



<http://www.diva-portal.org>

This is the published version of a paper published in *BioResources*.

Citation for the original published paper (version of record):

Bi, R., Wang, Y., Oinonen, P., Henriksson, G. (2016)

A Method for Studying Effects on Lignin-Polysaccharide Networks during Biological Degradation and Technical Processes of Wood.

BioResources, 11(1): 1307-1318

Access to the published version may require subscription.

N.B. When citing this work, cite the original published paper.

Permanent link to this version:

<http://urn.kb.se/resolve?urn=urn:nbn:se:kth:diva-182267>

A Method for Studying Effects on Lignin-Polysaccharide Networks during Biological Degradation and Technical Processes of Wood

Ran Bi,^a Petri Oinonen,^b Yan Wang,^c and Gunnar Henriksson^{d,*}

Woody tissues consist primarily of a mixture of cellulose, hemicelluloses, and lignin. Covalent bonds between lignin and polysaccharides likely play a central role in determining the mechanical and physical properties of wood. Intact and defined lignin-polysaccharide networks have not been isolated in large quantities because of the recalcitrance of lignin, which demands harsh chemical treatments that alter its structure. This report presents a method for preparing large quantities of lignin-polysaccharide networks similar to those naturally present in wood based on the enzymatic oxidation of hemicellulose from Norway spruce. Fungal enzymes produced from various carbon sources were used to depolymerize these networks. The method was used for simulating “enzyme mining” – a concept in biorefineries, giving a possible explanation for its mechanisms.

Keywords: Lignin carbohydrate complexes; Wood; Model system; Biorefinery; Biodegradation

Contact information: a: Wallenberg Wood Science Centre, School of Chemical Engineering, Royal Institute of Technology, KTH, 10044 Stockholm, Sweden; b: Ecohelix AB, Teknikringen 38, 10044 Stockholm, Sweden; c: Basel Convention Regional Center for Asia and the Pacific, School of Environment, Tsinghua University, Beijing, 100084, China; d: Wallenberg Wood Science Centre (WWSC), Department of Fibre and Polymer Technology, Royal Institute of Technology, KTH, Teknikringen 56, 10044 Stockholm, Sweden; *Corresponding author: ghenrik@kth.se

INTRODUCTION

Lignocellulose is a major constituent of the cell walls of vascular plants, and as the most abundant biological material on Earth, it has enormous biological and technical importance (Fernando *et al.* 2006). It consists of a mixture of cellulose, other polysaccharides known as hemicelluloses, and the complex aromatic polymer lignin (Sjöström 1993). While cellulose and polysaccharides similar to hemicelluloses are found in many different life forms, including true plants (Brown 1985), lignin is only synthesized in vascular plants, although a similar polymer might be synthesized by certain red algae (Martone *et al.* 2009; Weng and Chapple 2010). Lignin gives vascular plant cell walls their “woody” properties, which enable water conduction, mechanical stiffness, and resistance to microbial degradation (Kutscha and Gray 1970).

The structure and unique bio-polymerization of lignin are well-suited for these functions; it is a web-shaped polymer with a partly random structure that is formed by un-catalyzed radical polymerization in which radicals are formed on phenolic structures on lignin by oxidation by laccases or peroxidases (Adler 1977; Richardson *et al.* 2000). During this process, reactive intermediates—quinone methides—are created. These structures can either absorb water or react with alcohol groups on polysaccharides. In the latter case, covalent bonds between lignin and polysaccharides are formed (Freudenberg

and Grion 1959).

In softwood, lignin covalently crosslinks different polysaccharides, such as the hemicelluloses xylan and glucomannan (Lawoko *et al.* 2006). A recent study demonstrated that hemicellulose molecules from Norway spruce often contain more than one lignin functionality, thereby allowing extensive crosslinking into large networks (Oinonen *et al.* 2015). Based on these observations, we have suggested a model for wood where lignin and especially hemicelluloses, but also cellulose, form large covalent networks (Fig. 1). Covalent bonds between lignin and pectin also exist (Henriksson 2009). In this model, lignin “cures” the cell wall by covalently crosslinking polysaccharides (Fig. 1), which enhances the functions and properties of wood (stiffness, low water absorption, and resistance to biological degradation). However, the properties of these molecules—how they are degraded and what significance they have for the biological degradation of wood in industrial processes including pulping and biorefining—are not well understood.

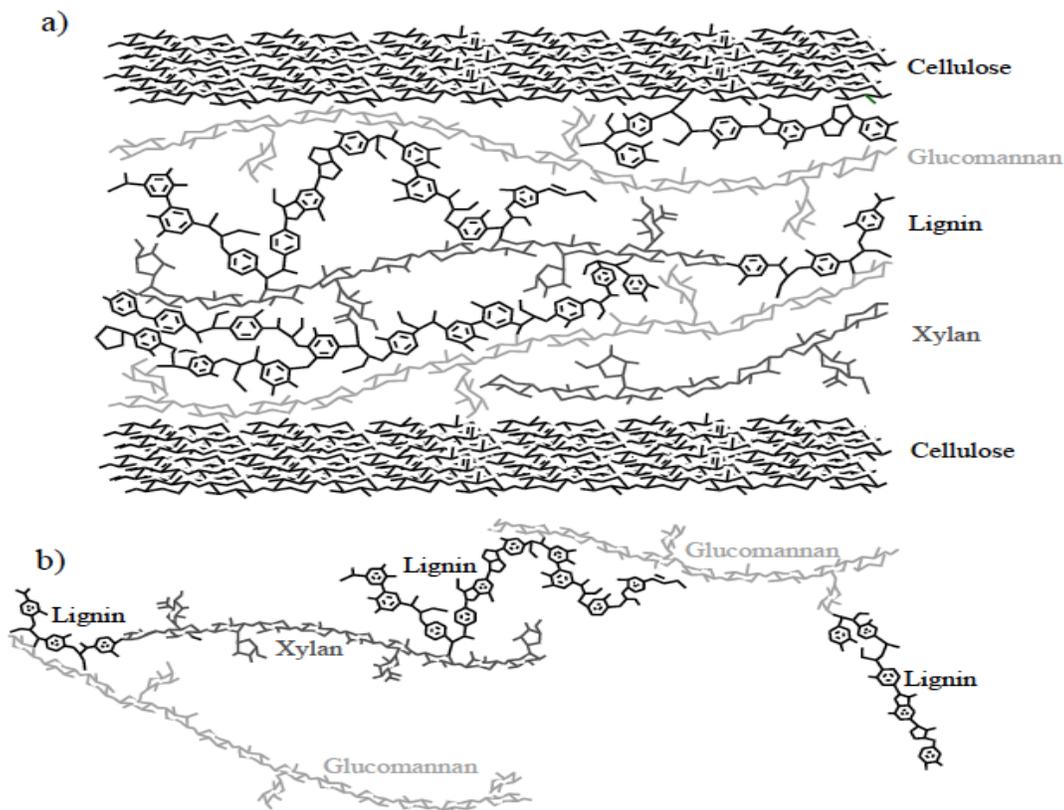


Fig. 1. A) A schematic representation of the organization of wood of at the molecular level, where lignin covalently crosslinks various polysaccharides in wood. B) A hypothetical “mixed” hemicellulose-lignin molecule, where lignin covalently crosslinks various hemicellulose chains, creating a molecule with xylan, glucomannan, and lignin functionalities.

Because of its complex and compact structure, wood is difficult for microorganisms to degrade (Sjöström 1993; Blanchette *et al.* 1997). Wood-degrading organisms often have complex and sophisticated enzyme systems for attacking lignin (Eriksson *et al.* 1990). Research on these degradation methods has been restricted mostly to pure isolated model compounds, which may not accurately represent the complexity of

wood structures. In biorefinery concepts based on lignocellulose, it is often difficult to extract high yields of polymers without intensive and partly destructive pretreatment of the wood (Mosier *et al.* 2005). However, the treatment of wood with hydrolyzing enzymes increases the extraction of polymers other than the enzymes' substrates (Azhar *et al.* 2011), and a possible explanation for this phenomenon is that "hybrid molecules" of lignin and polysaccharides (Fig. 1) are attacked. Furthermore, polysaccharide network degradation probably plays an important role in both chemical pulping and biological wood degradation (Lawoko *et al.* 2004). Thus, preparing these networks in an intact form could be useful for enzymatic mining studies.

By the oxidative coupling of phenols *via* the enzyme laccase, hemicelluloses carrying covalently bound lignin can be cross-linked (Oinonen *et al.* 2013). The raw material for this reaction can be obtained in large amounts, for example, from the process waters of mechanical pulping. These cross-linked polymers have properties that are interesting for many different technical applications (Oinonen *et al.* 2013).

In this study, "biomimetic" lignin-polysaccharide networks were used as tools to follow reactions in lignin polysaccharide networks during biological degradation and technical processes involved in pulping and biorefining.

EXPERIMENTAL

Materials

Process water from the thermomechanical pulping (TMP) of Norway spruce (*Picea abies*) was taken from the process stream of a Swedish TMP center. The sample was sequentially filtered with a 0.45- μm ceramic membrane (Kerasep module, Novasep, Pompey, France) and a 5-kDa cut-off membrane (Novasep). After filtration, the sample was freeze-dried. Lignoboost lignin was a kind gift from Professor Hans Theliander, Wallenberg Wood Science Center, Chalmers, Gothenburg, Sweden. Beech wood xylan was purchased from Apollo Scientific Limited (Manchester, UK). Locust bean gum mannan, cellulose type 50, xylan, and phenyl- α -D-mannopyranoside were purchased from Sigma (St. Louis, MO). Gamanase is a commercial enzyme product including many carbohydrate-degrading activities, which is particularly rich in 1,4- β -D-mannan mannohydrolase and β -glucuronidase activities (Redgwell *et al.* 2005). The Gamanase was a kind gift from Novozymes (Bagsværd, Denmark). All other chemicals were of analytical grade.

Methods

Production of enzymes

Several organisms isolated from soil appear to use lignin as their sole carbon source (Ran *et al.* 2012). One of these, *Phoma herbarum*, was cultivated on four different carbon sources: lignoboost lignin, xylan, galactomannan (locust bean, Sigma), and cellulose in shaking flasks. Each liter of cultivation medium contained 10 g of carbon source, 0.25 g of KH_2PO_4 , 1.0 g of NH_3NO_3 , 1.0 g of CaCl_2 , 0.25 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 1.0 mg of FeSO_4 . The medium pH was adjusted by NaOH to 5.0. Culture filtrates were collected after seven days.

In vitro synthesis of lignin polysaccharide networks

The TMP process water derived polysaccharides that were ultrafiltered were used to create the lignin polysaccharide networks. Laccase enzyme (NS51002, Novozymes, Bagsværd, Denmark) was used for the oxidative cross-linking of purified polysaccharides as described earlier by Oinonen and coworkers (2013). The reaction conditions were as follows: enzyme dosage 14 U/g (~1.4 mg/g substrate) of hydrolyzate, pH 5, reaction temperature 40 °C, reaction time 3 h, and hydrolyzate concentration 100 g/L. Pure oxygen gas was introduced to the samples during the reaction.

The cross-linked samples were separated into high- and low-molecular weight fractions by ultra-filtration (Solvent-Resistant Stirred Cell, Millipore, USA) with a cellulose membrane with a molecular weight cut-off of 30 kDa (PLTK07610, Millipore). Prior to ultra-filtration, the samples were diluted 1:10 by water. Ultra-filtration was conducted under nitrogen atmosphere (3 bar) with constant stirring and was continued until a retentate/permeate volume ratio of 1/10 was achieved. The retentate was further diluted 1:10 by water, and the ultrafiltration was repeated. After the repeated ultra-filtration, a total of three fractions were collected: a high-molecular weight retentate (high Mw) and two low-molecular weight permeate (low MW) fractions. The retentate fraction was used for degradation studies. The yield of high molecular weight lignin-polysaccharide networks through the filtration was 55%, consisting of 36 wt% carbohydrates (mainly galactoglucomannan), and 27 wt% lignin (Oinonen *et al.* 2013).

Treatment with culture filtrates of the soil organism Phoma herbarum

Cell-free culture filtrates (500 µL) of four different cultures were incubated with 400 µL of 50 mM sodium acetate buffer, pH 5, and 10 mg of synthetic lignin polysaccharide networks overnight, and the mixtures were analyzed on size exclusion chromatography (SEC) to detect depolymerization. The culture filtrate was heat-inactivated (90 °C, 20 min) prior to the control experiment.

LC bond model compound degradation by culture filtrates of galactomannan culture of the soil organism Phoma herbarum

Cell-free culture filtrates of galactomannan culture (500 µL) were incubated with 400 µL of 50 mM sodium acetate buffer, pH 5, and 10 mg of phenyl- α -D-mannopyranoside overnight, and the mixture was run on SEC to detect depolymerization. The culture filtrate was heat-inactivated (90 °C, 20 min) prior to the control experiment.

Treatment with commercial enzymes

Synthetic lignin polysaccharide networks (10 mg) were incubated with 10 µL of Gamanase in 50 mM sodium acetate buffer, pH 5, in a Thermomixer (Eppendorf, Hamburg, Germany) with shaking at 60 °C and 600 rpm for 24 h. The enzyme dosage was 1 VHCU/mg (1 µL/mg). The control experiment contained no Gamanase.

Size exclusion chromatography (SEC)

The alkaline SEC system consisted of a Rheodyne 7725 Manual Injector (Rohnert Park, CA) equipped with a 20-µL sample loop and a Waters 515 HPLC pump (Waters Corp., Milford, MA). It was operated at a flow rate of 1 mL/min. Three Tosoh TSKGel columns (G3000PW-G4000PW-G3000PW) and a TSKGel guard column (PWL 7.5 cm x 7.5 mm) were acquired from Tosoh Bioscience (Tokyo, Japan), and the detectors used were a Waters 2487 dual λ Detector, operated at 254 and 280 nm, and a Waters 410

Refractive Index Detector (both from Waters Corp., Milford, MA). The mobile phase was 10 mM NaOH in Milli-Q H₂O. Injection volumes of 20 µL were used for each sample analysis.

Component analysis of the samples

The carbohydrate and lignin compositions of the samples were determined by employing the hydrolysis conditions described in the TAPPI -standard method (TAPPI Test Methods 2003). The mono sugar analyses were performed with a HP 6890 series gas chromatography device with a BP-70 column (60 m, 0.32 µm I.D., and 0.25 µm film thickness). Anhydro corrections of 0.9 and 0.88 were used for the hexoses and pentoses, respectively. The ash content of the samples was measured according to the TAPPI-standard method (TAPPI Test Methods 1985).

RESULTS AND DISCUSSION

Synthesis of Water-Soluble Lignin-Polysaccharide Covalent Networks

The raw material for *in vitro* synthesis of lignin polysaccharide networks was prepared by cross-flow filtration of thermomechanical pulping process waters, resulting in a fraction that contained a high concentration of hemicelluloses. The hemicelluloses were treated with laccase, resulting in increased molecular weight (Table 1; Oinonen *et al.* 2013). The resulting molecules contained both lignin and hemicellulose, and may have been formed as suggested in Fig. 1. As the enzymatic reaction relies on the coupling of carbohydrate-attached aromatic moieties, these structures are accumulated into the high molecular weight fraction. This phenomenon explains the increased amount of lignin structures in the high molecular weight fraction. It is to be noted that in addition to lignin (Klason lignin), polysaccharides and ash the hydrolyzates likely also contain proteins, organic acids (incl. sugar acids), lipophilic substances, and lignin degradation products.

Table 1. Molecular Weight and Chemical Composition of Galactoglucomannans Isolated from Thermomechanical Pulping Process Waters before (TMP GGM) and after (TMP GGM hMw) Enzymatic Treatment and Fractionation

Sample	M_n (kDa)	M_w (kDa)	M_w/M_n
TMP GGM	14.8 ± 0.2	22.4 ± 0.1	1.5 ± 0.01
TMP GGM hMw	20.5 ± 0.1	59.5 ± 0.2	2.2 ± 0.01

(A)

	Lignin	Ash	Carbohydrates	Man	Glu	Xyl	Gal	Ara
	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)
TMP GGM	16	9	49	65	17	2	13	3
TMP GGM hMw	27	6	36	56	15	4	19	6

(B)

This method creates molecules that probably have similarities to molecules in wood (Lawoko *et al.* 2006). Structurally, material cross-linked by laccase is similar to the hypothesized natural network of carbohydrates and lignin (Oinonen *et al.* 2015). Thus,

this material is an interesting model system for lignin polysaccharide networks and is suitable for studying the biological and technical processes of wood.

The lignin content of different sizes of the material can be measured by chromatography using the ratio between UV absorbance and refraction index (Fig. 4). This data can be used to examine structural changes in lignin polysaccharide networks during different treatments. Two examples of how this system can be used in studies related to biorefineries and biology are described below.

Treatment with Commercial Enzymes used in Biorefinery Concepts

Wood and other lignocelluloses are abundant natural resources used in the production of renewable fuels and materials. Efficiency in biorefineries, where wood components are separated into their pure forms, is critical. However, LCC-networks are obstacles to efficient separation by extraction, as various wood components have quite different solubility properties (Liu *et al.* 2012).

Treatment with different commercial hemicellulases increases the yield and quality of material extracted from lignocellulose (Azhar *et al.* 2011). Surprisingly, the enzymes act not only on hemicelluloses but also on other polysaccharides and lignin (Azhar *et al.* 2011). In this study, the synthesized lignin polysaccharide networks were treated with the commercial culture filtrate Gamanase, which acts primarily on glucomannans, and the reactions were analyzed with SEC. As shown in Fig. 3 (panel c), the complex networks were degraded by the enzymes, and the UV absorption peaks associated with lignin were shifted to lower molecular weights. Because decreased molecular weight is associated with increased extractability, these results give a plausible explanation for earlier results (Azhar *et al.* 2011); namely, that an attack on one polymer, such as glucomannan, leads to increased extraction of another molecule, such as xylan or lignin (Fig. 2). Commercial culture filtrates rich in xylanase activity produced similar results (data not shown).

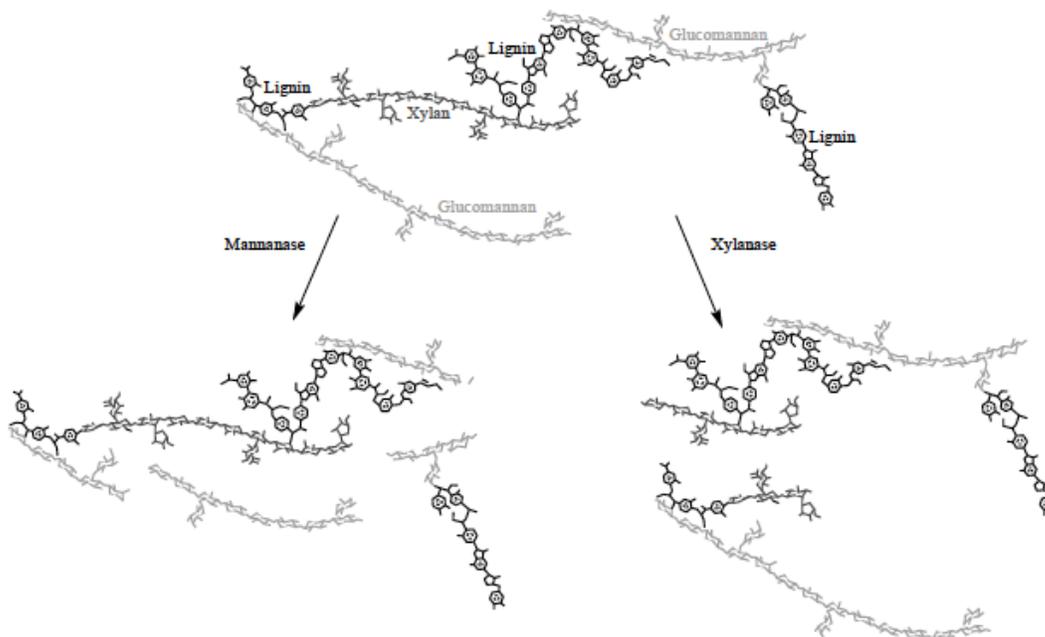


Fig. 2. Schematic presentation of how selective hemicellulases affect a “mixed” hemicellulose/lignin molecule. A selective enzyme attacking one hemicellulose affects the entire molecule and thereby also influences the solubility and extractability of lignin and other polysaccharides.

When the ratio between UV absorption and refraction index was examined (Fig. 4 C, D), the highest lignin content was initially located in the material of higher molecular weight. After treatment with Gamanase (Fig. 4D), the lignin shifted to lower molecular weight, indicating the presence of covalent bonds between lignin and polysaccharides. The purest lignin was obtained at approximately 24 min, as in the case of treatment with *Phoma* culture filtrate (see below). These results support the hypothesis that enzymatic treatment “unties” lignin polysaccharide networks, thereby releasing lignin and polysaccharides.

Treatment with Culture Filtrates of the Soil Organism *Phoma herbarum*

Wood is energy-rich but difficult for microbes to degrade. Lignocellulose is much more slowly degraded by molds, for example, than non-lignified tissues, such as potato peels (Eriksson *et al.* 1990). Lignin-polysaccharide networks probably play an important role in the relative resistance of wood, partly by making the wood structure very compact, but also by the complex and partly random covalent pattern of the lignin polysaccharide networks. Many wood-degrading organisms mount a concerted attack on wood with free radicals and specific hydrolases. White-rot fungi completely mineralize wood, while most other wood-degrading organisms leave a residue consisting of chemically modified lignin and covalently bound oligosaccharides (Eriksson *et al.* 1990; Ten Have and Teunissen 2001). This material remains in soil as the main component of humus and as an energy source utilized by soil microorganisms. A number of these organisms have been isolated (Ran *et al.* 2012); one of these, *Phoma herbarum*, was used in this study.

Cell-free culture filtrates of four different cultures of *P. herbarum* cultivated on different carbon sources—lignin, xylan, glucomannan, and cellulose—produced similar degradation patterns in synthetic lignin polysaccharide networks (data not shown). The galactomannan culture showed the strongest effect, with a peak shift indicating LCC network degradation at approximately 21.5 min for both UV and RI (Fig. 3, panel B). The increases in both peaks at 25 min and 27 min of UV signal (Fig. 3, panel B), respectively, might be attributed to degradation products. In many cases, the degradation of synthetic lignin polysaccharide networks by the soil organism *Phoma herbarum* and by the commercially produced enzyme Gamanase (see above) was very different. First, the shift in the UV absorbance peak at approximately 21.5 min was much more dramatic after the *Phoma herbarum* culture filtrate treatment than after the Gamanase treatment, indicating a much larger decrease in molecular weight. Secondly, the peak at approximately 25 min in the *Phoma herbarum* culture filtrate-treated lignin polysaccharide networks increased in the UV absorbance chromatogram but decreased in the RI chromatogram. This result suggested that the component represented by the peak was lignin rather than hemicellulose. At nearly the same time (25 min) in the Gamanase-treated lignin polysaccharide networks, the peak increased in both UV and RI chromatograms, which indicated less lignin in the product.

In the LC bond model, the *Phoma herbarum* galactomannan culture filtrate had the potential to enzymatically break LC bonds (Fig. 3, panel A). The reaction, comparing to the control experiment as detected by UV 280 nm detector, showed phenyl- α -D-mannopyranoside being consumed (peaks from 24 min to 26 min), and a large aromatic

peak at 32.5 min was formed as the result of the treatment. Phenyl glucoside bonds are believed to be common in lignin polysaccharide networks in wood.

The ratio of UV absorbance to RI clarified the differences in lignin content (Fig. 4). In the material treated with inactivated culture filtrate (Fig. 4A), the most lignin (UV absorbance) and the purest lignin (UV/RI) was present at the beginning of the chromatogram. After treatment with the *Phoma* culture filtrate (Fig. 4B), the lignin peak shifted left, *i.e.*, to a lower molecular weight. Interestingly, the purity decreased also, and a region with purer lignin developed at approximately 24 min.

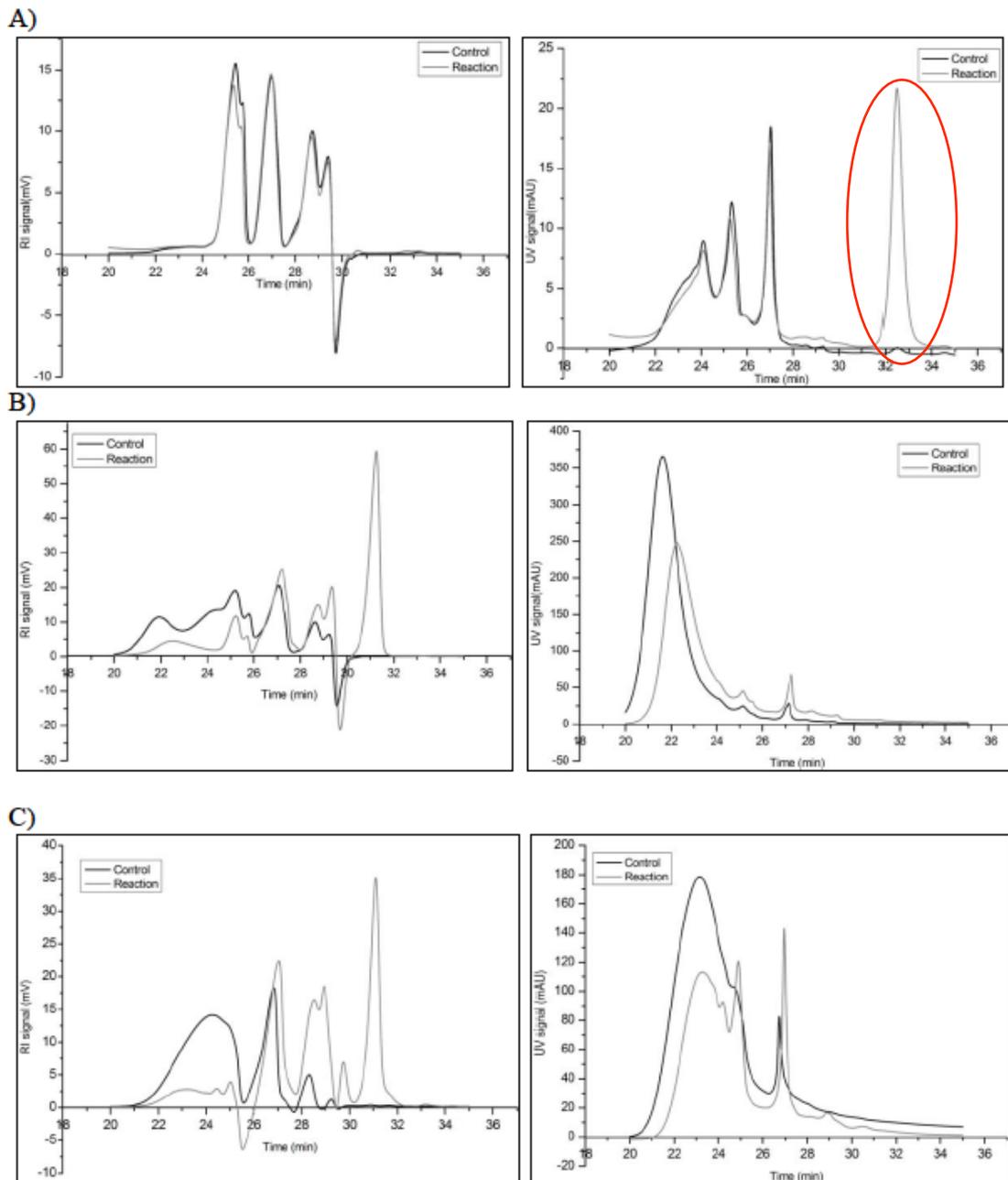


Fig. 3. Degradation of enzymatically produced lignin-hemicellulose networks and an LC bond model compound with *Phoma herbarum* galactomannan culture filtrate and Gamanase enzyme.

Left panels, refractive index; right panels, UV absorbance (280 nm). Solid lines, control experiments; dashed lines, experimental reactions. (A) Chromatogram of the LC bond model compound treated with *Phoma herbarum* filtrate. (B) Chromatogram of TMP GGM hMw treated with *Phoma herbarum* filtrate. (C) Chromatogram of TMP GGM hMw treated with Gamanase

The refraction index showed more dramatic effects, with increased peaks indicating lower molecular weights. These results suggested that the culture filtrate depolymerized carbohydrates in the lignin-hemicellulose hybrid molecules; furthermore, relatively pure lignin was released from the networks by direct attack on covalent bonds between lignin and polysaccharides or by degradation of carbohydrate chains proximal to those bonds. Filtrates of *Phoma herbarum* cultures with different carbon sources have different enzyme activities (Table 2; Ran *et al.* 2012), which may explain the observed differences in lignin polysaccharide networks degradation.

Table 2. Enzyme Activity in Culture Filtrates

Culture carbon source	Mananase Activity	Xylanase Activity	Endogluconase Activity	Lignin degradation Activity
Cellulose	+ ^a	+	+	- ^b
Galactomannan	+	+	+	-
Xylan	+	+	+	-
Lignin	+	+	+	+

^a Culture filtrates with certain enzyme activity

^b Culture filtrates without certain enzyme activity

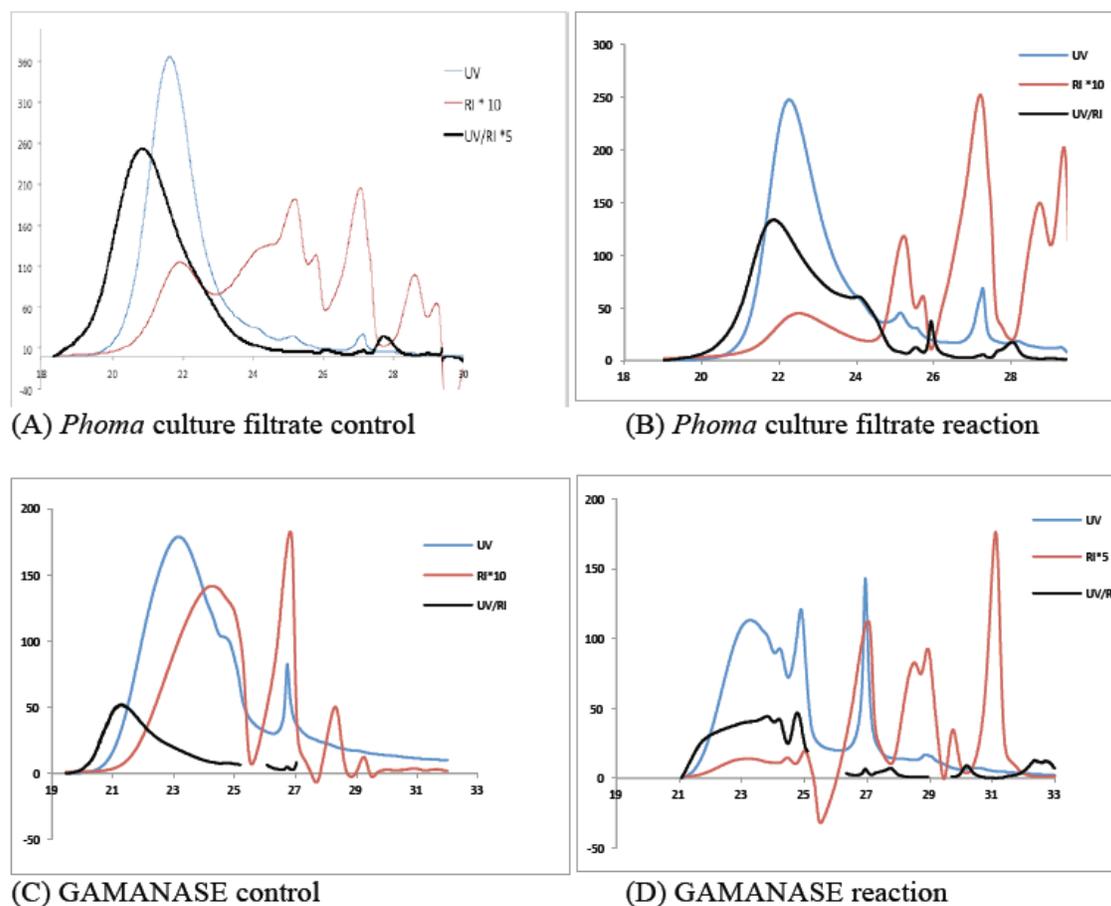


Fig. 4. Analysis of enzymatically produced lignin-hemicellulose networks treated with *Phoma herbarum* galactomannan culture filtrate or gamanase enzyme. Red lines, RI; blue lines, UV absorbance (280 nm); black lines, the ratio of UV/RI. (A) Negative control. *Phoma* culture filtrate was heat-denatured to deactivate enzymes. (B) Networks were treated with *Phoma* culture filtrate. (C) Negative control. Networks were treated with heat-denatured gamanase. (D) Networks were treated with gamanase.

CONCLUSIONS

1. Water-soluble lignin-polysaccharide networks can be synthesized *in vitro* from mechanical pulping process waters treated with laccase.
2. Degradation of these networks by enzymatic digestion can be examined by size exclusion chromatography, and changes in the molecular weights of lignin and polysaccharides can be followed specifically.
3. This model system can be used to follow reactions in wood during technical processes and biological degradation.
4. Hemicellulase treatment may partially degrade covalently bound lignin-polysaccharide networks in lignocellulose, leading to enhanced extraction of both polysaccharides and lignin.
5. The soil organism *Phoma herbarum* produces enzymes that attack lignin-polysaccharide networks, possibly by selectively breaking phenyl glucoside bonds.

6. Enzymatic attack on one polysaccharide might affect the degradation of other polysaccharides in hybrid hemicellulose-lignin molecules, and this effect is important for synergistic enzymatic biodegradation of plant cell walls.
7. This method can be used to screen enzymes for their ability to digest lignin-polysaccharide networks and to follow the degradation of lignin-polysaccharide networks during pulping and bleaching reactions.

ACKNOWLEDGMENTS

This work was supported by the Knut and Alice Wallenberg Foundation within the Wallenberg Wood Science Centre and by the Swedish Agency for Natural Sciences. Novozymes generously provided the enzymes.

REFERENCES CITED

- Adler, E. (1977). "Lignin chemistry – Past, present and future," *J. Wood Chem. Technol.* 11(3), 169-218. DOI: 10.1007/BF00365615
- Azhar, S., Wang, Y., Lawoko, M., Henriksson, G., and Lindström, M. E. (2011). "Extraction of polymers from enzyme-treated softwood," *BioResources* 6(4), 4606-4614. DOI: 10.15376/biores.6.4.4606-4614
- Bi, R., Spadiut, O., Lawoko, M., Brumer, H., and Henriksson, G. (2012). "Isolation and identification of microorganisms from soil able to live on lignin as carbon source and to produce enzymes which cleave the β -O-4 bond in a lignin model compound," *Cellulose Chemistry and Technology* 46(3-4), 227-242.
- Blanchette, R. A., Kreuger, E. W., Haight, J. E., Akhtar, M., and Akin, D. E. (1997). "Cell wall alterations in loblolly pine wood decayed by the white rot fungus *Ceriporiopsis subvermospora*," *J. Biotechnol.* 53(2-3), 203-213. DOI: 10.1016/S0168-1656(97)01674-X
- Brown, M. (1985). "Cellulose microfibril assembly and orientation: Recent developments," *J. Cell Sci.* Supplement 2, 13-32. DOI: 10.1242/jcs.1985.Supplement_2.2
- Eriksson, K. E. L., Blanchette, R. A., and Ander, P. (1990). *Microbial and Enzymatic Degradation of Wood and Wood Components*, Springer, Berlin. DOI: 10.1007/978-3-642-46687-8
- Fernando, S., Adhikari, S., Chauda, C., and Naveen, M. (2006). "Biorefineries: Current status, challenges, and future direction," *Energy Fuels* 20(4), 1727-1737. DOI: 10.1021/ef060097w
- Freudenberg, K., and Grion, G. (1959). "Contribution to the mechanism of formation of lignin and of the lignin-carbohydrate bond," *Chem. Ber.* 92(6), 1355-1363. DOI: 10.1002/cber.19590920618
- Henriksson, G. (2009). "Lignin," in: *Pulp and Paper Chemistry and Technology I Wood Chemistry and Wood Biotechnology*, M. Ek (ed.), De Gruyter, Berlin, pp. 121-145.
- Kutschka, N. P., and Gray, J. R. (1970). *Maine Agricultural Experiment Station Technical Bulletin 41*, University of Maine, Orono, ME.

- Lawoko, M., Berggren, R., Berthold, F., Henriksson, G., and Gellerstedt, G. (2004). "Changes in the lignin-carbohydrate complex in softwood kraft pulp during kraft- and oxygen delignification. Lignin-polysaccharide networks II," *Holzforchung* 58(6), 603-610. DOI: 10.1515/HF.2004.114
- Lawoko, M., Henriksson, G., and Gellerstedt, G. (2006). "Characterisation of lignin carbohydrate complexes (LCCs) of spruce wood (*Picea abies* L.) isolated with two methods," *Holzforchung* 60(2), 156-161. DOI: 10.1515/HF.2006.025
- Liu, S., Lu, H., Hu, R., Shupe, A., Lin, L., and Liang, B. (2012). "A sustainable wood biomass biorefinery," *Biotechnol. Adv.* 304(4), 785-810. DOI: 10.1016/j.biotechadv.2012.01.013
- Martone, P. T., Estevez, J. M., Lu, F., Ruel, K., Denny, M. W., Sommerville, C., and Ralph, J. (2009). "Discovery of lignin in seaweed reveals convergent evolution of cell wall architecture," *Curr. Biol.* 19(2), 169-175. DOI: 10.1016/j.cub.2008.12.031
- Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y. Y., Holtzapple, M., and Ladisch, M. (2005). "Features of promising technologies for pretreatment of lignocellulose biomass," *Bioresour. Technol.* 96(6), 673-686. DOI: 10.1016/j.biortech.2004.06.025
- Oinonen, P., Areskogh, D., and Henriksson, G. (2013). "Enzyme catalyzed cross-linking of spruce galactoglucomannan improves its applicability in barrier films," *Carbohydr. Polym.* 95, 690-696. DOI: 10.1016/j.carbpol.2013.03.016
- Oinonen, P., Zhang, L., Lawoko, M., Henriksson, G. (2015). "On the formation of lignin- polysaccharide networks in Norway spruce," *Phytochem.* 111, 177-184. DOI: 10.1016/j.phytochem.2014.10.027
- Redgwell, R.J., Schmitt, C., Beaulieu, M., and Curti, D., (2005). "Hydrocolloids from coffee: Physicochemical and functional properties of an arabinogalactan-protein fraction from green beans," *Food Hydrocolloid.* 19(6), 1005-1015. DOI: 10.1016/j.foodhyd.2004.12.010
- Richardson, A., Duncan, J., and McDougall, G. J. (2000). "Oxidase activity in lignifying xylem of a taxonomically diverse range of trees: Identification of a conifer laccase," *Tree Physiol.* 20(15), 1039-1047. DOI: 10.1093/treephys/20.15.1039
- Sjöström, E. (1993). *Wood Chemistry: Fundamentals and Applications*, Academic Press, Waltham, MA.
- TAPPI Test Methods (1985). "Ash in wood, pulp, paper and paperboard: Combustion at 525 °C," TAPPI T211 om-85.
- TAPPI Test Methods. (2003). "Carbohydrate composition of extractive-free wood and wood pulp by gas-liquid chromatography," TAPPI 249 cm-00.
- Ten Have, R., and Teunissen, P. J. M. (2001). "Oxidative mechanisms involved in lignin degradation by white-rot fungi," *Chem. Rev.* 101, 3397-3413. DOI: 10.1021/cr000115l
- Weng, J.-K., and Chapple, C. (2010). "The origin and evolution of lignin biosynthesis," *New Phytologist* 187(2), 273-285. DOI: 10.1111/j.1469-8137.2010.03327.x

Article submitted: September 22, 2015; Peer review completed: November 16, 2015;
Revised version received and accepted: December 1, 2015; Published: