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Detailed Analysis of Modifications in Lignin After Treatment With Cultures Screened for Lignin Depolymerizing Agents

Aarti Gidh,*[,1] Dinesh Talreja,[2] Todd B. Vinzant,[3] Todd Clint Williford,[1] and Alfred Mikell[2]

[1]Departments of Chemical Engineering; [2]Biology, University of Mississippi, University, MS 38677, E-mail: agidh@olemiss.edu; and [3]National Bioenergy Center, National Renewable Energy Laboratory, Golden, CO 80401

Abstract

Termites, beetles, and other arthropods can digest living and decaying wood plus other lignocellulosic plant litter. Microbial sources like other wood-eating insect guts and wastewater treatment sludge were screened for lignin depolymerization. Near infrared spectroscopy and atomic force microscopy (AFM) along with high-performance liquid chromatography (HPLC), were used to track changes in functional groups, size, shape, and molecular weight of lignin molecules during incubations. *Odontotaenius disjunctus* (Betsy beetle) guts dissected whole or separately as midgut, foregut, and hindgut, consumed corn stover but did not show lignin depolymerization. The sludge-treated lignin did show some reduction in molecular weight on the HPLC, particle size (350–650 nm initially to 135–220 nm by day 30) and particles per field on AFM. pH and the presence of nutrients had a substantial effect on the extent of depolymerization. Cultures in lignin and nutrients showed higher growth than cultures with lignin only. Colony characteristics within the beetle gut and the sludge were also evaluated.

Index Entries: Lignin; beetles; NIR; HPLC; AFM; depolymerization.

Introduction

Earth's most abundant biomass is produced by photosynthetic fixation of carbon dioxide in our biosphere, yielding approx 136×10^{15} g of dry plant material annually *(1)*. Lignin (20–30% dry weight) is conventionally defined as a complex hydrophobic network of phenyl propenoid units derived from the oxidative polymerization of one or more of three types of hydroxycinnamyl alcohol precursors—*p*-coumaryl, coniferyl, and sinapyl alcohols. Lignin can vary depending on the plant type or plant tissue, depending on the ratio of the monolignols and the degree of methoxylation *(2,3)*. Lignin is

*Author to whom all correspondence should be addressed.

particularly difficult to biodegrade, and it reduces the bioavailability of other cell-wall constituents including the predominant constituent, cellulose. Knowledge of these different constituents is essential for understanding the vastly different rates at which different plant materials decompose. Presently in bioethanol production, cellulose is separated from lignocellulose by dilute acidification, and then the cellulose is converted in two stages, enzymatic hydrolysis using cellulases and subsequent fermentation to ethanol. The acid hydrolysis and separation stages involve significant chemical use, generate waste streams, and require costly alloys, increasing capital costs.

With this method lignin cannot be isolated from lignocellulose without partial denaturation, because they are intimately linked with carbohydrate polymers and covalently linked to hemicelluloses *(4)*. Lignin was discovered in 1839 by Payen as the fraction of cell walls insoluble in acids, and since then progress has been made in understanding the mechanisms of lignin biodegradation. Ligninase (lignin peroxidase) is a powerful oxidant, which initiates lignin degradation by one electron oxidation. Lignin is also degraded by peroxidase and laccases of white-rot fungi. Although degradation of lignin by fungi has been reported, most bacteria degrade only low-molecular weight components of lignin but not the higher ones *(5)*. Most lignin loss reported from solid substrates is because of solubilization of lignin and not because of lignin degradation *(1)*. Bacterial lignin degradation has, however, been reported to be more specific than with fungal systems, an advantage, potentially leading to many industrial applications like vanillin, adhesives, binder for laminated or composite wood products, and so on *(6)*.

Few bacteria are able to degrade fully intact lignified wood cells on their own. Although, a large number of actinomycetes have been isolated from decayed wood, pure cultures cause only limited decay. Complete degradation of lignified cell walls probably requires a complex interaction and succession of a variety of bacteria and fungi. When successful, cell-wall erosion and delignification are some of the important processes that can be observed *(7)*.

For these reasons, bioprospecting for effective and alternate sources of lignin modification is important. Little work has been done on prokaryotes, in particular those derived from herbivore guts. Lignin-degrading filamentous bacteria have been isolated from the guts of wood-eating termites *(8)*. The occurrence of specific gut microbiota among the insects remains to be systematically studied, yet there is sufficient evidence of the presence of digestive symbionts for representative insect orders *(1,8)*. The most prominent examples are among the Coleoptera and Diptera. Coleopterans have actively fermenting gut microbiota, including cellulolytic and hemicellulolytic bacteria *(8)*. Wood infested by these beetles is usually well decomposed and falls apart readily *(9)*.

Lignin conversion involves complex reactions involving bond scission and functional group alteration. It is also important to track and quantify these alterations in enzyme-mediated depolymerization studies. Because of

complexity of the lignin structure, most work has involved the use of lignin model compounds. Lignin degradation by bacteria has been studied with various softwood, hardwood, or graminaceous lignin and lignocellulosic substrates *(6)*. The ^{14}C labeled lignins *(10,11)*, lignin model compounds *(12–14)*, and technical lignins like Kraft lignin *(15)* or lignosulfonates have also been studied.

Techniques employed to monitor lignin degradation have included ^{14}C-labeling *(16)*, dry weight analysis of solid substrates combined with the HPLC analysis of acid solublized substrate *(17)*, pyrolysis and Fourier transform infrared analysis *(18)*, microscopic examinations of substrates *(19)*, and protein and enzymatic activity assays *(20,21)*. Nuclear magnetic resonance (NMR) spectra *(22)* and gas chromatography-mass spectroscopy (GC-MS) *(23,24)* can be used for analysis of only lignin model compounds, their degradation products, and metabolites or to estimate bond linkages. The above listed techniques, however, do not give a comprehensive understanding of the changes to the parent lignin molecule.

Matrix-assisted Laser Desorption Ionization time-of-flight mass spectrometry has been used to detect lignin compounds up to 1.9 kDa with guaiacol as model substrate *(25)*. Although this is a useful method for compositional studies, this technique requires significant sample preparation and there are difficulties with ionization and desorption of higher molecular weight lignin molecules. Changes resulting from reactions during evaporation also cannot be ruled out *(26)*. In order to evaluate depolymerization of the parent lignins it is useful to monitor changes in molecular weight distributions. Molecular weight distributions for pure lignin substrates have been determined by means of various size exclusion columns using tetrahydrofuran or aqueous NaOH as eluents *(27)*.

This work had two objectives: investigating new bacteria for lignin depolymerization and new analytical protocols for measuring their effects. The source for new bacteria included consortia from wood-eating insect guts and some other sources for lignin depolymerization. The wood-eating insect in this initial investigation of choice was Betsy beetles, *Odontotaenius disjunctus*. (Order: Coleoptera). Dissection and serial plating techniques were optimized for beetles. We also explored the consortia within activated sludge. We have chosen to look at lignin from Kraft pulp black liquor, extracted from Jack Pine *(28)*. Lignin concentration in the liquor varies from 35.86% to 44.35% by weight. The high molecular weight, acid insoluble lignin that can be separated and is used in this work varies from 23.1% to 29.9% of the total liquor solids *(29)*. The smaller acid soluble fraction acts as a plasticizer for the larger lignin fraction. The kraft lignin purified is a very complex, highly chemically modified form of lignin which is very recalcitrant and thus more difficult to depolymerize then lignin purified by less stringent techniques. The screening incubations were analyzed only with HPLC for molecular weight shifts. The second objective involved development of protocols for atomic force microscopy (AFM) for monitoring

molecular conformations of lignin during incubation, and near infrared spectroscopy (NIR) for functional group tracking along with high-performance liquid chromatography (HPLC). These techniques proved very effective in validating our depolymerization results, and give a better understanding of the changes to lignin.

Materials and Methods

Dissection Technique

Betsy beetles were treated with ethyl alcohol to surface sterilize them. Then they were treated with boiling KOH for 1 min to soften the cuticle. The specimen was pinned through the dorsal portion of the thorax, to a suitable dissection tray filled with beeswax under a dissecting microscope with magnification up to ×10. Invertebrate saline kept the tissues moist and maintained correct osmotic conditions. The elytra (wing covers) were first raised using both the fine point forceps and microdissection scissors, thereby exposing the membranous wings and the soft underlying abdominal cuticle. Membranous wings were then cut off at their base. The soft abdominal tissue was gently lifted and a small incision was made in the cuticle directly under the forceps to expose the gut. Then, a cut was made along the entire length of the abdomen in a posterior to anterior direction and the cuticle was gently pulled upward to locate the gut. The unwanted organs were cleared away and the gut was removed and immediately placed in sterile invertebrate saline. Using a sterile scalpel, the gut was divided into foregut, midgut, and hindgut separately and added to sterile 10-mL invertebrate saline for serial dilution and plating (Fig. 1).

Serial Dilution and Plating

The foregut, midgut, and hindguts were processed separately using serial dilution technique with invertebrate saline (0.425% NaCl) as indicated below and dilutions 10^{-3}–10^{-8} were plated on tryptate soy agar (TSA) agar media plates and incubated at 30°C for 36–48 h. Distinct colonies were isolated and refreshed on TSA agar media again and colony characteristics like morphology, shape, and color and Gram nature were accessed *(30)*.

Dilutions: gut + 10-mL invertebrate saline = 10^{-1}

1 mL of 10^{-1} + 9 mL invertebrate saline = 10^{-2} up to 10^{-8}.

Propagation of Wastewater Treatment Sludge

Cultures in the sludge obtained from a wastewater treatment facility were initially grown with corn stover (National Renewable Energy Laboratories) and soytone peptone as substrate in a 3-L fermenter. After a week, 1 g/L lignin was added daily for the second week to replenish the carbon

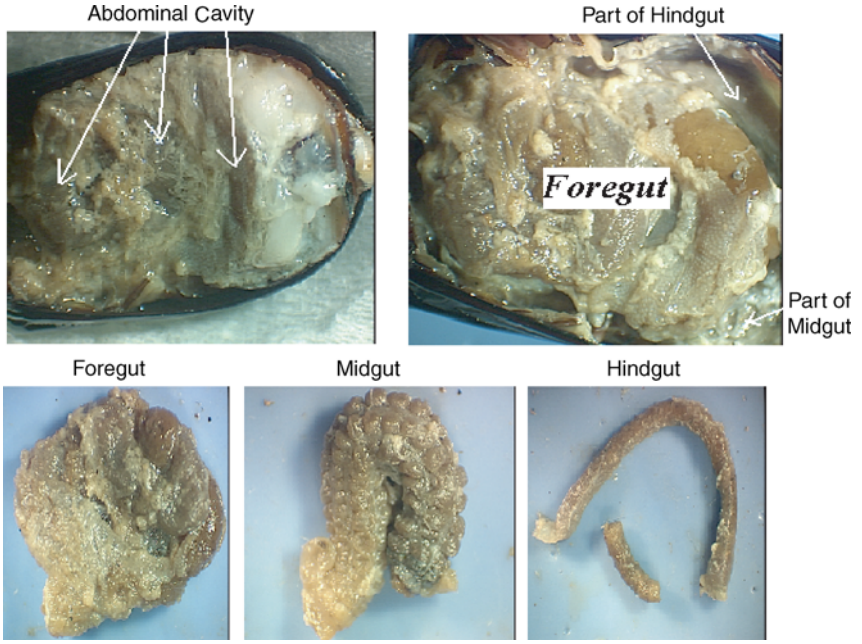

Fig. 1. Beetle dissection—abdominal cavity including the gut of Betsy beetle.

source and acclimatize the culture for a lignin substrate and the pH was maintained at 7.5 with HCl.

Screening Incubations

Batch screening incubation studies were conducted in duplicate. A 30-mL total reaction volume was used in 200-mL conical flasks covered with aluminum foil. Each liter of media consisted of 1 mL Cohen-Bazire's modified Hunters stock solution and 11 mL of 1:10 dilution Mineral base solution. Unlyophilized lignin (11.092 g/L) purified from Kraft black liquor was added to give a final concentration of 1 g/L. The media was prepared in bulk so that each flask would be the same containing 1 g/L lignin as the primary control (L). Lignin with just the nutrients and without the inoculum was used as the secondary control to ensure that the changes in the lignin profile were seen only because of the inoculum effects (M + L). Each flask, containing media, was then sterilized by autoclaving at 120°C for 45 min. The sterilized media was allowed to cool to ambient conditions for 1 d before inoculation. Propagated sludge and other inoculate sources were then added to make up 10% volume fraction (M + L + I). Beetles were inoculated in five forms as crushed whole, dissected with the whole gut, or separated as foregut, midgut, and hindgut. Inoculum was prepared in 30 mL of invertebrate saline. The other six sources investigated for lignin depolymerization were soil obtained from around rotting wood, worms, termites, deer dung, cow dung, and cow

rumen. The solid inoculum was diluted with deionized water to slurry. The entire sets of experiments were performed in duplicates for data validation, with static aerobic incubation at 32°C for 30 d.

Secondary sets of experiments were repeated with beetle gut inoculation with two different oxygen environments. Aerobic conditions were maintained as explained earlier and a set of tubes were also incubated in a limited oxygen environment (candle jar). Centrifuge tubes (50 mL) were placed in a desiccator with a burning candle, to develop limited oxygen environment when the candle extinguished. Hydrogen peroxide (150 µL) was added to some flasks to test for improved lignin depolymerization. Inoculation was with dissected beetle gut—foregut, midgut, and hindgut.

Samples were withdrawn every 3–4 d for HPLC analysis and molecular weight distribution tracking. Sample analysis was conducted using HPLC. The size exclusion column used was Asahipak GS-320 (Shodex). A 100-µL sample loop was used. 0.1 *M* NaOH (pH 12.0) at a flow rate of 0.5 mL/min was the mobile phase used. The detector connected in series was Dionex AD20 Absorbance Detector to track changes in molecular weight.

Detailed Analysis

The screening results indicated that the wastewater sludge depolymerized lignin. A detailed study was then conducted with the sludge to get more comprehensive data. Batch studies (200 mL) were conducted in duplicate with this culture. The reaction mixture contained by volume 5% unlyophilized lignin (final concentration—0.55 g/L) and 85% Yeast Malt extract media. pH was adjusted to 8.5 with 0.1 *N* NaOH before autoclaving. Inoculation, incubation, and sample withdrawal was conducted as described earlier. Mixtures of 0.55 g/L lignin without nutrients (L) and lignin with nutrients (Ym + L), both without inoculum, were used as primary controls to ensure the change seen was because of inoculation. Lignin without nutrient (L + I) and nutrient broth without lignin (Ym + I) both inoculated, and just inoculation in deionized water (I) were used as secondary controls, to study the effect of the nutrient broth and lignin on microbial growth. The final set had all three components (Ym + L + I). All six sets were conducted in duplicate for data verification.

Analysis, along with HPLC for lignin tracking, involved Vecco Multi-Mode AFM and Bruker EQUINOX© 55/S (Bruker Optics, Model No. 502) FT-IR Spectrophotometer equipped with OPUS© 2.2 software (Opus Supplies Limited). The spectra were collected in the NIR mode using a tungsten lamp source and an air-cooled GE-Diode detector with a quartz beam splitter. Lignin did not adsorb very well on the mica surface tried initially for AFM studies. The final concentration selected for lignin was 0.01 g/L and the surface selected was graphite. Bacterial colonies were counted for the initial and final day samples with Axiovert 25 CFL Microscope.

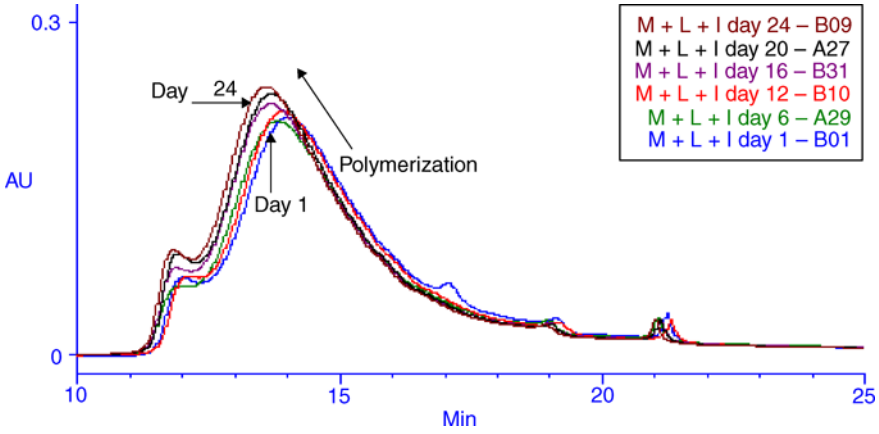

Fig. 2. Lignin polymerized with beetle gut inoculation. M + L + I indicates the flask contained nutrients, lignin, and inoculum.

Results and Discussion

Screening Incubations

Figure 1 shows the lignin profile overlays for flask inoculated with whole beetle gut. It is a representative diagram for the inoculum screening. Similar profiles were obtained for all different sources except the wastewater treatment sludge. Size exclusion columns elute the higher molecular fractions first followed by the lower molecular weight. Thus, a decrease in the earlier peaks with a consequent increase in the peaks eluting later, would indicate depolymerization. The six lignocellulose digester inoculum sources showed only polymerization with the tried fermentation conditions (Fig. 2). Lignin polymerized with beetle gut inoculation for both types of aerobic and limited oxygen environments. H_2O_2 seemed to bleach the samples from day 1 (Fig. 3). When beetle guts had earlier been applied to corn stover (10% dry solid content), a 7–8% total weight loss had indicated lignocellulose utilization and thus seemed a promising source for further investigation (data not shown). Morphology, shape, color, and Gram character of the bacterial, fungal, and yeast-like colonies isolated after beetle gut dissection are listed in Tables 1–3. Another batch was tried with the whole gut to see if the mutualistic behavior of the whole gut bacteria would improve depolymerization. The whole gut was able to achieve weight loss on corn stover incubations but when investigated with lignin showed only polymerization.

Figure 4 is a representative diagram for controls with only lignin during each set of experiments. Similar plots were obtained for lignin and nutrient (M + L) secondary controls. There was substantial overlap of the profiles over the entire incubation period without any change in the molecular weight distribution. This confirmed that any changes seen (polymerization or depolymerization) were because of the inoculum and

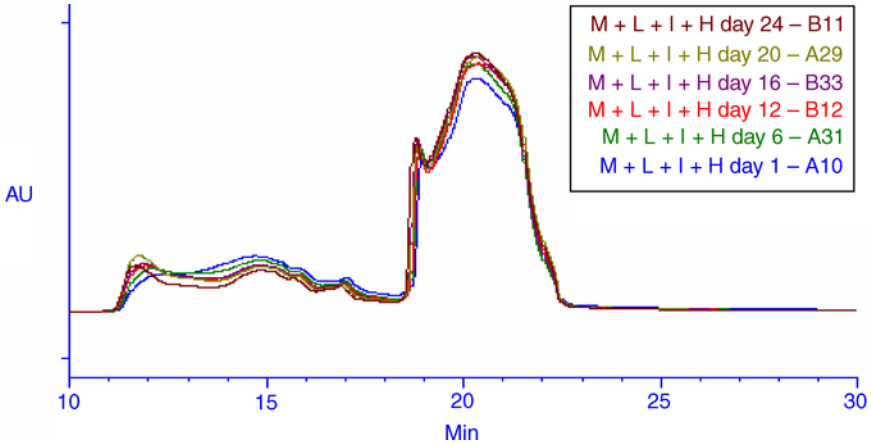

Fig. 3. Lignin bleached from day 1 in flasks supplemented with H_2O_2. M + L + I indicates the flask contained nutrients, lignin, and inoculum. H–H_2O_2.

Table 1
Colony Characteristics and Gram Character of Bacterial Colonies From the Foregut of *Odontotaenius disjunctus* (Betsy beetle)

Legend	Gram	Morphology	Color/Shape
A	+	Rods	Cream/rhizoid, filamentous, flat
B	–	Cocci	Red/circular, entire, convex
C	Nd*	Nd*	Filamentous fungus
D	+	Rods	Big white/circular, entire, flat
E	+	Cocci	Yellow/circular, entire, raised
F	–	Cocci	Cream/circular, entire, raised
G	+	Cocci	Tiny white/circular, entire, convex

Shapes are in the order: whole colony forms, Margin edge forms, form of elevation.
*Nd, not determined.

Table 2
Colony Characteristics and Gram Character of Bacterial Colonies From the Midgut of *Odontotaenius disjunctus* (Betsy beetle)

Legend	Gram	Morphology	Shape/Color
I	Nd*	Nd*	White/irregular, serrate, umbonate
II	–	Cocci	Red/circular, entire, convex
III	Nd*	Nd*	Filamentous fungus
IV	+	Rods	White/irregular, undulate, flat
V	+	Rods	Big white/circular, entire, flat

Shapes are in the order: whole colony forms, Margin edge forms, form of elevation.
*Nd, not determined.

that the lignin was stable. Lignin depolymerization was seen only with flasks inoculated with the wastewater sludge. Because of this a more comprehensive study was conducted on this inoculum source.

Table 3
Colony Characteristics and Gram Character of Bacterial Colonies From the Foregut of *Odontotaenius disjunctus* (Betsy beetle)

Legend	Gram	Morphology	Shape/Color
a	+	Rods	Translucent/circular, entire, flat
b	–	Rods	Yellow/irregular, undulate, raised
c	+	Cocci	Big off-white/circular, entire, convex
d	–	Cocci	Red/circular, entire, convex
e	+	Rods	Cream/rhizoid, filamentous, flat
f	–	Cocci	Tiny white/circular, entire, convex
g	Nd*	Nd*	Filamentous fungus

Shapes are in the order: whole colony forms, margin edge forms, and form of elevation.
*Nd, not determined.

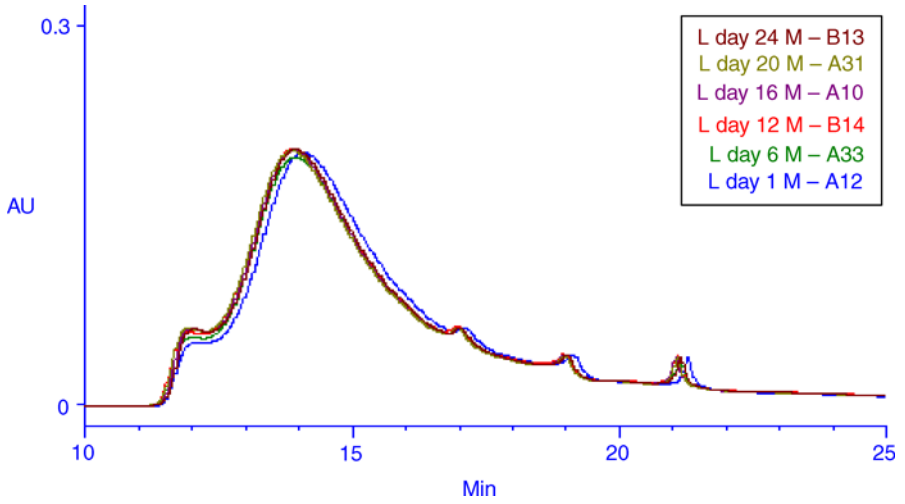

Fig. 4. Lignin primary control without any inoculum showed no molecular weight shifts. M indicates primary control from the midgut inoculum set.

Detailed Analysis for Sludge

Lignin depolymerization was conclusively seen only with the wastewater treatment sludge obtained from a brewery. Initially polymerization was observed as indicated by the increase in the higher molecular weight peaks eluting first (Fig. 4). But after 12 d depolymerization began. pH and the presence of yeast malt extract had a substantial effect on depolymerization. Lignin without yeast malt extract (L + I) showed only polymerization (Fig. 5), but lignin samples with yeast malt extract (Ym + L + I) showed depolymerization and degradation after initial polymerization (Fig. 4). The total area under the curve for the entire lignin profile also seemed to decrease with time indicating not only depolymerization but also complete decomposition of the lignin. Initial pH was adjusted to 8.5 with 0.1 *N* NaOH. pH declined

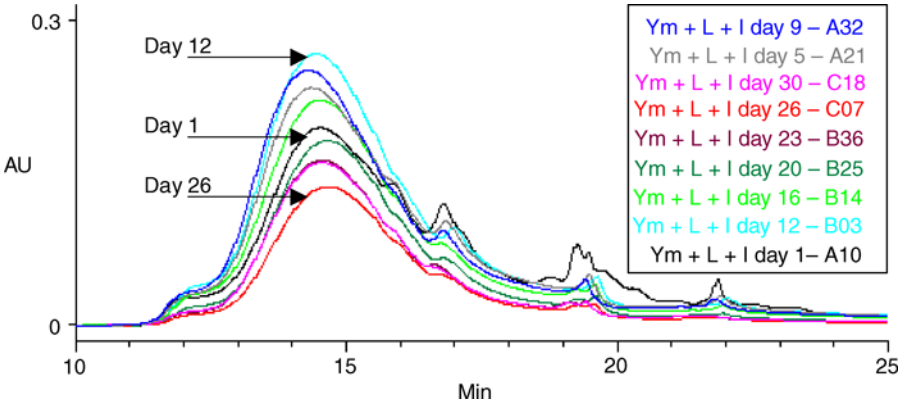

Fig. 5. Lignin polymerized and then depolymerized with wastewater treatment sludge.

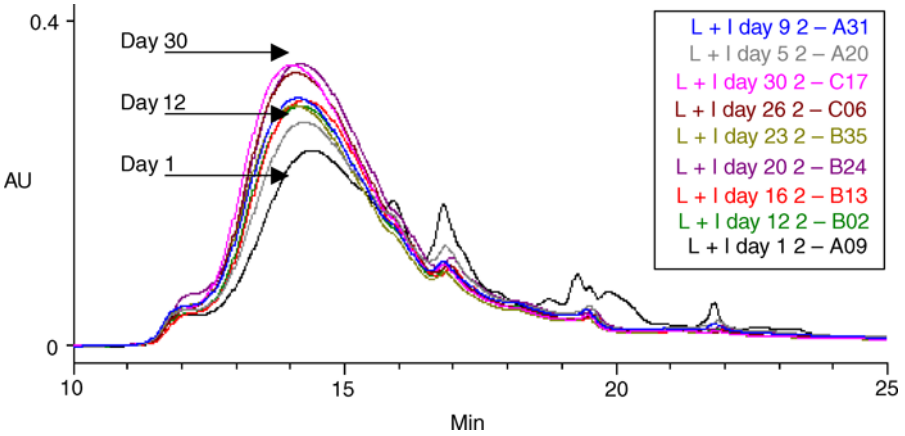

Fig. 6. Incubations without yeast malt extract did not show any depolymerization. Lignin only polymerized.

with incubation for flasks containing yeast malt extract (Ym). The final pH ranged from 4.0 to 4.5 in all the flasks containing Ym, whereas the pH in the other flasks maintained at 8–8.5. This might be another reason for no lignin depolymerization in flasks without Ym (Fig. 6).

An AFM protocol (never attempted before) was developed for lignin, as it was expected to give an idea of the molecular conformation of lignin. Mica was initially tried as a substrate, but a very weak adsorption was seen for lignin with the tip sweeping away the lignin. The substrate selected for AFM studies was graphite (hydrophobic). Graphite also gave a weak interaction with lignin in water or lignin with nutrients (M + L). But for a sample containing all three (lignin, nutrients, and inoculum), a very good, stable, and uniform picture was obtained which showed a monolayer of spherical lignin molecules at a concentration of 0.01 g/L (Fig. 7). The size range of the molecules (350–650 nm) seemed to match with the data (hydrodynamic and root mean square radius) that had been initially obtained for the same lignin with a light scattering detector characterization study *(28)*.

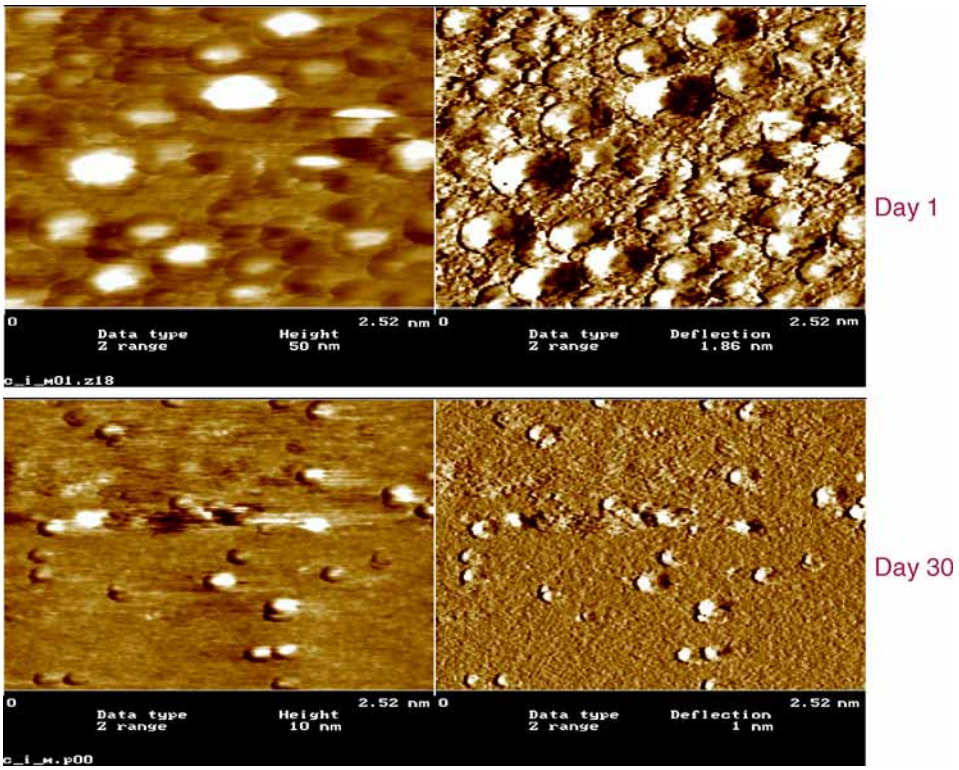

Fig. 7. AFM studies showed spherical lignin molecules.

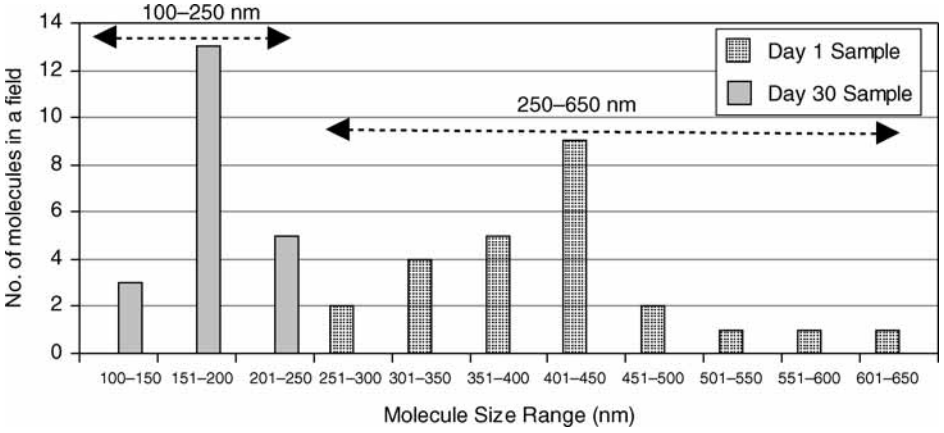

Fig. 8. Wastewater sludge depolymerization was further validated with AFM. Lignin depolymerized from a size ranging from 350 to 650 nm initially, to range from 135 to 220 nm by day 30.

Depolymerization of lignin for wastewater sludge seen on the HPLC was further confirmed with the AFM studies done on samples of days 1 and 30 (Figs. 7 and 8). The molecular size ranging initially from 250 to

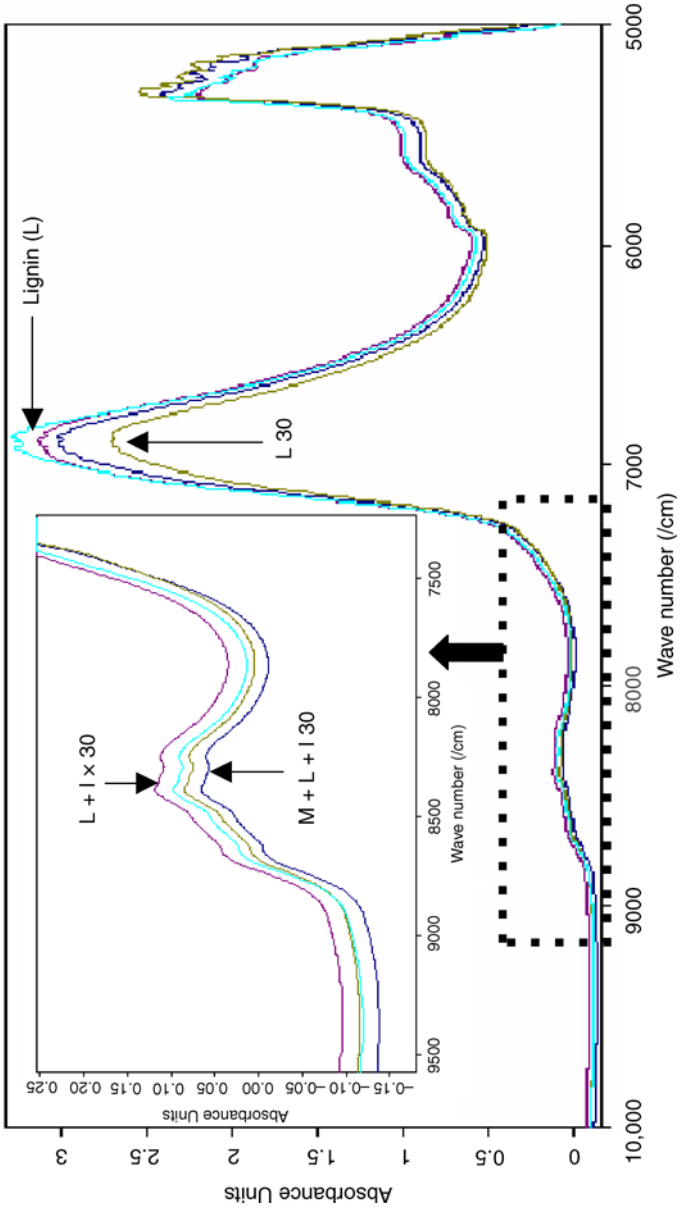

Fig. 9. NIR studies confirmed reduction in concentration of a range of functional groups. L, lignin; M, nutrient media; I, inoculum.

650 nm, reduced to range from 135 to 220 nm (Fig. 8). It can be seen that there is a reduction in not only the size but also the number of molecules, as was indicated with the HPLC results.

A NIR protocol was developed for lignin. A good R^2 of 0.99 was obtained for a range from Wave numbers 10,000.9–7497.8/cm. The functional groups that are covered in this wave number range are C—H, O—H, or N—H str. second overtone (CH, CH_2, CH_3, NH_2, HC=CH, OH, aromatic), 2X C—H str. + 2X C—C str. (Benzene, cyclopropane), 2X C—H str. + 2X C—H def. + $(CH_2)_n$—CH_2 (oil) and 2X N—H str. + 2X amide I (proteins). Figure 9 further validates the results seen on the HPLC and AFM that lignin was decomposed by the sludge. The calibrated zone (insert zoomed up) shows the curve for Ym + L + I is lower than the primary control L (day 1 and 30) indicating that there is an overall lowering in the functional groups.

The next step was to understand the characteristics of the colonies that depolymerized lignin. The colony count CFU for these flasks showed increased growth (20% higher) for inoculated flasks containing Ym + L + I, compared with flasks containing Ym + I or L + I only. There was no colony seen for dilutions of primary controls, indicating that there was no contamination in the flasks. Gram staining showed that there were only two bacterial species growing in all the flasks inoculated. They were white and off-white Gram positive rods. There were, however, slight modifications in both the species compared to the day 1 sample. The colonies from flasks containing only lignin (L + I) and both yeast malt extract and lignin (Ym + L + I) looked similar to those from day 1 inoculation (I). But the colonies from flasks containing only yeast malt extract (Ym + I) were slightly smaller compared to those from day 1 when seen thorough the microscope.

Conclusions

These studies conducted with lignin using analytical tools combining HPLC (UV detector), AFM, NIR, and Gram staining gave a more comprehensive idea regarding the changes that might be occurring to lignin during the incubations. Current studies with beetle guts and other lignin digester sources did not show depolymerization of lignin purified from kraft black liquor. One reason could be that this lignin has undergone many chemical modifications making it a high-molecular weight recalcitrant molecule.

Wastewater treatment sludge inoculum, however, did depolymerize and decompose even this form of lignin, reducing both the number and size of molecules as was validated on the HPLC, AFM, and NIR studies. AFM studies were very effective in studying molecular shape and also size reduction of lignin resulting from depolymerization. Lignin with inoculum gave a stable interaction and a very distinct picture of the lignin

monolayer. Presence of nutrients or Ym was required for depolymerization. Lack of nutrients caused only polymerization. But this could also be because the initial pH was readjusted to range around 4.5 which might be more conducive for the growth of the two white and off-white (Gram positive rods) bacterial isolated.

Beetle gut is an ideal system for lignocellulose processing combining all aspects of size reduction, lignocellulose separation, hydrolysis, saccharification, and eventual fermentation of the cellulose through the series of guts. The lignin separated is in a low-molecular weight solubilized form, which could then have many applications (vanillin, adhesives, binder for laminated or composite wood products, automotive plastics, and so on) and thus a more value-added product than in the present process when it is just used as fuel in the burners. A replication of this ideal system would be an excellent process for lignocellulose conversion to bioethanol, as also seen through the weight loss studies conducted with consortia separated from beetle gut (publication in preparation).

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