

## Requirement for a Growth Substrate During Lignin Decomposition by Two Wood-Rotting Fungi

T. KENT KIRK, W. J. CONNORS, AND J. GREGORY ZEIKUS\*

Forest Products Laboratory, Forest Service, U. S. Department of Agriculture, Madison, Wisconsin 53705,  
and Department of Bacteriology, University of Wisconsin, Madison, Wisconsin 53706\*

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Decomposition of  $^{14}\text{C}$ -labeled lignin to  $^{14}\text{CO}_2$  by the lignin-decomposing fungi *Phanerochaete chrysosporium* and *Coriolus versicolor* required a growth substrate such as cellulose or glucose. Growth with lignin as sole carbon addition to an otherwise complete medium was negligible.

We have recently developed a sensitive assay for lignin biodegradation based on the conversion of synthetic  $^{14}\text{C}$ -labeled lignins to  $^{14}\text{CO}_2$  (6). Initial studies demonstrated that  $^{14}\text{C}$ -labeled lignins were degraded by fungal cultures grown on wood meal and by the established microflora of a forest soil (6). Our ultimate research objectives are to delineate the nutritional, physiological, and environmental factors that affect the microbial decomposition of lignin, and, for that, a detailed study of degradation of the complex aromatic polymer by the white-rot, wood-destroying fungus *Phanerochaete chrysosporium* Burds. (3) is underway. We report here that catabolism of lignin by *P. chrysosporium* and by the much studied lignin-decomposing, white-rot fungus *Coriolus versicolor* (L. ex Fr.) Quel. requires a growth substrate; lignin alone is insufficient.

Experiments were conducted in 125-ml Erlenmeyer flasks. Flasks containing no  $^{14}\text{C}$ -labeled lignin were closed with cotton plugs. In the studies with  $^{14}\text{C}$ -labeled lignin, the flasks were closed with rubber stoppers equipped with ports for periodic aeration and collection of effluent gases. During incubation the flasks were flushed with sterile air for 15 min at 100 to 200 ml/min every third day, and effluent  $\text{CO}_2$  was trapped directly in scintillation fluid (6). Exchange of gases in the cultures was shown to be complete, and double trapping experiments demonstrated that all  $^{14}\text{CO}_2$  was in the first trap. (Extraction and combustion studies of spent cultures have shown that loss of  $^{14}\text{CO}_2$  or other volatiles during incubation under the conditions used here is negligible; most of the residual radioactivity can be extracted from the mycelial residues.)

Flasks contained, in a total volume of 10 ml, lignin, growth substrate, 1.0 ml of filter-sterilized basal medium, 1.0 ml of 0.1 M phthalate buffer, and water. Basal medium was the same

as used earlier (6) except that 0.5 g instead of 2.0 g of  $\text{NH}_4\text{NO}_3$  per liter was used. Flasks were seeded with 1.0 ml of a spore suspension ( $\sim 2.5 \times 10^6$  spores) from a 3-week-old malt agar slant of *P. chrysosporium* ME-446 or with 1.0 ml of a mycelial suspension ( $\sim 1$  mg, dry weight) of *C. versicolor* Madison 697. *P. chrysosporium* and *C. versicolor* were incubated at  $40^\circ\text{C}$  and pH 4.6 and  $27^\circ\text{C}$  and pH 5.0, respectively.

Unlabeled synthetic lignin (6), synthetic [ring- $^{14}\text{C}$ ]-labeled lignin (6), and spruce [*Picea glauca* (Moench) Voss]-milled wood lignin (1.4% total carbohydrate, 14.2%  $\text{OCH}_3$ ) (2) were used. Synthetic  $^{14}\text{C}$ -labeled lignin was added to culture vessels as sterile 8% solutions in dimethylformamide ( $<10 \mu\text{l/culture}$ ). Milled wood lignin (MWL) and unlabeled synthetic lignin were added as sterile suspensions in distilled water (5 or 20 mg/ml). The MWL was sterilized by passage of 2% solutions in 90% acetic acid through a sterile  $0.2\text{-}\mu\text{m}$  Fluoropore filter (Millipore Corp., Bedford, Mass.), followed by precipitation in sterile water, centrifugation, washing once in sterile 1%  $\text{Na}_2\text{SO}_4$  and then in sterile water, and resuspension in sterile water.

The radioactivity assay described previously (6) was used to measure the amount of  $^{14}\text{C}$ -labeled lignin converted to  $^{14}\text{CO}_2$ . Experiments were performed in duplicate or triplicate, and values are reported as averages.

Carbohydrates studied as growth substrates included cellulose from wood pulp ("Solka Floc SW40," Brown Co., Berlin, N.H.) and Whatman cellulose powder (no. S-18878, Sargent-Welch Co., Skokie, Ill.), wood gum xylan (Nutritional Biochemicals Corp., Cleveland, Ohio), D-xylose, D-glucose, and D-cellobiose. The last three substrates were added to cultures as filter-sterilized solutions, and the polysaccharides were sterilized by autoclaving dry in the flasks at  $121^\circ\text{C}$  for 20 min.

Growth of mycelia in the presence of MWL and/or glucose was determined as follows. Cultures were extracted overnight with 50% aqueous dioxane to remove most of the residual MWL, mycelia were recovered on asbestos pads in tared crucibles, and dry weights were determined. Because the extraction did not remove all of the MWL from the mycelia, growth was determined by measuring total reducing sugars in acid hydrolysates of the extracted mycelia. Pooled triplicate samples were hydrolyzed with 3 ml of 72% H<sub>2</sub>SO<sub>4</sub> at 30°C for 1 h and then with 4% H<sub>2</sub>SO<sub>4</sub> at 120°C for 1 h and neutralized with CaCO<sub>3</sub>. Reducing sugars were determined by the Nelson adaptation of the Somogyi method (7) and estimated as glucose. After 50% dioxane extraction, glucose-grown mycelia of *P. chrysosporium* and *C. versicolor* yielded 49.8 and 51.6% reducing sugars, respectively, in the above procedure.

When supplied with spruce MWL as the sole carbon and energy source, *P. chrysosporium* and *C. versicolor* degraded only a negligible amount of <sup>14</sup>C-labeled lignin to <sup>14</sup>CO<sub>2</sub> (Table 1). No measurable growth occurred. When synthetic lignin was substituted for MWL, similar results were obtained. If cellulose or another suitable substrate was substituted for the MWL, the <sup>14</sup>C-labeled lignin was degraded and growth occurred. Several carbohydrates related to the natural woody substrates of these fungi were examined for support of lignin degradation. The carbohydrates varied considerably in effectiveness, and the fungi differed in their responses to the individual compounds (Table 1). All of the carbohydrates supported good growth as ascertained visually.

The amount of lignin degraded to CO<sub>2</sub> depended on the amount of growth substrate provided during growth. Conversion of <sup>14</sup>C-labeled lignin to <sup>14</sup>CO<sub>2</sub> was monitored during growth with substrate quantities of MWL plus varying amounts of cellulose. The rate and extent of <sup>14</sup>CO<sub>2</sub> evolution depended on the amount of cellulose provided. Results with *P. chrysosporium* are shown in Fig. 1; similar results were obtained with *C. versicolor*. These data suggest that lignin decomposition ceased when cellulose was depleted. The relationship between the amount of cellulose supplied and the total lignin degraded to CO<sub>2</sub> was essentially linear and indicated that no lignin was degraded to CO<sub>2</sub> in the absence of cellulose.

Growth with MWL as sole carbon source was very slight or absent (Table 2). In these experiments the change in dry weight of extracted mycelium was determined after a 5-day incubation in buffered basal medium containing lignin and/or glucose. The trace of growth with

TABLE 1. Effectiveness of various carbohydrates as growth substrates for degradation of synthetic <sup>14</sup>C-labeled lignin

Growth substrates <sup>a</sup>	% Degradation <sup>b</sup> to <sup>14</sup> CO <sub>2</sub>	
	<i>P. chrysosporium</i>	<i>C. versicolor</i>
Milled wood lignin <sup>c</sup>	<0.1	<0.3
Solka Floc (cellulose)	19.7 ± 2.5	15.9 ± 0.2
Cellulose powder	10.4 ± 1.6	16.4 ± 3.6
D-Xylose	12.5 ± 2.6*	5.9 ± 1.8*
D-Glucose	4.9 ± 1.5*	12.2 ± 0.8*
D-Cellobiose	6.3 ± 2.1*	12.0 ± 3.0
Xylan	0.8 ± 0.2	4.4 ± 0.2

<sup>a</sup> Flasks contained basal medium, 0.01 M phthalate buffer, 1.0 mg of synthetic <sup>14</sup>C labeled lignin (31,000 dpm), and 100 mg of growth substrate in a final volume of 10 ml.

<sup>b</sup> Percentage of total <sup>14</sup>C recovered as <sup>14</sup>CO<sub>2</sub>, after 55 days of incubation with all growth substrates except xylan, which was terminated after 34 days with *P. chrysosporium*. Average values for two or three replicate cultures are given. Standard deviations are given for triplicate cultures; experimental ranges are given for duplicate (\*) cultures.

<sup>c</sup> Flasks contained 100 mg of MWL instead of carbohydrates.

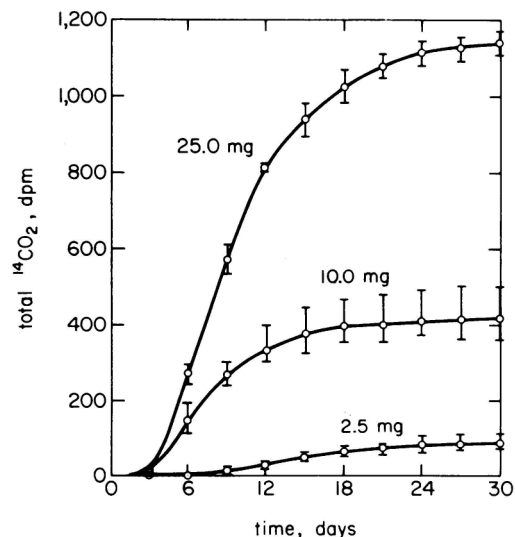


FIG. 1. Effect of cellulose addition on rate of lignin degradation by *P. chrysosporium*. Erlenmeyer flasks (125-ml) contained, in a total volume of 10 ml: basal medium, 0.01 M phthalate buffer, 25 mg of MWL, <sup>14</sup>C-labeled synthetic lignin (35,000 dpm), and 2.5, 10.0, or 25.0 mg of cellulose.

lignin as sole carbon addition was probably a reflection of the small amount of carbohydrate present in the MWL sample (1.4%). In other experiments it was shown that no growth occurred on substrate quantities of unlabeled synthetic lignin. Growth of *P. chrysosporium*, but not of *C. versicolor*, was stimulated by MWL. *P. chrysosporium* generally grows more rapidly than *C. versicolor*.

TABLE 2. Relation of fungal growth on lignin to growth substrate addition

Additions <sup>a</sup>	Growth, dry wt (mg) <sup>b</sup>	
	<i>P. chryso- sporium</i>	<i>C. versicolor</i>
MWL only	<0.2	<0.1
MWL + glucose	5.2	3.5
Glucose only	4.3	3.7

<sup>a</sup> Each 125-ml Erlenmeyer flask contained, in a total volume of 10 ml: basal medium; 0.01 M phthalate buffer; and 25 mg of MWL; 25 mg of MWL + 15 mg of glucose; 15 mg of glucose; or no addition. Cultures were incubated without shaking for 5 days.

<sup>b</sup> Dry weight of extracted mycelium corrected for adsorbed lignin. Values were determined from total reducing sugars in acid hydrolysates of pooled triplicate samples (see text).

These data demonstrate that certain fungi require a growth substrate to degrade lignin. Neither fungus used here grew significantly on spruce lignin or catabolized synthetic [ring-<sup>14</sup>C]-labeled lignin to <sup>14</sup>CO<sub>2</sub> in the absence of carbohydrates.

These results conflict with previous reports that *C. versicolor* and other white-rot fungi can grow on lignin as sole carbon source (5). A basis for the discrepancy may lie in differences in the methods used to determine growth and degradation or in the lignin preparations used. Whereas the MWL used here is representative of the total lignin in wood (4), the Brauns lignin used by earlier workers (5) is a low-molecular-weight, ethanol-soluble fraction that differs

substantially from the bulk of the lignin. Ander and Eriksson (1) reported recently that a commercial kraft lignin, which is a degraded industrial by-product of wood pulping, serves as substrate for growth of white-rot fungi but that cellulose stimulates growth and kraft lignin degradation.

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