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A Simple Method That Uses Differential Staining and Light Microscopy To Assess the Selectivity of Wood Delignification by White Rot Fungi

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Cryostat microtome sections of birch wood degraded by white rot fungi were examined by light microscopy after treatment with two stains: astra-blue, which stains cellulose blue only in the absence of lignin, and safranin, which stains lignin regardless of whether cellulose is present. The method provided a simple and reliable screening procedure that distinguishes between fungi that cause decay by selectively removing lignin and those that degrade both cellulose and lignin simultaneously. Moreover, morphological characteristics specific to selective delignification were revealed.

White rot fungi are unique in their ability to degrade lignin in wood efficiently, and because of this, great efforts have been made to elucidate their potential for application in the pulp and paper industry (4, 12). Promising results have been obtained with certain so-called selective white rot fungi, which are capable of removing lignin from the wood cell wall while leaving the cellulose fibers relatively intact (1, 16). However, there is tremendous variability among white rot fungi in how selectively they delignify wood. Some species, for example, can cause selective removal of lignin at one location and simultaneous removal of both lignin and cellulose at another location in the same wood sample. Even different strains of a single species can show considerable variation (6). Moreover, some white rot fungi seem to switch from selective to simultaneous degradation over time (18). Degradation patterns may also depend on environmental conditions. Therefore, because thousands of white rot fungi exist, it is important to have simple and reliable techniques for evaluation of selective delignification. In this study, we investigated the use of differential staining of wood microtome sections with safranin and astra-blue and found that this procedure readily detects selective delignification in white rot-degraded specimens.

Experimental procedures. *Ceriporiopsis subvermispora* CBS 347-63, *Dichomitus squalens* CBS 432-34, *Phanerochaete chrysosporium* ATCC 24725 (BKM F-1767), *Trametes* (*Coriolus*) *versicolor* CTB-863A, and *T. hirsuta* D-24 (a strain from our Institute) were used in this study. Birch wood (*Betula papyrifera* sapwood) blocks (1.6 by 1.6 by 0.6 cm) were placed in 25-ml glass flasks that contained 10 ml of vermiculite and 6 ml of a chemically defined medium (13) to increase fungal biomass formation and thus enhance degradation. We also conducted parallel experiments in which the medium was replaced by water. Inoculation, growth, and dry weight determination were carried out as previously described by Otjen et al. (19). Incubation times were 2 to 14 weeks, and several wood blocks were used for each incubation time. Uninoculated wood blocks served as controls. Additional samples were also taken from biopulping experiments with birch wood chips treated with *C. subvermispora* or *D. squalens* for 3 to 6 weeks in a 20-liter bioreactor (16).

Wood blocks with weight losses closest to the average weight loss of the replicates from each incubation time were soaked in water under a vacuum and frozen. Cross and tangential cryosections (15 μm thick) of the whole wood blocks were obtained with a Leitz cryostat microtome at −18°C. The cryosections were transferred to glass slides, covered with coverslips, and thawed. Staining with 1% aqueous safranin-O (color index no. 50240; Sigma catalog no. S-2255) and subsequently with 1% aqueous astra-blue (Merck catalog no. 1278) was carried out at room temperature for 3 to 5 min without removing the coverslip. The sections were rinsed with water after each staining step and then embedded in glycerol and examined under a Leitz Orthoplan light microscope equipped with a 35-mm camera using Kodak Gold 100 color film.

General assessment of the method. With a cryostat microtome, good sections were obtained without prior cutting of the wood samples into smaller pieces. Thus, after appropriate staining, the overall delignification throughout a degraded wood sample could be determined in less than 0.5 h. A number of staining techniques, including some that employ safranin and astra-blue, have been described by von Aufsess et al. (20, 21) to visualize the degree of lignification in woody tissues but have not previously been used to assess the selectivity of fungal delignification. Differential staining with astra-blue and safranin proved to be ideally suited for screening in our studies because it gave simple yes-or-no answers and the results did not depend on staining conditions and section thickness. This can be explained by the fact that safranin does not interfere with astra-blue. Astra-blue, a phthalocyanin dye (14), shows affinity for cellulose and is incorporated into cellulose fibers only in the absence of lignin (15, 20), whereas the basic dye safranin (14) stains lignin regardless of whether cellulose is present. The net effect was that the degraded wood cell walls clearly stained either blue or different shades of red. Rapid screening of fungi capable of complete delignification was possible even at a low magnification simply by comparing the ratio of blue to red areas. Moreover, most of the details were retained on color prints, which greatly facilitated analysis of data when numerous samples had to be compared. In addition to its usefulness for screening of fungi, the staining technique also revealed a number of details, which are discussed below.

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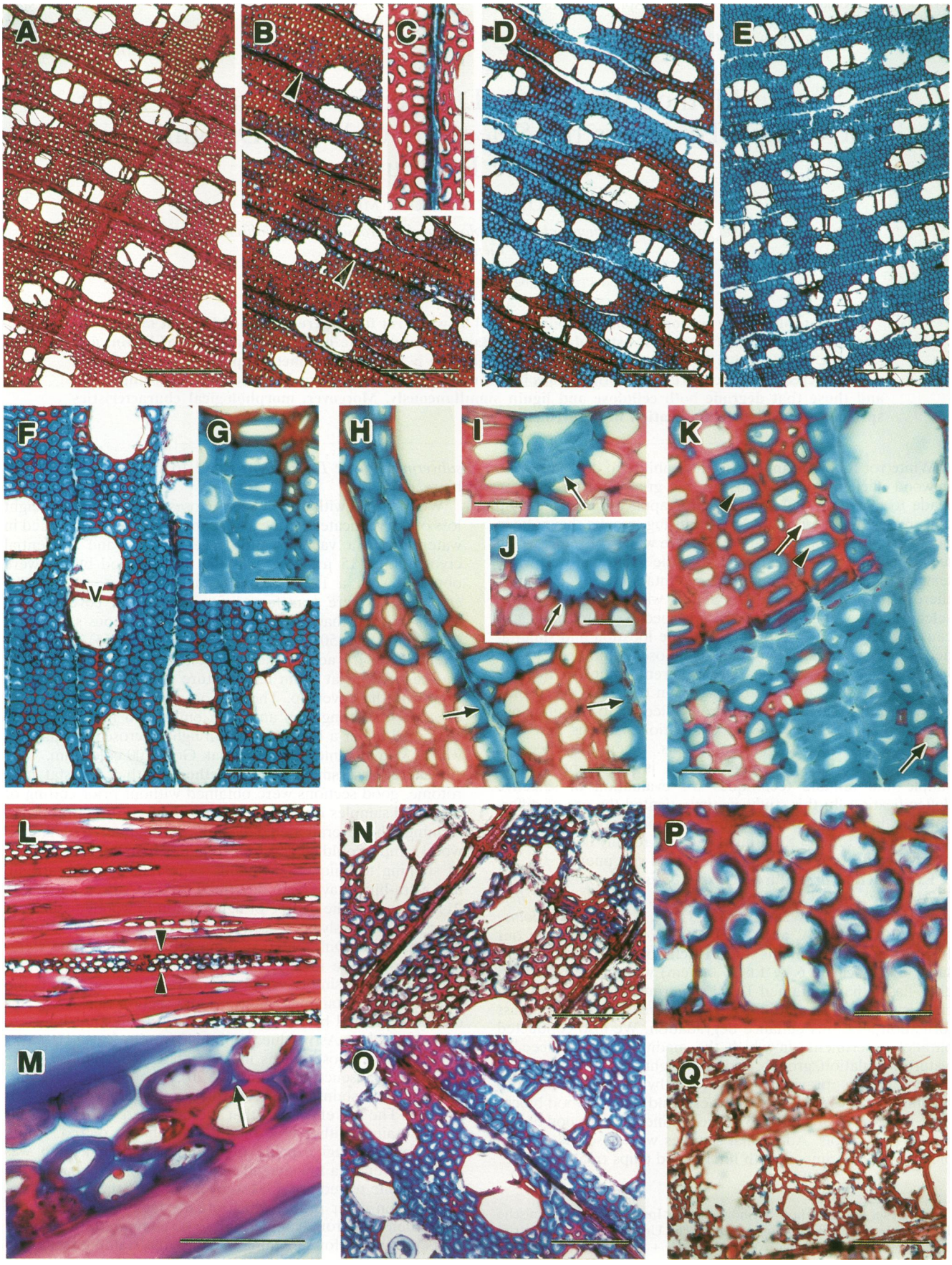
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Previously, scanning electron microscopy, in combination with chemical analysis, has been the method of choice for screening for selective delignification (3, 19). Light microscopy of decayed wood specimens has not been considered useful for this purpose, even though it has been widely used to study white rot decay in general (2, 7, 21–23), and has recently been used to assess the degree of fibrillation in mechanical wood pulps (5). Our results suggest that light microscopy is considerably more sensitive than scanning electron microscopy because it directly shows delignification, whereas scanning electron microscopy shows only fiber separation that occurs as a consequence of advanced delignification (3, 9, 19).

Delignification by *C. subvermispora* and *D. squalens*. Figure 1A to E shows the course of selective delignification by these two fungi over time. First signs of delignification were observed after only 2 weeks (1.5 to 2% weight loss), when ray cells (arrowheads) started to turn blue throughout the wood block (Fig. 1B and C). Delignification of fibers became more obvious after 3 to 4 weeks (with about 5% weight loss; data not shown), and best results were obtained at 10 to 20% weight loss with both fungi (Fig. 1D and E). At higher weight losses, the sections tended to disintegrate because of extensive fiber separation.

Delignification seemed to start randomly throughout a wood block (Fig. 1D), and in the course of time the sizes of delignified areas increased until they finally fused together into large areas of delignified wood, as shown in Fig. 1E (*D. squalens*; 20% weight loss). Figure 1F shows more details at a higher magnification. It also shows the remarkable resistance of vessels (V) to decay (8). In this example, the middle lamellae were still present, holding the fibers together, but in general most sections with this degree of weight loss lacked middle lamellae (Fig. 1G) and disintegrated upon thawing.

Figure 1H to M demonstrates in more detail the heterogeneity of delignification patterns and shows how wood cell walls were attacked in different ways. Since ray cells were generally colonized and delignified rapidly, the fibers attached to them were often attacked first. Figure 1H shows an example in which delignification started from the ray cells and then proceeded into the secondary wall (S2 layer) of adjacent fibers (arrows).

Figure 1K, by contrast, shows concentric delignification of fibers starting from the lumen surface (arrowheads), probably caused by hyphae growing in the lumen of these cells. A blue delignification zone indicating extensive delignification can be seen, but it is also noteworthy that the safranin staining in these cells had changed from pink to red. Previous work (20) has shown that safranin stains highly lignified wood fibers pink, whereas less lignified fibers stain red. This result, therefore, indicates gradual lignin loss from the lumen towards the middle lamella.

Delignified and intact fibers were often found next to each other and clearly delimited along the middle lamella (Fig. 1H and arrows in Fig. 1K), which suggests that diffusion of the delignifying fungal agents through the middle lamella was inhibited or delayed. After longer incubation times, the middle lamella dissolved and delignification spread into adjacent fibers (arrows in Fig. 1I and J). Thus, delignification of the S2 layer of a fiber does not always start at the lumen surface, as had been previously suggested (9).

Figure 1L and M shows tangential sections showing the degradation of ray cells. In rays (arrowheads in Fig. 1L), degradation of the cell corners preceded delignification of the secondary walls (arrow in Fig. 1M), whereas the opposite was observed with fiber cells, as shown above. This suggests that lignin composition rather than lignin content is responsible for the resistance of the cell corner to degradation. In fact, ray cells contain more syringyl-type lignin than do other cell types (17) and syringyl-type lignins are more susceptible to white rot (10).

Delignification by *P. chrysosporium*. Delignification by *P. chrysosporium* was significantly different from the process with *C. subvermispora* and *D. squalens*. Figure 1N provides an average image at 17% weight loss. Note that the weight loss of this sample was about the same as for *D. squalens* in Fig. 1E. Patchy blue delignification zones and simultaneous degradation of cellulose and lignin were observed, but no accumulation of completely delignified fibers and fiber separation, as seen with *C. subvermispora* or *D. squalens*, was apparent. After longer decay times (10 weeks and 25% weight loss), typical selective delignification was observed in some areas of the wood sections (Fig. 1O) but occurred rarely. At higher magnifications, secondary walls undergoing delignification often appeared swollen and detached from the middle lamella (Fig. 1P). This could have been, in part, a sectioning artifact, but it certainly reflects the fact that considerable damage to the fiber structure occurred.

It is curious, in the light of our results, that chemical analysis has shown *P. chrysosporium* BKM F-1767 to be highly selective. Compared with that of *C. subvermispora* and *D. squalens*, the ability of *P. chrysosporium* to delignify wood fibers completely, as indicated by astra-blue staining, was very limited. Otjen et al. (19) obtained a similar result by electron microscopy and suggested that delignification by *P. chrysosporium* might be characterized by incomplete loss of lignin uniformly throughout the wood block. This would be difficult to detect with the staining method used in this study because astra-blue stains only extensively delignified fibers. Another explanation for the apparent discrepancy between chemical and microscopical analyses could be that *P. chrysosporium* partially destroys the fiber structure by converting cellulose to other storage polysaccharides rather than mineralizing it (16). The chemical analysis of glucose in degraded wood samples would not show whether it originated from delignified wood fibers or from polysaccharides produced by the fungus. The available data do not rule out this possibility, and further research is needed to clarify the situation.

Delignification by *T. versicolor* and *T. hirsuta*. Even at 30% weight loss, these typical simultaneous white rot fungi showed

FIG. 1. Delignification of birch wood by white rot fungi. (A) Noninoculated control. (B to G) Delignification by *C. subvermispora* (B to D) and *D. squalens* (E to G) over time; (B and C) 2 weeks and 2% weight loss; (D) 6 weeks and 13% weight loss; (E) 6 weeks and 20% weight loss; (F and G) higher magnification of panel E showing resistance of vessels (V) and middle lamellae (F) and fiber separation (G). (H to M) Birch wood taken from a biopulping reactor after 5 weeks of treatment with *C. subvermispora* showing the heterogeneity of delignification patterns; (H to J) delignification of fibers starting from rays (arrows in panel H) and spreading into adjacent fibers (I and J); (K) fiber delignification starting from the lumen (arrowheads) and intact fibers surrounded by attacked fibers (arrows); (L and M) delignification of ray cells (arrowheads in panel L) in which cell corners were delignified faster than the secondary walls (arrow in panel M). (N to P) *P. chrysosporium* showing simultaneous (N) and selective (O) degradation of birch wood; (N) 6 weeks and 17% weight loss; (O) 10 weeks and 25% weight loss; (P) loss of fiber integrity because of fungal degradation. (Q) Simultaneous white rot caused by *T. versicolor* after 6 weeks and 30% weight loss. Bars: 250 (A, B, D, and E), 100 (C, F, L, N, O, and Q), and 25 (G to K, P, and M) μm.

only narrow delignification zones (Fig. 1Q). Complete delignification of secondary walls or fiber separation was never observed. These "negative" controls demonstrated the usefulness of the staining method for distinguishing different decay types.

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