APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 1976, p. 714-717

Vol. 31, No. 5
Printed in U.S.A.

Microbial Degradation of Lignocellulose: the Lignin Component

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Received for publication 20 November 1975

A new procedure was developed for the study of lignin biodegradation by pure or mixed cultures of microorganisms. Natural lignocelluloses were prepared containing ^{14}C in primarily their lignin components by feeding plants L-[U-^{14}C]phenylalanine through their cut stems. Lignin degradation was observed in numerous soils by monitoring evolution of $^{14}CO_2$ from [^{14}C]lignin-labeled oak (*Quercus albus*), maple (*Acer rubrum*), and cattail (*Typha latifola*). An organism (*Thermonospora fusca* ATCC 27730) that is known to degrade cellulose but not lignin was shown to grow on lignocellulose in the presence of [^{14}C]lignocelluloses without evolution of $^{14}CO_2$. A known lignin degrader (a white-rot fungus, *Polyporus versicolor*) was shown to readily evolve $^{14}CO_2$ from damp ^{14}C-labeled cattail and ^{14}C-labeled maple.

Lignin is a structural polymer of vascular plants that performs functions essential to their survival (16). Lignin gives plants rigidity and binds plant cells together in such a manner as to impart resistance towards impact, bending, and compression. Lignin plays an essential role in water/nutrient transport by acting as a barrier to permeation of water across cell walls of xylem tissue. Lignified tissues also act as barriers to prevent invasion of pathogenic microorganisms.

Lignin is a vital component of the biospheric carbon cycle since it ranks second only to cellulose in abundance as a naturally occurring biopolymer (6). Much has been learned during the past 30 years concerning the structure and biosynthesis of this complex, aromatic polymer (16). Corresponding progress in the study of microbiological degradation of lignin has been minimal, for the reasons summarized by Kirk et al. (9-11). Here we describe a method for study of lignin degradation by microorganisms either in pure culture or in natural habitats such as soil. The procedure involves preparation of natural lignocelluloses that contain ^{14}C primarily in their lignin components (as opposed to the ^{14}C-labeled synthetic polymers of Kirk et al. [11] and Haider and Trojanouski [5]). Microbial degradation of the lignin component of these natural lignin-cellulose complexes was observed in numerous soils and in pure cultures of a thermophilic, cellulolytic bacterium and a white-rot fungus by trapping $^{14}CO_2$ liberated during the decay process.

MATERIALS AND METHODS

Preparation of [^{14}C]lignocelluloses. The lignin components of various lignocelluloses were selectively labeled with ^{14}C by feeding plants aqueous solutions of L-[U-^{14}C]phenylalanine through their cut stems (2, 7). Labeled plant material was Wiley-milled to pass through a 40-mesh screen and extracted by the following procedure (12) to remove unincorporated phenylalanine and other extractable compounds: (i) washed with water at 80 C for 4 h; (ii) refluxed in 1:1 benzene-ethanol for 4 h, repeated once; (iii) refluxed with ethanol for 2 h, repeated until the ethanol remained colorless; and (iv) washed with water at 80 C for 2 h, repeated once. Extracted lignocelluloses were dried overnight at 50 C. Previous investigators have shown that phenylalanine is an efficient lignin precursor in many plant species (1, 2, 7). We have indirectly confirmed these observations. [^{14}C]lignocelluloses were prepared as described above from maple (*Acer rubrum*), oak (*Quercus albus*), and cattail (*Typha latifola*). These extractive-free lignocelluloses were analyzed for distribution of ^{14}C by a modified Klason fractionation procedure (12, 15). Klason lignin is the water-insoluble material remaining after digestion of lignified tissue with cold 72% H_2SO_4, dilution, and refluxing with dilute acid. The Klason procedure is the standard method by which plant materials are analyzed for lignin content (15). In all cases at least 90% of the incorporated ^{14}C in the extracted tissues was located in the lignin (acid-insoluble component (oak, 90%; maple, 95%; cattail, 93%). These values for percent incorporation of label into lignin components of our extractive-free lignocelluloses are minimal values since part of a plant's lignin is known to be acid soluble (13, 15, 16).

Counting of radioactivity. ^{14}C was quantified by

liquid scintillation counting (LSC) techniques. The instruments used were a Nuclear-Chicago Mark II LSC system and a Beckman model LS-200B spectrometer. Two counting/trapping fluids were utilized for trapping and quantifying $^{14}CO_2$. The first contained in each 130 ml of toluene: PPO (2,5-diphenyloxazole), 3.0 g; POPOP [1,4-bis-(5-phenyloxazolyl)benzene], 0.00375 g; methanol, 120 ml; and 2-aminoethanol, 30 ml. The second contained in each 100 ml of toluene: BBOT [2,5-bis-2-(5-tertbutylbenzoxazoyl)thiophene], 0.4 g; 2-aminoethanol, 20 ml; and methanol, 80 ml. Quenching was determined by the samples channels ratio method. Specific radioactivities of [^{14}C]lignocelluloses were determined by counting 1 to 5 mg of finely ground, extracted lignocellulose on LSC pads in 15 ml of LSC fluid. The three lignocelluloses had the following specific radioactivities (disintegrations per minute per milligram): maple, 3.2×10^3; oak, 9.4×10^3; and cattail, 3.0×10^3. Specific radioactivities were determined by using a quench curve, and values were confirmed by completely combusting known amounts of each [^{14}C]lignocellulose at >600 C and counting released $^{14}CO_2$ in the LSC/trapping solution.

Biodegradation of [^{14}C]lignocelluloses. Soil samples were placed in sterile flasks equipped with sealed ports for periodic aeration and flushing of the gas phase. Reaction vessels contained soil, mineral salts buffer (3) or water, and [^{14}C]lignocellulose as indicated in the figure legends. $^{14}CO_2$ evolved during incubation of individual flasks was flushed from the vessel every 12 to 48 h with a stream of sterile, CO_2-free air (10 ml/min) and trapped directly by bubbling the effluent through 15 ml of LSC/trapping fluid. Flushing and trapping of evolved $^{14}CO_2$ were shown to be quantitative. For incubation conditions, refer to the figure legend.

For experiments with the cellulolytic thermophile *Thermomonospora fusca* (3,4; ATCC 27730 = NCIB 11185), the mineral salts medium of Crawford et al. (4) was supplemented with 1 g of yeast extract (Difco) per liter. To 125 ml of medium in a sealed, ported 500-ml flask were added 750 mg of an 18% lignin pulp (pulp no. 4, reference 3) and 30 mg of ^{14}C-labeled oak (2.8×10^5 dpm) or 30 mg of ^{14}C-labeled maple (1.2×10^5 dpm). This suspension was inoculated with the spores from one stock slant of *T. fusca* and incubated in a New Brunswick incubator/shaker at 55 C and 175 rpm. The gas phase within the flask was flushed through $^{14}CO_2$ trapping/counting solution at frequent intervals to prevent oxygen depletion at the thermophilic incubation temperature. The experiments were then repeated with cold, extractive-free oak and maple in place of the lignocellulosic pulp.

To examine degradation of ^{14}C-labeled cattail and ^{14}C-labeled maple by the lignin-degrading fungus *Polyporus versicolor*, 10 mg (3.0×10^4 dpm) of damp, sterile ^{14}C-labeled cattail or 10 mg of damp, sterile ^{14}C-labeled maple (3.2×10^4 dpm) was placed in the bottom of a sterile, sealed 250-ml flask. This was inoculated with a small piece of fungal mycelium. Incubation was at 30 C. The gas phase within the vessel was flushed through $^{14}CO_2$ trapping/counting solution every 40 to 48 h.

RESULTS AND DISCUSSION

Observed evolution of $^{14}CO_2$ during soil-mediated degradation of ^{14}C-labeled oak, ^{14}C-labeled maple, and ^{14}C-labeled cattail is summarized in Fig. 1A through C, respectively. All soils (cf. figure legend) were high in organic matter, and 1:1 soil-water suspensions gave pH values between 7 and 8. They were collected July through September and were used fresh. For comparative purposes, Fig. 1D illustrates the observed evolution of $^{14}CO_2$ during soil-catalyzed degradation of L-[^{14}C]phenylalanine. Soils sterilized with formaldehyde did not catalyze evolution of $^{14}CO_2$.

When *T. fusca* was grown on lignocellulose (pulp no. 4, reference 3) in the presence of ^{14}C-labeled oak or ^{14}C-labeled maple, the thermophile grew very well as evidenced by macroscopic growth. After 14 days, 48% of the total lignocellulose (dry weight basis) had been solubilized, and another 8% had been incorporated into the bacterial cell mass (cf. reference 3 for methods used to calculate these percentages). Despite this pronounced degradation of lignocellulose, almost no $^{14}CO_2$ was evolved from these cultures (0.54% of added ^{14}C of the labeled oak at 261 h and 1.6% of added ^{14}C of the labeled maple at 242 h; cattail was not examined in this regard). Similar results were obtained when cold, extractive-free oak or maple replaced the lignocellulosic pulp. These observations are in accord with previous conclusions that *T. fusca* (ATCC 27730) is actively cellulolytic but does not degrade the lignin fraction of lignocelluloses (3). Also, these observations offer additional proof that the ^{14}C label in our lignocellulose preparations is not significantly incorporated into the carbohydrate fraction of the ligin-polysaccharide complex.

Inoculation of damp ^{14}C-labeled cattail or ^{14}C-labeled maple with a pure culture of the white-rot fungus *P. versicolor* resulted in substantial evolution of $^{14}CO_2$ after an initial, short (48-h) lag period. Approximately 47% of the ^{14}C provided in 10 mg of ^{14}C-labeled cattail was evolved as $^{14}CO_2$ after 184 h of incubation. Similar results were obtained with ^{14}C-labeled maple. This rapid degradation of lignin by a pure culture of *P. versicolor* confirms results of Kirk et al. (11), who examined degradation of ^{14}C-labeled synthetic lignins (see below) by certain other white-rot fungi. Several soft-rot fungi have also been shown to release $^{14}CO_2$ from ^{14}C-labeled synthetic lignins (5). The catabolic rates observed in the present case, however, are considerably faster than those observed by Kirk et al. (11) and Haider and Trojanouski (5).

Data summarized in Fig. 1 indicate substan-

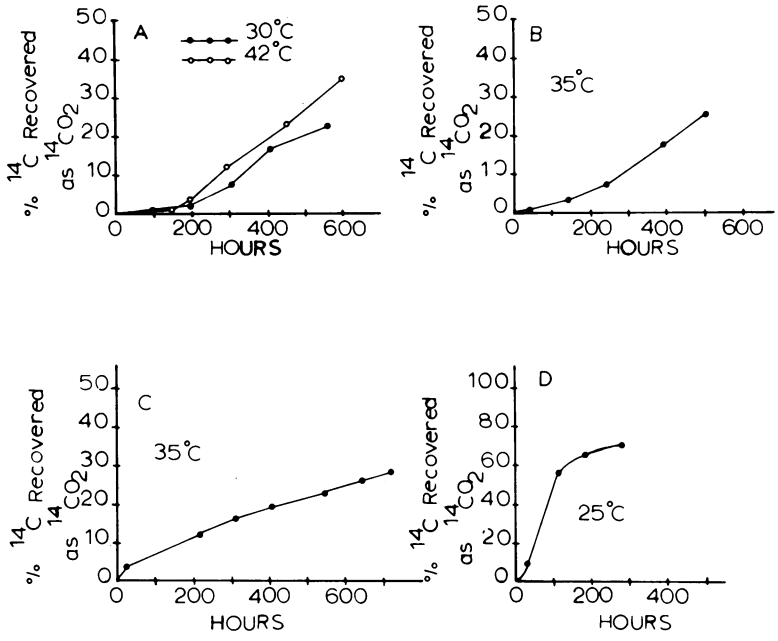

FIG. 1. *Conversion of [^{14}C]lignocelluloses and [^{14}C]phenylalanine to $^{14}CO_2$ by soil microorganisms. (A) Incubations were performed in the dark at the indicated temperatures in 500-ml, sealed flasks that contained 125 ml of sterile mineral salts medium (3), 750 mg of an 18% lignin pulp (pulp no. 4, reference 3), and 30 mg of ^{14}C-labeled oak (2.8 × 10^5 dpm). Flasks were inoculated with 1.0 g of soil taken from an active compost and incubated in an environmental shaker at 175 rpm. The flasks were reaerated, and their gas phase was flushed through $^{14}CO_2$ trapping/counting fluid every 15 to 30 h. (B) Incubation was in the dark at 35 C in a 250-ml, sealed flask that contained 1.0 g of forest soil (top 2 cm), 10 ml of sterile water, and 10 mg of ^{14}C-labeled maple (3.2 × 10^4 dpm). The flask was shaken, and its gas phase was sampled as described in (A) above. (C) Conditions were as in (B) above except that 25 mg of ^{14}C-labeled cattail (7.5 × 10^4 dpm) replaced the ^{14}C-labeled maple and the soil sample came from the edge of a cattail marsh. (D) Conditions were as in (A) above except that 100 mg of cold L-phenylalanine plus 2.2 × 10^5 dpm of L-[U-^{14}C]phenylalanine replaced the lignocellulose.*

tial decomposition of the various lignins by the microflora of different soils. The percentages shown for conversion of [^{14}C]lignocelluloses to $^{14}CO_2$ are actually minimum values since much lignin may be degraded without conversion to CO_2 by the process of humification (8, 14). Some ^{14}C will be incorporated into cell mass of soil microorganisms, decreasing further the observed rates of lignin degradation. Our observations also confirm previous indications (11) that lignin degradation by soil microorganisms is a relatively slow process. The recalcitrance of lignin is particularly evident when its rate of degradation is compared with the rate of degradation of phenylalanine (Fig. 1D), the low-molecular-weight precursor of lignin. After 300 h of incubation at 35 C, only about 10% of the label in 10 mg of maple lignocellulose had been evolved as $^{14}CO_2$ (Fig. 1B), whereas more than 65% of the label in 100 mg of phenylalanine had been evolved as $^{14}CO_2$ after only 200 h of incubation at 25 C. Phenylalanine degradation was even faster when incubations with soil were carried out at 35 C. The degradation pattern was similar to that observed at 25 C except that the lag phase prior to a maximal $^{14}CO_2$ evolution rate was shortened by about 24 h, resulting in a 75% total recovery of label as $^{14}CO_2$ at about 175 h. Although rates of lignin degradation that we have observed are slow, they are more rapid than those reported in a forest soil by Kirk et al. (11). These authors used a synthetic [^{14}C]lignin for their experiments, a "dehydrogenation polymerisate" (DHP). Such synthetic polymers are true lignins (11, 16); however, they are unnatural substrates since they are completely free from carbohydrates. Thus, it is not surprising that the lignin components of our lignocelluloses are decomposed more rapidly in soil than are DHPs, since our lignocelluloses are in their natural condition, complexed with polysaccharides. The cellulose in our substrates may, in fact, speed lignin degradation by acting as a substrate and an additional source of energy for lignocellulose degraders in soil. Evidence indicates that lignin degradation by the representative lingin-decomposing fungi *Phanerochaete chrysosporium* and *Coriolus*

versicolor requires a readily metabolizable cosubstrate such as cellulose (T. Kent Kirk, personal communication). Synthetic lignins are prepared with ^{14}C placed at specific points in the lignin molecule (rings, methoxyls, or side chains) and are irreplaceable for studies of degradation of specific chemical structures in the lignin polymer (11). Our labeling procedure lacks the versatility of the more complex DHP synthesis. However, it may be possible to produce specifically labeled, natural [^{14}C]lignin-labeled lignocelluloses by feeding plants specifically labeled lignin precursors (5; see below).

Kirk and Chang (10) pointed out that it is desirable to establish a minimum value in terms of the percentage of ^{14}C-labeled lignins evolved as $^{14}CO_2$ to ascribe lignin-degrading activity to a given organism or mixed culture. Our experiments with *T. fusca*. which is not a lignin degrader but is a very efficient cellulose degrader, indicate that a minimum value of approximately 2% conversion to $^{14}CO_2$ is acceptable in studies using [^{14}C]lignocelluloses such as ours.

Natural lignocelluloses containing ^{14}C in their lignin components will be of obvious value in studies of lignin biodegradation. They will be of particular value in studies of the turnover of lignin in natural environments, especially the study of humification (8, 14). [^{14}C]lignocelluloses will be of value to investigators who screen large numbers of microorganisms for the ability to decompose lignin. Data obtained with these natural substrates will be more relevant to actual degradative processes in natural environments than similar data obtained with model polymers or other isolated lignins. Also, our [^{14}C]lignocelluloses are much easier to prepare than are [^{14}C]DHPs, which require complex organic syntheses (11).

Haider and Trojanouski (5) prepared natural, extractive-free [^{14}C]lignin-labeled lignocelluloses by feeding maize seedlings ^{14}C-labeled ferulic acid. The lignin in these lignin-cellulose complexes was degraded to $^{14}CO_2$ by both white- and soft-rot fungi, and the rate of lignin degradation was approximately the same as that of [^{14}C]DHP degradation (~2% in 16 days). These authors used ferulic acid labeled specifically in the methyl group or the 3′ position, producing what was assumed to be $O^{14}CH_3$ and [3′-^{14}C]lignins. Thus, it appears that natural, [^{14}C]lignin-labeled lignocelluloses, like [^{14}C]-DHPs, can be prepared with ^{14}C placed at specific points in the lignin molecule. This might be accomplished by using specifically labeled lignin precursors such as phenylalanine or ferulic acid. However, additional characterization of specifically labeled, natural lignins is required before it is assumed that no plant-mediated, molecular rearrangements of the labeled precursors have occurred during lignin biosynthesis.

ACKNOWLEDGMENTS

This investigation was supported by grants from George Mason University, Fairfax, Va., and the Graduate School, University of Minnesota, Twin Cities.

We wish to thank T. K. Kirk of the U.S. Department of Agriculture Forest Products Laboratory, Madison, Wis., for providing a culture of *P. versicolor*.

LITERATURE CITED

1. **Barnoud, F., T. Higuchi, J. P. Joseleau, and A. Mollard.** 1964. La biosynthèse des lignines dans les tissues végétaux cultivés in vitro: role de précurseur de la L-phenylalanine. C.R. Acad. Sci. 259:4339–4341.
2. **Brown, S. A., and A. C. Neish.** 1955. Shikimic acid as a precursor in lignin biosynthesis. Nature (London) 175:688–689.
3. **Crawford, D. L.** 1974. Growth of *Thermomonospora fusca* on lignocellulosic pulps of varying lignin content. Can. J. Microbiol. 20:1069–1072.
4. **Crawford, D. L., E. McCoy, J. M. Harkin, and P. Jones.** 1973. Production of microbial protein from waste cellulose by *Thermomonospora fusca*, a thermophilic actinomycete. Biotechnol. Bioeng. XV:833–843.
5. **Haider, K., and J. Trojanouski.** 1975. Decomposition of specifically ^{14}C-labelled phenols and dehydropolymers. Arch. Microbiol. 105:33–42.
6. **Harkin, J. M.** 1967. Lignin—a natural polymeric product of phenol oxidation, p. 243–321. *In* A. R. Battersby and A. I. Taylor (ed.), Oxidative coupling of phenols. Marcel Dekker, Inc., New York.
7. **Higuchi, T., Y. Ito, and I. Kawamura.** 1967. p-Hydroxyphenyl-propane component of grass lignin and role of tyrosine-ammonia lyase in its formation. Phytochemistry 6:875–881.
8. **Hurst, H. M., and N. A. Burges.** 1967. Lignin and humic acids, p. 260–286. *In* A. O. McFaren and G. Peterson (ed.), Soil biochemistry. Marcel Dekker, Inc., New York.
9. **Kirk, T. K.** 1971. Effects of microorganisms on lignin. Annu. Rev. Phytopathol. 9:185–210.
10. **Kirk, T. K., and H. M. Chang.** 1975. Decomposition of lignin by white rot fungi. II. Characterization of heavily-degraded lignin from decayed spruce. Holzforschung 29:56–64.
11. **Kirk, T. K., W. J. Connors, R. D. Bleam, W. F. Hackett, and J. G. Zeikus.** 1975. Preparation and microbial decomposition of synthetic [^{14}C]lignins. Proc. Natl. Acad. Sci. U.S.A. 72:2515–2519.
12. **Moore, W. E., and D. B. Johnson.** 1967. Procedure for the analysis of wood and wood products. U.S.D.A. Forest Service, Forest Products Laboratory, Madison. Wis.
13. **Musha, Y., and D. A. I. Goring.** 1974. Klason and acid-soluble lignin content of hardwoods. Wood Sci. 7:133–134.
14. **Oglesby, R. T., R. F. Christman, and C. H. Driver.** 1967. The biotransformation of lignin to humus-facts and postulates. Adv. Appl. Microbiol. 9:111–184.
15. **Pearl, I. A.** 1967. The chemistry of lignin. Marcel Dekker, Inc., New York.
16. **Sarkanen, K. V., and C. H. Ludwig (ed.).** 1971. Lignins: occurrence, formation, structure and reactions. Wiley-Interscience, New York.