions involving HO^\cdot maybe facilitated by the fungal sheath. In addition to its proposed role as transport facilitator of decay agents (6), the sheath may provide a medium for controlled oxidative reactions.

Metals and Chelators

Because Fenton's reagent has been shown to mimic brown-rot degradation of cellulose, assays for metals, chelators, and metallo-proteins have been used in

attempts to determine the role of metals in brown-rot oxidative processes.

Electron spin resonance spectroscopy has been used in an effort to detect and follow changes in the oxidation states of paramagnetic metals during brown-rot decay (12, 14). Changes in iron were not detected. Because iron is highly reactive in biological systems, it is usually tightly bound. This may account for difficulties in detecting iron in the wood samples. A comprehensive study of iron oxidation states using ESR has yet to be made. Iron is expected to be complexed at the low pH levels (pH3-pH4) that occur during brown-rot decay, most likely by a fungal siderophore.

Extracellular siderophores have been isolated from culture filtrates of white- and brown-rot wood decay fungi (16). The low-molecular weight, phenolate-type siderophores have a high affinity for binding iron and can bind a wide variety of metals, including manganese (16).

Iron-containing proteins have recently been found in culture filtrates of brown-rot fungi (4). When incubated with ferrozine, the proteins gave an absorbance at 560 nm which disappeared after treatment with one equivalent of H_2O_2 , an indication that the Fe_2^+ was oxidized to Fe^{3+} by the Fenton reaction.

Changes in manganese were observed with ESR after inoculation of susceptible species of wood with the brown-rot fungus *P. placenta* (12, 14). These changes were manifest during the decay process as increases in the size of the sextet spectra that is specific for Mn^{2+} (Fig. 2). The increases in Mn^{2+} spectra correlated with wood susceptibility to brown-rot decay. Little or no increase was found in wood species resistant to brown-rot decay (14). The chemical basis of the

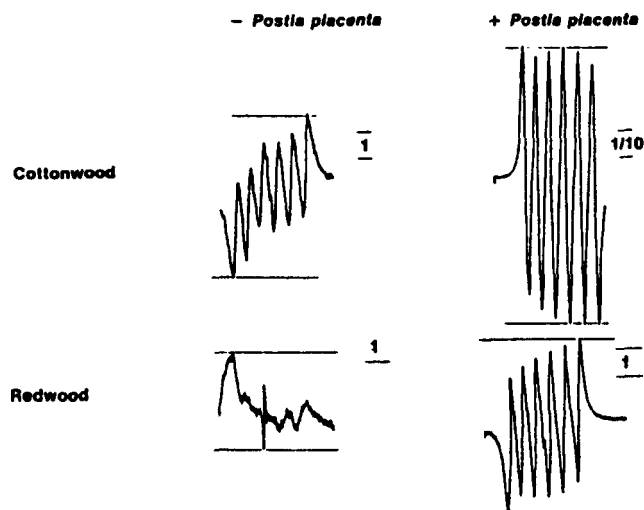


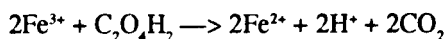
Figure 2. Electron spin resonance signal of Mn^{2+} in cottonwood and redwood, with and without a colonization by the brown-rot fungus *Postia placenta*.

manganese change was not determined. In our investigations for a manganese role in brown-rot decay we have treated wood with substances typically found in culture filtrates, including oxalic acid.

Brown-rot fungi secreted oxalic acid into decayed wood and into culture media (11). The role of the extracellular oxalic acid is not known. We found that oxalic acid changed the manganese ESR signal in wood (Illman and Englebert, unpublished data). After treatment of white fir wood slivers with oxalic acid, the Mn^{2+} ESR signal increased. The chemical basis of the oxalic acid effect on manganese is being investigated.

The increased manganese signal may be a result of lowered pH with acid solubilization of the metal. Microbial oxalic acid can dissolve and thereby mobilize manganese in geochemical sedimentations. Oxalic acid from several species of wood decay fungi can mobilize calcium from glass and concrete (9). Alternatively, the increase in the Mn^{2+} ESR signal may be due to chelation of the metal. Oxalate is a known chelator of several elements, including manganese, calcium, and potassium (9).

Schmidt *et al.* (19) predicted a role for oxalic acid in brown-rot decay. They reported that oxalic acid reduced Fe^{3+} , the species normally present in wood, to Fe^{2+} , the active form in Fenton's reagent (19).



Oxalic acid may be an important low molecular weight chelator or have other roles in the brown-rot system. However, no direct evidence has been reported for the effects of oxalic acid on wood cellulose oxidation.

CONCLUSION

Brown-rot fungi cause rapid depolymerization of wood cellulose by an oxidative mechanism that generates HO· or other reactive species. Evidence supports a Fenton-Haber-Weiss-type system that is initiated by an oxidative depolymerization agent or agents. The depolymerization agent is expected to be a low molecular weight metal chelate that initiates oxidation of wood substrates in a reaction that results in oxidative depolymerization of cellulose. The capacity of fungi to produce extracellular oxidative metabolites is expressed when the organisms are maintained under specific culture conditions, especially nitrogen and carbohydrate limitation. Transport of the extracellular agent to a site of action in the tracheid wall is most likely facilitated by the fungal sheath.

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