EFFECT OF WOOD PARTICLE SIZE ON FUNGAL GROWTH IN A MODEL BIOMECHANICAL PULPING PROCESS

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ABSTRACT

The pretreatment of aspen wood chips with white-rot fungus has been evaluated as a way of making biomechanical pulp. Our study addressed (1) whether wood particle size (chip size) affects the growth pattern of the attacking organism, and (2) whether the difference in particle size between chips and coarse pulp is related to the availability of wood polymers to the fungus. We qualitatively evaluated the growth of *Phanerochaete chrysosporium* BKM-F-1767 on aspen wood using standard industrial 6- and 19-mm chips and coarse refiner mechanical pulp. Scanning electron microscopy revealed a slight increase in the number of hyphae in the 19-mm chips compared to that in the 6-mm chips, but no major morphological differences in cellulose or lignin loss. Dense aerial hyphal growth occurred around the chips, but not around the coarse pulp. The fungus appeared to attack the coarse pulp from both outside and within the fiber wall. Hyphae within both the middle lamella and the cell lumina attacked the cell walls. The fungus eroded the chip cell walls and their constituents primarily from the wood cell lumen outward. After only 3 weeks of fungal treatment, both chips and coarse pulp showed marked localized cell-wall thinning and fragmentation as well as generalized swelling and relaxing of the normally rigid cell-wall structure. We conclude that particle size has only a minor effect on fungal growth on wood under conditions such as those likely to be used in a commercial biopulping process.

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INTRODUCTION

Pretreatment of wood chips with the white-rot fungus *Phanerochaete chrysosporium* BKM-F-1767 has been evaluated as a method of producing mechanical pulp (Bar-Lev et al. 1982; Eriksson and Vallander 1982; Eriksson and Kirk 1985; Myers et al. 1988) with less energy (Eriksson and Kirk 1985; Myers et al. 1988) and reduced effluents (Eaton et al. 1980; Eriksson and Kirk 1985). These studies have increased our understanding of the effects of fungi on wood under conditions likely to be used in a biopulping process. In studies at the Forest Products Laboratory, *P. chrysosporium* BKM-F-1767 grew prolifically on 6-mm aspen chips and produced many hyphae. Once the fungal colony had established contact with and obtained nutrient from the wood chips, it increased in size. The organism covered the chip surfaces and entered wood fiber and vessel lumina, where it secreted degradative enzymes, which diffused along the cell lumen surfaces and into the cell-wall layers (Sachs et al. 1989). Questions have arisen about whether aspen wood particle size (chip or coarse pulp) affects the growth pattern of *P. chrysosporium* and whether the difference between chips and coarse pulp might relate to the relative fungal attack of two important cell-wall constituents (lignin and cellulose). The present study was undertaken to qualitatively evaluate growth of *P. chrysosporium* BKM-F-1767 on standard industrial 6-mm and 19-mm aspen chips, and 540-ml Canadian Standard Freeness (CSF) pulp produced from mechanically fiberized 6-mm wood chips.

MATERIALS AND METHODS

Fungal treatment of wood chips

Phanerochaete chrysosporium BKM-F-1767 was grown on aspen (*Populus tremuloides* Michx.) chips supplemented with a chemically defined liquid medium (Myers et al. 1988). Approximately 60 g (oven-dry basis) of 6-mm chips and 60 g (oven-dry basis) of 19-mm chips were supplemented with liquid medium (2.5% glucose, 0.025% nitrogen, minerals, and vitamins) at 60% final wood moisture content. The supplemented chips were then steam sterilized (121 C), inoculated (Myers et al. 1988), and incubated in cotton-stoppered 500-ml Erlenmeyer flasks under ambient conditions; the chips were maintained at ±39 C and 70% relative humidity (RH). All flasks in this study were treated as seed flasks; that is, sterile chips or media were inoculated under a sterile air-flow hood with three sections (1 by 0.5 cm) each of potato dextrose agar from plates with actively growing fungus. At selected intervals, the chips were removed from the flasks. The chips were collected at the end of each week for a period of 6 weeks. Fungal activity was terminated by immersing the colonized chips in a 70% ethanol solution for 15 min.

Fungal treatment of coarse pulp

Coarse pulp was prepared from raw wood chips at the Forest Products Laboratory. The pulp was prepared from 6-mm chips fiberized in a single pass in a

305-mm-diameter single rotating disk refiner at atmospheric pressure. The coarse pulp slurry was caught in a container and dewatered in a bag-lined vacuum crock. The coarse pulp had a Canadian Standard Freeness (CSF) of 540 ml.

Fungal treatment of the coarse pulp was similar to that of the chips. The sterile pulp medium was inoculated in a sterile air-flow hood with three sections (1 by 0.5 cm) each of potato dextrose agar from plates of actively growing *P. chrysosporium* BKM-F-1767. Inoculated coarse pulps were collected at the end of each week for a period of 6 weeks. Fungal activity was terminated as described for the chips.

Microscopy

Sample preparation for scanning electron microscopy.—Dehydration, critical point drying, mounting of dried chips and pulp on aluminum stubs, and vacuum coating with approximately 100 Å of gold have been described previously (Sachs et al. 1989). Samples were examined with a JOEL 840[3] scanning electron microscope using 20 kV.

Sample preparation for transmission electron microscopy.—Wood chip samples were cut into 1.0- by 1.0- by 0.4-mm segments. These segments and small samples of coarse pulp were fixed individually in 2% potassium manganese oxide in distilled water for 1 h, rinsed in distilled water, and dehydrated in Quetol 651 (Kushida 1974) as described previously (Abad et al. 1988), thereby fixing and staining the lignin in the cell walls. The intensity of such staining has been shown to reflect lignin concentration (Blanchette et al. 1987). Thin sections (80 to 120 nm) for transmission electron microscopy (TEM) were sectioned with a diamond knife and then examined with an Hitachi 600 transmission electron microscope.

RESULTS AND DISCUSSION

Fungal action on wood chips

Although the role of microorganisms in biodegradation of wood has been known for some time, understanding of the specific mechanisms of degradation is limited. Similarly, although the use of microorganisms as pretreatments is known to improve mechanical pulp (Myers et al. 1988), the specific mechanisms responsible for such improvement are not known. In our study, *P. chrysosporium* BKM-F-1767 successfully grew across the chip surfaces and penetrated them in both 6-mm- and 19-mm-long chips. Threadlike hyphae formed an aerial network around the chips. Mycelia were prominent more frequently on the surface than in the interior of the chips; likewise, more slender threadlike hyphae were formed on the surface than in the interior. The enzymes produced by the hyphae appeared to have diffused into the cell wall and to have eroded the wall and other cell-wall constituents outward—from the cell lumen toward the middle lamella (Figs. 1 and 2). In response to the enzymatic attack, the cell walls swelled and partially collapsed; localized areas of thinning and fragmentation were also prevalent (Fig. 3). Also, along some cell-wall lumina, enzymes produced troughs along the length of

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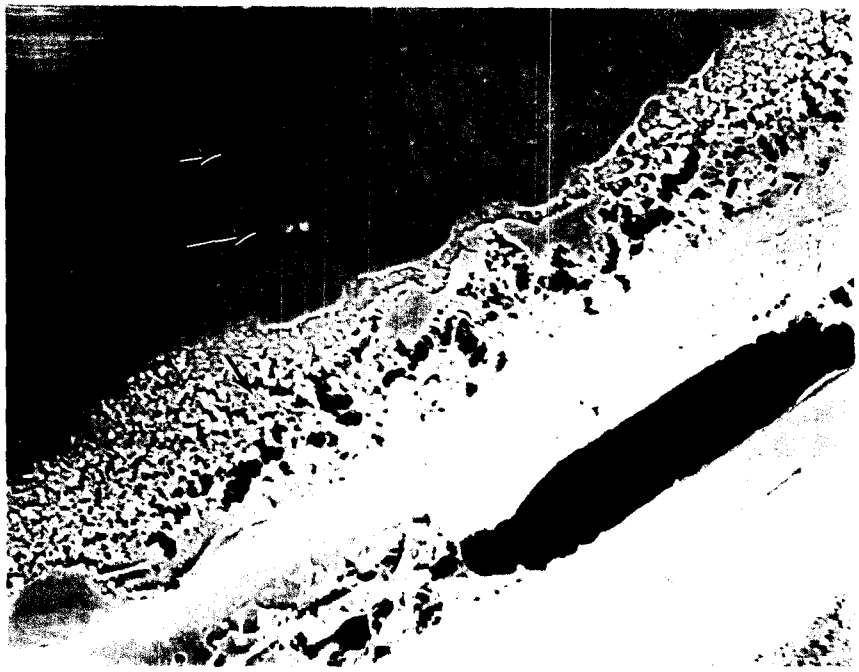

FIG. 1. Scanning electron micrograph of enzyme-etched cell wall (unlabeled arrow). Other arrows show cell lumen (CL) and secondary cell wall (S_2). (×6,000)

the hyphae. Bore holes in the cell walls were formed by enzymes produced at the tips of the hyphae. However, few hyphae were observed within the cell wall itself.

Lignin serves as a binding agent to hold cells together and to impart rigidity to the cell. Lignin occurs throughout the cell wall; it is deposited between and within microfibrils during and after cell-wall thickening (Parham 1983). The distribution of lignin across the cell wall is not the same for all species or for all types of wood within a species (Kutscha and Gray 1970). Lignin may be found in all layers of the cell wall, including the middle lamella, primary wall, and secondary wall (S_1, S_2, S_3) (Wardrop 1965; Sachs 1965). The distribution of lignin within the cell wall can be observed in TEM with potassium manganese oxide fixation (Bland et al. 1971).

In our study, the formation of bore holes and cell-wall degradation by *P. chrysosporium* strongly suggest that the cellulase- and lignin-degrading enzymes of this organism effectively acted upon the cellulose and lignin constituents of the cell wall. The rate of thinning of the cell wall appeared to be influenced by the rate of lignin decay (Fig. 4). The fungal-treated wood sections showed several distinct changes when compared to the untreated sections. First, the lignin was degraded (loss of potassium manganese oxide staining) from the lumen toward the middle lamella. Second, the band of lignin loss, made apparent by potassium manganese oxide staining, appeared to be influenced by the rate of secretion of fungal enzymes; thus, lignin loss varied considerably as a result of differences in hyphal contact and length of exposure. Density staining for the degraded lignin apparently varied considerably as a result of lignin decay, even where the lack of holes or gaps revealed that cellulose had not been removed (Fig. 5).

FIG. 2. Transmission electron micrograph of (A) cell wall nearly normal in width and (B) cell wall progressively thinner in width after lignin and cellulose removal. Lignin degradation is apparent within the cell wall as reduced potassium manganese oxide (arrows). (×12,000)

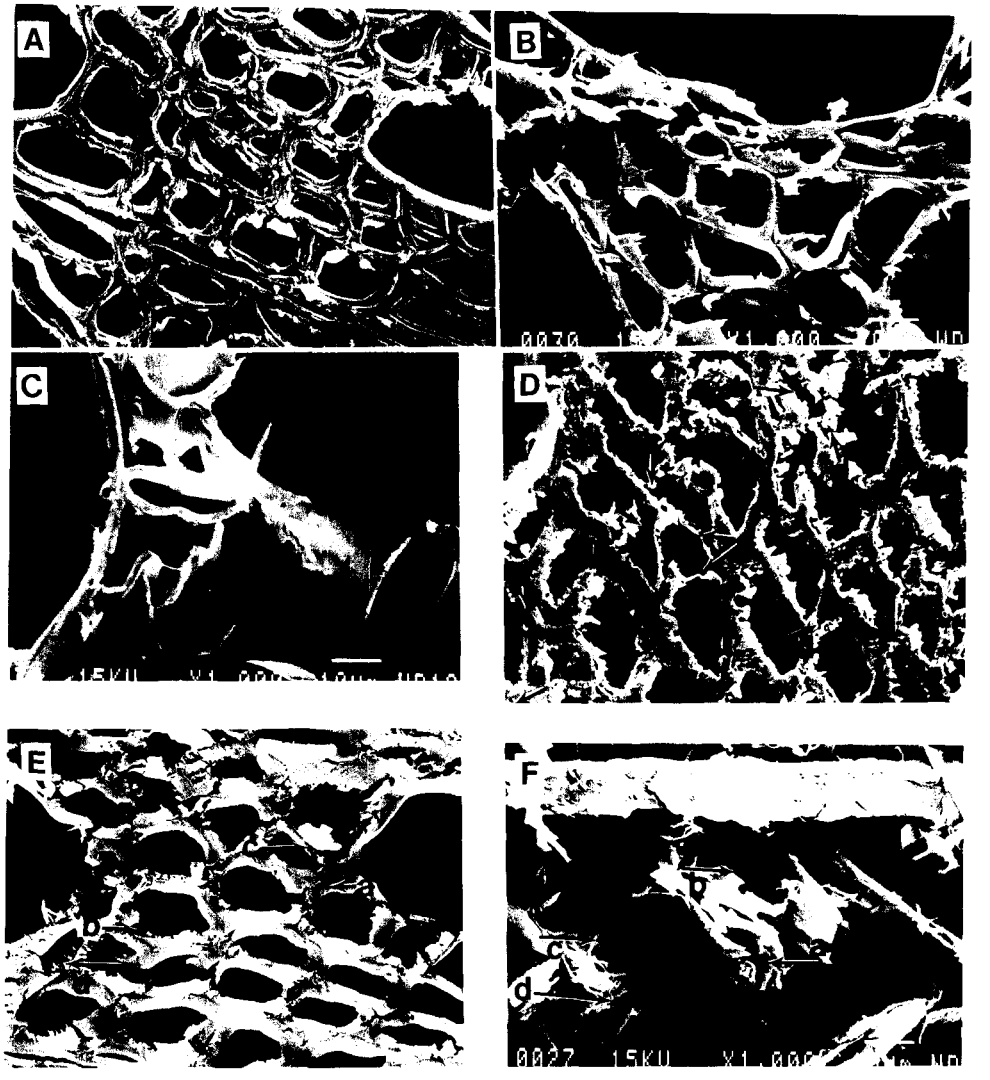

FIG. 3. Normally rigid wood cell structure within an aspen chip (A, B) and aspen coarse pulp (C) modified by fungal pretreatment used in a bench-scale biomechanical pulping process (D–F). Modifications included cell-wall swelling (a), enzymatic softening or relaxing, resulting in partial collapse of cell structure (b), localized areas of wall thinning (c), and fragmentation (d). (×1,000)

The chips as well as their component wood cells retained their overall shape. Chip length appeared to influence only the biomass of organism observed in the wood cell. Because the surface area of the 19-mm chip was greater than that of the 6-mm chip, more biomass was found on the surface and interior of the 19-mm chips. No major differences were observed between the two chip sizes, and the degree of attack and degradation appeared similar. The fungus formed more hyphae around the chips and penetrated the interior of the chips during the 6-week incubation period. With each successive week, more lignin and cellulose of the secondary wall were progressively reduced.

FIG. 4. Transmission electron micrograph of chip stained with potassium manganese oxide. Lignin degradation within cell wall without loss of cellulose (C) after 6-week fungal growth period. CC, cell corner; CML, compound middle lamella. (×11,000)

FIG. 5. Transmission electron micrograph of chip cross section stained with potassium manganese oxide. Lignin density variation (arrows) apparent in early stage of fungal attack. (×16,000)

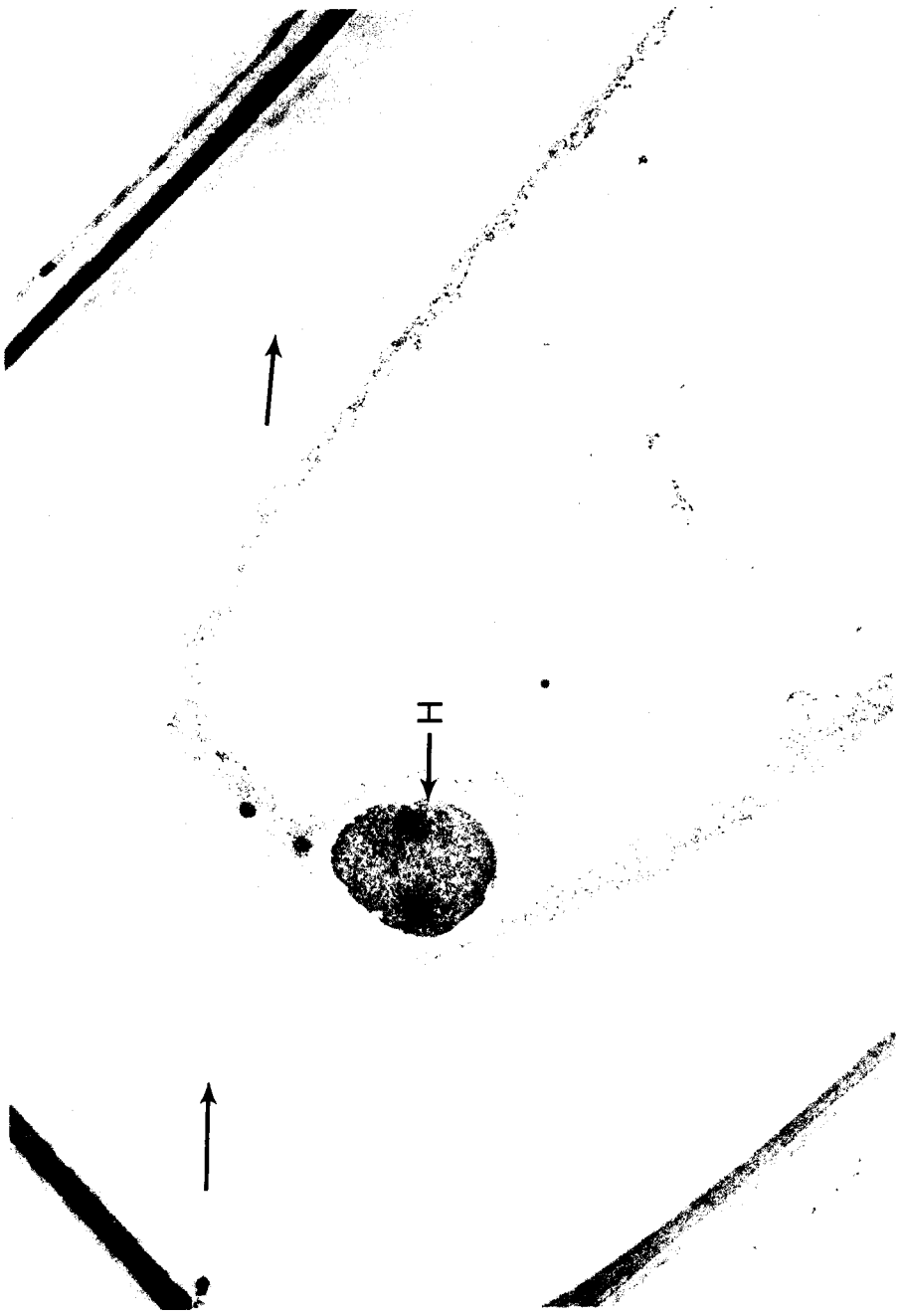

FIG. 6. Transmission electron micrograph of cross section of coarse pulp fiber stained with potassium manganese oxide. Considerable lignin decay in cell wall after 6 weeks' pretreatment with white-rot fungus (arrows). H, hypha cross section. (×11,000)

FIG. 7. Transmission electron micrograph of coarse wood pulp fibers. Cross section of chip with hyphae (H) in the secondary walls. Secondary walls and parts of middle lamella are delignified. (×7,000)

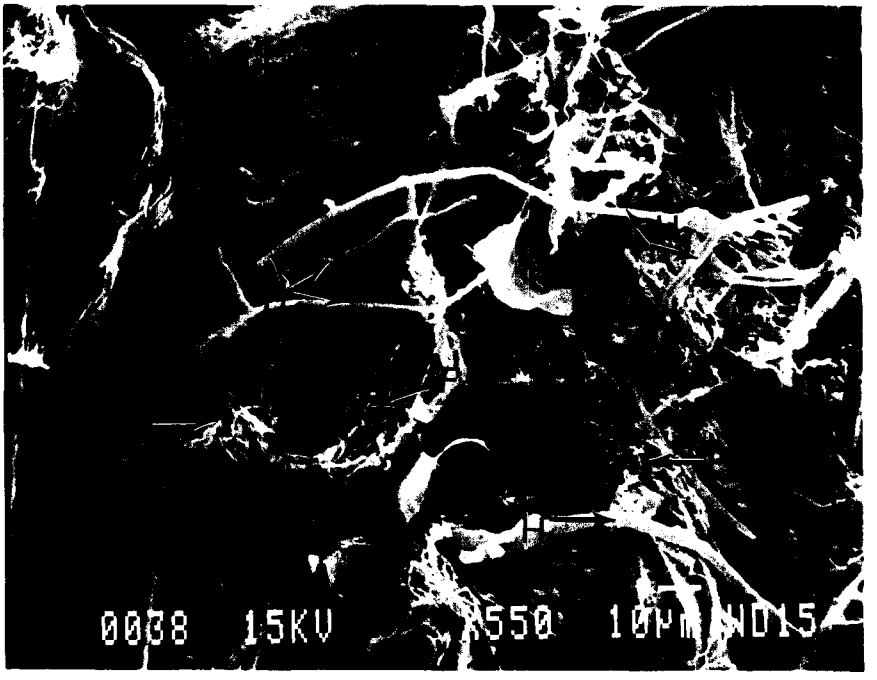

FIG. 8. Scanning electron micrograph of hyphae (H) attacking the surface of coarse pulp fibers (F). (×550)

Fungal action on coarse pulp

Fiber morphology, shape and size, and fiber wall architecture directly influence fiber flexibility and conformability (Parham 1983). Recent studies showed that satisfactory pulps with good fiber flexibility can be produced from biologically pretreated chips (Sachs et al. 1989). The question is whether coarse pulp will be attacked with the same or greater vigor with which *P. chrysosporium* BKM-F-1767 attacks chips.

In our study, we observed that lignin, in combination with the carbohydrates in the pulp fiber wall, retained the fine network originally observed in the chip cell walls (Fig. 4). Much lignin had been removed after 6 weeks (Fig. 6), and the remaining lignin may have been softened as a result of the high temperature used in mechanical pulping (Parham 1983). The coarse pulp was apparently attacked with greater vigor than were the chips because the fungus was able to move readily into the lumen of the fiber and to attack the secondary walls of pulp fibers from within the fiber (Fig. 5). Hyphae appeared to grow preferentially within separations formed in the fiber wall (Fig. 7), where the fungus attacked the surface of the fiber (Fig. 8). These separations, which were caused by mechanical pulping, were a preferred location for fungal attack. This difference in rate of fungal entry into chips and pulp presumably explains why a network of hyphae did not form around the pulp fibers as it had with the chips. However, lignin and cellulose reduction during the 6-week interval appeared to be similar for both coarse pulp and chips. As we observed with chips, more lignin and cellulose of the secondary wall in the pulp were progressively reduced with each successive week. Cross sections of the

fungus inhabiting pulp fibers showed changes such as swelling, partial collapse, localized areas of thinning, and fragmentation of the secondary walls (Fig. 4). Such fungal colonization of coarse aspen pulp fibers with *P. chrysosporium* lessens the amount of energy required to refine the pulp and to increase the strength properties of the resultant paper (Leatham and Myers In press).

CONCLUSION

Scanning and transmission electron micrography were used to investigate the effect of particle size on fungal growth patterns. The effect of pretreatment of aspen chips and coarse pulp with the white-rot fungus *P. chrysosporium* was evaluated by observing the loss of lignin and, by comparison, the distribution of cellulose in microsections of chips and pulps stained with potassium manganese oxide. Greater amounts of fungal growth occurred on wood surfaces than in the internal structure of the wood. The fungus attacked the coarse pulp more vigorously than the wood chips. The lignin-depolymerizing enzymes of the fungus gained early access to the lignin, which was initially decayed from the lumen of the wood cell outward. Of the two cell-wall constituents studied, lignin was decayed at a slightly faster rate than was cellulose. We conclude that pretreatment of chips and coarse pulp with *P. chrysosporium* shows promise as a way of making biomechanical pulp. Our work also indicates that particle size has only a minor effect on fungal growth patterns.

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