APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1992, p. 1121–1127
0099-2240/92/041121-07$02.00/0

Vol. 58, No. 4

Lignocellulose Degradation during Solid-State Fermentation: *Pleurotus ostreatus* versus *Phanerochaete chrysosporium*

ZOHAR KEREM, DANA FRIESEM, AND YITZHAK HADAR*

The Otto Warburg Center for Biotechnology in Agriculture, Faculty of Agriculture, The Hebrew University, Rehovot 76-100, Israel

Received 8 November 1991/Accepted 17 January 1992

Lignocellulose degradation and activities related to lignin degradation were studied in the solid-state fermentation of cotton stalks by comparing two white rot fungi, *Pleurotus ostreatus* and *Phanerochaete chrysosporium*. *P. chrysosporium* grew vigorously, resulting in rapid, nonselective degradation of 55% of the organic components of the cotton stalks within 15 days. In contrast, *P. ostreatus* grew more slowly with obvious selectivity for lignin degradation and resulting in the degradation of only 20% of the organic matter after 30 days of incubation. The kinetics of ^{14}C-lignin mineralization exhibited similar differences. In cultures of *P. chrysosporium*, mineralization ceased after 18 days, resulting in the release of 12% of the total radioactivity as $^{14}CO_2$. In *P. ostreatus*, on the other hand, 17% of the total radioactivity was released in a steady rate throughout a period of 60 days of incubation. Laccase activity was only detected in water extracts of the *P. ostreatus* fermentation. No lignin peroxidase activity was detected in either the water extract or liquid cultures of this fungus. 2-Keto-4-thiomethyl butyric acid cleavage to ethylene correlated to lignin degradation in both fungi. A study of fungal activity under solid-state conditions, in contrast to those done under defined liquid culture, may help to better understand the mechanisms involved in lignocellulose degradation.

Lignin is the second most abundant biopolymer in nature. The complex structure of lignocellulose prevents the economical use of plant polysaccharides or the potential use of lignin derivatives as chemical feedstocks (10) or ruminant feed (32, 43). Recently, great effort has been invested towards an understanding of lignin biodegradation mechanisms (11, 13, 31). The most effective lignin biodegraders are the white rot fungi, belonging to the *Basidiomycetes*. The most widely studied of these is *Phanerochaete chrysosporium* (11, 13, 16, 22). Studies have been focused on the physiology, enzymology, and molecular genetics of the biodegradation process. It appears that a key enzyme for polymer fragmentation by *P. chrysosporium* is lignin peroxidase (11, 13, 15).

The edible mushroom *Pleurotus ostreatus* has been shown to degrade lignin (1, 32, 43). However, efforts to detect lignin peroxidase activity or lignin peroxidase genes in *P. ostreatus* under several culture conditions have been unsuccessful (20, 38, 42). Sannia et al. (38) suggested that a lignin metabolism significantly different from that reported for other white rot fungi may exist in at least some species of fungi. *P. ostreatus* has been found to produce laccase, a copper-containing phenol oxidase (32, 37). Laccase (EC 1.14.18.1) is an extracellular phenol oxidase produced by basidiomycetes and has a role in lignin biodegradation (15, 25, 38). It was shown to cause some oxidative degradation of lignin and to catalyze the polymerization of small phenolic molecules arising from this degradation (5, 15).

P. ostreatus is generally cultivated on wheat straw. Platt et al. (32) suggested the use of cotton stalks as a substrate, cotton being an important field crop worldwide. The amount of cotton stalks in India, for example, is 10 million tons per year (3), of which 5 tons/ha remain in the field and must be ploughed under the soil surface. In addition to wasting a potential bioresource, this treatment may lead to an increase in cotton diseases and pests, as well as to difficulties in cultivation due to slow decomposition in the soil.

Agricultural wastes containing lignocellulose can be upgraded by solid-state fermentation with white rot fungi. Solid-state fermentation requires less energy input than liquid fermentation, making its potential application of interest. Moreover, lignin degradation is affected by the culture conditions, among which aeration is highly important (35, 43). Leisola et al. (23) suggested that the sensitivity of lignin degradation by *P. chrysosporium* to oxygen makes it imperative that the culture not be oxygen limited prior to monitoring other parameters. Solid-state fermentation conforms to this preliminary demand.

The present report describes a comparative study of the two white rot fungi *P. chrysosporium* and *P. ostreatus* as regards their physiology and lignocellulose degradation during solid-state fermentation.

MATERIALS AND METHODS

Fungi. The following fungal strains were used in this study: *P. ostreatus* Florida f6 (6), *P. chrysosporium* BKM, and *Sclerotium rolfsii* Sacc. ATCC 26325.

Substrate. Cut cotton plants (stalks) were obtained in September 1989 from a cotton field after defoliation and harvest of the cotton fibers. The cotton stalks were dried in a 60°C oven and then ground to pass a 2-mm-pore-size screen in a Wiley mill. Table 1 summarizes the composition of the untreated substrate composed of cotton stalks and sorghum seeds. Lignin and cellulose contents in the cotton stalks were similar to those in hardwoods (28, 35), while the C/N ratio in the cotton stalks was much lower.

Preparation of labeled lignin. [^{14}C-lignin]lignocellulose was prepared by the method of Crawford and Crawford (8) with the following modifications. Freshly cut, 40-cm-long cotton branches with three leaves remaining were put in a solution of 100 ppm 8-hydroxyquinoline and kept under alternating light and dark periods for 10 days. The treatment with

* Corresponding author.

TABLE 1. Composition of untreated cotton stalk substrate[a]

Component	Concn (% [wt/wt] of dry substrate)
Soluble glucose	1.0 ± 0.05
Total soluble sugars	3.8 ± 0.03
Crude protein	6.5 ± 0.2
True protein	2.3 ± 0.01
Cellulose	47 ± 3
Lignin	22 ± 2
Fibers	68 ± 3
Ash	6.4 ± 0.1
C/N ratio	40

[a] The substrate was composed of 2% (wt/wt) dry sorghum seeds mixed with cotton stalks.

8-hydroxyquinoline has been included to prevent bacterial contamination of the stems, thus prolonging the incubation period. Aqueous L-[U-^{14}C]phenylalanine (Amersham International, Buckinghamshire, England) was fed as a precursor for lignin biosynthesis. At the end of the incubation period, the leaves were discarded and the branches were homogenized in a pepsin-HCl solution (pH 2) and incubated for two 20-h periods (36); then a series of Soxhlet extractions were performed, as described by Crawford (10) and by Colberg and Young (7). The resultant lignocellulose showed a specific activity of 22×10^3 dpm/mg, as determined by the acetyl bromide method of Johnson et al. (17) and by the method of Morrison (27). The ^{14}C-labeled lignocellulose was treated with NaOH as described by Colberg and Young (7), and 68% of the whole labeling was solubilized by this procedure, in agreement with the results (7, 9) for a Douglas fir preparation.

Solid-state fermentation. Both fungi were grown in the substrate described above. A typical preparation consisted of 100 g of dry substrate in a polystyrene bag (50 by 50 cm) moistened with 200 ml of deionized water (corresponding to an oven-dry weight of 100 g on the basis of an experimentally determined value of 65% moisture content of the final substrate). The bags were sealed with cotton plugs to facilitate air transfer, autoclaved twice for 60 min each at 121°C, and cooled prior to inoculation with 4 g (50% moisture) of 6-day sorghum spawn per bag (44). After inoculation, the bags were incubated at 30°C for *P. ostreatus* and at 39°C for *P. chrysosporium*. Two aeration treatments were applied during solid-state fermentation by *P. chrysosporium*. In one, the atmosphere in the incubator was enriched by a constant flow of pure oxygen at a rate of 100 ml/min. A second incubator served as the untreated control. Several times during the fermentations (roughly every 3 days), three bags were randomly chosen and used for the different assays. For a study of [^{14}C-lignin]lignocellulose mineralization, the fermentation was performed in 3 g of wet substrate in a 20-ml beaker, which was sterilized and inoculated proportionally to the above procedure. Radioactive substrate with a total activity of 66×10^4 dpm (30 ± 0.1 mg of the preparation described above) was added to each beaker, and the beakers were then sealed in a 300-ml biometer flask (4) with two gas-tight caps. *P. ostreatus* and *S. rolfsii* were incubated at 30°C; *P. chrysosporium* was incubated at 38°C. Each flask was flushed every 2 days for 1 min with wet, sterile atmospheric air. Radioactive CO_2 was trapped for 3 h prior to aeration on a filter paper (2 by 10 cm) soaked with 0.5 ml of 7 N NaOH, and radioactivity was counted in Hionic-Fluor (Packard Instruments, Downers Grove, Ill.) scintillation liquid. To determine water-soluble radioactivity, 10 ml of water was added to each sample, and after a 1-h soak, samples were centrifuged for 15 min at 20,000 × *g*. Supernatant was counted as above.

Determination of CO_2 evolution. Duplicate 6-g (fresh weight) samples of inoculated substrate were placed in 100-ml glass bottles equipped with a gas-tight cap, and the bottles were sealed for 1 h. A 1-ml air sample was taken with a syringe to determine evolved CO_2 concentration by gas chromatography (GC; GC model 580 [Gow-Mac Instrumentation Co.], equipped with a Porapak Q column and a thermal conductivity detector). The results were expressed as milliliters of CO_2 per gram (dry weight) of substrate per hour.

Hydrolysis of FDA. Hydrolytic activity was measured by using a modified Schnurer-Rosswall (39) method. Fluorescein diacetate (FDA) (Sigma Chemical Co., St. Louis, Mo.) was dissolved in acetone (analytical grade) and stored as a stock solution (2 mg ml^{-1}) at −20°C. Duplicate 6-g (fresh weight) samples of inoculated substrate were dispersed in 100 ml of sterile 60 mM phosphate buffer solution (pH 7.6), and FDA was added to a final concentration of 10 μg ml^{-1}. The suspensions were incubated in 250-ml Erlenmeyer flasks at 30°C on a rotary shaker (120 rpm). One-milliliter samples were removed from the suspensions at 15-min intervals and centrifuged for 1 min in a microcentrifuge (no. 2021; Heitich Zentrefugen, Tuttlingen, Germany) to remove solid particles. The amount of free fluorescein was measured as A_{494} and expressed as milligrams of fluorescein released from FDA within 60 min of its addition to the fermentation medium suspension.

KTBA. 2-Keto-4-thiomethyl butyric acid (KTBA) cleavage to ethylene was measured by using a modification of the method of Pryor and Tang (34). KTBA was dissolved in deionized water to a concentration of 0.4 mM. Duplicate 6-g (fresh weight) inoculated substrate samples were dispersed in 25 ml of the solution, which was agitated at the growth temperature in 100-ml glass bottles sealed with a gas-tight cap. One-milliliter air samples were taken at zero time and after 2 h to determine the concentration of evolved ethylene per hour by GC (with a GC model 3300; Varian, Palo Alto, Calif.). The GC was equipped with a Porapak N column and a flame ionization detector. Results were expressed as milliliters of ethylene per gram (dry weight) of substrate per hour.

Water extractions. Deionized water in 100-ml aliquots was combined with 25 g ± 10 mg (fresh weight) from each individual bag in 250-ml Erlenmeyer flasks. The flasks were rotated at 200 rpm to achieve homogeneous suspensions and then centrifuged at 10,000 × *g* to separate water from substrate. For a better separation, supernatant was vacuum filtered through no. 1 frittered glass. Extracts were kept at −20°C.

Glucose determination. Glucose concentration in the water extracts was determined with Glucostat reagent (PGO enzymes; Sigma) in accord with the manufacturer's directions. Preboiled extract served as the background determination for each sample. Total extractable sugars were determined with an anthrone reagent (40), using glucose as a standard.

Protein determination. Protein concentration in the water extracts was determined with Coomassie blue 250 reagent by the method of Sedmac and Grossberg (41). Crude protein in the untreated substrate was determined by the Kjeldahl method, using a Kjeltec 1030 (Tecator, Höganäs, Sweden). True protein in the untreated substrate was determined with

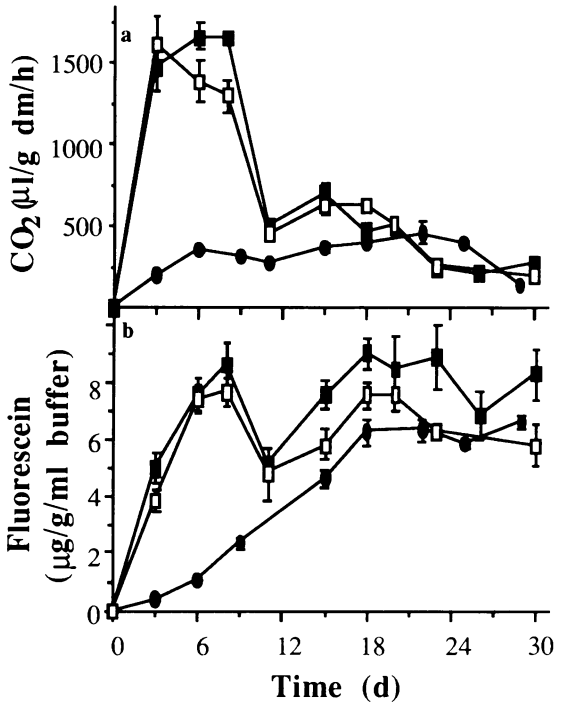

FIG. 1. Fungal activities on cotton stalks during solid-state fermentation. (a) Respiration. (b) FDA hydrolysis. ●, *P. ostreatus*; ■, *P. chrysosporium* grown under atmospheric air; □, *P. chrysosporium* grown under an O_2-enriched atmosphere. Bars represent standard errors.

a high-pressure liquid chromatography amino acid analyzer (model LS 1811; Biotronik Ltd.) after acid hydrolysis.

Enzyme assays. Laccase (EC 1.10.3.2) was assayed with the substrate 2,6-dimethoxyphenol in 0.1 M phosphate buffer (pH 6.0). The reaction was stopped after 10 min with dimethyl sulfoxide, and absorbance was recorded at 468 nm. The results are shown in units based on an assay with commercial laccase (Sigma). Carboxymethyl cellulase (EC 3.2.1.4) was assayed with the substrate carboxymethyl cellulose (32).

Substrate analysis. The dry weight of the rotted substrate was determined after drying in a 60°C oven. The dried sample was used for a determination of cellulose and acid-insoluble (Klason) lignin by the Goering-Van Soest method (14) and for ash determinations. Organic matter loss in the degradation process was calculated by comparing ash content in the rotted and dried substrates.

RESULTS

Fungal activities (related to growth). A time course study was performed to compare solid-state fermentation by *P. chrysosporium* with that by *P. ostreatus* on cotton stalks. Respiration and esterase activities (as measured by FDA hydrolysis) were monitored to evaluate fungal growth and colonization. The respiration rate of *P. ostreatus* increased during the first 6 days of incubation, reaching a constant rate of 270 μl of CO_2 per g per h (Fig. 1a). Much higher respiration rates were recorded in *P. chrysosporium* for both aeration treatments, reaching a maximum of 1,600 μl of CO_2 per g per h on day 3 and then decreasing to 500 μl of CO_2 per g per h on day 12. CO_2 evolution rates remained at low levels

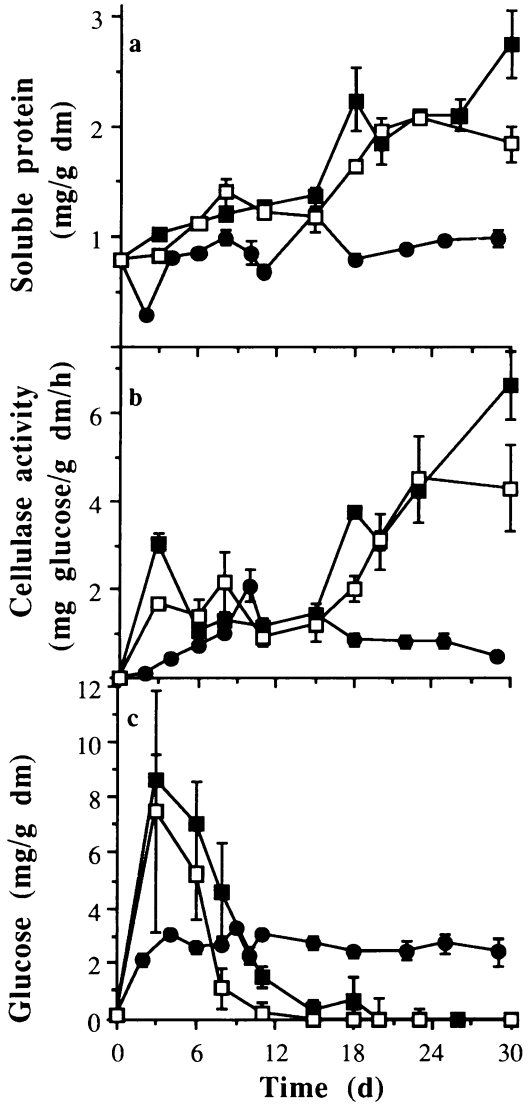

FIG. 2. (a) Protein content in water extract of fungus-substrate bulks. (b) Cellulases in the water extract expressed as milligrams of glucose hydrolyzed from carboxymethyl cellulose. (c) Glucose in the water extract. Bars represent standard errors. See legend to Fig. 1 for explanation of symbols.

during the last 8 days of the experiment. In the beginning, esterase activity increased faster in *P. chrysosporium* fermentation than in *P. ostreatus* fermentation. However, after 18 days, the two fungi leveled off at similar activity levels (Fig. 1b). Respiration and esterase activities did not follow the same trends. *P. chrysosporium* respiration dropped sharply, while FDA hydrolysis remained at a high constant level throughout the fermentation period under both aeration treatments. This could result from the stability of extracellular enzymes or leakage from fungal cells. During *P. ostreatus* fermentation, respiration and FDA hydrolysis leveled off after 6 and 18 days, respectively.

Soluble protein and fungal activities related to lignocellulose degradation. The initial concentration of water-soluble protein in cotton stalks was 0.8 mg/g (dry weight). This concentration did not change throughout the growth of *P. ostreatus*, while in both *P. chrysosporium* treatments extractable pro-

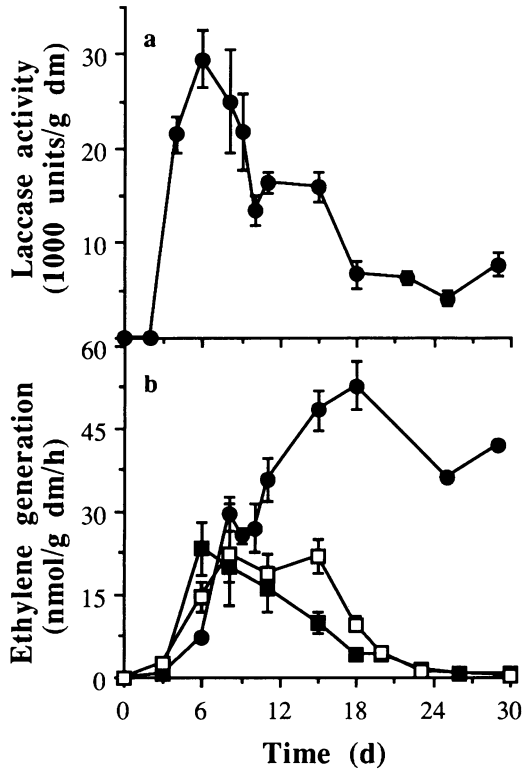

FIG. 3. Lignin degradation-related activities. (a) Laccase activity in water extract expressed as units based on commercial laccase. (b) Ethylene cleaved from KTBA by fungi on the cotton stalk substrate. Bars represent standard errors. See legend to Fig. 1 for explanation of symbols.

tein levels reached 1 mg/g (dry weight) within the first 3 days of incubation (Fig. 2a). In *P. chrysosporium*, an increase in the water-extractable protein concentration was also observed after day 15, probably because of cell lysis or the release of proteins adsorbed to the cotton stalk fibers. Cellulase activity in a water extract of the *P. chrysosporium* treatment increased during the first 3 days (Fig. 2b) and then decreased slightly to a steady level of 1.5 mg of glucose per g of substrate until day 15. At this time, higher cellulase activity was observed. A different trend was observed in the *P. ostreatus* treatment, in which cellulase activity gradually increased until day 8 and then leveled off. Determination of glucose concentration in the same extracts (Fig. 2c) showed a sharp increase of glucose in the *P. chrysosporium* fermentations for the first 3 days and then a decrease to undetectable levels on day 15. In the *P. ostreatus* fermentation, soluble glucose concentrations increased slightly over the first 4 days, stabilizing at 3 mg per g of substrate until the end of the fermentation.

Laccase activity was only detected in the *P. ostreatus* treatment (Fig. 3a). Activity increased rapidly from days 2 to 6 and then decreased until day 18 to a quarter of the original activity per gram of substrate. Lignin peroxidase activity could not be detected in water extracts of the fermentation products of either fungus. Efforts to detect lignin peroxidase secretion by *P. ostreatus* in a liquid medium under conditions favoring lignin degradation failed.

KTBA degradation to ethylene was studied on the colonized cotton stalk samples. No activity was observed during

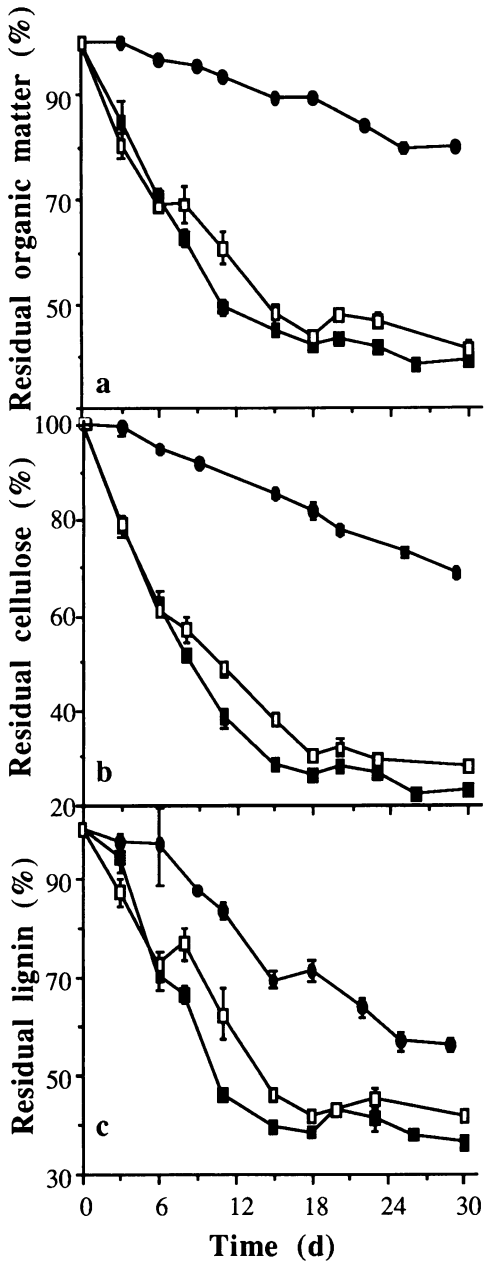

FIG. 4. (a) Kinetics of cotton stalk degradation, (b) cellulose degradation, and (c) delignification during fungal solid-state fermentation. ●, *P. ostreatus*; ■, *P. chrysosporium* grown under atmospheric air; □, *P. chrysosporium* grown under an O_2-enriched atmosphere. Bars represent standard errors.

the first 3 days under either *P. chrysosporium* aeration treatment. Most of the activity was detected between days 6 and 15 (Fig. 3b). A different trend was observed in the *P. ostreatus* treatment (Fig. 3b). A steady increase was found until day 18 and activity remained high until the end of the fermentation period, at which time it was 2.5-fold that of the peak activity of *P. chrysosporium*.

Lignocellulose degradation. Growth of *P. chrysosporium* on cotton stalks resulted in the disappearance of 55% of the initial organic matter within 15 days of fermentation, under both aeration conditions (Fig. 4a). Klason lignin loss was

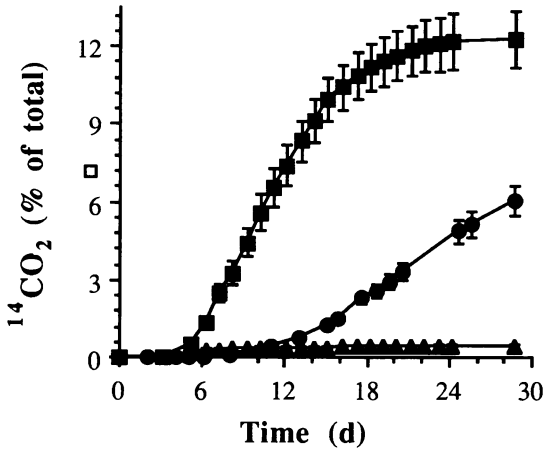

FIG. 5. Conversion of ^{14}C-lignin to $^{14}CO_2$ during solid-state fermentation of cotton stalks by *P. ostreatus* (●), *P. chrysosporium* (■), and *S. rolfsii* (▲). Each biometer flask contained 1 g of unlabeled substrate plus about 30 mg (66 × 10^4 dpm) of substrate containing ^{14}C-lignin. Bars represent standard errors.

rapid from days 3 to 12 and then slowed and essentially stopped on day 15. Cellulose degradation followed a similar pattern, resulting in a 70% decrease of the initial amount (Fig. 4b and c). Differences in degradation rates of lignin and cellulose were apparent between the two aeration treatments from days 9 to 15 of fermentation. From then on, degradation stopped and lignin and cellulose contents remained constant.

Growth of *P. ostreatus* resulted in the disappearance of only 20% of the original organic matter (Fig. 4a) at a degradation rate of 0.7% per day throughout the fermentation period. Lignin loss amounted to 45% of the original amount (Fig. 4b), being degraded steadily from day 6 to the end of the experiment. Loss of cellulose from the fermented cotton stalks was lower than that of lignin (Fig. 4b and c). Only 30% of the cellulose was degraded during the 30-day period. Initial lignin and cellulose contents in cotton stalks are presented in Table 1.

^{14}C-lignin mineralization. ^{14}C-lignin from cotton plants was used to evaluate lignin mineralization during solid-state fermentation. The labeled lignin was mixed with ground cotton stalks and then inoculated with various basidiomycetes. $^{14}CO_2$ was collected to estimate lignin degradation during fermentation. *S. rolfsii*, a non-lignin-degrading fungus, was used to verify labeling specificity. Only 0.25% of the total ^{14}C was released as $^{14}CO_2$ during fermentation, and another 0.25% was found in the water extract of the solid fermentation product after 23 days. In the solid-state fermentation of *P. chrysosporium*, the rate of $^{14}CO_2$ released increased from days 4 to 7, reaching a constant rate of 1% per day, which was maintained until day 13. The rate then slowed to day 22, at which point no additional $^{14}CO_2$ was released (Fig. 5). A total of 12% of the original labeling was released during the experiment by *P. chrysosporium* as $^{14}CO_2$ (Fig. 5). When *P. ostreatus* was grown on the same substrate, $^{14}CO_2$ release started on day 7. A constant release rate of 0.3% per day was observed from days 9 to 60 (data shown only up to day 27 [Fig. 5]). A total of 17% of the original labeling was released as $^{14}CO_2$ during the 60 days of fermentation. Water extracts of *P. chrysosporium* fermentation products after 25 days and of those of *P. ostreatus* after 45 days contained 9.1 and 9.2% of the original labelling, respectively. At these time points accumulated $^{14}CO_2$ released by the two fungi was 12%.

DISCUSSION

This study compares solid-state fermentation of cotton stalks by the two lignin-degrading fungi *P. chrysosporium* and *P. ostreatus*. Most of the research describing ligninolytic systems to date has been performed in liquid defined media (13). The works of Datta et al. (11) and Leisola et al. (23) emphasize the importance of working in solid-state conditions. *P. chrysosporium* ligninolytic systems have mainly been studied in a 100% O_2 atmosphere. Here no obvious or distinct differences were observed between O_2-enriched and atmospheric air aeration treatments. This could be a result of differences in oxygen transfer in solid substrate versus stationary liquid culture rather than being due to the partial pressure of oxygen. In solid-state fermentation, oxygen is therefore not a limiting factor for lignin degradation, which is in agreement with observations by Leisola et al. (23) and Ander et al. (2). Nor was the respiration rate affected by O_2 enrichment, supporting the conclusion that oxygen is not a limiting factor for growth in this system.

The more intensive and vigorous growth of *P. chrysosporium* was expressed by much higher respiration rates and FDA hydrolysis levels than those found in *P. ostreatus*, as well as by visual observation of mycelial biomass. These activities reached their maxima from days 3 to 9 of solid-state fermentation by the former, while maximal levels were obtained only after 10 to 18 days of fermentation by *P. ostreatus*.

Cellulase activity levels in water extracts of the fermentation products provided insight into the lignocellulose biodegradation process, partially explaining the higher selectivity for lignin degradation observed with *P. ostreatus*. High cellulase activity was evident from the first days of fermentation by *P. chrysosporium*, resulting in rapid cellulose degradation as well as high levels of free glucose (Fig. 2 and 4). Cellulases have a very high substrate affinity, which may reduce their extractability (29). From day 18 on, most of the cellulose had been degraded, resulting in the release of high cellulase activity.

When lignin degradation-related activities were measured, KTBA cleavage during *P. ostreatus* fermentation was found to correlate with both the period and rates of lignin degradation. However, at this stage we cannot verify whether this is purely an enzymatic activity or a chemical activity resulting from the generation of free radicals during lignin biodegradation (19). This activity was much higher in *P. ostreatus* than in *P. chrysosporium*. Laccase, a lignin degradation-related enzyme (32), was found only in *P. ostreatus* and was apparent mainly during colonization, with lower levels being detectable after 16 to 18 days.

Total organic matter loss by *P. chrysosporium* and *P. ostreatus* at the end of the fermentation period was 55 and 20%, respectively. A difference in selective lignin degradation by the two fungi was observed. Kamra and Zadrazil (18) defined the term "process efficiency" as the ratio between residual lignin and residual organic matter. The ratio for *P. ostreatus* fermentation from days 6 to 18 was 1.3, increasing slowly to a steady value of 1.4 from day 25 until the end of the fermentation period. A constant value of 1.0 was maintained throughout fermentation by *P. chrysosporium*.

A direct determination of lignin is problematic. Acid-insoluble lignin content has been measured by the Goering-Van Soest method (14), but no single lignin determination

method has been found satisfactory to date (26). More accurate determinations can be achieved by monitoring radiolabeled lignin during biodegradation (8). However, one should bear in mind that only newly synthesized lignin is labeled. In our experimental system, solid-state fermentation of lignocellulose with added ^{14}C-lignin from cotton was performed. The specificity of the labeling was confirmed by monitoring solid-state fermentation of *S. rolfsii*, which cannot degrade lignin: only 0.5% of the ^{14}C labeling was released as either $^{14}CO_2$ or water-soluble compounds.

During solid-state fermentation by *P. chrysosporium*, the mineralization kinetics of the ^{14}C-lignin corresponded to that of lignin degradation as measured by the Goering-Van Soest method (14). Both activities ceased after 18 days of fermentation. Different kinetics in both parameters was observed for *P. ostreatus*, showing a slower rate and longer period of lignin degradation. *P. chrysosporium*'s mineralization rate from lignin to $^{14}CO_2$ was 2.5 times faster than that of *P. ostreatus*. The latter's mineralization rate in this system was similar to ^{14}C-ring-labeled dehydrogenative polymerizate (DHP) mineralization rates in a liquid culture, as observed for *P. ostreatus* by Platt et al. (33). The rate of mineralization from ^{14}C-lignin to $^{14}CO_2$ was 7.6 and 6.5 times slower than the rate of acid-insoluble lignin degradation by *P. chrysosporium* and *P. ostreatus*, respectively.

These observations show distinct differences in lignocellulose biodegradation by the two fungi. There is a time-dependent correlation between KTBA cleavage and lignin biodegradation in both, though the timing is different. KTBA cleavage activity points to the involvement of hydroxy (·OH) and phenoxy (·ϕ-OH) radicals in lignin degradation as was suggested by Kutsuki and Gold (21). Faison and Kirk (12) showed the existence of two excited oxygen species in the growth medium of *P. chrysosporium*. They showed the dependence of the lignin biodegradation mechanism in these oxygen species, using catalase and superoxide dismutase. The lack of lignin peroxidase activity in *P. ostreatus* cultures suggests that other enzymes may be responsible for free radical production by this fungus. Platt et al. (32) suggested the initiative role of laccase in *P. ostreatus* lignin degradation, making lignin degradation by this fungus dependent on the presence of laccase. Our results show higher laccase activity at the beginning of the lignin degradation period, subsequently decreasing to a steady level while lignin degradation rates remain constant. Laccase could act by both detoxifying compounds in the substrate (24) and oxidizing phenolic groups as an initial enzyme involved in the cleavage of side chains and aromatic rings of lignin phenolic moieties (15). *Dichomitus squalens* was also shown to degrade lignin while lacking lignin peroxidase (31). When it was grown in liquid culture under conditions favoring lignin degradation, high Mn-dependent peroxidase and laccase activities were evident. On the other hand, Perez and Jeffries (30) have shown that laccase and Mn-dependent peroxidase activities of *Phlebia brevispora* are not correlated with DHP-lignin degradation, while positive correlation was found with lignin peroxidase activity. Other enzymes of an oxidizing nature, such as the veratryl alcohol oxidases reported by Sannia et al. (38) on day 20 of a liquid culture of *P. ostreatus*, may play a role in lignin degradation.

ACKNOWLEDGMENT

We thank Ilan Chet for his excellent ideas and helpful discussions.

REFERENCES

1. **Agosin, E., B. Monties, and E. Odier.** 1985. Structural changes in wheat straw during decay by lignin degrading white-rot fungi in relation to improvement of digestibility for ruminants. J. Sci. Food Agric. **36:**925–935.
2. **Ander, P., K.-E. Eriksson, P. Mansson, and B. Petterson.** 1981. Lignin degradation by *Sporotrichum pulverulentum*: a new cultivation method to study fungal lignin degradation. Int. Symp. Wood Pulping Chem. **3:**71.
3. **Balasubramanya, R. H., D. Y. Pai, A. J. Saikh, and V. J. Khandeparker.** 1989. Biological softening of spent cotton-plant stalks for the preparation of pulp. Biol. Wastes **30:**317–320.
4. **Bartha, R., and D. Pramer.** 1965. Features of a flask and method for measuring the persistence and biological effects of pesticides in soil. Soil Sci. **100:**68–70.
5. **Betts, W. B., and J. E. King.** 1991. Oxidative coupling of 2,6-dimethoxyphenol by fungi and bacteria. Mycol. Res. **95:** 526–530.
6. **Block, S. S., G. Tsao, and L. Han.** 1959. Experiments in the cultivation of *Pleurotus ostreatus*. Mushroom Sci. **4:**309–325.
7. **Colberg, P. J., and L. Y. Young.** 1985. Anaerobic degradation of soluble fractions of [^{14}C-lignin]lignocellulose. Appl. Environ. Microbiol. **49:**345–349.
8. **Crawford, D. L., and R. L. Crawford.** 1976. Microbial degradation of lignocellulose: the lignin component. Appl. Environ. Microbiol. **31:**714–717.
9. **Crawford, D. L., S. Floyd, A. L. Pometto III, and R. L. Crawford.** 1977. Degradation of natural and Kraft lignin by the microflora of soil and water. Can. J. Microbiol. **23:**434–440.
10. **Crawford, R. L.** 1981. Lignin biodegradation and transformation. John Wiley & Sons, Inc., New York.
11. **Datta, A., A. Bettermann, and T. K. Kirk.** 1991. Identification of a specific manganese peroxidase among ligninolytic enzymes secreted by *Phanerochaete chrysosporium* during wood decay. Appl. Environ. Microbiol. **57:**1453–1460.
12. **Faison, B. D., and T. K. Kirk.** 1983. Relationship between lignin degradation and production of reduced oxygen species by *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **46:** 1140–1145.
13. **Farrell, R. L., K. E. Murtagh, M. Tien, M. D. Mozuch, and T. K. Kirk.** 1989. Physical and enzymatic properties of lignin peroxidase isoenzymes from *Phanerochaete chrysosporium*. Enzyme Microb. Technol. **11:**322–328.
14. **Goering, H. K., and P. J. Van Soest.** 1970. Forage fiber analysis, p. 1–19. Agricultural handbook no. 379. Agricultural Research Service, U.S. Department of Agriculture, Washington, D.C.
15. **Higuchi, T.** 1990. Lignin biochemistry: biosynthesis and biodegradation. Wood Sci. Technol. **24:**23–63.
16. **Holzbaur, E. L. F., A. Andrawis, and M. Tien.** 1991. Molecular biology of lignin peroxidase from *Phanerochaete chrysosporium*, p. 197–223. *In* S. A. Leong, and R. M. Berka (ed.), Molecular industrial mycology. Marcel Dekker, Inc., New York.
17. **Johnson, D. B., W. E. Moore, and L. C. Zank.** 1961. The spectrophotometric determination of lignin in small wood samples. Tappi J. **44:**793–797.
18. **Kamra, D. N., and F. Zadrazil.** 1986. Influence of gaseous phase, light and substrate pretreatment on fruit-body formation, lignin degradation and in vitro digestibility of wheat straw fermented with *Pleurotus spp.* Agric. Wastes **18:**1–17.
19. **Kelley, R. L.** 1988. Ligninolytic activity of *Phanerochaete chrysosporium* measured as ethylene production from α-keto-γ methylthiolbutyric acid, p. 79–82. *In* A. W. Wood, and S. T. Kellog (ed.), Biomass: lignin, pectin and chitin. Academic Press, Inc., San Diego.
20. **Kimura, Y., Y. Asada, and M. Kuwahara.** 1990. Screening of basidiomycetes for lignin peroxidase genes using a DNA probe. Appl. Microbiol. Biotechnol. **32:**436–442.
21. **Kutsuki, H., and M. H. Gold.** 1982. Generation of hydroxyl radicals and its involvement in lignin degradation by *Phanerochaete chrysosporium*. Biochem. Biophys. Res. Commun. **109:** 320–327.
22. **Lee, B., A. L. Pometto III, A. Fratzke, and T. B. Bailey, Jr.** 1991.

Biodegradation of degradable plastic polyethylene by *Phanerochaete chrysosporium* and *Streptomyces* species. Appl. Environ. Microbiol. **57**:678–685.

23. **Leisola, M., D. Ulmer, and A. Fiechter.** 1983. Problem of oxygen transfer during degradation of lignin by *Phanerochaete chrysosporium*. Eur. J. Appl. Microbiol. Biotechnol. **17**:113–116.
24. **Mayer, A. M.** 1987. Polyphenol oxidases in plants—recent progress. Phytochemistry **26**:11–20.
25. **Milstein, O., A. Haars, A. Majcherczyk, J. Trojanowski, D. Tautz, H. Zanker, and A. Huttermann.** 1988. Removal of chlorophenols and chlorolignins from bleaching effluent by combined chemical and biological treatment. Water Sci. Technol. **20**:161–170.
26. **Monties, B.** 1989. Lignins, p. 113–157. *In* J. B. Harborne (ed.), Plant phenolics. Academic Press Ltd., London.
27. **Morrison, I. M.** 1972. A semi-micro method for the determination of lignin and its use in predicting the digestibility of forage crop. J. Sci. Food Agric. **23**:455–463.
28. **Musha, Y., and D. A. I. Goring.** 1974. Klason and acid-soluble lignin content of hardwoods. Wood Sci. **7**:133–134.
29. **Ong, E., N. R. Gilkes, R. Anthony, J. Warren, R. C. Miller, Jr. and D. G. Killburn.** 1989. Enzyme immobilization using the cellulose-binding domain of a *Cellulomonas fimi* exoglucanase. Bio/Technology **7**:604–607.
30. **Perez, J., and T. W. Jeffries.** 1990. Mineralization of ^{14}C-ring-labeled synthetic lignin correlates with the production of lignin peroxidase, not of manganese peroxidase or laccase. Appl. Environ. Microbiol. **56**:1806–1812.
31. **Perie, F. H., and M. H. Gold.** 1991. Manganese regulation of manganese peroxidase expression and lignin degradation by the white rot fungus *Dichomitus squalens*. Appl. Environ. Microbiol. **57**:2240–2245.
32. **Platt, M. W., Y. Hadar, and I. Chet.** 1984. Fungal activities involved in lignocellulose degradation by *Pleurotus*. Appl. Microbiol. Biotechnol. **20**:150–154.
33. **Platt, M. W., J. Trojanovski, I. Chet, and A. Huttermann.** 1983. Differences in the degradation of specifically ^{14}C-labelled lignin model compounds within the *Pleurotus* genus group. Microbiol. Lett. **23**:19–21.
34. **Pryor, W. A., and R. H. Tang.** 1978. Ethylene formation from methional. Biochem. Biophys. Res. Commun. **81**:498–503.
35. **Reid, I. D., E. E. Chao, and P. P. S. Dawson.** 1985. Lignin degradation by *Phanerochaete chrysosporium* in agitated cultures. Can. J. Microbiol. **31**:88–90.
36. **Reid, I. D., and K. A. Seifert.** 1982. Effect of atmosphere of oxygen on growth and lignin degradation by white rot fungi. Can. J. Bot. **60**:252–260.
37. **Sannia, G., P. Giardina, M. Luna, M. Rossi, and V. Buonocore.** 1986. Laccase from *Pleurotus ostreatus*. Biotechnol. Lett. **8**:797–800.
38. **Sannia, G., P. Limongi, E. Cocca, F. Buonocore, G. Nitti, and P. Giardina.** 1991. Purification and characterization of a veratryl alcohol oxidase enzyme from the lignin degrading basidiomycete *Pleurotus ostreatus*. Biochim. Biophys. Acta **1073**:114–119.
39. **Schnurer, J., and T. Rosswall.** 1982. Fluorescein diacetate hydrolysis as a measure of total microbial activity in soil and in litter. Appl. Environ. Microbiol. **43**:1256–1261.
40. **Scott, T. A., and E. H. Melvin.** 1953. Determination of dextran with anthrone. Anal. Chem. **25**:1656.
41. **Sedmac, J. J., and S. E. Grossberg.** 1977. A rapid, sensitive and versatile assay for protein using Coomassie brilliant blue G250. Anal. Biochem. **79**:544–552.
42. **Waldner, R., M. S. A. Leisola, and A. Fiechter.** 1988. Comparison of ligninolytic activities of selected white-rot fungi. Appl. Environ. Microbiol. **29**:400–407.
43. **Zadrazil, F., and H. Brunert.** 1982. Solid state fermentation of lignocellulose containing plant residues with *Sporotrychum pulverulentum* Nov. and *Dichomitus squalens* (Krast.) Reid. Eur. J. Appl. Microbiol. Biotechnol. **16**:45–51.
44. **Zadrazil, F., and K. Grabbe.** 1983. Edible mushrooms, p. 146–187. *In* H.-J. Rehm, and G. Reed (ed.), Biotechnology. Verlag Chemie, Weinheim, Germany.