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Relationship between Fungal Biomass Production and the Brightening of Hardwood Kraft Pulp by *Coriolus versicolor*

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The white-rot fungus *Coriolus versicolor* increased the brightness of hardwood kraft pulp by two mechanisms depending on the concentration of available nitrogen. In low-nitrogen conditions, the brightening process was a chemical effect mediated by the fungus, associated with the removal of residual lignin in the pulp; kappa number was used as an indicator of lignin concentration. A five-day treatment in low-nitrogen conditions increased the brightness of hardwood kraft pulp from 36.2 to 54.5%, with a corresponding decrease in kappa number from 12.0 to 8.5, equivalent to a reduction in the lignin concentration from ca. 2.0% (wt/wt) to ca. 1.4% (wt/wt). Under these conditions, we concluded that the brightening of the pulp was a secondary metabolic event initiated after the depletion of available nitrogen. This method of brightening has been described as bleaching or biobleaching. By contrast, in high-nitrogen conditions, the brightening was a physical effect associated with the dilution of the dark pulp fibers by the relatively high levels of brighter fungal mycelium produced. Since this method of brightening was not evidently associated with lignin removal, it cannot be described as bleaching. In pulp samples brightened in high-nitrogen conditions, as brightness increased, there was a corresponding increase in kappa number. This observation was explained by the consumption of potassium permanganate by the fungal mycelium, which interfered with kappa number determinations at high fungal biomass levels.

Wood pulps are the starting materials for the production of paper. During the pulping process, the fibers in wood are separated by mechanical or chemical means or by a combination of the two. In kraft pulping, wood is cooked in sodium hydroxide and sodium sulfide, which dissolve much of the lignin present in the fibers (4). Not all of the lignin is dissolved, however, and that which remains has been highly modified by alkaline condensation reactions that give the pulp a characteristic brown color (16). The residual lignin can be removed by a subsequent bleaching treatment with chlorine-based chemicals (15). However, environmental concerns are leading scientists to seek alternative ways to eliminate, or at least reduce, the use of chlorine in bleaching.

White-rot fungi are the most attractive candidates for the removal of residual lignin from wood pulps by biological means. The ability of these microorganisms to depolymerize lignin substrates and to degrade lignin model compounds is well characterized (3, 7, 11), although relatively little is known about their ability to selectively remove lignin from wood pulps. Kirk and Yang (12) first recognized that white-rot fungi were able to partially delignify unbleached kraft pulp. However, in their studies, delignification was measured only after alkaline extraction. More recently, Tran and Chambers (22) observed delignification of hardwood kraft pulp by the white-rot fungus *Phanerochaete chrysosporium* without subsequent alkaline extraction, although this observation was made after a 10-day incubation period.

In an earlier phase of this research program, the results of a screening experiment indicated that after 5 days of incubation in aerated agitated cultures, the white-rot fungus *Coriolus versicolor* was able to substantially delignify unbleached hardwood kraft pulp, resulting in a significant increase of the pulp brightness without subsequent alkaline extraction (M. G. Paice, L. Jurasek, C. Ho, R. Bourbonnais, and F. Archibald, Tech. Assoc. Pulp Paper Ind. J., in press). In most white-rot fungi, including *C. versicolor*, lignin degradation is repressed during primary growth but still requires an exogenous energy source (10). Consequently, it proceeds best in nitrogen-limited conditions (13, 18). Thus, if pulp brightening is a direct consequence of lignin degradation, it should be affected in the same way by nitrogen supply. To test this hypothesis, we examined the effect of medium composition on the relationship between the growth of the fungus and brightening of the pulp.

MATERIALS AND METHODS

Organism. *C. versicolor* (L.:Fr.) Quél. ATCC 20869 was used.

Pulp. Hardwood kraft pulp was obtained from an eastern Canadian pulp mill. The species composition of the wood used to make the pulp was maple (46.6%), beech (14.4%), elm (4.5%), poplar (13.3%), basswood (6.7%), birch (7.7%), and softwood (6.9%). After the pulp was fluffed and screened to 0.25 mm in particle size, it was stored at 4°C until use.

Culture conditions. Cultures were maintained in full-strength (i.e., 50.0 g/liter) mycological broth liquid cultures (Difco, low pH product) containing 0.5% (wt/vol) hardwood kraft pulp. The primary inoculation of these cultures was with four 1-cm agar plugs from the growing edge of a 5-day 2% malt agar plate of *C. versicolor* incubated at 27.5°C. After inoculation, cultures were incubated in an air atmosphere (foam stoppers) at 25°C on a gyrotory shaker at 200 rpm (2.5-cm radius) in 200 ml of medium in 500-ml polymethylpentene Erlenmeyer flasks (Nalgene), each containing a 1-cm glass marble. Agitation with the marble prevented fungal pellet formation and resulted in growth as a fragmented hyphal suspension. After 5 days of incubation, cultures were maintained by serially subculturing 2.5 ml of the resultant hyphal suspension into a fresh flask (equivalent to a ca. 0.03% [wt/vol] inoculum).

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Cultures were maintained in this way for a maximum of 10 serial transfers before we started again from a fresh malt agar plate of the fungus.

Liquid maintenance cultures also provided the inoculum for the brightening studies described below. Unless otherwise stated, for these experiments, cultures were inoculated with 10 ml of unwashed 5-day-old liquid maintenance cultures, equivalent to a ca. 0.1% (wt/vol) inoculum.

Experiments to study the brightening of hardwood kraft pulp by *C. versicolor* were performed in a variety of different medium compositions as described below. In addition to mycological broth and glucose-soytone mixtures, defined media described by Dodson et al. (6) and Trudel et al. (23) were used. The culture conditions for all brightening studies were the same. After inoculation, 200 ml of the culture containing 2.0% (wt/vol) hardwood kraft pulp in 500-ml glass Erlenmeyer flasks were incubated in an air atmosphere (foam stoppers) at 25°C and 200 rpm (2.5-cm radius).

All brightening studies were set up with triplicate cultures.

Analytical Techniques. (i) Growth of the fungus in the presence of pulp. Total dry weight of cultures was determined by drying washed pulp (containing fungus) to a constant weight in a vacuum oven at 80°C. Chitin concentrations were determined by a two-stage method. First, chitin was acid hydrolyzed to release glucosamine residues (19), and then the concentration of glucosamine was determined colorimetrically by using 3-methyl-2-benzothiazole hydrochloride (1).

(ii) Preparation of handsheets and determination of brightness. Handsheets (i.e., sheets of paper produced from pulp samples in the laboratory) were made by suspending approximately 200 mg (equivalent dry weight) of washed pulp with or without fungus in 500 ml of distilled water and then, after mixing for 5 min with a Brookfield counterrotating mixer, pouring the pulp suspension through a Millipore 47-mm funnel with a steel mesh screen connected to a Buchner flask. Pulp samples were not dried before handsheets were made. The resultant handsheets were then allowed to dry in air for 24 h. Their brightness was measured as their reflectance at 457 nm relative to a barium sulfate standard by using a Perkin Elmer lambda 3B spectrophotometer fitted with a reflectance accessory.

(iii) Kappa number (indicator of lignin concentration). The kappa number is defined as the amount (milliliters) of 0.1 N $KMnO_4$ solution consumed by 1 g of moisture-free pulp under standard conditions (21) and is equivalent to approximately six times the weight percent lignin (12). In this study, kappa numbers were determined by the micro method (2). The kappa numbers of samples determined by both the micro method and the Tappi standard method were not significantly different (data not shown).

(iv) Zero span breaking length (indicator of pulp strength). Zero span breaking length is an index of the tensile strength of a pulp beaten to its maximum value under ideal conditions and is considered to be an excellent measure of the maximum strength of a pulp (20). Zero span breaking lengths were determined by the standard method (20).

(v) Nitrogen and glucose. Amino nitrogen concentration was measured with ninhydrin and calibrated with leucine (25) after alkaline hydrolysis (8), and glucose was determined with a Waters 410 differential refractometer after separation by high-pressure liquid chromatography on a Hamilton HC-75 column at 80°C eluted at 1.0 ml/min with distilled water.

TABLE 1. Effect of dilution of mycological broth on brightening of hardwood kraft pulp by *C. versicolor*[a]

Dilution of broth	Brightness (%)		
	Initial	Final	Increase
Distilled water control	31.8	33.2	1.4
Undiluted broth	31.8	37.4	5.6
2-Fold	31.8	38.9	7.1
5-Fold	31.8	45.2	13.4
20-Fold	31.8	33.5	1.7

[a] All cultures were incubated with *C. versicolor* for 5 days as described in the text. Undiluted mycological broth was 50.0 g/liter; dilutions were prepared by using the appropriate quantity of dessicated medium in a given volume of distilled water. The mean standard deviation for this experiment was ±3.0%.

RESULTS

Effect of growth medium composition on the physiology of pulp brightening. The physiology of the brightening of hardwood kraft pulp by *C. versicolor* was studied in mycological broth (Difco; low pH product) diluted fivefold and containing 2.0% (wt/vol) pulp. This dilution led to the maximum brightness increase of the pulp after a 5-day treatment with the fungus (Table 1). During an incubation of 6 days, the brightness of the pulp decreased marginally during the initial 24 h of culture and then continued to increase more or less steadily; the maximum increase occurred between days 1 and 2 (Fig. 1). The consumption of glucose was steady throughout, whereas both the pH and the amino-nitrogen concentration dropped markedly during the initial 2 days of culture and then fluctuated at approximately the same values for the rest of the period studied (Fig. 1).

The growth of the fungus in this experiment was determined by two methods: increase in total dry weight and increase in fungal cell wall chitin concentration (Fig. 2). Both of these methods have limitations and rely on a number of assumptions as described in the Discussion. However, both methods indicated that the growth of the fungus leveled off after 2 days of culture. The marginally higher level of growth indicated by the total dry weight data may have been due to the buildup of cell wall polysaccharides (14), which do not

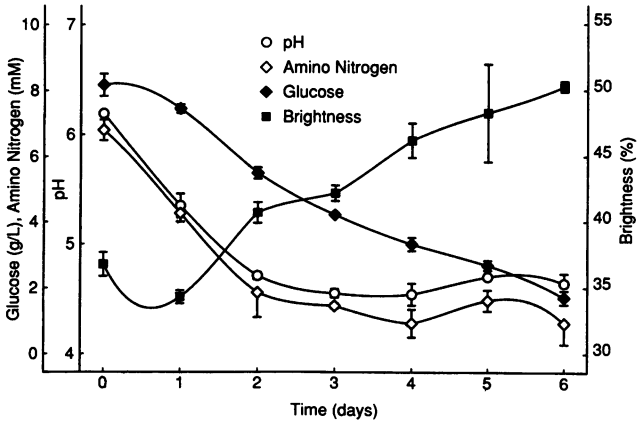

FIG. 1. Changes in the brightness of hardwood kraft pulp and in the pH and the concentration of glucose and amino nitrogen in the culture supernatant during incubation with *C. versicolor*. The growth medium was mycological broth diluted fivefold, and the initial pulp concentration was 2.0% (wt/vol). Error bars are standard deviations.

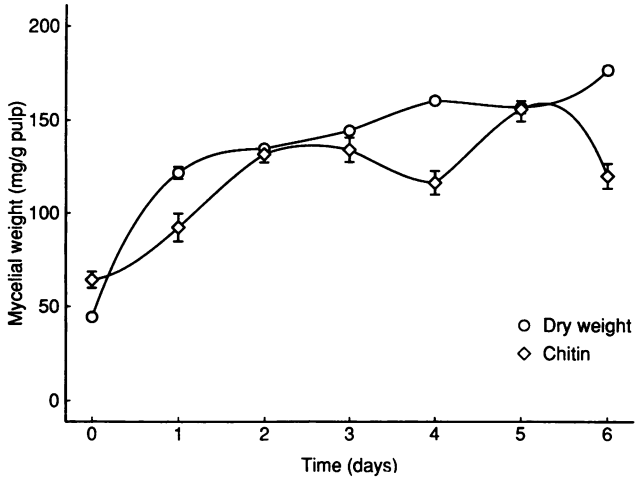

FIG. 2. Growth of *C. versicolor* in the presence of wood pulp as determined by the increase in total dry weight and the increase in chitin (determined as anhydroglucosamine). A conversion factor determined in the absence of wood pulp was 30.7 μg of chitin per mg (dry weight) of fungus. The growth medium was mycological broth diluted fivefold, and the initial pulp concentration was 2.0% (wt/vol).

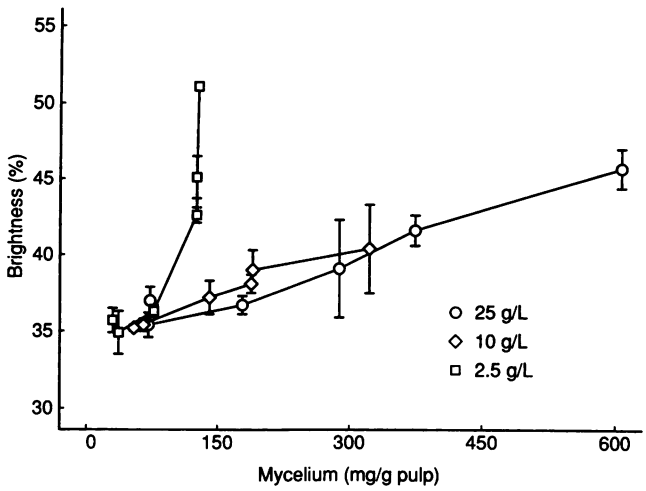

FIG. 4. Relationship between the brightness of hardwood kraft pulp and the fungal mycelium concentration (as determined by the increase in total dry weight) after incubation with *C. versicolor*. Treatment conditions were as described in the legend to Fig. 3.

contain chitin and are thus not detected by the chitin method of growth determination.

From the results of the chitin determination, we calculated that after 6 days of culture, the weight loss of the pulp was ca. 5% and that the final pulp and fungus mixture was ca. 10% fungus.

The medium used in this study, mycological broth, is a mixture of glucose and soytone. Using cultures containing the same initial glucose concentration but different concentrations of soytone, we found that the relationship between amino nitrogen supply and brightening of hardwood kraft pulp by *C. versicolor* was complex. At initial concentrations of 2.5, 10.0, and 25.0 g of soytone per liter, the relative brightness increase after 7 days of treatment with the fungus was 1.0:0.31:0.65, respectively (Fig. 3). From a plot of the resultant pulp brightness against the biomass produced in the different soytone concentrations (Fig. 4), we concluded that the mechanisms by which the fungus brightened the pulp were different depending on the initial concentration of soytone.

At a soytone concentration of 10 g/liter or higher, the brightness increase was proportional to the amount of mycelium produced. The addition of washed fungal mycelium grown in the absence of pulp to untreated pulp (Fig. 5) showed that at high fungal biomass levels the brightening of the hardwood kraft pulp was a physical effect due to the brightness of the fungal mycelium itself. At a concentration of 2.5 g of soytone per liter, where we assumed that nitrogen was growth limiting, the increase in the brightness was much greater than could be explained by

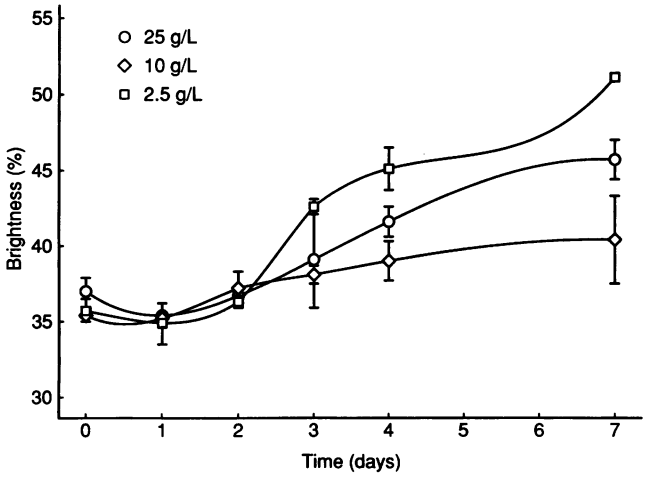

FIG. 3. Changes in the brightness of hardwood kraft pulp during incubation with *C. versicolor* in growth media containing different concentrations of soytone (25.0, 10.0 and 2.5 g/liter) and a single concentration of glucose (40.0 g/liter). The initial pulp concentration was 2.0% (wt/vol).

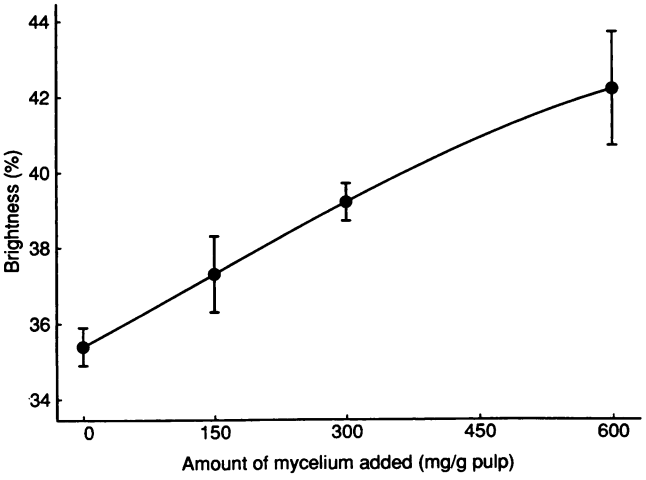

FIG. 5. Effect of the addition of washed mycelium of *C. versicolor* on the brightness of untreated hardwood kraft pulp. Mycelium was grown in full-strength mycological broth (low pH) and was washed three times with distilled water.

TABLE 2. Brightening of hardwood kraft pulp by *C. versicolor* in defined media and mycological broth[a]

Medium	Brightness (%)			Growth (mg increase per g of pulp)
	Initial	Final	Increase	
Mycological broth (diluted 5-fold)	32.5	50.3	17.8	87.5
High nitrogen[b]	30.7	39.4	8.7	63.7
Low nitrogen[b]	30.1	43.9	13.8	37.9
Low nitrogen[c]	31.6	45.3	13.7	45.0
Carbon limited[d]	31.7	38.8	7.1	−45.0
Nitrogen limited[d]	32.3	45.2	12.9	7.5

[a] All experiments were set up in triplicate where the mean standard deviation ranged between ±10.0 and ±0.1%. Cultures were inoculated with a washed inoculum at 0.02% (wt/vol); inoculum preparation and culture conditions were otherwise as described in the text. Cultures were incubated for 5 days.
[b] Medium was as described by Trudel et al. (23).
[c] Medium was as described by Trudel et al. (23), except containing no asparagine, with 2.5 mM ammonium nitrate.
[d] Medium was as described by Dodson et al. (6).

the brightness of the small amount of mycelium present, and we concluded that this was a true chemical effect mediated by the fungus.

Treatments of hardwood kraft pulp in defined media similarly indicated that significant brightening occurred in nitrogen-limited media (Table 2). The brightness increase in these cultures was less than that obtained in the mycological broth control cultures, suggesting that there may be scope for improved defined medium composition.

Determinations of zero span breaking lengths of pulps treated in different concentrations of soytone showed that at 2.5 g of soytone per liter there was a marginal loss of pulp strength, whereas at 10.0 g or more of soytone per liter this effect was much greater (Table 3).

Effect of fungal biomass on kappa number. Kappa numbers were determined on pulp samples brightened by the fungus. The relationship between pulp brightness and kappa number depended on the concentration of soytone supplied to the cultures. For pulp brightened in low-soytone medium, where relatively low amounts of fungal biomass were produced and the brightening was a chemical effect, the kappa number decreased with increasing pulp brightness, as expected (Fig. 6); i.e. as lignin was removed, the pulp became brighter. By contrast, for pulp brightened in high-soytone medium, where the brightness increase was apparently due to the brightness of the mycelium and was thus a physical effect, the kappa number increased with increasing pulp brightness (Fig. 6). This observation was explained by the consumption of potassium permanganate by the fungal mycelium alone (Fig. 7). The relatively high fungal biomass levels produced in high-soytone cultures significantly interfered with the determination of kappa number.

TABLE 3. Effect of fungal treatment on the zero span breaking length of hardwood kraft pulp[a]

Treatment	Zero span breaking length (km)	Fungal biomass (mg/g of pulp)
Untreated control pulp	14.3	
C. versicolor (40.0 g of glucose per liter) Soytone		
2.5 g/liter	12.2	125
10.0 g/liter	8.3	190
25.0 g/liter	8.7	374

[a] Cultures were set up as described in the text and were incubated for 4 days; then the pulp (containing fungus) was washed and dried before analysis.

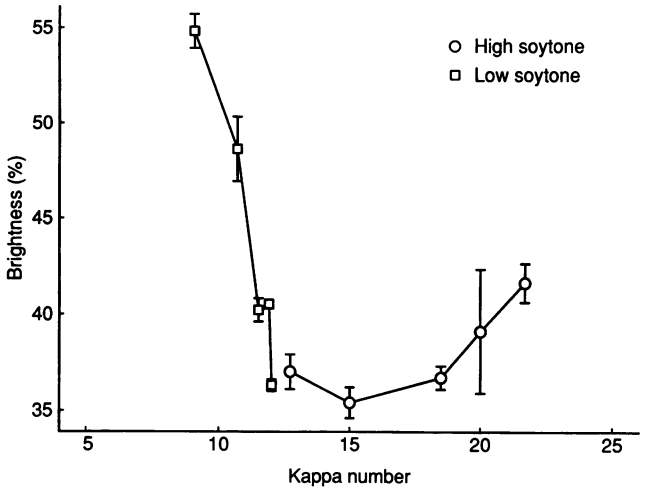

FIG. 6. Relationship between brightness and kappa number for hardwood kraft pulp incubated with *C. versicolor* with high and low soytone supply. High-soytone medium was 25.0 g of soytone per liter and 40.0 g of glucose per liter, and low-soytone medium was mycological broth diluted fivefold (equivalent to 2.0 g of soytone per liter and 8.0 g of glucose per liter). The initial pulp concentration was 2.0% (wt/vol).

DISCUSSION

Our results suggest that there are two ways by which *C. versicolor* can brighten pulp: (i) physically, by diluting the dark pulp fibres with brighter hyphae, and (ii) chemically, by degrading and/or decolorizing the residual lignin in the pulp. The physical effect is most important when large amounts of nitrogen are available and the fungus grows extensively. The

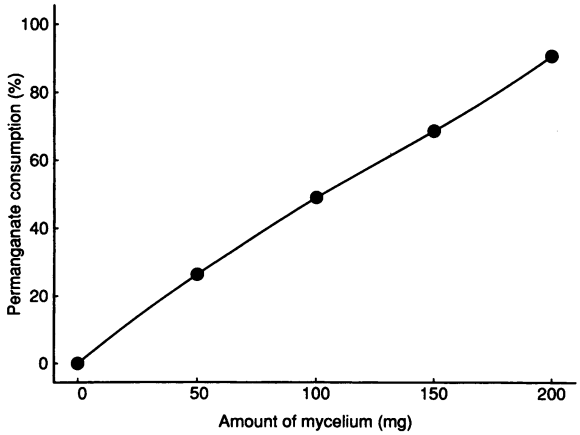

FIG. 7. Consumption of potassium permanganate by washed mycelium of *C. versicolor*. Consumption was determined in the absence of pulp with mycelium resuspended in 75 ml of distilled water. For comparison, with the same assay conditions but in the presence of pulp, ca. 0.4 g of dry weight pulp was necessary for 50% consumption of permanganate. A permanganate consumption of 100% corresponds to 200 μmol.

chemical effect is more important when fungal growth is limited, in this case presumably by the low nitrogen supply.

Soytone, an enzymatic hydrolysate of soybean meal (5), supplies not only nitrogen but also phosphate, sulfate, potassium, magnesium, a range of vitamins and trace elements, and some contaminating carbohydrates. We have assumed that nitrogen is the growth-limiting nutrient in our cultures, but it is possible that one of these other nutrients was actually limiting growth. Experiments performed in low-soytone concentrations showed that the amino-nitrogen concentration fell to a lower limit after 2 to 3 days of culture but in fact never ran out completely (Fig. 1). This observation emphasizes the difficulty in interpreting data obtained with a complex growth medium and the ninhydrin assay as an analytical tool. The results obtained with defined media, however (Table 2), suggest that nitrogen limitation does, indeed, lead to significant brightening of hardwood kraft pulp.

It has previously been shown that small amounts of mycelium (5 to 10% by weight) can be directly incorporated into kraft pulp without serious deleterious effects on the resultant paper strength properties (9). The effect of fungal biomass on pulp brightness was not reported (9). In this study, we found that for pulps brightened by *C. versicolor* in high and low soytone concentrations, there is a similar relationship between the fungal biomass concentration and pulp strength (at least for zero span breaking length) (Table 3). The important difference between the two sets of data, however, is that our samples were obtained after a fungal treatment resulting in a chemical brightening of the pulp at low fungal biomass concentrations, and the data previously reported were obtained by the direct mixing of fungus with unbleached kraft pulp in different ratios (9).

Interestingly, using the same microorganism under different culture conditions, Paice et al. (in press) concluded that any weight loss of the pulp was compensated for by fungal biomass production (hence the brightening was not accompanied by a significant increase in the total dry weight of the pulp and fungus mixture and was therefore a chemical effect and not a physical effect) and that the strength of the pulp was increased as a result of fungal treatment. Zero span breaking length was not one of the parameters they used to determine pulp strength.

Determinations of the growth of fungi in substrates such as wood pulps are not easy and generally rely on a number of assumptions (17). In this study, we assumed that the increase in total dry weight of pulps treated by the fungus was solely due to the growth of the fungus and that the weight of the pulp was constant throughout. Similarly, for the determination of fungal cell wall chitin concentration as an indicator of the degree of fungal growth in the wood pulp, we assumed that the chitin concentration of the fungal mycelium was constant and independent of growth rate and that the conversion factor determined in the absence of pulp was indeed relevant for cultures grown in the presence of pulp. These assumptions are undoubtedly open to criticism (1), and the absolute accuracy of the results in Fig. 2 must therefore be interpreted with a degree of caution.

Kappa numbers obtained for biologically treated pulps are affected by the degree of oxidation of the lignin structure (24). Nevertheless, this method is widely used in the industry to predict the amount of bleaching chemicals required by a pulp and was consequently chosen to give an indication of the lignin content of pulp samples treated by the fungus in this study. Our results show that consumption of potassium permanganate by fungal biomass is an additional consideration that must be taken into account when using the kappa number method for biologically brightened pulps.

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