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Biodeterioration Abstracts

Progress in understanding how brown-rot fungi degrade cellulose

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I. Introduction

Many types of organisms deteriorate wood, but brown-rot fungi are the most destructive to wood in service. These fungi adversely affect several strength properties before significant weight loss is detected (Wilcox, 1978). Richards (1954) found a loss in toughness of >50% by the time 1% weight loss had occurred. In a study by Kennedy (1958), a brown-rot fungus produced >50% loss in work to maximum load at 2% weight loss, Toole (1971) found that brown rot caused about 18% loss in compressive strength perpendicular to the grain at 2% weight loss. Similarly, Mizumoto (1965) found a 10% reduction in compressive strength parallel to the grain at 2% weight loss.

Brown-rot fungi primarily utilize the cellulose and hemicellulose components of wood. The effect of brown-rot fungi on wood strength properties reflects cellulose depolymerization. Shortly after colonizing wood, brown-rot fungi cause a sharp reduction in the degree of polymerization (DP) of cellulose (1800-2000 glucosyl units to 150-200 units) at low weight loss witbout removing the lignin (Cowling, 1961). However, brown-rot fungi do modify lignin, as indicated by demethylation and the accumultition of oxidized polymeric lignin degradation products (Jin *et al.,* 1990; Kirk, 1975). How this rapid cellulose depolymerization occurs is a perplexing biochemical question that has plagued researchers for years—cellulolytic enzymes are too large to penetrate the pores of wood and diffuse throughout the wood cell wall. Furthermore, the effect of cellulases on cellulose is different from the action of brown-rot fungi (Chang *et al.,* 1981; Phillip *et al.,* 1981).

Despite substantial effort to elucidate the biochemistry and physiology of the system employed by brown-rot fungi to degrade cellulose, the mechanisms by which these fungi penetrate wood cell walls and degrade cellulose are still not clear. In this paper, we present an overview of work to date on the decomposition of cellulose by brown-rot fungi. Particular attention is given to the effects of these fungi on cellulose ultrastructure, to production of degradative enzymes, and to possible involvement of oxidative degradative metabolites.

II. Ultrastructural changes and characterization of degraded cellulose

Using a pure form of cellulose, Highley and co-workers have conducted extensive microscopic and chemical studies on how brown-rot fungi degrade cellulose. The effects of inhibitors and cell wall substances other than cellulose on fungal growth patterns and analysis of breakdown products can be avoided by using a pure substrate. Highley (1977) described a solid substrate system for brown-rot fungal depolymerization of isolated cellulose, which has shed light on the chemistry and biochemistry of the depolymerization process. Isolated cellulose is degraded by placing it in contact with a wood or filter-paper inoculum over a soil or agar medium (Highley, 1977, 1988).

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1. Ultrastructural changes

Highley *et al.* (1983) used the solid substrate system to study degradation of purified cellulose by 11 brown-rot fungi using scanning and transmission electron microscopy (SEM and TEM, respectively). Erosion troughs or depressions in the fibre surface were not visible at points of hyphal contact (Fig. 1). However, the cellulose within the fibres was extensively degraded, regardless of hyphal invasion. This agrees with an earlier study (Liese, 1970) in which brown-rot fungi produced few changes in the surface of lignocellulose but caused advanced lysis of cell wall material, as evidenced by holes located beneath the lignocellulose surface.

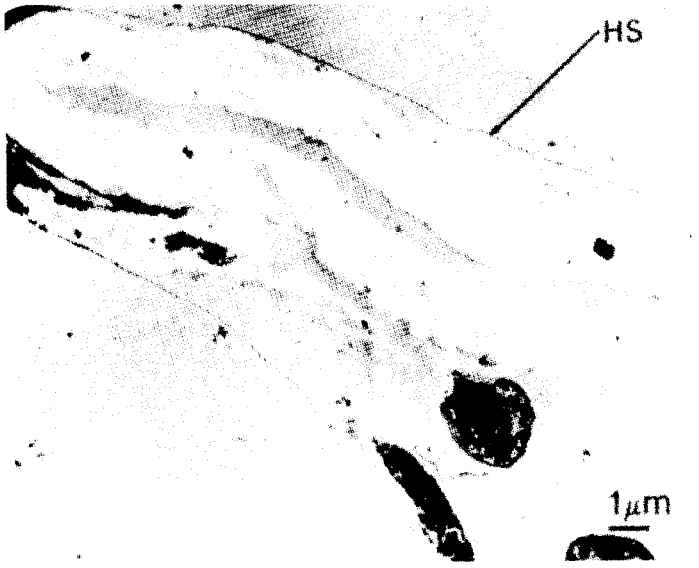

Fig. 1. Hyphal sheath (HS) of *Oligoporus placenta* enwrapped around several hyphae plus cellulose fibre. The depolymerizing agent apparently penetrated throughout the fibre wall at a distance from the hyphae, as evidenced by the degraded fibre.

This type of cellulose degradation is different from that observed in studies of most other cellulolytic microorganisms, in which a rather localized attack on cellulose occurs near cells of the invading organism (Basu & Ghose, 1962; Berg & Hofsten, 1976; Berg *et al.*, 1972). Distinct depressions are evident where cellulolytic bacteria contact the fibre surface, indicating that degradation occurs by means of cellulase bound to the cell envelopes (Berg *et al.*, 1972). Likewise, white-rot fungi in contact with cellulose fibres erode the fibre surface (Highley *et al.*, 1984). A localized attack on cellulose fibres also occurs with mould fungi such as *Trichaderma* spp. and *Aspergillus* spp. These fungi penetrate the fibre to the lumen (Basu & Ghose, 1962; Berg & Hofsten, 1976), and growth is abundant in the lumen and within the cell wall. Berg & Hofsten (1976) showed that *Trichoderma viride* moves through cellulose by means of surface-bound enzymes.

Isolated cellulases also produce a slow, localized erosion of cellulose near the point of contact (Eriksson, 1981; Porter *et al.*, 1960). Thus, morphological changes produced by brown-rot fungi grown on cellulose are quite different from those produced by most other cellulolytic organisms and isolated cellulolytic enzymes. Cellulolytic enzymes would be expected to produce a localized surface attack on cellulose because cellulases isolated thus far are too large to penetrate the microstructure of cellulose and because the affinity of cellulases to cellulose restricts their diffusion. Flournoy *et al.* (1991) found no pores >3.8 nm in sound or decayed wood, which would exclude known cellulases from brown-rot fungi. Strebotnik & Messner (1988, 1990) used TEM and immunogold labelling to study penetrability of enzymes into wood cell walls, Their studies showed that lignin peroxidase (M_r *c.* 42 000), ovalbumin (M_r *c.* 45 000) and myoglobin (M_r *c.* 16 700) are incapable of penetrating the cell walls of brown-rotted wood.

Direct contact between the living fungus and cellulose seems necessary for effective degradation of native-type cellulose by brown-rot fungi. Hyphae or hyphal sheath material is always observed in contact with degraded fibres (Highley *et al.*, 1983). Often, sheath material completely encircles cellulose fibres (Fig. 1). An extracellular sheath or matrix is common to cellulolytic fungi and bacteria. Similarly, in degradation of wood by brown-rot fungi, hyphal sheath structures are observed (1) on the surface of hyphae, (2) extending from hyphae on to the wood surface and covering the S_3 layer, and (3) penetrating into the wood cell wall layers (Green *et al.*, 1989b). Hyphal sheaths have been proposed to facilitate cellulolytic action by aiding transport of degradative agents to substrate and uptake of degradative products of cell wall dissolution (Green *et al.*, 1989b; Jutte & Sachs, 1976; Leightley & Eaton, 1980; Proctor, 1941; Schmid & Liese, 1965). Thus, without the hyphal sheath, the fungus may not be able to position the degradative enzymes near the substrate.

2. Characterization

To gain insight into the nature of the cellulase system involved in the depolymerization of cellulose by brown-rot fungi, Highley *et al.* (1989) chemically and physically characterized brown-rotted cellulose. All eight brown-rot fungi studied extensively depolymerized degraded cellulose exposed over soil or agar and increased alkali volubility before significant weight loss occurred. Molecular weight determinations of cellulose degraded by the brown-rot fungus *Oligoporus placenta (=Postia placenta)* gave a symmetrical DP distribution, with a fairly tight distribution around DP 200 (Fig. 2). Thus, random cleavage of all the cellulose must have occurred in the amorphous regions, not just at the fibre surfaces. The number average molecular weight was 188 and the weight average 335. This is close enough to a 1:2 ratio to be consistent with random cleavage. X-ray distraction analysis indicated a preferential attack by the fungus on smaller crystallites and amorphous regions of the cellulose, confirming earlier work (Cowling, 1961). 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 was not at C-6.

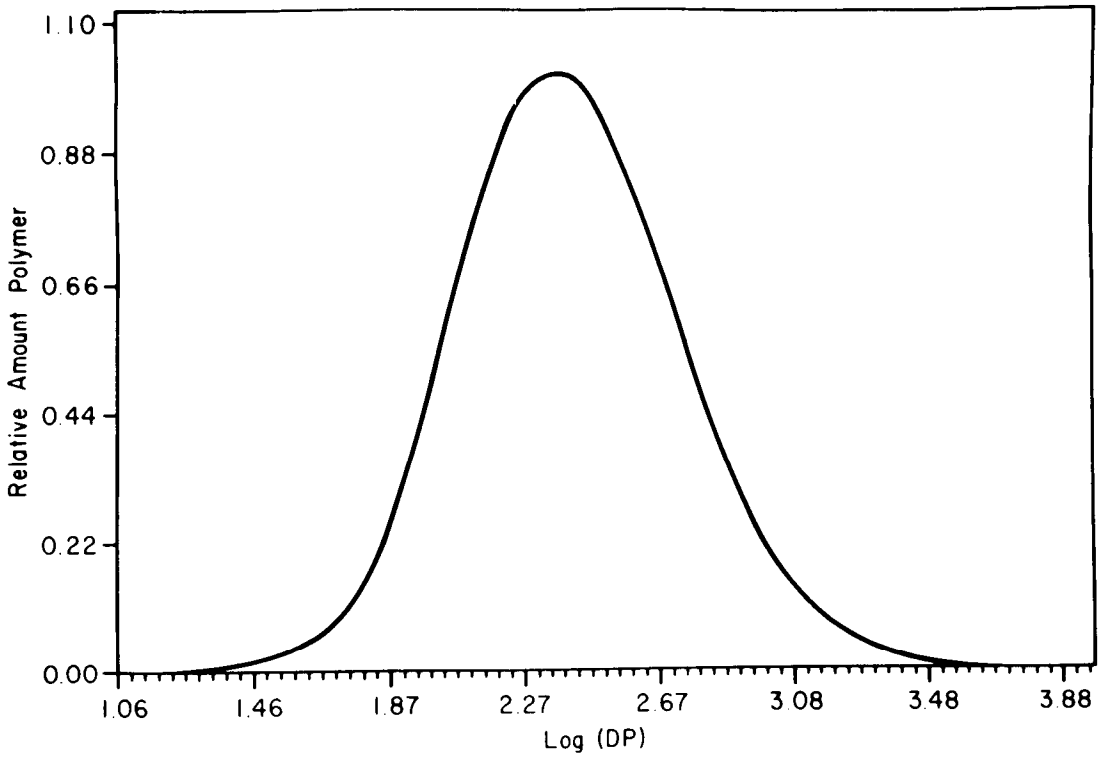

Fig. 2 Molecular weight distribution of cellulose decomposed by *Oligoporus placenta* shows symmetrical distribution, with fairly tight distribution around DP 200.

Kirk *et al.* (1991) further chemically characterized cellulose decayed by *O. placenta* and compared the degraded cellulose with cellulose depolymerized by (1) acid hydrolysis, (2) ferrous salts (Fe^{2+})-hydrogen peroxide (Fenton's reagent), and (3) periodate-bromium oxidation. The Fenton system, but not the other systems, mimicked the brown-rot system in all measured characteristics. Gas-chromatographic 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. Reaction of cellulose with Fenton's reagent generated the same sugar acids but not in the same amounts.

III. Production and characterization of cellulolytic enzymes

1. Cellulolytic activity of culture filtrates

Most cellulolytic organisms hydrolyse the β–1, 4-linkage of crystalline cellulose by the synergistic action of endo- β -l,4-glucanase and exocellobiohydrolase (exoglucanase). β -Glucosidase then breaks down oligosaccharides to glucose. Attack on partially degraded or amorphous cellulose can be carried out by partially deficient systems. Although brown-rot fungi extensively degrade cellulose in wood,

enzyme preparations from liquid cultures and decayed wood or cellulose appear to lack the exoglucanase component; they ineffectively degrade crystalline cellulose but do degrade cellulose that has been modified, such as carboxymethylcellulose (Herr *et al.,* 1978; Highley, 1973; Keilich *et al.,* 1970).

Crystalline cellulose in growing cultures of some brown-rot fungi, such as *O. placenta,* is not significantly degraded or utilized (Highley *et al.,* 1989). In contrast to *O, placenta, Coniophora puteana* is able to utilize cellulose in liquid culture (Highley *et al.,* 1989). However, in this study, DP of the residual cellulose decreased only slightly, and the attack of culture filtrates of *C. puteana* on cellulose was insignificant. In the same study, High Icy *et al.* failed to induce depolymerization and utilization of crystalline cellulose in liquid culture of *O. placenta* by increasing oxygen concentration and limiting nitrogen,

In contrast to other studies, Collet (1987) found that culture filtrates from a strain of *Gloeophyllum trabeum* DFP 8437 could effectively degrade both carboxymethylcellulose (endoglucanase activity) and Avicel [b] (exoglucanase activity). Collet (1984) also found strain variation among different isolates in ability to degrade Avicel. Evidently, the expression of exoglucanase activity in culture filtrates of brown-rot fungi is not a necessary requirement for cellulose decomposition isolates that do not have this activity are able to degrade isolated cellulose and cellulose in wood

The inability to obtain a filtrate from brown-rot cultures that are more active on crystalline cellulose than cultures studied thus far could be due to (1) instability or loss of essential cellulolytic components during isolation or (2) surface topology (configuration and arrangement of molecules on a surface). It is doubtful that cellulolytic enzymes would be inactivated because they must operate outside the cell; therefore, they must be stable (Goksoyr *et al.,* 1975). Exposure of cellulases from brown-rot fungi to classical enzyme inhibitors also verified their stability (Highley, 1975 *a).* Koenigs (1974 *a, b)* proposed that oxidases and activated oxygen species may be a component of the cellulolytic system of brown-rot fungi. These agents are unstable, and thus might be inactivated in culture filtrates.

As is the case for many brown-rot fungi, exoglucantise is also absent from culture filtrates of cellulolytic bacteria, and the filtrates cannot hydrolyse crystalline cellulose (Coughlan & Ljungdahl, 1988). Coughlan & Ljungdahl (1988) propose that locating enzymes on the cell surface may make possible the topological arrangement required for hydrolysis of crystalline substrates. The authors describe cellulolytic complexes (cellulAosomes), appropriately juxtaposed with respect to one another and with respect to cellulose, that account for the complete hydrolysis of cellulose without the need for exoglucanase. If the catalyst that causes cellulose decomposition diffuses from the mycelium and penetrates the entire secondary wall of wood, topology probably would not play an important role in cellulose decomposition by brown-rot fungi. However, if the recently discovered multistructural sheath that penetrates the cell wall of wood serves to deliver cellulolytic enzymes to the substrate, then topology could be important in facilitating cellulose decomposition.

The mycelium might have to be near the cell wall to produce high concentrations of a material that loosens the structure, making it susceptible to attack by the typical cellulose, Microscopic observation of wood degraded by brown-rot fungi indicated that at least one hyphal strand is in the lumen of each degraded wood cell (Wilcox, 1970), Furthermore, Green *et al.* (1989 *b*) found that the S_3 layer in brown-rotted wood was covered by hyphal sheath. A single hypha per wood cell appeared sufficient to reach the S_3 surface with hyphal sheath.

2. Cellulolytic activity in agar assays

One of the most common culture methods for determining cellulolytic activity of fungi is to monitor the clearing of cellulose in an opaque agar medium (Rautela & Cowling, 1966). As the fungi grow, they secrete cellulolytic enzymes that act to create a clear zone in the opaque medium beneath the growing culture. Clearing is due to dissolution of the cellulose substrate.

Rautela & Cowling (1966) studied the clearing of Walseth cellulose (phosphoric acid-swollen cellulose) by white-, brown- and soft-rot fungi. Most white- and soft-rot fungi cleared at least some cellulose, but none of the brown-rot fungi cleared cellulose. Nilsson (1974) used this assay to determine cellulolytic activity of 64 basidiomycetes and found that all the white-rot fungi were able to clear Walseth cellulose. In contrast to the white-rot fungi, most brown-rot fungi failed to clear the cellulose. The species of brown-rot fungi that cleared cellulose belonged to the family Coniophoraceae. Despite differences in ability of members and non-members of this family to clear Walseth cellulose, both types of fungi produced filtrates active on Walseth cellulose and carboxymethylcellulose (endoglucanase activity) but little or no activity on crystalline cellulose (exoglucanase activity) (Highley, 1980; King, 1966, 1968). Thus, the inability of several brown-rot fungi which are not members of the Coniophoraceae to clear Walseth cellulose is difficult to explain.

Zones produced by these non-members in cellulose-agar medium may be indistinct or difficult to identify. Smith (1977) found that dyed-cellulose powder as the substrate in agar medium greatly

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facilitated the detection of cellulolytic activity in several cellulolytic fungi. As cellulose bonds were broken, the dyed products diffused into the agar; the extent of colour developed indicated cellulolytic activity. The brown-rot fungi *O. placenta, Lentinus lepideus* and *G. trabeum,* which were unable to clear phosphoric acid-swollen cellulose in agar medium (Nilsson, 1974; Nilsson & Ginns, 1979), were able to break down cellulose in cellulose-azure agar (dyed phosphoric acid-swollen cellulose) and to release dyed-cellulose degradative products into the agar medium below (Highley, 1983). Thus, inability of these fungi to break down phosphoric acid-swollen cellulose in opaque or semi-opaque agar medium apparently cannot be attributed to failure to produce the required enzymes in an agar medium. In the dyed-cellulose assay, the cellulose was layered on top of the agar, rather than suspended in the agar as in the cellulose-clearing assay. This could account for the differences observed in the two assay methods. For cleared zones to be visible in cellulose-agar, the cellulolytic enzymes must diffuse beyond the edge of the fungal mycelium. Thus, the agar may retard movement of, may dilute, or may inactivate the cellulolytic system of the brown-rot fungi unable to clear cellulose agar. Somkuti (1974) found that clearing of cellulose-agar is not sensitive enough to detect the activity of weakly cellulolytic organisms. Therefore, another explanation for failure of certain cellulolytic fungi to produce visible clearing zones may be that cellulase activity is too weak to produce detectable clearing under the culture conditions of the assay.

In another study (Highley, 1988), three brown-rot fungi in the Coniophoraceae were able to solubilize dyed microcrystalline cellulose over an agar medium, indicating that they produced the full cellulolytic enzyme complement. None of the non-members of this family was able to solubilize dyed microcrystalline cellulose, which indicates that the fungi produced deficient enzyme systems under the test conditions. Collet (1984) found that some isolates of *Gloeophyllum* were able to solubilize dyed microcrystalline cellulose. However, Highley (1988) found that 47 isolates of *G. trabeum, G. sepiarium* or *O. placenta* were unable to solubilize dyed microcrystalline cellulose over an agar medium. Hegarty *et al.* (1987) reported that most brown-rot fungal isolates tested were capable of degrading dyed microcrystalline cellulose, but some strains had only weak activity. Thus, the ability of the brown-rot fungi to produce enzymes capable of degrading microcrystalline cellulose (exoglucanase activity) evidently varies with the particular strain of the species used. Production of exoglucanase is not related to the capacity of brown-rot fungi to degrade wood (Hegarty *et al.,* 1987), which means that the enzyme may not he required for cellulose decomposition by these fungi.

3. Effect of carbohydrate and nitrogen source

In contrast to most cellulolytic organisms, many brown-rot fungi produce cellulases on media containing a simple carbon source, such as glucose or cellobiose, as the only carbon source (Bailey *et al.,* 1969; Highley, 1973; Johansson, 1966; Keilich *et al.,* 1969; Reese & Levinson, 1952). Highley (1973) studied the effect of various carbohydrate sources in liquid media on endoglucanase production by *L. lepideus, G. trabeum* and *O. placenta, Gloeophyllum trabeum* was the most adaptable of the fungi studied: it could secrete cndoglucanase regardless of the carbohydrate source. *Lentinus lepideus* and *O. placenta* had trace amounts of growth and endoglucanase activity with cellulose as the only carbon source. Endoglucanase activity was significant in filtrates of these fungi grown in the presence of most other carbon sources.

Using the cellulose-azure agar method to determine endoglucanase activity, Highley (1988) found production of endoglucanase in the presence of simple sugars by all brown-rot fungi examined. Only the members of the Coniophoraceae *C. puteana, Leucogyrophana arizonica* and *L. olivascens* were capable of solubilizing dyed microcrystalline cellulose (exoglucanase activity), but glucose in the medium inhibited this activity. Nitrogen source and concentration had a variable effect on endoglucanase activity in cellulose-azure assays (Highley, 1983). Relatively good endoglucanase activity and fungal growth were obtained on all nitrogen sources; in most cases, concentration did not greatly affect activity. Different nitrogen sources produced optimum endoglucanase activity for each fungus tested.

4. Characteristics of cellulolytic enzymes

(a) Effect of inhibitors

The effect of classical enzyme inhibitors, phenolics, and extracts from wood on endoglucanase from the brown-rot fungi *O. placenta* and *G. trabeum* was studied by Highley (1975 *a).* Endoglucanase was not inhibited by sulfhydryl-binding compounds and metal-completing agents. Sulfhydryl groups apparently are not present at the active site, and the enzyme dots not require a metal for activity. Microbial cellulases are usually inhibited by metals such as copper, mercury, chromium, silver, lead and zinc. Sison *et al.* (1958) found that only mercury inhibited endoglucanase of the brown-rot fungus *Poria vaillantii.* Highley (1975 *a*) found that both mercury and silver inhibited brown-rot endoglucanase. Collet (1987) determined the effect of common wood-preservative metals—copper, chromium and arsenic—on cellulase activities of *G. trabeum* DFP 8437. When the data were

considered in relation to elemental concentrations in preservative treatment solutions, only chromium was present in quantities sufficient for complete inhibition of cellulase enzymes, and thus it may play a role in the toxicity of wood preservative formulations. Phenols have generally been reported to be inactive against cellulases in the unoxidized form (Byrde, 1963; Lyr, 1966; Mandels & Reese, 1963); except for sodium pentachlorophenate, phenols did not inhibit endoglucanase of brown-rot fungi. Upon oxidation, only catechol and vanillin inhibited endoglucanase of *O. placenta* and *G. trabeum.* Wood extracts from heartwood and sapwood of several species with and without peroxidase did not inhibit endoglucanase of the brown-rot fungi. Of several oxidizing and reducing agents tested, only sodium sulfide and copper sulfate pentahydrate plus potassium ferricyanide inhibited brown-rot endoglucanase (Highley, 1975 *b;* Sison *et al.,* 1958).

Thus, compared with other enzymes, cellulases from brown-rot fungi are quite stable to inhibitors and denaturing conditions. Extracellular carbohydrate-degrading enzymes of brown-rot fungi are usually heavily glycosylated. This accounts for their stability (Sharon & Lis, 1982). Tunicamycin, an antibiotic produced by *Streptomyces lysosuperificus,* prevents glycosylation of glycoproteins and can interfere with the secretion of these enzymes (Takatusuki *et al.,* 1975). The effect of tunicamycin on the production of extracellular carbohydrate-degrading enzymes of *O. placenta* was determined in liquid culture by Micales & Highley (1988). Production was inhibited and temperature and pH stability were decreased when the enzymes were formed in the presence of low concentrations of tunicamycin. The enzymes, therefore, may be produced in an active but nonglycosylated (or underglycosylated) form.

Highley & Micales (1990) studied the effect of several aromatic monomers in culture media on the production of β–1, 4-endoglucanase by *O. placenta* and *G. trabeum.* These brown-rot fungi differed in the formation of β–1, 4-endoglucanase in response to the aromatic compounds. In general, however, phenolics did not greatly inhibit production of endoglucanase in liquid cultures. Vanillin and catechol were the only phenolics that completely inhibited enzyme production by *O. placenta,* but they had little effect on enzyme production by *G. trabeum,* The only phenolic completely inhibitory to endoglucanase production from *G. trabeum* was 2,6-dimethoxyphenol.

Many physiological processes in eukaryotic organisms, such as enzyme secretion, are under the control of calcium. Calcium often mediates cellular processes through binding to specific proteins that serve as receptors. Of the calcium-binding proteins, calmodulin is the most widely distributed. Calmodulin antagonists bind with high affinity to calmodulin and thereby inhibit calmodulin-dependent enzymes. Hill & Waggener (1984) reported that the calmodulin antagonists chlorpromazine and trifluoperazine blocked secretion of β–1, 4-endoglucanase by *Trichoderma longibrachiatum* (= *T. reesei),* indicating that calmodulin may function as a regulatory agent in some critical stage in enzyme secretion. Highley (1989) found that several calmodulin antagonists decreased production of β–1, 4-endoglucanase and other extracellular carbohydrate-degrading enzymes of both white- and brown-rot fungi. Inhibition of enzyme production by the antagonists over the 3-week incubation period was about the same for the white-rot fungi, With the brown-rot fungi, however, the inhibitory effect decreased with time. Possibly, the calmodulin antagonists are inactivated by the brown-rot fungi with time.

The addition of nonionic surfactants, such as Tween 80 (polyethylene oxide sorbitan monooleate), has long been known to stimulate enzyme production and/or secretion in bacteria and fungi (Reese & McGuire, 1969). However, Micales (1990) found that Tween did not affect β–1, 4-endoglucanase from *O. placenta.* This author suggests that some factor other than secretion mechanisms, such as microelement or nitrogen availability, limits production of the enzyme.

(b) Physical and chemical properties

Very few data are available on the effects of temperature and pH on cellulases from brown-rot fungi. However, like cellulases (endoglucanases) from other fungi, brown-rot cellulases tend to be rather stable enzymes (Herr *et al.,* 1978; Highley, 1975 *b,* 1976; Keilich *et al.,* 1970). Endoglucanase from *G. trabeum* and *O. placenta* is stable over a temperature range of 23° to 60°C (Highley, 1975 *b*). The temperature optimum for endoglucanases from brown-rot fungi is relatively high: 70°C for endoglucanase from *G. trabeum* (Herr *et al.,* 1978); 60°C, *Phaeolus schweinitzii* (Keilich *et al.,* 1969) and *O. placenta* (Highley, *1975 b);* and 50°C, *C. puteana* (Keilich *et al.,* 1970).

Endoglucanases of brown-rot fungi are stable over the pH range of 2 to 7 (Highley, 1975 *b*). The pH optimum of endoglucanases of brown-rot fungi is usually 2 to 5 (Herr *et al.,* 1978; Highley, 1975 *b;* Keilich *et al.,* 1970). Activity drops substantially at pH 6. Stability of endoglucanases at low pH levels is unusual because most cellulases lose activity at pH 2 to 3. This stability at low pH may be necessary because of the acidic environment created by brown-rot fungi while decaying wood.

Very few attempts have been made to purify cellulolytic enzymes from brown-rot fungi. So-called purified cellulases from brown-rot fungi always contain other enzyme activities, particularly xylanase, and therefore may not be homogeneous. Because the various enzymes are difficult to separate, the term “cellulase complex” or “cellulase system” is often used.

The brown-rot fungus *Phaeolus schweinitzii* produced an endoglucanase with molecular weight of about 45000 daltons, but the purified enzyme was accompanied by mannanase and xylanase

activities (Keilich *et al.*, 1969). Herr *et al.* (1978) purified an endoglucanase from the brown-rot fungus *Lenzites trabea* (= *G. trabeum),* which had a molecular weight of 29000 daltons. The enzyme, however, also hydrolyses xylan. Highley *et al.* (1981) purified a carbohydrate-degrading complex from *O. placenta* active on both polysaccharides and glycosides with a molecular weight of 185000 daltons. In a later study, Green *et al.* (1989 *a)* separated the polysaccharide-degrading activities from the glycosidases in the enzyme complex of *O. placenta.* The molecular weight of the polysaccharide-degrading enzyme was about 45000 daltons; it had activity towards both carboxymethylcellulose and xylan.

IV. Nonenzymatic breakdown of cellulose

1. Low molecular weight agent

Accessibility of wood cellulose to brown-rot degradation remains a question. The relatively large enzymes that have been isolated from brown-rot fungi are too large to penetrate the wood to reach cellulose. In a study of pore size in sound and brown-rotted wood, Flournoy *et al.* (1991) reported that molecules greater than 6000 daltons were excluded from the pores. This result is in agreement with previous studies (Cowling, 1961; Cowling & Brown, 1969; Highley *et al.,* 1983). The TEM studies by Strebotnik & Messner (1988, 1990) demonstrated that enzymes with a molecular weight as low as 16700 daltons did not penetrate the cell walls of brown-rotted wood. Thus, either much smaller cellulase components or other agents that those isolated so far must also be involved in cellulose depolymerization.

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. Cellulose has been the substrate of choice in the search. Lignin is a candidate substrate for a fungal depolymerization agent. This polymer surrounds wood cellulose microfibres and is likely to come into contact with the fungal agent before cellulose. Lignin is chemically altered during brown-rot decay (Jin *et al.,* 1990; Kirk, 1975) and an oxidized polymeric lignin residue accumulates (Agosin *et al.,* 1989). The capacity of brown-rot fungi to degrade lignin model compounds has been correlated with their capacity to degrade cotton cellulose and wood (Enoki *et al.,* 1988). 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 microfibres and are removed from wood by brown-rot fungi (Illman & Highley, 1989).

2. Oxidative degradation

Oxidative depolymerization of cellulose is not an uncommon occurrence. Reports of oxidative depolymerization have accumulated in the literature from diverse research areas. Oxygen free radicals generated for mechanistic investigation by radiation (Phillips, 1980) and systems based on oxygen and metal ions (Herp, 1980) have led to depolymerization of polysaccharides, including cellulose.

An oxidative mechanism of depolymerization by brown-rot fungi has been proposed and accumulating data are lending support to the proposal. Highley (1977) demonstrated that cellulose is oxidized by the brown-rot fungus *O. placenta.* Decay of radiata pine *(Pinus radiata)* by the brown-rot fungus *G. trabeum* was shown to increase with increasing oxygen concentration (Agosin *et al.,* 1989). Brown-rot decay of Japanese cedar and Japanese beech wood was correlated with oxidative capacity of wood-containing fungal cultures as measured by oxidation of 2-keto-4-thiomethylbutyric acid in the media (Enoki *et al.,* 1988, 1989).

Partially characterized brown-rotted cellulose was found to be oxidized and depolymerized (Highley, 1977). Fungal-degraded cellulose and cellulose oxidized by hydrogen peroxide and ferrous salts had several similar characteristics. For both types of cellulose, the DP was drastically reduced (>90%) and volubility in 1% NaOH was similar (30%). Significantly, both types of cellulose had high and similar copper numbers and carboxyl contents. Uronic acids were not detected in acid hydrolysates of the brown-rotted cellulose; however, several other acids were separated (Highley *et al.,* 1989). Analysis of Fenton's oxidized cellulose yielded similar acids (Kirk *et al.,* 1991).

Although the mechanism may differ, the effects produced by brown-rot fungi on cellulose are similar to those of potassium superoxide (Thompson & Corbett, 1985). 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 fibre 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 small size of intermolecular spaces makes penetration by reactants difficult. However, small molecules, such as oxygen radicals, might penetrate. The rapidity of the degradation compared with that caused by acid

hydrolysis suggests that cellulose-metal complexes and oxidation reactions by oxygen radicals are involved in cellulose depolymerization by brown-rot fungi.

3. Oxidative depolymerization model

Many attempts to understand how brown-rot fungi depolymerize cellulose have been based on experiments designed to test a proposed model involving the production of reduced oxygen species. The model is based on evidence from fungal decay studies that oxidation occurs during early stages of decay and that wood pores are too small for known enzymes to access cellulose. The model predicts production of the hydroxyl radical from hydrogen peroxide and ferrous salts by the Fenton reaction. This oxygen free radical would cleave long-chain cellulose into smaller fragments.

The model structure began with the suggestion by Cowling & Brown (1969) that the depolymerization of cellulose under physiological conditions with hydrogen peroxide and Fe^{2+}, as described by Halliwell (1965), might also apply to the mechanism of brown-rot cellulose degradation. Several researchers have added to the model to account for data that might help explain an oxidative, nonenzymatic mechanism of depolymerization. Koenigs (1972, 1974 *a*) reported that some brown-rot fungi produce hydrogen peroxide and that wood contains enough iron for a possible involvement of the iron-peroxide Fenton system in cellulose degradation. Schmidt *et al.* (1981) reported that oxalic acid, which is secreted by brown-rot fungi (Takao, 1965), reduced Fe^{3+}, the species normally present in wood, to Fe^{2+}, the active form in Fenton's reagent.

Supporting and contradicting evidence for various segments (hypotheses) of the model has accumulated. Caveats exist for each piece of evidence but no experimental data have been conclusive. No agent from fungi has been demonstrated to depolymerize wood cellulose to the limit DP. However, no hypothesis of the model has been clearly eliminated. The Fenton-Haber-Weiss system continues to be indirectly implicated in brown-rot fungal depolymerization.

V. Detection of reduced oxygen species

1. Hydrogen peroxide production

The first reports of extracellular hydrogen peroxide production by brown-rot fungi (Koenigs, 1972, 1974 *a, b)* were not easily reproducible (Highley, 1981, 1982). Many researchers reported variable results in detecting hydrogen peroxide in cultures of brown-rot fungi (Highley, 1981, 1982, 1987; Koenigs, 1972), in contrast to easily detectable levels of hydrogen peroxide produced by white-rot wood-decay fungi (Highley, 1977). Detection systems were based on colour changes in media from oxidation of chromophores ranging from bovine hemoglobin or sheep's blood medium to catalase—aminotriazole. 3,3'-diaminobenzidine tetrahydrochloride (DAB), *o*- dianisidine, and titanium tetrachloride.

When solid culture medium was amended with a more sensitive detection system of 2,2'-azino-di-3-ethylbenthiazole-6-sulphonic acid (ABTS) and horseradish peroxidase, hydrogen peroxide was detected in two of thirteen brown-rot fungi and five of seven white-rot fungi tested. The ABTS system was used to study the effect of culture conditions on production of hydrogen peroxide (Highley, 1987). By altering the nitrogen supply in glucose-supported agar medium, six of thirteen brown-rot fungi produced hydrogen peroxide, as determined by medium colour. In agar medium with low nitrogen (0.02% NH_4NO_3) supported by glucose, mannose, xylose, cellobiose or malt extract, hydrogen peroxide was detected in 11 of 13 brown-rot fungi. All seven white-rot fungi in the study produced hydrogen peroxide (Highley, 1987).

Two of four brown-rot fungi gave a positive test for hydrogen peroxide in solid, wood-based culture medium amended with ABTS-peroxidase (Ritschkoff *et al.*, 1990). Hydrogen peroxide was not detected by an oxygen electrode system in culture filtrates of either white- or brown-rot fungi (Veness & Evans, 1989).

Because agar reportedly acts as a quencher of hydrogen peroxide (Thompson et *al.*, 1986), a liquid culture protocol was designed to test the time-dependent effects of nitrogen and carbohydrate limitation on production of hydrogen peroxide with the ABTS-peroxidase system (Illman *et al.*, 1989 *a;* Illman & Highley, in preparation). Production of hydrogen peroxide as measured by colour change in the culture medium was induced by nitrogen and/or carbohydrate starvation in 11 of 13 brown-rot fungi. The rate of colour change varied with each species, ranging from 4 to 40 days.

In addition to questions about the generation of hydrogen peroxide, the variable results call to question (1) the specificity and sensitivity of detection methods, (2) the variability of species and strains of a given species, (3) culture conditions for assays, and (4) lack of hydrogen peroxide accumulation.

Detection systems for hydrogen peroxide have yielded positive results in chemically defined solid, liquid and wood-based culture media of many brown-rot fungi under specific culture conditions. Such results have not been obtained consistently. Explanations may be found in the developmental

stage of the fungus. Hydrogen peroxide may be produced transiently, *i.e.,* in small concentrations for a short period at a specific hyphal age. If hydrogen peroxide is rapidly utilized or quenched by chemicals in the culture medium, it may not be detected consistently. Inoculum source may also influence detection by determining the stage of hyphal growth in a given test. Variable growth rates have been observed in adjacent mycelial plugs taken from agar stock plates (Illman, unpublished data).

The role of hydrogen peroxide as a diffusible depolymerization agent has been predicted (Cowling & Brown, 1969; Koenigs, 1974 *a, b)* and discussed (Illman & Highley, 1989; Kirk, 1983). Its role as *the* diffusible agent moving from tracheid lumen to an S_2 site of depolymerization has not been demonstrated. If produced, hydrogen peroxide may be generated in close proximity to its site of action, in or near the hyphal sheath. This view is supported by observations that brown-rot degradation of cellulose fibres and wood (Highley *et al.,* 1983, 1985) occurs when the sheath is in contact with these substrates. The proposed model of the sheath as facilitator of degradation also supports a model of time-dependent generation of hydrogen peroxide that is quenched by a number of site-specific oxidative reactions.

One-electron oxidation of 2-keto-4-thiomethylbutyric acid (KTBA) as measured by ethylene production was used to assay for oxidizing agents in brown-rot culture filtrates (Enoki *et al.,* 1989, 1990). The KTBA is nonspecific and is oxidized by the hydroxyl radical, cerric ammonium sulfate, or peroxidases in the presence of hydrogen peroxide. Enoki *et al.* (1989, 1990) reported that proteins from culture filtrates of brown-rot fungi tested produced ethylene after nicotinamide adenine dinucleotide (NADH) addition in the presence of oxygen, which was decreased by addition of catalase. Ethylene was produced when hydrogen peroxide was added under 100% N_2 atmosphere. The authors interpret the data to mean that the proteins are hydrogen peroxide dependent as well as capable of generating hydrogen peroxide.

The possibility that fungal autolysis is the source of hydrogen peroxide or other oxidizing agents responsible for positive results has not been ruled out. The most successful tests for hydrogen peroxide were under conditions of carbohydrate and/or nitrogen starvation, which could lead to autolysis (Morton, 1951), mimicking fungal action in wood (Cowling & Merrill, 1966).

2. Oxygen free radicals

The presumptive role of hydrogen peroxide is the generation of the hydroxyl radical in a reaction with a metal or a metal chelate. The superoxide radical is a candidate precursor for generation of hydrogen peroxide. Thus, assays for a depolymerization agent have been based on detection systems for oxygen free radials.

Several laboratories have used chemical assays to survey fungal cultures and rotting wood for the production of extracellular oxygen free radicals. Filtrates of 21-day-old cultures of brown-rot fungi were shown to bleach the spectrum of *p*- nitrosodium methylaniline, suggesting the presence of the hydroxyl radical (Highley, 1982). The assay is not a specific test for oxygen free radicals. Organic radicals and easily auto-oxidizable compounds can produce the same results (Bors *et al.,* 1979).

Veness & Evans (1989) used a silver oxidtition assay to test for the production of free radicals in liquid cultures. White-and brown-rot fungi were grown in silver-lined flasks, Desilvering of the mirror-type flask lining was read as a positive test for radical production. The authors interpreted the results to mean that free radical production had occurred. However, free radical production was only operative over very short distances because desilvering was apparent only when the mycelium was in direct contact with the fask lining. Desilvering occurred after 19 days with *Poria contigua,* whereas *Serpula lacrimans* grew up the walls of the flask without affecting the mirror. Several white-rot fungi desilvered the entire flask lining in contact with culture medium.

Several attempts have been made to determine radical production by employing oxygen radical inhibitors in fungal systems. Several different chelators and radical and peroxide-quenching agents had little effect on weight loss of cellulose or wood blocks by *O. placenta, G. trabeum* or *L. olivascens* (Highley, 1982; Highley & Murmanis, 1985) in soil-block cultures. In this experiment, the toxic chloramphenicols had no effect on wood decay. Weight loss was <2% with thiourea (1.0 M) and *c.* 5% with fluorouracil (0.001 M) compared with 64%, in the control (Highley, 1982).

Lundborg (1988) used radical scavengers in cultures of *Fomitopis pinicola.* The author suggests that depressions in cellulose agar plates where cellulose was most degraded were the results of hydroxyl radical formation. Addition of hydrogen peroxide and ferrous ions to uninoculated agar resulted in agar depressions (dissolved agar). Depression formation was inhibited in fungal cultures by radical scavengers.

A more direct detection of hydroxyl radical in brown-rot fungal systems was made by using electron spin resonance spectrometry (ESR) and a spin trapping technique (Illman *et al.,* 1988 *a,* 1989 *a).* The hydroxyl radical was detected in fungal inoculated wood using ESR and the radical spin trap 5,5-dimethylpyrroline- *N* -oxide (DMPO). The DMPO was used to derivative the hydroxyl radical to a more stable adduct radical that gives a specific, four-line ESR signal. The ESR signal of the hydroxyl radical—DMPO adduct was observed in wood slivers of Douglas fir and white fir when inoculated with *O. placenta* (Illman *et al.,* 1988 *a).* A hydroxyl radical—DMPO signal was also

detected in 10-day-old nitrogen-limited liquid cultures of *O. placenta* supported by 0.1% cellobiose or 0.1% glucose (Illman *et al.,* 1988 *a,* 1989 *a).* The chemical reaction or reactions generating the hydroxyl radical have not been determined.

Extracellular proteins from *G. trabeum* and *Tyromyces palustris* are reported to catalyse a one-electron oxidation as well as produce extracellular hydrogen peroxide (Enoki *et al.,* 1990). Proteins (separated by DEAE—Sepharose) from 30-day-old culture filtrates containing Japanese beech wood oxidized KTBA under an atmosphere of 100% O_2 but not 100% N_2. Addition of an electron donor nicotinamide adenine dinucleotide under 100'7.02 or hydrogen peroxide under 100% N_2 increased KTBA oxidation. Superoxide dismutase decreased oxygen—NADH oxidation by almost 50%, and catalase decreased oxidation almost completely. The radical scavengers guiacol and *N,N'* -dimethyl-4-nitrosoaniline (DMNA) decreased oxidation by about 80%.

The presumptive role of the hydroxyl radical as an agent to oxidize cellulose requires formation at its site of action. The hydroxyl radical is the most reactive of oxygen species, is nonspecific, and does not diffuse, as has been shown for hydrogen peroxide and the superoxide radical (Halliwell & Gutteridge, 1986). Reactions involving the hydroxyl radical may be facilitated by the fungal sheath, as proposed in the model by Green *et al.* (1990).

3. Metals and chelators

Fenton's reagent has been shown to mimic brown-rot degradation of cellulose (Highley *et al.,* 1989), implicating a role for a metal-catalysed Fenton-Haber-Weiss reaction in brown-rot wood decay. 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 spectrometry has been used to detect and follow changes in the oxidative states of paramagnetic metals during brown-rot decay (Illman *et al.,* 1988 *b,* 1989 *b).* Changes in low-spin iron could not be detected at room temperature, and changes in high-spin iron were not tested at low temperature (Illman *et al.,* 1988 *b).* A comprehensive study of iron oxidation states using ESR has yet to be made.

Changes in manganese were observed with ESR after inoculation of susceptible species of wood with the brown-rot fungus *O. placenta* (Illman *et al.,* 1988 *b,* 1989 *b).* These changes were manifested as increases in the size of the sextet spectra that are specific for manganese (Mn^{2+}). The ESR spectra for Mn^{2+} were taken over a 4-week period from fungal-inoculated white fir, Douglas fir, sweetgum and redwood. The increases in Mn^{2+} signals correlated with wood susceptibility to brown-rot decay. Little or no increase was found in wood species resistant to brown-rot decay (Illman *et al.,* 1989 *b).* The chemical basis of the Mn^{2+} change was not determined.

Oxalic acid is a low molecular weight fungal metabolite that is primarily responsible for the low pH (<3) of wood during brown-rot fungal decay (Takao, 1965). The low pH is conducive to the Fenton-Haber-Weiss reaction, which occurs more rapidly at lower pH levels (Halliwell & Gutteridge, 1986). Schmidt *et al.* (1981) demonstrated that oxalic acid can reduce Fe^{3+} to Fe^{2+} under certain conditions. These authors proposed that oxalic acid generates the Fe^{2+} for the Fenton system. We found that oxalic acid mobilizes Mn^{2+} in wood (Illman & Englebert, unpublished data). After treatment of white fir slivers with oxalic acid, the ESR signal for Mn^{2+} increased with increasing oxalic acid concentration. The chemical basis of oxalic acid effects is being investigated.

Iron is expected to be complexed at the low pH levels found in wood decay. Siderophores are the completing agents most often found to bind iron. Extracellular siderophores were detected in liquid and solid cultures of 10 basidiomycetous decay fungi (Fekete *et al.,* 1989; Jellison *et al.,* 1990). Both white- and brown-rot fungi produced the low molecular weight chelators. the phenolate-type siderophores had a high affinity for binding iron (Jellison *et al.,* 1990) and could bind a wide variety of metals (Hider, 1984).

Purified siderophores from cultures of the brown-rot fungus *G. trabeum* were able to carry out one-electron oxidation of KTBA (Chandhoke *et al.,* 1991). The oxidation rate was influenced by siderophore, iron, manganese, oxalate and pH. Degradation of cellulose azure in the presence of iron was shown to be directly correlated with siderophore concentration (Chandhoke *et al.,* 1991).

Iron-containing proteins were separated from culture filtrates of *G. trabeum* and *T. palustris* by DEAE—Sepharose (Enoki *et al.,* 1990). The proteins were determined by gel filtration to have molecular weights of 1650 to 2000 and were capable of oxidizing KTBA. When incubated with ferrozine, the proteins gave an absorbance at 560 nm that disappeared after treatment with one equivalent of hydrogen peroxide, an indication that the Fe^{2+} was oxidized to Fe^{3+} by the Fenton reaction.

VI. Conclusions

Wood degradation appears to involve two sequential (or concurrent) mechanisms, oxidation and hydrolysis. These appear to be tightly coupled reactions. Oxidation is most likely nonenzymatic, with hydrolysis catalysed by a complex of enzymes. A low molecular weight, diffusible element or

elements from brown-rot fungi is or are expected to have the capacity to generate oxygen radicals directly or to initiate a chain reaction resulting in their production. Evidence to date supports the proposal that brown-rot fungi, grown under specific culture conditions, produce reduced oxygen species (possibly including hydrogen peroxide and/or a transition metal chelate) in low concentration. Evidence points to a small (<6000 daltons) diffusible agent or system as the depolymerization factor with the capacity to set in motion a chemical reaction culminating in the oxidation of cellulose. The oxidative mechanism may result in the generation of the hydroxyl or other oxygen radical (or a metal lo-oxygen species) in close proximity to its site of action on cellulose. If hydrogen peroxide is the substrate for hydroxyl radical production, it is most likely produced in small amounts at a distinct stage of hyphal growth. It is quickly scavenged and does not accumulate. Given these criteria, an oxidative agent or agents is or are expected to have controlled transport from site of origin. The proposed model of the fungal sheath provides a vehicle for facilitating transport of degradative agents to the site of decomposition. Association of the cellulolytic enzyme complex with a hyphal sheath component could also Facilitate the hydrolysis of wood cellulose by appropriately juxtaposing the cellulolytic complex with cellulose.

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