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# Selective Delignification of Aspen Wood Blocks In Vitro by Three White Rot Basidiomycetes†

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**Aspen wood blocks were selectively delignified in the laboratory by *Ischnoderma resinosum*, *Poria medulla-panis*, and *Xylobolus frustulatus*. After 8 weeks only the outer surfaces of wood blocks were selectively delignified. The percentages of weight loss obtained after 4, 8, and 12 weeks showed that decay occurred at a relatively constant rate. Selectively delignified wood could be identified by using scanning electron microscopy only when lignin had been extensively removed from cell walls. *X. frustulatus* was able to form pockets of delignified wood throughout blocks after 12 weeks.**

Cellulose, the major component of wood by weight, is surrounded by lignin, a very complex polymer not readily degraded by most microorganisms (5). White rot basidiomycetes are one of the few groups of microorganisms that can degrade lignin. These fungi are capable of removing all of the cell wall components of wood, i.e., lignin, cellulose, and hemicellulose (4, 8). Some white rot basidiomycetes, however, selectively remove lignin and hemicellulose without degrading extensive amounts of cellulose (2). Cellulose fiber has many uses, and wood contains one of the largest reservoirs of cellulose in the world. Cellulose fiber can be used in making paper or as a feed for cattle or can be broken down and fermented to produce ethanol. However, for fungi to be considered for application to industrial biodelignification processes, it is important to understand how the degradation process takes place. In the past, individual white rot fungi have been chosen for lignin degradation studies largely because of their ability to grow rapidly on nutrient media (11). However, white rot fungi are known to degrade lignin in two micromorphologically distinct ways (3, 9, 12, 13). One type of decay is selective for lignin and hemicellulose removal. The other type of decay is nonselective and all cell wall components are removed simultaneously either directly around fungal hyphae, causing erosion troughs and holes, or uniformly from the cell lumen outward, causing a gradual thinning of the cell walls (13, 17). Many of the fungi that appear best suited for lignin removal (i.e., those that selectively remove lignin and hemicellulose) grow slowly on a nutrient medium and thus have been largely ignored in lignin degradation studies. In a recent study *Hirschioporus pargamenus*, a white rot fungus capable of selectively delignifying wood, removed almost all of the lignin from birch wood within 12 weeks, leaving cellulose and hemicellulose behind (Otjen, M. S. thesis, University of Minnesota, St. Paul, 1984). The progressive stages of lignin removal have not been demonstrated. The purpose of this study was to observe, at 4-week intervals, the process of selective delignification of aspen wood in vitro by three white rot fungi which cause selective delignification of wood in forests.

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## MATERIALS AND METHODS

A total of 150 wood blocks 0.7 cm thick were cut from a single 120-cm length of 1.6-cm square aspen wood (*Populus tremuloides* Michx.). The blocks were dried for 48 h at 105°C and were weighed to determine dry weight. They were then moistened with distilled water and placed in 1-ounce (30-ml) glass bottles (height, 5.0 cm; diameter, 3.5 cm) containing 10 ml of vermiculite and 5 ml of distilled water. The bottles were loosely capped, autoclaved for 30 min at 121°C, and cooled. *Ischnoderma resinosum* RAB-82-4, *Poria medulla-panis* RAB-82-5, and *Xylobolus frustulatus* RAB-82-11 were grown in glass vials (height, 7 cm; diameter, 2 cm) containing 5 ml of malt-yeast broth (15 g of malt extract [Difco Laboratories], 2 g of yeast extract [Difco], 1,000 ml of distilled water) for 3 weeks before wood block inoculation. The mycelial mats that grew on the surface of the broth were placed with sterile forceps directly onto the autoclaved blocks. Bottles containing inoculated wood blocks were incubated at 28°C and approximately 80% relative humidity.

Of the original 30 wood blocks inoculated with each isolate, 10 were observed after 4, 8, or 12 weeks. After incubation, the mycelium was carefully removed, and the blocks were dried for 48 h at 105°C and weighed. The percentage of weight loss was then determined for each block. A total of 30 uninoculated wood blocks incubated in the same manner as the inoculated blocks served as controls; 10 control blocks were observed after 4, 8, or 12 weeks.

Blocks were trimmed to a size of approximately 1 $cm^3$ from representative areas of affected wood, hand sectioned with a razor blade, and prepared for scanning electron microscopy. Blocks having the same dimensions were also infiltrated with distilled water by using low vacuum until they sank. The blocks were then mounted in water and sectioned radially, tangentially, or transversely by using a cryostat freezing microtome at −20°C. The sectioned cubes were then thawed, air dried in a desiccator, and mounted for scanning electron microscopy (14).

## RESULTS

After 4 weeks, radially sectioned wood blocks inoculated with *I. resinosum*, *Poria medulla-panis*, and *X. frustulatus* (Fig. 1a, b, and c, respectively) showed no visual evidence of decomposition when they were observed with a scanning electron microscope. After 8 weeks, decomposition was

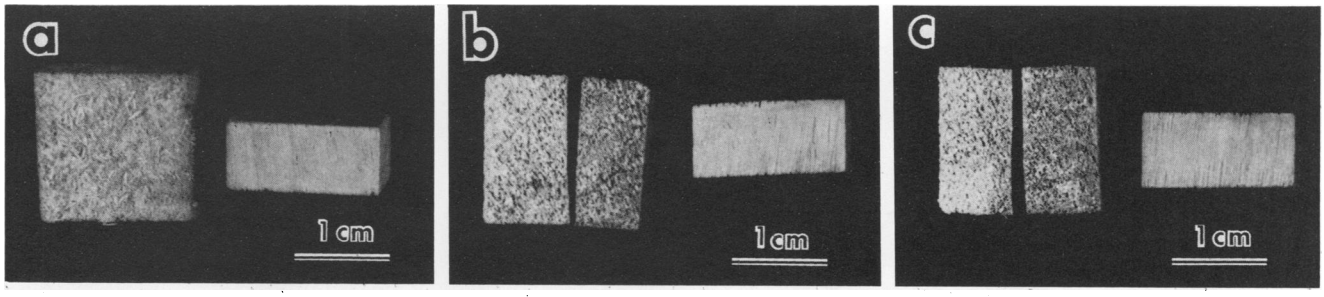


FIG. 1. Tangential and radial sections of wood blocks decayed for 4 weeks by *I. resinosum* (a), *Poria medulla-panis* (b), and *X. frustulatus* (c).

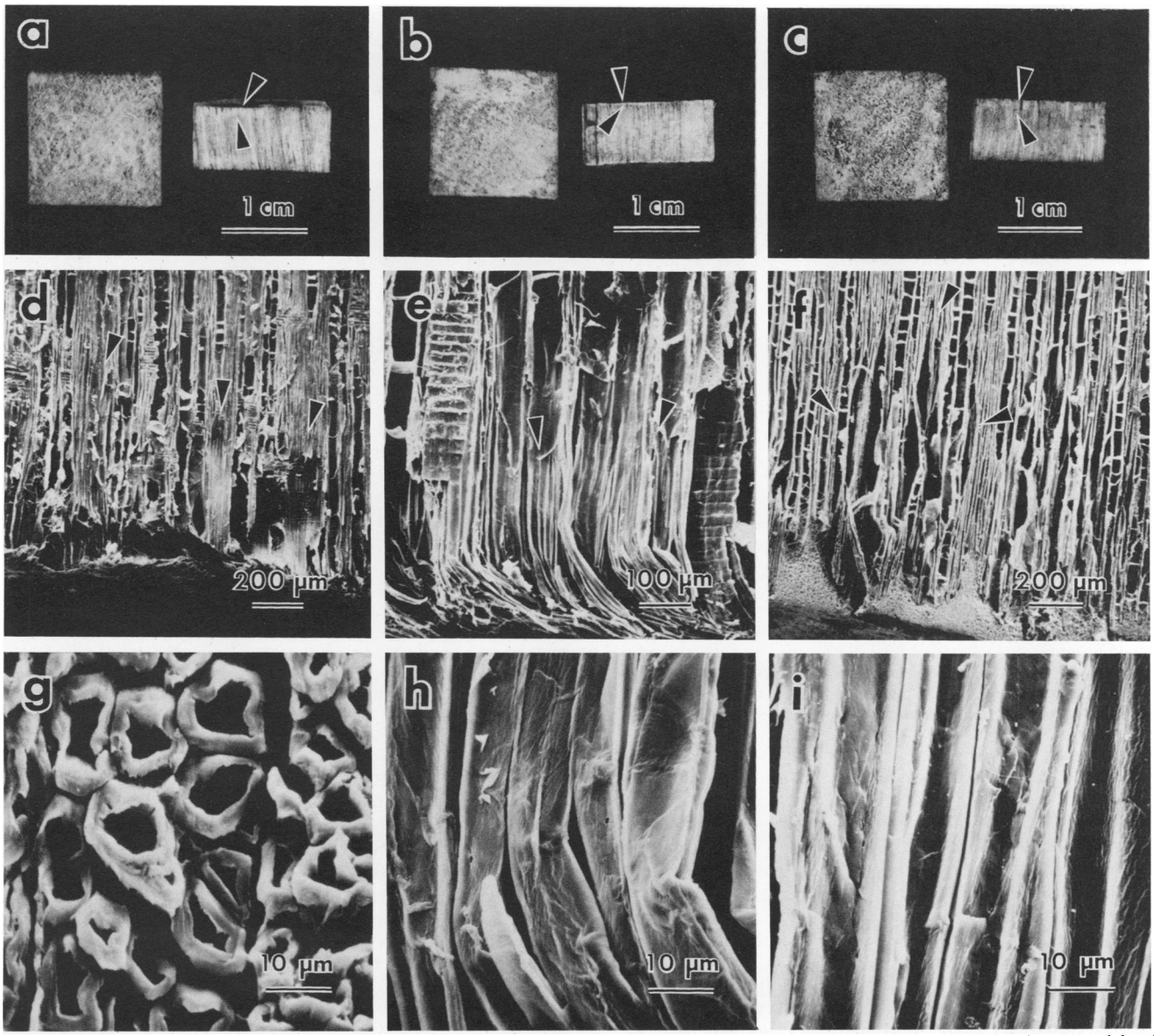


FIG. 2. Wood blocks 8 weeks after inoculation. (a through c) Whitening of the surfaces of wood blocks (arrowheads) caused by *I. resinosum*, *Poria medulla-panis*, and *X. frustulatus*, respectively. (d through f) Loss of structure at the surfaces of wood blocks decayed by *I. resinosum*, *Poria medulla-panis*, and *X. frustulatus* (arrowheads), respectively. (g) Transverse section of cells selectively delignified by *I. resinosum* on the surface of a wood block. (h) Separation of cells due to removal of middle lamellae by *Poria medulla-panis*. (i) Macrofibrillar structure of cellulose in cell walls in cells delignified by *X. frustulatus*.

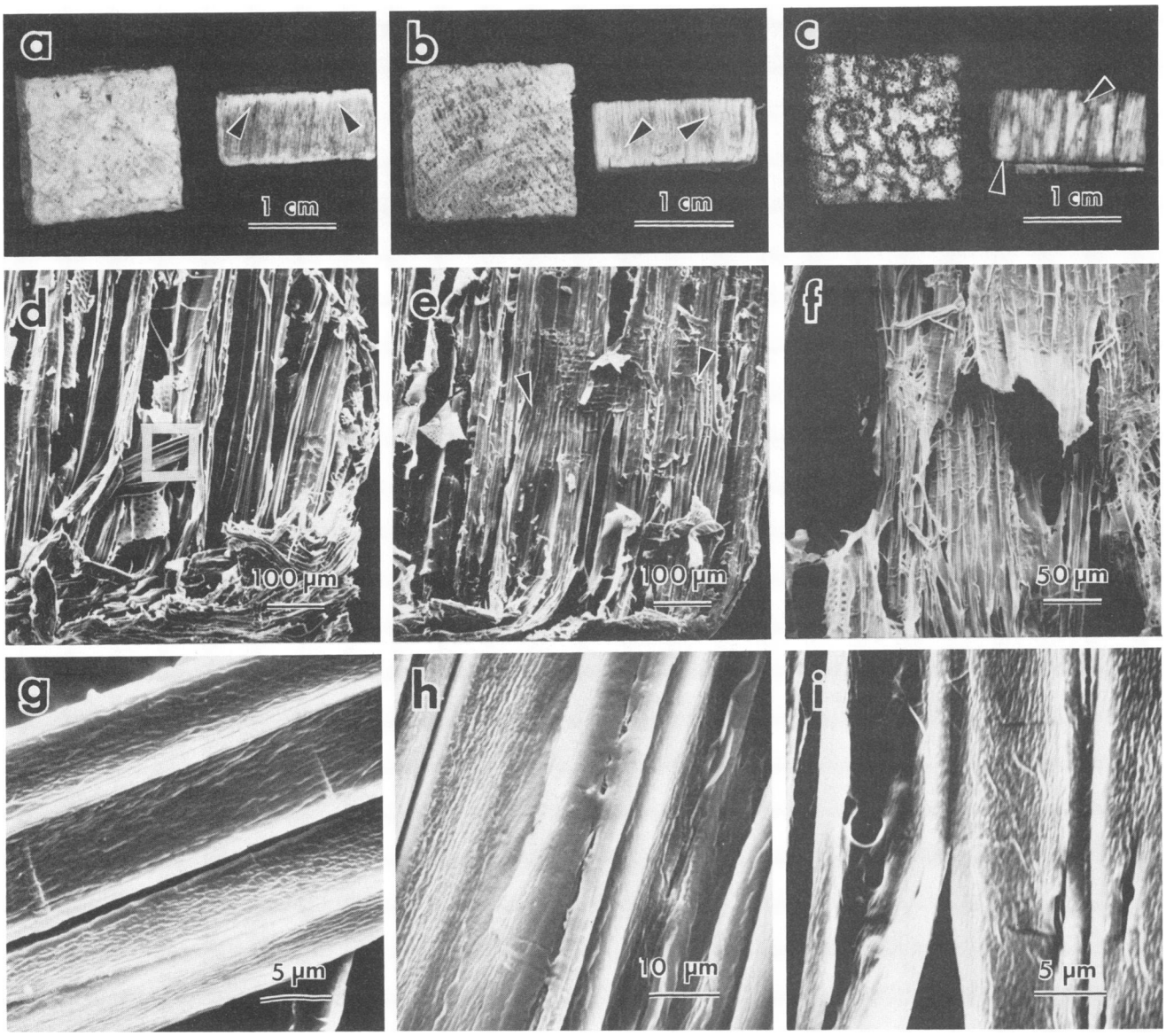


FIG. 3. Wood blocks 12 weeks after inoculation. (a and b) Wood blocks extensively delignified on their surfaces by *I. resinosum* and *Poria medulla-panis*, respectively. (c) Pockets of wood delignified by *X. frustulatus*. (d and e) Scanning electron micrographs of wood blocks decayed by *I. resinosum* and *Poria medulla-panis*, respectively. (f) Localized area of wood delignified by *X. frustulatus*. (g) Higher magnification of delignified cells from the area indicated in (d). (h) Cellulosic macrofibrils in aspen cell walls delignified by *Poria medulla-panis*. (i) Separation of cells delignified by *X. frustulatus*.

apparent due to the bleached appearance of the surfaces of the blocks (Fig. 2a through c). Observation of the same wood blocks with the scanning electron microscope showed the affected areas of wood to have a loss in structure (Fig. 2d through f arrowheads). Decayed wood had lost its integrity, and cells separated readily. All fungi appeared to cause selective delignification and separation of cells by removing lignified middle lamellae. Cellulose macrofibrils were visible in cells that separated (Fig. 2g through i).

At the end of 12 weeks, blocks inoculated with *I. resinosum*, *X. frustulatus*, and *Poria medulla-panis* were more extensively delignified than blocks observed after 8 weeks. Selective lignin removal was restricted to the periphery of wood blocks decayed by *I. resinosum* or *Poria medulla-panis* (Fig. 3a and b). *X. frustulatus* removed lignin from localized areas scattered throughout the wood blocks (Fig. 3c). This decay was similar to the incipient stages of pocket rot found in oak heartwood decayed by *X. frustulatus* in forests (15). Chemical analyses of selectively delignified wood could not be performed as the amount of delignified wood was not sufficient for a separate analysis. From scanning electron microscope observations and histochemical techniques, however, it was possible to confirm that wood was selectively delignified. Loss of structure (Fig. 3d through f), separation of cells, and exposure of cellulosic macrofibrils (Fig. 3g through i) were observed in affected portions of wood blocks. When blocks were stained with phloroglucinol hydrochloride (10), a stain that reacts with lignin to produce a red color, the affected areas of the blocks remained white, indicating an absence of lignin, whereas the apparently lignified tan portions of the blocks turned carmine red. Selective lignin removal was restricted to the periphery

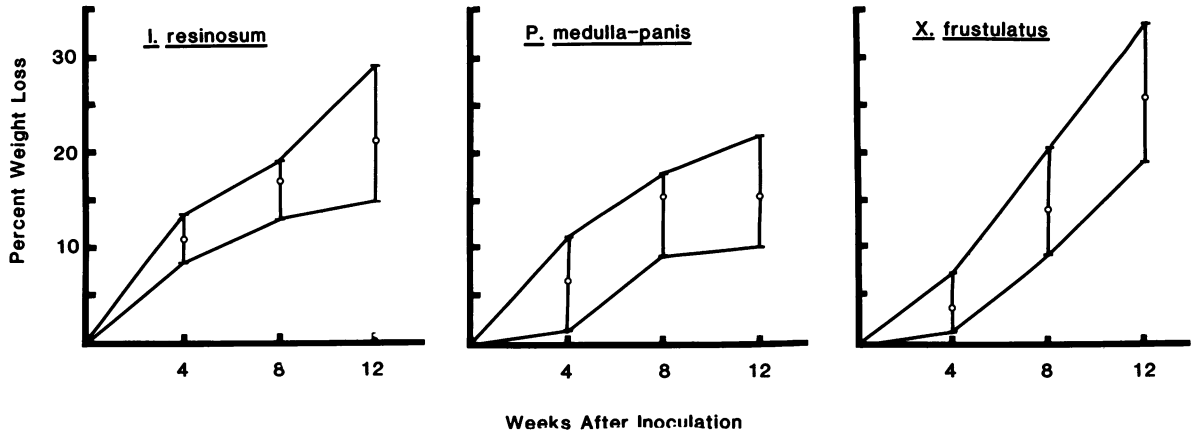


FIG. 4. Percentages of weight loss in aspen blocks caused by *I. resinosum*, *Poria medulla-panis*, and *X. frustulatus* after 4, 8, and 12 weeks. Circles indicate mean values, and bars represent ranges.

of wood blocks decayed by *I. resinosum* or *Poria medulla-panis*. *X. frustulatus* removed lignin from localized areas of wood blocks, producing a pattern similar to the degradation patterns observed in forests during incipient stages of pocket rot in oak heartwood (15). No decomposition was apparent in any of the control blocks at any time during the study.

The percentages of weight loss in the inoculated wood blocks are shown in Fig. 4. Based on observations of the ranges of weight loss values, decomposition appeared to occur steadily over time. After 4 weeks, the weight loss values ranged from 3 ± 2% (mean ± standard deviation) for *X. frustulatus* to 11 ± 3% for *I. resinosum*. At 8 weeks, the weight loss values were 14 ± 4% for *X. frustulatus*, 15 ± 3% for *Poria medulla-panis*, and 17 ± 2% for *I. resinosum*. After 12 weeks, *X. frustulatus* produced the highest weight loss (26 ± 4%). *I. resinosum* and *Poria medulla-panis* produced lower weight losses (22 ± 4 and 15 ± 5%, respectively).

## DISCUSSION

After 8 weeks, all three fungi were able to selectively delignify the surface cell layers of wood blocks. Although *X. frustulatus* was at first limited to the outer cells, it eventually (after 12 weeks) was able to selectively delignify localized pockets of wood throughout the blocks. Degradation was observed on all outer surfaces of wood blocks and not just the surface layer that supported the inoculum. It is possible that the increased availability of oxygen at the surface of the wood blocks enhanced the process of selective delignification in the laboratory. Recently, the involvement of reduced oxygen species, particularly $H_2O_2$, has been found to be necessary for the action of a ligninolytic enzyme produced by *Phanerochaete chrysosporium* (16). $H_2O_2$ production has been markedly enhanced in *Phanerochaete chrysosporium* by growing the fungus under 100% $O_2$ (6). Even though only small volumes of the wood blocks were selectively delignified by the three fungi used in this study, it may be possible to selectively delignify large quantities of wood with *I. resinosum* or *Poria medulla-panis* merely by chipping wood before inoculation to increase surface area or by providing higher $O_2$ concentrations in incubation chambers.

Before 8 weeks, decomposition could not be detected visually. At 4 weeks, averages ranging from 3 to 10% of the original weight of the wood blocks were removed by the fungi used in this study. The rates of decay appeared to be relatively constant for all three fungi tested. Our results indicate that decay was occurring before 4 and 8 weeks, but the gross micromorphological characteristics of the delignified wood could not be seen with the scanning electron microscope at this stage of decomposition. Only after substantial amounts of lignin are removed can scanning electron microscopy detect delignification (3). Meier (13) has shown that lignin can be removed from the cell lumen outward by fungi that selectively delignify wood; recent information (1) supports this view. Thus, even though no signs of selective delignification were visible after 4 weeks, the fungi may have begun degrading lignin within the secondary wall. The macroscopic white appearance of delignified wood and the separation of cells observed with the scanning electron microscope were not evident until the middle lamellae were degraded. This apparently occurred sometime after 8 weeks of degradation.

The percentage of weight loss by wood blocks inoculated with *X. frustulatus* was only 3 ± 2% after 4 weeks. This low percentage of decomposition may have been due to the method of inoculation. Since the inoculum used in this study consisted of a relatively large mat of mycelium, it is possible that the fungus was still utilizing the free nutrients present in the mycelial mat during the early stages of decay. Fenn and Kirk (7) have shown that lignin degradation is a secondary metabolic event in *Phanerochaete chrysosporium* which can be induced by limiting nitrogen. If *X. frustulatus* was utilizing simple sugars and nitrogen from excess nutrient medium remaining in the inoculum, the production of lignin-degrading enzymes could have been inhibited temporarily.

Numerous researchers have shown that white rot fungi can degrade wood in two micromorphologically distinct ways (1, 3, 12, 13). The hypha-bound ligninase produced by some fungi differs greatly from the diffusible ligninase produced by other white rot fungi. Very little actually is known about the differences between the ligninolytic enzymes produced by these fungi. Because little is known about the process of selective delignification, it is often ignored or overlooked in lignin degradation research. Much more research is needed to further define the constraints that restrict the process of selective delignification and to differentiate this process from the hypha-bound lignin degradation caused by other white rot basidiomycetes.

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