Direct biological bleaching of hardwood kraft pulp with the fungus *Coriolus versicolor*

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ABSTRACT *Nine fungal strains were tested for their ability to directly bleach hardwood kraft pulp under aerobic, agitated conditions. The white-rot fungus Coriolus versicolor produced the brightest pulp in five-day treatments. The pulp brightness increased by 15 points to 48% ISO, with a corresponding decrease in kappa number from 11.6 to 7.9. The handsheet strength properties were improved in the treated pulp, in spite of a viscosity drop indicating some cellulose chain cleavage. Most of the brightening effect occurred during the second day of the reaction, following an initial lag period. Combined fungal and chemical (DED) bleaching gave a pulp of 82% ISO brightness, whereas a conventional CEDED bleaching gave 88%. The bleaching effect of C. versicolor appears to be limited to hardwoods since experiments with spruce pulp failed to increase brightness.*

KEYWORDS
Bleaching
Brightness
Fungi
Hardwoods
Kraft pulp
Lignin
Microbiology

The white-rot fungi are generally acknowledged to be the most efficient microorganisms for depolymerization of lignin (*1*). *Phanerochaete chrysosporium* has been intensively studied in this regard, and a picture is now emerging of the lignin degradation process. Rapid colonization of lignocellulose fibers by hyphal invasion of the fiber lumen is followed by simultaneous removal of all wood components by extracellular fungal reagents (*2*). A hydrogen peroxide-dependent lignin peroxidase has been implicated (*3, 4*) in lignin degradation, at least when lignin model dimers are used as substrates. Other redox enzymes are probably also required for total conversion of lignin to carbon dioxide and water (*5*). Also, active oxygen species ($^1O_2$, H_2O_2, $OH^\bullet$, O_2^{-1}) and one-electron carriers (e.g. veratryl alcohol radical cation) may allow the modification of lignin in the middle lamella, which is inaccessible to enzymes originating in the lumen (*6*). Besides *Phanerochaete*, many other white-rot fungi, such as *Coriolus*, and *Pleurotus* species, can depolymerize lignin. Their physiology and enzymology may or may not resemble those of *Phanerochaete* (*7*).

Kraft pulp can be partly delignified by *Phanerochaete chrysosporium* when the fungal treatment is followed by alkaline extraction (*8*). More recently, a reduction in lignin content of hardwood unbleached kraft pulp has been observed without extraction (*9*), although prolonged (10-day) treatment in unagitated cultures was required. Whether lignin peroxidase is responsible for the observed effect is presently unclear—only qualitative data has been reported (*10*). However, another enzyme, xylanase, can aid delignification (*11, 12*), presumably by liberating lignin from a complex with hemicelluloses. Thus, if conventional bleaching is preceded by xylanase treatment, a brighter pulp will result.

Direct bleaching of pulp with microorganisms, i.e., without subsequent chemical treatment, has not been quantified until now. Thus, we decided to screen a selected group of microorganisms for their ability to increase the brightness of kraft pulp. We have found from this screening that the species *Coriolus versicolor* No. 52, previously identified as capable of chlorolignin decolorization from bleached kraft effluent from the first E stage (*13*), can bleach kraft pulp by up to 19 points.

Materials and methods

Strains

The following strains were screened:

- No. 52 *Coriolus versicolor* (ATCC 20869)
- No. 170 *Phellinus pini*
- No. 358 *Pleurotus eryngii*

I. The effects of nine fungal strains on brightness, lignin, and cellulose in unbleached hardwood kraft pulp

	Control (pulp only)	Strain #052	Strain #170	Strain #358	Strain #383	Strain #405	Strain #406	Strain #431	Strain #432	Strain #434
Before 2% extraction										
Brightness, % ISO	33.5	48.0	35.8	34.4	35.7	35.8	35.9	35.5	35.4	36.0
Kappa number	11.6	7.9	12	12	10.9	10.2	11.7	12.3	12.4	12.4
Viscosity, mPa*s	17	11.2	15.9	16	13.5	10.9	17.8	16.7	16.8	15.1
Klason lignin, %	1.1	0.7	1.4	1.6	1.1	1.4	1.3	1.6	1.6	2.0
UV lignin, %	0.68	0.63	0.69	0.67	0.58	0.61	0.63	0.63	0.67	0.49
After 2% extraction										
Brightness, % ISO	34.3	49.5	35.9	34.8	35.6	37.0	35.3	34.6	34.9	34.8
Kappa number	10.9	6.5	11.2	12.1	10.4	9.4	11.4	11.7	11.3	11.4
Viscosity, mP*s	15.3	10.4	14.6	14.4	13.4	10.9	16.8	15.8	15.2	14.4
Klason lignin, %	1.0	0.5	1.0	1.2	1.0	1.1	1.1	1.3	1.3	1.6
UV lignin, %	0.70	0.51	0.70	0.67	0.65	0.58	0.69	0.69	0.79	0.86

- No. 383 *Phanerochaete chrysosporium* (ATCC 24725)
- No. 405 *Pleurotus sajor-caju*
- No. 406 *Lentinus edodes*
- No. 431 (Cellulase-less mutant) *P. chrysosporium*
- No. 432 (Cellulase-less mutant) *P. chrysosporium*
- No. 434 *Aureobasidium pullulans.*

The numbers on the left refer to the Paprican culture collection. Strains 431 and 432 were kindly provided by K. E. Eriksson and C. Johnsrud, of STFI, Stockholm. Strain 170 originates from the collection of J. E. Purkynie University, Brno, and Strain 358 comes from F. Zadrazil, Institut fur Bodenbiologie, Braunschweig.

Standard culture conditions

Strains were maintained on malt agar slants. Malt agar plates were inoculated and grown for five days at 30°C. Three disks (about 1 cm in diameter) punched from the growing edge of the mycelium were used to prepare liquid inocula in 500-mL plastic shake flasks, each containing 200 mL of culture medium. The medium was mycological broth, 50 g/L (Difco Labs) consisting of a soybean digest, soytone, 10 g/L, and D-glucose, 40 g/L at pH 4.8. A glass marble was added to each flask to break up the mycelial disks and prevent pellet formation. Flasks were shaken (25°C, 200 rpm) for five days.

Unbleached washed kraft pulp was obtained from two eastern Canadian mills (hardwood, approximate kappa no. 12) or from the Paprican pilot plant (spruce, kappa no. 30). For pulp treatments with fungi, 30 g of pulp was sterilized (121°C, 20 min) in 1 L of deionized water in 4-L polypropylene screw-capped bottles containing sparging tubes for aeration. Liquid inoculum (about 80 mL) was added to each flask, and the total volume of the pulp suspension was made up to 2 L with deionized water. The bottles were shaken at 100 rpm, 30°C, with an air-flow of 2 L/min (humidified air) for up to five days. The pulp was then homogenized with a Brookfield counter-rotating mixer and filtered with fines recycle through a polyethylene macrofilter (Spectrum) of 290 μm mesh.

Pulp and paper testing

Handsheets were made, treated, and tested according to the standard methods of the Technical Section of the Canadian Pulp and Paper Association. The ISO brightness was determined at 457 nm with an Elrepho instrument.

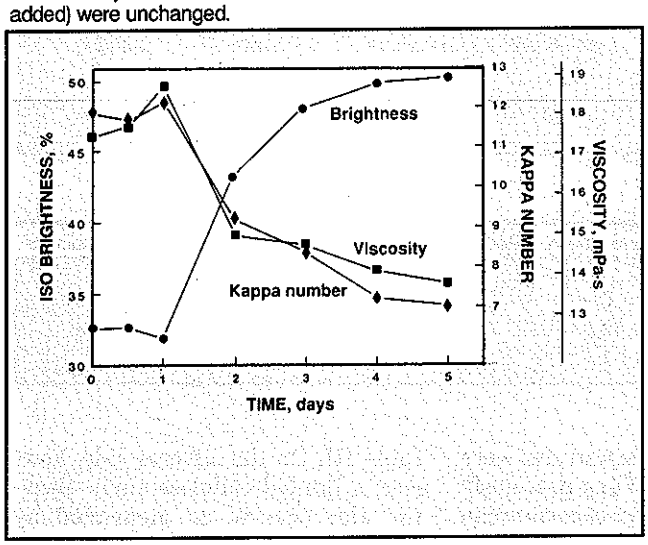
Chemical bleaching

Five-stage bleaching of pulp samples consisted of sequential chlorination (C), extraction (E), chlorine dioxide (D), extraction (E), and chlorine dioxide (D) stages. For samples previously treated with fungus (F), the first two stages were omitted. Chlorinations were carried out with a charge on pulp of 0.2 times the original kappa number at 25°C for 45 min at 3% consistency. Caustic extractions were performed at charges 0.11 times the original kappa for 90 min at 70°C with the pulp at 10% consistency. The dioxide treatments were done with chlorine dioxide and sodium hydroxide charges of 1% and 0.45% on pulp, respectively, while the pulp was at 10% consistency for 3 h at 60°C. For the second D stage, chlorine dioxide (0.45% charge) was added without sodium hydroxide. Upon completion of the chlorine dioxide oxidation, the pulp (1% consistency) was washed and soured with sulfur dioxide gas to a final pH of 5.

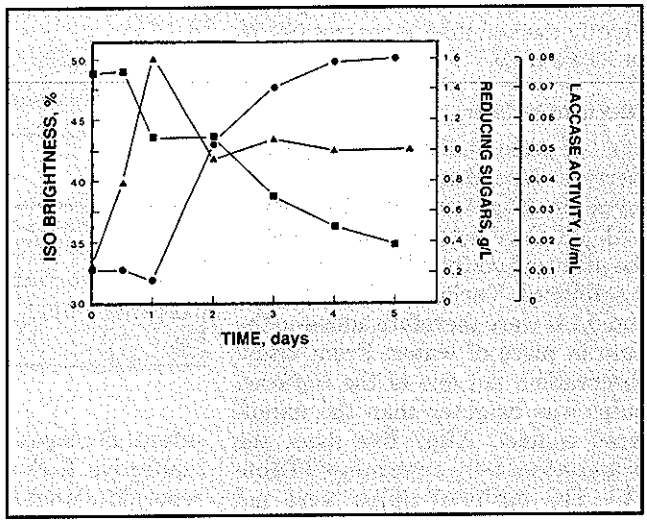
Enzyme assays

Xylanase and cellulase activities were measured by reducing sugar production from larchwood xylan and carboxymethyl cellulose solution, respectively (*14*). Reducing sugars were determined by the method of Miller (*15*). Laccase (phenol oxidase) and peroxidase were measured by the rate of oxidation of 2,2′ azinobis(3-ethylbenzothiazoline-6 sulfonic acid) (ABTS, Aldrich Chemicals) in the presence and absence, respectively, of 1*mM* hydrogen peroxide (*16*). Lignin peroxidase activity was measured by the oxidation of 0.4*mM* veratryl alcohol to veratraldehyde at pH 3.0 in the presence of 0.33*mM* hydrogen peroxide (*17*).

1. The effect of exposure to *C. versicolor* on the brightness, kappa number, and viscosity of unbleached hardwood kraft pulp. Control pulps (no fungus added) were unchanged.



2. Laccase production and reducing sugar consumption during exposure of pulp to *C. versicolor.* Brightness values from Fig. 1 are also shown.



II. Effect of 5-day *C. versicolor* treatment on unbleached kraft pulp from three mills

	Brightness, %	
	Control	*Treated*
Hardwood 1	33.5	48.0
Hardwood 2	33.2	47.7
Softwood	28.2	24.2

III. Physical properties of handsheets from 5-day *C. versicolor*-treated hardwood kraft pulp.

	Control	*C. versicolor-treated*
Freeness, mL	548	520
Basis wt, g/m²	60.1	60.1
Thickness, μm	119	113
Burst index, kPa·m²/g	1.05	1.53
Tear index, mN·m²/g	5.21	6.55
Breaking length, km	2.66	3.27
Stretch, %	1.33	1.74

Results

Screening of fungi

Samples of unbleached hardwood kraft pulp at 1.5% consistency were sterilized, inoculated with each of nine different fungi and incubated for five days with constant agitation and aeration. The resulting mixtures of pulp and fungi were homogenized, made into handsheets, and tested for brightness and lignin content (**Table I**). Relative to an uninoculated control pulp, the *C. versicolor*-treated pulp showed a marked increase in brightness. The increased brightness with *C. versicolor* was accompanied by a decrease in both kappa number and Klason lignin, indicating lignin removal from the pulp. However, the viscosity of the pulp decreased appreciably, indicating that cellulose attack had also occurred.

Extraction of the pulps with 2% NaOH following fungal treatment gave no increased brightness or kappa number decrease for the *C. versicolor*-treated pulp relative to the control. On the other hand, the pulp treated with *P. sajor-caju* did show a slightly increased brightness upon alkaline extraction.

Treatment of other unbleached kraft pulps. Unbleached kraft pulps from two other eastern Canadian mills were incubated with *C. versicolor* under the same conditions. The pulp originating from a mixed hardwood furnish increased in brightness from 33% to 48% ISO (**Table II**). However, a softwood (spruce) pulp showed no brightening.

Time study of *C. versicolor* bleaching reaction

Unbleached hardwood pulp was exposed to *C. versicolor* mycelium for various time periods of up to five days under the same conditions. Most of the brightening effect took place during the second day (**Fig. 1**) and was preceded by a one-day lag period. The decreases in kappa number and viscosity, in Fig. 1, paralleled each other and also showed the greatest change during the second day.

In spite of the viscosity loss, the handsheet properties of five-day-treated pulp from this experiment indicated strength improvements (**Table III**); the tear, breaking length, burst, and stretch values all increased.

Enzymes in solution during bleaching

The following enzyme activities were monitored in supernatants during the time course experiment in Fig. 1: laccase, peroxidase, veratryl alcohol peroxidase, xylanase, and cellulase. Only laccase activity was detected, and this reached a maximum after one day (**Fig. 2**). No cellulase activity was found in the supernatant in spite of the viscosity loss of the pulp. Cellulase production may be repressed by the reducing sugar (D-glucose) initially introduced with the inoculum (Fig. 2). Alternatively, cellulase and other enzymes may adhere strongly to the pulp and would therefore remain undetected in these assays.

The culture supernatant from the five-day-treated pulp was sterilized by filtration and then added to fresh sterilized unbleached pulp. This cell-

free mixture was agitated and aerated for five days, as for the fungal treatment. The resulting pulp was slightly darker than a control (32.5% vs. 34.4% ISO).

Effects of buffers

The lag period before the brightness increase dependent on *C. versicolor* mycelium (Fig. 1) might be associated with pH since between days 1 and 2, the pH dropped from 7.0 to 4.6, where it stabilized. Various buffers at pH 5 and 0.1*M* were therefore added to the pulp in place of water. From visual observation, not one of the buffered pulps was brighter than the unbuffered control. After five days, the brightnesses were determined (**Table IV**). The buffered pulps were not brighter, and, in the case of citrate, the buffer was inhibitory to bleaching action.

IV. Effect of 0.1M buffer addition on the bleaching of pulp by five-day treatment with *C. versicolor*

	Control (unbuffered)	*Dimethyl-succinate*	*Citrate*	*Acetate*
Brightness, % ISO	44.1	45.1	33.9	42.5
Final pH, after bleaching	4.6	4.6	4.9	5.2

V. Effect of glucose concentration on bleaching of pulp by five-day treatment with *C. versicolor*

Glucose conc., mM	0	10	25	40	50
Brightness, % ISO	53.2	55.5	56.3	56.0	59.3

Effect of added glucose

Different concentrations of glucose were added to the standard mixture of mycelium and pulp. After a five-day incubation, a significant increase in brightness was found at higher glucose concentrations (**Table V**). These results were obtained in Fernbach glass flasks, which allowed more agitation of the suspensions than in plastic 4-L bottles.

Combined fungal and chemical bleaching

One objective for fungal bleaching would be to replace chlorine used in chemical bleaching of kraft pulp. A pulp bleached by a conventional sequence (CEDED) was therefore compared with a pulp where the initial chlorination and extraction were replaced by the fungal treatment (**FDED, Table VI**). The fungal treatment left more lignin in the pulp, which probably accounts for the six-point difference in brightness. Initial experiments to determine the yield of the fungal treatment indicated that it was essentially a quantitative conversion. Any pulp loss was compensated by increased fungal biomass.

Discussion

The initial screening of fungi showed that *C. versicolor* (No. 52) was the most effective at direct brightening of the unbleached hardwood pulp. Under the culture conditions employed here, several *P. chrysosporium* strains were ineffective. Kirk and Yang previously reported (*8*) that *P. chrysosporium* can reduce the kappa number of softwood kraft pulp when combined with alkaline extraction. Tran and Chambers (*9*) noted a similar effect with hardwoods after 10-day treatment without alkaline extraction. However, both results were obtained with nitrogen-limited, unshaken cultures at 39°C. Our culture conditions for screening were selected on the basis of simplicity of design for oxygen exchange (agitated cultures) and pulp preparation (pulp in water). Some mycological broth nutrients were carried over with the 15% (volume/volume) fungal inoculum. The cultures were therefore not initially nutrient limited—the metabolic state of *C. versicolor* (primary or secondary) during bleaching is the subject of a forthcoming article (*18*). *C. versicolor* was ineffective on softwood kraft pulp, and this may be related to the fact that *C. versicolor* predominantly attacks hardwoods in nature.

Unlike *P. chrysosporium* or xylanase-treated pulps (*8, 11*), the brightening by *C. versicolor* was not enhanced by alkaline extraction. Since kappa and Klason values decreased following *C. versicolor* treatment alone, it appears that there is a fairly complete mineralization or at least solubilization of lignin in this case. However, both kappa and Klason values (Table I) should be interpreted with caution here, since fungal biomass may interfere with the assays, and the lignin contents are at the limits of detection by the Klason technique.

The strength of the biologically bleached pulp increases, as shown in Table III. On the adverse side, however, a limited cellulose chain cleavage also occurs, as measured by viscosity reduction. Since cellulases could not be detected, the question then arises as to whether a nonspecific catalytic agent, such as hydroxyl radical, is responsible for both lignin and cellulose degradation. If degradation of lignin and cellulose were caused by different reagents, then there could be an opportunity to improve the reaction specificity by separating the active components.

The rate and extent of kraft pulp bleaching with *C. versicolor* depend on a number of factors, some of which have been studied here. Glucose addition affects the extent of bleaching—as was found previously with color removal from bleached kraft effluent (*13*). Laccase and peroxidase have been implicated in lignin oxidation (*19*), and laccase is indeed abundantly produced during bleaching (Fig. 2). However, the extracellular fluid from bleaching cultures was not by itself capable of bleaching. Either there is a rapid turnover of secreted enzymes by the fungus or cell-associated enzymes or other systems are required. Laccase may be one enzyme in the biodegradative pathway, but it is evidently not sufficient *per se* for the bleaching observed here.

Conclusions

The results show that there is some potential for bleaching kraft pulp with microorganisms. Combined with conventional bleaching, bleaching with microorganisms could mean savings in chlorine savings or even the introduction of chlorine-free bleaching. In the future, some effort will be

VI. Combined fungal and chemical treatment of pulp. Initial brightness = 32.7% ISO

	Control pulps		*C. versicolor* treated pulp
	(CEDED)	*(DED)*	*(FDED)*
Brightness (5 day), % ISO	...	...	50.1
Extraction brightness, % ISO	46.9	...	...
First D stage			
Brightness, % ISO	81.7	51.9	62.7
Kappa number	0.9	...	4.3
Viscosity, mPa*s	11.8	...	13.2
Second D stage			
Brightness, % ISO	88.1	71.9	82.3
Viscosity, mPa*s	11.2	...	12.6

devoted to optimizing the process. The biochemical mechanism of bleaching will be studied, since this could lead to more rapid bleaching, especially if an active component can be isolated from the fungus.□

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We thank F. Lafortune and F. Young for enthusiastic and expert technical assistance. This work was supported in part by a financial contribution from the National Research Council of Canada, CA-949-6-0011.

Received for review Sept. 28, 1988.

Accepted Dec. 16, 1988.

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