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EFFECT OF BROWN-ROT FUNGI ON CELLULOSE

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INTRODUCTION

Brown-rot fungi cause the most destructive form of wood decay. These fungi secrete agents that bring about a rapid depolymerization of cellulose (to the "limit" degree of polymerization [DP]) before significant loss of wood substance occurs; in both cases attack initially is in the amorphous regions. Acid hydrolysis has a similar effect on cellulose as do several oxidants. How brown-rot fungi accomplish this feat is a perplexing biochemical question. Enzyme preparations from many brown-rot fungi can significantly degrade only cellulose that has been modified. such as carboxymethylcellulose (Highley, 1973. 1977a). Most likely the system producing the initial depolymerization of cellulose is nonenzymatic because even the smallest enzymes are far too large to penetrate the intersticies of the amorphous regions. Secretion of acids strong enough to hydrolyze the cellulose is unlikely. Cowling and Brown (1969) suggested that an oxidative system employing Fenton's reagent ($Fe^{+2}+H_2O_2$), which generates hydroxyl radical might be used by brown-rot fungi. Later Koenigs demonstrated that cellulose in wood is depolymerized by Fenton's reagent. Support for an oxidative system was provided by Highley (1977b) who obtained evidence that brown-rotted cellulose contains carbonyl and carboxyl groups.

To gain further insight into the nature of the biochemical agent involved in the initial depolymerization of cellulose by brown-rot fungi we studied (1) ability of several brown-rot fungi to depolymerize chemically pure cellulose under various cultural conditions and (2) physical and chemical properties of brown-rotted cellulose.

MATERIALS AND METHODS

Culture Procedures

Fungi. The fungal isolates used were: Coniophora puteana (Schum. :Fr.) Karst. (MAD-515), Fomitopsis meliae (Undew.) Gilbn. (FP 105065-sp.) Gloeophyllum trabeum (Pers.:Fr.) Murr. (MAD-617). Laetiporus sulphureus (Bull .:Fr.) Bond et Sing. (SH-27-R), Lentinus lepideus Fr. (MAD-534), Leucogyrophana arizonica Ginns (RLG-9902-sp.), L. olivescens (Berk. and Curt.) Ginns and Weresub (FP 104339). Postia placenta (Fr.) M. Lars. et Lomb. (MAD-698) [syn: Poria placenta (Fr.) (Cke.)], and Serpula incrassata (Berk. et Curt.) Donk (MAD-563).

Degradation of cellulose over an agar or soil medium. Degradation of cellulose (purified cotton type A-600, Hercules) by the fungi was determined by the American Society for Testing and Materials soil-block procedure (1971) or an agar method. For the agar method, 8-oz. French square bottles containing 25 ml of a basal salts medium (Highley, 1973) solidified with 1.5% agar were used as degradation chambers. Cellulose samples were aseptically inserted into bottles and placed upon preinoculate 2.54-cm squares of filter paper laid over glass triangles resting on the agar medium. Cellulose was also placed in contact with the agar medium to determine the effect of water saturation on degradation. The percentage of weight loss of cellulose (50-mg samples) was determined by comparing air-dry weights (27°C and 70% relative humidity) before and after exposure to the fungi. The average degree of polymerization (DP) was measured viscometrically (Cowling, 1960) with cupriethylendiamine hydroxide solvent (Ecusta Paper Corp.). The solubility in 1% sodium hydroxide was determined according to the TAPPI Standard T 4m-44 (Technical Association of the Pulp and Paper Industry, 1944).

Liquid cultures. Cellulose (0.15 g) in 15 ml of basal solution (Highley, 1973) was subjected to decomposition by P. placenta and C. puteana in 125-1111 Erlenmeyer flasks at (a) three oxygen levels (0.2, 0.4, and 1.0 atm) with and without the addition of 0.1% cellobiose or (b) atmospheric oxygen with 0.02% nitrogen substituted for 0.2% nitrogen in the basal solution with and without the addition of 0.1% cellobiose. Flasks were inoculated with 1 ml of mycelial suspension and incubated for up to 2 months in stationary culture. In the oxygen concentration experiments, the test flasks were flushed every 2 or 3 days for 15 min with 100 ml of the appropriate oxygen-nitrogen mixture per minute. After incubation, mycelium and cellulose were collected by filtration with suction. Mycelium was manually separated from the cellulose.

The filtrates were tested for the presence of H_2O_2 with titanium (Wolfe, 1962) and ABTS-peroxidase (Muller, 1984). The filtrates without cellobiose were also assayed for reducing sugars (Nelson. 1944). The DP (Cowling, 1960). reducing capacity (Nelson, 1944). and weight loss of residual Cellulose were determined.

Characterization of Brown-Rotted Cellulose

Preparation of cellulose. Cellulose was exposed to P. placenta in chambers (1-liter jars) patterned after the standard ASTM soil-block procedure (American Society for Testing and Materials, 1971). The cotton was placed in chambers on previously inoculated pine feeder strips (0.3 x 2.9 x 3.5 cm. with the long axis in the grain direction). After incubation. mycelium was separated from residual cellulose, and the cellulose was washed with 0.1 N NaOH. Microscopic examination showed that not all mycelium was removed. The DP and weight loss of the residual cellulose were determined as before. Cellulose was degraded to 20% weight loss for characterization except for x-ray diffraction analysis.

X-ray diffraction of brown-rotted cellulose. X-ray diffraction (Caulfield and Moore, 1974) was used to examine cellulose degraded to a 60% weight loss by P. placenta in soil-block tests.

Molecular weight distribution of brown-rotted cellulose. Molecular weight distributions of the degraded cellulose were determined by the Institute of Paper Chemistry (Appleton. Wis.). This was accomplished by producing cellulose tricarbanilate derivatives for gel permeation chromatography (Schroeder and Haigh, 1979). The samples were analyzed on Stryrogel columns calibrated by a dispersion-compensated universal technique (McCrackin, 1977).

Carboxyl content and uronic acids. The carboxyl content of the cellulose was estimated by the methylene blue method (Technical Association of the Pulp and Paper Industry, 1963). Uronic acid content (oxidation at C-6) was determined after acid hydrolysis (Scott, 1979).

Fourier transform infrared spectroscopy. The Fourier transform (FTIR) spectra of control and rotted cellulose were recorded both with the diffuse reflectance methods and using KBr pellets to determine the presence of carboxyls in brown-rotted cellulose. Samples were also treated with $NaBH_4$ to determine the presence of carbonyl groups other than carboxyl.

RESULTS

Cultural Studies

Cellulose exposed to brown-rot fungi in contact with wood over soil was extensively depolymerized, and solubility in alkali increased before any weight loss occurred (Table 1). No weight loss of cellulose occurred at 2 weeks, and over half of the brown-rot fungi did not produce weight loss at 4 weeks. Even at 8 weeks, two of the brown-rot fungi, Laitoporus sulphureus and Lentinus lepideus, did not produce weight loss although the cellulose was extensively degraded as indicated by decrease in DP and increase in alkali solubility.

Weight loss produced by the brown-rot fungi in cellulose over soil and agar in the absence of wood is given in Table 2. Only P. placenta required glucose as a "starter" sugar to degrade isolated cellulose. With the brown-rot fungi, the degradative rate of cellulose between the two tests did not differ greatly except for G. trabeum which utilized cellulose much more slowly over agar than over soil.

The effects of the cosubstrates on cellulose decomposition by the brown-rot fungi were quite variable, and often large differences were observed between agar and soil-block tests (Table 3). Possible reasons for differences between the two tests are: (1) the cellulose samples sometimes become waterlogged in agar tests even though not in contact with agar, perhaps by capillary action. and (2) soluble sugars were incorporated into the medium in agar tests and added to cellulose samples in soil block tests. The small amount of sugar added to cellulose samples in the soil block test would soon be used up while the much larger amount in agar tests would not.

C. trabeum, L. lepideus, and P. placenta were unable to utilize cellulose saturated with water by placement in contact with agar (Table 4). However, the three coniophoroid brown-rotters, C. puteana, L. arizonica, and L. olivescens utilized the cellulose, and DP of residual cellulose was substantially decreased.

Analysis of residual cellulose from P. placenta liquid cultures grown under various oxygen concentrations or at a low nitrogen concentration showed that the fungus was unable to produce cellulose degrading agents in the liquid cultures. P. placenta did not produce wight loss of cellulose, and DP of residual cellulose differed little from that not exposed to the fungus (Table 5). Reducing capacity of the cellulose was not increased nor did reducing sugars increase in

Table 1. Weight Loss, Degree of Polymerization (DP), and Alkali Solubility of Cellulose Exposed to Brown-Rot on Wood in Soil Block Test.

Fungi	Time cellulose exposed[a]								
	2 weeks			4 weeks			8 weeks		
	Weight loss (%)	DP	1% NaOH solubility	Weight loss (%)	DP	1% NaOH solubility	Weight loss (%)	DP	1% NaOH solubility
Postia placenta	0	1.072	4.7	0	450	13.4	52	[b]<196	36.8
Serpula incrassata	8	240	11.7	22	212	22.8	80	240	32.3
Fomitopsis melise	0	684	12.1	7	996	7.0	14	250	21.1
Laitoporus sulphureus	0	500	2.4	0	884	5.0	0	196	21.4
Gloeophyllum trabeum	0	620	11.1	10	250	27.1	36	250	36.4
Lentinus lepideus	0	1,354	3.8	0	982	5.7	0	304	14.5
Coniophora puteana	0	212	14.5	0	360	20.4	37	[b]<196	29.9
Leucogyophana arizonica	0	396	15.3	16	286	27.6	24	578	16.8

[a]DP of unexposed cellulose 2,250 and solubility in 1% NaOH 0.90%. three replications.

[b]Flow in viscometer too fast to determine DP with amount of cellulose used.

Table 2. Weight Loss of Cellulose Exposed to Brown-Rot Fungi in Absence of Wood[a] in Soil-Block and Agar Tests.

Fungi	Weight loss of cellulose in soil-block test (wk)[b]						Weight loss of cellulose in agar test (wk)[b]					
	4		8		12		4		8		12	
	+G1	-G1	+G1	-G1	+G1	-G1	+G1	-G1	+G1	-G1	+G1	-G1
Postia placenta	0	0	20	0	55	0	0	0	25	0	47	0
Gloeophyllum trabeum	20	6	51	41	66	41	0	0	0	0	17	15
Coniophora puteana	0	12	9	16	32	41	0	0	30	16	63	27
Leucogryophana arizonica	0	0	13	16	41	47	0	0	10	28	64	33

[a]Cellulose samples were placed on filter paper strips inoculated with the decay fungus, three replications.

[b]In soil block tests, glucose was added to the cellulose by soaking in a 1% glucose solution. In agar tests, glucose was incorporated into the agar medium to make a final concentration of 1%.

Table 3. Effect of Carbohydrate Cosubstrates on Cellulose Utilization by Brown-Rot Fungi in Soil Block and Agar Tests[a]

Carbohydrate Cosubstrate	Weight loss of cellulose[b]							
	P. placenta		*G. trabeum*		*C. puteana*		*L. arizonica*	
	Soil	Agar	Soil	Agar	Soil	Agar	Soil	Agar
Glucose	55	47	61	17	64	63	41	64
Mannose	68	62	90	21	40	50	57	67
Galactose	28	53	73	13	27	19	33	60
Arabinose	64	40	90	19	32	3	30	100
Xylose	48	39	50	15	32	11	30	43
Cellobiose	64	59	67	0	48	65	33	100
Xylan	0	0	80	6	53	62	70	61
Holocellulose	65	9	68	6	47	28	20	81
None	0	0	53	15	32	27	46	33

[a]Cellulose samples were placed on filter paper strips inoculated with the decay fungus. three replications.

[b]In soil-block tests, soluble sugars were added to the cellulose by soaking in a 1% solution of the sugar. In agar tests, sugars were incorporated into the agar medium to make a final concentration of 1%. Insoluble cosubstrates (100 mg) were placed directly on the filter paper inoculation strips. Duration of the test was 12 weeks.

Table 4. Weight Loss and Degree of Polymerization (DP) of Cellulose in Contact with Agar Exposed to Brown-Rot Fungi for Twelve Weeks[a].

Fungi	Glucose		No glucose	
	Weight loss	DP	Weight loss	DP
	(%)		(%)	
Gloeophyllum trabeum	0	658	0	600
Lentinus lepideus	0	983	0	983
Postia placenta	0	884	0	828
Leucogryophana arizonica	23.3	304	66.7	268
Leucogryophana olivescens	28.6	286	70.0	212
Coniophora puteana	15.3	552	36.7	552

[a]Cellulose samples were placed on agar inoculated with the decay fungus. three replications.
[b] DP of uninoculated cellulose was 1872.

Table 5. Effect of Oxygen Concentration on Degree of Polymerization of Cellulose by P. placenta Grown in Liquid Media.

Oxygen concentration (atm)	Carbon Source	Degree of polymerization			
		Exposed to P. placenta		Control	
		4 weeks	8 weeks	4 weeks	8 weeks
0.2	Cellulose	1,604	1,400	1,946	1,872
	Cellulose + 0.1% Cellobiose	1,408	1,240	1,842	1,858
0.4	Cellulose .	1,720	1,824	1,432	1,872
	Cellulose + 0.1% Cellobiose	1,590	1,230	1,840	1,952
1.0	Cellulose	1,812	1,468	1,618	1,666
	Cellulose + 0.1% Cellobiose	1,740	1,146	1,858	1,840

filtrates. Hydrogen peroxide was not detected in any of the culture filtrates with either the titanium reagent or the ABTS–peroxidase reagent.

Contrary to P. placenta, C. puteana was able to utilize cellulose in liquid culture when grown at 0.2 and 0.4 atm of O_2 (Table 6). However, DP of residual cellulose decreased only slightly. Hydrogen peroxide was not detected in culture filtrates and reducing sugars did not increase in filtrates.

Characterization of Brown–Rotted Cellulose

X–ray defractometer traces of the undegraded cotton cellulose control and the cellulose degraded by P. placenta showed no marked differences. The patterns are clearly indicative of cellulose I (native cellulose). These patterns can be used to provide measures of crystallinity index (Segal et al., 1959) and crystallinity size (Caulfield, 1971) (Table 7). The differences are not large, but it appears that the brown–rotted cellulose is more crystalline and has larger crystallites. This may result from a preferential attack on the smaller crystallites and amorphous regions of the cellulose.

Molecular weight determinations by HPLC of carbanilate derivatives gave a symmetrical DP distribution with a fairly tight distribution around DP 200 (Fig. 1). Thus, random cleavage of all the cellulose must have occurred in the amorphous regions, not just at the fiber surfaces. The number average molecular weight was 188 and the weight average 335. This is close enough to a 1:2 ratio to be consistent with random cleavage.

Undegraded cellulose did not show carbonyl bands with FTIR diffuse reflectance (Fig. 2). Carbonyls were present in the brown–rotted cellulose. When reduced with $NaBH_4$, carbonyls were still present, but in lower concentrations. This result suggests that acidic groups were present in the brown–rotted cellulose. The KBr pellets was too opaque for quantitative determinations, and diffuse reflectance was not quantitative.

No uronic acids were detected in acid hydrolysates, showing that acid groups that were present were not in C–6 (i.e., they had to be on the chain ends).

The presence of acid groups in the brown–rotted cellulose was verified by the methylene blue method. The NaOH–washed brown–rotted cellulose was found to contain about 1 carboxyl for 214 glucose units.

Table 6. Effect of Oxygen Concentration on Weight Loss and Degree of Polymerization (DP) by C. puteana in Liquid Media.

Oxygen Concentration (atm)	Carbon Source	Time of incubation			
		4 weeks		8 weeks	
		Weight loss	DP	Weight loss	DP
		(%)		(%)	
0.2	Cellulose	16.0	1,072	29.3	920
	Cellulose + 0.1% Cellobiose	12.3	1,752	24.1	760
0.4	Cellulose	20.0	1,072	34.0	1,030
	Cellulose + 0.1% Cellobiose	18.7	1,044	28.5	798
1.0	Cellulose	0	1,916	0	1,604
	Cellulose + 0.1% Cellobiose	0	1,848	0	1,366

values for uninoculated controls are given in Table 4.

Table 7. X-ray Diffraction of Brown-Rotted Cellulose.

Cellulose	Crystallinity index	Crystallite size
	(%)	(A)
Brown-rotted cellulose	82.7	60
Control cellulose	76.3	54

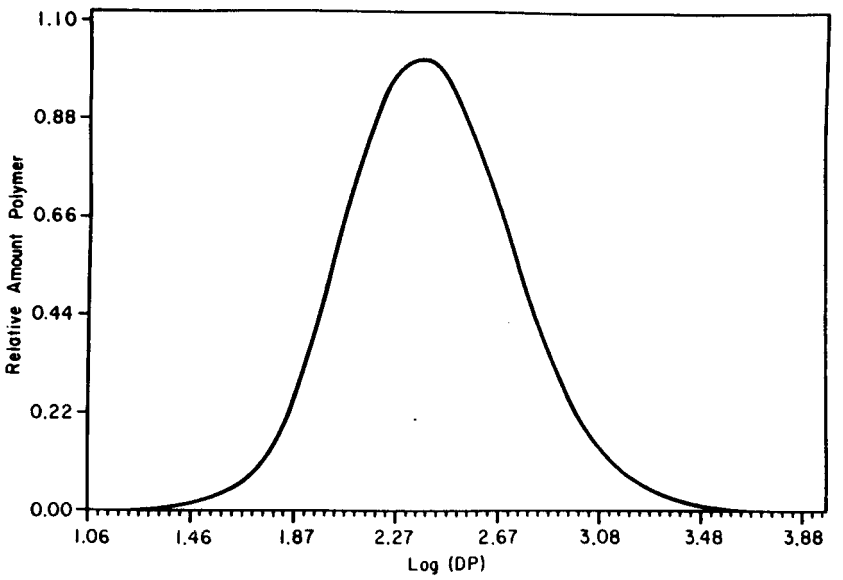

Figure 1. Molecular Weight Distribution of Brown-Rotted Cellulose.

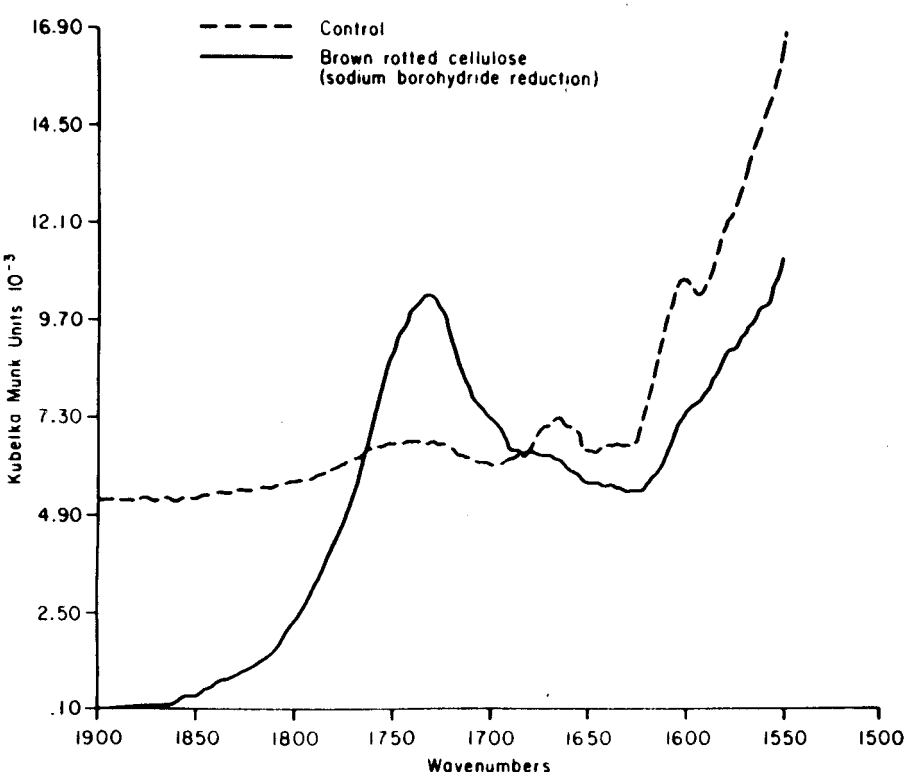

Figure 2. Fourier transform infrared spectra of undegraded cellulose (---) and brown-rotted cellulose treated with sodium borohydride (-).

DISCUSSION

Results from the degradative studies of cellulose over soil or agar medium indicate that the cellulolytic system of the various brown–rot fungi is similar. Rates of cellulose utilization differed but all produced large decreases in DP and increases in alkali solubility at low weight loss when cellulose was exposed over a soil or agar medium. Only P. placenta required a "starter sugar" to degrade cellulose.

Cellulose saturated with water by placement in contact with agar–medium or in liquid culture was utilized by the coniophoroid brown rotters (C. puteana, L. arizonica and L. olivescens). indicating that they produced the Cull cellulose–degrading complement. The nonconiophoroid brown–rotters tested were unable to utilize cellulose under these conditions, which indicates that they produced a deficient cellulose–degrading system. It is difficult to speculate why this difference in cellulose utilization by the two types of brown rotters occurs because degradative studies indicates a similar cellulolytic system. Possibly the cellulose-degrading agents of the nonconiophoroid brown–rotters are quickly destroyed in liquid cultures while the coniophoroid brown–rotters have means to prevent this. The difference in utilization of cellulose by the two types of brown–rotters deserves further attention because of the clues it might give to the nature of the brown–rot cellulose–degrading agent.

The results confirm earlier observations that brown–rot fungi preferentially attack the amorphous areas of cellulose, leaving a highly crystalline oxidized residue. Carboxyl groups were found in the cellulose molecule after brown–rot degradation. We are currently using gas chromatography and mass spectroscopy to identify the acids recovered after hydrolysis of the brown–rotted cellulose.

The mechanisms may differ, but the effects produced by brown–rot fungi on cellulose are similar to those of potassium superoxide (Thompson and Corbett, 1985). Both rapidly decrease the DP of cellulose to about 200 and show symmetrical molecular weight distributions, which indicate random cleavage of all the cellulose, not just the fiber surfaces. The ratio of number average DP to weight average DP of both is about 1:2, which is also consistent with random cleavage. Since both types of degradation reach a limit DP similar to that obtained after acid hydrolysis, degradation must proceed through the amorphous regions. The small size of intermolecular spaces makes penetration by reactants difficult. However, small molecules, such as oxygen radicals, might penetrate. The rapidity of the degradation compared with acid

penetrate. The rapidity of the degradation compared with acid hydrolysis makes it likely that cellulose–metal complexes and oxidation reactions by oxygen radicals are involved in cellulose depolymerization by brown–rot fungus.

The chemical reactions involved in cellulose degradation by the brown–rot fungi are as yet unknown, but by identifying the cleaved end residues in brown–rotted cellulose, it should be possible to speculate about the nature of the degrading agent. Identification of the degrading agent will serve as a foundation for the development of new methods of wood protection.

SUMMARY

To gain further understanding of the nature of the cellulose depolymerizing agent or agents involved in the initial depolymerization of cellulose by brown–rot fungi we determined the ability of several brown–rot fungi to degrade cellulose under various cultural procedures, and chemically and physically characterized the brown–rotted cellulose. All the brown–rot fungi extensively depolymerized and increased alkali solubility of the degraded cellulose exposed over soil or agar before significant weight loss occurred. Only the coniophoroid brown–rot fungi were able to utilize cellulose saturated with water by placement in contact with agar or in liquid culture medium. Analysis of the molecular weight distribution of brown–rotted cellulose indicated a fairly tight distribution centered around DP 200. From X–ray diffraction analysis it appears that there was a preferential attack on the smaller crystallites and amorphous regions of the cellulose by the fungus, confirming earlier work. Infrared spectroscopy and carboxyl determinations with methylene blue showed that carboxyl groups were present in the degraded cellulose. Uronic acids were not detected in acid hydrolysates of the brown–rotted cellulose, indicating that oxidation was not at C–6. However, several acids were separated and are currently being studied.

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