APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 1984, p. 585–587
0099-2240/84/030585-03$02.00/0

Vol. 47, No. 3

Degradation of Lignin by *Cyathus* Species

THOMAS P. ABBOTT* AND DONALD T. WICKLOW

Northern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Peoria, Illinois 61604

Received 10 November 1983/Accepted 20 December 1983

The ability of 12 *Cyathus* species to degrade ^{14}C-labeled lignin in kenaf was studied. The sum of ^{14}C released into solution plus ^{14}C released into the gas phase over a 32-day fermentation period was used to determine average daily rates of lignin biodegradation. *Cyathus pallidus*, *C. africanus*, and *C. berkeleyanus* delignified kenaf most rapidly. *C. canna* showed the greatest preference for lignin degradation over other plant components, and its rate of lignin degradation was only slightly lower than the three most active species. The apparent ability of fungi to metabolize low-molecular-weight lignin breakdown products correlated well with their overall delignification rates. *C. stercoreus* metabolized degradation products of lignin from wheat straw better than those from kenaf lignin, based on the amount of low-molecular-weight products left in solution.

Lignin is a cross-linked, highly aromatic polymer that acts as a structural adhesive, holding cellulose microfibrils together for composite-like strength. The intimately bound adhesive and fiber must be separated for the individual plant components (cellulose, hemicellulose, lignin) to be used as fermentation substrates or in many other applications. Selective bioconversion of lignin to soluble chemicals could maximize the value of the lignin and free up the other two components. *Cyathus* fungi are ecologically specialized in the breakdown of plant components, since they are found in nature on decaying plant matter.

Cyathus stercoreus (Schw.) de Toni NRRL 6473, isolated from aged and fragmented cattle dung, effected substantial losses in lignin during a fermentation of wheat straw (9). The resultant biodegradation products were primarily high-molecular-weight lignin-carbohydrate complexes and carbon dioxide, with small amounts of aromatic and aliphatic acids (1). Lignin-carbohydrate complexes are also found in autohydrolysis products (7) and bacterial degradation products (4). The large majority of *Cyathus* species are recorded from leaf litter, old wood, or twigs, whereas *C. stercoreus*, which is of worldwide distribution, occurs only on dung or in heavily manured soil (9). *Cyathus* species having unique substrate preferences may differ in their ability to attack the substructures of native lignins and lignocellulose complexes.

In the present study, lignin biodegradation by 12 *Cyathus* species is compared by monitoring ^{14}C release into gas phase and water solution from kenaf (*Hibiscus cannabinus* L.) containing ^{14}C-labeled lignin. The fraction of soluble biodegradation products having molecular weights greater than 1,000 (by membrane filtration) also was determined. A companion study in which unlabeled materials and other analyses were used has been submitted for publication elsewhere (D. T. Wicklow, R. Langie, S. Crabtree, and R. W. Detroy, in press).

MATERIALS AND METHODS

Selection of *Cyathus* strains. Twelve species of *Cyathus* were obtained from the Agricultural Research Service Culture Collection (NRRL). These included: *Cyathus africanus* Brodie NRRL 6519, on prunings of *Cupressus* sp., Mt. Kilimanjaro, Tanzania (= Brodie 66120); *Cyathus berkeleyanus* (Tul.) Lloyd NRRL 6520, on old wood, Guadeloupe (= Brodie 6694); *Cyathus bulleri* Brodie NRRL 6521, on old wood, Guadeloupe (= Brodie 6680a); *Cyathus canna* Lloyd NRRL 6522, on sawdust, Turrialba, Costa Rica (= Brodie 1238); *Cyathus earlei* Lloyd NRRL 6523, on soil, Veracruz, Mexico (= Brodie 1286); *Cyathus helenae* Brodie NRRL 6524, on old stems, Mountain Park, Alberta, Canada (= Brodie 1500; ATCC 28392); *Cyathus julietae* Brodie NRRL 6526, on old wood, Jamaica (= Brodie 6641); *Cyathus limbatus* Tul. NRRL 6527, on old wood, Guadeloupe (= Brodie 6688); *Cyathus pallidus* Berk & Curt. NRRL 6529, on wood trash, Port Antonio, Jamaica (= Brodie 6661); *Cyathus pygmaeus* Lloyd NRRL 6530, on dead *Artemisia* sp., Idaho (= Brodie 66133); *Cyathus stercoreus* (Schw.) de Toni NRRL 6473, on aged cow dung, Michigan; *Cyathus striatus* (Huds.) Willd. ex Pers. NRRL 6532, on riverbank, Edmonton, Alberta, Canada (= Brodie 68037). Brodie (2) notes that *Cyathus* strains isolated from sporocarps found on soil cannot be cultured on mineral soils free of organics but are probably growing on decayed plant material in the soil.

Lignin-containing substrates and fermentation. ^{14}C-labeled kenaf lignin was prepared by root-feeding [^{14}C]phenylalanine to 115-day-old plants (1; T. P. Abbott and C. James, in press). Kenaf plants were sectioned and oven dried in air at 45°C to constant weight. Ground plant sections were hexane and water extracted and then treated with protease in phosphate buffer. Washed and freeze-dried kenaf was used in this study. Duplicate samples (0.1 g) of kenaf were weighed into 6-ml vials, 1 ml of water was added, and the vials were covered loosely with a Teflon-lined septum and an aluminum cap. Before the vials were sealed, they were autoclaved for 15 min, cooled, and inoculated with a 0.25-cm^2 plug removed from a fungal colony grown on potato-glucose-agar in petri dishes (7 days; 25°C). Less than 2% of the ^{14}C-labeled lignin was solubilized by the sterilization procedure.

Fermentations kept at 25°C were flushed with a slow air bleed for 10 min at 1- to 3-day intervals, and evolved gases were collected in scintillation fluid (1). Samples (10 μl) of solubles were removed with a sterile microsyringe after the air flush and counted in scintillation fluid. The 10 μl of liquid in the fermentation was replaced with sterile water. After 32

* Corresponding author.

days, the fermentations were frozen until analyzed. Soluble components that passed through a Whatman no. 541 filter paper were further separated on a YM-2 membrane (Amicon Corp.) to determine percent high- (>1,000-molecular-weight, nominal) and low-molecular-weight fractions. Washed residue was oven dried in a vacuum for 4 h at 70°C, and ^{14}C content was determined by combustion in an O_2 atmosphere to CO_2, which was trapped and counted in scintillation fluid as described in reference 1.

RESULTS AND DISCUSSION

Typical $^{14}CO_2$ and water-soluble ^{14}C release rates are shown in Fig. 1A and B. Many investigators (3, 6) consider $^{14}CO_2$ evolution from fungally degraded ^{14}C-labeled lignin to be an assay of the rate of lignin biodegradation. Freer and Detroy (5) have shown that $^{14}CO_2$ and ^{14}C release into the soluble phase are both significant when measuring lignin degradation. With *C. striatus*, neither the rate of gaseous evolution nor the rate of release of ^{14}C into the solution equals the rate of lignin biodegradation (Fig. 1A). In the case of *C. berkeleyanus* (Fig. 1B), both $^{14}CO_2$ release and solubilized ^{14}C have similar rate patterns up to about 24 days, when soluble ^{14}C content decreases and ^{14}C gas evolution continues at a steady rate. The cumulative sum of water-soluble ^{14}C and the labeled gases is probably the best indication of total lignin biodegradation, because ^{14}C incorporated into fungal hyphae was not measured. We believe the two processes characterized by total ^{14}C release and gaseous ^{14}C release are initial biodegradation of the lignin macromolecule into soluble material and metabolism of these solubles to $^{14}CO_2$, respectively. The lag in cumulative CO_2 generation behind ^{14}C solubilizaion supports this view. Some of the species are less able to metabolize the generated water-soluble species to CO_2 (e.g., *C. striatus*; Fig. 1A) than others (e.g., *C. berkeleyanus*; Fig. 1B) based on this interpretation. Even when generation of soluble counts (measured in disintegrations per minute) levels off (24 to 30 days; Fig. 1B), $^{14}CO_2$ and other gases continue to be generated. This would be expected if the fungus has decreased active vegetative growth and lignin degradation but continues to metabolize the soluble species, which then decrease in concentration (Fig. 1B). From a practical standpoint, water-soluble products would have more value than CO_2 as a by-product of lignin degradation.

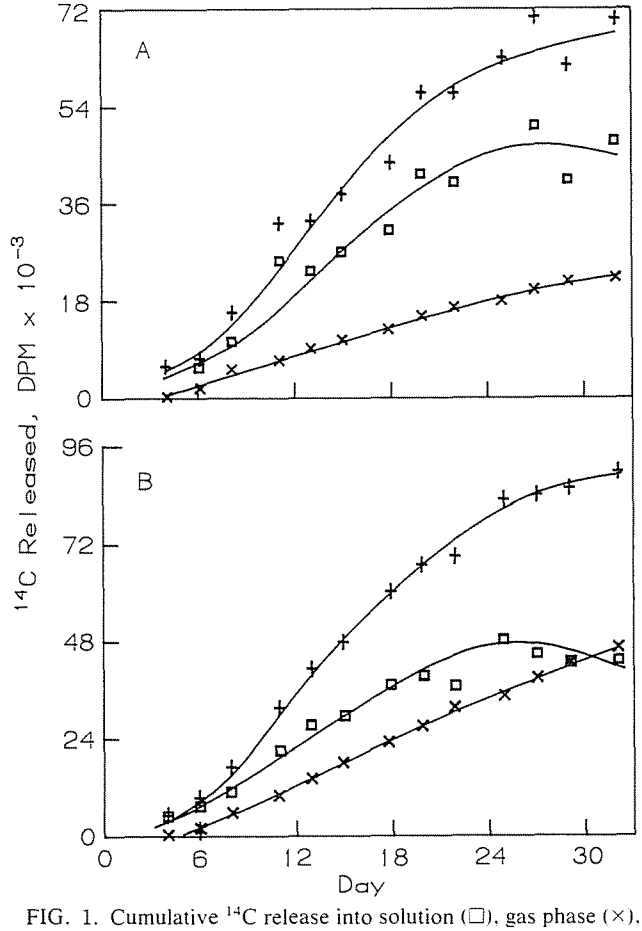

FIG. 1. Cumulative ^{14}C release into solution (□), gas phase (×), and total (+) for *C. striatus* (A) and *C. berkeleyanus* (B) attack on kenaf lignin.

Lignin degradation is compared in Table 1 for 12 *Cyathus* species by percentage of ^{14}C released per day into the gas-plus-liquid phase. On the basis of the most rapid delignification, the most effective species were, in decreasing order, *C. pallidus*, *C. africanus*, and *C. berkeleyanus*.

The raw data that yield the arithmetic means given in Table 1 were analyzed for variance to determine least significant differences. The measurements indicated highly significant ($P < 0.01$) variation among species. When the difference of two means in Table 1 exceeds the least significant difference, the means differ significantly ($P < 0.05$) (8). The various parameters listed in Table 1 were then tested statistically for direct correlation. In addition, the average daily $^{14}CO_2$ generation and average daily ^{14}C release into solution for the 12- to 21-day period also were tested statistically. The average daily $^{14}CO_2$ generation and average daily soluble ^{14}C release over the 32-day period correlate well (correlation constants, 0.983 and 0.980) with the 12- to 21-day values but have lower variance; thus, the 12- to 21-day values were not used or reported further. The fact that 32-day averages correlate well with the 12- to 21-day averages demonstrates that neither early ability to grow fast and cover the substrate nor senescence periods are reflected in the comparative rates of lignin biodegradation.

There are no significant differences in the rate of release of ^{14}C into solution by the 10 most active species. The rate of generation of $^{14}CO_2$ by *C. pallidus* is significantly higher than that of seven other active species and by *C. berkeleyanus* is significantly higher than that of five other active species. The most rapid lignin degraders in the genus *Cyathus* are, therefore, those which can best metabolize to CO_2 the lignin breakdown products that are released into solution. The correlation between the average daily $^{14}CO_2$ generation and the overall ^{14}C release rate is 0.946. As we have pointed out, however, the overall lignin degradation rate must include the rate of generation of soluble species that account for 25 to 70% of the degradation products. Neglecting their measure leads to erroneous conclusions.

The dominance of high-molecular-weight degraded lignin fractions over low-molecular-weight fractions in the soluble phase is more evident in *C. stercoreus*-biodegraded straw (1) than in *C. stercoreus*-biodegraded kenaf. The low-molecular-weight fraction averages 5 to 10% of the overall water-soluble products from biodegraded wheat straw, compared with 25% from biodegraded kenaf. Since *C. stercoreus* is adapted to metabolism of grass lignin breakdown products, we believe the higher proportion of unmetabolized lignin breakdown products in *C. stercoreus*-degraded kenaf is a

TABLE 1. ^{14}C-labeled biodegradation products

Species	Rate of ^{14}C release (%/day)[a]			% ^{14}C left in solution		Observed mycelial density[d]	^{14}C in fermented solids (dpm/mg)[e]
	Total ^{14}C	$^{14}CO_2$	Soluble species	Low mol wt[b]	High mol wt[c]		
C. pallidus	1.02	0.51	0.51	6.91	9.28	D	1,870
C. africanus	0.89	0.36	0.53	3.68	13.3	M	2,704
C. berkeleyanus	0.88	0.46	0.42	4.29	9.07	D	1,933
C. stercoreus	0.80	0.34	0.46	3.63	11.0	M–D	2,545
C. canna	0.76	0.30	0.45	4.65	9.69	D	1,836
C. julietae	0.71	0.22	0.49	5.01	10.7	D	2,304
C. helenae	0.70	0.24	0.46	5.90	8.81	M	2,388
C. striatus	0.71	0.22	0.49	6.08	9.52	M	2,224
C. bulleri	0.66	0.26	0.39	3.79	8.80	M	2,323
C. limbatus	0.65	0.25	0.40	4.23	8.46	M	2,552
C. pygmaeus	0.18	0.05	0.13	0.72	3.48	S	2,943
C. earlei	0.16	0.05	0.11	1.05	2.62	S	2,933
Control	0.09	0.03	0.07	1.08	1.16		3,348
LSD[f]	0.20	0.17	0.14	2.37			531

[a] Percentage of total ^{14}C in the labeled lignin in each sample over 32 days.
[b] Less than 1,000 molecular weight as determined by membrane filtration.
[c] Greater than 1,000 molecular weight as determined by membrane filtration.
[d] D, Dense; M, moderate; S, sparse.
[e] dpm/mg, Disintegration of ^{14}C per minute per milligram of sample.
[f] LSD, Least significant difference ($P < 0.05$) at two means.

result of differences in the structure of the substrate lignin. The faster rate of CO_2 generation from *C. stercoreus* on wheat straw (0.45% of ^{14}C-labeled lignin per day) over that from kenaf (0.34% of ^{14}C-labeled lignin per day) supports this conclusion.

Six of the species tested, *C. stercoreus*, *C. julietae*, *C. helenae*, *C. striatus*, *C. bulleri*, and *C. limbatus*, showed no statistically significant differences in any of the factors measured, suggesting a common response to culture conditions as well as a common mechanism of lignin degradation and metabolism of initial degradation products.

Some idea of the relative attack on other plant components versus attack on lignin can be gained by comparing the ^{14}C concentration (disintegrations per minute per milligram) in the recovered fermentation solids with the ^{14}C concentrations in the starting material. The ^{14}C concentrations in residues are all lower than in starting kenaf except for the control (Table 1). In *C. pygmaeus* and *C. earlei*, in which little or no growth or degradation was observed, counts are slightly lowered due to preferential solubilization of a small percentage (5%) of lignin by air oxidation and to dilution of solids by fungal mass and media added on inoculation. Nonetheless, good duplication of the residual ^{14}C concentration of these two species serves as a base for comparing the other values. The species with the highest preference for lignin degradation is *C. canna*, followed by *C. pallidus* and *C. berkeleyanus*.

C. pallidus is the most rapidly delignifying fungus of the group tested, but *C. africanus* or *C. stercoreus* might be the most useful organisms in the fermentation of kenaf because both had appreciable rates of delignification but, at the same time, left a larger proportion of ^{14}C as soluble products. Now that the static fermentation has been characterized, it would be interesting to develop a method of continuous extraction of degradation products for two reasons: to trap intermediate breakdown products, which might further enlighten our understanding of fungal attack on lignin, and possibly to recover more of the lignin as soluble breakdown products rather than as CO_2. Our experience is that the soluble lignin-carbohydrate breakdown products remain soluble and adhesive if they are freeze-dried as the sodium salt but become intractable if dried by other means. Those biologically generated products will be further characterized.

ACKNOWLEDGMENTS

We acknowledge Robert Langie and Jeff Hardy for ably performing much of the daily sampling and analyses. William Kwolek calculated the statistical parameters.

LITERATURE CITED

1. **Abbott, T. P., C. James, and R. D. Plattner.** 1983. Products of wheat straw biodegradation by *Cyathus stercoreus*. p. 267–284. American Chemical Society symposium series 214. American Chemical Society, Washington, D.C.
2. **Brodie, H. J.** 1975. The bird's nest fungi. University of Toronto Press, Toronto.
3. **Crawford, D. L., R. L. Crawford, and A. L. Pometto III.** 1977. Preparation of specifically labeled ^{14}C-(lignin)- and ^{14}C-(cellulose)-lignocelluloses and their decomposition by the microflora of soil. Appl. Environ. Microbiol. **33**:1247–1251.
4. **Crawford, D. L., A. L. Pometto III, and R. L. Crawford.** 1983. Lignin degradation by *Streptomyces viridosporus*: isolation and characterization of a new polymeric lignin degradation intermediate. Appl. Environ. Microbiol. **45**:898–904.
5. **Freer, S. N., and R. W. Detroy.** 1982. Biological delignification of ^{14}C-labeled lignocellulosics by basidiomyetes: degradation and solubilization of the lignin and cellulose components. Mycologia **74**:943–951.
6. **Kirk, T. K., E. Schultz, W. J. Connors, L. F. Lorenz, and J. G. Zeikus.** 1978. Influence of culture parameters on lignin metabolism by *Phanerochaete chrysosporium*. Arch. Microbiol. **117**:277–285.
7. **Milstein, O., Y. Vered, J. Gressel, and H. M. Flowers.** 1981. Biodegradation of wheat straw lignocarbohydrate complexes (lcc) II. Eur. J. Appl. Microbiol. Biotechnol. **13**:117–127.
8. **Steele, R. G. D., and J. M. Torrie.** 1980. Principles and procedures of statistics, 2nd ed. McGraw-Hill Book Co., New York.
9. **Wicklow, D. T., R. W. Detroy, and B. A. Jessee.** 1980. Decomposition of lignocellulose by *Cyathus stercoreus* (Schw.) de Toni NRRL 6473, a "white rot" fungus from cattle dung. Appl. Environ. Microbiol. **40**:169–170.