

Lignocellulose pretreatment in a fungus-cultivating termite

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Depolymerizing lignin, the complex phenolic polymer fortifying plant cell walls, is an essential but challenging starting point for the lignocellulosics industries. The variety of ether- and carbon-carbon interunit linkages produced via radical coupling during lignification limit chemical and biological depolymerization efficiency. In an ancient fungus-cultivating termite system, we reveal unprecedentedly rapid lignin depolymerization and degradation by combining laboratory feeding experiments, lignocellulosic compositional measurements, electron microscopy, 2D-NMR, and thermochemolysis. In a gut transit time of under 3.5 h, in young worker termites, poplar lignin sidechains are extensively cleaved and the polymer is significantly depleted, leaving a residue almost completely devoid of various condensed units that are traditionally recognized to be the most recalcitrant. Subsequently, the fungus-comb microbiome preferentially uses xylose and cleaves polysaccharides, thus facilitating final utilization of easily digestible oligosaccharides by old worker termites. This complementary symbiotic pretreatment process in the fungus-growing termite symbiosis reveals a previously unappreciated natural system for efficient lignocellulose degradation.

lignin | carbohydrate | NMR | symbiosis | age polyethism

Lignin is a heterogeneous plant cell wall polymer derived primarily from hydroxycinnamyl alcohols via combinatorial radical coupling reactions (1). Its depolymerization is challenging, making lignin a major barrier to gaining access to stored energy within lignocellulosic materials (2). Woody biomass, such as that contained in pine and poplar trees, represents the most abundant renewable energy resource in our terrestrial ecosystems. Because of a high content of lignin, a complex polymer that contains ether- and carbon-carbon linkages between monomer-derived units (1, 3), biotechnological efforts to use this material for bioenergy require expensive chemical, physical, or biological pretreatment processes to partially delignify lignocellulose to allow access to plant polysaccharides (2).

Termites are remarkably efficient at degrading woody biomass (4). Within hours, they digest 74–99% of cellulose and 65–87% of hemicellulose, leaving the lignin-rich residues as feces; that is, they process far more efficiently than other herbivores that can digest the less-lignified forages, such as grasses, leaves, and forest litter (5). Among termites, the subfamily Macrotermitinae form a monophyletic group of diverse species that originated in Africa and have cultivated specialized fungi (*Termitomyces* spp.) in their nests using woody or litter-based biomass for about 31 million years, and are able to almost completely decompose lignocellulose (6, 7). These termites enable the consumption of more than 90% of dead plant tissues in some arid tropical areas (7, 8), and thus play central ecological roles in Old World (sub)tropical carbon cycling (Fig. 1 *A* and *B*) (8). Throughout their evolutionary history, fungus-cultivating termites have evolved in mutualistic association with multiple microbial symbionts, not only with the lignocellulose-digesting basidiomycete fungi *Termitomyces*

spp., but also with gut microbiota and the bacterial community associated with their fungus comb (8, 9). The Macrotermitinae termite–fungus–bacterial symbiosis is a system that enables the extensive conversion of plant materials (9, 10). In addition, the plant substrate processing in fungus-cultivating termites is completed by the termite worker castes with complex age-related labor division (genera *Odontotermes* and *Macrotermes*) (11, 12) (Fig. 1*C*). Older workers forage for plant material and transport it to the nest, but gain their nutrition by consuming the mature, protein-rich fungus comb. In contrast, young workers ingest the undegraded plant material provided by their older nestmates together with fungal nodules (containing asexual spores), and use their feces, which are rich in fungal spores and lignocellulosic substrates, to build new fungus combs (Movie S1). The fungus comb has a turnover time of ~45 d (13) (Fig. 1*D*), making fungus-cultivating termites an efficient system for bio-degradation of lignocellulose (7). Moreover, the evolution of elaborate symbiotic associations, together with age polyethism in plant substrate processing, likely contributes to the ecological success of the Macrotermitinae, in particular the *Odontotermes* genus that represents more than half of the described Macrotermitinae species (14), and are dominant wood and grass degraders in southeast Asia (14).

Significance

Fungus-cultivating termites are icons of ecologically successful herbivores in (sub)tropical ecosystems, cultivating *Termitomyces* fungi for overcoming the rigid lignin barrier of wood resources. To date, research on these ectosymbiotic fungi has only identified laccases, rather than the typical ligninolytic peroxidases. Using 2D gel-state NMR, we chemically tracked the fate of lignin from the original poplar wood throughout the complex food-processing system in a farming termite. We found young worker termites rapidly depolymerize and degrade even the most recalcitrant wood lignin structures, facilitating polysaccharide cleavage by symbiotic fungi. These results suggest that the natural systems for lignin degradation/pretreatment are far beyond the systems currently recognized and are potential sources of novel ligninolytic agents, enabling more efficient plant cell wall utilization.

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Table 1. Two-dimensional NMR Integral data for interunit linkages relative to the lignin methoxyl, methoxyl analyses results, and aromatic unit ratios

Unit	Poplar wood	Fresh comb	Middle-aged comb	Mature comb
β -Ether A	0.121	0.087	0.037	0.037
Phenylcoumaran B	0.004	—	—	—
Resinol C	0.016	0.007	—	—
Spirodienone SD	0.005	—	—	—
OMe content/sample (%) [*]	4.5	2.9	1.9	1.4
OMe content/lignin (%) [†]	20.4	15.2	15.7	15.7
OMe decrease (%) [†]	—	25	23	23
S/G ratio	1.68	5.23	6.61	16.39
PB/(S+G) ratio	0.10	0.07	0.04	0.07

All integral data for lignin methoxyl corrected for actual OMe/lignin obtained from OMe analysis (*SI Appendix, SI Materials and Methods*). —, not detected.

^{*}Based on the dry weight of material analyzed.

[†]Based on the Klason lignin + acid-soluble lignin.

microbiome—including its dense *Termitomyces* mycelial mass, replete with laccases, and harboring unique bacterial lineages—has the ability to extensively mineralize aromatics (39, 40).

The aliphatic regions of the heteronuclear single-quantum coherence (HSQC) spectra (Fig. 3) provide details regarding the distribution of interunit linkages in the lignin structure. The lignin compositions in the fungus comb were strikingly different from those in the original poplar, even in the fresh comb (Table 1). The β -ether units **A** decreased by 28% going from the wood to the fresh fungus comb, followed by an additional 57% from fresh to the middle-aged comb, but no further depletion in the mature fungus comb. The resinol units **C** also showed a substantial decrease with 56% depletion in the fresh fungus comb and complete removal by the middle-aged comb. Phenylcoumarans **B**, spirodienones **SD**, and cinnamyl alcohol end-units **X1** had also essentially disappeared before the material made it to the fresh fungus comb (Fig. 3*B*). Methoxyl analysis revealed that the fresh fungus-comb sample had lost ~25% of its original methoxyls; no further depletion was seen in the progression through the middle-aged and mature fungus combs. The HSQC spectra of the digested wood from the young worker termites shows divergent behavior compared with wood after white- and brown-rot decay also analyzed by 2D-NMR spectroscopy (32, 33). For example, white-rotted wood typically shows simultaneous disappearance of lignin and polysaccharide moieties, whereas brown-rotted wood retains much of the lignin aromatics with some depletion of aliphatic side-chains. Here, our findings reveal that, even during the rapid transit time for the material in the termite gut (~3.5 h), the lignin is significantly depolymerized. Indeed, ring methoxyl groups are appreciably removed, and lignin sidechains are extensively cleaved, leaving a residue (fresh fungus comb) almost completely devoid of various condensed units that are identifiable by their characteristic carbon-carbon linkages. Apparently, the enzymes in young worker termite guts work in conjunction with intestinal microbiota to depolymerize lignin. Moreover, the comparative studies of fresh and middle-aged fungus comb showed that continuous cleavage of lignin sidechains is occurring throughout this process, but with no further depletion in the mature fungus comb, implying the bacterial lineages in the fresh comb derived from the feces of young workers may have similar lignin-degrading abilities to those in the gut, and thus subsequently contribute to the complete lignin depolymerization (39, 40).

Confirmation of Lignin Depolymerization and Degradation by Pyrolysis-GC-MS and Tetramethylammonium Hydroxide-Pyrolysis-GC-MS. To establish whether a significant decrease in aromatic units had occurred during the fungus-cultivating termite pretreatment, the original poplar wood and fungus comb samples were subjected to pyrolysis (Py)-GC-MS, the pyrograms of which, with their numbered peaks identifying lignin-derived phenols, are shown in Fig. 4*A*. Differences in the peak profiles of phenolic compounds

derived from the lignin moieties in the pyrograms from fungus-comb samples versus those from the original poplar wood reveal details consistent with the NMR structural analysis. For example, the amounts of phenolics released from the fresh, middle-aged, and mature fungus-comb lignin (1 mg total per sample) were 0.13 mg, 0.07 mg, and 0.05 mg, compared with 0.23 mg in the original poplar sample. Moreover, the H/G/S/PB ratios were 5:31:64:0, 8:40:50:0, and 15:28:57:0 compared with 2:23:76:2 in the poplar wood sample (*SI Appendix, Table S5*). Interestingly, as a common intermediate in β -ether degradation pathways (22), the vanillic acid (peak 28) was highly abundant in pyrograms from the fresh fungus comb (8.3%), represented only minor amounts in the middle-aged comb (2%), and was completely absent from the mature comb, suggesting that the major β -ether cleavage takes place in the young workers' gut, but is followed by a further cleavage by the fresh comb microbiome, again suggesting that activity is retained in the feces.

Additional support for our findings of the cleavage of lignin ethers during the rapid passage through the young worker termite gut is provided by investigating pyrolysis of the original poplar wood and fungus-comb samples in the presence of tetramethylammonium hydroxide (TMAH) (Fig. 4*B*). TMAH present during pyrolysis esterifies acids (and methylates phenols) with remarkable efficiency (41), and has been used to analyze insect-digested and fungal-rotted wood (37, 42). Ether cleavage resulted in the enhanced production of 3,4-dimethoxybenzoic acid methyl ester (peak 32) and 3,4,5-trimethoxybenzoic acid methyl ester (peak 42); an increase in the G-derived ester peak 32 to aldehyde peak 20 and the S ester peak 42 to aldehyde peak 33 ratios is indicative of lignin depolymerization (37, 42). These G-monomer ratios from the fresh, middle-aged, and mature fungus comb were 0.64, 1.08, and 1.88 compared with 0.12 from the poplar wood (*SI Appendix, Table S6*). Similarly, S-monomer ratios were 4.53, 6.87, and 10.50 compared with 1.62 from the original poplar wood (*SI Appendix, Table S6*). TMAH-Py-GC-MS results therefore demonstrate the remarkable degree of ether cleavage that has occurred during the gut passage in young worker termites, and are in good agreement with NMR and Py-GC-MS data.

Preferential Carbohydrate Utilization and Polysaccharide Cleavage. Insight into the polysaccharide conversion in this termite system was gained from HSQC NMR spectra together with high-performance anion-exchange chromatography (HPAEC) analysis. The polysaccharide anomeric regions of the spectra from fungus combs showed distinct differences from those within the original poplar wood (*SI Appendix, Fig. S5*). For the fresh comb sample, the absence of 4-*O*-MeGlcA (labeled U1) and α -L-Araf sidechain units of hemicelluloses and a slight decrease in the acetylated xylan hemicellulose, as exemplified by reduction in the level of the 2,3-di-*O*-Ac- β -D-Xylp (2) contours (Fig. 3 and *SI*

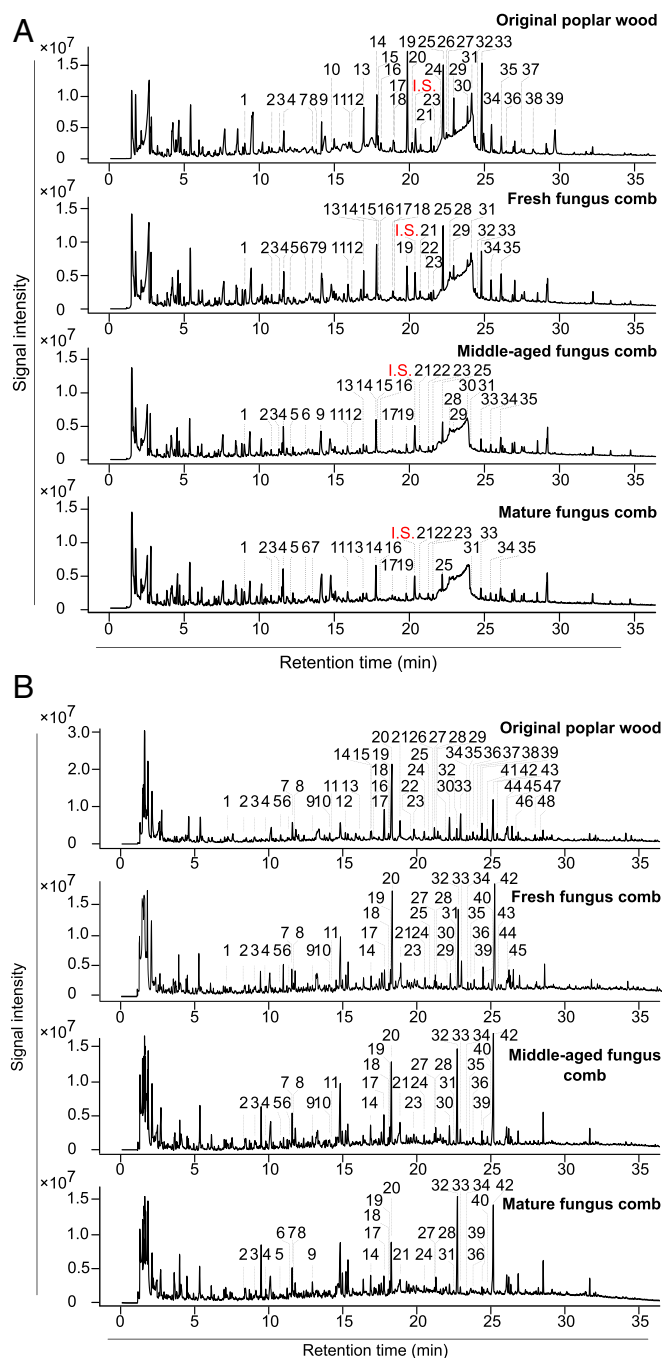


Fig. 4. Thermochemolysis profiles of cell walls from samples. (A) Py-GC-MS profiles. Poplar wood, fresh fungus comb, middle-aged fungus comb, and mature fungus comb with added internal standard (I.S.). The peaks labeled are identified phenolics-derived compounds. See *SI Appendix, Table S5* for peak identification and relative molar areas. The structures of the labeled peaks are shown in *SI Appendix, Fig. S7*. (B) TMAH-Py-GC-MS profiles. Poplar wood, fresh fungus comb, middle-aged fungus comb, and mature fungus comb. The peaks labeled are identified phenolics-derived compounds. See *SI Appendix, Table S6* for peak identification and relative molar areas. The structures of the labeled peaks are shown in *SI Appendix, Fig. S8*.

Appendix, Fig. S5), shows that the young workers mainly make use of the wood cell wall's branched sugars substituted along hemicellulosic backbones, suggesting that the young workers preferentially release the more easily accessible sugars from the sidechains during the rapid transit time. Dominant members of

the bacterial family *Lachnospiraceae* are present in the young workers of *O. formosanus* (39); such microbiota are ascribed essential and specific roles in both deconstruction and fermentation of noncellulosic poly- and oligosaccharides that have been interpreted as evolutionary adaptations for the rapid digestive niche (43). The enrichment in cellulose (β -D-Glcp), as a result of a decrease in xylan (β -D-Xylp) in the middle-aged and mature combs, was evident from the HSQC spectra. Consistent with this interpretation, the HPAEC analysis revealed that the glucose/xylose ratios in the middle-aged and mature combs increased to 3.80 and 8.20, compared with 2.27 in the original poplar wood (*SI Appendix, Table S1*). These data suggest that the fungus-comb microbiome preferentially uses xylose and other hemicelluloses, leaving most of the cellulosic substrates.

For the middle-aged and mature fungus comb, strong increases in the intensities of the reducing endgroups both in xylan (α -D-Xylp-R) and mannan (α -D-Manp-R) were also observed. In addition to these NMR spectra, comparisons of the total amount of carbohydrates in hydrolyzed comb samples (*SI Appendix, Table S1*) indicated that significant insoluble material weight losses were observed as the comb matured, as revealed by the HPAEC analysis of hydrolyzed extractive-free fraction from the comb (*SI Appendix, Table S7*). In going from the middle-aged comb to the mature comb, 69% of the insoluble carbohydrates were lost (see extract-free fraction in *SI Appendix, Table S7*). Together with the limited amount of soluble monosaccharides detected in the nonhydrolyzed extractive fractions, large amounts of soluble oligosaccharides were expected in the extractives. Although HPAEC analysis using an oligosaccharides column showed none of the typical 1,4- β -D-glucooligosaccharide and 1,4- β -D-xylooligosaccharide compounds, two unknown compounds had accumulated in the extractive phase of the mature comb sample (*SI Appendix, Fig. S6*). Attempts to identify these compounds were not successful (details provided in *SI Appendix, SI Materials and Methods*). Consistent with previous reports that *Termitomyces* fungi are genetically enriched in polysaccharide backbone-degrading enzymes and depleted in oligosaccharide-splitting enzymes (9, 16), our results demonstrate that the fungus-comb microbiome contributes to significant polysaccharide cleavage/depolymerization, leaving the mature comb enriched in oligosaccharides, particularly cellulosic oligomers/polymers.

The ecological success of fungus-cultivating termites derives from their striking ability to effect the almost complete decomposition of a broad range of plant-derived biomass, including litter, grasses, and lignin-rich woods. In this study, we show highly efficient deconstruction of wood in a fungus-cultivating termite *O. formosanus*, which sheds considerable new light on the delignification in the fungus-cultivating termite symbiosis. Indeed, both our NMR and thermochemolysis data compellingly reveal that the rapid first gut passage through young workers considerably depolymerizes and degrades lignin. Far beyond the young termite's traditionally held role to simply mix asexual spores and lignocellulosic substrates (11, 28), these observations align with previous studies, providing evidence that lower termites (nonfungus-cultivating species) enable lignin sidechain oxidation (37, 44). After the first gut-passage step, the lignin degradation products in the woody residue are continuously cleaved/removed by the fungus-comb microbiome. Finally, our combined polysaccharide analyses demonstrate a complementary cooperation in polysaccharide conversion among symbionts in *O. formosanus*, consistent with recent genetic evidence from another fungus-cultivating species *Macrotermes natalensis* (9). In total, these findings establish the role of the fungus comb as the external digestive system for the fungus-cultivating termite superorganism.

Conclusion

Woody biomass has substantial potential for diverse lignocellulose industries, but its lignin makes it particularly recalcitrant, requiring the use of expensive pretreatments to depolymerize lignin. Although the white-rot/brown-rot fungi have been viewed as

the paradigm for wood decay, our findings add to the growing body of contention that ectosymbiotic fungi of fungus-cultivating insects (ants and termites) are likely to lose the key delignification potential throughout evolutionary modifications (45). In the fungus-cultivating termite symbiotic system, lignin depolymerization takes place during the rapid passage through the pH-neutral gut of young worker termites (46) in a process that is striking in its speed and efficiency at destroying the traditionally considered most-recalcitrant C–C-bonded lignin structural units, thereby facilitating efficient degradation of the substrate by processes subsequently occurring via the fungus-comb microbiome. This efficient woody biomass bio-pretreatment comprised of step-wise delignification and cleavage of polysaccharides under mild conditions, therefore has considerable biotechnological potential.

Experimental Procedures

We qualitatively and quantitatively examined the mechanisms of biological pretreatment of poplar wood's lignin and carbohydrates through each stage

of the plant substrate flow using our well-developed laboratory rearing colonies of *O. formosanus* (12). Specifically, we: (i) combined feeding experiments through laboratory rearing colony and follow the passage of material throughout substrate processing; (ii) applied scanning electron microscopy to determine changes in plant morphology; and (iii) used chemical measurements of lignocellulosic composition, as well as 2D-NMR, Py-GC-MS, and TMAH-Py-GC-MS analyses of lignocellulosic structure to track the fate of poplar wood's lignin and carbohydrates. All detailed experimental procedures are provided in *SI Appendix, SI Materials and Methods*.

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