Chemistry of Natural Compounds, Vol. 36, No. 4, 2000

BIOTRANSFORMATION OF ASPEN LIGNIN BY THE FUNGUS *Trametes villosus*

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UDC 547.992:582.284

Quantitative ^{1}H and ^{13}C NMR spectroscopies demonstrate that biotransformation of aspen wood by the fungus Trametes villosus *results in oxidation and destruction of lignin with cleavage of C–C alkyl–alkyl bonds in side chains and partial demethoxylation in addition to cleavage of lignocarbohydrate bonds. New C_{ar}–O–C bonds form while lignin is being destroyed at alkyl—alkyl bonds. Cleavage of rings and destruction of C_{ar}–C bonds was not observed.*

Key words: lignin, biotransformation, *Trametes villosus* fungus.

The mechanism of microbiological destruction of lignin has been reviewed in many publications [1-6]. However, this issue is still unresolved. One of the serious problems arising during a study of the biodegradation of this natural polymer is the difficulty of its isolation. Many early investigations concerned the microbiological action on such preparations as alkaline lignin, Klason lignin, and Brauns lignin [1]. However, these are modified compared with the natural form. Such industrial lignins as hydrolyzed lignin and lignosulfonates are readily available and therefore widely used in research [7]. Questions about the degradation of industrial lignins are undoubtedly important in an ecological sense and deserve investigation. However, they give no information about the biodestruction of lignins in wood.

The biodestruction of native lignin and lignin isolated from wood decomposed by microorganisms is of special interest. In this instance, lignin obtained by the Bjorkman method (mechanically ground lignin, MGL) is considered to be the most authentic compared with that in the starting material [8].

Two principal mechanisms have been proposed by which microorganisms can utilize lignin: 1) depolymerization of lignin macromolecules with release of monomeric and dimeric fragments; 2) dearomatization of the polymer via cleavage of the ring and subsequent destruction of the aliphatic chain [2]. Both mechanisms may be combined in the biodegradation of lignin.

We studied lignins isolated from aspen wood damaged by the fungus *Trametes villosus*.

Bioprocessing of the wood for two weeks, one month, and two months produced samples with various mass losses (ML). The distribution of ML showed that the scatter of ML increases with increasing incubation time (Fig. 1). Whereas after two weeks (average ML 4.96%) 80% of the samples lie in the narrow distribution range 4-6%, after one month (average ML 8.77%) only 52% of the cubes lie in the range of 7-9% ML. After two months (average ML 15.88%), the difference between the minimal and maximal ML was 9.62%. This is more than two times greater than the corresponding value for the 2-week samples. The plot of distribution over masses had no clear maxima.

By eliminating samples with borderline values of ML, the total number of which was less than 6%, we isolated from the remaining cubes biolignins 1-3, respectively, by a modified Bjorkman method (see Experimental). For comparison, we obtained lignin from healthy aspen wood (LGW).

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0009-3130/00/3604-0411$25.00

TABLE 1. Number of C Atoms in Structural Fragments of Lignin Macromolecules Isolated from Aspen Wood According to ^{13}C NMR (Calculated per Aromatic Ring, Relative Experimental Uncertainty <6.4%)

Structural fragment	Aspen LGW	Duration of fungus action			Range of CS integration, δ, ppm from TMS, assignment
		2 weeks	1 month	2 months	
C=O	0.130	0.325	0.252	0.247	210-185; C=O ketones
CHO	2.610	0.059	0.000	0.000	197-185; COH aldehydes
COO	0.403	0.562	0.797	0.841	185-164; COO carboxylic acids
C=O acetate	0.000	0.340	0.238	0.000	169.7; C=O acetyls of carbohydrates
C_{ar}-O					
1	0.117	0.103	0.112	0.148	162; C-4 in *H*, *H′*
2	0.052	0.236	0.238	0.161	160-154; C-3, 4 in *G*, *G′*with α-C=O
3	1.066	0.901	1.189	0.989	153-151; C-3, 5 in *S*; C-3 in *G* with 5-5-bonds
4	0.572	0.754	0.713	0.718	151-143; C-3, 4 in *G*, *G′*; C-3, *5* in *S′*
5	0.539	0.450	0.587	0.494	138.1; C-4 in *S*
C_{ar}-C	1.184	1.433	0.979	1.212	143-125; 120.3; C-1 in *G*, *G′*, *S*, *S′*, *H*, *H′*; C-5 in 5-5, β-5, C-1 in β-1
CH_{ar}	0.456	0.369	0.602	0.557	131.5; C-1 in *H*, *H′*, 129-128; $CH_β$ in olefinic fragments, 125-117; C-6 in *G*, *G′*
CH_{ar}					
1	0.508	0.414	0.392	0.482	117-114; C-5 in *G*, *G′*, C-3, 5 in *H*, *H′*
2	0.325	0.296	0.210	0.334	112-110; C-2 in *G*, *G′*
3	0.208	0.281	0.363	0.421	109-106; C-2, 6 in *S*, *S′*with α-C=O
4	0.859	0.754	0.615	0.483	106-103; C-2, 6 in *S*, *S′*
C-1	0.000	0.488	0.392	0.062	102-95; C-1 in xylose of xylans
OCH	2.751	1.271	1.342	1.397	90-65; $C_α$, $C_β$ in α-O-4, β-O-4, $C_α$-OH, $C_β$-OH
OCH_2	1.379	1.211	1.258	1.212	75-59; $C_γ$ in β-β; CH_2 in $COOCH_2$ fragments, $C_γ$ in β-O-4, β-5, α-O-4
OCH_3	1.444	1.315	1.370	1.311	57-55; OCH_3 in Ar-OCH_3
CH	0.650	0.206	0.251	0.123	55-52; $C_β$ in β-β, β-1
C_{alk}	0.325	0.635	0.238	0.841	35-10; CH, CH_2, CH_3-groups of saturated hydrocarbon chains
CH_3	0.000	0.340	0.238	0.000	21.2; CH_3-group in acetyl fragment
f_a	0.461	0.406	0.429	0.485	163-103; fraction of C atoms in preparation (degree of preparation aromaticity)
f_a'	0.461	0.515	0.519	0.515	Typical degree of lignin aromaticity

S, *G*, *H* are etherified; *S′*, *G′*, *H′* are unetherified syringyl-, guaiacyl-, and *p*-hydroxyphenylsubstituted structures, respectively.

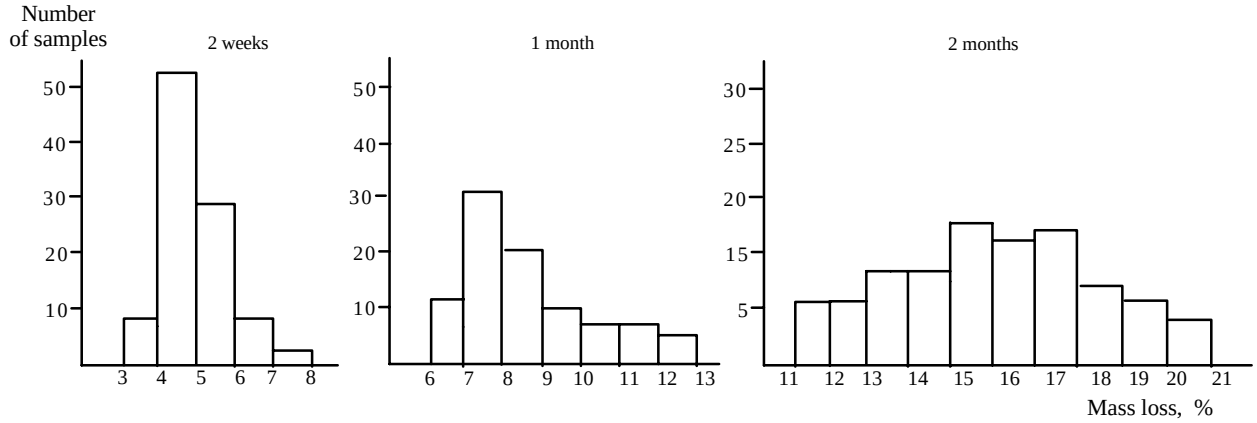

Fig. 1. Mass-loss distribution of aspen wood samples treated with the fungus *Trametes villosus*.

TABLE 2. Distribution of H Atoms Among Structural Groups of Lignin Isolated from Aspen Wood Treated with the Fungus *T. villosus* at Various Incubation Times (in Fractions, Relative Experimental Uncertainty 4.6%)

Structural group	Duration of fungus action				Range of ^{1}H NMR spectra, δ, ppm from TMS
	Aspen LGW	2 weeks	1 month	2 months	
$H_{OH,CHO}$	0.017	0.019	0.025	0.013	13.0-8.5; H atoms of phenolic OH and aldehyde CHO-groups
H_{ar}	0.136	0.148	0.207	0.147	8.5-6.3; H atoms of aromatic rings
$H_{O\text{-}alk}$	0.718	0.710	0.624	0.664	6.3-2.6; H atoms of –OCH, $–OCH_2$, and $–OCH_3$-groups and olefinic fragments
H_{acet}	0.000	0.033	0.110	0.000	2.0-1.8; H atoms of acetyl CH_3-groups
H_{alk}	0.131	0.098	0.034	0.176	1.8-0.5; H atoms of CH, CH_2 and CH_3-groups not bonded to O atoms

TABLE 3. Number of Functional Groups, Fragments, and Bonds in Aspen Lignin per 100 Aromatic Rings

Functional group, fragment, bond	Duration of fungus action				Relative uncertainty, %
	Aspen LGW	2 weeks	1 month	2 months	
C=O	7	32	25	24	4.5
CHO	7	6	0	0	4.5
COO	40	56	80	84	6.7
$OH_{phenolic}$	29	40	39	18	12.0
OCH_3	144	131	137	131	4.5
S, S′	58	52	58	52	12.0
G, G′	28	27	21	27	12.0
H, H′	14	10	11	14	4.5
H*(by difference)	0	11	10	7	
C_{ar}-C	118	143	97	120	6.7
C_{ar}-O-C	60	74	108	99	22.0
S with α-C=O	15	14	18	21	9.0
$\sum C_{side\ ch.}$ (without C_{alk})	556	363	389	382	9.5

S, S′, G, G′, H, H′ are the same as in Table 1.

We chose the method of quantitative ^{1}H and ^{13}C NMR spectroscopy for the study of processes accompanying biotransformation of lignin. These are the most informative for similar specimens. The microbiological action of *T. villosus* on wood was accompanied by the destruction of its macrocomponents. This made it easier to isolate the lignin. Lignins isolated from the biologically destroyed wood were lignocarbohydrate complexes containing from 3 to 22% carbohydrates, in contrast with lignin from healthy wood.

Due to the fact that primarily xylose was identified in acid hydrolysates of biolignins, it can be hypothesized that their carbohydrate complex is a xylan. The chemical shifts (CS) of the resonance signals characteristic of xylan in the ^{13}C NMR spectra at 102-95 (C′-1, anomeric C), 76-72 (C′-2, C′-3, C′-4), and 64-63 ppm (C′-5) clearly confirm the presence of the carbohydrate (Table 1) [9]. Xylans of biolignins **1** and **2** are acetylated. This is consistent with the resonance signals in the ^{1}H NMR spectra for CH_3-fragments of acetyls with CS 1.93 ppm (Table 2) [10] and signals in the ^{13}C NMR spectra with CS 169.7

and 21.1 ppm, which correspond to C=O and CH_3 C atoms of acetyls (see Table 1) [9]. The ratio xylan:acetyl in biolignins **1** and **2** was 1:0.7 and 1:0.6, respectively. Lignocarbohydrate bonds were actively cleaved and deacetylation was effected by *T. villosus*, especially upon incubation for more than a month. Biolignin **3** contained 2.8% xylan that was already deacetylated.

The action of *T. villosus* on aspen lignin was accompanied primarily by oxidation. The fungus increased already in two weeks the number of hydroxyls and carbonyls in biolignins. However, further incubation decreased their content, probably because they participate in secondary reactions (Table 1). The C-α and C-β atoms of side chains were oxidized to C=O groups. They increased by 1.8-2.5 times more than in LGW. Oxidation involved the α-C atom of the side chain only in the syringyl (*S*) fragments. This was consistent with resonance signals in the range 109-106 ppm, which belong to C-2 and C-6 atoms in *S* and *S′* (nonetherified syringyl) rings. Oxidation of the α-C atom in guaiacyl (*G*) fragments was not observed. The spectra lacked signals characteristic of C-6 and C-2 of the aromatic ring with α-CO substitution in *G* fragments at 123-122 and 112 ppm, respectively.

Aldehydes present in LGW and biolignin **1** were probably oxidized to carboxyls, as a result of which they are absent in biolignins **2** and **3**. However, the biolignins lacked free COOH groups (the PMR spectra do not contain their signals). Therefore, it can be assumed that they are either decarboxylated or esterified. The latter reaction is probably one of the reasons that the number of ester bonds in biolignins increases.

In addition to oxidation, *T. villosus* destroys already in one month of incubation C–C alkyl—alkyl bonds in side chains. Thus, the average side-chain length in biolignins **1-3** is 1.4-1.5 times shorter than in aspen LGW. The number of C atoms in the side chains is correlated with the loss of –OCH and $–OCH_2$ groups (Table 1) of C-α, C-β, and C-γ atoms of a phenylpropane aliphatic chain. Degree of aromaticity increased due to decreasing of aliphatic chain in biolignines.

In addition to cleavage of C–C alkyl—alkyl bonds in biolignins **2** and **3**, the number of aryl—alkyl and aryl—aryl ether bonds increased by 1.6-1.8 times. However, C_{ar}–C bonds in the biolignins remained. In the initial incubation period, they even increased (biolignin **1**). However, not more than one bond to the aromatic ring remained. Obviously C_{ar}–C_α bonds were not cleaved.

The number of *S*, *G*, and *H* fragments (*p*-hydroxyphenylsubstituted) in lignins **1-3** changed insignificantly compared with LGW. Biolignins **1** and **3** had approximately the same ratio of these fragments. However, biolignin **2** differed from all lignins by a very low content of the *G* ring for the same *S*-ring content as LGW (Table 3). Compared with aspen LGW, 3,4-dihydroxysubstituted aromatic fragments (H^*) appeared in all biolignins, possibly because of demethylation.

Thus, biotransformation of aspen wood by the fungus *Trametes villosus* oxidizes and destroys lignin with cleavage of C–C aklyl—alkyl bonds of side chains and partial demethoxylation in addition to cleavage of lignocarbohydrate bonds. In addition to destruction of lignin macromolecules at alkyl—alkyl bonds, new C_{ar}–O–C bonds form. Ring opening and destruction of C_{ar}–C bonds are not observed.

EXPERIMENTAL

Aspen wood in 3×2×0.5 cm cubes was extracted for six days by alcohol:benzene (1:1), dried in air, and weighed. It was soaked for 24 h in distilled water and sterilized three times with flowing steam (for 1 h). The cubes were placed on grown mycelium of *T. villosus* (Lloyd) Kreisel 0276 (supplied by AOOT VNIIGIDROLIZ, St. Petersburg) and incubated at 33°C. After two weeks and one and two months, 105 cubes were cleaned of mycelium, held for 15 min at 60°C to inactivate the fungus, and dried. The ML of the wood was determined. The average ML was calculated without including borderline values. The cellulose content in the starting wood and treated samples was determined by the Kuerschner method; the lignin, by the Komarov method [11] (Table 4).

Lignin of ground healthy (LGW) and destroyed wood [biolignins **1** (two weeks incubation), **2** (one month), and **3** (two months)] was isolated by the literature method [12]. Elemental composition of the isolated lignins, %: LGW, C 58.54, H 6.51; **1** (ML 4.96%), C 53.09, H 6.35; **2** (ML 8.77%), C 54.45, H 5.11; **3** (ML 15.88%), C 43.96, H 5.78.

^1H and ^{13}C NMR spectra were recorded on a Bruker WP-200 SY spectrometer at working frequencies 200.1 (^1H) and 50.13 MHz (^{13}C). The spectral widths were 7000 (^1H) and 20,000 Hz (^{13}C). ^{13}C NMR spectra with broad-band proton decoupling were recorded after 10,000 scans for 20-30% lignin solutions in DMSO-d_6. Broad-band decoupling was turned on during the relaxation delay of 2.5 sec. Chromium acetylacetonate (0.02 M) was used as the relaxation agent.

TABLE 4. Change of Chemical Composition of Aspen Wood Damaged by the Fungus *T. villosus*

Incubation time	Mass loss, %	Component content, %		Component loss	
		lignin	cellulose	lignin	cellulose
Healthy wood	-	18.18	52.62	-	-
2 weeks	4.96	18.80	54.83	1.71	0.95
1 month	8.77	17.63	56.12	11.55	2.70
2 months	15.88	16.50	52.48	23.65	16.10

^{1}H NMR spectra were recorded for 2-5% solutions of lignins in HMPA-d_{18} with a preliminary estimation of the content of water H atoms solvated by the solvent. The delay between pulses was 4 sec. The relative uncertainty of the integration was 3%. The relative uncertainty of the estimate of the number of structural parameters determined directly from the ^{13}C NMR spectra was 6.5-9.6%. Chemical shifts were assigned according to the literature [13-15].

Functional groups, fragments, and bonds (n_X) in lignins were calculated from the number of carbohydrates by the literature method [15-17], where the quantitative calculations of the ^{13}C NMR spectra were based on the fraction of C atoms in aromatic rings of the preparation (degree of aromaticity f_a) being equivalent to six C atoms in an aromatic ring: $n_X = q_X \cdot 6/f_a$ and q_X is the fraction of C atoms of the determined functional group or fragment. The number of carbohydrates in the lignins was calculated using [Carb] (mass %) = (gC-1·[C]·150)/12.01, where gC-1 is the fraction of anomeric C-1 of the carbohydrate and [C] is the content of carbohydrate in the preparation (elemental composition data).

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