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# Rapid Degradation of Isolated Lignins by *Phanerochaete chrysosporium*

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*Phanerochaete chrysosporium* degraded purified Kraft lignin, alkali-extracted and dioxane-extracted straw lignin, and lignosulfonates at a similar rate, producing small-molecular-weight (~1,000) soluble products which comprised 25 to 35% of the original lignins. At concentrations of 1 g of lignin liter$^{-1}$, 90 to 100% of the acid-insoluble Kraft, alkali straw, and dioxane straw lignins were degraded by 1 g of fungal mycelium liter$^{-1}$ within an active ligninolytic period of 2 to 3 days. Cultures with biomass concentrations as low as 0.16 g liter$^{-1}$ could also completely degrade 1 g of lignin liter$^{-1}$ during an active period of 6 to 8 days. The absorbance at 280 nm of 2 g of lignosulfonate liter$^{-1}$ increased during the first 3 days of incubation and decreased to 35% of the original value during the next 7 days. The capacity of 1 g of cells to degrade alkali-extracted straw lignin under optimized conditions was estimated to be as high as 1.0 g day$^{-1}$. This degradation occurred with a simultaneous glucose consumption rate of 1.0 g day$^{-1}$. When glucose or cellular energy resources were depleted, lignin degradation ceased. The ability of *P. chrysosporium* to degrade the various lignins in a similar manner and at very low biomass concentrations indicates that the enzymes responsible for lignin degradation are nonspecific.

The possibility of bioaltering lignin to commercial products has created a great interest in the study of the mechanism of lignin biodegradation. However, despite the large amount of research done in this field, relatively little has been reported concerning the comparative rate of biodegradation of various isolated lignin preparations.

Lundquist et al. (17) studied the degradation of Kraft lignin and lignin sulfonates in comparison to synthetic lignins by using the white rot fungus *Phanerochaete chrysosporium*. From Kraft and bleached Kraft lignin, 42% of the original $^{14}C$ was recovered as $CO_2$, as compared to 26 to 28% for the synthetic lignin and lignosulfonates. Incubation times of 40 to 78 days were used, although the lignosulfonates and bleached Kraft preparations underwent most of their decomposition in the first 20 days. This long incubation period could undoubtedly be decreased by using the media and culture conditions developed by this group (4, 12, 24).

Janshekar et al. (9) studied the degradation of various alkali-isolated straw and Kraft pine lignin preparations by six different fungi. With an incubation time of 14 days, most of the fungi degraded the straw lignin (16 to 50%), but only *P. chrysosporium* was found to substantially attack the Kraft lignin.

The rate of degradation of $^{14}C$-labeled lignin from wheat stems and poplar wood by *Pleurotus ostreatus* and *Pycnoporus cinnabains* 115 indicates that the lignins are degraded in a similar manner by both of these fungi (A. Hatakka and A. Uusi-Rauva, Proceedings of Journees Internationales d'Etudes de Groupe Polyphenols, in press).

The differences observed in the degradation of the two industrially obtained lignins (Kraft and lignosulfonates) and of the straw and Kraft lignin indicate that the rate and extent of lignin degradation may be dependent on the method of preparation and, to a lesser extent, the source of lignin. However, since ligninolytic cultures of *P. chrysosporium* metabolize a wide range of aromatic compounds (11), including synthetic polyguaiacol (2), the ligninolytic system may be regarded as relatively nonspecific and might be expected to degrade lignin-related compounds in a similar manner. Our aim was to determine whether *P. chrysosporium* could degrade various lignin preparations at the same rate and to the same extent under culture conditions optimized for lignin degradation (4, 12; M. S. A. Leisola, D. C. Ulmer, and A. Fiechter, Eur. J. Appl. Microbiol. Biotechnol., in press).

Kraft lignin and lignosulfonate with different modifications that occur during the pulping

process were chosen as substrates because, at present, they represent waste streams of little commercial value. In addition, straw lignin obtained by alkali solubilization and straw lignin obtained by dioxane extraction were used as a comparison. The former represents a product similar to Kraft lignin, whereas the latter represents a relatively unmodified substrate. Thus, the biodegradation pattern of the four types of lignins would give an indication as to whether degradation of lignin prepared in different ways or from different sources occurs by a common mechanism.

## MATERIALS AND METHODS

**Lignins.** Kraft pine lignin polymer (Indulin AT; Westvaco Co., Charleston, S.C.) was fractionated and purified by the method of Lundquist and Kirk (16). The organic-soluble, ether-insoluble fraction, KL-O, of this lignin was used for degradation studies. Alkali-isolated straw lignin was prepared by the method of Janshekar et al. (9), and dioxane-isolated straw lignin was prepared by the method of Odier and Monties (20). Lignosulfonate (Borresperse N) was obtained from Borregaard Co., Sarpsborg, Norway, and used without further purification (according to the manufacturer, it contains 95% lignosulfonates). Kraft and straw lignins were dissolved in 0.2 M KOH, and lignosulfonate was dissolved in deionized water.

**Organism.** The white rot fungus *P. chrysosporium* (ATCC 24725) was used throughout the work. The culture was maintained at room temperature on 2% (wt/vol) malt agar slants.

**Media and culture conditions.** The basal medium was prepared by the method of Kirk et al. (12), with 0.22 g of ammonium tartrate replacing the nitrogen source. This medium was filter sterilized with a 0.2-μm Pall ultipor disposable filter assembly. Mycelia for experimentation were taken from a chemostat with an effective volume of 4 liters; agitation, aeration, and dilution rates were 600 rpm, 0.5 vvm, and 0.015 $h^{-1}$, respectively.

Lignin degradation was studied in nonagitated cultures in 200-ml Erlenmeyer flasks under an atmosphere of oxygen. Gas flushing was performed as described by Kirk et al. (12). To each flask was added 5 ml of mycelial culture obtained from the chemostat and 0.2 ml of a lignin solution to give the desired concentration. For studies involving lower concentrations of cell biomass, cells were taken from the chemostat and diluted in basal medium without the added nitrogen source. Diluted cells (10 ml) were then added to each flask. The pH was adjusted to 4.5 with $H_3PO_4$. The cultures were buffered with 2,2-dimethylsuccinate at a concentration of 30 mM (3) and incubated at 38 to 39°C. Reference cells contained no added lignin and were incubated and treated like lignin-containing cultures.

**Analytical methods.** At the indicated intervals, the entire contents of the flasks were sonicated (sonifier B12; Branson Sonic Power Co., Danbury, Conn.; microtip probe; 50 w) for 30 s to form a homogeneous suspension which was subjected to the analyses described below.

**Glucose.** The concentration of glucose in the supernatant of a centrifuged sample was determined enzymatically with glucose oxidase by using a model 23A glucose analyzer (Yellow Springs Instrument Co., Yellow Springs, Ohio). The measuring range was 0 to 5 g $liter^{-1}$, and the sensitivity was 100 mg $liter^{-1}$.

**Gel permeation chromatography.** Gel permeation chromatography was performed with a Sephadex G-75 gel in a 1.6- by 87-cm column with 0.5% (wt/vol) NaOH as eluent. The standards used were blue dextran (molecular weight, $2 \times 10^6$), cytochrome *c* (molecular weight, 12,500), glucagon (molecular weight, 3,483), bacitracin (molecular weight, 1,432), and phenol (molecular weight, 94) (K. G. Forss and A. G. M. Fuhrmann, U.S. patent 4,105,606, 1978).

**Lignin assay.** Lignin was measured spectrophotometrically by a simplified modification of the method of Janshekar et al. (8). To 0.5 ml of sonicated culture was added 4.5 ml of 0.55% (wt/vol) NaOH. The mixture was sonicated for 30 to 60 s and then centrifuged. This treatment solubilized the lignin, resulting in a white mycelial pellet. Part of the supernatant was diluted, and total absorbance at 280 nm was measured. Part of the supernatant was used as a sample for gel permeation chromatography. To precipitate the acid-insoluble lignin, 2.5 ml of the supernatant (excluding lignosulfonate) was brought to a pH of 2.5 to 3.0 with HCl. The lignin was allowed to precipitate overnight and was then centrifuged and resolubilized in 0.5% (wt/vol) NaOH. After proper dilution, the absorbance at 280 nm was measured.

## RESULTS

**Degradation of different lignins.** The onset of ligninolytic activity and the extent of degradation for Kraft and straw lignins are shown in Fig. 1. Lignin was added to give a concentration of 1 g $liter^{-1}$. Results with lignosulfonate added in a concentration of 2 g $liter^{-1}$ to give an initial absorbance equivalent to that of the other lignins are shown in Fig. 2. For the Kraft lignin and two straw lignins, the patterns of degradation were very similar. After a 4-day lag period, the acid-insoluble lignin was rapidly degraded, and within 7 days, 90% of the acid-insoluble lignin had disappeared. Concomitant with the disappearance of the acid-insoluble lignin was an increase in the absorbance of acid-soluble products. After 7 days, the soluble absorbance represented 25% of that of the original straw lignins and 35% of that of the Kraft lignin.

The lignosulfonates went through a very pronounced color change during the first few days, possibly due to early oxidation reactions, which caused an increase in absorbance at 280 nm. This was followed by a rather constant decrease through day 10 of the experiment.

The glucose consumption rate, which is given for lignosulfonate, was similar in all cases and corresponded to 1 g $liter^{-1}$ $day^{-1}$. However, glucose was completely used by day 7 in the experiment with Kraft and straw lignins. This probably explains why degradation in this ex-

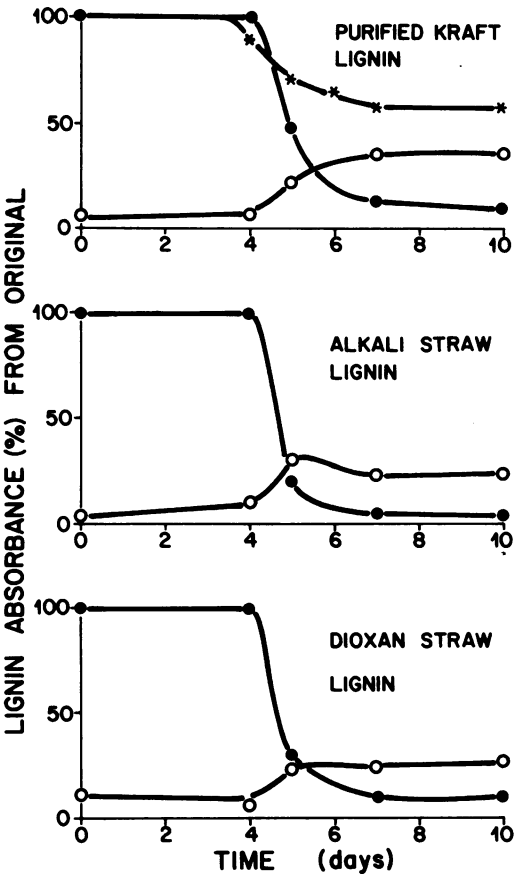


FIG. 1. Changes in acid-insoluble (●) and acid-soluble (○) absorbance of Kraft and straw lignins and redegradation pattern of acid-soluble products from Kraft lignin (*).

periment did not proceed to completion and why the soluble fraction was not degraded further.

The soluble products obtained from the Kraft lignin were subjected to redegradation (Fig. 1). The soluble fraction was degraded at a slower rate, and after 7 days, only 40% of the initial absorbance at 280 nm had disappeared.

The molecular weight distribution of the various lignins after 10 days of incubation is shown in Fig. 3 and 4. The pattern observed was again quite similar for all four of the lignins tested. The high-molecular-weight fraction (>3,000) of the lignins had virtually disappeared and was replaced by a distribution pattern with an average molecular weight of about 1,000. Although degradation of lignosulfonate proceeded a little slower than that of the other lignins, the high-molecular-weight fraction had disappeared by day 7 (data not shown). The chromatogram of the redegraded soluble fraction showed a general decrease over the entire range, with little change in distribution (Fig. 4).

**Cell capacity to degrade lignin.** Since 90% or more degradation was obtained during a relatively short active period for three of the lignins tested, it was decided to determine the maximal capacity of the cells to attack lignin. This was done by first increasing the lignin concentration of the cultures and then by using 1 g of lignin liter$^{-1}$ while decreasing the cell concentration. The results with various concentrations of the alkali straw lignin are shown in Fig. 5. At all the concentrations tested, up to 6 g liter$^{-1}$, there was rapid degradation of the acid-insoluble lignin. The onset of lignin degradation was 1 to 2 days later for the higher concentrations. This delay in ligninolytic activity was perhaps due to oxygen transfer problems caused by formation of a thick mycelium-lignin mat. The average amount of lignin degraded per day during the active period tended to increase with increasing lignin concentrations. However, to avoid oxygen transfer problems, we decided to keep the lignin concentration constant at 1 g liter$^{-1}$ and to decrease the amount of cells used to obtain a better estimate of lignin-biodegrading capacity.

The results of biodegradation of alkali straw lignin with various cell concentrations are shown in Fig. 6. This experiment was carried out with 10-ml-volume cultures that had been shown to be non-oxygen limiting for low biomass concentrations (Leisola et al., in press). Due to changes in biomass that occurred during the course of the experiment, the results are plotted according to the concentration of nitrogen present in the cultures. Nitrogen (2.4 mM) in the medium resulted in an initial biomass of 0.9 to 1.0 g liter$^{-1}$ in the bioreactor. Cultures with as little as 0.94 mM nitrogen degraded 1 g of lignin liter$^{-1}$ during a 2- to 3-day active period. At lower nitrogen values, and thus lower mycelial biomass concentrations, the lignin degradation rate was directly proportional to the amount of nitrogen present. Since alkali straw lignin contains 2 to 3% nitrogen (9), the slight increase in

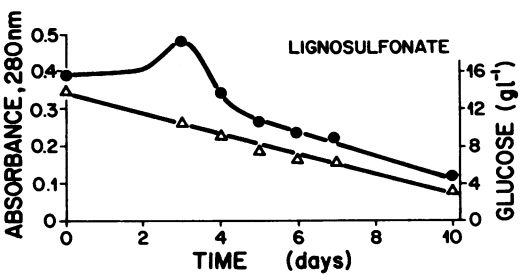


FIG. 2. Glucose consumption (△) and changes in absorbance (●) during degradation of lignosulfonates.

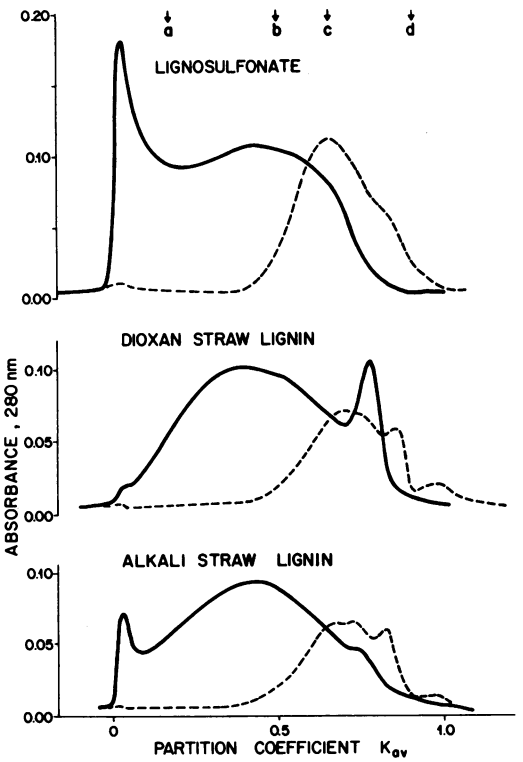


FIG. 3. Elution curves from gel filtration with Sephadex G-75 of original lignins (——) and soluble fraction after degradation for 10 days (------). Standards: a, cytochrome *c* (molecular weight, 12,500); b, glucagon (molecular weight, 3,483); c, bacitracin (molecular weight, 1,423); d, phenol (molecular weight, 94).

biomass concentrations and glucose consumption rates after the addition of 1 g of lignin liter$^{-1}$ to cultures with low concentrations of nitrogen in the medium indicated that the organism could use 0.14 mM of the nitrogen present in the lignin. The amount is included in the nitrogen values given. The amount of soluble products obtained after the complete disappearance of acid-insoluble lignin was 25 to 35%.

Glucose consumption by the dilute cultures during lignin degradation is also shown in Fig. 6. This consumption was dependent on the cell concentration and not on the amount of lignin degraded. From the data obtained, it was possible to approximate the capacity of nonagitated cultures of *P. chrysosporium* to degrade lignin (Table 1). The lignin degradation rate was calculated on the basis of the actual rate during the active ligninolytic phase. The glucose consumption during this active phase indicated that 1.0 g of glucose is needed to depolymerize and solubilize 1 g of lignin. Based on the amount of cell biomass present at a nitrogen concentration of 0.94 mM, 1 g of mycelial cells can bioalter 1.0 g of lignin day$^{-1}$, whereas 1 g of glucose is consumed simultaneously.

## DISCUSSION

The similarities in the degradation patterns and the final molecular weight distribution of the products from the various lignins is surprising, considering the differences in their physical and chemical properties caused by the various preparation procedures. Kraft lignin, produced by an alkaline pulping process, differs from natural lignin in that it undergoes aryl-alkyl cleavages and strong modification of side chains. It also undergoes various ill-defined condensation reactions (18). The resulting Kraft lignin is soluble as a salt but precipitates when in acid form. Lignin sulfonates, obtained by acidic sulfite pulping, also undergo some aryl-alkyl ether cleavages, but, more important, sulfonic acid groups are introduced in the position of the side chains (6, 7). The resulting lignosulfonates are completely water soluble in acid or salt form. The dioxane procedure may cause some changes in the chemical structure (1), but at the room temperatures employed here for extraction, these changes are expected to be minor (15).

The alkali straw lignin would be expected to differ from the Kraft lignin due to the initial differences in the composition of the materials. For example, soft wood lignin is comprised of approximately 90% guaiacyl units, whereas *Gramineae* species contain approximately 55% guaiacyl, 30 to 35% syringyl, and 10 to 13% coumaryl groups (19).

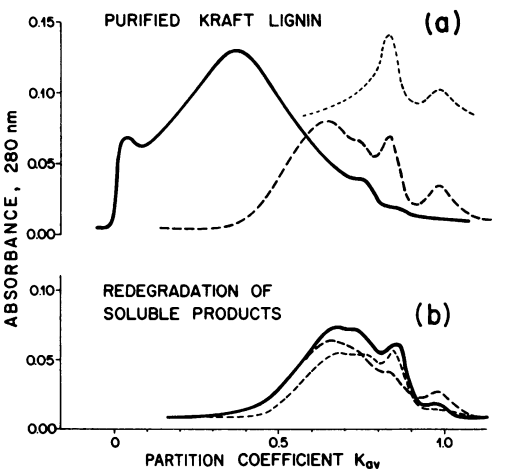


FIG. 4. Elution curves from gel filtration with Sephadex G-75. (a) Kraft lignin (——), after 10 days of degradation (------), and absorbing material produced by cells without lignin (------). (b) Soluble products from Kraft lignin after 10 days of degradation before use as a substrate (——) and after 6 days (------) and 10 days (------) of metabolism.

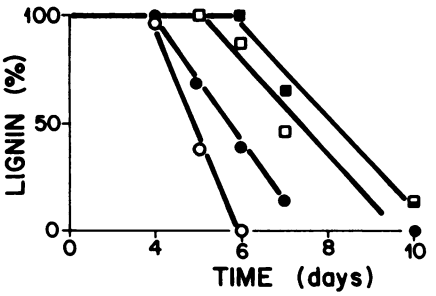


FIG. 5. Changes in acid-insoluble lignin during degradation with 1 (○), 3 (●), 4.5 (□), and 6 (■) g of alkali straw lignin liter$^{-1}$.

TABLE 1. Lignin degradation and glucose consumption rates during the active ligninolytic period with different mycelial concentrations and 1 g of alkali straw lignin liter$^{-1}$

| Nitrogen in medium (mM) | Mycelial dry wt (g liter$^{-1}$) | Lignin degradation rate (g liter$^{-1}$ day$^{-1}$) | Glucose consumption (g liter$^{-1}$ day$^{-1}$) | g of glucose per g of lignin degraded |
|---|---|---|---|---|
| 2.4 | 1.0 | 0.5 | 1.0 | 2 |
| 1.34 | 0.56 | 0.4 | 0.6 | 1.50 |
| 0.94 | 0.39 | 0.4 | 0.43 | 1.08 |
| 0.56 | 0.27 | 0.25 | 0.21 | 0.84 |
| 0.38 | 0.16 | 0.15 | 0.15 | 1.0 |

The extracted lignins should thus differ in the extent of cleavage and sulfonic acid incorporation in the aliphatic side chains, hydroxyl and methoxy group content, and degree of carbon-carbon binding between aromatic ring structures and between aromatic rings and aliphatic chains.

Our assay method does not distinguish between the degradation of the various groups of lignin, but measures the disruption of aromatic ring structures. However, the fact that we observed a similar rate of bioalteration and final molecular weight distribution of the bioaltered lignins indicates that the ligninolytic system functions in a similar way for the lignins despite their differences. Furthermore, the amount of $^{14}C$ released as $^{14}CO_2$ from various labeled lignins used by other authors ranges from 40 to 60% of the initial amount present (12, 17). Much of the remaining activity is found in the culture filtrate and a smaller amount associated with the cell residue (12). Since 25 to 35% of the lignin was bioaltered to soluble products in our work, it seems probable that 65 to 75% of the lignin is converted to $CO_2$.

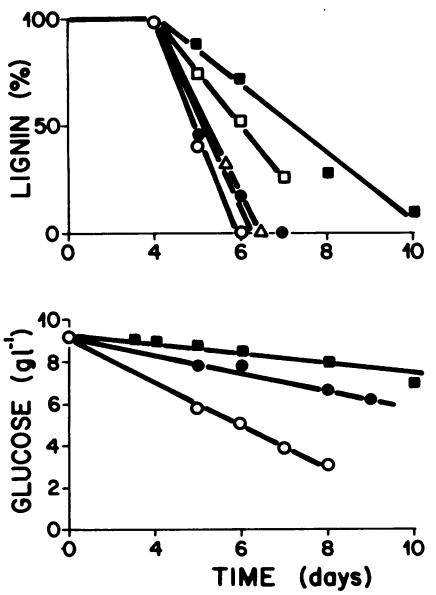


FIG. 6. Changes in acid-insoluble alkali straw lignin and glucose consumption during degradation with dilute mycelial cultures containing 2.4 (○), 1.34 (●), 0.94 (△), 0.56 (□), and 0.38 (■) mM nitrogen.

The molecular weight distribution of the bioaltered lignosulfonates obtained in our investigations differed from the results of Selin et al. (22). They found that the white rot fungus *Polystictus zonatus* polymerizes the lignosulfonates and that *Polyporus dichrous*, an atypical white rot fungus, causes a decrease throughout the molecular weight spectrum.

The chromatogram for Kraft lignin degraded by *P. chrysosporium* also differed from that previously reported by Janshekar et al. (8) for this fungus. Free lignin in the medium, measured after degradation was complete, had a molecular size distribution similar to that of the original lignin, whereas the cell-recovered lignin had undergone polymerization. Sundman et al. (23) found similar elution curves at 280 nm for Kraft bleach plant effluent and for the same effluent that had been decolorized by *P. chrysosporium*.

We believe that the difference in our results from those previously published is due to our avoidance of pronounced oxygen transfer problems caused by the formation of thick mycelial mats and a nonagitated mode of cultivation (Leisola et al., in press). By using more optimal experimental conditions, we could allow lignin degradation to proceed at a faster and probably maximal rate and allow degradation to approach completion.

The products in the small-molecular-weight region which account for increased absorbance at 280 nm have not been characterized but indicate that many water-soluble compounds are obtained during lignin degradation. The inability of *P. chrysosporium* to completely degrade the soluble products indicates that they are more

resistant to degradation than the original lignin under the conditions tested. Reid et al. (21) recently reported obtaining almost as much $^{14}C$ in water-soluble form as in $^{14}CO_2$ during degradation of [$^{14}C$]lignin-labeled aspen wood by *P. chrysosporium*. With the water-soluble radioactive material as a substrate, only one-third of the original radioactivity was converted to $CO_2$. The inability to completely degrade the water-soluble compounds was attributed to some diffusion away from the hyphae before they could be metabolized and the accumulation of more recalcitrant fragments. Krisnangkura and Gold (13) have found that guaiacyl lignin degraded by *P. chrysosporium* is enriched in biphenyl linkages and aryl-akyl C-C bonds, indicating that these bonds may be more resistant to attack. An abundance of these linkages in the water-soluble compounds may explain part of their recalcitrant nature.

The complete degradation of 1 g of lignin liter$^{-1}$ by low mycelial concentrations (1.0 to 0.16 g liter$^{-1}$) gives an indication of the lignin-degrading capacity of the cells and of the amount of glucose needed to achieve this degradation. A 1.0-g amount of glucose to bioalter 1 g of lignin is much less than has been previously reported. Kirk et al. (12) reported degradation of 5 mg of [ring-$^{14}C$]lignin to $CO_2$ with the utilization of approximately 100 mg of glucose in 21 days. Janshekar et al. (8) obtained a 13.5-mg decrease in the Klason lignin content of Kraft lignin, accompanied by the utilization of 100 mg of glucose.

At present, bioalteration of lignin requires an energy source and has been found to cease when glucose or cell energy reserves are depleted (unpublished data). Thus, the organism is probably unable to obtain energy from lignin. If energy were obtained from lignin, the rate of degradation would probably be sufficient so that it would be maintained in the absence of another energy source. To our knowledge, the maximal lignin degradation rate of 1.0 g day$^{-1}$ with 1 g of cells is fivefold greater than has been previously reported with this fungus (0.18 g day$^{-1}$) (10, 24). However, in the previous study, a gravimetric analysis and a different substrate were used; thus, the rates may not be directly comparable.

An enzyme system for lignin degradation would require either a wide range of specific enzymes to account for the differences in the lignins used or a few enzymes acting in an unspecific manner. The use of dilute cultures with only 0.94 mM nitrogen allows for a maximum concentration of 84 mg of protein liter$^{-1}$ (6.25 times the amount of N). Some of this nitrogen must be incorporated into the DNA and RNA of the cells, into membrane and cell wall proteins, and into the enzymes necessary for cell metabolism. The small amount of protein available for ligninolytic enzymes coupled with the similar pattern of degradation for the lignins studied support the hypothesis and recent evidence that hydroxyl radicals are involved in lignin degradation (5, 14). Relatively few enzymes would be required to generate the hydroxyl radicals, which could then attack a wide array of lignin-related compounds.

## ACKNOWLEDGMENT

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