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Mycelium Binding and Depolymerization of Synthetic ^{14}C -Labeled Lignin During Decomposition by *Phanerochaete chrysosporium*

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Summary

Earlier investigations with the white-rot wood-decomposing basidiomycete *Phanerochaete chrysosporium* elucidated the culture parameters that influence the decomposition of lignin to CO_2 . Optimized ligninolytic cultures produce $^{14}\text{CO}_2$ immediately on introduction of synthetic ^{14}C -lignins. The present study examined, with synthetic [ring- ^{14}C]-lignin, the degradative capacity of such cultures, and the interrelationship between mycelial binding of the lignin, its depolymerization, and its complete decomposition to CO_2 . The rate of CO_2 production increased with increasing lignin concentration through 1 mg/mg protein, the highest rate exceeding 8 μg of lignin/h/mg protein. The lignin underwent a degradation-dependent binding to the mycelium, and was gradually released on continued incubation. Random depolymerization of the lignin occurred simultaneously with its oxidation to CO_2 . Time required to reach maximal binding, depolymerization and rate of degradation to CO_2 increased with increasing initial concentration of lignin. Histidine and glutamate, repressors of the complete oxidation of lignin to CO_2 , also strongly suppressed mycelial binding and depolymerization. Lignin was not bound, depolymerized or oxidized to CO_2 in primary growth-stage cultures. These latter results indicate that the entire ligninolytic system is secondary metabolic.

*Myzel-Anlagerung an und Depolymerisation von synthetischem ^{14}C -markiertem Lignin während des Abbaues durch *Phanerochaete chrysosporium**

Zusammenfassung

In früheren Untersuchungen mit dem Weißfäule-Pilz *Phanerochaete chrysosporium* wurden die Kulturparameter geklärt, die zum Abbau des Lignins zu CO_2 führen. Optimale ligninolytische Kulturen ergeben $^{14}\text{CO}_2$ unmittelbar bei Einführung von synthetischen ^{14}C -Ligninen. In vorliegender Untersuchung werden mit synthetischem (Ring- ^{14}C)Lignin folgende Punkte behandelt: Abbau-Kapazität solcher Kulturen, die gegenseitigen Beziehungen zwischen Myzel-Anlagerung von Lignin, dessen Depolymerisation und dessen vollständigem Abbau zu CO_2 . Die Geschwindigkeit der CO_2 -Bildung wächst mit steigender Ligninkonzentration durch 1 mg/mg Protein, die höchste Geschwindigkeit übersteigt 8 μg Lignin/h/mg/Protein. Das Lignin unterlag einer abbaubehängigen Anlagerung an das Myzel und wurde graduell freigegeben bei fortgesetzter Inkubation. Wahllose Depolymerisation des Lignins trat gleichzeitig mit seiner Oxidation zu CO_2 ein. Die erforderliche Zeit für maximale Anlagerung, Depolymerisation und Abbaugeschwindigkeit zu CO_2 wächst mit steigender Lignin-Anfangskonzentration. Histidin und Glutamat, Repressoren der vollständigen Oxidation zu CO_2 , unterdrückten die Myzel-Anlagerung und die Depolymerisation stark, Lignin wurde nicht angelagert, depolymerisiert oder zu CO_2 oxidiert bei im Anfangsstadium befindlichen Kulturen. Letzterer Befund zeigt, daß das ganze ligninolytische System aus einer sekundären Stoffumwandlung entstanden ist.

Introduction

Research with the white-rot wood-destroying basidiomycete *Phanerochaete chrysosporium* Burds. has elucidated

the culture parameters important for the decomposition of ^{14}C -lignins to $^{14}\text{CO}_2$ (Jeffries et al. 1980; Kirk et al. 1978; Reid 1979). Immediately following addition of synthetic [ring- ^{14}C]-lignin to cultures optimized for ligninolytic activity, $^{14}\text{CO}_2$ is produced. Complete oxidation of aromatic carbon continues for several days, totalling 60–70% of the ^{14}C -lignin supplied.

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Whether the lignin is extensively depolymerized and converted to intermediate ("oligomeric") products during this decomposition has not been determined. Chemical and spectroscopic investigations have indicated that aromatic nuclei are cleaved while still bound in the polymer (Chua et al. 1982; Ellwardt et al. 1981; Kirk and Chang 1975). Further degradation of the cleaved rings could lead to early CO₂ production without significant depolymerization. It is, however, clear that this mode of degradation is not solely responsible for decomposition, because various low molecular weight aromatic compounds are also produced (Chen et al. 1982; Henderson 1955; Higuchi et al. 1955; Ishikawa et al. 1963).

The present study examined the relationship between depolymerization and CO₂ production from the aromatic carbons of lignin. During the investigation it was discovered that the lignin undergoes a degradation-dependent binding to the fungal mycelium. This binding was also investigated in relation to decomposition to CO₂. Finally, because previous studies have shown that L-histidine and L-glutamic acid cause a potent though transient suppression of lignin biodegradation by *Phanerochaete chrysosporium* (Fenn and Kirk 1981), their effects on depolymerization and mycelial binding were examined.

Materials and Methods

Lignins

Synthetic [ring-U-¹⁴C]-lignin with a specific activity of 9.2 x 10⁵ dpm/mg was prepared and used (Keyser et al. 1978; Kirk et al. 1975). Unlabeled synthetic lignin was also synthesized for these experiments (Kirk et al. 1975).

Fungi and Inoculum

Phanerochaete chrysosporium (strain BKMF-1767) was obtained from the Center for Forest Mycology Research, Forest Products Laboratory, Madison, Wisconsin, and was maintained at room temperature on 2% malt agar slants. Inoculum consisted of conidial suspensions filtered through glass wool ($A_{650nm} = 0.5/cm; \cong 2.5 \times 10^6$ spores/ml); the conidia were taken from 3- to 4-week-old slants, and exhibited > 90% viability.

Experimental Cultures

Experiments were conducted with 10-ml cultures in 125-ml Erlenmeyer flasks which were fitted with ports that permitted periodic flushing for monitoring the ¹⁴CO₂ evolved (Kirk et al. 1978). The culture medium contained the following (g/l): ammonium tartrate, 0.2; KH₂PO₄, 0.2; MgSO₄ · 7H₂O, 0.05; CaCl₂, 0.01; glucose, 10.0, trace element solution (10 ml, Kirk et al. 1978), and thiamine · HCl (1 mg). The culture was buffered at pH 4.5 with sodium 2,2-dimethylsuccinate (Fenn and Kirk 1979). The medium was made up at 10 x the required concentration and filter-sterilized. Glucose and buffer, both at 10 x the required concentration, were filter-sterilized separately.

The synthetic lignin, prepared as a suspension by precipitating a 10% w/v solution in N,N-dimethylformamide (DMF) into sterile distilled water, was added to 6.5-day-old cultures grown at 39°C under $\cong 100\%$ O₂. Synthetic lignin was added at 50 µg to 2,000 µg per culture, of which 50 µg was labeled ¹⁴C-lignin, and the balance was unlabeled.

Experiments similar to those described were also carried out with 6.5-day-old control cultures which had been autoclaved at 121°C for 20 minutes prior to addition of lignin.

Experiments with sodium azide (1 mM in the cultures), L-glutama-

te and L-histidine (both 2.86 mM in the cultures) were conducted by adding the compounds to 6-day cultures, 16 hours prior to addition of the lignin.

All experiments were conducted in quadruplicate.

Harvesting

The ¹⁴CO₂ evolved was assayed at 6, 12, 24, and 48 hours after the addition of synthetic lignin by trapping in 10 ml of an ethanolamine-containing scintillation fluid (Kirk et al. 1975). Data were corrected for background radioactivity and for cocktail efficiency, which was determined with an internal standard of ¹⁴C-toluene (ICN, Irvine, California).

After the ¹⁴CO₂ had been trapped, the pH of the cultures was determined and adjusted to pH 2.0–2.5 with 2N HCl. Dioxane (10 ml) was added to each acidified culture. The flasks were stoppered and agitated at room temperature overnight. The mycelia were then separated from the culture fluid by centrifugation, and radioactivity in the dioxane:water-solubles was determined by counting 1-ml samples in a dioxane/naphthalene scintillation fluid (Kirk et al. 1975).

The mycelium was solubilized in 5 ml of TS—1 tissue solubilizer (RPI Corp., Elk Grove, Illinois) and held at 50°C overnight. The radioactivity was determined after addition of 5 ml of a toluene solution (consisting of 4 g 2,5-diphenyloxazole (RPI) and 100 mg p-bis-[2-(5-phenyloxazolyl)]-benzene (RPI) per l of toluene). The counting efficiency of the solutions was determined regularly by use of an internal standard of ¹⁴C-toluene, and corrections made.

Gel Permeation Chromatography

The aqueous dioxane extracts of the quadruplicate samples were pooled and the volume reduced to 1.0 ml on a rotary evaporator (< 40°C). The samples were then analyzed by gel permeation chromatography by the descending method on a 1.2 x 53 cm column of Sephadex LH-20 with aqueous dioxane (dioxane:H₂O 9:1) as eluting solvent. Flow rate was approximately 16 ml per hour. The elution pattern was monitored by UV at 280 nm, and 3.5-ml fractions were collected. The radioactivity trace indicating molecular size distribution was obtained by counting 1.0 ml samples of the fractions in the dioxane/naphthalene scintillation fluid.

Results

Distribution of Lignin Carbon

Table 1 summarizes the distribution of ¹⁴C at various times following addition of the radiolabeled lignin to ligninolytic cultures. Each culture received approximately 5 x 10⁴ dpm of ¹⁴C, in a total of 50, 250, 500, 1,000, or 2,000 µg of lignin. Cultures were terminated 6, 12, 24, and 48 hours after lignin addition, and the distribution of ¹⁴C among CO₂, dioxane:water-soluble C, and mycelium-bound C was determined. Total ¹⁴C recovered varied from 88 to 103 percent, but was usually near 100 percent. From the ¹⁴C values the distribution of total weight of lignin carbon was calculated (Table 1).

Maximal evolution of CO₂ and the highest rate of its production from lignin occurred at the highest concentration of lignin administered (2,000 µg/culture) (Fig. 1). Other experiments showed that even 4,000 µg/culture did not saturate the ligninolytic system. The time required to develop highest rates of CO₂ production increased with increasing lignin concentration. Thus, the rate of CO₂ production from cultures receiving 50 µg total lignin was maximal essentially from the time of lignin addition,

Table 1. Radioactivity present in cultures as CO_2 , as dioxane:water-soluble C and mycelium-bound C

Synthetic lignin ^a $\mu\text{g}/\text{culture}$	^{14}C Distribution after degradation ^b	6 Hours		12 Hours		24 Hours		48 Hours	
		%	μg^c	%	μg	%	μg	%	μg
250 (Autoclaved control cultures) ^d	CO_2	0	0	0	0	0	0	0	0
	Dioxane:water solubles	89 ± 6	223	90 ± 5	225	86 ± 3	215	82 ± 4	205
	Mycelium-bound	$3 \pm < 0.1$	8	5 ± 1	13	7 ± 1	18	8 ± 2	20
	Total	92 ± 6	231	95 ± 6	238	93 ± 4	233	90 ± 6	225
50	CO_2	3 ± 1	2	9 ± 3	4	18 ± 2	9	33 ± 4	16
	Dioxane:water solubles	53 ± 6	27	58 ± 8	29	54 ± 3	27	52 ± 2	26
	Mycelium-bound	38 ± 6	19	30 ± 9	15	18 ± 3	9	$12 \pm < 1$	6
	Total	94 ± 12	48	97 ± 20	48	90 ± 8	45	97 ± 7	48
250	CO_2	$1 \pm < 0.1$	1	7 ± 1	18	12 ± 2	29	32 ± 3	80
	Dioxane:water solubles	74 ± 5	186	59 ± 2	147	52 ± 4	129	53 ± 1	133
	Mycelium-bound	22 ± 5	55	31 ± 8	78	33 ± 10	83	15 ± 3	39
	Total	97 ± 10	242	97 ± 11	243	97 ± 16	241	101 ± 7	252
500	CO_2	$1 \pm < 1$	3	1 ± 1	6	10 ± 4	48	29 ± 5	146
	Dioxane:water solubles	72 ± 6	360	74 ± 12	368	50 ± 2	250	$50 \pm < 1$	249
	Mycelium-bound	20 ± 3	102	23 ± 9	114	28 ± 5	138	20 ± 7	102
	Total	93 ± 9	465	98 ± 22	488	88 ± 11	436	99 ± 12	496
1,000	CO_2	$< 1 \pm < 0.1$	1	1 ± 1	10	4 ± 2	41	18 ± 5	176
	Dioxane:water solubles	84 ± 6	836	69 ± 11	694	53 ± 7	525	53 ± 1	529
	Mycelium-bound	10 ± 1	103	22 ± 5	218	36 ± 7	365	30 ± 2	300
	Total	94 ± 7	940	92 ± 17	922	93 ± 16	931	101 ± 8	1,005
2,000	CO_2	$< 0.1 \pm < 0.1$	2	$< 1 \pm < 0.1$	7	2 ± 1	46	12 ± 1	233
	Dioxane:water solubles	86 ± 13	1,727	75 ± 5	1,506	59 ± 9	1,185	58 ± 6	1,164
	Mycelium-bound	13 ± 5	268	17 ± 5	335	35 ± 6	691	34 ± 1	670
	Total	99 ± 18	1,996	92 ± 10	1,848	96 ± 16	1,928	103 ± 8	2,067

^a 50 μg as ^{14}C -lignin in all cultures.

^b Each value is the mean \pm standard deviation of four replicate cultures. The entire experiment was repeated with essentially the same results.

^c Calculations (μg of total lignin converted) are based on the original ^{14}C -lignin added, taking into account dilution by unlabeled lignin. Values are means \pm 1 standard deviation for 4 replicate cultures.

^d Cultures were heat-treated for 20 min at 121°C prior to lignin addition.

whereas cultures receiving 1,000 or 2,000 μg exhibited their highest rates after 24 hours. The maximum rate exceeded 8 μg of lignin to $\text{CO}_2/\text{h}/\text{mg}$ of protein (protein = 900-1000 $\mu\text{g}/\text{culture}$: Fenn and Kirk, 1981), and occurred between 24 and 48 h after addition of 2,000 μg of lignin. Longer incubation times presumably would have given even higher rates at the highest concentrations. Autoclaved cultures evolved no $^{14}\text{CO}_2$.

The amount of mycelium-bound lignin carbon increased to 28-38 % of the total administered and then declined in all cases (Fig. 2). Thus, the total weight of bound lignin carbon increased with increasing amount added to the cultures (Table 1). Time to reach this maximum percentage, however, increased with increasing lignin concentration (Fig. 2). Cultures receiving 50 μg of lignin exhibited maximum bound lignin carbon after 6 hours, whereas cultures receiving 2,000 μg exhibited maximum binding after 24 hours. Attempts to free the bound carbon by detergent or

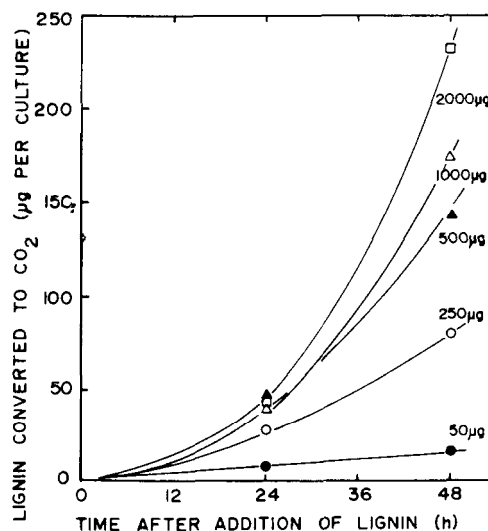


Fig. 1. Evolution of total CO_2 from lignin at different initial concentrations of lignin.

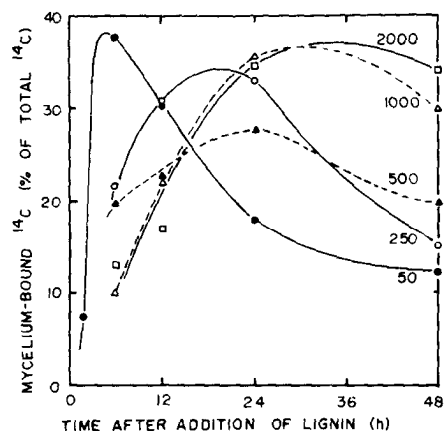


Fig. 2. Time course of binding of lignin to mycelia as affected by initial concentration of lignin.

enzyme treatments (protease, glucosylase) were not successful. Autoclaved cultures exhibited less than 8% binding. In the absence of added dioxane, the amount of non-extractable ("bound") lignin in cultures receiving 250 µg of lignin was 84% after 6 hours (as compared to 22% in the presence of dioxane, Table 1). This decreased to 31% and 35% by 24 and 48 hours, respectively.

The amount of dioxane:water-extractable lignin carbon decreased by 24 hours to 50 to 60% of the total originally added, irrespective of the amount of lignin, and remained nearly constant through 48 hours (Table 1). Again, the time required to reach this minimum increased with the amount of lignin added; only the 50 µg cultures had reached the minimum by the first harvest at 6 hours.

Molecular Size Distribution

Figure 3 shows the molecular size distribution of the original lignin as determined with Sephadex LH-20, with dioxane:water (9 : 1) as solvent. Most of the lignin was excluded from the gel indicating that the original lignin was of MW > 800. A very similar elution curve was obtained using LH-20 with DMF containing 0.1 M LiCl as solvent, indicating that intermolecular association effects were negligible (Connors et al. 1980).

Incubation of the lignin in ligninolytic cultures markedly altered the elution profile (Fig. 4). Total material recovered in the dioxane:water solubles in each treatment was subjected to column chromatography, and no corrections were made for the amount added. Thus quantitative changes in elution profiles (Fig. 4) reflect losses of CO₂ and losses due to mycelium binding, as well as changes in molecular weight. Loss of high molecular weight material and appearance of low molecular weight material occurred during incubation at all concentrations of lignin. These changes were clearly correlated with formation of CO₂, and, as in CO₂ formation, the time required to effect these changes increased with increasing amount of lignin added. Thus, marked changes were apparent already after 6 hours in the 50 µg cultures. Autoclaved cultures did not alter the elution profile of the ¹⁴C lignin (250 µg/cultures).

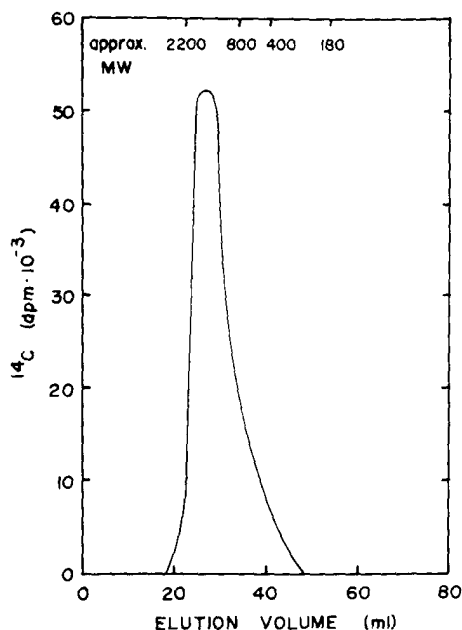


Fig. 3. Molecular size distribution of nondegraded synthetic (ring-¹⁴C)-lignin determined with a 1.2 x 53 cm column of Sephadex LH-20, with dioxane/water (9:1) as solvent.

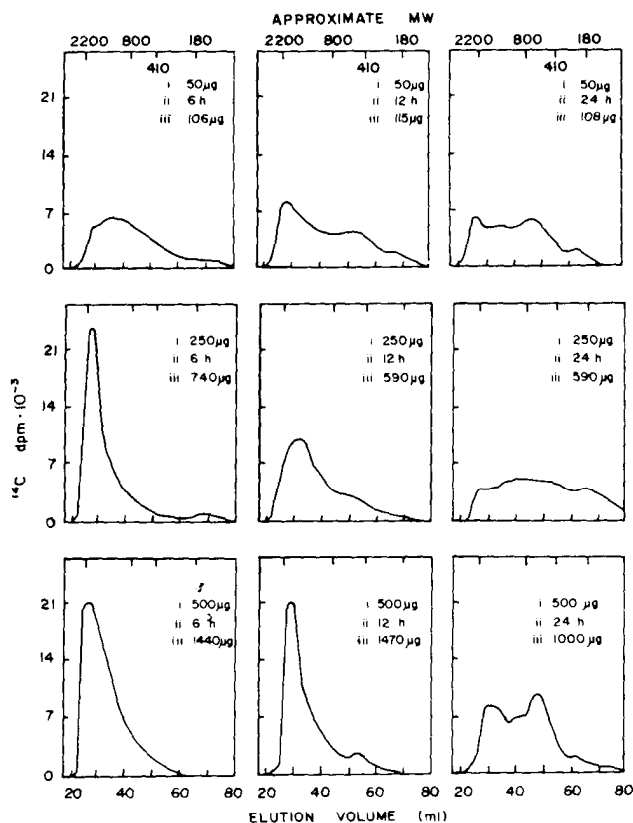


Fig. 4. Molecular size distribution of the dioxane-water solubles from ligninolytic cultures. Chromatographic procedure was as in Fig. 3. Legends with each chromatogram refer to: i) Total lignin in each culture, 50 µg of which was ¹⁴C-labeled; ii) Time of incubation after addition of lignin to 6.5-day-old cultures; iii) Calculated total amount of lignin recovered from quadruplicate cultures. The total was applied to the column.

Elution profiles from gel chromatography were also monitored by UV absorbance at 280 nm. The ^{14}C and $A_{280\text{nm}}$ elution patterns differed markedly (Fig. 5); UV absorbance did not accurately monitor lignin carbon. Experiments showed that control cultures containing no lignin exhibited a build-up of UV-absorbing materials of the pattern seen in Figure 5.

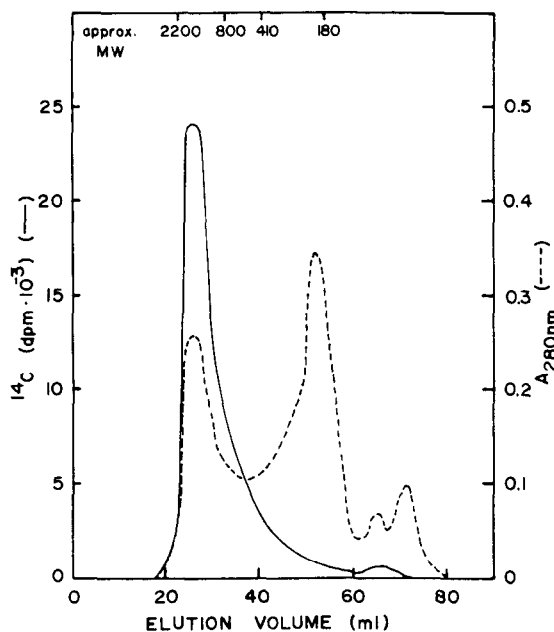


Fig. 5. Elution profile of dioxane/water extract as determined by radioactivity and by $A_{280\text{nm}}$. Extract was from four replicate cultures, each having received 250 μg of total lignin and incubated for 6 hours. Chromatographic conditions were as in Fig. 3.

Effect of Suppressors on Lignin Degradation

The effect of histidine on the distribution of ^{14}C in cultures receiving 250 μg of lignin is shown in Figure 6A and is included in Table 2. Addition of the amino acid caused almost total suppression of $^{14}\text{CO}_2$ production for 24 hours, during which time binding of ^{14}C to the mycelium was negligible - similar to that in autoclaved controls (cf. Table 1). Between 24 and 48 hours, ligninolytic activity reappeared (3.8% $^{14}\text{CO}_2$ by 48 h), and the amount of mycelium-bound ^{14}C increased markedly to 22%. Significant depolymerization accompanied degradation to CO_2 and mycelial binding between 24 and 48 hours (Fig. 6B).

Even after 48 hours, the histidine-treated cultures had not affected the lignin to the extent that control cultures had after 12 hours.

L-Glutamate gave similar results to L-histidine, but the suppressive effect was shorter-lived (Table 2). Six hours after addition of ^{14}C -lignin (total 250 μg) to glutamate-treated cultures, $^{14}\text{CO}_2$ production was negligible, and binding was low. After 12 hours, some $^{14}\text{CO}_2$ had appeared, but binding was still low, and depolymerization was minimal. After 24 hours, binding and $^{14}\text{CO}_2$ production had increased and depolymerization was apparent. Sodium azide essentially completely inhibited binding, depolymerization and $^{14}\text{CO}_2$ production (Table 2).

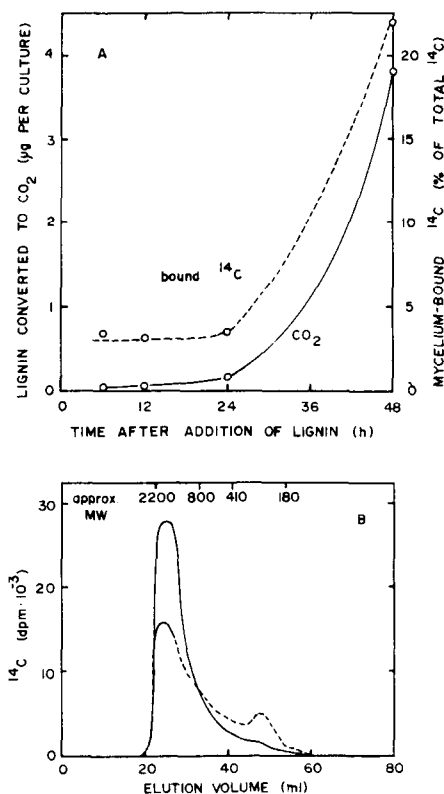


Fig. 6. Effect of L-histidine on the ^{14}C distribution in cultures receiving 250 μg of total lignin per culture.

(A) Evolution of $^{14}\text{CO}_2$ and mycelium binding for different incubation times.

(B) Molecular size distributions of the dioxane-water solubles after 24 and 48 hours of incubation.

Table 2. Distribution of ^{14}C in CO_2 , in dioxane:water solubles and in mycelium in cultures following treatment with suppressors or azide, and in primary growth stage cultures

Treatment ^a	Synthetic lignin ($\mu\text{g}/\text{culture}$)	6 Hours (%)				12 Hours (%)				24 Hours (%)			
		CO_2	Dioxane: water solubles	Mycelium-bound	Total	CO_2	Dioxane: water solubles	Mycelium-bound	Total	CO_2	Dioxane: water solubles	Mycelium-bound	Total
None (controls)	250	$1^{\pm 0.1}$	$74^{\pm 5}$	$22^{\pm 5}$	$97^{\pm 10}$	$7^{\pm 1}$	$59^{\pm 2}$	$31^{\pm 8}$	97	$12^{\pm 2}$	$52^{\pm 4}$	$33^{\pm 10}$	$97^{\pm 16}$
L-Histidine	250	0	$97^{\pm 1}$	$3^{\pm <1}$	$100^{\pm 1}$	$<0.1^{\pm <0.1}$	$98^{\pm 5}$	$4^{\pm 2}$		$<1^{\pm <1}$	$94^{\pm 5}$	$7^{\pm 4}$	$101^{\pm 9}$
L-Glutamate	250	0	$88^{\pm 5}$	$6^{\pm 2}$	$94^{\pm 7}$	$1^{\pm 1}$	$89^{\pm 10}$	$12^{\pm 6}$	102	$2^{\pm <1}$	$85^{\pm 9}$	$16^{\pm <1}$	$103^{\pm 10}$
Sodium Azide	50	0	$84^{\pm 15}$	$5^{\pm 3}$	$89^{\pm 18}$	-	-	-		0	$94^{\pm 1}$	$5^{\pm <1}$	$99^{\pm 1}$
Growth-stage culture	250	0	$96^{\pm 0}$	$3^{\pm <1}$	$99^{\pm <1}$	0	$96^{\pm 3}$	$2^{\pm 1}$	98	$0.2^{\pm <0.1}$	$84^{\pm 3}$	$12^{\pm 5}$	$97^{\pm 8}$

^a L-Histidine and L-glutamate (both 2.86 mM in culture), and sodium azide (1 mM in culture) were added in 1 ml of water 16 h prior to lignin addition to 6-day-old cultures. Control cultures (6.5-days-old) received water only. Growth stage cultures were 1.5-days old.

Effect of Primary Growth-Stage Cultures

Growing (1.5-day-old) cultures did not degrade lignin to CO₂, did not depolymerize it, and did not bind it during 12 hours (Table 2). In comparison, 6.5-day-old (control stationary phase) ligninolytic cultures evolved 7% CO₂ and bound 31% of the ¹⁴C in 12 hours.

Discussion

The ligninolytic system was not "saturated" even by 4000 µg of lignin per culture. Because the lignin is insoluble, however, the weight of lignin supplied to the cultures is not necessarily indicative of the amount of accessible substrate. Increasing the amount of lignin probably provided more accessible lignin surface under the conditions employed. Similar rate enhancement might be obtained at lower concentrations of lignin by increasing its dispersion in the medium. Our unpublished work has shown that the rate of degradation is the same for the highest and lowest molecular weight fractions (Sephadex LH-60) of the lignin used here. The increased rates at higher lignin concentrations, therefore, were not attributable to increased provision of low molecular weight components.

Our results establish that decomposition of lignin to CO₂ and its depolymerization occur simultaneously in cultures of *P. chrysosporium*. Radiolabeled products of lower molecular weight than the original lignin appeared soon after addition of the ¹⁴C-lignin to ligninolytic cultures and simultaneously with ¹⁴CO₂ production. The progressive changes in molecular size distribution as seen by gel permeation chromatography indicate random depolymerization of the substrate with continuous metabolism of the lower molecular weight products. An earlier study also demonstrated production of low molecular weight radiolabeled products from ¹⁴C-lignin in cultures of *P. chrysosporium* (Kirk et al. 1978). That study, however, involved 35-day incubations and did not examine progressive changes in molecular size distribution.

As pointed out in the Introduction, research has provided evidence that aromatic rings are cleaved while still bound in the polymer (Chua et al. 1982; Ellwardt et al. 1981, Kirk and Chang 1975). It is possible, therefore, that some degradation to CO₂ – perhaps via low molecular weight aliphatics – might occur without formation of "oligomeric" products of intermediate molecular size. In the present work, however, there was no obvious degradation of aromatic carbon to CO₂ prior to depolymerization.

Polymerization of lignin on incubation with lignin-degrading fungi has been reported (Brunow et al. 1978; Haars and Huttermann 1980; Haars et al. 1982; Huttermann et al, 1977; Iwahara 1980). In the present study, an increase in high molecular weight radiolabeled material was observed between 6 and 12 hours in cultures receiving 50 µg of lignin (Fig. 4). Whereas this observation is consistent with repolymerization of low molecular weight degradation products, it is also possible that the increase in high molecular weight material simply reflected the high molecular weight release of mycelial-bound lignin during this period. UV absorbance could not be used as an accurate monitor

of the changes in molecular size distribution of lignin in cultures of *P. chrysosporium*. UV monitoring of lignin may also give spurious results in other microbial systems, making appropriate controls imperative.

Much of the ¹⁴C originally added was bound by the mycelium and was not recoverable for chromatographic analysis. The capacity of the mycelium to bind lignin, like its capacity to degrade the polymer to CO₂, was not saturated at 2,000 µg/culture.

Binding of the lignin was associated with degradation. Cultures heatkilled or treated with azide did not degrade lignin and did not bind it significantly. Cultures treated with histidine or glutamate to repress ligninolytic activity (Fenn et al. 1981; Fenn and Kirk 1981) also did not bind lignin. After a transient repression by the amino acids, degradation and binding proceeded. These results clearly establish a direct relationship between binding and degradation. The apparent connection between attainment of maximum rate of degradation to ¹⁴CO₂ and maximum percent binding is in accord with this relationship.

Although it is possible that binding preceded degradation, it is more plausible that the degradation created groups that reacted with the mycelium or promoted a tight association with it. This conclusion is reached because it is unlikely that the histidine and glutamate would have affected binding per se. That the bound lignin could not be extracted into dioxane:water or freed by detergent or enzyme treatments suggests that it may have been covalently bound to the mycelium.

Another possible, although unlikely, explanation for "binding" is that the lignin was simply polymerized to dioxane:water insolubility. Polymerization would be expected to result from the action of phenol-oxidizing enzymes (Huttermann 1977). However, because the activities of these enzymes are insensitive to histidine and glutamate, the amino acids could not have prevented "binding" due to enzymatic polymerization. Lignin incubated in filtrates of ligninolytic cultures, which exhibit slight phenol-oxidizing activity (Keyser et al. 1978), yielded no dioxane:water-insoluble material. The maximum percentage of lignin that became mycelium-bound was similar (~ 30-40%) regardless of the amount added to the cultures. This suggests that an equilibrium was established between the degradation-dependent binding and a degradation-dependent release of bound material. Because the degradative system was not "saturated" by the concentration of lignin used here (as discussed above), the equilibrium was not affected by the amount of lignin added. It would be expected that the highest molecular weight lignin was bound preferentially because it would have more sites for binding; attempts to resolubilize the material for assessment of its molecular weight, however, were unsuccessful.

The bound lignin was eventually released, presumably as a result of degradation. The large amount of lignin (84%) initially associated with the mycelium in the absence of dioxane reflects water-insolubility as well as binding. Further incubation resulted in water solubilization as a result of degradation. Reid et al. (1981) have reported the

production of water-soluble degradation products from lignin during decay of aspen wood by *P. chrysosporium*.

Binding of kraft lignin by mycelia of *Aspergillus* and *Penicillium* spp. was reported by Tono et al. (1968), but the mechanism was not investigated. Binding by lignin-degrading basidiomycetes has apparently not been reported previously.

The results show for the first time that histidine and glutamate suppress depolymerization of lignin as well as its oxidation to CO₂. This indicates that the entire ligninolytic system is suppressed, and that these amino acids may be particularly useful in further research. Previous studies showed that L-glutamate causes a biochemical repression of ligninolytic activity as measured by oxidation of ¹⁴C-lignin to ¹⁴CO₂ (Fenn et al. 1981). The repression is a general one affecting secondary metabolism. Shimada et al. (1981) showed that L-glutamate addition suppresses the enzymatic reactions involved in the synthesis of the secondary metabolite veratryl alcohol in ligninolytic cultures of *P. chrysosporium*. Results here showed that the lignin was not bound, depolymerized, or oxidized to CO₂ in young, primary-stage cultures. Taken together, these results indicate that the entire ligninolytic system is probably secondary metabolic.

Conclusions

The following conclusions can be reached concerning the degradation of synthetic lignin in ligninolytic cultures of *Phanerochaete chrysosporium*:

1. The cultures can oxidize synthetic lignin to CO₂ at rates exceeding 8 μg of lignin/mg protein/h.
2. Lignin undergoes a transient degradation-dependent binding to the mycelium.
3. Lignin is randomly depolymerized as it is oxidized to CO₂.
4. Histidine and glutamate suppress mycelium binding of lignin and its depolymerization, as well as its oxidation to CO₂.
5. The entire ligninolytic system is probably secondary metabolic.

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