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A biopulping mechanism: Creation of acid groups on fiber[1]

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Abstract

We investigated how biopulping modifies chemical and physical properties of wood and how these changes affect the properties of the resulting fiber. Mechanical and chemical testing revealed wood cell changes during 2 weeks of colonization by *Ceriporiopsis subvermispora*. Typical mechanical properties, such as modulus of elasticity and maximum load, tracked reductions in energy needed for mechanical refining to pulp. The data indicate the fiber saturation point of spruce increased from 29% to 42% during biopulping. At the same time, titratable acid groups increased up to 62%. Chemical analysis showed that oxalic acid esters were produced in the wood during biopulping in sufficient amounts to account for the increase in acid groups. The benefits of biopulping – energy savings and increased handsheet strength – as well as other physical property changes are consistent with the mechanism we propose: biopulping increases the acid group content of wood.

Keywords: acid group; biopulping; *Ceriporiopsis subvermispora*; mechanical properties; oxalate ester; oxalic acid.

Introduction

During biopulping, the white-rot fungus *Ceriporiopsis subvermispora* or *Phanerochaete chrysosporium* is grown on wood chips for 10 to 15 days. When these chips are used to produce mechanical pulp, energy for refining is reduced 25% to 35% and the sheet strength properties are typically improved 20% to 40%. Lignin depolymerization was originally assumed to be responsible for biopulping, but the oxidative enzymes are too large to penetrate the secondary wall, even after 2 weeks of fungal treatment (Blanchette et al. 1997). Direct enzymatic action is thus unlikely. Alternatively, the production of low molecular weight diffusible oxidants (Goodell et al. 1997; Johannnes and Majcherczyk 2000; Hammel et al. 2002) such as hydroxyl radical (Backa et al. 1993; Tanaka et al. 1999) has been proposed. The oxidant/radical mechanism is consistent with the chemical changes typically observed after biopulping. Typically there is increase in porosity (Srebotnik and Messner 1994) and weight loss of all components, especially lignin and extractives (Guerra et al. 2003). Rapid degradation of β-O-4 lignin linkages are observed during the first 30 days of fungal decay (Guerra et al. 2002). Large numbers of metabolites have been isolated (Ferraz et al. 2001), including oxalic acid, most of which are expected to participate as metal chelators or oxidation intermediates (Dutton and Evans 1996; Hofrichter 2002; Reading et al. 2003).

Previous work in our laboratory suggested that the fiber saturation point (FSP) of wood increases during biopulping treatment (personal observation). Creating acid groups on wood carbohydrates is an excellent way to increase FSP and paper strength while reducing refiner energy for pulping (Katz et al. 1981; Scallan 1983). This is commonly achieved by adding NaOH during refining, which saponifies esters and results in increased carboxylic acids in the pulp, in addition to the normal swelling with NaOH. The acid groups draw water into the pulp by the osmotic pressure of their counter ions, resulting in refiner energy savings and improved bonding (Katz et al. 1981; Laine and Stenius 1997; Hammar et al. 2000). If the acid groups are added to lignin, as is the case with sulphonation, bonding improves but refining energy generally increases (Salmen 1995; Westermark and Hatakeyama 1996).

We investigated how biopulping fungal treatment modifies the chemical and physical properties of the wood and how these changes affect the properties of the resulting fiber. Physical measurements such as stiffness and toughness were used since both are quite sensitive to incipient white-rot decay (Wilcox 1978); lowering these values in wood should affect refining energy consumption. Modulus of elasticity (MOE) was investigated to help understand the mechanisms of decay. Results from these mechanical tests suggested that acid groups and FSP might be increased. Therefore, titratable acids were measured. Since oxalic acid is widely produced by wood decay fungi (Akamatsu et al. 1994; Young and Akhtar 1998) and oxalic acid cooking produces similar results to biopulping (Akhtar et al. 2002), oxalate esters were investigated as a potential source of acid groups.

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Materials and methods

Fungal treatment reactors were prepared by placing 1 kg fresh industrial chips of white spruce (*Picea glauca*), aspen (*Populus tremuloides*), loblolly pine *(Pinus taeda)*, or eucalyptus (50/50 mix of *Eucalyptus grandis* and *Eucalyptus salignia*) in a 20-l incubator and treating with steam for 10 min. After cooling, water (to 50% solids, w/w), corn steep liquor (0.5% w/w on dry wood), and inoculum were added, and the mixture was incubated for 2 weeks. *C. subvermispora* and *Gloeophyllum trabeum* were added as blended mycelia (0.0005% w/w) and grown at 27°C. *P. chrysosporium* RP–78 was inoculated as spores [5×10^8 (kg dry wood)$^{-1}$] and grown at 39°C. Pulp for titration was refined with a single pass using a 30.5-cm-diameter laboratory atmospheric refiner. Handsheet pulps were refined at atmospheric pressure to 100 ml Canadian standard freeness (CSF) for aspen, one pass at 103 kPa followed by atmospheric refining to 400 ml CSF for eucalyptus or 50 ml CSF for pine and spruce. Energy consumption was recorded with a watt meter attached to the refiner motor.

Mechanical tests

One white spruce and one quaking aspen were harvested for mechanical test samples in August, cut to 130-cm sections, and immediately frozen. All samples were handled to prevent decay and drying below saturation. Repeated freezing did not change mechanical properties. All mechanical tests were done with specimens treated with *C. subvermispora* L14807-SS3 for 2 weeks.

Mechanical tests included crush, impact, tension, and dynamic mechanical analysis (DMA). Crush specimens ($n=12$, 1.9-cm cube) were compressed between parallel plates at 1 mm min^{-1}, and data were analyzed with the "universal macro" from the Engineering Mechanics Laboratory of the Forest Products Laboratory. Impact tests ($n=12$, 1.9-cm cube) were performed with a Dynatup 8250 impact tester (Instron Corp, Canton, MA, USA) using a chisel tip across the grain at 2.3 m s^{-1} with a mass of 6.1 kg. Tension specimens ($n=8$, 1.3 cm longitudinal by 1.3 by 10 cm) were clamped in flat grips, leaving a 5-cm free span. Grips were separated at 1.45 mm min^{-1}.

All the above-described measurements were done in radial (pith to bark) and tangential loading. DMA measurements were made at 20°C with 0.02% strain on a 1.9-cm free span ($4.0\times1.3\times0.3$ cm longitudinal) with a DMTA V (Rheometric Scientific, Piscataway, NJ, USA). Since DMA is nondestructive, the change in properties for each sample was measured ($n=5$). Elastic and loss moduli by DMA are reported at 1.6 and 4.0 Hz, respectively. The sensitivity of mechanical tests to fungal degradation was determined using the signal-to-noise ratio (S/N); the change in average value divided by the standard deviation.

Colonization

DMA samples were removed from the freezer and sectioned to 20 μm on a sliding microtome, then mounted onto a glass slide in glycerine–ethanol 1:1. Lumina of axial tracheids crossed by a line oriented at 45° with respect to the rays were examined for hyphae with a microscope at 400× power. Tallies were kept for cells in which hyphae were present or absent for 3 linear millimeters of cells at each time point (approximately 200 cells).

Moisture interactions

A glove box was attached to the DMA instrument to evaluate moisture effects. Saturated salt solutions were used to control relative humidity in the box containing the untreated and 14-day treated specimens. Samples were equilibrated, weighed, and tested inside the box and were at equilibrium moisture content for each humidity condition. The moisture–strength equation for wood had two unknown values, FSP and strength at 12% moisture, which were determined by a least squares fit.

Acid group content

Coarse pulp was extracted following Tappi T-264-88 with the substitution of toluene/ethanol (32:68 w/w ratio) for benzene/ethanol. Extracted pulp was used for conductometric titrations since extractables might obscure fiber observations. The extracted pulp was alternately soaked and rinsed in 0.1 M HCl (total time 18 h). After rinsing with deionized water, samples were freeze dried.

Conductivity titrations and analyses were by a modification of the method of Katz (Katz et al. 1984). Titration of 1.4 g pulp in 1 l of 0.001 M KCl with 3 ml of 0.1 M KOH was followed over 1 h with pH and conductivity measurements. HCl (20 μmol) was added at the start of the titration to extend the initial strong acid portion of the conductivity curve.

Extraction of acetate and oxalate

Milled wood or pulp samples (0.1 g ± 0.01) were weighed into 15-ml polypropylene centrifuge tubes. Extractant (0.1 M NaOH or 0.05 M H_2SO_4, 2 ml) was added, the contents well mixed, and the acidic samples left at room temperature for 96 h. The basic samples were heated for 1 h at 65°C and left to stand for 96 h at room temperature. Equivalent results in extraction of acetate esters were obtained with incubation of 0.5 or 2 h at 65°C and base concentrations of 0.05 or 0.5 M. The tubes were centrifuged (4000 *g*, 15 min), and the supernatant solution was transferred to a microcentrifuge tube and centrifuged at 21 000 *g* for 10 min. The supernate of the acid treatment was filtered through a 0.45-μm filter into a GC/HPLC sample vial and sealed. The basic supernate was acidified by mixing 15 μl of 50% H_2SO_4 ml^{-1} extract in a new tube and centrifuged. The supernatant solution was filtered into GC/HPLC vials as described in the previous text. All samples were prepared and run in duplicate for both the acid and the base treatments.

Acetate was determined by gas chromatography using a Hewlett Packard 5890A GC equipped with a flame ionization detector and a Porapac Q (Alltech Associates, Deerfield, IL, USA) glass column (6.35×915 mm). Helium (70 ml min^{-1}) was used as the carrier gas and 1-μl samples were injected via an automated sampler. Acetate standards were prepared and run before and after samples. The purpose of conducting the analysis for acetate was to show that the reaction conditions were sufficient to hydrolyze a large fraction of the native acetyl esters to acetic acid. The base treatment resulted in liberation of a large quantity of acetate, whereas the acid treatment resulted in little acetate liberation (Table 1). The acetate esters determined by base extraction (Table 1) agreed with literature values of acetyl content (Isenberg et al. 1980). Acetate values changed by less than 10% with fungal treatment, indicating that fungal treatment did not significantly change the effectiveness of the extraction method.

Oxalic acid was determined by HPLC using detection at 210 nm. Samples (80 μl) were injected into an Ion-300 organic acid analysis column (Phenomenex, Torrance, CA, USA) with 0.005 N H_2SO_4 eluant at 0.5 ml min^{-1} and 67°C. Oxalic acid standards were prepared and run before and after samples. Equal amounts of oxalate were extracted with the acid and base procedure when applied to *C. subvermispora* hyphal cell mass, indicating that the presence of fungal biomass did not significantly affect our conclusions.

Table 1 Acetate extracted from control samples inoculated with *C. subvermispora*[a].

Species	Acetate (mmol kg^{-1})	
	Total	Free
Pine	324	29
Spruce	324	45
Aspen	923	65
Eucalyptus	692	30
Pooled SD	29	15

[a] Samples were frozen immediately after inoculation. Total acid was determined by base extraction, free acid determined by acid extraction. Data represent mean for wood type. Number of determinations: pine, 2; spruce, 10; aspen, 10; eucalyptus, 6. SD = standard deviation.

Identification of oxalate

GC/HPLC samples were tested for the presence of oxalate. Samples (GC/HPLC) from acid extraction, base extraction, and oxalic acid HPLC standards were neutralized to pH 3 by the addition of 1 M sodium tartarate. The neutralized samples were treated with 0.04 units of oxalate decarboxylase (1 unit = 1 μmol oxalate converted to formate and CO_2 min^{-1}; Sigma Chemical Co., St. Louis, MO, USA) for 1 h at room temperature. The samples were then acidified, centrifuged, and filtered, then analyzed by HPLC. Oxalate presence was confirmed by the disappearance of the HPLC peak coincident with the elution of oxalic acid standards.

Results and discussion

Mechanical strength changes

For all mechanical tests on treated wood, a general downward trend in maximum load, MOE, and energy absorbed occurred as treatment progressed (Table 2). In almost every measure, spruce was more degraded by fungal treatment than was aspen, although refiner energy savings for these species was very similar. Mechanical properties changed by up to 58%. Loss modules, as measured by DMA, had the highest S/N (Table 2). The average S/N values for the impact and tension tests (data not shown) were 1 for aspen and 3 for spruce. Spruce tangential specimens had the best overall S/N. Figure 1 shows the degradation of loss modulus and MOE over time, as well as extent of colonization. Most degradation in MOE occurred in the second week of treatment, although a downward trend in both moduli was suggested as early as the second day of incubation. The early changes in MOE and loss modulus shown in Figure 1 suggest that the fungus produces changes in wood cell walls soon after colonization.

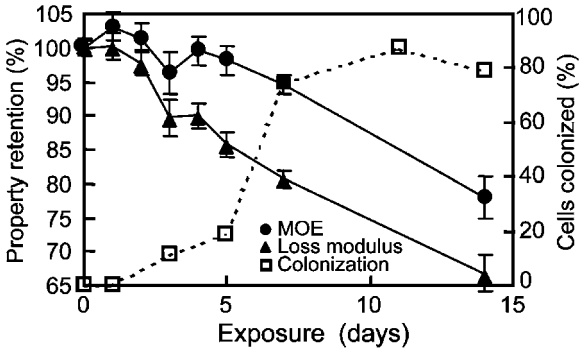

Figure 1 Tangential elastic and loss moduli of spruce with fungal treatment as measured by DMA (90% confidence intervals, $n = 4$). Cell colonization on right axis.

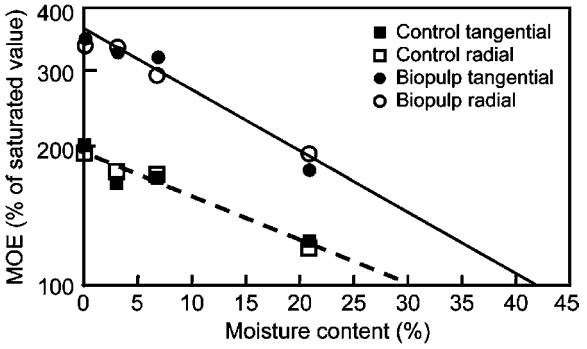

Figure 2 Spruce MOE with moisture content (log scale), as percentage of MOE at saturation.

Effect of moisture

DMA measurements made at various humidity conditions are plotted in Figure 2. The lines represent the general moisture–strength equation for wood (Forest Products Laboratory 1999):

Table 2 Loss in properties and signal-to-noise (S/N) ratio associated with 14-day treatment with *C. subvermispora.*

Sample		Crush test			
		MOE		Max load	
		Δ	S/N	Δ	S/N
Aspen	Tangential	16	4	21	6
	Radial	28	6	31	7
Spruce	Tangential	31	5	35	16
	Radial	55	8	58	12
		DMA			
		MOE		Loss modulus	
		Δ	S/N	Δ	S/N
Spruce	Tangential	22	12	33	21

Δ = percentage of decrease, S/N = change/standard deviation.

Table 3 Acid group content determined by conductometric titrations of pulps.

Wood type	Fungus	Total acid (mmol kg^{-1})		
		Control	Treated	Change
Pine	*C. subvermispora*	99	129	30
	G. trabeum	99	83	−16
Spruce	*C. subvermispora*	96	121	25
Aspen	*C. subvermispora*	80	112	32
Eucalyptus	*C. subvermispora*[a]	52	74	22
	P. chrysosporium	56	59	3
Pooled SD		6	6	8

[a] Pulp was not extracted before titration.

$$P = P_{12}\left(\frac{P_{12}}{P_g}\right)^{\left(\frac{12-M}{FSP-12}\right)} \quad (1)$$

where P is a mechanical property, and subscripts 12 and g denote 12% moisture content and green, respectively. M is moisture content in percent. FSP is the original definition of fiber saturation point (Tiemann 1906), where an inclined line representing the logarithm of strength-moisture content relationship of semi-dry wood intersects a horizontal line representing the strength of green wood. The lines indicate that the FSP of spruce increased from 29% (control) to 42% moisture content after fungal treatment. The line for untreated wood (dashed line in Figure 2), when extrapolated to 41% moisture content (average value after fungal treatment), predicts a 23% decrease in MOE as a result of the added water. This compares to an observed decline in MOE of 22% (Table 2). This agreement suggests that the mechanical property changes observed in biopulping are in large part due to an increase in FSP.

The increase in FSP suggests a change in the acid group content of the pulp. The titration data in Table 3 indicate significant increases of 22 to 32 mmol kg^{-1} in acid groups after effective fungal treatment. A 25-mmol kg^{-1} increase in acid groups on spruce pulp would be expected to increase FSP by 14% (Katz et al. 1981). DMA (Figure 2) indicated an actual increase of 13%, which is in good agreement.

Energy savings during refining appear to be related to acid group generation. Typical energy savings with *C. subvermispora* were near 30%, though generally lower for eucalyptus (19%) (Table 4). *C. subvermispora* generated fewer acid groups on eucalyptus than on other species (Table 3). On eucalyptus, *P. chrysosporium* generated almost no acid groups and produced only 9% energy savings. The brown-rot fungus *G. trabeum* reduced acid group content.

In general, an increase in acid groups also leads to an increase in handsheet tensile strength and better response to beating (Katz et al. 1981; Laine and Stenius 1997). We found the same relationship in biopulped fiber (Tables 3 and 4). For *C. subvermispora*, the increase in acid corresponded to the increase in strength. *P. chrysosporium* grown on eucalyptus had a much smaller increase in acid groups than did the *C. subvermispora* treatment, and handsheet tensile strength was increased only 2.1 N m g^{-1} compared to 5.2 N m g^{-1} with *C. subvermispora.* Another possible result of acid groups would be to modify fracture patterns during refining. Biopulped chips require less energy to attain the same freeness, so fiber length and surface properties may also be affected.

Higher FSP in the carbohydrate fraction of the pulp results in refiner energy savings and improved fiber bonding (Scallan 1983; Leask 1987). If the acid groups are added to lignin, as is the case with sulphonation, bonding increases but refiner energy use generally increases as the lignin becomes more plastic (Salmen 1995; Westermark and Hatakeyama 1996). Therefore, we suspect the acid groups are generated primarily in the carbohydrates. Thus, the data suggest that acid group generation and the resultant increase in FSP are largely responsible for the change in MOE of the wood and the increased sheet strength. The breakage of β-O-4 linkages in lignin (Guerra et al. 2002) also contributes to

Table 4 Oxalate extracted from wood samples after inoculation (control) and 14-day incubation with *C. subvermispora*.

Species		Oxalate[a] (mmol kg^{-1})		Tensile strength (N m g^{-1})	Energy saved (%)
		Total	Free		
Pine	Control	12	5	20	28–34
	Change	+87	+23	+9	
Spruce	Control	13	3	32	28–34
	Change	+66	+17	+7	
Aspen	Control	21	ND	19	26–30
	Change	+59		+8	
Eucalyptus	Control	49	6	6	18–20
	Change	+31	+21	+5	
Pooled SD		24	12		

[a] Total acid determined by base extraction; free acid determined by acid extraction. Data represent mean for wood type. Number of determinations: pine, 2; spruce, 10; aspen, 10; eucalyptus, 6. ND = not determined, due to migration of acid-extractable peak at time of oxalate elution. Handsheet tensile strength and refiner energy savings (compared to control) are reported for a representative sample of each wood species.

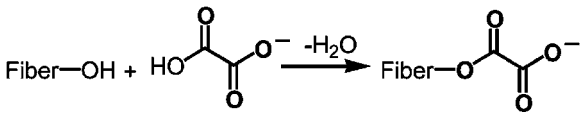

Figure 3 Hypothetical attachment of oxalate esters to wood fiber.

higher FSP by allowing the wood to swell. The total biopulping effect is therefore likely a combination of these factors.

Identification of acid groups

Acids could be created in the wood by oxidation or they could be added to the wood by the fungus. As shown in Figure 3, one end of oxalic acid (or other dicarboxylic acid) could be covalently attached to the wood or pulp via an ester linkage, leaving the second carboxylic acid group free to interact with water. We developed a procedure to saponify and quantify esters and compared this result to the quantity that is freely extractable by acid in the wood samples. Calcium oxalate and free oxalic acid are extracted by both acid and base extraction procedures; however, base would, by saponification, also extract oxalate that was bound as esters and can thus be considered the total oxalate in the sample. The verification of this procedure is discussed in section Materials and methods.

Base-extractable oxalate was greater than acid-extractable oxalate for all fungal treatments where quantification was possible (Table 4). Only those pulps with large total oxalate increases were accompanied by large energy savings and increases in handsheet tensile strength. We propose that oxalate esters such as those in Figure 3 are formed during fungal treatment, and the resulting acid groups produce a significant portion of the biopulping effect by increasing the FSP of biopulped wood.

We analyzed for total oxalate during the incubation of *C. subvermispora* on aspen and spruce to determine if the time course for oxalate formation would parallel physical changes. For both aspen and spruce, the base-extractable oxalate increased dramatically between 11 and 14 days of incubation (Figure 4), the same period during which physical properties of the wood decreased (Figure 1) and refiner energy consumption typically decreased (data not shown). An increase in base-extractable oxalate is also shown for *P. chrysosporium* grown on spruce. The energy savings and improvement in handsheet tensile strength are consistent with increases in total oxalate values.

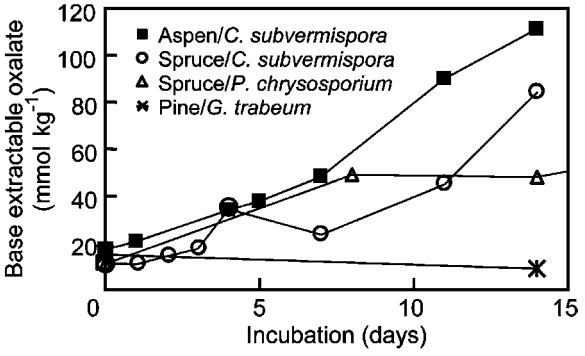

Figure 4 Total extractable oxalate from wood after incubation. Data represent mean of duplicate samples.

Oxalate extractable by acid also increased (Table 4) but was lower in amount and could be influenced by the treatment of the sample. A series of spruce chips treated 0, 7, 10, and 14 days with *C. subvermispora* were analyzed directly after refining to pulp. The reduction in acid- and base-extractable oxalate resulting from refining was the same for the 14-day treatment. This supports our hypothesis that some oxalate can be washed out, but most is covalently linked to the fiber and not removed by mechanical action and washing.

The number of oxalic acid groups added exceeded the observed increase in acid groups. There are two possible explanations for this apparent disparity: (1) some oxalate molecules were esterified on both ends, resulting in decreased acid groups, or (2) the extensive acid washing and extraction treatment for the titrations removed some water-soluble polymers, resulting in an underestimation of fiber-bound acid content.

The addition of chemical units by esterification to the wood substrate is unusual. Oxalate esters have been previously reported in fungally degraded bagasse lignin (Hattori et al. 1993), but their presence was attributed to ring opening reactions. Biological treatments usually create acids by oxidizing or depolymerizing a substrate or by excreting acid. The mechanism of ester formation and the exact location of the esters have not been elucidated but are the subject of ongoing investigations.

Conclusions

Mechanical properties testing established DMA as a useful method for studying fungal treatments. DMA measurements at various moisture contents showed that the softening of wood with fungal treatment is an effect of increased FSP. Time course data showed that mechanical property changes occur very soon after colonization. Conductometric titrations established that fungal treatments that produce good biopulping also generate about 25 mmol kg^{-1} of acid groups. Chemical analyses found about 50 mmol kg^{-1} increase in oxalate esters with treatment.

The data suggest that biopulping fungi esterify oxalic acid to the wood, leaving some free carboxylic acid groups bound to the fiber. These acid groups increase FSP. The increased hydration of the wood results in refiner energy savings and more flexible fibers, which yields better bonding in handsheets. It is possible that the primary action during biopulping is not lignin depolymerization but rather esterification of oxalate to carbohydrates in wood.

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