EVALUATION OF PHYSICAL CHANGES IN WOOD DURING COLONIZATION BY *CERIPORIOPSIS SUBMERVISPORA*

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ABSTRACT

Mechanical, chemical, and light microscopic methods were used to observe wood cell wall changes during colonization by *Ceriporiopsis submervispora.* Maximum crushing load, dynamic mechanical analysis (DMA), and quantitative Simon's staining were found to be the most useful methods for tracking biopulping action. MOE and loss modulus trended downward within 2 days of inoculation, even though few cells were colonized by that time. Quantitative Simon's staining results were highly correlated with refiner energy savings.

INTRODUCTION

Biopulping is a process in which a selectively delignifying fungus is used to soften wood chips before mechanical refining and papermaking, resulting in energy savings, pitch reduction, and better strength properties. We are interested in how the biopulping process modifies the chemical and anatomical features of the wood and how these changes affect physical and chemical properties. This initial work surveyed physical methods that could be used to indicate the extent and nature of the biopulping action. It was intended to help us focus future efforts. Mechanical refining energy is the key variable in biopulping, but it does not lend itself to understanding fundamental mechanisms. Therefore, we evaluated different mechanical and staining procedures for sensitivity to biopulping action.

During mechanical refining, fibers are repeatedly squeezed between steel bars, resulting in physical separation of the cells and "developing" the fiber (forming fractures within the fiber wall to improve flexibility and removing the compound middle lamella and Sllayers). The great majority of energy expended in mechanical refining goes to fiber development (1). We focused on mechanical properties perpendicular to the longitudinal axis in order to minimize the strength contribution of cellulose microfibrils, which are unaffected by biopulping. We reasoned that the reduced stiffness of the wood after biopulping would correlate with reduced refining energy and increased flexibility of the resulting fiber. Stiffness is quite sensitive to decay (2) and can be evaluated non-destructively. Toughness measurements such as impact bending (2) or work to maximum crushing load are said to be very sensitive measures of incipient white rot.

After evaluating mechanical methods, we chose dynamic mechanical analysis (DMA) to determine the time course of physical property degradation.

Another tool for evaluating the extent of biopulping is Simon's staining (3). Simon's stain is a differential stain used to evaluate pore structure and degree of fibrillation in wood. The smaller blue dye has access to small voids, whereas the larger orange dye out-competes the blue dye in adsorption but cannot access small voids. Untreated wood or fiber stains blue; fibrillated or accessible wood stains orange. We used Simon's stain in light microscopy (LM) to study cell wall porosity and also evaluated a quantitative method to determine if it could predict refiner energy savings.

EXPERIMENTAL

One white spruce *(Picea glauca)* and one quaking aspen *(Populus tremuloides)* tree were harvested in August, cut to 130-cm sections, and immediately frozen. All samples were frozen and/or stored in plastic bags with moist towels at all handling stages to prevent decay and drying below saturation. Control specimens were returned to the freezer after cutting. Treated specimens were placed with chips in 20-L bioreactors and partially sterilized with atmospheric steam for 10 minutes. After cooling, water, corn steep liquor (0.5% on dry wood), and blended *Ceriporiopsis submervispora* L14807-SS3 (0.0005%) were added. Incubation was carried out at 31°C for 2 weeks. After incubation, samples were frozen until testing.

Mechanical tests include crush, impact, tension, speed of sound, and DMA. Specimen dimensions are given in table 1. ("Radial" means load applied from pith to bark.) Crush specimens were compressed between parallel plates at 1 mm/minute. Crush data were analyzed with the "universal macro" from FPL's Engineering Mechanics Laboratory. Impact tests were performed with a Dynatup 8250 impact tester (Instron Corp, Canton, MA) using a chisel tip impacting across the grain at 2.3 m/s with a mass of 6.1 kg. Tension specimens were clamped in flat grips, leaving a 5-cm free span. Grips were separated at 1.45 mm/min. Compression sound wave velocity 'was calculated from the slope in time of transit

Table 1: Specimen dimensions (cm)

	Radial	Tan.	Long.
DMA, LM	1.3	4.0	0.3
Impact, Crush	1.9	1.9	1.9
Tension R (T)	10 (1.3)	1.3 (10)	1.3
Speed of Sound	0.5	30	1.0

measurements from 3 to 30 cm on a ppm-5 dynamic modulus tester. DMA measurements were taken at 20°C with 0.02% strain on a 1.9-cm free span with a DMTA V (Rheometric Scientific, Piscataway, NJ). The DMA measured elastic and loss moduli. Sensitivity of mechanical tests to fungal degradation was determined using the *t* statistic, which is the change in average value divided by the pooled standard deviation.

To observe changes in mechanical properties during the first week of decay, replicate flasks containing two tangential spruce DMA specimens and 100 g pine chips were made for sampling at 1, 2, 3, 4, 5, 7, and 14 days (four samples at each point). Properties of the seven control samples changed due to steaming, and these changes were subtracted from all treatments. Since DMA is nondestructive, the change in properties for each sample was measured. Elastic and loss moduli were recorded at 1.6 and 4.0 Hz, respectively.

Quantitative Simon's staining was used to evaluate the influence of biopulping on fiber porosity, reflecting swelling and crack formation during refining. Fifty milliliters of aqueous solution containing 0.01% blue dye, 0.01% orange dye, and 1% NaCl was added to 20 mg fiber and held at 75°C for 48 hours, followed by dye stripping with pyridine:H_2O, as described by Yu (5). The strippings were analyzed for the ratio of orange:blue dye with uv/vis spectroscopy. Fibers for staining (750–812 csf) were made by single pass atmospheric refining. Refiner energy savings data are based on energy consumption during the entire refining process, with final freeness of 50 (spruce), 100 (pine and aspen), or 400 csf (eucalyphls).

Microscopy samples were removed from the freezer and sectioned on a sliding microtome at 20 µm thickness in accordance with standard microtechnique protocols; the samples were unfixed and were not embedded. Sections were subjected to Simon's staining, phloroglucinol staining, or were mounted in glycerine–ethanol 1 : 1.

Colonization of axial tracheids was determined by light microscopic evaluation of sections mounted in glycerine–ethanol, Only axial tracheids, the lumina of which were crossed by a 200 µm long-line oriented at a 45° angle with respect to the rays, were counted. Fifteen such 200 µm lines in both the earlywood and latewood of multiple growth rings were analyzed such that three linear millimeters of cells were evaluated for each time point. Tallies were kept for those cells in which hyphae were present or absent.

Cells wherein mechanical damage to the section could not be distinguished from fungal hyphae were tallied separately and not included in the colonization calculations.

RESULTS/DISCUSSION

Colonization was studied by counting lumina containing at least one hypha. At 3 days, only 10% of cells contained hyphae; at 7 days there was 75% colonization. The wood surface turned distinctly darker between 3 and 4 days incubation. This coloration penetrated 1mm into the specimen by the 7th day, but not significantly further by the 14th day.

In all the mechanical test methods used, spruce *(Picea glauca)* was more degraded by biopulping than aspen. In crush, tension, impact, and speed of sound testing, mechanical properties (elastic modulus, max loads, energy absorbed, and speed of sound) typically changed by 25–50%,with t statistics ranging from 2 to 7. Maximum crushing strength was the best indicator out of this group of tests for spruce and aspen (table 2). DMA measurements of elastic and loss moduli in spruce tangential specimens yielded t statistics of 10 and 13, respectively.

Table 2: *t* statistic (% change) in max crush strength

	Radial	Tangential
Spruce	12.5 (58)	16.3 (35)
Aspen	7.15 (31)	5.84 (21)

to inoculation reduced MOE (and we expect other parameters) by about 10%. The values reported in the preceding paragraph are a sum of the steaming and fungal degradation effects and so overestimate the biopulping effects. The finding that toughness or energy properties were not the most sensitive indicator of degradation, as the literature suggested, might be due to the fact that *C. submervispora* is a selective delignifier. Its mode of decay is therefore somewhat different from that of other white rotters.

Figure 1 shows the degradation of loss modulus and elastic modulus of steamed and inoculated DMA samples compared to steamed controls. A downward trend in both moduli is suggested as early as the 'second day of incubation, when approximately 5% of cells contained hyphae. The very early changes in mechanical properties and almost linear decay in loss modulus from day 2–14is intriguing. It suggests that some portion of the cell wall is degraded very early in the decay process.

The change in Simon's staining orange/blue (O/B) dye uptake ratio was plotted vs. refiner energy savings (figure 2), indicating a strong correlation despite different species and incubation times. The increase in orange uptake indicates increased porosity

in the cell wall. This is probably due to cell wall swelling and dissolution of cell wall components,

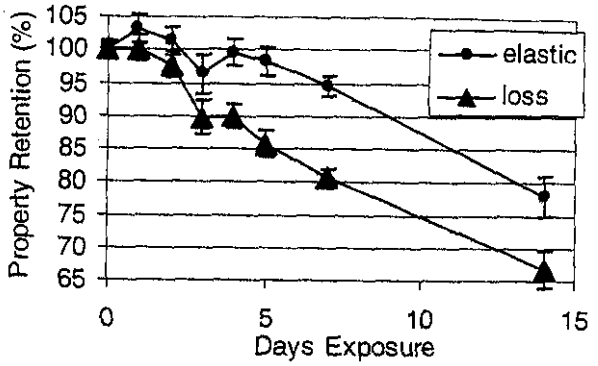

Figure 1: Elastic and loss moduli with biopulping as measured by DMA (90% confidence intervals)

which left a porous network. Forming such cracks is a major part of fiber development, so it seems reasonable that Simon's stain is a good predictor of energy savings. The strong correlation and a $% coefficient of variation for the Simon's stain method suggest that it may be a valuable tool for quantifying changes in cell wall structure during biopulping. It also has the potential to be used as a process control method if analysis time could be shortened.

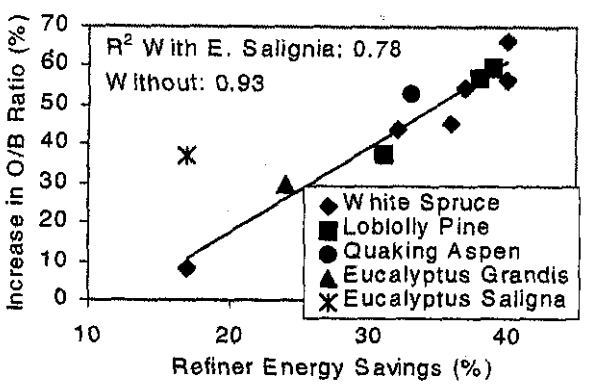

Figure 2: Increase in orange/blue ratio of Simon's stain uptake vs. refiner energy savings

LM examination of 20µm cross sections with Simon's stain (15 min on a hot plate before viewing) showed that in untreated wood, only mechanical damage due to sectioning was light orange, undamaged wood was solidly blue. After 2 weeks of *C. submervispora* growth on spruce, wen carefully sectioned wood showed considerable mechanical damage. Damaged, cells stained intensely orange. Many ray parenchyma cells and most of the earlywood cell walls were completely orange. In contrast, only the S3 and portions of the S2—the portions of the cell wall closest to the lumina—in latewood were orange. The latewood may resist orange stain because the physical and chemical structures were not sufficiently degraded to allow the orange dye to penetrate. Another interpretation is that 15 min was not enough time for the large orange dye to fully diffuse into those spaces during staining.

Phluoroglucinol stains normal lignin but does not react with oxidized lignin (5). The pattern of phluoroglucinol staining was almost identical to the orange portion of Simon's stain. From this we infer that there is little or no time lag between oxidation and opening of pores.

CONCLUSIONS

We have shown that the DMA and quantitative Simon's stain are sensitive tools for evaluating changes in wood during biopulping. Loss and elastic moduli began to decrease almost immediately upon inoculation. Statistically significant changes, were evident by the third day, when only 10% of cells were colonized. An almost linear decrease in loss modulus from day 2 to 14 indicated that there may be some fungal agents produced early in the decay process that act quickly to degrade cell walls, resulting in lower mechanical properties.

Quantitative Simon's staining showed a strong correlation between accessibility of the fiber to a large orange dye molecule after a single refiner pass and energy savings after an entire refining treatment. This suggests that increasing the porosity of the cell wall is a significant physical property change brought on by biopulping.

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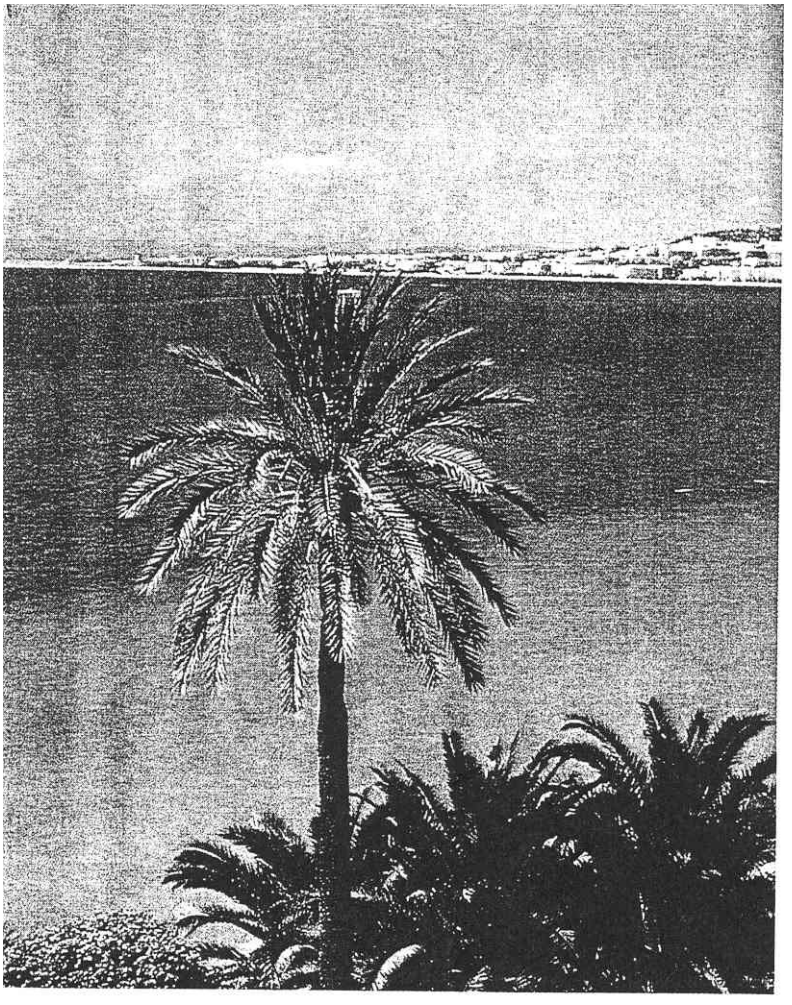

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